Enzymatic Hydrolysis of Defatted Soy Flour by Three Different Proteases and their Effect on the Functional Properties of Resulting Protein Hydrolysates

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Abstract

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Commercial defatted soy flour (DSF) was dispersed in distilled water at pH 7 to prepare 5% aqueous dispersion. Soy protein hydrolysates (SPH) were obtained by enzymatic hydrolysis of the DSF using three different proteases (Flavourzyme 1000 L, Novozym FM 2.0 L and Alcalase 2.4 L FG). The highest degree of hydrolysis (DH 39.5) was observed in the presence of protease Flavourzyme. SPH were used for measuring functional properties (foaming stability, gelation). Treatment with Flavourzyme improved foaming of proteins of DSF. Foaming stability was low in the presence of Novozym. Proteases treated DSF showed good gelation properties, mainly in the case of treatment with Flavourzyme. SDS-PAGE analysis showed that after enzyme addition to the 5% aqueous dispersion of DSF each enzyme degraded both β-conglycinin and glycinin. In general, the basic polypeptide from glycinin showed the highest resistance to proteolytic activity. The most abundant free amino acids in the hydrolysates were histidine (30%), leucine (24%) and tyrosine (19%) in the case of the treatment with proteases Alcalase and Novozym, and arginine (22.1%), leucine (10.6%) and phenylalanine (12.9%) in the case of the treatment with Flavourzyme.

Keywords: defatted soy flour; proteases; enzymatic hydrolysis; functional properties; foam stability; gelation; protein; SDS-PAGE

Proteins are essential food components because they are a source of amino acids needed for growth and maintenance and provide functional properties to foods. Commercially available protein foods are obtained from a range of animal and plant sources and are used as functional ingredients (PERIAGO *et al*. 1998). Due to the increasing costs and limited supplies of animal proteins, and since vegetable protein is the most abundant source of protein on the Earth, a number of vegetable proteins such as alfalfa leaf, cottonseed, winged bean, peanut and soya have been investigated for possible incorporation into formulated foods (ACHOURI *et al*. 1999; SZE-TAO & SATHE 2000).

Functionality, applied to food ingredients, is defined as any property on which the utility of those foods depends (CEPEDA *et al*. 1998). For special foods, such as those destined for children, old people or athletes, food proteins are hydrolysed. In general, food proteins are hydrolysed for many reasons ranging from the improvement of nutritional and functional properties, texture characteristics to the removal of odour, flavour, and toxic or antinutritive components. The most commonly used proteins in hydrolysis treatments are casein, whey and soy protein (PERIAGO *et al*. 1998).

Soybean is an important crop because its seed contains high concentrations of protein and oil (LIANG 1999). As a protein source for nonruminants, the amino acid profile of soybean meal could benefit from higher amounts of S-containing amino acids, methionine and cysteine. About 70% of the protein in soybean seed is present as storage proteins (SP) glycinin and β-conglycinin, which are also called 11S and 7S proteins, respectively. Glycinin accounts for about 60% of SP and β-conglycinin for the remaining 40%. Glycinin is composed of five subunits whose concentration of S-amino acids ranges from 3 to 4.5%. In contrast, β-conglycinin, which is composed of three subunits (α´ [76 kDa], α [72 kDa], β [53 kDa]), contains less than 1% of S-containing amino acids. The

mature β-subunit of β-conglycinin contains only one cysteine residue and no methionine among about 470 amino acids (SEXTON *et al*. 1998; MARSMAN *et al*. 1997; SHIMOYAMA *et al*. 1998; HETTIARACHCHY & KALAPATHY 1998).

Compared to acid or alkali hydrolysis, enzymatic hydrolysis of protein, using selective proteases, provides more moderate conditions of the process and few or no undesirable side reactions or products. In addition, the final hydrolysate after neutralization contains less salts and the functionality of the final product can be controlled by selection of specific enzymes and reaction factors (CHIANG *et al*. 1999; MADSEN *et al*. 1997; DARWICZ *et al*. 2000). Enzymatic modification of proteins using selected proteases to split specific peptide bonds is widely used. The peptides produced have a smaller molecular size than proteins. Thus, their functional properties are different: increased solubility over pH range, decreased viscosity, and significant changes in foaming, gelling and emulsifying properties. The functional properties of hydrolysed proteins are governed to a large extent by their molecular size and their hydrophobia (TURGEON *et al*. 1992).

Soy protein hydrolysates have found applications in specialized adult nutritional formulas, usually in combination with other protein hydrolysates or intact proteins (MAHNOUD 1994). Enzyme modifications of soy proteins could offer the second or third generation of products that might allow an even broader range of utilization. Actually, in one area this is already achieved by using a pepsin-digested soy protein product to make a whipping protein for egg white replacement. However, there has been little published information on any systematic study of the effect of enzyme treatment on the various functional properties of soy proteins, such as emulsification, gelling properties and foaming. Perhaps one reason is that there are no well-accepted methods available to measure some of these functional properties and, it is also difficult to correlate the results of measurements in a model system with what happens in a complex food system (PUSKI 1975).

In this study, soy flour protein was hydrolysed with three different proteases, and an attempt was made to measure the change in some functional properties. The changes in chemical properties were also determined.

MATERIALS AND METHODS

Materials: Commercial defatted soy flour HP (48 g protein per 100 g of flour) was obtained from Raiffeisengenossenschaft OSTTIROL, Lienz (Austria). The enzymes used were Flavourzyme 1000 L MG, Alcalase 2.4 L and Novozym FM 2.0 L (Novo Nordisk, Bagsvaerd, Denmark). Alcalase 2.4 L is an endopeptidase from *Bacillus licheniformis*, with subtilisin Carlsberg as a major enzymic component, having the specific activity of 2.4 Anson Units (AU) per gram. One AU is the amount of enzyme that under standard conditions digests haemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product which gives the same colour with the Folin reagent as one milliequivalent of tyrosine released per minute. Flavourzyme 1000 MG is an exopeptidase and endoprotease complex with an activity of 1 LAPU/g. One LAPU (Leucine aminopeptidase unit) is the amount of enzyme that hydrolyses 1 μmol of leucine-*p*-nitroanilide per minute. Novozym FM 2.0 L is a proteolytic enzyme prepared by submerged fermentation of a selected strain of *Bacillus licheniformis*. The enzyme component in Novozym FM 2.0 L is subtilisin A (= subtilisin Carlsberg), which is endoproteinase of the serine type. Its declared activity is 2.0 AU/g.

Proteolytic activity of enzyme: The proteolytic activity of proteases was determined using bovine casein (1% solution, pH = 8) as a substrate according to BERGMAYER (1974). The substrate solution (1 ml) was incubated at 50°C for 5 min, and 0.2 ml of enzyme solution and 0.8 ml of phosphate buffer were added (pH = 8). The mixture was incubated at 50°C for 20 min, 3 ml of 5% aqueous trichloroacetic acid (TCA) solution was added, and the resulting precipitate was removed by centrifugation (3000g per 10 min). Enzyme activity was measured as the amount of solubilized protein in 5% trichloroacetic acid and the free amino acids released after hydrolysis, both quantified spectrophotometrically using Lowry's technique at 620 nm (LOWRY *et al*. 1951) and ninhydrin method at 570 nm (AWOLUMATE 1983) with slight modification, respectively (Table 1).

Table 1. Proteolytic activity (PA) of the enzymes determined by a laboratory method according to BERGMAYER (1974)

Enzyme	PA_a (nkat/ml)	PA_L (nkat/ml)
Alcalase	150.3	22.4
Flavourzyme	496.1	53.9
Novozym	155.9	18.9

a – using ninhydrin reagent
L – using Lowry's technique

Enzymatic hydrolysis: Prior to enzymatic treatment a suspension of soy flour was prepared by adding 450 ml of distilled water to 22.5 g of flour. The suspension was adjusted to pH 7, preheated to 40°C, placed in water bath (40°C) and the enzyme was added. The enzymatic hydrolysis was conducted at 40°C for 8 hrs. Aliquots (75 ml) were taken after 10, 30, 60, 120, 240 and 480 min. The enzymes were added in such amounts to have equal proteolytic activity. Then the mixtures were heated at 85°C for 15 min to inactivate the enzyme.

Chemical analysis: Concentrations of peptides were determined according to LOWRY *et al*. (1951) with BSA

as a standard. Trichloroacetic acid (3 ml, 5%) and phosphate buffer (1 ml, pH 8) were added to the hydrolysate mixture (1 ml) and after 15 min at room temperature the precipitate was removed by centrifugation at 3000 g per 15 min. The precipitate was solubilised in 5 ml of 10% NaOH and used to determine the amount of protein. The supernatant was directly used to determine the amount of liberated proteolytic products by the Folin reagent and expressed as milligrams of tyrosine per ml. The amounts of liberated amino acids were determined with ninhydrin reagent.

Degree of hydrolysis (DH): Degree of hydrolysis was determined as the ratio of released amino acids in supernatant using the ninhydrin reagent to the amount of total amino acids obtained after acid hydrolysis of the protein:

$$DH\ (\%) = \frac{h}{h_{tot}} \times 100$$

Amino acid analysis: 0.2 mg of DSF were hydrolysed with 6N HCl at 110°C for 24 h (GERKE *et al.* 1985). Amino acid distribution of DSF and its hydrolysates were determined with an automatic amino acid analyzer AAA 339 (Mikrotechna, Praha). The separation was carried out using an ion exchanger OSTION LG ANB according to the modified ion exchange resin method of SPECKMAN *et al*. (1958). The analysis of amino acid mixture was carried out in the presence of sodium citrate buffers; pH 3.25; 4.25 and pH 9.5 were used for amino acid analysis at temperatures 50 and 60°C.

SDS-PAGE: SDS-PAGE was performed on 10% gel slabs that were prepared and run with sodium dodecyl sulphate using the discontinuous buffer system of LAEMMLI (1970). Protein standards were obtained from Sigma Chemicals (St. Louis, MO) and from Boehringer Mannheim GmbH (Mannheim, Germany) to identify degraded to breakdown products protein molecules (molecular masses from 20.0 to 97.4 kDa). The samples were dissolved in the sample buffer that consisted of 10mM Tris-HCl, pH 8.0, 1mM EDTA, 1% SDS, 5% β-mercaptoethanol, 15% glycerin, and bromphenol blue.

Functional properties: Foam stability (FS) was assessed using the method of HORIUCHI *et al.* (1978). Fifteen millilitres of the sample (obtained after enzymatic hydrolysis and heated at 85°C) were measured out into a 50 ml cylinder with a stopper and shaken horizontally for 30 s (15 cm amplitude of shaking). Shaking was repeated three times successively. The volume of foam was measured. The resultant solution was allowed to stand until the foam volume decreased to a half of its initial level and the foam subsidence velocity (mm/min) was determined from the time of subsidence of a half of the foam volume. Next, the time of subsidence of 100 mm of the foam layer was calculated from this foam subsidence velocity.

Gelation was determined by the method of OBATOLU & COLE (2000). Sample dispersions (obtained after enzymatic hydrolysis and heating at 85°C) of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20% (v/v) were prepared in 5 ml distilled water. The test tubes containing the suspensions were heated for 1 h in a boiling water bath followed by rapid cooling under cold running tap water. The test tubes were cooled for 2 h at 40°C. The least gelation concentration was determined when the sample from the inverted test tube did not fall or slip.

RESULTS AND DISCUSSION

The free amino acids/free tyrosine ratio (AA/Tyr) expresses the amount of released amino acids during hydrolysis per unit of tyrosine and it indicates the tyrosine content in amino acids. Different values of the AA/Tyr ratio of different proteases showed protease specificity related to hydrolysis of the bonds involving tyrosine (Table 2). During soy flour hydrolysis, this ratio was dependent on hydrolysis time. The treatment of soy flour with Alcalase showed the highest increase in amino acids during the first 120 min of hydrolysis (Fig. 1). Later the amount of released amino acids increased moderately. It can been seen in hydrolysis in the presence of Fla-

Table 2. Degree of hydrolysis of 5% dispersion of defatted soy flour at 40°C and pH = 8 with different proteases

Protease	Hydrolysis time (min)						DH_{AA} (%)
	10	30	60	120	240	480	
	AA/Tyr						
Alcalase	4.0	4.0	4.0	4.8	5.3	4.3	35.1
Flavourzyme	3.7	4.3	5.8	6.1	7.9	8.0	39.5
Novozym	3.2	3.4	3.0	4.0	4.5	5.1	33.3

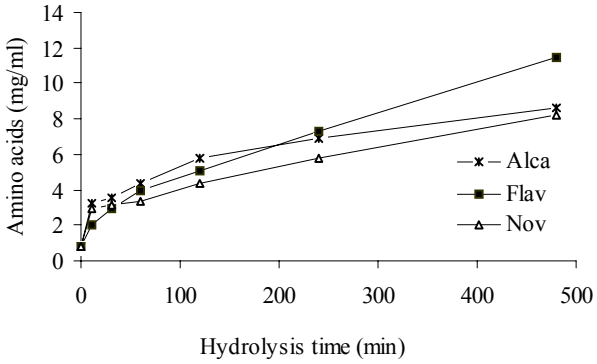

Fig. 1. The amount of relased amino acids during hydrolysis of 450 ml 5% solution of SDF in the presence of three different proteases

vourzyme that an increase in amino acids is more significant than in the case of Alcalase or Novozym. Somewhat ambiguous behaviour was observed in the initial stage of soy flour hydrolysis in the presence of Novozym. From the beginning it released more amino acids, however after 10 min of hydrolysis a decrease in amino acids content was recorded. Later the amount of amino acids still increased but not more than in the case of Flavourzyme treatment of DSF.

The amounts of tyrosine released by Alcalase, Flavourzyme and Novozym are given in Fig. 2. It can be seen that in all protease treatments the amount of released tyrosine increased. A significant increase was recorded during hydrolysis in the presence of Flavourzyme.

The degree of hydrolysis (DH) obtained for the hydrolysates produced by three different proteases after 8 hours of DSF hydrolysis is presented in Table 2. The highest degree of hydrolysis was attained in the presence of Flavourzyme.

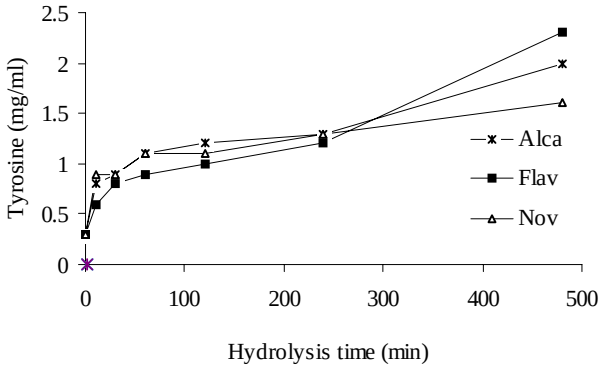

Fig. 2. The amount of tyrosine during hydrolysis of 450 ml 5% solution of SDF in the presence of three different proteases

Foaming properties of protein from unhydrolysed and hydrolysed soy flour are represented in Figs. 3 and 4. Soy proteins were suggested to have poor foaming properties due to their large, compact structure (GERMAN *et al.* 1985). Enzyme treatment increased the height of foam (HF), but foam stability was markedly decreased in each instance. The foem stability of the unhydrolysed protein of DSF was 148 min/100 mm while that of the hydrolysates in the presence of Alcalase, Flavourzyme and Noozym after 8 hrs was 17.2,32.4 and 2.8 min/100 mm, respectively. The paper by TURNER (1969) indicated that to make a stable foam partially hydrolysed protein is needed to increase the foam expansion and some larger protein components are needed to stabilize the foam. The foaming ability of protein improved in hydrolysis in the presence of Alcalase but foam stability decreased. When the smaller peptides produced after hydrolysis were separated by ultrafiltration, the foam stability of permeate was improved (PANYAM & KILARA 1996). In the present study, there are no larger protein components present in the hydrolysates so that they could not stabilize the foam. Enzymatic hydrolysis of soy proteins can be used to produce a product with good whipping properties (TURNER 1969; GUNTHER 1972).

The gelation properties of hydrolysates are shown in Table 3. The gelation concentration for the unhydrolysed 5% dispersion of defatted soy flour was 6%. Hydrolysates treated with Alcalase showed better gelation properties (gel concentration was 2%) during the first 60 min of hydrolysis than the corresponding intact protein. After 60 min of hydrolysis, the hydrolysates did not form any gel. The hydrolysates treated with Flavourzyme showed better gelation properties during 240 min of hydrolysis. Not even in the case of treatment with Alcalase did the final hydrolysate form any gel. The hydrolysis in the presence of Novozym did not significantly improve

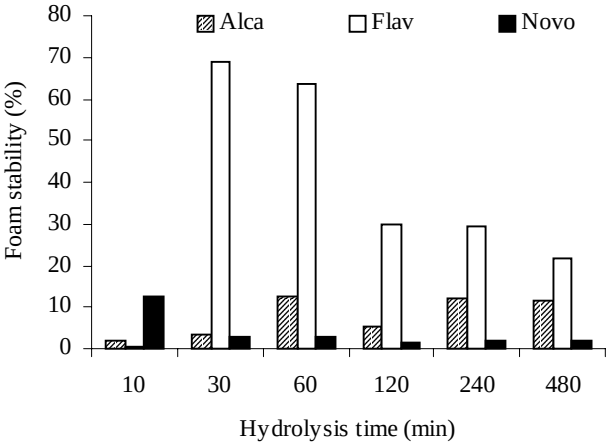

Fig. 3. Foam stability of hydrolyzed SDF

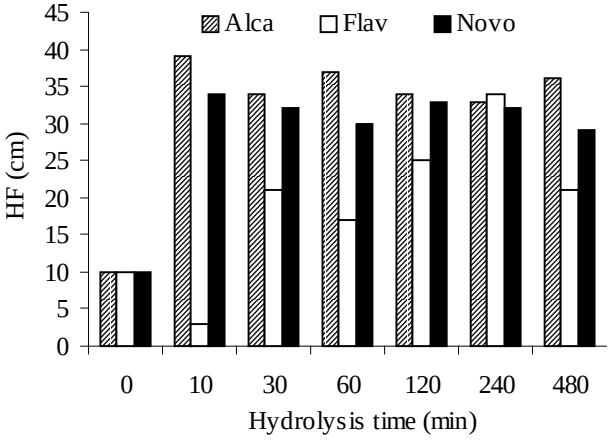

Fig. 4. Hight of foam (HF) of unhydrolyzed and hydrolyzed SDF

Table 3. Gelation properties of unhydrolysed soy flour and its hydrolysates

Hydrolysis time (min)	v/v (%) 2	4	6	8	10	12	14	16	18	20
Alcalase										
0	+	+	++	++	++	++	++	+	+	+
10	++	+	+	+	+	+	+	+	+	+
30	++	+	+	+	+	+	+	+	+	+
60	++	++	+	+	+	+	+	+	+	+
120	+	+	+	+	+	+	+	+	+	+
240	+	+	+	+	+	+	+	+	+	+
480	+	+	+	+	+	+	+	+	+	+
Flavourzyme										
0	+	+	++	++	++	++	++	+	+	+
10	++	++	++	++	++	++	++	++	+	+
30	++	++	++	+	+	+	++	++	++	++
60	++	+	+	++	++	+	+	+	++	++
120	++	++	+	+	+	+	++	++	+	+
240	++	++	++	+	+	+	+	+	+	+
480	+	+	+	+	+	+	+	+	+	+
Novozym										
0	+	+	++	++	++	++	++	+	+	+
10	+	++	++	++	++	++	++	++	++	++
30	+	+	+	+	++	++	++	+	+	+
60	+	+	+	++	++	+	+	+	+	+
120	++	++	+	+	+	+	+	+	+	+
240	–/+	–/+	+	+	+	+	+	+	+	+
480	+	+	+	+	+	+	+	+	+	+

–/+ slight turbidity of the sample
\+ the sample slipped from the inverted test tube
++ the sample did not fall from the inverted test tube

gelation except in the hydrolysates obtained after 10 and 120 min of hydrolysis (gelation concentration was 4 and 2%, respectively).

To study the effect of different enzymes on protein breakdown in defatted soy flour, SDS-PAGE was performed on the residues after enzymatic hydrolysis of DSF. The result is shown in Fig. 5, where lane 5 shows the starting material, and lanes 2–4 the results obtained after incubation with Novozym (lane 4), Flavourzyme (lane 3), and Alcalase (lane 2) for 8 h. The main attention was focused on two main storage proteins β-conglycinin and glycinin. β-conglycinin shows three components, α, α´ and β subunits, while glycinin consists of acidic (A) and basic (B) polypeptides (ROMAGNOLO *et al.* 1990; MARSMAN *et al.* 1997).

It can be seen in the residues from defatted soy flour that substantial protein breakdown could be observed for all enzyme incubations. The protein profile was drastically changed, so that it was concentrated only at the bottom end of the gel corresponding to low molecular weight. After 8 hrs of incubation with all proteases, the three subunits from β-conglycinin and A polypeptide from glycinin were fully degraded. Only a small residual amount of B polypeptide could be observed. A part of the degraded protein disappeared in a large band just below the B subunit. Both β-conglycinin and glycinin were effectively degraded by all enzymes.

In general, it can be concluded that β-conglycinin is easy to degrade, whereas glycinin showed higher resistance to the proteolytic activity. These findings are in agreement with those in other papers (KIM *et al.* 1990; MARSMAN *et al.* 1997). Within the glycinin fraction it appeared that B polypeptide was degraded more slowly than A polypeptide. The relatively high resistance of B polypeptide to the proteolytic activity can also be explained by the fact that these polypeptides have a ten-

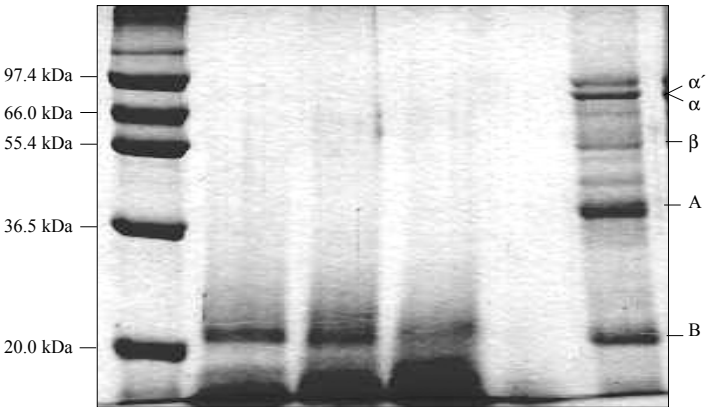

Fig. 5. SDS-PAGE of hydrolysates after reduction by 2-mercaptoethanol

The hydrolysates were obtained after hydrolysis of 5% solution of defatted soy flour (5) in the presence of proteases Novozym (4), Flavourzyme (3) and Alcalase (2) after 8 hrs. Lane 1: protein standards – phosphorylase b (97.4); glutamate dehydrogenase (55.4); bovine serum albumin (66.0); lactate dehydrogenase (36.5); trypsin inhibitor (20.0)

dency to form large insoluble complexes which make them less susceptible to enzyme hydrolysis (YAMAUCHI *et al.* 1991).

The amino acid distribution of defatted soy flour before and after enzymatic treatment is shown in Table 4. The difference in amino acid distribution was significant between hydrolysates treated with Alcalase and Novozym, and hydrolysate treated with Flavourzyme. In general, the most abundant amino acids were histidine (approx. 30%), leucine (approx. 24%) and tyrosine (approx. 19%) in the case of treatment with proteases Alcalase and Novozym, and arginine (22.1%), leucine (10.6%) and phenylalanine (12.9%) in the case of treatment with Flavourzyme. These differences are due to a different character of Flavourzyme. Flavourzyme is produced by fermentation of a selected strain of *Aspergillus oryzae* and contains both endoprotease and exopeptidase activities, so its ability to release more free amino acids is higher than in the

Table 4. Effect of enzymatic treatment on the amino acid distribution (%) of soy defatted flour in the presence of three different proteases

Amino acid	Content of amino acids in unhydrolysed soy flour	Contribution of amino acids in hydrolysates		
		Alcalase	Flavourzyme	Novozym
Essential				
His	2.7	**30.1**	8.1	**27.9**
Ile	4.1	1.5	3.98	1.3
Leu	8.1	**23.2**	**10.6**	**24.1**
Lys	7.2	–	6.4	–
Met	0.7	–	1.8	–
Cys	–	–	–	–
Phe	4.6	–	**9.2**	–
Tyr	2.8	**17.1**	8.6	**19.2**
Thr	4.0	–	3.6	–
Trp	–	–	–	–
Val	4.2	6.1	5.9	3.6
Non-essential				
Ala	4.3	1.4	3.6	1.5
Arg	**8.7**	6.1	**22.1**	8.7
Asp	**12.7**	3.2	2.8	2.9
Glu	**21**	5.4	4.5	5.9
Gly	4.3	1.8	1.3	1.7
Pro	5.3	–	–	–
Ser	5.4	–	7.4	

case of serine endoprotease Alcalase or Novozym. The results of this study show a specificity of used proteases as to released amino acids.

In general, the enzymatic treatment of defatted soy flour with three different proteases Flavourzyme, Alcalase and Novozym improved the foaming and gelling properties. It can be concluded from the results that the most effective protease was Flavourzyme.

This fact is relevant for the production of protein hydrolysates since the choice of used protease determines both the functional and nutritional properties, which is of great importance with respect to food application of protein hydrolysates.

It is clear that the enzymatic hydrolysis can be a way to improve the functional and nutritional properties of the products.

Abbreviations

DSF	defatted soy flour
SPH	soy protein hydrolysates
DH	degree of hydrolysis
SP	storage protein
AU	Anson Unit
LAPU	leucine aminopeptidase unit
TCA	trichloroacetic acid
BSA	bovine serum albumin
AA/Tyr	free amino acids:free tyrosine ratio
FS	foam stability
HF	height of foam
SDS-PAGE	electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate
EDTA	ethylenediaminetetraacetic acid

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Súhrn

HRČKOVÁ M., RUSŇÁKOVÁ M., ZEMANOVIČ J. (2002): **Enzýmová hydrolýza sójovej odtučnenej múky v prítomnosti troch rôznych proteáz a ich účinok na funkčné vlastnosti konečných proteinových hydrolyzátov**. Czech J. Food Sci., **20**: 7–14.

Z komerčnej odtučnenej sójovej múky (SjOM) sme pripravili 5% roztok rozpustením v destilovanej vode o pH 7. Hydrolyzáty sme získali enzýmovou hydrolýzou roztoku SjOM v prítomnosti troch rôznych proteáz (Flavourzyme 100 L, Novozym FM 2.0 L a Alcalase 2.4 L FG). Najvyšší stupeň hydrolýzy (DH 39,5) bol v prítomnosti proteázy Flavourzyme. Hydrolyzáty sme použili na stanovenie funkčných vlastností – stability peny a tvorby gélu. Po hydrolýze v prítomnosti Flavourzyme sa penivé vlastnosti bielkovín SjOM zlepšili. V prítomnosti Novozym bola stabilita peny nízka. Hydrolyzáty SjOM mali lepšie gélové vlastnosti, najmä v prípade Flavourzyme. SDS-PAGE analýza ukázala, že hydrolýzou 5% roztoku SjOM v prítomnosti všetkých proteáz došlo k degradácii aj β-konglycinínu aj glycinínu. Vo všeobecnosti zásaditý polypeptid glycinínu bol rezistentnejší voči proteolytickej aktivite použitých proteáz. V hydrolyzátoch použitím proteáz Alcalase a Novozym sa v najväčšom množstve vyskytovali aminokyseliny histidín (30 %), leucín (24 %) a tyrozín (19 %), v prípade proteázy Flavourzyme mali najväčšie zastúpenie arginín (22,1 %), leucín (10,6 %) a fenylalanín (12,9 %)

Kľúčové slová: odtučnená sójová múka; proteázy; enzýmová hydrolýza; funkčné vlastnosti; stabilita peny; tvorba gélu; proteín; SDS-PAGE

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