

APPLICATION OF PANCREATIC ENZYMES IN HYDROLYSIS OF EGG-WHITE PROTEINS

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Key words: ovoalbumin, enzymatic hydrolysis, antioxidant activity, trypsin, chymotrypsin

The hydrolysis of egg-white protein precipitate (EWPP) with trypsin, chymotrypsin, and elastase was studied. The peptides obtained were determined for their antioxidative properties. Chymotrypsin caused the most extensive degradation of EWPP. The degrees of hydrolysis (DHs) of the one-hour hydrolysates were 77.6%, 55.8%, and 43.3% for chymotrypsin, trypsin, and elastase, respectively. Only small peptides with molecular weights of 5-10 kDa (74.2%) and <3 kDa (25.6%) were found in the one-hour hydrolysate with chymotrypsin, whereas products with higher molecular weights (78 kDa, 37 kDa, 34 kDa, and 78 kDa, respectively) as well as the small peptides were produced with elastase and trypsin. In the ensuing hours the rate of hydrolysis slowed and the DHs of the final hydrolysates were 95.8%, 95.3%, and 79.9% for trypsin, chymotrypsin, and elastase. The RP-HPLC profiles of the final hydrolysates showed differences in the hydrophobicity of the generated peptides. All hydrolysates exhibited weak scavenging activity of DPPH free radicals.

INTRODUCTION

The rapid growth of biotechnology, agriculture, and the food industry leads to the generation of large amounts of high-quality protein by-products that may be used for human consumption. By-products, such as whey proteins, soybean proteins (β -conglycinin and glycinin), collagen, porcine plasma, and by-products of the fish industry, can be converted into value-added products with improved functional and nutritional properties by enzymatic hydrolysis [Gibbs *et al.*, 2004; Hyun & Shin, 2000; Je *et al.*, 2007; Kim *et al.*, 2007; Li *et al.*, 2007]. Enzymatic hydrolysis is a process with mild conditions, is easily controlled, and allows obtaining products with well-defined features. The solubility, emulsification, foaming, and gelation properties of the hydrolysate depend on the enzyme's specificity and the degree of hydrolysis (DH) [Clemente, 2000; FitzGerald & O'Cuinn, 2006]. However, the application of enzymes can increase the cost of the process, which is why cheap sources are preferred in this respect. One of the best sources of proteinases are pancreases, which are by-products of the meat industry. Trypsin, chymotrypsin, and elastase are pancreatic serine proteases with molecular weights of about 25 kDa which work under slightly alkaline conditions. At the same time they differ significantly in their specificity, which results in distinct products of protein degradation [Żelazko *et al.*, 2007].

Hen egg is an important source of many substances which have wide and novel applications in medicine and as preservative agents in food production [Kijowski *et al.*,

2000; Trziszka *et al.*, 2006]. Biologically active substances contained in egg white, for example lysozyme, cystatin, avidin, conalbumin, and ovomucin, have recently drawn the attention of researchers [Ibrahim *et al.*, 1996; Li-Chan *et al.*, 1995; Trziszka *et al.*, 2004]. Several highly efficient and useful methods (crystallization, precipitation, membrane filtration, and anion exchange or affinity chromatography) for their isolation and purification have been developed [Hiidenhovi, 2007; Sokolowska *et al.*, 2007; Trziszka *et al.*, 2006]. Two of the by-products of these processes are ovoalbumin and conalbumin. Ovoalbumin is the main protein of egg (54% of egg dry matter). It is a 45-kDa glycoprotein which does not exhibit any biological activity. However, it has been shown that in enzymatic hydrolysis, bioactive peptides are released from the primary structure of this protein. Ovokinin, a vasorelaxing octapeptide derived from the pepsin digestion of ovoalbumin, has been shown to significantly lower the systolic blood pressure of spontaneously hypertensive rats [Fujita *et al.*, 1995]. ACE-inhibitory, antimicrobial, and antioxidant activities have been reported for ovoalbumin hydrolysates [Davalos *et al.*, 2004; Miguel *et al.*, 2006; Pellegrini *et al.*, 2004]. Ovotransferrin antimicrobial peptide (OTAP-92) is a 92-amino-acid cationic fragment of hen ovotransferrin. This peptide showed strong bactericidal activity against both Gram-positive and Gram-negative strains and also to insect defensins [Ibrahim *et al.*, 1998].

The purpose of this study was to investigate the application of pancreatic enzymes in the hydrolysis of egg-white protein left during the course of isolation of cystatin and lysozyme

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and to determine their antioxidative properties (DPPH radical scavenging activity).

MATERIALS AND METHODS

Materials

Egg-white protein precipitate (EWPP) was obtained in our laboratory as the by-product of the lysozyme and cystatin isolation method according to Sokołowska *et al.* [2007]. TPCK-treated bovine trypsin T-8003 Sigma, bovine α -chymotrypsin type II C-4129 Sigma, elastase type I from porcine pancreas E-0258 Sigma, and 5% picrylsulfonic acid, trinitro-benzene sulfonic acid (TNBS) were purchased from Sigma Chemicals Co., trichloroacetic acid was purchased from Ubichem, acetonitrile was purchased from Lab-Scan, and trifluoroacetic acid (TFA), sodium dihydrogen phosphate, and disodium hydrogen phosphate were from Fluka.

Enzymatic activity

Trypsin, α -chymotrypsin, and elastase activities were determined in reaction with 1% casein as the substrate according to Kakade *et al.* [1970]. Each enzyme was dissolved in 20 mmol/L HCl with 80 mmol/L CaCl₂ to a final protein concentration of 4 to 20 μ g/mL. One mL of 1% casein (in 0.2 mmol/L Tris-HCl buffer, pH 8.0) was preincubated at 37°C for 5 min and the reaction was started by adding 1 mL of an enzyme solution. After 10 min the reaction was stopped by adding 3 mL of 5% trichloroacetic acid. Then the tubes were centrifuged 4500×g, 15 min, 20°C). The absorbance of the supernatants was measured at 280 nm. One unit of enzymatic activity of these enzymes (U) corresponded to that amount of enzyme which is capable of hydrolyzing 1% casein under reaction conditions and giving an increase in absorbance at 280 nm of 0.1.

Protein assay

Protein concentration was determined spectrophotometrically at both 280 and 235 nm. The formula $(A_{235} - A_{280}) / 2.51$ (mg/mL) was used to calculate their concentrations [Whitaker & Granum, 1980].

Enzymatic hydrolysis

The egg-white precipitate was dissolved in the reaction buffer (0.2 mol/L Tris-HCl with 20 mmol/L CaCl₂, pH 7.8) to a final protein concentration of 1.38 mg/mL. Hydrolysis was started by applying enzyme solutions (enzymes were dissolved in 20 mmol/L HCl with 80 mmol/L CaCl₂; 2 U per 1 mg of substrate) and the reaction was carried out at 37°C for 7 h. It was stopped by heating at 100 °C for 10 min. Then the hydrolysates were stored at 4 °C.

Degree of hydrolysis (DH%)

DH was expressed as the percentage ratio of protein soluble in 10% trichloroacetic acid (TCA) to total protein [Polanowski, 2005]. Next, 10% TCA was added to the hydrolysates (1:1). After 1 h of incubation at 4°C the samples were centrifuged (4500×g, 15 min, 20°C). The concentration of the trichloroacetic acid-soluble product in the supernatant

was measured spectrophotometrically [Whitaker & Granum, 1980].

$$DH = \frac{\text{mg soluble protein after hydrolysis}}{\text{mL}} - \frac{\text{mg soluble protein before hydrolysis}}{\text{mL}} \times 100\%$$

Determination of free amino group content

The concentration of the free amino groups was determined according to Kuchroo *et al.* [1983]. The hydrolysate (2 mL) diluted with 0.1 mol/L borate buffer was mixed with 50 μ L of TNBS reagent (0.03 mol/L). The samples were incubated in the dark for two hours at a room temperature. The reaction was stopped by adding 2 mL of 0.1 mol/L sodium phosphate containing 1.5 mol/L sodium sulfate, and the absorbance was measured at 420 nm. A blank was prepared with water instead of hydrolysate. The results were expressed as μ mol/Gly/g by reference to a standard curve prepared with defined concentrations of glycine.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)

The samples of hydrolysates were mixed in the mobile phase A (1:1), centrifuged (13.000×g, 15 min, 20°C) and applied (100 μ L) onto a Zorbax XDB-C₁₈ column (4.6 × 250 mm, particle size 5 μ m, Agilent). The operation conditions were: analysis time 55 min, T=30 (C, flow rate 1 mL/min, gradient 2% B/min, mobile phases A: water with 1% (v/v) trifluoroacetic acid (TFA) B: acetonitrile with 1% (v/v) trifluoroacetic acid (TFA). Detector was set 235 nm.

Determination of the molecular weight distribution of the hydrolysates

The molecular weights of the peptides in the hydrolysates were determined by gel chromatography on a Zorbax GF-250 column (4.6 × 250 mm, particle size 4 μ m, Agilent). The elution buffer was composed of 0.2 mol/L phosphate buffer and 0.2 mol/L NaCl (pH 7.0). The flow rate was 0.5 mL/min and the absorbance was monitored at 230 and 280 nm. The column was previously standardized using proteins: ribonuclease A (13.7 kDa), β -lactoglobulin (18.4 kDa), egg albumin (45 kDa), lysozyme (14.4 kDa) and ovotransferrin (78 kDa).

Antioxidant activity as scavenging of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The antioxidant activity was measured according to the method described by Yen & Chen [1995] with slight modifications. The tested samples were dissolved in water to a final volume of 1 mL and mixed with 1 mL of ethanol (98%). The reaction was started by adding 0.5 mL of 0.3 μ mol/L DPPH in ethanol. The mixtures were left for 30 min at a room temperature and the absorbance of the resulting solution was measured at 517 nm. Radical scavenging activity of the peptides was expressed as μ mol Trolox × 10⁻²/mg protein by reference to a standard curve prepared with defined concentrations of Trolox.

Statistical analysis

All assays were conducted in triplicate. The results were analysed using the Statistica 7.0 program Analysis of Variance (ANOVA). Significance was set at the level of p≤0.05.

RESULTS AND DISCUSSION

The substrate, containing ovoalbumin (45 kDa) and ovotransferrin (78 kDa), was obtained as a by-product in the proteinaceous agent of an antimicrobial activity isolation protocol [Graszkiewicz *et al.*, 2007; Sokołowska *et al.*, 2007]. This inexpensive and simple procedure allows obtaining a mixture of cystatin, lysozyme, and ovomucoid which can find application in food technology or medicine. The simultaneously generated ethanol precipitate of proteins containing denatured proteins has not shown any interesting functional properties (data not shown). Furthermore, these proteins are known to be strong allergenic factors. Therefore we proposed enzymatic hydrolysis as a method for utilizing egg-white protein precipitate (EWPP). Hydrolysis of the EWPP was performed using the pancreatic serine proteinases trypsin, chymotrypsin, and elastase. The progress and kinetics of the hydrolysis were analysed by monitoring the degree of hydrolysis (DH) and free amino group content.

The DHs obtained for each enzyme are presented in Figure 1. The hydrolysis of egg-white protein precipitate (EWPP) with the pancreatic enzymes was the most extensive during the first 60 mins and then slowed down, indicating that the maximum cleavage of peptides occurred within the first hour of hydrolysis. Chymotrypsin caused a higher DH of EWPP in one-hour hydrolysates than trypsin and elastase. The respective DHs of EWPP with these enzymes were 77.6%, 55.8%, and 43.3%. Similar kinetics of protein substrate degradation with various proteolytic enzymes was observed by other authors [Graszkiewicz *et al.*, 2007]. After one hour the rate of hydrolysis with chymotrypsin and elastase slowed down, whereas trypsin maintained a rapid rate for three hours and eventually reached the final level of DH with chymotrypsin. The final DHs of the seven-hour hydrolysates were 95.8%, 95.3%, and 79.9% for trypsin, chymotrypsin, and elastase, respectively, confirming that all the studied enzymes can efficiently hydrolyse EWPP.

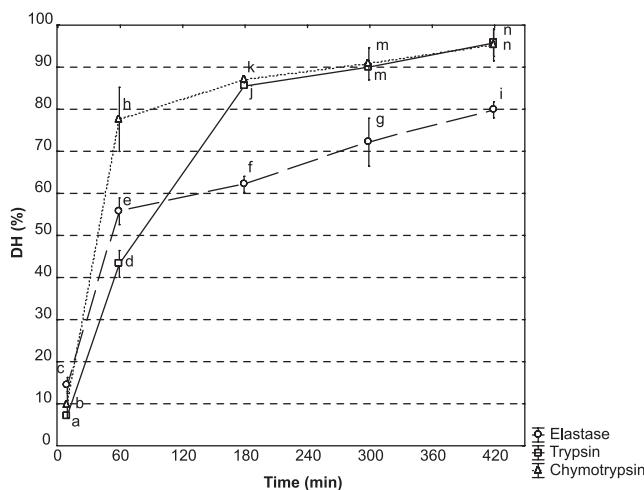


FIGURE 1. Changes in DH (degree of hydrolysis) of EWPP (egg-white protein precipitate) during hydrolysis by (○) elastase, (□) trypsin, and (△) chymotrypsin (a,b,c,...- the same letter in indices of means shows no significant differences at $p \leq 0.05$).

The progress of hydrolysis was also confirmed by determination of the free amino group content (Figure 2). The greatest increase in their concentration was also observed during the first hour of hydrolysis with all the studied enzymes. The most intensive degradation of EWPP occurred using chymotrypsin. The level of the free amino groups determined in the one-hour hydrolysate with chymotrypsin was 4.2 times higher than in the ten-minute hydrolysate, whereas in the hydrolysates with trypsin and elastase the increases in the free amino groups during this period were by factors of 3 and 2.5, respectively. After the first hour of hydrolysis, the rate of degradation of EWPP with each pancreatic enzyme slowed down, finally reaching 1487 $\mu\text{mol Gly/g}$, 1521 $\mu\text{mol Gly/g}$, and 1631 $\mu\text{mol Gly/g}$ in the hydrolysates with elastase, trypsin, and chymotrypsin, respectively.

The molecular weight distributions of the one- and seven-hour hydrolysates were characterised using size-exclusion chromatography on a Zorbax GF-250 (Agilent) column. The products of EWPP degradation obtained in each hydrolysate differed in molecular mass (Table 1). Degradation with chymotrypsin resulted in the smallest peptides. Already after one-hour degradation the peptides obtained exhibited molecular masses ranging from 10-5 kDa (74.2%) to less than

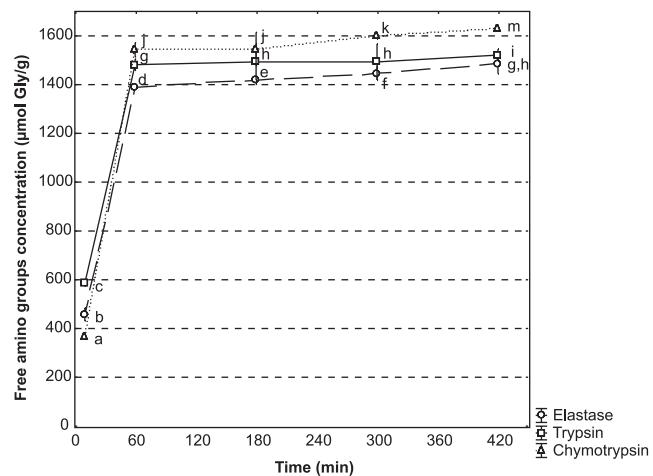


FIGURE 2. Content of free amino groups after hydrolysis of EWPP (egg-white protein precipitate) by (○) elastase, (□) trypsin, and (△) chymotrypsin (a,b,c,...- the same letter in indices of means shows no significant differences at $p \leq 0.05$).

TABLE 1. Relative concentration (%) of peptides generated by pancreatic enzymes after EWPP hydrolysis.

Enzyme	Time (h)	Molecular masses of peptides in EWPP hydrolysates					
		78 kDa	37 kDa	34 kDa	10 kDa	5 kDa	< 3 kDa
Elastase	1	22.9	ND	18.2	ND	58.5	0.427
	7	9.8	50	ND	28.2	10.1	1.8
Trypsin	1	6.55	ND	ND	ND	86.9	6.6
	7	ND	ND	ND	ND	92.8	7.2
Chymotrypsin	1	ND	ND	ND	ND	74.2	25.6
	7	ND	ND	ND	ND	60.8	39.2

ND- not detected

3 kDa (25.6%). In the hydrolysates with trypsin and elastase, in addition to the products with small molecular masses, larger peptides (9.5 kDa, 34 kDa, 37 kDa, and 78 kDa) were also observed. The more extensive hydrolysis of EWPP with chymotrypsin may be attributed to its broader specificity to cleave peptide bonds. Chymotrypsin hydrolyses peptide bonds with aromatic amino-acid residues at the P1 position with high efficiency as well as peptide bonds with Leu and Met at P1, but at much lower efficiency. Whereas trypsin exhibits affinity only to basic amino-acid residues such as Lys and Arg, pancreatic elastase hydrolyses peptide bonds located next to hydrophobic amino acids such as Ala or Gly [Żelazko *et al.*, 2007].

The presence of a 78-kDa protein, corresponding to ovotransferrin, was indicated in the hydrolysates with elastase as well as the one-hour hydrolysate with trypsin. This is in line with results obtained by Miguel *et al.* [2006], who reported that peptides obtained during crude egg-white hydrolysis were derived mainly from ovoalbumin, whereas ovotransferrin was much more resistant to proteolysis.

RP-HPLC of the final (seven-hour) hydrolysates was performed (Figure 3). The peptide profiles of the degraded EWPP confirmed the different cleavage patterns of the investigated proteolytic enzymes.

All the products of the enzymatic treatment of EWPP were eluted from 10 to 53% of acetonitrile. However, the distribution of peaks were significantly different.

The antioxidative capacities of the EWPP hydrolysates were expressed as free-radical scavenging activity with a stable DPPH radical (Figure 4). In a previous study [Graszkiewicz *et al.*, 2007], we showed that the degradation of EWPP with trypsin generates peptides with antioxidant activity. In the present study the hydrolysates with trypsin also showed the highest levels of antioxidant activity, amounting to 0.9, 2.9, and 3.8 $\mu\text{mol Trolox} \times 10^{-2}/\text{mg}$ after 10 min, 1 h, and 7 h, respectively. After 1 h of hydrolysis with chymotrypsin, the peptides exhibited antioxidant activity of 1.8 $\mu\text{mol Trolox} \times 10^{-2}/\text{mg}$, whereas elastase was able to generate peptides capable of scavenging DPPH radical (0.96 $\mu\text{mol Trolox} \times 10^{-2}/\text{mg}$) after 5 h of EWPP hydrolysis. Other authors [Gibbs *et al.*, 2004; Kim *et al.*, 2007] showed that this effect may be

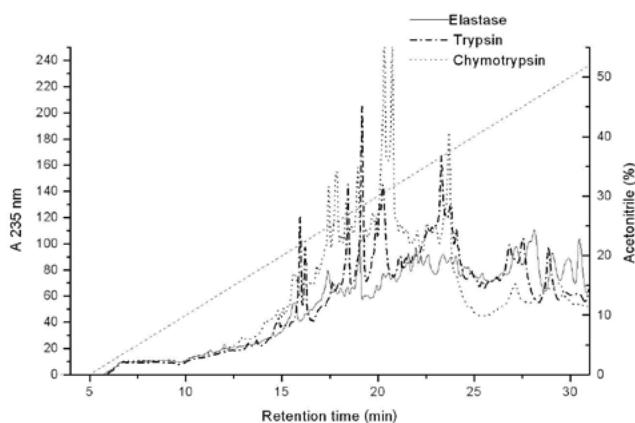


FIGURE 3. RP-HPLC chromatograms of EWPP (egg-white protein precipitate) 7- hour hydrolysates with elastase (—), trypsin (— · —), and chymotrypsin (····). Y_1 - absorbance was monitored at $\lambda=235$ nm, Y_2 - acetonitrile percentage concentration. The conditions of chromatography are described under the figure.

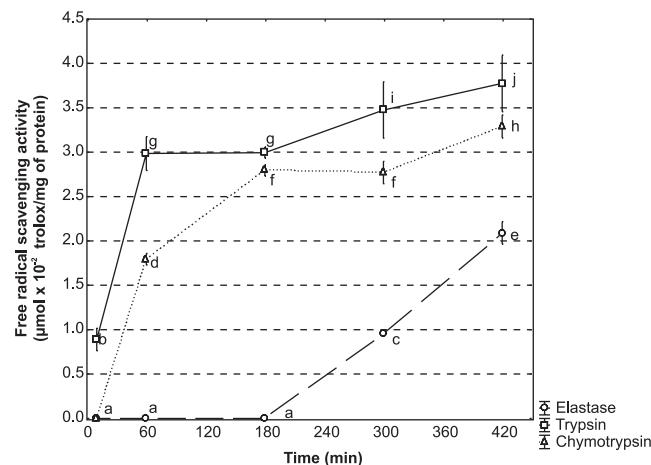


FIGURE 4. Free radical (DPPH) scavenging activity of enzymatic hydrolysates of EWPP (egg-white protein precipitate). The hydrolysates prepared with (○) elastase, (□) trypsin, and (△) chymotrypsin (a,b,c.....the same letter in indices of means shows no significant differences at $p\leq 0.05$).

related to the DHs of the hydrolysates as well as to the quality (amino-acid composition) of the peptides released during hydrolysis. They suggested that DH may highly affect the antioxidant activity of the hydrolysates and that smaller peptides have a higher level of radical scavenging [Kim *et al.*, 2007].

CONCLUSIONS

1. Of all the pancreatic enzymes applied in this investigation, chymotrypsin caused the highest degree of hydrolysis (DH) of egg-white protein precipitate (EWPP) in one-hour hydrolysates.

2. The products of EWPP degradation obtained in each hydrolysate differed in their molecular masses. Degradation with chymotrypsin resulted in the smallest peptides. Ovotransferrin was resistant against elastase and partly (one-hour) against trypsin.

3. The RP-HPLC peptide profiles of the degraded EWPP confirmed the different cleavage patterns of the investigated proteolytic enzymes, indicating the different hydrophobicity of the hydrolysate products obtained from the EWPP.

4. The hydrolysis of EWPP using trypsin generates peptides with the highest antioxidant activity in comparison with chymotrypsin and elastase.

ACKNOWLEDGMENTS

This study was financed under a scientific project no. R1201401 (2007-2010).

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Received February 2009. Revision received and accepted September 2009.

