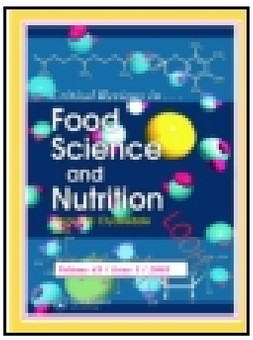

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REVIEW

Food-related transglutaminase obtained from fish/shellfish

Yi Zhang and Benjamin K. Simpson

Department of Food Science and Agricultural Chemistry, McGill University, Québec, Québec, Canada

ABSTRACT

In the past two decades, there has been an upsurge of interest in transglutaminase (TGase) research due to their immense value in food applications to improve the quality as well as functional and nutritional attributes of food. Besides, TGases have been involved in new biomaterial development and shown to have potential in various biomedical applications. Presently, TGases from microbial sources (and some animal sources) are the main forms of the enzyme used in industrial applications. Nonetheless, there are disadvantages with the commercial TGases currently in use for food processing, such as the low activity, low yield, high cost, consumer aversions on their safety, and difficult in activity recovery, among others. Fish and shellfish TGases are promising alternatives for use in food industries due to their cheap sources, high yield and special characteristics such as cold activity and thermal-lability. This review presents at the onset, the catalytic mechanism of TGase based on updated research; compares the enzymatic properties of fish/shellfish TGases with their animal, plant and microbial counterparts; summarizes the unique properties of fish/shellfish TGases related with food usage; and discusses the current and potential applications of fish/shellfish TGases in foods.

KEYWORDS

Transglutaminase; fish; shellfish; food; protein

Introduction

Transglutaminase (a.k.a. TGase) with the systematic name protein-glutamine-γ-glutamyltransferase, is assigned Enzyme Commission number EC 2.3.2.13, where EC 2 denotes they are transferases; EC 2.3 indicates they are acyl transferases; and EC 2.3.2 indicates that they are aminoacyl transferases. TGase catalyzes modification of protein molecules via covalent inter- and intra-molecular cross-linkages between the γ-carboxamide group of mid-chain glutamine residues with the free NH_2 group of peptide-bound primary amines or the ε-NH_2 group of lysine residues (Li et al. 2013), as well as directly with water for deamination. This γ-glutaminyl-peptide/amine-γ-glutamyl transferase cross-linking reaction is pH-dependent and can form isopeptide bonds within or between polypeptide chains. It can also result in integration of polyamines into proteins through glutamine side chains (Wilhelm et al. 1996). These behaviors of TGases have significant impacts on food quality such as texture, gelation stability, food consistency and mouthfeel among others. Thus, TGases have been used in a plethora of food applications in the food industry for the manufacture of baked goods, dairy products, muscle food products, imitation food products, as well as fruit and vegetable products (Fernandes 2016; Kieliszek and Błażejak 2017; Yokoyama et al. 2004). The outline of this review is summarized in Figure 1.

TGases from terrestrial animal, plant, and microorganisms

TGases are ubiquitous in living organisms, i.e., animal, plant and microorganisms (bacteria, fungi and algae); and they show structural as well as subtle functional differences. They also differ in their availability for commercial applications based on their sources.

TGases have been characterized in vertebrates, with the first mammalian TGase reported in guinea pig liver (Clarke et al. 1959) and shown to catalyze integration of polyamines into glutamine residues of protein molecules (Wilhelm et al. 1996). Animal TGases described so far have been shown to be of nine different types (eight active and one inactive). The eight active forms are Factor XIII, TGM1, TGM2, TGM3, TGM4, TGM5, TGM6, and TGM7, and the inactive form is designated as Band 4.2 (Luciano and Arntfield 2012; Thangaraju et al. 2017). They are reported to be Ca^{2+} dependent and they all share identical active sites with a catalytic triad comprised of cysteine, histidine and aspartate (or cysteine, histidine, and asparagine) despite having different primary structures (Beninati and Piacentini 2004). Factor XIII is found in blood plasma where it catalyzes cross-linking of fibrin leading to blood coagulation (AriëNs et al. 2002; Laki and Lorand 1948). Factor XIII is a tetramer of 320 kDa with two A and two B subunits. The A subunits contain the catalytic sites while B subunits are considered as

CONTACT Benjamin K. Simpson benjamin.simpson@mcgill.ca Department of Food Science and Agricultural Chemistry, McGill University, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, Québec, H9X 3V9, Québec, Canada.

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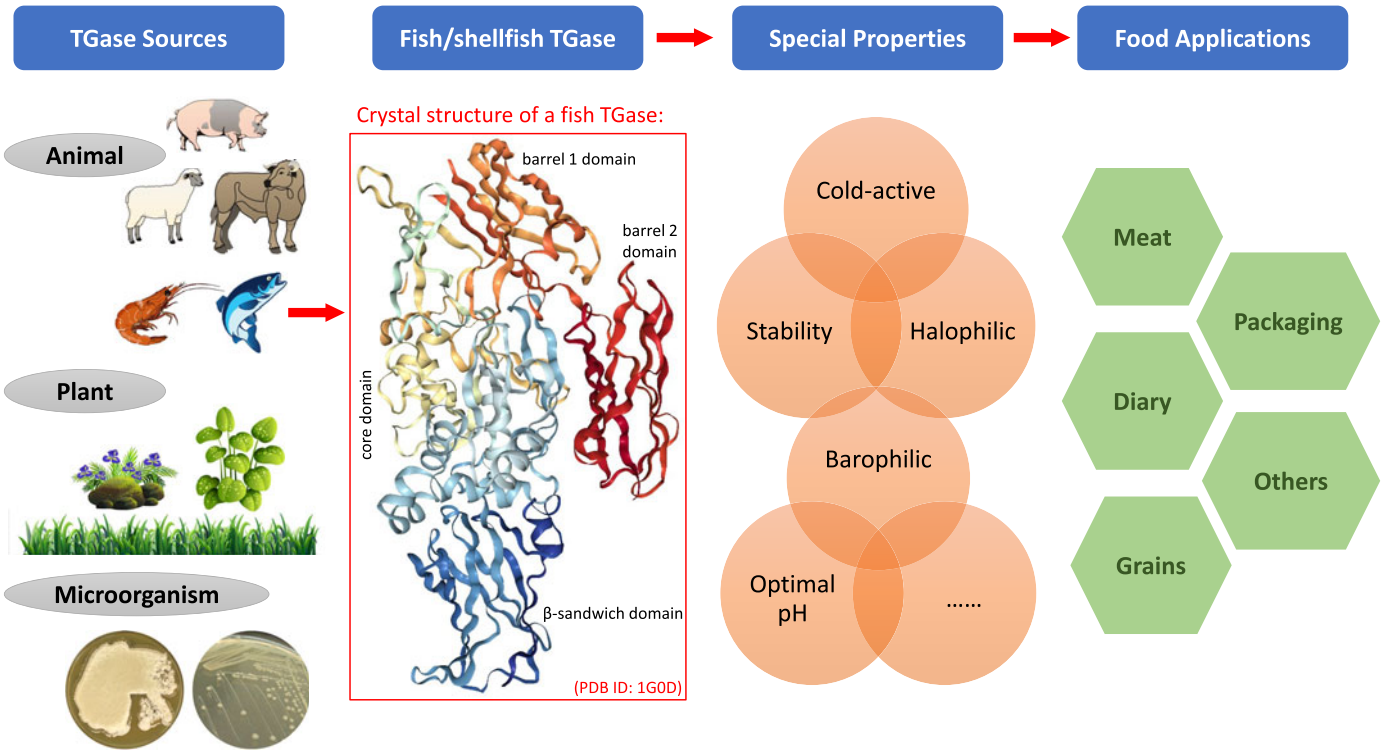

Figure 1. Outline of the review on food-related fish/shellfish TGase.

carriers of the A subunits (Radek et al. 1993). TGases have been reported in the tissues and body fluids of several vertebrate and invertebrate animals (Table 1), where the TGases obtained from fish/shellfish are listed in Table 2.

TGases have also been well characterized from several higher and lower plant sources (Table 1). Plant TGase was first reported in *Pisum sativum* seeds (Icekson and Apelbaum 1987), and has since been reported in other plant parts including the leaves, seeds and roots of sugar beet (*Beta vulgaris*), soybean (*Glycine max*), broad bean (*Vicia faba*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) chrysanthemum (*Chrysanthemum morifolium*), white lupine (*Lupinus albus*), rice (*Oryza sativa*), maize (*Zea mays*), Jerusalem artichoke (*Helianthus tuberosus*), and rosemary leaves (*Rosmarinus officinalis*) (Aribaud et al. 1995; Bernet et al. 1999; El-Hofi et al. 2014; Falcone et al. 1993; Kang and Cho 1996; Lilley et al. 1998; Siepaio and Meunier 1995; Signorini et al. 1991). In plants, TGases participate in photosynthesis (Della Mea et al. 2004), cell death (Del Duca et al. 2014) and pollination (Gentile et al. 2012). Plant TGases have low homologies with animal or microbial TGases but similar catalytic sites also comprised of the catalytic triad Cys–His–Asp as found in rice (Campos et al. 2013), as well as activation by the requisite Ca^{2+} concentration (El-Hofi et al. 2014). Plant TGase activity can be stimulated by Ca^{2+} ions; however, plant TGases do not have an absolute requirement for Ca^{2+} ions as do animal TGases. A study by Kang and Cho (1996) in fact suggested that plant TGases may be inhibited by Ca^{2+} ions at concentrations greater than 2 mM (Serafini-Fracassini et al. 1995). Other investigators have also shown Ca^{2+} ions to have an inhibitory effect at concentrations greater than 2 mM (Aribaud et al. 1995; Kang and Cho 1996). The sequences of plant TGases have little homology with their animal counterparts, except for the catalytic triad (Serafini-Fracassini and Del Duca 2008). Nonetheless, plant TGases have not been extensively characterized with respect to structure and function as compared with animal or microbial TGases; nor have their applications for food modifications been fully explored (Luciano and Arntfield 2012). This is probably due to TGases being present in low amounts in the various plant parts thus far studied (Serafini-Fracassini and Del Duca 2008).

The exorbitant costs of producing TGases from animal and plant sources inspired a search for TGases from microbial sources (Kieliszek and Misiewicz 2014). Microbial TGases (or mTGases) constitute the most abundant types of commercially available TGases. They have been reported from several sources (Table 1). The first mTGase (also the first non-animal TGase) was purified from a soil bacterium *Streptoverticillium* sp. S-8112 (Ando et al. 1989). mTGases have been found in several bacteria (Table 1), and they generally have low molecular weight (MW), and are monomeric protein molecules with MW of about 38 kDa (Ando et al. 1989). The mTGases show structural differences compared to their counterparts from animal and plant sources. Their function is independent of Ca^{2+} and GTP (guanosine-5′-triphosphate) and they also have much broader substrate specificity and lower deamidation activity (Kashiwagi et al. 2002; Ohtsuka et al. 2001). Because of their availability in relatively larger quantities and higher degree of purity, mTGases are being exploited as multipurpose modern research tools for manifold applications in the food and allied industries, such as a cross-linking agent for bonding proteins, peptides, or smaller molecules and other polymers together, and/or for site-specific covalent fusion of therapeutic proteins to polyethylene glycol to produce derivatives

Table 1. TGases from selected animal, plant, and microorganisms.

Species	Part	References
Animal source		
Bovine	Snout	Buxman and Wuepper (1976)
Canine heartworm (*Dirofilaria immitis*)	–	Chandrashekar et al. (1998)
Chicken	Gizzard	Puszkin and Raghuraman (1985)
Filaria worm (*Brugia malayi*)	–	Devarajan et al. (2004)
Filaria worm (*Onchocerca volvulus*)	–	Lustigman et al. (1995)
Frog	Liver	Assisi et al. (1999)
Guinea pig	Liver	Folk and Cole (1965)
Human	Platelet	Puszkin and Raghuraman (1985)
Porcine	Skin	Ando et al. (1988)
Rabbit	Liver	Abe et al. (1977)
Rat	Brain	Ohashi et al. (1995)
Plants source		
Apple (*Malus domestica*)	Pollen	Del Duca et al. (1997)
Barley (*Hordeum vulgare*)	Leaves; roots	Lilley et al. (1998)
Chrysanthemum (*Chrysanthemum morifolium*)	Leaves	Aribaud et al. (1995)
Garden pea (*Pisum sativum*)	Seeds	Icekson and Apelbaum (1987)
Jerusalem artichoke (*Helianthus tuberosus*)	Tubers	Serafini-Fracassini and Del Duca 2008)
Maize (*Zea mays*)	Calluses; chloroplasts	Bernet et al. (1999)
Rosemary leaves (*Rosmarinus officinalis*)	Leaves	El-Hofi et al. (2014)
Sugar beet (*Beta vulgaris*)	Leaves	Signorini et al. (1991)
Soybean (*Glycine max*)	Leaves	Kang and Cho (1996)
Tobacco (*Nicotiana tabacum*)	Flowers	Serafini-Fracassini et al. (2002)
Wheat (*Triticum aestivum*)	Leaves; roots	Lilley et al. (1998)
White lupine (*Lupinus albus*)	Seedlings	Siepaio and Meunier (1995)
Microbial sources		
Bacillus circulans		De Souza et al. (2006)
Bacillus subtilis		Kobayashi et al. (1998)
Enterobacter sp. C2361		Bourneow et al. (2011)
Physarum polycephalum		Klein et al. (1992)
Streptomyces hygroscopicus		Cui et al. (2007)
Streptomyces lydicus		Faergemand et al. (1997)
Streptomyces mobaraense		Yang et al. (2011)
Streptomyces netropsis		Yu et al. (2008)
Streptomyces platensis		Lin et al. (2006)
Streptococcus suis		Yu et al. (2015)
Streptoverticillium ladakanum		Ho et al. (2000)

with improved clinical properties (Fontana et al. 2008; Maso et al. 2018). In order to meet the growing demands for the enzyme, recombinant TGases are being produced via genetic manipulations in host microorganisms such as *Aspergillus*, *Bacillus*, *Escherichia coli*, and yeast (Cubiro et al. 2007; Li et al. 2013; Liu et al. 2011; Salis et al. 2015; Yu et al. 2008).

Production of TGase for research and commercial use

There has been extensive research conducted on the preparation of TGases from animal and plant tissues as well as from traditional microorganisms. Purified TGase can be obtained using neutral salt or cold solvent precipitation techniques followed by chromatographic separation methods. Ion exchange chromatography, especially weak anion exchangers such as diethylaminoethyl (DEAE)-Sephacel have been widely used to purify TGase from animal and plant tissues such as rat and tilapia (Tseng et al. 2008; Worratao and Yongsawatdigul 2005). Gel filtration chromatography using resins such as Sephacryl S-200 HR and Sephacryl S-300 HR have also been used for TGase purification from threadfin bream liver and squid gill (Hemung and Yongsawatdigul 2008; Nozawa et al. 2001a). Affinity chromatography is efficient based on specific interaction(s) between the enzyme and its ligand (substrate, inhibitor or co-factor). In this regard, casein-CNBr activated Sepharose 4B column, phenylalanine-CH Sepharose 4B column, and GTP-agarose column have been applied to purify TGase from rat liver and guinea pig liver (Achyuthan and Greenberg 1987; Brookhart et al. 1983; Croall and DeMartino 1986).

Microbial fermentation has shown great value in industrial preparation of TGase. *Streptomyces* spp. and *Bacillus* spp. strains are commonly used to produce mTGase (Bahrim et al. 2010; Cui et al. 2007; Zilhao et al. 2005) by the successive steps of screening, fermentation, centrifugation and purification (Kieliszek and Misiewicz 2014). In particular, since the demand for TGase is growing due to its potential applications in food processing, biotechnological and medical industries (Hitomi et al. 2016; Santhi et al. 2017), recombinant TGases from mammals, plants and bacteria have been investigated and produced in various host organisms (Li et al. 2019; Yang and Zhang 2019). A TGase from Pacific white shrimp has been expressed in *E. coli* for immunoregulation study (Zheng et al. 2018). Genetic modification techniques have been used to alter TGases to improve their catalytic properties. For example, a novel TGase engineered using rational mutagenesis and random mutagenesis exhibited higher specific activity than its wild type counterpart (Yokoyama et al. 2010); and a new thermostable TGase with 12-fold higher half-life at 60 °C compared to the natural one was produced from random and saturation mutagenesis (Buettner et al. 2012).

Commercial TGases are available as pharmaceutical, analytical and food grades. Pharmaceutical grade TGase

Table 2. Enzymatic properties of TGases obtained from fish/shellfish.

Fish/shellfish source	MW (kDa)	Activity (U/mg)	pI	Opt pH	Opt temp (°C)	Stability at 50 °C	Opt [Ca^{2+}] (mM)	References
Antarctic krill (*Euphausia superba*)	76	53.52	–	8–9	0–10	–	10	Zhang et al. (2017)
Atka mackerel (*Pleurogrammus azonus*)	–	1.10	–	–	–	–	–	Nozawa et al. (1997)
Bigeye snapper (*Priacanthus hamrur*)	73–95	47.44	–	–	37	89%	50	Binsi and Shamasundar (2012)
Botan shrimp (*Pandalus nipponensis*)	–	22.90	–	–	–	–	–	Nozawa et al. (1997)
Carp (*Cyprinus carpio*)	73–95	49.33	–	–	37	87%	50	Binsi and Shamasundar (2012)
Cod (*Gadus morhua*)	–	–	–	–	–	–	–	Malinowska-Pańczyk and Kołodziejska 2018)
Crayfish (*Pacifastacus leniusculus*)	86	–	–	8.5	22	–	–	Sirikharin et al. (2018)
European sardine (*Sardina pilchardus*)	–	–	–	–	35	26%	–	Batista et al. (2002)
Gray mullet (*Mugil cephalus*)	–	–	–	7.7	35	–	–	Lee et al. (1998)
Indian oil sardine (*Sardinella longiceps*)	73–95	66.35	–	–	37	75%	50	Binsi and Shamasundar (2012)
Japanese oysters (*Crassostrea gigas*)	84	156.6	–	8.0	40	60%	–	Kumazawa et al. (1997)
Lobster (*Homarus americanus*)	200	–	5.9	–	–	–	–	Myhrman and Bruner-Lorand (1970)
Rainbow trout (*Oncorhynchus mykiss*)	–	1.17	–	–	–	–	–	Nozawa et al. (1997)
Red sea bream (*Pagrus major*)	78	–	–	9.0–9.5	55	84%	0.5	Yasueda et al. (1994)
Salmon (*Salmo salar*)	–	–	–	–	–	–	–	Malinowska-Pańczyk and Kołodziejska 2018)
Scallop (*Patinopecten yessoensis*)	–	2.2	–	8.0	–	–	50	Nozawa et al. (1997)
Squid (*Todarodes pacificus*) gill	94	216.3	–	7.5–8.0	20	80%	50	Nozawa et al. (2001a)
Threadfin bream (*Nemipterus* SP.) liver	95	3920	–	8.5–9.0	50	–	1	Hemung and Yongsawatdigul (2008)
Tilapia (*Oreochromis mossambicus*)	73-95	69.14	–	–	50	97%	50	Binsi and Shamasundar (2012)
Tropical tilapia (*O. niloticus*)	85	196.9	6.53	7.5	50	–	1.25	Worratao and Yongsawatdigul (2005)
Walleye pollack (*Theragra chalcogramma*) liver	77	–	–	9.0	50	–	–	Kumazawa et al. (1996)
Comparison with other common sources								
Guinea pig liver	85	–	–	7.0–7.5	–	–	–	Connellan et al. (1971; Larre et al. (1993)
Human blood (Factor XIII)	300	–	–	–	–	–	–	Bishop et al. (1990)
Streptoverticillium mobaraense	40	–	8.9	6.0–7.0	50	74%	–	Ando et al. (1989)

products are usually expensive with relatively low yield. Examples are Factor VIII and Factor IX that function as blood clotting agents, and have been manufactured by recombinant gene technology with a market value estimated at >2 billion USD (Schmid et al. 2016). Analytical grade TGase includes lyophilized TGase powder isolated from guinea pig liver (≥1.5 U/mg protein) or recombinant guinea pig liver, human, and mouse TGase products distributed by Sigma-Aldrich Co. and AbCam Inc. Food grade TGase products have received more attention in recent years because of its preferred utilization for food texture modification (Nawong et al. 2016). ACTIVA® TGase produced by microbial fermentation by Ajinomoto Co., Japan, and Saprovia L® by PMT Trading Co., Poland, are the two most popular types in the food industry. Production of industrial TGase is rising in Asia, Europe and North America.

Catalytic mechanism

TGase catalyzes transfer reaction between the acyl groups in glutamine and the amine groups in alkyl amines to form a cross-linked molecule N^5-alkyl-glutamine with ammonia molecule as co-product. The substrate recognition mechanism by TGase is still less well understood. However, there is common consensus that TGase exhibits strong selectivity for glutamine residues in protein substrates which act as acyl donor. Studies with tissue TGase (tTGase, tTG, or TG2) and Factor XIII TGase (FXIII) suggest preferred amino acids sequences of 4-12 amino acids residues containing glutamine (Hitomi et al. 2009; Sugimura et al. 2006). Lysine is the usual acyl acceptor for the cross-linkage reaction catalyzed by tTGase and FXIII, and these two TGases have clear preference for the residues preceding lysine in proteins, which are serine, alanine, leucine, tyrosine and asparagine (Grootjans et al. 1995). Various groups like carboxyl groups, aromatic groups and saccharides can be incorporated into glutamine containing proteins with appropriate primary amines as spacers (Ohtsuka et al. 2000). Different from tTGase, mTGase has broader tolerance to both natural and non-natural acyl-acceptor substrates such as some esterified α-amino acids (Gundersen et al. 2014).

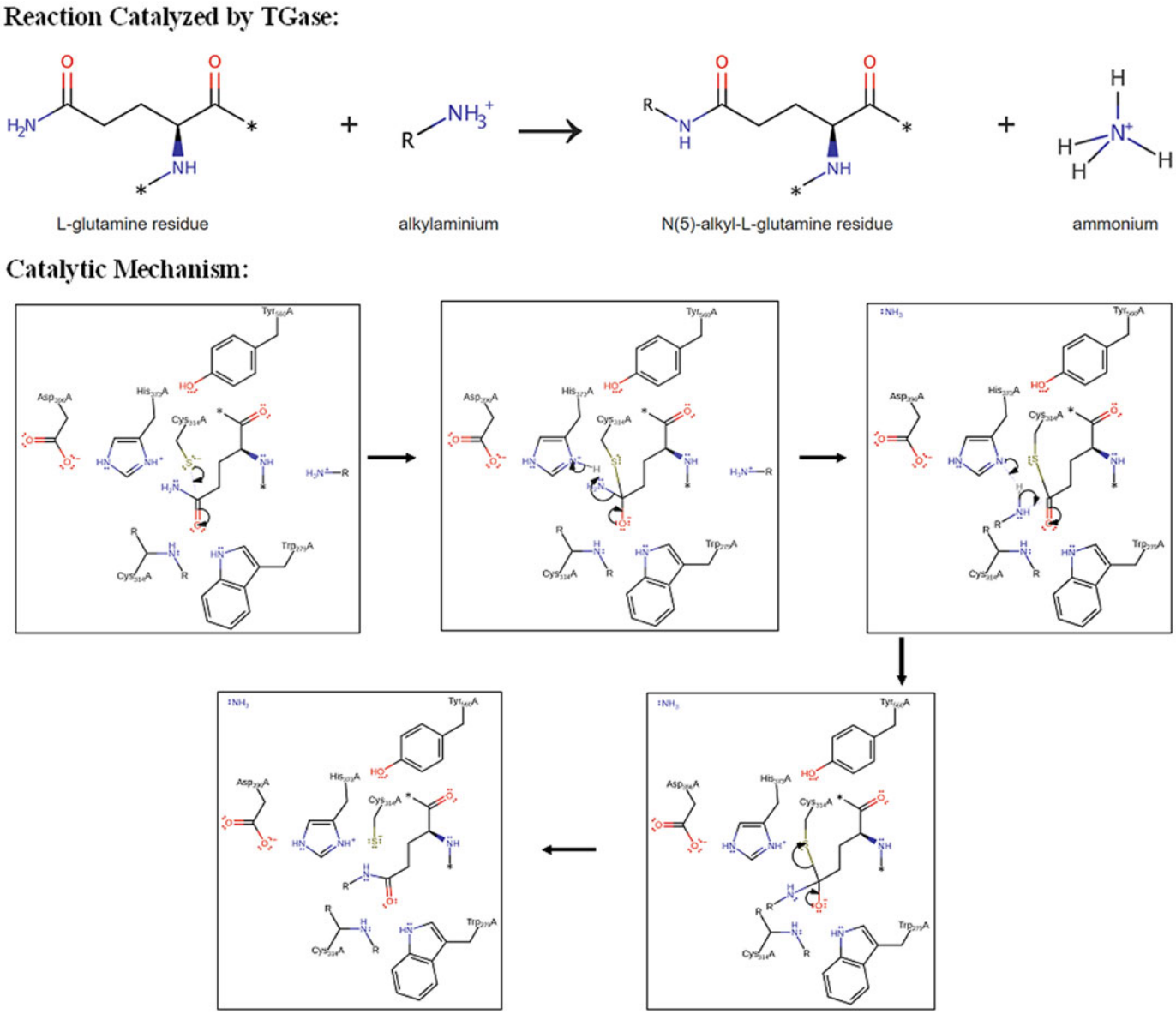

Figure 2. Catalytic reaction and process of tissure TGase.

The catalysis process of TGase is closely related to its three-dimensional structure. Components such as Ca^{2+}, guanine nucleotides (i.e., GTP, GDP, guanosine 5′-diphosphate), GMP (guanosine 5′-monophosphate) can modulate both structure and function of TGase. For example, the transamidation activity of tTGase is allosterically activated by Ca^{2+} but inhibited by GTP, GDP and GMP. Thioredoxin has been shown to activate extracellular tTGase (Jin et al. 2011). Crystal structures of TGases derived from humans including Factor XIII, human TGase 2 and TGase 3 (Ahvazi et al. 2002; Han et al. 2010; Yee et al. 1994), red sea bream fish liver tTGase (Noguchi et al. 2001) and *Streptomyces mobaraense* TGase (Kashiwagi et al. 2002) have been reported. Taking red sea bream liver TGase as an example, it contains 4 domains with a total of 695 amino acid residues (Noguchi et al. 2001), as shown in Figure 1. The β-sandwich domain consists of one α-helix and two four-stranded sheets; the core domain consists of 11 α-helices and 12 β-sheets. Barrels 1 and 2 domains consist of seven β-sheets, respectively; and the active site is Cys_{272}–His_{332}–Asp_{355} located in the core domain (Noguchi et al. 2001). Due to the high similarity between fish/shellfish TGase and human TGase, their postulated catalysis process is basically the same, and the stepwise catalytic mechanism is shown in Figure 2 obtained from the M-CSA website (https://www.ebi.ac.uk/thornton-srv/m-csa/) (Ribeiro et al. 2018). Firstly, three calcium ions bind to residues in the core domain, which can either drag the structure or rotate the structure slightly (Ahvazi et al. 2002) under low concentration of guanine nucleotides such as GTP and GDP (Ahvazi et al. 2004). Then a substrate glutamine residue arrives in the acyl binding site located around the two-barrel domains which probably causes large conformational change of TGase. The structural change damages the disulfide bonds between Cys_{272} and Cys_{333} around the active site, as well as the hydrogen bond between Cys_{272} and Tyr_{515} to totally expose the active site (Noguchi et al. 2001). TGase then switches from a compact inactive form to an extended active

conformation. Next, the glutamine residue arrives at the active site in the core domain, and the thiolate of Cys from the catalytic triad attacks the carbonyl carbon of glutamine (Keillor et al. 2014), as shown in the first square in Figure 2, and this process is considered to be the rate-limiting step (Iismaa et al. 2003). The formed oxyanion on glutamine initiates the elimination of ammonia as shown in the second square. The His residue from the catalytic triad deprotonates the other substrates such as lysine, primary amines or water, which attacks the thioester carbonyl of the acyl-enzyme intermediate (Siegel and Khosla 2007), as shown in the third square. The oxyanion initiates the elimination of Cys to produce the final product and generate the native state of TGase, as shown in the fourth and fifth in Figure 2.

The comparison on the structures of red sea bream TGase, human Factor XIII, and human TGase 3 binding of calcium shows that their structures are similar, and their active sites are conserved in amino acids sequence. However, there are a few structural and electrostatic differences especially in the catalytic sites, which may cause the easier activation, broader substrate specificity and lower inhibitor selectivity for tTGase (Noguchi et al. 2001). Tissue TGase has been demonstrated to catalyze various reactions based on the substrates type, making it also function as a deamidase (Ohtsuka et al. 2001), GTPase (Fesus and Piacentini 2002), kinase (Mishra and Murphy 2006), and protein disulfide isomerase (Hasegawa et al. 2003).

Inhibition mechanism

TGase inhibition by various agents has been investigated and classified as: i) reversible and competitive inhibition, where the inhibitors bear structural resemblance with TGase substrates such that they can also bind or occupy the active binding pocket of TGase. Examples of inhibitors of this kind include GTP (Bergamini et al. 1987), cystamine (Jeitner et al. 2005), and cinnamoyl triazole (Pardin et al. 2008), all of which have been shown to inhibit TGase by serving either as amine replacement or catalytic site cover; ii) reversible and noncompetitive inhibition of TGase by which the inhibitors such as N-Me-casein (Case and Stein 2007) and the oligopeptide SQAETYR (Yang et al. 2011) could bind to TGase away from the binding site and impede catalyzes; and iii) irreversible inhibition of TGase where the inhibitors such as 4-aminopiperidine (Prime et al. 2012), dihydroisoxazole (Choi et al. 2005) and α, β-unsaturated amides (de Macédo et al. 2002), covalently bind with TGase. Such inhibitors normally contain reactive chemical groups that attack the cysteine in the active site in the presence of nucleophilic atoms (Siegel and Khosla 2007). In addition, extreme heat treatment can cause irreversible unfolding of the higher orders of structures of the enzyme and cause irreversible enzyme inactivation. At temperatures above 60 °C, TGase starts to unfold with the rearrangements mainly in helices (Aprodu et al. 2013) followed by aggregation of the enzyme molecules (Nury and Meunier 1990).

TGase inhibitors are normally small, designed and synthesized compounds, which were developed since the 1980s. A peptidomimetic irreversible inhibitor of TGase has been used in clinical trials for celiac disease treatment (Keillor and Apperley 2016). Moreover, tTGase inhibitors are useful in biological model studies on numerous diseases such as celiac sprue (a disorder of the digestive tract that causes an intolerance to gliadin), neurodegeneration and cancer, suggesting that such inhibitors are of therapeutic significance for human health (Chen et al. 2015; Lee et al. 2015; Wilhelmus et al. 2014). Natural and endogenous TGase inhibitors are uncommon in nature, except for a few examples, such as a component in bovine, goat, sheep and human milks (Jong et al. 2003) and a high molecular weight substance produced by *Streptomyces lavendulae* Y-200 (Ikura et al. 2000) that was shown to have TGase inhibitory activity.

Importance of discovering TGases from fish/shellfish

Problems with currently-used TGases

The animal TGase currently in use is predominantly obtained from guinea pig liver. The extraction and purification procedures are invariably intricate, tedious, and the enzyme instability during purification results in low yields and high cost (El-Hofi et al. 2014; Zhu et al. 1995). These drawbacks coupled with environmental issues on the use of animals as source materials for producing TGases have diverted attention to microbial and recombinant TGases as alternatives or replacements. However, microorganisms are not always the perfect source. Some microorganisms produce unsafe toxic metabolites and/or antibiotics during fermentation, which may be co-extracted with the enzymes. Furthermore, the culture media such as the peptone and yeast extract for TGase-producing *Streptoverticillium* strains is expensive (Kieliszek and Misiewicz 2014); and the activity of TGase produced by microorganisms has no clear superiority to the ones derived from fish and shellfish. For instance, activities of TGase from *Streptomyces* sp. polar strains and recombinant *E. coli* strains were 0.43 U/mL and 22 U/mg protein, respectively (Bahrim et al. 2010; Liu et al. 2011), which is lower compared with TGases characterized from threadfin bream (*Nemipterus* SP.) liver, squid (*Todarodes pacificus*) gill and tropical tilapia (*O. niloticus*) with activities of 3920, 216.3, and 196.9 U/mg protein, respectively (Hemung and Yongsawatdigul 2008; Nozawa et al. 2001a; Worratao and Yongsawatdigul 2005). Microbial strains for TGase production can be native, variants or genetically engineered, and they must be nonpathogenic, nontoxigenic, and stringently evaluated for safety for food use, which is tedious and expensive to achieve. Generally, microorganisms that are considered safe for enzyme preparation include *Aspergillus niger*, *Bacillus subtilis* and yeast. The genetically modified microorganisms (GMOs) for producing enzymes need to be identified and characterized; and the inserted DNA and related techniques need to be investigated and described (Sewalt et al. 2016). Such safety evaluations are considerably costly. Moreover, the GMOs must be stable without strain drift, and the manufacturing must be in good practice and maintained hygienically and consistently to

prevent any contamination. Use of GMOs for producing TGase usually aims to improve the productivity and enzymatic characteristics such as thermal stability and optima pH, for better adoption for industrial utilization. However, enzymes expressed from GMOs may not be active because of protein folding and misfolding, which are influenced by both the intrinsic amino acid sequence and various factors from cellular milieu (Dobson 2003). Apart from the activity problem, enzymes from GMOs cannot be guaranteed to have the expected enzymatic characteristics. A lyase from *Photobacterium profundum* SS9 showed high pressure tolerant property but lost this property after expression in *E. coli* (Phillips et al. 2011). Recombinant TGases from GMOs are relatively less studied, but Pinkas et al. (2007) stated that it is the natural conformation of different TGases that determine their activation, catalysis and inherent functions.

Specialty enzymes from aquatic habitats

The aquatic and terrestrial habitats differ in important respects, such as temperature range, pH, salinity, pressure and oxygen tension, etc. The temperature of the aquatic habitat depends on both the latitude and depth. The average surface water temperature hovers around 17 °C with a high estimated at about 30 °C in the tropics to a low of −2 °C near the poles (National Oceanic and Atmospheric Administration 2018). The salinity of ocean ranges from 3.3–3.7%, while pH ranges from 7.5–8.5 (United States Environmental Protection Agency 2011). The average hydrostatic pressure in ocean is 38 MPa, and it increases by about 1 atmosphere for every 10 m depth (Vossmeyer et al. 2012). Organisms inhabiting normal and extreme marine and freshwater habitats have evolved various strategies to enable them adapt to the particular environments, such as structural and functional differences of their biomolecules, e.g., nucleic acids and proteins (including enzymes) (Rothschild and Mancinelli 2001). The adaptive strategies of enzymes from fish/shellfish acclimated to cold temperature regimes such as Arctic, sub-Arctic and Antarctic zones include: high catalytic activity at low temperatures made possible by their higher flexible structures and lesser binding tenacity with substrates; higher degree of conformational complementarity with their substrates; and high susceptibility to heat inactivation (Cummings and Black 1999). Cold-active enzymes are also excellent processing aids. Examples of commercially available cold-adapted enzymes include the protease and savinase from Novozyme Co. Moreover, food industries in Japan, EU and USA have exploited cold-active enzymes such as proteases and TGases from marine species for manufacturing food products such as fish peptones for biomass production, fish paste and heat-gelled products such as kamaboko, itatsuki, satsuma-age (Cavicchioli et al. 2002). Furthermore, enzymes from some marine organisms, e.g., alginate lyase from deep sea *Agarivorans* sp. (Kobayashi et al. 2009), proteases from deep sea fungi (Damare et al. 2006) and lipase from marine sponge *Ircinia* sp. (Su et al. 2015), have been shown to be adapted to mildly alkaline pH, and optimally stable and active at neutral to weakly alkaline pH (pH 7–9). This has been attributed to a relatively higher basic to acidic amino acid ratios (Simpson and Haard 1984). However, the intercellular pH of the marine species (sources of the enzymes), is usually different from what prevails in their surroundings, for reasons such as differences in cell surface charges and Na^+/H^+ membrane transport proteins (Sarethy et al. 2011). Such "alkaline enzymes" have potential usage in food industry with respect to catalysis in high alkaline conditions, as well as in laundry and detergent industry. Salt tolerant or halophilic enzymes found in some marine species, such as an α-amylase isolated from marine bacteria were shown to contain a high proportion of the polar amino acids serine and threonine, as well as acidic amino acids like aspartic acid and glutamic acid, which contribute to enzyme surface hydration, electrostatic interactions and salt bridge building (Delgado-García et al. 2012). Furthermore, halophilic enzymes tend to be thermostable, and active/stable over a broad pH range (Aygan and Arıkan 2008; Prakash et al. 2009). They also require relatively high salt (NaCl or KCl) concentrations above 1 M to be active and stable (Sellek and Chaudhuri 1999), which suggest structural and functional integrity in low water activity or organic solvent milieu. Halophilic enzymes (including various proteases, lipases, amylases, cellulases, chitinases, and xylanases) can find applications in the food and beverage industries for baked goods manufacture, brewing, cheesemaking, starch saccharification, and in high salt-containing fish sauce fermentations (Li and Li 2009; Patel and Saraf 2015). Some halophilic enzymes also find uses in other industries such as detergent, textile and tanning (Aygan and Arıkan 2008; Li and Li 2009; Ratnakar 2013), as well as biofuel (Pikuta et al. 2007; Wang et al. 2009) and pharmaceutical (Jaeger and Holliger 2010) industries. There are other extremozymes derived from aquatic species inhabiting great ocean depths that have been shown to be high pressure-tolerant or barophilic (also known as piezophilic). Such enzymes are found to have a high content of amino acids such as arginine, glycine, serine, aspartic acid and valine, but low in amino acids such as tryptophan, tyrosine and cysteine (Nath and Subbiah 2016). Examples of such enzymes include a dihydrofolate reductase isolated from the psychropiezophilic bacterium *Moritella profunda* that inhabits ocean depth with temperature and pressure of about 2 °C and 280 bar, respectively (Hay et al. 2009); and a tryptophan indole-lyase isolated from a marine piezophilic bacterium, *Photobacterium profundum* SS9 (Phillips et al. 2011). These enzymes were reported to retain full activity under hydrostatic pressure up to 100 MPa (Phillips et al. 2011); thus, they can be used in high pressure processing industries, such as a piezophilic protease in high pressure cooking for tenderizing meat, as well as formation of protein gels and starch granules (Demirjian et al. 2001; Simonin et al. 2012).

Fish/shellfish as source of TGase

The current sources of commercial TGase are limited to animal sources (mainly guinea pig liver) and microorganisms. TGases are needed for a plethora of applications in the food

processing, chemical, pharmaceutical, biotechnological, and the textile industries, among others. Thus, demand for TGases has been growing steadily in recent years (Lerner et al. 2017), resulting in intensified research to discover newer sources of the enzyme with high yield, purity and performance for industrial applications. Thus, fishery processing byproducts and discards including viscera, livers, heads, skins, blood, reproductive systems, etc., that are commonly discarded or put to low value use, are being investigated as potential sources of enzymes such as TGases. Fish viscera such as tuna viscera account for 10–28% of the whole animal (Herpandi et al. 2011), while shrimp viscera and shells account for at least 50–70% of the total body weight (Gortari and Hours 2013). It is conservatively estimated that about 34.8 million tons of fish waste are generated globally per year (Rebah and Miled 2013), which suggests that this abundantly available resource material could be accessed and used as source material for producing useful biochemicals including enzymes like TGases (Gildberg et al. 2000). Fish/shellfish enzymes that have been obtained from fishery byproducts include trypsin from yellowfin tuna (*Thunnus albacores*) spleen (Klomklao et al. 2006), lipase from the viscera of gray mullet (*Mugil cephalus*) (Aryee et al. 2007), amylase from viscera of hard clam (*Meretrix lusoria*) (Tsao et al. 2004), and many others; however, complete discussion of the various seafood enzymes is beyond the scope of this discourse and reference is made to the following reviews for additional information (Benjakul et al. 2010; Fernandes 2016; Klomklao et al. 2012; Nielsen and Nielsen 2012). TGases have similarly been investigated in the tissues and organs of various fish and shellfish species, e.g., from the muscle, blood, liver, heart and intestine in species such as carp, red sea bream, lobster, rainbow trout, scallop, squid and others (Table 2).

Enzymatic properties of fish/shellfish TGases

TGase is one of the two members of the transpeptidase family of enzymes, the other being γ-glutamyl transpeptidase (Myhrman and Bruner-Lorand 1970). A γ-glutamyl transpeptidase from lobster muscle was found to have similar characteristics to guinea pig liver TGase and human Factor XIII (Myhrman and Bruner-Lorand 1970), which represents the first evidence of fish/shellfish derived TGase. As shown in Table 2, there are reports describing the isolation and characterization of TGases from different fish/shellfish species (Binsi and Shamasundar 2012; Hemung and Yongsawatdigul 2008; Nozawa et al. 1997). Fish/shellfish TGases show significant sequence homology, conserved regions and similar structures because they share a common evolutionary ancestor from the ocean. For example, salmon TGase has 62.4% similarity in amino acid sequence to red sea bream TGase, while it showed only 43.5% and 34.4% of sequence similarities with guinea pig liver TGase and Factor XIII, respectively (Sano et al. 1996). The physiological function of TGase in fish/shellfish species include blood coagulation, muscle cell healing, tissue differentiation and development, immune response and stress response as well as behavior, movement, and viral entry (Arockiaraj et al. 2013; Chen et al. 2014; Junkunlo et al. 2019; Maningas et al. 2008; Nozawa and Seki 2002; Sirikharin et al. 2019). The basic properties of TGases from fish/shellfish species, guinea pig liver TGase, and human (Factor XIII) are compared in Table 2.

Overall structure

The sea bream TGase appears to be the only one among all the fish/shellfish TGases with its crystal structure fully characterized to disclose it as a monomer with a tertiary structure that is relatively loose and relaxed versus mammalian TGases (Noguchi et al. 2001). It is comprised of four domains and a catalytic triad active site (Cys–His–Arg) located in the α-helix, random coil and β-sheet, respectively; as well as a shallow and narrow shaped binding pocket for substrate. In recent years, bioinformatics approaches have been found to be useful for studying enzymes. It uses the obtained gene or amino acid sequences to analyze homology of enzymes from different sources, as well as to predict and study the enzyme structure, to obviate the high cost and limited access to X-ray crystallographic and nuclear magnetic resonance (NMR) methods for protein structural studies. TGases from salmon (*Onchorhynchus keta*) liver (Sano et al. 1996), freshwater crayfish (*Pacifastacus leniusculus*) (Wang et al. 2001), giant tiger shrimp (*Penaeus monodon*) (Chen et al. 2005), Chinese shrimp (*Fenneropenaeus chinensis*) (Liu et al. 2007), zebrafish (*Danio rerio*) (Deasey et al. 2012), white shrimp (*Litopenaeus vannamei*), and freshwater prawn (Arockiaraj et al. 2013) have been cloned and characterized by bioinformatics. For example, the predicted three-dimensional structure of prawn TGase was generated by I-TASSER program based on the homologous similarities in protein databases (Arockiaraj et al. 2013). From such bioinformatics studies, it was established that fish TGase exhibited high similarities with crustacean TGase such as white shrimp, and the conservation of amino acids mostly occurred in the core domain, based on multiple sequences alignments.

Activity

TGases from different fish/shellfish species show different catalytic properties and activities, which may be due to acclimation to different habitats. A common method for determining TGase activity from marine species is via fluorescence spectrophotometric measurement of incorporation of monodansylcadaverine (MDC) into *N*,*N*′-dimethylated casein (DMC) (Takagi et al. 1986). Variations in the purification and activity assays used by different researchers may result in differences that make their comparisons difficult. By using the same purification and assay methods for six fish/shellfish TGases, it was established that botan shrimp (*Pandalus nipponensis*) TGase had the highest specific activity of 22.9 U/mg, followed by TGases from carp (*Cyprinus carpio*; 8.6 U/mg), scallop (*Patinopecten yessoensis*; 2.2 U/mg), rainbow trout (*Oncorhynchus mykiss*; 1.17 U/mg),

atka mackerel (*Pleurogrammus azonus*; 1.1 U/mg) and squid (*Todarodes pacificus*; 0.046 U/mg) in decreasing order of activity (Nozawa et al. 1997). Another study found that TGase from Mozambique tilapia (*Oreochromis mossambicus*) showed highest specific activity of 69.14 U/mg, followed by TGases from Indian oil sardine (*Sardinella longiceps*; 66.34 U/mg), common carp (*Cyprinus carpio*; 49.33 U/mg) and bigeye snapper (*Priacanthus hamrur*; 47.44 U/mg) (Binsi and Shamasundar 2012).

Molecular weight

Molecular weights (MW) of TGases from different fish/shellfish species have been verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and results found that their MW ranged from 73 to 95 kDa, e.g., 77 kDa for walleye pollack (*Theragra chalcogramma*) liver TGase (Kumazawa et al. 1996) and 94 kDa for squid (*Todarodes pacificus*) gill TGase (Nozawa et al. 2001a). Recently, a TGase from Antarctic krill (*Euphausia superba*) showed MW of 78 kDa (Zhang et al. 2017) and a crayfish (*Pacifastacus leniusculus*) TGase had MW of 86 kDa (Sirikharin et al. 2018). TGase genes were cloned from fish/shellfish species by polymerase chain reaction (PCR), and the encoded amino acid sequence used to deduce the theoretical MW of these TGases, e.g., the MW of TGases from freshwater crayfish (*Pacifastacus leniusculus*) and Chinese shrimp (*Fenneropenaeus chinensis*) were calculated from their cDNAs as 86.03 kDa (Wang et al. 2001) and 84.96 kDa (Liu et al. 2007), respectively.

Isoelectric point

The isoelectric points (pI) of TGases were obtained by isoelectric focusing (IEF), where the purified TGase migrated in a pH gradient under the influence of electric current. The pI values of the TGases were determined as follows: tropical tilapia (*O. niloticus*) TGase, 6.53 (Worratao and Yongsawatdigul 2005); and for lobster TGase, 5.9 (Myhrman and Bruner-Lorand 1970); which are lower than that of TGase from guinea pig liver (pI 8.9). Besides, theoretical pI values can be deduced from the pK_a values of the constituent amino acids. By using gene cloning method, pI values were reported as 5.61 for Chinese shrimp (*Fenneropenaeus chinensis*) (Liu et al. 2007), and 5.5 for freshwater prawn (Arockiaraj et al. 2013).

pH optima and stability

At different pH values, the side chain residues of the amino acids in an enzyme may carry different charges. If the charge of enzyme and substrate molecules formed at certain pH values favor the catalytic reaction, the overall reaction is optimal at that particular pH value. As shown in Table 2, TGases are active within a relatively narrow pH range of neutral to slightly alkaline. Tropical tilapia (*O. niloticus*) TGase showed highest activity at pH 7.5 (Worratao and Yongsawatdigul 2005). Threadfin bream (*Nemipterus* sp.) liver TGase showed optimum activity from pH 8.5 to 9.0 (Hemung and Yongsawatdigul 2008). However, optimal pH values of enzymes depend on different factors such as nature of substrate, temperature, ionic strength, presence and absence of activators or inhibitors, etc. Thus, it is inaccurate to compare the optimal pH of TGases from different sources under different reaction conditions. Extreme pH (pH < 2 and pH > 12) environments can cause ionization of amino acid side chains and even unfolding of enzyme structure. The stable pH range of squid gill TGase is 7.5–9.0 at 25 °C within 1 h incubation, while TGase from microorganism *Streptoverticillium* is stable between the pH range of 5–9, when reaction was carried out for 10 min at 37 °C (Ando et al. 1989).

Thermal optima and stability

TGases derived from fish/shellfish display differences in temperature optima and thermal stabilities which may be related to their habitat temperatures (Ashie and Lanier 2000; Uresti et al. 2006). The optimal temperatures for TGases from selected marine species were reported as 4 °C for Antarctic krill (*Euphausia superba*) TGase (Zhang et al. 2017) and crayfish (*Pacifastacus leniusculus*) TGase (Sirikharin et al. 2018), 20 °C for squid (*Todarodes pacificus*) gill TGase (Nozawa et al. 2001a), 35 °C for both gray mullet (*Mugil cephalus*) (Lee et al. 1998) and European sardine (*Sardina pilchardus*) TGases (Batista et al. 2002), 37 °C for bigeye snapper (*Priacanthus hamrur*), Indian oil sardine (*Sardinella longiceps*) and carp (*Cyprinus carpio*) TGases, 40 °C for Japanese oysters (*Crassostrea gigas*) (Kumazawa et al. 1997), 50 °C for tropical tilapia (*O. niloticus*), and 55 °C for red sea bream (*Pagrus major*). The optimal temperatures for TGases from most species listed above were lower than those from terrestrial organisms which are usually around 50 °C (Ando et al. 1989; Motoki and Seguro 1998), suggesting that the habitat temperature plays an important in modulating enzyme function.

Nonetheless, TGases active at low temperatures invariably are less stable at high temperatures. For example, while the optimal temperature for tilapia TGase is 50 °C with 65% activity retention at 60 °C, carp TGase showed optimal temperature at 37 °C but only retained 29% activity at 60 °C (Binsi and Shamasundar 2012). Besides, the presence of calcium seems to improve the stability of TGase against thermal denaturation (Nozawa et al. 2001a). In general, TGase loses activity at temperatures above 70 °C (Yokoyama et al. 2004) accompanied with a conformational change from ordered secondary structure to an unordered structure (Cui et al. 2008). The thermal properties of enzymes to some extent determine their suitability for industry application; thus, it is necessary to investigate TGase activities in extreme cold and warm temperatures to provide the basic knowledge needed to rationalize their use in potential applications.

Activators

Calcium dependence is an important property for TGases from aquatic species, with the degree of dependence varying

with calcium concentration from 0.5 to 50 mM (Binsi and Shamasundar 2012; Hemung and Yongsawatdigul 2008; Yasueda et al. 1994). The wide difference may be due to species variation, but it is also likely that different reaction conditions and procedures used in assaying enzyme activities play a role. For example, a TGases reaction solution with high EDTA level and low NaCl concentration turned out to need more calcium ions to activate the enzyme (Faye et al. 2010; Nozawa et al. 2005). In addition, NaCl had an activating effect on marine invertebrate TGases, but not freshwater shellfish TGases (Nozawa et al. 2001b). This observation may be because of their open blood vascular system surrounded by high salt containing seawater. Moreover, increase of NaCl level could reduce the required amount of $CaCl_2$ to fully activate squid gill TGase; on the other hand, NaCl could rapidly inactivate the enzyme in the absence of substrates (Nozawa et al. 2001a). Metal ions, e.g., Sr^{2+} also activate TGases from marine, freshwater and guinea pig liver by inducing subtle conformation changes in the enzyme structure to expose the catalytic triad (Kumazawa et al. 1996; Worratao and Yongsawatdigul 2005).

Inhibitors

Various inhibitors have been studied for the inhibition of purified aquatic animal TGases. They include ethylene diamine tetra acetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), dithiothreitol (DDT), lysine-HCl, ammonium chloride (NH_4Cl), ammonium sulfate ($(NH_4)_2SO_4$), phenylmethylsulfonyl fluoride (PMSF), parachloro mercuri benzoate (PCMB), iodoacetic acid (IAA), and *N*-ethylmaleimide (NEM) (Hemung and Yongsawatdigul 2008; Nozawa et al. 2001a; Worratao and Yongsawatdigul 2005). Among these compounds, IAA, NEM and PCMB were the most potent inhibitors because these chemicals are sulfhydryl alkylating agents which reacts with the thiol group of cysteine in the active site of the enzyme. EDTA and EGTA are usually added into the extraction buffer to prevent the TGase from self-aggregation; however, they are well-known calcium ion chelating agents that prevent TGase transformation into its active structure. DDT shows slight to no interruption of TGase activity; and it is commonly included in the extraction buffer since it prevents metal catalyzed oxidation of cysteine at the active site of TGase (Kwon et al. 1994). Lysine-HCl serves as a competitive inhibitor for hydroxyl amine-HCl which acts as substrate in some activity assays (Binsi and Shamasundar 2012). NH_4Cl reduces TGase activity by generating excess ammonium ions to reverse the catalysis of TGase, because NH_4^+ is also a product of the acyl transfer reaction. PMSF, a well-known serine protease inhibitor, showed slight or no inhibition of TGase from aquatic animals, which may be because PMSF reacts with the histidine residue in the active site of fish TGase, but at the same time, it can prevent enzyme degradation by endogenous serine proteinases (Worratao and Yongsawatdigul 2005). Metal ions such as Cu^{2+}, Zn^{2+}, Fe^{2+}, and Co^{2+}, which have strong affinity for sulfhydryl groups can inhibit TGases from aquatic animals (Hemung and Yongsawatdigul 2008).

Food applications

Proteins are important food components that play important roles in the physicochemical properties of food. The exploitation of TGase to crosslink food proteins and thereby change their functional characteristics started in the early 80's (Ikura et al. 1980a, 1980b; Ikura et al. 1981); and this capacity of TGase to effect crosslinking could be exploited synergistically with various other food processing techniques. However, TGases have been shown to be heat labile and capable of losing up to 50% activity when heating for 30 min at 50 °C (Kieliszek and Misiewicz 2014). Thus, applying TGase in combination with traditional thermal processing may not be useful. Rather, TGase may be used in combination with emerging minimal food processing technologies such as ultra high pressure, microwave assisted processing, pulsed electric field, UV irradiation, ultrasonication and fermentation to improve food attributes such as gelation, water holding capacity, firmer texture, increased yield, and viscoelastic properties (Gharibzahedi et al. 2018). The first TGase used was from animal origin, and was able to cross-link important food proteins, such as casein and soy proteins. The modifications caused by TGase action also altered protein functionalities such as gelation, solubility and emulsifying properties (Motoki et al. 1984; Nio et al. 1986). However, industrial use of TGase at that time was practically impossible due to the high costs of enzyme extraction and purification methods. Despite this, a patent was published with commercial interests of using mammalian TGases for binding fish and meat parts (DeJong and Koppelman 2002). When Ando et al. (1989) identified the production of an extracellular TGase from a *Streptoverticillium* strain, the industrial utilization of this enzyme became feasible. Since then, several patents have been published regarding the identification, production, isolation and utilization of microbial TGases, but only Ajinomoto Co., Inc. (Tokyo, Japan) and Rohm Enzyme (USA) practically retain the oligopoly of the worldwide TGase commercialization (Cubiro et al. 2007).

All the three main reactions catalyzed by TGases, viz., 1) cross-linking between protein-protein; 2) crosslinking between protein-polyamine; or 3) reaction between protein-water resulting in the deamidation of the glutamyl residues of the protein, can be used for food transformation (DeJong and Koppelman 2002). Residues of glutamine and lysine must be exposed to the TGase for protein cross-linking reactions in a food system to occur. The reaction will continue if those two amino acids are available and spatially reachable to the enzyme. One of the main consequences of food protein cross-linkage is the formation of gels. Moreover, the strengthening of the protein network also results in tougher texture of the final product. TGase is also used to incorporate lysine in food products with nil to low lysine levels via isopeptide bond formation with glutamine residues. The result of this reaction can improve some protein

functionalities such as solubility, emulsification, foam formation and gelation characteristics (Hamada and Swanson 1994). Ohtsuka et al. (2001) successfully used TGase to deamidate 45% and 38% of the glutamine residues of casein and gliadin, respectively. TGases are also being used for biotechnological applications to produce novel biopolymer conjugates as biomaterials with improved and beneficial properties, such as increased stability and reduced toxicity, as well as for site-specific attachment of diverse compounds (e.g., bioactives, essential amino acids and lipids) to proteins.

TGase enzyme is listed as GRAS by the Food and Drug Administration (FDA). It is used in the food industry to improve texture, achieve better flavor, add nutrients, and assist processing. Due to the properties of TGase from fish and shellfish, it would be beneficial to use them to produce various food products. For example, one promising use of fish/shellfish TGase will be for the development of nutrient-dense food products at low temperatures, to replace the energy-dense foods available to consumers that are associated with adverse health impacts.

Meat

Among all food products, TGase has been more extensively used in meat and meat products (e.g., pork, beef, poultry meat, fish) to improve their functional properties, e.g., the water-holding capacity, thermal stability, texture, gelation, color, elasticity and flavor (Seighalani et al. 2017). First, myosin and actin can be cross-linked by TGase (Kahn and Cohen 1981), contributing to the manufacture of products such as sausages and ham. In addition, large amounts of meat bound to the animal carcasses are lost or sold as low value products. The meat scraps on the bones can be excised and restructured to form gel-like materials or pastes using TGase, then shaped and flavored for packaging as a value-added product. This is the basis of imitation seafood products, which are products derived from surimi gels (DeJong and Koppelman 2002). Microbial TGase has been used to restructure European hake (*Merluccius merluccius*) mince with optimal setting at 25 °C for 2 h using 15 g/kg NaCl and 300 U/kg TGase (Martelo-Vidal et al. 2016), and was found to increase hardness and water holding capacity in restructured fish products from pangasius (*Pangasianodon hypophthalmus*) fish prepared by hot setting process (Kunnath et al. 2015). Another application of mTGase in meat products is to readjust the characteristics lost in PSE (pale, soft, and exudative) meats. This type of meat is usually derived from stressed animals and is characterized by its light color, soft consistency and low water-hold capacity, leading to a product of reduced mouthfeel and acceptability. Olkiewicz et al. (2003) reported that mTGase was able to re-aggregate some of the released proteins, impacting positively on the consistency of the ham produced from PSE meat.

Fish/shellfish TGases application have been studied and showed potential in the meat processing industry. Cold setting or cold restructuring is a process used to reconstruct underutilized meat scraps from filleting, cuts, or trimmings, into value-added and customer acceptable meat products such as imitation or restructured seafood, restructured meat steaks, sausages, hams, etc. The processing requires low temperatures to maintain the heat labile essential components present in foodstuffs, curtail potential microbial contamination, and to reduce energy cost. Usually, supplementation with NaCl is recommended in some meat processing, e.g., surimi, to solubilize myofibrillar proteins, and improve flavor and texture. High pressure treatment can also be applied modify the texture, structure and function of meat proteins. Thus, TGase may be used at low temperatures in combination with low salt and pressure in some meat processing operations based on the hurdle concept, to lower the levels of the barriers (i.e., salt and pressure) that would be normally required to enhance product quality. The NaCl (2–4%) assisted gelation of surimi at low temperatures (5–40 °C) involves crosslinking of myosin catalyzed by endogenous transglutaminase (Motoki and Seguro 1998). According to Yongsawatdigul et al. (2002), TGase retained 44% activity in threadfin bream mince (original 99.6 U/g dry weight) after washing and dewatering, and promoted cross-linking in the mince during 2 h incubation at 25 °C and 40 °C . The increase in the breaking force and penetration distance of surimi product prepared from pacific whiting (*Merluccius productus*) mince, egg white and calcium lactate was attributed to endogenous TGase (Yin and Park 2015). It is well established that naturally present endoproteases adversely affect fish mince gelation, thus supplementation with exogenous TGase is necessary.

Dairy

TGase can use proteins, such as caseins and whey proteins, as substrates to improve emulsifying, foaming, and gelling properties of foods; as well as water and oil absorption capacity, and thermal stability of dairy products such as yoghurt, milk beverage, and cheese (Yang et al. 2016). TGase has been shown to enhance cross-links in the different milk caseins in decreasing order of reactivity as κ-casein $>$ α-casein $>$ β-casein (Tang et al. 2005), which has been attributed to differences in the peripheral position in the casein micelles (Sharma et al. 2001). Microbial TGase has been largely studied in dairy processing with respect to its effect on physicochemical properties. Ozer et al. (2007) showed that addition of mTGase to nonfat yogurt could enhance gel strength and reduce syneresis; and the yogurt thus produced had enhanced physical properties with better sensorial characteristics than the non TGase-treated yogurt. Addition of mTGase at a level of 0.03% in set-style yoghurt increased the viscosity, acidity, milk solid nonfat content, and storage time (Jooyandeh et al. 2015). TGase also improved the consistency index and pseudoplastic properties of ice cream at TGase concentration of 4 U/g protein at 56.8 °C for 90 min (Rossa et al. 2011). TGase also increased the resistance of ice cream stored at −25 °C to repeated thermal shocks (Kasprzyk et al. 2016). In addition, TGase was used to introduce plant proteins like soybean or peanut into dairy Panela

cheese to enhance its nutritive value and flavor (Salinas-Valdés et al. 2015).

There is no study found on the fish/shellfish TGase applied in dairy processing. In general, dairy products prefer cold processing and cold storage; however, few studies have been done in terms of the TGase use in dairy products under cold conditions.

Grains

Protein from grains are good substrates for cross-linkages by TGase in formulating products such as dough, noodles, pasta, tofu, meat substitutes, bread, etc. For instance, soy globulin-stabilized emulsion gel formation was induced by TGase at 20 U/g protein, and this was attributed to enhanced covalent cross-linking of the subunits of β-conglycinin and glycinin to form high molecular weight biopolymers (Tang et al. 2013). Gluten-containing products are also affected by TGase. Collar and Bollaín (2004) mixed mTGase in wheat flour dough and resulted in improved viscoelastic properties, stability and water-holding capacity in the final baked bread. TGase has also been used to induce crosslinks in wheat gluten, as well as glucosamine-/oligochitosan-glycation to result in a reduction of allergenicity (Zhang et al. 2016).

Cold setting is one of the gelation methods used in formulating products with soybean protein isolates. Adding mTGase at a level of 1–3 U/g soy protein during cold storage increased the gel strength, deformation, elasticity, and Newtonian viscosity of the cold soy gels (Soeda 2003), and was comparable to soybean gels usually prepared first at high temperatures, followed by cold-set process (Zhu et al. 2011) for the reason that the mTGase used have better action at high temperatures. Thus, fish/shellfish TGases may have advantages over mTGase in cold-setting of grain proteins as they can function at low temperatures.

Food packaging

Biodegradable plastic films intended for use for food packaging have been prepared from food protein molecules such as gelatin, pectin, whey protein, casein, zein, wheat gluten, soybean protein, egg white, and fish proteins, etc. The proteins have been combined with carbohydrate molecules such as pectin and chitosan using TGase to improve mechanical and barrier properties of the films, so as to provide physical protection, as well as reduce O_2 absorption and moisture loss (Porta et al. 2011). They have also been used to prepare composite films with other food grade materials such as food lipids (including essential oils), and nanoclay. The use of TGase in producing food packaging films is popular because it is perceived as safe, effective, and eco-friendly. Since year 2016, around 100 published articles reported packaging film formation facilitated by TGase. The films prepared by TGase possessed good properties in terms of tensile strength as well as water vapor and gas barrier properties, etc. Furthermore, fresh fruits and vegetables coated with TGase prepared gels exhibited less weight loss, and minimal microbial growth, etc. Cold adapted transglutaminase can be used to formulate gels to coat fruits and vegetables during cold storage, as well as to make more flexible biofilms that suffer less breakage problems at low temperatures. A cold-active TGase from Antarctic krill, *Euphausia superba* was used in cold-setting of gelatin gels at 4 °C with increased the gel strength, setting temperature, setting time and melting temperature with TGase addition of 0.1 U/mg (Zhang et al. 2017), and such gels could be further dehydrated into packaging films.

Other food applications

Fish/shellfish TGase can be used in other food applications for food safety and control. Fish/shellfish TGase can modify foods protein by crosslinking under certain conditions to change the protein structure and properties including allergenicity. Research has shown that TGase treatment reduced allergenicity of tropomyosin and glycinin (Wang et al. 2019b; Yang et al. 2019). In addition, TGase is involved in celiac disease, a chronic small intestinal immune-mediated enteropathy due to consumption of wheat gluten. Novel ELISA-based kit or biosensor designed with TGase (antigen) and anti-TGase (antibody) (Habtamu et al. 2019) is a development where fish/shellfish TGase has great potential to be applied. Immobilization of TGase could improve its stability and reusability for industrial applications. There have been research studies on immobilizing TGase on magnetic materials, nanomaterials, (Gajšek et al. 2019), that could also be used for immobilizing fish/shellfish TGases. TGase can also serve as crosslinker to immobilize other enzymes onto support materials, e.g., a TGase-catalyzed crosslinking of enterokinase onto amine-modified magnetic nanoparticles with high operational stability and remarkable reusability (Wang et al. 2019a), which suggests potential applications of fish/shellfish TGase for immobilization under special conditions such as low-temperature. Despite the potential of fish/shellfish as alternative sources of TGase for food processing, it is conceded that the traditional fish/shellfish harvest cannot be relied upon to sustain a steady supply of the enzyme for industrial purposes. Thus, fish/shellfish TGases with distinct properties may first be produced in microorganisms, then improved by protein engineering and computational approaches to be able to withstand industrial processing conditions, and further improved by immobilization for permit recovery and reuse.

Concluding remarks

Recent developments in TGase research point to growing interest in the enzyme for both fundamental and practical reasons. Traditional enzymes invariably do not tolerate industrial processing conditions well; thus, there is the fundamental need to produce novel TGases that can tolerate industrial settings and function effectively under those harsh conditions. This goal can be achieved by using advances in protein engineering combined with computational approaches to create novel TGases with improved catalytic

activity at both high and low temperatures, as well as improved thermostability, and pH tolerance to enable them to suit industrial settings. Practically, the functional properties of TGases have important implications for the fields of food science (e.g., food quality improvement, food safety, and new food product development), plant science (for process regulation, e.g., fertilization and photosynthesis) and health (in terms of development and control of non-communicable diseases such as certain cancer, Alzheimer disease, and reduced cholesterol levels). In the specific case of foods, some new perspectives and key areas for future research include the development of high-quality and nutritious foods. By virtue of its catalytic mechanisms, TGase can generate covalent crosslinks within and between naturally present food components such as proteins and carbohydrates to modify physicochemical characteristics of foods, such as texture, water holding capacity, gel forming ability, thermal stability, emulsifying capacity, elasticity and viscosity. Thus, TGases can be investigated for creating new foods with improved nutritional properties and human health benefits via incorporation of bioactives and essential components in foods to correct for deficiencies, creation of dietetic foods, and reduced allergenicity. TGases can also be evaluated for synergistic effects when combined with current and emerging minimal food processing techniques such as high-pressure processing, ultrasonication, microwave treatments and others to create novel food products with improved physicochemical and nutritional characteristics.

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