POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2003, Vol. 12/53, SI 2, pp. 171-177

LIMITED ENZYMIC HYDROLYSIS OF EXTRUDED SOY FLOUR AS A METHOD FOR OBTAINING NEW FUNCTIONAL FOOD COMPONENTS

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Key words: limited proteolysis, functional properties, soy protein, process optimisation, Alcalase

The influence of proteolysis of extruded soy flour (ExSF) with Alcalase conducted under different conditions upon functional properties of hydrolysates was studied. The results were summarised as response surfaces and contour plots. It follows that limited hydrolysis of extruded raw material allows one to obtain products with better functional properties in comparison with non-hydrolysed extrudate, with the exception of water holding capacity (WHC). With progressing hydrolysis, an increasing release of amino nitrogen was accompanied by an increase in solubility ($r=0.97$, $p<0.05$) and decreasing WHC ($r= -0.85$, $p<0.05$), as well as a decrease of maximum force in back extrusion test ($r= -0.96$, $p<0.05$). Changes in foaming and emulsifying properties were more differentiated. As a result of the process optimization, it can be concluded that the best protein hydrolysate, in terms of physicochemical properties, can be obtained from extruded soy flour by conducting the process for 90 min at 60°C, pH 8.3, water added to the raw material 13 g/g protein and with 8 mAU/g protein addition of Alcalase.

INDRODUCTION

Soybean is a particularly valuable source of protein, since its proteins have high biological value while its cost is relatively low. However, functional properties of soy protein preparations depend to a considerable degree on the methods of their production which affect the degree of protein denaturation and the presence of non-protein constituents [Wagner & Añón, 1990; Surówka, 1997]. Besides non texturised soy protein preparations produced on a large scale, texturised products chiefly by the extrusion method, become more and more common. Unique functional properties of extrudates are caused by the effect that high temperature, pressure and shear forces have upon protein and non protein constituents, while maintaining a limited access of water in extruder [Ledward & Mitchell, 1988].

Generally, it can be stated that limited proteolysis of protein preparations offers a possibility to obtain hydrolysates with enhanced functional properties. As a result of this process, molecular weight is reduced, the number of functional groups capable of ionisation is increased and a change in surface hydrophobicity occurs [Lahl & Braun, 1994; Darewicz *et al.*, 2000], which leads to the change in the functional properties of the system. This depends, however, not only on the enzyme used and conditions of proteolysis, but also on the kind of raw material and the method by which it was obtained. Such relationships were shown by Henn and Netto [1996], who conducted hydrolysis, with pancreatin, of 13 different commercially available soy protein isolates. According to these authors, reaching a 22% degree of hydrolysis under standard conditions required the time from 48 to 274 min, and profiles of molecular weights of peptides in the final products were much differentiated, which had a direct impact on their functional properties.

In the available literature no reports could be found on the use of enzymatic proteolysis for modifying functional properties of extruded soy protein preparations. A combination of these two processes has been applied up to now only in the production of soy sauce [Motoi *et al.*, 1982] and for dissolving the protein matrix of the extrudate from full-fat soy flour in order to obtain oil without using organic solvents [Pereira-Freitas *et al.*, 1997]. In addition, investigations were conducted on the effect of hydrolysed soy protein addition upon texturisation of soy flour and soy concentrate, showing its negative influence on the structure of extrudates [Dahl, 1986]. Sautier and Camus [1976], while studying digestibility of the extruded isolate, found that under *in vitro* conditions it is more easily digested than the non-extruded one.

The aim of the presented study was to demonstrate that the process of limited enzymatic hydrolysis of proteins from extruded soy flour with Alcalase would allow one to obtain novel products with favourably modified physicochemical properties, which would combine properties typical of extrudates as well as those characteristic for protein hydrolysates. They could find practical use as functional additives to food products.

MATERIALS AND METHODS

Materials. The starting material for the investigation consisted of defatted soy flour Nutrisoy 7 B (ADM, Decatur, IL, USA), with total protein content of 50.6% (N x 6.25). It was subjected to extrusion in an industry-standard twin-screw extruder equipped with screws in a high shear configuration. The obtained extrudate of soy flour (ExSF) was dried by the air-blast method at room temperature, and ground to pass a 0.2 mm screen.

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TABLE 1. The coded and uncoded levels of independent variables of proteolysis in the response surface design.

Combination	E:S ratio level		Time level	
	Coded	Uncoded (mAU/g)	Coded	Uncoded (min)
1	$-\sqrt{2}$	8	$-\sqrt{2}$	30
2	0	28	$-\sqrt{2}$	30
3	$\sqrt{2}$	48	$-\sqrt{2}$	30
4	-1	13.9	-1	65
5	1	42.1	-1	65
6	$-\sqrt{2}$	8	0	150
7	0	28	0	150
8	$\sqrt{2}$	48	0	150
9	-1	13.9	1	235
10	1	42.1	1	235
11	$-\sqrt{2}$	8	$\sqrt{2}$	270
12	0	28	$\sqrt{2}$	270
13	$\sqrt{2}$	48	$\sqrt{2}$	270

Food-grade alkaline bacterial protease Alkalase of *Bacillus licheniformis* (Novo Nordisk A/S) with an activity of 2.4 Anson Units/g was used for hydrolysis.

Determination of optimal conditions for hydrolysis. The extruded soy flour (ExSF) was mixed with 0.1 mol/L borate buffer, then the enzyme was added and the mixture was hydrolysed at a specific temperature in a water bath with constant agitation. Every 15 min, pH was adjusted using 4 mol/L NaOH. After a set time of proteolysis, an aliquot was taken for amino nitrogen assay, and the remaining mixture was heated to 90°C for 10 min in order to inactivate the enzyme, after which it was freeze-dried. The following parameters were varied in the hydrolysis process: pH at 0.2-0.4 intervals from 6.6 to 9.0, temperature at 5°C intervals from 35 to 70°C, and water added to the raw material in a ratio of 11.5, 13.3, 15.7, 19.0, 24.0, and 32.3 g H_2O/g protein. After an initial optimisation of pH, temperature and water addition, a response surface design was used with enzyme–substrate (E:S) ratio and time as independent variables, and with amino nitrogen, solubility, water holding capacity (WHC), emulsifying activity index (EAI), emulsion stability index (ESI), foaming capacity (FC) and back extrusion maximum force (BEMF) as dependent variables. A total of 13 various combinations of E:S ratio and proteolysis time were used (Table 1), nine according to the principles of a second order central composite design [Cochran & Cox, 1957] and four additional ones (combinations 1, 3, 11 and 13) with extreme E:S ratio and time. The model was fitted to each set data as follows:

$$Z_i = b_o + b_1 X + b_2 Y + b_3 X^2 + b_4 Y^2 + b_5 XY$$

where Z_i - a particular dependent variable; b_o – b_5, estimated regression parameters (b_o - constant, b_1, b_2 - parameters for linear terms, b_3, b_4 - parameters for quadratic terms, b_5 - parameter for interaction term); X - E:S ratio (mAU/g protein); Y - time (min).

Multiple regression analysis was completed for each model, and the variance was partitioned into linear, quadratic and interaction components in order to assess the relative significance of these components. The significance of the equation parameters for each dependent variable was assessed using t-test. Coefficients of determination (R^2) were also calculated. To evaluate the relationships between all examined characteristics, the relevant correlation coefficients at $p<0.05$ confidence level were computed. Statistical analyses and three-dimensional graphs with contour plots were generated from the regression equations over the range of variables tested using CSS Statistica package (StatSoft, Tulsa, OK., USA).

Analysis of hydrolysates. Protein was determined by the Kjeldahl method, and amino nitrogen using trinitrobenzenesulfonic acid (TNBS) [Adler-Nissen,1986].

Solubility and water holding capacity (WHC) were assessed in parallel. To this end, 2 g hydrolysate was weighed in a scaled centrifugal tube and, while stirring, deionised water was added to the total volume of 25 mL. Stirring was continued for 1 h and afterwards the tube contents were centrifuged (10 000 x g, 10 min). Next, 5 mL of the supernatant was taken, dried in vacuum (70ºC, 20 h) and the remains were weighed. The remaining supernatant was discarded and the tube was weighed together with the wet residue. WHC was expressed as the mass of water (in grams) held by 1 gram of hydrolysate, and solubility as the mass of the soluble substance released from 1g of hydrolysate.

Emulsifying properties were investigated by the turbidimetric method [Pearce & Kinsella, 1978] with slight modifications. To 20 mL of hydrolysate solution (7 g total protein/litre) in 0.1 mol/L phosphate buffer with pH 7.0, 6.7 mL soy oil was added and the mixture was homogenised for 1 min in a Waring blender at a maximum speed. Immediately after homogenisation and after 5 min, portions of 50 µL emulsion were taken, diluted with 10 mL of SDS solution (10 g/L), and at a wavelength of 500 nm turbidance values A_o and A_5, respectively, were measured on a Cecil Super Aquarius (UK) spectrophotometer in respect to the SDS solution used for diluting emulsion. Emulsifying activity index (EAI) was expressed, according to Pearce & Kinsella [1978] as the area of oil/water interface (m^2) which, under the given experimental conditions, can be formed by 1 g protein of the product, and the Emulsifying Stability Index (ESI) was calculated according to Qi *et al.* [1997] from the formula: $ESI = A_0 \cdot 5_{min} / (A_0 - A_5)$.

Foaming properties were determined by passing argon at a rate of V = 5.53 cm^3/s through 100 mL of 36 g/L water suspension of the hydrolysate and measuring the time t(s) required to reach the 1000 mL level by the produced foam. Immediately afterwards, the remaining suspension was drained off with a syringe and its volume v_1 (mL) was measured. The draining was repeated after 5 min, when a part of the foam was destabilised and released a volume v_2 (mL) of liquid. Two parameters of the foam were calculated:

(i) foaming capacity FC as the ratio (in percent) of the foam volume and the volume of gas used to produce it [Sorgentini *et al.*, 1995], $FC = (1000 - v_1) \cdot 100\% / V \cdot t$.

TABLE 2. Parameters for the second order polynomial equations developed for the effects of E:S ratio (mAU/g) (b_1, b_3) and time (min) (b_2, b_4) as well as interactions between them (b_5) on amino nitrogen release and functional properties of hydrolysates obtained from ExSF with Alcalase.

Dependent variable	Equation parameters						R^2 (%)
	constant	linear		quadratic		interactive	
	b_0	b_1	b_2	b_3	b_4	b_5	
Amino ntrogen (mmol/g)	4.367×10^{-3}	$3.581 \times 10^{-2**}$	$5.473 \times 10^{-3**}$	$-4.01 \times 10^{-4*}$	$-1.1 \times 10^{-5*}$	3.5×10^{-5}	96.1
Solubility (g/g)	3.990×10^{-1}	$7.302 \times 10^{-3**}$	$1.486 \times 10^{-3***}$	$-8.50 \times 10^{-5*}$	$-3.0 \times 10^{-6**}$	-1.0×10^{-6}	97.7
WHC (gH_2O/g)	2.280	$-2.756 \times 10^{-2**}$	1.574×10^{-3}	$3.20 \times 10^{-4*}$	$-6.0 \times 10^{-6*}$	-1.3×10^{-5}	95.3
EAI (m^2)	4.508×10^{-1}	$-8.356 \times 10^{-3*}$	$-9.77 \times 10^{-4*}$	6.90×10^{-5}	1.0×10^{-6}	$1.4 \times 10^{-5*}$	90.3
ESI (min)	8.138	-4.634×10^{-3}	$-2.081 \times 10^{-2***}$	5.72×10^{-4}	$8.8 \times 10^{-5***}$	$-1.7 \times 10^{-4*}$	88.7
FC (%)	1.014×10^{2}	$-7.793 \times 10^{-2*}$	-9.435×10^{-3}	8.40×10^{-4}	$3.7 \times 10^{-5*}$	$-3.97 \times 10^{-4***}$	97.9
BEMF (N)	8.051×10^{1}	$-7.901 \times 10^{-3*}$	$6.050 \times 10^{-4*}$	1.30×10^{-5}	$-3.0 \times 10^{-6*}$	$1.2 \times 10^{-6*}$	95.1

*, **, *** - effect significant at $p<0.05$, $p<0.01$ and $p<0.001$, respectively

(ii) foam stability as liquid drainage (LD_5), which is an index expressing in per cent the ratio of the amount of liquid released from the foam during 5 min since its formation and the amount of liquid contained in the foam immediately after finishing the aeration [Waniska & Kinsella, 1979; Johnson & Brekke, 1983], $LD_5 = v_2 \cdot 100\% / (100 - v_1)$.

Rheological properties were determined by the back extrusion test using a TA XT2 texture analyser (Stable Micro Systems, Surrey, UK) [Bourne, 2002; Surówka *et al.*, 2003]. To this end, in a cylindrical vessel (d=26 mm, h=100 mm), 27 mL of the previously prepared 138 g/L water suspension of the hydrolysate was placed, into which a cylindircal plunger made of stainless steel (d=25 mm, h=35 mm) was immersed. The plunger speed was 5 m/s. Values of the maximum force (N) of the back extrusion (BEMF) were recorded.

All determinations were performed in triplicate and the mean values were reported.

RESULTS AND DISCUSSION

The optimal pH for Alcalase activity on extruded soy flour, falls in the range given by the producer and amounts to 8.3. It was found that the intensity of hydrolysis at this pH reaches the maximum at 60°C. Greater amounts of water in the reaction mixture are usually conductive, by increasing its homogeneity and dilution of the hydrolysis products, to the increase of the proteolysis rate [Surówka & Fik, 1994]. However, when the substrate is insoluble, as in the case of extrudates, then a smaller addition of water leads to a better contact between the protein and the enzyme. Presumably this is why, in the present studies, a more intensive proteolysis was observed in more concentrated suspensions. In view of this, a technologically sound water addition of *ca*. 13 g/g protein was assumed.

The addition of enzyme is a decisive factor affecting the hydrolysis rate. Its magnitude depends, among others, on the kind of hydrolysed protein. Since the raw material for hydrolysis (ExSF) was previously subjected to extrusion, it may be assumed that its structure, consisting of denatured protein, is stabilised – besides hydrogen bonds, electrostatic and hydrophobic interactions – also by disulphide and other covalent bonds [Simonsky & Stanley, 1982; Ledward & Mitchell, 1988; Prudencio-Ferreira & Areas, 1993]. As shown by the investigations of Marsman *et al.* [1997], such soy protein, denatured as a result of extrusion, easily undergoes enzymic hydrolysis. Consequently, it would not require adding substantial amounts of enzyme. The magnitude of these additions and time of proteolysis, at pH, temperature and water addition mentioned previously, were optimised using response surface methodology. Selected functional properties of the hydrolysates were taken as optimisation criterion. Mean values of particular experimental data, response surfaces and contour plots showing the effect of independent variables on the amino nitrogen content as well as on the functional properties analysed are shown in Figures 1 and 2, respectively. The surfaces presented are a graphic representation of second order equations fitted to each set of data (Table 2). All equations except for ESI, had coefficients of determination (R^2) higher than 90% (Table 2). As it can be seen, increasing of the E:S ratio and time produced an increase in the amino nitrogen content in the hydrolysates (Figure 1). The shape of the response surface, and data given

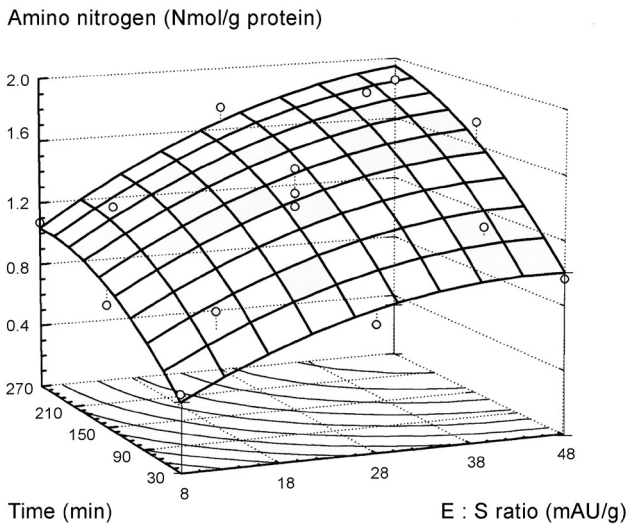

FIGURE 1. Contour plot and three dimensional response surface showing the effect of enzyme:substrate ratio and time of proteolysis on amino nitrogen release. Conditions of proteolysis: pH, 8.3; temperature, 60°C; water added to the raw material, 13 g/g protein.

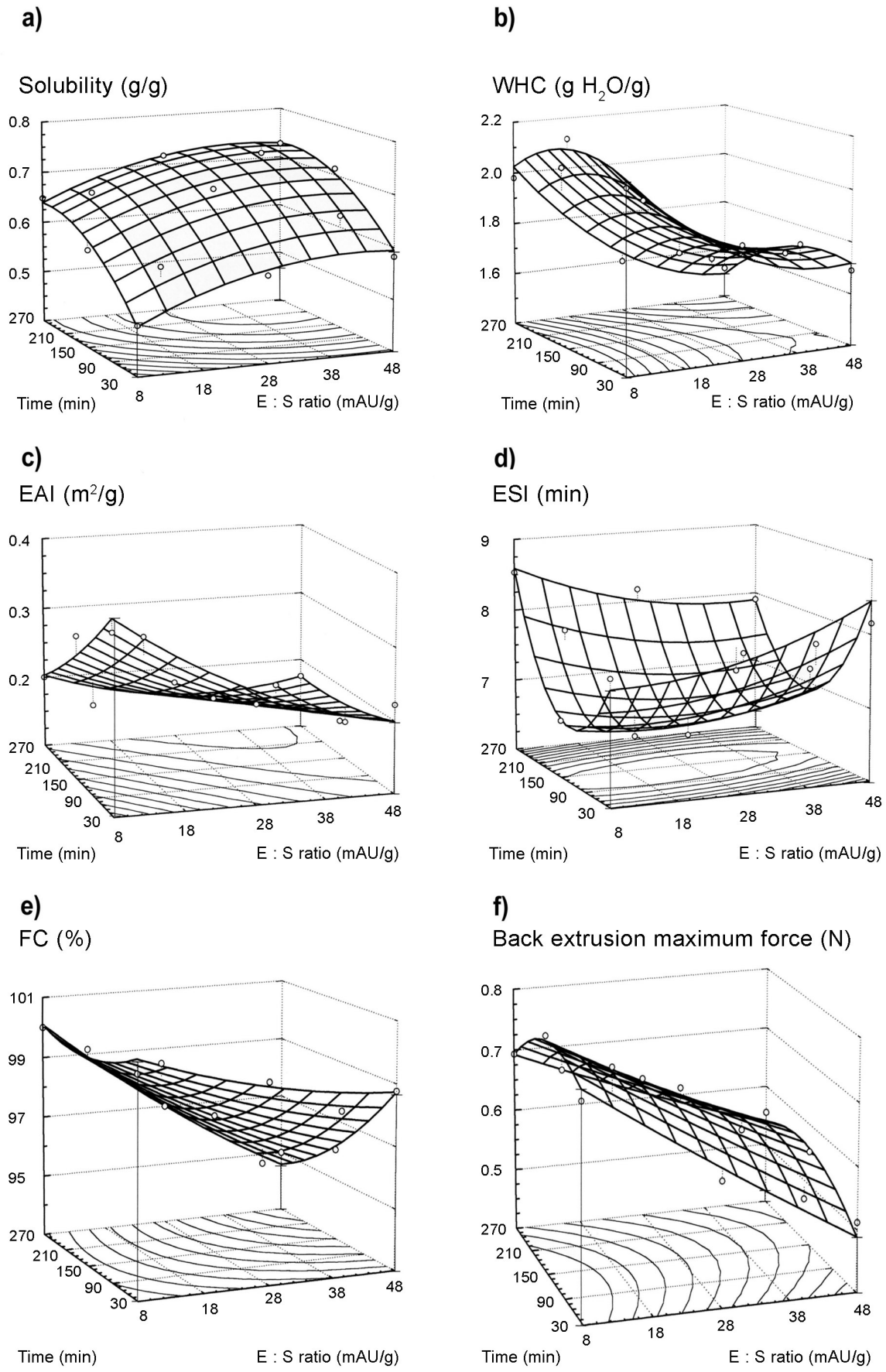

FIGURE 2. Contour plots and three dimensional response surfaces showing the effect of enzyme:substrate ratio and time of proteolysis on solubility (a), water holding capacity – WHC (b), emulsifying activity index – EAI (c), emulsion stability index – ESI (d), foaming capacity – FC (e), and back extrusion maximum force – BEMF (f) of hydrolysates. Conditions of hydrolysis as in Figure 1.

TABLE 3. Correlation coefficients ($p<0.05$) between amino nitrogen content and functional properties of hydrolysates from ExSF obtained with Alcalase.

	Amino nitrogen	Solubility	WHC	EAI	ESI	FC
BEMF	-0.58	ns	0.82	0.77	ns	0.67
FC	-0.94	-0.85	0.87	0.77	ns	
ESI	ns	ns	ns	ns		
EAI	-0.86	-0.87	0.84			
WHC	-0.85	-0.77				
Solubility	0.97					

ns – not significant

in Table 2 indicate that its increment had a positive linear and negative quadratic character.

As a rule, protein hydrolysates have high solubility indices [Frokjaer, 1994]. Also the hydrolysates obtained in this work revealed good solubility. Even a 30-min proteolysis with an E:S ratio of 8 mAU/g protein, led to nearly 80%, enhancement of this feature as compared to the value of 0.262 g/g characteristic for non hydrolysed extrudate (ExSF). Solubility of hydrolysates continued to grow with the progress of hydrolysis. This is illustrated in Figure 2a. The shape of the response surface shown here is determined, as with amino nitrogen content, by positive linear and negative quadratic effects in relation to both independent variables (Table 2), and high correlations were recorded between the amount of released $N\text{-}NH_2$ and solubility (Table 3).

Hydration properties of soy protein products depend on the protein content and the degree of its denaturation [Wagner & Añón, 1990; Sorgentini *et al.*, 1995]. Also, of some importance is the content of low molecular weight components such as oligosacharides and phytates [Surówka, 1997]. It can be supposed that peptides formed during proteolysis of proteins may also produce a decrease in WHC. The reason for this may be, partly the damage of the protein matrix and, partly the growth of solubility. This was confirmed in the presented studies, where WHC of hydrolysates was lower than that of the raw material prior to hydrolysis (2.29 g H_2O/g). Prolongation of the process expressed less pronounced influence than employing an increased enzyme addition on diminishing of this parameter. Response surface and contour plot illustrating variations of WHC as a result of proteolysis parameters are shown in Figure 2b. Particularly manifest is the negative linear and positive quadratic effect of the E:S ratio, and predominance of the negative quadratic effect of time which can be observed for higher values of this independent variable (Table 2). In the presented study, negative correlations between WHC and amino nitrogen level as well as solubility were found (Table 3).

According to the data found in the literature [Arai & Fujimaki, 1991] proteolysis can produce an enhancement of the emulsifying properties, especially emulsification capacities, although it is more difficult to maintain an appropriate stability of the emulsion. The results of the presented investigations on extruded material confirm this tendency. Namely, it turns out that, as a result of its 30-min hydrolysis using the smallest enzyme concentrations among those employed in the presented work, an increase in the emulsifying activity index (EAI) is observed, above the value of 0.223 m^2/g, which is characteristic for ExSF. However, further increasing of the Alcalase to protein ratio in the reaction mixture and time of the process leads to a decrease in this parameter (Figure 2c), which is connected with the statistically significant negative linear and positive interactive effects of these two independent variables (Table 2).

Stability of emulsions obtained from hydrolysates was slightly lower than that of emulsions obtained from non-hydrolysed extruded material (ExSF), for which ESI was equal to 9.44. The effect of E:S ratio was less pronounced than the time of proteolysis. The less stable emulsions were formed from hydrolysates obtained at medium time (Figure 2d), and the shape of the response surface is determined mainly by high statistically significant negative linear and positive quadratic effect of time, as well as negative interaction of time and Alcalase:protein ratio (Table 2). It follows from the observations made in the experiment that although enzymatic proteolysis of the ExSF diminishes the stability of obtained emulsions, this stability drop can be controlled by properly selected parameters of the process.

Modification through Alcalase resulted in hydrolysates with better foaming capacity (FC) than that observed for unmodified extrudate, for which this parameter was found to be 91.1%. The greatest improvement in FC, which was close to 100%, took place in hydrolysates obtained under such conditions, when the E:S ratio, had the smallest values among those employed in the experiment (Figure 2e). A marked degradation of this feature occurred with the increasing intensity of proteolysis resulting from simultaneous prolongation of its time and increase in the enzyme concentration. This finds a confirmation in the statistically highly significant negative interactive effect of E:S ratio with time (Table 2). This trend is also well reflected by negative correlation coefficients between the amino nitrogen level and FC (Table 3).

The percentage of liquid drained from the foam (LD_5), which is inversely related to foam stability, for unhydrolysed extrudate was equal to 90.9%, and expressed a drop by *ca*. 40% as a result of proteolysis conducted at minimal values

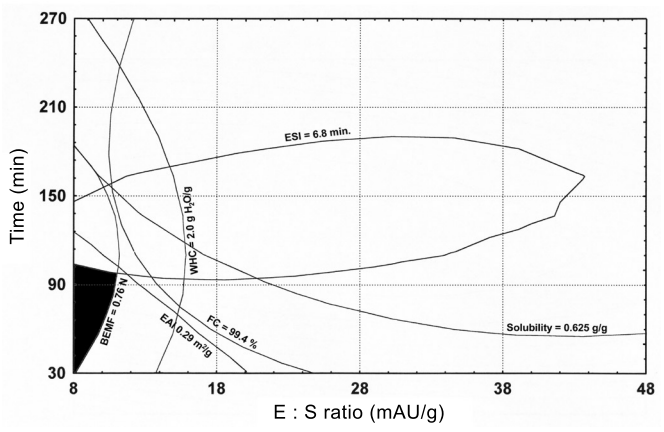

FIGURE 3. Superimposed contour plots for the response variables showing the region of optimal conditions for the proteolysis of ExSF with Alcalase. Abbreviations as in Figure 2.

of variable process parameters employed herein. Further progress of hydrolysis led to an increase in LD_5 (response surface not shown) and too deep hydrolysis resulted in almost complete loss of foam stability. Observations made during the investigations point to a conclusion that limited proteolysis of ExSF, with Alcalase employed in this work, leads to the release of peptides which contribute to the obtaining of foam with relatively good stability and in good yield. Excessive proteolysis causes, on the other hand, the formation of peptides, which are unable to satisfy requirements necessary for good foamability. Also other authors [Turner, 1969; Gunther, 1972] demonstrated that enzymatic proteolysis has advantageous effect on foaming properties of soy protein. Recently, Hračkova *et al.* [2002] observed that foaming ability of soy flour improved in hydrolysis in the presence of Alcalase, Flavourzyme and Novozyme, but foam stability decreased.

The back extrusion maximum force (BEMF) of unhydrolysed ExSF suspension amounted to 0.251 N. A substantial growth of this feature was observed as a result of proteolysis, however, as presented in Figure 2f, a further increase in E:S ratio has a pronounced negative effect. The shape of response surface is also determined by positive linear and negative quadratic effect of time (Table 2). Relevant literature indicates that even limited enzyme treatment reduced the viscosity of protein solutions [Arai & Fujimaki, 1991]. According to Urbański *et al.* [1982] viscosity of protein preparations is well correlated with WHC. Also the results of the present work indicate good correlation between these parameters ($r=0.82$; $p<0.05$).

Since the optimum response for each variable did not fall in exactly the same region of independent variables plane, constraints were set such that the selected time and E:S ratio would be kept within acceptable ranges of numerical values for each functional parameter of the products. Acceptable values for hydrolysates obtained from ExSF with Alcalase were set as follows: solubility 0.625 g/g, WHC 2.0 g H_2O/g, EAI 0.29 m^2/g, ESI 6.8 min., FC 99.4 %, and BEMF 0.76 N. The selected optimum conditions for the process will be based on the region which will satisfy the stated constraints. Superimposing the individual contour plots for the response variables results in the identification of a region which satisfied all constraints as shown in Figure 3. From this region, the E:S ratio of 8 mAU/g and time of 90 min can be proposed as optimal ones. The use of these parameters, when employing previously selected values of pH 8.3, a temperature of 60°C and water addition to the raw material 13 g/g protein, will allow one to obtain a hydrolysate with optimally modified functional properties.

CONCLUSIONS

Limited proteolysis of extruded soy flour, conducted with Alcalase, which can be achieved by employing parameters established in the presented work, will ensure obtaining of products which partly preserve the water holding capacity (WHC), while showing enhanced solubility and viscosity, good foaming properties and features conducive to the formation of emulsions. These products combine typical qualities of extruded material and those of protein hydrolysate, and hence they may find a potential application as novel functional ingredients, components of dietetic foods and fortifiers enriching concentrated soups and drinks in protein, as well as components of seasoning mixtures. Like other plant protein products, they may be also used in confectionery, and bread-making industries, as well as in meat processing industry.

ACKNOWLEDGEMENTS

The authors wish to thank the Novo Nordisk A/S, Denmark, for supplying Alcalase. This research was carried out within the project financed by the Polish State Committee for Scientific Research (Project No PBZ-KBN/021/P06/99/01).

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