Université de Montréal

Développement d'un système analytique pour la digestion de protéines, la séparation et la détection de fragments peptidiques

par

Éric Bonneil

Département de Chimie

Faculté des Arts et Sciences.

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Présentée par:
Éric Bonneil

A été évaluée par un jury composé des personnes suivantes:

Pr. Michel J. Bertrand....., président-rapporteur
Pr. Karen C. Waldron....., directrice de recherche
Pr. Michel Lafleur....., membre du jury
Pr. Peter Banks....., examinateur externe

Thèse acceptée le:.....

Sommaire

La cartographie peptidique est une technique comparative couramment utilisée pour l'analyse des protéines et l'identification de substitutions d'acides aminés ou de modifications post-transductionnelles dont certaines sont à l'origine de maladies graves comme l'anémie. Cette technique est constituée de trois étapes: le clivage enzymatique ou chimique de la protéine, la séparation et la détection des fragments peptidiques par une technique chromatographique. La cartographie peptidique de protéines provenant d'échantillons biologiques est une technique longue et fastidieuse. Afin de digérer les protéines, nous avons mis au point un microréacteur qui contient de la trypsine immobilisée sur un support solide. La solution de protéine est perfusée à travers le microréacteur où a lieu la digestion. Les fragments peptidiques sont collectés à la sortie du micro-réacteur et séparés par électrophorèse capillaire (CE). Ce microréacteur a montré une bonne reproductibilité des cartes peptidiques pour des concentrations de protéines assez élevées (0.08 mM). Cependant, le nombre de pics obtenus sur nos électrophérogrammes dans le cas de la β-caséine est supérieur au nombre de peptides attendus. Nous avons montré que ce phénomène est dû au fait que les échantillons commerciaux de cette protéine comportent plusieurs isoformes.

Dans les échantillons biologiques, les fragments peptidiques issus de la digestion de protéines sont souvent présents à des concentrations inférieures aux limites de détection usuelles par spectrophotométrie. Nous avons donc fabriqué un préconcentrateur fait de billes d'octadécylsilane (1 mm × 370 μm). Dans un premier temps, la solution de fragments peptidiques est perfusée à travers le préconcentrateur où les peptides sont retenus. Après connexion de celui-ci à un capillaire de séparation par électrophorèse capillaire, la désorption est effectuée en injectant à travers le préconcentrateur un petit volume d'une

solution d'acétonitrile (65 nl). Ce volume d'élution est ensuite poussé par pression dans le capillaire de séparation. Pour éviter les problèmes associés à la présence du préconcentrateur lors de la séparation, ce dernier est déconnecté du capillaire de séparation avant application du voltage. Nos études ont montré que l'étape de préconcentration induit un phénomène chromatographique qui se superpose à la séparation électrophorétique. En conséquence, les cartes peptidiques obtenues avec une étape de préconcentration sont très différentes de celles obtenues à des concentrations élevées. En dépit de ce phénomène, nous avons obtenu des cartes peptidiques de digestats de la β-caséine avec une bonne reproductibilité et avec des solutions de protéine à des concentrations de 400 nM.

Afin de minimiser la perte d'échantillon, les manipulations et le temps nécessaires pour obtenir la cartographie peptidique à partir de la solution de protéine à basse concentration, nous avons connecté le microréacteur, le préconcentrateur et le capillaire de séparation en un système continu. Malgré la perte de résolution et d'efficacité induite par le design du système et le relativement important volume de désorption injecté (60 nl), nous avons atteint un facteur de préconcentration de 500. Nous avons obtenu les cartes peptidiques de différentes protéines à des concentrations allant de 400 nM à 800 nM en quatre heures et ce de manière reproductible. De plus, notre système est réutilisable.

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Abbréviations

AH	Aqueous Humor
BGE	Background electrolyte
CE	Capillary Electrophoresis
cITP	Capillary isotachophoresis
HPLC	Chromatographie liquide haute performance
CLOD	Concentration limit of detection
CPG	Controlled pore glass
DITC	Diisothiocyanate
ÉC	Électrophorèse capillaire
EOF	Electroosmotic flow
f.s.	Fused silica
FITC	Isothiocyanate de fluorescine
HbA	Normal human hemoglobin
HbS	Sickle cell hemoglobin
K	Lysine
LOD	Limit of detection
LSB	leading stacking buffer
MEKC	Micellar electrokinetic chromatography
mPC-CE	Membrane preconcentrator on-line with CE
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MT-1	Metallothionein isoform 1
MT-2	Metallothionein isoform 2
ODS	Octadecylsilane
PTFE	Polytetrafluoroethylene
R	Arginine
RSD	Relative standard deviation
s.s.	Stainless steel
SDB	Polymeric styrene divinyl benzene
SPE	Solid-phase extraction;
SPE-CE	Solid-phase extraction capillary electrophoresis
TFE	Teflon
tITP	Transient isotachophoresis
TSB	trailing stacking buffer

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Chapitre 1

Introduction

1.1. Les Protéines

Les protéines sont au coeur de tous les processus biologiques. Elles catalysent l'ensemble des réactions chimiques à la base de la vie. Elles servent de régulateurs de ces réactions soit directement comme composants des enzymes, soit indirectement sous la forme de messagers chimiques appelés hormones ou comme récepteurs de ces hormones. Elles servent à transporter ou à emmagasiner des substances essentielles comme l'oxygène, les ions métalliques, le glucose et les lipides. Sous la forme de fibres ou d'autres associations contractiles, les protéines génèrent le mécanisme de nombreux processus incluant la séparation des chromosomes durant la division cellulaire ou bien le mouvement des yeux lorsque on lit. Des protéines comme la rhodopsine dans la rétine de l'oeil acquièrent l'information visuelle qui est transmise au cerveau par l'action des protéines des cellules nerveuses. Les protéines comme les immunoglobulines sont aussi partie intégrante du système immunitaire des mammifères et forment une défense biologique contre l'attaque des microbes, bactéries et autres virus. Elles sont aussi les éléments et les produits de l'information génétique. Les acides nucléiques sont pour la plupart une banque de données génétiques auxquelles les protéines doivent se conformer. Pour finir, elles ont aussi un rôle passif important comme le collagène par exemple qui produit les os, les tendons et ligaments.

La fonction de toutes les protéines est basée sur leur structure donc toute modification de la structure primaire ou séquence de la protéine va avoir une influence importante sur son activité biologique. Les modifications les plus courantes sont les substitutions d'acides aminés et les modifications post-translationnelles (1). Par exemple, c'est la substitution d'un résidu d'acide glutamique par un résidu de valine qui est à l'origine de l'anémie (2-4). Les modifications post-translationnelles, quant à elles, incluent

incluent le clivage protéolytique de liaisons peptidiques spécifiques ou bien la dérivation chimique des groupes fonctionnels sur les chaînes latérales ou sur les groupements amine et carboxylique terminaux. Ces dérivations, parmi les plus courantes, incluent les acétylations, glycosylations, méthylations, nucléotidylylations, et phosphorylations (5). Ces modifications post-transductionnelles sont souvent au coeur du fonctionnement spécifique d'une protéine. Par exemple, les glycoprotéines sont impliquées dans le processus de reconnaissance cellulaire (6). L'étude de ces modifications est essentielle et représente une part importante du projet protéome actuellement.

L'identification des modifications ou substitution d'acides aminés que la protéine a subi, est souvent effectuée en utilisant la technique d'analyse des acides aminés ou bien le séquençage par dégradation d'Edman (7). Ces procédés sont cependant longs et contraignants (8). La dégradation d'Edman, par exemple, ne permet l'analyse que d'un seul échantillon par jour. De plus, le séquençage direct n'est pas possible pour des protéines de plus de 80 résidus, ou dont l'extrémité amine est bloquée.

Une alternative séduisante pour détecter une modification de la structure primaire d'une protéine est la cartographie peptidique. Bien qu'elle ne donne pas d'information directe sur la mutation, elle permet de déceler celle-ci en un temps beaucoup plus court et avec une mise en oeuvre minimale. Dans cette thèse, nous nous sommes intéréssés à développer un système continu permettant de faire la cartographie peptidique à partir de la solution de protéine à basse concentration en y intégrant les étapes de digestion de la protéine, préconcentration, séparation et détection des peptides protéolytiques.

1.2. La cartographie peptidique

Cette technique consiste en le clivage spécifique de la protéine (digestion) puis la séparation et la détection des fragments peptidiques par une technique chromatographique. Une fois digérée, chaque protéine va donner un mélange de peptides qui lui est propre. La séparation ultérieure de ces peptides, par chromatographie liquide haute performance (HPLC), par exemple, va donner un chromatogramme caractéristique de la protéine (fig. 1.1). C'est pourquoi le chromatogramme obtenu représente ce que l'on appelle l'empreinte digitale ou carte peptidique de la protéine. On obtiendra le même chromatogramme chaque fois que la digestion et la séparation seront faites dans les mêmes conditions pour la même protéine pure.

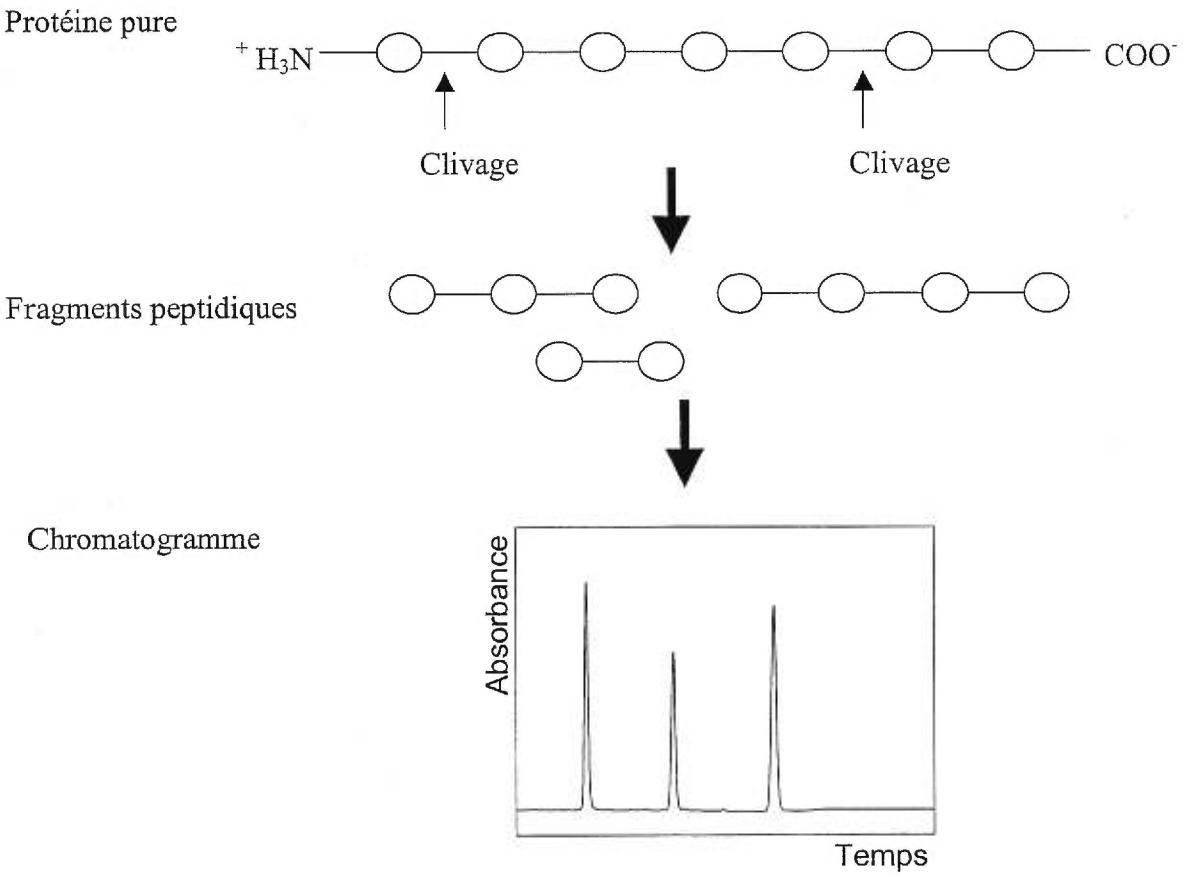

Figure 1.1. Schéma décrivant la cartographie peptidique.

La comparaison de ce chromatogramme avec celui de la protéine native permet de déceler d'éventuelles modifications de séquence. Le fragment peptidique portant la modification ou la mutation va avoir un temps de rétention dans le système de séparation différent de celui du peptide correspondant issu de la digestion de la protéine normale. On peut récupérer une bonne quantité du peptide modifié par collection de la fraction contenant le peptide d'intérêt après la séparation par HPLC. La modification ou mutation peut alors être localisée et identifiée en hydrolysant le peptide isolé, résidu par résidu d'après la technique séquentielle de la dégradation d'Edman (7, 9) ou par spectrométrie de masse (10-12). La technique de cartographie peptidique repose donc sur une excellente reproductibilité de la digestion et de la séparation des peptides. On doit donc utiliser des agents de clivage hautement spécifiques et une technique de séparation donnant des temps de rétention reproductibles des analytes dans le système.

1.2.1. *Les techniques de séparation*

Les premières cartes peptidiques furent générées par électrophorèse sur gel (13, 14) ou chromatographie sur couche mince (15-17). Actuellement, l'utilisation de l'HPLC pour le cartage des peptides s'est généralisée (18-25). Cette dernière possède l'avantage d'avoir une plus grande précision et une meilleure limite de détection que la chromatographie sur couche mince, et on peut collecter les fragments séparés pour faire le séquençage par dégradation d'Edman. Dans un même ordre d'idée, l'électrophorèse capillaire (CE) s'est révélée très efficace pour la cartographie peptidique (26-32). Elle est plus facile à mettre en oeuvre que l'électrophorèse sur gel et requiert une quantité injectée d'échantillon très faible (~10 nl). Les principes de séparation de l'HPLC et de la CE sont très différentes. La première s'appuie sur les interactions hydrophobes (lorsqu'on travaille en phase inverse) qui existent entre l'analyte et la phase stationnaire. La deuxième n'utilise pas de phase

stationnaire dans sa forme classique et la séparation est basée sur la différence des rapports charge sur masse des analytes lorsque ceux-ci sont soumis à un champ électrique. C'est pourquoi ces deux techniques sont souvent utilisées orthogonalement pour la cartographie peptidique pour pouvoir obtenir un deuxième degré d'information structurale (33-37). Cependant la CE donne une meilleure efficacité que l'HPLC; son utilisation est moins dispendieuse (elle utilise moins de solvants et les colonnes capillaires sont moins onéreuses que les colonnes HPLC) et enfin, elle utilise moins d'échantillon pour chaque injection. Comme la CE est en train de devenir une technique analytique de séparation à haute performance, toutes les cartes peptidiques présentées dans cette thèse ont été obtenues par CE.

1.2.2. *Les agents de clivage*

Comme mentionné ci-dessus, des agents de clivage très spécifiques sont requis pour obtenir des digestions reproductibles et donc des cartes peptidiques reproductibles. Ces agents de clivage sont de deux catégories: les enzymes protéolytiques et les réactifs chimiques (38). Dans ces deux catégories, les agents de clivage les plus utilisés sont ceux qui ne clivent uniquement que des séquences d'acides aminés données (tableau 1.1). On peut ainsi prévoir facilement le nombre de peptides que l'on va obtenir, si la séquence primaire de la protéine est déjà connue. De plus, la digestion produira un nombre relativement faible de peptides qui seront plus facilement séparables.

Tableau 1.1. Méthodes de digestion des protéines (38)

Agents de clivage enzymatique	**Clivage à "-"**	**Conditions**
Trypsine	Arg-X, Lys-X	pH 8-9; 37°C
α-Chymotrypsine	Trp-X, Phe-X, Tyr-X, Leu-X, Met-X, Ala-X	pH 7.5-8.5; 37°C
Pepsine	Trp-X, Phe-X, Tyr-X, Leu-X, Met-X, Ala-X, Leu-X-Leu	pH 2; 22 °C
Thermolysine	acides aminés hydrophobes	pH 8; 40°C
V8 protéase	Glu-X, Asp-X	pH 4 ou 8
Agents de clivage chimique		
Bromure de cyanure	Met-X	bas pH; 25°C
Hydroxylamine	Asn-Gly	pH 9.5; 45°C
Acide acétique dilué	Asp-Pro	pH 2.5; 40°C

Les clivages avec le bromure de cyanure ou la trypsine sont les deux méthodes de choix. Le nombre moyen de résidus de méthionine par protéine est de 2 % et le clivage à l'extrémité carboxylique de ce résidu est relativement facile. En ce qui concerne la trypsine, le nombre moyen de lysine et d'arginine par protéine est de 12 %. Ces deux méthodes produisent donc un nombre limité de peptides ce qui simplifie la prédiction de la cartographie peptidique et facilite la séparation. De plus, la trypsine peut digérer les protéines insolubles ou adsorbées sur un support. A l'inverse, l'utilisation des autres enzymes produit souvent un très grand nombre de peptides. Le clivage des résidus aromatiques de leur acides aminés adjacents n'est pas toujours quantitatif et des fragments

partiellement digérés sont parfois générés. Quant à l'hydroxylamine et l'acide acétique, ils sont peu utilisés du fait de la faible fréquence des séquences Asn-Gly et Asp-Pro (0.3 %). Dans notre système, nous avons donc utilisé la trypsine comme agent de clivage.

1.2.3. *Digestion enzymatique de protéines*

La plupart des protéines possèdent une structure secondaire: des portions de la protéine se retrouvent sous forme d'hélice α ou de feuillets β. De plus, si une protéine comporte dans sa séquence plus d'un résidu cystéine, il arrive alors que certains résidus cystéines forment entre eux des liaisons disulfures. Pour que la digestion enzymatique soit complète, il faut donc rompre ces liaisons disulfure et briser la structure secondaire de la protéine pour que tous les sites de clivage soient accessibles à l'enzyme. La protéine peut être "dépliée" par l'action d'un dénaturant comme l'urée à une concentration d'environ 8 M. Les ponts disulfure sont brisés par l'action du dithiothreitol (fig. 1.2), puis pour éviter leurs reformations, on fait réagir les atomes de soufre avec de l'iodoacétamide, selon les réactions suivantes:

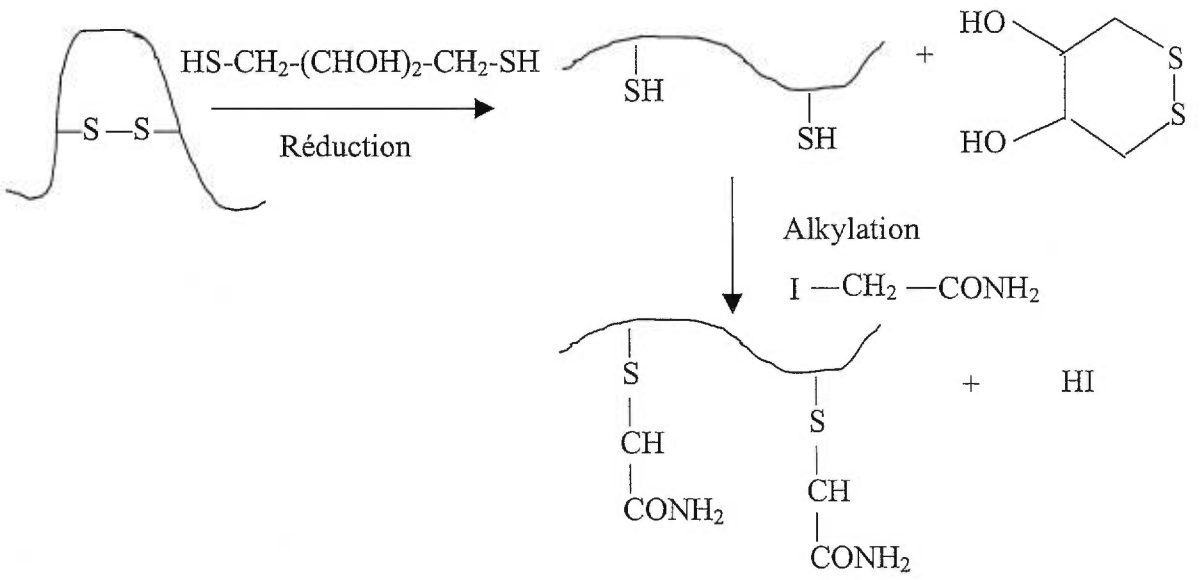

Figure 1.2. Schéma réactionnel de la réduction et l'alkylation des ponts disulfures (1).

Avant l'ajout de l'enzyme, l'échantillon doit être dilué dans un électrolyte basique (dans le cas de la trypsine) et volatile comme le mélange carbonate d'ammonium/urée. Cet électrolyte doit avoir un pH situé entre 8 et 9 qui est le pH où la trypsine a une activité maximum. De plus, la dilution doit être faite pour que la concentration finale d'urée dans l'échantillon n'excède pas 2 M. Cette concentration d'urée est suffisante pour aider la dissolution des protéines mais est insuffisante pour inhiber de manière significative l'action de la trypsine. La quantité d'enzyme ajoutée par la suite doit être de 1/25 (poids sur poids) par rapport à la quantité de protéine en solution. Ce rapport a été déterminé de facon à obtenir une digestion adéquate de la protéine avec une quantité minimale de trypsine. Pour des protéines supérieures à 60 kDa, ce rapport devrait être diminué à 1/50 pour que le rapport molaire protéine/enzyme soit d'au moins 10. Dans ce cas, les peptides provenant de l'autodigestion de l'enzyme sont rarement détectables.

Depuis quelques années, la technique de cartographie peptidique a connu une nette amélioration avec l'utilisation des enzymes immobilisés. Les enzymes immobilisés s'autoprotéolysent peu ou pas du tout, ont des temps de réaction beaucoup plus courts, utilisent moins de solvants et de réactifs, digèrent leurs substrats à de plus faibles concentrations que les solutions enzymatiques conventionnelles (39). Ils sont, d'autre part, facilement adaptables à des sytèmes de flux ce qui permet de les réutiliser.

1.2.4. *Les enzymes immobilisés*

Lorsqu'un enzyme est immobilisé soit dans une matrice ou à la surface d'un support quelconque, certains changements peuvent survenir dans le comportement de l'enzyme. Les facteurs affectant ce comportement sont nombreux et varient d'un enzyme à l'autre, je ne considèrerai dans cette section que les changements les plus communément observés.

Le profil de pH

Tous les enzymes ont une zone de pH dans laquelle ils ont une activité maximale. Lorsque l'enzyme est immobilisée cette zone peut être légèrement déplacée, dépendamment de la nature du support. Par exemple, si le support est négativement chargé, alors une forte concentration d'ions H^+ va s'accumuler à la frontière entre le support et la solution environnante. Cette accumulation d'ions hydrogène va entraîner une diminution du pH à la surface du support. Par conséquent, l'enzyme se trouve dans une région où le pH est plus bas que celui du reste de la solution. En conséquence, le pH apparent où l'activité de l'enzyme est maximale va sembler diminuer. Si le support est positivement chargé, le phénomène inverse se produit.

La cinétique

Après immobilisation, on observe généralement une augmentation de la constante de Michaëlis-Menten. Ce phénomène est dû habituellement à la charge du substrat et du support, aux effets de diffusion et dans quelques cas, à une modification de la structure tertiaire de l'enzyme.

La stabilité

Comme toutes les autres protéines, les enzymes sont sujets à la dénaturation par augmentation de la température, qu'ils soient libres ou immobilisés. Dans beaucoup de cas cependant, la diminution de l'activité se fait plus lentement avec un enzyme immobilisé et se produit également à des températures plus élevées. Il faut noter aussi que la stabilité opérationnelle d'un enzyme n'est pas que fonction de sa stabilité thermique, elle dépend aussi de la durabilité du support, du type d'inhibiteurs organiques rencontrés en solution ainsi que de leurs concentrations.

Ces enzymes sont utilisés dans des systèmes de flux ce qui permet de les réutiliser et de préserver la solution de fragments peptidiques de la présence de l'enzyme au moment de l'analyse. Les enzymes sont immobilisés soit sur les parois d'une colonne ou bien sur des billes qui sont empaquetées ensuite dans une colonne. Lorsque ces colonnes enzymatiques ont les dimensions d'un capillaire comme décrit dans la section suivante, ils sont communément appelées microréacteurs.

1.3. Les microréacteurs

Voyksner et al. (40) ont immobilisé différents enzymes— carboxypeptidase A,B et Y, chymotrypsine, thermolysine, trypsine et la protéase V8—sur des billes de verre à pores contrôlées. Les billes subissent au préalable une silanisation et une succinylation. Ensuite, la réaction d'immobilisation se fait en présence d'un dérivé carbodiimide à pH alcalin. Les billes sont ensuite empaquetées dans une colonne en acier en appliquant la technique de "wet packing" couramment utilisée pour remplir des colonnes pour l'HPLC (41-43). L'étude de la stabilité et de l'activité de ces enzymes sous différentes conditions de pH, de tampons et de solvants a montré que l'utilisation d'un pH supérieur à 10 provoque une perte d'activité irréversible pour tous les enzymes, ce qui n'est pas le cas pour le reste de la gamme de pH (40). Les auteurs ont également essayé de diluer leurs échantillons dans des solvants organiques avant de les digérer de manière à améliorer la résolution et la séparation des fragments peptidiques lorsqu'ils effectuent la cartographie peptidique par HPLC. Des solutions de 25 % d'isopropanol, d'acétonitrile et de tétrahydrofurane dans l'eau n'ont pas d'effets sur l'activité enzymatique. L'augmentation de la quantité de solvants organiques au-delà de 25 % entraîne une diminution progressive de l'activité enzymatique probablement à cause de modifications structurelles de l'enzyme. Le méthanol et l'éthanol entraînent quant à eux une perte d'activité d'un facteur de 2 pour la trypsine et

la carboxypeptidase B. L'utilisation d'acétone entraîne une dénaturation complète des enzymes et une totale perte d'activité.

Il semble également que le choix du tampon ait une très forte influence sur l'activité enzymatique. La trypsine et la protéase V-8 ne subissent pas de perte d'activité notable avec l'utilisation du tampon Tris ou des tampons ammonium (40). Par contre les carboxypeptidase A et B voient une réduction de leur activité de 40 à 60 % lorsque le tampon utilisé est de l'hydrogenocarbonate d'ammonium ou le bicarbonate d'ammonium.

Cobb et Novotny (35) ont construit des microréacteurs en empaquetant dans un tube de pyrex de la trypsine immobilisée sur un gel d'agarose. Après avoir placé leur réacteur verticalement, ils déposent 0.5-1 µl de leur solution de protéine (β-caséine) à 0.3 mg/ml. L'élution à travers le réacteur se fait à pression atmosphérique en ajoutant du tampon de digestion au sommet du réacteur. Le digestat est ensuite récolté à l'autre extrémité, lyophilisé puis dilué dans le mélange de séparation pour analyse. A cause de la lyophilisation, le tampon de digestion de choix dans ce cas est le carbonate d'ammonium qui possède la propriété d'être volatil. La digestion de si petites quantités de protéines n'est souvent pas possible en phase liquide. Pour digérer de faibles quantités de protéines, on doit effectuer des dilutions successives de la solution de protéine et de la solution d'enzyme pour maintenir le rapport enzyme/protéine à 1/25.

Cette approche est valable jusqu'à des quantités de quelques microgrammes de protéine mais au-delà la solution de protéine/trypsine est si diluée que la digestion de la protéine est fréquemment incomplète et sa reproductibilité extrêmement faible. Ceci est attribué au fait que la concentration de trypsine est bien inférieure à sa concentration optimale ce qui implique un ralentissement certain de la cinétique enzymatique. Une variation de la procédure de digestion en phase liquide pourrait induire l'utilisation de solutions de protéine diluées et de solutions de trypsine plus concentrées. Cela permettrait à

la concentration de trypsine d'être proche de sa concentration optimale. Mais dans cette situation, il est bien sûr impossible de maintenir le rapport protéine/enzyme propre à une digestion efficace (i.e. 1/25) et en fait la concentration de trypsine dépasse souvent la concentration de protéine. C'est une situation indésirable, car dans ce cas, l'autoprotéolyse de l'enzyme sera telle que le digestat consistera essentiellement en fragments peptidiques provenant de la digestion de la trypsine. Les auteurs ont opté pour une séparation par CE car l'un des avantages majeurs de cette technique par rapport aux autres est qu'un très faible volume d'échantillon est injecté pour chaque analyse. Ceci est particulièrement intéressant avec ce type de protocole qui inclut une lyophilisation et une dilution dans l'électrolyte de séparation car le volume d'échantillon requis en CE est de l'ordre de 5 µl dont seulement 10 nl sont injectés. Ceci permet de diminuer grandement les quantités de protéine digérée. Les auteurs se sont servis de la même technologie pour construire des microréacteurs de chymotrypsine (29). L'inconvénient majeur de leur microréacteur est que la migration de l'échantillon à travers le microréacteur doit se faire à la pression atmosphérique pour éviter l'extrusion du gel d'agarose du microréacteur. En conséquence, le temps de digestion est extrêmement long.

Amankwa et Kuhr (44) ont fabriqué un microréacteur à partir d'un capillaire de 50 µm de diamètre interne et de 50 cm de long. Ils ont immobilisé l'enzyme sur les parois par couplage biotine-avidine-biotine (figure 1.3). La préparation du microréacteur consiste d'abord en une silanisation des parois internes du capillaire avec le 3-aminopropyltriethylsiloxane. Ce traitement permet d'avoir des groupements aminés sur les parois du capillaire. Ceci facilite la dérivation de ces parois avec la NHS-LC-biotine par la création d'un lien amide. Le capillaire est ensuite traité avec l'avidine. L'immobilisation de l'enzyme s'effectue en perfusant à travers le capillaire une solution d'enzyme dérivé à

son extrémité amine avec de la biotine. La biotine et l'avidine ayant une grande affinité l'une envers l'autre ($K_d = 10^{-15}$ M), l'enzyme va se trouver immobilisé sur la paroi. Cette méthode d'immobilisation non-covalente permet d'utiliser des conditions extrêmes de pH, de température et différents solvants. Cette technique de couplage prend néanmoins 24 h avant d'obtenir le microréacteur.

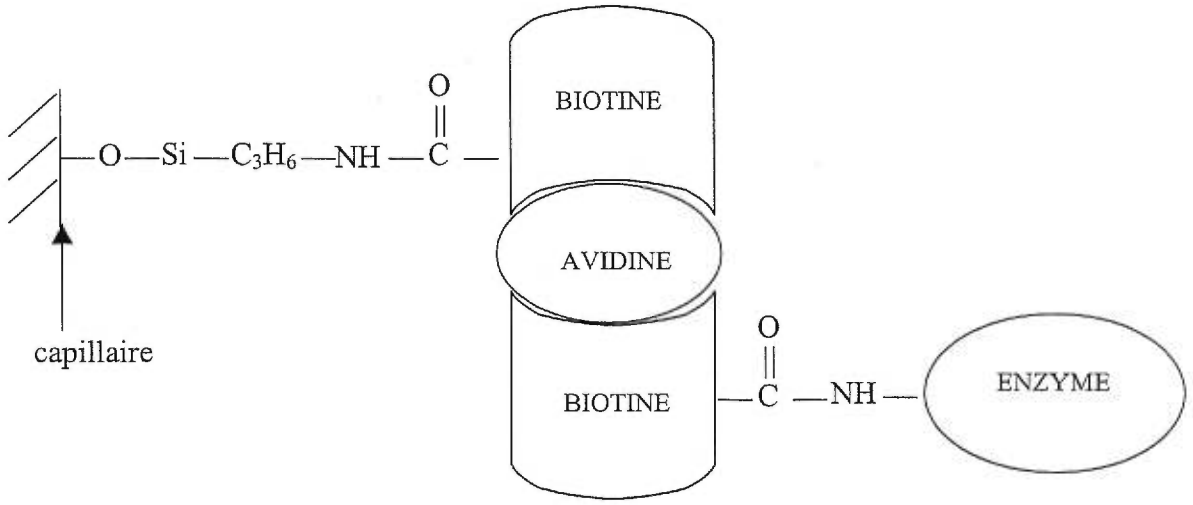

Figure 1.3. Diagramme schématique montrant l'immobilisation de l'enzyme par couplage Biotine-Avidine sur la surface d'un capillaire.

La digestion est simplement effectuée par perfusion de la solution de β-caséine dans le microréacteur. Malheureusement, du fait que l'enzyme soit essentiellement concentré sur les parois du capillaire, le flux de la solution doit être extrêmement lent (40 nl/min) de manière à ce que la protéine puisse interagir avec l'enzyme. Néanmoins, leur microréacteur peut être utilisé pendant plusieurs semaines sans baisse notable de l'activité.

Les auteurs ont ensuite effectué les digestions non pas dans un mode où la solution passe dans le microréacteur, non pas par application d'un voltage mais en mode statique en

immobilisant le microréacteur, bouché à chaque extrémité, sur un appareil piezzoélectrique (45, 46). La fréquence de la vibration augmente la vitesse de la digestion elle-même non pas par augmentation du transfert de masse mais par déstabilisation de la structure tertiaire des protéines (47). Il n'y aura pas d'augmentation de la vitesse de réaction pour des substrats sans structure tertiaire comme l'insuline ou la β-caséine. Les auteurs ont montré que pour le cytochrome c qui possède une structure tertiaire bien définie, la vitesse de digestion augmente avec la fréquence de la vibration.

Davis et al. (48) ont essayé de connecter un microréacteur empaqueté avec des billes d'enzymes selon la méthode de Voyksner et al. (40) à un appareil de HPLC. La migration de l'échantillon se fait grâce à la pompe HPLC et un système de valves placé entre le microréacteur et la colonne HPLC permet d'avoir un contrôle total sur la nature de la phase mobile. Les auteurs ont montré que la quantité de protéine digérée dépend fortement de la vitesse du flux à travers le réacteur. Plus le flux est rapide, plus la quantité de protéine digérée sera faible. De même, il semblerait que l'autoprotéolyse de l'enzyme soit directement fonction de la température et soit maximale à 37 °C. Un des inconvénients de leur technique est que la diffusion de l'échantillon dans leur système est extrêmement importante. Des aliquots de 1µl de protéine sont injectés et des études de recouvrement ont montré que 90 % de la quantité totale de peptides se retrouvent dans une fraction de 150 µl.

Récemment, une amélioration de la connection en ligne a été faite par Guzman (49) avec un microréacteur construit en liant covalament l'enzyme Staphylococcus aureus V8 sur un support en verre poreux. Le tout est placé dans une chambre bloquée par des frits. La digestion spécifique de la sous-unité α de la prolyl-4-hydroxylase a été montrée par comparaison avec les électrophérogrammes obtenus lorsqu'on fait passer la protéine dans des microréacteurs contenant soit du cytochrome *c*, soit de l'albumine du sérum de boeuf,

ou la protéase *staphylococcus aureus* V8. La digestion protéolytique ne fut observée qu'avec le réacteur contenant la protease *staphylococcus aureus* V8. Afin de diminuer leur limite de détection, les auteurs ont ajouté un autre réacteur entre le réacteur enzymatique et le capillaire de séparation. Ce réacteur contient des billes modifiées avec l'isothiocyanate de fluorescine (FITC) lié à des anticorps anti-FITC. Le but de cette approche est de dériver chimiquement les peptides produits par la digestion en ligne pour augmenter leur fluorescence et leur absorbance dans l'UV.

L'électrophorèse capillaire a prouvé qu'elle était une technique bien appropriée pour la cartographie peptidique. Sa très haute efficacité (N>100 000 plateaux théoriques) s'avère très utile pour la séparation de digestats qui contiennent habituellement plus d'une douzaine de peptides. Elle ne nécessite pas non plus l'utilisation de solvants organiques, qui comme on l'a vu plus haut peuvent entraîner une perte d'activité enzymatique. De plus, elle ne requiert qu'un très faible volume d'échantillons pour les analyses, du fait du faible diamètre interne du capillaire. Bien qu'avantageuse, les dimensions du capillaire sont la principale cause de la faible limite de détection de cette technique. A la différence de l' HPLC, la détection par absorption UV-VIS en CE se fait directement sur la colonne. D'après la loi de Beer-Lambert, l'absorbance varie linéairement avec le diamètre interne du capillaire. Comme le diamètre moyen d'un capillaire est de 50 μm, la limite de détection en CE se situe autour de 10^{-6} M. La cartographie peptidique d'échantillons biologiques pose un problème avec la configuration actuelle des systèmes en ligne microréacteur-capillaire de séparation. En effet, les protéines d'intérêt se trouvent à des concentrations allant du picomolaire au nanomolaire dans le sang, le sérum, les cellules, les tissus etc. Il faut donc incorporer une étape de préconcentration des fragments peptidiques entre le microréacteur et le capillaire de séparation afin d'augmenter la sensibilité de la détection des peptides.

Une alternative séduisante est l'incorporation d'un morceau de capillaire contenant un faible volume de phase stationnaire sur laquelle les peptides pourraient s'adsorber. En effectuant leur désorption dans un petit volume de solvant organique, qui sera injecté ensuite dans le capillaire de séparation, on est capable d'abaisser grandement la limite de détection. A ce titre, des améliorations de la sensibilité d'au moins deux ordres de grandeurs sont reportées couramment dans la littérature avec l'utilisation de ce type de préconcentrateur et ce, pour une grande variété d'échantillons allant de petites molécules organiques aux protéines. La section suivante présente une revue détaillée de la littérature concernant l'utilisation de ces préconcentrateurs afin d'augmenter la sensibilité de la détection. Dans le chapitre 2, les objectifs et l'approche expérimentale du projet sont mis en grandes lignes.

1.4. On-line solid phase preconcentration for sensitivity enhancement in capillary electrophoresis (Review)[1]

Abstract

Capillary electrophoresis (CE) is attractive for the analysis of biological samples because a few nanoliters of sample need only be injected. Indeed, optimal resolution is achieved when the injected volume is 1 % or less of the total capillary volume. Unfortunately, this advantage leads to severe detection limitations compounded by the fact that many analytes in a biological sample are present at very low concentrations. To overcome the detection sensitivity limitations of CE, non-specific on-line preconcentration has been employed in a variety of applications. This technique is based on inserting a small

[1] E. Bonneil et K.C. Waldron, *Journal of Capilary Electrophoresis* (sous presse)

quantity of reversed-phase material (e.g., C_{18} particles or membrane) near the inlet of the CE capillary for sample enrichment by solid-phase extraction. A detachable cartridge containing the solid phase is relatively simple to construct and permits the injection of large sample volumes (1–100 μl) into the capillary. Elution of adsorbed analyte in less than 100 nl of solvent permits a 100 to 1,000 fold improvement in the effective concentration limit of detection, depending on the hydrophobicity of the compound. Detection of analytes present in complex mixtures at concentrations as low as 20 amol/μl has been reported when using an on-line preconcentration device in conjunction with CE-mass spectrometry. In this review, the potential merits of the technique are described and some examples of direct analysis of biological samples without rigorous off-line pretreatment are given.

Indexing terms: Review; Sensitivity enhancement; On-line preconcentration; Solid-phase extraction-capillary electrophoresis; Peptides; Proteins.

1.4.1. *Introduction*

Over the past 18 years, capillary electrophoresis (CE) has shown great versatility for the analysis of many chemically diverse classes of compounds like inorganic ions, organic molecules, peptides, proteins, glycoproteins, oligosaccharides, oligonucleotides, DNA, RNA, and lipids. As a result, the use of CE in the industrial sector is becoming more widespread. Direct injection of sample into the narrow-bore capillary offers advantages ranging from its small sample volume requirements to its accommodation of diverse sample matrices. In some applications the small volume injected (typically < 20 nl) makes CE attractive, particularly for the direct determination of biological constituents in cells or tissues whose amount is too small for analysis by traditional laboratory techniques. More often, however, the limited loading capacity of analyte solutions in CE is a disadvantage.

Only 1 % or less of the total capillary volume should ideally be injected for optimum separation efficiency. Moreover, conventional absorbance detection suffers because the optical path length is more or less equal to the inner diameter of the capillary, i.e., 50 or 75 μm in most applications. Ultimately, these limitations result in poor concentration limits of detection (CLOD), a major problem when analyzing biological samples or preparations in which analyte are present at very low concentrations. To overcome this problem, a variety of approaches have been developed.

One obvious solution to achieve better CLOD is to improve the detection configuration. Several different approaches have been developed to this end, such as the Z-shaped detection cell (50-52), bubble-cell, rectangular capillary column (53) and T-shaped cell for post-column derivatization (54) to name just a few. However, the improvement in signal-to-noise ratio (S/N) is at best one order of magnitude and the above-mentioned techniques are often expensive or complicated to implement. Another solution to enhance CLOD is by using on-column techniques for focusing a large injection volume (e.g. 1 μl). The simplest of these is electrophoretic sample stacking based on a pH difference between the separation buffer and sample solution (55, 56). In this case, the concentration factor is modest; about 5 times. Stacking by field amplification is another approach to improve CLOD (57). In this technique, cations stack up in front of the sample plug and the anions stack up behind the sample plug, thereby focusing these two classes of analyte into narrower zones. However, when the local electroosmotic velocity of the sample plug is greater than the electroosmotic velocity of the separation buffer, the pressure difference caused by the mismatch in electroosmotic velocities will generate a laminar flow which may broaden the sharp zone generated by the stacking process and therefore reduce resolution (58). This laminar flow is proportional to both the resistivity and the volume of

injected sample (57). Transient isotachophoresis (tITP) is a third on-column technique used to preconcentrate analytes present at low concentrations. Reported preconcentration factors are as high as 700 (59). Unfortunately, sample loading in tITP preconcentration is limited to the total volume of the capillary (about 1 μl).

Coupling capillary isotachophoresis to CE (cITP-CE) for sample preconcentration has been reported by several researchers (60-62). This technique uses two individual capillaries connected together and is thus different from on-column tITP. The first capillary, (cITP), allows both concentration of analytes and removal of matrix components whereas the second capillary (CE) implements analyte discrimination. The cITP capillary can be relatively wide-bore (> 500 μm I.D.) to permit injection of a large volume of sample. When the analyte plug reaches the interface between the cITP column and the CE, a change in the location of applied potential is used to interrupt the electrical field in the ITP column. This interruption simultaneously creates a new electrical field between the terminating buffer and separating buffer in such a way that the analyte plug is injected into the CE capillary. Two detection systems are used, one to monitor the ITP separation and the other to detect the analytes separated by CE.

Unfortunately, there are some limitations to the coupled cITP-CE technique besides its complicated construction. First, the sample matrix can greatly influence the migration times of various zones so that timing of the transfer step is tricky. As well, detection of the cITP zone prior to transfer is difficult because samples may still be very dilute. Secondly, a compromise in buffer selection must be made; the cITP leading electrolyte ultimately becomes the separation buffer for the CE separation. This means that non-ideal separation conditions may be needed to ensure preconcentration. Thirdly, it may not be possible to transfer all solutes from the cITP separation to the CE capillary. This is

particularly true when solutes of widely varying mobilities are present in the sample. Finally, only cations or anions can be separated in a single run, but not both.

Recently, Quirino and Terabe (63) reported a 5,000-fold preconcentration of analytes using micellar electrokinetic chromatography. They used a special concentration effect that they call sweeping. Sweeping is a physical phenomenon that works well for all analytes that have a high affinity for the pseudo-stationary phase, i.e., micelles. Sweeping is described as the picking and accumulating of analytes by the pseudo-stationary phase, which fills the sample zone during application of voltage. The authors explain that the technique is analogous to using a broom to carefully carry along grains of rice scattered on the floor. Although this technique works for a wide range of organic compounds, it is limited by the total volume of the capillary and places special requirements on the sample matrix and separation buffer.

While the approaches mentioned above have improved the CLOD in many CE applications, these techniques may be inadequate for analysis of components from biologically-derived mixtures. Analytes in these a complex mixtures are, in many cases, likely to be present at concentrations lower than that which can be focused electrophoretically. An alternative and very classical approach to improving the CLOD in CE is the use of solid-phase extraction (SPE) for preconcentration (sample enrichment), a method employed routinely in HPLC-based analyses. Unfortunately, commercially available SPE cartridges for sample pre-treatment are designed for use off-line and require relatively large sample volumes both during the loading step (e.g., $\geq$ 1 ml) and elution step (e.g., 10 μl). While this technique is ideal for very large sample volumes and sample clean-up, off-line pretreatment of dilute solutions can lead to substantial sample losses due to adsorption on various surfaces (i.e., pipettes, vials, SPE column casing, frits, etc.) (64).

These losses are particularly pronounced for peptides and proteins. Other drawbacks to the use of these SPE devices with CE are their limited automation ability and relatively high solvent consumption (65).

To minimize sample handling and analyte losses, the use of a miniature SPE preconcentrator on-line with the separation capillary has been employed by several groups as described in detail in this review. These sample enrichment devices usually consist of an adsorptive phase packed at the inlet of the CE capillary. This technique has been used for either non-specific analyte preconcentration or for specific analyte preconcentration using affinity-based sorbents. Several reviews already exist concerning specific antigen-antibody preconcentration (66-69). In this review, we will focus on the use of non-specific preconcentration devices made with stationary phase materials packed as a bed of particles or in membrane format for sample enrichment in CE analysis.

1.4.2. *General approach*

One of the greatest advantages of the on-line SPE preconcentration technique for CE, where analyte adsorption and desorption take place near the inlet the separation capillary, is its rapid implementation and low cost. There are two types of sorbent most commonly used for SPE-CE: C_{18} or octadecylsilane (ODS)-based reversed phases and polymeric styrene-divinyl benzene (SDB), both of which have been used in either the packed bed (i.e., beads) or membrane format. Predominantly in the literature, C_{18} beads have been employed in the packed bed format and SDB as a membrane. However, this is not always the case. Regardless of sorbent chemical structure and format, most SPE preconcentrators are constructed and operated in the same fashion, as described below.

Fabrication of a typical preconcentrator is shown in Fig. 1.4. For construction of a packed bed C_{18}–based preconcentrator, a length of fused silica (f.s.) capillary tubing is

inserted into a piece of polyethylene or Teflon (PTFE) tubing. A frit, which is often a piece of PTFE membrane or glass fiber, is then inserted in the polymer tubing and pushed through so that it butts up against the end of the capillary tubing. Next, the adsorptive material is packed into the polymer tubing. Several approaches for this step have been described in the literature. Packing procedures similar to that used for wet-packing or dry-packing HPLC columns have been reported (70, 71). Another method is to gently tap the free end of the polymer tubing into a vial containing the C_{18} beads (72). Regardless of the packing method, the final solid-phase bed ranges from 0.3 to 1.5 mm in length, corresponding to a packed bed volume of 50 to 250 nl.

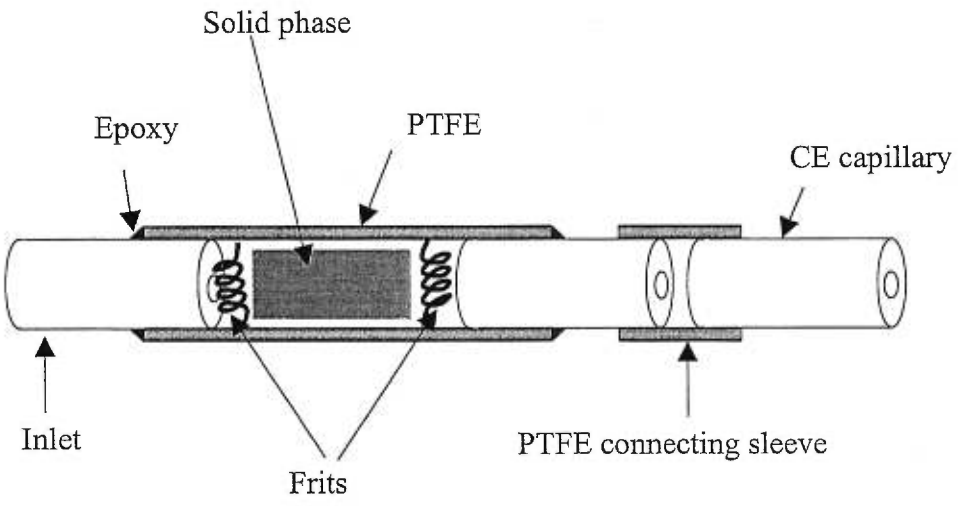

Figure 1.4. Schematic showing the basic design of a solid-phase preconcentrator cartridge on-line with capillary electrophoresis.

The sorbent is held in place with a second frit and a second piece of f.s. capillary, which is pushed up against the frit to close the SPE preconcentrator cartridge. The use of frits can be completely avoided if the stationary phase particles are sufficiently large, e.g., 40 μm in diameter. This is desirable since frits are believed to generate irreversible

adsorption of analyte, unnecessary backpressure and supplementary dead volume that leads to loss of efficiency (73). For fritless construction, the flow of solutions through the preconcentrator during loading and elution should be made at very low pressure to prevent the beads from entering the capillary (72). In several reports, epoxy has been used to seal the polymer tubing to the f.s. capillary pieces. If 375 μm I.D. polyethylene tubing (PE 20 from Intramedic Clay Adams) is used to construct the SPE preconcentrator cartridge, epoxy is not needed because the f.s. capillary fits snuggly inside the polyethylene tube. Essentially the same construction technique is applied when the sorbent itself is a membrane except that frits are obviously not required. The total volume of the SPE membrane-based cartridge is only about 15 nl. Finally, the solid-phase material is rinsed with an organic solvent (e.g., methanol or acetonitrile) and the cartridge is connected to the inlet of the separation capillary with a second piece of polymer tubing.

Typically, several tens of microliters of sample are loaded onto the preconcentrator by pressure. Analytes having sufficient hydrophobicity are retained on the sorbent for which they have a high affinity. The capillary is then rinsed with separation buffer to remove salts and other hydrophilic contaminants present in the sample matrix. The analytes are then eluted from the solid-phase material by injection of 50 to 200 nl desorption solution, which typically contains a very high percentage of organic solvent. Injection of a plug of CE separation buffer follows the desorption step to move the plug a small distance from the sorbent. Finally, high voltage is applied and the separation proceeds while the preconcentrator is left in place.

Despite the expected loss of efficiency and resolution due to the presence of solid-phase packing material in the separation capillary, the relatively high volume of desorption solution and the large amount of organic solvent used results in efficient analyte stacking. This stacking is due to the fact that analytes are now in a low conductivity zone relative to

the separation buffer which leads to an increase in the field strength across the organic solvent zone (74). The result is rapid migration of analyte through the organic solvent plug which ultimately leads to localized stacking. Consequently, it is common to see preconcentration factors of 100 to 500 reported for organic compounds and even 700 to 1,000 for proteins and highly hydrophobic peptides. For a selection of analytes reported in the literature, Table 1.2 lists the approximate preconcentration factor and CLOD after enriched by either packed bed or membrane-based SPE.

Table 1.2. Selection of drugs, peptides and proteins determined using solid-phase preconcentration on-line with capillary electrophoresis.

Analyte	Sorbent [a]	Detection Method	Preconcentration Factor [b]	Reported CLOD (M)	Reference
Angiotensin	C_{18}	UV	100	5×10^{-7}	[82]
	C_{18}	UV	1600	3×10^{-8}	[80]
	C_{18}	MS	180	2.5×10^{-9}	[88]
	SDB	MS	180	6.2×10^{-9}	[88]
	C_{18}	MS	5	1×10^{-6}	[86]
	SDB	MS	85	5×10^{-8}	[102]
Bradykinin	C_{18}	UV	800	6×10^{-8}	[80]
	C_{18}	UV	100	5×10^{-7}	[82]
	C_{18}	MS	5	1×10^{-6}	[86]
	SDB	MS	85	5×10^{-8}	[102]
CNBr digest of bacteriorhodopsin	C_{18}	UV	100	3.8×10^{-8}	[62]
Doxepin	C_{18}	UV	100	4×10^{-6}	[78]
NDA-Glycine	C_{18}	LIF	100	2×10^{-8}	[76]
Haloperidol analogs	C_{18}	MS	5	4×10^{-6}	[86]
	C_{18}	MS	n/r [c]	4×10^{-5}	[85]
	SDB	UV	n/r	4×10^{-7}	[103]
Leucin enkephalin	C_{18}	UV	100	9×10^{-7}	[82]
	C_{18}	UV	200	5×10^{-8}	[80]
	C_{18}	MS	180	4.8×10^{-8}	[88]
	SDB	MS	180	2.3×10^{-8}	[88]
	SDB	MS	n/r	9×10^{-8}	[103]

Luteinizing hormone releasing hormone	C_{18}	UV	100	4×10^{-7}	[82]
	C_{18}	UV	400	2×10^{-7}	[80]
	C_{18}	MS	5	1×10^{-6}	[96]
	SDB	MS	n/r	4×10^{-8}	[102]
Lysine C endopeptidase digest of apomyoglobin	C_{18}	MS	5	1×10^{-6}	[40]
α-Melanin-stimulating hormone	C_{18}	UV	100	9×10^{-7}	[82]
	C_{18}	UV	1600	5×10^{-8}	[80]
	C_{18}	MS	5	5×10^{-5}	[96]
metallothionein	C_{18}	UV	700	3×10^{-7}	[68]
Neurotensin	YMC basic beads	MS	250	2×10^{-11}	[97]
Papaverine	SDB resin	UV	100	$\sim 10^{-8}$	[74]
Propanolol	C_{18}	UV	100	5×10^{-7}	[75]
Sulfonylurea drugs	C_{18}	UV	500	$\sim 10^{-8}$	[69]
Synthetic peptides	SDB	MS	240	2×10^{-7}	[92, 101]
Tryptic digest of BSA	C_{18}	MS	n/r	1.8×10^{-5}	[91]
	C_{18}	MS	n/r	2×10^{-9}	[96]
Tryptic digest of β-casein	C_{18}	UV	500	2×10^{-7}	[70]
Tryptic digest of myosin I heavy chain kinase	Poros R2 beads	MS	n/r	2×10^{-8}	[98]

a Unless otherwise indicated, C_{18} sorbent was always used in the packed bed format (i.e. beads) and SDB in the membrane format.

b When not explicitly reported, this value was estimated as follows: volume loaded ÷volume desorbed × 100% recovery.

c Not reported (either the volume loaded or desorbed was not reported).

1.4.3. *Packed bed preconcentrators*

The concept of on-line preconcentration for CE was first reported by Guzman and co-workers in 1991 (75). In preliminary studies, antibody coupled to a solid support for the on-line extraction of analytes from urine and subsequent separation by CE was described. The following year, Debets et al. (76) constructed a microcolumn of SDB resin inside a rotary valve for the preconcentration of papaverine. Using the valve, their packed bed

preconcentrator could be switched on- and off-line with the separation capillary. This device was able to extract and concentrate extremely large sample volumes but required a liquid pump for sample loading and off-line switching to perform the CE separation, all of which added to the complexity of the apparatus. Unfortunately, the papaverine peak was extremely broadened if, after desorption, the preconcentrator remained on-line with the separation capillary. Band broadening was caused by several effects such as the large volume of the solid-phase bed, the dead volume of the injection valve and the disturbance of the EOF profile caused by backpressure from the preconcentrator. The authors were able to increase the efficiency dramatically by switching the preconcentrator off-line prior to starting the CE separation. However, this configuration was rather complex and has not since been used according to the literature.

The first report of an on-line preconcentrator based on C_{18} reversed phase material was by Cai and El Rassi (77), although it was neither a packed bed nor membrane format. These authors developed an open-tubular preconcentrator with octadecyl functions bound to the inner wall for use on-line with free solution CE. However, while this approach enabled the loading of larger sample volumes than conventional CE, the preconcentrator easily became saturated because there was so little reversed-phase material available.

The obvious next step was the introduction of a capillary packed with octadecylsilane (ODS) beads as a preconcentrator at the inlet of the separation capillary. In 1993, one of the first reports of this was by Jorgenson and co-workers (78) who fabricated an on-line ODS-gel preconcentrator for the enrichment of glycine labeled with naphtalene-2,3-dicarboxaldehyde and detection by laser induced fluorescence. Morita and Sawada (79) developed a dual capillary system and preconcentrator packed with protein-coated ODS to achieve a 100-fold improvement in detection limit for determining propanolol in serum. The same analyte, propanolol, was used to demonstrate on-column preconcentration in a

capillary commercialized by Millipore Waters Chromatography (Accusep C/PRP) that contained a 1-mm long packed bed of polymeric reversed phase material (80). Glass frits were scintered in place to retain the sorbent at the capillary inlet. The main drawback with this commercial preconcentrator is its permanent installation in a pre-cut capillary of pre-defined inner diameter and effective length, thus leaving no flexibility if the detection window breaks or the sorbent becomes fouled. Since 1993, several authors have fabricated and characterized detachable SPE preconcentrators for CE, as described in detail below, studying their influence on the separation process when connected on-line with the separation capillary.

As one of the pioneer groups in this field, Tomlinson et al. (81) showed that the C_{18} beads in the separation capillary dramatically slowed down the electro-osmotic flow (EOF). It seemed that the reduction of EOF was a function of the volume of solid-phase material and that EOF could be reversed if more than 125 nl of C_{18} solid phase were present. However, this phenomenon was only observed after washing the sorbent with ammonium acetate (i.e., ammonium cations) or when a peptide mixture had been loaded and eluted. The reversed EOF phenomenon was also observed by Landers' group when trying to preconcentrate synthetic peptides at low pH (82). After elution of the retained peptides from the sorbent and following application of an electric field under standard conditions (anode = inlet, cathode = outlet), no peptides were detected. However, the peptides could be forced to elute at the cathode when, after desorption, pressure and voltage were simultaneously applied.

Strausbauch et al. (82) proposed a hypothesis, described in Fig. 1.5, to explain the reversal of EOF in SPE-CE. Peptides are extracted from the sample matrix and adsorbed onto the solid-phase packing upon loading. Injection of a small volume of organic solution—the elution plug—is then made at the capillary inlet. This plug is pushed toward

then through the preconcentrator by continuous pressure injection of separation buffer. The peptides are thus desorbed (eluted) and enter the section of the capillary where separation takes place. Upon application of high voltage, electrophoresis starts and the peptides begin to stack at the cathodic interface between the organic plug and the separation buffer, which is at low pH. To explain EOF reversal, the authors propose that unrecovered peptide on the sorbent generates a cationic charge localized at the SPE cartridge. The formation of an electric double layer with counter ions from the buffer leads to anodic flow near the capillary inlet. This process is analogous to the reversal of EOF generally associated with coating the inner capillary walls with a cationic surfactant or polymer (83). As they are now in an aqueous environment, the previously eluted peptides are re-adsorbed on the SPE cartridge upon reversal of the EOF. This hypothesis was tested by switching the polarity of the power supply (82). Under these conditions, the peptides were detected. Reversal of the EOF could be suppressed by simultaneous application of voltage and pressure during the separation.

When analyzing mixtures of synthetic peptides (84) and peptide analogs derived from the neuroleptic agent haloperidol (85, 86), it was observed that C_{18} packed bed preconcentration on-line with CE increased the peak widths of certain peptides leading to some overlap of closely migrating peaks. Substantial peak tailing has also been reported (87). This has been attributed to several factors: the volume of sorbent and desorption solution; the analysis of relatively large amounts of component mixtures where analyte-analyte and analyte-wall interactions increased (88); the backpressure generated by the sorbent or frits (89); the voltage drop across the capillary due to the high resistivity of the elution plug (84). Loss of resolution dramatically increased with an increase of the sorbent bed volume or the desorption volume (82).

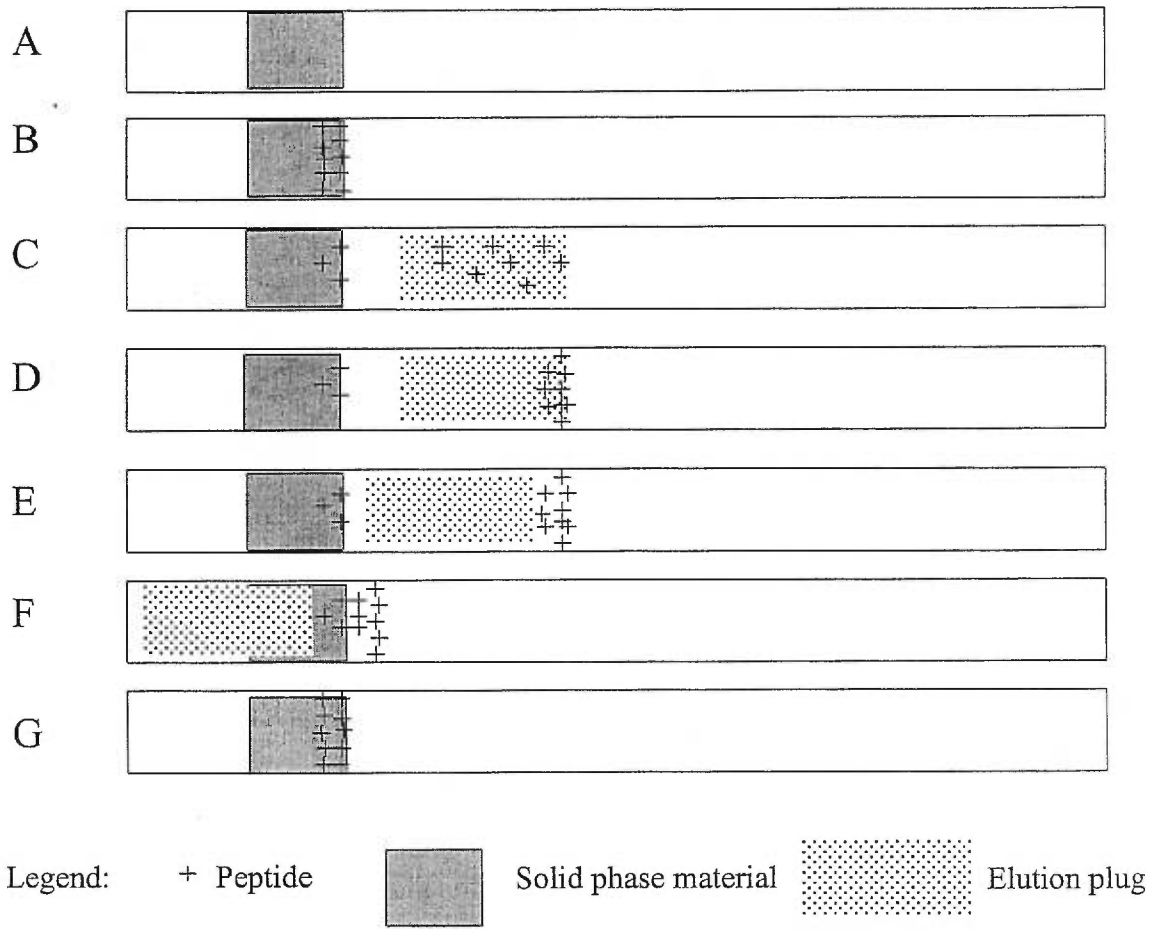

Figure 1.5. Schematic showing the proposed mechanism for electroosmotic flow (EOF) reversal and re-adsorption of peptides on a C_{18} packed bed preconcentrator. (A) Capillary and preconcentrator cartridge (SPE-CE) packed with C_{18} beads prior to sample loading. (B) SPE-CE system after loading peptide sample at low pH. (C) Hydrodynamic elution of peptides from sorbent by injection of a desorption solution plug (acetonitrile/aqueous acid) followed by hydrodynamic injection of separation buffer to push the plug past the sorbent. (D) Start of electrophoretic separation with analyte stacking at the cathodic interface between the organic solvent plug and buffer. (E) Migration of cationic peptides into the aqueous phase separation buffer and start of EOF reversal. (F) Migration of peptides toward SPE preconcentrator under the influence of a reversed EOF. (G) Re-adsorption of peptides on the sorbent while the elution plug exits the capillary inlet. (Adapted from reference [34]).

On the other hand, the opposite effect (i.e., improved resolution with SPE-CE) has also been demonstrated. For example, Strausbauch et al. (70) reported that while addition of sodium cholate improved the separation of seven sulfonylurea drugs by CE, this surfactant interfered with the solid-phase extraction process. Therefore, to directly analyze urine specimens by SPE-CE where sulfonylurea drug concentrations are far below the detection sensitivity of conventional CE, a phosphate-borate buffer without surfactant would be needed, compromising peak resolution. Nevertheless, in the SPE-CE mode, the resolution of previously unresolved peaks dramatically improved. This may provide evidence that, despite the small bed volume of the preconcentrator, some chromatographic process may occur during the desorption of the drugs from the solid-phase (70). In fact, we observed a very strong chromatographic effect for peptide mapping of β-casein by SPE-CE. β-casein is a protein whose digestion produces a mixture of hydrophobic and hydrophilic peptides. The peptide maps obtained from off-line preconcentration and discontinuous on-line preconcentration of the β-casein digest were dramatically different, as shown in Fig. 1.6 (adapted from Ref. (72)). After desorption and before high voltage application, the less hydrophobic peptides should be near the cathodic interface of the elution plug and the separation buffer whereas the more hydrophobic peptides will be near the anodic interface. The elution plug, which serves as the CE injection plug, will therefore not be uniform in composition across the length of the plug. Ultimately, this can modify the order of migration of the peptides compared to electrophoresis without SPE preconcentration.

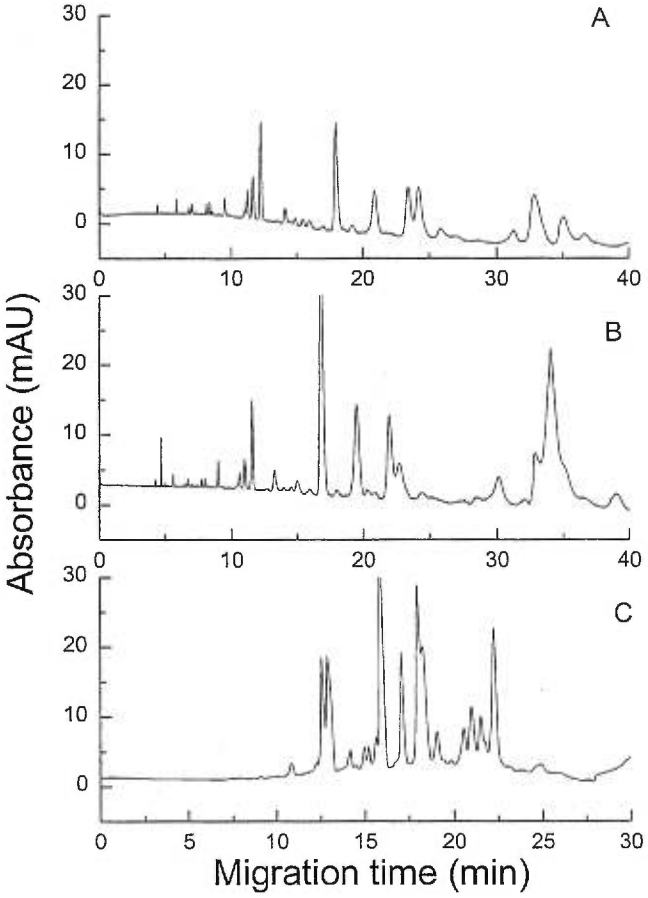

Figure 1.6. Electropherograms showing the chromatographic effect of C_{18} packed bed preconcentration on peptide mapping of a tryptic digest of β-casein. (A) 80 μM digest without preconcentration, 15 nl injected into CE system. (B) Off-line preconcentration of 8 μM digest (10-fold dilution of (A)) with 100 μl loaded, elution in 5 μl acetonitrile:buffer (90:10) and 15 nl injected into CE system. (C) Discontinuous on-line preconcentration of 0.8 μM digest (100-fold dilution of (A)), with 40 μl loaded, elution in ca. 65 nl acetonitrile:buffer (90:10) transferred quantitatively into the separation capillary. A 1.5-mm preconcentrator cartridge was used in (B) and (C). Separation was effected at 18 kV in 50 mM sodium phosphate buffer at pH 2.5.

It is assumed that peak height after an on-line preconcentration procedure will be peptide dependent (72, 82, 90). For example, the more hydrophobic the peptide, the more it is retained on the sorbent and enriched, thus the more intense the peak after desorption. Intuitively, one would think that the peak area of a compound preconcentrated from X ml of a solution at Y mol/l should be equal to the peak area of the same compound preconcentrated from $100 \cdot X$ ml of a solution at $Y/100$ mol/l. In other words, the same mass loaded should lead to the same peak area. However, it appears that extraction efficiency drops with analyte concentration (70), and this decrease is strongly dependent upon the compound hydrophobicity. The less hydrophobic the analyte, the more severe is its decrease in extraction efficiency. This loss could be counteracted by increasing the volume of sorbent. The larger volume of packed bed allowed for an increase in sensitivity, but if excessive, the authors note that resolution may be lost as a result of the increased elution volume required to release the adsorbed analytes (70).

Since the preconcentration factors for most organic compounds and peptides are roughly two orders of magnitude (see Table 1.2), higher factors than this are expected for protein enrichment. The C_{18}-based preconcentrator has been used to enhance the detection of rabbit metallothionein (MT) (71). This technique permitted detection of 1 μg/ml metallothionein, a 700-fold enhancement, with good resolution of the isoforms MT-1 and MT-2. Besides improving detection sensitivity, the preconcentrator can enhance specificity. Without use of the preconcentrator, a contaminating protein was present as a shoulder on the MT-2 peak for sheep metallothionein, thus distorting the apparent relative amounts of the isoforms (71). After on-line preconcentration, the relative amounts of MT-1 and MT-2 could be quantified without interference and were consistent with that observed by other techniques like liquid chromatography or micellar electrokinetic chromatography (MEKC).

Therefore, preconcentration was able to give a more accurate reflection of the true isoform composition.

As with HPLC, the size of C_{18} particles is expected to have an effect on adsorption, desorption and peak shape. Bateman et al. (91) compared packed bed preconcentrators made with 5 μm and 40 μm C_{18} beads. For both particle sizes, the packed bed was 1 mm long. The 5 μm beads yielded stronger analyte adsorption as expected because the adsorptive surface area is much higher than for the same bed length of 40 μm particles. The 5 μm beads also appeared to have better elution properties because tailing of the peaks was considerably less noticeable than for the 40 μm particles. On the other hand, memory effects were observed for the smaller particle size. In other words, the desorption yield was well below 100 %. It is worthy to note that the observed memory effect depends strongly on the analyte structure. Hydrophilic peptides do not induce a memory effect as they are completely desorbed with a single elution plug. However, with more hydrophobic analytes like proteins, the analyte could still be seen in subsequent elution plugs (92-94). Increasing the volume of the elution plug to obtain a complete elution would degrade the separation and peak shapes. An interesting possibility proposed by Thibault and co-workers (91) that takes advantage of incomplete elution of sample would be to carry out multiple analyses from a single sample injection. This would be particularly useful when SPE-CE is combined with tandem mass spectrometry (MS) in order to obtain peptide sequence information or for the acquisition of tandem mass spectra of co-eluting components. It is important to point out that the construction of a preconcentrator containing 5 μm C_{18} beads required incorporation of retaining frits, making fabrication of the device more time consuming.

(94). Unfortunately, some solid phase materials are not resistant to the electrolyte conditions imposed by tITP.

In order to further improve the analytical performance of the preconcentrator device, alternative sorbents have been investigated. Recently, Li et al. (90) compared C_{18} beads and an SDB membrane as adsorptive phases for the preconcentration of enkephalin peptides. The separations performed using the two types of sorbent yielded qualitatively similar electropherograms. There was a slight increase in sensitivity using the SDB membrane for some of the peptides studied, yet in other cases the C_{18} beads performed better. The only significant drawback of the SDB membrane was the lower linearity coefficient observed over the concentration range examined. For the most part, the loss of linearity occurred at the highest peptide concentration. This effect could possibly be a reflection of having reached the maximum adsorption capacity of the membrane.

Several different sorbents were investigated by Caprioli and co-workers for the preconcentration of peptide E (derived from proenkephalin A), neurotensin and five proteins using an on-line SPE transfer, off-line CE-MALDI/MS approach (99). The preconcentrators containing either C_1, C_8, C_{18} or YMCbasic particles were packed into 50 or 75 μm I.D. capillaries rather than a Teflon tube, leading to a packed bed volume of about 20 nl. A preconcentration factor of 500 with CLOD as low as 10 amol/μl for neurotensin was reported, although this was achieved by removing the SPE preconcentrator cartridge from the capillary before performing the separation. In agreement with results reported by other groups, the C_1 sorbent was best suited for preconcentration of proteins and the C_8, C_{18} and YMCbasic phases for peptides (99).

Recently, Herring and Quin (100) described a preconcentrator in which the packed bed was 20 μm diameter Poros R2 beads (from PerSeptive Biosystems) trapped in an

entanglement of high purity glass wool. This approach avoided the use of frits which, in combination with the large pore size of the particles (7,000 Å), led to very little backpressure. Another advantage of the Poros sorbent is its tolerance to extreme pHs unlike silica-based C_{18} beads. On the other hand, the authors reported the frequent formation of air bubbles in the preconcentrator which caused breakdown in the CE separation. Separation and detection of a tryptic digest of 20 nM myosin I heavy chain kinase was made by CE-ESI/MS (100). The authors report that the CLOD for their Poros-bead preconcentrator-CE-MS system is in the mid-picomolar range.

1.4.4. *Membrane preconcentrators*

The first group to report the use of a membrane-based preconcentrator with CE was that of Tomlinson and Naylor (95, 101). The philosophy behind development of membrane preconcentrators on-line with CE (mPC-CE) was to decrease or remove the problems associated with packed bed SPE-CE. With a membrane, for example, it is possible to minimize the preconcentrator volume at the capillary inlet end and avoid the use of frits. The most popular membrane in use is SDB. Initial reports on the use of SDB mPC-CE for the separation of a mixture of three similar peptides (SGINFEKL, SIINFEKL, SIINFEKLT), showed that resolution of these peptides was rather poor (95, 101-103). Fortunately, mPC-CE benefits greatly from the use of tITP to improve sample focusing. According to the authors, when combining mPC-CE or mPC-CE-MS with tITP under optimal conditions, no compromise in CE or CE-MS performance is noted (89).

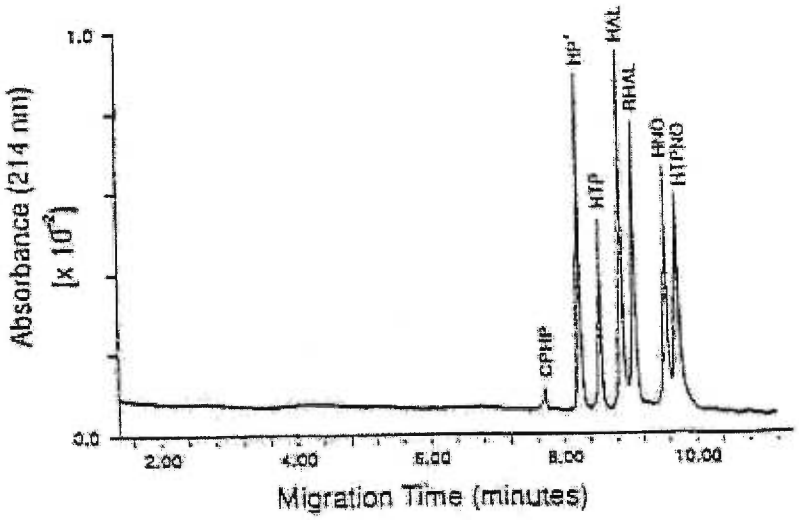

Figure 1.7. Electropherograms showing an example of SDB membrane preconcentration (mPC-CE) with analyte stacking for separation of a mixture of haloperidol (HAL) analogs. The sample (380 amol/nl in separation buffer) was loaded by pressure for 1 min. The mixture was eluted from the membrane in 50 nl of acetonitrile:methanol (50:50). Separation was effected at 30 kV (25 μA) in 50 mM ammonium acetate buffer containing 10 % methanol and 1 % acetic acid [57].

Unfortunately, tITP does not always improve peak efficiency. Tomlinson et al. (88, 95) noted that tITP applied to the mPC-CE separation of a nine-peptide mixture desorbed with 110 nl of methanol/water/trifluoroacetic acid had worse effects than without tITP for the same mixture desorbed after mPC-CE with 200 nl of the same desorption solution. The same authors have noted that increasing the concentration of the leading stacking buffer (LSB) generally improved the focusing of peptide zones when tITP was

employed (104). However, this improvement was often at the expense of component resolution. The mPC-CE performance was also found to be dependent on the trailing stacking buffer (TSB) volume. Essentially, the analyte elution plug needs to be pushed a reasonable distance away from the membrane to achieve optimal tITP performance. This is necessary to prevent analyte re-adsorption on the membrane. Like the C_{18} packed bed preconcentrator cartridge, there is a slight difference in hydrodynamic flow properties for each membrane cartridge. Therefore, it is necessary to systematically evaluate the volume ratios of each of LSB and TSB when used for the first time in a newly prepared mPC-CE capillary.

The smaller bed volume of membrane preconcentrators (ca. 15 nl) compared to C_{18} packed bed preconcentrators allows the elution volume to be minimized. This effect greatly enhances the efficiency and resolution as can be seen in Fig. 1.7 (adapted from Ref. (105)). Tomlinson et al. (105) were able to separate a mixture of haloperidol analogs in less than 10 min with excellent resolution of all components. The concentration of the sample was 380 amol/nl, which is an order of magnitude below the typical CLOD of conventional CE with UV detection. The authors indicate that the major contribution to the high efficiency of their separation was the low desorption volume needed (50 nl) with mPC-CE.

The versatility of mPC-CE lies in the range of different membranes available. The use of a novel poly-SDB reversed phase sulfonated membrane allowed the preconcentration of two hydrophobic metabolites of 3-phenylamaino-1,2-propanediol (PAP) as well as two polar metabolites, *p*-hydroxy PAP and *p*-hydroxy 3-phenylamino-*L*-alanine (106). The two polar metabolites were not retained on preconcentrators made with either C_2 or C_{18} derivatized membranes. From this point of view, an SDB membrane is recommended for the preconcentration of small organic molecules whereas C_8–based (107) or C_2–based (108, 109) impregnated membranes are recommended for the preconcentration of proteins.

Certain SDB resins and membranes are insensitive to pH, which is an additional advantage over traditional reversed phases bonded to silica beads or polymer resins. These latter supports are sensitive to low pH and even more so to high pH solutions. As a result, it has proved difficult to reproducibly regenerate the capillary wall, which is typically achieved by rinsing with 0.1 *M* NaOH, and the sorbent material after a separation (110).

The mPC-CE technique displays the same advantages as packed bed SPE-CE in that it has been used to remove salts, to remove other hydrophilic contaminants or detergents and to lyse cells. Detergents can be extremely difficult to remove from peptide mixtures even after many stages of purification. When MS detection is employed, surfactants can be problematic if they are still present in the final peptide analysis step because it results in significant suppression of the electrospray ionization (ESI)-MS ion current. In a similar example, the neuroleptic agent haloperidol and its analogs were separated from proteins in urine samples by the addition of $ZnSO_4$ (74). The authors used mPC-CE-MS to remove the $ZnSO_4$ and detect four of these peptides at concentrations normally undetectable by on-line CE-ESI-MS

Unlike packed bed SPE-CE, the loading time is much longer with mPC-CE. The on-line flow rate of the sample solution should not exceed 120-150 nl/min, which can lead to a few hours spent just on sample loading. Fortunately, these devices can withstand relatively high pressures. Hence, the loading and the washing step are often performed off-line (111). In this way, particles can be prevented from entering the separation column. The bi-directional flow of analyte solution and solvents through the mPC-cartridge is noteworthy. It has been the experience of Naylor and co-workers (112) that sample loaded in the direction opposite to which analytes are eluted into the CE capillary yields better performance than sample loaded in the forward direction. These authors believed this is due to the fact that adsorption occurs with little penetration into the membrane. Therefore,

analytes will be eluted by the leading edge of the elution solvent. This leads to efficient removal from the membrane and results in a less diffuse analyte zone within the mPC-CE capillary even before the high voltage is applied. This is certainly an advantage of off-line sample loading in membrane (111) and packed bed (72) preconcentration.

Previous studies have shown that considerable enhancement of CE-MS sensitivity is obtained by using capillaries with a reduced internal diameter (113). However, such small volume capillaries exacerbate the problems associated with poor concentration sensitivity in CE. Ultimately, only ultra-small sample injection volumes, sometimes as low as a few picoliters, could be analyzed without degradation of the separation performance. This limitation has essentially been overcome by the advent of mPC-CE technology since it is possible to use the impregnated membrane to trap analytes at the inlet of the capillary. Initially, the use of on-line mPC-CE-MS with reduced diameter capillaries presented a significant challenge. The flow rate in a 25 μm I.D. capillary is extremely low at a given applied pressure. It would be very time consuming to load the preconcentrator with 100 μl of sample and rinse with separation buffer. However, the removable mPC-CE cartridge design lends itself to sample loading and clean-up prior to its connection onto the separation capillary.

Naylor and Tomlinson's group (104, 112) have developed a pressurized bomb apparatus to introduce biological samples onto a membrane preconcentrator before its connection to a CE-MS system. An Eppendorf tube containing either analyte solution or rinsing buffer are inserted into a Perspex pressured bomb and subjected to 40-60 psi pressure. This permits transfer of the contents of the Eppendorf tube via a fused silica capillary into the mPC cartridge. With this pressure, ca. 100 μl of solution can be passed through the mPC-CE cartridge in less than 5 min. Their system permits bi-directional flow

of analyte solution and solvents through the mPC-cartridge which, as described above, yields better CE performance. The same authors note that a potential limitation of mPC-CE-MS analysis in narrow bore capillaries is the development of efficient tITP conditions after analyte elution from the mPC cartridge at the onset of electrophoresis. Reagent concentrations and volumes that are optimal for larger bore capillaries tend to completely fill a capillary of smaller I.D. The authors showed that it is possible to adapt conditions defined for large bore capillaries to narrow bore capillaries by increasing the concentration of the LSB for a smaller volume. Moreover, the volume of TSB is more critical in narrow bore than wide bore capillaries. The authors obtained optimum concentrations and volumes for the LSB, elution solvent and TSB for the analysis of MHC class I peptides originating from the lysis of EL-4 cells (104). Unfortunately, it seems that these conditions require optimization for each newly prepared mPC-CE capillary.

The versatility of mPC-CE-MS for the analysis of in-vivo derived compounds is highlighted by studies on the determination of adduct formation sites in proteins (114), the separation of haloperidol metabolites in urine (107), the analysis of MHC class I peptides (111) and the separation of complex protein-based mixtures like aqueous humor (AH), which is the fluid that surrounds the eye (108, 109). Proteins in AH are known to be present at low concentrations in a complex matrix of high salt. Loading of 1 µl of AH onto a C_2–based membrane enriches the proteins and removes salts during the rinsing step. Using optimal conditions for protein elution from the membrane, post-elution focusing by tITP and Polybrene-coated capillaries coupled on-line with MS detection, numerous proteins were characterized by CE (Fig. 1.8 (109)) by directly injecting human AH samples from patients having various ocular pathologies (109). mPC-CE-MS was found to be an excellent technique for analyzing AH component proteins with a mass range of 4,000 to 70,000 Da. Larger proteins known to be present in AH were not detected. This may be due

to irreversible adsorption on the membrane. Recovery of the protein standard seemed to be much lower than for biologically derived protein mixtures suggesting that the sample matrix plays an important role in the adsorption and desorption processes during mPC-CE. However, the difficulty in analyzing complex protein mixtures is the optimization of both the desorption and the separation (108).

Another problem arises because the response of proteins to organic solutions varies from one protein to another. Normally a high organic component in the elution solvent is desirable; however, proteins tend to be denatured or precipitate in solvents containing ≥40% acetonitrile. Addition of 1% acetic acid or 0.02% trifluoroacetic acid aided the dissolution of proteins so that up to 80 % acetonitrile could be used without the formation of a precipitate.

Interestingly with mPC-CE, the use of 80% acetonitrile in water yields no significant qualitative and quantitative differences to 80% acetonitrile modified with 1% acetic acid in water. The added effect of turbulence and mixing of elution solvent with separation buffer in the small dead volumes that exist in an mPC-CE cartridge may also aid the dissolution of proteins as they are eluted from the membrane.

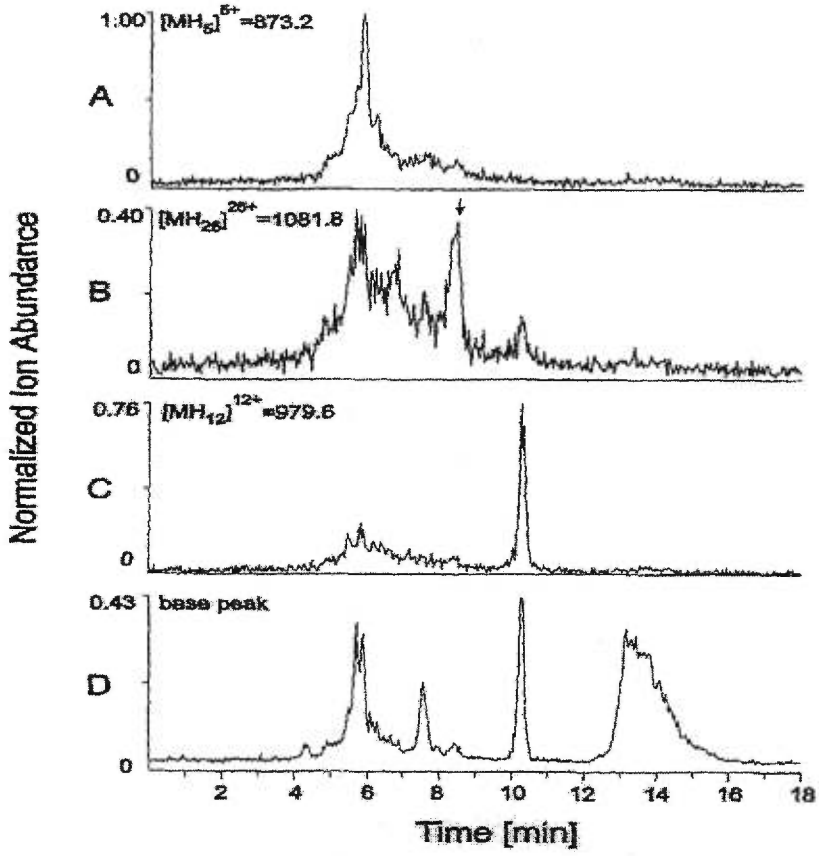

Figure 1.8. Electropherograms showing an example of a C_2–impregnated membrane for the mPC-CE-MS analysis of aqueous humor (AH) from the eye of a patient with PEX syndrome. Shown are the total ion current traces from ubiquitous proteins with masses of (A) 4360.0 Da, unidentified; (B) 28096.0 Da apolipoprotein A1; (C) 11727.1 Da, β-2 microglobulin; (D) base peak plot. The loaded volume of sample was 1μl. Elution was made in 60 nl of acetonitrile:water (80:20) followed by 240 nl of buffer for spatial separation from the membrane before tITP focusing. Separation was effected at –15 kV, with simultaneous application of pressure at 0.5 psi, in 5 % acetic acid–2 mM ammonium acetate buffer using a 50 μm × 80 cm Polybrene–coated capillary [60].

1.4.5. *Conclusions*

Despite the fact that the technique is still in its infancy, non-specific on-line preconcentration can overcome the limitations of poor concentration detection sensitivity in CE. From a technical standpoint, on-line preconcentrators are appealing not only because their fabrication is simple, rapid and inexpensive but also because analyte adsorption relies on the well-documented solid-phase extraction process. Preconcentration factors reported range from 5 to 1,000 and allow the detection of compounds at concentrations as low 20 amol/μl (BSA tryptic digest). Furthermore, the coupling of preconcentrators to CE-MS has been demonstrated and is directly applicable to studies that require the structural characterization of analytes extracted at low concentration from complex sample matrices. Because of its excellent sensitivity, the direct injection of biological samples is now possible without tedious off-line sample cleanup. This advantage tends to increase the efficiency of analyte recovery and also reduce human exposure to potentially toxic materials.

Nevertheless, the on-line preconcentration technique suffers from several drawbacks. First, the preconcentrator induces a modification of the EOF. This has been shown to be severe enough at low pH for packed beds that it induces the reversal of EOF and re-adsorption of peptides on the sorbent. A decrease in resolution is often noticed with preconcentrators due to the presence of the sorbent in the separation column and the volume of desorption solution, which is larger than ideal for high efficiency separations. However, reproducibility of preconcentration and CE separation has been demonstrated to be excellent when optimal conditions are employed. Optimization of the desorption step and subsequent separation of the analytes is relatively easy when conventional CE is used. Addition of a supplementary tITP focusing step, which seems to be needed with membrane preconcentrators, requires careful attention when optimizing the different electrolytes used

for the LSB, elution buffer and TSB. Finally, this technology affords practitioners of CE and CE-MS a means of analyte extraction and clean-up prior to determination.

1.5. Objectifs du projet

Le but de cette thèse est de construire un système en ligne comprenant un microréacteur enzymatique, un préconcentrateur et un système artisanal d'électrophorèse capillaire. Les objectifs de ce travail concernent en premier lieu la diminution du temps nécessaire à l'étape de digestion, l'augmentation de la fiabilité des cartes peptidiques qui nécessitent la diminution de l'autoprotéolyse. Au vu de la littérature sur les microréacteurs, cette technologie peut remplir ces objectifs. Nous nous proposons donc de construire un microréacteur garni de billes de trypsine. Nous en effectuerons la caractérisation en terme de temps de digestion et de reproductibilité des cartes peptidiques. Ce microréacteur sera ensuite connecté à un préconcentrateur pour améliorer la sensibilité de la cartographie peptidique par électrophorèse capillaire. Nous en étudierons l'influence sur la carte peptidique et sur la reproductibilité de celle-ci. Nous évaluerons également l'amélioration de la sensibilité obtenue. En troisième lieu, nous effectuerons la connection en ligne de ces deux éléments avec un appareil de CE. Cette dernière partie comprendra une étude de reproductibilité, une évaluation de l'amélioration de la sensibilité. Une description plus détaillée décrivant la méthodologie du projet est faite dans le chapitre suivant.

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Chapitre 2

Méthodologie

2.1. Introduction

La technique de cartographie peptidique classique est une technique relativement longue et fastidieuse. La digestion en phase liquide demande 24 h en général (le temps de digestion dépend de la protéine) (1). L'utilisation de microréacteurs avec enzyme immobilisé a permis d'améliorer la fiabilité de la technique en minimisant l'autolyse de l'enzyme et en diminuant le temps de digestion. Malheureusement, les deux principaux types de microréacteurs existants—microréacteur garni d'un gel de trypsine (2, 3) ou sur les parois duquel l'enzyme est immobilisé (4)—ne permettent pas encore l'utilisation de flux élevés et donc le développement de systèmes avec "high throughput".

Du fait du manque de sensibilité de l'électrophorèse capillaire, la cartographie peptidique d'échantillons réels requiert des étapes de concentration (enrichissement d'échantillon) qui sont encore souvent faites par lyophilisation (5). Cette étape allonge le temps total d'analyse et peut entraîner des pertes d'échantillons sur les parois des tubes du lyophilisateur. De plus, la redissolution du produit lyophilisé à faible quantité est difficile.

Notre but était donc de construire un système analytique en ligne qui permette de réaliser la cartographie peptidique rapidement et à des concentrations inférieures à celles atteignables normalement en électrophorèse capillaire. Ce système en ligne pourrait également diminuer la perte d'échantillon sur les parois des vials ainsi que le nombre de manipulations de l'expérimentateur, deux aspects qui contribuent à la perte de sensibilité de la cartographie peptidique. De plus, l'utilisation d'enzyme immobilisés permettrait une réutilisation de ce système pour des digestions subséquentes. Il devrait donc prévoir trois éléments; un microréacteur dans lequel s'effectuera la digestion de la protéine, un préconcentrateur afin de collecter et de concentrer les fragments peptidiques dilués et un appareil d'électrophorèse capillaire pour séparer les fragments collectés et établir la carte

peptidique de la protéine. Le développement d'un tel système en continu est un défi analytique. Donc, nous avons construit et caractérisé le microréacteur et le préconcentrateur séparément avant de les intégrer, comme il est expliqué dans les sections 2.2 à 2.5. Les performances et les influences de chaque élément du système respectives sur les cartes peptidiques ont ainsi été évaluées.

2.2. Le microréacteur

Au chapitre trois, nous présentons la construction simple et rapide d'un microréacteur. Brièvement, nous avons utilisé de la trypsine immobilisé via le diisothiocyanate sur des billes de verre à pores controlées (CPG) (fig. 2.1), un produit disponible commercialement dont nous avons rempli par pression un tube de silice fondue (diamètre interne de 830 μm) pour créer un microréacteur enzymatique.

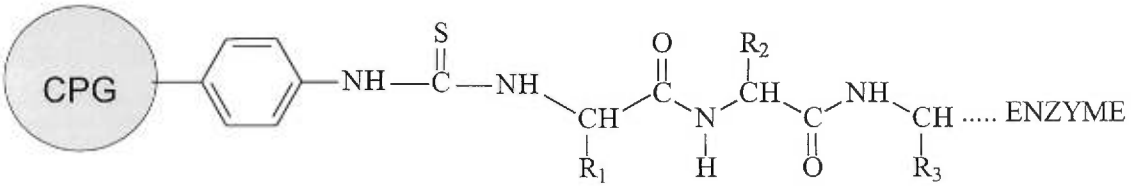

Figure 2.1. Schéma de la trypsine immobilisée sur des billes de verre de diamètre 200 μm.

Cette configuration, décrite dans la section 3.2 de cette thèse, présente plusieurs avantages. Tout d'abord la distribution de l'enzyme dans le microréacteur est beaucoup plus uniforme qu'avec les autres techniques. Ceci favorise grandement le contact enzyme-protéine par rapport à la configuration d'Amankwa et al. (4, 6) où l'enzyme est immobilisé

directement sur les parois du capillaire et le contact est limité par la diffusion de la protéine. Notre méthode permet donc une diminution du temps de digestion parce qu'on peut faire passer la protéine plus rapidement dans le microréacteur que dans le cas d'Amankwa et al. Les billes CPG-trypsine peuvent être facilement maintenues à l'intérieur du microréacteur en connectant celui-ci avec des capillaires de plus faible diamètre interne. C'est également un avantage par rapport au microréacteur garni de gel de trypsine (2, 3) à cause du gonflement du gel qui empêche l'écoulement de la protéine dans le microréacteur, et de l'extrusion possible du gel.

Le déplacement de la solution de protéine dans le microréacteur se fait également par différence de pression. La mise en oeuvre d'un tel système est très simple. Il suffit d'appliquer une pression au vial contenant la solution de protéine et dans laquelle trempe l'extrémité du microréacteur. Les détails sur les pressions appliquées sont présentées à la section 3.2. Du fait que son enveloppe est constituée d'un capillaire en silice fondue, une migration de la protéine par voltage (électrophorèse) à travers le microréacteur pourrait être envisagée. Nos études préliminaires avec ce type de configuration n'ont pas abouti. Pendant l'électrophorèse, l'effet Joule a genèré une grande quantité de chaleur qu'il était difficile de contrôler. Cette chaleur a donc pu nuire à l'activité de l'enzyme (optimale à 37 °C). Pour finir, du fait que l'enzyme est une protéine chargée, l'application d'un champ électrique pourrait modifier sa conformation et donc diminuer son activité.

La séparation des fragments tryptiques par CE, a été effectuée à pH 2.5 avec du tampon phosphate comme précedemment utilisée par Righetti et al. (7). Ces conditions donnent une bonne résolution et une bonne efficacité des pics. La détection a été effectuée par absorption dans l'UV à 200 nm de manière à détecter tous les peptides tryptiques issus de la digestion puisque la liaison peptidique absorbe à cette longueur d'onde. Bien que la fluorescence soit une technique plus sensible, elle nécessite une étape de dérivation

chimique supplémentaire qui peut s'avérer problématique. En effet, un même peptide peut être dérivé avec plusieurs molécules de l'agent fluorescent ce qui peut produire plusieurs pics sur la carte peptidique (8).

Pour caractériser les performances de ce microréacteur, nous avons choisi deux protéines : l'insuline chaîne B oxydée et la β-caséine. L'insuline chaîne B est une petite protéine, qui après digestion avec la trypsine ne donne que deux fragments tryptiques. Nous avons utilisé cette protéine pour tester l'étendue de l'autoprotéolyse en comparaison de la digestion en phase liquide avec trypsine soluble. L'apparition de pics supplémentaires dus à ce phénomène est aisément identifiable lorsque la digestion de la protéine ne génère que deux pics. Nous avons observé beaucoup moins de pics supplémentaires dans le cas de la digestion sur phase solide.

La β-caséine est une protéine du lait qui possède trois sites de phosphorylation sur les résidus Ser16, Ser18, Ser19, Ser20. Elle ne possède pas de ponts disulfures, et n'est pas glycosylée. La séquence primaire de la β-caséine est connue depuis longtemps (214 acides aminés, voir fig. 3.3) et il y a des exemples de sa carte peptidique par CE dans la littérature (2, 6, 7, 9). Elle semble donc être un bon choix pour tester la reproductibilité de la digestion avec notre système. En premier lieu, nous avons optimisé la longueur de notre microréacteur en effectuant des digestions de solutions de β-caséine à différentes concentrations dans des microréacteurs de différentes longueurs. Nous avons retenu pour nos études ultérieures la longueur de 26 cm qui nous a donné le plus grand nombre de pics (fragments peptidiques) et les hauteurs de pics les plus importantes. Bien sur, nous aurions pu réduire la longueur du microréacteur, si nous avions réduit aussi la quantité de protéine à digérer, mais nous étions limités par la sensibilité de l'électrophorèse capillaire avec détection UV. Donc, nous avons utilisé une quantité de protéine assez importante.

La cartographie peptidique est une technique comparative. Elle nécessite une bonne reproductibilité des temps de migration puisque c'est cette comparaison qui permet la détermination des modifications post-transductionnelles ou génétiques. A ce titre, les cartes peptidiques obtenues avec notre microréacteur ont montré une reproductibilité acceptable des temps de migration. Malheureusement, nous avons obtenu un nombre de pics correspondant aux peptides tryptiques supérieur au nombre attendu. Ce phénomène a déjà été observé pour la β-caséine (7, 9) et d'autres protéines (4, 10-12). Nous avons proposé différentes explications à ce phénomène mais une étude ultérieure présentée dans le chapitre 4 démontre que les échantillons de β-caséine n'étaient pas purs. En fait, la β-caséine présente plusieurs isoformes qui ajoute des pics supplémentaires à la carte peptidique.

Nous avons également noté des variations dans les hauteurs relatives de pics comme observé dans la littérature (6, 11). Les limitations possibles avec notre design pourrait être une perte d'échantillon du fait des pores des billes, qui pourrait expliquer ce phénomène. Ceci dépend essentiellement de l'affinité des peptides tryptiques pour les billes. C'est un désavantage par rapport au microréacteur ou l'enzyme est greffé sur les parois internes du capillaire. Ce point est également abordé dans le chapitre suivant. Aussi, la durée d'utilisation a été estimée au vu de la modification des cartes peptidiques avec la diminution d'activité de l'enzyme attendue après de nombreuses réutilisations.

2.3. Le préconcentrateur

La stratégie que nous avons choisi dans cette étape est la construction d'un préconcentrateur à billes de silice greffé d'une phase stationnaire C-18 comme précédemment décrit dans l'introduction. Nous nous sommes tournés vers cette configuration car elle est relativement facile à mettre en oeuvre. De plus, comme expliqué

dans l'introduction, les préconcentrateurs à base de billes de C-18 permettent une adsorption d'un plus grand volume d'échantillons (13), ce qui peut s'avérer un avantage lorsqu'on a affaire à des mélanges complexes comme les digestats tryptiques. Du fait que l'efficacité chromatographique de ces préconcentrateurs n'est pas concernée dans le cas d'une désorption (comme c'est le cas pour les Zip-Tip^{TM} ou les cartouches d'extraction sur phase solide) la méthode de fabrication du préconcentrateur est faite par insertion directe des billes dans le tube en Téflon. Ces billes sont maintenues en place aux deux extrémités, non pas par des frits qui peuvent entraîner une adsorption irréversible des peptides, mais par deux morceaux de capillaire de diamètre interne équivalent à celui des billes. Ainsi, elles ne devraient pas pénétrer dans le capillaire lors de la désorption par pression. Le design de ce système est présenté en détail au chapitre 5.

À cause de l'important volume de la solution de désorption (50-250 nl) par rapport aux volumes typiquement injectés en CE (1-10 nl) (14-17), les cartes peptidiques établies par CE étaient faites à bas pH. L'intérêt est qu'alors, le flux électroosmotique est suffisamment faible à pH 2.5 pour que le volume d'éluant (solution organique) qui absorbe dans l'UV à 200 nm n'interfére pas avec la carte peptidique.

Un effet indésirable observé avec l'utilisation de préconcentrateur connectés en ligne avec le capillaire de séparation est l'inversion de la direction du flux électroosmotique à bas pH (18). Ceci entraîne une réadsorption des peptides sur la phase stationnaire. Pour éviter ce problème nous avons construit un système qui a permis la déconnection du préconcentrateur avant la séparation par CE.

Une étude de reproductibilité du préconcentrateur est présentée dans le chapitre 5. Des cartes peptidiques engendrées à partir de trois préconcentrateurs différents étaient produites ainsi que celles obtenues à partir d'un même préconcentrateur. Du fait des possibles variations de pression de retour engendrée par la compression éventuelle de la

phase stationnaire (billes de C-18) au fur et à mesure des utilisations, nous avons utilisé une méthode de normalisation des temps de migration des pics décrite par Li et al. (19). La normalisation sera faite avec 2 pics (le premier et le dernier) d'un électrophérogramme choisi comme standard et le temps de migration corrigé des autres électrophérogrammes par rapport au standard choisi obéira à l'équation suivante:

$$t_{corrigé} = [1/t_{m1}-1/\gamma \times (1/t'_{m1}-1/t)]^{-1} \quad \text{avec } \gamma = (1/t'_{m1}-1/t'_{m2})/(1/t_{m1}-1/t_{m2}) \qquad (2.1)$$

où t_{m1}: temps de migration du premier pic de l'électrophérogramme choisi comme standard;

t_{m2}: temps de migration du dernier pic de l'électrophérogramme choisi comme standard;

t'_{m1}: temps de migration du premier pic de l'électrophérogramme à normaliser;

t'_{m2}: temps de migration du dernier pic de l'électrophérogramme à normaliser;

t: temps "brut" de l'électrophérogramme à normaliser.

L'équation (2.1) est basée sur le fait que la mobilité électrophorétique d'un peptide donné est constante à un pH donné et que les variations des temps de migration viennent des changements dans le flux électroosmotique.

Du fait que la rétention sur ce type de préconcentrateur dépend de la polarité des peptides, nous avons étudié l'influence de ce phénomène pendant l'étape de préconcentration sur la carte peptidique et sa reproductibilité. Les peptides les plus hydrophobes devraient être les plus retenus et donc devraient donner le signal le plus intense dans la carte peptidique après désorption. En ce sens, la β-caséine est une protéine de choix dans cette étude car elle donne lieu, après digestion à une grande variété

d'hydrophobicité des peptides tryptiques. Les effets de rétention chromatographique sont discutés en détail dans le chapitre 5.

Nous avons étudié également l'effet du volume de désorption et la quantité de phase stationnaire utilisée sur l'allure des cartes peptidiques. Par exemple, plus le volume de désorption est important, plus grande est la perte de résolution et d'efficacité selon la théorie de la CE. La quantité de phase stationnaire quant à elle, devrait avoir une influence sur la carte peptidique en termes de hauteurs relatives des pics. Si elle n'est pas suffisante pour adsorber tous les peptides, ne seront retenus que les peptides les plus hydrophobes au détriment des peptides les plus hydrophiles qui resteront en solution. Ces aspects sont également discutés au chapitre 5.

Pour finir, nous avons évalué l'augmentation de la sensibilité obtenue avec l'utilisation de cette technique en effectuant la préconcentration de plusieurs aliquots de digestats tryptiques de même volume mais à des concentrations décroissantes. Nous avons estimé alors la concentration la plus basse que notre système nous permet d'atteindre pour obtenir une carte peptidique complète.

2.4. Le système en ligne

Malgré les avantages de notre approche pour améliorer la cartographie peptidique que constituent le microréacteur et le préconcentrateur, nous pourrions raffiner notre démarche en connectant en ligne ces deux éléments avec un appareil d'électrophorèse capillaire. Nous devrions ainsi limiter au maximum la perte d'échantillon provoquée par la collection après digestion et après concentration dans différents vials. Dans le même ordre d'idée, le nombre de manipulations de l'expérimentateur en serait réduit ce qui permettrait un gain de temps appréciable. Le schéma d'un tel système est présenté à la figure 2.2.

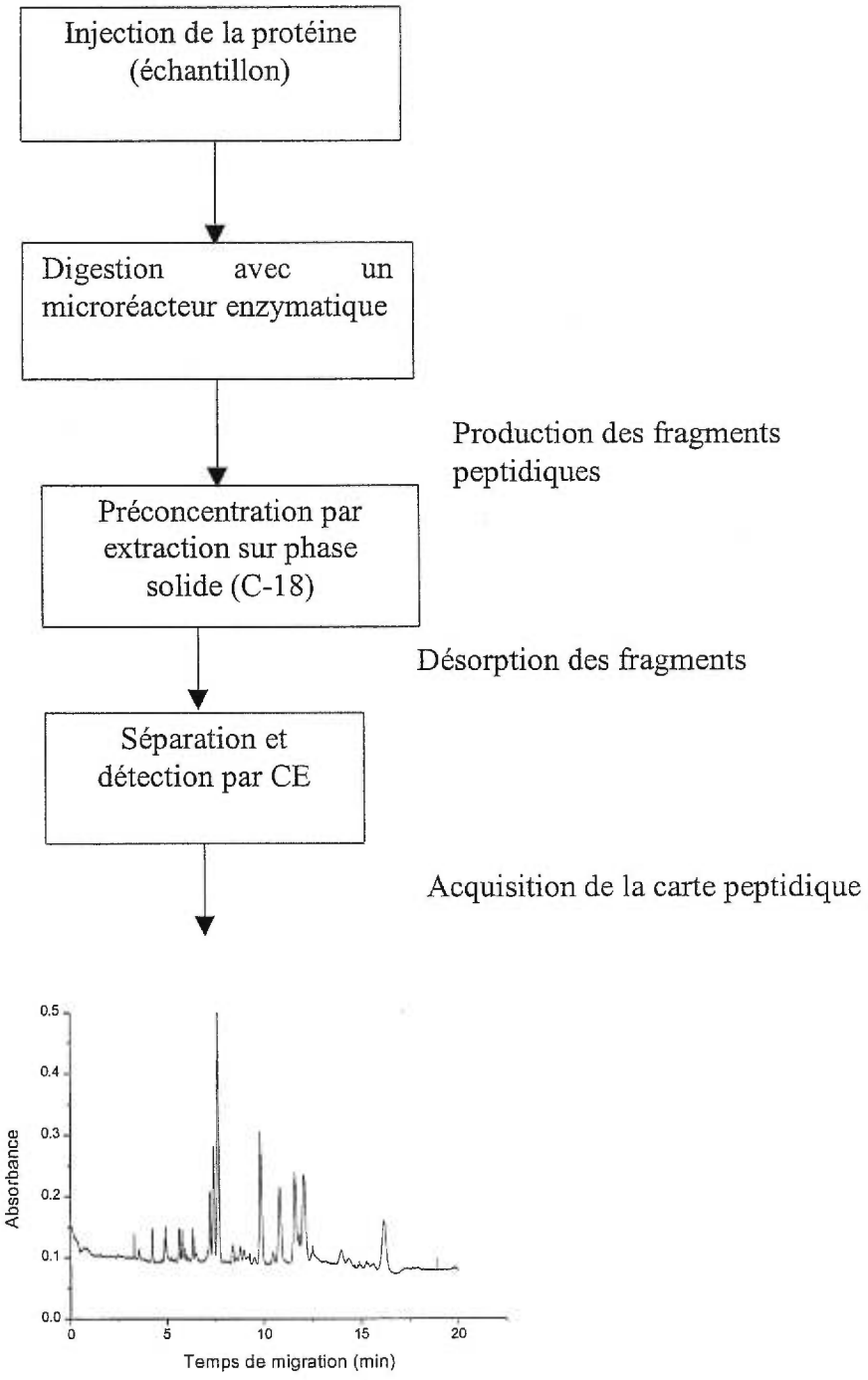

Figure 2.2. Représentation schématique du système en ligne.

Notre objectif était donc de construire un système de telle manière qu'après l'injection de la solution de protéine dans le microréacteur, l'expérimentateur puisse obtenir directement la carte peptidique sans autre manipulation de l'échantillon. Ses interventions ne se feront qu'au niveau de l'injection directe de la solution organique de désorption des peptides tryptiques du préconcentrateur.

En ce qui a trait à la connection en ligne de microréacteur à un système de séparation analytique, il n'existe que peu d'articles sur la question. La connection avec un appareil HPLC est relativement facile. Il suffit de connecter le microréacteur à la boucle d'injection du système chromatographique (12, 20). La connection avec un système d'électrophorèse est plus problématique du fait qu'on utilise la pression pour deplacer la solution de protéine dans le microréacteur et du voltage utilisé en CE.

Récemment, la connection en ligne d'un microréacteur enzymatique avec un capillaire de séparation a été faite par le groupe de Kuhr (4, 21). Le schéma de leur système est présenté à la figure 2.3. La migration de l'échantillon dans le microréacteur se fait par application d'un voltage comme en CE classique. Les deux capillaires ne sont pas connectés directement l'un à l'autre mais sont séparés par un espace de 100 μm environ dans une cellule de Teflon. Cela permet de pouvoir changer le tampon de digestion pour l'électrolyte de support une fois le digestat dans le capillaire de séparation. La méthode de Kuhr est une amélioration majeure puisque l'autre système continu décrit par Nashabeh et El Rassi en 1992 implique la connection directe des deux capillaires (22). Il en résultait que le tampon de digestion servait aussi d'électrolyte de support ce qui pouvait énormément nuire à la qualité de la séparation. L'inconvénient majeur de la configuration de Kuhr est l'alignement des deux capillaires. Si les deux capillaires ne sont pas parfaitement alignés, il peut se produire des turbulences dans le flux du liquide ce qui se traduit par un

élargissement des pics et une perte de résolution de l'électrophérogramme (carte peptidique).

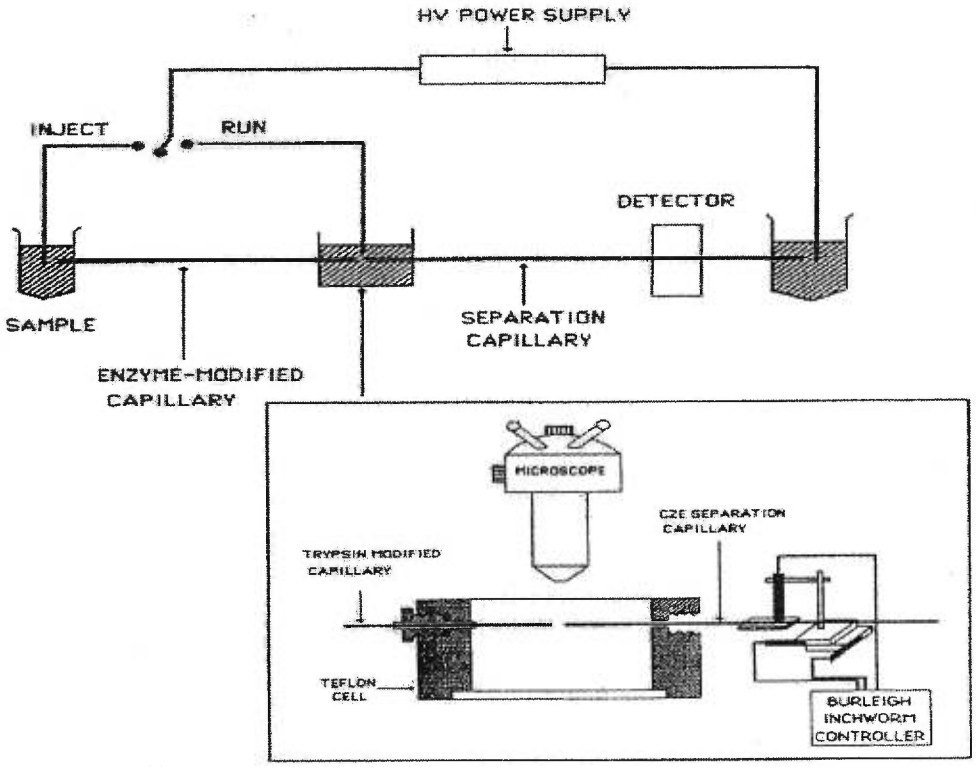

Figure 2.3. Diagramme schématique du système continu constitué du microréacteur ("enzyme modified capillary") et du capillaire de séparation (4).

Notre système en ligne est présenté au chapitre 6. Les différents éléments de l'appareil sont séparés par des valves qui permettent d'évacuer le tampon de digestion après l'adsorption des peptides tryptiques sur la phase stationnaire, d'injecter la solution de désorption, de rincer le capillaire de la CE après l'obtention de la carte peptidique sans que le tampon de séparation ne pénètre dans le microréacteur. Ces valves sont de très faibles

diamètres internes afin de minimiser les pertes par diffusion dans le système. La performance du système en ligne est évaluée pour quelques protéines.

2.5. Conclusion

Le microréacteur décrit dans le chapitre suivant améliore de facon conséquente le temps de digestion en comparaison de ceux obtenus avec d'autres types de microréacteurs présentés dans la littérature (8 h. au minimum). Sa conception est assez rapide puisque la trypsine est vendue déjà immobilisée et prête à servir. Le microréacteur présente également une bonne résistance à la pression.

Bien que précedemment décrit dans la littérature, le préconcentrateur constitué de billes de C-18 en ligne avec la CE n'a encore jamais été caractérisé d'une facon rigoureuse pour la cartographie peptidique. Les caractérisations faites à ce jour l'ont été avec des peptides assez hydrophobes, ce qui facilite grandement les conditions d'étude. Nous l'appliquons spécifiquement à la cartographie peptidique avec une certaine variété de peptides tryptiques. Les conditions expérimentales seront optimisées pour obtenir les meilleurs cartes peptidiques possibles.

Finalement, nous présentons un système complet reliant le microréacteur au préconcentrateur, lui-même connecté à un capillaire de séparation. Un tel système n'a encore jamais été présenté dans la littérature. Ce système permet une diminution du temps nécessaire à l'obtention de cartes peptidiques à basses concentrations ainsi que le nombre de manipulations. À long terme, l'automatisation et le contrôle par ordinateur du système de cartographie peptidique seront faits.

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Chapitre 3

Reproducibility of a Solid-Phase Trypsin Microreactor for Peptide Mapping by Capillary Electrophoresis

E. Bonneil, M. Mercier et K.C. Waldron, *Analytica Chimica Acta* **2000,** *404 (1)*, 29-45.

Abstract

We describe the rapid, facile fabrication of an immobilized-protease microreactor and its characterization for peptide mapping by capillary electrophoresis (CE). A 30-cm-long fused silica capillary was dry-packed with trypsin-immobilized controlled pore glass (CPG) beads. A 40-μl solution of protein standard (β-casein or insulin chain B) was perfused through the microreactor for ca. 2 h by applying low pressure at the inlet. Digest was collected at the microreactor outlet and the tryptic fragments were separated by CE and detected by UV absorbance using a diode array detector. Tryptic peptide maps obtained by solid-phase digestion in the microreactor were compared to those obtained by traditional, liquid-phase methods to evaluate the microreactor's performance and extent of trypsin autoproteolysis (self-digestion). The effect of substrate flow rate through the packed bed of trypsin-CPG (i.e., the residence time) and effect of substrate concentration were also investigated. In general, digestion conditions in the microreactor were very reproducible, whereas the CE separation conditions were found to contribute the most to migration time variability in peptide mapping. Reproducible peptide maps were obtained for 40-μl aliquots of a 2-mg/ml β-casein solution. The activity of the immobilized enzyme was high enough to ensure reusability of this microreactor for at least 10 digestions.

Abbreviations: CPG, controlled pore glass; DITC, diisothiocyanate; BGE, background electrolyte; EOF, electroosmotic flow; f.s., fused silica; s.s., stainless steel; TFE, Teflon; SPE, solid-phase extraction; RSD, relative standard deviation.

3.1. Introduction

Peptide mapping remains a widely used tool for the identification and characterization of proteins. This procedure consists of enzymatic (proteolytic) or chemical cleavage of the protein into a number of smaller peptide fragments followed by separation and detection of the peptides. The most common analytical separation techniques used for peptide mapping are HPLC [1-3] and, more recently, capillary electrophoresis (CE) [4-7]. CE has drawn attention to itself from many fields because of its simplicity, speed, high separation efficiency and lower consumption of sample and solvent than HPLC. Regardless of the separation method, the resulting peak profile gives, for each protein, a unique fingerprint called a peptide map. The subsequent use of mass spectrometry, either coupled to HPLC [8] or CE [9], greatly enhances the ability to identify the peptide fragments and thus the parent protein.

Peptide mapping is essentially a qualitative, comparative technique. Any variation in the chromatogram or electropherogram, as subtle as the change in migration time of a single peak, is an indicator that modification or substitution of one or more amino acids has occurred [10, 11]. Hence the peptide maps of proteins can reveal small differences between two otherwise similar proteins. Applications of peptide mapping are numerous, but its utility is especially recognized in the verification of primary structure of proteins produced by recombinant DNA technology [12] and in the detection of post-translational modification [13].

Trypsin is a widely used proteolytic enzyme, commonly used for protein cleavage because of its high specificity and ability to digest insoluble or adsorbed protein. Trypsin cleaves peptide bonds at the carboxylate-terminal side of lysine and arginine residues. Essentially, proteolysis can be performed in two ways: by homogeneous or by solid-phase

digestion. The homogeneous mode relies on dissolution of lyophilized enzyme and protein substrate in a buffer, with a protein-to-enzyme ratio of 25:1, and reaction at 37°C for 24 h [14]. The solid-phase mode is achieved by passing the sample protein solution over or through a bed of enzyme that has been immobilized on a solid support [15]. The use of immobilized enzymes has several advantages. Firstly, their rate of denaturation or inactivation is less than with free enzymes, which often quickly lose their catalytic activity and can lead to a lack of reproducibility in the peptide map. Immobilized enzyme kinetics are typically faster [15] and easier to control. Also, they are well adapted to use in flow systems, thus amenable to automation. Secondly, immobilized enzymes undergo fewer secondary reactions like autoproteolysis, which contribute to background peaks in the peptide map. Thirdly, immobilized enzymes are re-usable. Finally, because the protein of interest is typically isolated and purified in small amounts from biological samples, there is need to establish a rapid, reliable technique to obtain peptide maps from nanomole and lower quantities of protein.

Immobilized RNAses and proteases have been previously used in a flow-through microreactor format [4, 15-17]. Trypsin bound to agarose gel was the first such system to receive attention in the field of CE [4, 18]. A microreactor made from a small-bore glass tube was easily filled with the gel. Unfortunately, the low backpressure tolerance of agarose gel drastically limited the flow rate of the protein solution perfused through the reactor. In an alternative microreactor design, Amankwa et al. immobilized enzyme on the inner surface of a 50-μm i.d. fused silica (f.s.) capillary via biotin-avidin-biotin coupling chemistry [19, 20]. The strong coupling constant of this method makes the immobilized enzyme structurally robust to any flow rate and enables the use of extreme pHs, temperatures and solvent systems. Unfortunately, the biotin-avidin coupling procedure is

lengthy (more than one day). Moreover, because enzyme is coated on the capillary walls, a very low flow rate (e.g., 40 nl/min) was needed to permit time for diffusion of protein sample to the immobilized enzyme. This led to an overall digestion time of 8 h [20]. Kuhr's group later found that the proteolysis reaction rate in biotin-avidin-based enzyme microreactors could be enhanced by applying low-power acoustic vibration to the capillary, with digestion carried out in a batch-wise procedure, i.e., not as a flow-through system [21, 22]. More recently with this device, efficient tryptic digestions of large proteins have been carried out in as few as 30 min [23].

The immobilization of enzyme on controlled pore glass (CPG) is an alternative solid-phase method that provides an improvement in robustness for high-pressure perfusion as well as providing a high surface area for enzyme-protein contact. Voyksner et al. used a large bioreactor of this kind (2.1 mm i.d.) but observed a lack of reproducibility in the packing [24]. Improved packing was made by Davis et al. who used smaller silica beads (10-μm particles) as solid support in a microscale protease reactor [25]. The problem in this latter case was a high degree of enzyme autoproteolysis. Nonetheless, the use of enzyme-bound beads in a microreactor is attractive for its simplicity. In particular, the packed-bed flow-through design can be easily automated. This principle has been applied using Poroszyme™ protein digestion cartridges in conjunction with HPLC-based peptide mapping [12, 26, 27]. Unfortunately, the volumes used with these cartridges are not compatible with the very small volumes encountered in CE.

In this paper, we report the fabrication and characterization of a f.s. capillary (530 μm i.d.) microreactor dry-packed with commercially available enzyme-immobilized CPG beads in which trypsin is bound via diisothiocyanate (DITC) coupling. The advantage of using a commercial immobilized enzyme product lies in the convenience of having pre-

assayed enzyme activity and product quality control. Assembly of the system used for dry-packing the microreactor was carried out in 30 min and the packing procedure itself took less than 1 min. We demonstrate the utility of this microreactor for the rapid peptide mapping of two test substrates: oxidized insulin chain B and phosphorylated β-casein. Peptide maps of 20 nmol of insulin chain B or β-casein were obtained in 100 and 150 min, respectively. These times were an improvement compared to homogeneous digestion (i.e., 24 h) or that reported using other types of enzyme microreactor (i.e., 8 h). Because peptide mapping is a comparative technique, the performance and reproducibility of the microreactor for mapping β-casein are highlighted.

3.2. Experimental Section

3.2.1. Reagents and Materials

Ammonium carbonate, sodium phosphate (monobasic and dibasic) and phosphoric acid were purchased from Anachemia (Montréal, QC, Canada). Tryptophan, β-casein (phosphorylated), insulin chain B (oxidized), TPCK-treated trypsin (activity: 11,000 units/mg), TPCK-treated trypsin attached to DITC–controlled pore glass (80–120 mesh; 700 Å average pore size; activity: 8,300 units/g), methanol and ethanol were obtained from Sigma (St. Louis, MO, USA). Pre-purified argon was obtained from Praxair (Montréal, QC, Canada). In-house distilled water was purified by passing it through a multi-cartridge Millipore water filtration/deionization system before use. Microcentrifuge tubes (1-ml and 0.6 ml) and disposable pipette tips (1-ml and 0.2-ml) were bought from Fisher Scientific (Montréal, QC, Canada). Fused silica (f.s.) capillary tubing, Teflon (TFE) tubing (1/16 in o.d. × .040 in i.d.), nylon syringe filters of 0.2-μm pore size, Wheaton 5–ml V-vials and the capillary rinse kit were obtained from Chromatographic Specialties (Brockville, Canada). A

solid–phase extraction (SPE) cartridge (VWR, Montréal, QC, Canada) was kindly donated by Dr. M. Bertrand. Swagelok™ fittings and unions were bought from Laurentian Valve & Fitting (St.-Laurent, QC, Canada). A stainless steel (s.s.) HPLC column (5 cm × 3/16 in i.d. × 1/4 in o.d.), with frits and stationary phase removed, was obtained from the University of Montréal teaching laboratory.

3.2.2. Microreactor Fabrication

The microreactor was prepared by dry–packing, a common technique used to make chromatography columns. Figure 3.1 shows the basic experimental setup for packing trypsin-DITC-CPG beads into a f.s. capillary (30 cm × 530 μm i.d. × 800 μm o.d.) under sonication provided by an ultrasonic membrane disrupter (Model 300 Dismembrator, Fisher Scientific, St.-Laurent, QC, Canada). To prevent aggregation during packing, the packing material was conditioned for 2 h with ethanol vapors by placing the beads and a few drops of ethanol into a closed tube. The s.s. HPLC column served as a reservoir for packing the trypsin–CPG beads. The bottom 1 cm of a 1-ml pipette tip was cut off and inserted into the s.s. column to funnel the beads into the microreactor capillary. The inlet (top) of the microreactor capillary was coupled to the s.s. reservoir with the aid of a reducing Swagelok union (1/4 in to 1/16 in) and a 1-cm length of Teflon tubing that was slipped over the end of the capillary (see Fig. 3.1). A drop of 5-minute epoxy (TM/MC LePage, Ontario, Canada) was used to seal the Teflon sleeve to the f.s. capillary. Similarly, a Swagelok union (1/16 in) was attached to the outlet of the capillary via a Teflon sleeve. This Swagelok union was fitted with the filter removed from the SPE cartridge in order to retain the beads during the packing procedure.

The preconditioned trypsin–CPG beads (ca. 70 mg) were put into the s.s. reservoir, which was connected to the argon source. The entire reservoir–microreactor assembly and

the ultrasonic probe were then submerged into a 6-litre container filled with water. The ultrasonic action served to increase the uniformity of the packing. An argon pressure of 20 psig was applied for 1 min under sonication to pack the capillary with ca. 50 mg of the trypsin–CPG beads. The capillary assembly was then removed from the water, disconnected from the s.s. reservoir and outlet Swagelok fitting, and the Teflon covered ends were cleaved off leaving a 28–cm long microreactor. This procedure packed the beads tightly enough to prevent them from falling out during the subsequent manipulations.

In order to provide a 37°C environment for tryptic digestion, a simple condenser was constructed in our glassblowing shop using a glass tube (26 cm × 8 mm i.d. × 10 mm o.d.) and two corks, as shown in Figure 3.2. A straight pin was used to pierce the corks and make holes through which the microreactor capillary was inserted. Transfer capillaries (inlet: 20 cm × 530 μm i.d. × 800 μm o.d.; outlet: 20 cm × 250 μm i.d. × 400 μm o.d.) were then connected to each end of the microreactor by coupling them with a Teflon sleeve and epoxy as described above.

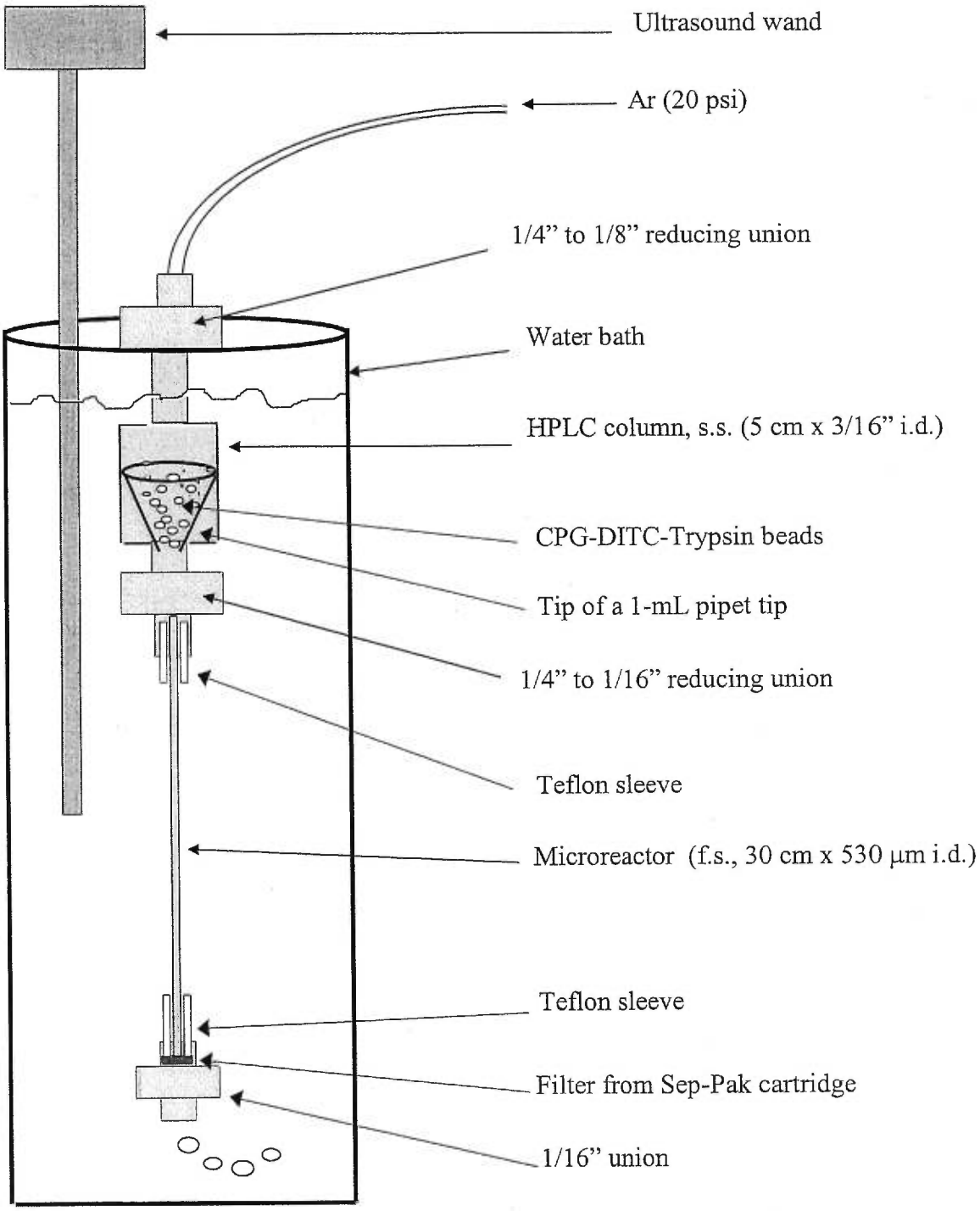

Figure 3.1. Diagram showing arrangement of the trypsin-microreactor packing system (see experimental section 3.2 for details).

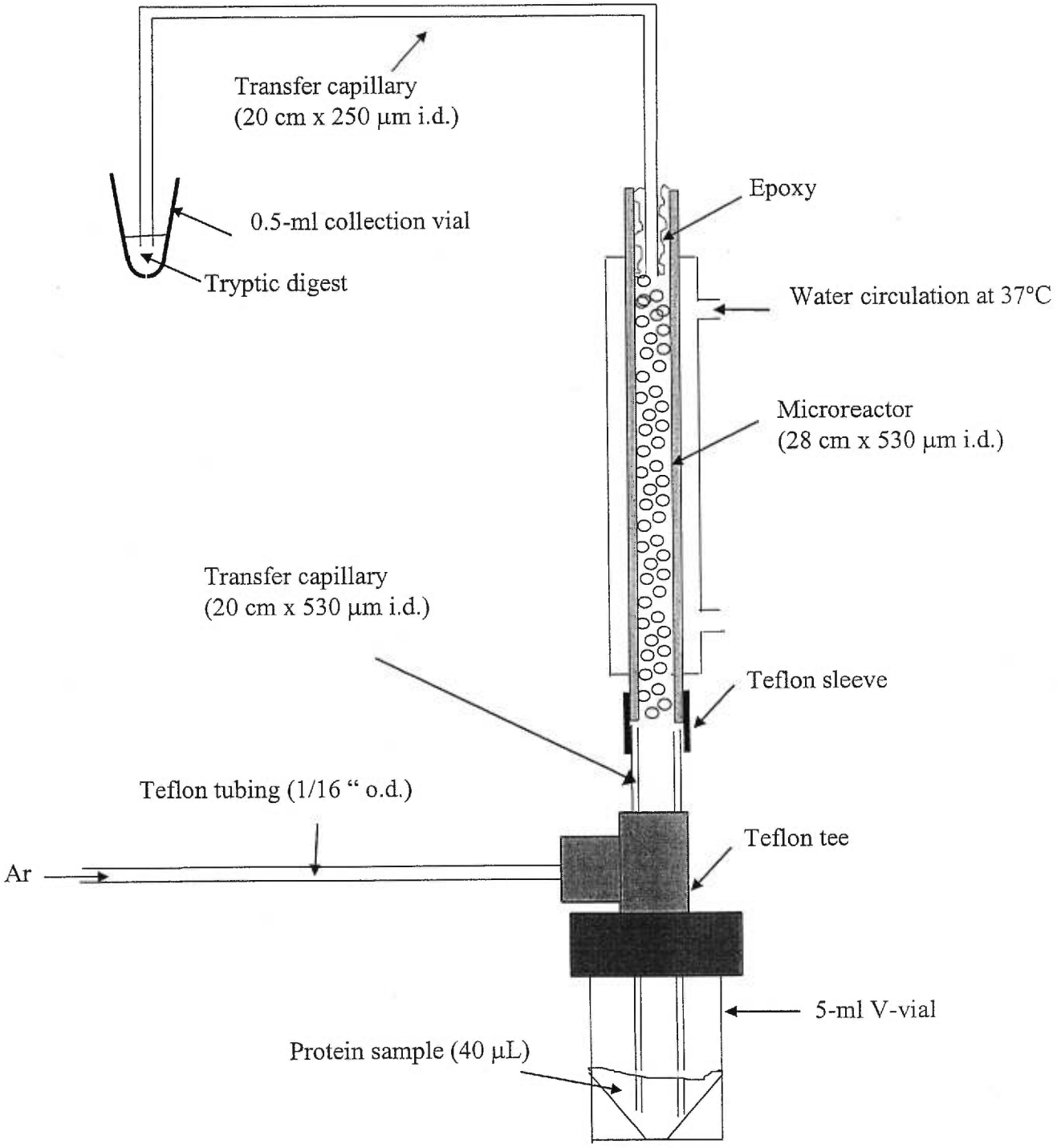

Figure 3.2. Diagram showing the set-up for digestion in the immobilized-trypsin microreactor (see experimental sections 3.2.2 and 3.2.3 for details).

A few beads were removed from the outlet (top) of the microreactor so that the transfer capillary could be inserted about 1 mm. The inlet (bottom) transfer capillary was connected to the capillary rinse kit, which consisted of a 5–ml vial fitted with a tee, a capillary tube adapter and a gas pressurization line. We substituted the rinse kit vial with a Wheaton V-vial that was conical inside. The outlet (top) transfer capillary was inserted into a 0.6-ml microcentrifuge tube (through a hole in the cap) for collection of the digest. The effective (heated) length of the microreactor was 26 cm. A 1.5-litre circulating water bath (Messgeräte–Werk Lauda, Germany) was used to control the temperature of protein digestion.

3.2.3. Tryptic Digestion in the Microreactor

The conditions for tryptic digestion was chosen based on the procedure of Matsudaira [14]. A buffer solution of 70 mM ammonium carbonate, pH 8.2, was prepared in purified water. About 100 μl of this buffer was put into the Wheaton V-vial for rinsing the microreactor before digestion and for measuring the flow rate. Perfusion of buffer through the microreactor was controlled by adjusting the pressure of Ar (< 1 psig) inside the vial using a 0-30 psig regulator. The flow rate was calibrated by weighing, and correcting for density, the volume of buffer collected in the microcentrifuge tube during the first 30 min of applied pressure. After rinsing, the microreactor was flushed with argon for ca. 15 min to remove the rinsing solution. Protein samples (insulin chain B or β-casein) were dissolved in digestion buffer at concentrations ranging from 0.5 to 5 mg/ml and 0.2 mM tryptophan was added as an internal standard. A 40–μl aliquot of sample was put into the Wheaton vial and perfused through the microreactor at the same pressure (flow rate) used for rinsing. The digest was collected (~35 μl, determined by its mass) and analyzed by CE.

3.2.4. Homogeneous Tryptic Digestion

Tryptic digestion was performed following the procedure of Matsudaira [14] with slight modification. Protein sample was incubated at 37°C with TPCK-treated trypsin (enzyme:protein = 1:25) in 100 µl of 100 mM ammonium carbonate, pH 8.2, for 24 h without stirring. Tryptophan (0.2 mM) was added to the protein/trypsin solution before digestion to serve as an internal standard. The digest was analyzed directly by CE.

3.2.5. CE Separations

Separations were performed on a HP^{3D}CE Instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array detector. All separations were performed in f.s. capillary of 50 µm i.d., 400 µm o.d. Total capillary length was 48.5 cm with an effective length of 40 cm. Background electrolyte (BGE) separation buffers were prepared in purified water and the pH was adjusted by altering the ratio of monobasic sodium phosphate to phosphoric acid. All BGEs were passed through a 0.22–µm pore size syringe filter prior to filling the buffer reservoirs in the HP^{3D}CE instrument. The capillary was rinsed with 0.1 M NaOH at 1 bar for 2 min followed by a BGE rinse (2 min) prior to sample analysis. Injection was accomplished by application of 50 mbar for 5 s at the inlet end of the capillary. Detection[2] was achieved on–column at 8.5 cm from the outlet by burning off the polyimide coating with a gentle flame.

3.3. Results and Discussion

3.3.1. Microreactor Fabrication and Operation

Using the set-up described in Fig. 3.1, a capillary-based trypsin microreactor could be constructed in 30 min. The resulting 28-cm-long microreactor contained ca. 50 mg

[2] Detection was made at 200 nm.

typsin-DITC-CPG beads and about 26 cm of its length was heated by water circulation to 37°C, the optimum temperature for tryptic digestion [14]. This procedure has the advantage of being inexpensive, simple and fast compared to the method of immobilizing enzyme to the capillary walls via biotin-avidin chemistry, which takes more than one day of coupling and conditioning before the microreactor can be used [19, 20]. Shorter microreactors having lengths of 10-cm and 15-cm (effective heated portion) were also made and tested, but very poor peptide maps[3] resulted indicating that an insufficient quantity of enzyme was present in these devices[4].

Rapid digestion in the 28-cm-long microreactor was achieved with a simple, flow-through system in which protein solution was perfused through the microreactor by low pressure (< 1 psig) argon (Fig. 3.2). The extent of enzymatic digestion taking place was first evaluated by comparing peptide maps of the microreactor products (i.e., solid-phase digestion) to the products obtained from homogeneous (i.e., liquid-phase) tryptic digestion. We expected the maps to be similar for both cases, with slightly more peaks appearing in the liquid-phase case due to autoproteolysis of trypsin, whose sequence is given in Fig. 3.3A. To this end, a large protein (phosphorylated β-casein, Fig. 3.3B) and a small protein (insulin chain B, Fig. 3.3C) were chosen as test substrates to compare performance of the enzyme microreactor to homogeneous digestion.

3.3.1.1. Beta-casein

β-Casein is a well-characterized, highly hydrophilic phosphoprotein [28], expected to yield fourteen peptides and two free amino acids upon cleavage by trypsin as shown in

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[3] Fewer and smaller peaks were seen.

[4] Reducing the amount of protein to make it compatible with the reduced quantity of enzyme would have sacrified detection sensitivity.

Fig. 3.3B [2]. Figs. 3.4A and B show the peptide maps of products from liquid-phase and solid-phase digestion, respectively, of 5 mg/ml β-casein. The peak marked "W" corresponds to tryptophan, the internal standard added prior to digestion for normalization of CE migration times[†]. Peaks labeled with the same numbers in Figs. 3.4A and B indicate identical UV spectra based on diode array data, thus identical peptides, which confirmed the similarity of the two maps for migration times up to 20 min. At migration times greater than this, the identification became much more difficult because all these peaks had essentially the same UV spectra. Overall, the microreactor was able to produce in 2½ h a digest similar to that carried out in liquid-phase in 24 h. In both cases, the overall enzyme activity was more-or-less the same, 220 units for liquid-phase versus 360 units for solid-phase, considering that the required mole ratio of enzyme to substrate should be 1:25 [14]. A blank solid-phase digestion (i.e., no β-casein), shown in Fig. 3.4C, suggested that autoproteolysis of immobilized trypsin did not contribute significantly to the peptide map obtained using the microreactor.

Only 14 peptides were expected for β-casein based on its primary structure. However, more than 26 peaks could be counted in the maps obtained by both methods of digestion. Some of these excess peaks in the case of homogeneous digestion (Fig. 3.4A) arose from trypsin autoproteolysis[5].

[†] The following equation was used to normalize migration times to an electropherogram randomly chosen as the standard: $t' = (1/t'_W - 1/t_W + 1/t)^{-1}$, where t' is the normalized migration time; t'_W is the migration time of W in the standard electropherogram; t_W and t are the migration times of W and the tryptic peptide, respectively, in the electropherogram to be normalized.

[5] In preliminary studies, blank liquid phase digestion showed extra peaks arising from trypsin autodigestion.

(A) Trypsin sequence

VDDDDKIVGGYTCGANTVPYQVSLNSGYHFCGGSLINSQWVVSAAHCYKSGIQVR

LGEDNINVVEGNEQFISASKSIVHPSYNSNTLNNDIMLIKLKSAASLNSRVASISLPT

SCASAGTQCLISGWGNTKSSGTSYPDVLKCLKAPILSDSSCKSAYPGQITSNMFCAG

YLEGGKDSCQGDSGGPVVCSGKLQGIVSWGSGCAQKNKPGVYTKVCNYVSWIKQ

TIASN

(B) β-Casein sequence and theoretical tryptic cleavage sites

R ELEELNVPGEIVESLSSSEESITR INK K IEK FQSEEQQQTEDELQDK IHPFAQT
↑ ↑ ↑↑ ↑ ↑
QSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK VK EAMAPK HK
↑↑ ↑ ↑
EMPFPK YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSV
↑
LSLSQSK VLPVPQK AVPYPQR DMPIQAFLLYQEPVLGPVR GPFPIIV
↑ ↑ ↑ ↑

(C) Insulin chain B sequence and theoretical tryptic cleavage sites

FVNQHLCGSHLVEALYLVCGER GFFYTPK A
(P1) ↑ (P2) ↑

Figure 3.3. Primary structures of (A) the proteolytic enzyme trypsin, (B) β-casein and (C) insulin chain B, oxidized. Arrows indicate the expected (theoretical) tryptic cleavage sites.

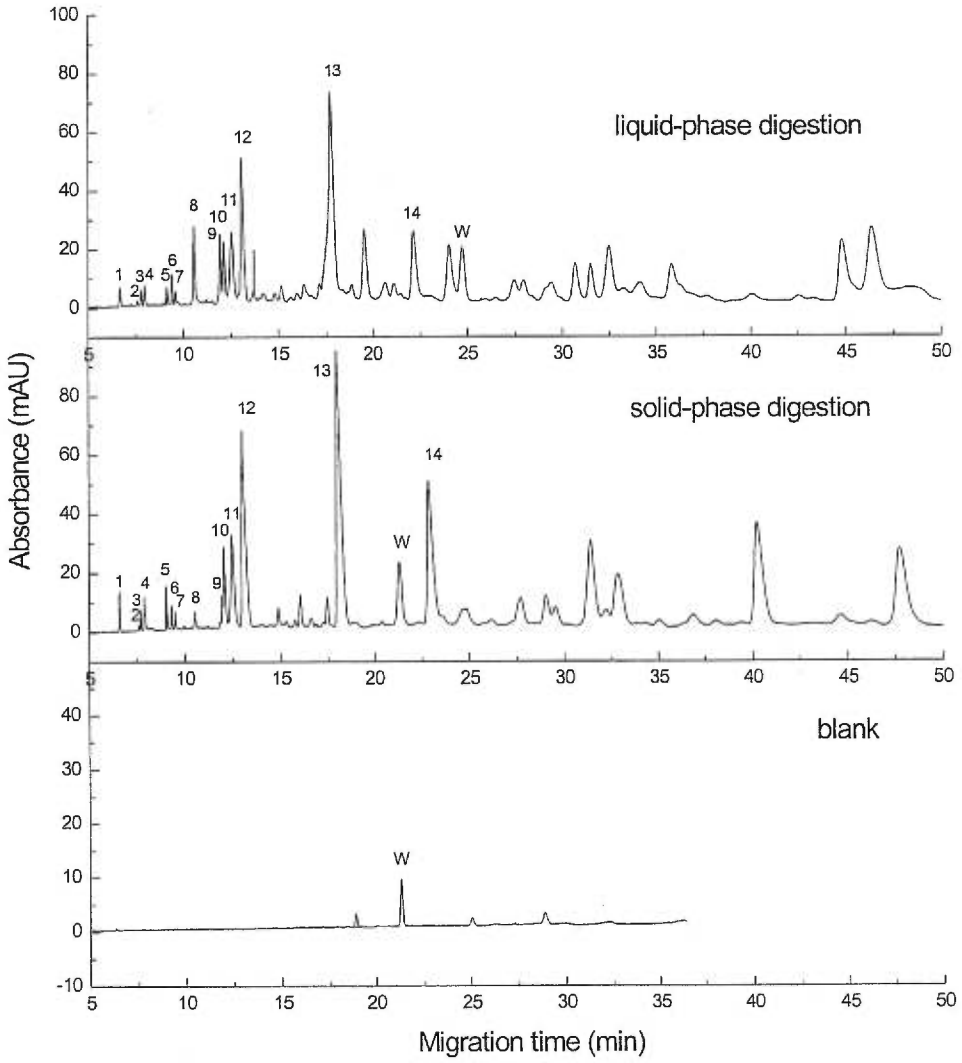

Figure 3.4. Electropherograms showing the peptide maps of (A) a homogeneous digestion of 5 mg/ml β-casein solution, (B) a solid-phase digestion in the microreactor of 5 mg/ml β-casein and (C) a blank solid-phase digestion with no β-casein. In the latter two cases, perfusion of the protein and blank solution were made at 0.15 μl/min initial flow rate. Separations were carried out in 50 mM sodium phosphate buffer, pH 2.5, at 15 kV. Injections were made at 50 mbar for 5 s. Peaks labeled with the same number in both electropherograms indicate identical UV spectra from the diode array data. The peak labeled "W" indicates the internal standard (tryptophan) added prior to digestion.

It is important to point out that autoproteolysis of free trypsin (cleavage at R and K residues) differs from that of immobilized trypsin, which is attached to the DITC-CPG at its N-terminal α-amino group and at each lysine ε-amino group. Therefore, only cleavage at arginine will produce fragments that contribute to autoproteolysis peaks in the peptide map[6].

Another contribution to the excess peaks may arise from incomplete digestion of β-casein. In fact, trypsin hydrolyses arginyl and lysyl peptides bonds at variable rates. For example, the presence of an acidic residue (i.e., D or E) following R or K lowers the rate of hydrolysis [2, 29] and there are three such sequences in β-casein. This could account for the failure to completely hydrolyze the protein, leading to multiple partial digestion products and, thus, extra peaks. In addition, the 7th tryptic fragment of β-casein contains an asp-ser bond susceptible to hydrolysis under certain conditions, which would lead to two peptides more than expected [2]. A third contribution to excess peaks may be the presence of several isoforms of β-casein leading to several superimposed peptide maps [30]. Finally, the fairly high concentration of β-casein used (i.e., 5 mg/ml) may have been a contributing factor to the presence of undigested peptides. Despite the large difference in digestion times for the two methods, the electropherograms in Figs. 3.4A and B suggest that the solid-phase digestion was at least as efficient as the liquid-phase digestion because the peaks were slightly larger in the former case.

Assignment of peaks in the β-casein peptide maps was impossible because peptide standards for the fragments from either β-casein or trypsin were not readily available[7].

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[6] It is rather unlikely that all the lysine residues would be involved in binding to the beads. However, this binding should reduce the number of peptide fragments coming from autoproteolysis.

[7] In fact, assignments of peaks could have been done by mass spectrometry.

Therefore, evaluation of the true extent of digestion was impracticable to quantify. On the other hand, only two peptides (P1 and P2, see Fig. 3.3C) should result from digestion of the smaller test protein, insulin chain B, and these fragments could be identified by their peak spectra. Therefore, a better evaluation of the extent of solid-phase relative to liquid-phase digestion could be made using insulin chain B as a substrate, keeping in mind that proteolysis of such a small protein is facile compared to β-casein.

3.3.1.2. Insulin chain B

Fig. 3.5 shows electropherograms of oxidized insulin chain B before digestion (Fig. 3.5A), its peptide map after liquid-phase digestion for 120 min (100 μl at 0.75 mg/ml, Fig. 3.5B) and after solid-phase digestion for 100 min (40 μl at 0.75 mg/ml, perfused through the microreactor at an initial flow rate of 0.2 μl/min, Fig. 3.5C). The impurity peak in Fig. 3.5A, which was present in the lot of lyophilized protein standard used, was suspected to be a modified form of insulin chain B.

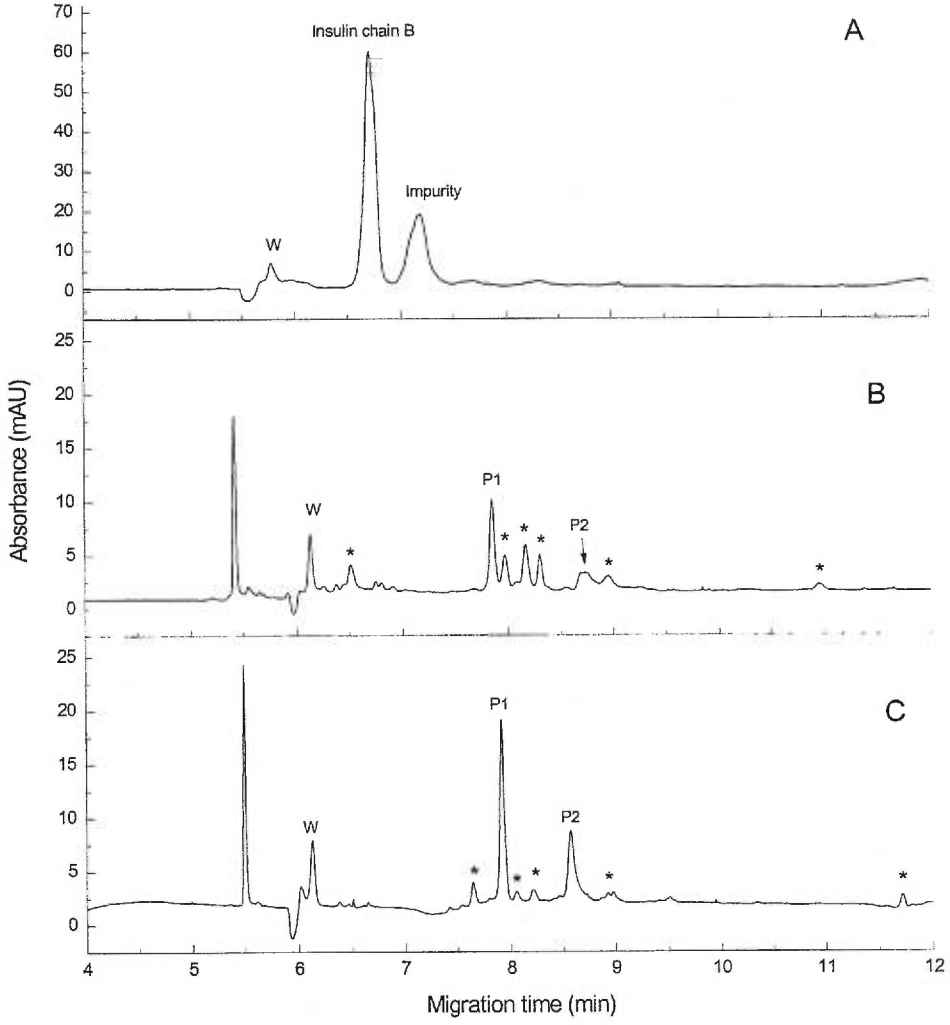

Figure 3.5. Electropherograms showing the protein standard (A) insulin chain B, oxidized (1.5 mg/ml) before digestion and its peptide maps after (B) homogeneous digestion with trypsin and (C) solid-phase digestion in the microreactor with perfusion of protein solution made at 0.2 μl/min (initial rate) through the microreactor. Separations were carried out in 50 mM sodium phosphate buffer, pH 6.9, at 13 kV. Injections were made at 25 mbar for 5 s. Peptide fragments P1 and P2 correspond to those in Fig. 3.3C. Peaks labeled with asterisks are unidentified background and autoproteolysis products.

In both the homogeneous and solid-phase cases, the peaks for insulin chain B and its impurity disappeared from the electropherogram, suggesting that both were totally digested. Therefore, digestion in the microreactor was deemed at least as efficient as liquid-phase (homogeneous) digestion with trypsin. Peaks marked with asterisks in Figs. 3.5B and C were attributed to either trypsin autoproteolysis products or partial digestion products. For example, if cleavage of some molecules at only lysine occurred, a large fragment equivalent to P1 + P2 would result. Similarly, cleavage at only arginine would produce a fragment equivalent to P2 + alanine. These events would generate two peaks to those expected. The unidentified peak at 5.4 min in Figs. 3.5B and C corresponds to a cationic species eluting before the electroosmotic flow (EOF), which may have come from digestion of the insulin impurity. Evidence for this was based on the absence of this peak when a particular lot of insulin chain B lacking the impurity was digested (data not shown).

In comparing liquid-phase and solid-phase (i.e., microreactor) digestion of insulin chain B, less ambiguous identification of the peptides could be made in the latter case even though a small background of non-specific peaks still existed. These results, and those using β-casein, convinced us that the immobilized enzyme microreactor was digesting substrate as well as or better than the traditional, liquid-phase method of proteolytic digestion. All further studies were carried out using β-casein, a more realistic substrate to characterize the microreactor and evaluate peptide mapping reproducibility.

3.3.2. Factors affecting microreactor performance for β-casein

3.3.2.1. Perfusion flow rate

Clearly, substrate residence time in the microreactor affects the extent of digestion.

Therefore, β-casein was perfused through the microreactor at various flow rates to see the effect on peptide mapping. As described in experimental section 3.2.3, flow rate was calibrated during the microreactor rinse step and is thus equivalent to the perfusion rate of sample measured during the initial 30 min of digestion. Since the flow rate during the final 30 min of digestion is fast because of reduced resistance to flow in the microreactor and transfer capillaries, the average flow rate is always higher than the reported initial flow rate.

At 0.25 μl/min initial flow rate, only 9 peaks were seen, all of which had low signal intensities. By decreasing the initial flow rate to 0.17 μl/min, the number of peaks increased to 23, as did the heights of these peaks. Further decreasing the flow rate of substrate through the microreactor to 0.15 μl/min produced 26 peaks, about half of which were more intense than those obtained at 0.17 μl/min. Digestion of the sample protein by trypsin relies on the sufficient contact time between the enzyme and substrate. In a flow-through system, this time is primarily governed by two modes of mass transfer: diffusion and convection. At low flow rates, these two modes are cooperative, so proteolytic digestion is optimized. Further decreasing the initial flow rate below 0.15 μl/min provided little or no enhancement in the appearance of peptide maps and simply increased the overall time required to digest 40 μl of sample. Therefore, this flow rate was employed in subsequent studies unless otherwise indicated.

3.3.2.2. Sample concentration

The effect of initial protein sample concentration on peptide map reproducibility is shown in Fig. 3.6 for concentrations of β-casein ranging from 0.5 to 5 mg/ml. Digestions were carried out on the same microreactor starting with the lowest sample concentration. Each of the maps in Fig. 3.6 has a similar appearance for the first 25 min with the exception of the internal standard, W, in Fig. 3.6A. Assignment of W was confirmed by its UV

spectrum. Early elution of this peak was reproducible in all digestions of 5 mg/ml β-casein but the reason for this was unknown[8]. For CE separation at pH 2.5, the peaks at migration times longer than 25 min are probably large peptides that come from incomplete digestion or from autoproteolysis of the aging immobilized trypsin. Despite the low background observed in the blank (Fig. 3.4C), autoproteolysis is irreproducible and comparison of electropherograms for migration times greater than 25 min was not reliable. In addition, separations in Fig. 3.6 were not carried out on the same day, which may account for the large variation in migration times > 25 min as shown in section 3.3.3.

Calibration curves of peak area as a function of β-casein concentration were made for the peaks labeled 1, 2 and 3 in Fig. 3.6 to verify that digestions were independent of concentration in the range studied. These curves, presented in Fig. 3.7, showed good linearity (correlation coefficients > 0.98) and confirmed to us that the extent of digestion in the microreactor was not a function of substrate concentration for 0.5 to 5 mg/ml β-casein. Although peptide fragment sensitivity varied widely (slopes of 66, 145 and 208 mAU^2/mg/ml for peaks 1, 2 and 3, respectively), digestions were complete[9] for the concentrations studied. The fact that 1 to 2 mg/ml appears, in Fig. 3.6, to be the minimum β-casein concentration required for reliable peptide mapping is founded in the poor detection limits for CE with UV absorbance. Sample and product losses due to handling during the digestion and mapping steps, as discussed below, contribute to the ca. 50 μM (1 mg/ml) limit of sample protein necessary for accurate peptide mapping.

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[8] The shift of W to shorter migration time was intially thought to be a function of protein concentration, but the liquid-phase digestion (Fig. 3.4) was also made at 5 mg/ml β-casein and W did not elute early in this case. Complexation with a peptide is unlikely because this would have been caused the UV spectrum of the W peak to be modified, which was not seen.

[9] Digestions were in fact assumed to be complete because of the excellent linearity in peptide fragment (product) peak area as a function of protein (substrate) concentration.

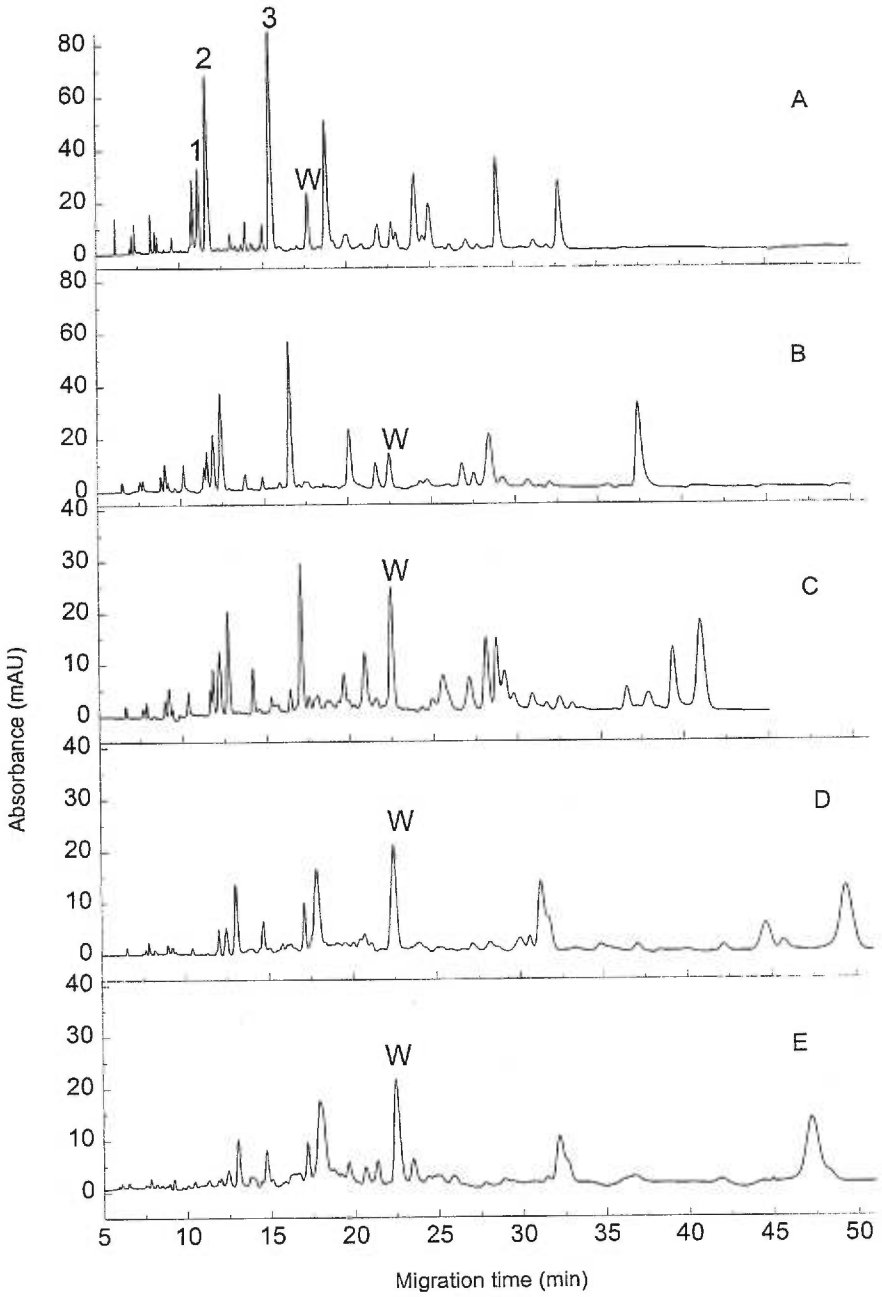

Figure 3.6. Electropherograms comparing peptide maps of digests obtained from the microreactor for various initial concentrations of β-casein sample: (A) 5 mg/ml, (B) 3 mg/ml, (C) 2 mg/ml, (D) 1 mg/ml and (E) 0.5 mg/ml. Perfusion of the protein solutions was made at 0.15 μl/min (initial rate). Separation conditions were the same as in Fig. 3.4. Peaks labeled 1, 2 and 3 refer to the graphs in Fig. 3.7.

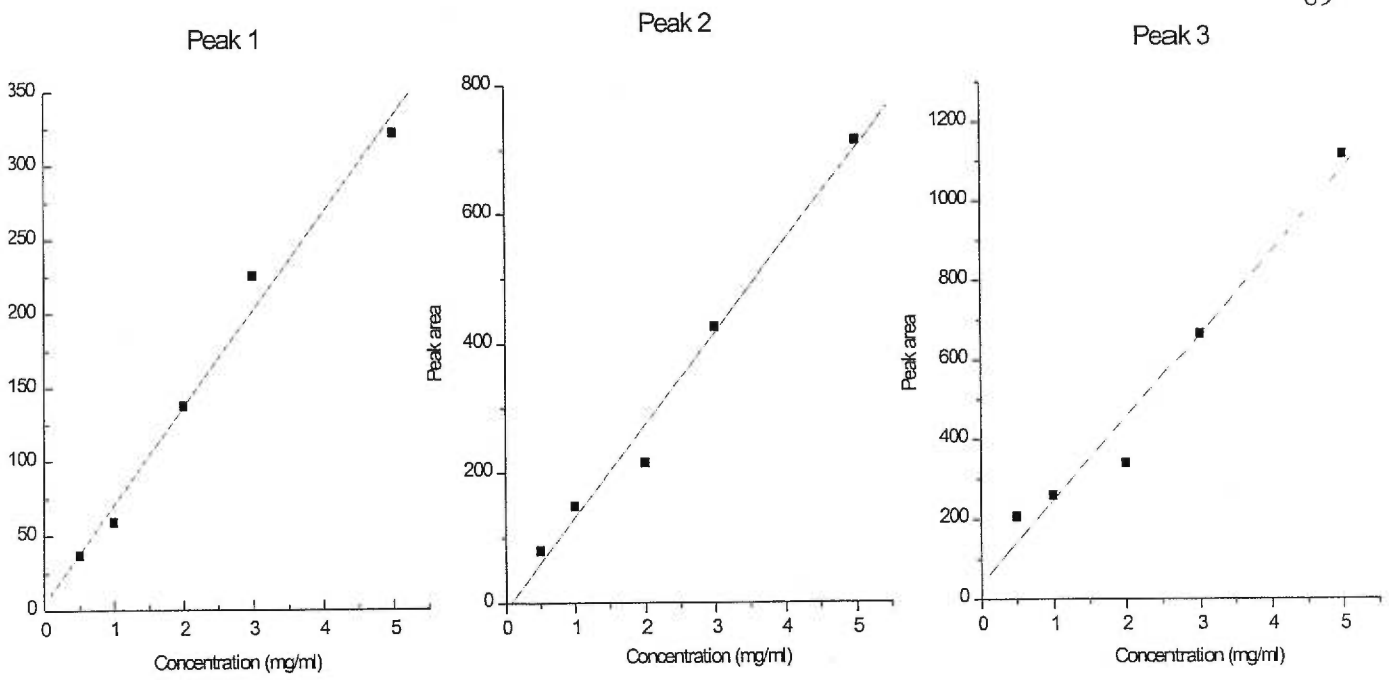

Figure 3.7. Graphs showing peak area as a function of β-casein concentration for peaks labeled 1, 2 and 3 in Fig. 3.6. Linear regression correlation coefficients of the calibrations curves are as follows: peak 1, R = 0.993; peak 2, R = 0.992; peak 3, R= 0.984.

3.3.2.3. Sample loss during digestion

As mentioned in the experimental section, only 35 µl of product was recovered after digestion, representing a 12% loss of sample in the microreactor. We believe that the pores of the solid support (CPG beads), the interstitial spaces between these loosely packed beads and adhesion to the inner walls of the 5-ml sample vial were jointly responsible for the unrecovered 5 µl. In fact, the microreactor dead volume is ca. 20 µl, because the beads are packed so loosely, and the transfer capillaries have a combined volume of 55 µl. Therefore, volume losses during sample perfusion through the system are not that surprising.

To investigate whether non-specific binding in the column was occurring, i.e., retention of certain peptide fragments by adsorption on the beads or microreactor walls, we mapped sequential fractions of the digest exiting the microreactor. A very low initial flow rate of β-casein solution was used (0.1 μl/min) to exaggerate conditions under which adsorption might occur. Fig. 3.8A shows the peptide map of the initial 11 μl of digest collected from the microreactor after 2½ h. Fig. 3.8B shows the peptide map of the next 7 μl of digest collected 75 min later, and so on until no more solution eluted from the microreactor. The last 9 μl were collected in only 8 min because this volume was already in the outlet transfer capillary and negligible back pressure led to a very fast flow rate.

The four electropherograms in Fig. 3.8 had more-or-less the same peak pattern, with additional peaks appearing in the first peptide map. If there had been non-specific adsorption of either sample protein or certain peptides inside the microreactor, we would have expected to see fewer peaks and peaks of lower intensity in the fraction that eluted first (Fig. 3.8A) compared to later eluting fractions. This phenomenon is not uncommon in CE and HPLC, where a protein has to be injected several times before it can be detected at the end of the column. Therefore, the results in Fig. 3.8 suggest that retention of protein sample or peptide fragments by non-specific interaction with either the trypsin-CPG beads or the capillary was negligible. Similar results were reported by Ronnenberg et al. who used a much larger immobilized-enzyme reactor and a dextrose gel-based solid support instead of CPG [17]. Adsorption on the microreactor wall and in the transfer capillaries was expected to be negligible anyway because at pH 8 the capillary walls and peptides are negatively charged and repel each other. Further experiments to quantify of of digest recovery in the microreactor are underway in our laboratory.

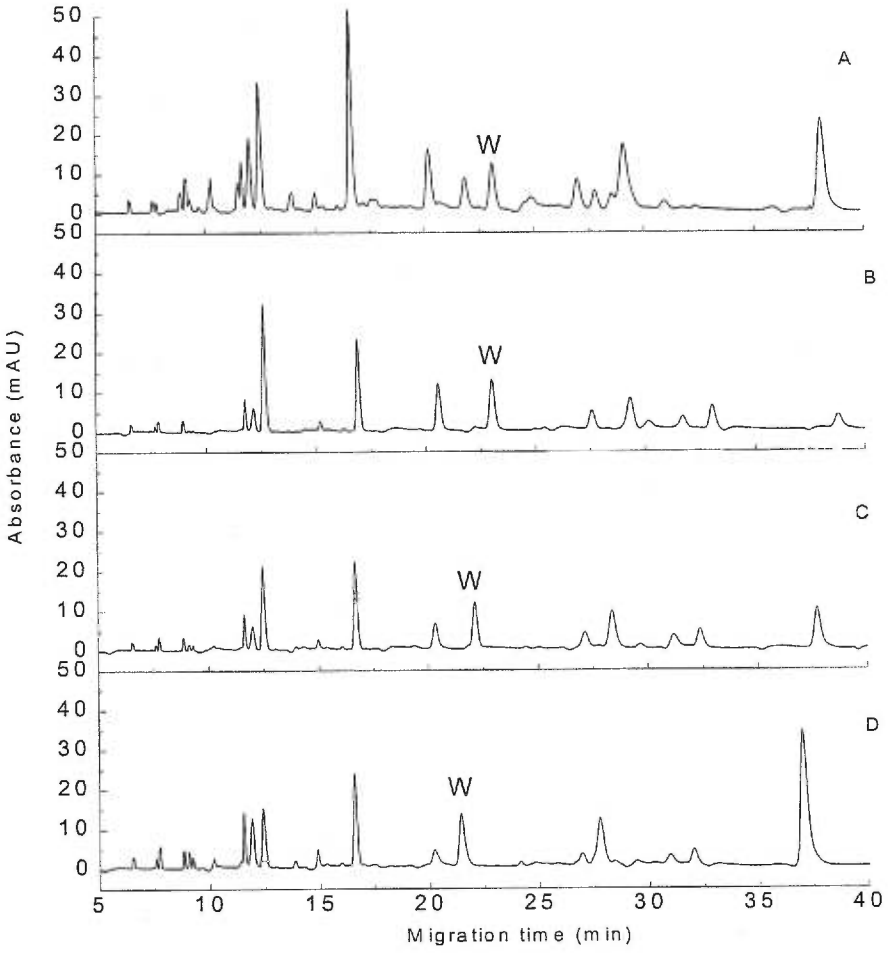

Figure 3.8. Electropherograms comparing fractions of the same β-casein (3 mg/ml) digest eluting sequentially from the microreactor. Fractions were as follows: (A) 11 μl of digest collected after 150 min, (B) 7μl of digest collected 75 min later, (C) 8 μl collected 75 min later and (D) 9 μl collected 8 min later. The initial perfusion rate of the protein solution was 0.1 μl/min. Separation conditions were the same as in Fig. 3.4.

In all experiments performed with the microreactor, the volume of protein sample was 40 μl. Clearly, using half this amount (i.e., 1 nmol β-casein) would have resulted in tryptic digestions taking less than 1 h. However, considering the 5-μl loss during digestion, initial sample volumes of less than 20 μl would have made manipulation of the microreactor products difficult. Most CE instruments require at least 20 μl of sample for injection, even though only 10 nl of this is analyzed. In experiments where sample volume was greater that 40 μl, much longer digestion times were encountered without improvement in the peptide maps.

3.3.3. Reproducibility of peptide maps for β-casein

3.3.3.1. Stability of separation conditions

Since peptide mapping is a technique based primarily on comparison of electropherograms or chromatograms, a high degree of migration time reproducibility is required of the separation method employed. To test the stability of our chosen CE separation conditions, three analyses of the same 40-μl digest (2 mg/ml β-casein solution) were performed over a period of 24 h (Fig. 3.9). The separation capillary was uncoated and we did not make attempts to try to suppress the EOF, which was very low at the working pH of 2.5. At this pH, the capillary wall was essentially neutral and the peptide fragments were positively charged, minimizing interaction between the two. The absence of peak tailing suggested that peptides did not adsorb on the capillary wall. Visual inspection of the three electropherograms in Fig. 3.9 show good migration time reproducibility for peptides eluting before 25 min, after which time map comparison became difficult.

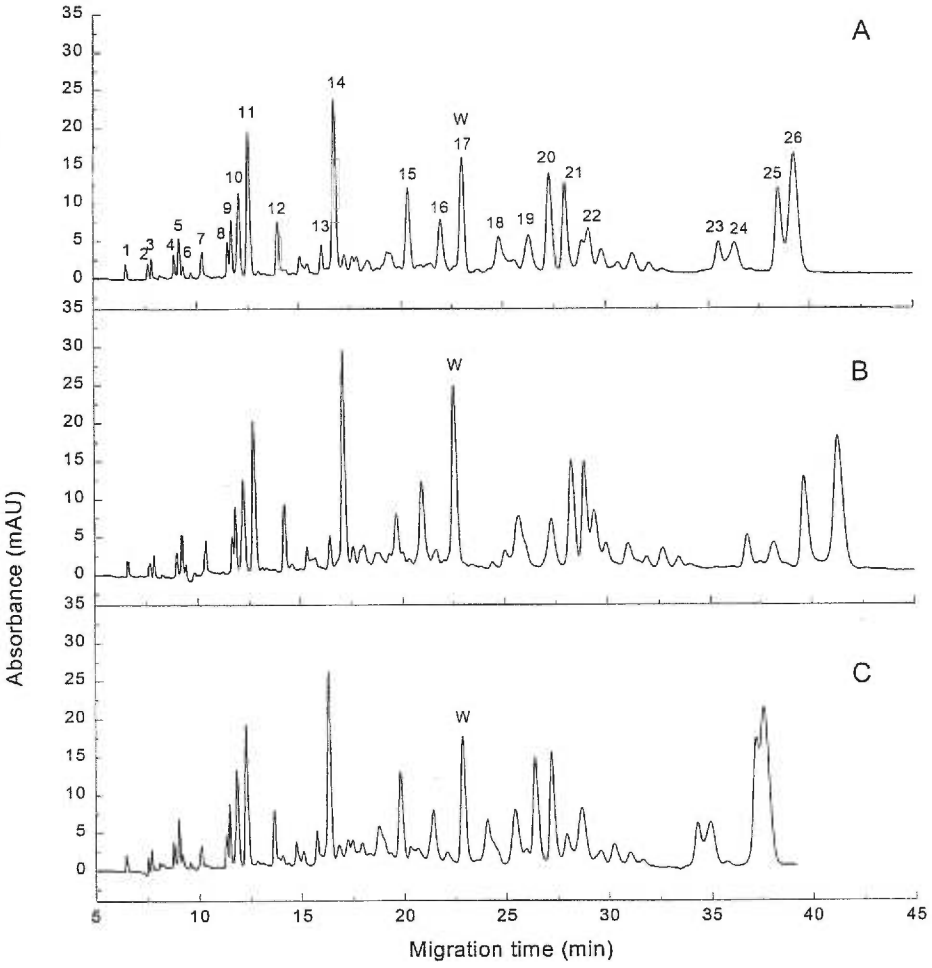

Figure 3.9. Electropherograms showing the reproducibility of separation conditions over a 24-h period by comparing peptide maps of the same β-casein digest. CE separations were carried out at (A) 10 a.m. on day 1, (B) 5 p.m. on day 1 and (C) 11 a.m. on day 2. Perfusion of the protein solution (40 μl, 2 mg/ml β-casein) was made at an initial rate 0.15 μl/min. Separation conditions were the same as in Fig. 3.4. Numbered peaks correspond to those in Table 2-1.

Table 2-1.

Table 3.1. Reproducibility of CE separation conditions in peptide mapping for a given β-casein digest

Peak number [a]	A [b] (min)	B (min)	C (min)	Mean migration time (min)	RSD of migration time (%) [c]
1	6.56	6.61	6.48	6.55	1.00
2	7.63	7.64	7.56	7.61	0.96
3	7.82	7.9	7.73	7.81	1.33
4	8.88	8.36	8.76	8.66	1.18
5	9.13	9.21	9.04	9.12	1.38
6	9.33	9.41	9.21	9.31	1.22
7	10.29	10.38	10.07	10.24	1.39
8	11.50	11.63	11.27	11.46	1.58
9	11.68	11.81	11.44	11.64	1.52
10	12.06	12.18	11.84	12.02	1.61
11	12.52	12.72	12.24	12.49	1.74
12	13.92	14.23	13.78	13.97	2.04
13	16.11	16.49	15.72	16.10	2.25
14	16.74	17.09	16.35	16.72	2.29
15	20.31	19.67	19.83	19.93	1.69
16	21.97	20.58	21.43	21.32	2.36
17	23.00	22.52	22.82	22.78	1.06
18	24.84	25.62	24.12	24.86	3.26
19	26.26	27.26	25.37	26.29	3.52
20	27.28	28.25	26.40	27.31	3.39
21	28.03	28.88	27.26	28.05	3.02
22	29.19	29.43	28.68	29.1	1.25
23	35.47	36.82	34.34	35.54	3.55
24	36.27	38.10	34.91	36.42	4.43
25	38.43	39.63	37.19	38.41	3.17
26	39.17	41.30	37.53	39.3	4.74

[a] Peak numbers correspond to those labeled in Fig.3.9.

[b] A: Electropherogram A

[c] For three separations of the same sample (digest) made within a 24-h period.

Table 3.1 shows the migration time reproducibility for peaks numbered in Fig. 3.9. A maximum RSD of 4.3 % for the three analyses was higher than expected, although the long time between separations should be taking into consideration. In addition, the resolution of peak pairs 23/24 and 25/26 varied considerably in the three maps presented in Fig. 3.9. For peptide mapping, reproducibility in migration time is more important than peak height or area, which gave RSD values ranging from 1.9 to 26 % and 2.5 to 31 %, respectively. These results were disappointing, particularly since care was taken to store the digest at 4°C between runs and to ensure it was brought to room temperature before injection into the CE system. Percent RSD did not improve when either peak heights or areas were normalized to tryptophan, the internal standard.

The presence of 26 peaks indicates that the digestion was either incomplete, sample impurities were present, or that β-casein isoforms were present. For these experiments, the protein standard was used as is, without further purification before digestion. Clearly, these problems make comparison of maps difficult when, for example, confirmation of a modified protein relies on detecting a change in migration time of only one peak. The possible presence of impurities or isoforms underlines the fact that protein purity should be verified to make sure a unique sample is being mapped.

Deviations in migration time typically originate from changes in buffer pH and, therefore, EOF. Although a low pH buffer (i.e., low EOF) and temperature-controlled environment were used for these experiments, migration time variability might be further minimized by adding a buffer replenishment step before every run. Unfortunately, these measures do not address the poor reproducibility in peak height and area observed in Fig. 3.9. As the next section shows, reproducibility in migration times between peptide maps of the same standard protein was improved by carrying out CE separations within 3 h instead

of 24 h.

3.3.3.2. Microreactor reproducibility

The reproducibility of tryptic digestion in a given microreactor was tested by comparing peptide maps of three different digestions made on the same device. In each case, 40 μl of a 2 mg/ml β-casein solution was perfused through the microreactor. The three digestions were carried out over a period of several days and the collected microreactor products were stored at –20°C until they could be analyzed by CE. The electropherograms presented in Fig. 3.10 were run consecutively on the same day to minimize migration time variability arising from the CE separation procedure.

By visual inspection of the three peptide maps in Fig. 3.10, those in B and C are clearly more alike than A. Quantitatively, Table 3.2 shows that the reproducibility in migration times was good, with an RSD of < 3 % over the entire elution window and an RSD of < 2 % for peaks eluting before 23 min. On the other hand, peak heights and areas showed very poor reproducibility, with RSD values ranging from 1.3 to 47 %. Considering the results obtained above (Fig. 3.9 and Table 3.1), migration time reproducibility was greatly improved for separations carried out sequentially as opposed to over a 24-h period.

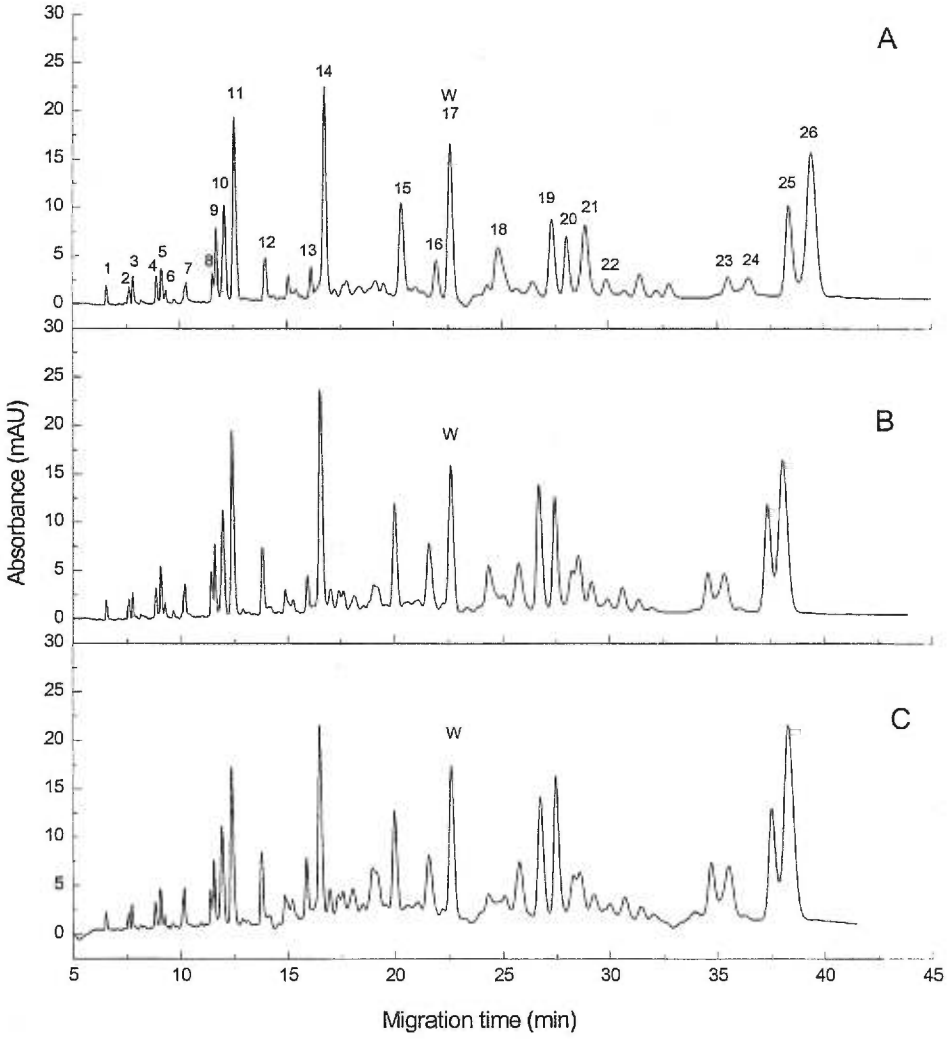

Figure 3.9. Electropherograms showing the reproducibility of digestion in a given microreactor by comparing the peptide maps from three protein samples. Three different β-casein solutions (40 µl at 2 mg/ml) were perfused through the same microreactor at an initial flow rate of 0.15 µl/min and the collected digests were stored at –20°C. CE separations were carried out sequentially using the conditions described in Fig. 3.4. Numbered peaks correspond to those in Table 3.2.

Table 3.2. Reproducibility of a trypsin microreactor for digestion of β-casein with peptide mapping by CE.

Peak number [a]	A(min)	B (min)	C (min)	Mean time (min)	RSD of migration time (%) [b]
1	6.58	6.54	6.48	6.53	0.71
2	7.59	7.60	7.55	7.58	0.73
3	7.80	7.81	7.70	7.77	0.73
4	8.87	8.87	8.76	8.83	0.85
5	9.08	9.19	9.03	9.1	0.87
6	9.40	9.35	9.15	9.3	0.89
7	10.25	10.30	10.09	10.21	1.02
8	11.42	11.47	11.26	11.38	1.10
9	11.68	11.74	11.37	11.60	1.09
10	12.06	12.06	11.89	12.00	1.13
11	12.53	12.48	12.28	12.43	1.13
12	14.08	13.97	13.70	13.91	1.35
13	16.09	16.09	15.72	15.96	1.51
14	16.83	16.79	16.35	16.66	1.52
15	20.34	20.29	19.75	20.13	1.76
16	22.04	21.94	21.30	21.76	1.82
17	22.63	23.00	22.31	22.65	1.59
18	24.56	24.85	23.05	24.15	2.11
19	27.30	26.24	25.34	26.29	2.33
20	28.20	27.30	26.40	27.3	2.18
21	29.00	28.10	27.04	28.05	2.19
22	29.96	29.21	28.40	29.19	2.00
23	35.48	35.59	33.89	34.99	2.59
24	36.54	36.63	34.74	35.97	2.73
25	38.35	38.40	36.65	37.8	2.69
26	39.22	39.20	37.40	38.6	2.91

[a] Peak numbers correspond to those labeled in Fig. 3.10.

[b] For three digestions made on the same microreactor. CE separations were run on the same day.

Likewise, RSD in peak height and area were only slightly higher than in Fig. 3.9 yet the peptide maps corresponded to three different digests. While the same commercial lot of β-casein was used for all three samples, the batches were not the same. If the lyophilized β-casein standard were not uniform, batch to batch differences would account for the increased RSD in peak heights and areas, such as the large variation in peak pattern for peaks 18 to 22 (Fig. 3.10). In general, we can conclude that the separation conditions were mostly responsible for peptide map variability and that the microreactor and digestion conditions were very reproducible.

In comparing peptide maps from any two microreactors, for the same lot of β-casein sample, reproducibility (i.e. RSD in migration time, peak height and peak area) was the same as that observed in Fig. 3.10. In general, a microreactor with an effective length of 26 cm contained more than enough trypsin to digest the quantities of β-casein tested in this study. However, as a given microreactor aged, the quality of peptide maps deteriorated in that the number of background peaks and their intensities increased. This became most problematic for protein sample sizes of ≤ 2 mg/ml. In comparing 15 digestions made on the same microreactor over a period of 10 days, the last 4 peptide maps showed a steady incline in the intensities of background peaks. The origin of this "chemical background" was probably degradation of the immobilized trypsin, even though the microreactor was stored at –20°C when not in use. These results encouraged us to limit the number of digestions on one microreactor to about 10^{10}.

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[10] As the microreactor was stored dry and at 4°C between digestions, we assumed that degradation was a function of the number of uses and not its age in days since its initial fabrication.

3.7. Conclusions

We have described a convenient and economical way to make a capillary-sized microreactor based on immobilized trypsin for peptide mapping by CE. Unlike most previous reports on the construction and use of microreactors for peptide mapping, we have presented data on the repeated use and reproducibility of such a device, albeit for a single protein sample. Our results show that reproducible peptide maps of the protein standard β-casein can be obtained for sample concentrations ranging from 1 to 5 mg/ml, provided that the CE separations comparing two maps are run consecutively. Solid-phase digestion produced peptide maps with fewer trypsin autoproteolysis peaks than the homogeneous (liquid-phase digestion) case thus simplifying the maps. The flow rate of protein substrate perfused through the microreactor was shown to have an important influence on the resultant peptide map: sufficient contact time between enzyme and substrate was obtained for flow rates of ≤ 0.15 μl/min, measured over the initial 30 min of digestion. We also found that non-specific binding of peptide fragments in the microreactor was negligible, although there was a 12 % loss of sample volume between sample introduction and collection of microreactor products due to excessive dead volume in the system. Nonetheless, we were able to obtain a peptide map of β-casein with good confidence in a total of about 3 h for 40 μl of protein, from sample introduction to map completion.

Further experiments are underway to test the microreactor with other proteins, especially hydrophobic samples. Clearly, protein size and tertiary structure will dictate microreactor performance. To decrease the quantity of protein required for peptide mapping (i.e., improve map sensitivity), we have optimized a solid-phase extraction (SPE) pre-concentrating device that can be employed prior to CE separation [31]. Coupling the

microreactor, SPE preconcentrator and CE on-line will minimize sample loss due to handling and further improve peptide mapping sensitivity.

Acknowledgments

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3.7. References

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Chapitre 4

Origin of extra peaks in the β-casein tryptic digest map

Eric Bonneil et Karen C. Waldron soumis à *Journal Of Chromatography B*

Abstract

Peptide mapping is essentially a comparative technique for protein characterization that relies on the high reproducibility and specificity of enzyme proteolysis. Digestion of proteins has been carried out using a microreactor packed with covalently immobilized trypsin, which is known to yield little autoproteolysis. From this point of view, α-lactalbumin tryptic maps obtained by capillary electrophoresis were reproducible in terms of migration time (< 4 % RSD) and specificity, such that the correct number of expected peaks (i.e., tryptic fragments) was obtained, as shown in this work. On the other hand, phosphorylated β-casein digestion consistently produced extra peaks in the peptide map with respect to the expected number of tryptic fragments. In this paper, we show that this phenomenon is directly linked to the presence of isoforms in the protein sample rather than to an incomplete digestion of β-casein by immobilized trypsin.

Keywords: capillary electrophoresis; peptide mapping; trypsin; α-lactalbumin; β-casein; enzyme microreactor.

4.1. Introduction

Peptide mapping remains a widely used tool for the identification and characterization of proteins. Briefly, this procedure consists of enzymatic or chemical cleavage of the protein into smaller peptide fragments followed by their separation and detection. HPLC [1-4] and more recently capillary electrophoresis (CE) [5-7] are the most common analytical separation techniques used for peptide mapping. The major advantages of CE are its simplicity, low cost, low sample and solvent consumption and high efficiency. Regardless of the separation method, the elution pattern or "map" (i.e. electropherogram or chromatogram) represents a fingerprint of the protein, which can be compared to maps of protein standards for its identification or for the determination of post-translational modifications.

High specificity of the cleavage reagent is essential to get reliable and reproducible peptide maps. From this point of view, trypsin is typically the enzyme of choice for peptide mapping because it cleaves peptide bonds only at the carboxy terminal side of lysine and arginine residues. Conventional peptide mapping is often performed in homogeneous solution where reaction times can be very long (24 h) and maps suffer from background peaks generated by enzyme autoproteolysis. Solid-phase protein digestion using trypsin immobilized in a microreactor yields little or no autoproteolysis and can achieve digestion in one-tenth the time or less than by homogeneous liquid-phase digestion [7-10].

We recently characterized the performance of a microreactor packed with trypsin immobilized on controlled-pore glass (CPG) beads [11]. This microreactor allowed us to obtain peptide maps from 40 μl aliquots of the protein β-casein with good reproducibility in terms of migration time (RSD < 4.7 %). Bovine β-casein is a fairly small milk protein (208 residues) with a pI of 4.5, no disulfide bridges, an open conformation and average

hydrophobicity. Complete cleavage by trypsin theoretically generates 16 fragments, of which 2 are single amino acids and cannot be detected by the mapping procedure. However, when trying to map the digest of β-casein, we consistently obtained many more than the expected 14 peaks, regardless of the batch of protein substrate or immobilized enzyme used [12]. Amongst the proposed sources of these extra peaks, incomplete digestion was deemed the most likely.

It has been assumed that incomplete digestion is related to either the trypsin conformation, which is constrained due to its solid-phase nature, or the substrate conformation while resident in the microreactor. Therefore, a more difficult protein, α-lactalbumin, was digested in the microreactor to determine if the enzyme or substrate was responsible for the extra peaks. α-Lactalbumin is a 123-amino acid protein containing disulfide bridges. In order to maximize the digestion efficiency, this protein needs to be denatured prior to digestion, requiring unfolding of the tertiary structure and cleavage of disulfide bonds. Although reproducibility of the peptide map depends partly on the efficiency of the denaturation process, we achieved a good reproducibility for α-lactalbumin peptide maps (RSD < 4 %) as described in the work presented here. Moreover, we were able to clearly identify 12 peaks, which equals the number of peptide fragments expected for complete digestion of α-lactalbumin. Therefore, the microreactor did not seem to be responsible for the additional fragments seen with β-casein. In fact, further investigation into the source of the extra peaks showed that they were directly linked to the purity of the β-casein sample, as described in the following sections.

4.2. Experimental

4.2.1. Materials

Ammonium carbonate, monobasic sodium phosphate and phosphoric acid were purchased from Anachemia (Montréal, Canada). Urea, TPCK-treated trypsin attached to diisothiocyanate DITC-controlled pore glass (80-120 mesh; 700 Å average pore size; activity: 8,300 units/g), dithiothreitol, iodoacetamide, phosphorylated β-casein and α-lactalbumin were purchased from Sigma (St Louis, MO, USA). In-house distilled water was passed through a multi-cartridge Millipore water filtration/deionization system before use. Microcentrifuge tubes (1-ml and 0.6 ml) and disposable pipette tips (1-ml and 0.2-ml) were brought from Fischer Scientific (Montréal, Canada). Fused silica (f.s.) capillary tubing, nylon syringe filters of 0.22-μm pore size, 5-ml Wheaton V-vial and the capillary rinse kit were obtained from Chromatographic Specialties (Brockville, Canada).

4.2.2. Preparation of α-lactalbumin and β-casein for digestion

Denaturation and reduction of α-lactalbumin was carried out according to Matsudaira [13] as follows: 1.1 mg of α-lactalbumin was dissolved in 200 μl of 0.5 M ammonium carbonate buffer. Next, 50 μl of 45 mM dithiothreitol was added and the solution was incubated at 50°C for 15 min. After cooling to room temperature, 50 μl of 0.1 M iodoacetamide in water was added to the solution and left at room temperature for 15 min. Finally, the solution was brought up to 1 ml with pure water to give a final concentration of 1.1 mg/ml α-lactalbumin. Preparation of phosphorylated β-casein simply involved dilution of 2 mg of the protein in 1 ml of 0.1 M ammonium carbonate buffer. Previous studies in our laboratory have showed that denaturation by heating (protein unfolding) has no effect on the tryptic peptide map of β-casein.

4.2.3. Digestion procedure

For both substrates, a 40-µl aliquot of protein solution was digested in a 30-cm long microreactor as described previously [11]. Briefly, trypsin immobilized on CPG beads was dry-packed into a 30 cm × 530 µm i.d. fused silica capillary to create a proteolysis microreactor. The microreactor was then inserted into a small 26 cm-long condenser to provide a 37°C environment for tryptic digestion. Transfer capillaries (250 µm i.d.) were connected to each end of the microreactor for introduction of protein sample (inlet) and collection of peptide fragments (outlet). The inlet transfer capillary was connected to a capillary rinse kit, adapted with a small-volume V-vial such that a 40 µl aliquot of the protein solution could be perfused through the microreactor at an average flow-rate of 0.1 µl/min. This rate was controlled by adjusting the flow of argon pressurizing (~1 psi) the V-vial.

4.2.4. Preparation of β-casein for direct analysis

Phosphorylated β-casein solutions were made by dissolving the protein in either 0.1 M ammonium carbonate buffer, pH 2.5, or 0.1 M ammonium carbonate pH 2.5/ 6 M urea. The final protein concentration was 5 mg/ml.

4.2.5. Instrumentation

CE separation of β-casein and α-lactalbumin tryptic digests was performed on a SpectroPHORESIS 100 (Thermo Separation Products, Mississauga, Canada) equipped with a variable wavelength UV detector. Analog output from the detector was digitally converted and acquired on a personal computer using Maxima 820 software (Dynamic Solutions, Division of Millipore). Direct CE analysis of β-casein was performed on a $HP^{3D}CE$ system (Hewlett-Packard, Palo Alto, CA, USA), equipped with a diode array

detector and high sensitivity detection cell. For all separations, detection was carried out at λ=200 nm. Stored electropherograms were imported as data files into a Microcal Origin (Northampton, MA, USA) spreadsheet for plotting.

4.2.6. CE separations

Background electrolyte (BGE) buffers for CE separation were prepared in Millipore-purified water and the pH was adjusted by altering the ratio of monobasic sodium phosphate to phosphoric acid. All BGE solutions were passed through a 0.22-μm pore size syringe filter just prior to use. Separations were performed in f.s. capillary of 75 μm i.d., 400 μm o.d. and 45 cm effective length. The capillary was rinsed with 0.1 M NaOH followed by BGE, each at 1 bar for 2 min prior to sample analysis. Injection was accomplished by application of 50 mbar for 5 s at the capillary inlet for separations made using the HP^{3D}CE system and application of vacuum for 0.25 s at the capillary outlet for separations made using the SpectroPHORESIS system.

4.3. Results and Discussion

α-Lactalbumin is a bovine milk protein containing 123 residues of which 8 are cysteine. There are 4 disulfide bridges in the protein's native form, which occur between cys 6 and cys 120, cys 28 and cys 111, cys 61 and cys 77, cys 73 and cys 91. Complete digestion of this protein is expected to give 12 peptides and 2 amino acids given the 13 tryptic cleavage sites. When digested in our microreactor without prior cleavage of these disulfide bridges, α-lactalbumin gave very few peaks (data not shown) indicating that protein digestion was incomplete. This is typically the case when peptide mapping is carried out on proteins containing one or more disulfide linkages, which either prevent access of the enzyme to every scissile bond or leave some of the cleaved peptide fragments

linked together [7]. In some cases, substrate denaturation with urea can be used to make the cleavage sites accessible to enzyme, although α-lactalbumin has no α-helical or β-sheet regions so this procedure was not used.

To achieve complete digestion of the protein, disulfide bridges must be cleaved to make every tryptic cleavage site accessible to the enzyme. Therefore, S-S bonds were reduced with dithiothreitol followed by alkylation of the sulfide groups with iodoacetamide according to Matsudaira's procedure [13]. Fig. 4.1 shows the peptide maps of three different batches of α-lactalbumin (1.1 mg/ml), each batch of which underwent cleavage of disulfide bonds prior to digestion. The 12 expected peptides were detected, as numbered in Fig. 4.1, suggesting that digestion of α-lactalbumin was complete. Since the single amino acid fragments produced by tryptic digestion have almost zero molar absorptivity at 200 nm, they were not detected. Immobilized enzyme is known to yield little autoproteolysis [11], so no peaks due to trypsin were expected. A blank analysis in which the same procedure was followed but without addition of α-lactalbumin confirmed that the denaturation reagents did not generate peaks in the peptide map.

Migration time reproducibility is of utmost importance in peptide mapping because this is largely a comparative technique for protein characterization. That is, the tryptic map obtained from the protein of interest is compared to that of a known or standard protein analyzed in the same way. The peptide maps resulting from three separate digestions (Fig. 3-1) of α-lactalbumin after its denaturation compared quite favorably to one another with relative deviations (RSD, n = 4) in migration time for the twelve peaks ranging from 1.3% for the earlier eluting peaks to 4.0% for the latter eluting peaks. It should be noted that these maps (electropherograms) were not obtained the same day so run-to-run variations in migration time can be attributed to day-to-day fluctuations in the weak electroosmotic flow.

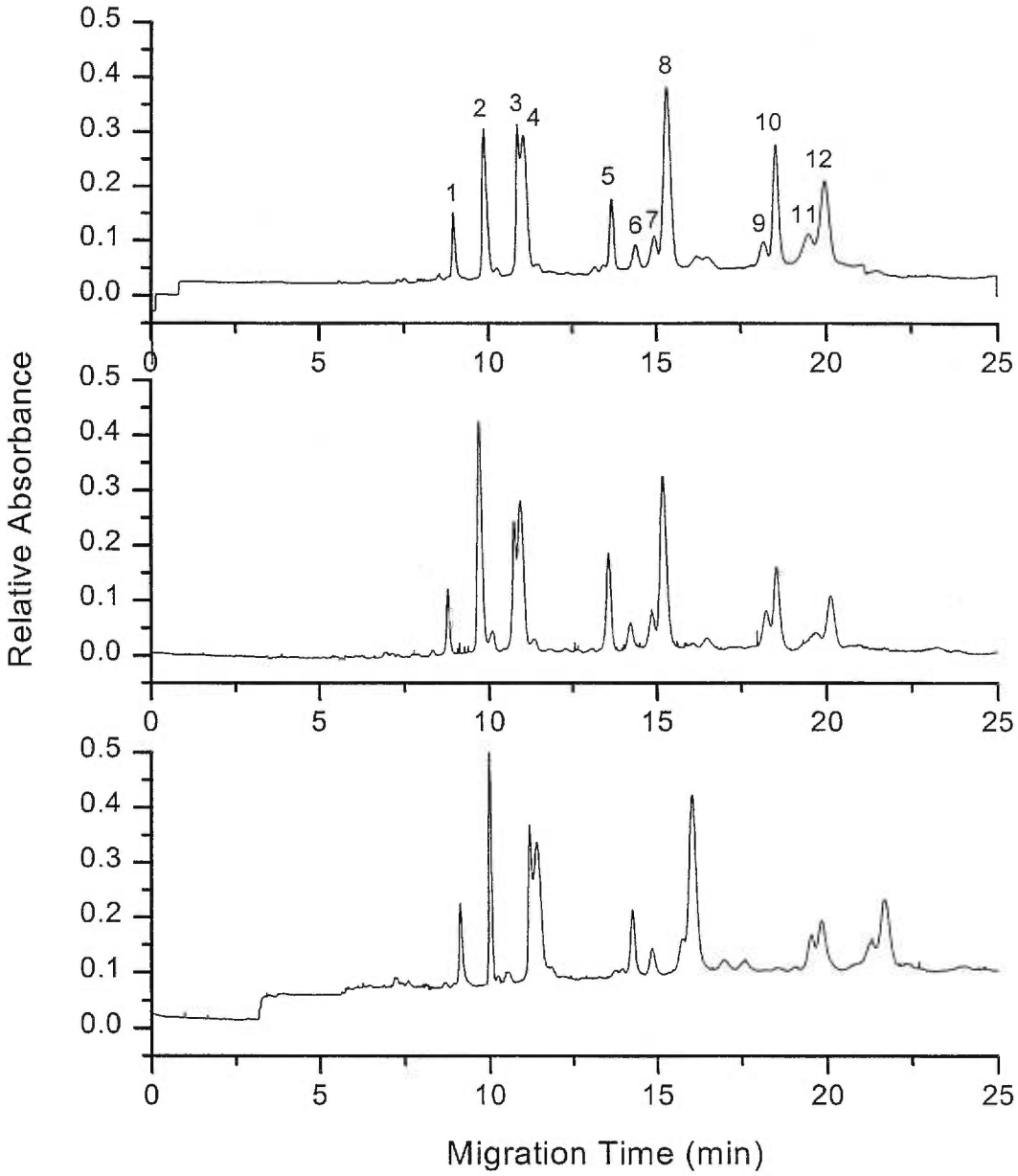

Figure 4.1. Electropherograms showing the reproducibility of peptide mapping. Each panel represents the tryptic map of a different batch of α-lactalbumin (1.1 mg/ml). Digestions were carried out by perfusion of 40 μl protein solution at 0.1 μl/min through a 30-cm long immobilized-enzyme microreactor. Separations were carried out at 20 kV in 30 mM sodium phosphate buffer, pH 2.5. Sample was injected (15 nl) by vacuum applied to the capillary outlet for 0.35 s.

Variations in peak height were significantly higher, up to 40% RSD as we have reported previously [11], but this is not problematic since peptide mapping is typically a qualitative rather than quantitative method. Peak height (or area) variations between maps were assumed to arise from various sources: errors in weighing the sample protein, losses due to sample handling during the denaturing and digestion steps, variability in the volume of digest electrophoresed since a semi-manual hydrodynamic injection method was used. The results for α-lactalbumin convinced us that the nature of the immobilized trypsin microreactor was not at the source of extra peaks in the β-casein map (Fig. 4.2). Therefore, the conformation of β-casein was proposed as the source of the extra peaks. However, heat denaturation of β-casein, a protein with no disulfide bridges, had no effect on the number of peaks seen in the β-casein maps (data not shown).

β-Casein is found in milk in its phosphorylated form, which contains five phosphoserine residues [14]. It has been previously used for peptide mapping [7, 10, 15, 16] because its primary structure is well known. The open conformation and lack of disulfide bridges in β-casein meant it could be digested immediately after dilution in an ammonium carbonate buffer without further pretreatment. Fig. 4.2 shows the electropherogram of the digest of a sample of phosphorylated β-casein (2 mg/ml). Tryptic digestion of this protein was expected to give 14 peptides and 2 amino acids, similar to the case of denatured α-lactalbumin. However, at least 18 peaks appeared in the peptide map of β-casein and this phenomenon of extra peaks was discovered to be quite common for the digestion of β-casein. In fact, up to 11 extra peaks can be counted in the map from digestion of β-casein generated using non-covalently immobilized trypsin [16, 17]. In our studies, a given lot of β-casein produced the same extra peaks, i.e., peptide fragments, after digestion in the trypsin microreactor regardless of the concentration digested.

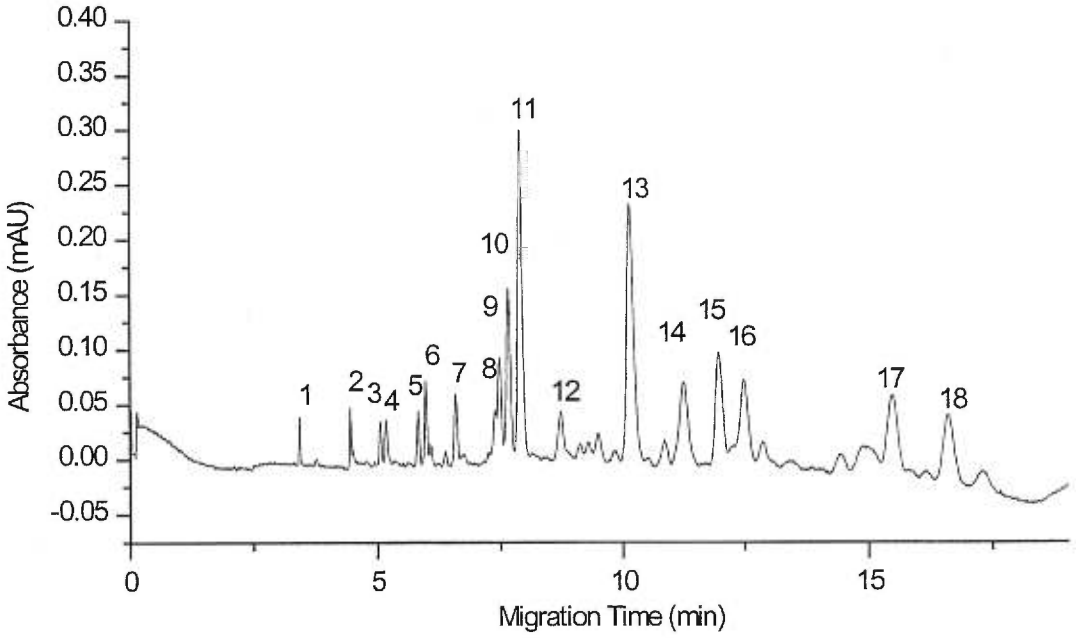

Figure 4.2. Electropherogram showing the map of phosphorylated β-casein (2 mg/ml). Digestion was carried out by perfusion of 40 µl protein solution at 0.1 µl/min through a 30-cm long immobilized-enzyme microreactor. Separation was carried out at 25 kV in 50 mM sodium phosphate buffer, pH 2.5. Sample was injected (15 nl) by vacuum applied to the capillary outlet for 0.35 s.

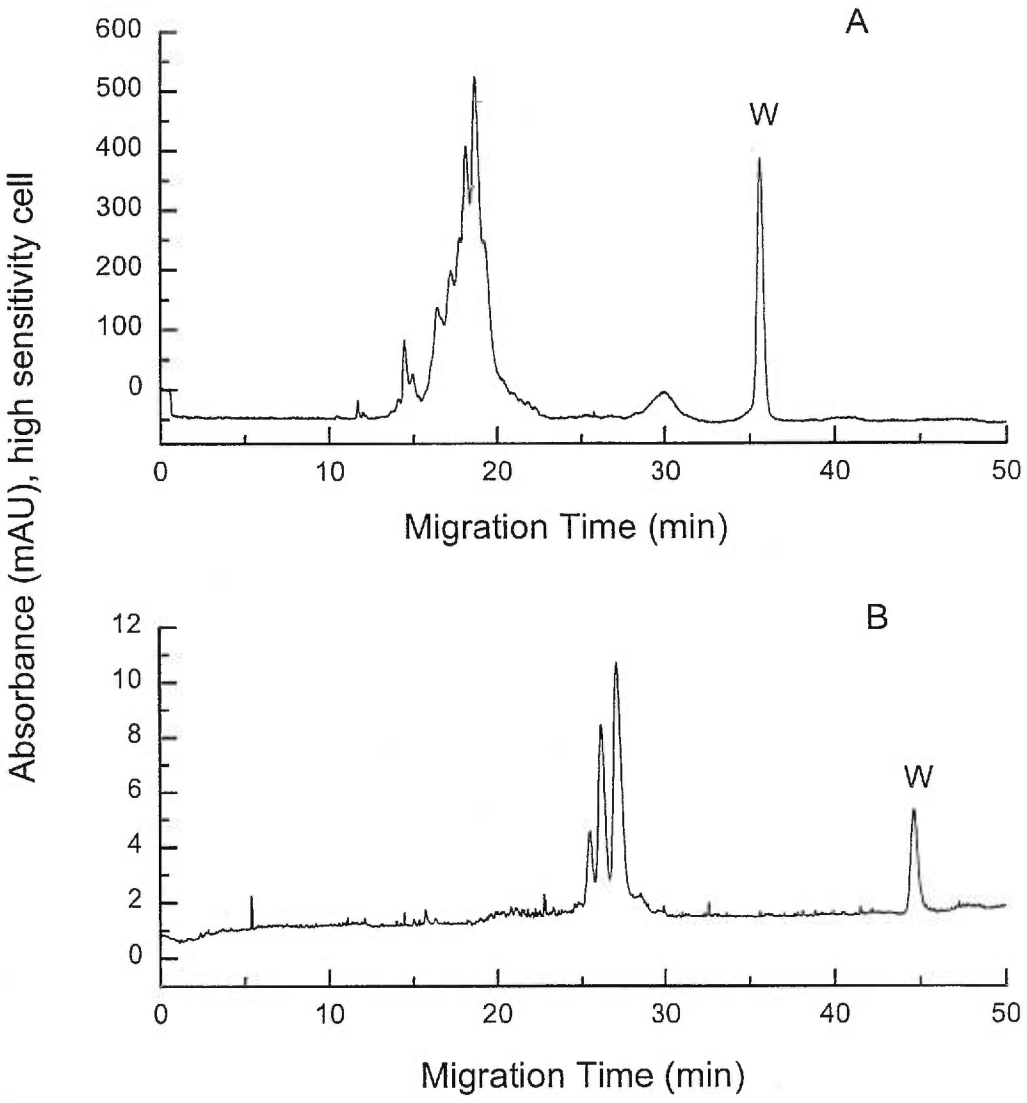

Figure 4.3. Electropherograms of phosphorylated β-casein (5 mg/ml in 100 mM ammonium carbonate) in (A) 10 mM sodium phosphate buffer, pH 2.5 and (B) 10 mM sodium phosphate, 8 M urea, pH 2.5 with 0.2 mM tryptophan added as the internal standard. Separations were carried out at 30 kV on an HP^{3D}CE system equipped with high sensitivity detection cell. Sample was injected by pressure (50 mbar) applied to the capillary inlet for 5 s.

Migration time reproducibility of β-casein maps was similar to those of α-lactalbumin, with migration time RSDs (n =3) varying from 1.5 % to 4.3 % regardless of how many extra peaks were generated.

As mentioned earlier, we initially believed that incomplete digestion was responsible for these extra peaks since trypsin can hydrolyze arginyl (R) and lysyl (K) bonds at variable rates. For example, the presence of an acidic residue (i.e. D or E) following R or K lowers the rate of hydrolysis [15, 18] and there are three such sequences in β-casein. However, this argument is unconvincing as a source of extra peaks because the residence time of protein substrate in the microreactor is long, about 120 min, and because α-lactalbumin has two such sequences yet its digestion did not yield corresponding extra peaks.

The presence of extra peaks in the β-casein map suggested that the sample was not composed of a unique primary structure. Therefore, we electrophoresed β-casein prior to its digestion to evaluate the sample purity. Fig.4.3A reveals that the β-casein standard was indeed impure, generating a group of partially resolved peaks. This protein is reported to form micelles in solution [19], which would lead to peak broadening in a CE separation but would not necessarily lead to the pattern observed in Fig. 4.3A. On the other hand, the peak pattern does imply that several forms of phosphorylated β-casein might be present. In fact, β-casein samples are prepared from milk in which at least 6 genetic variants of this protein are known to be present (Table 4.1) [19-21]. Because they differ mostly in the number of basic residues, genetic variants of β-casein have been easily separated in a hydrophilic coated capillary in low ionic strength, low pH viscous buffers containing 6–8 M urea [19, 20, 22, 23].

Fig. 4.3B shows the analysis of a phosphorylated β-casein sample under denaturing conditions. The presence of three distinct peaks confirmed that our β-casein standard was probably composed of several variants of this protein. The higher viscosity of the urea-containing buffer compared to that without (Fig. 4.3A) explains the increased migration time of the internal standard (tryptophan) and protein peaks seen in Fig. 4.3B. Also, because background absorbance of urea is high at the detection wavelength, the peaks in Fig. 4.3B appear smaller than for the same concentration of sample in the absence of urea. Unlike previously reported separations for analysis of milk, we did not use a hydrophilic coated capillary, viscous polymer-based buffer or low pH. Therefore, it is possible that more than three variants were present in the standard and simply not resolved from one another, which would explain to some extent the generation of so many additional peaks in the tryptic map.

Polymorphism is typical for milk proteins and arises most commonly from amino acid substitutions, deletion of an amino acid sequence or post-translational modification [24]. Several authors showed that the relative amount of each variant can vary in milk from one cow to another [19, 20]. Each variant listed in Table 4.1 differs by at least one amino acid and in some cases this is a lys or arg residue implying additional tryptic cleavage sites. Therefore, the presence of variants A1, A2, A3, B, C and E in the same sample would generate a total of 10 supplementary peaks. Although we did not investigate further which variants were present in our sample, we concluded that the superimposed peptide maps of several variants of phosphorylated β-casein were responsible for generating more peaks (i.e. peptides) than expected for a sample standard that was supposedly of homogeneous composition.

Table 4.1. Positions and amino acid substitutions for some of the genetic variants found in bovine β-casein [a]

Variant	Position and modification with respect to variant A2 [b]						Tryptic fragments extra to variant A2
	35	36	37	67	106	122	
A2	SerP [c]	Glu	Glu	Pro	His	Ser	n/a
A1				His			1
A3					Gln		1
B				His		Arg	3
C	Ser		Lys	His			3
E		Lys					2

[a] Table comprises data from references [19-21].

[b] Variant A2 represents the native sequence.

[c] Phosphorylated serine.

4.4. Conclusion

Peptide mapping is a qualitative technique needing high reproducibility in terms of relative migration time of each peptide. Obviously, this technique is greatly facilitated when protein digestion is complete since the number of peaks can be predicted if the primary structure is known. As a protein identification tool, the unknown peptide map must be compared to that of a standard. Consequently, the standard's purity is of primary importance. Because of its presence in milk, β-casein is becoming one of the most studied proteins. However, this protein is a victim of polymorphism caused by either changes in the DNA sequence that constitutes the protein gene or by post-translational modifications. It is not uncommon to find in the literature peptide maps of β-casein showing several more

peaks than expected. Although authors claim incomplete digestion as the cause of the supplementary peaks, our study shows that the presence of several variants (i.e. isoforms) in the original standard sample is most likely responsible for this phenomenon.

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Chapitre 5

Characterization of a solid-phase extraction device for discontinuous on-line preconcentration in capillary electrophoresis-based peptide mapping

E. Bonneil et K. C. Waldron, Journal of Chromatography B **1999**, *736 (1-2)*, 273-287.

Abstract

Peptide mapping by capillary electrophoresis (CE) with UV detection is problematic for the characterization of proteins that can only be obtained at low micromolar concentrations. Dilution of peptide fragments during digestion of the protein can further reduce the detection sensitivity in peptide mapping to the point where analysis at sub-micromolar concentrations is not possible. A remedy to this problem is preconcentration (sample enrichment) of the proteolytic digest by solid-phase extraction (SPE). To minimize non-specific adsorptive losses during sample handling, on-line SPE-CE is preferred. However, packed-inlet SPE-CE is not always feasible due to either instrument or sample limitations. We describe here a simple method of preconcentration by discontinuous on-line SPE-CE, specifically applied to peptide mapping in low-pH separation buffer after protein digestion in a solid-phase enzyme microreactor. The SPE-CE system does not require application of a low pressure during electrophoretic separation to overcome reversed electroosmotic flow because the preconcentrator device is disconnected from the separation capillary before the electric field is applied. Up to a 500-fold preconcentration factor can be achieved with this device, which can be reused for many samples. Parameters such as the volume of desorption solution, the adsorption/desorption (chromatographic) process, reproducibility of packing the SPE preconcentrator and effects of sample concentration on the peptide map are investigated.

Keywords: solid-phase extraction; preconcentration; peptide mapping; capillary electrophoresis.

Abbreviations: SPE-CE, solid-phase extraction capillary electrophoresis; CPG, controlled pore glass; f.s., fused silica.

5.1. Introduction

Over the past seventeen years capillary electrophoresis (CE) has proven very effective for peptide and protein analysis amongst many other applications. CE has particularly established its capability in the area of peptide mapping [1-4]. This comparative technique is useful for confirming the identity of a protein which, for example, can intervene in diseases like Hepatitis C [5] and gastric cancer [6]. Peptide mapping essentially consists of site-specific cleavage of the protein by an enzyme, followed by separation and detection of the ensuing peptide fragments. As a consequence, the post-translational modification of a protein can be detected by a shift in migration time of the peptide bearing that alteration. The limitation of this technique is that biological samples often yield low concentrations of proteins in relatively small volumes after extraction and purification, making the peptide fragments created by proteolysis difficult to detect.

The advantages associated with CE arise from one important characteristic of the capillary—its micrometer dimensions, which allow for efficient dissipation of Joule heating. Therefore, separations can be performed at high electric field strengths and thus are often faster than by HPLC. However, injected sample volumes in CE must be extremely small (1-10 nl) to avoid column overloading. This is advantageous when only very small volumes of analytes are available for analysis. In this respect, CE is convenient for peptide mapping of small volume samples. However, low concentrations of analyte rather than very small sample volumes are more commonly encountered. Compounding this problem, on-column UV absorption detection of only a few nanolitres over an optical path length of 50-100 μm severely handicaps visualization of the protein digestion fragments. To overcome this detection limitation, various chemical and instrumental techniques have been developed. For example, injection techniques to enhance concentration include analyte stacking [7-10] and field amplification [11]. In these cases, analyte zones are stacked or

focused due to differing ion mobilities in various field strengths or chemical environments. Instrumental methods to improve the sensitivity of detectors involve increasing the optical pathlength by means of using a bubble cell, Z-cell and other devices [12-14]. Chemical methods that produce extraordinary detection enhancement for CE can be achieved by laser induced fluorescence. Unfortunately, the limitation of this technique is the relative inefficiency of the reaction between the fluorescent derivatization reagent and many analytes [15].

Another approach to improve detectability in CE is to undertake extensive off-line sample pretreatment and concentration. However, substantial losses of analytes to exposed surfaces can occur for most organic molecules and especially biopolymers like proteins and peptides. Also, extensive handling of concentrated solutions of proteins or peptides can lead to denaturation, aggregation and eventually precipitation and sample loss. Traditional preconcentration (sample enrichment) methods often suffer from a lack of convenience or unsuitability with either the CE system employed or the type of analyte under consideration. As a result, many researchers have recently focused on the development and use of solid-phase extraction (SPE)–based preconcentration methods coupled on-line with CE [16-31]. For example, Guzman et al. [16] constructed a concentrator device that consisted of a bed of an immunoaffinity resin and was coupled to a CE capillary for analysis of specific analytes. Debets et al. [17] described a micro pre-column that could be switched on-line and off-line with the separation capillary by means of a valve that contained the solid phase material. A packed-inlet capillary developed by Waters Company (Accusep C/PRP capillary), which contained C18 (octadecylsilane) reversed-phase HPLC packing scintered in place, was used to improve the sensitivity of CE analysis of pharmaceuticals [20]. Landers' group [25, 26], as well as that of Tomlinson et al. [23] and Beattie et al. [21], designed a detachable preconcentrator cartridge containing C18 beads.

While SPE-CE is efficient at concentrating hydrophobic compounds, this method suffers from several drawbacks: reversal of the electroosmotic flow (EOF) at low pH [32], memory effects [33] and peak broadening [34]. In the past five years, Naylor's group has developed and implemented a membrane-based preconcentrator for use on-line with CE (mPC-CE) [22-24, 35-45]. The philosophy behind the development of mPC-CE was to decrease or remove potential problems associated with SPE-CE. Using a membrane, for example polymeric styrene divinyl benzene copolymer (SDB), it is possible to minimize the bed volume at the capillary inlet as well as avoid the need for frits. As a result, a smaller volume of desorption solution is used in comparison to that needed with preconcentration using a packed bed of C18 beads. The smaller desorption volume should greatly increase peak efficiency in the electropherogram [36]. Li et al. [30] made a comparison of the two sorbents, C18 beads and SDB membrane, for the preconcentration of hydrophobic peptides. The most significant difference noted was a slight increase in sensitivity with the SDB membrane for most of the peptides. The only drawback of the SDB membrane was the lower coefficient of linearity observed over the analyte concentration range examined. Non-linearity at higher concentrations was attributed to the membrane having reached its maximum adsorption capacity.

Our previous experience in developing a capillary-sized protein microsequencer [46] in which both adsorptive membranes and porous silica bead cartridges were studied showed that the small, packed bed of silica beads had a higher adsorptive capacity for polypeptides than the membrane. In addition, the packed bed was better equipped to trap larger sample volumes. Therefore, for preconcentration of protein digests, we have chosen to investigate the use of a C18–bead packed bed for SPE-CE, similar to that used by Landers' group [25, 26, 32, 47] and Beattie et al. [21]. Most papers describing the development of SPE-CE techniques use well-behaved samples to demonstrate the utility of the device. In this paper,

we describe the preconcentration of a more complex mixture of polar and non-polar peptides (e.g., a tryptic digest), highlighting the effect of the preconcentrator fabrication (i.e., packing), the volume of desorption solution, the peptide concentrations and the adsorption/desorption (chromatographic) process on the peptide map. The tryptic peptides are obtained by digestion of protein sample in an immobilized-enzyme microreactor described elsewhere [48].

5.2. Experimental

5.2.1. Materials

Acetonitrile (MeCN), Methanol (MeOH), controlled pore glass–diisothiocyanate–trypsin (CPG-DITC-trypsin) and bovine β-casein, dephosphorylated, were purchased from Sigma (St Louis, MO, USA). Monobasic sodium phosphate and ammonium acetate were purchased from Anachemia (Montreal, Canada). C18 beads (40 μm diameter) were purchased from Aldrich (Milwaukee, MI, USA). A 5 psig gauge and valve were purchased from Labcor (Anjou, Canada). Vials (20 ml) were purchased from Fischer Scientific (Nepean, Canada). Swagelock connectors, a reducing union, 0.2 μm pore nylon syringe filters and Teflon tubing were purchased from Chromatographic Specialties (Brockville, Canada). Fused silica (f.s.) capillary tubing (75 μm i.d. × 350 μm o.d.) and f.s. Innerlok™ capillary connectors were obtained from Polymicro Technologies (Phoenix, AZ, USA). Polyethylene tubing (0.38 mm i.d., Intramedic No. 7405 Clay Adams) and syringes (30 ml and 1 ml) were purchased from VWR-Canlab (Mississauga, Canada). In-house distilled water was purified with a multi-cartridge Millipore water filtration/deionization system before use.

5.2.2. Buffer and sample preparation

A 50 mM sodium phosphate, pH 2.5, buffer prepared from Millipore-purified water and filtered (0.2 μm pore membrane) was used for all CE separations and manipulations involving the SPE preconcentrator. Elution of peptides from the preconcentrator was achieved using a solution of acetonitrile–50 mM sodium phosphate at pH 2.5 (90:10 v/v). The peptide sample was obtained from tryptic digestion of β-casein using an immobilized-enzyme microreactor, described in detail elsewhere [48]. Briefly, a 40 μl solution of β-casein was perfused at 0.15 μl/min through a microreactor (28 cm × 530 mm i.d.) packed with CPG-DITC-trypsin beads. Collection of the peptide fragments (~ 36 μl) into a 500 μl vial was made after approximately 2.5 h.

5.2.3.CE instrumentation

Separations were carried out on a SpectroPHORESIS 100 (Thermo Separation Products, Mississauga, Canada) equipped with a variable wavelength UV detector. In this work, detection of peptides was carried out at λ=200 nm. Separations were performed in a 75 μm i.d. uncoated f.s. capillary with an effective length of 45 cm. The capillary was rinsed with 0.1 M NaOH for 2 min followed by a buffer rinse for 2 min prior to sample injection. Analog output from the detector was either recorded on an Chromjet Reporting Integrator (Thermo Separation Products) or digitally converted and acquired on an IBM-PC computer using Maxima 820 software (Dynamic Solutions, Division of Millipore). Stored electropherograms were imported as data files from the Maxima software into a Microcal Origin (Northampton, MA, USA) spreadsheet for plotting.

5.2.4. SPE preconcentrator fabrication and operation

Construction of the SPE preconcentration device was based primarily on that described by Landers and coworkers [25, 26] and Beattie et al. [21]. Essentially, the preconcentrator consists of a short (1 cm) piece of polyethylene tubing which contains a 50-225 nl bed (i.e., 0.25 to 1.5 mm length) of C18 beads held in place by f.s. capillary tubing at both ends. To make this device, a 6 cm piece of 75 μm i.d. f.s. capillary was first inserted into one end of the polyethylene tube. By gently tapping the free end of the polyethylene tubing into an Eppendorf tube containing the C18 beads, several beads can be trapped inside the tubing and held in place by inserting the second short piece (3 cm) of f.s. capillary tubing. The C18 beads were conditioned by exposure to methanol vapors for 2 min prior to packing to eliminate electrostatically induced clumping. Care was taken to keep the packing loose in order to avoid a high back-pressure during sample loading and elution, even though the C18 bed length was ≤ 1.5 mm. The exact length of the bed was measured using a microscope. No frits were needed as bed supports because the C18 beads stayed firmly inside the cartridge once the f.s. tubing was inserted. Finally, the SPE preconcentrator cartridge was rinsed with approximately 10 volumes of methanol by using a 1 cc disposable Tuberculin syringe that had been adapted with a f.s. Innerlok™ connector to fit the 75 μm i.d. f.s. capillary tubing. In the above-mentioned literature references, the SPE preconcentrator was directly attached to the separation capillary and remained in place throughout sample loading, desorption and CE separation. For reasons discussed in Section 5.3, this was not feasible and we thus had to resort to the procedure that follows. A summary of the times required for preconcentrator-cartridge fabrication and the loading/desorption steps is given in Table 5.1.

Sample adsorption: loading of the tryptic digest was achieved by flushing the peptide solution (40 or 100 µl) through the SPE preconcentrator cartridge manually at a rate of approximately 8 µl/min using the same type of 1 cc syringe/Innerlok system described above in the rinsing step.

Discontinuous on-line desorption: elution of the tryptic digest was achieved using a home-built liquid delivery system based on air pressurized by a manually operated 30 cc syringe (Fig. 5.1). CE separation buffer and desorption solvent were delivered from a 20 ml glass vial (Kimble Glass Inc., Vineland, NJ, USA) having a screw cap through which a 1.3 cm diameter hole had been drilled out. A PTFE (Teflon) disc, 1.5 cm in diameter, 0.2 cm thick, was tapped with two threaded holes: one for a 3/8 in connector and the other for a 1/16 to 1/32 in reducing union. This disc fit snugly between the glass vial and screw cap, sealed by an o-ring to make the device gas tight. The 3/8 in connector was linked by 60 cm of 0.04 in i.d. Teflon tubing to a 30 kPa gauge (Cole-Parmer) and a 30 cc syringe. The reducing union was connected to the 6 cm end (inlet) of the SPE preconcentrator cartridge, such that the f.s. capillary could be threaded through to the bottom of the glass vial, which contained either 10 ml of pH 2.5 CE separation buffer or 10 ml of desorption solution (acetonitrile–50 mM phosphate at pH 2.5, 90:10 v/v). The other end (outlet) of the SPE preconcentrator was either left open (first step, below) or connected to the separation capillary (second step) via a sleeve of polyethylene tubing.

Table 5.1. Times required for fabrication and manipulation in discontinuous on-line preconcentration CE

Procedure	Time
Precondition C18 beads with methanol	2 min
Preconcentrator fabrication and packing followed by 20 s methanol wash	2 min
Sample loading (40 μl tryptic digest) by syringe	5 min
Connect preconcentrator inlet to liquid delivery system; 20 s buffer rinse	40 s
Connect preconcentrator outlet to CE capillary inlet; change delivery vial to acetonitrile–50 mM phosphate (90:10 v/v); 5 s desorption	20 s
Change delivery vial to buffer and transfer elution plug into CE capillary	50 s
Disconnect CE capillary inlet from preconcentrator and immerse into buffer reservoir	20 s
CE separation	25 - 35 min

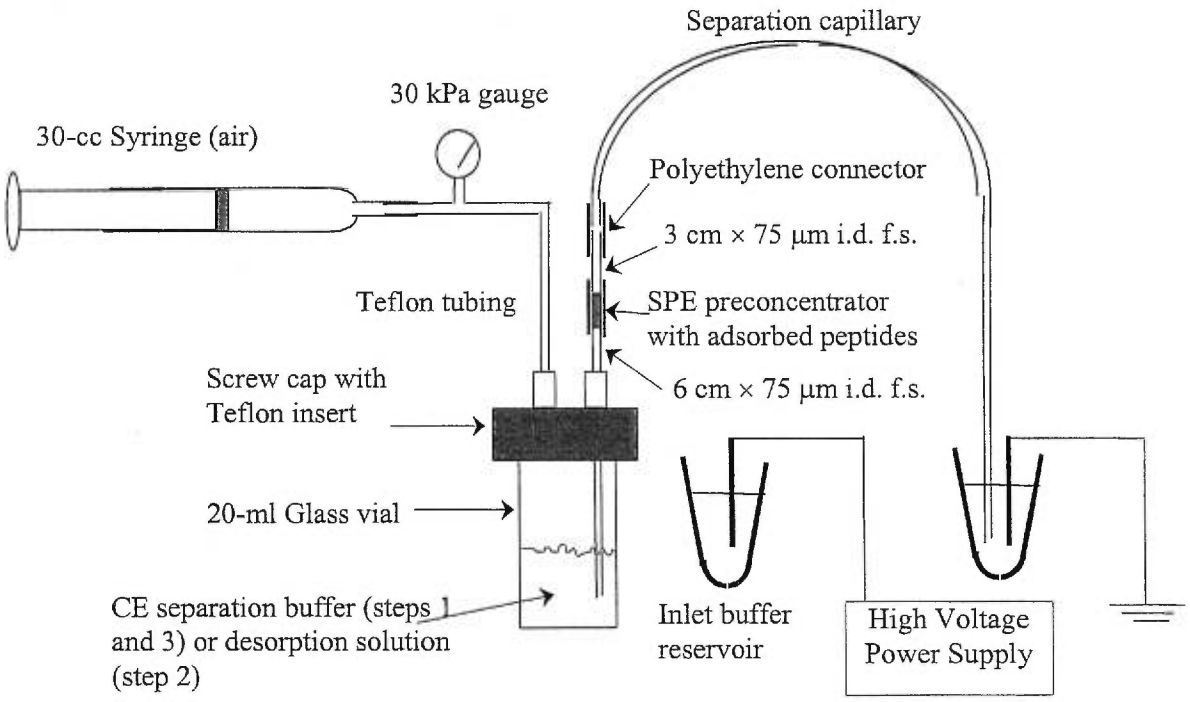

Figure 5.1. Schematic representation of the SPE preconcentrator–CE system used for discontinuous on-line desorption. Details of its operation can be found in Section 2.4. Briefly, once sample has been loaded onto the SPE preconcentrator cartridge, desorption takes place as follows: (1) the preconcentrator inlet (6 cm f.s. tube) is connected to the liquid delivery system and the device is rinsed with separation buffer for 20 s, (2) the separation capillary inlet is attached to the preconcentrator outlet (3 cm f.s. tube) and tryptic peptides are eluted from the preconcentrator in 65 ± 15 nl desorption solution by pressurization for 5 s, (3) separation buffer is pressurized for 45 s to push the elution plug well into the separation capillary, (4) the separation capillary is disconnected from the polyethylene connector and dipped into the inlet buffer reservoir to commence separation.

In the first step, the SPE preconcentrator cartridge (containing adsorbed peptide) was flushed with separation buffer by applying a pressure of 25 kPa for 20 s inside the glass vial by manually activating the piston of the 30 cc syringe. In the second step, the separation capillary inlet was attached to the outlet of the preconcentrator and the glass vial containing separation buffer was replaced with one containing desorption solution, which was perfused through the preconcentrator by applying a pressure of 25 kPa for 5 seconds (to deliver 65 ± 15 nl). In the third step, the glass vials were quickly switched back so that buffer solution could be used to push the *entire* desorption plug completely into the separation capillary inlet by applying 25 kPa for 45 seconds. Finally, the separation capillary was disconnected from the SPE preconcentrator cartridge and dipped into the inlet buffer reservoir of the CE system and the high voltage applied. Although the steps involved in discontinuous on-line desorption required several manual manipulations, they could be carried out rapidly (see Table 5.1) and easily.

Off-line desorption: Elution of the tryptic digest was achieved using, for the most part, the same system as above except that the 30 cc syringe and 30 kPa gauge were replaced by an argon tank with a two-stage pressure regulator. The SPE preconcentrator was not connected to the separation capillary. Instead, after adsorption and rinsing with separation buffer, the outlet of the SPE preconcentrator was inserted into a 0.5 ml polypropylene microcentrifuge tube. By applying a pressure of 35 kPa argon in the 20 ml glass vial, the desorption solution was flushed through the SPE preconcentrator and the first 5 µl of this solution was retrieved at the cartridge outlet, in the microcentrifuge tube. This tube was then placed in the CE system carousel and approximately 15 nl were injected for separation.

5.3. Results and Discussion

Critical in peptide mapping is the reproducibility of maps (electropherograms) for a given protein. In order to achieve this, we constructed an immobilized trypsin-based microreactor for protein digestion [48]. Trypsin is a highly specific enzyme that cleaves proteins at the C-terminal side of lysine and arginine residues, resulting in a relatively simple series of peptide fragments from the parent protein sample. The protein used in this study was dephosphorylated bovine β-casein, which has a relatively open conformation (i.e., no disulfide bridges) to facilitate digestion by trypsin. One of the major advantages of digestion in the solid-phase microreactor compared to solution phase proteolysis is the lack of trypsin autoproteolysis products, thereby simplifying the peptide map and improving its reproducibility [48-50]. We are therefore working under conditions that should result in highly reproducible maps. To demonstrate this, Fig. 5.2 shows the tryptic peptide maps, without preconcentration, of three aliquots of β-casein (80 μM) digested in three different microreactors.

As seen in Fig. 5.2, the reproducibility of the digests is good in terms of relative peak height and relative migration time. Our previous study on the trypsin microreactor [48] showed that absolute peak heights and areas varied up to 47% when comparing three peptide maps. Nonetheless, absolute migration times displayed < 3% variation in that study, which is sufficient for peptide mapping applications. It should be noted that the peak pattern in Fig. 5.2 differs from that seen in reference [48] because an old batch of immobilized trypsin and the phosphorylated form of β-casein were used for the previous study.

The goal of this work was to generate reproducible peptide maps from dilute protein solutions by means of SPE-CE with a C18-based cartridge after first digesting the protein

in our enzyme microreactor. To this end, several parameters were investigated such as the influence of chromatographic processes on the peptide map (i.e., adsorption), the volume of desorption solution, the volume of the solid-phase bed (i.e. the length of the SPE preconcentrator cartridge), and the sample concentration. It is important to point out here that unlike previous SPE preconcentrator designs (e.g., [21, 25, 26]), frits were not used to retain the solid-phase material in the cartridge. It has been suggested that bed supports (i.e., frits) can cause excessive dead volume, irreversible adsorption and loss of efficiency during preconcentration [51]. The only solid-phase material that we investigated was 40 μm diameter C18 beads, primarily to avoid high backpressures and the need for frits.

5.3.1. Influence of the Chromatographic Process on Peptide Mapping

In our first attempt to investigate the effect of chromatographic processes arising from the SPE preconcentrator, we left the device attached to the capillary inlet during separation, similar to that described by Landers and co-workers [25, 26] and Beattie et al. [21]. We wanted to compare the peptide map of an 80 μM β-casein sample to one in which the same digest was diluted 100 times, then loaded (40 μl) on the preconcentrator cartridge. A 65 ± 15 nl plug of the desorption solution (acetonitrile–phosphate buffer, 90:10 v/v) was injected and pushed past the preconcentrator to elute the peptides from the C18 sorbent. Upon application of high voltage across the SPE-CE system, no peptides were detected. This phenomenon has also been reported by Strausbauch et al. [32] who postulate that peptide eluting from the C18 sorbent is carried into the separation capillary and then re-adsorbed shortly after application of the electric field due to reversal of EOF in the capillary.

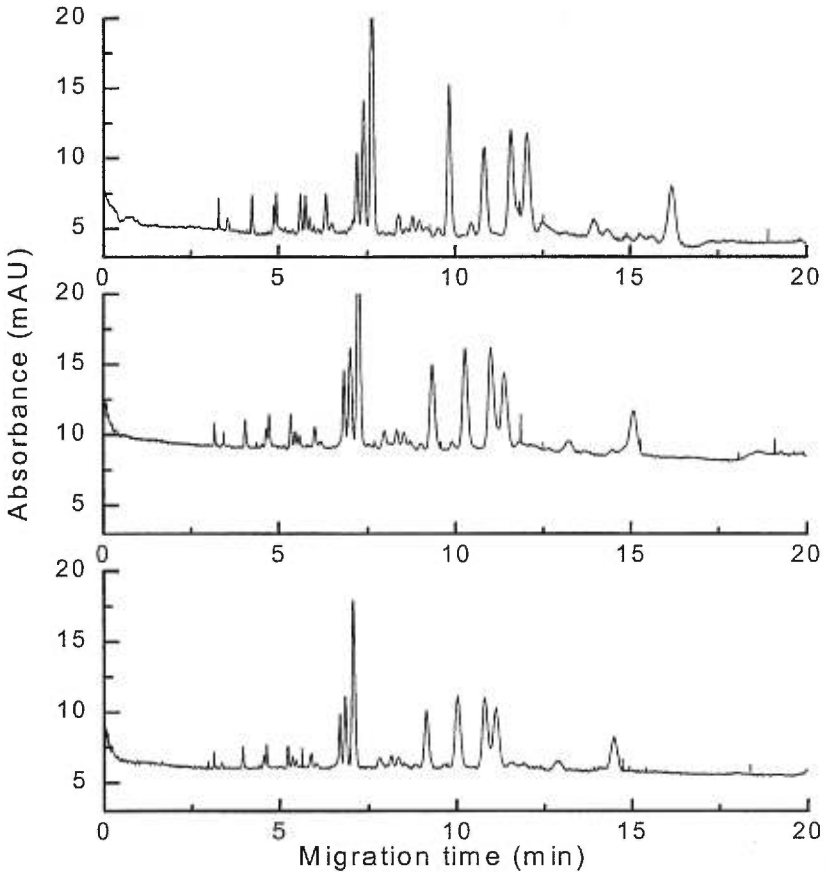

Figure 5.2. Electropherograms showing the reproducibility of peptide mapping without preconcentration. Each panel represents the tryptic map of a different aliquot of 80 μM of dephosphorylated β-casein in 100 mM ammonium carbonate, pH 8.1. Digestion was carried out by perfusion of 40 μl protein solution at 0.1 μl/min through an enzyme microreactor. Separations were carried out at 25 kV in 50 mM sodium phosphate buffer, pH 2.5. Sample was injected by vacuum at the capillary outlet for 0.35 s (15 nl injected).

Essentially, the peptide begins to electrophoretically migrate at the cathodic interface of the desorption and buffer solutions. However, the field strength is not constant throughout the capillary due to the presence of the desorption plug so a strong reversed EOF is generated in the region of the SPE preconcentrator. Strausbauch et al. [32] believe that unrecovered peptide bound to the solid phase material creates a cationic charge on its surface, leading to the localized reversal of the EOF. They were unable to restore the cathodic EOF, even after rigorous washing of the SPE device. The same group observed highly suppressed EOF (> 85 min) for preconcentration of peptides using the C/PRP capillary from Waters. Their solution was to apply a small pressure of 3.5 kPa concomitant with application of high voltage during the CE separation.

Inversion of the EOF in our studies only occurred at low pH. While CE separation at higher pH where the cathodic EOF is stronger might overcome flow reversal, this is not the optimum pH for peptide mapping. In addition, Chien and Burgi [11] reported that a mismatch of local EOF velocities in such a discontinuous pH buffer system can create mixing and a laminar back-flow at the interface of the buffers, seriously degrading resolution and peak shape. Therefore, we chose to retain the low-pH separation buffer (pH 2.5) to match the desorption solution pH. In off-line preconcentration studies (data not shown), the desorption solution was found to give better overall peptide maps when it contained 10% sodium phosphate buffer (pH 2.5) rather than 10% dilute HCl, which was used in other SPE-CE studies [32]. We found that HCl in the desorption solution caused unstable and irreproducible baselines at separation voltages over 15 kV.

Application of a small pressure to ensure a positive EOF as described by Strausbauch et al. [32] is not feasible with all CE instruments, can lead to additional peak broadening and would have to be optimized depending on the sample, thus making it a potentially time-consuming step. Therefore, to circumvent the reversed EOF problem, we

decided to disconnect the SPE preconcentrator cartridge from the capillary inlet just prior to separation (Fig. 5.1). By performing this "discontinuous on-line desorption" process, where 100 % of the volume desorbed is transferred into the separation capillary, we could reach a higher preconcentrator factor than with traditional off-line SPE preconcentration. Figure 5.3 compares three peptide maps: (1) without any sample enrichment (80 μM digest, Fig. 5.3A), (2) with off-line preconcentration (80 μM digest diluted 10-fold, Fig. 5.3B), and (3) with discontinuous on-line preconcentration (80 μM digest diluted 100-fold, Fig. 5.3C). These three electropherograms have been plotted on the same scale to demonstrate relative peak heights. Off-line preconcentration (Fig. 5.3B) was achieved by loading 100 μl of the digest (diluted to 8 μM) onto the solid phase followed by elution in 5 μl of the desorption solution (i.e., a 20-fold predicted preconcentration factor), from which 15 nl was injected for CE separation. Discontinuous on-line preconcentration (Fig. 5.3C) was achieved by loading 40 μl of 800 nM digest onto the SPE device followed by elution in 65 ± 15 nl desorption solution, directly into the separation capillary (i.e., an approximately 500-fold predicted preconcentration factor).

In comparing Figs. 5.3A and 5.3B (without and with preconcentration, respectively), some variations in the relative peak heights can be seen. Obviously, the peak height of each peptide depends strongly on its hydrophobicity. The more hydrophobic the peptide, the more it is retained on the sorbent and, hence, preconcentrated to a higher degree. While the predicted equivalent concentration in Fig. 5.3B is 160 μM (i.e., 80 μM ÷10-fold dilution × 20-fold preconcentration), several peaks were not 2× higher than the 80 μM sample (Fig. 3A). Recovery from the SPE preconcentrator, like enrichment, varies greatly with the hydrophobicity of each peptide. Our early studies of off-line desorption using leu-enkephalin as a model peptide showed 79 % recovery [52]. This value agrees

with that found by Bateman et al. [33]. These authors also reported a preconcentrator memory effect where subsequent application of elution solvent provided variable amounts of peptide ranging from 20 to 40 % of the first elution plug, although this effect was more severe for 5 μm than 40 μm C18 particles.

In the case of discontinuous on-line preconcentration (Fig. 5.3C), the peptide map of the same tryptic digest is completely different. The hydrophobicity of each peptide has an influence on the map not only in terms of the quantity of peptide retained on the sorbent and its absolute recovery from the sorbent but also in terms of its desorption rate. A highly hydrophobic peptide has a lower desorption rate than a less hydrophobic peptide, leading to a modification of the overall migration time. The SPE elution plug, which is also the CE injection plug, will not be uniform in composition across the length of the plug. In essence, a reversed-phase chromatographic process becomes superimposed on the CE separation. Such a large chromatographic effect was not seen by Strausbauch et al. [32], who simply noted a slight increase in resolution between two highly hydrophobic model peptides after preconcentration.

The presence of such a large quantity of elution solvent (65 ± 15 nl) in the separation capillary should bring about an additional stacking effect on top of the theoretical preconcentration factor. The predicted equivalent concentration in Fig. 5.3C should be ca. 310 μM taking into account an average recovery of 79 % but not considering any transient stacking effects.

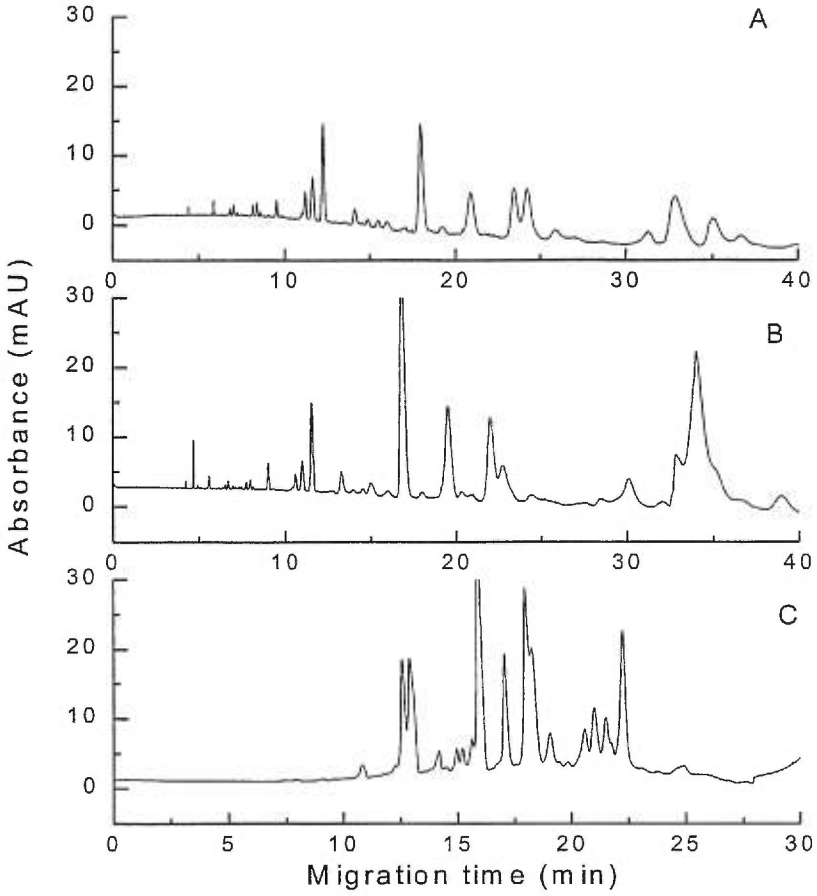

Figure 5.3. Electropherograms showing the effect of preconcentration on the peptide map. (A) 80 μM β-casein solution without preconcentration; (B) off-line preconcentration of a 10-fold dilution of the same 80 μM β-casein solution, with 100 μl loaded and elution in 5 μl desorption solution; (C) discontinuous on-line preconcentration of a 100-fold dilution of the same 80 μM β-casein solution, with 40 μl loaded and elution in 65 ± 15 nl desorption solution (90% MeCN/50 mM phosphate buffer at pH 2.5). A 1.5 mm SPE preconcentrator cartridge was used in (B) and (C). In all three cases, separations were carried out at 18 kV in 50 mM sodium phosphate buffer at pH 2.5. Injection times were 0.35 s by vacuum for (A) and (B).

While we do not have sufficient information (i.e., peptide standards or diode array data) to match up the peaks from Fig. 5.3A to Fig. 5.3C to determine the true preconcentration factor, there is clearly a large increase in the areas of several peaks in Fig. 5.3C. To determine if we could increase the elution window observed in Fig. 5.3C and improve sensitivity, we investigated the effect of increasing the desorption volume for the discontinuous on-line preconcentration system.

5.3.2. Influence of the Volume of Desorption Solution on Peptide Mapping

Fig. 5.4 shows the influence of tripling the desorption solution volume for discontinuous on-line preconcentration peptide mapping. The same tryptic digest (80 μM, diluted 100-fold) and loaded sample volume (40 μl) were used in Figures 5.4A and B, whereas the desorption solution volumes were 175 ± 20 nl and 65 ± 15 nl, respectively. Clearly, the volume of the elution plug has a strong influence on the peptide map in terms of peak shape and resolution. Within the large elution plug, the electric field is reduced due to the presence of organic solvent [53]. Therefore, the separation, which depends on differential electrophoretic mobility, is affected. Moreover, the diffusion process is much greater when the injection plug is so large. These two processes are commonly responsible for the lack of good peak efficiency and resolution in peptide mapping, highlighting the fact that elution volume optimization is necessary. For an SPE cartridge 0.3 mm in length, Strausbauch et al. [32] found that the optimum volume of desorption solution was 130 nl, about 2.5 times greater than the volume of C18 sorbent.

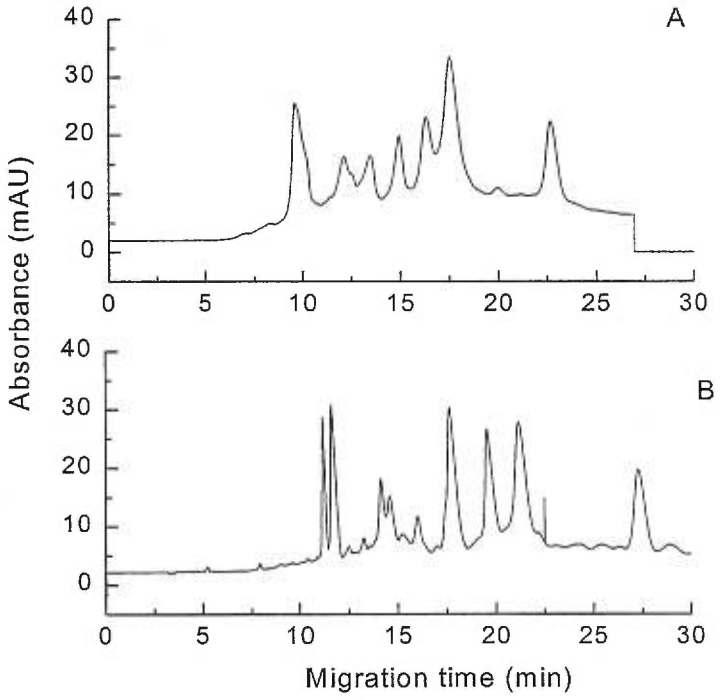

Figure 5.4. Electropherograms showing the effect of tripling the volume of desorption solution for the same 800 nM tryptic digest (100-fold dilution of 80 μM β-casein digest) in which 40 μl have been loaded on a 1.2 mm SPE cartridge for discontinuous on-line preconcentration. (A) Elution in 65 ± 15 nl desorption solution; (B) elution in 175 ± 20 nl desorption solution. Separations were carried out at 18 kV in 50 mM phosphate, pH 2.5.

In their work, peak sensitivity was compromised for smaller volumes than this. Interestingly, these authors also reported that no reversed EOF was observed when the volume of desorption solution was < 130 nl. In our case, the volume of desorption solution was 3.5 times *smaller* than the C18 bed (225 nl), yet we still observed reversed EOF when the SPE preconcentrator was on-line (i.e., as a packed-inlet) with CE. The results in Fig. 5.4 show that increasing the desorption solution volume to 175 ± 20 nl severely degrades resolution and does not significantly enhance peak heights or areas as hoped.

5.3.3. Influence of the Preconcentrator Cartridge Length on Peptide Mapping

Figure 5.5 shows the influence of SPE preconcentrator length (i.e., quantity of solid phase material) on the peptide map for the same tryptic digest of β-casein. Aliquots of 40 μl from an 80 μM digest diluted to 800 nM were loaded onto a series of SPE preconcentrators of lengths ranging from 0.5 mm to 1.5 mm. With a length of 0.5 mm, only 5 small peaks could be seen (Fig. 5.5A). Essentially, there was insufficient sorbent for retention of the tryptic peptides, with only a small fraction of the most hydrophobic peptides being adsorbed. Increasing the preconcentrator cartridge length to 1 mm (Fig. 5.5B) led to increased peak heights, but not a significant increase in the number of peaks. While these two factors depend on the volume of solid phase in the preconcentrator cartridge, they also depend on the relative analyte hydrophobicity [29]. Further increasing the preconcentrator length to 1.5 mm (Fig. 5.5C) produced more peptide peaks with, for the most part, increased heights.

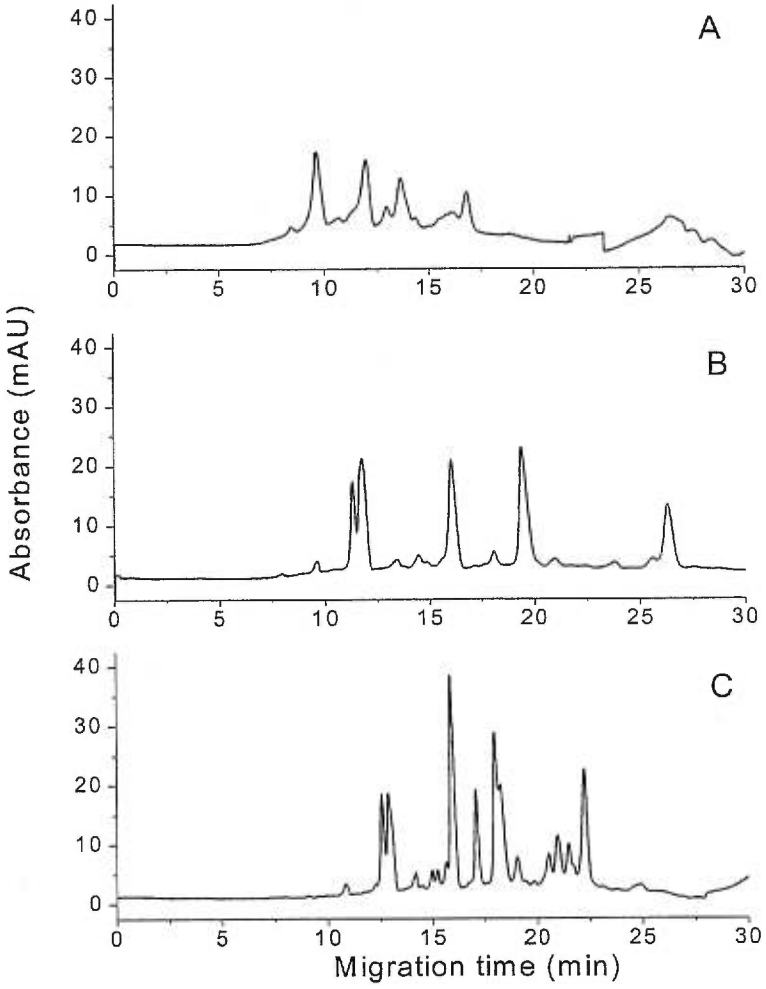

Figure 5.5. Electropherograms showing the effect of SPE preconcentrator cartridge length on the peptide map of 800 nM β-casein tryptic digest (80 μM solution diluted 100-fold). (A) 0.5 mm long preconcentrator; (B) 1 mm long preconcentrator; (C) 1.5 mm long preconcentrator. In each case, 40 μl tryptic digest was loaded onto the SPE preconcentrator and peptides were eluted in 65 ± 15 nl desorption solution. Separations were carried out at 18 kV in 50 mM sodium phosphate buffer, pH 2.5.

The peaks at 12 to 13 min in Figs. 5.5B and 5.5C showed no change in height when increasing the preconcentrator length from 1 to 1.5 mm. These are likely the most hydrophobic peptides, retained just as well on the shorter as on the longer preconcentrator cartridge. As can be seen in Fig. 5.5, the overall length of the preconcentrator affects the pattern of peaks in the peptide map because the chromatographic process during desorption is a function of the length of C18 stationary phase. On the other hand, it was expected that peptide maps obtained using the shorter preconcentrator would more closely resemble those without preconcentration. This was not the case. Therefore, a length of 1.5 mm was determined to be the best amount of sorbent in the SPE preconcentrator cartridge.

5.3.4.Packing Reproducibility

To evaluate the reproducibility of packing the SPE preconcentrator, which is essentially a measure of the efficiency of fabricating the device, we compared the cartridge-to-cartridge variation for three preconcentrators. Figure 5-6 shows three peptide maps in which 40 μl aliquots of the same digest (80 μM, diluted 100-fold) were loaded onto three different preconcentrators. Although the preconcentrator cartridge length as well as the desorption and separation conditions were identical in each of the three cases, large variations in the peak migration times from Fig. 5-6A to 5-6C can be seen. While relative peak heights are similar for the three peptide maps, resolution is vastly different and absolute migration times vary from 18% for the first peak to 31% for the last peak. The differences seen in Fig. 5-6 arise from the packing procedure. For example, a tighter packing induces a higher backpressure, which is problematic during the desorption step and when pushing the elution plug into the separation capillary. In both these operations, a fixed pressure is applied for a specified time interval to deliver a certain volume. In the case of desorption, a 50–80 nl variation in elution solvent volume, which is also the injection

volume, arises leading to variable resolution of peaks. During transfer of this volume into the separation capillary, the elution plug does not travel as far into the capillary when backpressure from the SPE cartridge is high making the effective length of separation longer. Therefore, the migration times appear longer. This is the case for the electropherogram in Fig. 5-6C. On the other hand, if the packing is too loose, then a 1.5 mm SPE cartridge will have less overall sorbent material than a cartridge of the same length packed tightly. As a result, the chromatographic process will be different. In addition, the volume of desorption solution will not be compatible, which probably accounts for the poor resolution seen in the peptide map in Fig. 5-6A. While several discrepancies exist between peptide maps made with different SPE preconcentrators, this is much less of a problem when the same preconcentrator is reused as described in the next section.

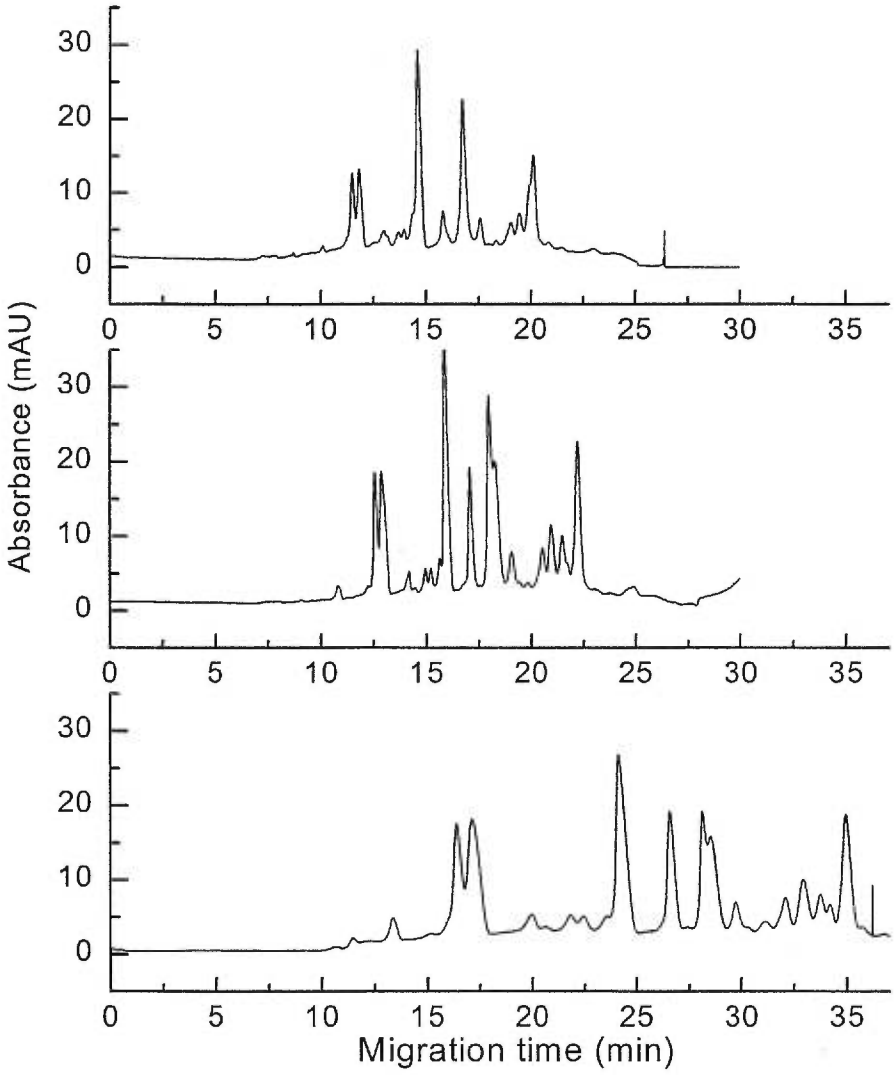

Figure 5.6. Electropherograms showing the reproducibility of preconcentrator packing on peptide maps of 800 nM β-casein tryptic digest (80 μM solution diluted 100-fold) for three different SPE preconcentrator cartridges (panels A, B and C), each 1.5 mm in length. Loading, elution and separation conditions were the same as in Fig. 5.5.

5.3.5. Reusability of a Preconcentrator Cartridge for Peptide Mapping

The reproducibility of peptide maps made using the same SPE preconcentrator (i.e., reusability) is shown in Fig. 5.7. In each case, 40 μl aliquots of the same digest (80 μM diluted to 800 nM) were loaded onto the preconcentrator. The three electropherograms in Fig. 5.7 have been presented with offset time scales to show their similarities in terms of peak height and peak pattern. Differences in absolute migration times are manifested somewhat as a compression or expansion of the elution window, but mostly as a displacement of the elution window. These differences arise from the method by which the SPE-CE system is pressurized. A slight change in the solid phase packing organization after repeated sample loadings can lead to a tighter packing, thus increased backpressure. As mentioned in Section 5.3.4, the elution plug is not pushed as far into the capillary when backpressure is higher. The overall result is longer absolute migration times because the effective length of the capillary, from injection plug to detector, is longer. Even though the same desorption and separation conditions were used in each case, the large deviation in desorption solution volume (65 ± 15 nl) accounts for the differences in resolution seen amongst the three peptide maps in Fig. 5.7. On the other hand, relative migration times are very reproducible. Table 5.2 presents the mean migration times and the RSD in migration time for the three maps in Fig. 5.7 using a two-peak normalization method [54]. The first and last peak were used for migration time correction, leading to migration time RSDs of less than 2.5 %, which is sufficient for detection of protein modifications by comparison of two peptide maps.

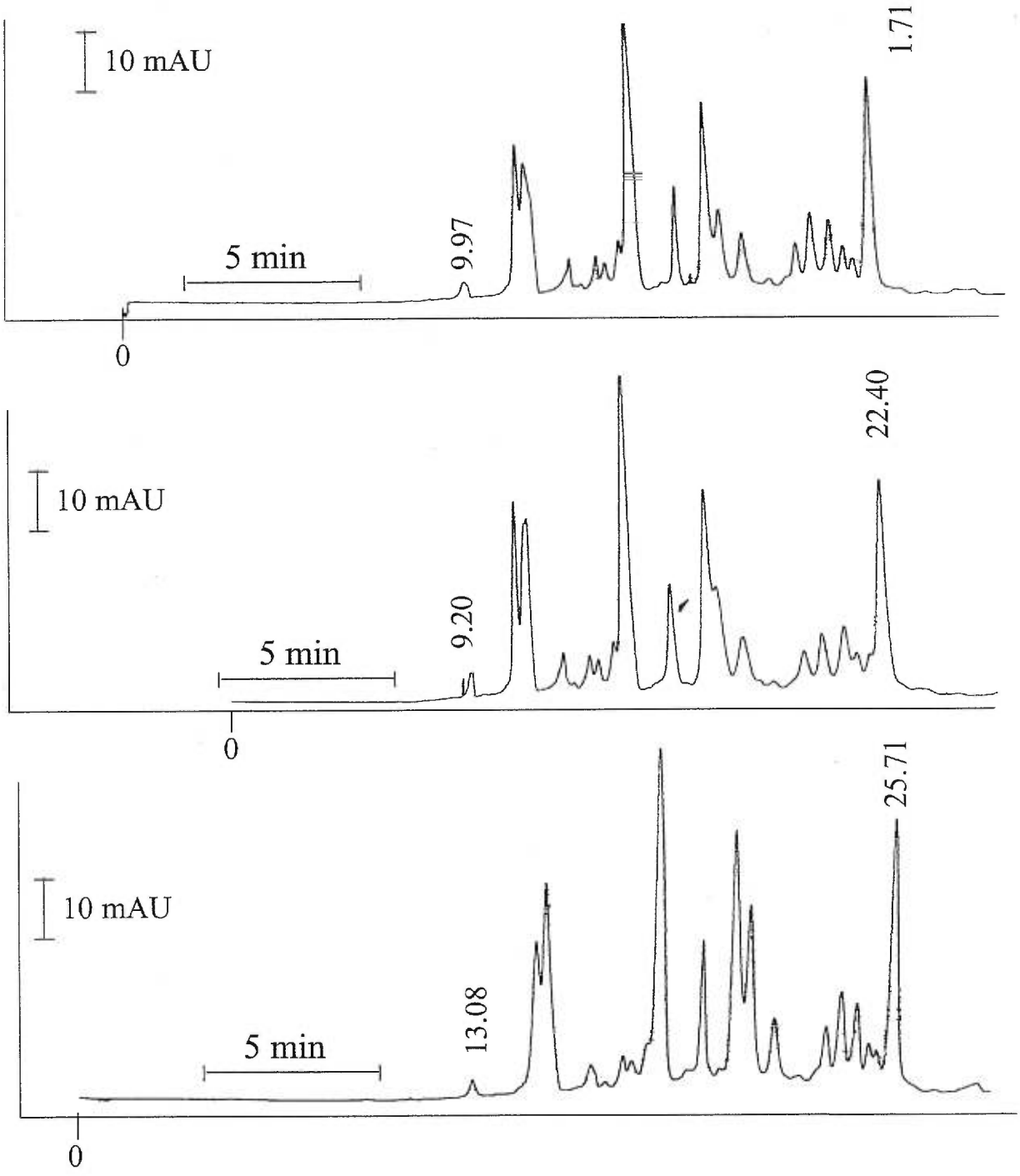

Figure 5.7. Electropherograms showing the reusability of the same SPE preconcentrator (1.5 mm long) for peptide mapping of an 800 nM β-casein tryptic digest (80 μM solution diluted 100-fold). Loading, elution and separation conditions were the same as in Fig. 5.5. † Data were originally recorded on an integrator and were thus scanned to offset the images for comparison.

Table 5.2. Relative migration time precision for three peptide maps obtained using the same SPE preconcentrator

Peak Number [a]	E.A (min)	E. B (min)	E. C (min)	Mean migration time (min) [b]	Migration time RSD (%) [b]
1	9.2	9.2	9.2	9.2	0
2	10.7	10.6	10.8	10.7	1.1
3	10.9	11.0	11.0	11.0	0.47
4	12.1	12.2	12.3	12.3	0.87
5	12.9	13.0	13.2	13.0	2.4
6	13.1	13.1	13.4	13.3	1.7
7	13.3	13.4	13.8	13.6	2.3
8	14.1	14.2	14.3	14.2	0.52
9	15.6	15.6	15.7	15.7	0.20
10	16.6	16.7	16.7	16.7	0.29
11	17.1	17.1	17.2	17.2	0.17
12	17.9	18.0	18.0	18.0	0.27
13	19.6	19.8	19.8	19.7	1.9
14	20.1	20.2	20.3	20.2	1.7
15	20.7	21.1	21.2	20.9	2.0
16	21.3	21.4	21.6	21.4	1.5
17	21.7	22.0	22.0	21.8	1.6
18	22.4	22.4	22.4	22.4	0

[a] Numbers correspond to sequential peaks in Fig. 5.7.

[b] Migration times were corrected using a two-peak normalization method [54].

One SPE preconcentrator device was used for up to 30 runs (data not shown), demonstrating its robustness of the peptide maps. Displacement of the elution windows with respect to each other is discussed in the text.

5.3.6. Effect of Sample Concentration on Preconcentrator Performance

The effect of initial peptide sample concentration on the SPE preconcentrator performance was investigated. The SPE-CE peptide maps for concentrations ranging from 800 to 100 nM of the same tryptic digest of β-casein (initial concentration of 80 μM) are shown in Figure 5.8. The same preconcentrator cartridge was used in the 4 experiments, starting with the lowest concentration sample. Clearly, a peptide-specific correlation between sample concentration and detectability exists. There is a bias towards larger peptides, which are (1) typically more hydrophobic than small peptides, thus better retained by the SPE preconcentrator, and (2) often have a higher molar absorptivity that small peptides, so signal intensity is higher. From the electropherogram in Fig. 5-8D, loading 100 nM appears to be the minimum concentration of β-casein digest that can be mapped with our discontinuous on-line SPE preconcentrator design.

Strausbauch et al. [32] performed a similar study, although they increased the injected volumes of peptide standards as concentrations decreased so that mass loadings were equivalent. They found that SPE-CE is concentration dependent, with loss of sensitivity at low concentrations (~ 400 nM) likely being caused by adsorptive losses during sample manipulation. Our results concur with theirs, although we did not adjust injected volumes as concentration changed. Instead, we used a constant injected volume the choice of 40 μl because this is the volume of digest exiting the enzyme microreactor.

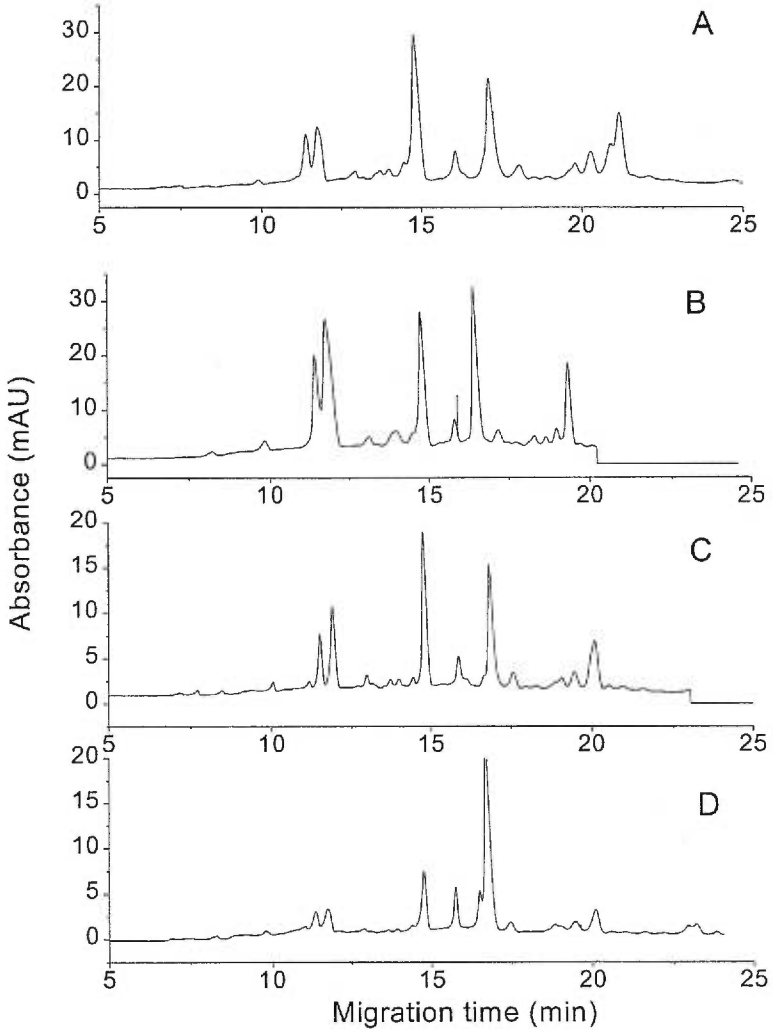

Figure 5.8. Electropherograms showing the effect of sample concentration on SPE-CE for peptide maps of various dilutions of an 80 μM β-casein tryptic digest. (A) 800 nM (100-fold dilution); (B) 400 nM (200-fold dilution); (C) 200 nM (400-fold dilution); (D) 100 nM (800-fold dilution). Loading, elution and separation conditions were the same as in Fig. 5.5. The same preconcentrator cartridge, 1.5 mm long, was used in all four experiments.

On one hand, Strausbauch et al. [32] reported that the retentive capacity of their SPE cartridge (0.3 – 0.7 mm long) was not exceeded when loading up to 288 μl peptide (780 ng). This implies that we might improve peptide mapping sensitivity if the volume of protein sample loaded into the microreactor (currently only 40 μl) prior to SPE-CE was increased. On the other hand, attaining good peptide maps for lower protein concentrations may not be achievable due to the concentration dependence of SPE-CE.

For example, we examined the difference between the SPE-CE peptide maps of a 400 nM sample of β-casein (Fig. 5.9A) versus an 80 μM sample of β-casein for which the peptide fragments were diluted to 400 nM (i.e., 200-fold) before SPE-CE (Fig. 5.9B). The same SPE cartridge and desorption and separation conditions were used to obtain the peptide maps shown in Fig. 5.9. While the relative migration window is the same for the two electropherograms, that in Fig. 5.9B is shifted towards zero due to variability in the distance the elution plug is pushed into the separation capillary as the C18 packing tightens. Significant differences in relative peak height for the two peptide maps are seen in Fig. 5.9. The smaller peaks obtained for the digest of 400 nM protein (Fig. 5.9A) are likely due to sample losses in either the enzyme microreactor or the handling between digestion and preconcentration. At such low concentrations and small volumes (i.e. 40 μl loaded), any loss of peptide inside the microreactor due to adsorption on the CPG-trypsin beads or on the capillary wall will dramatically reduce the recovery of peptides. Further studies are underway to estimate recovery from the microreactor.

The peptide maps in Fig. 5.9 differ from that in Fig. 5.8B (also 400 nM) for the following proposed reasons. First, the SPE cartridge length was 0.3 mm shorter for the maps obtained in Fig. 5.9 than in Fig. 5.8, which implies a slight variation in the chromatographic process. Secondly, we used a different batch of dephosphorylated β-

casein, which may contain impurities in the form of β-casein isoforms. For example, in studies where the protein was denatured with urea and electrophoresed in a bare f.s. capillary, we found that β-casein had several isoforms (data not shown). Presumably there are batch-to-batch differences in the quantity of the major isoform with respect to the total amount of protein. Finally, according to the label, the protein sample was approximately 80% dephosphorylated, which meant that variability in the quantity of phosphorylated versus dephosphorylated β-casein can probably occur between batches. Unfortunately, we were not equipped to further purify the protein before enzymatic digestion, which is highly desirable for high precision mapping. Otherwise, the electropherogram represents superimposed peptide maps of each unique protein in the sample, provided their concentrations are sufficient for detection. The fact that we have observed several differences, mostly in relative peak height, for peptide maps of different batches of β-casein digested using the same microreactor (data not shown) strongly suggests that our protein sample does not have a unique composition.

5.4. Conclusions

We have presented here a rigorous study of several operational aspects of a discontinuous on-line desorption SPE-CE system for mapping the peptides of dephosphorylated β-casein. This protein presented some complications with respect to evaluating peptide map reproducibility because it was found to have isoforms. However, the choice of using a peptide map for evaluating the performance of the SPE-CE device was deliberate, in that the complex mixture of hydrophobic and hydrophilic peptides provided a more realistic sample than the typically used peptide analogs or drugs. In particular, our results are the first to point out the severe effect of the chromatographic process from SPE-CE, which is superimposed on the electrophoretic separation.

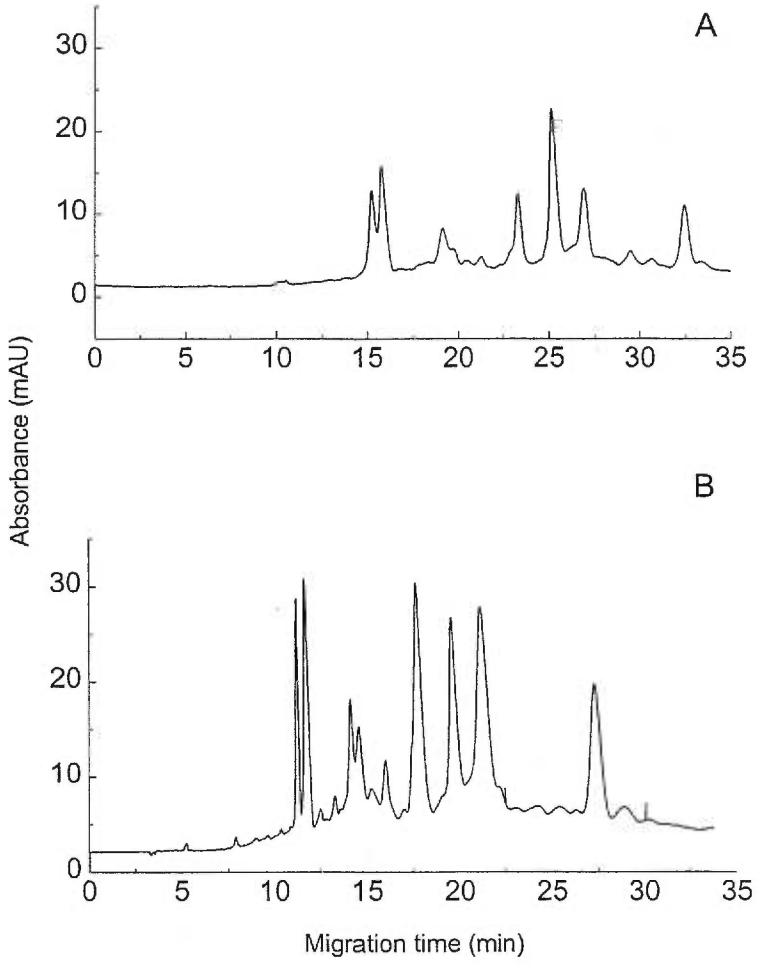

Figure 5.9. Electropherograms comparing peptide maps for dilution before and after protein digestion followed by SPE-CE. (A) Tryptic digest of 400 nM β-casein and (B) a 200-fold dilution of a tryptic digest of 80 μM β-casein (i.e., 400 nM sample). Loading, elution and separation conditions were the same as in Fig. 5.5. The preconcentrator cartridge was 1.2 mm long.

While the quality of peptide maps was good for a 1.5 mm long bed of C18 beads and a 65 ± 15 nl volume of desorption solution, our results clearly show that a more precise method for metering the desorption solution, independent of the SPE cartridge backpressure, is necessary. We are currently testing an on-line SPE-CE design using micro valves to insure accurate and precise delivery of desorption solution. On the other hand, the current design of our discontinuous on-line desorption system (Fig. 5.1) could easily be adapted for delivery of buffer and desorption solution from 2 ml sample vials that fit into the CE instrument sample carousel to create a more compact system.

Acknowledgements

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Chapitre 6

On-line system for peptide mapping by capillary electrophoresis at sub-micromolar concentrations

Eric Bonneil et Karen C. Waldron, soumis à *Talanta* (accepté avec corrections mineures)

Abstract

Peptide mapping has been widely used for the identification of modified proteins involved in certain diseases. Despite the fact that capillary electrophoresis (CE) has been shown to be a powerful tool for the separation and detection of tryptic peptide fragments after protein digestion, this technique lacks sensitivity for mapping proteins isolated in small quantities from biological samples. Consequently, it has been necessary to preconcentrate the protein before adding the proteolytic enzyme for digestion in solution. These experimental steps are quite long, labor intensive and require a lot of sample handling. In this paper, we describe an on-line system allowing digestion of the protein, followed by preconcentration, separation and detection of the tryptic fragments in four hours. Despite a loss of efficiency and hence resolution induced by the multiple valve design of the system and rather large desorption volume (60 nl), up to an 800-fold preconcentration factor was achieved for cytochrome *c*. Moreover, our system showed fairly good reproducibility between peptide maps and could be reused for several samples.

Keywords: preconcentration; solid-phase extraction; peptide mapping; capillary electrophoresis; protein digestion; trypsin microreactor; insulin chain B; cytochrome *c*; β-casein;

Abbreviations: CE, capillary electrophoresis; HbA, normal human hemoglobin; HbS, sickle cell hemoglobin; LOD, limit of detection; EOF, electroosmotic flow; SPE-CE, solid-phase extraction-capillary electrophoresis.

6.1. Introduction

Peptide mapping was first developed in 1958 by Ingram to pinpoint the difference between normal human hemoglobin (HbA) and sickle cell hemoglobin (HbS), responsible for sickle-cell anemia [1-3]. Ingram showed by peptide mapping that the difference between these two proteins came from the replacement of a glutamic acid residue in HbA by valine in HbS. This was the first time that an inherited disease was shown to arise from a specific amino acid modification in a protein. Amino acid substitutions and post-translational modifications (e.g., phosphorylation) alter the charge-to-mass ratio of peptides bearing such changes. During electrophoresis, this induces a change in migration time of the modified peptide compared to the normal peptide. The transformation of classical electrophoresis to CE in 1981 has proven it to be a powerful tool for peptide mapping, capable of identifying modified proteins [4-8].

Conventional enzymatic digestion of a protein is performed in solution with a reaction time of up to 24 h [9]. This method often produces autoproteolysis of the enzyme, leading to non-substrate related background peaks. To avoid autoproteolysis and render the enzyme reusable, immobilized enzymes have been employed in a microreactor format through which the protein solution is perfused and undergoes digestion [10-12]. In a previous report by us [13], we described the characterization of a trypsin-based microreactor that allowed protein digestion in a few hours. To insure good detectability after CE separation, we used fairly high protein concentrations in that work (80 μM), which are not representative of the dilute solutions often obtained after isolation and purification of proteins from biological samples.

In CE, the narrow bore capillary allows injection of about 10 nl, which is convenient for biological sample analysis. Paradoxically, this advantage leads to major

drawbacks. Most commercial CE detectors rely on on-column UV absorption. Therefore, the optical pathlength is essentially equal to the inner diameter of the capillary: 25 to 75 μm. These dimensions pose a severe limitation to the Beer-Lambert law. The concentration limit of detection (CLOD) in CE, which ranges from 10^{-5} to 10^{-6} M, is thus poorer than in high performance liquid chromatography (HPLC), a complimentary technique used for peptide mapping. Unfortunately, protein concentrations in biological samples (blood, plasma, cells) can be in the sub-micromolar range. At these concentrations, tryptic fragments represented in the peptide map are undetectable by conventional CE with UV absorbance detection.

A variety of techniques have been developed over the past ten years to improve detection sensitivity in CE, not only for peptide mapping applications. Z-shaped cells [14, 15] produce the best sensitivity enhancement—one order of magnitude—but remain expensive. It is possible to improve CLOD using less expensive techniques like field amplification, isoelectric focusing and isotachophoresis, which are methods of analyte stacking [16-18]. However, since these physico-chemical techniques are carried out in a conventional CE capillary, the maximum sample volume that can be analyzed cannot exceed the total volume of the capillary, typically 1 to 2 μl. Coupling capillary isotachophoresis to CE has been shown to enhance the CLOD [19, 20], but this method is difficult to implement and has other limitations.

Off-line preconcentration methods, which can lower the CLOD several fold, are often precluded because of polypeptide aggregation or adsorptive losses to the surfaces of vials and pipets. A good alternative to this has been the development of on-line solid-phase extraction (SPE-CE) methods involving either C_{18}-bead or membrane preconcentrators. This technique was first pioneered by Guzman *et al.* [21] for the enrichment of metabolites

in urine. It has since been applied to a large variety of compounds like peptides [22-25], proteins [26, 27], and even to peptide mapping [28, 29]. In most of the cases, sensitivity enhancements were at least two orders of magnitude.

We recently characterized a preconcentration system specifically adapted for peptide mapping [30]. Maps of β-casein digests diluted to 400 nM were obtained with good resolution and reproducibility. The current report describes the on-line coupling of our immobilized-enzyme microreactor, SPE preconcentrator and CE in an effort to improve mapping sensitivity by minimizing sample handling that leads to peptide losses. Because of problems related to the preconcentrator (reversal of electroosmotic flow (EOF) at low pH) as described in reference [30], we have designed our on-line system in such a way that the preconcentrator is not part of the separation capillary, unlike most configurations reported in the SPE-CE literature. Consequently, the preconcentrator should not interfere with the separation process. In this paper, the on-line peptide mapping system performance is evaluated for low concentrations of insulin chain B, cytochrome *c* and β-casein.

6.2. Experimental

6.2.1.Materials

Acetonitrile, methanol, bovine dephosphorylated β-casein, cytochrome *c*, insulin chain B and TPCK-treated trypsin attached to DITC-controlled pore glass (80-120 mesh, 700 Å average pore size; activity, 8,300 units/g) were obtained from Sigma (St. Louis, MO). Monobasic sodium phosphate, phosphoric acid and ammonium carbonate were purchased from Anachemia (Montréal, QC). C_{18}-derivatized beads (40 μm diameter) were purchased from Aldrich (Milwaukee, MI). A 5-psig gauge and valve were purchased from Labcor (Anjou, QC). Prepurified argon was obtained from Praxair (Montréal, QC).

Swagelock connectors, a reducing union, 0.2 μm-pore nylon syringe filters, Teflon tubing (1/16″ o.d. × 0.040″ i.d.), Wheaton 5-ml V-vials and the capillary rinse kit were purchased from Chromatographic Specialties (Brockville, ON). Various sizes of fused silica capillary tubing (75 μm i.d. × 350 μm o.d.; 250 μm i.d. × 350 μm o.d.; 530 μm i.d. × 820 μm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ). Polyethylene tubing (0.38 mm i.d., Intramedic No. 7405 Clay Adams) and syringes (30-ml and 1-ml) were purchased from VWR-Canlab (Mississauga, ON). In-house distilled water was purified with a multi-cartridge Millipore water filtration/deionization system before use. Vials, 20 ml, were purchased from Kimble Glass Inc. (Vineland, NJ). A 12-port multiposition valve with electric actuator (on load from Prof. Dovichi, Univ. of Alberta, Canada) and a 4-port 2-position valve with 60 nl internal loop as well as 1/32″ and 1/16″ removable fused silica adapters were from Valco (Houston, TX). An Upchurch PEEK shut-off valve and micro-tee for 0.025″ tubing were purchased from SPE Inc. (Concord, ON).

6.2.2. Buffer and sample preparation

Sodium phosphate buffer prepared from Millipore-purified water was used for all CE separations and manipulations involving the on-line system. The pH was adjusted to 2.5 with phosphoric acid. The separation buffer was always filtered through a 0.2 μm-pore syringe filter before use. Elution of peptides from the C_{18} preconcentrator was achieved using a solution of acetonitrile:phosphate buffer, 90:10 (v/v).

6.2.3. CE instrumentation

Separations were carried out on a homemade CE system equipped with a variable wavelength UV detector (Dionex, Sunnyvale, CA) adapted in-house for on-capillary detection. In this work, detection of peptides was carried out at λ=200 nm. Analog output

from the detector was digitally converted and acquired on a personal computer using Maxima 820 software (Dynamic Solutions, Division of Millipore). Stored electropherograms were imported as data files into a Microcal Origin (Northampton, MA) spreadsheet for plotting.

6.2.4. Microreactor and preconcentrator development

Fabrication and characterization of the microreactor [13] and preconcentrator [30] are described in detail elsewhere.

6.2.5. Construction of the on-line system

Components of the on-line system are shown in Fig. 6.1. We substituted the rinse kit vial with a Wheaton V-vial that was conical inside for use as a sample vessel. Before installation in the on-line system, the microreactor (16-cm long) was rinsed with several column volumes of ammonium carbonate buffer then inserted into a condenser, as previously described [13], to provide a 37°C environment for tryptic digestion. A 20-cm, 530-μm i.d. fused silica capillary tubing served as a transfer line from the sample V-vial to the microreactor, where it was connected by means of 1-cm sleeve of Teflon tubing (1/16″ o.d. × 0.04″ i.d.), sealed in place with 5-min epoxy. The outlet of the microreactor was then connected to the inlet port of the multiposition valve via a second transfer line of 250 μm i.d. × 30-cm fused silica tubing.

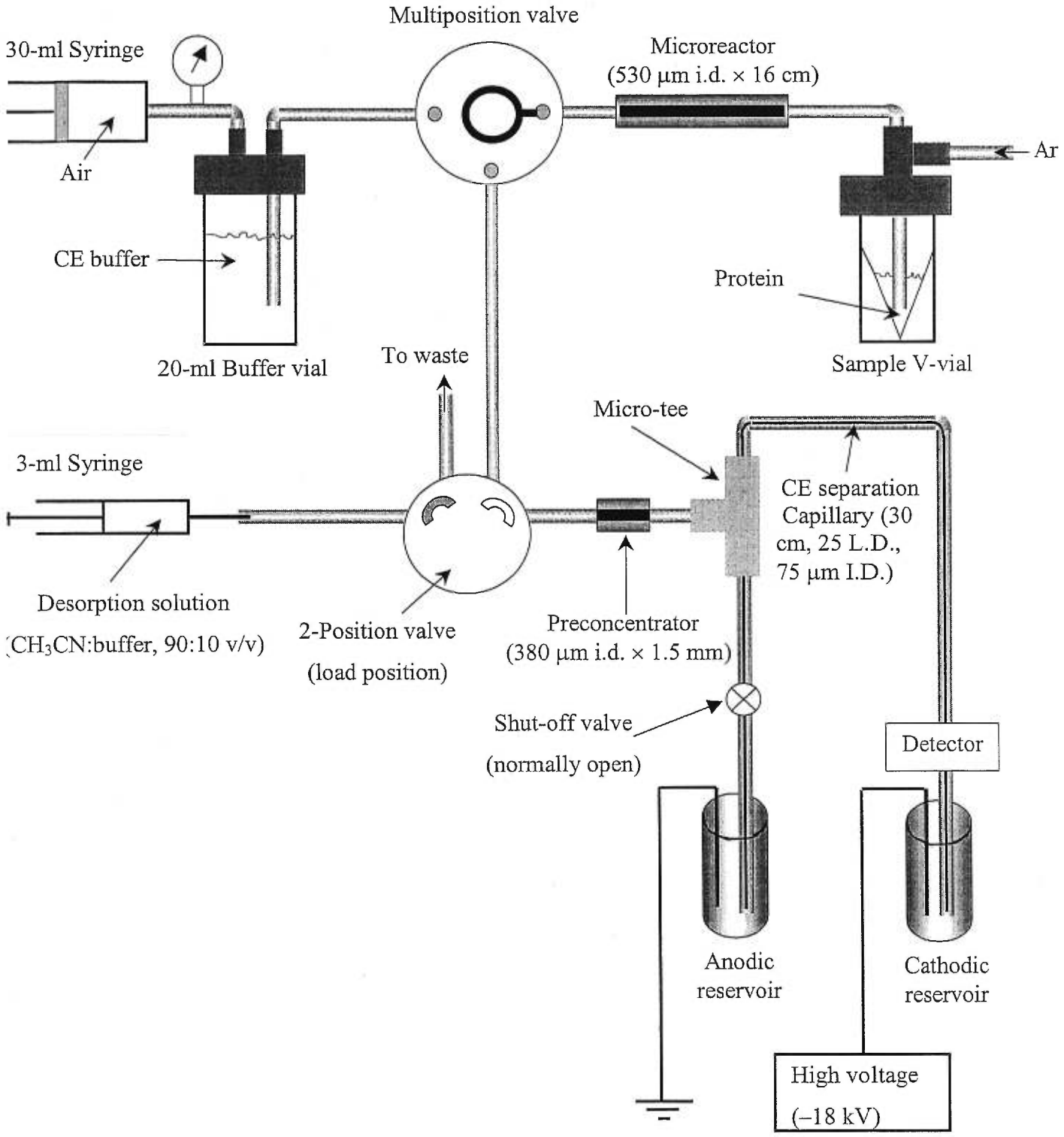

Figure 6.1. Schematic of the on-line peptide mapping system (see Section 6.2 for details).

CE separation buffer was delivered from a 20-ml glass vial whose screw cap was adapted with a Teflon disk tapped with two threaded holes to allow pressurization via one port and liquid delivery via the other (see [30]). Air pressurization of the 20-ml vial was achieved using a 30-ml syringe attached to the vial by a 60-cm length of 0.04″ i.d. Teflon tubing into which a 5-psig gauge was inserted to monitor the rate of pressurization. Separation buffer was transferred from the vial to the multiposition valve via 10 cm of 250-μm i.d. fused silica capillary.

The outlet port of the multiposition valve was connected to one inlet of the 2-position valve via 15 cm of 250 μm i.d. fused silica capillary. To the other inlet, a 7-cm length of Teflon tubing (0.04″ i.d.) was used to connect a 3-ml syringe containing the desorption solution (acetonitrile:buffer, 90:10). One outlet of this 2-position valve went to waste whereas the other was connected to the preconcentrator cartridge (1.5 mm packed bed of C_{18} stationary phase) by a 4-cm length of 75 μm i.d. fused silica capillary, as shown in Fig. 6.1. The configuration of the 2-position valve allowed delivery of precisely 60 nl of desorption solution to the preconcentrator by switching this valve back and forth. The outlet end of the preconcentrator was connected to the separation capillary by means of a micro-tee having an internal (swept) volume of 30 nl. The preconcentrator was flushed with several column volumes of methanol before its installation in the on-line system. Finally, a small-volume shut-off valve, normally open, was inserted at the grounded (anodic) end of the separation capillary as shown in Fig. 1 to insure that the plug of desorbed peptides would flow into the separation capillary toward the cathode instead of toward the anode during transfer from the preconcentrator.

6.2.6. Operation of the on-line system

The on-line system, from sample V-vial to separation capillary outlet, was initially flushed with air in the absence of protein sample to relieve backpressure generated by liquid-filled flow paths. The preconcentrator was then temporarily disconnected from the micro-tee to further reduce backpressure and to prevent the digestion buffer from entering either branch of the separation capillary. Protein solution (40-100 μl) was placed in the sample V-vial, which was then pressurized by argon (1 to 2 psig) to perfuse the sample through the enzyme microreactor to effect digestion. The average perfusion rate was approx. 0.5 μl/min. The tryptic peptide fragments (the digest) were routed through the multiposition valve and 2-position valve to the preconcentrator where adsorption on the C_{18} solid phase took place. Continuous argon pressure (1-2 psig) was applied until no more liquid (i.e., digest buffer) exited the preconcentrator.

At this stage, the preconcentrator was re-connected to the micro-tee and the multiposition valve was switched to the CE buffer (20-ml vial) position. The 2-position valve was switched to the *inject* position to allow the 60-nl loop, the preconcentrator and both branches of the separation capillary to be flushed with CE buffer. This flushing step was achieved by manually activating the piston of the 30-ml syringe, which generated a pressure of approx. 4 psig inside the 20-ml vial. Flushing continued until buffer could be seen exiting the cathodic and anodic ends of the capillary (approx. 2 min). The shut-off valve was then closed.

Next, the 2-position valve was switched to the *load* position and the 60-nl loop filled with desorption solution (acetonitrile:buffer, 90:10 v/v). Switching this valve back to the *inject* position followed by application of 3.5 psig for approx. 30 s to the 20-ml vial allowed the 60-nl plug of desorption solution followed by buffer to be pushed through the

preconcentrator and directly into the cathodic branch of the capillary, a distance of about 5 cm from the tee. Finally, the shut-off valve was re-opened and high voltage applied at the cathodic end to commence electrophoresis of the elution plug already situated in the separation capillary. The on-line mapping procedure took 4 h, from protein sample introduction to completion of the separation (i.e., acquisition of the peptide map).

6.3. Results and discussion

6.3.1. On-line system design

The peptide mapping system shown in Fig. 6.1 comprises several components, each of which was first studied in detail off-line before its integration on-line. For example, digestion of protein in a 28-cm microreactor packed with trypsin beads was optimized, as described in previous reports [13, 31], for substrate concentrations in the order of 80 to 200 μM. Under these conditions, we obtained fairly reproducible peptide maps. Since the size of microreactor required (i.e. quantity of immobilized enzyme) depends on the quantity or concentration of protein to be digested, a 16-cm long microreactor (with 14 cm heated to 37°C) was deemed sufficient in the present study for digestion of sub-micromolar concentrations of protein solution.

The solid-phase extraction (SPE) preconcentrator was coupled to the CE capillary in a pseudo on-line fashion for the following reasons. True on-line preconcentration, where the solid-phase bed is inserted into the CE capillary, has demonstrated great efficiency for the analysis of many biological samples without further pretreatment [32-36]. Nonetheless, some drawbacks are associated with in-column preconcentrators. For example, reversal of the EOF at low pH results in re-adsorption of the peptides on the solid phase [37, 38] [25]. Contributions to band broadening, loss of resolution, and backpressure generated by the

solid phase have also been reported as disadvantages to C_{18} beads packed into the capillary inlet [39, 40].

Therefore, to minimize the influence of the SPE device on the CE separation, we designed a system in which the preconcentrator was not placed in the same capillary where electrophoresis was to take place. A micro-tee was used to couple the preconcentrator to the separation system. Unfortunately, this design required the insertion of a shut-off valve within the separation capillary, situated between the anodic end and the micro-tee. This shut-off valve was needed to ensure that the elution plug was pushed toward the cathodic end of the capillary during the desorption process. In the absence of this valve, the elution plug either took the path of least resistance, i.e. toward the anodic buffer reservoir, or traveled into both branches of the separation capillary generating excessive band broadening and occasionally peaks indicative of two independent injection plugs. Several alternate configurations were investigated (i.e. narrower bore tubing for the anodic branch) in the hope of eliminating the shut-off valve, but to no avail. The use of larger bore tubing (250 μm) for the anodic branch in conjunction with the shut-off valve was explored as an alternative to disconnecting the preconcentrator from the micro-tee during digestion and adsorption. However, this configuration also caused part of the elution plug to be directed toward the anodic branch of the separation capillary even when the shut-off valve was closed.

In our recent report describing the characterization of an SPE-CE device [30], we used up to 80 nl of acetonitrile:buffer solution for desorption after sample loading. Imprecise metering of this volume contributed to irreproducibility between peptide maps. Therefore, we incorporated a 60-nl fixed volume loop into the present system for precise and reproducible injection of the acetonitrile:buffer desorption solution.

To verify the efficacy of desorption in this small volume, we preconcentrated 50 μl of 400 nM insulin chain B (undigested), an hydrophobic polypeptide. Desorption from the SPE cartridge (in 60 nl acetonitrile:buffer, 90:10 v/v) was carried out as described in Section 6.2.6 and the elution plug was pushed past the CE detector hydrodynamically (no voltage applied). The large peak observed (data not shown) was attributed to insulin chain B plus acetonitrile, the latter of which absorbs slightly at 200 nm. Injection and detection of a second, third and fourth 60-nl elution plug produced a small peak of identical height each time. By running a blank, this peak was confirmed to be acetonitrile each time and not residual insulin chain B. Therefore, we were confident that 60 nl of desorption solution could efficiently elute peptide fragments from the preconcentrator cartridge.

6.3.2. Insulin chain B: effectiveness of on-line mapping

To test the efficacy of our on-line system, we digested and mapped a simple protein standard: insulin chain B, oxidized. Tryptic digestion of insulin chain B theoretically gives two relatively hydrophobic peptides, each bearing aromatic residues. Therefore, they should be easily adsorbed on the SPE cartridge and easily detectable at 200 nm. Fig. 6.2A shows the peptide map for digestion of 50 μl of 400 nM insulin chain B. The two major peaks at 22 and 30 min corresponded to the two expected peptide fragments and the four smaller peaks at 11, 13, 15 and 17 min to impurities. These assignments were made based on a sample blank, which is shown in Fig. 6.2B, and previous digestions of insulin chain B [13]. The peaks at 11 and 15 min in the sample blank were assumed to be impurities coming from either the digestion buffer or autoproteolysis of the enzyme.

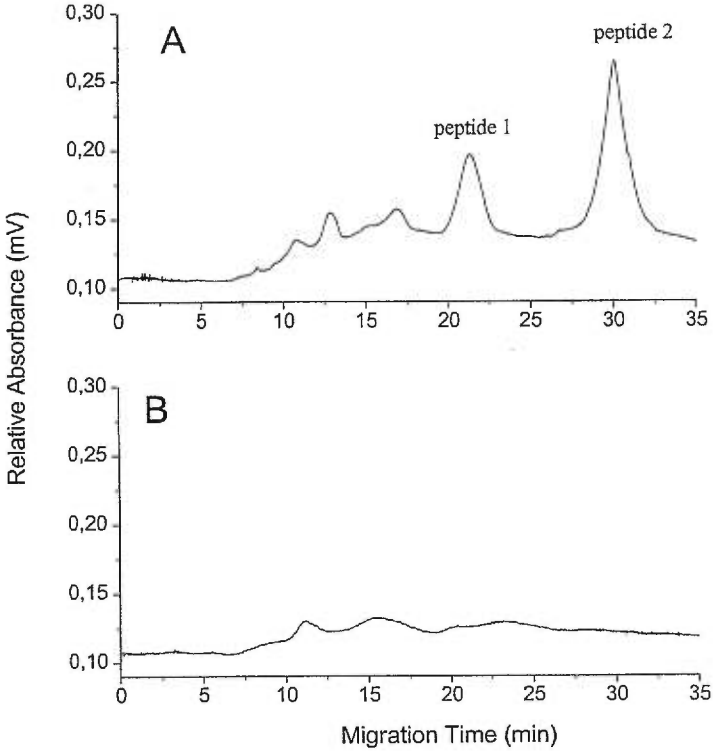

Figure 6.2. On-line digestion and preconcentration of insulin chain B and a blank sample: (A) 50 µl of 400 nM insulin chain B solution; (B) 50 µl of 0.1 M ammonium carbonate (blank). Perfusion of the sample (or blank) through the microreactor was made at 0.5 µl/min. Desorption was made with 60 nl of acetonitrile:30 mM sodium phosphate buffer, pH 2.5, (90:10 v/v) at 3.5 psig for 35 s. Separations were carried out in 30 mM sodium phosphate buffer, pH 2.5, by applying -18 kV to the cathodic buffer reservoir.

Unfortunately, the disadvantage of non-specific preconcentration (i.e. adsorption on C_{18} or styrene divinyl benzene support) compared to specific preconcentration (i.e. antigen-antibody interactions) is that all trace impurities presenting a certain hydrophobicity will also be preconcentrated and appear as peaks in the peptide map. Our previous studies [13] showed that the digestion of Sigma Co. insulin chain B sample actually produced three peptides. Because this manufacturer's standard was used without further purification, the peak at either 13 or 17 min in Fig. 6.2A was assumed to correspond to a modified form of insulin chain B, as seen in previous work. Reducing the concentration of protein substrate to 50 nM still gave clearly defined peaks at 22 and 30 min for the two tryptic fragments (signal-to-noise greater than 9; data not shown). However, the impurity peak at 13 min had a much higher relative peak height compare to the tryptic peptides for 50 nM substrate than for either 100 or 400 nM. This result suggested that the impurity was unrelated to the insulin chain B sample. The peak at 17 min was too small to detect in the peptide map of 50 nM insulin chain B and was thus assumed to be a sample-related impurity.

While the theoretical enrichment factor was 800 (50 μl protein sample ÷ 60 nl digest electrophoresed), we estimated only a 500-fold preconcentration from the data for insulin chain B based on our CE system's detection limit of about 10^{-5} M for peptides. Sample was likely lost during the on-line mapping procedure due to adsorption on the transfer tubing, valves, microreactor and micro-tee. Moreover, the peak efficiency was poor ($N < 1000$ theoretical plates). We believe this was due to two factors. First, the transfer lines and micro-tee introduced supplementary dead volume post desorption leading to extra band broadening. Secondly, pushing the elution plug into the separation capillary induced a certain backpressure which, after opening the shut-off valve, caused the elution plug to

"slip back" several millimeters toward the anodic end. This operation led to further dilution of the injection plug and, thus, further band broadening.

6.3.3. Cytochrome c: preconcentration factor for on-line mapping

Figure 6.3A shows the peptide map of 100 μl of 800 nM cytochrome *c* obtained using the on-line system. For comparison, Fig. 6.3B shows the peptide map of 80 μM cytochrome *c* digested off-line in an immobilized trypsin microreactor (28-cm long) without SPE preconcentration prior to CE separation. While the number of peaks was similar in the two electropherograms, the migration pattern was quite different because preconcentration followed by desorption in 90% acetonitrile induced a chromatographic process superimposed on electrophoretic migration [25, 30]. Adsorption on the solid-phase device is highly peptide dependent: hydrophobic peptides will be preferentially enriched over the more hydrophilic peptides. Ultimately, relative peak heights after desorption will be modified. Moreover, the peptide map is a function of the desorption rate of each peptide such that the elution plug is not homogeneous. This will modify the migration time of the analytes relative to electrophoresis performed without on-line preconcentration.

Peak heights varied between Fig. 6.3A and B due to the enrichment factor. In electropherogram A, 80 pmol of protein sample (800 nM × 100 μl) was digested, preconcentrated, then desorbed in 60 nl. Therefore, the equivalent concentration in the elution plug was theoretically 1.3 mM, assuming 100% recovery after preconcentration. This represents a maximum preconcentration factor of 1600. In electropherogram B, the initial cytochrome *c* concentration was 0.08 mM. Consequently, the peaks in Fig. 6.3A tropherograms should in reality be roughly 16 times higher than

average peak height for the seven major peaks in Fig. 6.3A and B was 0.18 and 0.046 mV, respectively, demonstrating a preconcentration factor of 4. Comparing average peak areas,

a preconcentration factor of > 8 was achieved. These values are far from the theoretical factor of 16 for several reasons. First, previous studies on preconcentration of a hydrophobic peptide showed that recovery was only about 79 % [30]. Second, severe band broadening in the maps obtained on-line greatly reduces the utility of using peak heights for comparison purposes. Third, there were many opportunities for peptide losses through adsorption to components in the on-line flow path, which would also reduce the quantity of peptide detected compared to the expected amount. Given the experimental data, we more likely attained an 800-fold concentration factor for most of the tryptic peptides.

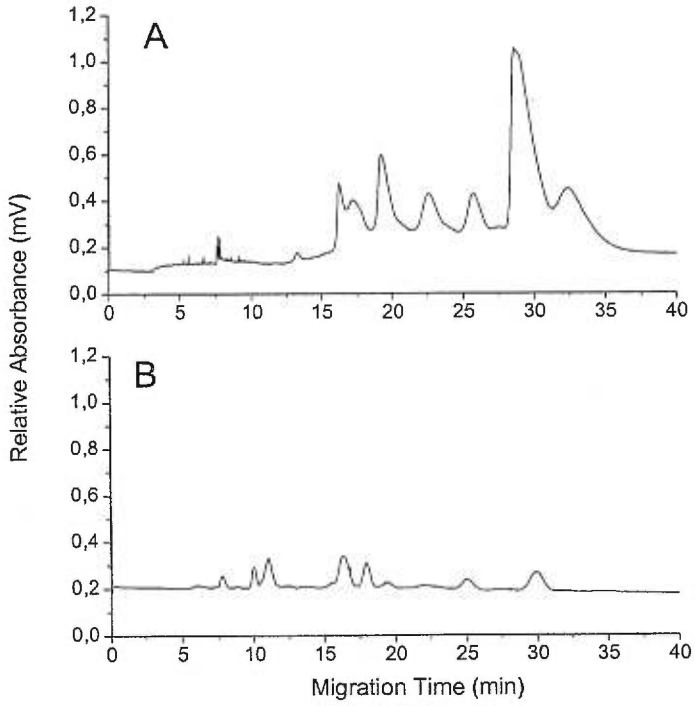

Figure 6.3. Influence of the on-line system on the peptide map of cytochrome *c*: (A) 100µl of 800 nM cytochrome *c* solution digested and preconcentrated with the on-line system. Desorption was made with 60 nl of acetonitrile:30 mM sodium phosphate buffer, pH 4.6 (90:10 v/v), at 3.5 psig for 30 s; (B) 40 µl of 80 µM cytochrome *c* digested off-line using a 28-cm long microreactor and no preconcentration of tryptic fragments with 10 nl injected into the CE. Separations in both cases were carried out in 30 mM sodium phosphate buffer, pH 4.6, at 18 kV applied potential.

6.3.4. Reproducibility

We examined the reproducibility of peptide maps obtained with our on-line system using 100-μl aliquots of 800 nM cytochrome *c* (Fig. 6.4). For each digestion, a new microreactor and a new preconcentrator were installed. Previous studies on the individual components of the on-line system [13, 30] showed that reproducibility in maps improved several fold when either the same microreactor or the same preconcentrator were reused. Therefore, the three electropherograms in Fig. 4 represent the worst case scenario starting point to examine the performance of the system. Nonetheless, the maps were fairly reproducible in terms of peak height and relative migration time, as can be seen in Fig. 6.4. Differences in migration time between maps in Fig. 6.4 arise from the slightly different backpressure induced by each preconcentrator, even though care was taken to loosely pack each of the three cartridges studied. Because the pressure and duration required to move the elution plug into the separation capillary was the same for all three trials, slight backpressure differences resulted in a different distance traveled by the elution plug. This means that each map corresponded to a different effective length of electrophoretic separation, which translated into irreproducible separation efficiency and resolution between the three maps. On the other hand, repeated use of the same microreactor and preconcentrator cartridge gave much better map reproducibility, as can be inferred from the data presented in the next section.

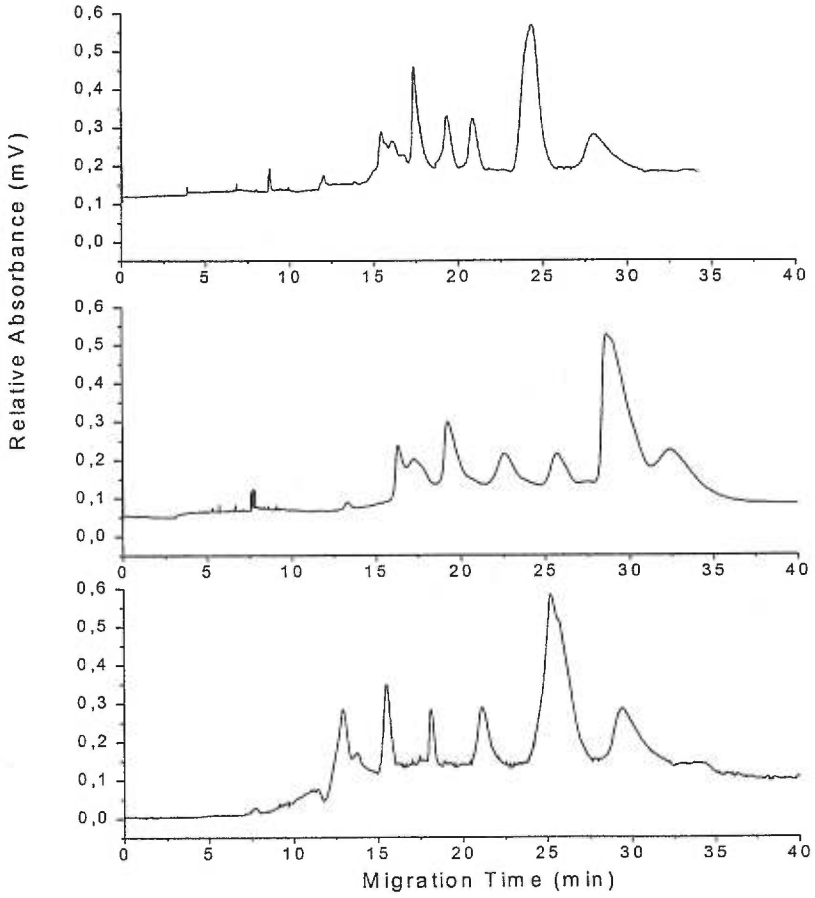

Figure 6.4. Reproducibility of peptide maps obtained with the on-line system for 100 µl aliquots of cytochrome *c* solution at 800 nM. In each case, a different microreactor and preconcentrator were used. Desorption was made with 60 nl of acetonitrile:30 mM sodium phosphate buffer at pH 4.6 (90:10 v/v), at 3.5 psig for 30 s. Separation conditions were the same as in Fig. 6.3.

6.3.5. Effect of initial sample concentration

On-line peptide maps for initial protein concentrations ranging from 100 to 800 nM are presented in Figure 6.5. Unlike the data presented in Section 6.3.4, the same microreactor and preconcentrator were used in these 3 experiments, in which the lowest concentration sample was mapped first. There is a predicted correlation between sample concentration and detectability that is peptide specific. For example, larger peptides or those containing many aromatic side chains are typically the most hydrophobic so their signal intensity should be the highest at 200 nm. In Fig. 6.5C, the later eluting peptides were just barely detected at 100 nM for 100 μl injected initial protein sample.

Subjecting larger volumes of protein standard to the on-line mapping system in order to improve peptide detection in the maps was not attempted based on a literature report that SPE-CE performance was concentration dependent [40]. Strausbauch et al. showed that adjusting the volume to compensate for the decrease in concentration did not restore peak sensitivity in the electropherogram [40]. In our case, adsorptive losses during sample manipulation and the preconcentration process also contributed to an overall loss of sensitivity. Therefore, any further improvements in peptide mapping sensitivity may require an additional preconcentration step prior to substrate digestion.

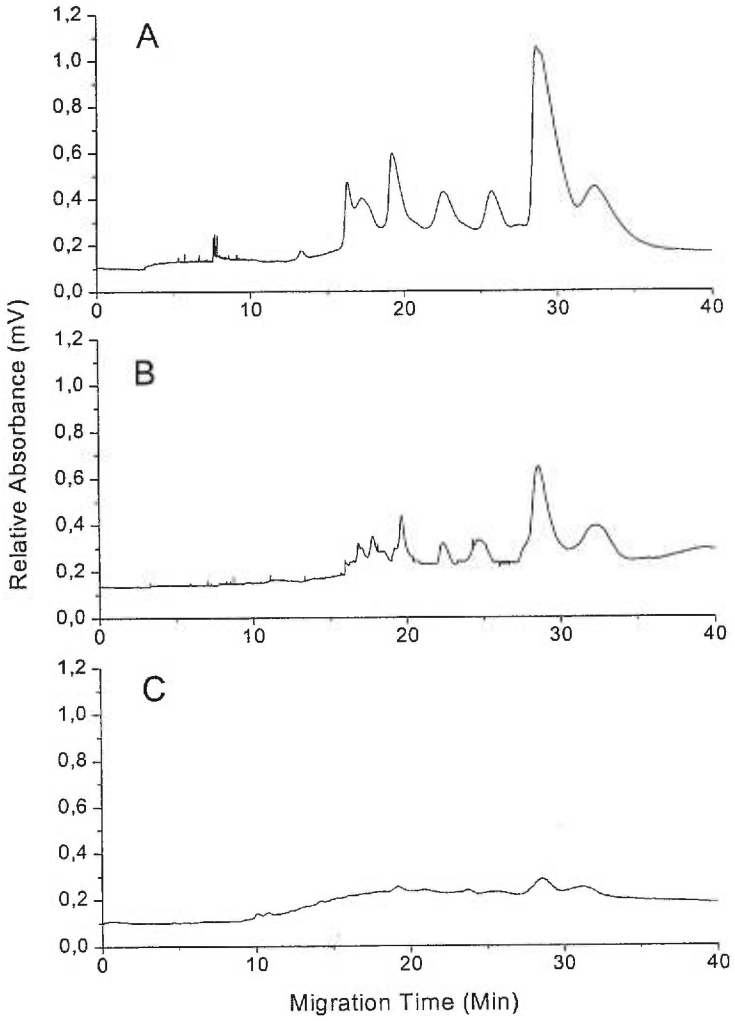

Figure 6.5. Influence of substrate concentration on the peptide map obtained using the on-line system for 100 µl cytochrome *c* at (A) 800 nM; (B) 400 nM; (C) 100 nM. Desorption and separation conditions were the same as in Figure 6.4.

6.3.6. Contribution of backpressure to poor peak efficiency

As expected, resolution of peaks in the peptide map suffered when digestion and preconcentration were made on-line. This loss of resolution resulted from a combination of excessive dead volume and backpressure generated by system components and by the pressure needed to flow the desorption plug through the system. To probe these effects, two preconcentrators were fabricated, one with loosely packed C_{18} beads and one with tightly packed C_{18} beads. The time t taken for the elution plug to pass the detection window when mobilized *only* by pressure at 3.5 psig (no electric field applied) was first determined for the loosely packed preconcentrator installed on the on-line system. Then, the tightly packed preconcentrator was installed and the pressure needed to push the plug past the detection window in the *same* time interval t was evaluated. This pressure was determined to be 7 psig. Therefore, it was presumed that a given elution plug would be at the same starting position x in the separation capillary when using either the loosely packed preconcentrator and desorption/injection at 3.5 psig × 30s or the tightly packed preconcentrator and desorption/injection at 7 psig × 30 s. This procedure allowed us to compare two peptide maps made with the same effective CE separation length but different contributions to backpressure from the on-line system.

Fig. 6.6 shows the effect on map resolution of having a higher backpressure in the system due to a tightly packed preconcentrator and elevated desorption pressurization. In Fig. 6.6A, the backpressure associated with the tightly packed preconcentrator led to much poorer resolution in the peptide map of 800 nM cytochrome *c* compared to the map obtained using a more loosely packed preconcentrator and, hence, lower backpressure (Fig. 6.6B).

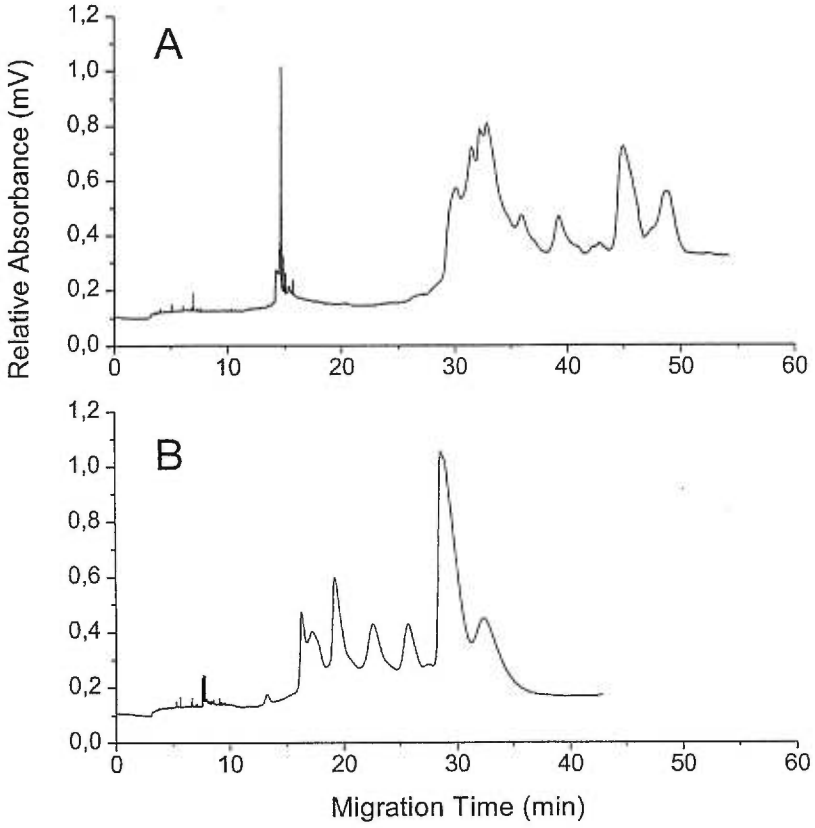

Figure 6.6. Influence of the pressure applied during desorption on the peptide map of 100µl of 800 nM cytochrome *c*: (A) preconcentration on a tightly packed preconcentrator after digestion on the on-line system. Desorption was made with 60 nl of acetonitrile:30 mM sodium phosphate buffer at pH 4.6 (90:10 v/v), at 7 psig for 30 s; (B) preconcentration on a loosely packed preconcentrator. Desorption was made as in (A) but at 3.5 psig for 30 s. Separation conditions were the same as in Fig. 6.3.

The higher backpressure (Fig. 6.6A) caused the injected plug to "slip" back into the anodic branch of the capillary once the shut-off valve was opened and voltage was applied. This slippage led to a longer effective migration length to the detector and dispersion of the injection plug, resulting in severe loss of resolution for early eluting peptides in the map. At the low pH of separation, the electroosmotic flow is weak and thus unable to overcome this effect of slippage. Therefore, care had to be taken to use a loosely packed preconcentrator and to push the elution plug inside the separation capillary at low pressure to reduce band broadening. This means care must be taken to build a preconcentrator with fairly loose packing.

In a previous paper, we characterized the preconcentrator using a "discontinuous on-line system" [30]. In this procedure, the outlet of the digest-loaded preconcentrator was butt-connected to the anodic end of the separation capillary. The peptides were eluted directly into the separation capillary and then pushed a few millimeters further toward the cathodic end by pressurization with separation buffer. The preconcentrator was then disconnected from the separation capillary whose anodic end was immersed into the pH 2.5 separation buffer reservoir to begin electrophoresis. Fig. 7 compares the peptide maps of β-casein tryptic digest (40 μl at 800 nM) preconcentrated using the discontinuous on-line system (Fig. 7A) versus the on-line system (Fig. 7B). Discontinuous preconcentration gave quite sharp peaks with good resolution compared to the on-line system, which showed a similar peak pattern but much poorer resolution. Desorption conditions were almost identical in both cases: 65 nl and 60 nl for Fig. 7A and 5B, respectively, using acetonitrile:buffer, 90:10 (v/v). Also, the preconcentrator lengths were the same for both (1.5 mm). These results demonstrate that neither the preconcentrator itself nor desorption volume are responsible for the large loss of resolution.

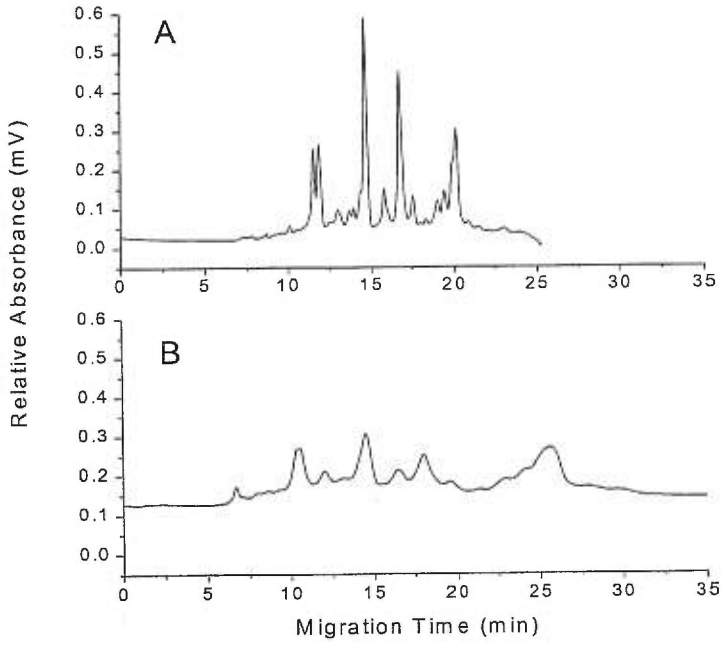

Figure 6.7. Comparison of the on-line mapping system versus off-line mapping using the individual components: (A) peptide map of 40 μl of 800 nM β-casein after digestion in the microreactor followed by discontinuous on-line preconcentration of the tryptic peptides. Desorption was made with 65 ± 15 nl of acetonitrile:50 mM sodium phosphate buffer at pH 2.5 (90:10 v/v). Separation was carried out in 50 mM sodium phosphate buffer, pH 2.5, at 18 kV; (B) peptide map of 40 μl of 800 nM β-casein digested and preconcentrated in the on-line system. Desorption was made with 60 nl acetonitrile:30 mM sodium phosphate buffer at pH 2.5 (90:10 v/v). Separation was carried out in 30 mM sodium phosphate buffer, pH 2.5, at 15 kV applied potential.

Instead, the backpressure and injection plug dispersion resulting from the micro-tee and shut-off valve configuration are to blame for the poor peak efficiency seen in the on-line mapping system. We are currently investigating new designs for SPE-CE components of the on-line system as well as the automation of several of the on-line steps.

6.4. Conclusion

We have constructed an on-line system for mapping the tryptic peptides of insulin chain B, cytochrome *c* and β-casein at sub-micromolar concentrations. This system required minimum sample handling after the initial introduction of 50 to 100 μl substrate solution. Reproducible peptide maps were obtained within 4 hours and the on-line system was reusable for several analyses. The lowest substrate concentrations used in this study did not yield sufficient signal-to noise for the tryptic peptides by conventional CE with UV-absorbance detection. As expected, sensitivity depends strongly on the hydrophobicity of each peptide because this governs the extent of preconcentration. Consequently, tryptic peptides of insulin chain B were still detectable at 50 nM whereas cytochrome *c* tryptic peptides were barely detectable at an initial substrate concentration of 100 nM.

Despite fairly good reproducibility of the maps, the resolution and efficiency were poor compared to conventional CE. This was foremost because of backpressure generated by the preconcentrator and small internal volumes of the micro-tee, separation capillary and 60-nl injection loop, which led to inconsistent transfer of the elution plug into the separation capillary as well as backward flow once the shut-off valve was opened. These effects combined with dead volumes created by the transfer tubes caused extensive band broadening in the peptide map and, ultimately, a loss of resolution. To minimize the backpressure effect, elution plug injection should be made at the lowest pressure possible or

by electroosmosis whereby an electric field applied across the preconcentrator and separation capillary could mobilize the elution plug into the separation capillary.

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Chapitre 7

Conclusion

7.1. Conclusion générale

Comme bon nombre de maladies graves entraînent la modification d'une ou plusieurs protéines, la cartographie peptidique a une place prépondérante dans la détection et l'identification survenues sur les protéines concernées. La technique classique utilisant des enzymes solubilisés génère des peptides supplémentaires dues à l'autoprotéolyse de l'enzyme, nécessite de longues étapes de digestion et de préconcentration et entraîne de la perte d'échantillons du fait de nombreuses manipulations. Par conséquent, le développement de systèmes "en continu" permettant la digestion et la préconcentration sur phase solide représentent un atout important dans ce domaine. Cette thèse a décrit le développement progressif d'un tel système: un microréacteur enzymatique, un préconcentrateur et une étape de séparation.

Le Chapitre 2 a présenté la construction et la caractérisation d'un microréacteur pour la digestion enzymatique de protéines. Tout d'abord, ce microréacteur est d'une réalisation simple et facile. La trypsine immobilisée sur des billes de verre peut être empaquetée sous pression dans un capillaire en une minute. Ce microréacteur est ensuite inséré dans un tube de verre dans lequel circule de l'eau à 37 °C, température d'activité enzymatique maximum de la trypsine. Il est ensuite connecté à un système pouvant être mis sous pression afin de faire migrer la solution de protéine à travers. La caractérisation fut faite en utilisant une protéine facilement hydrolysable: la β-caséine. Cette protéine ne comporte pas de ponts disulfures et est connue pour avoir une conformation ouverte. La carte peptidique de cette protéine obtenue avec le microréacteur en 3 h est très similaire à celle obtenue en 24 h avec la trypsine en solution homogène. Elle comporte également moins de pics dus à l'autoprotéolyse de l'enzyme, phénomène fréquent lorsqu'on utilise la trypsine en solution. Une étude ultérieure menée avec un peptide comme l'insuline nous a

permis de montrer qu'il y a très peu d'autoprotéolyse avec l'enzyme immobilisé. Nous avons obtenu des cartes peptidiques reproductibles pour des concentrations allant de 1 à 5 mg/ml (40-200 μM) à condition que les analyses par CE soient consécutives. Nous avons montré que le débit de la solution de protéine à travers le microréacteur a une grande influence sur la carte peptidique. Le débit de 0.15 μl/min utilisé pour un microréacteur de 28 cm de long a été maximisé pour que le temps de contact entre la protéine et l'enzyme soit suffisant afin que la digestion puisse se produire.

La β-caséine devrait donner 14 pics sur un électrophérogramme enregistré à 200 nm qui correspondent à 14 fragments peptidiques, après digestion avec la trypsine. Or nous avons toujours observé un nombre supérieur de pics pour cette protéine ce qui semble être causé par une digestion incomplète. Ce phénomène a aussi été observé dans différentes autres publications ou le nombre de pics pour la digestion de la β-caséine varie de 16 à 30 (1-3). Du fait que la cartographie peptidique est une technique comparative, une digestion totale est souvent préférée à une digestion partielle afin de supprimer toute ambiguïté sur l'identité de la protéine. Afin de découvrir si les pics supplémentaires décelés sur la carte peptidique de la β-caséine provenaient d'une digestion incomplète de la protéine ou de la protéine elle-même, nous avons reproduit la même digestion avec une protéine de taille équivalente comme l'alpha-lactalbumine. L'alpha-lactalbumine possède plusieurs résidus cystéines et donc des ponts disulfures. Avant digestion il nous a fallu rompre ces ponts disulfures et dériver le groupement thiol sur la chaîne latérale de chaque cystéine afin d'empêcher la reformation de ces ponts. En dépit de cette étape supplémentaire, la digestion de l'alpha-lactalbumine faite dans les mêmes conditions que la β-caséine nous a conduit au nombre de pics attendus avec une assez bonne reproductibilité comme montré dans le chapitre 4. Une analyse subséquente de la β-caséine non digérée dans un milieu

dénaturant nous a permis de distinguer clairement trois pics à des temps de migration très proches. Ceci prouve que la protéine est présente dans l'échantillon de départ sous trois formes (ex. séquences primaires) au moins. En fait, une recherche dans la littérature (4-6) nous a appris que la β-caséine provenant du lait de boeuf est présente sous la forme de plusieurs variants génétiques (A1, A2, A3, B, C, D, E) à des concentrations variables d'un échantillon à l'autre. C'est sans doute pourquoi, nous avons obtenu un nombre de pics supérieur à celui attendu.

Notre système de digestion ne nous permet pas d'avoir des cartes peptidiques de la β-caséine exploitables à des concentrations inférieures à 40 μM. Du fait que certaines protéines impliquées dans les maladies sont à des concentrations allant du nanomolaire au picomolaire dans les échantillons biologiques, nous nous sommes intéressés à un moyen d'abaisser notre limite de détection. Afin de minimiser la perte d'échantillon sur les parois des divers tubes et les étapes de préparation, nous avons d'emblée écarté les méthodes classiques de préconcentration (lyophilisation, extraction sur phase solide avec des cartouches commerciales, etc.). Nous nous sommes tournés vers la préconcentration "en-ligne". Cette technique est employée depuis quelques années avec succès pour la concentration de divers analytes: molécules organiques, peptides, protéines avec des facteurs de préconcentration pouvant aller jusqu'à 1000 (7-9). Nous avons donc construit un préconcentrateur dont la phase stationnaire est constituée de billes de silice greffées d'une phase de C-18 dont nous avons testé les capacités avec des digestats tryptiques dilués de la β-caséine.

De nombreux auteurs ont rapporté dans leurs articles que cette technique d'enrichissement d'échantillon, bien qu'efficace, est à l'origine de plusieurs problèmes comme l'élargissement des pics, la perte de résolution et dans certaines conditions

l'inversion du flux électro-osmotique (10, 11). Pour minimiser ces problèmes, nous l'avons utilisé dans une configuration où le préconcentrateur serait déconnecté du capillaire de séparation avant l'application du voltage. Ce système nous a donné d'excellents résultats pour la carte peptidique de la β-caséine à des concentrations allant de 200 nM à 800 nM (chapitre 5). Malgré tout, la carte peptidique des protéines après préconcentration est très différente de celle obtenue à plus haute concentration directement après digestion. Nous avons attribué ce phénomène au préconcentrateur lui-même. En effet, la vitesse de désorption des peptides, de même que la hauteur des pics dépend de l'affinité de ceux-ci pour la phase stationnaire C-18, employé comme adsorbent dans la cartouche de préconcentration.

Bien que nous ayons obtenu des cartes peptidiques ayant une bonne résolution entre chaque pic, nous avons montré que le volume de désorption pouvait avoir une grande influence sur celle-ci. De même, la longueur du préconcentrateur (i.e. le volume de phase stationnaire) modifie également la carte peptidique en terme de hauteur de pics et de leurs nombres. Plus il y a de phase stationnaire, plus grande sera la quantité de peptides retenus.

En vue d'une automatisation éventuelle, nous avons connecté "en-ligne" le microréacteur, le préconcentrateur et le capillaire de séparation. Avec ce système, nous sommes capables d'obtenir la carte peptidique d'une protéine, quelques heures après son injection, à une concentration normalement indétectable par UV avec un appareil conventionnel. Nous avons obtenu la carte peptidique du cytochrome *c* à 400 nM (chapitre 6) avec un facteur de préconcentration de 800.

7.2. Critique et perspectives

L'objectif de cette thèse etait le développement et la caracterisation d'un système analytique complet permettant d'obtenir directement la carte peptidique d'une protéine à

faible concentration. Ce système devait répondre à plusieurs critères. En premier lieu, le temps de digestion devait être grandement diminué. La concentration de protéine devait être relativement basse de manière a refléter les concentrations de protéine rencontrées dans les échantillons réels. En troisième lieu, le nombre de manipulations devait être limités dans l'objectif de gain de temps et de minimisation de la perte d'échantillon. Pour ce faire, nous avons donc développé et caractérisé séparément les différents composants de ce système: un microréacteur pour la digestion des protéines, un préconcentrateur pour améliorer la sensibilité de la détection. Nous avons ensuite connecté ces deux éléments en-ligne à un appareil de CE à détection d'absorption dans l'UV. Nous avons finalement caractérisé ce système au complet.

Le microréacteur que nous avons construit nous a permis d'effectuer des digestions de solutions relativement concentrées de protéines en 2.5 h ce qui est une nette amélioration par rapport aux microréacteurs décrits dans l'introduction ou le temps minimal reporté pour les digestions est de 8 h.

Cependant, pour des aliquots de 40 µl de solution de β-caseine injectés dans le microréacteur, seulement 35 µl furent récupérés suggérant qu'une partie de la solution est restée emprisonnée dans les pores des billes de trypsine ou à cause du volume mort de notre système de digestion. Bien que la perte d'échantillon n'ait pas été quantifiée dans le cas de microréacteur garni de gel de trypsine, ceci constitue un des désavantages de notre système en comparaison des microréacteurs où l'enzyme est greffé sur les parois de capillaire.

La déviation standard relative que nous avons observe pour nos cartes peptidiques après digestion avec le microréacteur, varie de 1 à 4 %. Ces valeurs sont un peu supérieures à celles obtenues par Cobb et Novotny qui sont inférieures à 1,5 % (1,18). En premier lieu, nos séparations s'étalent sur un temps relativement long. En ce cas, la déviation standard

relative augmente graduellement avec le temps de migration du fait de l'échauffement progressif du capillaire. Nous aurions pu améliorer notre reproductibilité en controlant la température à l'intérieur du capillaire ou en reconditionnant plus régulièrement le capillaire avec une solution d'hydroxyde de sodium 0,1 M avant chaque séparation. Cependant, cette légère différence de temps de migration ne devrait pas empêcher la détection de modifications post-transductionnelles comme les phosphorylations qui induisent un changement de charge ou les glycosylations qui induisent un important changement de masse. Également, toute modification génétique impliquant un changement de charge du peptide sera facilement détectable. Cependant, les substitutions d'acide aminé ne donnant lieu qu'à une modification de la masse du peptide ne pourront être détectées avec certitude.

Le préconcentrateur fait de billes de C-18 que l'on a utilisé nous a permis d'abaisser la limite de détection de deux ordres de grandeur pour la carte peptidique de la β-caséine. Cela représente une nette amélioration de la sensibilité de la cartographie peptidique. Une telle amélioration a déjà été rapportée dans la littérature pour la préconcentration de peptides comme mentionné au chapitre 1. D'après nos résultats, il semble que la longueur du préconcentrateur a une influence critique sur la carte peptidique de même que le volume de désorption. Ces deux paramètres seront à optimiser plus rigoureusement en fonction du nombre de fragments tryptiques et de leur hydrophobicité.

Notre système en-ligne a rempli constitué des deux éléments précédemment cités et d'un appareil de CE nous a permis de remplir les objectifs décrits plus haut. Le temps total pour obtenir la carte peptidique d'un échantillon de cytochrome c à une concentration de 400 nM a été réduit à 3 h sans autre manipulation que celles que l'injection de la solution de protéine et la solution de désorption. Il y a cependant plusieurs limitations à notre système tel que décrit dans cette thèse. En premier lieu, il convient de noter que la perte de résolution due à la connexion en-ligne qui génère une pression de retour, entraîne une perte

de sensibilité et pourra s'avérer problématique dans le cas de protéines donnant de nombreux peptides. Il pourrait être difficile de déceler les peptides portant des modifications post-transductionnelles ou génétiques.

Une modification intéressante serait d'adapter le système en-ligne de façon à pouvoir effectuer la désorption en appliquant un champ électrique. On devrait ainsi pouvoir minimiser l'élargissement des pics dû à la pression de retour. En fait, il faudrait placer une électrode avant la valve d'injection de la solution de désorption. Cette électrode délivrerait un haut voltage. La cathode à la sortie du capillaire de séparation serait mise à la terre. On aurait alors un champ électrique entre les deux électrodes qui permettrait à la solution de désorption de passer à travers le préconcentrateur et de pénétrer dans le capillaire de séparation. Ceci fait, on couperait le voltage à l'électrode supplémentaire et on appliquerait un champ électrique aux bornes du capillaire de séparation. Le second avantage de cette configuration est que la valve sur le capillaire de séparation ne serait désormais plus nécessaire.

Une autre configuration à essayer est d'installer une interface à "flux stoppé" comme celle décrite par Jorgenson (12, 13). Cette technique de couplage de deux colonnes capillaires permet à une fraction de l'échantillon de la première colonne d'être injectée dans la colonne 2. Nous pourrions alors augmenter la résolution de la carte peptidique à cause du plus faible volume injecté (>20 nl). Cependant, nous diminuerions notre facteur de préconcentration puisque seulement 20 à 25% de l'échantillon désorbé du préconcentrateur serait injecté.

Nous n'avons pas caractérisé notre système avec des échantillons réels car les limites de détection obtenues ne concurrencent pas encore les concentrations de protéine d'intérêt que l'on retrouve normalement dans des échantillons biologiques comme les marqueurs pour le cancer (10^{-9}-10^{-12} M). Malgré cela, un tel système n'a encore jamais été

rapporté dans la littérature. La seconde génération de notre système en ligne sera testé avec des protéines réelles et non des protéines standards.

La limite de détection de la CE pourrait encore être abaissée en utilisant une détection par fluorescence. Dans ce cas, la dérivation pourrait être faite en incorporant un réactif fluorescent dans la solution de désorption. La plupart de ces réactifs sont solubles en phase organique. La dérivation serait effectuée au moment de la désorption et donc impliquerait que le réactif fluorescent réagisse rapidement et controler la réaction pour éviter la multiple dérivation. Le pH de séparation devrait être modifié puis que la fluorescence dépend du pH de même que la réaction de dérivation. De même, la possibilité d'interaction entre la phase stationnaire et ces réactifs devrait être étudiée et minimisée de manière à pouvoir réutiliser le préconcentrateur.

Un développement ultérieur qui serait d'un intérêt primordial pour l'identification des peptides serait le couplage avec un appareil de spectrométrie de masse (MS). Le couplage de la SPE-CE avec cette technique a déjà fait l'objet de plusieurs articles et est bien connue maintenant (14-17). Elle permettrait une identification sans ambiguïté des pics, la localisation et la nature de tous types de modifications. De plus, la détermination de la séquence primaire par MS exige presque toujours une étape préliminaire de la cartographie peptidique. Donc, notre système en-ligne serait bien adapté pour cette application.

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