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Purification of bovine liver transglutaminase by gel filtration

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Abstract

Transglutaminases (TGases) are enzymes that catalyze transfer of acyl group and covalent crosslinks formation between peptide-bound glutaminyl residues and amino groups. TGases have many industrial applications and have been purified from various sources. TGase was purified from the bovine liver extract by gel filtration on Sephacryl S-200 HR column. TGase activity was measured using CBZ-L-glutaminylglycine & hydroxylamine and the enzyme was characterized with respect to its response to different temperatures, pHs and salt concentrations. TGase was purified by yield 36.7%, had a weight 74 kDa, a high pH (pH = 8) and temperature (45 °C) optimum. The enzyme was observed to be stable at temperatures below 55 °C and was stable within a narrow pH range of 6.5–8.0. Purified TGase showed Ca^{2+} dependent characteristics and tended to retain activity at a high NaCl concentration. These results revealed that purified TGase can be used as a potential alternative to other sources.

Keywords: Transglutaminase, Bovine liver, Purification, Gel filtration, Optimum condition

Introduction

Transglutaminases (TGase) (EC 2.3.2.13) are a widely distributed group of enzymes that belong to the group of transferases. TGases catalyze the acyl transfer reaction between a free amine group (e.g., in a protein or peptide-bound lysine or an amine) and the γ-carboxy amide group of proteins or peptide bound glutamine thus leading to the modification of proteins [1]. When an ε-amino group of a peptide bound lysine acts as a acyl acceptor, isopeptide bond is formed between the glutamine and lysine residues in them, introducing both inter- and intramolecular covalent cross-links, resulting in the polymerization of the proteins [1–3].

TGase was first discovered by Heinrich Waelsch more than 40 years ago as a liver enzyme; incorporating amines into proteins [4]. Initial research on the applications of TGases started with the isolation of enzymes from mammalian tissues and body fluids [5–7]. These enzymes showed the possibility to modify the functional properties in milk caseins and soybean globulins in the early 1980s [8–10].

TGases have been purified from various animal tissues or organs (such as the liver and hair follicle of guinea pig, pig plasma and human epidermis, erythrocyte, plasma and placenta, fish muscle) [5, 11–14], plants [15, 16] and microorganisms [17, 18]. Until late 1980s, guinea pig liver TGase was the only commercially available TGase [19]. However, its scarce source in addition to the laborious purification procedure entailed extremely high prices on the market and the use of TGases as texture enhancer in foods resulting in a low attractiveness for potential industrial applications [9, 10].

For industrial applications, large scale production of the enzymes is necessary. Recently, efforts have been made to obtain TGases from microorganisms and used widely as a functional enzyme within various branches of the food industry. Fermentation of TGases derived from microorganisms makes it possible to achieve mass production of them from cheap substrates [9]. However, separation and purification of TGases from plant and animal tissues are still in their infancy.

Other approach is extraction and purification of the TGase from the tissues or body fluids of food-use

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animals, such as fish, swine, and cattle [20]. Now in Europe, blood factor XIII, a certain type of TGase is extracted commercially from the blood of swine and cattle at slaughterhouses [21].

On the other hand the search for new enzymes with novel properties or which displays better characterization than the ones documented is a growing trend and holds a pivotal position in the future of enzyme technology. The overall rational for this study was to discover new source of TGase for potential use to meet the growing demands for enzymes in commercial applications. In this research, TGase from the bovine liver was selected for preliminary purification and characterize with respect to its response to pH and temperature, in addition to its response to different calcium and sodium chloride concentrations.

Materials

The following materials: CBZ-glutaminyl glycine, hydroxylamine, L-glutamic acid γ-mono hydroxamate and reduced glutathione were purchased from Sigma Chemical Co. Bovine serum albumin (BSA), trichloroacetic acid (TCA), ferric chloride hexa hydrate, were purchased from Merck Chemical Co. All other reagents and chemicals were of analytical grade.

Experimental methods

Sample preparation and enzyme purification

Fresh bovine liver was received from a local slaughterhouse transported on ice to the laboratory. The tissues were washed by flushing with distilled water and then TGase was purified based on the method described by Worratao and Yongsawatdigul [22] with a slight modification. Approximately 4 g of liver sample were homogenized in 12 ml of extraction buffer (0.05 M Tris–HCl, 0.05 M NaCl, 0.025 M EDTA and 0.01 M DTT, pH 7.5) at 8,000 rpm in ice bath for 3 min using a Yellow Line DI 18 basic homogenizer. The homogenate was centrifuged first at 3000 rpm, 5 min and then two times at 13,000 rpm for 30 min both at 4 °C in a Sigma ultracentrifuge. The supernatant solution was loaded on the gel filtration column. The Sephacryl S-200 HR column (1 × 87 cm) was packed as per the manufacturer's manual (GE Healthcare). The columns Void Volume determined by blue dextran, which was about 25 ml. About 1.2 ml of the homogenate was loaded onto the column equilibrated with E (elution) Buffer (0.01 M Tris–HCl pH 7.5 containing 0.1 M NaCl, 0.005 M EDTA and 0.002 M DTT) and washed at a constant (2 ml/min) flow rate with the same buffer. Fractions of 1.5 ml were collected and the protein content and TGase activity of each fraction was monitored. The fractions with any activity were pooled and concentrated into 2 ml by a Millipore ultrafiltration membrane (cut-off 30 kDa).

Characterization of purified TGase

The purified TGase was characterized with respect to pH and temperature (stability and optimum) in addition to effect of Calcium and sodium Chloride salts.

The thermo stability of TGase was studied by incubating the enzyme extract for 60 min at various temperatures at 5 °C intervals from 5 to 70 °C at pH 6.0 and their activity was assayed. Also the effect of temperature on enzyme activity was determined at the same temperatures. The activity was estimated as a percentage, taking maximum change in absorbance as 100%.

The effect of pH on enzyme stability and activity was determined using various buffers. The enzyme activity was measured after incubation at 37 °C for 5 min in buffer solutions of various pHs. Also the effect of pH on the TGase stability was determined by incubating the enzyme in different pHs for 30 min at 37 °C. Activity was estimated as a percentage, taking the maximum pH change in absorbance as 100%.

The effect of Na and Ca ions was investigated by varying the concentration of $CaCl_2$ and NaCl in the reaction mixture at 0–8 and 0–0.015 M range respectively.

Protein determination

Concentration of protein was determined by the Lowry method [23], using bovine serum albumin (BSA) as a standard.

Assay of TGase activity

The enzyme activity was assayed according to the method of Folk [5]. It was based upon the measurement of peptide-bound γ-glutamyl hydroxamate formed from N-carbobenzoxy (CBZ)-L-glutaminyl glycine and hydroxylamine as the substrates in the presence of the enzyme and Ca^{2+}.

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

And then in the presence of TCA a red color iron complex was formed with the produced hydroxamic acid. About 0.5 ml of the sample was mixed with 0.5 ml of the R (reaction) mixture (200 µl of 1.0 M Tris–acetate buffer, 150 µl of 0.1 M CBZ-L-Glutaminylglycine, 50 µl of 0.1 M $CaCl_2$, 50 µl of 2.0 M hydroxylamine and 50 µl of 0.2 M reduced glutathione; pH 6.0) and incubated for 10 min at 37 °C. Then 0.5 ml of S (stop) solution (equal volume of 3 M HCl, 12% (w/v) TCA and 5% (w/v) ferric chloride solution in 0.1 M HCl) was used to terminate the reaction and the absorbance of the supernatant was measured at 525 nm immediately. A standard curve was drawn using γ-mono-hydroxamic acid L-glutamate.

One enzyme activity unit was defined as the amount of enzyme that catalyzed the formation of 1.0 µmol of peptide-bound γ-glutamyl hydroxamate in each minute at pH 6.0 and at 37 °C. The specific activity was defined as the number of activity units per milligram of protein.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE under reducing conditions was carried out according to the method described by Laemmli [24]. Electrophoresis was conducted at a constant voltage of 120 V in 10% polyacrylamide gels and the gels were stained with Coomassie Brilliant Blue R-250 overnight.

Results

Enzyme purification

About 1.2 ml of the homogenate was applied to the column and eluted. The concentrated fraction of each peak (Fig. 1) was tested for protein and TGase activity and fractions 12–23 (totally 17 ml) that had any detectable activity were collected, pooled and concentrated into 2 ml by ultrafiltration membrane. The purification results (Table 1) revealed about twofold increase in specific activity and a 36.7% recovery yield. SDS-PAGE of the fractions revealed one major band at 74 kDa indicating the preparations were completely homogenous.

Effect of temperature on enzyme

The thermo-stability of bovine TGase after 60 min incubation at various temperatures is shown in Fig. 2. The enzyme was stable at temperatures below 55 °C but was gradually inactivated at higher temperatures. There was no activity observed at refrigerator temperature (4 °C). The activity seems to decrease steadily after 50 °C. The optimal temperature of the bovine TGase was at 45 °C. The activity seems to decrease steadily after 50 °C and was no activity observed not only at 70 °C but also at refrigerator temperature (4 °C).

Effect of pH on enzyme

The enzyme was stable between pH 6.5 and 8.0 when incubated for 30 min at 35 °C. There was 70% residual activity above pH 7, however losing of activity in the acidic (below pH 4 and alkaline (above pH 8.5) regions was remarkable (Fig. 3). Bovine TGase could acylate its substrate over a relatively broad pH range (4.0–10.0). At low pH (acidic) enzyme seemed to be inactive. The TGase exhibited higher activity within the pH range of 6–8 with an optimum at pH 8.0.

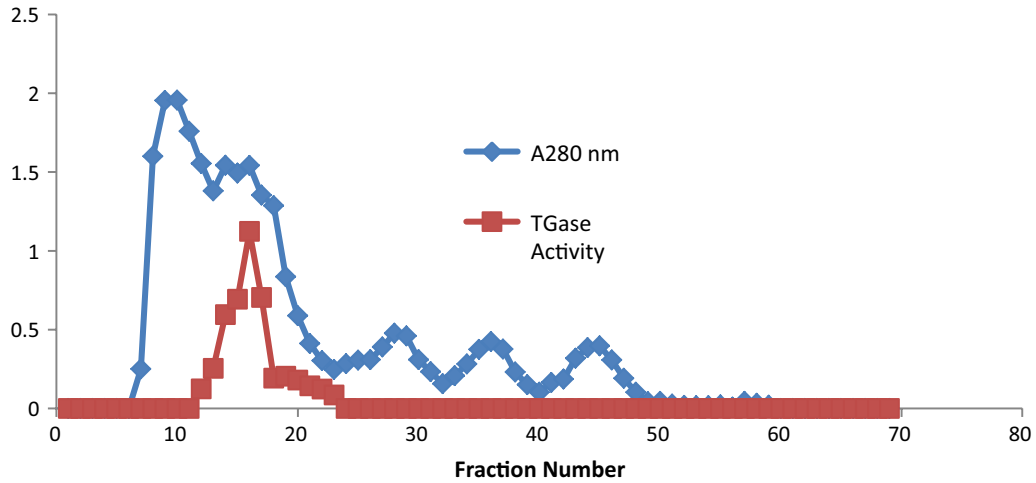

Fig. 1 A 280 nm of fractions eluted from the size exclusion column after extract loading

Table 1 Summary of procedures employed for purifying of the TGase from the liver of bovine

Purification step	Total volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Activity (U/ml)	Total activity (IU)	Spcific activity (U/mg)	Recovery (%)
After homogenization	1.2	69.33	83.2	9.94	11.93	0.143	100
After gel filtration	17	0.91	15.47	0.26	4.42	0.28	37
After ultrafiltration	2	7.1	14.2	2.19	4.38	0.31	36.7

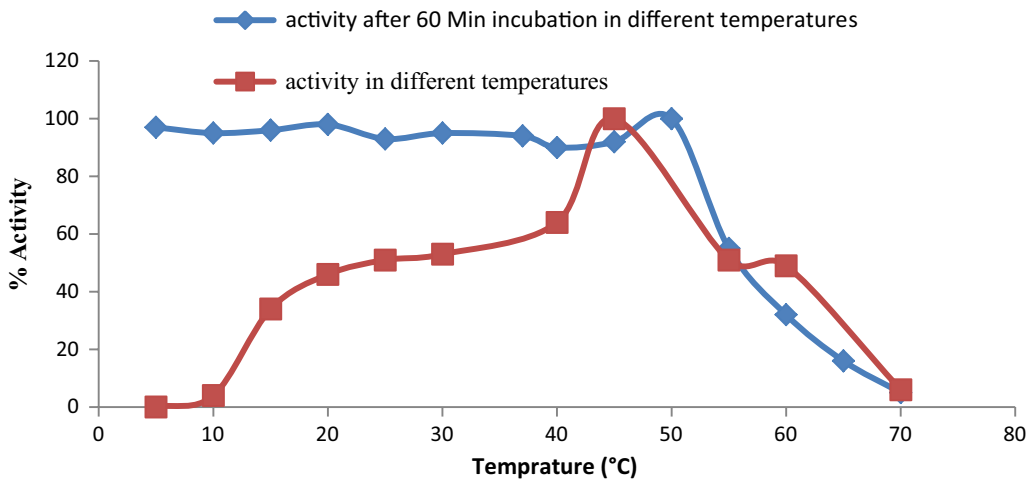

Fig. 2 Relative percent of enzyme activity in different temperatures and after 60 min incubation in different temperatures (assay is performed at pH 6.0 with CBZ-L-glutaminylglycine and hydroxyl amine as substrates)

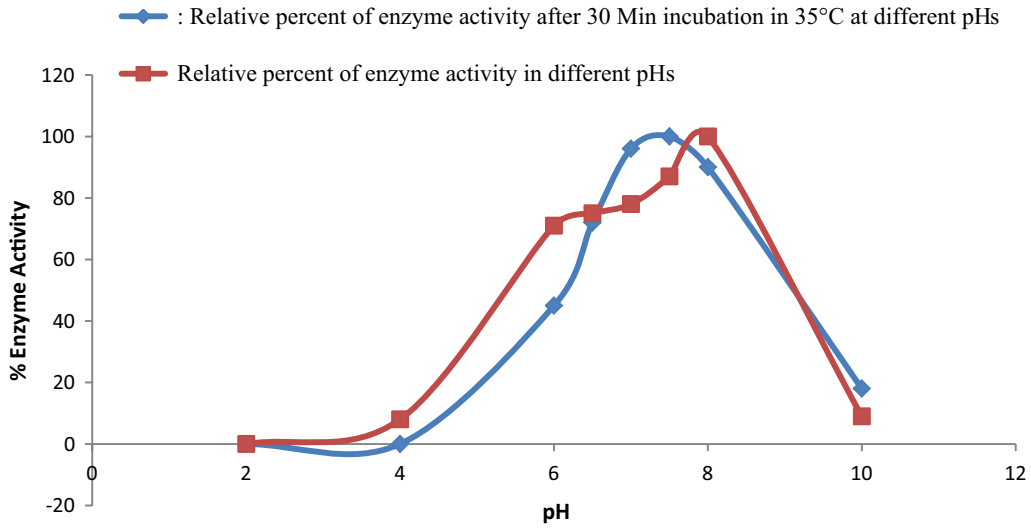

Fig. 3 Relative percent of enzyme activity in different pHs and activity after 30 min incubation in 35 °C at different pHs (KCl—HCl pH 2.0, citrate–phosphate pH 4.0–6.0, Tris–HCl pH 8.0, and carbonate–bicarbonate pH 10.0) (assay is performed in 37 °C with CBZ- L-glutaminylglycine and hydroxyl amine as substrates)

Effect of calcium and sodium chloride

Purified TGase like other mammalian TGases showed an absolute requirement for calcium ions (Fig. 4) [25] and not only no activity observed in the absence of Ca^{2+}, but also the activity increased with Ca^{2+} concentration (Fig. 4) and at 0.006 M reached the maximum. These results indicate that purified TGase is Ca^{2+} dependent. NaCl slightly reduced the activity of purified TGase. A residual activity of 78% was found at 0.015 M NaCl.

SDS-PAGE of TGase

The purity and MW of TGase was examined via SDS–polyacrylamide gel electrophoresis versus molecular weight standards. Appearance of a single band indicating that the prepared enzyme was exhibited a purity of more than 80%. Based on the log MW of the BSA and its relative mobility (R_f), the molecular weight of TGase was determined about 74 kDa (Fig. 5).

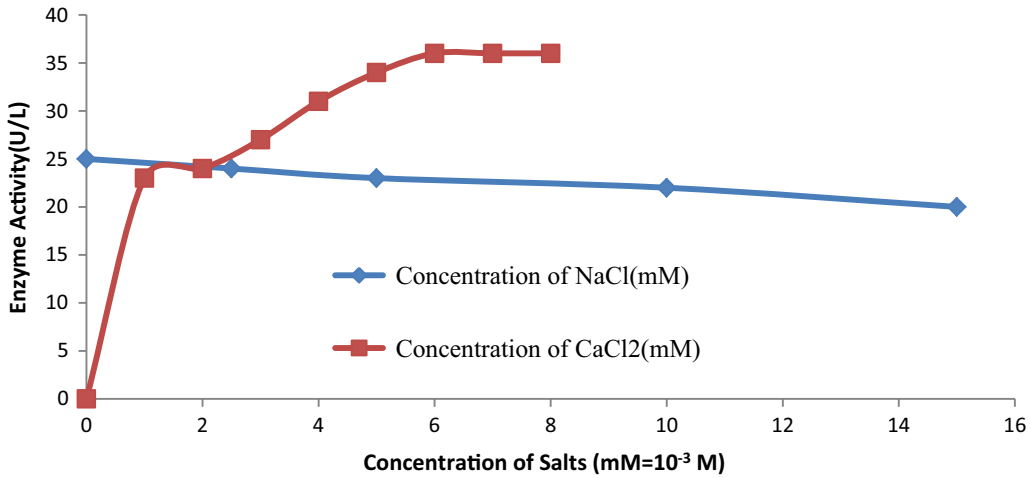

Fig. 4 Effect of different $CaCl_2$ (0–0.008 M) and NaCl (0–0.015 M) concentrations on the activity of purified bovine liver TGase. (assay is performed in 37 °C and pH 6.0 with CBZ- L-glutaminylglycine and hydroxyl amine as substrates)

Discussion

Liver TGase is one of the most widely studied tissue-type TGase although its physiological role is clearly unknown. The rat liver TGase activity is associated with the hepatocytes envelope and may participate in cell to cell contact by forming covalently cross-linked matrices of proteins [26]. Now, TGase is widely used in industries as a protein glue and texture enhancer and its production should be increased. TGase is purified from various animal tissues or organs, plants and microorganisms. Therefore waste tissues such as livers in slaughterhouses could be a novel source from environmental view (Table 2). On the other hands a few researchers have studied and reported different purification methods and yields.

In this research, observed yield (36.7%) was much lower than reported for other sources [14, 22, 33]. Araki [34] showed that TGase activity vary in different sources and species. Also we should keep it in mind that liver tissue was received from a local slaughterhouse and transported to Lab on ice and according to Leblanc [35] and Vihelmsson (1997) [36], endogenous TGases decreases rapidly after catch.

Effect of temperature on the TGase (activity and stability)

The thermal stability of purified TGase is shown in Fig. 2. It was observed to be quite stable below 50 °C and approximately 90% of the initial activity of enzyme was retained after 60 min of incubation at 40 °C. However at higher temperatures, the stability decreased with the TGase losing 60% of activity at 70 °C. The higher thermal stability of bovine TGase may be related to the higher habitat temperature of the animal.

The optimal temperature of bovine TGase was between 40 and 45 °C. There was no activity observed at refrigerator temperature (4 °C) and the activity seems to decrease steadily after 50 °C. This could be due to possible denaturation of the enzyme and a subsequent reduction in its catalytic activity.

Carp TGase showed optimum activity at 30 °C [37], Pollock liver TGase between 35 and 50 °C [38] and red sea bream liver between 55–60 °C [12]. A microbial TGase obtained from *Streptoverticillium mobaraense* and commercialized by Ajinomoto Co., showed a temperature optimum at 50 °C [39]. An optimal temperature of 50 °C is reported for tropical tilapia [22], threadfin bream liver [33] and walleye pollack liver [30]. Slightly higher optimum temperature of 55 °C was reported in red sea bream liver TGase [12] while TGase from Japanese oyster, Pollock liver and scallop exhibited the best activity between 35 and 50 °C [30, 38]. Therefore optimal temperature of TGase tends to vary with sources and as Lee [40] reported, could be related to the species habitat temperature.

Effect of pH on the TGase (activity and stability)

The effect of pH on the stability of the purified TGase was evaluated by measuring enzyme activity after incubation at various pH conditions for 30 min. TGase was stable within a narrow pH range of 6.5–8.0 (Fig. 3). This result was similar to those obtained by Nozawa [38] who reported squid gill TGase to be stable at pH 7.5–9.0.

The TGase extract exhibited higher activity within the pH range of 6–8 with an optimum at pH 8.0. These results are consistent with those obtained by Worratao [22] and Nozawa [38] who reported an optimum pH of

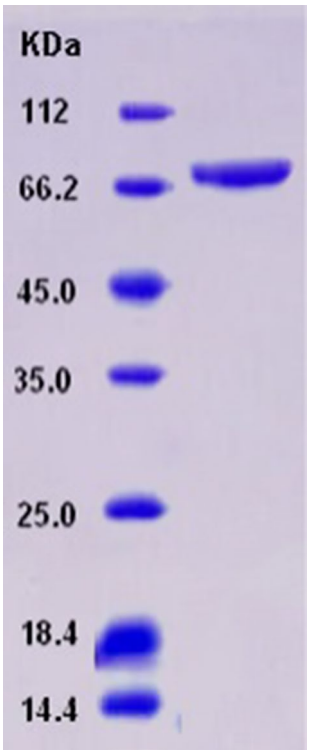

Fig. 5 SDS-PAGE pattern showing the purification of the bovine liver TGase. Lane 1: Molecular Weight Markers, lane 2: purified TGase (74 KDa) from the gel filtration chromatography. The concentration of the separating gel was 10%. Migration was from top to bottom

7.0–7.5 for tilapia muscle TGase and 7.5–8.0 for squid gill, respectively. Especially a relatively high pH optimum (8.5–9.0) was found in red sea bream TGase [12], threadfin bream liver [33], and walleye pollack liver TGase [30].

Effect of calcium and sodium chloride on the activity of TGase

Observations indicated purified TGase like other mammalian TGases has an absolute requirement for Ca^{2+} ions and its activity is Ca^{2+} dependent (Fig. 4) [25]. The enzyme has no activity in the absence of Ca^{2+}, and its activity increased with Ca^{2+} concentration so at 0.006 M reached the maximum. For showing full activity, TGases from different sources need to various Ca^{2+} concentrations. TGases from limulus hemocyte and guinea pig liver, for example, needed 0.008 and 0.010 M Ca^{2+}, respectively [32]. TGase purified from red sea bream liver, for full activation, required only 0.0005 M of Ca^{2+} [12]. Ahvazi [41] reported for activating each human TGase III which is the cytoplasmic TGase expressed in stratified squamous epithelia, three Ca^{2+} ions is required. The Ca^{2+} may induce enzyme conformational changes which lead to exposing the active site cysteines (Cys) to a substrate [7]. Noguchi [42] has postulated the

Table 2 Different sources employed for TGase purification

Source	References
Mammals	
Human plasma factor XIII	Folk [27]
Bovine factor XHIa	Folk [27]
Guinea pig liver	Folk [27]
Rabbit liver	Abe et al. [11]
Plant	
Pea seedlings	Icekson et al. [15]
Alfalfa	Kuehn et al. [16]
Microbial	
S. mobaranese	Ando et al. [18]
Physarum polycephalum	Klien et al. [28]
S. ladakanum	Tsai et al. [17]
Seafoods	
Red sea bream liver	Yasueda et al. [12]
Carp muscle	Kishi et al. [29]
Walleye Pollack liver	Kumazawa et al. [30]
Lobster muscle	Myhrman et al. [31]
Japanese oyster	Kumazawa et al. [13]
Limulus hemocyte	Tokunaga et al. [32]
Scallop, Botan shrimp, Squid, Rainbow Trout, Atka mackerel	Nozawa et al. [14]

Ca^{2+} binding to a special site on the surface of red sea bream TGase, resulting in structural and conformational changes. Subsequently, tyrosine covering the catalytic Cys is removed. Then the acyl group donor can bind with the Cys and forming an acyl-enzyme intermediate at the active site.

The activity of purified TGase slightly decreased in the presence of NaCl so at 0.015 M NaCl, 78% of total activity was found (Fig. 4). Nozawa [38] showed the activity of TGases from carp, rainbow trout and atka mackerel are not sensitive to NaCl up to 0.5 M, although this sensitivity was observed in marine invertebrates such as botan shrimp, scallop and squid TGases. Also NaCl can activate TGase from Japanese oyster and hemocyte [13, 38]. These results indicate that bovine TGase can be used in food products containing up to 0.015 M NaCl ignoring significant loss in activity.

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Authors' contributions
The design and implementation of this research was the responsibility of SMHR and AK, ZK helped him in the preparation of materials and instruments. All authors read and approved the final manuscript.

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