EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF TRANSGLUTAMINASE FROM THE LIVER OF BLUEFISH (*Pomatomus saltatrix*)

BY

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ABSTRACT

Transglutaminases (EC 2.3.2.13) are a group of thiol enzymes that catalyse acyl-transfer reaction in which the γ-carboxyamide groups of peptide-bound glutaminyl residues are the acyl donors. They cause post-translational modification of proteins mainly by protein to protein cross-linking, but also through acyl transfer reaction and deamidation of glutamine residues.

Crude liver and flesh extracts of bluefish (*Pomatomus saltatrix*) were investigated to ascertain the effects of storage time and temperature on the stability and activity of transglutaminase (TGase). TGase activity was measured and the enzyme was subsequently characterized using CBZ-L-glutaminylglycine and hydroxylamine as substrates. Frozen bluefish liver and flesh extracts had higher specific activities (0.321 Units and 0.230 Units respectively) in comparison to refrigerated liver and flesh extracts (0.124 Units and 0.071 Units respectively) at the end of a 30 day storage period with the frozen liver extract retaining the highest stability. The optimum temperature for the crude bluefish liver TGase reaction with CBZ-L-glutaminylglycine and hydroxylamine was between 40°C and 45 °C. The enzyme was stable at temperatures below 55°C, beyond which it lost activity progressively. The crude enzyme extract was active within the pH range of 6.0-7.5, with an optimum pH of 7.0, and was stable from pH 6.5-8.0.

TGase was partially purified from the frozen liver extract of bluefish by gel filtration on Sephacryl S-200 HR. The partially purified extract was further characterized with respect to its response to temperature and pH. The effects of sodium as well as calcium chloride and other divalent cations, and the inhibitory effects of various chemicals on the activity of the partially purified TGase were also investigated. The partially purified bluefish TGase had an optimum temperature of 40°C via the reaction with CBZ-L-glutaminylglycine and hydroxylamine. The enzyme was observed to be stable at temperatures below 50°C and approximately 90% of the initial TGase activity was retained at the end of a 30 min incubation period. The partially purified bluefish TGase had a pH optimum of pH 7.5 and was stable within a narrow pH range of 7.0 - 8.0.

The partially purified enzyme showed requirement for calcium (Ca^{2+}) ions for activity and no activity was observed in the absence of Ca^{2+}. The replacement of Ca^{2+} by other divalent

cations such as Mg^{2+}, Mn^{2+}, Ba^{2+}, Zn^{2+} and Fe^{2+} produced various levels of activity with the enzyme, albeit less than that achieved with Ca^{2+}. Increasing NaCl concentrations, 0 - 15mM, did not seem to have an enhancing effect on the activity of partially purified bluefish TGase. TGase was inhibited by sulfhydryl alkylating agents (monoiodoacetic acid (IAA) and N'-ethylmaleimide (NEM)).

RÉSUMÉ

Les transglutaminases (CE 2.3.2.13) sont un groupe d'enzymes thiol qui catalysent la réaction du transfert d'acyl, dans laquelle les groupes γ-carboxyamide des residus glutaminyl reliés aux peptides sont les donateurs d'acyl. Ils provoquent la modification post-translationnelle des protéines, surtout par la trans-liaison d'une protéine à une autre protéine, mais aussi par reaction avec le transfert d'acyl et la deamidation des résidus de glutamine.

Le foie et les extraits de chair du poisson bleu (Pomatomus saltatrix) ont été testés pour vérifier les effets de la durée et la temperature de stockage sur la stabilité et l'activité de l'enzyme transglutaminase (TGase). L'activité de TGase a été mesurée et l'enzyme a été par la suite caractérisée en utilisant le CBZ-L-glutaminylglycine et l'hydroxylamine comme substrats. Le foie et les extraits de chair congelés du poisson bleu avaient les plus hautes activités spécifiques (0.321 Unités et 0.230 Unités respectivement) en comparaison avec le foie et les extraits de chair frigorifiés (0.124 Unités et 0.071 Unités respectivement) à la fin d'une période d'entreposage de 30 jours, avec l'extrait de foie congelé retenant la plus haute stabilité. La température optimale pour la reaction du TGase de l'extrait brut du foie du poisson bleu avec le CBZ-L-glutaminylglycine et l'hydroxylamine était entre 40 et 45 °C. L'enzyme était stable aux températures en dessous de 55°C, au-delà duquel l'activité est reduite progressivement. L'extrait d'enzyme brut était actif entre le pH 6.0 à 7.5, avec un pH optimum de 7.0 et était stable de pH 6.5 à 8.0.

La TGase a été partiellement purifiée de l'extrait de foie congelé du poisson bleu, par filtration a l'aide du gel Sephacryl S-200 HR. L'extrait partiellement purifié a été davantage caractérisé en ce qui concerne l'effet de la température et du pH. Les effets de sodium aussi bien que de chlorure de calcium et d'autres cations divalent, et les effets inhibiteurs de différents produits chimiques, sur l'activité du TGase partiellement purifiée ont aussi été enquêtés. La TGase partiellement purifiée du poisson bleu avait une température optimale de 40°C en utilisant le CBZ-L-glutaminylglycine et l'hydroxylamine comme substrats pour la caractérisation. On a noté que l'enzyme été stable aux températures en-dessous de 50°C et environ 90 % de l'activité initiale de TGase a été retenu à la fin de la période d'incubation de 30 minutes. La TGase du poisson bleu avait un pH optimum de 7.5 et était stable à une étroite gamme de pH, de 7.0 - 8.0.

L'enzyme a montré l'exigence pour les ions de calcium (Ca^{2+}) pour l'activité et aucune activité ont été observés en l'absence de Ca^{2+}. Le remplacement de Ca^{2+} par d'autres cations divalent, comme le Mg^{2+}, Mn^{2+}, Ba^{2+}, Zn^{2+} et Fe^{2+} a produit différents niveaux d'activité avec l'enzyme, mais bien moins que le niveau d'activite noté avec les ions de calcium (Ca^{2+}). L'augmentation de la concentration de NaCl, de 0 a 15 mM, semble avoir aucun effet améliorant sur l'activité de la TGase partiellement purifiee du poisson bleu. La TGase a été inhibé par les agents alkylation sulfhydrylique (acide monoiodoacétique) et N'-ethylmaleimide.

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LIST OF ABBREVIATIONS/NOMENCLATURE

TGase	Transglutaminase(s)
EC	Enzyme Commission
GL	ε-(γ-glutamyl) lysine cross-links
CD	Celiac disease
MDC	Monodansylcadaverine
TCA	Trichloroacetic acid
CBZ	N-carbobenzoxy
nm	Nano meter
mM	Millimole
µmol	Micromole
mL	Milliliter
kDA	Kilo Dalton
U	Units of enzyme activity
°C	Degree celsius
pH	Potential of hydrogen (the logarithm of the reciprocal of hydrogen- ion concentration in gram atoms per liter)
s,min,h,d	second, minute, hour, day
BSA	Bovine serum albumin
Tris	Tris (hydroxymethyl) aminomethane
HCl	Hydrochloric acid
Ca^{2+}	Calcium ions

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate
NEM	N-Ethylmaleimide
IAA	Monoiodoacetic acid
$(NH_4)_2SO_4$	Ammonium sulfate
MW	Molecular weight
MWCO	Molecular weight cut-off
PAGE	Polyacrylamide gel electrophoresis
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
R_f	Relative mobility of proteins

CHAPTER I

GENERAL INTRODUCTION

Transglutaminases [TGases, EC 2.3.2.13] are a family of enzymes that catalyze cross-linkages between protein molecules by formation of covalent bonds between γ-carboxyamide group of glutamine and the ε-amine group of lysine. They can modify proteins by means of amine incorporation, cross-linking and deamidation. TGases are widely distributed in nature and have been found in various animal tissues, microrganisms, plants, invertebrates, amphibians, fish and birds. The ability of TGase to modify protein functionality by covalent cross-linking has generated enormous interest.

In protein-containing food systems, the cross-linking reaction proceeds prior to the other reactions. The transglutaminase mediated cross-linkage of protein units causes various physical changes in food proteins, and it is generally believed that endogenous TGase plays an important role in spontaneous gelation of surimi pastes at low temperature, thus resulting in increased "set" gel strength upon cooking (Seki *et al.*, 1990; Yongsawatdigul *et al.*, 2002). This discovery has led to further studies of the content and nature of endogenous TGase in seafood as well as the evaluation of TGases from other sources in seafood application as a processing aid for quality improvement and product development (Ashie & Lanier, 2000). Recently, TGases have captured peoples' interest due to their attractive potential application in immobilization of enzymes and textile industries.

In comparison to mammalian enzymes, which have been extensively studied and applied in food processing and food production, studies and application of marine enzymes are still in their infancy. The aquatic environment contains a wide variety of genetic material thus representing an enormous potential for discovering different enzymes (Raa, 1990). Serving as important food sources, fish and shellfish comprise the largest number of species with the widest biological diversity. However, each year, large quantities of fish processing waste are produced worldwide. Processing discards from fisheries [in particular the viscera, head, bones and frame, stick-water and effluent from

fish processing] in the 1992-2001 period, are estimated to be 7.3 million tones of the total weight of the catch and these are generally dumped in-land or hauled into the ocean (Kelleher, 2005). Approximately 30% of total landings may be considered as underutilized, bycatch, unconventional, or unexploited (Venugopal & Shahidi, 1995). These discards from fish processing along with fish bycatch pose disposal issues.

Fish processing discards as well as underutilized species are an important source of useful biochemicals such as enzymes (Wilke *et al.*, 1986). In recent years, recovery and characterization of enzymes from fish and aquatic invertebrates have taken place and this has led to the emergence of some interesting new applications of these enzymes in food processing (Shahidi & Kamil, 2001). The potential advantages of enzymes from fish and aquatic invertebrates, as compared to mechanical or chemical methods, include the development of gentle enzymatic methods as alternatives to mechanical or chemical treatments which may often damage the product and reduce product recovery (Gildberg, 1993). There is also no need to perform toxicological testing of the raw materials in order to demonstrate their safety because they are obtained from edible animal tissues (Wasserman, 1984). Some of these enzymes have high catalytic efficiency at low temperatures due to the unique habitat of the fish. This is significant because processing at low temperatures protects heat labile essential components, reduces energy costs and minimizes microbial growth and proliferation (Haard, 1998; Simpson & Haard, 1987a). Furthermore, extraction of enzymes from fish and shellfish processing wastes and their utilization in the food industry may contribute significantly to reducing local pollutions issues (Raa, 1997). However the seafood industry is beset with its own predicaments; the seasonal nature of the source material, the "fishy" odour associated with the offal, among others, has resulted in relatively fewer studies and thus posing challenges for utilization of seafood processing wastes.

The search for new enzymes with novel properties or which displays better characteristics than the ones documented is a growing trend and holds a pivotal position in the future of enzyme technology. The prospective biotechnological applications of these versatile fish transglutaminases are enormous thus making it necessary to fully exploit methods in order to enhance the value of seafood processing discards.

The overall rational for this study was to discover new sources of TGases for potential use to meet the growing demands for enzymes in commercial applications. In this context, TGases from the liver and muscle of bluefish (*Pomatomus saltatrix*) were selected for preliminary stability studies, purification, and characterization. The specific objectives were:-

1. To determine the optimum source of TGase by conducting preliminary stability studies and characterization on crude bluefish liver and muscle (*Pomatomus saltatrix*) TGase
2. To extract and partially purify TGase from the liver of bluefish (*Pomatomus saltatrix*)
3. To characterize the TGase fraction from the liver of bluefish (*Pomatomus saltatrix*) with respect to its response to pH and temperature, in addition to its response to calcium and sodium chloride, as well as other divalent cations and inhibitors.

CHAPTER II

LITERATURE REVIEW

2.1. TRANSGLUTAMINASES (EC 2.3.2.13)

2.1.1. Definition and Classification of Transglutaminase

Transglutaminases (TGase) belong to the group of enzymes known as transferases. They catalyze the acyl-transfer reaction in which γ-carboxyamide groups of peptide-bound glutamine residues serve as acyl donors (Folk, 1980). Transglutaminases are a widely distributed group of enzymes that have been classified and assigned a systematic name R- glutamine-peptide: amine γ-glutamyl-transferase (EC 2.3.2.13) in the Enzyme Commision Nomenclature System (Aboumahmoud & Savello, 1990; Zhu *et al.*, 1995). Due to similarities in the catalytic triad and reaction mechanism, TGases (EC 2.3.2.13), papain (EC 3.4.22.2) and papain-like cysteine proteases are classified within the same superfamily in the Structural Classification of Proteins (SCOP) database (Fesus & Piacentini, 2002).

2.1.2. Historical Background

Transglutaminase, which forms part of a large family of protein-remodelling enzymes was first disovered by Heinrich Waelsch and his colleagues more than 40 years ago as a liver enzyme; incorporating amines into proteins (Fesus & Piacentini, 2002). In 1959 Clarke *et al.*, introduced the term transglutaminase (TGase) to describe the transamidating activity observed in guinea-pig liver. Later studies carried out by Pisano *et al.*, (1969) demonstrated that transamidation is brought about by enzymes which cross-link proteins through an acyl-transfer reaction.

Since TGases are widely distributed in various living organisms, they fulfill a great variety of biological functions. Initial research on the applications of TGases started with the isolation of enzymes from mammalian tissues and body fluids (Folk & Cole, 1966; Ikura *et al.*, 1985b; Jiang & Lee, 1992) and until the late 1980's, guinea pig liver

was the only commercially available source of TGases (Jaros *et al.*, 2006). TGases derived from guinea pig liver or bovine plasma showed the possibility to modify the functional properties in milk caseins and soybean globulins in the early 1980s (Motoki & Seguro, 1998). However due to its scarce source and the laborious purification procedure, the use of TGases as texture enhancer in foods resulted in a low attractiveness for potential industrial applications (Zhu *et al.*, 1995, 1999). Furthermore, guinea pig TGases required calcium for activation thus leading to protein precipitation in some food systems containing casein, soybean globulin or myosin (Seguro *et al.*, 1996b).

Due to the difficulties encountered with mammalian TGases, efforts were made to obtain TGases by genetic manipulation of microorganisms such as *Escherichia coli.* However none of these enzymes have been commercialized due to the lack of public acceptability for their use as food additives (e.g., texture enhancement in particular food systems) (Motoki & Kumazawa, 2000; Yokoyama *et al.*, 2004).

Recently, efforts have been made to obtain TGases from microorganisms. In 1989, TGase was isolated from *Streptoverticillium* S-8112 by Ando *et al.* There are several reasons why microbial TGases have gained commercial applicability; (1) since bacteria excrete the enzyme into the cultural broth, cell disruption is not necessary and subsequently the purification of TGase proves to be easier (Seguro *et al.* 1996b; Motoki & Kumazawa 2000; Yokoyama *et al.* 2004); (2) unlike mammalian TGases, microbial TGases are calcium independent (Ando *et al.*, 1989), and show lower substrate specificity compared to mammalian TGases (de Jong *et al.,* 2001; de Jong & Koppelman, 2002; Shimba *et al.*, 2002). Because of these advantages and the possibility of low-cost mass production by traditional fermentation technology, microbial TGases is now widely spread as a functional enzyme within various branches of the food industry.

2.1.3. Transglutaminase Catalyzed Reactions

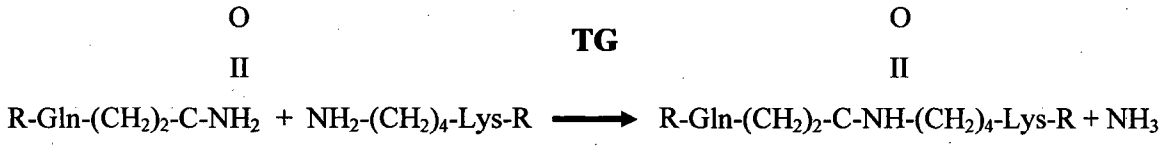
Transglutaminases are Ca^{2+} dependent enzymes that catalyze acyl transfer reaction between a free amine group (e.g. protein or peptide-bound lysine) and the γ-carboxyamide group of proteins or peptide bound glutamine thus leading to the modification of proteins.

These enzymes display a high degree of specificity with regard to the amine donor substrate and also in the selection of lysine side chains in proteins with which they react. They do not react with the free amino group of glutamine; but rather target the γ-carbonylamide function in the side chain of glutamine residues in protein substrates. TGases seem to react best with glutamine (donor) residues in unstructured flexible regions of proteins, often in the N and C terminal domains, but always in endo-positions (Lorand & Stenberg, 1976). However, they show poor specificity for the acyl- acceptor amine group, which can be either the ε- amino group of peptidyl lysine or a primary amine (Folk, 1980).

When an ε- amino group of peptide bound lysine acts as a acyl acceptor, isopeptide bonds are formed between the glutamine and lysine residues in proteins, thus introducing both inter- and intramolecular covalent cross-links, resulting in the polymerization of proteins (Folk, 1980; Ichinose *et al.*, 1990; Lorand & Conrad, 1984). Cross-linking of proteins resulting in the formation of high molecular weight polymers seems to be the most dominant reaction in nature for TGases. Two other important reactions catalyzed by TGases are acyl transfer reactions and deamidation (Fig. 1).

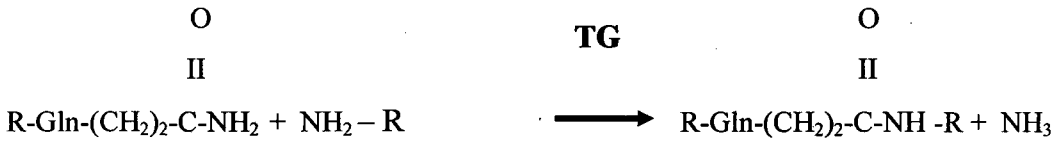
Acyl transfer reactions are catalyzed by TGases between the γ-carboxyamide groups of peptide-bound glutamine (acyl donors) and a variety of primary amines (acyl acceptors). In the absence of primary amines in the reaction system, water becomes the acyl acceptor and the γ-carboxamide groups of glutamine residues are deaminated, becoming glutamic acid residues and ammonia (Zhu *et al.*, 1995). All three, cross-linking, acyl transfer and deamidation reactions can be used to modify the functional properties of food proteins.

(A) **Cross-linking**



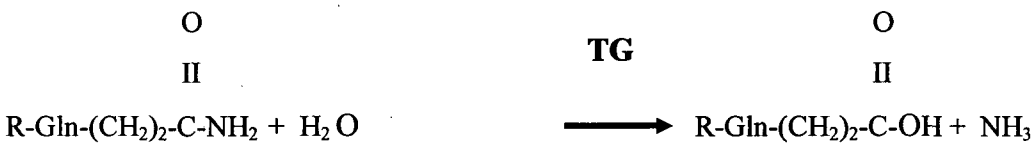
Glutamyl residue Lysyl residue

(B) Amine incorporation



Glutamyl residue Amine

(C) Deamidation



Glutamyl residue

FIGURE 1: REACTIONS CATALYZED BY TRANSGLUTAMINASE

(A) Cross-linking reaction; **(B)** Acyl transfer reaction; **(C)** Deamidation.

(Source: Ozrenk, 2006)

2.1.4. Sources of Transglutaminases

TGases have been purified from various animal tissues or organs, such as the liver and hair follicle of guinea pig, pig plasma and human epidermis, erythrocyte, plasma and placenta, fish, plants and microorganisms as illustrated in Fig. 2 & Table 1.

Until late 1980's, guinea pig liver TGase was the only commercially available TGase (Jaros *et al.*, 2006). However, its scarce source in addition to the laborious purification procedure entailed extremely high prices on the market, resulting in a low attractiveness for potential industrial applications (Zhu *et al.* 1995, 1999).

Recent publications on TGases in the meristemic tissue of etiolated plants such as *Pisum sativum*, sprouts of *Helianthus tuberosus* tubers, alfalfa, seedlings of lupine, chrysanthemum, soybean leaves, and angiosperms show that TGase activity exists in plant tissues as well (Serafini-Fracassini *et al.*, 1995; Kang & Cho, 1996; Tsai *et al.*, 1996). However, separation and purification of transglutaminase from plant and fish tissue are still in their infancy.

Fermentation of transglutaminases derived from microorganisms makes it possible to achieve mass production of transglutaminase from cheap substrates (Zhu *et al.*, 1995).

2.1.5. General Properties of Transglutaminases

TGases are thought to be either a cytosolic enzyme (type II) (Folk & Chung, 1973) or in some organisms, partially bound to membranes (type I) in the lysosomes or mitochondria (Juprelle-Soret *et al.*, 1984). Depending on its source, TGases might exist either as a monomer, dimer or tetramer. For example, TGase from guinea pig liver is a monomer while that isolated form the mold *Physarum polycephalum* is a dimer.

Enzymatic and structural properties of the TGase have been well characterized from mammalian sources (Ikura *et al.*, 1988; Ichinose *et al.*, 1986; Phillips *et al.*, 1990; Nakanishi *et al.*, 1991). The enzymes originating from marine organisms including fish, crustaceans, and echinoderms, for example, red sea bream liver (Yasueda *et al.*, 1994, 1995), carp dorsal muscle (Kishi *et al.*, 1991), limulus hemocyte (Tokunaga *et al.*, 1993), lobster muscle (Myhrman & Bruner-Lorand, 1970), and sea urchin eggs (Cariello *et al.*, 1984) have been reported.

2.1.6. Search for Transglutaminases with Industrial Applications

For industrial applications, large scale production of the enzyme is necessary. There are three general approaches to developing industrially useful TGase from various sources and by different methods as illustrated in Table 2.

The first approach is to extract and purify the enzyme from the tissues or body fluids of food-use animals, such as cattle, swine, and fish (Motoki & Seguro,1998). Factor XIII, a certain type of TGase is extracted commercially from the blood of cattle and swine at slaughter in Europe (Wilson, 1992). However the blood enzyme is rarely utilized in food manufacture, as (1) a specific protease called thrombin is required to activate the enzyme and (2) the red pigmentation is often detrimental to product appearance (Motoki & Seguro, 1998).

The second approach is to obtain the enzyme by means of genetic manipulation using host microorganisms, such as *Escherichia coli.*, *Bacillus*, yeast, and *Aspergillus* (Motoki & Seguro, 1998). Some of these biotechnology researches include guinea pig liver TGase in *E.coli* (Ikura *et al.*, 1988), human factor XIIIa in yeast (Bishop *et al.*, 1990) and fish TGase in *E.coli* (Yasueda *et al.*, 1995). However, none of these TGases has been commercialized due to factors such as food regulations and consumer acceptance (Motoki & Seguro, 1998).

The third approach is to screen for TGase producing microorganisms and mass produce the enzyme by traditional fermentation technology. Screening for enzyme-producing strains was carried out in Nagoya, Japan in collaboration with Amano Pharmaceutical Co., (Motoki & Seguro, 1998). During these screening studies, a variant strain of *Streptoverticillium mobaraense* was found to produce extracellular TGase-like enzymes and was called microbial transglutaminase (Washizu *et al.*, 1994).

2.1.7. Industrial Applications of Transglutaminase

TGase mediated cross-linking of proteins has dramatic effects on their physical and chemical properties. This has triggered the use of these biocatalysts in a wide range of industrial sectors, from cosmetics to the food industry. In addition, and arising from the involvement of this group of enzymes in many physiological and pathological processes, they have found important applications in the pharmaceutical industry

(Arrizubieta, 2007). Their biotechnological potential is best reflected in the rapidly growing number of patent applications regarding this group of enzymes (Griffin *et al.*, 2002).

The first assay to test possible industrial applications of TGases was carried out to modify food proteins using mammalian enzymes (Matheis & Whitaker, 1987). Despite the interest in these enzymes for modulating food rheological properties, limited supply hindered their commercial utilization. The situation changed in 1989 with the purification of the microbial TGase from *Streptomyces mobaraensis* (Ando *et al.*, 1989) which could be produced relatively cheaply by fermentation methods. Subsequently, the application of TGase in industry, and especially in the food industry, began to increase at a steady rate. Sixteen years later, the microbial transglutaminase obtained from *Streptomyces* is still the only commercial source of TGase (Arrizubieta, 2007).

2.1.7.1. Canadian Food Regulations

The use of transglutaminases in food has been approved for use in the United States, Japan and some European countries. In Japan the use of microbial TGase as an innovative ingredient for food processing had increased due to its unique characteristics (Motoki & Kumazawa, 2000). In the United States, transglutaminase has been affirmed as Generally Recognized As Safe (GRAS) by an independent panel of scientific experts.

In Canada, enzymes used in the manufacture of foods are considered food additives and must be listed in Division 16 of the *Food and Drug Regulations* before they are permitted to be used in food products and at the present time, there is no provision in the Regulations for the use of the enzyme transglutaminase in foods marketed in Canada.

A proposal to approve the use of transglutaminase obtained from the microorganism *Streptoverticillium mobaraense* strain S-8112 in unstandardized foods such as prepared fish products, cheese products, cream and processed cheese products, simulated meat products, yogurt and frozen dairy desserts in Canada was issued on June 21, 2003 in the Canada Gazette Part I.

On November 12, 2007 Health Canada amended regulations in order to permit the use of the transglutaminase enzyme obtained from *Streptoverticillium mobaraense* strain S-8112 to enhance the texture of the above listed foods.

2.1.7.2. Transglutaminase Applications in Food Processing

The demand for high-quality food proteins has increased over the past two decades. Particularly regarding consumers' acceptance and preferences, methods for modifying the techno-functional properties of proteins are of increasing interest in order to develop convenience foods and health foods.

Some techno-functional properties of proteins that can be altered by transglutaminases are texture, stability (regarding temperature, syneresis and emulsification), water binding, foaming, viscosity, elasticity and gelation (Dube *et al.*, 2007). Specific applications have been reported for meat, fish (Seguro *et al.*, 1995), dairy (Lorenzen and Schlimme,1998; Jaros *et al.*, 2006), egg (Kato *et al.*, 1991), bread and bakery products (Gerrard *et al.*, 2001), and in soybean processing (Nonaka *et al.,* 1996).

Ajinomoto Co. of Japan were the first to develop and market TGase for food applications under the trade name Activa^{TM}. An overview of the application possibilities for TGase in food processing is illustrated in Table 3.

2.1.7.2.1. Seafood Products

In manufacturing fish gel products such as kamaboko (fish cake) and imitation crab leg meat products from *surimi* (fish paste), several kinds of bonds, such as hydrophobic interactions, disulfide bonds, hydrogen bonds and others are formed (Niwa *et al.*,1993). Endogenous transglutaminases (TGases) in fish flesh may form ε-(γ-glutamyl) lysine (GL) crosslinks in fish proteins (Sato *et al.*, 1992; Kumazawa *et al.*, 1993) thus participating in textural changes of fish sol during processing (Seki et al., 1990). The use of commercial transglutaminases in the food industry started with the manufacturing of *surimi* products in Japan.

Sakamoto *et al.*, (1995) investigated the influence of added microbial TGase in surimi manufacture and relationship between GL crosslink formation and the strength of the surimi gels. The result obtained showed that the breaking strength and the amount of cross-linkage increased while the monomer myosin heavy chain decreased correspondingly with the transglutaminase content. Furthermore addition of microbial TGase was effective in increasing gel strength.

However the effect of transglutaminase on surimi gel differs with the type of fish (Soeda *et al.*, 1996). Due to its cross linking properties, care has to be taken to add appropriate amounts in order to avoid excessive cross-linkage (Kuraishi *et al.*, 2001). The texture can be improved by adjusting the amount of transglutaminase and the reaction conditions.

There are other applications for transglutaminase in seafood. (1) It is used to prevent changes in the texture of retorted products and frozen foods by soaking the raw materials in a solution of transglutaminase and caseinate (Kurakata *et al.*, 1995). This effect may be useful for retorted or frozen seafood products, such as shrimps, which must maintain their textural properties. (2) Transglutaminase is also used to treat salted cod roe in Japan; a firm and chewy texture is obtained by soaking the roe overnight in a curing solution containing transglutaminase.

2.1.7.2.2. Meat Products

Restructuring fresh meat is one of the transglutaminase applications that already exists and is applied on an industrial scale (de Jong & Koppelman, 2002). Structural meat proteins like myosin and actin can be crosslinked by TGases (Kahn & Cohen, 1981).

TGase can be used in combination with fibrinogen and thrombin in producing restructured meat below 10°C with an overnight reaction time (Wijngaards & Paardekooper, 1988). An alternative approach for restructuring of fresh meat is the use of a combination of bacterial transglutaminase and casein (Kuraishi *et al.*, 1997). Binding is achieved by cross-linking of the substrate protein gel (fibrin or casein) resulting in the formation of a protein gel between the meat parts and cross-linking of this gel with proteins of the meat particles surface (de Jong & Koppelman, 2002). In this way, small pieces of meat are bound together to larger pieces with shapes that can be adjusted. The advantage of both the enzymatic procedures is that the bite, taste, and flavor of the meat are retained (de Jong & Koppelman, 2002).

The gel-forming capability of meat proteins after addition of transglutaminase is used extensively in the preparation of sausages and ham. The formation of cross-links in meat products like ham and sausage strengthen the protein network to prevent shattering during slicing (Kuraishi *et al.*, 1998).

Transglutaminases can also be used in meat products to partially mimic the effects of salts that are added to improve the water holding capacity, binding, consistency, and overall texture (Kuraishi *et al.*, 2001). Meat pieces, including minced meat, can also be bound together by microbial TGase without sodium chloride and phosphates, resulting in "healthy" meat products (Motoki & Seguro, 1998).

2.1.7.2.3. Wheat Products

2.1.7.2.3.1. Bakery

Gluten proteins in wheat flour are the dominant proteins that characterize dough properties and play an important part in the quality of bread. TGase cross-links gluten proteins thus resulting in the formation of high molecular weight polymers (Larre *et al.*, 2000). The formation of these polymers results in a stronger gluten network and, subsequently, changes the physico-chemical properties and the rheological behavior (Larre *et al.*, 2000).

Application of TGase to dough results in an increased volume, improved structure of the bread (Wijngaards *et al.*, 1997), and improved the crumb strength (Gerrard *et al.*, 2001). The height of puff pastry and croissant volume increased after the addition of the enzyme (Gerrard *et al.*, 2001).

Moore *et al.*, (2006), studied the effect of TGase on the quality and structure of gluten free bread. The results revealed that gluten-free breads tend to be of a better overall quality with the formation of a stable protein network and it is possible to produce such a stable network within a gluten-free bread system with the application of TGase in combination with the correct protein substrate and enzyme addition level (Moore *et al.*, 2006). Protein network formation is imperative in relation to the quality of gluten-free breads. Because gluten is responsible for the good quality visco-elastic structure in wheat bread, mimicking gluten by creating a protein network with TGase and various proteins may in fact improve the quality and structure of gluten-free breads (Moore *et al.*, 2006).

2.1.7.2.3.2. Noodles and Pasta

Transglutaminase is utilized widely in the production of noodles and pasta in Japan (Kuraishi *et al.*, 2001). With the addition of transglutaminase it was noted that the texture

of various noodles like the Chinese noodles, udon (Japanese noodle made from wheat flour), soba (made from buckwheat), and pasta improved (Kuraishi *et al.*, 2001). The cross-linkings introduced by transglutaminase are heat-stable, therefore, the firmness and elasticity of the noodles are retained for longer even after cooking (Kuraishi *et al.*, 2001). Furthermore transglutaminase strengthens the structure of the dough, thus reducing the amount of solid contents released into the boiling water thereby reducing the starch loss, stickiness and bulkiness of the pasta/noodles thus benefiting both the manufactures and consumers (Kuraishi *et al.*, 2001).

Sakamoto *et al.*, (1996) found that microbial transglutaminase treatment of noodles and pasta prevented deterioration in textures after cooking, and improved the firmness and elasticity of the product, even when low-grade flours were used.

2.1.7.2.4. Dairy Products

Publications describing the use of TGases in cross-linking reactions of food proteins have had a strong focus on milk proteins. Casein, the major protein in milk which has no capability of gel formation even upon heating, has been shown to be a very good substrate for transglutaminase, while the globular whey proteins have been shown to be poor substrates (de Jong & Koppelman, 2002). When TGase is added to untreated milk, only the caseins are cross-linked while the whey proteins are left untouched (de Jong & Koppelman, 2002). This property makes it possible to apply TGase in food products containing casein.

One of the major areas of the use of TGase in dairy products is in the production of yogurt. The enzyme is added either prior to the fermentation process or simultaneously with the starter culture (Lorenzen & Schlimme, 1998). The major effects of transglutaminase, when used in yogurt production are an increase in firmness, viscosity and improved water-holding capacity that results in reduced syneresis (Lorenzen *et al.*, 1999). Another type of dairy product where the use of TGase has a great potential is in cheese making. The curd yield can be increased by the cross-linking process (de Jong & Koppelman, 2002).

Experiments have also been carried out on quality improvements in frozen dairy desserts (Miyamaoto & Kanbara, 1994). TGase has been found to improve the quality of

low-fat non sugar ice cream, making it smoother, softer and without the "icy" texture which often damages the quality (Miyamaoto & Kanbara, 1994). Transglutaminase helps stabilize the structure of the ice-cream by inhibiting the growth of ice crystals (Kuraishi *et al.*, 2001).

2.1.7.2.5. Soy Products

Soy proteins are widely used in prepared foods such as sausages, ham, and kamaboko (surimi gel) because the gelation of soy proteins in food processing provides various textural properties (Kuraishi *et al.*, 2001). Tofu is a major soy product in Asia. The use of transglutaminase in tofu manufacturing is another practical example. Transglutaminase treated tofu has an increased water-holding capacity, good consistency, and a silky and firmer texture (Kuraishi *et al.*, 2001). The cross-links formed by transglutaminase are thermostable and many of the physical properties are retained even after retort treatment (Kuraishi *et al.*, 2001). Studies prove that tofu treated with transglutaminase, forms more stable covalent crosslinks, and subsequently is able to hold more water in spite of temperature changes (Nonaka *et al.*, 1996).

2.1.7.2.6. Bioavailability of Cross-linked Proteins

Transglutaminase catalysed reactions can be used to modify the functional properties of food proteins. As mentioned earlier, transglutaminase has been used to catalyse the cross-linking of a number of proteins, such as milk preotin casein, soy proteins, gluten, myosin and actomyosin. It can be further used to modify proteins to improve their nutritional and functional properties (Huang *et al.*, 1994), increase shelf life and reduce allergenicity (Zhu *et al.*, 1995).

The modification of food proteins by transglutaminase may help protect lysine in food proteins from various chemical reactions and produce food proteins of higher nutritive value through cross-linking of different proteins containing complementary limiting essential amino acids (Motoki & Seguro, 1994).

The amino acid composition of plant proteins widely varies due to plant diversity, cultivation area, different function of proteins in the organism (e.g. storage proteins), purification, and process technology (isoelectric precipitation, membrane filtration). Plant proteins frequently lack one or more amino acids that are required for fulfillment of the

FAO/WHO standard (FAO & WHO, 1989) for a well-balanced amino acid composition in human nutrition.

Ikura *et al.*, (1981) investigated the feasibility of the incorporation of amino acids into food proteins through TGase catalysed acyl-transfer between the γ-carboxylamide groups of peptide-bound glutamine residues and primary amino groups. D- and L-lysine and most of the methyl or ethyl esters and amides of L-amino acids are suitable amine substrates. The incorporation of methionine ethyl ester into bovine casein components and soybean proteins using TGase resulted in an increase in the methionine content ranging from 50% to 250% when compared to the starting material (Ikura *et al.*, 1981). Ikura *et al.*, (1981) also successfully incorporated lysine into wheat gluten.

2.1.7.3. Transglutaminases in Health and Disease

2.1.7.3.1. Medical Applications

The interest in these enzymes is further stimulated by their involvement in several pathological processes such as tumour growth and metastasis, arteriosclerosis, neurodegenerative diseases, skin pathology and autoimmune conditions such as celiac disease, cancer, and tissue fibrosis (Griffin *et al.*, 2002). Some of these conditions are alleviated by supplementation with exogenous TGase. But in many cases, related molecules, such as TGase inhibitors or antiTGase-specific antibodies, find application as therapeutic or diagnostic agents.

The first medical application of a TGase was the use of FXIII in blood clotting products to control bleeding during surgery and as a general tissue adhesive. In addition, FXIII has found application in substitutive therapy of congenital or acquired deficiencies in FXIIIA that result in severe bleeding due to lack of fibrin clot stabilization. Tissue transglutaminase, or TGase2, has also received commercial interest as a tissue adhesive and as a cell adhesion protein in medical implants (Collighan *et al.*, 2002).

The effect of transglutaminases on wheat gluten in the treatment of celiac disease (McDevitt & Winkler, 1999) has been examined by Moore *et al.*, (2006). Celiac disease (CD) is a chronic enteropathy caused by the intake of gluten proteins from widely prevalent food sources such as wheat, rye, barley, and possibly oats. The ingestion of gluten induces an inflammatory response resulting in the destruction of the villous

structure of the small intestine (Shan *et al.*, 2002). Currently, the only effective treatment for CD is the strict lifelong renunciation of gluten-containing foods (Moore *et al.*, 2006). Gluten is the major structure-forming protein present in wheat bread and is responsible for the visco-elastic properties. Thus ingredients that have the ability to mimic the properties of gluten need to be used.

The introduction of novel proteins into foods carries risks of eliciting allergic reactions in individuals sensitive to the introduced protein and of sensitizing susceptible individuals. Since proteins are good substrates for TGases Yamauchi *et al.*, (1991) were able to develop a method for reducing the allergenicity of some food proteins and/or peptides. Casein (23 kDa) was treated with transglutaminase at 25°C for 20 h in water to manufacture cross-linked casein (approx. 90 kDa), which was less allergenic.

A material promoting absorption of minerals in the human body was developed by Noguchi *et al.* (1992). It was prepared by deaminating casein by treating it with transglutaminase. The material promotes absorption of minerals in intestine and can be used in the food industry and for medicines, for instance in mineral supplement formulations for adults, children and infants.

Hypertrophic scarring occurs in a significant number of patients following surgery or serious burns. A higher percentage of type III collagen due to elevated levels of TGase activity is associated with these scars (Arrizubieta, 2007). The use of putrescine as a TGase inhibitor has been patented for treatment of the scar tissue (Dolynchuk & Bowness, 1999). This patent led to a topical cream which has been proven to significantly reduce hypertrophic scarring in clinical trials (Arrizubieta, 2007).

Clarification of the role of TGase in these processes is a very active area of research and there is strong evidence that TGase enzymes or their inhibitors offer big potential as therapeutic agents for these processes, and in some cases medical applications have already been patented (Steinert *et al.*, 2000).

2.1.7.3.2. Cosmetic Application

The important roles that TGases play in epidermal tissue differentiation determines that modulation of the activity of these enzymes will affect the structure of the skin and can be used to improve skin appearance (Arrizubieta, 2007). Topical

preparations consisting of TGase and one or more of the corneocyte proteins are proposed to form a protective layer on the surface of hair, skin and nails (Green & Dijan, 1996). It has been suggested that appearance of wrinkles associated with ageing can be retarded by the potentiation of TGase1 expression by the topical application of plant extracts (Megata, 2004). In addition, TGases can be used to covalently bind various primary amine containing compounds such as antimicrobials, UV-absorbers, anti-inflammatory substances, antioxidants, colouring agents, perfumes and insect repellants to skin, nails and hair surfaces (Richardson *et al.*, 1996). A method has been described for curl retention in hair and lashes based on the action of the enzyme TGase (Mammone & Popescu, 2004).

2.1.7.4. Other Applications

Many authors have described applications of this enzyme as an enzymatic crosslinking agent useful for the generation of analytical tools for immunoassays or biosensors. In this context, it can be used to conjugate proteins (Tanaka *et al.*, 2004) and create hapten-antibody conjugates (Meusel, 2004), labelling of proteins (Josten *et al.*, 2000), or to immobilize proteins onto solid surfaces (Kamiya *et al.*, 2005).

2.1.7.4.1. Applications in Textile and Leather Industry

Treatment of leather with TGase, preferably together with a glutamine and/or lysine containing polymer such as keratin or casein, has a beneficial effect on the subsequent dyeing and colour properties of leather (Collighan *et al.*, 2002). Transglutaminase treatment is also shown to improve resistance to abrasion and collagenase activity (Addy *et al.*, 2005).

The treatment of wool textiles with TGase has been shown to improve the qualities of finished garments such as shrink resistance, wettability, reduced felting tendency, improved softness, tensile strength retention, improved stretch, improved dye uptake, dye wash-fastness, and protection from proteolytic detergents (McDevitt & Winkler, 1999; Cortez *et al.*, 2005).

2.2. ENZYMES AND COLD ADAPTATION

The cold environment represents a large portion of environment on our planet, with approximately 70% of the total earth surface covered by water with temperatures not exceeding 5°C (An & Visessanguan, 2000). In deep seas, marine organisms live at depths of 4,000 m. Despite the strong negative effect of low temperatures on biochernical reactions, extremophiles (i.e., organisms inhabiting these environments), breed, grow and survive successfully at rates comparable to those achieved by closely related species living in temperate environments (An & Visessanguan, 2000).

At extreme environmental conditions such as cold temperatures and pressure and in a multicomponent system, proteins undergo modifications (Shoichet *et al.*, 1995; Jaenicke & Bohm, 1998) and structural alterations to compensate for the reduced/changed thermal energy in their environment. Considerable studies have been conducted to elucidate the molecular and structural basis for cold adaptation (Toyota *et al.*, 2002; Aghajari *et al.*, 2003). These studies revealed that all active site residues involved in catalysis are strictly conserved between homologous enzymes adapted to different temperatures; this implies that the fundamental reaction pathway is not modified.

An efficient mechanism devised by organisms living in "extremely" cold environments is reduced reaction rates (Gerday *et al.*, 1997). Since enzymes catalyze most of these reactions they are also cold adapted and important target for further adaptation (Jaenicke & Bohm, 1998). Cold adapted enzymes have adapted various mechanisms to enhance thermostability and retain high specific activity (Watanabe *et al.*, 199l; Somero, 1995; Kulakovaa *et al.*, 2003).

The commonly observed biochemical features of these enzymes are their ability to display higher specific activity, increased catalytic efficiency at such low temperatures and significantly higher thermo-lability at higher temperatures than their mesophilic counterparts (Herbert, 1992; Feller & Gerday, 1997; Gerday *et al.*, 2000). Cold adapted enzymes from fishes have also been documented to be more susceptible to thermal denaturation than homologous temperate enzymes (Simpson & Haard, 1987a).

In order for enzymes to function at extreme temperatures, they require conformational adjustments and various hypotheses based on studies with thermophiles

have proposed increased flexibilities in the active sites (Lonhienne *et al.*, 2000; Kulakovaa *et al.*, 2003). These include weakening of intramolecular forces which maintains structural integrity; and increased solvent interaction which will invariably result in enzyme instability thus showing a positive relationship between thermal stability and temperature. The increase in flexibilities: - the main structural feature of cold adapted enzymes (Aghajari *et al.*, 1998) improves substrate accessibility to the catalytic site at low temperatures and energy cost. However enzyme inactivation, due to an increase in flexibilities may not inevitably pave way to enzyme unfolding (Gerday *et al.*, 1997); only enzyme-substrate interaction may be modified with the increased flexibility. Crystallography, biochemical and biophysical studies by different groups (Collins *et al.*, 2003; Georlette *et al.*, 2003) revealed much higher catalytic efficiencies, improved turn over numbers (k_{cat}) at the active sites of cold adapted enzymes than mesophilic homologues at lower temperatures. Others investigators have speculated the correlation between molecular flexibility and cold activity (Gerday *et al.*, 1997; Kulakovaa *et al.*, 2003), hence there appears to be an interdependence between flexibility, stability and activity.

Applications of cold adapted enzymes range from the production of sweeteners and detergents to the diagnosis of infectious diseases (Zeikus *et al.*, 1998). These enzymes are both novel and invaluable. The obvious advantages of cold adapted enzymes in biotransformation include: reduction in energy consumption and overall energy cost, wear and tear, and unwanted side reactions. Additionally the destruction or loss of heat labile flavor or color components during production can be fairly minimized with these enzymes.

2.3. THE SEAFOOD INDUSTRY

2.3.1. Fish and Fish Quality

Fish constitutes a major food source for humans. Marine species comprise approximately half of total global biodiversity, ranging between 3 to 500 million different species thereby offering an enormous resource for novel compounds (DeVries & Beart, 1995). Of these however only an estimated <1% have been screened for potential use(s) (Colwell, 1993).

About one third of the catch of fish in the world is thrown back into the sea (Raa, 1997). Furthermore, processing discards from fisheries account for as much as 70–85% of the total weight of the catch and these have been generally dumped in-land or hauled into the ocean (Shahidi, 1994). Approximately 30% of total landings may be considered as underutilized, bycatch, unconventional, or unexploited (Venugopal & Shahidi, 1995). However with the ever-increasing population, limited land size and resources, it has become morally and economically imperative to utilize these resources for isolating value-added components.

In recent years, recovery and characterization of enzymes from fish and aquatic invertebrates have taken place and this has led to the emergence of some interesting new applications of these enzymes in food processing. During the past two decades, considerable effort has also been made for investigating the new possibilities offered by enzymes originating from fish and aquatic invertebrates. Furthermore, extraction of enzymes from fish and shellfish processing wastes and their utilization in the food industry may contribute significantly to reducing local pollutions, where it creates an aesthetic as well as an ethical problem (Raa, 1997).

There is an increasing awareness of potential biochemical and pharmaceutical artifacts from fishery by-products, especially enzymes (DeVries & Beart, 1995). Due to the prospective commercial nature, marine biotechnology is one of the most actively developing areas and detailed research is needed to fully maximize this potential.

Currently industrial enzymes for the food industry are derived from animal by-products, plants and generally recognized as safe (GRAS) microorganisms with a few from fishery byproducts. Though microorganisms offer diverse source of enzymes, their relatively low incorporation into industrial applications have been partly due to consumer perceptions as well as their tedious and costly toxicological evaluation. Thus it is to be expected that marine enzymes, with rigorous research will be incorporated in biotransformation reactions, and make significant contributions to industrial applications.

2.3.2. Bluefish *(Pomatomus saltatrix)*

2.3.2.1. Occurrence, Habitat, Feeding and Mating

Bluefish (*Pomatomus saltatrix*) are coastal pelagic species with a worldwide subtropical distribution (Juanes *et al.*, 1996; Goodbred & Graves, 1996). Commonly known as chopper, tailor, snapper, elf, skipjack, greenfish and blue, they are found both inshore and offshore, as well as in many parts of the ocean, usually in continental shelf waters of temperate zones. It is the sole species of the Pomatomidae family and is closely related to the jacks, pompanos and roosterfish. Bluefish is an important recreational and commercial fish.

They are migratory marine fish, found worldwide in temperate and tropical waters around continental shelves and estuaries. They are absent only from the northern and central Pacific Ocean. In the Western Atlantic, they occur from Nova Scotia southwards through Brazil, Uruguay and Argentina, including Bermuda, the Gulf of Mexico, the Bahamas, and northern Cuba (Oliver *et al.*, 1989). They are also found off Africa, and in the Mediterranean and Black Seas.

Bluefish are warm water fish and are never found in any numbers in temperatures lower than about 14°-16°C. They are generally found in bays and sandy bottomed near-shore waters. Migrating fish may be encountered in as much as 200 foot (60 m) depths. Depending on conditions such as water temperature and atmospheric pressure, bluefish may be found nearly anywhere in the water column, from just above the bottom to just below the surface.

Bluefish are moderately proportioned fish, with a broad, forked tail. The spiny first dorsal fin is normally folded back in a groove, as are its pectoral fins. It can be recognized by its distinct colouration: grayish blue-green dorsally, fading to white on the lower sides and belly. They commonly range in size from seven inch (18 cm) "snappers" to as much as forty pounds (18 kg), though fish heavier than twenty pounds (9 kg) are exceptional.

Bluefish are voracious, cannibalistic, predatory fish. Depending on area and season, they favor menhaden and other sardine-like fishes (Clupeidae), jacks

(Scombridae), weakfish (Sciaenidae), grunts (Haemulidae), striped anchovies (Engraulidae), shrimp and squid (Buckel *et al.*, 1999).

2.4. EXTRACTION AND PURIFICATION OF TRANSGLUTAMINASES

The purification of TGases is known to be difficult, since they have a propensity to form irreversible aggregates under native conditions (Wilhelm *et al.*, 1996). Though all types of TGases share functional and structural similarity, they differ in their molecular and immunological characteristics, thus demanding different purification procedures. Most separation procedures employ combinations of ion-exchange and size-exclusion chromatography. Other chromatographic methodologies such as hydrophobic-interaction, affinity, adsorption, and metal chelating chromatography may also be applied. Furthermore electrophoretic separation methods such as preparative isoelectric focusing, SDS-PAGE and zone electrophoresis are also used.

2.4.1. Mammalian

Purification from mammalian sources has mainly involved the use of guinea pig liver (Brqokhart *et al.*, 1983). The unfrozen liver was homogenized, centrifuged and the soluble fraction was subjected to chromatography on DEAE-cellulose. The enzyme was then precipitated with protamine followed by extraction using ammonium sulfate and gel filtration on agarose. This procedure gave a 250-fold purification and 15-30% recovery (Abe *et al.*, 1977).

In a modified procedure by Brookhart *et al.*, (1983), the soluble fraction obtained from centrifugation was subjected to QAE-Sephadex ion exchange chromatography followed by adsorption onto a hydroxylapatite column and subsequent elution of various fractions. In order to obtain a pure enzyme, the partially purified enzyme was exhaustively dialyzed and finally subjected to affinity chromatography on a phenylalanine - sepharose 4B column. The overall yield for the enzyme was 31%. To obtain a 70% recovery, Brookhart *et al.*, (1983) suggests proceeding directly to affinity chromatography after centrifugation and filtration of the homogenate.

2.4.2. Microbial

The fermentation procedure for the production of transglutaminase is in principle the same for the various microorganisms (Ando *et al.*, 1989, 1992; Motoki *et al.*, 1989).

Common carbon sources that serve as the nutritional medium are glucose, sucrose, starch, glycerine and dextrin while the inorganic and organic nitrogen sources are NH_4NO_3, $(NH_4)_2SO_4$, urea, $NaNO_3$, NH_4Cl,soya, rice, maize, wheat or wheat flour, bran, defatted soya bean, maize-steep liquid, peptone, meat extract, casein, amino acids and yeast extract (Zhu *et al.*, 1995). The culture undergoes an aerobic fermentation at temperatures ranging between 25°C and 35°C, and the fermentation time is dependent on the culture conditions and determined by the highest transglutaminase activity that can be achieved, which is normally 2-4 days (Zhu *et al.*, 1995). Since microbial transglutaminase is an extra-cellular enzyme, it is dissolved in the fermentation broth and separated using conventional methods of enzyme purification (Zhu *et al.*, 1995).

In a procedure followed by Ando *et al.*, (1989), the microorganisms were separated from the fermentation broth by centrifugation followed by concentration of the supernatant by ultra filtration. In order to obtain pure enzyme, the concentrate was then subjected to ion exchange and gel filtration chromatography. The total recovery of TGase activity was approximately 42%. In the modification by Gerber *et al.*, (1994), the ultrafiltration and gel-filtration steps were omitted. The total recovery of TGase activity was approximately 40%.

Cui *et al.*, (2007), isolated transglutaminase from a *Streptomyces hygroscopicus* strain and purified the enzyme from its culture broth by ethanol precipitation, dialysis and successive chromatographic separation on CM-Cellulose and Sephadex G-75 (gel filtration). The final yield was 21.1 % with a 30 purification fold.

2.4.3. Plant

TGase activity has been demonstrated in plant tissues. Falcone *et al.*, (1993) extracted the enzyme from etiolated sprout apices, slices of tuber medullary parenchyma, leaves and flower buds of *Helianthus tuberosus*. Extraction of the tissue was done using an ice-cold mortar followed by filtration through layers of cheese cloth and centrifugation

and finally purification by gel filtration chromatography on a Sephadex column (Falcone *et al.*, 1993).

Kang and Cho (1996) extracted and purified transglutaminase from mature leaves of soybean (*Glycine max*). Mature leaves of the plant were homogenized in chilled electric blender and filtered through layers of gauze and clarified by centrifugation. The supernatant was further purified by chromatography on DEAE-Sepharose, Blue Sepharose CL-6B, ω-aminohexyl agarose and α-casein agarose (affinity) columns. The purification results of transglutaminase are summarized in Table 4.

2.4.5. Fish and Other Marine Species

Fish transglutaminase is generally prepared by homogenization of the tissue followed by centrifugation and filtration to obtain a TGase rich supernatant and then depending on the extent of purification required; this supernatant is then subjected to a series of precipitation reactions with ammonium sulfate followed by dialysis and chromatography (Kishi *et al.*, 1991; Yasueda *et al.*, 1994).

For example, purification from red sea bream involves anion exchange chromatography on DEAE-Sephacel followed by cation exchange on a CM-Sepharose column after elution and finally dialysis of the fraction. Further purification on a heparin-Sepharose column resulted in a 14% yield (Yasueda *et al.*, 1994).

Partial purification of the enzyme from carp (*Cyprinus caprio*) muscle required two different column chromatographies; DEAE-cellulose (ion exchange) and Sephacryl S-300 (gel filtration) column chromatographies (Kishi *et al.*, 1991). The TGase preparation was purified over 156 fold with a 25.5% yield.

TGase from tropical tilapia (*Oreochromis niloticus*) was purified to electrophoretic homogeneity using successive chromatographies on DEAE-Sephacel (ion exchange), Sephacryl S-4 HR (gel filtration) and HiTrap Heparin affinity column. This three step chromatographic separation protocol resulted in more complete purification with a 12.9 % yield and a purification fold of 69.8 as shown in Table 5 (Worratao & Yongsawatdigul, 2005).

Nozawa *et al.*, (1997) extracted TGases from the muscle of various fishes such as carp, rainbow trout, and atka mackerel; and marine invertebrates such as scallop, botan

shrimp and squid. The homogenate was then partially purified by successive chromatographies on DE-52 (anion exchange) and Sephacryl S-300 (gel filtration) columns. The purification yield for each species is illustrated in Table 6.

A novel approach that makes the prospects for commercial production of fish TGases rather encouraging is the cloning and expression of red sea bream TGase activity in *Escherichia coli* (Yokoyama *et al.*, 1998). The process involves cloning an expression plasmid for the production of red sea bream TGase in *E.coli* cells. These transformed cells are then grown under aerobic conditions and the synthesis of TGase is induced when the level of tryptophan in the growth medium is depleted. The growth and synthesis of the TGase is continued for approximately 20 h and monitored by optical density measurements at 660 nm (Yokoyama *et al.*, 1998). In order to obtain the enzyme, the cells are centrifuged, suspended in an extraction buffer, and subjected to ultrasonication at 4°C for cell lysis; these cell lysates are futher centrifuged at 4°C to separate the soluble and insoluble fractions (Yokoyama *et al.*, 1998). It was observed that production levels increased up to a maximum at 32-37°C. However most of the enzymes produced at 32°C formed aggregates and were thus inactive. In order to suppress the polypeptide aggregation, production strains containing both the heat shock chaperones and expression plasmids were constructed. This enabled the increase of soluble TGase from 10% to 50 % in addition to a four fold increase in activity (Yokoyama *et al.*, 1998).

2.5. TRANSGLUTAMINASE CHARACTERIZATION

TGases isolated from muscles of various seafood species generally exists in a monomeric form (Yasueda *et al.*, 1994; Tokunaga *et al.*, 1993; Kumazawa *et al.*, 1996). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used methodology to determine the molecular weight of enzymes.

The molecular weights of tropical tilapia, red sea bream, Alaskan pollack liver and crustacean (limulus hemocyte) TGase have been reported as 85 kDa (Worratao and Yongsawatdigul 2005), 78 kDa (Yasueda *et al.*, 1994), 77 kDa (Kumazawa *et al.*, 1996), and 86 kDa (Tokunaga *et al.*, 1993), respectively. The molecular weight of marine species has been found to be generally greater than that of other tissue TGases, including guinea pig liver (76.6 kDa), human placenta (76.6 kDa), and rat liver (80 kDa) (Abe *et*

al., 1977; De Backer-Royer *et al.*, 1992; Ikura *et al.*, 1988). Microbial TGases are monomeric proteins too and their molecular weight was reported to be approximately 38 kDa (Ando *et al.*, 1989).

The pI of tissue TGases, such as guinea pig liver TGase, rabbit liver TGase, human placenta TGase, were in the range of 4.5–5.4 (Abe *et al.*, 1977; De Backer-Royer *et al.*, 1992; Folk & Cole, 1966) while that of tilapia TGase was about 6.53 (Worratao and Yongsawatdigul 2005). The pI of microbial TGase is about 7.2–8.9 (Ando *et al.*, 1989; Tsai *et al.*, 1996).

2.5.1. Measuring Transglutaminase Activity

Transglutaminase activity assays are based on its enzymatic function, that is, the covalent binding of a primary amine to a peptide-bound glutamine in a calcium-dependent reaction. Several principles for following the activity of transglutaminase have been utilized based on incorporation of amine, disappearance of amino groups, increase in molecular weight, formation of NH_3, or measurement of the functional effects, i.e., viscosity or gel strength.

Amine incorporation assays have been used by many. It entails measurement of the rate of incorporation of radio-labelled amine putrescine, fluorescent-labelled primary amine monodansyl cadaverine or hydroxamate (Folk & Cole, 1966; Folk & Chung, 1985). The colorimetric hydroxamate procedure was also utilized by Ando *et al.*, (1989) in their screening for microbial transglutaminase.

The principle of following the amount of remaining amino groups was used by Ikura *et al.* (1980a) by the trinitrobenzenesulfonate methodology. Huang *et al.*, (1994) too followed the amount of free amino acids by measurement of fluorescence intensity in their study of the kinetics of immobilized transglutaminase.

Ikura et al., (1980a) followed the increase in molecular weight by SDS-polyacrylamide gel electrophoresis. This procedure is a more qualitative measurement compared to the other methods (Nielsen, 1995).

DeBacker-Royer *et al.*, (1992) and Faergemand (1993) described the detection of release of NH_3. They used an assay based on the conversion of NADPH to $NADP^+$ in the glutamate dehydrogenase catalyzed production of glutamate from NH_3 and β-

ketoglutarate (Faergemand, 1993). Another way of detecting NH_3 is by usage of an NH_3–selective electrode. However this has proven to be limited to certain substrates only (Nielsen, 1995).

The general problem with the methods for determination of activity is that they do not necessarily correlate with each other. The degree of correlation depends on type of TGase substrate and conditions.

2.5.2. Enzymatic Properties of Transglutaminases

2.5.2.1. Optimum Temperature

The optimal temperature of the purified TGase varied with the source (Table 7). For example, the optimal temperature of the purified tilapia TGase for the catalytic reaction of monodansylcadaverine (MDC) ranged from 37 to 50°C (Worratao & Yongsawatdigul, 2005), TGase from scallop, Japanese oyster, and pollock liver exhibited optimum activity between 35 and 50°C (Kumazawa *et al.*, 1996, 1997; Nozawa & Seki, 2001) while the optimal temperature of red sea bream liver TGase was between 55 and 60°C (Yasueda *et al.*, 1994). Transglutaminase of carp showed the highest activity from 50°C to 55°C decreasing to 33% of its highest activity at 60°C (Tsukamasa *et al.*, 2002).

The optimum temperature for enzymatic activity in the case of microbial TGases (derived from a variant of *Streptomyces mobaraensis*) was 55°C and complete activity was maintained for 10 min at 40°C, but lost within a few minutes at 70°C (Yokoyama *et al.*, 2004). Enzymatic activity was retained at near-freezing temperatures. Transglutaminases from mammalian sources on the other hand are much more sensitive to higher temperatures. For example, Clarke *et al.*, (1959) demonstrated that the activity of guinea pig liver TGases rapidly decreased at temperatures above 40°C.

2.5.2.1. Optimum pH

TGases from other fish species as illustrated in Table 7, showed pH optima between 8.0 and 9.5 (Kumazawa *et al.*, 1996, 1997; Nozawa *et al.*, 1997; Nozawa & Seki, 2001; Yasueda *et al.*, 1994). Crude and purified tilapia TGase showed optimal pH at 7.5 and decreased slightly between pH 8 and 9 (Worratao & Yongsawatdigul, 2003, 2005). Carp, Japanese oyster and scallop TGases showed pH optima at 8.0 which were

very similar to guinea pig and rat liver TGases (Nozawa *et al.*, 1997). The highest pH values were observed for red sea bream liver TGase, whose optimal pH was 9.0 - 9.5 (Yasueda *et al.*, 1994).

As opposed to marine TGases, microbial TGases are considered to be stable over a wider pH range. Though these TGases showed some activity at pH 4 or 9, the optimum pH for microbial TGase activity was found to be between 5.0 and 8.0 (Ando *et al.*, 1989).

2.5.2.1. Effect of Calcium Ions ($CaCl_2$)

TGases have been widely described as Ca^{2+} dependent enzyme, but isolation from new sources indicates that the requirement of Ca^{2+} ions is not universal. For instance, microbial transglutaminases are totally independent of Ca^{2+}, and in this respect are quite different from mammalian TGases (Yokoyama *et al.*, 2004).

Mammalian and fish TGases show an absolute requirement for calcium ions to catalyze monodansylcadaverine (MDC) incorporation (Yongsawatdigul *et al.*, 2002). Purified tilapia's TGase activity increased with Ca^{2+} concentration and reached a maximum at 1.25 mM (Worratao & Yongsawatdigul, 2005). Optimal Ca^{2+} concentrations for TGase from red sea bream liver, Japanese oyster, scallop, and pollock liver were at 0.5, 25, 10, and 3 mM, respectively (Kumazawa *et al.,* 1996, 1997; Nozawa *et al.,* 2001; Yasueda *et al.*, 1994).

2.5.2.1. Effect of NaCl on Transglutaminase Activity

The TGase activity of freshwater marine species such as tropical tilapia (Worratao & Yongsawatdigul, 2005), carp dorsal muscle (Kishi *et al.*, 1991) and Japanese oyster (Kumazawa *et al.*, 1997) decreased with increasing NaCl concentrations. High concentration of NaCl could induce conformational changes in the enzyme molecule, resulting in a decrease of TGase activity (Kishi *et al.*, 1991; Kumazawa *et al.*, 1997). However, NaCl significantly increased TGase activity from saltwater marine species, such as scallop, botan shrimp (*Pandalus nipponensis*), and squid (Nozawa *et al.*, 1997). These results suggest that the optimal conditions for enzyme activity are closely related to the environmental habitat of the aquatic species. Since tilapia is a freshwater species, tilapia TGase would physiologically function at relatively low NaCl thus having lower activity at high NaCl concentrations.

2.5.2.1. Effect of Inhibitors on Transglutaminase Activity

TGase from aquatic species are completely inhibited by chelating agents such as ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) in addition to sulfhydryl alkylating agents such as ρ-chloromercuribenzoic acid (PCMB), N-ethylmaleimide (NEM), and iodoacetamide (IAA) (Ha & Iuchi, 1998; Kumazawa *et al.*, 1997; Nozawa & Seki, 2001; Tokunaga *et al.*, 1993; Yasueda *et al.*, 1994). Since sulfhydryl alkylating agents react with a thiol group, the results support the view that marine TGases contain thiol groups at the active sites.

2.5.2.1. Substrate Specificity of Transglutaminases

Different enzymes display specificity differences towards substrate proteins. This constitutes an important aspect of their biological function and it is also relevant to their biotechnological applications. In general, transglutaminases have stringent specificity for the glutamine substrate whereas the specificity for the primary amine substrate is broader: i.e., TGase absolutely requires protein or peptide bound glutamine, in which both the α-amino and α-carboxyl groups of glutamine are in peptide linkage for activity (Ashie & Lanier, 2000). The nature of the amino acids surrounding the sensitive glutamine residues and the degree of the latters' exposure to solvent appear to be the main determinants of substrate specificity (Coussons *et al.*, 1992).

When comparing the substrate specificity of TGases that are presently available for application in food industry, TGase from *Streptoverticillium mobaraense* was found to react with a larger number of substrate proteins than the plasma and erythrocyte TGases from mammalian blood (de Jong *et al.*, 2001). Table 8 summarizes information on substrate specificity of some TGases with regard to food proteins

In summary, transglutaminases have the ability to catalyze the formation of intra- and inter-molecule cross-links of proteins thus offering a means by which the food industry can alter protein functionalities thus improving the functional properties of food. At the moment there is only a limited selection of cross-linking enzymes in the market. Hence, transglutaminase represents an essential enzyme for protein modification in food processing and novel food development in the future (Table 9). Applications involving

TGase in the development of novel foods are emerging. There may be many applications in the incorporation of various amines, amino acids, lysine-containing peptides, glutamine-containing peptides and heterologous polypeptides (Fig. 3). As a safe and mild way to modify the properties of prepared foods, the applications of transglutaminase will no doubt continue to expand.

CHAPTER III

PRELIMINARY CHARACTERIZATION OF CRUDE BLUEFISH (*Pomatomus saltatrix*) TRANSGLUTAMINASE EXTRACT

ABSTRACT

Crude transglutaminase was extracted from the muscle and liver of bluefish (*Pomatomus saltatrix)* and tested for activity. Frozen liver supernatant had the highest activity at 0.321 Units. Frozen crude liver supernatant TGase was active between 45°C and 55°C and the optimum temperature was determined to be at 50°C. The enzyme was stable at temperatures up to 55°C beyond which it lost activity progressively. The TGase fraction was active within the pH range 6.0 to 7.5, with an optimum pH at 7.0, and was stable from pH 7.0-8.0.

3.1. INTRODUCTION

Transglutaminases [TGases, EC 2.3.2.13] belong to a family of enzymes which catalyze acyl transfer reactions. Tissue-type transglutaminase (TGase; EC 2.3.2.13) is a calcium-dependent enzyme that catalyzes the cross-linking and amine incorporation of proteins by an acyl transfer reaction between γ-carboxyamide groups of a glutamine residue and primary amino groups (Folk 1980; Greenberg *et al.* 1991). TGase is widely found in living organisms, such as mammals, plants, microorganisms, and various tissues of fish and aquatic invertebrates (Ando *et al.,* 1989; Icekson and Apelbaum 1987; Wong *et al.,* 1990; Yasueda *et al.,* 1995). Recently, TGases has captured peoples' interest due to its attractive potential application in (i) food industries; to improve functional properties of food proteins (Zhu *et al.*, 1995; Motoki & Seguro, 1998), (ii) immobilization of enzymes (Josten *et al.*, 2000) and (iii) textile industries (Cortez *et al.*, 2005).

Seki *et al.* (1990) were the first to show the presence of TGase in fish muscle and surimi extracts. The enzyme has been suggested to be concerned with gel formation in fish muscle or surimi paste (Yongsawatdigul *et al.*, 2002; Benjakul *et al.*, 2003). The possibility of using TGase to improve functional and rheological properties of food proteins has been extensively investigated. Though TGases have been studied and isolated from several marine species such as carp, rainbow trout and atka mackerel, tilapia, scallop, botan shrimp and squid, the enzymatic and structural properties of marine vertebrate TGases have not yet been extensively characterized as the mammalian and microbial counterparts.

The characteristics of transglutaminase from Bluefish (*Pomatomus saltatrix*), a comestible food material, has not yet been reported. The objective of this work is to make a preliminary characterization of bluefish transglutaminase in terms of its temperature and pH optimum as well as the influence of refrigeration and freezing of the crude extract on the stability of this enzyme.

3.2. MATERIALS

3.2.1. Chemicals

The following chemicals:- CBZ-glutaminylglycine, trizma acetate, trizma HCl, hydroxylamine, L-glutamic acid γ-monohydroxamate and reduced glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA), trichloroacetic acid, ferric chloride hexahydrate, calcium chloride, hydrochloric acid, sodium carbonate, copper sulphate and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). All other reagents and chemicals were of analytical grade.

3.2.2. Biological Samples

Frozen bluefish (*Pomatomus saltatrix*) was purchased from Poissonnerie O.C.N. Import fish shop (Jean Talon West, Montreal), kept on ice and transported to the laboratory. The fish was cleaned and manually eviscerated upon arrival at the laboratory. Fish muscle and liver was carefully collected, cleaned by flushing with distilled water to remove any adhered blood, weighed and crude bluefish transglutaminase was immediately extracted (section 3.3.1.) and stored at 4°C and -20°C

3.3. EXPERIMENTAL METHODS

3.3.1. Preparation of Crude Transglutaminase Extract

The crude TGase was extracted based on the method described by Worratao and Yongsawatdigul (2005) with a slight modification. About 100 grams of flesh and 20 grams of liver samples were homogenized separately in 4 volumes of extraction buffer, (50 mM trizma-HCl, 50 mM NaCl, 25 mM EDTA and 10 mM DTT, pH 7.5) at 10,000 rpm for 1.5 min using a Polytron PT 3000 homogenizer (Brinkmann Instruments). The two homogenates were then centrifuged at 20,000 g for 60 min at 4°C in an IEC Multi RF high performance centrifuge. The supernatant solutions of bluefish flesh and liver samples were carefully removed from the centrifuge bottles without disturbing the pellet and filtered through eight layers of cheesecloth. The supernatant was for various attributes called crude TGase extract and characterized.

3.3.2. Protein Determination

Concentrations of total protein in the TGase extracts were determined by the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin (BSA) as a standard. Color development during the Lowry assays took place over 15 min at room temperature (approximately 24°C), and absorbance values were measured using a Beckman DU800 UV/Vis spectrophotometer at 540 nm wavelength.

3.3.3. Assay of Transglutaminase Activity

$$\text{CBZ-Gln-Gly} + \text{Hydroxylamine} \xrightarrow{\text{TGase}} \text{CBZ-Gln-Gly-Hydroxamate}$$

The transglutaminase activities in each of the crude TGase fractions was assayed in triplicate according to the method of Folk and Cole (1966) (colorimetric hydroxamate procedure) with a slight modification. The method is based upon the measurement of peptide-bound γ-glutamyl hydroxamate formed from N-carbobenzoxy (CBZ)-L-glutaminylglycine and hydroxylamine as the substrates in the presence of the enzyme and

Ca^{2+}. An iron complex was formed with the resulting hydroxamic acid in the presence of TCA, which produced a color change from yellow to red.

About 500 µl of the sample extract was added to 500 µl of the reaction mixure (200 µl of 1.0 M Trizma-acetate buffer, 150 µl of 0.1 M CBZ-L-Glutaminylglycine, 50 µl of 0.1 M $CaCl_2$, 50 µl of 2.0 M hydroxylamine and 50 µl of 0.2 M reduced glutathione; pH 6.0), mixed and incubated at 37°C for 10 min in a water bath (Haake refrigerated bath/circulator D1-G, HAAKE Mess-Technik GmbH u. Co, Germany). To stop the reaction 500 µl of Reagent B (equal volume of 3 M HCl, 12 % (w/v) TCA and 5 % (w/v) ferric chloride solution in 0.1 M HCl was added. The samples were centrifuged at 15,000 g for 10 min in an IEC Multi RF centrifuge, and the absorbance of the supernatant was measured at 525 nm wavelength at room temperature (approximately 24°C) in a Beckman DU 800 UV/Vis Spectrophotometer. A calibration curve was separately prepared using 10 mM γ-mono-hydroxamic acid L-glutamate standard solution instead of the enzyme solution (Fig. 9B).

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1.0 micromole (µmol) of peptide-bound γ-glutamyl hydroxamate per min from CBZ-Glutaminylglycine and hydroxylamine at pH 6.0 and at 37°C. For instance, the specific activity was defined as the number of units of activity per milligram of protein.

3.4. CHARACTERIZATION OF CRUDE TRANSGLUTAMINASE

The crude TGase extracts from the flesh and liver of bluefish were characterized with respect to stability over time as well as sensitivities to pH and temperature. All measurements of enzymatic activity were done in triplicates.

3.4.1. Effect of Storage Time and Temperature on the Stability and Activity of Crude Muscle and Liver TGases.

The stability of the crude enzyme extracts over time was assessed by measuring the activity over a 30 d period. Tubes containing 5 mL of the supernatant crude liver and muscle TGase extracts were stored at refrigerated (4°C) and frozen temperatures (-20°C).

Transglutaminase activity assay was carried out on the refrigerated and frozen samples on 1st, 3rd, 5th, 7th, 10th, 15th, 20th, 25th and 30th day. The same reaction mixture and reaction conditions as described in section 3.3.3 were used. The relative activities were estimated as a percentage, taking maximum activity as 100%.

3.4.2. Effect of Temperature on Enzyme Activity

The effect of temperature on crude bluefish TGase activity was determined at 5°C intervals from 5°C to 70°C. In each assay, 500 µl of the crude enzyme extract was added to 500 µl of the reaction mixture described previously (section 3.3.3). The mixture was incubated for 10 min at room temperature (24 °C) before measuring the absorbance at 525 nm (DU 800 Spectrophotometer, Beckman Coulter, Inc. USA). The activity was estimated as a percentage, taking maximum change in absorbance as 100 %.

3.4.3. Effect of Temperature on Enzyme Stability

The thermostability of crude transglutaminase extract was studied by incubating the enzyme extract for 60 min at various temperatures from 5°C to 70°C, at 5°C intervals. At the end of the incubation period the enzyme extract was assayed for residual activity as previously described (section 3.3.3).

3.4.4. Effect of pH on Enzyme Activity

The effect of pH on crude transglutaminase activity was determined using various buffer solutions ranging from pH 2.0 to pH 10.0. The following buffers; potassium chloride - HCl pH 2.0, citrate-phosphate pH 4.0-6.0, tris-HCl pH 8.0, and carbonate-bicarbonate pH 10.0, were used with the reaction mixture described previously (section 3.3.3). The enzyme activity was measured after incubation at 37°C for 10 min in a water bath (Haake refrigerated bath/circulator D1-G, HAAKE Mess-Technik GmbH u. Co, Germany). Activity was estimated as a percentage, taking the maximum pH change in absorbance as 100 %.

3.4.5. Effect of pH on Enzyme Stability

The effect of pH on the stability of the crude extract was determined by incubating the crude transglutaminase extract in buffer solutions of various pH ranging from 2.0 to 10.0 for 30 min at 37°C in a water bath (Haake refrigerated bath/circulator D1-G, HAAKE Mess-Technik GmbH u. Co, Germany). At the end of the incubation period, aliquots were taken and transglutaminase activity was assayed spectrophotometrically (DU 800 Spectrophotometer, Beckman Coulter, Inc. USA). In each assay, 500 µl aliquot of the incubated enzyme extract was added to 500 µl of the reaction mixture described previously (section 3.3.3).

3.5. RESULTS AND DISCUSSION

3.5.1. Stability of TGases Activity in Bluefish Tissues

The stability of the crude bluefish TGase extract was established by freezing (-20°C) and refrigerating (4°C) aliquots of liver and muscle supernatant solutions and testing them for activity over a period of 30 days. Fig. 4 shows the percentage of TGase activity remaining for frozen and refrigerated bluefish liver and muscle extracts over a 30 day period. Transglutaminase activity in both the liver and flesh extracts declined rapidly during storage at refrigerated temperature (4°C) with the refrigerated flesh losing approximately 50 % of activity during the first 15 days. The frozen (-20°C) flesh and liver seemed to have higher activities with the liver retaining maximum activity at the end of 30 days. The order of TGase activity per ml of solution at the end of 30 days, when assayed at 37°C can be arranged as follows: frozen liver supernatant (0.321 Units) > frozen flesh supernatant (0.230 Units) > refrigerated liver supernatant (0.124 Units) > refrigerated flesh supernatant (0.071 Units).

These results are consistent with those of Leblanc *et al.*, (1999) and Folk and Cole (1966), who reported freezing of supernatant solution at -20°C to prevent loss of TGase activity. Since the results indicated frozen liver supernatant to have the highest stability/activity, subsequent experiments were conducted with the frozen liver aliquots.

In a study of TGase activities in some fish species, Nozawa *et al.* (1997), reported specific activity of carp, rainbow trout and atka mackerel to be 0.36, 0.01 and

0.14 Units/mg protein respectively when incubated at 25°C for 20 min using MDC and succinylated casein as substrates. The differences in TGase activity values obtained for bluefish and those reported for other marine species maybe due to the differences in definition of the unit of TGase activity and assay conditions used. Most results reported so far have been based on the more sensitive fluorometric method using monodansyl cadaverine and dimethylated casein as substrates (Tsukamasa *et al.*, 2002; Tagaki *et al.*, 1986; Kumazawa *et al.*, 1997; Nozawa *et al.*, 1997; Worratao & Yongsawatdigul, 2005) as opposed to the present experiment that used the colorimetric hydroxamate assay procedure (Folk & Cole, 1996) with benzyloxy-carbonyl-L-glutaminylglycine and hydroxylamine as substrates. The differences in results may also be due to different measuring conditions such as reaction period and reaction temperature. Furthermore, Araki and Seki (1993) have shown that TGase activities vary between different fish species.

3.5.2. Effect of Reaction Temperature on the Activity and Stability of Crude Bluefish TGase

The optimal temperature of crude bluefish TGase was between 40°C and 45°C with benzyloxy-carbonyl-L-glutaminylglycine and hydroxylamine as substrates (Fig. 5). There was no activity observed at refrigerated temperature (4°C). The activity seems to decrease steadily after 50°C. This could be due to possible denaturation of the enzyme and a subsequent reduction in the catalytic activity of the TGase. The optimal temperature of crude fish TGase tends to vary with sources and as Lee *et al.*, (1998) reported, could be related to the species habitat temperature. Pollock liver exhibited optimum activity between 35 °C and 50°C (Nozawa & Seki, 2001), while that of red sea bream liver was between 55 and 60°C (Yasueda *et al.*, 1994). Carp TGase showed optimum activity at 30°C (Tsukamasa *et al.*, 2002). A microbial transglutaminase derived from *Streptoverticillium mobaraense*, commercialized by Ajinomoto Co., showed a temperature optimum at 50°C (Batista *et al.*, 2002).

The thermal stability of crude bluefish TGase is shown in Fig. 6. The enzyme was stable at temperatures below 55°C but was gradually inactivated at higher temperatures. The higher thermal stability of TGase could be related to the higher habitat temperature

of the marine species. However because the crude form of the enzyme extract was used in this study, the possibility of several enzymes in the observed activity cannot be excluded.

3.5.3. Effect of pH on the Activity and Stability of Crude Bluefish TGase

Bluefish TGase acylated benzyloxy-carbonyl-L-glutaminylglycine and hydroxylamine over a relatively broad pH range (2.0 - 10.0). At low pH (acidic conditions) crude bluefish TGase seemed to be inactive. The TGase extract exhibited higher activity within the pH range of 6.0 – 7.5 with an optimum at pH 7.0 and 7.5. Beyond pH 7.5, the activity of the enzyme decreased gradually. The optimum pH of fish liver TGase seems to be in the basic region (Fig. 7). This is shared by TGases from other sources (Worratao & Yongsawatdigul, 2003; Nozawa *et al.*, 2001).

The enzyme was stable between pH 6.5 - 8.0 when incubated at 35°C for 30 min, with benzyloxy-carbonyl-L-glutaminylglycine and hydroxylamine as substrates. There was 70% residual activity above pH 7, however a marked loss of activity was observed in the acidic region below pH 4 and in the alkaline region above pH 8 (Fig. 8).

3.6 CONCLUSIONS

The following conclusions can be made for crude blefish TGase extract based on the studies described in this chapter.

1. This study has shown the presence of transglutaminase (TGase) activity in bluefish flesh and liver tissues. Liver tissue appeared to be a richer source of TGases than flesh tissue. Crude bluefish TGase extract was more stable at frozen temperatures (-20°C) and the activity decreased rapidly when TGase extract was stored at refrigerated temperatures (4°C).
2. The enzyme exhibited an optimum temperature between 40 °C and 45°C and was stable at temperatures below 55°C. Activity decreased rapidly beyond this temperature. The optimal temperature of crude fish TGase tends to vary with sources and could be related to the species habitat temperature (Lee *et al.*, 1998).
3. The optimum pH of bluefish for the catalytic reaction of hydroxylamine and N-CBZ-Gln-Gly was observed at neutral to slightly alkaline pH (7.0-7.5), which is the optimum pH of fish liver TGase (Hemung & Yongsawatdigul, 2008). Bluefish

TGase was stable in the pH range 6.5-8.0 with a remarkable loss of activity being observed below pH 4 and above pH 8.

TGase isolation and further purification are necessary in order to study among other things individual enzymes directly involved in the effects observed to be able to preclude other enzymes with overlapping specificities or activities.

CHAPTER IV

PARTIAL PURIFICATION AND CHARACTERIZATION OF TRANSGLUTAMINASE FROM BLUEFISH (*Pomatomus saltatrix*) LIVER

ABSTRACT

Transglutaminase was partially purified from the liver of bluefish (*Pomatomus saltatrix)* using gel filtration on Sephcryl S-200 column. The partially purified extract was characterized with N-carbobenzoxy (CBZ)-L-glutaminylglycine and hydroxylamine as substrates. The optimum temperature of the enzyme was determined as 50°C. The enzyme was stable between 5-50°C beyond which it lost activity progressively. The TGase fraction was active within the pH range of 7.0 – 8.0 with an optimum pH of 7.5, and was stable from pH 7.0-8.0. The partially purified bluefish TGase showed an absolute requirement for calcium ions, with no activity being detected in the absence of calcium ions. TGase activity was unaffected by NaCl up to 0.6 M and reduced to 78% at 1.5 M NaCl. Bluefish TGase was strongly inactivated by sulfhydryl alkylating agents; N-ethylmaleimide (NEM), iodoacetamide (IAA), and ammonium sulfate ($(NH_4)_2SO_4$) suggesting the presence of a thiol group at the active site.

4.1. INTRODUCTION

Transglutaminase is a transferase enzyme whose systematic name is protein-glutamine γ-glutamyltransferase (TGase; EC 2.3.2.13). The enzyme catalyzes the acyl transfer reaction in which the γ-carboxyamide groups of glutamine residues in proteins, peptides and various primary amines, act as acyl donors and primary amino groups including ε-amino groups of lysine residues, which are either peptide-proteins bound or free lysine, act as the acyl acceptors (Folk, 1980).When acceptors are ε-amino groups of lysine residues, the formation of ε-(γ-glutamyl) lysine (GL) linkages occurs both intra-

and inter-molecularly (Folk, 1980; Greenberg *et al.*, 1991). This introduces covalent cross-linkages between the ε-amino groups of lysine residues and the γ-carboxyamide group of a glutamine residue in a protein molecule (Folk, 1980; Greenberg *et al.*, 1991). These bonds are stable and resistant to proteolysis (Joseph *et al.*, 1994). Therefore, TGase has been widely studied to improve functional properties of various food proteins (Jiang *et al.*, 2000).

TGases have been studied and isolated from several fish species. The biochemical characteristics of the enzyme showed the Ca^{2+}-dependent characteristics. Nozawa *et al.* (1997) characterized the partially purified TGases from various fish (carp, rainbow trout and atka mackerel) and found the different effects of salt on activity when compared with marine invertebrates (scallop, botan shrimp and squid). TGase from freshwater fish muscle was also purified from carp (Kishi *et al.* 1991) and tropical tilapia (Worratao and Yongsawatdigul 2005). Besides muscle, another good tissue TGase source is liver. Yasueda *et al.*, (1994) purified TGase from red sea bream liver and demonstrated that fish liver TGase appears to have a potential in improving the textural properties of protein gels.

The investigation of the biochemical characteristics of bluefish liver TGase is essential not only from the enzymology viewpoint but also for food applications. Optimum conditions in food application are mainly determined from the biochemical properties of the enzyme. In order to clarify the biochemical characteristics of bluefish TGase, the enzyme was partially purified from bluefish liver and the biochemical properties of the partially purified TGase were investigated.

4.2. MATERIALS

4.2.1. Chemicals

The following chemicals: - CBZ-glutaminylglycine, trizma acetate, trizma HCl, hydroxylamine, L-glutamic acid γ-monohydroxamate and reduced glutathione were purchased from Sigma Chemical Co. (St. Louis, MO). Ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), trichloroacetic acid (TCA), ferric chloride hexahydrate, calcium chloride, hydrochloric acid, sodium carbonate, copper sulphate and

sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). Sephacryl S-200 HR was purchased from GE Healthcare, Bio-Sciences AB. Dithiothreitol (DTT) was purchased from Fluka BioChemica (Buchs, Switzerland). Sodium dodecyl sulfate (SDS), coomassie brilliant blue R-250 was purchased from Bio-Rad (Richmond, Calif., USA). Standard protein kits for SDS-PAGE were purchased from GE Healthcare, Bio-Sciences AB. All other reagents and chemicals were of analytical grade.

4.2.2. Biological Samples

Frozen bluefish (*Pomatomus saltatrix*) was purchased from Poissonnerie O.C.N. Import fish shop (Jean Talon West, Montreal), kept on ice and transported to the laboratory. The fish was cleaned and manually eviscerated upon arrival at the laboratory. Fish liver was carefully collected, cleaned by flushing with distilled water to remove any adhered blood, weighed and crude bluefish transglutaminase was immediately extracted as described in section 4.3.1and stored at -20°C.

4.3. EXPERIMENTAL METHODS

4.3.1. Preparation of Crude Transglutaminase Extract

The crude TGase was extracted based on the method described by Worratao and Yongsawatdigul (2005) with a slight modification. Approximately 100 grams of liver samples were homogenized in 4 volumes of extraction buffer (50 mM Trizma-HCl, 50 mM NaCl, 25 mM EDTA and 10 mM DTT, pH 7.5) at 10,000 rpm for 1.5 min using a Polytron PT 3000 homogenizer (Brinkmann Instruments). The homogenate was then centrifuged at 20,000 g for 60 min at 4°C in an IEC Multi RF high performance centrifuge. The supernatant solution of bluefish liver sample was carefully removed from the centrifuge bottles without disturbing the pellet and filtered through eight layers of cheesecloth.

4.3.2. Partial Purification of Bluefish Transglutaminase

4.3.2.1. Sephacryl S-200 Chromatography.

The Sephacryl S-200 column was prepared as per the manufacturer's protocol (GE Healthcare, Bio-Sciences AB). About 20 mL of the crude liver homogenate was applied onto a Sephacryl S-200 (GE Healthcare, Bio-Sciences AB) column (1.5 x 80 cm) equilibrated with 10 mM tris–HCl buffer (pH 7.5) containing 0.1 M NaCl, 5 mM EDTA and 2 mM DTT (Buffer A). The sample was eluted as per the method of Worratao and Yongsawatdigul (2005) at a constant flow rate of 2 mL/min. Fractions of 6 ml were collected with a fraction collector (Model 2110 Fraction Collector, BioRad Laboratories, Mississauga, ON). The protein content of each fraction collected from the elution was monitored at 280 nm, and TGase activity was routinely measured at 525 nm with CBZ-L-glutaminylglycine and hydroxylamine as the substrates. The active fractions were pooled and concentrated into 1.5 ml by an ultrafiltration membrane with a MWCO cut-off 30 kDa (Centriprep Model YM, Millipore, Billerica, Mass., USA).

4.3.3. Assay of Transglutaminase Activity

$$\text{CBZ-Gln-Gly} + \text{Hydroxylamine} \xrightarrow{\text{TGase}} \text{CBZ-Gln-Gly-Hydroxamate}$$

The transglutaminase activity in each of the collected fractions was assayed in duplicates according to the method of Folk and Cole (1966) (colorimetric hydroxamate procedure) with a slight modification. The method is based upon the measurement of peptide-bound γ-glutamyl hydroxamate formed from N-carbobenzoxy (CBZ)-L-glutaminylglycine and hydroxylamine as the substrates in the presence of the enzyme and Ca^{2+}. An iron complex was formed with the resulting hydroxamic acid in the presence of TCA, which produced a color change from yellow to red.

About 500 µl of the sample extract was added to 500 µl of the reaction mixure (200 µl of 1.0 M Trizma-acetate buffer, 150 µl of 0.1 M CBZ-L-Glutaminylglycine, 50 µl of 0.1 M $CaCl_2$, 50 µl of 2.0 M hydroxylamine and 50 µl of 0.2 M reduced glutathione; pH 6.0), mixed and incubated at 37 °C for 10 min in a water bath (Haake refrigerated

bath/circulator D1-G, HAAKE Mess-Technik GmbH u. Co, Germany). To stop the reaction, 500 µl of Reagent B (equal volume of 3 M HCl, 12 % (w/v) TCA and 5 % (w/v) ferric chloride solution in 0.1 M HCl was added. The samples were centrifuged at 15,000 g for 10 min in an IEC Multi RF centrifuge, and the absorbance of the supernatant was measured at 525 nm wavelength at room temperature (approximately 24 °C) in a Beckman DU 800 UV/Vis spectrophotometer. Separately, a calibration curve was prepared using 10 mM γ-mono-hydroxamic acid L-glutamate standard solution instead of the enzyme solution (Fig. 9B).

One unit of enzyme activity was defined as that amount of enzyme that catalyzes the formation of 1.0 micromole (µmol) of peptide-bound γ-glutamyl hydroxamate per min from CBZ-Glutaminylglycine and hydroxylamine at pH 6.0 and at 37°C. The specific activity was defined as the number of units of activity per milligram of protein.

4.3.4. Protein Determination

Concentration of total protein in the TGase extracts were determined by the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin (BSA) as a standard. Color development during the Lowry assays took place over 15 min at room temperature (24°C), and absorbance values were measured using a Beckman DU800 UV/Vis spectrophotometer at 540 nm wavelength.

4.4. CHARACTERIZATION OF PARTIALLY PURIFIED TRANSGLUTAMINASE

The partially purified TGase extract from the liver of bluefish was characterized with respect to stability over time as well as sensitivites to pH and temperature. All measurements of enzymatic activity were done in duplicates.

4.4.1. Effect of Temperature on Enzyme Activity

The effect of temperature on the activity of the partially purified enzyme extract was determined at 5°C and there after at 10°C intervals from 10°C to 70°C. In each assay, 500 µl of the enzyme extract was added to 500 µl of the reaction mixture (section 4.3.3).

The mixture was incubated for 10 min before measuring the absorbance at 525 nm in a Beckman DU 800 Spectrophotometer (Beckman Coulter, Inc. USA). The activity was estimated as a percentage, taking maximum temperature activation as 100 %.

4.4.2. Effect of Temperature on Enzyme Stability

The thermostability of transglutaminase was studied by incubating the enzyme extract at various temperatures (i.e., 5°C, 10°C, 20°C, 30°C, 40°C, 50°C, 60°C, and 70 °C) for 60 min. At the end of the incubation period the enzyme extract was assayed for residual activity as previously described (section 4.3.3.).

4.4.3. Effect of pH on Enzyme Activity

Optimal pH was determined by incubating the partially purified transglutaminase fraction in various buffers: pH 6.0–6.5, 100 mM acetate buffer; pH 7.0–7.5, 50 mM tris–HCl; and for pH 8.0–9.0, using 50 mM borate buffer with the reaction mixture described previously (section 4.3.3). The enzyme activity was measured in duplicates after incubation at 37°C for 10 min in a water bath (Haake refrigerated bath/circulator D1-G, HAAKE Mess-Technik GmbH u. Co, Germany). The results were expressed as percentage of activity obtained at pH 7.5. The effect of pH on bluefish TGase was not studied below 6.0 because no activity was observed in the crude extract below pH 6.0 (Fig. 7).

4.4.4. Effect of pH on Enzyme Stability

The effect of pH on the stability of the partially purified enzyme extract was determined by incubating the transglutaminase fraction in buffer solutions of various pH ranging from 6.0 to 9.0 for 30 min at 37°C in a water bath (Haake refrigerated bath/circulator D1-G, HAAKE Mess-Technik GmbH u. Co, Germany). At the end of the incubation period, aliquots were taken and the remaining transglutaminase activity was assayed spectrophotometrically at 525 nm (DU 800 Spectrophotometer, Beckman Coulter, Inc. USA). In each assay, 500 µl aliquot of the enzyme extract was added to 500 µl of the the reaction mixture (section 4.3.3). The results were expressed as percentage of activity obtained at a pH where the maximum activity was observed (pH 7.5).

4.4.5. Effect of Calcium Chloride ($CaCl_2$)

The effect of calcium ions was investigated in duplicates by varying the concentration of $CaCl_2$ in the reaction mixture described previously (section 4.3.3.). The effect of $CaCl_2$ on TGase activity was tested at 0-5 mM. The reaction mixture (section 4.3.3) was incubated with 500 µl of the enzyme extract at pH 6.0 and 37°C for 10 min before measuring the activity spectrophotometrically at 525 nm at room temperature of approximately 24°C.

4.4.6. Effect of Sodium Chloride (NaCl)

The effect of sodium ions was investigated in duplicates by varying the concentration of NaCl in the reaction mixture described previously (section 4.3.3.). The effect of NaCl on TGase activity was tested at 0-10 mM. The reaction mixture was incubated with 500 µl of the enzyme extract at pH 6.0 and 37°C for 10 min before measuring the activity spectrophotometrically at 525 nm at room temperature of approximately 24°C.

4.4.7. Effect of Divalent Ions

To investigate if other divalent ions were able to activate TGase, the assays were prepared by substituting $CaCl_2$ with $MnCl_2$, $MgCl_2$, $ZnCl_2$, $FeCl_2$ or $BaCl_2$ at 15 mM. The reaction mixture (section 4.3.3.) was incubated with 500 µl of the enzyme extract at pH 6.0 and 37°C for 10 min. The relative activity was estimated as a percentage, taking maximum activation as 100 %.

4.4.8. Effect of Inhibitors

The effects of IAA, $(NH_4)_2SO_4$ and NEM on TGase activity were investigated at a concentration of 10 mM. The reaction mixture (section 4.3.3.) containing the inhibitor was incubated with 500 µl of the enzyme extract at pH 6.0 and 37°C for 10 min. The relative activity was calculated as the percentage of the remaining activity. The activity of the sample without the inhibitor was considered to be 100%.

4.4.9. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) is a widely used technique for the study of the size of bimolecules including RNA, DNA and proteins. The fundamental principal is based on mobility of charged particles in an electrical field. The rate of movement depends on the field strength and the number of charges. Proteins possess surface charge due to the presence of acidic and basic amino acids.

In SDS-PAGE, electrophoresis is performed in the presence of the anionic detergent sodium dodecyl sulphate (SDS). First, SDS binds to proteins at a constant ratio and results in denaturation. Second, SDS swamps the protein with negative charges. Therefore, the treatment with SDS removes effect of charge density and shape and separation is then solely based upon molecular weight.

SDS-PAGE separation was carried out according to the method originally described by Laemmli (1970).

4.4.9.1. Sample Preparation and SDS Polyacrylamide Gel Electrophoresis

The samples and standards (low range, GE Healthcare) were electrophoresed under reducing conditions. The enzyme extract was mixed with the sample buffer at 2:1 (v/v) (the sample buffer consisted of bromophnol blue 0.01% (w/v), 25% glycerol, 5% β-mercaptoethanol, and 2% SDS in 62.5 mM tris-HCl, pH 6.8), Bio-Rad Laboratories (Hercules, CA), and denatured for 10 min at 100°C. A total of 20 µl of this mixture was loaded unto Tris-HEPES-SDS- precast polyacrylamide mini gels (8 cm x 5.8 cm x 1 mm) consisting of 4% stacking gel. Electrophoresis was conducted at a constant voltage of 120°V using the Mini Protean III® electrophoresis system, Bio-Rad Laboratories (Hercules, CA) with tris-HEPES-SDS (0.025M tris, 0.192 M HEPES and 0.1% SDS, pH 8.0) as running buffer for 1 h and 30 min. The gels were stained with 0.025g Coomassie Brilliant Blue R-250 in methanol/water/glacial acetic acid (4.5:4.5:1.0 v/v) overnight, and destained with the same composition of solution but without the Coomassie Brilliant Blue Dye until the background stain was removed.

4.4.9.2. Molecular Weight Determination

The molecular mass of the fractions were estimated from their electrophoretic mobilities with reference to the low molecular weight standards (phosphorylase b-97.0 kDa, bovine serum albumin-66.0 kD, ovalbumin- 45 kDa, carbonic anhydrase-30 kDa, soybean trypsin inhibitor-20.1 kDa and α-lactalbumin – 14.4 kDa), GE Healthcare, Bio-Sciences AB.

The migration of each protein was divided by the distance travelled to the tracking dye. The distance migrated is measured for the tracking dye and for each of the protein bands. The relative mobility of the proteins (relative to the dye front) is denoted as R_f. The R_f is then calculated as follows:

R_f = Distance migrated by protein / Distance migrated by dye

A curve was generated by plotting the log of the molecular weight of the standards vs. the relative mobility (R_f), and used to determine molecular weights of unknown proteins.

4.5. RESULTS AND DISCUSSION

4.5.1. Enzyme Extraction and Partial Purification

An aliquot (20 mL) of the crude bluefish homogenate was applied to the Sephacryl S-200 column equilibrated with Buffer A as described under experimental methods (section 4.3.2.). Four major peaks (Fig. 10) were obtained all of which were tested for protein and TGase activity. Fractions containing the highest TGase activity were pooled and used for further tests.

The stepwise purification of TGase extracted from the bluefish liver homogenate is summarized in Table 10. A few workers have studied marine TGases and have reported different purification methods and yields. The purification scheme (Table 10) reveals a 1.15 fold increase in specific activity and a 2.4% recovery when the crude sample was applied to the Sephacryl S-200 column. These observed values are lower than those reported for other marine TGases (Hemung & Yongsawatdigul, 2008; Worratao & Yongsawatdigul, 2005; Nozawa *et al.*, 1997). However, TGase purification done in the above mentioned studies, used successive chromatographic procedures where gel filtration using Sephacryl S-200 was either the second or final purification step thus

decreasing the total protein content in the fractions and subsequently increasing the specific activity. Since successive chromatographic steps were not used in this study, comparison of results with previous studies would be inaccurate.

Low yields are often inevitable when working with fish material. Factors responsible for the low yields could be: - (i) low TGase content in the starting material. The bluefish liver that was used was purchased from a local store in Montreal. The bluefish sold in Montreal, is caught off the Boston, Massachusetts shore. The fish is transported to Montreal on ice and is stored in a freezer upon arrival for an undefined period of time. According to Vihelmsson (1997), endogenous TGases decreases rapidly after catch, and is almost completely destroyed by freezing. Leblanc *et al.* (1999), advocate homogenization to occur within 3 days of the animal sacrifice in order to obtain the highest TGase activity; (ii) low stability of enzyme extract or (iii) losses at various purification steps. SDS polyacrylamide electrophoresis of the Sephacryl S-200 fraction revealed 1 major band at (55.3 kDa) and 4 minor bands (Fig. 17) indicating the preparations were not completely homogenous.

4.5.2. Effect of Temperature on Enzyme Activity

The optimal temperature of the bluefish liver TGase was at 40°C (Fig. 11), similar to that obtained for crude bluefish TGase (Fig. 5). No TGase activity was detected at 70°C (Fig. 11). An optimal temperature of 50°C have been reported for tropical tilapia (Worratao and Yongsawatdigul 2005), threadfin bream liver (Hemung & Yongsawatdigul, 2008) and walleye pollack liver (Kumazawa *et al.,* 1996). Slightly higher optimum temperature of 55°C was reported in red sea bream liver TGase (Yasueda *et al.*, 1994) while TGase from scallop, Japanese oyster, and Pollock liver exhibited the optimum activity between 35 and 50°C (Kumazawa *et al.*, 1996, 1997; Nozawa & Seki, 2001). The optimal temperature of the purified TGase tends to vary with sources and as Lee *et al.* (1998) reported, could be related to the species habitat temperature.

4.5.3. Effect of Temperature on Enzyme Stability

The thermal stability data of bluefish TGase after incubation at various temperatures are shown in Fig. 12. Bluefish TGase was observed to be quite stable below

50°C and approximately 90% of the initial activity of TGase was retained after 60 min of incubation at 40°C. However at higher temperatures, the stability decreased with the TGase losing 60% of activity at 70°C. It can be speculated that the higher thermal stability of TGase was probably due to the higher habitat temperature of bluefish.

4.5.4. Effect of pH on Enzyme Activity

Partially purified bluefish TGase showed an optimum pH between 7.0 and 8.0, with an optimal pH of 7.5 (Fig. 13). The optimal pH was similar to that of crude bluefish TGase (7.0 – 7.5) (Fig. 7). These result are consitent with those obtained by Worratao and Yongsawatdigul (2002) and Nozawa *et al.*, (2001) who reported an optimum pH of 7.0 – 7.5 for tilapia muscle transglutaminase and 7.5–8.0 for squid gill, respectively. The optimum pH of fish liver TGase seems to be in the basic region (Hemung & Yongsawatdigul, 2008). A relatively high pH optimum (8.5 – 9.0) was found in red sea bream TGase (Yasueda *et al.* 1994), threadfin bream liver (Hemung & Yongsawatdigul, 2008), and walleye pollack liver TGase (Kumazawa *et al.* 1996).

4.5.5. Effect of pH on Enzyme Stability

The effect of pH on the stability of the partially purified enzymes was evaluated by measuring enzyme residual activity after incubation at various pH conditions for 30 min. Bluefish TGase was stable within a narrow pH range of 7.0 – 8.0 (Fig. 14). This result was similar to those obtained by Nozawa *et al.*, (2001) who reported squid gill TGase to be stable at pH 7.5-9.0.

4.5.6. Effect of Calcium Chloride ($CaCl_2$) and Other Divalent Cations

Partially purified bluefish TGase showed an absolute requirement for calcium ions (Fig.15), which is a typical characteristic of mammalian and fish TGases (Yongsawatdigul *et al.*, 2002). The enzyme showed no activity in the absence of Ca^{2+}, and the activity increased with Ca^{2+} concentration and reached the maximum at 1–5 mM Ca^{2+} (Fig. 15). These results indicate that partially purified bluefish TGase is Ca^{2+} dependent. TGases from various sources require different Ca^{2+} concentrations for full activation. For example, TGase from limulus hemocyte and guinea pig liver required 8

and 10 mM Ca^{2+}, respectively (Togunaga *et al.,* 1993). Red sea bream liver TGase needed only 0.5 mM of Ca^{2+} for full activation (Yasueda *et al.* 1994). Ahvazi *et al.*, (2002) reported that three molecules of Ca^{2+} were required to activate human TGase 3, which is the cytosolic TGase expressed in stratified squamous epithelia. It was postulated that the calcium ion induced conformational changes in the enzyme, which consequently exposed the cysteine located at the active site to a substrate (Jiang & Lee, 1992). Noguchi *et al.* (2001) have reported that the calcium ion binds to a binding site of red sea bream TGase molecule, resulting in conformational changes. Subsequently, Tyr covering the catalytic Cys is removed. Then, the acyl donor bind with the Cys at the active site, forming an acyl-enzyme intermediate.

The replacement of Ca^{2+} by other ions (Er^{3+}, Sm^{3+},Tb^{3+} and Lu^{3+}) could recover the activity of human TGase 3 (Ahvazi *et al.*,2002). Thus, other appropriate ions could be used to activate the enzyme even though TGase showed a Ca^{2+} dependent characteristic. Based on this study's results, Mn^{2+}, Ba^{2+} and Fe^{2+}could not completely replace Ca^{2+} for TGase activation (Table 11). The ions might not be able to induce a proper TGase conformation for catalytic reaction. Relative to Ca^{2+}, TGase activity was moderately inhibited by Mg^{2+}. Bluefish TGase was completely inhibited by Zn^{2+}, similar to TGase from other marine species (Jiang & Lee, 1992; Nozawa *et al.*, 2001; Nozawa & Seki, 2001; Tokunaga *et al.*, 1993; Tsai *et al.*, 1996). It is well known that Zn^{2+} has strong affinity towards sulfhydryl groups at the active site of TGase (Nozawa *et al.*, 1997). These results indicate that bluefish TGases could possess thiol group at the active site, similar to other tissue-type TGases (Nozawa *et al.*, 2001; Nozawa & Seki, 2001).

4.5.7. Effect of Sodium Chloride (NaCl)

NaCl slightly reduced the activity of partially purified bluefish liver TGase (Fig. 16). A residual activity of 78% was found at 15 mM NaCl. Nozawa *et al.*, (1997) reported that 0.5 M NaCl had no effect on TGase activity from carp, rainbow trout and atka mackerel, while the enhancing effect of NaCl was found in marine invertebrates such as scallop, botan shrimp and squid TGases. NaCl also activated TGase from hemocyte and Japanese oyster (Kumazawa *et al.*, 1997; Nozawa *et al.*, 2005). TGase is secreted extracellularly when the muscle of marine invertebrate is injured in order to

participate in the wound-healing process. Therefore, the enzyme from these sources is normally exposed to high NaCl content of the environment during its catalytic reaction (Nozawa *et al.,* 1997). Unlike the enzyme from the invertebrate, the activity of TGases from marine fish was not activated by NaCl, but retained at high NaCl concentrations (Hemung & Yongsawatdigul, 2008). The TGase activity of tropical tilapia, a freshwater fish, was markedly inhibited by NaCl (Worratao and Yongsawatdigul, 2005).

The effect of NaCl on TGase activity seems to relate to the environmental habitat of each species, which directly governs enzyme evolution. It should be noted that the TGase activity of the bluefish liver exhibits a high activity at 2 mM NaCl, which is a typical salt content (≈1.8% NaCl) used to solubilize muscle protein during the preparation of fish protein gels (Lanier, 2000). Protein solubilization enables the exposure of available reactive amino residues for TGase. These results indicated that bluefish TGase could be applied to a food product containing NaCl up to 15 mM without significant loss in activity.

4.5.8. Effect of Inhibitors

The enzyme was completely inhibited by the sulfhydryl alkylating agents, monoiodoacetic acid (IAA) and partially inhibited by *N'*-ethylmaleimide (NEM) (Table 12). These results strongly support the assumption that bluefish liver TGase possesses thiol groups at the active site. $(NH_4)_2SO_4$ is a well-known inhibitor acting as the product inhibitor and drastically reduced the activity of the bluefish liver TGase. Presence of ammonium seems to interfere with the activity assay (Leblanc *et al.*, 1999). Basically, TGase reacts with γ-carboxyamide groups of glutamine and releases ammonia. The excess of ammonium ions prevent further progress of the reaction (Takagi *et al.*, 1986).

4.5.9. Molecular Weight Determination

4.5.9.1. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Bluefish TGase

The purity of bluefish TGase was examined via SDS-polyacrylamide gel electrophoresis. The SDS-PAGE data represented in Fig. 17, shows the results for the protein standards (lane St.) versus the fractions from the main purification step. It is

evident from Fig. 17 that the most of the impurities were removed from fraction S-1 to fraction S-2,3 after the gel filtration step.

Based on the molecular weight of the protein standards, a standard curve of relative mobility (R_f) versus log of molecular weights was prepared and used to determine the molecular weight of bluefish TGase (Fig. 18)

Despite purification, bluefish liver TGase still contained one major band with Mw of 55.3 and four minor protein bands at lower Mw (Fig. 17) indicating that the preparation wasn't completely homogenous. This molecular weight of bluefish TGase is low compared to other TGases reported from other fish species (Table 7).

4.6. CONCLUSIONS

The following conclusions are made for bluefish liver TGase based on the studies described in this chapter.

1. Partially purified bluefish TGase, like its crude counterpart had a high temperature optimum (40°C). This finding was similar to other fish TGases such as tropical tilapia (Worratao and Yongsawatdigul 2005), threadfin bream liver (Hemung & Yongsawatdigul, 2008) and walleye pollack liver (Kumazawa *et al.*, 1996). The enzyme was found to be stable at temperatures below 50°C beyond which the activity seemed to decrease appreciably.
2. Bluefish TGase is acid labile, similar to TGase from other marine species and TGase-like enzymes from other marine organisms.
3. Partially purified TGase obtained from bluefish liver showed Ca^{2+}- dependent characteristics. The enzyme activity was inhibited by metal ions (Mn^{2+}, Zn^{2+}, Ba^{2+} and Fe^{2+}) and various TGase inhibitors.
4. The enzyme tended to retain activity at a high salt concentration.

These results revealed that TGase from the bluefish liver could be used as a potential alternative improving the textural properties of fish muscle proteins. Further purification would enable more efficient utilization of bluefish liver as a potential source of TGase.

CHAPTER V

GENERAL CONCLUSIONS AND RECOMMENDATIONS

5.1. GENERAL CONCLUSIONS

This study was conducted in two parts. In the first half, crude TGase was extracted from the muscle and liver of bluefish and was further analyzed to determine its stability. The crude TGase extract was then characterized with respect to temperature and pH. In the second section of the study, TGase was extracted and partially purified from the liver of bluefish and was further characterized with respect to various physical and chemical properties, such as temperature, pH, effects of various divalent cations and inhibition effects on the activity of the partially purified TGase. Based on experimental data and related analysis and discussion, the following interpretations were made.

i. Liver tissue appeared to be a better source of TGases than flesh tissue. Crude bluefish TGase extract was more stable at frozen temperatures (-20°C) and the activity decreased rapidly when refrigerated (4°C).

ii. Crude bluefish TGase extract exhibited an optimum temperature between 40°C and 45°C and was stable at temperatures below 50°C. The higher thermal stability of TGase could be a result of higher habitat temperature of bluefish.

iii. The optimum pH of crude bluefish liver extract for the catalytic reaction of hydroxylamine and N-CBZ-Gln-Gly was observed in the pH range of 7.0-7.5, Bluefish TGase was stable in the pH range 6.5-8.0 with a remarkable loss of activity being observed below pH 4 and above pH 8.

iv. The enzyme obtained after gel filtration migrated as one major band and four minor bands in sodium dodecyl sulphate polyacrylamide gel.

v. The optimum temperature for partially purified TGase was at 40°C. The enzyme was stable at temperatures below 50°C.

vi. The optimum pH of partially purified bluefish TGase for the catalytic reaction of hydroxylamine and N-CBZ-Gln-Gly was between pH 7.0 – 8.0 which is the optimum pH of fish liver TGase (Hemung & Yongsawatdigul, 2008). Bluefish TGase was stable in a narrow pH range from 7.0 – 8.0.

vii. Partially purified bluefish TGase showed an absolute requirement for calcium ions and no enzyme activity was observed in the absence of Ca^{2+} ions. Other divalent cations namely Mn^{2+}, Ba^{2+}, $Zn^{2+}$and Fe^{2+}could not replace Ca^{2+} for TGase activation.

viii. Unlike marine invertebrates, activity of the partially purified bluefish TGase was not activated by NaCl, but rather retained at high NaCl concentrations.

ix. The partially purified bluefish TGase completely inhibited by the sulfhydryl alkylating agents, IAA and partially inhibited by NEM. Product inhibitor, $(NH_4)_2SO_4$, reduced the activity of bluefish TGase.

5.2. RECOMMENDATIONS FOR FUTURE STUDIES

In order to better understand the biochemistry/physiology of bluefish transglutaminase, further purification needs to be conducted in order to get a homogenous extract. Future studies are recommended to focus on the following aspects:

Purified bluefish liver TGase should be used to investigate the cross-linking abilities in food systems to see if it could be a promising alternative to microbial TGases in an attempt to develop new foods with unique proteins and processing methodologies.

The amino acid composition of bluefish TGase should be determined. From the amino acid composition data, the average hydrophobicity can be calculated which gives an indication of the contribution of hydrophobic residiues to stability (Bigelow, 1967). Furthermore, a comparison of the ratio of acidic to basic amino acid residues of bluefish TGase with other TGases might help provide some insights into the pH effect on bluefish TGase activity and/or stability.

N-terminal amino acid sequence analysis of purified bluefish TGase extract should be conducted to enable comparison of bluefish TGase homology with other known TGases.

Extensive studies need to be under taken on the kinetics of TGases. Kinetics of the enzyme give an idea about the impact the enzyme will have in the industry in terms of its application. Finally the practical applications of marine TGases need to be emphasized to disclose the potential advantages of the enzyme in commercial applications.

TABLE 1: Sources of transglutaminase

Source	Reference
Mammal	
Human plasma factor XIII	Folk, (1983)
Bovine factor XIIIa	
Guinea pig liver	Folk, (1983)
Rabbit liver	Abe *et al.*, (1977)
Plant	
Pea seedlings	Icekson and Apelbaum, (1987)
Alfalfa	Kuehn *et al.*, (1991)
Microbial	
S. mobaranese	Ando *et al.*, (1989)
Physarum polycephalum	Klien *et al.*, (1992)
S. ladakanum	Tsai *et al.*, (1996)
Seafoods	
Red sea bream liver	Yasueda *et al.*, (1994)
Carp muscle	Kishi *et al.*, (1991)
Walleye Pollack liver	Kumazawa *et al.*, (1996)
Lobster muscle	Myhrman and Bruner-Lorand, (1970)
Japanese oyster	Kumazawa *et al.*, (1997)
Limulus hemocyte	Tokunaga *et al.*, (1993)
Scallop	Nozawa *et al.*, (1997)
Botan shrimp	Nozawa *et al.*, (1997)
Squid	Nozawa *et al.*, (1997)
Rainbow Trout	Nozawa *et al.*, (1997)
Atka mackerel	Nozawa *et al.*, (1997)

(Source: Ashie & Lanier, 2000)

TABLE 2: Approaches for developing industrially useful transglutaminases from various sources

Method	TGase Name	Source	Host Micororganism	Remarks	Reference
Extraction	Factor XIII	Bovine/Pork Blood		Commercialized	Wilson (1992); Jiang and Lee (1992)
	PTGase	Soybean Leaf			Kang and Cho (1996)
	GTGase	Guinea Pig Liver			Brookhart *et al.*, (1983)
	FTGase	Bream Liver, Oyster			Yasuda *et al.*, (1994); Kumazawa *et al.*, (1997)
Fermentation	MTGase	*Streptoverticillium mobaraense*		Commercialized	Ando *et al.*, (1989)
	MTGase - I	*Streptomyces* sp.			Ando *et al.*, (1992)
	MTGase - B	*Bacillus* sp.			Kobayashi *et al.*, (1998b)
	MTGase - P	*Physaram polycaphalum*			Klein *et al.*, (1992)
Gene Technology	rBTGase	*Bacillus* subtilis	*E. coli*		Kobayashi *et al.*, (1998a)
	rFactor XIII	Human Blood	*Saccaromyces* sp.		Bishop *et al.*, (1990)
	rGTGase	Guinea Pig Liver	*E. coli*		Yokoyama *et al.*, (1998)
	rFTGase	Bream Liver	*E. coli*		Ikura et al., (1998)
	rMTGase	*Streptoverticillium mobaraense* var.	*E. coli*		Takehana *et al.*, (1994)
	rMTGase	*Streptoverticillium mobaraense* var.	*Streptomyces lividans*		Washizu *et al.*, (1994)
	rPTGase	*Phytophotora* sp.	*Aspergillus* sp.		Bech *et al.*, (1996)

(Source: Motoki & Kumazawa, 2000)

TABLE 3: Overview of applications of transglutaminase in food processing

Source	Product	Effect	Reference
Meat	Beef gels	Improved water-holding capacity and textural parameters	Pietrasik and Li-Chan (2002)
	Pork gels	Effect on colour and water-binding properties	Jarmoluk and Pietrasik (2003)
	Meat products	Improved texture, appearance and structural and binding properties	Wilson (1993)
Fish	Surimi	Improved the quality and gel-forming	Tsai *et al.*, (1996); Jiang *et al.*, (2000)
Wheat	Pastry	Improved the lift of puff pastry & sensory quality	Gerrard *et al.*, (2000); Hozova *et al.*, (2003)
	Croissant	Increased shipping and shelf life	
Soybean	Polymers	Increased solubility and hydrophobicity	Yildirim *et al.*, (1995, 1996)
	Tofu	Improved texture	Kwan and Easa (2003)
Proteins	Cross-linked proteins	Improved the amino acid composition	Ikura *et al.*, (1981)
Wool	Cross-linked proteins	Increased in wool yarn and fabric strength	Cortez *et al.*, (2004)
Vegetables and Fruits	Celery	Food preservation	Takagaki *et al.*, (1991)

(Source: Ozrenk, 2006)

TABLE 4: Purification results of transglutaminase from leaves of soybean (*Glycine max*)

Step	Total protein (mg)	Yield (%)	Purification (-fold)
Crude extract	4484	100	1.0
Ammonium sulphate Precipitation (50-70 %)	886	50	2.5
DEAE-Sepharose	332	16	2.2
Blue-Sepharose CL-6B	4.3	9	95
Ω-Aminohexyl agarose	0.8	6	326
A-Casein agarose	0.01	1.1	4941

(Source: Kang & Cho, 1996)

TABLE 5: Purification results of transglutaminase from tropical tilapia (*Oreochromis niloticus*)

Purification step	Total protein (mg)	Yield (%)	Purification (fold)
Crude extract	514	100	1
DEAE-Sephacel	12.8	30.0	12.0
Sephacryl S-200	9.94	17.1	8.86
Hitrap Heparin	0.95	12.9	69.8

(Source: Worratao & Yongsawatdigul, 2004)

TABLE 6: Purification of transglutaminases from various marine organisms: A summary

Species	Step	Yield (%)
Scallop	Extract	100
	DE-52 cellulose	76
	Sephacryl S300	37
Botan Shrimp	Extract	100
	DE-52 cellulose	17
	Sephacryl S300	14
Squid	Extract	100
	DE-52 cellulose	4
	Sephacryl S300	1
Carp	Extract	100
	DE-52 cellulose	57
	Sephacryl S300	24
Rainbow Trout	Extract	100
	DE-52 cellulose	89
	Sephacryl S300	21
Atka Mackerel	Extract	100
	DE-52 cellulose	6
	Sephacryl S300	2

(Source: Nozawa *et al.*, 1997)

TABLE 7: Characteristics of some marine transglutaminases

Source	Mol Wt. (kDa)	Optimum Temp. (°C)	Optimum pH	Reference
Red sea bream liver	78	55	9.0-9.5	Yasueda *et al.*, (1994)
Carp muscle	80	-	-	Kishi *et al.*, (1991)
Walleye Pollack liver	77	50	9.0	Kumazawa *et al.*, (1996)
Lobster muscle	200	-	-	Myhrman and Bruner-Lorand, (1970)
Japanese oyster	84/90	40/25	8.0	Kumazawa *et al.*, (1997)
Limulus hemocyte	86	-	-	Tokunaga *et al.*, (1993)
Scallop	80	-	-	Nozawa *et al.*, (1997)
Botan shrimp	80	-	-	Nozawa *et al.*, (1997)
Squid	80	-	-	Nozawa *et al.*, (1997)
Rainbow Trout	80	-	-	Nozawa *et al.*, (1997)
Atka mackerel	80	-	-	Nozawa *et al.*, (1997)

(Source: Ashie & Lanier, 2000)

TABLE 8: Substrate specificity with regard to food proteins of some selected transglutaminases

	Erythrocyte TGase	**Plasma TGase**	**Bacterial TGase**
α-lactalbumin	±	±	++
β-lactoglobulin	-	±	++
BSA	+	+	++
Casein	++	++	++
Hemoglobin	-	±	±
Myosin	-	++	++
Soy glycinin	++	-	++

(Definition of symbols: -, no cross linking; ±, slow cross linking; +, moderate crosslinking; ++, fast cross linking).

(Source: de Jong *et al.*, 2001)

TABLE 9: Application and effect of transglutaminase on non-meat food proteins

Protein	Origin of TGase	Effect	Reference
Soy Proteins	Guinea pig liver	Protein polymerization	Ikura *et al.*(1980)
	Bovine plasma	Protein polymerization	Kurth & Rogers (1984)
	Human placenta	Gel formation	De Backer-Royer *et al.* (1992)
	Streptomyces sp	Gel formation	Nonaka *et al.*(1989)
	Bacterial	Protein polymerization	Basman *et al.* (2002)
Milk Proteins	*Streptomyces* sp	Formation of α-casein gels	Nonaka et al.(1989)
		Improved strength of Na-caseinate gels	Sakamoto et al(1994)
		Improved acid-induced gelation of miceellar casein	Schorsch et al.(2000)
		Altered Ca-sensitivity, hydrophobicity and viscosity of casein micelles	O'Connel & de Kruif (2003)
	Guinea pig liver	Polymerization of α-lactalbumin & β-lactoglobulin	Aboumahmoud & Savello(1990)
	Streptomyces lydicus	Polymerization of α-lactalbumin & β-lactoglobulin	Faergemand *et al.*(1997)
	Streptomyces mobaraense	Increased surface shear viscosity of Na-caseinate & casein protein film	Faergemand *et al.*(1999)

Milk Proteins	*Streptomyces mobaraense*	Improvement of water vapour permeability of a casein-gelatin film	Chambi & Grosso (2006)
		Improved gel formation rate, rheological properties and gel microstructure of acidified caseinate gels	Myllarinen *et al.*(in press)
		Improved physical and sensory properties of non-fat yogurt	Ozer *et al.*(2007)
		Cross-linking of micellar caseins in the presence of glutathionine and without heat pre-treatment	Bonisch et al.(2007)
Egg White Proteins	Bacterial	Protein polymerization & improved gelation	Lim *et al.*(1998)
Cereal Proteins	*Streptomyces* sp	Polymerization of glutenin subunits, reinforcement of gluten network	Larre *et al.*(2000)
		Polymerization of oat globulin, altered textural properties, improved water & fat binding	Siu *et al.*(2002)
		Improved thermal stability of gluten from damaged flour	Caballero *et al.*(2005)
	Bacterial	Wheat & barley protein polymerization	Basman *et al.*(2002)

(Source: Nielsen, 1995)

TABLE 10: Summary of partial purification procedure of the transglutaminase fraction from the liver of bluefish (*Pomatomus saltatrix)*

Purification steps	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (U)[a]	Specific activity (U/mg)[b]	Recovery (%)	Purification (fold)
Crude	360	6.810	2451.16	0.1099	39.56	0.016	100	1
Gel Filtration[c]	11	4.710	51.82	0.0865	0.952	0.0184	2.4	1.15

[a] Activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of peptide-bound γ-glutamyl hydroxamate per minute from CBZ-Glutaminylglycine and hydroxylamine at pH 6.0 and at 37°C.

[b] Specific activity is reported as unit of enzyme activity per milligram of protein

[c] Fractions 29, 30, 31, 32 and 33 were pooled

TABLE 11: Effect of divalent ions on the activity of bluefish liver transglutaminase

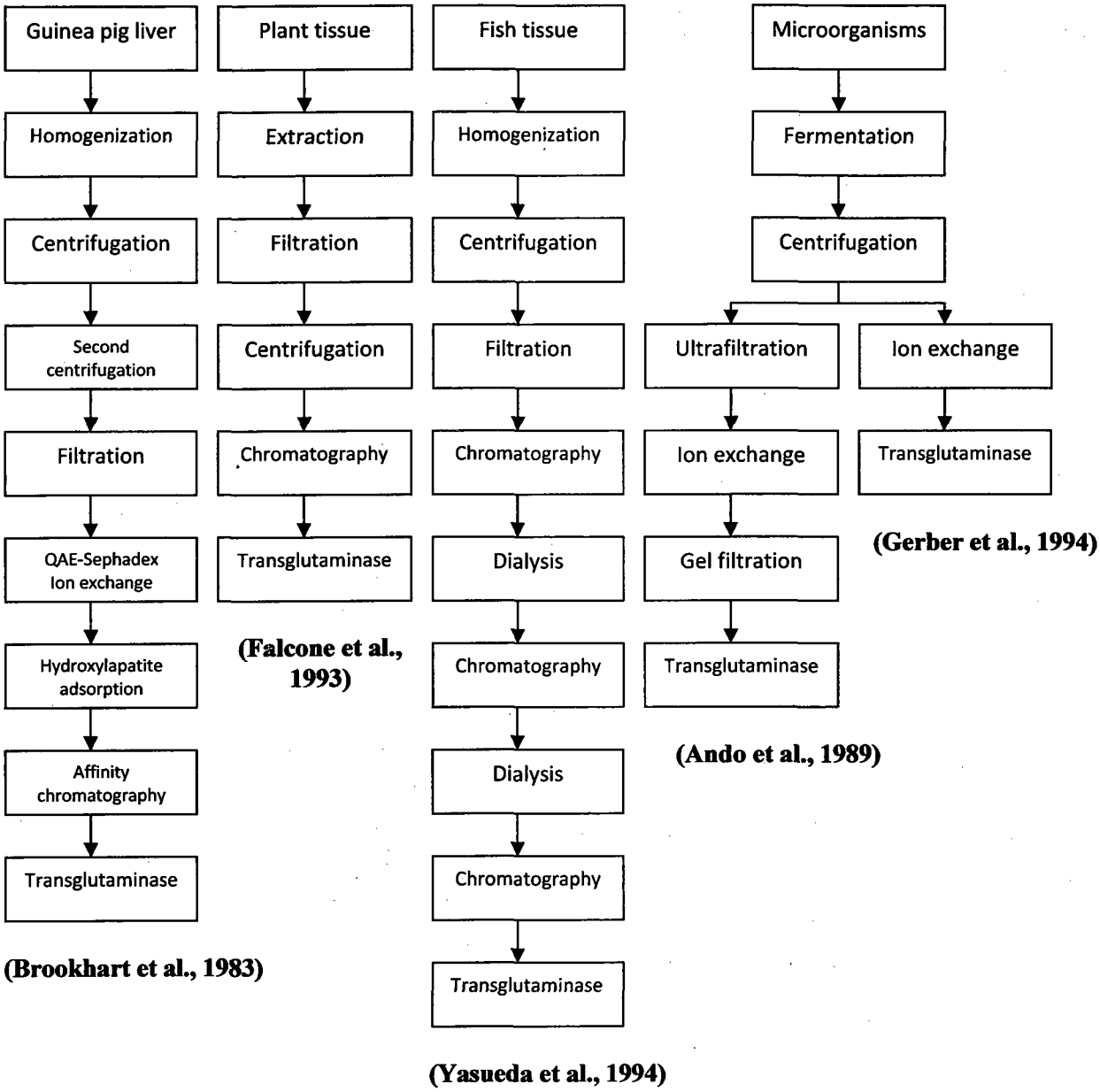
Ions	Remaining Activity (%)
Control (Ca^{2+})	**100.00 ± 3.7**
***Mg^{2+}**	**43.7 ± 6.73**
***Ba^{2+}**	**3.33 ± 6.73**
***Zn^{2+}**	**0**
***Mn^{2+}**	**1.79 ± 0.00**
***Fe^{2+}**	**6.34 ± 0.00**

* The reactions were carried out without Ca^{2+}.

TABLE 12: Effect of inhibitors on the activity of bluefish liver transglutaminase

Inhibitors	Remaining Activity (%)
Control (Ca^{2+})	**100.00 ± 1.7**
IAA	**6.4 ± 2. 3**
NEM	**21.06 ± 0.70**
$(NH_4)_2SO_4$	**26.1 ± 0.56**

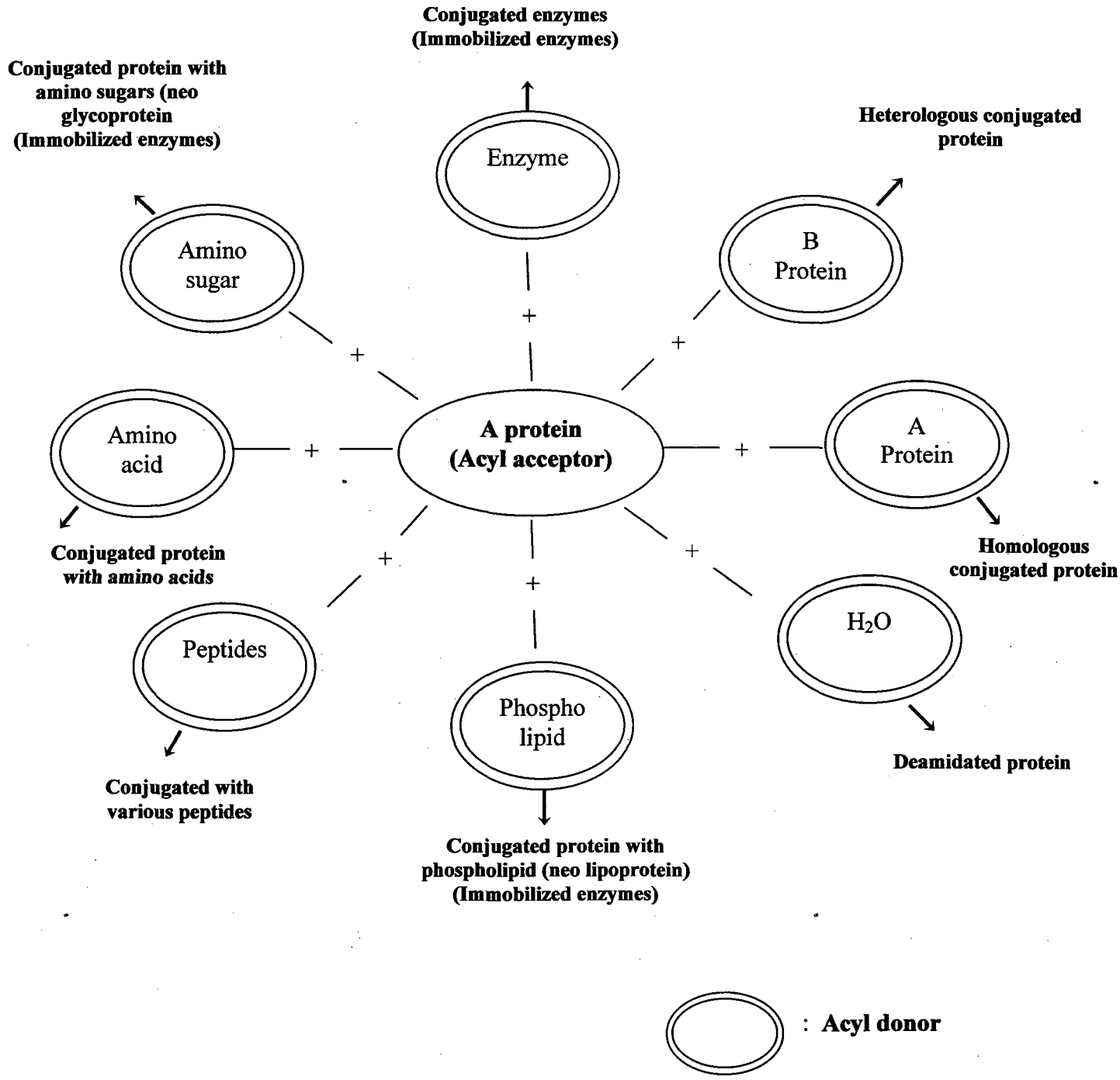
FIGURE 2: Process chart of transglutaminase production from various sources



(Source: Zhu *et al.*, 1995)

FIGURE 3: Future applications of transglutaminase

(A single-lined oval in the middle indicates a protein to be modified, and doubled-lined ovals indicate substances to be incorporated).



(Source: Motoki & Seguro, 1998)

FIGURE 4: Effect of storage time and temperature on the stability of crude muscle and liver TGase. The enzyme extract was stored at -20°C and 4°C. Residual activity was assayed with 0.1 M CBZ-L-glutaminylglycine and 2.0 M hydroxylamine as substrates at pH 7.0. (■) Frozen liver, (●) Frozen flesh, (▲) Refrigerated liver, (♦) Refrigerated flesh.

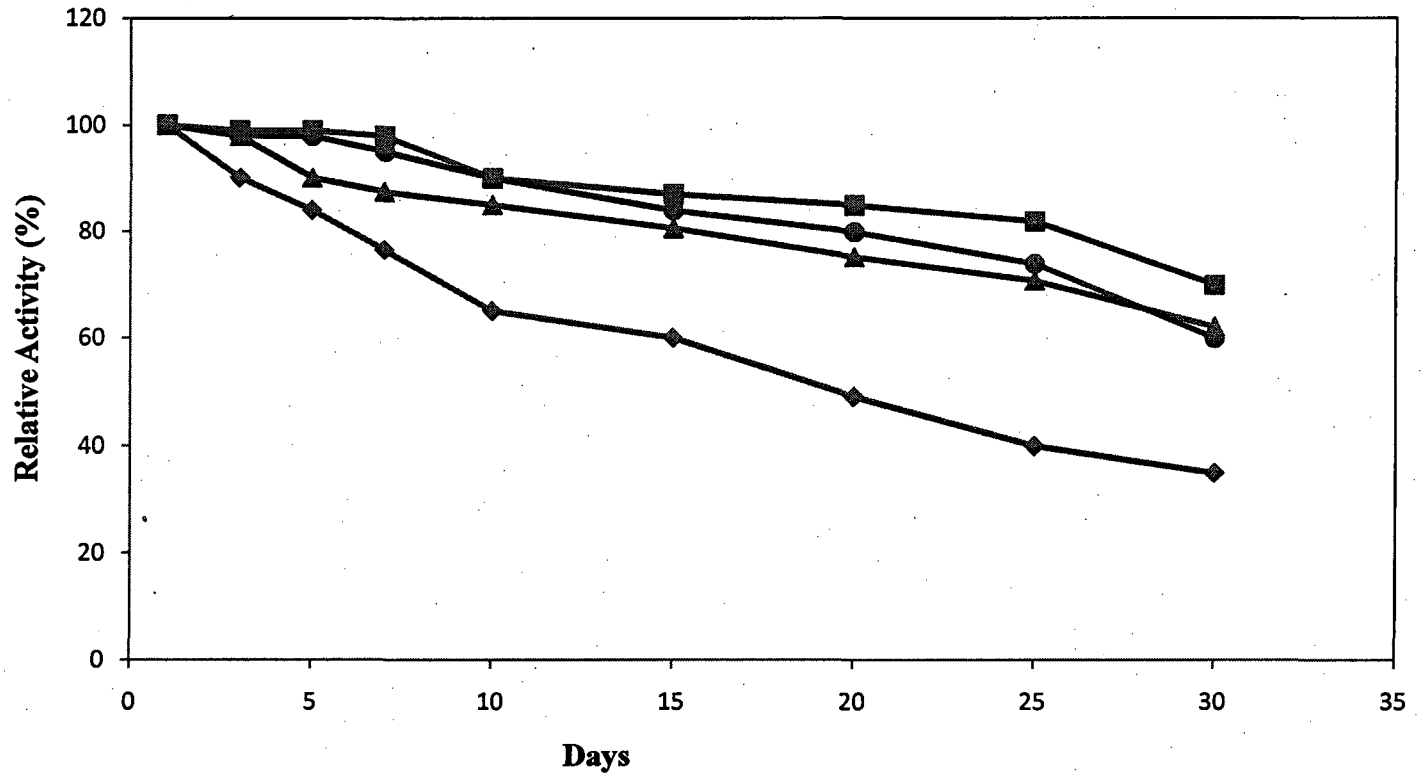


FIGURE 5: Effect of temperature on crude bluefish liver TGase activity. Activity was evaluated using 0.1 M CBZ-L-glutaminylglycine and 2.0 M hydroxylamine as substrates at pH 7.5 at indicated temperatures for 10 min.

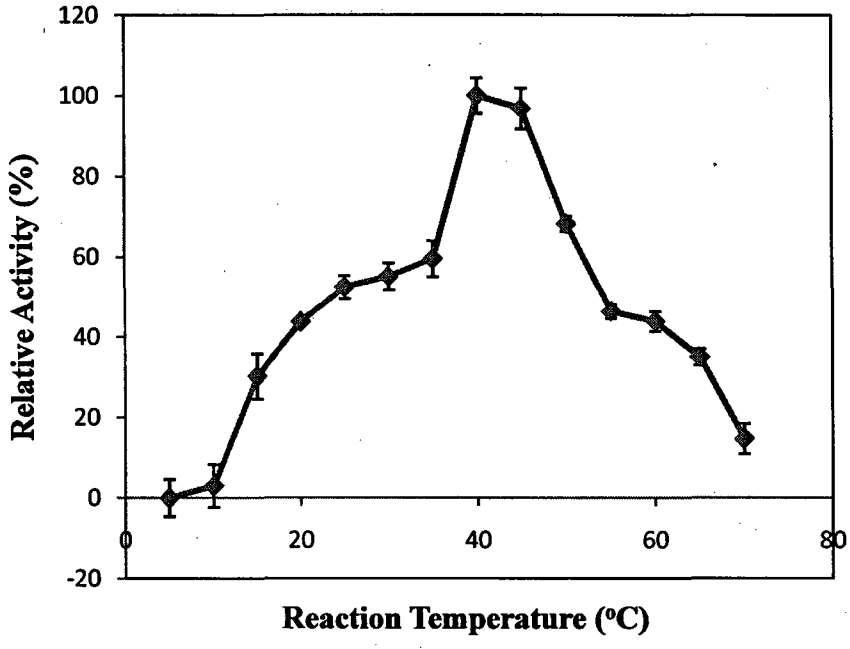


FIGURE 6: Effect of temperature on the stability of crude bluefish liver TGase. Residual activity was evaluated using 0.1 M CBZ-L-Glutaminylglycine and 2.0 M hydroxylamine as substrates at pH 7.5 at indicated temperatures for 60 min

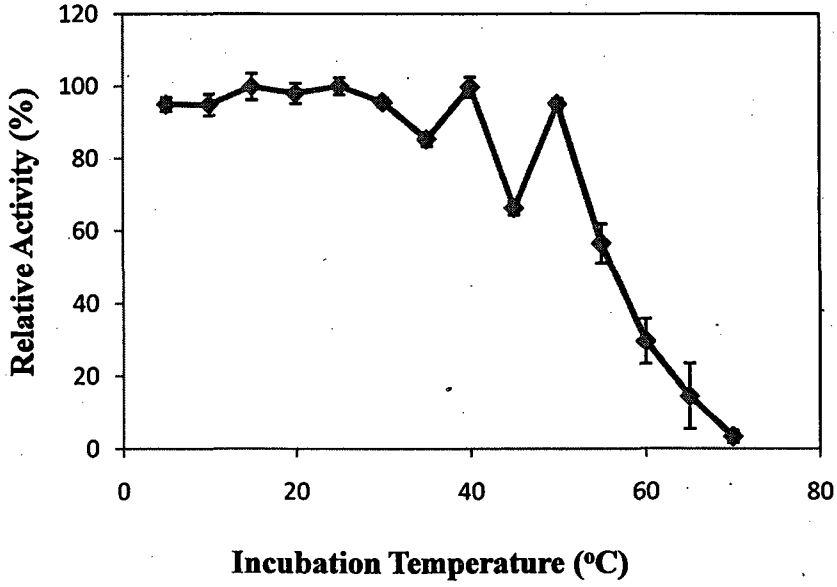


FIGURE 7: Effect of pH on crude bluefish liver TGase activity with various buffers from pH 2.0 to 10 at 35°C

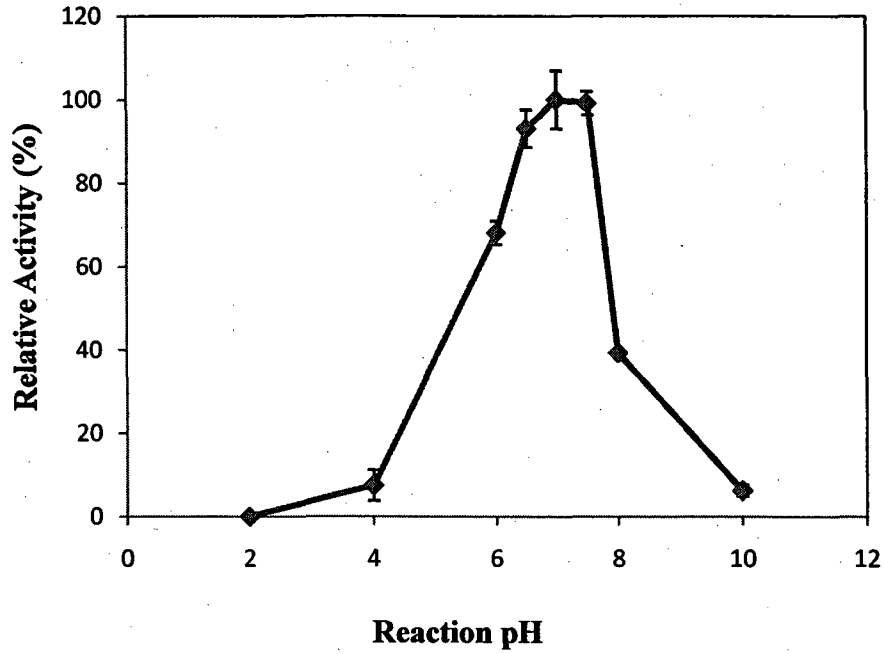


Figure 8: pH stability of crude bluefish liver TGase after 30 mins of incubation in various buffers with pH varying from 2.0 to 10 at 35°C

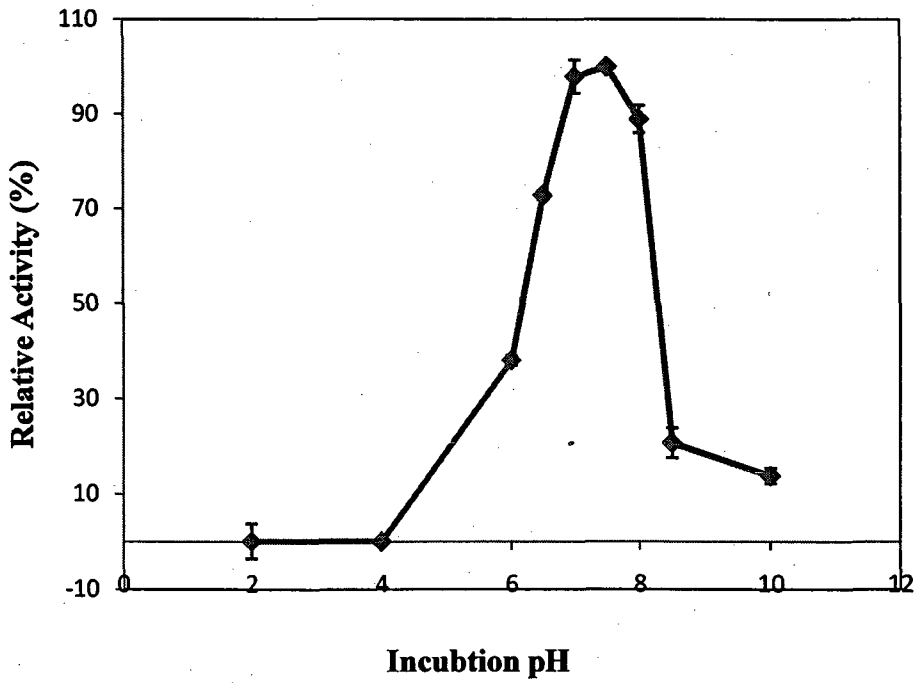
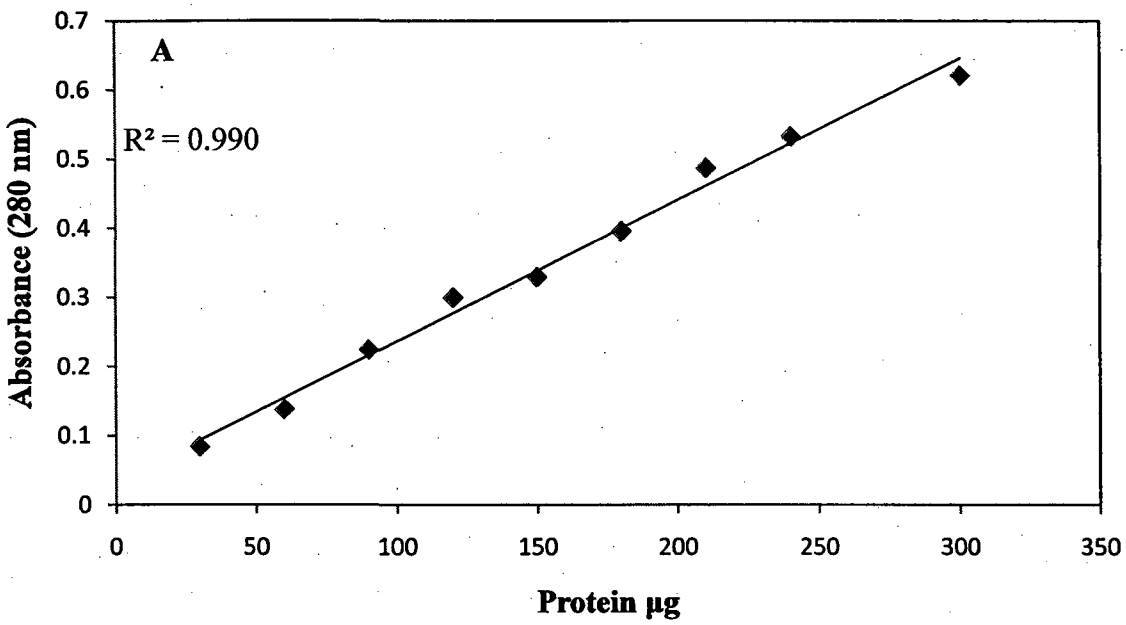


FIGURE 9: Calibration curve for protein content determination using bovine serum albumin (BSA) as standard **(A)**, standard curve for TGase activity determination **(B)**.



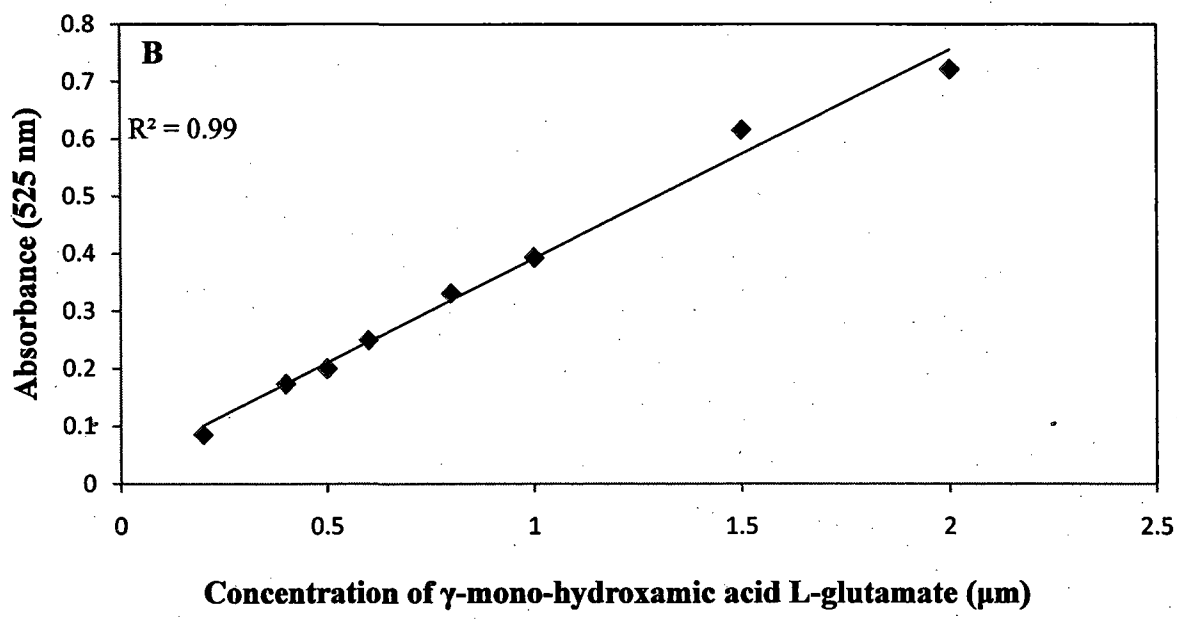


FIGURE 10: Elution profile of bluefish TGase on Sephacryl S-200 HR column. Sample volume: 15 mL. Column dimensions: 1.5 x 80 cm. Flow Rate: 2 mL/min. Elution procedure: Tris-HCl buffer, pH 7.5.

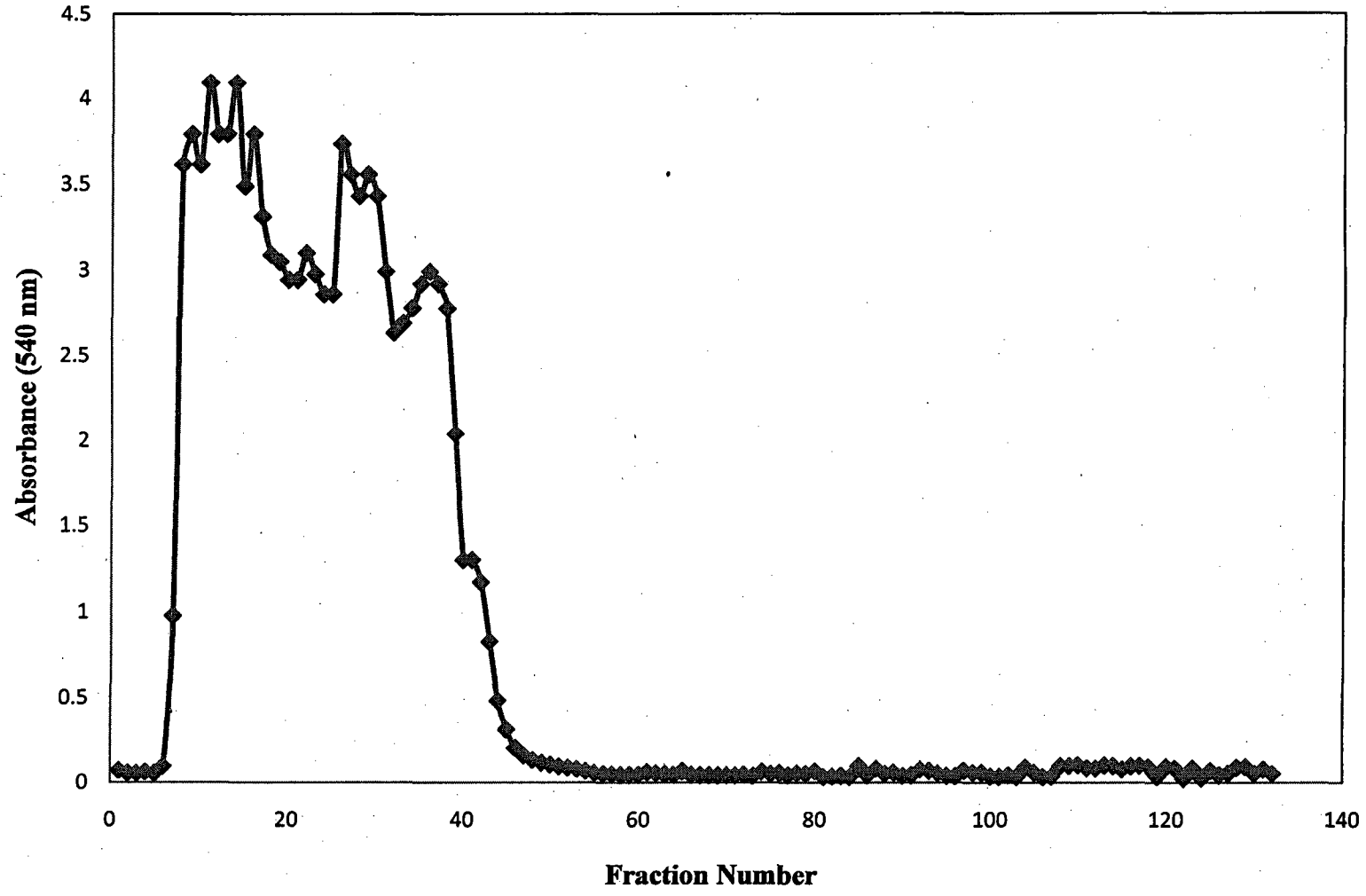


FIGURE 11: Effect of temperature on partially purified bluefish liver TGase activity. Activity was evaluated using 0.1 M CBZ-L-glutaminylglycine and 2.0 M hydroxylamine as substrates at pH 7.5 at indicated temperatures for 10 min

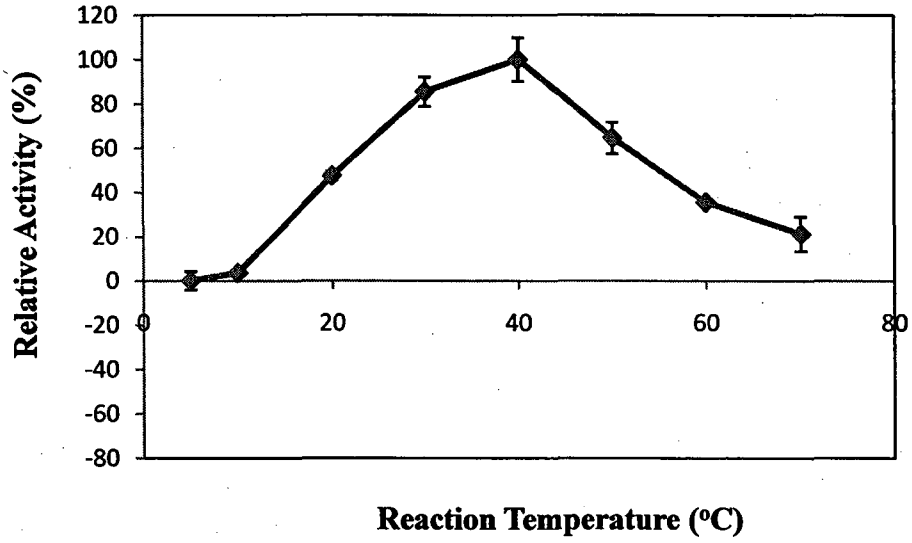


FIGURE 12: Effect of temperature on the stability of purified bluefish liver TGase. Residual activity was evaluated using 0.1 M CBZ-L-Glutaminylglycine and 2.0 M hydroxylamine as substrates at pH 7.5 at indicated temperatures for 10 min

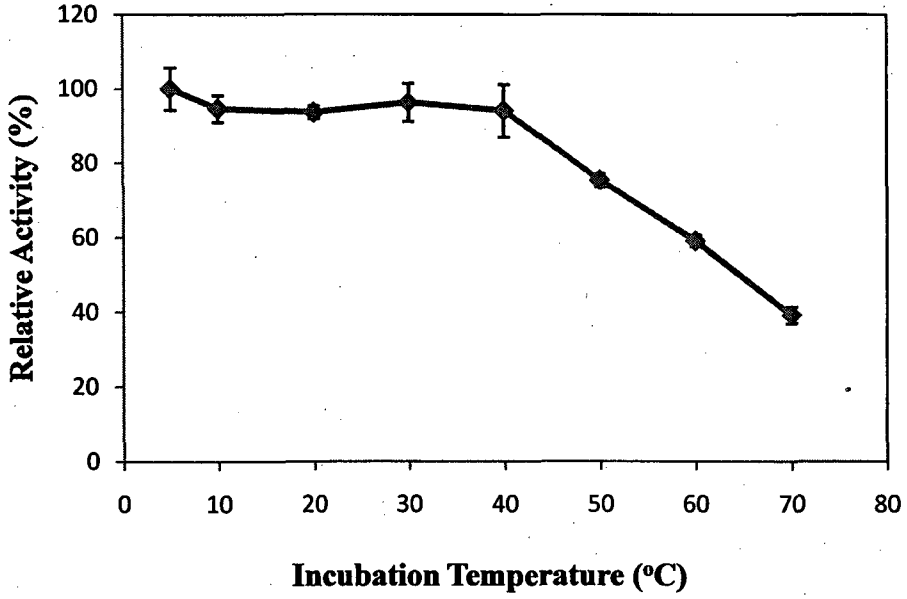


FIGURE 13: Effect of pH on the activity of the partially purified bluefish liver TGase activity with various pH from 6.0 to 9.0 at 35°C

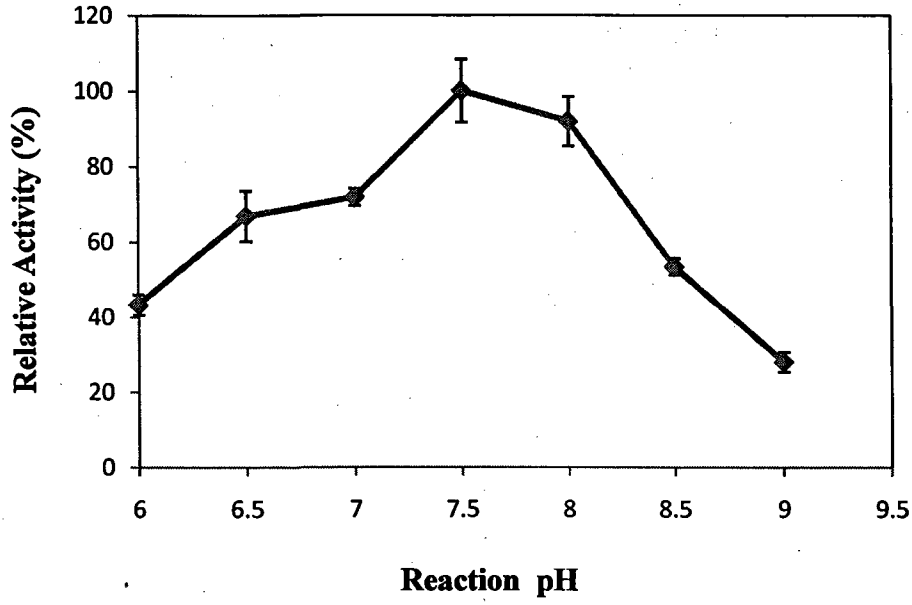


FIGURE 14: pH stability of partially purified bluefish liver TGase after 30 min of incubation in various buffers with pH varying from 6.0 to 9.0 at 35°C

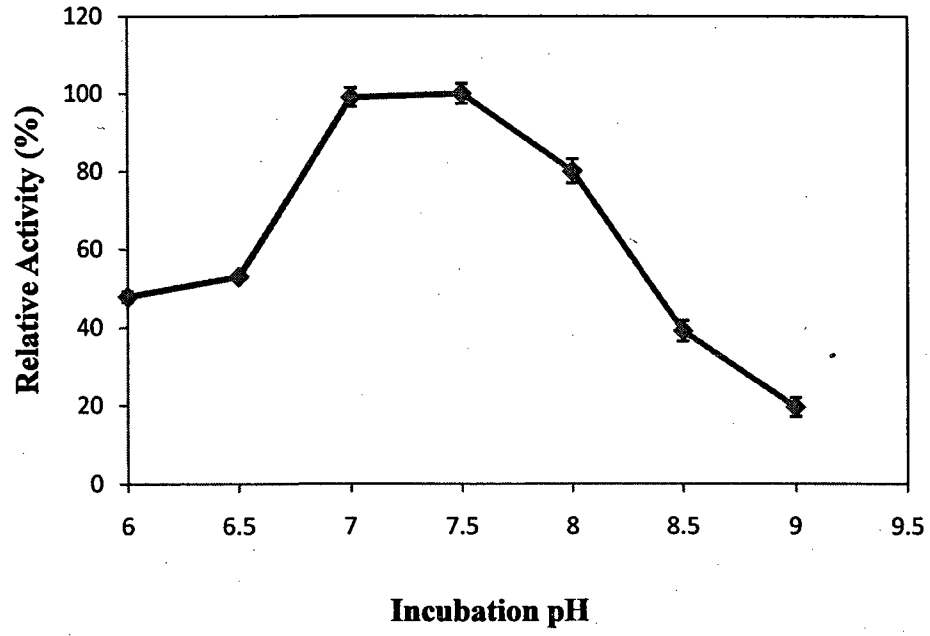


FIGURE 15: Effect of $CaCl_2$ on the activity of partially purified bluefish liver TGase

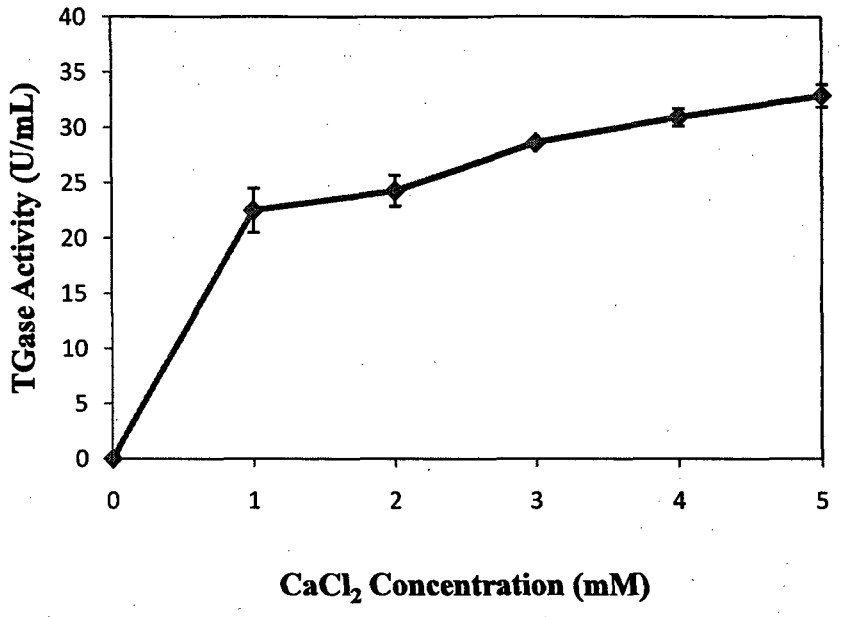


FIGURE 16: Effect of NaCl on the activity of partially purified bluefish liver TGase

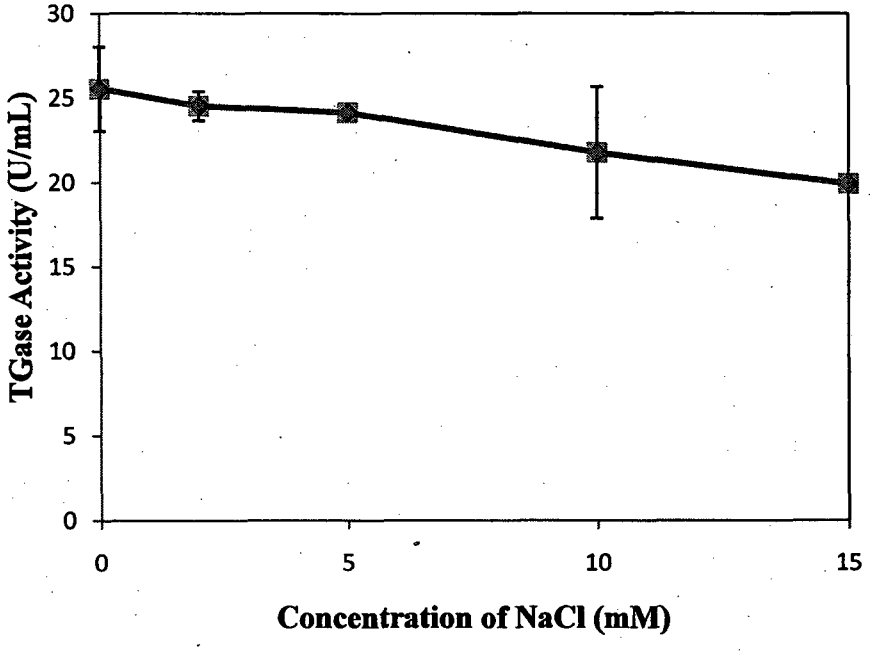


FIGURE 17: SDS-PAGE of the fractions obtained during purification of transglutaminase from the liver of bluefish.
Lane St., Standard proteins: phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kD), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Lane S-1, crude homogenate proteins; Lane S-2 and S-3, Gel filtration fraction.

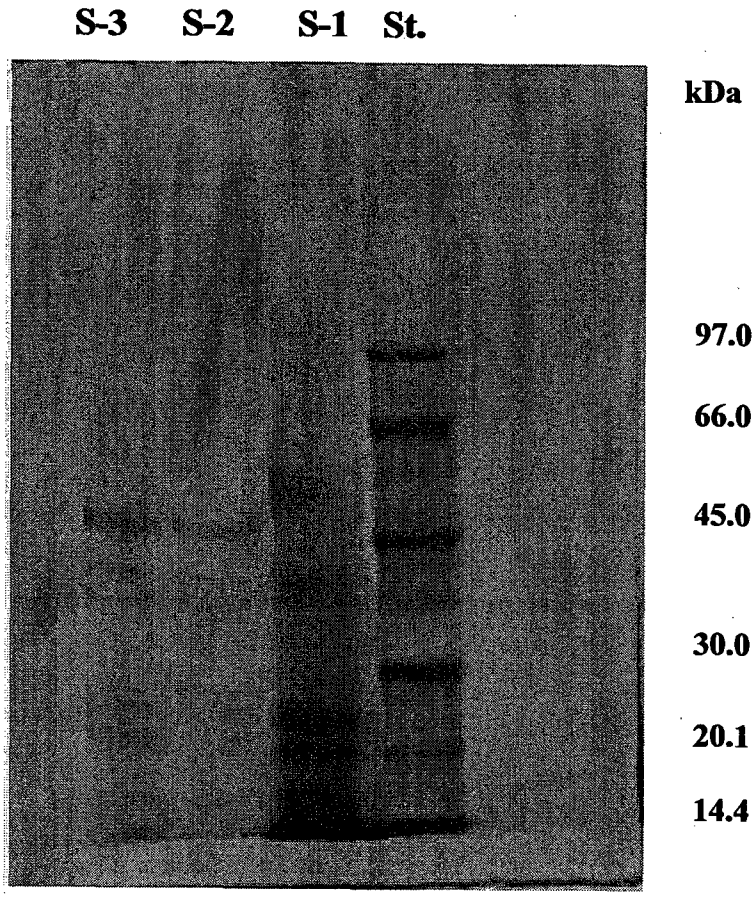
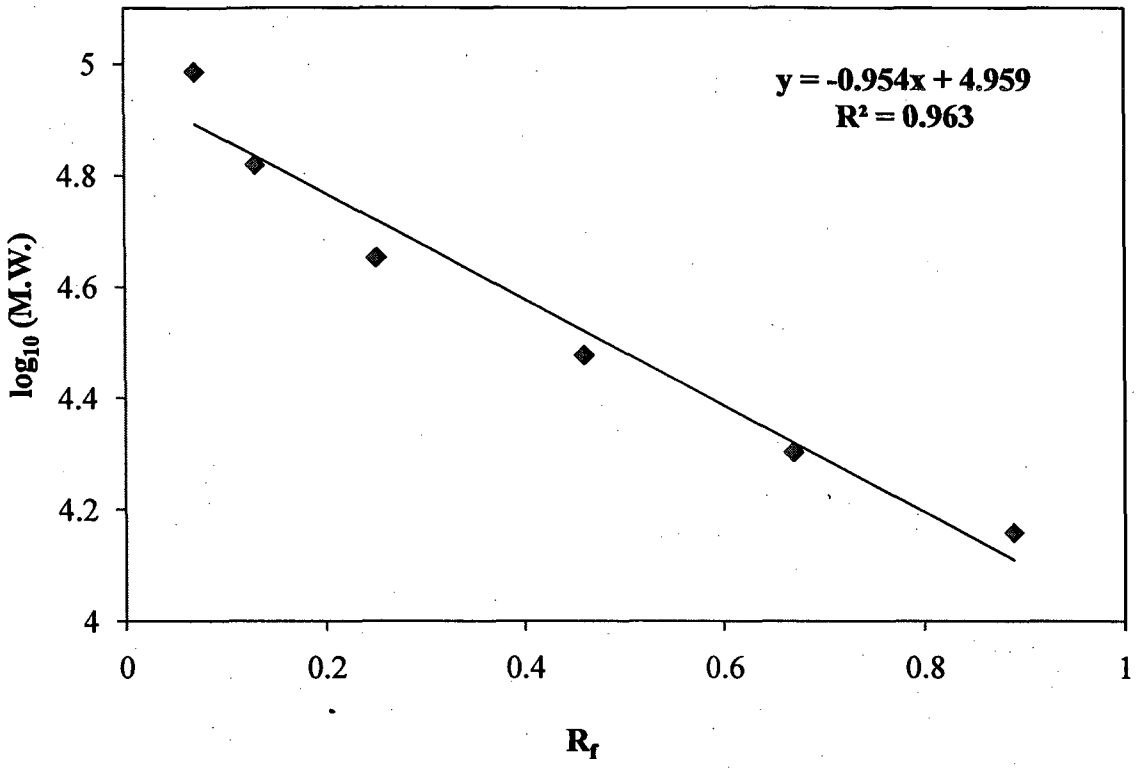


FIGURE 18: Standard curve for molecular weight determination by SDS-PAGE



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