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Functional properties of pumpkin (*Cucurbita pepo*) seed protein isolate and hydrolysate

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Abstract: Pumpkin seed protein isolate (PSPI) was enzymatically hydrolysed by pepsin to obtain pumpkin seed protein hydrolysate, PSPH. Investigation of the solubility, interfacial and emulsifying properties of both PSPI and PSPH was conducted under different conditions of pH (3–8) and ionic strength (0–1 mol dm^{-3} NaCl). PSPI had the lowest solubility, *i.e.*, isoelectric point (pI) at pH 5. PSPH had higher solubility than PSPI over the whole range of tested pH and ionic strength values. The decreases in the surface and interfacial tension evidenced that both PSPI and PSPH adsorb at the air/protein solution and oil/ /protein interfaces of the solution. Emulsions (20 % oil in water) stabilized by 10 g dm^{-3} PSPI or PSPH solution were prepared at pH 3, 5 and 8 and ionic strength of 0 and 0.5 mol dm^{-3} NaCl. PSPH stabilized emulsions from coalescence at all tested pH and ionic strength values. PSPI was able to stabilize emulsions at pH 3 and 0 mol dm^{-3} NaCl, and at pH 8 regardless of ionic strength, while emulsions at pH 5 and both 0 and 0.5 mol dm^{-3} NaCl and at pH 3 when the ionic strength was increased separated into an oil and a serum layer immediately after preparation. All emulsions were susceptible to creaming instability.

Keywords: plant proteins; natural emulsifiers; enzymatic hydrolysis; oilseed proteins.

INTRODUCTION

Proteins from vegetable origin are an alternative to animal proteins for food and cosmetics applications due to the renewability of the raw material and widespread and variety of sources (especially legumes, cereals and oilseeds).[1] Oilseeds, despite the fact that they are primarily grown as a source of oil, are rich in proteins. Moreover, after oil extraction, the protein content in an oil cake, which is a by-product of the oil extraction process, can increase up to 60 %, making it a valuable material for protein extraction.[2]

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As one of the oilseeds, pumpkin (*Cucurbita* sp.) seed has 36 % of proteins,[3] while the protein content in the oil cake can increase up to 65 %.[4] The major fraction of these proteins is represented by cucurbitins. Cucurbitins are 12S globulins composed of six similar subunits with molecular weights of 54 kDa, and thus, the total molecular weight of cucurbitin is about 325 kDa.[5,6] Cucurbitins are accompanied by 2S albumins, proteins with a molecular weight of 12.5 kDa. Albumins are composed of two chains, a small and a large one, with molecular weights of 4.8 and 7.9 kDa, respectively.[7,8] The 12S and 2S protein fractions together make 59 % of total crude protein content in pumpkin seed.[9]

Pumpkin seed proteins are desirable ingredients in food products because they are reported to be health improving on several levels, *i.e.*, they were found to have anti-microbial and anti-carcinogenic effects, to inhibit mechanisms of blood coagulation and to alleviate the detrimental effects associated with protein malnutrition.[10,11]

Thus, plant proteins are increasingly used as unconventional sources of proteins to perform functional roles in food formulations.[12] However, the low solubility of plant proteins in acidic media complicates their utilization in foodstuffs of moderate acidity (citric beverages, dressings, *etc.*),[13] especially when the required functional properties, *e.g.*, foaming and emulsifying properties, depend on solubility.[14] The solubility of pumpkin seed proteins was reported to be very low (< 20 %) at pH < 5.[15]

One of the most efficient means of increasing protein solubility as well as improving the functional properties of oilseed proteins is to subject them to enzymatic hydrolysis.[2] Functional properties of particular interest include improved solubility, particularly at pH near the isoelectric point (pI), enhanced emulsifying properties, as well as enriched biological activities.[16] Namely, modification of proteins by partial enzymatic hydrolysis is accompanied by: 1) a decrease in molecular weight, 2) an increase in number of ionisable groups and 3) exposure of previously concealed hydrophobic groups at the interface. These effects can well modify the conformation and structure of proteins, thus changing the solubility, surface characteristics and emulsifying properties.[12,17,18]

Hitherto, only a few investigations on pumpkin seed protein hydrolysates have been conducted. Vaštag *et al.*[4] reported pumpkin seed protein hydrolysates to have antioxidant and ACE-inhibitory activities and investigation of Peričin *et al.*[19] showed that enzymatic hydrolysis is an effective tool for obtaining highly soluble pumpkin seed proteins.

The aim of this study was to investigate the effect of different environmental conditions (pH and ionic strength) on functional properties such as solubility, interfacial and emulsifying properties of pumpkin seed protein isolate (PSPI) in comparison to pumpkin seed protein hydrolysate (PSPH).

EXPERIMENTAL

Materials

Pumpkin (*Cucurbita pepo*) seed oil cake was obtained from "Agrojapra", Bosnia and Herzegovina. It was stored at a temperature of 4 °C and ground in a coffee grinder before use. Sunflower oil was obtained from "Vital", Serbia. Demineralised water was used as a solvent. Pepsin (0.7 FIP-U mg^{-1}) was obtained from Sigma (St. Louis, MO, USA). All other used chemicals were obtained from "Centrohem d.o.o.". Serbia, and were of at least extra pure quality. Buffer solutions were prepared by mixing 0.2 mol dm^{-3} disodium hydrogenphosphate and 0.1 mol dm^{-3} citric acid in proportions defined for each pH.

PSPI preparation

Pumpkin seed protein isolate (PSPI) was prepared by alkali extraction with isoelectric precipitation. Firstly, the ground pumpkin seed oil cake was defatted with hexane (mass ratio 1:5) in two stages and air-dried at room temperature. The defatted pumpkin cake was suspended in alkali solution at pH 10.00, which was set by 1 mol dm^{-3} NaOH, at room temperature to allow protein dissolution. After 30 min of gentle stirring, the slurry was filtered. The dissolved proteins in the filtrate were precipitated by adjusting pH to 5.00 with 1 mol dm^{-3} HCl. The precipitate was separated from the liquid phase by centrifugation (Sorvall^{TM} RC 5B centrifuge) at 4 °C and 10000 rpm for 20 min and dried at 30 °C for 48 h. Finally, the dried protein precipitate was ground in a coffee grinder to obtain PSPI powder.

Enzymatic hydrolysis

PSPI suspension (10 g dm^{-3}) was prepared by suspending the required amount of PSPI in a solution of pH 3, which was set and controlled by the addition of 1 mol dm^{-3} HCl. Enzymatic hydrolysis was performed in a batch reactor at 37 °C and an enzyme to substrate ratio of 0.02 g per 1 g. The reaction conditions were set so that degree of hydrolysis was 19±1 % at the end of reaction. Enzymatic hydrolysis was completed after 90 min. The hydrolysed suspension was then vacuum filtered and dried using a "Büchi 190" spray drier at an inlet temperature of 120 °C and outlet temperature of 70 °C.

*Determination of the degree of hydrolysis (*DH*)*

The same volumes of hydrolysed suspension and trichloroacetic acid (0.44 mol dm^{-3}) were mixed and incubated at 4 °C for 30 min. Thereafter, the mixture was centrifuged (Eppendorf mini Spin Plus, 14500 rpm, 10 min).[4] The obtained 0.22 mol dm^{-3} trichloroacetic acid soluble protein fraction and the hydrolysate mixture without addition of trichloroacetic acid were each analyzed to determine the protein content by the method of Lowry *et al.*[20], using bovine serum albumin as the standard protein. The *DH* was calculated as the ratio of the 0.22 mol dm^{-3} trichloroacetic acid soluble proteins to total proteins in the hydrolysate, expressed as a percentage.

Determination of PSPI and PSPH solubility

PSPI and PSPH suspensions of different concentrations (c_{susp}, 10–100 g dm^{-3}) were prepared by suspending the required amount of PSPI in buffer solutions of different values of pH (3–8) and ionic strength, I_c (0–1 mol dm^{-3}). The suspensions were placed in a water bath at 50 °C for 1 h under constant stirring, in order to enable dissolution of the proteins. Soluble proteins were separated from undissolved particles by Sartorius membrane filtration (filter pore size 0.45 μm) to obtain PSPI or PSPH solutions. The concentration of dissolved proteins in the protein solution, c_{sol}, was determined by the Lowry *et al.* [20] method and was expressed as g dm^{-3}. The PSPI and PSPH solubility, *s*, was calculated as:

$$s = 100\frac{c_{\text{sol}}}{c_{\text{susp}}} \quad (1)$$

Tensiometry

The surface tension (air–water interface) and interfacial tension (oil–water interface) of PSPI solutions were determined at 25 °C according to the Du Noüy ring method using a Sigma 703D tensiometer (KSV Instruments Ltd., Finland). The ring was immersed in PSPI solution (20 mL) and the surface was left to equilibrate for 10 min. For the interfacial tension measurements, after ring immersion in the solution, 20 mL of sunflower oil was carefully added on top of the solution surface and the interface was left to equilibrate for 10 min. Upon surface–interface equilibration, the surface/interfacial tension was measured. The reported values of surface and interfacial tension are the mean values of at least three measurements. Surface tension of buffer solutions of pH 3–8 was 71.7±0.4 mN m^{-1}. The interfacial tension of the buffer solutions of pH 3–7 was 28.4±0.9 mN m^{-1}, whereas, it was 25.7±1.0 mN m^{-1} at pH 8.

Preparation and characterization of emulsions

Emulsions of 20 mass% sunflower oil in water were prepared by dispersing 6 g of sunflower oil in 24 g of a continuous phase by means of an Ultraturrax T–25 (Janke & Kunkel, Germany) homogenizer at 10000 rpm for 10 min at 25 °C. The compositions of the continuous phase were varied by changing the ionic strength (0 and 0.5 mol dm^{-3}) and pH (3, 5 and 8), while the concentration of the PSPI and PSPH solution was kept constant at 10 g dm^{-3}. The volume weighted mean diameter ($d_{4,3}$) of oil the droplets in the emulsions was determined using a Mastersizer micro particle analyzer (Malvern Instruments Ltd., UK). Buffer solutions which corresponded to the measured emulsions were used to collect the background data. Emulsions were dosed so that the obscuration stayed between 10 and 20 %. During the measurements, the pump rotation speed was kept at 1500 rpm. The reported $d_{4,3}$ values are the average value of at least three measurements.

Stability of emulsions was evaluated by the creaming test. Emulsions were transferred into 10 mL sealed graduated glass cylinders immediately after preparation and were left for 14 days at room temperature to observe their creaming behaviour. During time, the emulsions separate into an emulsion cream (top) and an emulsion serum (bottom) layer. Changes in a creaming index, *CI*, were visually monitored, where the creaming index is:

$$CI = 100\frac{V_{\text{S}}}{V_{\text{E}}} \quad (2)$$

where V_{S} and V_{E} are the volumes of the serum layer and the emulsion, respectively.

RESULTS AND DISCUSSION

Solubility

The solubility characteristics of protein are among the most important functional properties since many functional performances of proteins depend upon their capacity to go into solution initially.[2] Therefore, the solubility of PSPI and PSPH as a function of pH were investigated first (Fig. 1).

Figure 1 shows that PSPI had the lowest solubility at pH 5, which is reported to be the isoelectric point for the majority of food proteins.[13] PSPI solubility increased as the pH of the solution is increased towards more alkaline or decreased towards more acidic conditions.

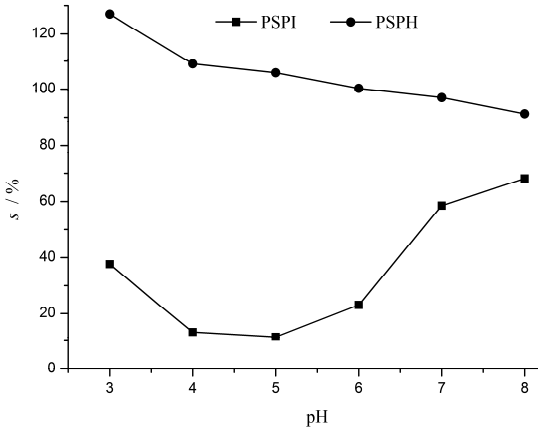

Fig. 1. Influence of pH on the solubility of PSPI and PSPH; c_{susp} = 10 g dm^{-3}, I_c = 0 mol dm^{-3}.

The enzymatic hydrolysis improved the solubility of PSPI at all the tested pH values. It is noteworthy that the lower was the solubility of PSPI, the more it was increased by enzymatic hydrolysis, which was especially true for pH values close to the p*I* of PSPI (Fig. 1). Similar results were observed for proteins from oilseed flour mixture, chickpea, peanut, *Brassica carinata* and barley and for sodium caseinate.[16,21–24] The increase in protein solubility as a result of enzymatic hydrolysis could be attributed to the reduction of the molecular size and the increase in hydrophilic character of proteins, exposing more polar groups to the aqueous environment.[2,12,21]

The influence of ionic strength on PSPI and PSPH solubility at three different pH values (3, 5 and 8) is shown in Fig. 2. Depending on the pH of PSPI and PSPH solutions, two distinct effects, salting-in and salting-out, could be observed.

The salting-out effect, *i.e.*, a decrease in solubility with increasing ionic strength was observed at pH 3 for PSPI and at pH 3 and 5 for PSPH. On the contrary, the addition of NaCl up to 0.5 mol dm^{-3} for PSPI and 1 mol dm^{-3} for PSPH at pH 8 brought about increase in solubility of about 10 %, indicating a slight salting-in effect. Influence of ionic strength on the solubility of proteins is the result of the complex interplay of various effects, such as electrostatic interactions, ion specific effects, hydrophobic effects, *etc.*[24,25]

Interfacial properties

Interfacial properties of PSPI and PSPH solutions of different pH values, ionic strengths and protein concentrations were investigated. Influence of pH on surface and interfacial tension of PSPI and PSPH solutions is shown in Fig. 3.

PSPI had the lowest interfacial tension at pH 5, *i.e.*, at the isoelectric point. Namely, low protein solubility at pI, which is a result of increased hydrophobic interactions, force protein molecules to minimize unfavourable interactions by aligning themselves at the interface.[26] On the contrary, on increasing the pH above or

decreasing it below pI, the protein molecules become more hydrated and solubility increases. Consequently, protein drive towards the interface is mitigated as evidenced by the increase in the surface tension of PSPI solutions (Fig. 3).

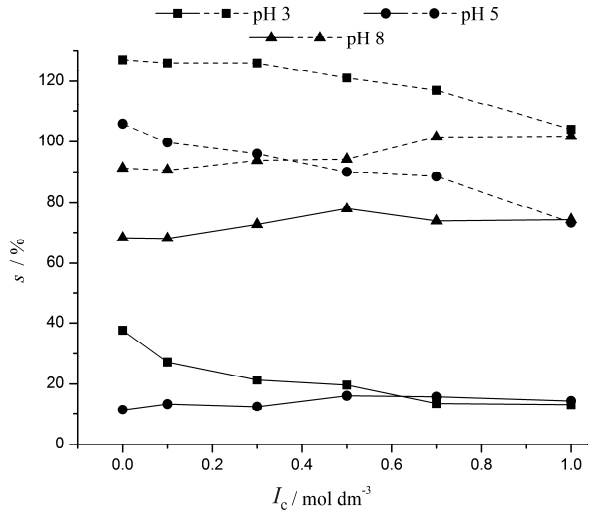

Fig. 2. Influence of ionic strength on the solubility, *s*, of PSPI (solid lines) and PSPH (dash lines); c_{susp} = 10 g dm^{-3}.

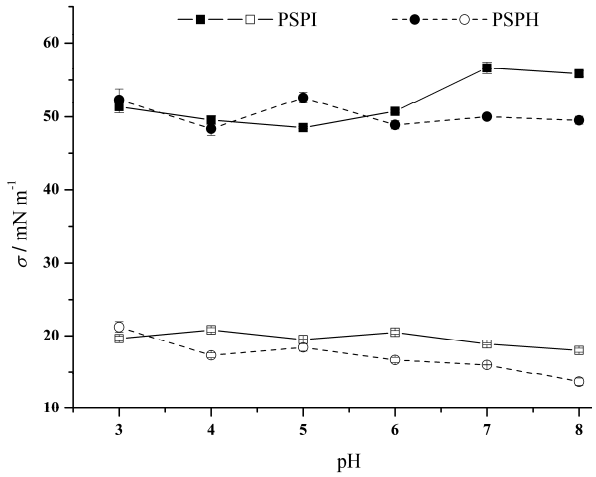

Fig. 3. The influence of pH on the surface and interfacial tension of PSPI (solid lines) and PSPH (dash lines); I_c = 0 mol dm^{-3}, NaCl, c_{sol} = 1 g dm^{-3}. Filled symbols represent surface and the open symbols interfacial tension.

Influence of PSPH on surface and interfacial tension was comparable to that of PSPI except at pH > 6 when PSPH was more effective in decreasing surface and interfacial tension, indicating that the hydrophobic amino acids might

become exposed during hydrolysis, resulting in unfolded structures and increased hydrophobicity of the hydrolysates.[16,17]

Influence of the PSPI and PSPH solution concentration on the surface and interfacial tension at three different pH values (3, 5 and 8) is shown in Fig. 4a and 4b, respectively. As expected, the higher is the concentration of the PSPI or PSPH solution, the lower are the surface and interfacial tension, regardless of pH. Nevertheless, PSPH showed more pronounced decrease in surface and interfacial tension, especially at lower solution concentrations (c_{sol} < 0.1 g dm^{-3}), when compared to PSPI.

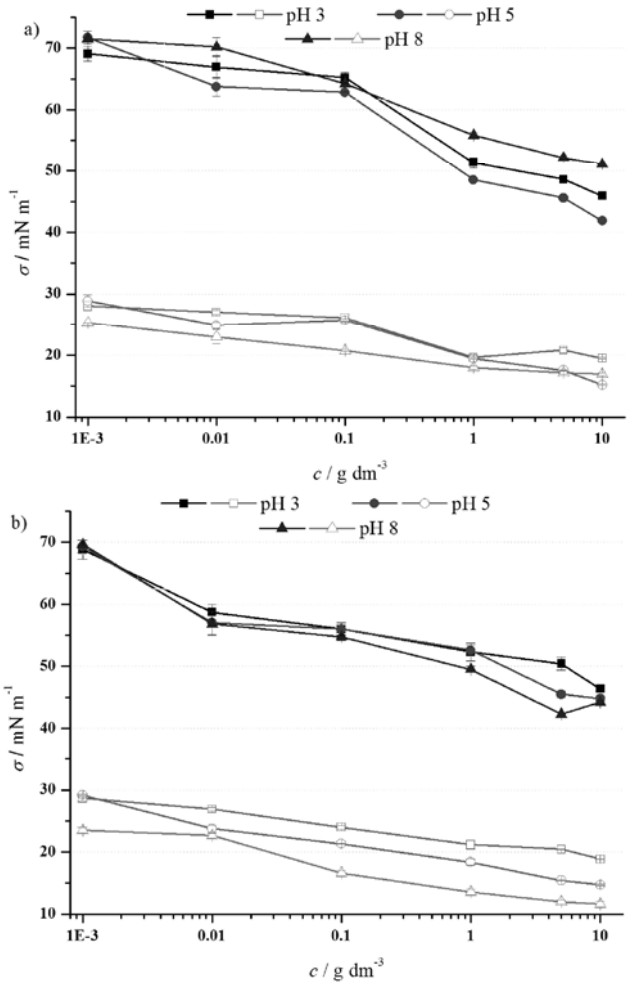

Fig. 4. Influence of solution concentration, c_{sol}, on the surface and interfacial tension of: a) PSPI; and b) PSPH solution; I_c = 0 mol dm^{-3} NaCl. Filled symbols represent the surface and open symbols the interfacial tension.

The influence of the ionic strength on the surface and interfacial tension of 1 g dm^{-3} PSPI and PSPH solutions at pH 3, 5 and 8 are presented in Fig. 5. Increasing the ionic strength had a minimal effect on the surface and interfacial tension of PSPI and PSPH solutions (±1.5 mN m^{-1}) at all tested pH values, except for the PSPI solution at pH 3. Since the addition of NaCl at pH 3 significantly decreased PSPI solubility (Fig. 2), *i.e.*, increased hydrophobic interactions and decreased protein hydration, it was expected that the PSPI solution would be more effective in decreasing the surface and interfacial tension upon increasing the ionic strength at pH 3. In addition, the lowest surface and interfacial tension of PSPH solution at pH 8 could be because of its lowest solubility at pH 8 (see Fig. 1).

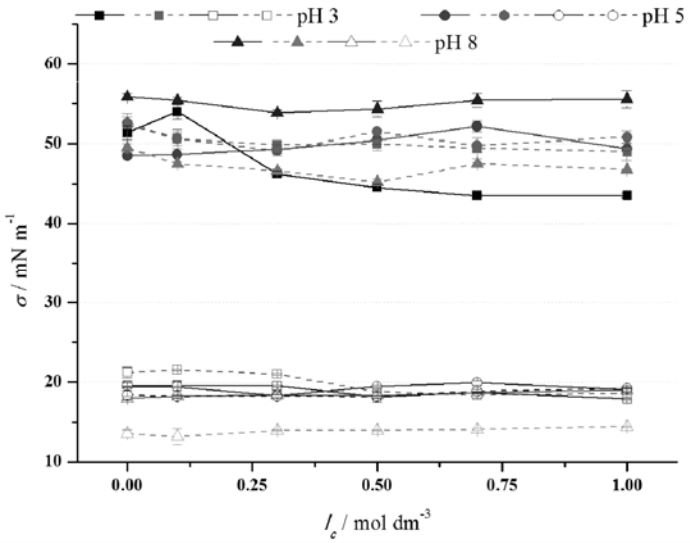

Fig. 5. Influence of ionic strength on surface and interfacial tension of PSPI (solid lines) and PSPH (dash lines); c_{sol} = 1 g dm^{-3}. Filled symbols represent the surface and open symbols the interfacial tension.

Emulsifying properties of PSPI and PSPH

Emulsions of 20 % sunflower oil in protein solution (10 g dm^{-3}) were prepared at pH 3, 5 and 8 without or with 0.5 mol dm^{-3} NaCl. The volume weighted mean droplet diameter, $d_{4,3}$, of the PSPI and PSPH stabilized emulsions without and with 0.5 mol dm^{-3} NaCl in the continuous phase is illustrated in Fig. 6a and b, respectively. Stable emulsions when PSPH was used as emulsifier were obtained at all tested pH and ionic strength values while the PSPI stabilized emulsions were only stable at pH 3 and without NaCl and at pH 8 regardless of the ionic strength. PSPI failed to stabilize emulsions at pH 5 regardless of the ionic strength and at pH 3 when the ionic strength was increased and hence, the

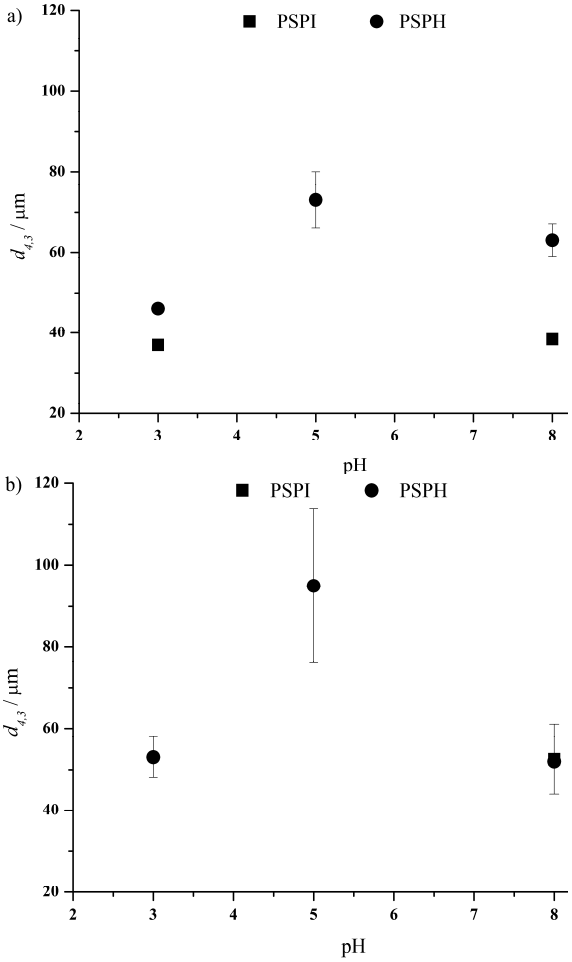

Fig. 6. Mean volume weighted droplet diameter, $d_{4,3}$, of emulsions stabilized by 10 g dm^{-3} PSPI and PSPH: a) without NaCl, and b) with 0.5 mol dm^{-3} NaCl in the continuous phase.

oil separated immediately after emulsion preparation. It is noteworthy that oil separation occurred even though interfacial study showed that both PSPI and PSPH adsorb at the air–protein and oil–protein solution interfaces under all tested conditions (pH, c_{sol} and ionic strength), as evidenced by the decrease in the surface and interfacial tension (Figs. 3–5). The inability of PSPI to stabilize emulsions at pH 5 is attributed to insufficient electrostatic repulsion between the emulsion droplets, which led to droplet aggregation/flocculation and coalescence. Namely, proteins contain many ionisable groups that alter the electrical characteristics of the interface of oil droplets once adsorbed.[27] These ionisable groups could be positively or negatively charged depending on the pH of the solution. At p*I*, the net protein charge is zero and thus electrostatic repulsion between droplets of the emulsion is minimal, resulting in emulsion instability. On the other hand, as the pH increases above or decreases below the pI value, negative or positive

charge takes preponderance leading to enhancement of the emulsion stability due to increased electrostatic repulsion between the droplets.

The $d_{4,3}$ values of the emulsions stabilized by either PSPI or PSPH depended on pH of the continuous phase (Fig. 6a and b). However, the $d_{4,3}$ values for the PSPI stabilized emulsions (pH 3 without NaCl and pH 8 without or with 0.5 mol dm^{-3} NaCl) were smaller than the $d_{4,3}$ for emulsions stabilized by PSPH under the same environmental conditions (pH and ionic strength).

All emulsions were susceptible to creaming instability. Creaming index, *CI*, of the emulsions stabilized by PSPI and PSPH are shown in Fig. 7a and b, res-

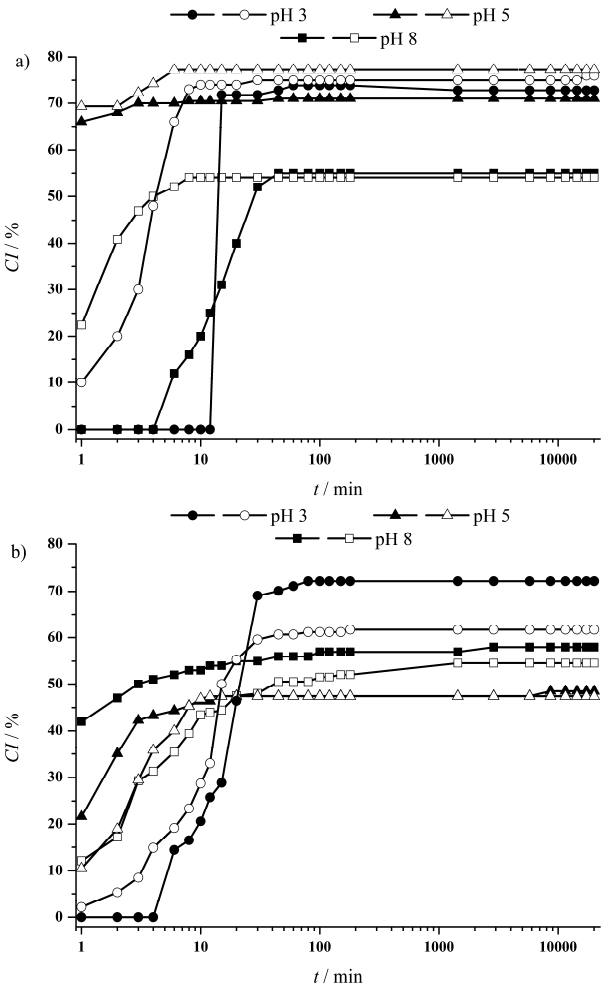

Fig. 7. Creaming index, *CI*, of emulsions stabilized by 10 g dm^{-3} of: a) PSPI; and b) PSPH during 14 days of storage. Filled symbols indicate I_c = 0 mol dm^{-3} NaCl, and open symbols I_c = 0.5 mol dm^{-3} NaCl.

pectively. All emulsions separated to a cream and a serum layer within 20 min after preparation as a consequence of relatively large droplet size and low viscosity of the continuous phase. The *CI* of the PSPH stabilized emulsions at pH 3 when 0.5 mol dm^{-3} of NaCl was added to the continuous phase and at pH 5 regardless of ionic strength were lower than the *CI* of PSPI stabilized emulsions under the same conditions of pH and ionic strength, indicating improved stability of the emulsions with PSPH in the continuous phase.

CONCLUSIONS

The effect of enzymatic hydrolysis on the functional properties of PSPI at different values of pH (3–8) and ionic strength (0–1 mol dm^{-3}) was investigated. The solubility of PSPH was significantly increased when compared to solubility of PSPI over whole range of tested pH and ionic strength values. Solubility was especially improved at pH values close to the pI of PSPI. Both PSPI and PSPH decreased surface and interfacial tension. Stable PSPH emulsions were obtained irrespective of the pH and ionic strength of the continuous phase, in contrast to PSPI, which gave stable emulsions only at pH 3 and 8 in 0 mol dm^{-3} NaCl and at pH 8 in 0.5 mol dm^{-3} NaCl. The $d_{4,3}$ values of emulsions stabilized by PSPH were larger when compared to those of PSPI stabilized emulsions. The obtained results suggest that due to its high solubility, PSPH may be better suited than PSPI for many food formulations, especially in conditions of acidic pH and increased ionic strength.

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И З В О Д

ФУНКЦИОНАЛНЕ ОСОБИНЕ ПРОТЕИНСКИХ ИЗОЛАТА И ХИДРОЛИЗАТА ДОБИЈЕНИХ ИЗ СЕМЕНА ТИКВЕ (*Cucurbita pepo)*

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Протеински хидролизат семена уљане тикве (PSPH) добијен је путем ензиматске хидролизе протеинског изолата семена уљане тикве (PSPI) пепсином. Испитиван је утицај услова средине као што су pH (3–8) и јонска јачина (0–1 mol dm^{-3}) на растворљивост и површинска и емулгујућа својства PSPI и PSPH. PSPI је најмање растворљив на pH изоелектричне тачке (pI), тј. на pH 5. У односу на PSPI, растворљивост PSPH је повећана при свим испитиваним условима pH и јонске јачине. Присуство PSPI или PSPH у раствору изазвало је смањивање површинског и међуповршинског напона што указује на то да се и изолат и хидролизат адсорбују на међуповршинама ваздух/протеински раствор и уље/протеински раствор. Припремљене емулзије са 20 % уља у води на pH 3, 5 и 8, и при јонској јачини од 0 и 0,5 mol dm^{-3} стабилизоване су раствором PSPI или PSPH (10 g dm^{-3}). PSPH је спречио коалесценцију емулзија при свим испитиваним условима pH и јонске јачине. PSPI је стабилизовао емулзије на pH 3 при 0 mol dm^{-3} NaCl и на pH 8 без обзира

на јонску јачину док су се емулзије припремљене на pH 5 и 0 и 0,5 mol dm^{-3} NaCl и на pH 3 при повећаној јонској јачини раздвојиле на уљану и серум фазу одмах након припреме. Све емулзије су биле подложне гравитационој нестабилности.

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