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RESEARCH ARTICLE

PROPERTIES AND APPLICATION OF MICROBIAL TRANSGLUTAMINASE PRODUCED FROM A NEWLY ISOLATED STRAIN OF *STREPTOMYCES* SP.

Dalia H. Eshra, Samer M. El-Iraki and Taiseer M. Abo Bakr*
Food Science and Technology Department, Faculty of Agriculture, El-Shatby, Alexandria University, Alexandria Egypt.

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Abstract

The aim of the present work is to study some physical properties of microbial transglutaminase (MTGase) produced from a newly isolated strain of *Streptomyces* sp. Also, its application in producing *Shamy* bread from a mixture of wheat and corn flours was investigated. The results revealed that the enzyme exhibited optimum activity at 45°C; it retained about 80% of the initial activity after incubation for one hour at this temperature. The optimum activity was at pH 6.5 and was stable at this pH for one hour.

The cross-linking effect of the enzyme was tested through cross-linking of wheat dough prepared for *Shamy* bread. The results indicated that the enzyme have a cross- linking effects towards the free amino and thiol groups of wheat dough. The free amino groups of the wheat dough were 0.538 µM/ mg flour without adding the enzyme. It decreased to 0.378 µM/ mg flour when treated with the enzyme; the percent of reduction was 29.74. Mixing corn flour with wheat flour decreased the free amino groups of the resulted dough. It decreased to 0.248 µM/ mg flour in the sample treated with the enzyme and containing 30% corn flour with percentage of reduction 34.39.

In general, increasing the percentage of corn flour in the blends treated with MTGase decreases the free thiol groups. The free thiol groups of the wheat dough (without enzyme) were 11.222µM/ g flour, it decreased to 10.342 µM/ g flour when treated with the enzyme, with percentage of reduction 7.849 %. When the levels of corn flour increased to 30% the free thiol groups decreased and the percent of the reduction increased to 8.575. It was noted that the prepared *Shamy* bread from dough containing 70% wheat flour and 30% corn flour and treated with the enzyme have a higher volume with pronounced improvement in the general appearance and quality than that without the enzyme.

Corresponding Author:- Taiseer Abo Bakr.
Address:- Food Science and Technology Department, Faculty of Agriculture, El-Shatby, Alexandria University, Alexandria, Egypt.

Introduction:

Transglutaminase (TGase) (EC 2.3.2.13) is an enzyme that catalyses an acyl transfer reaction using peptide – bond glutamine residues as acyl doners and several primary amines as acyl acceptors (Yokoyama et al., 2004).
The enzyme is active over a wide range of temperatures and stable between pH 5 and 9 which is the range for most food processing (Kuraishi et al., 2001). With respect to substrate specificity; most food proteins, legume globulins, wheat, egg yolk and milk caseins, as well as many other albumins could be cross linked by MTGase (Nonaka et al., 1997).

MTGase is capable of gelling concentrated solutions of proteins such as soybean, milk, beef, chicken and fish proteins. Also, two or more different proteins can be covalently conjugated to produce new proteins with novel functionalities (Nielsen, 1995; Zhu et al., 1995).

The characteristics of MTGase obtained from various microorganisms vary even among strains. MTGase from *Streptomyces libani* showed slightly lower optimum reaction temperature and thermal stability than from *Streptomyces mobaraensis* (Umezawa et al., 2002).

The enzyme has many applications in the food industry because its effect of crosslinking which is useful in texturization, foaming and emulsifying properties as well as for improvement of nutritional properties.

Several studies reported that the use of TGase improves baking properties and products quality. The action of enzyme reinforces the protein network structure changing the viscoelastic properties of the dough (Larre et al., 2000). Gerrad et al. (2001) reported that addition the enzyme to dough improved its stability and loaf volume, as well as, improved the lift of puff pastry. Many applications showed that TGase increased the crumb strength of baked loaves and improved the water absorption of the dough, which lowering processing costs for commercial baking (Dube et al., 2007). Ap et al. (2011) reported that the enzyme modified chemical and functional properties of glutenins fraction of proteins, improving dough strength and bread volume. The addition of 1.0% TGase increased both foam stability and emulsion activity of cake batter. Also, it had the maximum specific volume and the softest texture (Wang et al., 2013).

Corn flour is one of the most valuable cereal flours from a nutritional point of view. However, corn flour is unable to hold gas produced during fermentation for baked products. So, the aim of the present work is to study some physical properties of microbial transglutaminase produced from a newly isolated strain of *Streptomyces* sp. Also, its application in producing *Shamy* bread from blends of wheat and high levels from corn flour was investigated.

Materials and Methods:-

Microorganism:-

The actinomycetes strain used in the present study was isolated from soil samples collected from Alexandria Governorate, Egypt. The strain was identified as *Streptomyces diastaticus* as reported in the previous paper (Eshra et al., 2015)

Chemicals and reagents:-

N-carboxybenzoyl-L-glutaminyl-glycine (CBZwas purchased from Sigma-Aldrich, Co., USA. All the other chemicals were of analytical grade.

Preparation of tryptic casein hydrolyzate (TCH):-

Twenty grams of alkaline soluble casein were dissolved in 100 ml Tris-buffer pH 8.0, and then 0.2 g of trypsin (E-Merck) were added and incubated for 18 h at 37°C. The mixture was boiled for 5 min, cooled in ice bath and centrifuged at 3000 rpm for 20 min and preserved at - 4°C. The activity of equal volumes of pure enzyme solution (Ajinomoto Activa WM MTGase) was measured separately by both the prepared TCH and CBZ. A correction factor was calculated to convert the enzyme units measured by TCH to the more common units measured by the synthetic substrate CBZ.The correction factor was measured for each prepared batch of the tryptic casein hydrolyzate. The average of this factor was ranged between1.42 to 1.55.

MTGase Activity:-
The enzyme activity was measured using the colorimetric hydroxamate procedure using TCH and CBZ as substrates. The absorbance was measured at 525 nm (Spectronic 20, Bausch & Lomb, USA). One unit of MTGase activity was defined as the amount of enzyme which causes the formation of one micromole of hydroxamic acid per min at 37°C. A calibration curve was prepared using γ-glutamic acid γ-monohydroxamate (Macedo *et* al., 2007).

Effect of Temperature:-
The effect of temperature on the enzyme activity was tested at temperatures ranging from 25 to 60°C at pH 6. The relative activity was determined by maximal activity of the enzyme at a specific temperature as 100% (Cui et al., 2007).

Thermal stability was determined by incubating the enzyme at 25 to 60°C for 1 hr. The percentage of stability was determined as follow:

$$\% \text{ stability} = \frac{\text{Residual units}}{\text{Initial units}} \times 100$$ (Cui et al., 2007).

The effect of pH on the enzyme activity was determined using 50 mM citrate buffer (pH 4.0 – 6.5) and 50 mM Tris-HCL buffer pH (7.0-9.0) after 30 min at 35°C.To check the pH stability, the enzyme was incubated with the previous buffers at 37°C for 1 hr and the enzyme activity was determined (Cui et al., 2007).

***Shamy* bread making:-**
Shamy bread was prepared as described by Gujral and Rosell (2004) with some modifications. The following ingredients were used: 500 g wheat flour (72% extraction), 450 ml water, 15 g active dry yeast, 10 g salt and 40 g sugar (yeast was previously mixed with sugar and dissolved in 30 ml water). All ingredients were mixed in the Nouval dough mixer (Image, Egypt) for 5 min. MTGase was incorporated at level of 100 units/ 500 g wheat flour.

The resulted dough was left to ferment for about 20 min at 30°C, and then divided into pieces. The pieces were arranged on a wooden board and left to ferment for about 60 min at the same temperature. The fermented dough pieces were flattened. The flattened loaves were baked at 220°C for 5 min in muffle furance (Vulcan ™ A-550 Yucaipa, California, USA) 10-15 min. The loaves of bread were allowed to cool then packed in polyethylene bags.

The effect of using MTGase on *Shamy* bread containing different levels of corn flour was studied. Corn flour was added at levels of 10, 20, 30, 40 and 50% based on the weight of wheat flour. The same method was used for *Shamy* bread prepared from wheat flour only.

Quantification of free amino groups:-
Free amino groups in control and MTGase-treated dough samples were determined by the method described by Nielsen *et al.* (2001). The O-phthaldialdehyde (OPA) 40 mg was dissolved in 1 ml of ethanol. In a separate solution, 1.905 g of di-sodium tetraborate decahydrate and 50 mg of sodium dodecylsulfate were dissolved in 40 ml of distilled water. The two solutions were mixed and the volume brought to 50 ml with distilled water. The OPA reagent was stored in an opaque bottle in a refrigerator. One part of 2- mercaptoethanol was mixed with 21.27 parts of the OPA reagent just before use in the assay. Supernatant of each sample was obtained by mixing 100 mg dough with 90µl of distilled water, adding 1 ml 0.1 M HCl, mixing the suspension on a vortex mixer for 10 min and then centrifuging (Cell Refrigerated Centrifuges, Bunsen) at 16000 × g for 5 min. To 50 µL of the dough supernatant, 250 µl of the OPA reagent containing 2-mercaptoethanol was added. Absorbance at 340 nm was recorded for the mixtures after 2 min using a UV-spectrophotometer (Thermospectronic,UV- visible Spectrophotmeters , Helios). The results were calculated against a serine standard curve (Gujral and Rosell, 2004).

Quantification of free thiol groups:-
Free thiol groups in control and dough samples –treated with MTGase were determined with a procedure using Ellman's reagent (Rao et al., 2002). Tris-glycine (Tris-Gly) buffer (pH 8.0) was prepared by dissolving 10.4 g Tris, 6.9 g glycine and 1.2 g ethylenediamine tetraacetic acid (EDTA) in 1 l of distilled water. A solution (GuHCl/ Tris-Gly) containing 5 M guanidine hydrochloride (GuHCl) in Tris-Gly buffer was prepared. Ellman's reagent contained 4 mg of 5,5' dithiobis-2-nitrobenzoic acid in 1 ml of Tris-Gly buffer pH 8.0. To obtain supernatant, 200 mg of dough was added to 1 ml of (GuHCl/ Tris-Gly) solution, mixed on a vortex mixer for 10 min, and centrifuged (Cell

Refrigerated Centrifuges, Bunsen) at 16000 × g for 5 min. (GuHCl/ Tris-Gly) solution (150 µl) and Ellman's reagent (50 µl) were added to 100µl of the supernatant and the absorbance measured at 412 nm (Spectronic 20, Bausch & Lomb, USA). The results were calculated against a cysteine standard curve (Gujral and Rosell, 2004).

Results and Discussion:-

Effect of temperature:-

The results presented in Table (1) indicated that the enzyme exhibited optimum activity at 45°C. At 60°C, 52.0% of the maximum enzyme activity was detected. Decreasing the temperature to 25°C conserved only 48.0 % of the maximum activity. The enzyme retained about 80% of the initial activity after incubation for one hour at this temperature. The optimum activity was at pH 6.5 and was stable at this pH for one hour. Ho et al. (2000) reported that the optimal temperature of TGase enzyme purified from *Streptoverticillium ladakanum* was 40°C. It conserved more than 90% of its activity even after 30 min incubation at 35°C. Cui et al. (2007) mentioned that the optimum activity of MTGase from *Streptomyces hygroscopicus* was at 37-45°C. Also, they reported that the purified enzyme maintained full activity after incubation for 30 min at 20°C. When the temperature was above 50°C, the enzyme was rapidly inactivated and preserved only 7% of the initial activity when it was exposed to 60°C for 30 min.

Table 1:- Effect of temperature on MTGase activity produced from *Streptomyces diastaticus.*

Temperature (°C)	Relative Activity (%)	Stability (%)
25	48.00	97.15
30	54.00	98.06
35	65.33	100.00
40	82.67	96.15
45	100.00	80.77
50	70.67	67.31
55	54.00	48.08
60	52.00	40.38

Effect of pH:-

The optimum activity was at pH 6.5. The activity decreased rapidly at alkaline pH, but it decreased gradually at acidic side being only 36.36% of its maximum activity at pH 4.0. MTGase was stable at pH 6.5 for one hour. At pH 5.0 the retained activity was about 50% of the initial activity. The activity decreased greatly below pH 5.0 and above 8.5 (Table 2).

Table 2: Effect of pH on MTGase activity produced from *Streptomyces diastaticus*.

pH	Relative activity (%)	Stability (%)
4.0	36.36	16.39
4.5	48.48	37.70
5.0	48.48	50.82
5.5	68.18	65.57
6.0	86.36	78.69
6.5	100.00	100.00
7.0	81.82	90.16
7.5	48.48	81.97
8.0	18.18	55.74
8.5	9.09	44.26
9.0	3.0	29.51

These results are in agreement with other authors (Cui et al., 2007) who reported that, the enzyme exhibited optimum activity in a range of pH 6.0- 7.0. Also, they declared that, the purified MTGase was stable within a wide range of pH 5.0- 8.0 at 10°C. The enzyme was stable at pH 5.0- 7.0 after 30 min of incubation at 37°C and about 50% activity was retained at pH 8.0.The activity decreases greatly outside this pH range.

Attest the cross-linking effect of the prepared enzyme:-

The attest of the occurrence of cross-linking and/ or its effect have to be proofed prior to its application in food. The cereal products mainly bread is one of the common applications of MTGase in food industry. So, the cross-linking effect will be tested through cross-linking of wheat dough prepared for *Shamy* bread making. The amount of the enzyme used was 100 enzyme units per 500 g of the flour used. The *Shamy* bread was prepared as described in the materials and methods section.

The free amino groups which representing the direct cross-linking by forming a new iso-peptide bonds and the free thiol groups which indicate the indirect effect refer to the oxidation of free sulfhydryl group and formation of disulphide bonds were investigated. The changes of the free amino and thiol groups content will be used as a tool for tracing the cross-linking occurred.

The results indicated that the enzyme have a cross-linking effects towards the free amino and thiol groups .These results are in agreement with those reported by all authors used MTGase (Gujral and Rosell, 2004 ; Ahn et al., 2005).

Tracing the effect of MTGase in dough prepared from blends of wheat and corn Flours:-

The following experiments were carried out to investigate the possibility of incorporating corn flour into wheat flour at higher levels for making *Shamy* bread with the aid of MTGase cross-linking. Corn flour was mixed with wheat flour at levels of 10, 20, 30, 40, and 50 % of the weight of wheat flour.

Free amino groups:-

The data in Table (3) revealed that the free amino groups of the dough. The results indicated that the enzyme have a cross- linking effects towards the free amino and thiol groups of wheat dough. The free amino groups of the wheat dough were 0.538 µM/ mg flour without adding the enzyme. It decreased to 0.378 µM/ mg flour when treated with the enzyme; the percent of reduction was 29.74. Mixing corn flour with wheat flour decreased the free amino groups of the resulted dough. It decreased to 0.356 µM/ mg flour, in dough containing 10 % corn flour and treated with the enzyme. Increasing the level of corn flour to 30% caused a decrease in the free amino group to 0.248 µM/ mg flour with reduction of 34.39%. The cross-linking effect was 0.142 and 0.130 µM/ mg flour in the blends containing of 10% and 30% corn flour, respectively. When the level of corn flour increased to 50%, a slight decrease in the cross-linking effect was observed. Generally increasing the corn wheat to 30% did not affect or have a slight positive effect on the enzyme activity.

Table 3:- Effect of prepared MTGase on the free amino groups in dough of wheat and corn flour blends

Flour blends	Control	Free amino groups (µM/ mg flour)		
		After treatment "Residual"	Cross-linking effect	Reduction (%)
Wheat flour 100%	0.538	0.378	0.160	29.74
Wheat flour 90% + Corn flour 10%	0.498	0.356	0.142	28.51
Wheat flour 80% + Corn flour 20%	0.414	0.288	0.126	30.43
Wheat flour 70% + Corn flour 30%	0.378	0.248	0.130	34.39
Wheat flour 60 % + Corn flour 40%	0.288	0.208	0.080	27.78
Wheat flour 50% + Corn flour 50%	0.258	0.184	0.074	28.68

Gujral and Rosell (2004) reported that when rice flour was treated with different concentrations of MTGase, a progressive decrease in the amount of free amino groups was observed on addition of MTGase up to 1.0%. Beyond that concentration no significant differences in the amount of free amino groups were detected. This could be due to the disappearance of the lysine groups exposed to the enzyme effect. Ahn et al. (2005) indicated that, the amount of free amino groups in TGase-treated wheat, barley and soy flours and their blends were decreased with TGase treatment.

Free thiol groups:-

The data in Table (4) indicated that, the free thiol groups of the control samples (without enzyme) in the dough of wheat and corn flour blends decreased with increasing the percentage of corn flour.

Table 4:- Effect of prepared MTGase on the free thiol groups in dough of wheat and corn flour blends

Flour blends	Control	Free thiol groups (µM/ g flour)		
		After treatment	Cross-linking effect	Reduction %
Wheat flour 100%	11.222	10.342	0.88	7.849
Wheat flour 90% + Corn flour 10%	10.861	10.008	0.853	7.854
Wheat flour 80% + Corn flour 20%	9.411	8.604	0.807	8.575
Wheat flour 70% + Corn flour 30%	9.103	8.626	0.477	5.240
Wheat flour 60 % + Corn flour 40%	8.612	8.211	0.401	4.656
Wheat flour 50% + Corn flour 50%	8.114	7.834	0.28	3.573

In general, increasing the percentage of corn flour in the blends treated with MTGase decreased the free thiol groups. The free thiol groups of the wheat dough (without enzyme) were 11.222µM/ g flour, it decreased to 10.342 µM/ g flour when treated with the enzyme, with reduction of 7.849 %. The free thiol groups of the dough (without enzyme) contain 10% corn flour was 10.861 µM/ g flour, it decreased to 10.008 µM/ g flour when treated with the enzyme.When the levels of corn flour increased to 30% the free thiol groups decreased from 9.103 µM/ g flour in the untreated sample (control) to 8.626 µM/ g flour in the sample treated with the enzyme. Increasing the level of corn flour caused a gradually decrease in the free thiol groups. At a level of 50% corn flour the cross-linking was 0.28 µM/ g flour with a reduction reached 3.573 % in the sample treated with the enzyme.

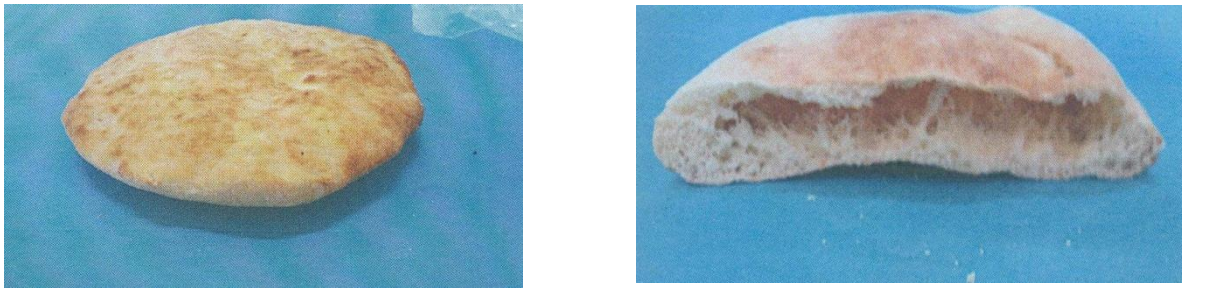
Gujral and Rosell (2004) declared that, there was a significant decrease in the free thiol groups content of rice flour treated with TGase. The decrease in thiol groups was more significant at a TGase concentration of 0.5% compared with 1 and 1.5%. The reduction in thiol groups concentration suggests the formation of disulphide bonds is most likely favored by the proximity of the cross-linked polypeptide chains.

Ahn et al. (2005) mentioned that, significant decrease in the number of free thiol groups of the blended samples (wheat, barley and soy flour) up on TGase treatment. Sulfur-containing amino acids may come close to each other during the TGase reactions leading to the formation of disulfide bonds via oxidation.
Ap et al. (2011) reported that the enzyme modified chemical and functional properties of glutenins fraction of proteins, improving dough strength and bread volume.

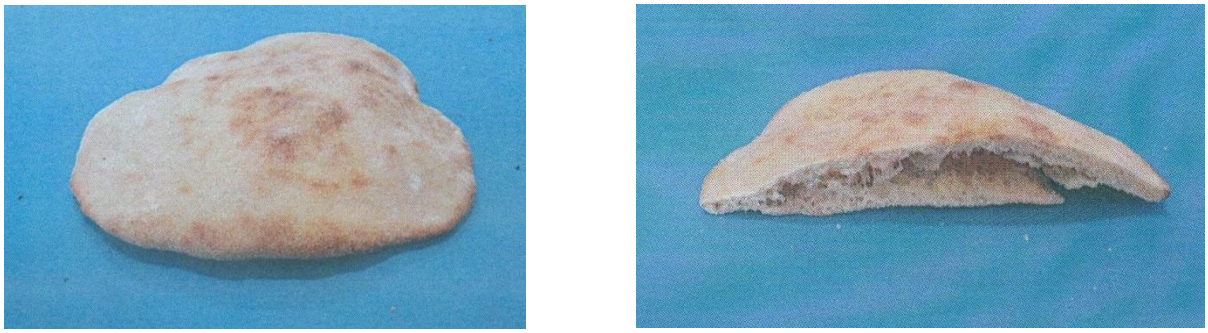
Application of MTGase in *Shamy* bread using wheat and corn flour blends:-
Wheat flour is the main flour used for production of bread and many other bakery products. Wheat flour contains proteins, mainly gluten which is the dominant proteins that characterize dough properties and play an important part in the quality of bread. Disulfide bridges between gluten proteins strengthen the gluten network, which is beneficial for the quality of the baked loaf (Kaufmann et al., 1986).

Corn flour is one of the most valuable cereal flours from a nutritional point of view. However, corn flour is unable to hold gas produced during fermentation for baked products. The properties of corn flour are very different from wheat flour this is because of the absence of gluten.

The previous results of tracing the cross-linking effect revealed from the free amino and thiol groups (Tables 3 and 4) indicated that the maximum effect was observed when using a flour blend contains 70% wheat and 30% corn flour. So this blend was used for making *Shamy* bread as indicated in the materials and methods section.
Figure (1) shows a complete and cross section of the prepared bread. The figure indicated that, the prepared loaf from the dough treated with the enzyme have a higher volume with pronounced improvement in the general appearance and quality than that when no enzyme was used.



With MTGase



Without MTGase

Figure 1:- Baking performance of dough prepared from 70% wheat flour and 30% corn flour with and without the purified enzyme

De Jong and Koppelman (2002) stated that, articles describing the use of TGase in cross-linking of proteins are mostly focused on one single type of protein. The cross-linking reaction can however also be applied to the glutamines and lysine of two different types of proteins. By coupling two proteins with different structures, totally new functionalities can be created.

Basman et al. (2003) reported that, bread from TGase-treated samples prepared from different blends of wheat and barley flours had higher loaf volumes and better crumb and crust characteristics, and were apparently softer than those from untreated samples up 30% barely flour supplementation level. The increases in loaf volumes of TGase-treated samples might be the result of TGase transforming weak gluten into a strong one due to TG-catalyzed cross-linking reactions between and within wheat and barley proteins.

Gujral and Rosell (2004) reported that, the specific volume of rice bread which treated with MTGase increased by increasing the concentration of MTGase up to 1%, but negative effects was observed at higher concentrations. They attributed this behavior to the ability of the TGase to transform weak gluten into a strong one and suggest that at high TGase concentrations excessive cross-linking produced over-strong dough. Ap et al. (2011) reported that the enzyme modified chemical and functional properties of glutenins fraction of proteins, improving dough strength and bread volume.

Conclusion:-

It can be concluded that the prepared enzyme have a cross-linking effects towards the free amino and thiol groups. Also, it is possible to produce *Shamy* bread with a good quality from a blend of wheat flour mixed with a high level of corn flour reached to 30% with the aid of the prepared enzyme. This is due to the cross-linking effect of the enzyme. The maximum level of corn flour which used previously was 20% only without using the enzyme.

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