ISSN 1330-9862
doi: 10.17113/ftb.55.03.17.5061

scientific note

Pepsin-Assisted Transglutaminase Modification of Functional Properties of a Protein Isolate Obtained from Industrial Sunflower Meal

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Received: November 15, 2016
Accepted: May 4, 2017

Summary

The utilization of industrial sunflower meal to produce protein-rich products for the food industry is an alternative approach for better and more efficient use of this agricultural by-product. Sunflower meal proteins possess specific functional properties, which however need improvement to broaden their potential as supplements for delivering high-quality products for human nutrition. The aim of the study is to evaluate the combined influence of low-degree pepsin hydrolysis and transglutaminase (TG) modification on industrial sunflower meal protein isolate functionality at pH=2 to 10. Three TG-modified pepsin hydrolysates with the degree of hydrolysis of 0.48, 0.71 and 1.72 % were produced and named TG-PH1, TG-PH2 and TG-PH3, respectively. All three TG-modified pepsin hydrolysates exhibited improved solubility at pH between 3.5 and 5.5 as the highest was observed of TG-PH3 at protein isoelectric point (pI=4.5). Sunflower meal protein isolate and TG-modified sunflower meal protein isolate had greater solubility than the three TG-modified hydrolysates at pH<3 and >7. Significant improvement of foam making capacity ($p<0.05$) was achieved with all three TG-modified pepsin hydrolysates in the entire pH area studied. Pepsin hydrolysis of the protein isolate with the three degrees of hydrolysis did not improve foam stability. Improved thermal stability was observed with TG-PH3 up to 80 °C compared to the protein isolate (pH=7). At 90 °C, TG modification of the protein isolate alone resulted in the highest thermal stability. Pepsin hydrolysis followed by a treatment with TG could be used to produce sunflower protein isolates with improved solubility, foam making capacity and thermal stability for use in the food industry.

Key words: industrial sunflower meal, pepsin, protein hydrolysates, transglutaminase modification, functional properties

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Introduction

Sunflower is an economically important oil-bearing crop which is primarily used for production of vegetable oil. In 2012, sunflower oil production reached 15.22 million tonnes worldwide (*1*) and was further projected to increase due to enhanced consumer demand for *trans*-fat-free unsaturated fats (*2*) and its potential value as a feedstock for biodiesel generation (*3*). Either for food or technical purposes, the oil extraction results in a substantial quantity of sunflower meal, which may reach up to 30 % of the initial amount of the used sunflower seeds (*4*). Currently, this by-product is used as a protein source in the feed industry. However, its application in animal nutrition is limited due to high fibre content (*5*). To avoid adverse performance effect, sunflower meal inclusion in broiler and swine diets should not exceed 16 and 20 %, respectively (*4,6*). The overproduction and accumulation of excessive amounts of unutilized sunflower meal causes higher storage or disposal expenses leading to overall decrease in net profit margin.

The utilization of industrial sunflower meal for generation of protein-rich products for the food industry is an alternative approach for better and more efficient use of this agricultural by-product. Sunflower meal proteins have a high nutritive value. They do not contain antinutritional compounds and exhibit well-balanced amino acid composition with the exception of a low level of lysine (*7*). Sunflower meal proteins possess specific functional properties, which however need improvement to broaden their potential as supplements for delivering high-quality products for human nutrition (*8,9*).

Major challenges in preparation of sunflower meal-derived protein products and their subsequent application in the food industry are related to the alteration of protein characteristics due to technological parameters of oil production and sunflower seed pretreatment. Industrial sunflower meal is produced after treatment of sunflower seeds with high temperature and organic solvents which, in most cases, decrease the nutritive value of sunflower meal proteins and reduce their functional properties (*10*). Enzymatic treatment of plant-derived proteins with pepsin, trypsin or Alcalase® is a common approach aiming at improvement of functional properties (*11–13*). For example, Martinez *et al.* (*14*) improved foaming capacity of sunflower protein by limited enzymatic digestion (degree of hydrolysis 1.5 %) with Alcalase. Karayannidou *et al.* (*15*) reported that limited proteolysis of sunflower protein isolate with trypsin was very efficient in stabilizing emulsions and foam. By using a sequential two-step enzymatic digestion with chymotrypsin and carboxypeptidase, Bautista *et al.* (*16*) obtained sunflower protein hydrolysates with decreased allergenicity and a high Fischer ratio.

Transglutaminase (TG) catalyzes the cross-linking of proteins by formation of ε-(γ-glutamyl)-lysine bonds (*17*). TG is predominantly used to alter the functionality of proteins of animal origin and has a wide application in the dairy industry and meat and fish processing (*18*). However, successful application of TG to alter plant protein functionality for the production of tofu, bread and other wheat products has also been reported (*19,20*). Ivanova (*9*) demonstrated the potential of microbial TG to improve thermal stability and foam making capacity of proteins isolated from industrial sunflower meal. In a later study, it was observed that limited hydrolysis of sunflower meal protein isolate with pepsin facilitated TG reaction and resulted in approx. 4-fold faster polymerization than unhydrolyzed counterpart (*21*). However, to the best of our knowledge, no information about the effects of preceding enzymatic hydrolysis and cross-linking with TG on the functional properties of industrial sunflower meal protein isolates is available in literature. The aim of the current study is to evaluate the combined influence of low-degree pepsin hydrolysis and TG modification on sunflower protein isolate functionality in a wide pH range (from 2 to 10). Solubility, thermal stability, foam capacity and stability, and emulsifying activity and stability of three TG-modified pepsin hydrolysates with low degree of hydrolysis (0.48, 0.71 and 1.72 %) were evaluated.

Materials and Methods

Materials

Sunflower meal was obtained from a local company (Biser Oliva, Stara Zagora, Bulgaria). It was produced after thermal treatment of sunflower seeds at 110 °C followed by oil extraction with hexane at 55 °C. Microbial TG (Activa® WM) was kindly provided by Ajinomoto Co., Inc. (Tokyo, Japan) for research purposes. All reagents used in the study were of analytical grade and bought from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of protein isolate

Proteins were extracted with 10 % NaCl (pH=6) and isolated by isoelectric precipitation at pH=2.5 with 6 M HCl as described by Ivanova *et al.* (*22*). The protein precipitate was collected by centrifugation at 1800×*g* for 15 min (MPW 251; Medical Instruments, Warsaw, Poland), washed three times with 100 mL of HCl (pH=2.5), dried by lyophilization (Lyovac GT2; Leybold Heraeus, Köln, Germany) and stored for further analyses.

Enzymatic modification of protein isolate

Protein isolate (2.0 %) was dissolved in 70 mL of distilled water and hydrolyzed with pepsin (EC 3.4.23.1; 6 U/g protein) at 40 °C for 15, 45 and 120 min. The pH was maintained at 1.8 by adding 0.1 M HCl when needed. The reaction was stopped by increasing the pH to 7.5 with 1 M NaOH, after which TG (EC 2.3.2.13; 5 U/g protein) was added to the reaction mixture. TG modification occurred for 2 h at 40 °C. Inactivation of TG was achieved by adding 1 % N-ethylmaleimide (*23*).

Degree of hydrolysis of pepsin proteolysates was evaluated after removal of unhydrolyzed protein by precipitation with 10 % trichloroacetic acid (final concentration) and subsequent centrifugation for 20 min at 1800×*g* (MPW 251; Medical Instruments). It was calculated by the following equation:

$$DH=\frac{\gamma_{\alpha-N}}{\gamma_N}\cdot 100 \qquad /1/$$

where DH is the degree of hydrolysis (%), $\gamma_{\alpha\text{-N}}$ is the concentration of soluble α-amino nitrogen in the supernatant (mg/mL), and γ_N is the concentration of total nitrogen in the sample used in the assay (mg/mL). The α-amino nitrogen in the supernatant was estimated by ninhydrin method and glycine was used to generate a standard curve (*24*). Total nitrogen was determined by Kjeldahl's method (*25*).

Solubility

Protein solubility was determined as described by González-Pérez and Vereijken (*7*) with some modifications. Proteins were dispersed in water to a final concentration of 4 mg/mL. The pH values were varied in the range from 2.0 to 8.5 with increments of 0.5 by using either NaOH or HCl. After 2 h at room temperature, the suspension was centrifuged for 15 min at 1800×*g* (MPW 251; Medical Instruments). Soluble protein in the supernatant was evaluated by biuret method (*26*). Bovine serum albumin was used for generation of a standard curve. The protein solubility was calculated by the following equation:

$$\text{PS}=\frac{m_{\text{ps}}}{m_{\text{tp}}}\cdot 100 \qquad /2/$$

where PS is protein solubility (%), m_{ps} is the mass of soluble protein in the supernatant (mg), and m_{tp} is the mass of total protein in the used sample (mg).

Foam capacity and stability

Foam capacity and stability were determined as described by Sze-Tao and Sathe (*27*) with some modifications. An aliquot of 20 mL of protein solution (0.5 mg/mL) was whipped for 70 s in a graduated cylinder as described by Ivanova *et al.* (*28*). Foam capacity was determined by volume increase immediately after whipping and was calculated by the formula:

$$\text{FC}=\frac{(V_2-V_1)}{V_1}\cdot 100 \qquad /3/$$

where FC is foam capacity (%), V_1 is the volume of protein solution before whipping (mL) and V_2 is the volume of the solution after whipping (mL). The influence of pH on foaming properties was tested by varying the pH from 2 to 10 with increments of 2 units using NaOH or HCl. The foam stability was defined as the volume of the foam that remained after 60 min at room temperature (23 °C) and was calculated by the following equation:

$$\text{FS}=\frac{V_{\text{t60}}}{V_{\text{t0}}}\cdot 100 \qquad /4/$$

where FS is foam stability (%), V_{t60} is the volume of the foam that remained after 60 min (mL), and V_{t0} is the volume of the foam immediately after whipping (mL).

Emulsifying properties

Emulsifying activity and emulsion stability were determined as described by Neto *et al.* (*29*). A volume of 5 mL of protein solution (0.5 mg/mL) was homogenized with 5 mL of food-grade sunflower oil for 60 s at 1000 rpm by using a homogenizer (T18 Ultra Turrax Basic; IKA®--Werke GmbH & Co.KG, Staufen, Germany). The emulsion was centrifuged for 5 min at 1800×*g* (MPW 251; Medical Instruments) and the volume of the emulsified layer was recorded (*28*). The emulsifying activity was calculated by the following equation:

$$\text{EA}=\frac{V_{\text{el}}}{V_{\text{T}}}\cdot 100 \qquad /5/$$

where EA is emulsifying activity (%), V_{el} is the volume of the emulsified layer (mL), and V_T is the volume of the total content of the tube (mL).

Emulsion stability was established after heating at 80 °C in a water bath (WNB 29; Memmert GmbH+Co.KG, Schwabach, Germany) for 30 min. The emulsion was cooled down to room temperature (23 °C) and centrifuged at 1800×*g* for 5 min (MPW 251; Medical Instruments). Emulsion stability was calculated by the following equation:

$$\text{ES}=\frac{V_{\text{el30}}}{V_{\text{el0}}}\cdot 100 \qquad /6/$$

where ES is the emulsion stability (%), V_{el30} is the volume of emulsified layer after 30 min of heating (mL), and V_{el0} is the volume of the emulsified layer before heating (mL). NaOH or HCl was added to protein solutions to modulate the pH from 2 to 10 with increments of 2 where appropriate.

Thermal stability

Thermal stability was determined as described by Kato *et al.* (*30*). Aliquots of 5 mL of protein solutions (2 mg/mL) were adjusted to either pH=7.0 or 8.0 and were heated for 20 min at temperatures varying from 50 to 90 °C with increments of 10 °C. After cooling to room temperature (23 °C), the turbidity of the solutions was measured at λ=500 nm (Spekol 11; Carl Zeiss Jena, Jena, Germany). Distilled water was used as a control. Thermal stability was calculated by the following equation:

$$\text{TS}=\frac{(S_{\text{Ti}}-S_{\text{RT}})}{S_{\text{RT}}}\cdot 100 \qquad /7/$$

where TS is thermal stability (%), S_{Ti} is the sample turbidity at a specific temperature, and S_{RT} is the sample turbidity at room temperature (23 °C).

Statistical analysis

Data are presented as mean values of at least three independent experiments±standard deviation (S.D.). Statistical evaluation was performed by one-way analysis of variance (ANOVA) using Statgraphics Centurion statistical program (v. XVI; StatPoint Technologies, Inc., Warrenton, VA, USA). Mean differences were established by Fisher's least significant difference test for paired comparison with a significance level at α=0.05.

Results and Discussion

To evaluate a combined influence of pepsin and transglutaminase (TG) enzymatic modifications on protein functionality, sunflower meal protein isolate was initially hydrolyzed with pepsin for 15, 45 and 120 min. Respective hydrolysates, PH1, PH2 and PH3, were characterized with low degree of hydrolysis (DH), namely 0.48, 0.71

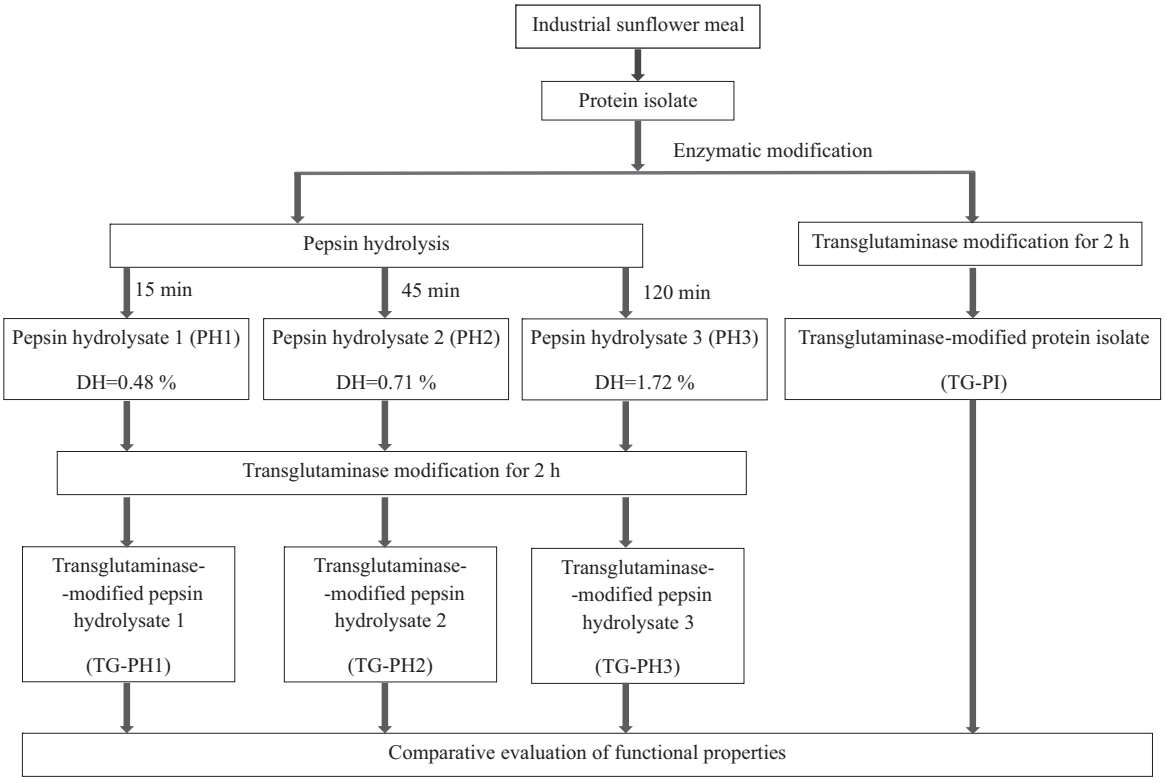

Fig. 1. Flow chart for comparative evaluation of functional properties of sunflower meal protein isolate enzymatically modified with pepsin and transglutaminase. DH=degree of hydrolysis

and 1.72 %. The following treatment with TG resulted in the preparation of TG-modified pepsin hydrolysates, named TG-PH1, TG-PH2 and TG-PH3, respectively. Their functional properties were evaluated and compared to the functional properties of TG-modified protein isolate (TG-PI) and untreated protein isolate. The design of the study is schematically presented in Fig. 1.

Solubility of sunflower protein isolates

Good solubility is usually required for a protein to have good functional properties (*31*). In our study, improved solubility at pH=3.5–5.5 was achieved with the three TG-modified pepsin hydrolysates and the most pronounced effect was observed with TG-PH3 (Fig. 2). Approximately 3- and 3.5-fold increase in TG-PH3 solubility was observed compared to TG-PH2 and TG-PH1, respectively. Compared to protein isolate and TG-modified protein isolate, the solubility of TG-PH3 increased more than 15-fold at pH= 4.5. As previously determined in our laboratory, the protein isolate was rich in sulfur-containing amino acids (amino acid score 99.14 %) (*32*) and, therefore, could serve as a food additive to balance these specific amino acids in human diets if appropriate solubility is provided (*33*). Limited pepsin hydrolysis of protein isolate combined with TG treatment resulted in structures with good solubility at pH=4.0–6.0.

The results implied that limited hydrolysis of the protein with pepsin decreased the influence of pH on protein solubility at the isoelectric point (pI=4.5). Apparently, pepsin hydrolysis of protein isolate is a prerequisite step for the following TG modification to obtain more hydrophilic structures with improved solubility. In a previous study, the hydrolysis of sunflower meal protein isolate with pepsin followed by TG treatment resulted in the increase of the amount of 200-kDa protein fractions at the expense of fractions with higher molecular mass (*21*). Our results are in agreement with the results of Walsh *et al.* (*34*) showing increased solubility of TG-cross-linked products of soy protein isolate Alcalase™ hydrolysates. Flanagan and FitzGerald (*35*) also observed improved solubility of sodium caseinate at around pH=4.6 after combined enzymatic treatments with Protamex and TG compared

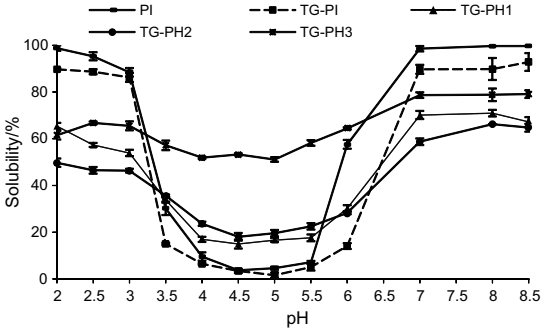

Fig. 2. Influence of pH on the solubility of sunflower protein isolate (PI) and sunflower protein pepsin digests after treatment with transglutaminase (TG). TG-PI=TG-modified protein isolate, TG-PH1, 2 and 3=TG-modified pepsin hydrolysates with degree of hydrolysis (DH) of 0.48, 0.71 and 1.72 %, respectively

to either TG-modified protein or sodium caseinate hydrolysate alone. Improved solubility of enzymatic digests of gluten by chymotrypsin, papain, pronase and pepsin followed by a TG treatment over a wide pH range was reported by Babiker *et al.* (*36*).

In general, protein solubility depends on molecular mass, overall charge and surface hydrophobicity. Higher solubility can be expected of proteins with lower molecular mass, higher molecular charge and low surface hydrophobicity. According to Hassan *et al.* (*37*), TG treatment of proteins decreases surface hydrophobicity due to partial deamination of glutamine and asparagine. In our study, the decrease in surface hydrophobicity was most probably compensated by the increase in molecular mass after protein polymerization and the solubility pattern of TG--PI followed the one of untreated protein isolate (Fig. 2). Protein isolate and TG-modified protein isolate, however, expressed greater solubility than TG-PH1, TG-PH2 and TG-PH3 at pH≤3 and ≥7, which determines their better practical application in formulations of food with highly acidic, neutral or slightly alkaline pH.

Alteration of foam capacity and stability by enzymatic modification

Foam capacity followed the overall trend observed for protein solubility. Protein isolate expressed the lowest foam making capacity at around protein pI (pH=4 and 6), which is related to low molecular charge and the formation of high-molecular-mass aggregates (Fig. 3). The highest foam making capacity of protein isolate, observed at pH=2 (47.7 %), is probably due to dissociation of sunflower protein globulins to monomers, thus contributing to increase in foam volume (*38*). The modification of protein isolate with TG slightly increased the foam making capacity at pH=4 and 6 which, however, did not exceed 40.0 %. Significant improvement ($p<0.05$) was achieved with all three TG-modified pepsin hydrolysates (TG-PH1, TG--PH2, TG-PH3), which resulted in foam making capacity varying from 52 to 66 % in the entire pH range studied. Although no direct comparison can be made because of differences in the used substrates and evaluation methods, similar observations were reported by Babiker (*39*). The foam capacity of the soy protein and chymotrypsin--based soy protein hydrolysate after polymerization with TG increased from 500 to 590 μv/cm respectively, as measured by electrical conductivity. In an earlier study, Babiker *et al.* (*36*) also established an improvement of foaming properties after TG polymerization of gluten hydrolysates prepared with chymotrypsin, papain, pronase and pepsin. Flanagan and FitzGerald (*40*) reported 1433 % foam expansion of the sodium caseinate product obtained after hydrolysis with *Bacillus* proteinase and TG polymerization.

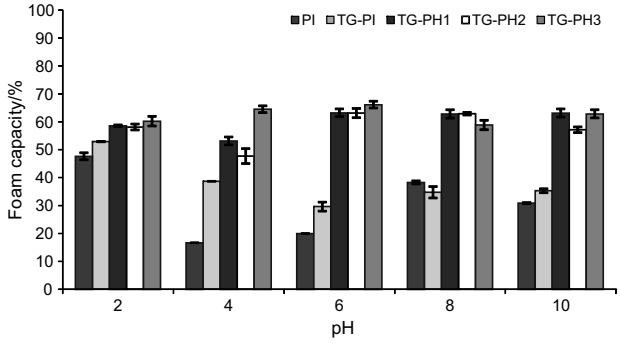

Fig. 3. Foam capacity at different pH values of sunflower protein isolate (PI) and sunflower protein pepsin digests after treatment with transglutaminase (TG). TG-PI=TG-modified protein isolate, TG-PH1, 2 and 3=TG-modified pepsin hydrolysates with degree of hydrolysis (DH) of 0.48, 0.71 and 1.72 %, respectively

In contrast to foam making capacity, pepsin hydrolysis of the protein isolate with three degrees of hydrolysis (0.48, 0.71 and 1.72 %) did not improve the foam stability (Fig. 4). In fact, the highest degree of hydrolysis (1.72 %) gave the lowest foam stability at pH=6. Inverse relationship between the degree of hydrolysis and foam stability of protein hydrolysates was attributed to the decrease in the amount of larger protein component required for foam stabilization (*41*). A similar trend was observed by Kong *at al.* (*42*) and Wouters *et al.* (*43*) while studying wheat gluten hydrolysates, and by Guan *et al.* (*44*) and Larré *et al.* (*45*) for hydrolysates of oat bran protein concentrate and rapeseed protein isolate, respectively.

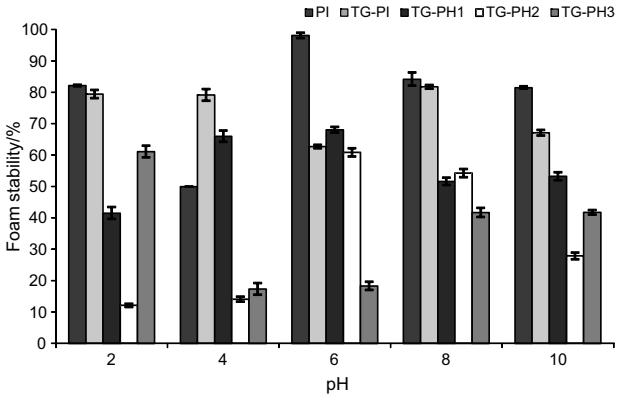

Fig. 4. Foam stability at different pH values of sunflower protein isolate (PI) and sunflower protein pepsin digests after treatment with transglutaminase (TG). TG-PI=TG-modified protein isolate, TG-PH1, 2 and 3=TG-modified pepsin hydrolysates with degree of hydrolysis (DH) of 0.48, 0.71 and 1.72 %, respectively

Our results were not in agreement with the results reported by Babiker (*39*), who observed increased foam stability of the chymotrypsin-digested soy protein polymerized with bacterial TG. This may be due to differences in the substrates used in the study as well as differences of the protein profiles of the hydrolysates obtained with pepsin and chymotrypsin. Jung *et al.* (*46*) revealed that porcine placenta was barely hydrolyzed by pepsin, resulting in peptides with molecular mass (*M*) greater than 7 kDa, while chymotrypsin produced peptides with broad ranges of *M*, from 1 to 20 kDa. Although high molecular complexes are necessary for stabilization of foam, excessive aggregation may impede the formation of a viscoelastic protein film at the air-water boundary (*47*).

Influence of enzymatic treatment on emulsifying properties

Overall, no statistically significant improvement of the emulsifying properties of enzymatically modified sunflower protein isolate was achieved compared to the unmodified counterpart (Tables 1 and 2). Chobert *et al.* (*48*) suggested that low molecular mass peptides may have too low amphiphilic capacity to exhibit technologically satisfying emulsifying properties. Although small-size peptides are advantageous in the migration and interface absorption, they seem inefficient for stabilizing emulsions most probably due to improper unfolding and reorientation at the interface (*49*). Reductions in emulsifying properties of enzymatic hydrolysates of blends of groundnut flour and sorghum meal and defatted groundnut flour were reported by Ahmed and Ramanatham (*50*) and Subba Rau and Srinivasan (*51*) respectively. Our results agreed with Hu *et al.* (*52*), who did not observe any effect of TG on emulsifying activity of peanut protein isolate except at pH=4 when TG-modified protein isolate exhibited the highest emulsifying activity (53.8 %, Table 1).

Influence of temperature on protein stability

Thermal stability was studied at pH=7 and 8 because of the relatively high solubility of all protein isolates at these pH values (Fig. 2) and their potential practical application in food systems. Reaction pH is an important factor influencing protein heat stability since it affects protein charge, conformation and sulfhydryl reactivity upon aggregation (*53*). Up to 80 °C (pH=7), TG-PH3 exhibited lower increases in turbidity and, therefore, better thermal stability than that of protein isolate (Table 3). The thermal stability of TG-PH3 was better at 50 and 60 °C than of TG--modified protein isolate. Babiker (*39*) reported improved thermal resistance of chymotrypsin-digested soy protein up to 60 °C after TG treatment. The lower-degree pepsin hydrolyses (0.48 and 0.71 %) followed by TG polymerization (TG-PH1 and TG-PH2) resulted in reduced thermal stability compared to unmodified protein isolate at all studied temperatures. The reason may be the formation of less hydrophilic structures at pH=7 and 8 (Fig. 2), which in general, leads to a decrease in protein stability (*54*). Similar trends in thermal stability of TG-modified sunflower protein isolates, predigested with pepsin, were also observed at pH=8 (Table 4).

Improvement of heat stability after TG modification of the sunflower protein isolate alone compared to unmodified protein isolate was observed only at higher thermal treatments (70, 80 and 90 °C) at pH=7 (Table 3). Decreases in turbidity at 90 °C of pigeon pea and hyacinth bean protein isolates after TG treatment compared to the native

Table 1. Emulsifying activity of sunflower protein isolate (PI) and sunflower protein pepsin digests after treatment with transglutaminase (TG) at different pH values

	Emulsifying activity/%				
	pH				
	2	4	6	8	10
PI	$(52.9\pm2.5)^{aA}$	$(44.7\pm1.7)^{bB}$	$(53.6\pm3.6)^{aA}$	$(54.5\pm3.4)^{aA}$	$(55.7\pm3.46)^{aA}$
TG-PI	$(50.6\pm0.0)^{dAB}$	$(53.8\pm0.0)^{bcA}$	$(55.5\pm0.4)^{aA}$	$(53.3\pm0.0)^{cAB}$	$(54.2\pm0.42)^{bAB}$
TG-PH1	$(51.6\pm2.3)^{bAB}$	$(46.6\pm0.0)^{cB}$	$(45.3\pm0.0)^{cB}$	$(46.0\pm0.9)^{cC}$	$(56.2\pm0.00)^{aA}$
TG-PH2	$(44.7\pm2.6)^{bB}$	$(44.7\pm2.6)^{bB}$	$(46.6\pm0.0)^{abB}$	$(50.0\pm0.0)^{aBC}$	$(50.0\pm0.00)^{aBC}$
TG-PH3	$(50.0\pm4.7)^{aAB}$	$(9.7\pm1.0)^{bC}$	$(48.3\pm2.3)^{aB}$	$(50.0\pm0.0)^{aBC}$	$(48.5\pm2.09)^{aC}$

Mean values in a row with the same lower case letter do not differ significantly ($p\geq0.05$).
Mean values in a column with the same capital letter do not differ significantly ($p\geq0.05$).
TG-PI=TG-modified protein isolate, TG-PH1, 2 and 3=TG-modified pepsin hydrolysates with degree of hydrolysis (DH) of 0.48, 0.71 and 1.72 %, respectively

Table 2. Emulsifying stability of sunflower protein isolate (PI) and sunflower protein pepsin digests after treatment with transglutaminase (TG) at different pH values

	Emulsifying stability/%				
	pH				
	2	4	6	8	10
PI	$(93.2\pm2.7)^{bcB}$	$(100.0\pm0.0)^{aA}$	$(90.3\pm1.9)^{cB}$	$(95.7\pm0.2)^{abA}$	$(93.6\pm0.2)^{abAB}$
TG-PI	$(97.2\pm0.1)^{bA}$	$(100.0\pm0.0)^{aA}$	$(100.0\pm0.0)^{aA}$	$(96.2\pm1.8)^{bA}$	$(97.5\pm1.3)^{bAB}$
TG-PH1	$(100.0\pm0.0)^{aA}$	$(71.4\pm4.1)^{bAB}$	$(100.0\pm0.0)^{aA}$	$(93.7\pm2.8)^{abA}$	$(88.9\pm0.0)^{abB}$
TG-PH2	$(100.0\pm0.0)^{aA}$	$(57.1\pm3.1)^{bAB}$	$(100.0\pm0.0)^{aA}$	$(100.0\pm0.0)^{aA}$	$(100.0\pm0.0)^{aA}$
TG-PH3	$(100.0\pm0.0)^{aA}$	$(15.0\pm1.1)^{dB}$	$(73.2\pm2.5)^{bC}$	$(100.0\pm0.0)^{aA}$	$(56.2\pm8.8)^{cC}$

Mean values in a row with the same lower case letter do not differ significantly ($p\geq0.05$).
Mean values in a column with the same capital letter do not differ significantly ($p\geq0.05$).
TG-PI=TG-modified protein isolate, TG-PH1, 2 and 3=TG-modified pepsin hydrolysates with degree of hydrolysis (DH) of 0.48, 0.71 and 1.72 %, respectively

Table 3. Thermal stability of sunflower protein isolate (PI) and sunflower protein pepsin digests after treatment with transglutaminase (TG) at pH=7

Temperature/°C	Turbidity/%*				
	PI	TG-PI	TG-PH1	TG-PH2	TG-PH3
50	$(4.2\pm1.9)^{b}$	$(4.2\pm0.3)^{b}$	$(4.7\pm0.0)^{b}$	$(43.9\pm3.2)^{a}$	$(0.8\pm1.2)^{c}$
60	$(5.4\pm0.2)^{c}$	$(4.4\pm1.6)^{c}$	$(18.3\pm2.3)^{b}$	$(48.1\pm2.7)^{a}$	$(1.7\pm2.4)^{d}$
70	$(9.9\pm0.5)^{c}$	$(4.9\pm0.2)^{d}$	$(28.2\pm7.2)^{b}$	$(72.5\pm0.7)^{a}$	$(4.8\pm6.8)^{d}$
80	$(15.2\pm0.8)^{c}$	$(6.9\pm2.5)^{d}$	$(39.9\pm6.3)^{b}$	$(73.5\pm0.7)^{a}$	$(6.6\pm0.0)^{d}$
90	$(18.1\pm0.8)^{c}$	$(10.3\pm0.2)^{d}$	$(45.9\pm9.2)^{b}$	$(75.0\pm0.0)^{a}$	$(17.0\pm0.8)^{c}$

*Thermal stability was evaluated as the increase of sample turbidity expressed in percentage.
Mean values in a row with the same lower case letter do not differ significantly ($p\geq0.05$).
TG-PI=TG-modified protein isolate, TG-PH1, 2 and 3=TG-modified pepsin hydrolysates with degree of hydrolysis (DH) of 0.48, 0.71 and 1.72 %, respectively

Table 4. Thermal stability of sunflower protein isolate (PI) and sunflower protein pepsin digests after treatment with transglutaminase (TG) at pH=8

Temperature/°C	Turbidity/%*				
	PI	TG-PI	TG-PH1	TG-PH2	TG-PH3
50	$(4.7\pm0.3)^{b}$	$(4.9\pm1.4)^{b}$	$(15.8\pm1.5)^{a}$	$(14.0\pm3.6)^{a}$	$(2.3\pm4.7)^{c}$
60	$(7.7\pm0.0)^{bc}$	$(6.1\pm1.4)^{bc}$	$(16.6\pm8.6)^{ab}$	$(24.2\pm3.1)^{a}$	$(2.3\pm0.0)^{d}$
70	$(11.7\pm0.3)^{b}$	$(6.7\pm1.1)^{c}$	$(22.5\pm3.1)^{a}$	$(26.3\pm3.9)^{a}$	$(4.35\pm6.1)^{c}$
80	$(13.9\pm0.0)^{b}$	$(9.3\pm0.0)^{b}$	$(36.7\pm6.6)^{a}$	$(31.5\pm1.7)^{a}$	$(11.5\pm1.3)^{b}$
90	$(20.9\pm3.7)^{cd}$	$(13.1\pm0.7)^{d}$	$(55.3\pm11.9)^{a}$	$(41.2\pm4.3)^{ab}$	$(28.8\pm1.7)^{bc}$

*Thermal stability was evaluated as the increase of sample turbidity expressed in percentage.
Mean values in a row with the same lower case letter do not differ significantly ($p\geq0.05$).
TG-PI=TG-modified protein isolate, TG-PH1, 2 and 3=TG-modified pepsin hydrolysates with degree of hydrolysis (DH) of 0.48, 0.71 and 1.72 %, respectively

proteins were observed by Ali *et al.* (*55*). According to Siu *et al.* (*56*) and O'Sullivan *et al.* (*57*), elevated temperatures denature proteins and facilitate TG cross-linking to form aggregates with more compact and heat-stable structures. Ryan *et al.* (*53*) reported that 10 °C difference in heating substantially influenced the degree of polymerization as the maximum level could be reached in 10 min at 85 °C (neutral pH), while up to 8 h may be needed for aggregate formation at 70 °C due to slower denaturation and diffusion rates. Most probably, neutral pH additionally contributes to the increased heat resistance of the protein aggregates *via* formation of disulfide bonds, which are diminished at the expense of noncovalent associations maintaining the aggregates at higher pH values (*58*). In our study, at pH=8 no statistically significant differences in thermal stability between TG-modified protein isolate and protein isolate were established (Table 4). Thermal treatment is a common approach in food processing, and knowledge on thermal stability of the protein isolates would facilitate their potential application as food ingredients.

Conclusions

Major challenges in preparation of sunflower meal-derived protein products and their application in the food industry are related to protein characteristics and changes due to processing parameters during oil production. This study demonstrated the application of low-degree pepsin hydrolysis combined with transglutaminase (TG) treatment for the improvement of specific functional properties of a protein isolate prepared from industrial sunflower meal. The results implied that hydrolysis of the protein with pepsin decreased the influence of pH on protein solubility at isoelectric point (pI=4.5). If greater solubility at pH<3 and >7 is needed, enzymatic modification of protein isolate should be avoided. Modification of protein isolate with TG could be useful in the improvement of foam making capacity at pH=4 and 6. The effect could be enhanced by a preceding pepsin hydrolysis, as evidenced by foam making capacity of TG-PH1, TG-PH2 and TG-PH3, which remained between 52 and 66 % in the entire studied pH range. Improved heat resistance of protein isolate at the lower range of temperatures studied (up to 80 °C, pH=7) could be achieved by pepsin hydrolysis of protein isolate to DH of 1.72 % and consequent TG treatment (TG-PH3), while at the higher temperatures (80 and 90 °C), TG modification alone is sufficient for improvement of this characteristic. Data obained in this study could be helpful for production of sunflower protein isolates with desired functional properties.

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