Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
2015, volume 100, livre 4, pp. 82-95
First National Conference of Biotechnology, Sofia 2014

TRANSGLUTAMINASE MODIFICATION OF SUNFLOWER PROTEINS

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Keywords: sunflower protein isolates, transglutaminase, OPA, SDS-PAGE, gel-filtration chromatography

Abstract: Transglutaminase modification of proteins is used in the food industry to improve their functional properties. The enzyme catalyses cross-linking of proteins by formation of ε-(γ-glutamyl) -lysine bonds, which leads to the formation of protein aggregates with net structure and altered functional properties.

In this research, the potential of microbial transglutaminase to modify proteins isolated from industrially produced sunflower meal was explored. The influence of pH and the longevity of reaction at two temperatures (40°C and 60°C) were studied. Cross-linking process was monitored via the amount of free amino groups and electrophoretic mass distribution of proteins by using ortho-phtalaldehyde analysis (OPA) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) respectively. Results demonstrated that the amount of free amino groups during the polymerization reaction decreased as indicated by decreased absorption at 340 nm when using OPA analysis. The formation of high-molecular weight proteins (MW > 150kDa) gradually increased at the expense of low-molecular weight protein fractions (MW < 50 kDa). The formation of protein fractions with molecular weight between 50 and 150 kDa increased at the beginning of enzymatic reaction followed by a gradual decrease. Molecular aggregates with molecular weights of approximately 400 kDa appeared 2 h after the beginning of cross-linking at 40°C and pH 7-8 and after 4h at pH 9. At 60°C, they were observed 10 to 60 min after the start of the reaction depending on pH values.

INTRODUCTION

Sunflower is one of the most important oil crops in the world (Žilić et al., 2010). In Bulgaria, sunflower seeds are a major source for industrial production of vegetable oil. After oil extraction from sunflower seeds, sunflower meal remains

as a by-product with a high protein content. It may reach up to 66%, depending on seed pre-treatment and the type of technology used for the oil production (Dorrell and Vick, 1997). Currently, sunflower meal is mainly used as a feed ingredient, which, however, is limited due to its high fibre content (Raza et al., 2009).

Sunflower proteins possess good nutritive and functional properties and could potentially be used in human nutrition as a valuable source of sulphur-containing amino acids (Pawar et al., 2001; González-Pérez, 2003). However, seed processing and oil extraction procedure may denature proteins and worsen their functional properties (González-Pérez and Vereijken, 2007; Salgado et al., 2011). As a result, the preparation of sunflower protein isolates with high quality as well as their application in food industry is restrained.

Various chemical and enzymatic modifications could be applied to improve the quality of sunflower proteins (Sanchez and Burgos, 1997; Taha et al., 2014). For example, partial hydrolysis of sunflower proteins increased their solubility (Yust et al., 2003). Hydrolysates which contained peptides with antioxidant activity (Ren et al., 2010), positive antihyperglycemic and hepato-protective effect as well as reductive serum cholesterol and triacylglycerols capacity were obtained after enzyme modification of the sunflower protein isolates with alcalase and flavourzyme (Taha et al., 2014). By using limited hydrolysis with trypsin Sanchez and Burgos (1997) obtained modified sunflower proteins with improved gelling properties.

A transglutaminase (EC 2.3.2.13, R-glutaminyl-peptide: amine γ-glutamyl-transferase, TGase) is an enzyme which is widely used to modify functional properties of food proteins. The enzyme catalyses cross-linking of proteins by formation of ε-(γ-glutamyl) -lysine bonds, which leads to the formation of protein aggregates with a net structure and altered functional properties such as improved water absorption and temperature stability as well as increased emulsifying and gelling capacities (Ren et al., 2010). Proteins modified with TGase differ from temperature induced gels by their properties. They are preferred structuring agents in food formulations and appropriate ingredients for the preparation of highly stable gelled emulsions and edible films (Motoki and Kumazawa, 2000). Furthermore, newly developed ε-(γ-glutamyl) -lysine bonds do not decrease the nutritive value of the modified protein since they are metabolized in the human organism (Yokoyama et al., 2003; Nahid et al., 2010).

TGase modification of food proteins has been studied since the sixties by using various substrates such as casein, α – lactalbumin and β – lactoglobulin, egg proteins, actin and miosine (De Barros Soares et al., 2004; Shleikin et al., 2011). However, few plant proteins, including those of soy and wheat were modified with a TGase and used in food industry for the production of tofu, bread and other wheat products (Dube et al., 2007; Schäfer et al., 2007). Some studies on TGase modification of peanut and pea proteins were performed as well (Clare and Sanders, 2010). According to Schäfer et al. (2007), limited

application of TGase on plant proteins may be due to relatively low content of lysine.

Currently, published data on TGase modification of sunflower proteins are scarce. According to Dube et al. (2007), this may be due to relatively high levels of phenolic compounds in sunflower seeds which either inhibit the enzyme or reduce the amount of the available for the reaction lysine by interacting with proteins. The same authors postulate that TGase modification of sunflower proteins, if possible, could improve their functional properties especially gelling capacity.

The purpose of our study was to explore the potential for modification of sunflower protein isolate, prepared from industrial sunflower meal, with TGase under different conditions including pH, temperature and longevity of enzymatic reactions.

MATERIALS AND METHODS

Protein isolate preparation

Sunflower meal, provided by a local oil factory, was used as a source for preparation of the protein isolate used in this study. It was obtained by extraction with 10% NaCl followed by isoelectric precipitation of the extracted proteins at pH 2.5 (Ivanova et al. 2011, 2012).

Transglutaminase

A commercially available enzyme product (Activa® WM) with activity 100 U/g was used for modification of the sunflower protein isolate. It was kindly provided by Ajinomoto Foods Europe, S.A.S Department ADC, France under the form of white powder that contained 99% maltodextrin and 1% active TGase, isolated from *Streptomyces mobaraensis*.

Transglutaminase modification

All enzymatic reactions were performed with a protein isolate containing 2% protein and TGase which activity was equal to 5 U/g at either 40 or 60ºC. The range of pH varied from 7 to 9. The progress of the TGase modification was monitored by ortho-phtalaldehyde analysis (OPA) and alterations of the fractional composition of proteins evaluated by either sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or gel-filtration chromatography on Sephadex G200. A control containing a thermally inactivated TGase (95ºC for 30 min) was included in all experiments.

Ortho-phtalaldehyde analysis (OPA)

Ortho-phtalaldehyde analysis (OPA) was performed as described by Church et al. (1983). Briefly, 20 µl reaction mixture containing 2% protein isolate was mixed with 2ml OPA reagent. The mixture was remained for 2 min at room temperature, and the absorption was subsequently measured with a spectrophotometer (Carl Zeiss, Jena, Germany) at 340 nm. The reduction of

the absorption indicated a decrease of free amino groups due to the progress of the polymerization reaction.

SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970) with an omniPAGE mini Cleaver electrophoresis (Model CVS10DSYS). The separating gel contained 10% acrylamide and 0.13% bis-acrylamide. The stacking gel was prepared with 6% acrylamide and 0.08% bis-acrylamide. Visualization of gels was realized with 0.2% Coomassie Brilliant Blue R-250 dye (SERVA Electrophoresis GmbH, Germany). Protein standards with molecular weights ranging from 6 500 to 200 000 Da were used to determine the molecular weight of protein fractions. Data were analysed by using a SameSpots software for 2D Gel Analysis (BioStep GmbH, Germany).

Gel-filtration chromatography

Gel-filtration chromatography separation was performed on Sephadex G200 by using a gel-filtration chromatography system (FPLC) of Pharmacia Biotech Inc. (GE Healthcare Bio-Sciences, USA). Aliquots of 4ml 2% protein isolate were loaded to the chromatography column (height 70cm, diameter 2.6cm) to separate. The proteins were eluted with distilled water (pH 7.5) with velocity 20 ml/h. The absorption of collected fractions (10ml each) was measured at 280 nm.

RESULTS AND DISCUSSION

The choice of experimental temperature (40°C и 60°C) and pH (from 7 to 9) was based on optimal conditions for the activity of TGase isolated from *Streptomyces mobaraensis* as well as data published by others (Dube et al., 2007; Ionescu et al., 2008; Nahid et al., 2010) The range of pH was also in accordance with data on the solubility of the protein isolate previously established in our laboratory (Ivanova et al., 2013). Figures 1 and 2 represent the dynamics of the TGase modification of the sunflower meal protein isolates for 4 h as monitored by OPA. All experiments were performed in triplicates. Each data set represents an average mean of the results generated from three independent experiments ± standard deviations. Data were considered significant at $p < 0.5$. Data indicated a progress in cross-linking of the protein isolate under all experimental conditions as evidenced by decreases in the absorption measured at 340 nm. At 40°C, the decrease in the absorption was more substantial during the first hour of transglutaminase treatment, after which it remained unaltered (Fig. 1). At 60°C, after the first hour of fast transglutaminase treatment, the process continued with lower speed during the entire period of observation (Fig. 2).

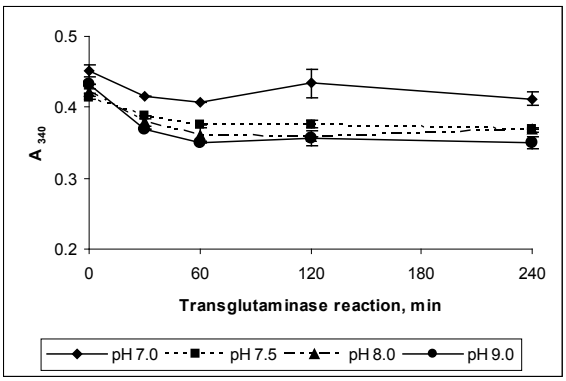


Figure 1. Ortho-phtalaldehyde analysis of transglutaminase modification of sunflower meal protein isolate at 40°C.

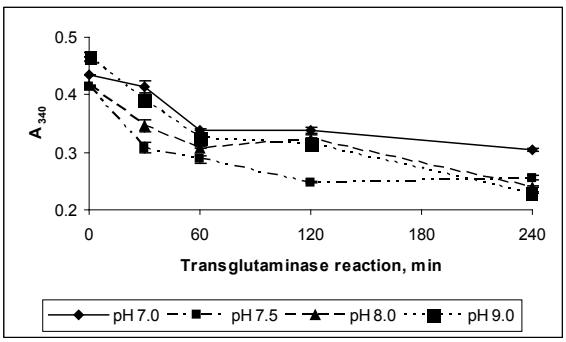
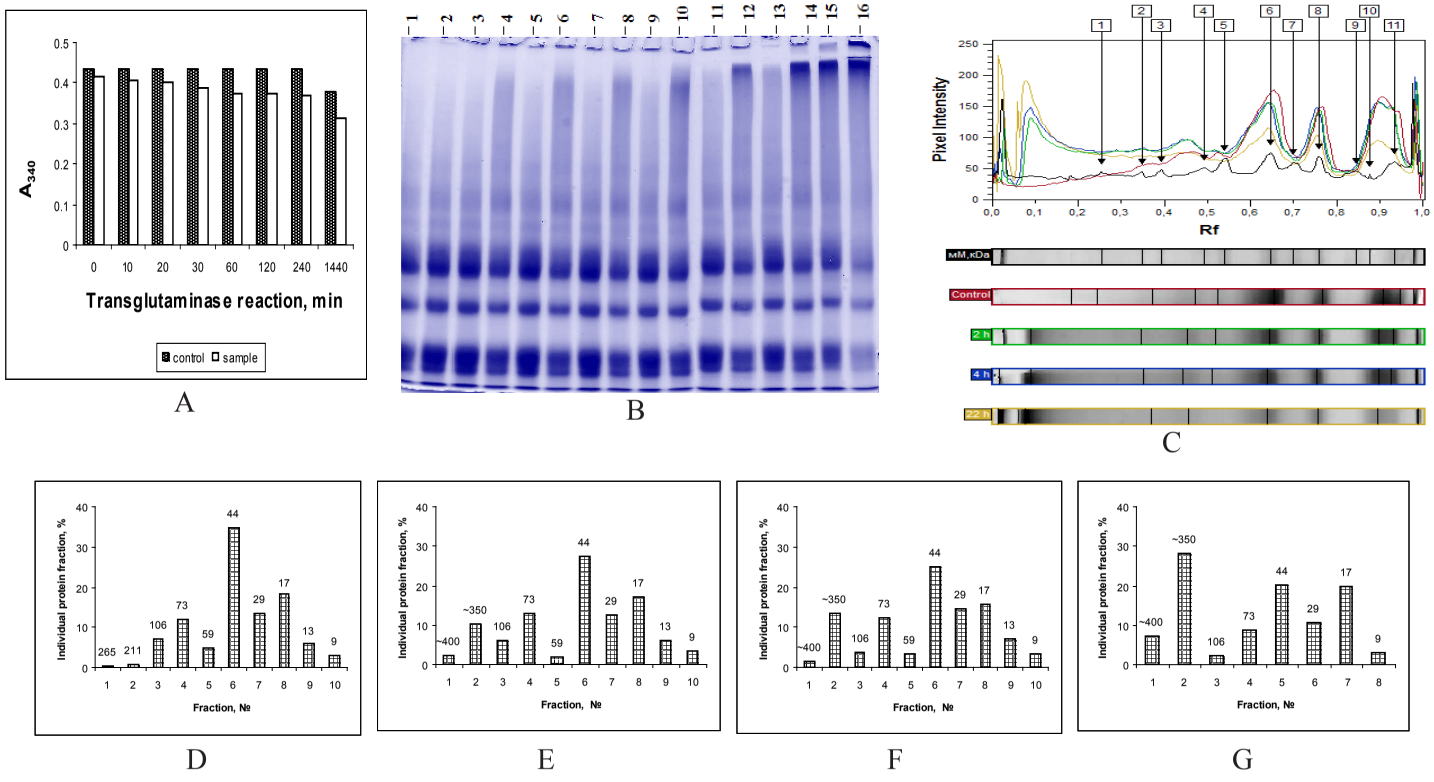


Figure 2. Ortho-phtalaldehyde analysis of transglutaminase modification of sunflower meal protein isolate at 60°C.

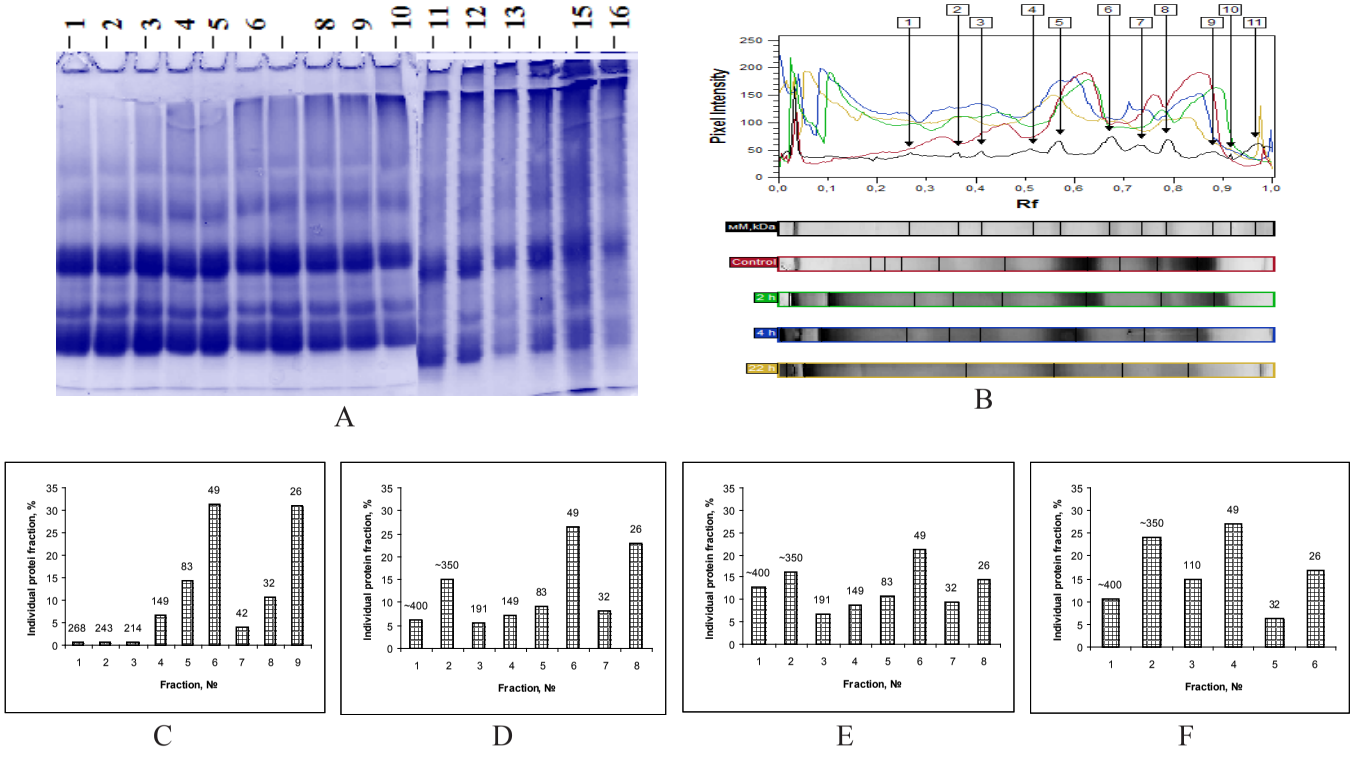
Since the trend in the transglutaminase treatment at the chosen experimental pH values is similar (Fig. 1 and Fig. 2), SDS-PAGE data generated only at pH 7.5 are presented in this article.

Figure 3B demonstrates the modification of the protein isolate with TGase at 40°C (pH 7.5) as monitored by SDS-PAGE for 22 h. Fractional profiles of the samples after 2, 4 and 22 h enzymatic activities (Fig. 3C) and the content of the individual protein fractions in the total protein profile expressed in percentage (Fig.3 D, E, F and G) were also determined. OPA analysis was also conducted under the same conditions (40°C, pH 7.5) for a comparative reason (Fig. 3A). For easier understanding, protein fractions were conditionally separated into three groups: a low molecular weight fraction with MW up to 50 kDa (LMF), a fraction with a medium molecular weight from 50 to 150 kDa (MMF) and a high molecular weight fraction with MW above 150 kDa (HMF).



*Numbers above columns indicate molecular weights of protein fractions

Figure 3. Transglutaminase modification of sunflower meal protein isolate at 40°C and pH 7.5: **(A)** – Ortho-phtalaldehyde analysis; **(B)** – SDS-PAGE (1,3,5,7,9,11,13,15 – controls, 2,4,6,8,10,12,14,16 – samples modified for 0,10,20,30,60,120, 240 and 1440 min); **(C)** - Fractional profiles of a control (0 min) and a sample modified for 2, 4 and 22 h (120, 240 и 1440 min); **(D, E, F and G)** – Content of individual protein fractions in the total protein profile determined at time 0 h (D), 2 h (E), 4 h (F) and 22 h (G) of enzyme modification.



*Numbers above columns indicate molecular weights of protein fractions

Figure 4. Transglutaminase modification of sunflower meal protein isolate at 60°C and pH 7.5: **(A)** – SDS-PAGE (1,3,5,7,9,11,13,15 – controls, 2,4,6,8,10,12,14,16 – samples modified for 0,10,20,30,60,120, 240 and 1440 min); **(B)** - Fractional profiles of a control (0 min) and a sample modified for 2, 4 and 22 h (120, 240 и 1440 min); **(C, D, E and F)** – Content of individual protein fractions in the total protein profile determined at time 0 h (C), 2 h (D), 4 h (E) and 22 h (F) of enzyme modification.

OPA exhibited gradual decrease in the absorption, which remained constantly lower than the control. After 1 h enzyme modification, SDS-PAGE demonstrated low defined HMF. Two hours after the onset of the reaction, the HMF was well defined and visible. After 22 h, a decrease in the amount of free amino groups was still recorded by OPA. Some HMFs were not able to move into the stacking gel at that time. Software analysis of electrophoregrams demonstrated the formation of two new HMFs with molecular weights of approximately 350 and 400 kDa after 2 h of enzyme modification at the expense of low- and medium weight molecular fractions. Samples modified for 4 and 22 h also exhibited increase of HMF in parallel with a decrease of low- and medium weight molecular fractions. Similar results were generated by SDS-PAGE at pH 7.0 and pH 8.0 (data are not presented). At pH 9.0, the reaction proceeded at a low speed. At 60°C, HMFs appeared for a short time – for 10 min to 1 hour.

Table 1 and Table 2 represent the dynamics in the content of individual protein fractions (LMF, MMF, HMF) during transglutaminase treatment at 40°C and 60°C respectively. The content of HMF increased at the expense of LMF under all experimental conditions. The percentage of MMF increased at the beginning of cross-linking process followed by a decrease. However, it should be noted, that proteins with molecular weights of approximating 400 kDa and above were not correctly determined since they were located at the boundary of the two electrophoretic gels. Some of them did not even migrate to the stacking gel. Regardless, the trend of formation of protein aggregates with high molecular weights under the activity of TGase was unambiguously documented.

Table 1. Dynamics of the content of individual protein fractions during transglutaminase modification of protein isolate at 40°C.

Sample	Content of protein fractions*,%											
	pH 7.0			pH 7.5			pH 8.0			pH 9.0		
	LMF	MMF	HMF	LMF	MMF	HMF	LMF	MMF	HMF	LMF	MMF	HMF
0 h	79.2	19.8	1.0	74.9	23.8	1.3	95.1	4.4	0.5	92.7	5.8	1.5
2 h	65.4	22.3	12.3	66.7	20.9	12.5	73.4	9.7	16.8	85.4	9.9	4.7
4 h	63.8	18.3	17.9	65.8	19.2	15.0	67.3	9.7	23.0	78.9	9.8	11.3
22 h	52.3	11.9	35.9	62.6	2.3	35.1	57.3	16.3	26.4	77.8	0.0	22.2

*Content of individual protein fractions was determined as a percentage of total protein content.
LMF denotes low molecular weight fraction; MMF denotes medium molecular weight fraction; HMF denotes high molecular weight fraction.

Table 2. Dynamics of the content of individual protein fractions during transglutaminase modification of a protein isolate at 60°C.

Sample	Content of protein fractions*,%								
	pH 7.0			pH 8.0			pH 9.0		
	LMF	MMF	HMF	LMF	MMF	HMF	LMF	MMF	HMF
0 h	77.1	20.8	2.2	76.7	17.4	5.9	83.2	13.9	2.9
2 h	57.3	16.2	26.5	65.4	18.1	16.5	57.1	18.4	24.5
4 h	45.1	19.5	35.5	53.4	15.0	31.6	51.4	15.2	33.4
22 h	50.1	14.9	34.9	52.9	17.2	30.0	54.4	0.0	45.6

*Content of individual protein fractions was determined as a percentage of total protein profile.
LMF denotes low molecular weight fraction; MMF denotes medium molecular weight fraction; HMF denotes high molecular weight fraction.

Some differences in molecular weights of individual fractions before enzyme modification were established as a pH function. At pH 7.0 and pH 7.5 differences were negligible and were probably due to slight fluctuations in electrophoretic conditions. At pH 8.0 and pH 9.0 variations in the content of individual fractions at the onset of the reaction were more distinctive. The content of LMF increased substantially at the expense of MMF. This phenomenon was probably due to conformational changes of sunflower proteins which occur in alkaline media (González-Pérez et al., 2004).

Similar results on TGase modification of proteins with various origins have already been published. De Baros Soares et al. (2004) reported the formation of high molecular casein aggregates as a result of TGase activity after 2 h at 37°C and pH 7.0. Soy protein isolates demonstrated alteration in molecular weight distribution of individual fractions after 1 h of the beginning of the enzymatic reaction, which was more pronounced 2 h later on SDS-PAGE electrophoregram. According to Clare et al. (2007), the reduction of the absorption of peanut proteins at 340 nm as a result of TGase activity appeared after 30 min at 37°C and pH 8.0, while well defined high molecular fractions on electrophoregram were visible 1 h after the beginning of transglutaminase treatment. Ionescu et al. (2008) established that most satisfying results of TGase modification of myofibrillar proteins could be obtained at 35°C for 1 h.

In our study, OPA, although fast and convenient, appeared to not be the most appropriate method to monitor the progress of TGase protein modification. Modified protein samples exhibited lower absorption at 340 nm when compared to the control under all experimental conditions. However, differences in the absorption values were relatively small and fluctuating, especially at the higher pH values and 60°C. It may be due to the relatively small lysine amount which was available for enzymatic reaction as well as the presence of phenols. They have maximal absorption in the same range and, even in small amounts, could interfere with the assay. Schäfer et al. (2007) reported similar difficulties while using OPA to monitor TGase modification of pea and soy proteins.

OPA and electophoregrams of control samples, where inactivated TGase was added, indicated that some alteration with the protein sample occurred as evidenced by the reduction of the absorption at 340 nm and the appearance of HMF (Fig. 3 and Fig. 4). At 40°C and pH 7.5, the alteration of the control sample was recorded 5 h after the addition of inactivated TGase. To identify the reasons leading to the observed changes of the control sample, the potential for reactivation of the inactivated TGase when used for longer time was investigated. It was determined that no reactivation of TGase occurred under studied conditions. In addition, control sample which contained water instead of TGase also appeared modified after 22 h as evident from the fractional protein profile of the sample. It may be due to either interactions among amino acid radicals, or between proteins and other components such as carbohydrates and phenols which, although in

small amounts, were present in the protein isolate. According to Sinz (2003) and Friedman (1999) interactions between lysine radicals and those of alanine and cysteine are possible and lead to formation of intra-molecular and cross-linked products with higher molecular weights. Furthermore, the alkaline environment and higher temperatures favour the reaction between lysine and carbohydrates (Hurrell et al., 1976).

Figure 5 represents the results of gel-filtration chromatographic distribution of the sunflower protein isolate before (time 0) and after 2 and 22 h of TGase modification. Before modification, the protein isolate was consisted of 5 fractions (1, 2, 3, 4 and 5) with the following elution volumes 110, 170, 210, 270 and 300 ml They were reduced to 4 fractions (1', 2', 3' and 4' with elution volume 110, 270, 320 and 340 ml respectively) after 2 h of transglutaminase treatment where the main fraction appeared with an elution volume of 110 ml. 22 h later, the enzymatic modification of the protein isolate resulted in 3 fractions (I, II and III) as the main fraction was eluted from the column immediately after the empty column volume. Taking into account that the separation capacity of Sephadex G200 is 800 kDa, it could be assumed that the MW of the proteins in the first fraction was close to that one.

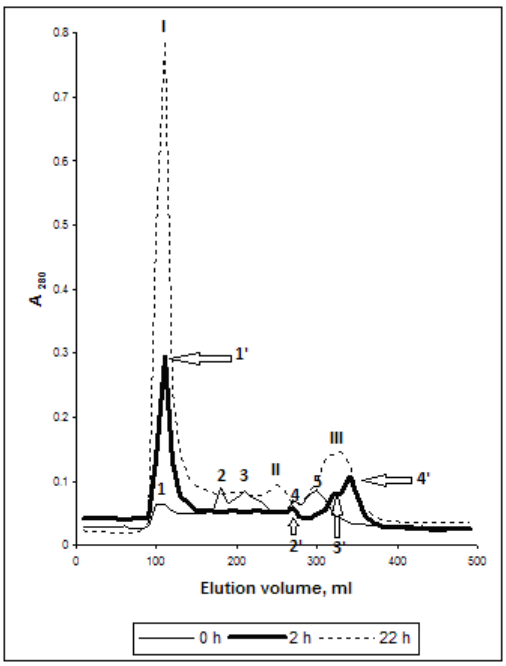


Figure 5. Gel-filtration chromatography of sunflower meal protein isolate on Sephadex G200 after 2 h and 22 h of transglutaminase modification.

CONCLUSIONS

Data generated in our study are a convincing evidence for the successful utilization of a bacterial TGase to modify a protein isolate obtained from the industrial sunflower meal. Fractionation of proteins with SDS-PAGE and gel-filtration chromatography on Sephadex G200 indicated the formation of high molecular aggregates 2 h after enzyme modification at 40ºC and pH 7.0– 8.0 and 4 h at pH 9.0. At 60ºC, HMF appeared more rapidly (from 10 min to 1 h) depending on pH.

Control samples underwent changes which were not related to the activity of the enzyme. At 40ºC and pH 7.5, they were observed 5 h later. Therefore, the most appropriate longevity for modification of sunflower meal protein isolate with bacterial TGase under the conditions used in our experiments was from 2 to 4 h.

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