Mehta K, Eckert R (eds): Transglutaminases.
Prog Exp Tum Res. Basel, Karger, 2005, vol 38, pp 174–191

Transglutaminases as Biotechnological Tools

Loredana Mariniello, Raffaele Porta

Department of Food Science, University of Naples 'Federico II',
Naples, Italy

For their capacity to cross-link protein substrates, transglutaminases have always attracted a wide interest from both scientific and applied points of view. Since 1957, when Clark et al. [1] described a transamidating activity in guinea pig liver and introduced the term 'transglutaminase' (TGase), research on this class of enzymes has been growing, reaching a very consistent number of published reports (around 400 per year) during the last five years.

The fields of research are very broad, from human health to industrial applications in different sectors. Such a large range of interest is related to the existence of different isoforms of TGase which are widely distributed in different organisms, such as bacteria [2], plants [3], invertebrates [4], vertebrates including amphibians [5], fish [6] and birds [7]. For most of them the physiological role has been established, while for others further studies are still needed. Moreover, some TGase isoforms have attracted a large interest as potential biotechnological tools because of their different substrate specificity and since they are easier to be manipulated outside of their natural environment. For example, keratinocyte TGase, which needs a complex post-translational modification to be fully active [8], was never suggested for biotechnological applications. But, for its implication in lamellar ichthyosis [9], this molecular form of the enzyme was only proposed as a possible target for gene therapy. On the other hand, Factor XIIIa, despite the fact of being the first isoform to be used to modify protein and peptide substrates [10], is nowadays used as a therapeutic agent to treat severe pathology, as fatal bleeding, since its role in this disease has been well assessed. Also the so called 'tissue' TGase (type 2), the ability of which to modify biological properties of some peptides and proteins has been well established [11–21], is currently used in biomedicine

and specifically for the diagnosis of an autoimmune pathology like the celiac disease, where the enzyme was suggested to be the major autoantigen [22]. In the last decade *Streptoverticillium mobaraense* isoform, a TGase of microbial origin possessing wide substrate specificity, calcium independence and high thermostability, has been largely utilized as a biotechnological tool, but mostly in the food field [23, 24].

In this chapter we describe the main applications of the multiple TGase molecular isoforms in different sectors, from biomedicine to cosmetics, from food to leather and textile industries.

Biomedicine

The applications of TGase in biomedicine are directly related to the studies on the physiological role of each enzyme isoform, on the identification of new endogenous and exogenous substrates, and on the enzyme capability to modify the biological properties of the proteins able to act as acyl donor and/or acceptor substrates.

Investigations on the physiological role of Factor XIII have allowed to design products for therapeutic use of this isoenzyme. As it is well known, coagulation Factor XIII (fibrin stabilizing factor) is a TGase that circulates in blood plasma as an inactive heterotetramer consisting of two catalytic A and two regulatory B subunits (A_2B_2), both of which are synthesized and secreted by the liver. Factor XIII is converted into the fully active enzyme (Factor XIIIa) by a thrombin-mediated proteolysis occuring in the final stage of the blood coagulation cascade. Once produced, Factor XIIIa cross-links fibrin aggregates both stabilizing them against mechanical stress and proteolytic degradation and incorporating proteinase inhibitors into the fibrin clot. Factor XIIIa activity suggested the design of products with blood clotting properties to be used for bleeding control during surgery. Thus, the aim to produce fibrin sealants was reached by different enterprises promoting new therapeutic preparations, like Tissucol kit (by Baxter) and Tisseel kit (by Immuno), both containing Factor XIII and used for the treatment of postsurgery hemostatis. Also the 'tissue' TGase has been proposed as biological glue because of its capability to cross-link proteins occurring in the extracellular matrix. Its use in promoting cell adhesion for the coating of medical implants has been, in fact, recently patented [25].

Other commercial products related to Factor XIII involve its administration in patients affected by Factor XIII deficiency, a very rare bleeding disorder either inherited or acquired [26] which is characterized by the production of defective and unstable blood clots in response to tissue damage. Replacement therapy in Factor XIII deficiency has been proved to be generally straightforward owing to

the low levels of Factor XIII required to control bleeding. Nowadays two commercial products are available for substitutive therapy with plasma-derived Factor XIII (Factor XIII from Bio Products Laboratory and Fibrogammin P from Centeon).

Further application of TGase in biomedicine is related to the therapeutic treatment of the 'excessive or hypertrophic scarring', a pathological state characterized by the occurrence of hypertrophic scars following a dermal insult, such as surgery, grafting, trauma and severe burns. Hypertrophic scars are characterized by being thick, red, painful and itchy and can cause functional deficits when they occur across a joint. In the wound healing process 'tissue' TGase plays a role in the production of ε-(γ-glutamyl)lysine cross-links with the formation of insoluble collagen matrices. In hypertrophic scarring 'tissue' TGase is found to be overexpressed resulting in both an overhealing process and an excess of collagen deposition. After assessing the effectiveness of polyamines as alternative acyl acceptor substrates of 'tissue' TGase [27], Procyon Biopharma Incorporation developed Fibrostat®, a topical cream containing putrescine. The development status of Fibrostat® is currently in phase IIb of clinical trial to further evaluate the safety and the effectiveness of the product [28].

Moreover, the progress in the knowledge of the etiology of the celiac disease has pointed out the importance of 'tissue' TGase as an effective diagnostic tool. In fact, the identification of 'tissue' TGase as the major autoantigen of celiac disease, against which the endomysial antibody is directed [22], led to more extensive understanding of the pathogenesis of this immunologically mediated intolerance to wheat gliadins. These findings also allowed to change the technique by which the diagnosis of the disease was made. In fact, previous methods based on the identification of antiendomysium antibodies have been replaced by the immunological detection through ELISA tests of IgA autoantibodies against 'tissue' TGase. First generation tests, identifying IgA class anti-'tissue' TGase antibodies, used the enzyme purified from guinea pig liver as antigen [29–31]. However, since several studies demonstrated significant differences in the performance of the guinea pig enzyme compared to the human isoform, human TGase antigen-based kits have been recently developed. Most of them contain a recombinant form of the enzyme, expressed either in *E. coli* or in *Baculovirus*/insect cell systems, together with antibodies raised against both types of the recombinant forms.

Another pathology related to a member of the TGase family is the lamellar ichthyosis, a disfiguring skin disease characterized by an abnormal epidermal differentiation and a reduced cutaneous barrier function. Since it has been established that lamellar ichthyosis patients possess a defective keratinocyte TGase (type 1) gene [9], several studies have been carried out in the attempt to design gene therapy-based medical approaches to restore TGase 1 activity.

Both retroviral and plasmid vectors have been used for delivering engineered molecules and two different procedures have been investigated to optimize cutaneous gene delivery. The first one involves grafting of primary keratinocytes derived from lamellar ichthyosis affected patients and cell transformation with normal TGase 1 [32]. The second, representing a less labor intensive approach, consists in the direct injection of the vector harboring normal TGase 1, as naked DNA, into intact skin [33]. The unsatisfactory results obtained with both systems indicate that further studies are needed to apply gene therapy to this kind of disease.

A role played by TGase in some neurodegenerative disorders, such as Alzheimer's, Huntington's and Parkinson's diseases, has been also suggested [34]. The involvement of the enzyme has been hypothesized since some proteins related to these pathologies have been proven to act as TGase substrates and an increase in TGase activity was observed. For example, 'huntingtin', the protein product of the mutated gene responsible for Huntington's disease, is known to be characterized by the presence of polyglutamine stretches in its aminoacid sequence. In vitro studies demonstrated that this protein is able to act as substrate of 'tissue' TGase and that an elevated enzyme activity occurs in the affected cerebral regions [35]. It has been demonstrated that administration of the TGase inhibitor cystamine caused an improvement in patient survival as well as in the symptoms associated with neurodegeneration [36].

Cosmetics

Applications in this field are, in general, related to TGase ability to covalently bind specific compounds containing primary amino groups to keratinocyte proteins known to act as acyl donor substrates. Thus, a method to deliver a large variety of compounds (i.e. sunscreens, antimicrobials, either skin or hair conditioning agents, anti-inflammatory and antioxidants drugs, colorants, perfumes, insect repellents) has been patented [37]. Moreover, topical preparations containing TGase and one or more proteins occurring in the stratum corneum of the skin (like involucrin, loricrin, cornifin) have also been described. The effect of these products is related to the formation of a protective film useful for the care of hair, skin and nails [38]. Another topical preparation, containing an inhibitor of TGase and possessing the property to alter the rate of mammalian hair growth, has been patented. This kind of product could be of interest to remove undesired hair in specific parts of the human body [39]. Finally, microparticles containing TGase-substrate reactive groups, which can be cross-linked to the skin surface by either endogenous or exogenous TGase, represent an additional pharmaceutical proposal [40]. However, even

though to our knowledge the products related to the aforementioned patents are not at the moment on the market, it is predictable that a large number of skin care preparations derived from investigations in this field will be available soon.

Food

Most preliminary studies addressed to the TGase-catalyzed modifications of proteins of food interest have been carried out with the enzyme purified from guinea pig liver or bovine plasma. The limited supply of these isoforms and the high costs of their production inhibited the development of technologies involving TGase in food processing to enhance texture and emulsion properties of protein-based foods.

In 1989 a microbial TGase was isolated from *Streptoverticillium sp*. and its characterization indicated that this isoform could be extremely useful as a biotechnological tool in food industry [41]. In fact, microbial TGase was shown to be active over a wide range of temperatures and stable between pH 5 and 9, as well as to possess a calcium-independent activity [42]. This isoform has been 'Generally Recognized As Safe' and its use is allowed as food additive. Ajinomoto Incorporation actually produces several preparations of microbial TGase that are commercialized with different names. They differ in stabilizer composition in relation to the type of food for the production of which they have been designed. For example, Activa WM, a powder which contains 1% TGase and 99% maltodextrins, was described to enhance the texturizing properties of meat-based foods and successfully used to prepare novel dairy products [43]. Conversely, Activa MP, which contains lactose besides maltodextrins as stabilizer, was suggested to be used in modifying milk protein-based foods as cheese or yogurt. To date, even though many typical oriental foods are already produced in the presence of TGase as adjuvant, the use of the enzyme is certainly destined to be spread out worldwide in the future. In agreement with the European legislation (Directive 89/107/EC), Ajinomoto declared that TGase could be considered as a processing aid and, thus, its presence does not need to be indicated in the finished products.

Seafood Products

The use of TGase in food industry started in Japan to prepare *surimi*-derived products (fish paste). One of this, *kamaboko*, is thought to date as far back as 1,100. To become *surimi*, fish is skinned, boned, repeatedly rinsed to eliminate any fishiness and pigments and ground into a paste. This odorless white paste is then mixed with a flavor concentrate made from real shellfish.

The paste is then formed, cooked and cut into the desired shapes. Since the presence of endogenous TGase has been detected in species of pollack, fish used for *surimi* [44], several experiments have been carried out to assess the effect of TGase on the physical properties of *surimi* gels [45–48]. There are no doubts that both endogenous fish TGase and exogenous microbial TGase are able to improve the texture of the raw materials by catalyzing the formation of ε-(γ-glutamyl)lysine cross-links between fish proteins. In particular, the addition of exogenous microbial TGase is certainly responsible for increasing elasticity and firmness of the *surimi* gel.

Meat Products

Also the use of TGase to prepare meat products is based on studies showing that several meat-related proteins are able to act as substrates of the enzyme. In fact, Factor XIIIa-catalyzed cross-link formation between fibrin and fibronectin, fibrin and actin, myosin and fibronectin, and myosin and actin was previously demonstrated [49]. Further studies reported the capability also of guinea pig 'tissue' TGase to modify meat proteins [10]. Finally, most recent investigations demonstrated the effectiveness of the microbial isoforms of the enzyme to produce different types of meat-based foods by using successfully beef, poultry and pork to prepare restructured products. For example, it has been described that the texture of chicken sausages, which originally showed a gel strength weaker in comparison with the pork sausages, was significantly improved by TGase. Thus, the enzyme offers the possibility of creating new poultry products with improved textural characteristics.

The most significant advantage in the utilization of the enzyme in meat processing is due to the ability of TGase to efficiently substitute salts and phosphates, generally used in the traditional procedures of meat binding and texturing. In the alternative process involving TGase, a mixture of the enzyme and sodium caseinate, added to the meat pieces, allows the formation of cross-links among the casein molecules and the resultant meat product can then be cooked without breaking apart [50]. Nowadays, this process is extensively used to restructure meat from many different sources and to produce corresponding food products that visually and texturally meet consumer demands.

Dairy Products

Modifications of milk proteins by TGase have been extensively studied. The ability of milk proteins to act as TGase substrates was preliminary investigated by using both the enzyme purified from guinea pig liver and the blood coagulation Factor XIII, while more recent studies involved the utilization of the microbial isoform of the enzyme. Experiments carried out using single milk proteins allowed to establish that caseins are effective substrates for

TGase, even if αs1-, β- and κ-casein react differently with the enzyme depending on the isoform used. In fact, Ikura et al. [51] reported a lower reactivity of κ-casein, compared to αs1- and β-caseins, with the guinea pig liver enzyme, whereas α-casein was shown to act less effectively as acyl donor substrate for the blood coagulation enzyme in comparison with both β- and κ-caseins [52]. More recently, Cozzolino et al. [43] demonstrated no significant differences among the different caseins by using microbial TGase, the preferred isoform both to produce new protein ingredients and to change the texture of food products.

Most studies have also demonstrated the effectiveness of TGase to prepare novel yogurts. Kuraishi et al. [24] reported that a yogurt made in the presence of TGase possesses improved gel strength and viscosity as a consequence of its enhanced water-holding properties. Moreover, microbial TGase has been recently proposed for producing whey protein-enriched cheeses by adding the enzyme during the manufacturing process [43]. The obtained new products showed an increased hardness and deformability, depending on the amount of the enzyme used, as well as increased protein content. Development of this kind of dairy production is desirable since it would represent a way for the re-utilization of dairy plant by-products, thus contributing to decrease the environmental pollution due to the whey protein disposal.

Soy Products

Soy proteins are of great interest in the world food industry since they are widely used as ingredients in a variety of western products, such as sausages and ham, as well as being the basic component of typical eastern foods. Their importance is related to their ability to undergo gelation after thermal treatment. It is well known that gelled proteins provide some useful textural properties to different foods. It has been extensively studied how TGase-mediated polymerization of both soy 7S and 11S globulins influences soy protein gel properties. Chanyongvorakul et al. [53] and Kang et al. [54] reported that TGase-induced 11S globulins gels are more rigid and elastic than the corresponding thermally induced gels. The authors proposed that rigidity might be due to an extensive cross-linkage, since it was possible to influence the protein textural quality by varying enzyme concentration. These molecular studies suggested the utilization of TGase in the manufacturing of *tofu*, the major soy product in Asia. The use of TGase, together with magnesium chloride acting as coagulant, provides a *tofu* with a smoother, firmer texture. These new properties depend on an enhanced breaking strength compared to that of *tofu* obtained in the absence of the enzyme. *Tofu* prepared with TGase also exhibits an increased water-holding capacity, probably because of the presence of more stable covalent cross-links that hold more water despite the temperature changes.

Cereal-Based Products

Wheat is known as one of the most important cereals for human nutrition and many reports indicate that wheat proteins are able to act as TGase substrates. Porta et al. [55] demonstrated the reactivity of different cereal proteins as acyl donor substrates for 'tissue' TGase and that wheat globulins, glutenins and gliadins were more effective than prolamins from oat, maize and rice. Different authors reported similar results by using the microbial isoform [56]. The rheological properties of gluten modified following the formation of ε-(γ-glutamyl)lysine cross-links were also investigated. Viscoelasticity of TGase-treated gluten, as well as its sensitivity to thermal processing, was reduced compared to that of the unmodified gluten. Therefore, the enzymatic treatment was shown to cause a considerable reinforcement of the network. These studies have promoted the use of TGase to prepare noodles and pasta in Japan. TGase, added when flour and other ingredients are mixed, confers to both pasta and noodles a firmness higher than that of untreated products, indicating that this characteristic depends on the enzyme amount [24]. In addition, since the cross-links introduced by TGase are heat stable, firmness and elasticity of noodles and pasta are retained even after cooking.

The use of the enzyme showed beneficial effects also on breadmaking. In fact, TGase improves dough elasticity and its utility in breadmaking is similar to that of the oxidizing improvers [57].

Finally, recent studies investigated the possibility to modify rice flour proteins with the aim to use this important cereal for breadmaking. In its natural conditions rice flour is used only to make unfermented baked products since rice proteins are unable to hold gas produced during fermentation. Conversely, Gujral and Rosell [58] have demonstrated that TGase-modified rice proteins provide a protein network effective in holding gas produced during fermentation.

Edible Films

An increasing interest toward edible films has been registered in the last few years mostly for their potential use in food industry. First of all, edible films can represent an alternative to the chemically synthesized polymeric films that are, nowadays, widely used for packaging. Since latter films are not environment friendly, development of films constituted by edible and biodegradable components is strongly advisable. Moreover, edible films have substantial possibilities to enhance stability and quality of foods. Their functional efficiency strongly depends on the nature of the components that can be, typically, hydrocolloids and/or lipids. Many scientific papers and patents refer to films constituted by different proteins, like collagen, casein, wheat gluten and whey and soy proteins, whereas most of the effects of TGase treatment on film properties have been studied using the guinea pig purified

enzyme. Utilization of whey proteins for the production of packaging films was investigated by Mahmoud and Savello [59, 60]. Additional studies were carried out using αs1-casein [61], 11S globulin [62] and egg white proteins [63]. Cross-links introduced by TGase confer on films a precise network that influences mechanical and permeability properties, reflecting characteristics at microstructural level. Smoother surface and higher homogeneity are features found in microbial TGase-synthesized film made with whole soy flour and pectin [64]. Such films exhibit also an increased strength, are less flexible, and less permeable to oxygen, carbon dioxide and water vapour [65]. It is worthy to note that TGase-mediated polymerization modifies, among the proteins occurring in soy flour, the soybean vacuolar protein, known to possess allergenic properties and that can be neutralized also by the Maillard reaction [66].

Finally, edible films have also been proposed as vehicles to carry substances to monitor and/or to influence the quality of wrapped foods. In this way, edible films may represent an active packaging and its use might be extended outside the food sector if the vehiculated substances are specific drugs [10].

Nutritional Aspects of TGase-cross-linked Proteins

The evaluation that so many applications of TGase exist in food processing raises questions about the nutritional value of the proteins containing isopeptide bonds. In this respect bioavailability and digestibility of the glutamine-lysine cross-links deserve to be considered. It has been established that a number of isopeptide bonds occur in many tissues of different animals that are commonly eaten. One explanation of this phenomenon is related to the presence of different endogenous TGase isoforms responsible for the cross-links formation in uncooked foods. But also the processed foods, including the Japanese *kamaboko*, ham, fried chicken, grilled pork, and hamburger, were found to contain γ-glutamine-ε-lysine isopeptide bonds. In particular, cooked foods have an higher content of isopeptide bonds compared to raw food, probably because at the beginning of the process endogenous TGases become more active and are able to better catalyze the formation of cross-links.

On the other hand, enzymes able to hydrolyze the γ-glutamine-ε-lysine dipeptide, which is not susceptible to gastrointestinal proteolysis, have been described. In particular, kidney was shown to be provided with the enzyme γ-glutamylamine cyclotransferase [67], while a different γ-glutamyl transferase was demonstrated to be present mainly in intestinal brush-border membranes and blood [68]. Lysine, which is an essential amino acid, is generated from the cleavage operated by these two enzymes and is readily available and nutritionally beneficial. As a matter of fact, Seguro et al. [69] demonstrated that

rats fed with TGase-cross-linked caseins grew as much as control rats fed with unmodified caseins.

Leather and Textile Industries

The use of TGase also is of interest for other industrial sectors. In the leather industry casein is used to coat leather through a process that involves hardening agents such as aldehydes, isocyanates or aziridine. Because of the high toxicity of these agents, for both operators and environment, new and different methods are demanded. In this respect the application of TGase-modified casein as a coating has been patented [70] and its wider use in leather manufacturing is to be desired.

More recently, studies have been carried out demonstrating the importance of TGase also for the wool finishing industry [71]. To overcome both felting and shrinkage problems of wool fibers, chemical processes are currently used. Most of them involve acid chlorination of the wool goods or the application of permonosulphuric acid. Although these methods concur to confer a significant level of shrink-resistance to the wool, they are of high environmental impact due to the toxicity of the reagents used. On the other hand, the alternative technique based on the use of proteases to prevent shrinkage, a problem occurring after repeated laundering, results in an undesired reduction of wool fibre strength and weight. Cortez et al. [72] have demonstrated, by using both guinea pig liver and microbial TGases, that the enzyme increases tensile strength of the wool products, in some cases completely reverting the loss caused by a previous proteolytic treatment. Protease pre-treatment enhances the effect of subsequent TGase treatment since it causes an increase in accessibility of the fibres to protein cross-linking. Finally, even in the absence of exogenous TGase addition, fibre matrix is stabilized, beside the most abundant disulphide bonds, also by $\varepsilon(\gamma$-glutamyl)lysine bonds catalyzed mainly by keratinocyte and epidermal TGases that normally confer resistance to hair and wool.

Cortez et al. [72] have also demonstrated that both TGase isoforms may be used to incorporate the primary amine substrate fluorescein cadaverine into wool fibres. This result suggests a possible use of the enzyme also to incorporate functional agents as antimicrobials, water repellents and perfumes, as far as they are provided with an alkylamino side group [72]. It is worthy to note that these studies have underlined the different substrate specificities of microbial and tissue TGases towards wool proteins. In fact, microbial enzyme was used at a protein concentration 20 times higher than that of guinea pig liver TGase. Only the availability of recombinant 'tissue' TGase obtained at low costs, hence competitive with the microbial isoform,

will allow its wider use as a biotechnological tool, becoming more popular in this field of interest.

Analytical Biotechnology Applications

Avidin-biotin technology attracts a great interest because of its ability to replace many tests employing radioactively labeled materials. Thus, this system is frequently utilized to identify proteins which have been biotinylated. It has been demonstrated that both acyl acceptor and donor TGase substrates are still able to be modified by the enzyme after they have been previously biotinylated. Often the biotinylated molecules are useful to demonstrate whether a peptide or a protein is a TGase substrate. Josten et al. [73] have used biotinylated compounds endowed with an acyl acceptor amino group to obtain biotinylated antibodies. In particular, by using microbial TGase for the biotinylation of a monoclonal IgG against the herbicide 2,4-dichlorophenoxyacetic acid, they demonstrated that the biotinylated antibody exhibited the appropriate biological activity.

TGase has also been proposed to synthesize products useful for immunochemical assays. Testing of antibodies against small molecules, such as haptens, is routinely performed by ELISA techniques. For accurate results, it is essential to provide an efficient coupling of the hapten to a protein carrier in order to obtain conjugates to be used to coat the microplates in which immunoassays are carried out. As long as hapten is provided with an acyl acceptor group, it can be incorporated through TGase into an acyl donor substrate. Josten et al. [74] have demonstrated the effectiveness of microbial TGase to cross-link an aminofunctionalized hapten to casein, a typical acyl donor substrate for the enzyme, which is currently used to coat plates for immunoassays. Similar results were obtained by other authors by using 'tissue' TGase [61]. Enzymatic synthesis of the conjugates is advisable since chemical procedures are time and labor consuming and the degree of conjugation is hard to achieve. Conversely, enzymatic catalysis is highly reproducible and needs reduced times for conjugation.

The use of microbial TGase for the development of biosensors has also been successfully exploited. In particular, the microbial enzyme was used to prepare a protein matrix, constituted by TGase-cross-linked casein or fibrinogen, onto which model enzymes like glucose oxidase or lactate oxidase were entrapped [75].

It was demonstrated that lactate enzyme sensors, obtained by microbial TGase-mediated immobilization, exhibited a higher storage and operational stability compared to sensors prepared by chemical cross-linking through

glutaraldehyde. In contrast to other enzyme membranes, prepared by entrapment of enzymes in hydrogels, the TGase-mediated network remains homogeneous during drying in air with beneficial effects on the reproducibility of the enzyme sensors.

Other Applications

TGase was recently also proposed as a tool to synthesize glycosylated proteins to be employed in different sectors, from food industry to medical field. Thus, microbial TGase has proved to be effective to prepare trypsin-oligosaccharide conjugates with improved stability properties of the proteolytic enzyme. Trypsin, widely used in food manufacturing and processing industry, is able to act as acyl donor for TGase and to incorporate different types of amino-derived cyclodextrins, compounds previously employed as physical additives to increase the solubility and the catalytic properties of various enzymes in organic media. In particular, TGase-synthesized trypsin-cyclodextrin conjugates have been shown to exhibit significantly improved both specific esterolytic activity and kinetic constants, besides being more resistant to autolytic degradation at alkaline pH and to heat inactivation [76, 77].

The effectiveness of glycosylation through TGase in the medical field was exploited to modulate the biological properties of interleukin-2, a lymphokine with important immunoregulatory functions [78]. In particular, polyethyleneglycol modified with an alkylamine straight chain was used as acyl acceptor substrate. Interleukin-2, acting as glutamine donor substrate, showed an improved capability to survive in the blood circulation of treated rats after TGase modification. In fact, unmodified interleukin-2 showed an half life of only 5.5 min, while TGase glycosylated lymphokine exhibited an half life almost 40 times higher [78].

Conclusions

In this chapter we describe a quite wide panorama on the most revelant biotechnological applications of TGases. The potentiality offered by this class of enzymes in creating many different products is shown in figure 1. Due to their capacity of synthesizing isopeptide bonds, homo- and heteropolymers may be formed with the consequence of obtaining products with new or improved features. Biological activities of proteins and peptides can be also influenced by incorporating polyamines and/or aminosugars. In addition, a different performance can be induced in a protein by its partial TGase-catalyzed deamidation

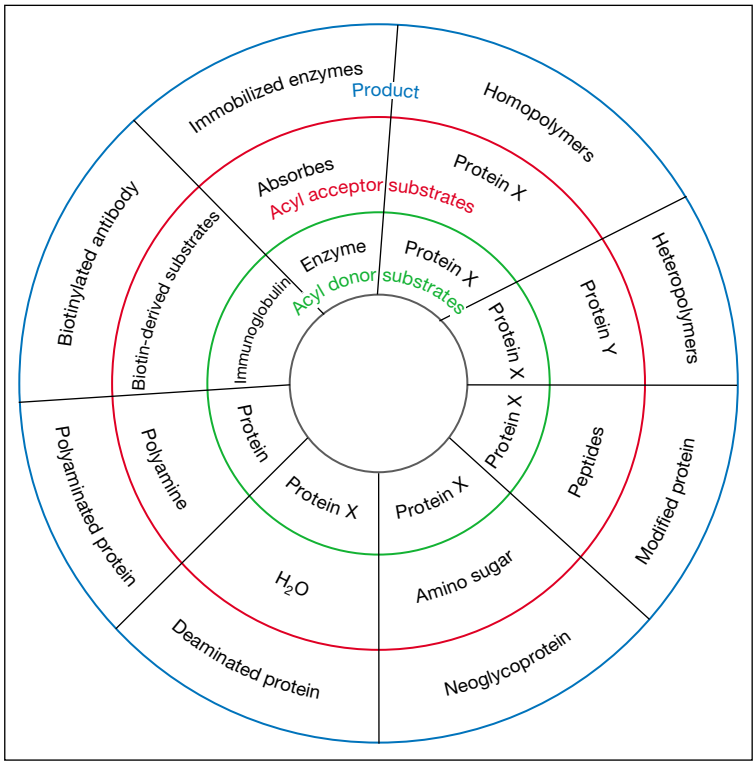

Fig. 1. Examples of different reactions catalyzed by TGase. The internal circle contains the acyl donor substrates, the central circle contains the acyl acceptor substrates, whereas the external circle contains the corresponding products.

converting one or more glutamine residues in glutamic acid(s). Finally, the use of the enzyme has been successfully exploited in analytical biotechnology with the aim both to synthesize effective biotinylated antibodies and to immobilize enzymes acting as biosensors. In many of these applications, the use of TGase resulted alternative to chemical modification methods that often give rise to toxicity problems for users and provoke environmental pollution.

In conclusion, the reported studies indicate that TGase-based methodologies are destined to have further important applications in many different industrial sectors. Thus, the development of novel inexpensive sources of the enzyme represents a crucial point for future research studies. To date, the most convenient TGase as a biotechnological tool is the enzyme purified from *Streptoverticillium mobaraense*, even though this isoform seems to be useful mainly in the food sector. For different applications, the production in large

amounts of other isoforms, i.e. Factor XIII or 'tissue' TGase, is needed. One promising approach to obtain these enzymes at low costs involves the genetic manipulation using heterologous hosts. By this way, the expression of recombinant human Factor XIII was obtained using both *E. coli* [79, 80] and yeast [81] systems as bioreactors. In the same manner, the recombinant 'tissue' TGase has been produced in *E. coli* [82, 83] and *Baculovirus*/insect cells [84] and used as reliable antigen for the diagnosis of celiac disease. However, the high costs of production suggest to find alternative systems. More recently, plants have been proposed as effective hosts for the biosynthesis of mammalian proteins [85]. Gao et al. [86] engineered tobacco plants producing Factor XIIIa with the aim to obtain a recombinant isoform which could replace Factor XIII occurring in the commercially available therapeutic products. Similarly, *Nicotiana tabacum*-derived cells have been recently investigated as bioreactor for the production of human 'tissue' TGase. A catalytically active form has been efficiently produced and the partially purified enzyme shown to be effective in recognizing anti-TGase antibodies present in celiac patient antisera [87]. Thus, the perspective that new transgenic sources may provide large amounts of the different TGase isoforms useful for various biotechnological applications should be considered at the moment more than a simple possibility.

References

1 Clarke DD, Mycek MJ, Neidle A, Waelsh H: The incorporation of amines into proteins. Arch Biochem Biophys 1957;79:338–354.
2 Kanajl T, Ozaki H, Takao T, Kawajiri H, Ide H, Motoki M, Shimonishi Y: Primary structure of microbial transglutaminase from *Streptoverticillium* sp. Strain S-8112. J Biol Chem 1993;268: 11565–11572.
3 Villalobos E, Santos M, Talavera D, Rodrìguez-Falcòn, Torné JM: Molecular cloning and characterization of a maize transglutaminase complementary DNA. Gene 2004;336:93–104.
4 Singh RN, Mehta K: Purification and characterisation of a novel transglutaminase from filarial nematode *Brugia Malayi*. Eur J Biochem 1994;225:625–634.
5 Zhang J, Masui Y: Role of amphibian egg transglutaminase in the development of secondary cytostatic factor in vitro. Mol Reprod Dev 1997;47:302–311.
6 Yasueda H, Kumazawa Y, Motoki M: Purification and characterization of a tissue-type transglutaminase from red sea bream (*Pagrus major*). Biosci Biotechnol Biochem 1994;58: 2041–2045.
7 Puszkin EG, Raghuraman V: Catalytic properties of a calmodulin-regulated transglutaminase from human platelet and chicken gizzard. J Biol Chem 1985;260:16012–16020.
8 Chakravarty R, Rice RH: Acylation of keratinocyte transglutaminase by palmitic and myristic acids in the membrane anchorage region. J Biol Chem 1989;264:625–629.
9 Huber M, Rettler I, Bernasconi K, Frenk E, Lavrijsen SP, Ponec M, Bon A, Lautenschlager S, Schorderet DF, Hohl D: Mutations of keratinocyte transglutaminase in lamellar ichthyosis. Science 1995;267:525–528.
10 Nielsen PM: Reaction and potential industrial applications of transglutaminase. Review of literature and patents. Food Biotechnol 1995;9:119–156.

11 Cordella-Miele E, Miele L, Mukherjee AB: A novel transglutaminase-mediated post-translational modification of phospholipase A2 dramatically increases its catalytic activity. J Biol Chem 1990;265:17180–17188.
12 Persico P, Calignano A, Mancuso F, Marino G, Pucci P, Esposito C, Mariniello L, Porta R: Substance P inactivation by transglutaminase in vitro. Peptides 1992;13:151–154.
13 Porta R, Metafora S, Esposito C, Mariniello L, Persico P, Mancuso F, Peluso G: Biological activities of a major protein secreted from the rat seminal vesicles after structural modification catalyzed by transglutaminase in vitro. Immunopharmacology 1993;25:179–188.
14 Peluso G, Porta R, Esposito C, Tufano MA, Toraldo R, Vuotto ML, Ravagnan G, Metafora S: Suppression of rat epididymal sperm immunogenicity by a seminal vesicle secretory protein and transglutaminase both in vivo and in vitro. Biol Reprod 1994;50:593–602.
15 Esposito C, Mancuso F, Calignano A, Di Pierro P, Pucci P, Porta R: Neurokinin receptors could be differentiated by their capacity to respond to the transglutaminase-synthesized γ-(glutamyl^5) spermine derivative of substance P. J Neurochem 1995;65:420–426.
16 Tufano MA, Porta R, Farzati B, Di Pierro P, Rossano F, Catalanotti P, Baroni A, Metafora S: Rat seminal vesicle protein SV-IV and its transglutaminase-synthesized polyaminated derivative Spd_2-SV-IV induce cytokine release from human resting lymphocytes and monocytes in vitro. Cell Immunol 1996;168:148–157.
17 Mancuso F, Calignano A, Cozzolino A, Metafora S, Porta R: Inhibition of zymosan-induced air-pouch inflammation by rat seminal vesicle protein and by its spermidine derivative. Eur J Pharmacol 1996;312:327–332.
18 Filippelli A, Esposito C, Falciani M, Costa C, Cozzolino A, Rossi F, Porta R: Transglutaminase-synthesized spermine derivative of substance P recognizes rat portal vein neurokin-3 receptors. Life Sci 1997;60:403–411.
19 Mancuso F, Costa C, Calignano A, Mariniello L, Rossi F, Porta R, Esposito C: Transglutaminase-synthesized γ-(glutamyl^5)spermidine derivative of substance P is a selective tool for neurokinin-2 receptors characterization. Peptides 1998;19:683–690.
20 Esposito C, Costa C, Amoresano A, Mariniello L, Sommella MG, Caputo I, Porta R: Transglutaminase-mediated amine incorporation into Substance P protects the peptide against proteolysis in vitro. Regul Pept 1999;84:75–80.
21 Mancuso F, Porta R, Calignano A, Di Pierro P, Sommella MG, Esposito C: Substance P and its transglutaminase-synthesized spermine derivative elicit yawing behavior via nitric oxide in rats. Peptides 2001;22:1453–1457.
22 Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO, Schuppan D: Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat Med 1997;3:797–801.
23 Zhu Y, Rinzema A, Tramper J, Bol J: Microbial transglutaminase – a review of its production and applications in food processing. Appl Microbiol Biotechnol 1995;44:277–282.
24 Kuraishi C, Yamazaki K, Susa Y: Transglutaminase: Its utilization in the food industry. Food Rev Int 2001;17:221–246.
25 Griffin M, Heath D, Christian P: Medical implant materials. 2001 World Patent: W001/85224 A1.
26 Board PG, Losowsky MS, Miloszewski KJ: Factor XIII: Inherited and acquired deficiency. Blood Rev 1993;7:229–242.
27 Dolynchunk K: Inhibition of tissue transglutaminase and (ε-γ-glutamyl)lysine crosslinking in human hypertrophic scar. Wound Repair Regen 1996;4:16–20.
28 Dolynchunk KN, Ziesmann M, Serletti JM: Topical putrescine (Fibrostat) in treatment of hypertrophic scars: Phase II study. Plast Reconstr Surg 1996;97:117–123.
29 Dieterich W, Laag E, Schopper H, Volta U, Ferguson A, Gillett H, Riecken EO, Schuppan D: Autoantibodies to tissue transglutaminase as predictors of celiac disease. Gastroenterology 1998;115:1317–1321.
30 Sulkanen S, Halttunen T, Laurila K, Kohlo KL, Korponay-Szabo IR, Sarnesto A, Savilahti E, Collin P, Maki M: Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. Gastroenterology 1998;115:1322–1328.
31 Sollid LM, Scott H: New tool to predict celiac disease on its way to the clinics. Gastroenterology 1998;115:1584–1586.

32 Choate KA, Kinsella TM, Williams MI, Nolan GP, Khavari PA: Transglutaminase 1 delivery to lamellar ichthyosis keratinocytes. Hum Gene Ther 1996;7:2247–2253.
33 Choate KA, Khavari PA: Direct cutaneous gene delivery in a human genetic skin disease. Hum Gene Ther 1997;8:1659–1665.
34 Kim SY, Jeitner TM, Steinert PM: Transglutaminases in disease. Neurochem Int 2002;40:85–103.
35 Karpuj MV, Garren H, Slunt H, Price DL, Gusella J, Becher MW, Steinman L: Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei. Proc Natl Acad Sci USA 1999;96:7388–7393.
36 Karpuj MV, Becker MW, Springer JE, Chabas D, Youssef S, Pedotti R, Mitchell D, Steinman L: Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine. Nat Med 2002;8:143–149.
37 Bailey P, Riohardson NK, Pocalyko DJ, Schilling KM: Covalent bonding of active agents to skin, hair or nails. 1996 US Patent: US5490980.
38 Green H, Dijan P: Cosmetic containing corneocyte proteins and transglutaminase, and method of application. 1996 US patent: US55225336.
39 Handelman JK, Shander D, Funkhouser MG: Alteration of rate and character of hair growth. 1992 World patent: WO9211007.
40 Compton BJ, Corey GD, Dijan P, Green H: Linkage of agents using microparticles. 2002 World patent: WO0207707.
41 Ando H, Adachi M, Umeda K, Matsuura A, Nonaka M, Uchio R, Tanaka H, Motoki M: Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric Biol Chem 1989;53:2613–2617.
42 Yokoyama K, Nio N, Kikuchi Y: Properties and applications of microbial transglutaminase. Appl Microbiol Biotechnol 2004;64:447–454.
43 Cozzolino A, Di Pierro P, Mariniello L, Sorrentino A, Masi P, Porta R: Incorporation of whey proteins into cheese curd by using transglutaminase. Biotechnol Appl Biochem 2003;38:289–295.
44 Kumazawa Y, Nakanishi K, Yasueda H, Motoki M: Purification and characterization of transglutaminase from walleye pollack liver. Fish Sci 1996;62:959–964.
45 Sakamoto H, Kumazawa Y, Toiguchi S, Seguro K, Soeda T, Motoki M: Gel strength enhancement by addition of microbial transglutaminase during onshore surimi manufacture. J Food Sci 1995;60:300–304.
46 Seguro K, Kumazawa Y, Ohtsuka T, Toiguchi S, Motoki M: Microbial transglutaminase and ε-(γ-Glutamyl)Lysine crosslink effects on elastic properties of Kamaboko gels. J Food Sci 1995; 60:305–311.
47 Kumazawa Y, Numazawa T, Seguro K, Motoki M: Suppression of surimi gel setting by transglutaminase inhibitors. J Food Sci 1995;60:715–717.
48 Soeda T, Sakai T, Toiguchi S: Effects of microbial transglutaminase on the texture of surimi gels prepared from various kinds of fishes. Nippon Shokuhin Kagaku Kogaku Kaishi 1996;43: 787–795.
49 Kahn DR, Cohen I: Factor XIIIa-catalyzed coupling of structural proteins. Biochim Biophys Acta 1981;668:490–494.
50 Kuraishi C, Sakamoto J, Yamazaki K, Susa Y, Kuhara C, Soeda T: Production of restructured meat using microbial transglutaminase without salt or cooking. J Food Sci 1997;62:488–490.
51 Ikura K, Kometani T, Yoshikawa M, Sasaki R, Chiba H: Cross-linking of casein components by transglutaminase. Agric Biol Chem 1980;44:1567–1573.
52 Traorè F, Meunier JC: Cross-linking of caseins by human placental FXIIIa. J Agric Food Chem 1991;39:1892–1896.
53 Chanyongvorakul Y, Matsumura Y, Nonaka M, Motoki M, Mori T: Physical properties of soy bean and broad bean 11S globulin gels formed by transglutaminase reaction. J Food Sci 1995;60: 483–489.
54 Kang IJ, Matsumura Y, Ikura K, Motoki M, Sakamoto H, Mori T: Gelation and gel properties of soybean glycinin in a transglutaminase-catalyzed system. J Agric Food Chem 1994;42:159–165.
55 Porta R, Gentile V, Esposito C, Mariniello L, Auricchio S: Cereal dietary proteins with sites for cross-linking by transglutaminase. Phytochemistry 1990;29:2801–2804.

56 Basman A, Koksel H, Ng KW: Effects of transglutaminase on SDS-PAGE patterns of wheat, soy, and barley proteins and their blends. J Food Sci 2002;67:2654–2658.
57 Gerrard JA, Fayle SE, Wilson AJ, Newberry MP, Ross M, Kavale S: Dough properties and crumb strength of white pan bread as affected by microbial transglutaminase. J Food Sci 1998;63: 472–475.
58 Gujral HS, Rosell CM: Functionality of rice flour modified with microbial transglutaminase. J Cereal Sci 2004;39:225–230.
59 Mahmoud R, Savello PA: Mechanical properties of and water vapor transferability through whey protein films. J Dairy Sci 1992;75:942–946.
60 Mahmoud R, Savello PA: Solubility and hydrolyzability of films produced by transglutaminase catalytic crosslinking of whey protein. J Dairy Sci 1993;76:29–35.
61 Motoki M, Aso H, Seguro K, Nio N: αs1-casein film prepared using transglutaminase. Agric Biol Chem 1987;51:993–996.
62 Yildrim M, Hettiarachchy NS: Properties of films produced by cross-linking whey protein and 11S globulin using transglutaminase. J Food Sci 1998;63:248–252.
63 Lim L, Mine Y, Tung MA: Transglutaminase cross-linked egg white protein film: Tensile properties and oxygen permeability. J Agric Food Chem 1998;46:4022–4029.
64 Mariniello L, Di Pierro P, Esposito C, Sorrentino A, Masi P, Porta R: Preparation and mechanical properties of edible pectin-soy flour films obtained in the absence or presence of transglutaminase. J Biotechnol 2003;102:191–198.
65 Di Pierro P, Mariniello L, Giosafatto CVL, Masi P, Porta R: Solubility and permeability properties of edible pectin-soy flour films obtained in the absence or presence of transglutaminase. Food Biotechnol, in press.
66 Babiker EE, Hiroyuki A, Naotoshi MM, Iwata H, Ogawa T, Bando N, Kato A: Effects of polysaccharide conjugation or transglutaminase treatment on the allergenicity and functional properties of soy protein. J Agric Food Chem 1998;46:866–871.
67 Fink ML, Chung SI, Folk JE: γ-Glutamylamine Cyclotransferase: Specificity toward ε-(L-γ-glutamyl)-L-Lysine and related compounds. Proc Natl Acad Sci USA 1980;77:4564–4568.
68 Maister A, Tate SS, Griffith OW: γ-Glutamyl transpeptidase. Methods Enzymol 1981;77: 237–253.
69 Seguro K, Kumazawa Y, Kuraishi C, Sakamoto H, Motoki M: The ε-(γ-glutamyl)lysine moiety in cross-linked casein is an available source of lysine for rats. Nutrition 1996;126:2557–2562.
70 Rasmussen L, Mollgaard A, Petersen BN, Sorensen NH: Method for casein finishing of leather. 1996 World Patent: WO9413839.
71 Cortez J, Bonner PLR, Griffin M: A method for enzymatic treatment of textiles. 2002 World Patent: WO0204739.
72 Cortez J, Bonner PLR, Griffin M: Application of transglutaminases in the modifications of wool textiles. Enzyme Microb Technol 2004;34:64–72.
73 Josten A, Haalck L, Spener F, Meusel M: Use of the microbial transglutaminase for the enzymatic biotinylation of antibodies. J Immunol Methods 2000;240:47–54.
74 Josten A, Meusel M, Spener F: Microbial transglutaminase-mediated synthesis of hapten-protein conjugates for immunoassays. Anal Biochem 1998;258:202–208.
75 Josten A, Meusel M, Spener F, Haalck L: Enzyme immobilization via microbial transglutaminase: A method for the generation of stable sensing surfaces. J Mol Catal B Enzym 1999;279: 57–66.
76 Villalonga R, Fernàndez M, Fragoso A, Cao R, Di Pierro P, Mariniello L, Porta R: Transglutaminase-catalyzed synthesis of trypsin-cyclodextrin conjugates: Kinetics and stability properties. Biotechnol Bioeng 2003;81:732–737.
77 Villalonga R, Fernàndez M, Fragoso A, Cao R, Mariniello L, Porta R: Thermal stabilization of trypsin by enzymic modification with β-cyclodextrin derivatives. Biotechnol Appl Biochem 2003;38:53–59.
78 Sato H: Enzymatic procedure for site-specific pegylation of proteins. Adv Drug Deliv Rev 2002;54:487–504.
79 Broad PG, Pierce K, Coggan M: Expression of functional coagulation factor XIII in *Escherichia coli*. Thromb Haemost 1990;63:235–240.

80 Lai T-S, Santiago MA, Achyuthan KE, Greenberg CS: Purification and characterization of recombinant human coagulation factor XIII A-chains expressed in *E. coli*. Protein Expr Purif 1994;5: 125–132.
81 Bishop PD, Teller DC, Smith RA, Lasser GW, Gilbert T, Seale RL: Expression, purification and characterization of human Factor XIII in *Saccaromyces cerevisiae*. Biochemistry 1990;29: 1861–1869.
82 Shi Q, Kim S-Y, Blass JP, Cooper ALJ: Expression in *Escherichia coli* and purification of hexahistidine-tagged human tissue transglutaminase. Protein Expr Purif 2002;24:366–373.
83 Gillet SM, Roberto A, Chica A, Keillor JW, Pelletier JN: Expression and rapid purification of highly active hexahistidine-tagged guinea pig liver transglutaminase. Protein Expr Purif 2004;33:256–264.
84 Osman AA, Richter T, Stern M, Conrad K, Henker J, Brandsch C, Zimmer KP, Mothes T: Production of recombinant human tissue transglutaminase using the *Baculovirus* expression system, and its application for serological diagnosis of coeliac disease. Eur J Gastroenterol Hepatol 2002;14:1217–1223.
85 Giddings G, Allison G, Brooks D, Carter A:. Transgenic plants as factories for biopharmaceuticals. Nat Biotechnol 2000;18:1151–1155.
86 Gao J, Hooker BS, Anderson DB: Expression of functional human coagulation factor XIII A-domain in plant cell suspensions and whole plants. Protein Expr Purif 2004;37:89–96.
87 Sorrentino A, Schillberg S, Fischer R, Rao R, Porta R, Mariniello L: Recombinant human tissue transglutaminase produced into tobacco suspension cell cultures is active and recognizes autoantibodies in the serum of coeliac patients. Int J Biochem Cell Biol; in press.

Dr. Loredana Mariniello
Department of Food Science, University of Naples 'Federico II'
Parco Gussone, Portici, IT–80055 Naples (Italy)
Tel. +39 0817754867, Fax +39 0817755116, E-Mail loredana.mariniello@unina.it