Dynamics of Evening Primrose Protein Hydrolysis*

J. GOŁĄBCZAK**, J. STRĄKOWSKA, and A. KONSTANTYNOWICZ

Institute of Technical Biochemistry, Faculty of Biotechnology and Food Sciences, Technical University of Łódź, PL-90 924 Łódź
e-mail: jgolab@mail.p.lodz.pl

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Evening primrose protein isolate prepared by proteolysis was digested with trypsin at pH 8 and 50 °C. Effects of enzyme and substrate concentrations and of digestion time on the degree of protein hydrolysis were determined. Experimental results were used to develop a mathematical model describing the path of enzymatic cleavage of evening primrose protein. According to this model, the process starts with the peptide bond hydrolysis followed either by formation of a stable enzyme-substrate complex or the peptide bond resynthesis, at the constant initial enzyme to substrate concentration ratio ($\rho(\mathrm{E})_0/\rho(\mathrm{S})_0$) and evening primrose protein concentrations of 20 to 50 g dm^{-3}. Optimum proteolysis conditions ($\rho(\mathrm{S})_0 = 20$ g dm^{-3}, $\rho(\mathrm{E})_0/\rho(\mathrm{S})_0 = 2$ %, 5 h) provided the highest degree of protein hydrolysis of 28—30 %. Enzymatic digestion enhanced the content of low relative molar mass of peptides (easily absorbable in small intestine) in evening primrose protein hydrolyzate and increased its solubility in water.

Searching for novel, nonconventional sources of food constituents, including proteins, and development of new techniques of raw material processing, providing the highest nutritional value, have been recent trends in food production. Tremendous advances in chemical, physical, and enzymatic methods of production of protein with improved nutritional properties from such new sources have been observed [1, 2].

Evening primrose cake is undoubtedly a nonconventional protein source. It is uselessly accumulated by Polish pharmaceutical industry (approximately 90 t per year). De-fatted cake contains 23—25 mass % of protein, and a protein isolate derived from the cake contains as much as 74—76 mass % of protein. Evening primrose protein is rich in sulfur amino acids, in contrast to the majority of plant proteins [3]. Therefore, it can be used as a fodder supplement, eliminating deficiency in sulfur amino acids. However, the bioavailability of evening primrose protein isolate was found to be limited due to a high content of dietary fibre [4]. The isolate was enzymatically modified by proteolysis to improve its digestibility. Partial proteolytic cleavage has been widely applied to improvement of nutritional value of proteins [5]. Enzymatic digestion carried out at optimized conditions leads to a rise in a content of peptides with low relative molar mass, M_r, provides satisfactory amino acid profile and attractive functional properties.

EXPERIMENTAL

Evening primrose protein isolate was digested with trypsin from porcine pancreas (985 U mg^{-1}, ICN Biomedicals, USA). Total nitrogen content in raw material was determined by the Kjeldahl method [6] and the protein content was calculated based on this assay ($w(\mathrm{N}) \times 6.25$). Protein hydrolysis was performed at a temperature of 50 °C for 8 h. Initial substrate concentration (the protein isolate suspended in 0.1 M borate buffer with pH = 8.0) $\rho(\mathrm{S})_0$ ranged from 15 to 50 g dm^{-3}. Initial enzyme to protein isolate concentration ratio, $\rho(\mathrm{E})_0/\rho(\mathrm{S})_0$, ranged from 0.5 to 3.3 %. On completion of protein hydrolysis, the enzyme was inactivated by incubation at 70 °C for 20 min and the reaction mixture was lyophilized. Degree of hydrolysis, DH, was calculated based on the amine nitrogen content, estimated by the Sörensen method [6].

Amino acid profiles of evening primrose protein isolate and its enzymatic digests were determined using the amino acid analyzer AAA-400 (Ingos, Czech Republic). Protein (10 mg cm^{-3}) was oxidized with peroxyformic acid at 50 °C for 10 min [7] and hydrolyzed with 6 M-HCl at 110 °C for 24 h under vacuum. Remaining protein was precipitated with 5-sulfosalicylic acid, collected by centrifugation (8000 *g*, 15 min), and discarded.

M_r of peptides contained in the protein isolate and that contained in protein digests was evaluated by

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**The author to whom the correspondence should be addressed.

Table 1. Variation of the Degree of Hydrolysis of Evening Primrose Protein Isolate with the Trypsin to Substrate Ratio

Trypsin to substrate ratio, $\rho(E)_0/\rho(S)_0$/%	Substrate concentration, $\rho(S)_0$/(g dm^{-3})	
	20	35
	Degree of hydrolysis, DH/%	
0.5	21.2	16.2
1.0	24.3	18.7
2.0	31.2	22.8
3.3	32.8	24.9

Tricine/SDS-polyacrylamide gel electrophoresis (Tris-Tricine/SDS-PAGE, 16.5 % polyacrylamide) [8]. Electrophoresis (Mini-Protean 3, Bio-Rad, USA) was conducted under reducing conditions (sample buffer supplemented with 50 g dm^{-3} dithiotreitol, pH 6.8). After electrophoresis the gels were fixed in the methanol—acetic acid—water mixture ($\varphi_r = 5 : 1 : 4$) for 0.5 h, stained with 0.025 % Coomassie brilliant blue R-250 (in 10 % acetic acid) for 2 h, and washed with 10 % acetic acid.

Solubility of the evening primrose protein isolate and its trypsin digests was assayed according to *Walsh et al.* [9]. Samples (0.5 g) were suspended in 50 cm^3 of deionized water, and the pH of each suspension was adjusted to 4.5 or 7.0, using 0.1 M-NaOH or 0.1 M-HCl, respectively. Sample suspensions were shaken for 30 min at room temperature (25 ℃) and centrifuged at 8000 *g* for 15 min. Soluble nitrogen in the supernatant was determined using the Kjeldahl procedure. Solubility was expressed as the percentage of nitrogen content in supernatant *vs.* the total nitrogen content in the protein suspension.

Mathematical model of enzymatic hydrolysis of evening primrose protein was developed. Classical Michaelis—Menten model enriched with an intermediate complex was applied for description of this process. The intermediate complex reflected reaction of peptide bond re-synthesis, which occurred concomitantly to hydrolytic cleavage of these linkages.

RESULTS AND DISCUSSION

Digestibility of the rich in protein (75 mass %) evening primrose protein isolate was improved by digestion with trypsin. To evaluate the effect of enzyme concentration on the degree of protein hydrolysis (Table 1) the process was carried out for 8 h at protein isolate concentrations of 20 g dm^{-3} and 35 g dm^{-3} and at four different mass ratios $\rho(E)_0/\rho(S)_0$ of 0.5 %, 1.0 %, 2.0 %, and 3.3 %. It was found that the increase of the $\rho(E)_0/\rho(S)_0$ value from 0.5 to 3.3 % caused a relatively weak rise of DH, from 21.2 to 32.8 %. The latter finding, *i.e.* a small change in a rate of peptide bond

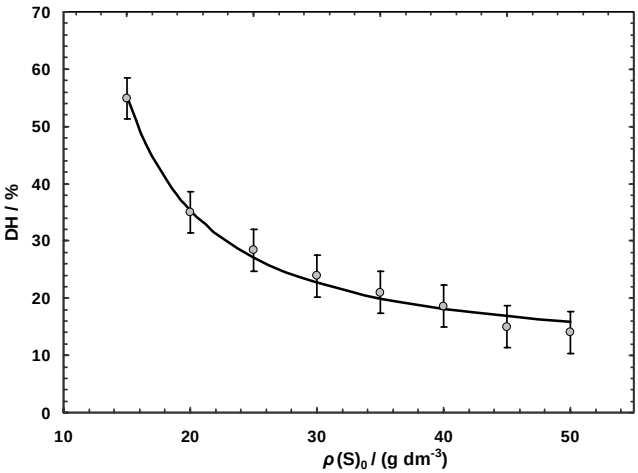

Fig. 1. Variation of the degree of hydrolysis of evening primrose protein isolate with the substrate concentration $\rho(S)_0$.

cleavage in response to a significant enhancement of the enzyme content in reaction mixture, was consistent with the results of other researchers [10] who determined the relationship between DH of soybean protein isolate (4.0 %) and amount of alcalase (subtilisin Carlsberg) applied in concentrations of 0.2—1.2 mass %.

The optimum $\rho(E)_0/\rho(S)_0$ value was found to be 2.0 %. It provided the highest degree of protein hydrolysis independently of the substrate concentration. Further increase of the ratio $\rho(E)_0/\rho(S)_0$ to 3.3 % resulted in only a minor rise of DH (by approximately 1—2 %) and was economically unfeasible.

To estimate the influence of concentration of evening primrose protein isolate on a degree of its hydrolysis, the isolate was digested with trypsin for 6 h at the $\rho(E)_0/\rho(S)_0$ value of 2 %. Substrate concentration was shown to be the crucial parameter of the process (Fig. 1). An increase of the substrate concentration, $\rho(S)_0$, from 15 g dm^{-3} to 50 g dm^{-3} caused a more than three-fold drop of DH. The relationship between $\rho(S)_0$ and DH was hyperbolic for a constant ratio of $\rho(E)_0/\rho(S)_0$. Mathematically, this could be expressed using the equation

$$\mathrm{DH} = a\left(\frac{\rho(S)_0}{\rho(S)_0 - b}\right)^c \qquad (1)$$

where a, b, and c are the model parameters.

This model was fitted to experimental data. Coefficients a, b, and c were determined by the least-squares method. For $\rho(E)_0/\rho(S)_0$ equal to 2 %, values of these coefficients were: $a = 0.09434$, $b = 0.0002347$, and $c = 1112.7$.

Mathematical model of enzymatic hydrolysis of evening primrose protein was based on experimental results. The classical Michaelis—Menten model was enriched with the intermediate complex, reflecting reactions of peptide bond re-synthesis, which occurred

Table 2. Model Parameters Identification with Residual Error of Fitting

Data set	1	2	3	4	5
$\rho(S)_0$/(g dm^{-3})	20	35	35	35	50
$\rho(E)_0/\rho(S)_0$/%	2	2	1	0.5	2
k_1/(dm^3 g^{-1} s^{-1})	672.3	2160	1800	2222	206.7
k_2/s^{-1}	0.0000	0.5800	0.0116	0.0113	0.0000
k_3/s^{-1}	0.0063	1.598	2.254	0.1504	0.0014
k_4/(dm^3 g^{-1} s^{-1})	0.0000	0.0000	0.0000	0.0000	0.0000
k_5/(dm^3 g^{-1} s^{-1})	1986	6705	7886	9718	1640
k_6/s^{-1}	0.0011	0.0000	0.0000	0.0000	0.0733
k_7/s^{-1}	0.0108	0.0010	0.0007	0.0008	200.9
k_8/(dm^3 g^{-1} s^{-1})	0.0000	0.0000	0.0000	0.0000	0.0000
rms deviation/%	1.19	0.60	0.46	0.28	0.20
Michaelis—Menten kinetics					
Hydrolysis, k_{M+}	9.39×10^{-6}	6.78×10^{-6}	1.01×10^{-3}	1.26×10^{-3}	7.28×10^{-5}
Re-synthesis, k_{M-}	5.94×10^{-6}	0.123	0.15×10^{-6}	9×10^{-8}	8×10^{-8}

concomitantly to hydrolysis of these linkages, according to the scheme

$$\mathrm{E+S} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{C1} \underset{k_4}{\overset{k_3}{\rightleftarrows}} \mathrm{P+E} \underset{k_6}{\overset{k_5}{\rightleftarrows}} \mathrm{C2} \underset{k_8}{\overset{k_7}{\rightleftarrows}} \mathrm{S+E}$$

where E represents enzyme, S substrate, C1 unstable complex formed by E and S, P is reaction product, C2 unstable complex formed by E and P, and k_1—k_8 represent corresponding reaction rate constants.

The model includes equations presenting the mass balance of substrate and enzyme for reaction carried out at a constant volume of the reaction mixture. The complete mathematical model is composed of three differential equations, reflecting the reaction dynamics in a closed system.

$$\frac{\mathrm{d}\rho(\mathrm{C1})}{\mathrm{d}t} = (\rho(\mathrm{E})_0 - \rho(\mathrm{C1}) - \rho(\mathrm{C2})) \cdot \cdot (k_1(\rho(\mathrm{S})_0 - \rho(\mathrm{C1}) - \rho(\mathrm{C2}) - \rho(\mathrm{P})) + + k_4\rho(\mathrm{P})) - (k_2 + k_3)\,\rho(\mathrm{C1}) \quad (2)$$

$$\frac{\mathrm{d}\rho(\mathrm{C2})}{\mathrm{d}t} = (\rho(\mathrm{E})_0 - \rho(\mathrm{C1}) - \rho(\mathrm{C2})) \cdot \cdot (k_5\rho(\mathrm{P}) + k_8(\rho(\mathrm{S})_0 - \rho(\mathrm{C1}) - \rho(\mathrm{C2}) - - \rho(\mathrm{P}))) - (k_6 + k_7)\,\rho(\mathrm{C2}) \quad (3)$$

$$\frac{\mathrm{d}\rho(\mathrm{P})}{\mathrm{d}t} = -(k_4 + k_5)\,(\rho(\mathrm{E})_0 - \rho(\mathrm{C1}) - \rho(\mathrm{C2})) \cdot \cdot \rho(\mathrm{P}) + k_3\rho(\mathrm{C1}) + k_6\rho(\mathrm{C2}) \quad (4)$$

Eqns (*2*—*4*) are Riccati-type nonlinear equations. Solution of this set of differential equations, *i.e.* numerical integration for the given initial conditions, was carried out using the local linearization method. This method is almost independent of the size of integration step. Exact values of the reaction rate constants, k_1—k_8, were determined using the least-squares method for fitting of time trajectories, followed by generating a set of nonlinear equations and solving it with the gradient method. Data fitted with relatively small deviation are exhibited in Table 2. Proposed model proved to be able to recognize among different initial experimental conditions, which is shown in Fig. 2. According to this model, hydrolysis of peptide bonds is followed either by formation of the relatively stable enzyme—product complex or re-synthesis of peptide linkages. It was found that for the constant substrate concentration, $\rho(S)_0$, changes of the ratio $\rho(E)_0/\rho(S)_0$ within the applied range resulted in a weak variation of the course of protein hydrolysis. Mathematical model allows determining the enzyme concentration and the time of hydrolysis necessary to attain the desired hydrolysis degree of evening primrose protein with known initial concentration.

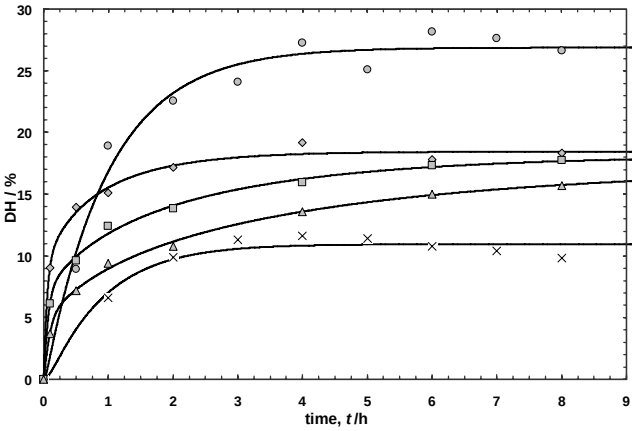

Fig. 2. Dynamics of the evening primrose protein hydrolysis for different protein concentrations $\rho(S)_0$ and enzyme to protein ratios, $\rho(E)_0/\rho(S)_0$: 20/2 (●), 35/2 (■), 35/1 (▲), 35/0.5 (◆), and 50/2 (×).

Published data [10, 11] indicate that at low protein concentration (20—50 g dm^{-3}) peptide bond hydrolysis is the favoured reaction. Protein concentration rise of 100—600 g dm^{-3} triggers the peptide bond

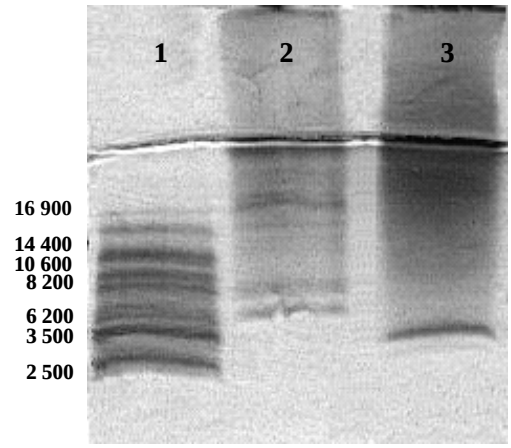

Fig. 3. Tris-Tricine/SDS-PAGE analysis: lane 1 – molecular mass standards; lane 2 – evening primrose protein isolate (relative molecular mass of peptides: 5400—21800); lane 3 – trypsin hydrolyzate (relative molecular mass of peptides: 4400—5100).

Table 3. Amino Acid Composition of Evening Primrose Protein and its Trypsin Digest

Amino acid	Protein isolate	Hydrolyzate
	Amino acid content/g per 100 g of protein	
Alanine	4.4	3.6
Arginine	3.9	3.9
Aspartic acid	9.6	8.2
Glutamic acid	20.7	28.4
Glycine	7.9	12.1
Histidine	2.9	3.2
Isoleucine	4.4	3.1
Leucine	8.2	7.8
Lysine	1.4	1.0
Methionine	3.2	2.6
L-Cysteine	3.2	0.8
Phenylalanine	2.2	4.2
Proline	4.1	4.4
Serine	6.0	8.2
Tyrosine	3.8	0.9
Threonine	8.4	3.7
Valine	5.7	4.6

re-synthesis, the rate of which surpasses that of the peptide hydrolysis. Occurrence of the latter reaction in diluted solutions of evening primrose protein isolate is not surprising because of reduced water activity in these solutions, resulting from a relatively high content of dietary fibre (15 mass %). Low water activity is known to promote synthesis reactions [12].

Molecular mass of peptides present in the evening primrose protein isolate and that of products released by the isolate digestion with trypsin (6 h, $\rho(S)_0 = 20$ g dm^{-3}, $\rho(E)_0/\rho(S)_0 = 2$ %) was determined by Tris-Tricine/SDS-PAGE (Fig. 3). In contrast to the raw material, which contained 5 different fractions of peptides with relative molecular mass from 5400 to 21800, its enzymatic digest with DH of 30 % contained 2 fractions of peptides of molecular mass of 4400 and 5100 (two bands at the bottom of the gel) and unresolved products of relative molecular masses higher than 17000 (intensive smear visible on the gel).

Amino acid profiles of the raw material and its hydrolyzate were also different. Enzymatic cleavage at alkaline pH brought a drop in the sulfur amino acid content (Table 3). 100 g of the evening primrose protein isolate contained 3.2 g of methionine and 3.2 g of cysteine. Enzymatic modification reduced the Cys content 4 times, and that of Met decreased to 2.6 %.

Treatment with trypsin elevated the solubility of this material at pH 4.5 and 7.0, from 2.0 % and 13.2 % to 16.8 % and 87.7 %, respectively.

These results are consistent with observations of other researcher groups [13, 14]. Digestion of evening primrose protein isolate with trypsin increased the content of peptide fractions with low molecular mass and the substrate solubility, thus improving the bioavailability of this nonconventional protein source.

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