Université de Montréal

New high through-put assays for detecting Transglutaminase activity

Par

Wajih Ben tahar

Département de Chimie

Faculté des Arts et Sciences

Mémoire présenté à la Faculté des Études Supérieures

en vue de l'obtention du grade de

Maître ès Sciences (M.Sc.)

En Chimie

Avril, 2008

Université de Montréal

Faculté des Études Supérieures

Ce mémoire intitulé:

New high through-put assays for detecting Transglutaminase activity

Présenté par :

Wajih Ben tahar

A été évalué par un jury composé des personnes suivantes :

Président-rapporteur	Professeur Pelletier. Joelle N.
Membre du jury	Professeur Lortie. Robert.
Directeur de recherche	Professeur Lubell, William D,
Codirecteur de recherche	Professeur Keillor. Jeffrey W.

Mémoire accepté le :

Acknowledgments

I wish to express my sincere gratitude to my supervisors, Prof. William D. Lubell and Prof. Jeffrey W. Keillor for giving me the opportunity to be a member of their group. Thanks also for their help, guidance and enthusiastic support during my master thesis studies.

I would like to express my sincere gratitude to my former co-workers, Dr Claudio Gnaccarini, Hassan Iden, Dr Gil Fridkin, and Dr Tarek Kassem for pleasant times in the lab, helpful discussions and comments concerning chemistry, and to all present and former members of the Lubell and Keillor groups for maintaining a friendly and pleasant atmosphere.

I wish to express my sincere gratitude to Mildred Bien Aimé for help with technical issues, for enjoyable and interesting discussions concerning life and especially for her spirit.

I would like to acknowledge the assistance of Dalbir Sekhon for HPLC analyses, Dr Alexandra Furtos and Mrs Karine Venne for mass spectral analysis, as well as the Natural Sciences and Engineering Research Council of Canada (NSERC), for financial support.

Last but not least I am grateful to my parents, wife and brothers for their love, support, encouragement and patience.

Résumé

Depuis une décennie, l'industrie pharmaceutique s'intéresse de très près à la synthèse de principes actifs constitués de peptides. Ces composés ont l'avantage d'être hautement spécifiques, présentent de faibles problèmes toxicologiques et s'accumulent peu dans les tissus. Cependant, il existe un réel défi pour les sociétés de mettre au point des synthèses peu couteuses, moins polluantes et plus propres. Une synthèse peptidique par voie enzymatique serait une alternative. La transglutaminase (TGase) enzyme a Ca^{2+} dépendance qui catalyse le transfert d'un groupement glutamyle d'un donneur acyl, la glutamine à un accepteur d'acyle, généralement une lysine, semblerait être un candidat idéal dans cette approche. Cette enzyme catalyse la formation de la liaison amidique entre les aminoacides et les peptides et fonctionne dans l'eau de façon quasi-irréversible. Les mimes de TGase sont prometteuses dans ce domaine de recherche. Dans ce type de projet, le développement de méthodes de détection de l'activité TGase des mutants est d'importance.

Dans ce travail sera présenté le développement de deux nouvelles méthodes fluométriques à haut débit pour la détection de l'activité de la TGase. Ces méthodes présentent de nombreux avantages : elles sont tout d'abord très sensibles à faibles concentration en enzyme et substrats. Ces méthodes sont reproductibles et peuvent être utilisées dans des milieux biologiques plus complexes (lysat).

Le premier test est basé sur la mesure fluorométrique de l'activité de la TGase et a été validé sur la transpeptidation d'un substrat donneur préalablement synthétisé contenant une partie N-terminale fluorophore avec un accepteur (une amine biotynilée). Après traitement

avec la TGase, le mélange est fixé à une streptavidine immobilisée et l'activité de la TGase est mesurée par l'augmentation du signal de fluoresence.

Le second test est basé sur l'observation du phènoméne de FRET (définie comme un transfert d'énergy non radiative suite à une interaction dipole-dipole entre deux molécules fluorescentes) suite à la ligation par la TGase d'un donneur acyle marqué avec le fluorophore Tokyo Green à une amine primaire marquée avec la 7-hydroxy-coumarine.

La synthèse des substrats, l'optimisation des tests et leurs applications dans l'étude de l'activité de la TGase et leurs possibles applications dans des criblages à haut débit sont présentées dans ce mémoire.

Mots clés : Transglutaminase, FRET, fluorimétrie, enzyme, cinétique, synthèse sur support solide, lysat, criblage à haut débit.

Abstract

During the last decade, interest in the synthesis of peptides as drugs has grown, due to their high specificity, low toxicology problems, and minimal accumulation in tissue. Environmentally sound economical synthesis of peptides represents a challenge considering the limitation of current chemistry. Enzymatic peptide synthesis may serve as an alternative for efficient peptide synthesis. Transglutaminase (TGase) is a Ca^{2+} dependent enzyme that catalyzes the transamidation of an acyl donor glutamine to an acyl acceptor, lysine. This enzyme represents as interesting candidate for peptide synthesis, because it already catalyzes amide bond formation between amino acids and peptides, in aqueous solution. Methods for the detection of mutant TGase activity are needed to study this enzyme for peptide synthesis.

The present study describes the development of two new high-throughput fluorometric methods for the detection of TGase activity. These methods are sensitive at low concentration of enzyme and substrate. They are also reproducible and effective in biological media expressing enzyme (lysate).

The first assay is based on the fluorometric measurement of TGase activity. Transpeptidation involves an amide donor substrate possessing an N-terminal fluorophore moiety and an acceptor amine substrate containing biotin. After treatment with TGase, the mixture is fixed to immobilized streptavidine, the resin is washed and TGase activity is monitored by the increase of fluorescence signal.

The second assay is based on the observation of the FRET (Fluorescence Resonance Energy Transfer) effect upon ligation by TGase of a donor acyl substrate labelled with the fluorophore Tokyo Green to a primary amine labelled with 7-hydroxycoumarin.

The synthesis of organic substrates and assay optimization have been performed to study TGase activity.

Key Words: transglutaminase, FRET, fluorometry, enzyme, kinetics, solid phase synthesis, lysate, high-through put screening, quench.

Table of contents

Chapter 3: a continous FRET based assay for tissue transglutaminase

Chapter 4: Concluding Statements and Future Work

List of Figures

List of Tables

Chapter 1

Chapter 2

List of Schemes

Abbreviations

$[\alpha]_D$	optical rotation
Ala	alanine
Asn	asparagine
Asp	aspartic acid
Amp	ampicillin
Arg	arginine
Boc	*tert*-butoxycarbonyl
BSA	bovine serum albumine
t-butyl	*tert*-butyl
bp	boiling point
br	broad (spectral)
Bz	benzoyl
Cbz	benzyloxycarbonyl
Chl	chloramphenicol
°C	celsius
Cys	cysteine
d	doublet
dd	doublet of doublets
D	aspartic acid
Da	Dalton
DMPDA	N,N-dimethyl-1,4-phenylenediamine

DMAP	4-Dimethylaminopyridine
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DCC	dicyclohexyl carbodiimide
DIC	1,3-diisopropylcarbodiimide
DIEA	diisopropylethylamine
DMF	*N,N*-dimethylformamide
E. coli	*Escherichia coli*
EC	Enzyme commission
EDC.HCl	1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
FRET	free resonance energy transfer
HBTU	O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
HOBt	N-Hydroxybenzotriazole
HATU	2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium
Fmoc	9-fluorenylmethyloxycarbonyl
G	glycine
GABA	γ-aminobutyric acid
GDH	glutamate dehydrogenase
Gly	glycine
Glu	glutamic acid

h	hours
HATU	O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOAt	1-hydroxy-7-azabenzotriazole
His	histidine
HOBt	1-hydroxy 1H-benzotriazole
HRMS	high-resolution mass spectrometry
IR	infrared
IPTG	isopropyl-β-D-thiogalactoside
INC	incorporation
LB	Luria-Bertani
J	coupling constant
K_d	dissociation constant
Lys	lysine
K_m	Michaelis constant
μ	micro
m	multiplet (spectral)
M	molar
mg	milligram
min	minutes
MHz	megahertz

mL	millilitre
mmol	millimole
mp	melting point
MS	mass spectrometry
MW	molecular weight
N	asparagine
NEt_3	triethyl amine
nm	nanometers
NMR	nuclear magnetic resonance
OD	optical density
ppm	part per milliom
Phe	phenylalanine
Pro	proline
QLPF	Gln-Leu-Pro-Phe
rpm	rotation per minute
s	singlet
SPPS	solid-phase peptide synthesis
t	triplet
Thr	thryreonine
TB	terrific broth
TGase	transglutaminase
Tris	tris(hydroxymethyl)methane
TTGase	Tissue TGase

TFA	trifluoroacetic acid
TLC	thin layer chromatography
TBTU	O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate
U	units
λ_{em}	emission wavelength
λ_{ex}	excitation wavelengh

CHAPTER 1

INTRODUCTION

1.1 Peptides

1.1.1 Introduction

A peptide is a molecule formed from the coupling of two or more α-amino acids via an amide bond resulting from the condensation of a carboxylic group of one and the amino group of the other. Peptides containing more than 50 amino acids are called proteins. Peptides play an important role in regulating many physiological processes, and can be used in various treatments for diseases such as the peptide insulin, which is used to treat diabetes.

1.1.2 Peptides as Drugs

Peptides represent promising candidates for drug discovery due to their high specificity, high affinity, low toxicity, and low accumulation in tissues. The first peptide based drug to be administered therapeutically was insulin.[1] To date, insulin remains one of the most successful peptide based drugs with insulin sales of $ 7 billion (US) in 2005, and expected to reach $ 14 billion (US) by 2010.[2]

Solution phase synthesis was the method originally used for making peptides; however, this method presented many problems involving purification, solubility as the size of the peptide grew, reaction times, and low yields. In 1963, a new strategy for peptides synthesis, so called solid-phase peptide synthesis (SPPS) was pioneered by Bruce Merrified,[3] and had a great impact on the chemistry for making peptides by overcoming the problems cited above. Five years later, the solid phase synthesis of oxytocin, [4] and diamino oxytocin were reported.[5] In fact, this method brought a 20-30 fold increase in the number of analogues

that could be synthesized compared to the solution method. The solid phase technique has become the most commonly used process for peptide synthesis, moreover, solid-phase chemistry has established itself as a useful tool in the field of organic synthesis. Bruce Merrifield was awarded the chemistry Nobel Prize in 1984 for the development of a simple and ingenious method for obtaining peptides and proteins, his original paper is the 5th most highly cited paper in the Journal of the American Chemical Society.[3]

The SPPS method has become commonplace for research in drug discovery.[6] Over the last decade, investment of the pharmaceutical industry in peptide-based therapeutics has expanded, prompted by the advances in the understanding of the genetics of diseases and drug delivery, as well as developments in peptide synthesis. The global market of therapeutic peptides in 2007 was valued at around $1 billion (US), and is predicted to significantly increase over the next decade.[7] In fact, interest in peptide drugs has recently intensified with the approval of the peptide-based drug Fuzeon (T-20) an anti-HIV treatment by the Roche Company. Peptides are currently being developed for therapeutic applications such as, allergy, asthma, cancer, and diabetes.

1.1.3 Limitations

To date, compared with the total number of drugs that have been delivered to market, peptide based-drugs are considered marginal. As articulated by pioneers in the field, [7] the key development of peptides as drugs is contingent on the following facts:

- Improvement in manufacturing to increase yields and reduce costs.

- Enhanced synthesis of diverse peptide analog libraries to screen agonist drug targets.
- Modification of peptides with non-natural amino acids to increase stability.

In fact, in spite of the advantages reported above, there are many issues facing this field of research that need to be solved in order to gain an increased market for peptides as drugs. One of the major problems in this field is the high cost of production of peptide drugs. Roche's anti-HIV Fuzeon (T-20), the present synthetic peptide-drug paradigm, costs more than $20 000 (US) per person a year which restricts its general use. As previously mentioned, the solid phase strategy is the method of choice for peptide synthesis. This method has two major problems:

- High cost: Coupling reagents and protected amino acids are expensive, commonly used in excess, and rarely recycled
- Pollution: Protection, deprotection and washing steps generate relatively large quantities of waste per procured product.

Investment in this field is needed to resolve these two problems: high cost and pollution. An increased peptide market demands resolution of these issues to provide affordable products in an environmentally friendly manner.

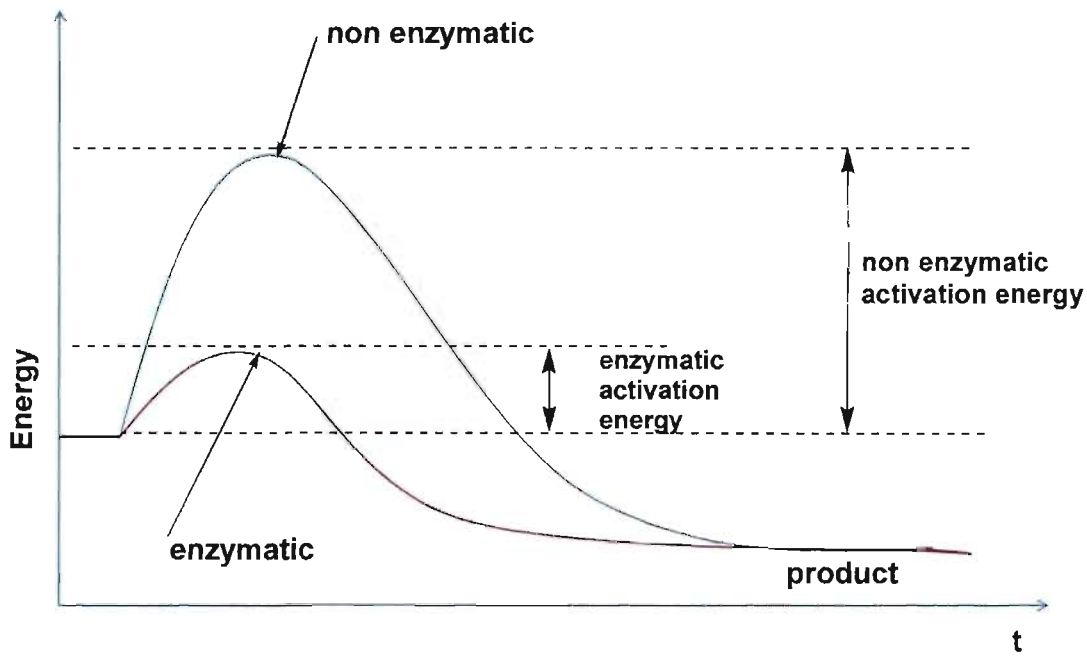
In order to find an efficient way of synthesizing peptides in low cost and with less pollution, the pharmaceutical industry, as well as food companies have been investigating other methods for large scale peptide synthesis that meet the following criteria:

- Improvement in the time and yield to manufacture peptides.

- Reduced cost of the synthesis.
- Less pollution.

Enzymes are available biocatalysts that function in aqueous conditions and more efficiently by lowering the activation energy barrier ($\Delta G^{\neq}$) of a reaction, thus allowing the reaction to proceed much faster without affecting the equilibrium of the reaction (Figure 1.1). Industry can consider biocatalysts to be a potential strategy for efficient peptide synthesis. The proteases have been the principal targeted family of enzyme for amide bond formation between α-amino acid residues. [8]

Figure1.1 Difference between enzymatic and non enzymatic reaction



1.1.4 Proteases

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions, industrial and pharmaceutical applications. [8, 9, 10] Proteases can also be used as biocatalyst for peptide bond formation via a kinetically or thermodynamically controlled approach. However, in thermodynamically controlled approach the hydrolysis of the formed peptide was favoured reducing than the yield of the reaction. [11] More research was focused in favouring the kinetically approach. [12, 13, 14] For example, subtiligase, a double mutant of subtilisin BPN', has been shown to have many uses, from the total synthesis of RNase A to the semisynthesis of a variety of other proteins via the coupling of esterified peptides onto the N-termini of proteins in aqueous media and in high yield. [15] Moreover, asprgillus proteases were found to be efficient for peptide coupling when using the carbamoyl ester as acyl donor. [14] The two major drawbacks of proteases as peptide biocatalyst are substrate specificity and hydrolysis of the growing peptide, which respectively lower the flexibility and yields of this method. [16] Enzyme transglutaminase (TGase) thus has been pursued as an alternative for enzyme-catalyzed peptide coupling.

1.2 Transglutaminase

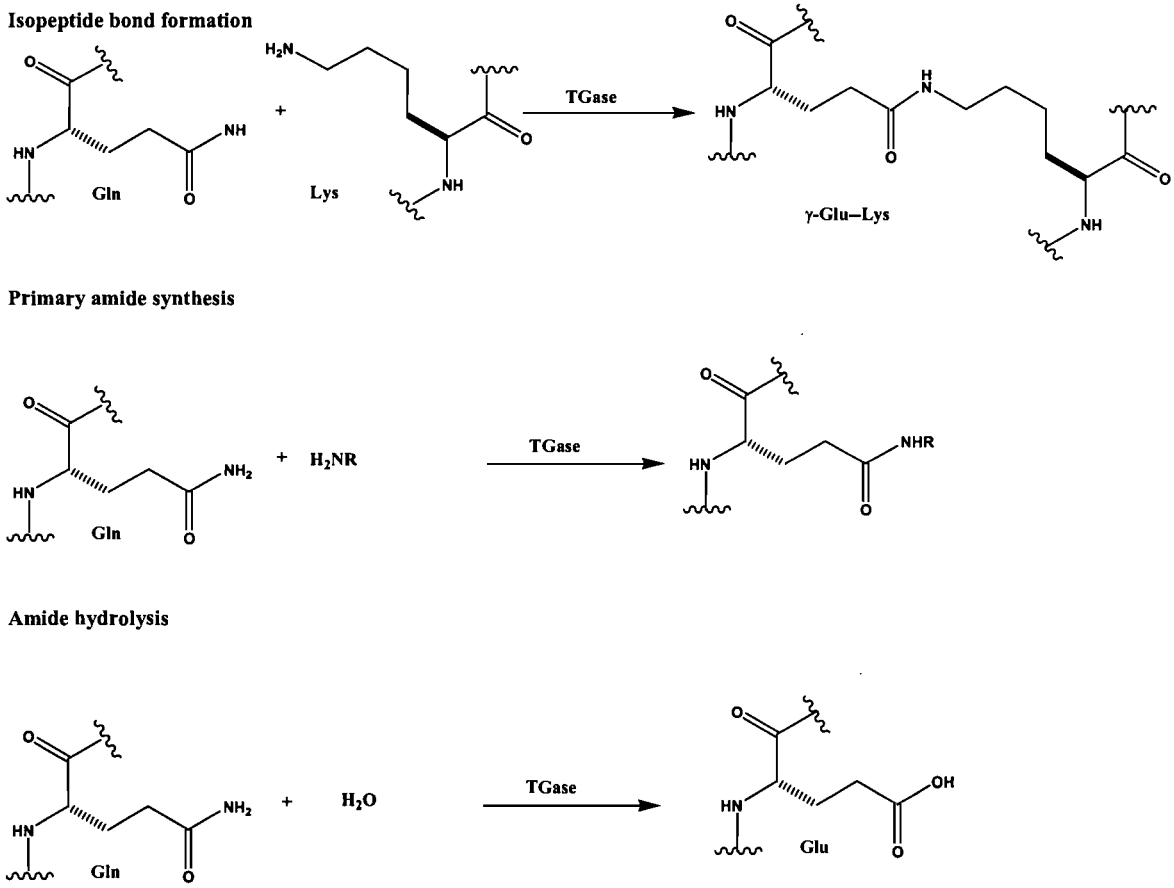
1.2.1 Definition

Transglutaminase (TGases) are a class of Ca^{2+}-dependent enzymes that catalyze the cross-linking of proteins through the formation of isopeptide bonds between reactive lysine and glutamine residues in proteins. An aminotransferase enzyme, TGase catalyses the transamidation of glutamine-containing proteins with a variety of other primary amine

nucleophiles (scheme 1.1). [17] The so formed isopeptide bonds exhibit resistance to proteolytic degradation as well as physical-chemical degradation.

TGase is widely used in several different industrial products and processes. Advances in biotechnology have enabled this enzyme to be developed for processes and have made these enzymes a viable option for chemical catalysis. In particular, the microbial TGase (MTGase) derived from the *Streptoverticillium mobaraense* in the fermentation of sorghum straw hydrolysates has been used in the food industry for catalyzing the cross-linking of many food proteins such as caseins, soybean globulins, gluten, actin, myosins, and egg proteins, through the formation of an Ɛ-(γ-glutamyl)lysine bond. Used as well for the incorporation of primary amines into proteins, MTGase is notable because it is a calcium independent enzyme of low molecular weight, which is smaller than of other known TGases. [18, 19, 20] Efforts continue to focus on identifying novel and alternative microbial sources for production of TGase analogs with greater substrate specificity and enhanced utility in (iso) peptide synthesis.

Scheme 1.1 Transglutaminase Catalyzed Reactions



1.2.2 Mechanism of action

TGase follows a modified "Ping-Pong" mechanism described by Folk, J in 1969 (Figure 1.2),[21] involving two steps, acylation followed by a deacylation.

- **Acylation step:**

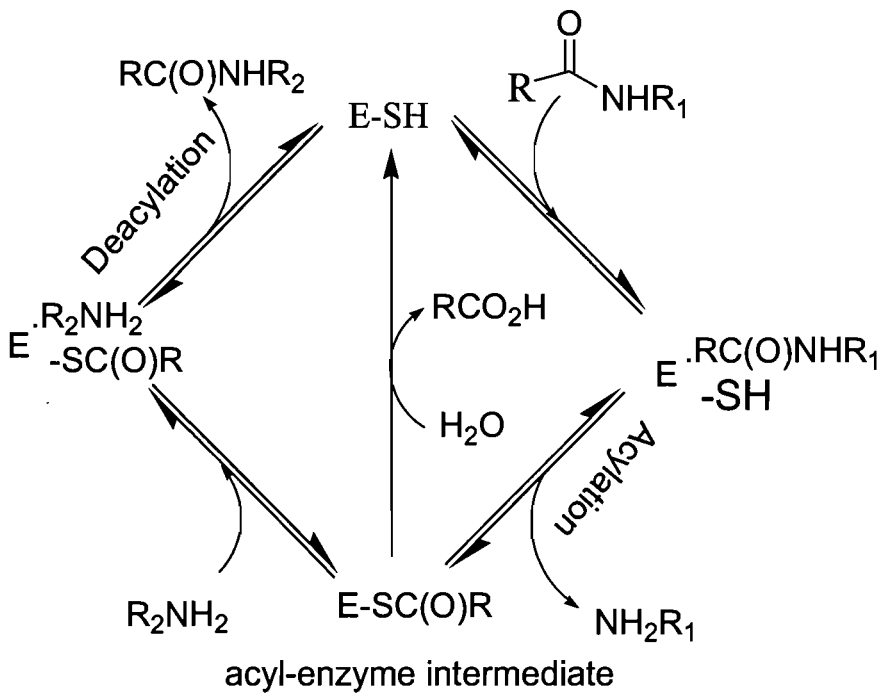
The acyl donor is bound at the active site of the enzyme forming the first Michaelis complex ready for the acylation step.

The catalytic cysteine thiol attacks the γ-carboxamide group of the donor substrate (glutamine residue), forming the acyl-enzyme intermediate and releasing one equivalent of ammonia.[22]

- **Deacylation step:**

When the acyl-enzyme intermediate is formed, a competition occurs between hydrolysis with water forming glutamic acid (slow reaction), and the aminolysis reaction of the intermediate with lysine forming an isopeptidic bond or with a primary amine forming an amide bond.

Figure 1.2: Modified Ping-Pong Mechanism of TGase



The Keillor laboratory has studied reactions catalyzed by the guinea pig liver TGase and demonstrated that the catalytic mechanism involves general base catalysis by characterisation of the transition state using kinetic methods. [22]

The TGases family have been identified from different sources such as bacteria, invertebrate, and plants. [23] From mammalians, a total of eight different TGase enzymes have been identified (Table 1.1):

- **Tissue transglutaminase (TG2):**

Full length human TG2 is 687 amino acids with a predicted molecular mass of ~ 77 kDa. TG2 is well conserved, with human TG2 being 80-90% homologous to bovin, mouse and guinea pig TG2. [24] The most studied TGase, TG2 exists in the majority of tissues, transcription of TG2 is increased by retionoic acid, it plays a role in the extra-cellular matrix development, apoptosis and neural differentiation. [25] Beside having transpeptidation activity, TG2 also acts as a kinase, [26] protein disulfide isomerase, and deamidase by the deamidation of gliadin peptides thus playing an important role in the pathology of coeliac disease. [27] TG2 has also been linked to inflammation, neurodegenerative diseases, Huntington's disease, [28] and tumor biology. [29]

Table 1.1: The transglutaminase (TGase) family.

TGase	Synonyms	Gene	Size aa (kDa)	Function	Location
Factor XIII	Fibrin stabilizing factor	F13A1	732 (83)	Blood clotting and wound healing	Cytosol, extracellular
Band 4.2	Erythrocyte membrane protein	EPB42	690 (72)	Structural protein in erythrocytes—no activity	Membrane
TGase 1	Keratinocyte TGase	TGM1	814 (90)	Cornified envelope assembly in surface epithelia	Cytosol, membrane
TGase 2	Tissue TGase	TGM2	686 (80)	Cell death/differentiation, adhesion, matrix assembly	Cytosol, nucleus, membrane, cell surface, extracellular
TGase 3	Epidermal TGase	TGM3	692 (77)	Cornified envelope assembly in surface epithelia	Cytolsol
TGase 4	Prostate TGase	TGM4	683 (77)	Semen coagulation in rodents	Unknown
TGase 5	TGase X	TGM5	719 (81)	Epidermal differentiation	Nuclear matrix, cytoskeleton
TGase 6	TGase Y	TGM6	Unknown	Unknown	Unknown
TGase 7	TGase Z	TGM7	710 (80)	Unknown	Unknown

Source: *Journal of investigative dermatology*, **2005**, *124*, 481-492.

1.2.3 Physiological transglutaminase

TGase is implicated in different biological processes, including cell adhesion,[30] apoptosis,[31] formation of the extra cellular matrix,[32] blood coagulation.[33] Deregulation of transglutaminase activity leads to different physiological disorders and diseases.[34]

1.2.3.1 Alzheimer's disease

The formation of the abnormal protein structures of Alzheimer's disease have been suggested to be catalyzed by TGase because insoluble isoforms of TGase colocalized with plaques and tangles 30-50 times greater than normal brain tissue.[35]

1.2.3.2 Huntington's disease

Huntington's disease (HD) results from genetically programmed degeneration of brain cells, called neurons, in certain areas of the brain. Elevated TGase activity has been found in the affected areas. The implicated protein, Huntington, has been found to be a good substrate for the TG 2 enzyme.[36]

1.2.3.3 Celiac disease

Celiac disease is a digestive disease that damages the small intestine and interferes with absorption of nutrients from food. Celiac disease is precipitated by the ingestion of gliadin, a proteinaneous component of wheat gluten. The presentation of fragments of gliadin cross-linked to TG 2 results in antibodies against gliadin, TG 2 and the cross-linked proteins.

1.2.3.4 Cataract formation

A cataract is an opacity that develops in the crystalline lens of the eye or in its envelope and may cause blindness. Cataracts are distinguished by a marked elevation of intracellular Ca^{2+}, which may act to stimulate TGase activity either directly or indirectly suggesting involvement of TGase in cataract formation. [37]

1.2.3.5 Atherosclerosis

Atherosclerosis involves the build-up of a waxy deposit on the inside of blood vessels. TGase may play a role in the formation of the atherosclerotic plaques by catalyzing the incorporation of lipoprotein (a) (TGase substrate) into these structures, creating insoluble atherosclerotic tissue. [38]

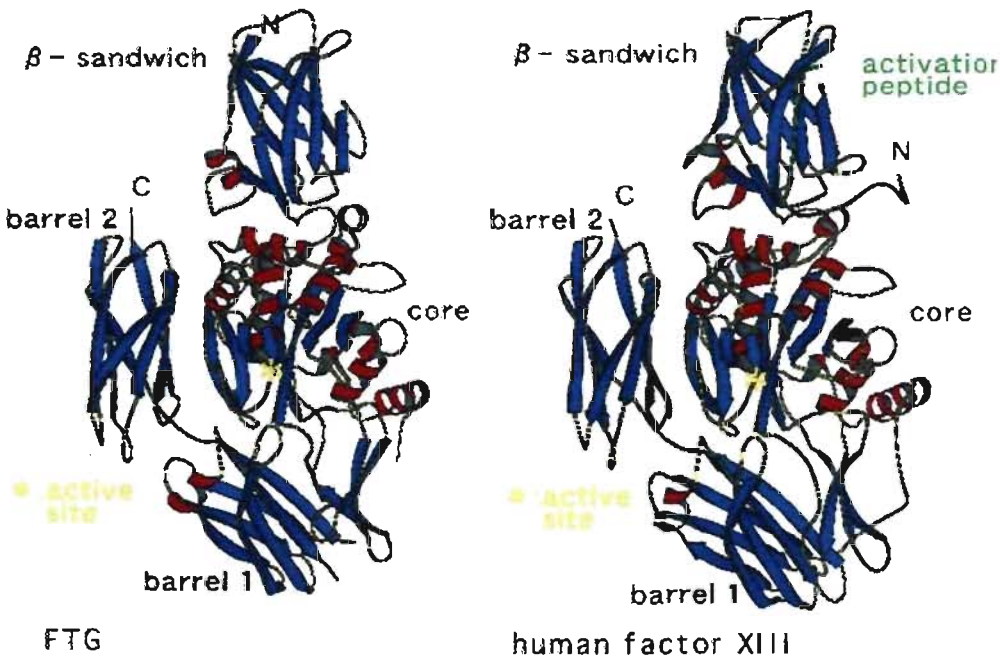
1.2.4 Transglutaminase structure

To have a good understanding of the structure-function relation for enzyme activity, it is very important to know the three-dimensional crystalline structure of the enzyme. Having this structure could help researchers to better understand the enzyme, to design efficient inhibitors of the enzyme, and to assess the important domains for the activity of the enzyme.

To date, the structures of four different vertebrate TGases have been resolved: the factor XIII TGase furnished the first three-dimensional structure, [39] followed by red sea bream fish TGase, [40] human tissue TGase (TGase 2), [41] and the epidermal human TGase. [42]

In the X-ray structures, the enzyme is composed of a β-sandwich at the N-terminal containing the residues Gly-6 to Phe-134, the catalytic α/β core containing the residues Asn-135 to Thr-461, the β 1 barrel covering residues Arg-472 to Ser-583 and β 2 barrel at the C-terminal covering residues Thr-584 to Lys-684. The active site belongs to the catalytic α/β core (composed of α-helices and β-sheets). Conserved among TGases, the active site is formed by three residues, a cysteine, a histidine and an aspartic acid. Red sea bream TG2 and guinea pig liver TGase share around 80% sequence homology at the active site (Figure 1.3).[43]

Figure 1.3: Resemblance of the Overall Structure of Factor XIII and Red Sea Bream TG2.



The red sea bream catalytic triad, composed of Cys-272, His-332 and Asp-355, is similar to the active site of the widely studied cysteine proteases. The catalytic residues Cys and His

are close enough in proximity to form a hydrogen bond. The calcium ion modifies the protein conformation, providing the active form of the enzyme, which is a complex with the metal-ion. From this structural information, we can infer information concerning the structure and conformation of substrates.

1.2.5 Transglutaminase binding

As mentioned earlier, TGase catalyzes the cross-linking of proteins through the formation of isopeptide bonds between the side-chains of lysine and glutamine residues. TGase shows very high specificity with respect to the electrophilic substrate, and L- glutamine is usually required for enzyme recognition.[44] The L-glutamine residue lies usually between the third amino acid from the N-terminal and the second amino acid from the C-terminal of the peptide substrate. [45] The presence of certain amino acids at the C-terminal as well as a benzyloxycarbonyl group at the N-terminal facilitate the recognition of the electrophilic substrate. For example Cbz-L-Gln-Gly is a good substrate for TGase, and was used as starting structure for the designing probes for kinetic studies of TGase. [46] Another peptide sequence, which has been demonstrated as a good substrate for TGase is PQPQLPY from the gluten protein. This sequence exhibits a high affinity for TGase (Km= 30 uM). [47, 48] Moreover, the EAQQIVM sequence, from the N-terminal of fibronectin, serves as a TGase substrate. [49]

TGase tolerates a wider range of nucleophilic substrates: [46] primary alkylamines, such as monoprotected putrescine, and cadaverine. [50] The steric bulk of the nucleophilic substrate may limit the acylation step.

1.3 Aim of the Project

The general goal of this research project is to develop novel TGases that are capable of efficiently catalyzing peptide coupling for economical and environmentally sound peptide synthesis. Specifically, this project aims to develop a series of new assays for high-through put detection of TGase activity. These assays take advantage of the sensitivity of fluorescent probes and are designed to be flexible for studying a variety of TGase mutants.

Methods for monitoring TGase activity, either in its purified form or within complex solutions, are fundamental for the study of TGase and for research towards modifying its activity. The Keillor group has previously reported assays for measuring the potency of TGase, by monitoring enzyme-catalyzed release of a chromophoric anilide, [51] *p*-nitrophenol [22] or 7-hydroxycoumarin leaving group attached to the electrophilic substrate. [52] Although sensitive, these methods have the inherent disadvantage of requiring the measurement of a chromophoric product or leaving group. Assays that detect released ammonia from amide donor substrates [53-55] may provide better mechanistic insight into the enzyme activity than those which measure release of alcohols from activated ester substrates. Assays detecting the formation of the acyl-enzyme intermediates have been complemented by those that measure the formation of the final product. Recently, two very sensitive methods that detect the formation of isopeptidic bonds were reported. Both are based on the increase of fluorescence of a dansyl group on the acceptor and donor substrates. In the first case, the fluorescence increases as a result of π-stacking of the two dansyl moieties after ligation [56] and in the second case as a result of inclusion within the hydrophobic environment of casein. [57] These assays detect the native transamidation reaction. The nature of these assays,

however, is not compatible with the wide variation in the structural characteristics of the substrates. An assay for *in situ* TGase activity measurements based on protein biotinylation has also been developed. In this method human *neuroblastoma* cells were preincubated with 5-(biotinamido) pentylamine, the cells were lysed and the homogenate was added to a microtiter plate. By addition of horseradish peroxidase conjugated streptavidin and *o*-phenylenediamine dihydrochloride the presence of proteins into which 5-(biotinamido) pentylamine had been incorporated was detected by measuring the absorbance at 492 nm. The activity of TGase *in situ* was calculated as percentage of basal activity.[58] As it relies on native protein as the acyl donor substrate, it precludes modification of the acyl donor substrate. Therefore, while all these methods are useful to characterize the wild type enzyme or for screening inhibitors, they are not suitable for screening TGase reactivity toward unnatural substrates. The development of two new high through-put assays for screening mutant TGases are described herein:

- ✓ **A fluorometric assay for the detection of transglutaminase activity:**

This method is based on the high affinity of biotin to the streptavidin protein. The idea is to detect the increase of fluorescence featuring transpeptidation of a donor substrate containing N-terminal fluorophore moiety with an acceptor amine substrate containing biotin. After treatment with TGase, the mixture is affixed to streptavidin beads and TGase activity is monitored by the increase of the fluorescence signal. The optimization of this assay and its application in studying TGase activity will be described.

✓ **A continuous FRET based assay for transglutaminse activity:**

Fluorescence resonance energy transfer (FRET), is an interaction of two fluorophores, in which an excited-state donor molecule transfers energy nonradiatively to an acceptor chromophore. A FRET-based assay for detection of ligation of a donor amine fluorophore moiety with an acceptor substrate containing another fluorophore has been designed. After treatment with TGase, the activity is monitored by decrease in emission of the donor fluorophore and concomitant increase of the acceptor fluororophore signal.

These assays were designed to meet two major criteria:

- High sensitivity: in order to detect activity of pure TGase and TGase in lysate.
- High throughput: in order to test a large number of mutants in a short time.

Moreover, these assays have the advantage of detecting the ε-(γ-glutaminyl) lysinyl bond formed upon ligation catalyzed by the pure enzyme, and may be suitable for high-through put screening of TGase activity in lysate.

1.5 References

1- F. G. Banting, C. H. Best., The internal secretion of the pancreas. *J. Lab. Clin. Med.* **1922**, *7*, 251-256.

2- http://www.metabolic.com.au/files/SXH7P6WRYW/UpdateOralPeptideDelivery Platform_20Sept2007.pdf.

3- R. B. Merrifield, Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149-2154.

4- Manning, M., Synthesis by the Merrifield method of a protected nonapeptide amide with the amino acid sequence of oxytocin. *J. Am. Chem. Soc.* **1968**, *90*, 1348-1349.

5- H. Takashima, V. Du Vigneaud, R. B. Merrifield, Synthesis of deaminooxytocin by the solid phase method. *J. Am. Chem. Soc.* **1968**, *90*, 1323–1325.

6- A. Loffet., Peptides as Drugs: Is There a Market?. *J. Peptide Sci.* **2002**, *8*, 1–7.

7- http:/www.in-pharmatechnologist.com/news/ng.asp?n=57290-peptide-market-finally. (See Annex page 86).

8- U. C. Banerjee , R. K. Sani, W. Azmi, R. Soni., Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochem.* **1999**, *35*, 213–219.

9- R. Gupta, Q. K. Beg, P. Lorenz., Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol.* **2002**, *59*, 15-32.

10- H. Ishikawa, K. Ishimi, M. Sugiura, A. Sowa, N. Fujiwara., Kinetics and mechanism of enzymatic hydrolysis of gelatin layers of X-ray film and release of silver particles. *J. Fermentation. Bioeng.* **1993**, *76*, 300-305.

11- M. Lobell, M. P. Schneider., Pronase catalysed peptide syntheses. *J. Chem. Soc. Trans. 1*, **1998**, 319-325.

12- W. C-H, G. M. Whitesides., Enzymes in synthetic organic chemistry (pergamon, oxford). 1994, 46-59.

13- D. Kumar . T. C. Bhalla., Microbial proteases in peptide synthesis: approaches and applications. *Appl Microbiol Biotechnol.* **2005**, *68*, 726–736.

14- T. Miyazawa, M. Hiramatsu, T. Murashima, T. Yamada., Utilization of Proteases from *Aspergillus* Species for Peptide Synthesis via the Kinetically Controlled Approach. *Biocatal. Biotransfor.* **2003**, *21*, 93-100.

15- T. K. Chang, D. Y. Jackson, J. P. Burnier, J. A. Wells., Subtiligase: A tool for semisynthesis of proteins. *Proc. Natl. Acad. Sci. USA.* **1994**, *91*, 12544-12548.

16- M. Thormann, S. Thust, Hans-Jörg, Hofmann, F. Bordusa., Protease-Catalyzed Hydrolysis of Substrate Mimetics (Inverse Substrates): A New Approach Reveals a New Mechanism. *Biochemistry.* **1999**, *38*, 6056-6062.

17- T. Ohtsuka, A. Sawa, R. Kawabata, N. Nio, and M. Motoki., Substrate Specificities of Microbial Transglutaminase for Primary Amines. *J. Agric. Food. Chem.* **2000**, *48*, 6230-6233.

18- M. Motoki, K. Seguro., Transglutaminase and its use for food processing, *Trends Food. Sci. Technology.* **1998**, *9*, 204-210.

19- Z. Pietrasik, E. C. Y. Li-Chan., Binding and textural properties of beef gels as affected by protein, k-carrageenan and microbial transglutaminase addition. *Food Res. Int.* **2002**, *35*, 91-98.

20- P. C. Lorenzen, H. Neve, A. Mautner, E. Schlimme., Effect of enzymatic cross-linking of milk proteins on functional properties of set-style yoghurt , *Int. J. Dairy Technol.* **2002**, *55*, 152-157.

21- J. E. Folk., Mechanism of action of guinea pig liver transglutaminase. VI. order of substrate addition. *J. Biol. Chem.* **1969,** *244*, 3707-3713.

22- A. Leblanc, C. Gravel, J. Labelle, J.W. Keillor., Kinetic studies of guinea pig liver transglutaminase reveal a general-base-catalyzed deacylation mechanism. *Biochemistry.* **2001,** *40*, 8335-8342.

23- M. D. Mea, D. Caparrós-Ruiz, I. Claparols, D. Serafini-Fracassini, J. Rigau., AtPng1p. The First Plant Transglutaminase. *Plant Physiol.* **2004**, *135*, 2046-2054.

24- V. Gentile, M. Saydak, E. A. Chiocca, O. Akande, P. J. Birckbichler, K. N. Lee, J. P. Stein, and P. J. Davies., Isolation and characterization of cDNA clones to mouse macrophage and human endothelial cell tissue transglutaminases. *J. Biol. Chem.* **1991**, *266*, 478-483.

25- R. B. Maccioni, N. W. Seeds., Transglutaminase and neuronal differentiation. *Mol. Cell. Biochem.* **1986,** *69*, 161-168.

26- S. Mishra, L. J. Murphy., Tissue transglutaminase has intrinsic kinase activity: identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase. *J. Biol. Chem.* **2004**, *279*, 23863-23868.

27- W. Sakly, V. Thomas, G Quash, S, El Alaoui., A role for tissue transglutaminase in alpha-gliadin peptide cytotoxicity. *Clin. Exp. Immunol.* **2006**, *146*, 550-558.

28- M. V. Karpuj, M. W. Becher, L. Steinman., Evidence for a role for transglutaminase in Huntington's disease and the potential therapeutic implications. *Neurochem. Int.* **2002**, *40*, 31-36.

29- J. S. Palumbo, K. A. Barney, E. A. Blevins, M. A. Shaw, A. Mishra, M. J. Flick, K. W. Kombrinks, K. E. Talmage, M. Souri, A. Ichinoseand J. L. Degen., Factor XIII transglutaminase supports hematogenous tumor cell metastasis through a mechanism dependent on natural killer cell function. *J. Thrombosis. Haemostasis.* **2008**, *6*, 812-819.

30- Gentile, V. Thomazy, M. Piacentini, L. Fesus, P. J. A. Davies., Expression of tissue transglutaminase in balb-C 3T3 fibroblasts: effects on cellular morphology and adhesion. *J. Cell. Biol.* **1992**, *119*, 463-474.

31- S. Oliverio, A. Amendola, F. Di Sano, M.G. Farrace, L. Fesus, Z. Nemes, L. Piredda, A. Spinedi, M. Piacentini., Tissue Transglutaminase-dependent posttranslational modification of the retinoblastoma gene product in promonocytic cells undergoing apoptosis. *Mol. Cell. Biol.* **1997,** *17*, 6040-6048.

32- D. Aeschlimann, O. Kaupp, M. Paulsson, Transglutaminase-catalyzed matrix cross-linking in differentiating cartilage., identification of osteonectin as a Major glutaminyl substrate. *J. Cell Biol.* **1995**, *129*, 881-892.

33- Thung-S. Lai, Thomas F. Slaughter, Keith A. Peoples, and Charles S. Greenberg., Site-directed mutagenesis of the calcium-binding site of blood coagulation factor XIIIa. *J. Biol. Chem.* **1999**, *274*, 24953-24958.

34- S-Y. Kim, T. M. Jeitner, P. M. Steinert., Transglutaminases in disease. *Neurochem. Int.* **2002**, *40*, 85-103.

35- S. Kim, Philip Grant, Jeung-Hoon Lee, Harish C. Pant, and Peter M. Steinert., Differential expression of multiple transglutaminases in human brain. Increased expression and cross-linking by transglutaminases 1 and 2 in alzheimer's disease. *J. Biol. Chem.* **1999**, *274*, 30715-30721.

36- M. V. Karpuj, M. W. Becher, L. Steinman., Evidence for a role for transglutaminase in Huntington's disease and the potential therapeutic implications. *Neurochem. Int.* **2002**, *40*, 31-36.

37- J. E. Folk, J. S. Finlayson., The epsilon-(gamma-glutamyl) lysine crosslink and the catalytic role of transglutaminases. *Adv. Protein Chem.* **1977**, *31*, 1-133.

38- M. A. Romanic, A. J. Arleth, R. N. Willette, E. H. Ohlstein., Factor XIIIa cross-links lipoprotein(a) with fibrinogen and is present in human atherosclerotic lesions. *Circ. Res.* **1998**, *83*, 264-269.

39- V. C. Yee, L.C. Pedersen, I. L. Trong, P. D. Bishop, R.E. Stenkamp, D. C. Teller., Three-Dimensional Structure of a Transglutaminase: Human Blood Coagulation Factor XIII. *Proc. Nat. Acad. Sci. U.S.A.* **1994**, *91*, 7296-7300.

40- K. Noguchi, K. Ishikawa, K. Yokoyama, T. Ohtsuka, N. Nio, E. Suzuki Crystal Structure of Red Sea Bream Transglutaminase. *J. Biol. Chem.* **2001**, *276*, 12055-12059.

41- S. Liu, R. A. Cerione, J. Clardy., Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity. *Proc. Nat. Acad. Sci. U.S.A.* **2002**, *99*, 2743-2747.

42- B. Ahvazi, K. M. Boeshans, P. M. Steinert., Crystal Structure of Transglutaminase 3 in Complex with GMP: structural basis for nucleotide specificity. *J. Biol. Chem.* **2004,** *279*, 26716-26725.

43- T. Ohtsuka, M. Ota, N. Nio, M. Motoki., Comparison of substrate specificities of transglutaminases using synthetic peptides as acyl donors. *Biosci., Biotechnol. Biochem.* **2000**, *64*, 2608-2613.

44- J. E. Folk, P. W. Cole., Transglutaminase: mechanistic features of the active site as determined by kinetic and inhibitor studies. *Biochim. Biophys. Acta.* **1966**, *122*, 244-264.

45- J. E. Folk, S. I. Chung., Transglutaminase. *Methods in Enzymology.* **1985**, *113*, 358-375.

46- J. E. Folk., Mechanism and basis for specificity of transglutaminase-catalyzed epsilon-(gamma-glutamyl)-lysine bond formation. *Adv. Enzymol. Relat. Areas Mol. Boil.* **1983**, *54*, 1-56.

47- K. Choi, M. Siegel, J. L. Piper, L. Yuan, E. Cho, P. Strnad, B. Omary, K. M. Rich, C. Khosla., Chemistry and biology of dihydroisoxazole derivatives: selective inhibitors of human transglutaminase 2. *Chem. Biol.* **2005**, *12*, 469-475.

48- F. Hausch, T. Halttunen, M. Maki, C. Khosla., Design, synthesis, and evaluation of gluten peptide analogs as selective inhibitors of human tissue transglutaminase. *Chem. Biol.* **2003**, *10*, 225-231..

49- H. Sato, N. Yamada, N. Shimba, Y. Takahara., Unique substrate specificities of two adjacent glutamine residues in EAQQIVM for transglutaminase: identification and characterization of the reaction products by electrospray ionization tandem mass spectrometry. *Anal. Biochem.* **2000**, *281*, 68-76.

50- Mary Lynn Fink, Yvonne Y. Shao, Gilbert J. Kersh., A fluorometric, high-performance liquid chromatographic assay for transglutaminase activity. *Anal. Biochem.* **1992**, *201*, 270-276.

51- P. de Macédo, C. Marrano, J.W. Keillor., A direct continuous spectrophotometric assay for transglutaminase activity. *Anal. Biochem.* **2000**, *285*, 16-20.

52- S. M. F. G. Gillet, J. N. Pelletier, J. W. Keillor., A direct fluorometric assay for tissue transglutaminase. *Anal. Biochem.* **2005**, *347,* 221-226.

53- A. R. Barnes, J. K. Sugden., Comparison of colourimetric methods for ammonia determination. *Pharm. Acta Helv.* **1990**, *65*, 258-261.

54- S. Sheng, J. J. Kraft, S. M. Shuster., A specific quantitative colorimetric assay for L-asparagine. *Anal. Biochem.* **1993**, *211*, 242-249.

55- N. Day, J. W. Keillor., A continuous spectrophotometric linked enzyme assay for transglutaminase activity. *Anal. Biochem.* **1999**, *274*, 141-144.

56- T. M. Jeitner, H. -L. Fuchsbauer, J.P. Blass, A.J.L. Cooper, A sensitive fluorometric assay for tissue transglutaminase. *Anal. Biochem.* **2001**, *292*, 198-206.

57- A. Case, J. Ni, L.-A. Yeh, R.L. Stein., Development of a mechanism-based assay for tissue transglutaminase-results of a high-throughput screen and discovery of inhibitors. *Anal. Biochem.* **2005**, *338*, 237-244.

58- J. Zhang, M. Lesort, R. P. Guttmann, G. V. W. Johnson., Mudulation of the in situ activity of tissue transglutaminase by calcium and GTP. *J. Biol. Chem.* **1998**, *273*, 2288-2295.

CHAPTER 2

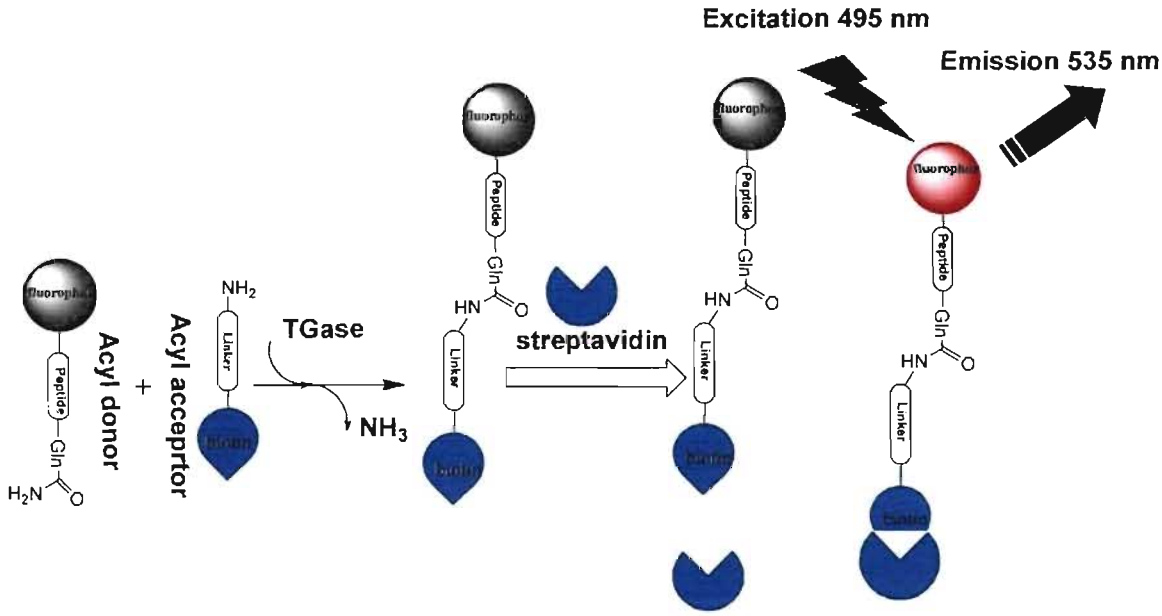
A fluorometric assay for the detection of transglutaminase activity.

2.1 Introduction

The attachement of biotin to various chemical sites, so called biotinylation, can be used as an important laboratory technique for studying various processes, including protein localization, protein-protein interactions, DNA transcription and replication. [59, 60, 61] Biotin binds tightly to the tetrameric protein avidin (as well as streptavidin and neutravidin), with a dissociation constant K_d in order of 10^{-15} mol/L. In different biotechnological applications, biotin is added to a molecule of interest for fixation to a supported streptavidin, and subsequent measurements of activity.

Herein, we present a discontinuous assay with synthetic donor and acceptor substrates. In the assay reaction, TGase catalyzes the formation of a conjugate between a synthetic acyl-donor substrate, bearing the Tokyo Green fluorophore, [62] and an amine acceptor substrate bearing a biotin group. The resulting biotinylated fluorophore-labelled product is then trapped with immobilized streptavidin. Unreacted substrates are removed by washing and subsequent fluorescent measurement allows sensitive detection of activity. For ease of application, the method was adapted to use in 96-well microtiter plates. This assay is highly sensitive, allowing detection of as little as 8.25 μU of TGase activity from a sample of purified enzyme, yet it is also sufficiently flexible to be used for detection of as little as 91 mU of TGase activity in a complex biological sample. While this assay was validated using native-like substrates, both substrates can be readily modified to allow screening for TGase reactivity toward unnatural substrates (Figure 2.1).

Figure 2.1: Model of streptavidin beads assay of detection of TGase activity.



2.2 Materials and methods

2.2.1 Enzyme preparation

Recombinant guinea pig liver TGase was over-expressed and purified from *Escherichia coli* as previously published. [63] The purified TGase solution used in all assays had a specific activity of 14 U/mg in 25 mM Tris-acetate (pH 7) according to the hydroxamate activity assay, [64] in which Cbz-Gln-Gly and hydroxylamine are used as acyl-donor and acyl-acceptor substrates respectively.

2.2.2 Preparation of crude bacterial lysate

First day: For expression of recombinant guinea pig liver TGase, 4 mL of Terrific Broth (TB), [65] containing 100 μg/mL ampicillin (Amp) and 30 μg/mL chloramphenicol (Chl) was

inoculated with *E. coli* XL1-Blue harbouring the plasmids pDnaKJ (Chl-resistant) and pQE-32 TGase (Amp-resistant). *E. coli* XL1-Blue harbouring pDnaKJ only, inoculated in TB + Chl, was used as a control cell line not expressing TGase. All cultures were incubated for 17 h at 37 °C with shaking at 240 RPM in a C25 Incubator Shaker (New Brunswick Scientific, Edison, NJ. USA).

Second day: Aliquots of 1.5 µL of pre-culture were used to inoculate 1.5 mL of fresh TB medium containing the appropriate antibiotics in wells of a 48-well plate (maximum volume: 6 mL/well) at a ratio of 1/20. The bacteria were propagated at 37 °C with shaking at 240 RPM. When the OD (600 nm) reached approximately 0.5, over-expression of TGase was induced by addition of IPTG to a final concentration of 1 mM. The plate was then incubated at 28 °C with shaking at 800 RPM for 20 hrs in a Titer Plate Shaker (Lab-Line Instruments, Inc.).

Third day: The plate was centrifuged for 30 min at 4°C and 3000 RPM (Allegra^{TM} 6R Centrifuge). The supernatant was discarded. The bacterial cell pellets were stored at – 80 °C for 10 min, thawed, resuspended in 200 µL of CelLytic^{TM} B cell lysis reagent (Sigma) and incubated at 28 °C for 20 min. The crude bacterial lysates thus obtained and used for subsequent tests were determined by hydroxamate test to show an activity of ~18 U/mL

2.3 Synthesis and kinetics for acyl donor substrate

Recognition of the acyl donor *in vivo* is restricted to the γ-carboxyamide of glutamine within a relatively restricted subset of sequence contexts. The first peptide sequence of interest was Gln-Gly. In fact, it was demonstrated that the Cbz-Gln-Gly is a substrate

for the TGase enzyme. [44] Different fluorophores (dansyl, fluoresecein, carboxy Tokyo Green) were attached at the N-terminal of Gln-Gly, and none of these peptides were recognized by the enzyme, as initially suggested by the molecular modeling study by Chica et al. [66] This was attributed to the importance of the N-terminal group for recognition by the TGase enzyme. Furthermore, the C-terminal group exhibits opposing properties, in that it is less tolerated as a recognition site by the enzyme. The alternative tetrapeptide Gln-Leu-Pro-Phe was selected as a known [67] acyl-donor substrate for TGase and conjugated to one of three fluorophores - Tokyo Green, [62] fluorescein isothiocaynate and dansyl - by way of an aminocaproate spacer. Three biotinylated amines were also explored as acyl-acceptor substrates for isopeptide bond formation with the acyl-donor substrate.

In order to verify if the identity of the fluorophore affected the extent of productive binding of the donor substrate to TGase, acyl-donor substrates **11a-c** were prepared by a solid phase synthetic strategy (Scheme 2.1). Tokyo Green was chosen as a fluorophore (in compound **11a**) because it is characterized by a high quantum yield (Φ = 0.93) [62] and possesses a carboxylate that is suitably disposed to allow peptide coupling. The two additional fluorophores, dansyl chloride and fluorescein isothiocyanate, were also tested (yielding **11b** and **11c**, respectively) because they are commercially available and equally amenable to coupling reactions. Donor substrates **11a-c** were found to have low K_M values of 28.7 ± 6.1 μM, 13.1 ± 1.8 μM, and 72.5 ± 6.0 μM (Table 2.1), respectively, using the DMPDA method. [51]

scheme 2.1 Solid-phase support synthesis of different acyl donors.

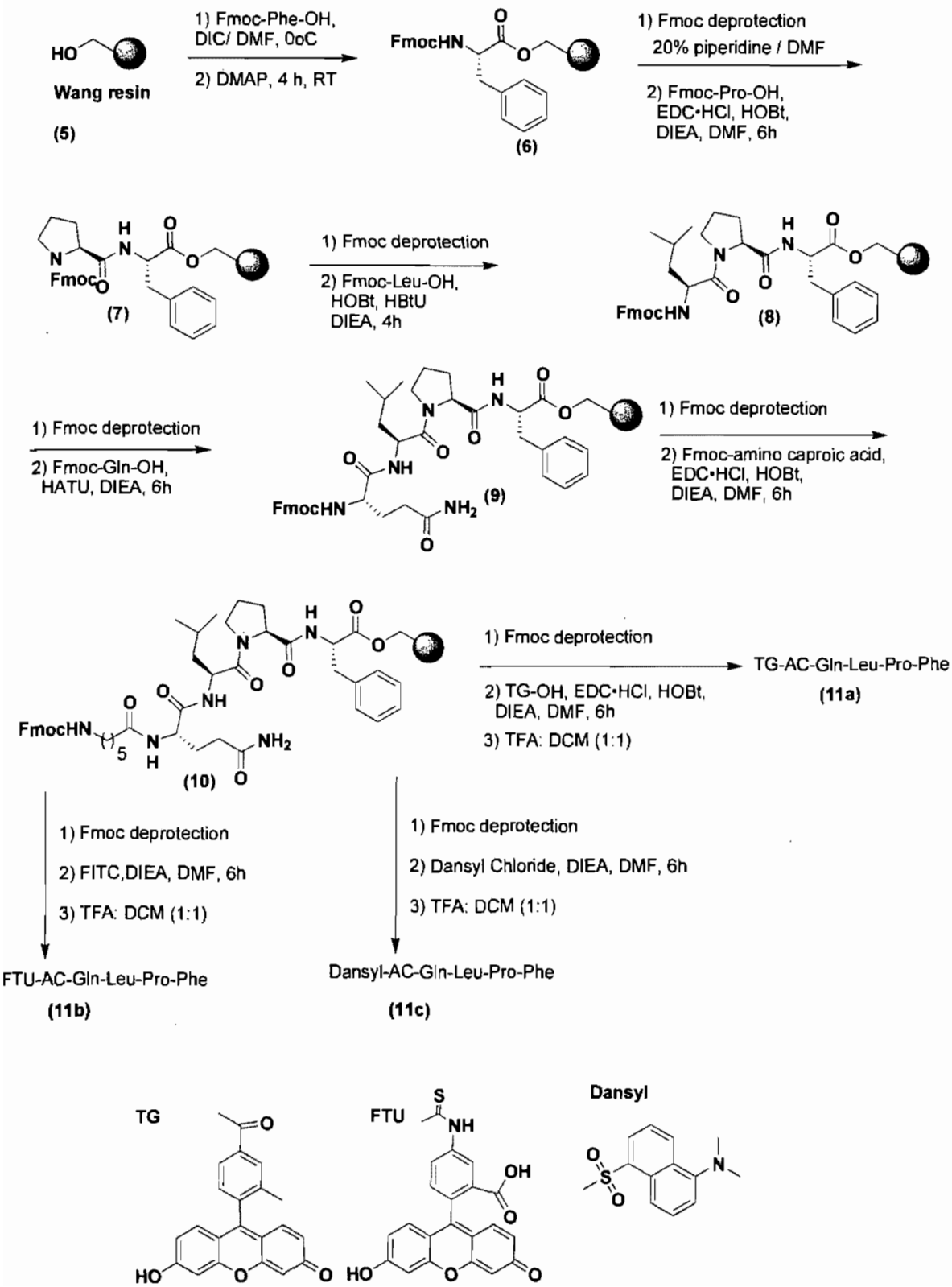


Table 2.1: Kinetic constants for TGase with various N-terminal derivative peptides

substrate	K_m (μM)	V_{max} (μmol.min^{-1})
Dansy-Ɛ-Ac-Gln-Leu-Pro-Phe	72.5 ± 6.0	219 ± 39.6
[a] TG-Ɛ-Ac-Gln-Leu-Pro-Phe	28.6 ± 6.1	282 ± 29.8
[a]IF-Ɛ-Ac-Gln-Leu-Pro-Phe	13.1 ± 1.8	290 ± 14.7

[a] TG) 4-Carboxy-Tokyo Green, IF) isocyanate fluorescein, Ac) amino caproyl.

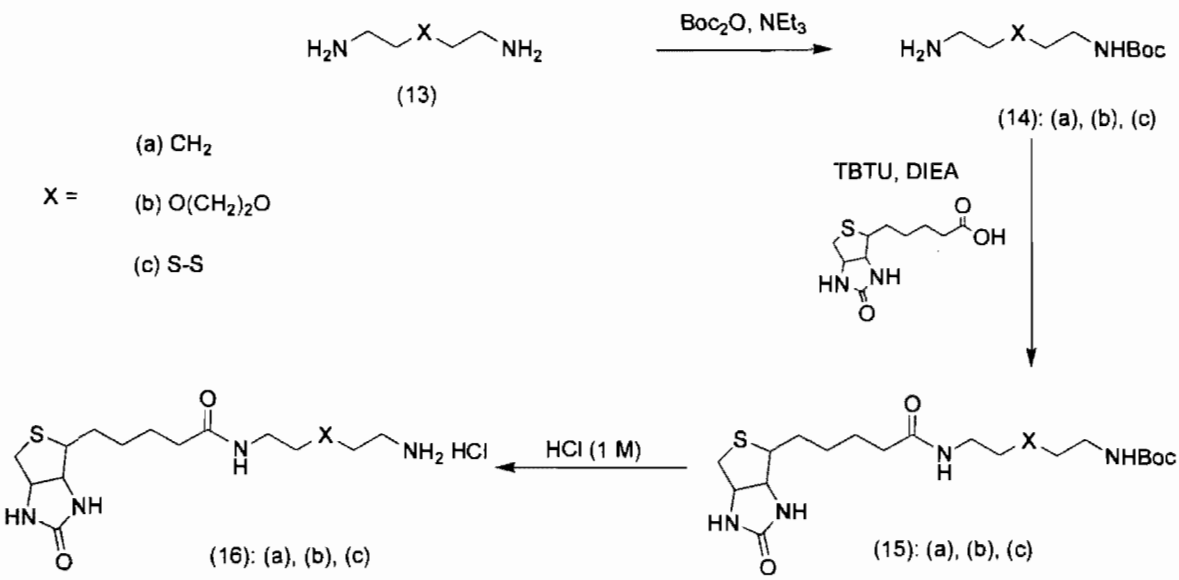
2.4 Synthesis and kinetics for acyl acceptor substrate

Biotinylated amines **16a-c** were synthesized by coupling biotin to their corresponding diamines **13a-c** (Scheme 2.2). The shortest spacer used was 1,5-diaminopentane **13a**. Tris(ethylene glycol)-1,8-diamine **13b** was both longer and more polar, while 2-(2-amino-ethyldisulfonyl)-ethylamine **13c** was of length similar to **13a** but contained a cleavable disulfide bond. Compounds **16a-c** were tested as acceptor substrates using the Cbz-L-Glu(O-*p*-nitrophenyl)Gly assay [23] and found to have K_M values of 2.3 ± 0.4 mM, 11.3 ± 3.7 mM, and 13.6 ± 4.6 mM, respectively (Table 2.2). Thus, the different spacers tested all provided productive binding in the same order of magnitude, validating their use in the coupling reaction.

Table 2.2: Kinetic constants for TGase with various acyl acceptor

substrate	K_m (mM)	V_{max} (mmol.min^{-1})
16 a	2.3 ± 0.4	43.4 ± 2.4
6 b	11.3 ± 3.7	4.4 ± 0.6
16 c	13.6 ± 4.6	9.9 ± 1.7

Scheme 2.2: Synthesis of different acyl acceptors



2.4.1 Materials

The buffer solution in all assays gave a final concentration of 100 mM Tris•HCl (pH 8), 0.6 mM EDTA, and 10 mM $CaCl_2$. The washing buffer solution was composed of 100 mM sodium phosphate (pH 8), 100 mM NaCl, and 0.1% SDS. Water was purified using a Millipore BioCell water purification system. Streptavidin beads (Pierce) were supplied as a suspension capable of binding 15-28 μg biotin/mL of settled resin (a capacity corresponding to ~1-3 mg biotinylated BSA/mL of resin) on a support of 6% crosslinked beaded agarose.

2.5 Assay procedure using a purified enzyme

2.5.1 Methods

All assays were performed in triplicate. Donor substrate **11a**, **b**, or **c** (10 μL of 0.6 mM stock solution), acceptor substrate **16a**, **b**, or **c** (10 μL of 0.6 mM stock solution), 80 μL of buffer solution, and 90 μL of water were combined in a final volume of 190 μL. The reaction was initiated by addition of 25 μg (10 μl) of purified TGase [63] or an equivalent volume of 25 mM tris-acetate assay buffer for the blank. Following incubation for 30 min at 25 °C, 10 μl of the streptavidin bead suspension was added. The mixture was incubated for 30 min at 25 °C. The tubes were centrifuged for 1 min at 1400 RPM (micro centrifuge). The supernatant was discarded. The pellets were washed by resuspension in washing buffer (200 μL) and centrifuged as above. The pellets were washed twice more, resuspended in washing buffer (100 μL) and transferred to a polystyrene 96-well plate. Enzyme activity was detected using a Perkin Elmer Bio Assay Reader (HTS 7000) using λ_{exc} = 485 nm, λ_{em} = 535 nm for substrates **11a** and **11b**, and λ_{exc} = 360 nm, λ_{em} = 535 nm for substrate **11c**, by measuring the increased fluorescence signal relative to the blank.

Reduction of the disulfide-bridged acyl-acceptor substrate

Substrates **11a** and **16c** were incubated for 60 min at 25 °C (30 μM **11a**, 30 μM **16c**, 100 mM Tris-HCl, 10 mM $CaCl_2$, 0.6 mM EDTA, and 350 mU TGase in a total volume of 200 μL), after which 10 μL of streptavidin bead suspension was added. The mixture was incubated for a further 60 min. The tubes were centrifuged for 1 min at 1400 × RPM (microcentrifuge) and the supernatant discarded. The resin was washed twice by

adding 200 μL of wash buffer, centrifuging for 1 min at 1400 × RPM (microcentrifuge) and discarding the supernatant. Finally the beads were suspended in 100 μL of wash buffer for treatment under one of the following conditions:

1 100 μL of 0.1 M DTT was added, followed by 15 min incubation, centrifugation and removal of the supernatant for fluorescence detection.
2 100 μL of 0.1 M DTT was added, followed by 15 min incubation, and the entire mixture was used for fluorescence detection.
3 100 μL of water was added, followed by centrifugation and removal of the supernatant for fluorescence detection.
4 100 μL of water was added, and the entire mixture was used for fluorescence detection.

2.5.2 Results and discussion

In our fluorescent assay for TGase, using synthetic donor and acceptor substrates, the formation of a conjugate between a fluorescent acyl-donor substrate and an amine acceptor substrate bearing a biotin group was catalyzed by TGase. The resulting biotinylated fluorophore-labelled product was trapped with immobilized streptavidin.

- **Assay sensitivity with different acyl acceptors**

To evaluate the effect of the acceptor substrate spacer on assay sensitivity, acyl donor substrate **11a** was incubated with acyl acceptor substrates **16a-c**, respectively, at final concentrations of 30 μM, in the presence or absence of TGase (Figure 2.2). The

acceptor substrates **16a-c** gave different levels of fluorescence that did not correlate with their K_M values. For example, the highest and lowest fluorescence measurements were exhibited by the substrate harbouring the polyethylene glycol **16b** and disulfide **16c** spacer, respectively, that exhibited very similar K_M values.

Acceptor substrate **16c** gave the highest signal and contains a disulfide spacer, which also offered the potential for 'reversible' biotinylation by reductive cleavage of the disulfide bond. This allowed us to verify whether quantitation of the fluorescent conjugate directly on the streptavidin beads provided a maximum signal or whether some loss of fluorescence was suffered. Thus, following enzymatic coupling of the donor and acceptor substrates **11a** and **16c**, trapping on the streptavidin-coated beads and washing, reduction using D,L-dithiothreitol (DTT) and separation by centrifugation allowed measurement of the released fluorophore in the absence of the beads (Table 2.3).

The intensity of the fluorescence measured for the released fluorophore (+DTT, before or after centrifugation) was comparable to that measured directly on the beads (-DTT, before centrifugation). A low level of fluorescence was apparently released from the beads even in the absence of DTT (-DTT, after centrifugation). Nonetheless, fluorescence measurement directly on the beads was not significantly hampered and the additional steps required to release the fluorophore were not deemed to be justified. As compound **16c** yielded the highest fluorescence after coupling to the donor substrate **11a** (Figure 2.2), it was retained as the most suitable acceptor substrate for further measurements.

Figure 2.2: Comparison of assay sensitivity using acyl acceptor substrates **16a-c**, which differ by their spacer. Substrate **11a** was the acyl donor. Results are provided for triplicates. The error bars represent the standard deviation from the mean.

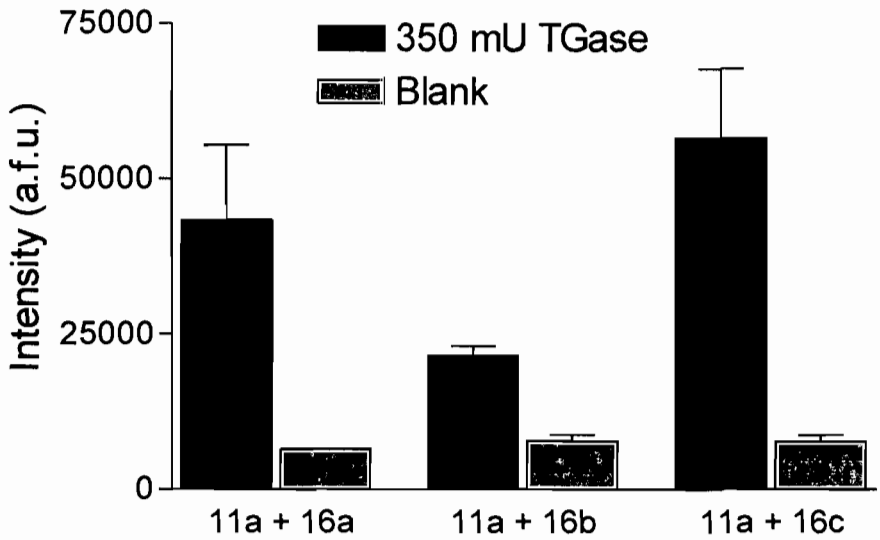


Table 2.3: Fluorescence resulting from the coupling of **11a** and **16c**, measured on streptavidin beads or following release.

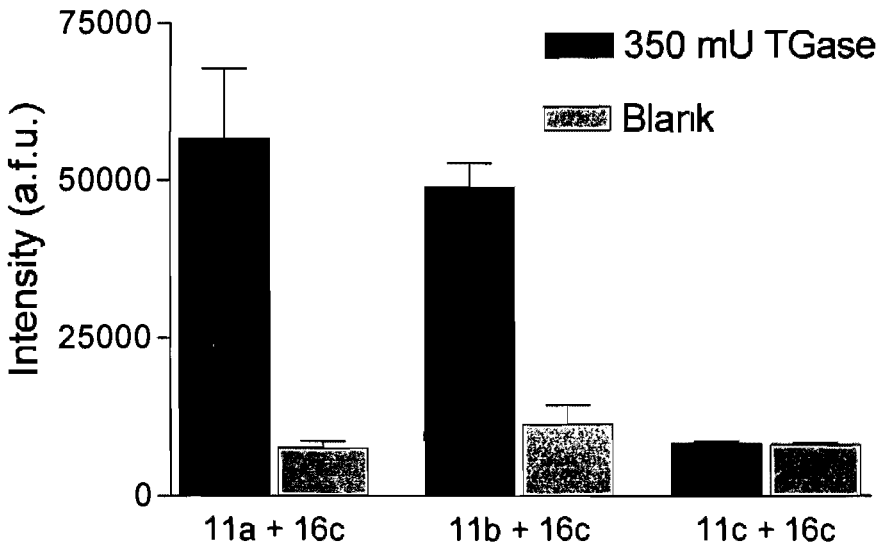
	Fluorescence (a.f.u.)[a]	
	+ DTT	- DTT
Before centrifugation	6.3×10^4	4.6×10^4
Supernatant after centrifugation	4.2×10^4	1.0×10^4

[a] $\lambda_{ex} = 485$ nm, $\lambda_{em} = 535$ nm

- **Assay sensitivity with different acyl donors**

To evaluate the effect of the fluorophore on assay sensitivity, acyl-acceptor substrate **16c** was incubated with the acyl-donor substrates **11a-c** and purified TGase. Substrate **11c** was shown to be an insufficiently intense fluorophore and was not retained for further testing. The substrates **11a** and **11b** showed significant enhancement of signal compared to the control, but the fluorescence of the blank for **11b** was significantly higher than that of **11a**. Among these acyl donor substrates, **11a** gave the best results and was therefore chosen for subsquent tests (Figure 2.3).

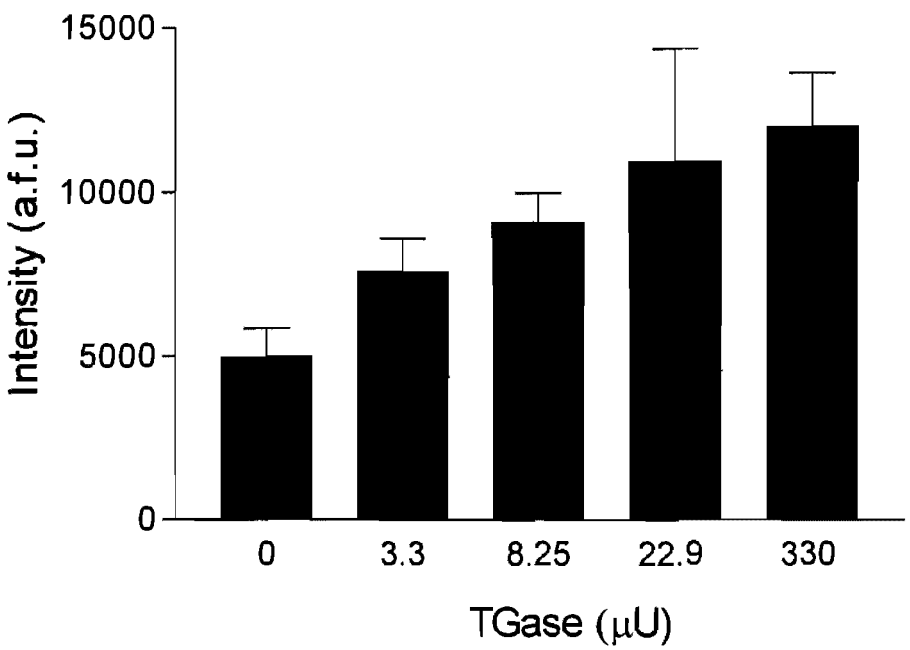
Figure 2.3: Assay sensitivity with acyl donor **11a-c** and acyl acceptor substrate **16c** . Results are provided for triplicates. The error bars represent the standard deviation from the mean.



- **Limit of detection**

The limit of detection of the assay with respect to TGase concentration was then determined using different concentrations of purified TGase with acyl donor **11a** and acyl acceptor **16c** (see Experimental). While as little as 3.3 μU of purified TGase provided a signal that was detectable above the background of the blank, we assess conservatively that the minimum amount of enzyme reliably detected corresponded to 8.25 μU (Figure 2.4). This is lower than other previously reported limits of detection,[48] making this the most sensitive tissue TGase assay reported to date, to the best of our knowledge.

Figure 2.4: Limit of detection of the assay with respect to TGase concentration. Results are provided for triplicates. The error bars represent the standard deviation from the mean.



2.6 Assay procedure using crude bacterial lysate

2.6.1 Methods

All assays were performed in triplicate. Donor substrate **11a** (10 μL of 6 mM stock solution), acceptor substrate **16c** (10 μL of 6 mM stock solution), 80 μL of buffer solution, and 90 μL of water were combined in a final volume of 190 μL. The reaction was initiated by addition of 5-10 μL of crude bacterial lysate expressing TGase, or bacterial lysate void of the TGase expression plasmid as a blank. Following incubation for 60 min at 25 °C, a fraction of the solution (10 μL) was added to a mixture of 180 μL of washing buffer and 10 μL of streptavidin bead suspension. The mixture was incubated for 30 min at 25 °C, and centrifuged for 1 min at 1400 RPM (microcentrifuge). The pellets were washed three times as described above, resuspended in washing buffer (100 μL) and transferred to a polystyrene 96-well plate for analysis of enzyme activity by measuring fluorescence relative to the blank, as described above.

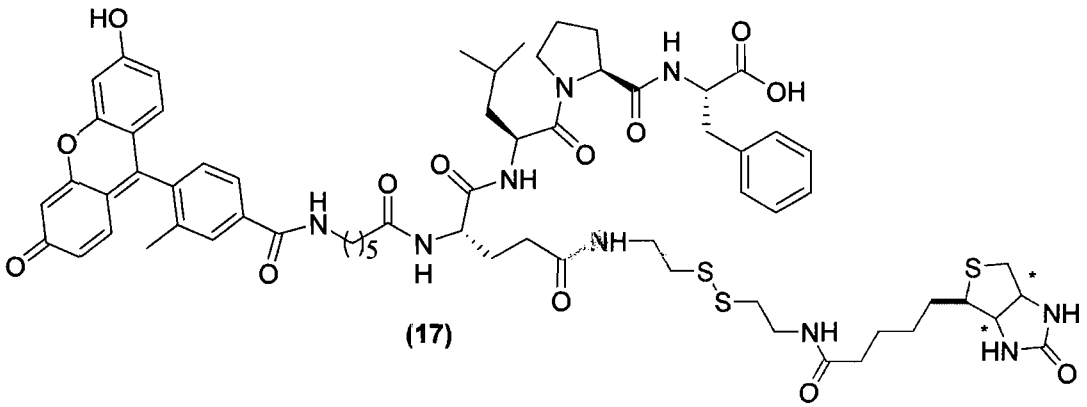
2.6.2 Results and discussion

To be of enhanced practical value, an enzymatic assay should also be amenable to detection of activity in complex biological samples. The coupling of acyl donor **11a** and acyl acceptor **16c** was thus undertaken using crude lysate of *E. coli* expressing recombinant TGase, using as a negative control the lysate of *E. coli* void of the expression plasmid. When analyzing TGase activity in complex biological samples, assays that measure only the first step of the transamidation may give artificially higher activities due to competing hydrolysis of the acyl donor substrate. In contrast to such

assays which report the extent of acylation of the enzyme, detection of fluorescence in the present assay is a consequence of formation of an amide (Figure 2.5).

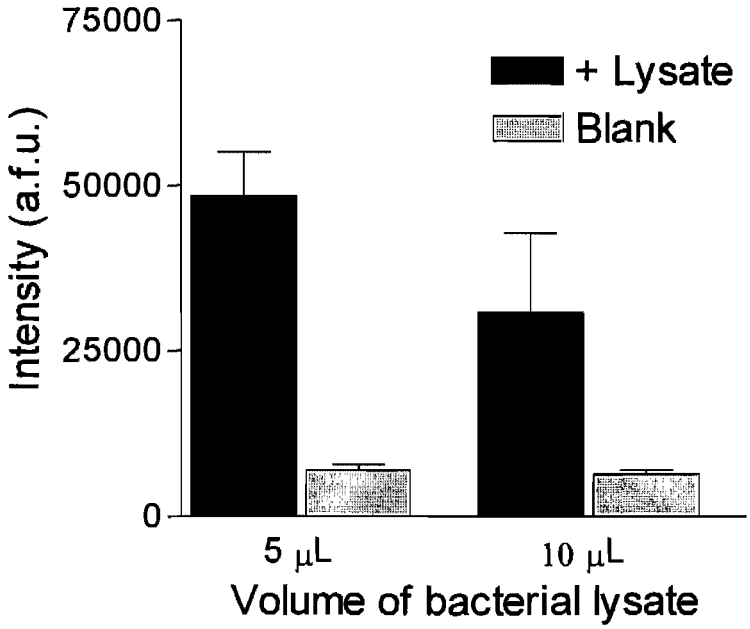
Notably, TGase activity was detected with high sensitivity in lysate (Figure 2.6); however, the intensity of signal was not proportional to the amount of lysate used, over the limited range that was tested. Although more lysate provides more enzyme, alternative acceptor amine substrates present in the crude cellular lysate may compete with the acceptor substrate **16c**, ultimately decreasing the amount of biotinylated fluorophore product. Ultimately we found that a 40-fold dilution of lysate in substrate solution provided a reliable and reproducible means of detecting low levels of TGase activity in crude lysate.

Figure 2.5: Product **17** formed by TGase ligation of acyl donor **11a** and acyl acceptor **16c**



(17)

Figure 2.6: Detection of TGase activity in crude bacterial lysate. Results are provided for triplicates. The error bars represent the standard deviation from the mean.



2.7 Conclusion

In summary, an assay for the detection of the TGase activity has been developed with the highest sensitivity yet reported. This assay has proven to be effective for detection using crude bacterial lysate as well as purified TGase. Because both donor and acceptor substrates are synthetic, we are currently investigating their modification for application

to high throughput screening of libraries of mutant TGase having potentially different substrate specificity.

2.8 Experimental section

General

All solvents used for reactions were purified with solvent filtration systems (Glass Contour, Irvine, CA). 4-Carboxy-Tokyo Green was synthesized according to a published procedure. [59] Wang resin and amino acids were purchased from Nova Biochem except for Fmoc-Gln-OH, obtained from Advanced ChemTech, and Fmoc-ε-aminocaproic acid, obtained from Bachem Chemical. All other reagents were obtained from Sigma-Aldrich. ^{1}H- and ^{13}C-NMR spectra were recorded on Bruker NMR spectrometers.

Reactor tubes for solid-phase peptide synthesis were obtained from Alltec Associates Inc. and shaking was performed on a reciprocating shaker (SK-300 Jeio Tech). All resins were swelled in DMF and washing steps were performed using CH_2Cl_2 and DMF (EMD Chemicals) using five DMF washes followed by three CH_2Cl_2 washes. Purification of all peptides was performed using a preparative HPLC method. Mass spectral data (MS, LCMS) were all obtained using two different solvent systems (MeOH/water; acetonitrile/water) at the Centre Régional de Spectrométrie de Masse de l'Université de Montréal.

General procedure for Fmoc deprotection: Wang resin (0.81 mmol/g) purchased from Nova Biochem was treated with a mixture of 20% piperidine in DMF, shaken for 30 min, filtered and washed as described above. Deprotection was verified by positive Kaiser test on a sample of a few beads.[68] The level of loading of the amino acid on the resin after the first coupling step was used as the resin loading capacity for all subsequent steps, as determined by spectroscopic measurement of the UV absorbance of

the piperidine dibenzofulvalene adduct.

Peptide sequence purity was performed on an aliquot of resin using LCMS-MS in two differents solvant system: method A: 40-80% acetonitrile, H_2O, 0.1% formic acid, 0.5 mL/ min, λ= 214 nm, Agilent LCMSD, column: C6 Phenyl, 3H, 50 × 4.6 Gemini. Method B: 60-90% MeOH, H_2O, 0.1% formic acid, 0.5 mL/ min, λ= 254 nm, Agilent LCMSD, column: C6 Phenyl, 3H, 50 × 4.6 Gemini.

Kinetics

Kinetic parameters for all donor substrates were determined independently using the *N,N*-dimethyl-1,4-phenylenediamine (DMPDA) method,[47] using a range of donor substrate concentrations of 0.5 mM to 20 mM and a constant acceptor substrate concentration of 20 mM. Kinetic parameters for acceptor substrates were obtained using the chromogenic donor substrate, Cbz-L-Glu(O-*p*-nitrophenyl)Gly,[17] using a range of acceptor substrate concentrations of 2 mM to 30 mM and a constant donor substrate concentration of 0.108 mM (5-fold apparent K_M). Initial rates were measured as a function of varied substrate concentration and kinetic parameters were determined from non-linear regression to the hyperbolic Michaelis-Menten equation.

Determination of Peptide sequence purity was performed on an aliquot of resin using LCMS-MS in two differents solvant system: method A: 40-80% acetonitrile, H_2O, 0.1% formic acid, 0.5 mL/ min, λ= 214 nm, Agilent LCMSD, column: C6 Phenyl, 3H, 50 × 4.6 Gemini. Method B: 60-90% MeOH, H_2O, 0.1% formic acid, 0.5 mL/ min, λ= 254 nm, Agilent LCMSD, column: C6 Phenyl, 3H, 50 × 4.6 Gemini.

Synthesis of the donor substrates.

Supported Fmoc-L-phenylalaninate ***6***

In a 20-mL round-bottom flask, Fmoc-Phe-OH (2.116 g, 7.12 mmol, 8 eq) was dissolved in a minimum volume of dry DMF (5 mL) with magnetic stirring, chilled to 0 °C, treated with DIC (557 μL, 3.56 mmol, 4 eq), and stirred for 15 min, when a white precipitate was observed. The solution was then filtered through a fritted funnel into a 20-mL syringe tube equipped with a stopcock and a Teflon filter containing 2 g of Wang resin (0.81 mmol/g), swollen in DMF, followed by 22 mg (0.178 mmol, 0.2 eq) of DMAP. After shaking for 4 h, the resin was filtered and washed with DMF (5 × 5 mL) followed by DCM (3 × 5 mL). The loading was calculated to be 0.525 mmol/g, corresponding to a 59 % yield. The remaining free hydroxyl functionalities were capped by treating the resin with a mixture of acetic anhydride/pyridine/DCM (1/2/2) and shaking for 2 h. The resin was washed as described above, dried over a vacuum pump and stored at 0 °C.

Supported Fmoc-L-prolinyl-L-phenylalaninate 7

In a 20-mL syringe tube equipped with a stopcock and a Teflon filter, resin **6** (2.34 g, 0.77 mmol) was deprotected according to the general procedure for Fmoc removal. The amino ester resin was then swollen in the minimum volume of DMF (10 mL), treated with EDC•HCl (741.6 mg, 3.85 mmol), HOBt (478.1 mg, 3.5 mmol), Fmoc-Pro-OH (1.24 g, 3.5 mmol) and DIEA (0.672 mL, 3.85 mmol), shaken for 6 h, filtered and washed as described above, after which the resin exhibited a negative Kaiser test.

Method A: 81.8 % purity, method B: 91.2 % purity.

Supported Fmoc- L-leucinyl-L-prolinyl-L-phenylalaninate ***8***

As described above for resin **6**, resin **7** was placed in a syringe tube, the Fmoc group was removed and the dipeptide resin was treated with HOBt (478 mg, 3.5 mmol), HBTU (1.32 g, 3.5 mmol), Fmoc-Leu-OH (1.24 g, 3.5 mmol) and DIEA (1.34 mL, 7 mmol) in 10 mL of DMF, shaken for 4 h, filtered and washed, after which the resin exhibited a negative Kaiser test. Method A: 79.6 % purity, method B: 100% purity.

Supported Fmoc-L-glutaminyl-L-prolinyl-L-leucinyl-L-phenylalaninate ***9***

As described above, in the same reactor tube used in the last step, resin **8** was first treated under the general Fmoc deprotection protocol and then swollen in DMF and coupled to Fmoc-Gln-OH (1.55 g, 4.2 mmol) using HATU (1.59 g, 4.2 mmol) and DIEA (1.75 mL, 8.4 mmol) with shaking for 6 h. After filtration and washing as described above, the resin exhibited a negative Kaiser test. Method A: 80.2 % purity, method B: 94.5 % purity.

Supported Fmoc-ε-aminocaproyl-L-glutaminyl-L-prolinyl-L-leucinyl-L-phenylalaninate ***10***

As described above, in the same reactor tube used in the last step, resin **9** was first treated under the general Fmoc deprotection protocol and then swollen in DMF (13 mL) and coupled to *N*-Fmoc-ω-amino caproic acid (1.237g, 3.5 mmol) using EDC•HCl (741.6 mg, 3.85 mmol), HOBt (478.1 mg, 3.5 mmol), and DIEA (0.672 mL, 3.85 mmol) with shaking for 6 h. After filtration and washing as described above, the resin

exhibited a negative Kaiser test. Method A: 66.1 % purity, method B: 85.0 % purity.

Tokyo Green Peptide 11a. Resin **10** (500 mg, 0.16 mmol) was placed in a 12-mL syringe tube, swollen in 6 mL of DMF for 20 min, filtered, treated with 4 mL of a solution of 20% piperidine in DMF, and shaken for 30 min. The resin was filtered and washed with DMF (5 × 5 mL) followed by DCM (3 × 5 mL), when a positive Kaiser test was observed. The resin was swollen with DMF (10 mL) and coupled to Tokyo Green (**4**, 56 mg, 0.16 mmol) using EDC•HCl (46.3 mg, 1.5 mmol), HOBt (26.22 mg, 1.2 mmol), DMF (3.2 mL) and DIEA (43 µl, 0.24 mmol) with shaking for 6 h. The resin was filtered and washed with DMF (5 × 5 mL) followed by DCM (3 × 5 mL), when a negative Kaiser test was observed. The resin was treated with a 1:1 solution of TFA/DCM (5 mL), shaken for 2 h, filtered and washed three times each with TFA (3 mL) and DCM (5 mL). The collected filtrate and washings were combined and placed on a rotary evaporator to remove the volatiles. The residue was dissolved in water, and freeze-dried. The crude material was purified by preparative HPLC on Prevail C18 (250 × 22 mm, 5 micron) column using 30-80 % acetonitrile in water as eluant, a flow rate of 15 mL/min and the detector set at 214 nm. The collected fractions were freeze-dried to give the desired fluorescent peptide **11a** (40 mg) as an orange powder. HRMS m/z ($M+H^+$): calcd 945.4393. found 945.4394.

Fluoresceine peptide 11b. Resin **10** (300 mg, 0.105 mmol) was placed in a 12 mL syringe tube, swollen in 5 mL of DMF for 20 min, filtered, treated with 3 mL of a solution of 20% piperidine in DMF, and shaken for 30 min. The resin was filtered and washed with DMF (5

× 5 mL) followed by DCM (3 × 5 mL), when a positive Kaiser test was observed. The resin was swollen with DMF (10 mL) and coupled to fluorescein isocyanate (163.6 mg, 0.42 mmol) using DIEA (74 μL, 0.42 mmol) with shaking for 6 h. The resin was filtered and washed with DMF (5 × 5 mL) followed by DCM (3 × 5 mL), when a negative Kaiser test was observed. The resin was treated with a 1:1 solution of TFA/DCM (5 mL), shaken for 2 h, filtered and washed three times each with TFA (3 mL) and DCM (5 mL). The collected filtrate and washings were combined and placed on a rotary evaporator to remove the volatiles. The residue was dissolved in water, and freeze-dried. The crude material was purified by preparative HPLC on Prevail C18 (250 × 22 mm, 5 μL) column using 20-80 % acetonitrile in water as eluant, a flow rate of 15 mL/min and the detector set at 214 nm. The collected fractions were freeze-dried to give fluorescent peptide **11b** (56 mg) as a red powder. HRMS m/z ($M+H^+$): calcd 1006.4015. Found 1006.4027.

Dansyl Peptide 11c. Resin **10** (520 mg, 0.182 mmol) was placed in a 12 mL syringe tube, swollen in 5 mL of DMF for 20 min, filtered, treated with 3 mL of a solution of 20% piperidine in DMF, and shaken for 30 min. The resin was filtered and washed with DMF (5 × 5 mL) followed by DCM (3 × 5 mL), when a positive Kaiser test was observed. The resin was swollen with DMF (10 mL) and coupled to dansyl chloride (149 mg, 0.55 mmol) using DIEA (96μL, 0.55 mmol) with shaking for 4 h. The resin was filtered and washed with DMF (5 × 5 mL) followed by DCM (3 × 5 mL), when a negative Kaiser test was observed. The resin was treated with a 1:1 solution of TFA/DCM (5 mL), shaken for 2 h, filtered and washed three times each with TFA (3 mL) and DCM (5 mL). The collected filtrate and washings were combined and placed on a rotary evaporator to remove the volatiles. The

residue was dissolved in water, and freeze-dried. The crude material was purified by preparative HPLC on Prevail C18 (250 × 22 mm, 5μ) column using 40-80 % acetonitrile in water as eluant, a flow rate of 15 mL/min and the detector set at 214 nm. The collected fractions were freeze-dried to give the fluorescent peptide **11c** (60 mg) as a green powder. HRMS m/z ($M+H^+$): calcd 850.4168. Found 850.4173.

Synthesis of (5-amino-pentyl)-carbamic acid tert-butyl ester **(14a).**

Di-*tert*-butyl dicarbonate (2.13 g, 9.8 mmol) in 40 mL of 9:1 dioxane/water was added to a solution of 1,5-diaminopentane (**13a**, 2 g, 19.6 mmol) in 60 mL of 9:1 dioxane/water over a period of 4 h. The solution was stirred at room temperature overnight and concentrated. The residue was taken up in 40 mL of water. The white precipitate corresponding to the *N*,*N'*-di-Boc-1,5-diaminopentane was removed by filtration through a fritted glass funnel, and the filtrate was extracted with CH_2Cl_2 (4 × 30 mL). The combined organic extracts were concentrated. The product was purified by silica gel chromatography (90:10 CH_2Cl_2/methanol). Evaporation of the collected fractions provided *N*-(Boc)-1,5-diaminopentane **14a** (1.4 g, 6.95 mmol, 71%) as a dense liquid; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.63 (s, 1H), 3.1 (dd, 2H, J = 6.0, J = 12.4), 2.68 (t, 2H, J = 6.7), 1.75 (s, 3H), 1.49-1.52 (br, 3H), 1.42 (S, 9H), 1.27-1.36 (br, 2H). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 156.85, 79.86, 42.77, 41.29, 33.99, 30.72, 29.23, 24.86. HRMS m/z ($M+H^+$): calcd for $C_{10}H_{22}N_2O_4$ 203.1754. found 203.1761.

Synthesis of {5-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-pentyl}-carbamic acid tert-butyl ester **(15a)**.

A solution of D-biotin (153 mg, 0.625 mmol) in 3 mL of DMF was treated with amine **(14a)** (151 mg, 0.751 mmol), HOBt (103 mg, 0.751 mmol), EDC•HCl (181 mg, 0.938 mmol) and DIEA (1.2 mL, 0.938 mmol) and stirred at room temperature for 4 h, when TLC showed disappearance of the starting amine (R_f = 0, solvent: 5% MeOH/95% $CHCl_3$) and appearance of a new product (R_f = 0.1, solvent: 5% MeOH/95% $CHCl_3$). The volatiles were removed by evaporation, and the residue was dissolved in 110 mL of EtOAc and washed with brine (3 × 20 mL) then saturated sodium bicarbonate (3 × 20 mL), and dried over $MgSO_4$. The organic layer was evaporated to give amide **15a** (167.1 mg, 61 %) as a white powder:; ^{1}H NMR (400 MHz, MeOD) δ 4.51 (br, 1H), 4.32 (br, 1H), 3.33 (br, 1H), 3.2 (m, 3H), 3.04 (m, 2H), 2.95 (dd, 1H, *J* = 5, *J* = 12.7), 2.73 (d, 1H, J=12.7), 2.21 (t, 2H, J=7.4), 1.75 (m, 1H), 1.36 (m, 2H), 1.64 (m, 3H), 1.51 (m, 5H), 1.45 (s, 9H), ^{13}C NMR (400 MHz, MeOD) δ 174.2, 164.3, 156.7, 78.0, 61.6, 59.9, 55.2, 39.5, 39.3, 38.5, 35.1, 28.8, 28.3, 28.0, 27.0, 25.1, 23.4. HRMS m/z ($M+H^+$): calcd 429.2535 for for $C_{20}H_{36}N_4O_4S$. found 429.2533.

Synthesis of 5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid (4-amino-butyl)-amide hydrochloride salt **(16a)**

To a solution of carbamate **15a** (100 mg, 0.23 mmol) in MeOH (3 mL), aqueous HCl (1M, 0.4 mL) was added and the mixture was stirred for 2h. The volatiles were removed under vacuum to afford amine hydrochloride **16a** as a yellow gel in quantitative yield (83.9 mg):

^{1}H NMR (400 MHz, CD_3OD) δ 4.50 (br, 1H), 4.32 (br, 1H), 3.32 (br, 1H), 3.2 (m, 3H), 2.93 (m, 3H), 2.68 (d, 1H, J = 8.9), 2.21 (t, 2H, J = 9.7), 1.67 (m, 4H), 1.58 (m, 3H), 1.44 (m, 3H). HRMS m/z ($M+H^+$): calcd 329.2006 for $C_{15}H_{28}N_4O_2S$. found 329.2002.

{2-[2-(2-amino-ethoxy)-ethoxy]-ethyl}-carbamic acid tert-butyl ester ***14b****.*

To a solution of Et_3N (9.41 mL, 67.5 mmol) in MeOH (200 mL), tris(ethylene glycol)-1,8-diamine (5 g, 33.7 mmol) and di-*tert*-butyl dicarbonate (7.36g, 33.7 mmol) were added. The reaction was stirred for 1 h. The volatiles were evaporated and the residue was dissolved in 200 mL of CH_2Cl_2, washed with a saturated solution of Na_2CO_3 (2 × 150 mL), dried over Na_2SO_4 and filtered. The volatiles were evaporated and the residue was purified by silica gel chromatography (CH_2Cl_2/MeOH/NH_4OH 55/4/0.2) to afford carbamate **14b** (2.68 g, 32%) as an oil; ^{1}H NMR (d_6-DMSO, 300 MHz) δ 1.37 (s, 9H, $3CH_3$), 1.81 (br. s, 2H, NH_2), 2.64 (t, J = 5.75, 2H, CH_2NH_2), 3.06 (m, 2H, CH_2NH-Boc), 3.35 (t, J = 5.79, 2H, $CH_2CH_2NH_2$), 3.38 (t, J = 6.3, 2H, CH_2CH_2NH-Boc), 3.49 (s, 4H, $O(CH_2)_2$), 6.81 (br. t, 1H, NH-Boc). ^{13}C NMR (d_6-DMSO, 75 MHz) δ 29.47, 40.97, 42.63, 70.49, 70.81, 70.85, 74.34, 78.79, 156.87. HRMS m/z ($M+H^+$): calcd for $C_9H_{24}N_2O_4$ 203.1754. found 203.1761.

[2-(2-{2-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-ethoxy}-ethoxy)-ethyl]-carbamic acid tert-butyl ester ***15b****.*

To a solution of amine **14b** (500 mg, 2.01 mmol) and DIEA (770 mL, 4.42 mmol) in anhydrous DMF (50 mL), D-biotin (540 mg, 2.21 mmol) and TBTU (905 mg, 2.82 mmol) were added and the reaction mixture was stirred at room temperature for 18 h. The volatiles were evaporated. The residue was dissolved in EtOAc (100 mL), washed with a saturated

solution of Na_2CO_3 (2 × 100 mL), dried over Na_2SO_4, filtered and evaporated. The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH/NH_3 55/4/0.4) to afford amide **15b** (954 mg, 1.15 mmol, 57% yield) as a white solid: mp: 102 °C; ^{1}H NMR (d_6-DMSO, 300 MHz) δ 1.15-1.66 (m, 15H, $3CH_2$, $3CH_3$), 2.06 (t, J =7.31, 2H, CH_2C=O), 2.55-2.81 (m, 2H, CH_2S), 3.02-3.12 (m, 3H, CH_2NH-Boc, CHS), 3.17 (m, 2H, CH_2NHC=O), 3.34-3.40 (2t, 4H, $2CH_2CH_2N$), 3.48 (s, 4H, O-$(CH_2)_2$), 4.10-4.14 (m, 1H, CHN), 4.28-4.32 (m, 1H, CHN), 6.38 (s, 1H, NH), 6.45 (s, 1H, NH), 6.78 (br. t, 1H, NH-Boc), 7.85 (br. t, 1H, NHC=O). ^{13}C NMR (d_6-DMSO, 75 MHz) δ 26.57, 29.33, 29.52, 36.38, 39.73, 40.96, 41.16, 56.73, 60.50, 62.34, 70.47, 70.75, 70.82, 78.89, 156.88, 164.04, 173.43. HRMS m/z (M+Na^+): calcd for $C_{21}H_{38}N_4O_6S$ 271.1628. found 271.1624.

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid {2-[2-(2-amino-ethoxy)-ethoxy]-ethyl}-amide hydrochloride salt ***(16b).***

Employing the same procedure described above for the synthesis of amine hydrochloride **16a**, carbamate **15b** (60 mg, 0.14 mmol) was quantitatively converted to amine hydrochloride **16b** which was isolated as an amorfous solid: mp: 111 °C; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.41-1.82 (m, 6H, $3CH_2$), 2.25 (t, J=2.25, 2H, CH_2C=O), 2.72-3.00 (m, 2H, CH_2S), 3.14 (br. t, 2H, CH_2NHC=O), 3.22-3.28 (m, 1H, CHS), 3.39 (t, J = 5.6, 2H, CH_2), 3.58 (t, J = 5.6, 2H, CH_2), 3.68 (s, 4H, O$(CH_2)_2$), 3.73 (t, J = 5.1, 2H, CH_2), 4.35-4.40 (m, 1H, CH), 4.55-4.59 (m, 1H, CH). ^{13}C NMR (CD_3OD, 75 MHz) δ 26.89, 29.50, 29.75, 36.69, 40.25, 40.71, 40.96, 56.99, 62.09, 63.76, 67.92, 70.64, 71.30, 71.39. HRMS m/z (M+Na^+): calcd for $C_{16}H_{30}N_4O_4S$ 497.2404. Found 497.2401.

*[2-(2-Amino-ethyldisulfanyl)-ethyl]-carbamic acid tert-butyl ester (**14c**).*

This compound was prepared according to the same procedure described to make carbamate **14b** above using Et_3N (9.41 mL, 67.5 mmol), MeOH (200 mL), cysteamine disulfide (5.12 g, 33.72 mmol), and di-*tert*-butyl dicarbonate (7.36, 33.72 mmol). Purification by silica gel chromatography (CH_2Cl_2/MeOH/NH_3 55/4/0.2) gave carbamate **14c** (3.03 g, 12.00 mmol, 29% yield) as yellow solid: mp: 92 °C; ^{1}H NMR (d_6-DMSO, 300 MHz) δ 1.37 (s, 9H, t-Bu), 2.68-2.80 (m, 6H, NCH_2CH_2S, NCH_2CH_2S, $NH_2CH_2CH_2S$), 3.19 (m, 2H, SCH_2CH_2NH-Boc), 7.00 (br t, 1H, NH). ^{13}C NMR (d_6-DMSO, 300 MHz) δ: 29.51, 38.94, 40.73, 42.21, 43.36, 79.04, 156.79. HRMS m/z ($M+H^+$): calcd for $C_9H_{20}N_2O_2S_2$ 253.1039. Found 253.1040.

*(2-{2-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-ethyldisulfanyl}-ethyl)-carbamic acid tert-butyl ester (**15c**).*

This compound was prepared from amine **14c** (1.41 g, 5.59 mmol), DIEA (2.14 mL, 12.3 mmol), D-biotin (1.4 g, 5.8 mmol) and TBTU (2.5 g, 7.84 mmol) using the same protocol as described for amide **15b**. Purification by silica gel chromatography (CH_2Cl_2/MeOH/NH_3 55/4/0.4) afforded amide **15c** (1.44 g, 3.02 mmol, 54% yield) as a white solid: mp: 112 °C; ^{1}H NMR (d_6-DMSO, 300 MHz) δ 1.23-1.66 (m, 15H, $3CH_2$, $3CH_3$), 2.06 (t, J = 7.33, 2H, CH_2C=O), 2.69-2.85 (m, 6H, $3CH_2S$), 3.06-3.12 (m, 1H, CH-S), 3.19 (m, CH_2, CH_2NH-Boc), 3.31 (m, 2H, CH_2NHC=O), 4.10-4.14 (m, 1H, CH), 4.28-4.32 (m, 1H, CH), 6.37 (br. s, 1H, NH), 6.43 (br. s, 1H, NH), 6.99 (br. t, 1H, NH-Boc), 7.98 (br. t, 1H, NH). ^{13}C NMR (d_6-DMSO, 75 MHz) δ 26.53, 29.33, 29.52, 36.44, 38.65, 38.81, 39.12, 40.68, 56.72, 60.48,

62.32, 79.10, 156.81, 164.00, 173.47. HRMS m/z ($M+H^+$): calcd for $C_{19}H_{34}N_4O_4S_3$ 479.1815. found 479.1819.

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid [2-(2-amino-ethyldisulfanyl)-ethyl]-amide hydrochloride salt (**16c**).

Employing the same procedure described above for the synthesis of amine hydrochloride **16a**, carbamate **15c** (300 mg, 0.63 mmol) was quantitatively converted to amine hydrochloride **16c** which was isolated as a solid: mp: 119 °C; ^{1}H NMR (d_6-DMSO, 300 MHz) δ 1.22-1.65 (m, 6H, $3CH_2$), 2.06 (t, 2H, $CH_2C=O$), 2.53-2.83 (m, 4H, CH_2S, CH_2S), 2.94 (t, J = 6.51, 2H, $NH_2CH_2CH_2S$), 3.01-3.10 (m, 3H, CHS, CH_2NH_2), 3.27-3.34 (m, 2H, $CH_2NHC=O$), 8.11 (t, 1H, NHC=O), 8.17 (br. s, 3H $-NH_3$). ^{13}C NMR (d_6-DMSO, 75 MHz) δ 26.54, 29.33, 29.48, 35.17, 35.29, 36.42, 38.50, 39.15, 41.15, 56.73, 60.52, 62.36, 164.05, 173.61. HRMS m/z ($M+H^+$): calcd for $C_{14}H_{26}N_4O_2S_3$ 379.1291. found 379.1276.

2. 9 References:

59- A. Viens, U. Mechold, H. Lehrmann, A. Harel-Bellan, V. Ogryzko., Use of protein biotinylation in vivo for chromatin immunoprecipitation. *Anal. Biochem.* **2004**, *325*, 68-76.

60- M. Fernandez-Suarez, T. S. Chen, A. Y. Ting., Protein-Protein Interaction Detection in Vitro and in Cells by Proximity Biotinylation. *J. Am. Chem. Soc.* **2008**, *130*, 9251-9253.

61- D. M. Peters, J. B. Griffin, J. S. Stanley, M. M. Beck, J. Zemplini., Exposure to UV light causes increased biotinylation of histones in Jurkat cells. *Am J Physiol Cell Physiol.* **2002**, *283*, C878-C884.

62- L. F. Mottram, S. Boonyarattanakalin, R. E. Kovel, B. R. Peterson., The Pennsylvania Green fluorophore: A hybrid of Oregon Green and Tokyo Green for the construction of hydrophobic and pH-insensitive molecular probes. *Org. Lett.* **2006**, *8*, 581-584.

63- S. M. F. G. Gillet, R. A. Chica, J. W. Keillor, J. N. Pelletier., Expression and rapid purification of highly active hexahistidine-tagged guinea pig liver transglutaminase. *Protein. Expr. Purif.* **2004**, *33*, 256-264.

64- J. E. Folk, S. I. Chung., Transglutaminases. *Methods Enzymol.* **1985**, *113*, 358-364.

65- K.D. Tartoff, C.A. Hobbs., Improved media for growing plasmid and cosmid clones. *Bethesda Res. Lab. Focus*, 1987, **9**, 12.

66- Roberto. A. Chica, Paul Gagnon, Jeffrey W. Keillor and Joelle N. Pelletier., Tissue transglutaminase acylation: proposed role of conserved active-site Tyr and Trp resisues revealed by molecular modeling of peptide substrate binding. *Protein Sciences*, **2004**, *13*, 979-991.

67- C.-W. Lin, A.Y. Ting., Transglutaminase-catalyzed site-specific conjugation of small-molecule probes to proteins in vitro and on the surface of living cells. *J. Am. Chem. Soc.*, **2006**, *128*, 4542-4543.

68- E. Kaiser, R.L. Colescott, C.D. Bossinger, P.I. Cook., Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.*, 1970, **34**, 595-598.

Chapter 3

A continuous FRET based assay for tissue Transglutaminase

3.1 Introduction

Fluorescence resonance energy transfer (FRET) is a useful contemporary technique for studying proximity effects of macromolecules. The FRET phenomenon involves the non-radiant energy transfer from an excited fluorophore donor to an acceptor molecule through a dipole-dipole interaction. [69] The acceptor may in turn release the energy at a different wavelength. The energy transfer efficiency is dependant upon many factors, such as the spectral overlap, the relative orientations and the distance between the donor and acceptor pair. [70, 71] FRET is detected at a 10 to 100 Å distance between the donor and acceptor pair contingent on orientation, as expressed by the Forster value R_o (equation 3.1). [72]

$R_o = 9.78 \times 10^3 (k^2 n^{-4} Q_D J(\lambda))^{1/6}$ (Å) (equation3.1).

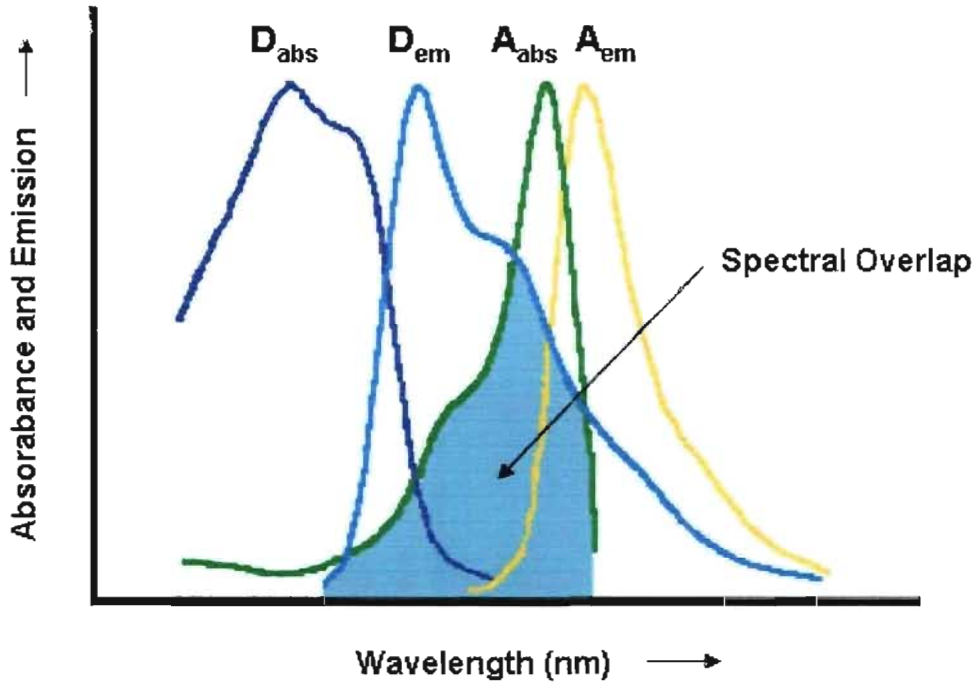
where k^2 describes the donor/acceptor transition dipole orientation and can range in value from 0 (perpendicular) to 4 (collinear/parallel). The refractive index "n" is ascribed a value of 1.4 for biomolecules in aqueous solution; Q_D is the quantum yield of the donor in the absence of the acceptor; $J(\lambda)$ is the overlap integral which represents the degree of spectral overlap between the donor emission and the acceptor absorption (Figure 3.1). The values for $J(\lambda)$ and R_o increase with higher acceptor extinction coefficients and greater overlap between the donor emission spectrum and the acceptor absorption spectrum.

FRET efficiency is determined by either the steady-state (Equation 3.2) or the time resolved measurement (Equation 3.3). In the following equations, F is the relative donor fluorescence intensity in the absence (F_D) and presence (F_{DA}) of the acceptor, and τ is the fluorescent lifetime of the donor in the absence (τ_D) and presence (τ_{DA}) of the acceptor.

$E = 1 - F_{DA} / F_D$ (Equation 3.2).

$E = 1 - \tau_{DA} / \tau_D$ (Equation 3.3).

Figure 3.1: FRET dependence on the overlap integrals.



Source : *Plant Methods*, 2006, **2**, 12.

FRET has been widely used in enzymatic activity detection assays. [70, 71] for example, the activity of proteases, [73] and phosphodiesterases [74] have been monitored by the disappearance of FRET signal on enzyme-catalyzed cleavage of the fluorophore pair linked the substrate.

TGase catalyzes isopeptide bond formation. The donor and acceptor substrates were thus labeled each with one member of a FRET pair of fluorophores that could engage in energy transfer when brought in close proximity. The FRET pair of 7-hydroxycoumarin (which is excited at 400 nm and emits at 460 nm) and Tokyo Green or fluorescien thiourea (which are typically excited at 490 nm and emits at 515 nm) has previously been employed as

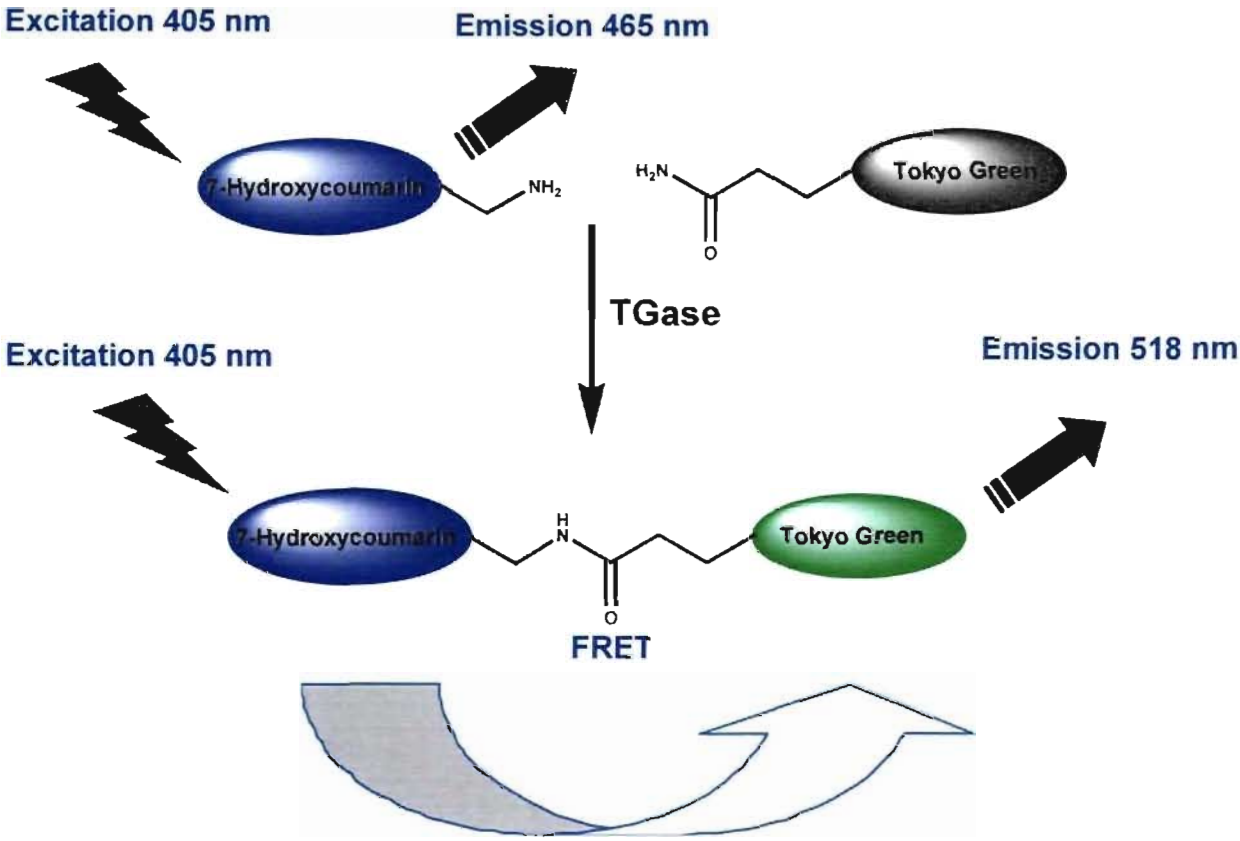
indicator of DNA structure in solution [75] as well as in protein labeling studies. [76] Donor and acceptor substrates were thus labeled with these two fluorophores. Upon TGase-induced isopeptide bond formation, a fluorescence decrease at 460 nm was observed (emission of the 7-hydroxycoumarin) as well as fluorescence increase measured at 515 nm (emission of Tokyo Green and Fluorescein thiouea). The fluorescence decrease of 7-hydroxycoumarin (λ_{exc} 405 nm, λ_{em} 460 nm) was effectively monitored using purified enzyme with high sensitivity (4 mU enzyme concentration). This method was also effective in a more complex biological system of lysates of *E. coli* that express TGase enzyme. Furthermore, this method was applied to 96-well microtiter plate format, for high-through put screening.

3.2 General

Enzyme preparation

Recombinant guinea pig liver TGase was over-expressed and purified from *E. coli* following the published procedure. [63] The purified TGase solution used in all assays was shown to have a specific activity of 14 U/mg (2.5 mg/mL) in 25 mM of tris-acetate (pH 7) buffer using the hydroxamate activity assay, [64] in which Cbz-Gln-Gly and hydroxylamine were used as acyl-donor and acyl-acceptor substrates, respectively.

Figure 3.2: Scheme of FRET for Tokyo Green/7-hydroxycoumarin carboxylic acid pairs



Preparation of crude bacterial lysate

Lysate was prepared using the same protocol as described in chapter 2.

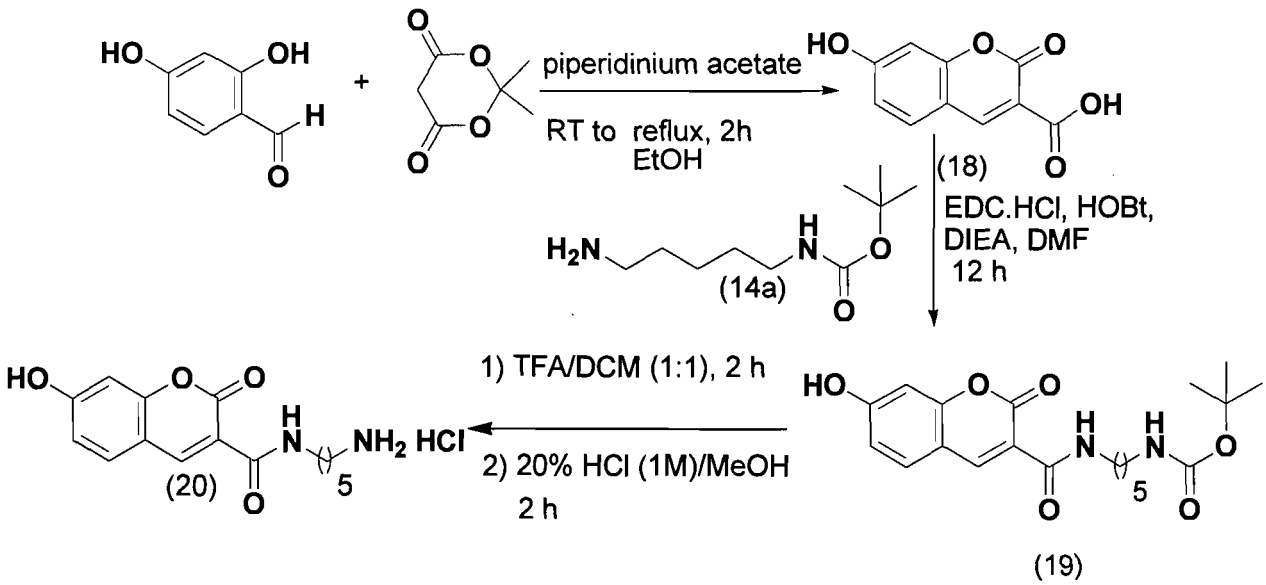
Material

The buffer solution in all assays was tris.HCl 100 mM, EDTA 0.6 mM, 10 mM $CaCl_2$, (pH 8). The washing buffer solution was a mixture of 100 mM phosphate, 100 mM NaCl, and 0.1% SDS (pH 8). Water was purified using a Millipore BioCell water purification system.

3.3 Synthesis of the acyl acceptor substrate

7-Hydroxy-3-coumarin carboxylic acid was prepared as reported in the literature, [72] and was then coupled to mono-protected *N*-Boc-diamine **14a**. Boc deprotection of **19** was performed using trifluoro acetic acid in DCM. The TFA salt was exchanged with 1 M HCl solution to provide the hydrochloride salt for enzymatic studies (Scheme 3.1).

Scheme 3.1: Synthesis of the acyl acceptor substrate



3.4 Synthesis of the acyl donor substrate

Peptides **11a** and **11b**, as previously described in Chapter 2, were chosen as acyl donor candidates due to their high affinities to TGase. Furthermore, they offered better spectral overlap with 7-hydroxy-3-coumarin for FRET. [76, 77]

3.5 FRET assay using purified tissue TGase

3.5.1 Methods

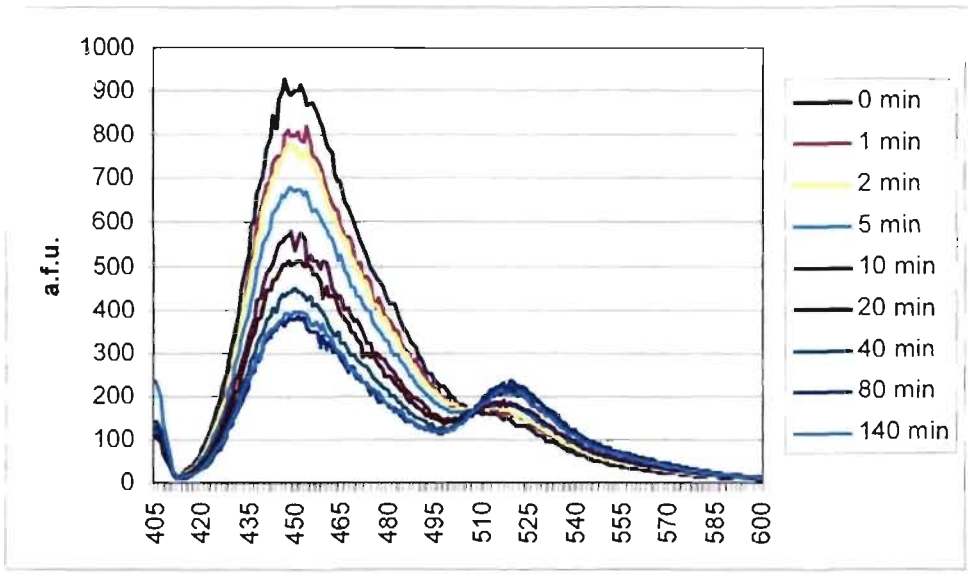
All assays were performed in triplicate. Acyl donor substrate **11a** or **b** (10 μL, 0.6 mM) and acyl acceptor substrate **20** (10 μL, 0.6 mM) were combined in 80 μL of buffer solution, 12 μL of DMF, and 78 μL of water at a final volume of 190 μL. The reaction was initiated by addition of 25 μg (10 μL) of purified TGase or an equivalent volume of 25 mM tris-acetate buffer was added for the blank. Fluorescent spectra were measured after 1, 2, 5, 10, 20, 40, 80 and 140 min of incubation. At each time, an aliquot of the parent solution (7 μl) was diluted with a mixture of 200 μL MeOH, 200 μL buffer, and 200 μL water. The decrease in fluorescent signal corresponding to enzyme activity was measured relative to the blank solution using a Perkin Elmer Bio Assay Reader (HTS 7000). Excitation and emission wavelengths were respectively λ_{exc} = 405 nm and λ_{em} = 465 nm, and the gain was set at 52 units.

3.5.2 Results and discussions

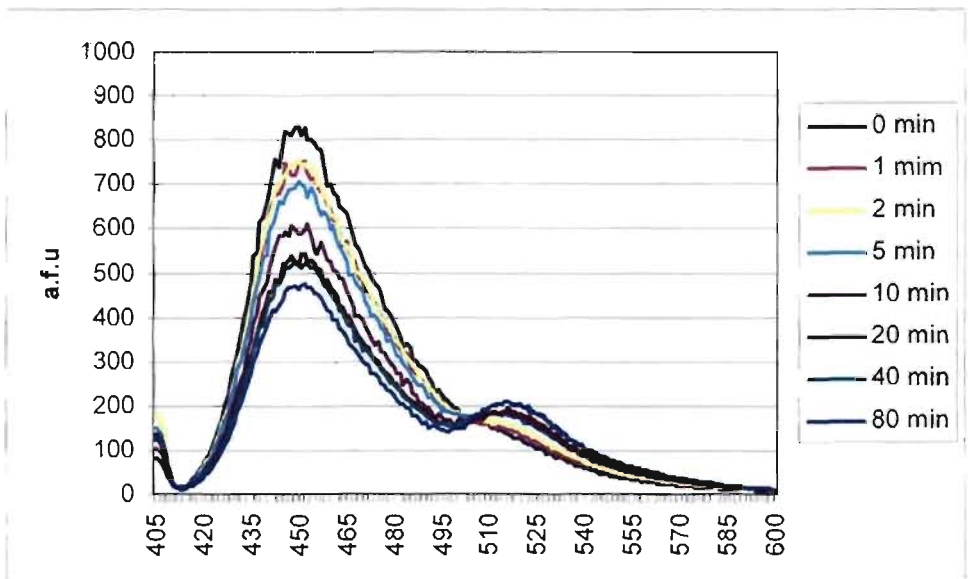
The change of fluorescence was measured at different times after incubation of **11a** or **11b** with **20** in the presence of TGase. Excitation at 405 nm caused a continuous emission decrease at 460 nm corresponding to the decrease of the fluorescence of 7-hydroxy-coumarin carboxylic acid. Simultaneously, a continuous increase of fluorescence at 518 nm corresponded to the fluorescence emission of Tokyo Green for a typical fluorescence resonance energy transfer (Figure 3.4).

Figure 3.4: FRET assay sensitivity with acyl donor **11a-b** and acyl acceptor 20 using purified enzyme

a- Acyl acceptor **20** / acyl donor **11a**



a- Acyl acceptor **20** / acyl donor **11b**



In the spectra, the fluorescence decrease at 465 nm was greater than the increase at 515 nm for both the Tokyo Green and fluorescein labeled peptides. In light of this fact, changes in fluorescence were measured at 465 nm. The relative decrease of fluorescence due to non specific quenching was presumed to be negligeable because the same sample of lysate was used for all experiments.

The decrease in fluorescence at 465 nm, and increase of fluorescence signal at 515 nm were observed to cross at 510 nm, when the spectra were measured at different incubation times. This specific wavelength is called the isosbestic point and is characteristic in spectroscopy of species having the same extinction coefficients (at the same concentration). It is a consequence of having a two-state system; the transamidation reaction is consistent with this two-state system (substrates vs product, with no appreciable intermediate state).

The decrease of fluorescence at 465 nm was greater in the case of the 7-hydroxycoumarin/ Tokyo Green pair (a decrease of 510 units after 80 min) relative to that of the 7-hydroxycoumarin/ fluorescein thiourea pair (a decrease of 300 units after 80 min). The fluorescence increase was similarly more pronounced for the 7-hydroxycoumarin/ Tokyo Green pair relative to the 7-hydroxycoumarin/ fluorescein thiourea pair. The conditions used for the measurements may influence the sensitivity of the assay. For example, fluorescence emission of fluorescein thiourea possessing a carboxylic acid at the 1' position of the aromatic system is greatly influenced by pH because of lactam formation. Tokyo Green, which has a greater quantum yield and no carboxyl functionality at the 1' position, gave a stable signal over a wide range of pH. [62]

In order to confirm that this assay was in fact measuring fluorescence change resulting from enzymatic formation of the final product formation, and not due to the formation of a precipitate that could not be detected by the naked eye, control experiments were performed.

- Spectrometry:

LCMS-MS analysis (0-40% acetonitrile, H_2O, 0.1% formic acid, 0.5 mL/ min, λ= 214 nm, Agilent LCMSD, column: C6 Phenyl, 3H, 50 × 4.6 Gemini) was performed on sample aliquots from enzymatic and non enzymatic reactions using acyl donor **11a** (Figure 3.5) and acyl acceptor **20**. From the non enzymatic sample spectrum, a single peak with a molecular weight of 945.4 g/mol (7.15 min retention time) corresponding to the acceptor substrate **11a**, was observed. From the enzymatic sample, a peak with a molecular weight of 1218.1 g/mol (7.26 min retention time) corresponding to the exact mass of the amide product **21** was observed (Figure 3.6) without trace of **11a**.

Figure 3.5: Product formed upon transamidation by TGase

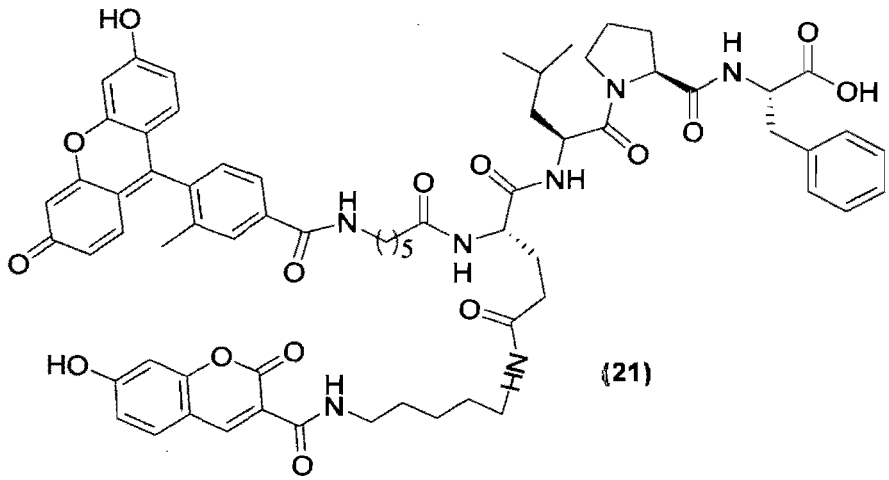
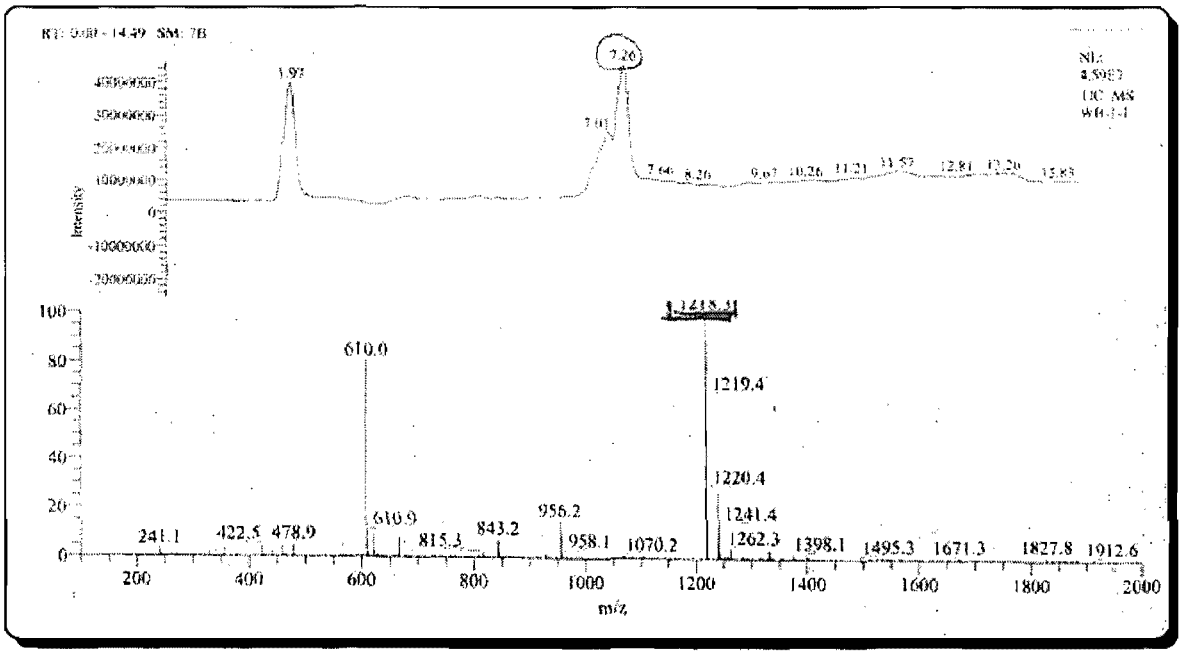
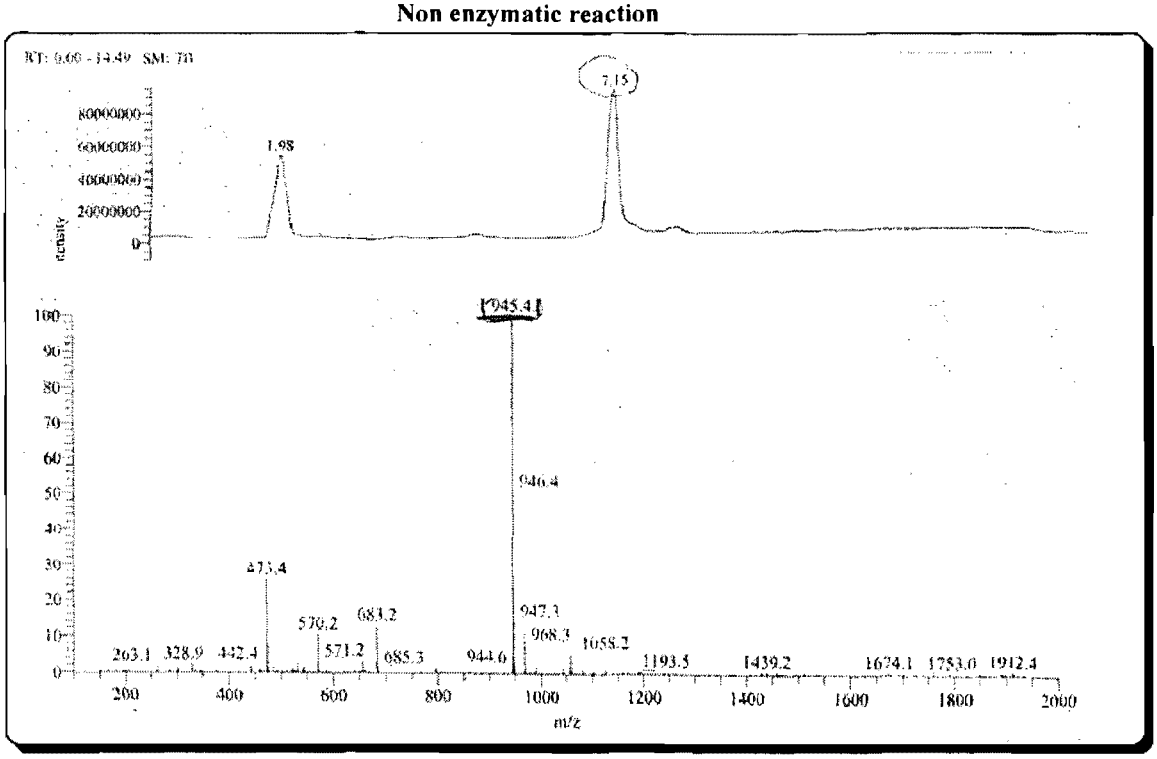


Figure 3.6: LCMS spectrum of the enzymatic and non enzymatic reactions

Enzymatic reaction



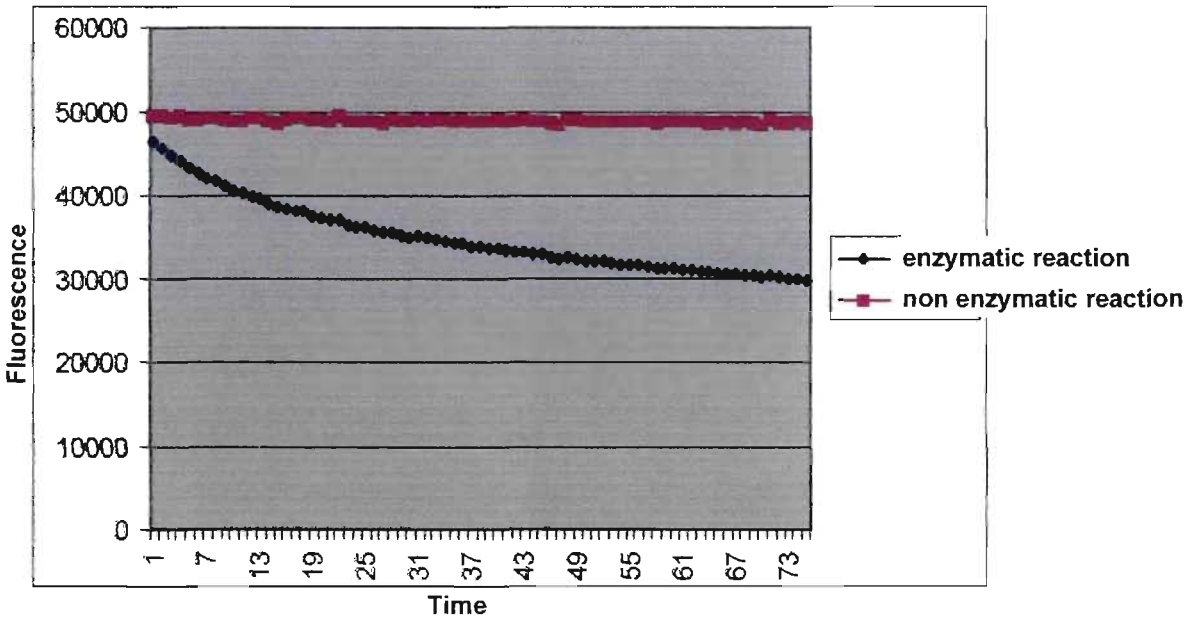
Non enzymatic reaction



3.5.3 Limits of detection

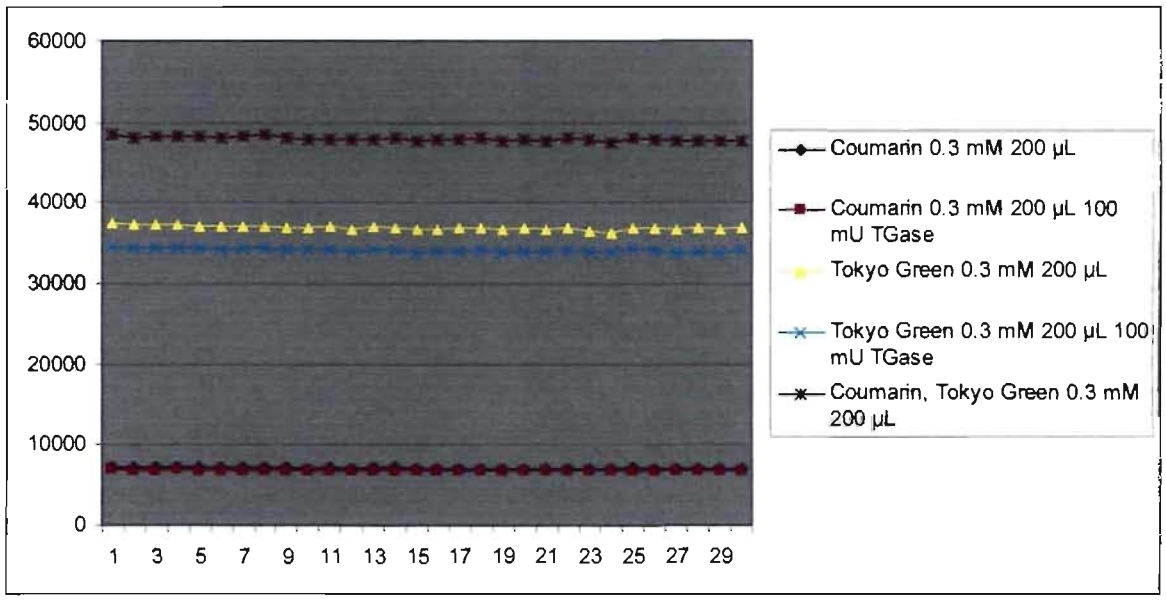
To further demonstrate the practical value of the FRET assay in plate format, a continuous experiment as described in the methods section was performed under enzymatic (35 U/mL) and nonenzymatic conditions (Figure 3.7) using purified TGase. As clearly shown, the time-dependent decrease in fluorescence was pronounced.

Figure 3.7: Application of the FRET assay on plates.



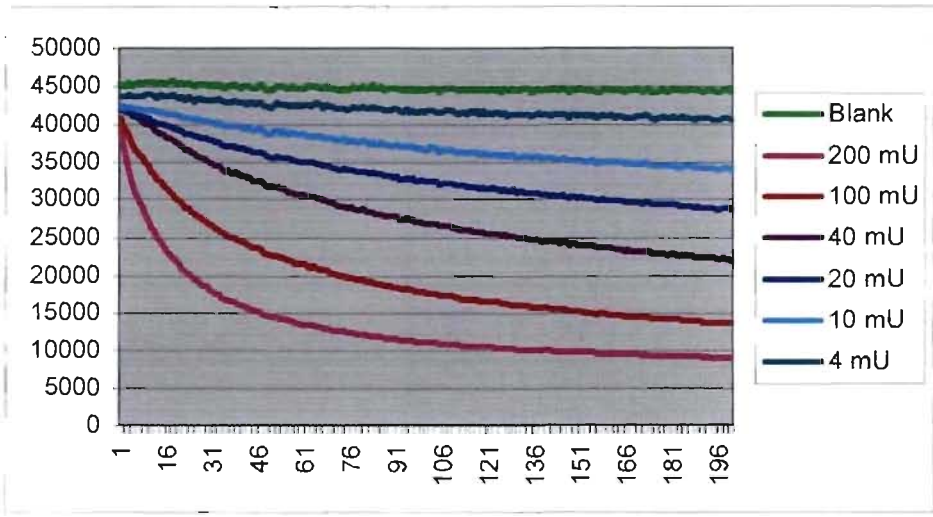
In order to determine that continuous fluorescence decrease was solely due to FRET, the donor and the acceptor substrates were separately incubated with and without enzyme (Figure 3.8). The spectra exhibited no decrease of fluorescence, in the absence of the FRET pair.

Figure 3.8: Control test for the FRET assay on plate



The limits of detection of TGase by the assay with respect to concentration was determined using various concentrations of purified enzyme with acyl donor **11a** and acyl acceptor **20**, as described under Methods. For correlation of the signal with enzymatic activity, the decrease of fluorescence at 465 nm was followed as shown for a typical kinetic experiment with different amounts of purified enzyme (Figure 3.9). The FRET effect upon ligation by TGase could be followed kinetically and continuously using this assay. The minimum amount of enzyme reliably detected at different enzyme concentrations corresponded to 4mU (Figure3.9).

Figure 3.9: Limit of detection of the FRET assay



3.6 FRET assay using crude bacterial lysate

3.6.1 Methods

All assays were performed in triplicate. Acyl donor substrates **11a** and **11b** (10 μL, 0.6 mM) were respectively incubated with the acyl acceptor substrate **20** (10 μL, 0.6 mM) in 80 μL of buffer solution and 12 μL DMF, giving a final volume of 200 μL. The reaction was initiated by addition of lysate (2.5 μL, 0.90 U/mL) or an equivalent volume of the lysate of *E. coli* cells void of the expression plasmid, as the blank. Enzyme activity was measured by monitoring the decrease of the signal of fluorescence relative to the blank using a Perkin Elmer Bio Assay Reader (HTS 7000) using λ_{exc} = 405 nm and λ_{em} = 465 nm for substrate **11a** and **11b** with the gain set at 52 units.

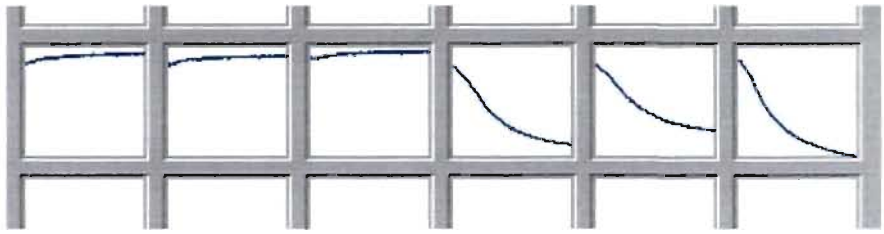
3.6.2 Result and discussion

Detection of enzymatic activity in complex biological samples is of practical value for high-throughput screening. The coupling of acyl donor **11a** and acyl acceptor **20** was thus studied using crude lysate of *E. coli* expressing recombinant TGase. Expression of TGase was performed in small volume cultures in 48 well plates as described under Methods Section. The lysate of *E. coli* void of the expression plasmid was used as a negative control. From each culture, cellular lysate of 200 μL were obtained, from each lysate an aliquot of 2.5 μL (~ 2.5 mU) was removed and incubated with substrate. Enzymatic substrate ligation in lysate samples was monitored using a plate reader, by following the decrease in fluorescence at 465 nm (Figure 3.10) over a period of 130 min.

When analyzing TGase activity in complex biological samples, assays that measure only the first step of the transamidation may give artificially higher activities due to competing acyl donor hydrolysis. In contrast to such assays which report the extent of acylation of the enzyme, detection of fluorescence in the present assay is a consequence of formation of an amide **21** as earlier demonstrated (Figure 3. 6).

As shown in Figure 3.10, the experiment was executed in triplicate. In the first three wells were incubated the lysates of cells that did not express the TGase: the fluorescence remained constant. In the last three wells, the lysates with TGase showed a great decrease in fluorescence.

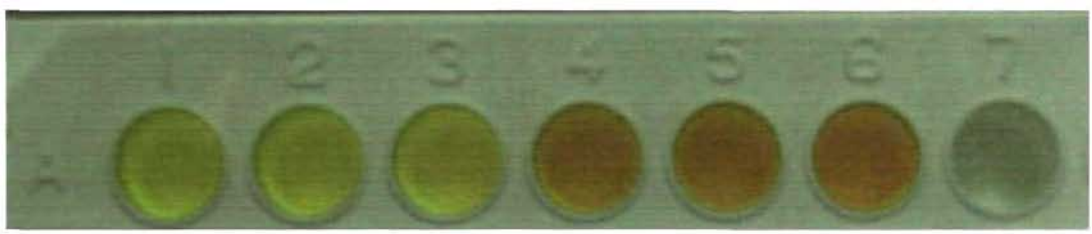
Figure 3.10: Continuous FRET assay using crude bacterial lysate



The assay was shown to be reproducible and sensitive for the detection of the TGase activity (Figure 3.10).

The spectral change caused by the FRET effect was visible to the naked eye (Figure 3.11). The yellow color that was observed in the absence of TGase changed to orange in presence of TGase.

Figure 3.11 Sensitivity of the FRET assay: (**A1-3**: 10 μL of 0.6 mM of acyl donor **11a**, 10 μL of 0.6 mM of acyl donor **20**, 80 μL Buffer, 100 μL H_2O; **A4-6**: 10 μL of 0.6 mM of acyl donor **11a**, 10 μL of 0.6 mM of acyl donor **20**, 80 μL Buffer, 97.5 μL H_2O, 2.5 μl lysate).



3.7 Conclusion

A FRET assay has been developed for monitoring TGase activity. The reproducibility, sensitivity in a series of control experiments and efficiency of this continuous assay were demonstrated. Moreover, the assay proved sensitive for detecting TGase activity in lysate. This method offers potential for high-throughput screening of TGase mutants for new activities.

3.8 Experimental section

Synthesis of the FRET donor substrate

Synthesis of 7-hydroxy-coumarin-3-carboxylic acid **(18)**

A mixture of 2,4-dihydroxyl benzaldehyde (2g, 20 mmol), Meldrum's acid (2.89g, 20 mmol), piperidinium acetate (58 mg, 0.4 mmol) and ethanol (10 mL) was stirred at room temperature for 20 min, and then heated at reflux for 2 h. The reaction mixture was allowed to cool down to room temperature, then chilled in an ice bath for 1h. The crystallized product was filtered, washed three times with ethanol, and dried in vacuo yielding **18** (3.4 g, 83%) as a brown powder; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.66 (s, 1H), 7.73 (d, 1H, *J*= 8.5), 6.84 (d, 1H, *J*= 8.4), 6.73 (s, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.3, 164.1, 157.7, 157.1, 149.3, 132.1, 114.1, 112.7, 110.7, 101.9.

(5-Amino-pentyl)-carbamic acid tert-butyl ester **(14a)**.

A solution of di-*tert*-butyl dicarbonate (2.13 g, 9.8 mmol) in 40 mL of 9:1 dioxane/water was added over a period of 4 h to a solution of 1,5-diaminopentane (2 g, 19.6 mmol) in 60 mL of 9:1 dioxane/water. The solution was stirred at room temperature overnight and concentrated under rotory evaporated. The residue was taken up in 40 mL of water. The white precipitate corresponding to the *N,N'*-di-Boc-1,5-diaminopentane was removed by filtration through a fritted glass funnel, and the filtrate was extracted with CH_2Cl_2 (4×30 mL). The combined organic extracts were concentrated. The product was purified by silica gel chromatography (90:10 CH_2Cl_2/methanol). Evaporation of the collected fractions provided *N*-(Boc)-1,5-diaminopentane **14a** (1.4 g, 6.95 mmol, 71%) as a dense liquid: bp 97 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.63 (s, 1H), 3.1 (dd, 2H, *J* = 6.0, *J* = 12.4), 2.68 (t,

2H, J = 6.7), 1.75 (s, 3H), 1.49-1.52 (br, 3H), 1.42 (S, 9H), 1.27-1.36 (br, 2H). ^{13}C NMR ($CDCl_3$, 300 MHz) δ 156.85, 79.86, 42.77, 41.29, 33.99, 30.72, 29.23, 24.86. HRMS m/z ($M+H^+$): calcd for $C_{10}H_{22}N_2O_4$ 203.17540. found 203.1761.

Synthesis of {5-[(7-hydroxy-2-oxo-2H-chromene-3-carbonyl)-amino]-pentyl}-carbamic acid tert-butyl ester **(19).**

A solution of acid **18** (30 mg, 0.15 mmol) in 3 mL of DMF was treated with amine **14a** (31 mg, 0.15 mmol), HOAt (21 mg, 0.15 mmol), EDC•HCl (29 mg, 0.15 mmol) and DIEA (0.027 mL, 0.15 mmol) and stirred at room temperature for 4 h, when TLC showed the disappearance of the starting amine (Rf = 0, solvent: 5% MeOH/95% AcOEt) and appearance of a new product (Rf = 0.2, solvent: 5% MeOH/95% AcOEt). The volatiles were removed by evaporation. The residue was treated with 5 mL of MeOH, when a white precipitate was formed. The precipitate was recovered by filtration onto a fritted funnel, washed with 3× 2 mL of MeOH, and dried under vacuum overnight yielding amide **19** (50.1 mg, 87%) as white solid: mp 176.3 °C; ^{1}H NMR (DMSO d_6, 400 MHz) δ δ 11.16 (s, 1H), 8.77 (s, 1H), 8.61 (t, 1H, J= 5.6), 7.81 (d, 1H, J= 8.6), 6.88 (dd, 1H, J=8.6, J=2.2), 6.8 (d, 1H, J=2.1), 6.73 (br, 1H), 3.18 (d, 1H, J= 4.8), 2.91 (q, 2H, J= 12.8, J= 6.6), 1.51 (m, 2H), 1.36 (s, 9H), 1.28 (br, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.9, 162.7, 162.4, 157.5, 156.9, 149.2, 133.2, 115.6, 115.1, 112.4, 103.1, 78.6, 30.5, 30. 1, 29.5, 25.0. HRMS m/z ($M+Na^+$): calcd for $C_{20}H_{26}N_2O_6$ 413.1683. found 413.1695.

7-Hydroxy-2-oxo-2H-chromene-3-carboxylic acid (5-amino-pentyl)-amide **(20).**

A solution of carbamate **19** (20 mg, 0.06 mmol) in a mixture of TFA/DCM (1mL/1mL) was stirred for 2 hours. The volatiles were removed under vacuum. A salt exchange was performed dissolving the residue twice in 2 mL of 1M HCl and freeze-drying. Amine hydrochloride **20** was isolated as a yellow gel in quantitative yield (19.26 mg); λ_{ex}= 400 nm, λ_{em}= 448 nm. ^{1}H NMR (DMSO d_6, 400 MHz) δ 11.16 (s, 1H), 8.77 (br, 1H), 8.61 (t, 1H, J= 5.6), 7.81 (d, 1H, J= 8.6), 6.88 (dd, 1H, J=8.6, J=2.2), 6.8 (d, 1H, J=2.1), 6.73 (br, 1H), 3.18 (d, 1H, J= 4.8), 2.91 (q, 2H, J= 12.8, J= 6.6), 1.51 (m, 2H). HRMS m/z ($M+H^+$): calcd for $C_{15}H_{19}N_2O_4$ 291.1341. found 291.1339.

References

64- 10th Spiers Memorial Lecture. Transfer mechanisms of electronic excitation": T. Forster, Discuss. *Faraday Soc.* **1959**, *27*, 7.

65- K. Kikuchi, H. Takakusa, T. Nagano, Recent advances in the design of small molecole-based FRET sensors for cell biology. *Trends Anal. Chem.* **2004**, *23*, 407-415.

66- K.E. Sapsford, L. Berti, I.L. Medintz, Materials for fluorescence resonance energy transfer analysis: beyond traditional donor-acceptor combinations. *Angew. Chem. Int. Ed.* **2006**, *45*, 4562-4588.

67- T. Foster. *Ann. Phys.* **1948**, *2*, 55.

68- A.K. Konstantinidis, P.L. Richardson. K.A. Kurtz, R. Tripathi, C.-M. Chen, P. Huang, J. Randolph, D. Towne, J. Donnelly, U. Warrior, T. Middleton, W.M. Kati, Longer wavelength fluorescence resonance energy transfer depsipeptide substrates for hepatitis C virus NS3 protease. *Anal. Biochem.* **2007**, *368*, 156-167.

69- Y. Kawanischi, K. Kikuchi, H. Takakusa, S. Mizukami, Y. Urano, T. Higuchi, T. Nagano, Design and synthesis of intramolecular resonance-energy transfer probes for use in ratiometric measurements in aqueous solution. *Angew. Chem. Int. Ed.* **2000**, *39*, 3438-3440.

70- T. Mitsui, H. Nakano, K. Yamana, Coumarin–fluorescein pair as a new donor–acceptor set for fluorescence energy transfer study of DNA. *Tetrahedron Lett.* **2000**, *41*, 2605–2608.

71- T. Komatsu, K. Kikuchi, H. Takakusa, K. Hanaoka, T. Ueno, M. Kamiya, Y. Urano, T. Nagano, Design and synthesis of an enzyme activity-based labeling

molecole with fluorescence spectral change. *J. Am. Chem. Soc.* **2006**, *128*, 15946-15947.

72- A. Song, X. Wang, K.S. Lam, A convenient synthesis of coumarin-3-carboxylic acids via Knoevenagel condensation of Meldrum's acid with *ortho*-hydroxyaryl aldehydes or ketones. *Tetrahedron Lett.* **2003**, *44*, 1755-1758.

Chapter 4

Concluding Statements and Future Work

4.1. Conclusion

In order to develop a new library of mutant transglutaminases, which could be efficient biocatalysts for peptide coupling reactions, a series of cycles of enzyme mutations should be performed using different methods, such as PCR or gene shuffling. This necessitates the development of the appropriate enzyme expression methods for the mutants and the development of efficient methods for screening mutants for desired activity.

Our project aim was to develop new methods for screening overexpressed TGase enzyme. For this goal two fluorometric methods have been developed for detecting activity of purified TGase as well as TGase in biological media. The first method was based on the great affinity of biotin to streptavidin or avidin protein. TGase catalyzes the reaction of a biotinylated amine as the γ-glutamyl acceptor substrate and a short peptide - QLPF - labeled with a fluorescent moiety as the γ-glutamyl donor substrate. After the reaction, the conjugate was fixed on streptavidin-coated beads and excess unreacted substrate was washed away, allowing quantitation of the activity by fluorescence measurement. This method was used to detect the activity of as little as 8.25 μU of purified enzyme, and was demonstrated to be applicable for detection of the activity from crude cellular lysates. This assay exhibits remarkable sensitivity, and may tolerate modifications of the fluorophore and biotin labeled substrates for potential screening to identify TGase mutants that recognize unnatural substrates.

The second method was based on the fluorescence resonance energy transfer, (FRET) properties of a selected pair of fluorophores.

Donor and acceptor substrates labelled respectively with the chromophores Tokyo Green and 7-hydroxy coumarin, were used to detect TGase-catalyzed formation of an amide bond between these two substrates, by an observable FRET effect. On excitement of the system at 405 nm, fluorescence at 460 nm for the 7-hydroxy coumarin residue decreases with a concurrent increase in fluorescence at 515 nm for the emission of the Tokyo Green chromophore. On monitoring the decrease of fluorescence of the coumarin chromaphore (λ_{exc} 405 nm, λ_{em} 460 nm), the activity of 4 mU purified enzyme could be measured . Moreover, this FRET-based assay has been used to examine TGase activity in the lysate of *E. coli* expressing the enzyme in a 96-well microtiter plate format, and may thus be suitable for high-throughput screening.

4.2 Comparison of Methods

The developed methods each contain different sensitivity, reproducibility and applications for high-throughput screening of enzyme activity.

❖ Sensitivity

The biotin-based method can detect a minimum enzyme activity of 8.25 μU of purified enzyme. The FRET assay gives a sensitivity of 4 mM. Thus, the biotin method is much more sensitive than the FRET method.

❖ Reproducibility

In all cases, experiments were repeated in triplicates, and both methods indicated a very good reproducibility making theses methods applicable for high-throughput screening.

❖ Application for high-throughput screening

An important criteria for method development in high-throughput screening is the number of assays conducted in the same day. The biotinylated method may be more sensitive than the FRET method; however, it is a discontinuous assay, making it difficult to calculate kinetics, and is also time consuming due to the multiple washing steps. The FRET assay is a continuous method and thus better suited for kinetic analyses, as well as for rapid screening.

Numerically, a 96-well plate could be tested in the same day in the case of the bionylated assay. For the FRET assay, the 96-well plate could be tested in one hour. Moreover, this assay is valid for calculating kinetic parameters for both donor and acceptor substrates. Thus, the FRET method is a promising method for screening enzyme mutants for desired activities against a specific target.

4.3 Future Work

This work has been performed as a collaborative effort between members of the Lubell, Keillor and Pelletier groups in the Department of Chemistry at l'Universite de Montreal aimed at developing new mutant enzymes for the biocatalysis of peptide coupling reactions. Roberto Chica from the Keillor and Pelletier group has mutated transglutaminase by different biological techniques creating a promising library of enzyme mutants for the desired activity.

Future work would involve demonstrating that those developed methods may tolerate modifications of the fluorophores and biotin labeled substrates for potential screening to identify TGase mutants that recognize unnatural substrates. The modification of the amino acid residue Gln with Asn within the acyl donor substrate (from QLPF to NLPF) will be the

next step to test the degree of tolerance of the library towards this important modification. In fact, since Asn has one less carbon in the side chain, which is a relatively minor modification, the aim is to select the mutants that could achieve the transpeptidation between the asparagine residue and the primary amine. Selected TGase mutants will also be tested with a variety of donor and acceptor substrates in order to determine their specificity. Assay optimization will go hand in hand with the development of TGase mutants with alternative transamidation specificity.

Annex

in-Pharma Technologist.com
Breaking News on Pharmaceutical Technology

Peptide market finally ripening?

13-Jan-2005 - **Peptide drugs look like they may finally fulfil their promise, after years of what has largely been disappointment, if figures released in a new market research report prove accurate.**

Although peptide drugs have been on the market for decades - insulin being the most prominent example - it was not until 10 to 15 years ago that the pharmaceutical industry really started to work seriously on the development of a new generation of peptide-based therapeutics, prompted by advances in the understanding of the genetics of disease. But initial enthusiasm was soon dampened by the realisation that drug delivery technologies at that time were not up to the task of getting these relatively large compounds into the body effectively, while production could be complex and expensive.

Now, with drug delivery seeing enormous strides forward, the stage seems to be set for a renaissance in peptide drugs.

A just-published report by Frost & Sullivan notes that the global therapeutic peptides market is currently valued at around $1 billion (€756m), with Europe contributing about $300m of that total, and will at around 10.5 per cent a year between 2003 and 2010. This means that the European market for peptides will double in size by 2010 to $605m.

"The rising need for new therapeutic approaches combined with the potential of peptides as active pharmaceutical ingredients (APIs) for effective drug formulation is contributing to rapid market development," says the report. But it cautions that manufacturers will need to show they can scale-up their production process to meet demand.

One driver behind the resurgent interest in peptides has been the development of large synthetic and biological peptide libraries that, in combination with high-throughput screening processes, has enhanced the prospects of obtaining new drug candidates, according to the author of the report, F&S research analyst Himanshu Parmar.

The approval of new peptide-based drug products such as Roche's HIV treatment Fuzeon (enfuvirtide) is stirring interest among many pharmaceutical companies. Globally, more than 40 peptide-based products are commercially available with six in the registration process. In Europe, about four to six peptide based products are in the market, with 100 in the clinical stage and 150 in advanced preclinical phases.

Peptides used in drug formulation and clinical trials account for nearly 93 per cent of the European therapeutic peptides market with R&D comprising the remainder. Almost 60 per cent of the market comprises peptide-based therapeutics for the oncology segment followed by cardiovascular, infection and metabolic therapeutics.

Despite the significant progress that has been made, technological challenges relating to the effective delivery of peptides, their instability *in vivo* and short half-life remain critical challenges. Costly and inefficient large-scale manufacturing and purification processes are, moreover, hampering market growth; Fuzeon itself has a massive 106 separate synthesis steps in its production, which has contributed to the hefty price tag for the drug.

The F&S report notes that priorities in research are to optimise peptide delivery inside cells, tissue organs or body, develop synthetic peptides with increased stability and half life and realising peptidomimetic molecules that duplicate the structural and functional properties of biologically active peptides but are smaller and easier to work with.

Meanwhile, it suggests that the development of cost-competitive, bulk manufacturing strategies are the key to sustained market expansion. *"Modern and sophisticated formulation techniques, efficient and cost-effective scale up process and cutting-edge purification and separation methods with low cost and high throughput are fundamental to achieving high quality, economical products for commercial purposes,"* said Parmar.

Bachem is currently the leading manufacturer in the European therapeutic peptides market followed by UCB, PolyPeptide Laboratories, Peptisyntha and Diosynth. But other manufacturers are getting into the marketplace, including large chemical players like Clariant which recently started offering peptide manufacturing through an alliance with Indian company Jupiter Bioscience.

But escalating price sensitivity, competition and eroding prices are confronting all European manufactures. And as in the wider API sector, intensifying competition from low-cost Asian manufacturers by 2006-2007 is expected to trigger further price attrition in the peptide category.

"While manufacturers unable to compete on pricing will not be successful, they will also need to focus on competitive criteria such as quality, flexibility, reliability and implementation timeline," warned Parmar. *"Additional strategies could include investing in process improvements, building manufacturing plants in low-cost areas, improving product and service portfolios to build customer loyalty and entering into strategic alliances with manufacturers and pharmaceutical companies."*