Modification of Proteins from Evening Primrose by Transglutaminase*

J. GOLABCZAK**, J. STRAKOWSKA, and A. STAN

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

Received 1 April 2004

Dedicated to the 80th birthday of Professor Elemír Kossaczký

Application of transglutaminase for improvement of biological properties of evening primrose (*Oenothera paradoxa*) proteins was investigated. Proteins were extracted from defatted plant seeds being the waste material in pharmaceutical industry. The analysis of amino acids content of this protein extract proved the lysine deficiency. In order to increase its content, transglutaminase of guinea pig liver was employed. Low-degree papain hydrolyzate (DH = 7 %) of the protein extract and L-lysinium monochloride were used as the substrates for this reaction. This process resulted in an increase of lysine content from 1.3 % to 4.2 %. Transglutaminase has appeared to be efficient tool for modification of amino acid content in proteins.

Transglutaminase identified over forty years ago is still the enzyme, which is applied for industrial protein modification [1, 2]. Transglutaminase (TGase, protein-glutamine:amine γ-glutamyltransferase, E.C. 2.3.2.13) catalyzes acyl transfer between γ-carboxamide groups of protein-bound glutaminyl residues and primary amines [3]. When either protein-bound lysyl residues or lysine alone act as a substrate, inter- and intramolecular ε-(γ-glutamyl)lysine bonds can be formed. This cross-link resulting from transglutaminase-catalyzed reaction is highly stable and resistant to mechanical and proteolytic breakdown [4]. In the absence of amines, transglutaminase catalyzes hydrolysis of γ-carboxamide group of the glutaminyl residue (deamidation). Because of its isopeptidase activity transglutaminase can also hydrolyze $\gamma : \varepsilon$ isopeptides [5].

The catalytic activities and availability of cheap transglutaminase opened the way for production of proteins of unique functional properties and high nutritional value [2]. TGase-catalyzed cross-linking is used in dairy processing, processing of seafood (surimi), meat (sausages, ham), and noodles [6] to improve solubility, the emulsifying and foaming properties of protein hydrolyzates from gluten and soy [7, 8].

Transglutaminase can modify nutritional properties of proteins similarly as proteases used so far for this purpose. The enzyme was reported to be successful in increasing the lysine content in gluten [9]. *Bercovici et al.* [10] enriched casein with lysine by treating it with animal transglutaminase. L-Lysine or its oligomers served as amino acid donors in the process. The mole ratio of the lysine incorporated into the protein to overall casein was 5 : 1. The replacement of lysine with its tetrapeptide increased the level of incorporation to 22.5 : 1. Transglutaminase is able to incorporate into the protein structure not only lysine but also other amino acids, *e.g.* lysylmethionine and lysylarginine were used to supplement the methionine and arginine deficiency in casein [6].

In the presented work animal transglutaminase was used for incorporation of lysine into protein extract isolated from nonconventional source, namely from evening primrose seeds.

EXPERIMENTAL

Evening primrose proteins were isolated by alkaline extraction (0.05 M-NaOH) from ground seeds (0.125 mm "mesh" size) defatted with chloroform—methanol mixture ($\varphi_r = 1 : 1$). Pharmaceutical company "Agropharm" (Poland) supplied the defatted

*Presented at the 31st International Conference of the Slovak Society of Chemical Engineering, Tatranské Matliare, 24—28 May 2004.

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

seeds, a waste material in pharmaceutical industry. Total nitrogen content in raw material was determined by the Kjeldahl method and then protein content was calculated ($w(\mathrm{N}) \times 6.25$).

Composition of proteins extracted from the raw material was determined by separating the individual amino acids. Protein isolates (10 mg cm^{-3}) were oxidized with performic acid at 50 °C for 10 min [11]. Protein hydrolysis was performed in vacuum-tight glass flasks with 6 M-HCl at 110 °C for 24 h. Tryptophan content was determined after hydrolysis with 4 M-LiOH [12]. Residual proteins were precipitated with 5-sulfosalicylic acid and spun down (8000 × *g*, 15 min). Amino acid analysis was performed in the resulting supernatant with the use of analyzer AAA-400 (Ingos, Czech Republic).

Before transglutaminase-catalyzed reaction the protein isolate was subjected to partial hydrolysis with papain (30 000 USP U/mg, Roth). The enzyme activity of 1.04 jA g^{-1} was assayed by the Anson method [13]. Protein hydrolysis was performed at 50 °C for 1 h with $\{m(\text{protein}) : V(\text{water})\} = 0.05$ of the protein isolate in 0.2 M-phosphate buffer, pH 8.0, the enzyme to substrate ratio w_r was 1 : 100. Then, the enzyme was inactivated by incubation at 70 °C for 20 min and the reaction mixture was lyophilized. Degree of hydrolysis (DH/%) was calculated from the proportion of amine nitrogen content determined by the Sörensen method [14] and the total nitrogen content assayed by the Kjeldahl method [14].

L-Lysine incorporation into the evening primrose protein was catalyzed by transglutaminase from guinea pig liver (2 U, Sigma) using the method described by *Bercovici et al.* [10]. Protein hydrolyzate (DH = 7 %) obtained as described above and L-lysinium monochloride (Sigma) served as the substrates for transglutaminase reaction. The reaction mixture containing the protein solution (0.1 mg cm^{-3}), the amino acid (0.02 mg cm^{-3}), $\mathrm{CaCl_2}$ (5 mM), dithiotreitol (DTT, 10 mM), and transglutaminase (0.1 U cm^{-3}) in 0.1 M-Tris-HCl buffer, pH 7.5, was incubated at 37 °C in orbital shaker (300 min^{-1}) for 4 h and then 10 vol. % of 0.4 M-EDTA (pH 8.0) was added. The control sample was identical but the enzyme addition.

The amount of lysine incorporated into the protein was estimated from the decrease of free L-lysinium chloride concentration in the reaction mixtures. For this purpose protein was precipitated from the reaction and control samples using 96 % ethanol and isolated by centrifugation at 9000 × *g* for 15 min. The precipitates were thoroughly washed with 70 % ethanol to rinse out all free lysine and the supernatants were collected. This step was repeated 3 times to assure complete recovery of free Lys · HCl from the protein precipitate. Resulting supernatants were pooled, filtered by means of 0.22 μm membranes and then used for the final HPLC assay (Jupiter column 5μ C18300A, 250 mm × 4.6 mm, Phenomenex, fluorescence detector, Shimadzu). Detection of lysine was carried out using the linear gradient 8—16 % $\mathrm{CH_3CN}$ and 12.5 mM-phosphate buffer (pH 7.2) from 0 to 30 min, 0.27 % min^{-1} at a flow rate of 1 cm^3 min^{-1}, and then the new gradient 16—40 % $\mathrm{CH_3CN}$ and 12.5 mM-phosphate buffer (pH 7.2) from 30 to 60 min, 0.8 % min^{-1}, the same flow rate as previously. Under these conditions retention time for lysine was 52 min. The amount of L-lysine incorporated into protein was calculated using the following equation

$$Y = \left(1 - \frac{A_\mathrm{s}}{A_\mathrm{r}}\right) \frac{m}{B} \frac{M_\mathrm{Lys}}{M_{\mathrm{Lys} \cdot \mathrm{HCl}}} 100\ \% \qquad (1)$$

where Y represents the mass of lysine incorporated into 100 g of protein, A_s sample peak area, A_r reference peak area, m the initial amount of L-lysine · HCl, B the amount of protein in the reaction mixture, and M molar mass.

Independently, L-lysine content was determined by means of amino acid analyzer. For this purpose control and reaction mixtures containing the modified proteins were treated with sulfosalicylic acid ($\{m(\text{acid}) : V(\text{mixture})\} = 0.03$) and the resulting protein precipitates were spun down by centrifugation for 15 min at 8000 × *g*. This step was repeated twice to assure complete recovery of free Lys · HCl from the protein precipitate. Then, the collected supernatants were analyzed using amino acid analyzer. The amount of L-lysine incorporated into protein was calculated using the equation

$$Y = \left(1 - \frac{C_\mathrm{s}}{C_\mathrm{r}}\right) \frac{m}{B} \frac{M_\mathrm{Lys}}{M_{\mathrm{Lys} \cdot \mathrm{HCl}}} 100\ \% \qquad (2)$$

C_s and C_r being the amounts of L-lysine · HCl in 100 mm^3 of sample and reference mixture, respectively.

RESULTS AND DISCUSSION

In this work the incorporation of L-lysine into the proteins of evening primrose was carried out with the use of transglutaminase from guinea pig liver. Dry mass of ground evening primrose defatted seeds contained 23 % of proteins. The protein isolate obtained from this source with the use of 0.05 M-NaOH contained 74 % of proteins and it was used in further analyses without any additional purification. It should be noticed that under these conditions (*i.e.* 0.05 M concentration of NaOH), the efficiency of the alkaline extraction was almost 70 %, while the use of 0.02 M-NaOH gave much lower efficiency of this process (only 40 %).

The amino acid composition of evening primrose protein extract was analyzed and compared to that of soy protein (Table 1). The evening primrose protein

Table 1. Amino Acid Composition of Evening Primrose and Soy Protein

Amino acid	Evening primrose	Soy	FAO
	Amino acid content/g in 100 g of protein		
Ala	4.2	12.3	
Arg	3.9	5.8	
Asp	8.9	10.3	
Glu	19.4	17.8	
Gly	7.4	3.7	
His	2.8	2.1	
Ile	4.2	4.7	4.0
Leu	7.6	7.1	7.0
Lys	1.3	6.1	5.5
Met	2.9	0.9	
Cys/Met	5.8	3.9	3.5
Phe	2.2	5.2	
Pro	3.9	4.7	
Ser	5.6	4.4	
Tyr/Phe	5.8	8.5	6.0
Thr	7.8	5.2	4.0
Trp	7.0	1.0	1.0
Val	5.4	4.2	5.0
Sulfur	1.4	1.0	

extract is rich in sulfur amino acids (5.8 %), including methionine (2.9 %) but is poor in lysine (1.3 %); meanwhile, soy protein contains up to 6.1 % of lysine. These results were in good agreement with the data published by *Hudson* [15] and *Miric et al.* [16]. The authors reported lysine deficiency (about 2 %) and a high content of sulfur amino acids (11—14 %) in evening primrose protein. The amount of sulfur-containing amino acids in the evening primrose protein isolate obtained in the present study was two-fold lower than that one previously reported. This difference could result from relatively drastic method of protein isolate preparation used in our experiments.

The protein extract was partially hydrolyzed with papain to increase its solubility. Dry mass of hydrolyzate contained 58 % of protein, of which 1.3 % corresponded to lysine. Then, the L-lysine incorporation into protein was performed making use of transglutaminase from guinea pig liver. L-Lysinium chloride was employed as a source of lysine for this reaction. The process catalyzed by transglutaminase was controlled by determination of the decrease of L-lysine content in the supernatants obtained after removal of the modified proteins from reaction mixtures. It has been found that transglutaminase supplemented lysine deficiency in evening primrose protein extract and the L-lysine content in the modified proteins increased from the initial value of 1.3 % to 3.9—4.4 % (Table 2).

The results proved that tissue transglutaminase is efficient for improvement of amino acid composition of proteins including evening primrose protein. This enzyme could be recommended as an alternative to proteases routinely applied for this purpose.

Acknowledgements. This research study was financed by the State Committee for Scientific Research, Poland, Project No. PBZ-KBN/021/P06/99/19.

REFERENCES

1. Lorand, L., *Neuroch. Inst. 40*, 7 (2002).
2. Kołakowski, E. and Sikorski, Z. E., *Żywność. 2*, 5 (2001).
3. De Jong, G. A. H. and Koppelman, S. J., *J. Food Sci. 67*, 2798 (2002).
4. Chen, J. S. K. and Mehta, K., *Int. J. Biochem. Cell Biol. 31*, 817 (1999).
5. Fesus, L. and Piacentini, M., *Trends Biochem. Sci. 27*, 534 (2002).
6. Motoki, M. and Seguro, K., *Trends Food Sci. Technol. 9*, 204 (1998).
7. Babiker, E. E., Fujisawa, N., Matsudomi, N., and Kato, A., *J. Agric. Food Chem. 44*, 3746 (1996).
8. Babiker, E. E., *Food Chem. 70*, 139 (2000).
9. Motoki, M. and Nio, N., *J. Food Sci. 48*, 561 (1983).
10. Bercovici, D., Gaertner, H. F., and Puigserver, A. J., *J. Agric. Food Chem. 35*, 301 (1987).
11. Gherke, C. W., Wall, L. L., Absheer, J. S., Kaiser, F. E., and Zumwalt, R. W., *J. Assoc. Off. Anal. Chem. 68*, 811 (1985).
12. Lucas, B. and Sotelo, A., *Anal. Biochem. 109*, 192 (1980).
13. Anson, M. J., *J. Gen. Physiol. 22*, 79 (1939).
14. *Official Methods of Analysis.* Association of Official Analytical Chemists (AOAC). 16th Edition, Ch. 12, p. 7. Horowitz, Washington DC, 1995.
15. Hudson, B. J. F., *JAOCS 61*, 540 (1984).
16. Miric, M., Miletic, I., Lalic, Z., and Aksentijevic, S., *Hrana Ishrana 29*, 147 (1990).

Table 2. L-Lysine Amount Incorporated into Evening Primrose Protein Extract as Measured by HPLC and Amino Acid Analyzer AAA-400

	L-Lysine · HCl initial amount, m/mg	Protein amount, B/mg	Peak area, A/(a.u.)	Y/% Eqn (*1*)	L-Lysine · HCl content, $C \times 10^2$/(nmol mm^{-3})	Y/% Eqn (*2*)
Reference	0.4	2	8196	–	9.7333	–
Sample	0.4	2	6595	3.13	8.1638	2.58