Characterization of Protein's Enzymatic Hydrolysis Products

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Abstract

Commercial interest in obtaining preparations rich in amino acids has increased greatly as their applications in medicine, pharmacy, and cosmetics, in the industry of food and feed additives. As a result, the development of amino acid production technologies is very attractive, both from economic and ecological point of view. Enzymatic hydrolysis is a method that avoids damage of the chemical structure of amino acids and the problems related to the environment. The enzymes, efficient biocatalysts in the biotechnological industry, show a high specificity, require mild reaction conditions and determine the obtaining of secondary products in minimal quantity. Two enzymes were used in our work, a microbial product with proteolytic activity, Alcalase from B. licheniformis and a vegetable enzyme from the Carica papaya fruit, papain. The enzymes were used both in free form and immobilized by entrapment in silica gels (TEOS and TMOS precursors), and in calcium alginate. The kinetics of the hydrolysis reaction was determined over 72 hours. Casein hydrolysis can be efficiently accomplished by using the both proteolytic enzymes. Entrapment of enzymes does not destroy the activity of Alcalase and papain and allows their use in immobilized form as stable biocatalysts in protein hydrolysis reactions.

Index Terms—Enzymatic hydrolysis, immobilized enzymes, protease.

1. Introduction

The amino acids are used in numerous applications that concern human and animal needs. Amino acids are used as nutritional agents in animal feed, as food supplements for optimal functioning of human bodies. They are also used in: medicine, the pharmaceutical industry, the food industry, the cosmetic industry. The commercial interest in obtaining preparations rich in amino acids has increased greatly [1], [2].

Proteins are made up of 20 different amino acids, most of which are produced by the body. Amino acid molecules are chiral molecules and exist as two distinct geometric forms, called D and L enantiomers. Living organisms use only the L-enantiomer of the amino acids [*3*] - [5].

Amino acids can be obtained by chemical synthesis, fermentation and extraction and by hydrolysis of vegetable or animal proteins. Hydrolysis and fermentation methods are more economic than chemical synthesis methods [6].

Protein hydrolysis can be done chemically (acidic or basic hydrolysis) or enzymatically.

Chemical hydrolysis causes the racemization of amino acids. Also, the chemical hydrolysis leads to deterioration of the chemical structure of amino acids, but also to problems related to the environment. In order to protect the environment and to reduce pollution, methods that use reduced amounts of chemical reagents, reduced energy consumption and that lead to minimal amounts of secondary products, must be approached.

A very good alternative for obtaining enantiomerically pure amino acids is enzymatic biotransformation. The advantages of enzymatic hydrolysis are the high specificity of, the mild reaction conditions required, the possibility of optimizing the reaction and the minimal amount of secondary products [7].

By enzymatic hydrolysis of proteins optically pure L-amino acids are obtained. Using this method, the presence of inorganic components that could contaminate the amino acids during the obtaining process is avoided.

Microbial, vegetable and animal proteases are used efficiently to hydrolyse proteins. Proteolytic enzymes include a large number of enzymes that catalyse hydrolysis reactions of peptide bonds in proteins and peptides. In protein hydrolysis, all four classes of endopeptidases are used: Serine proteases (EC3.4.21), Cysteine proteases (EC3.4.22), Aspartic proteases (EC3.4.23), and Metallo proteases (EC3.4.24) [8].

Serine proteinases are a family of enzymes that contain a reactive serine residue in the catalytic triad Ser-His-Asp in the active site. The Subtilisins (EC 3.4.21.14) are a group of alkaline serine-proteinases secreted by *Bacillus* species. Alcalase is a non-specific serine proteinase, from the group of subtilisins, produced by cells of *Bacillus licheniformis* [9].

Cysteine proteinases contain a cysteine residue in the active site. An effective cysteine proteinase is papain. The active site of papain contains a thiol group involved in catalysis. Papain has both endo- and exopeptidase activity. Papain is obtained from the latex of the papaya tree (Carica papaya). Papain has a wide substrate specificity it catalyses the hydrolysis of proteins and peptides resulting from the action of other proteases [10].

Enzymes are very efficient biocatalysts, but for industrial applications their stabilization by immobilization can be very advantageous. Enzymes immobilized in insoluble matrices can be separated and reused for several times, the enzyme is not found in the final product. Among the immobilization methods, entrapment in porous polymer matrices has a number of advantages - the enzyme is blocked in an internal space of the matrix and therefore there are no steric problems associated with binding the enzyme to a support [11]. Entrapment in silica gels involves the immobilization of enzymes in an inorganic silica matrix obtained by the sol-gel technique at low temperature. The most widely used tetraalkoxysilanes in sol-gel processes are tetraethoxysilane TEOS ($Si(OC_2H_5)_4$) and tetramethoxysilane TMOS ($Si(OCH_3)_4$) [12], [13], [9].

Entrapment in calcium alginate matrix is a classic method of enzyme immobilization and it takes place with very good results [14].

Taking into account the performances of subtilisins and papain and the advantages of silica and alginate gels and in the protection and stabilization of the two enzymes, the purpose of this work was to hydrolyse proteins using the two free and immobilized enzymes. The protein used in the enzymatic hydrolysis was an animal protein, casein.

2. Materials and Methods

2.1. Materials

Casein, L-tyrosine, D,L-aspartic acid, L-glutamine, D,L-valine, D,L-phenylalanine, L-methionine, L-lysine, D,L-arginine, D,L-glycine, hexane, Folin-Ciocalteu's phenol reagent, bovine serum albumin (BSA), *Bacillus licheniformis* protease Alcalase were purchased from Merck. 3,5-Dinitrosalicylic acid (DNS), alginic sodium salt, tetramethoxysilane (TMOS), tetraethoxysilane (TEOS) were obtained from Fluka, ethanol and calcium chloride sicc. From Chimopar. Sodium and potassium tartrate and papain were purchased from Sigma - Aldrich. All the other chemicals were obtained from local suppliers or were commercially available reagent grade products and were used without further purification.

2.2. Assay of enzyme activity, protein content and amino acids resulted from hydrolysis

The **protease activity** was measured by UV-VIS spectrometry, according to the Anson method [15].

The **protein content** was assayed according to the Lowry method, using the Folin-Ciocalteu's phenol reagent and bovine serum albumin (BSA) as standard [16].

The resulting **L-tyrosine** from the enzymatic hydrolysis of casein was determined spectrophotometrically by measuring the absorbance at 280 nm.

The tyrosine units (TU) in the hydrolysis mixture were determined using the equation:

$$TU = \frac{(E_P - E_M)d_E}{V_E} \cdot \frac{V_H}{t_H} \cdot \frac{1}{the\ slope\ of\ the\ calibration\ line}$$

where:

E_P-E_M – the extinctions of the sample and the control, measured at 280 nm

d_E – the enzyme dilution

V_E – the volume of the enzyme solution used in the hydrolysis

V_H – total volume of the hydrolysis mixture (substrate, enzyme, reaction stopper reagent)

t_H – hydrolysis time

The **amino acids** resulting from the casein enzymatic hydrolysis reaction were determined by the ninhydrin (NHD) reaction [17], [18].

The casein enzymatic hydrolysis products were studied by **thin layer chromatography** using glass plates having deposed a thin layer of silica gel. Amino acid standard solutions (D,L-aspartic acid, L-glutamine, D,L-valine, D,L-leucine, D,L-phenylalanine, L-tyrosine, L-methionine, L-lysine, D,L -arginine, D,L-glycine) were prepared in water and had a concentration of 2%. The mobile phase was a mixture butanol: acetic acid: water=12:3:5. Amino acids were stained by spraying with 0.35% ninhydrin solution [18], [19].

2.3. Enzymes immobilization methods

For the immobilization of enzymes **in silicagels** the sol-gel method was used. The alkoxysilane precursors where TEOS and TMOS according to the method described before [20].

Entrapment in **alginate hydrogels** was done by mixing sodium alginate solution with enzyme solution and dropped and $CaCl_2$ solution, according to the method described in a previous study [20].

2.4. The kinetics of the casein hydrolysis reaction with free and immobilized proteases

The 1% casein solution was incubated with *Bacillus licheniformis* protease (pH 7) and papain (pH 6) in a ratio of 1:0.4 (40°C, 150 rpm), for up to 6 days. In order to eliminate the effects of

non-enzymatic casein hydrolysis, control samples were made - the enzyme solutions were replaced with phosphate buffer solutions. Samples were withdrawn from time to time and the content of proteins, L-tyrosine, amino acid content were measured. As well as samples for thin-layer chromatography of hydrolysis products were taken.

3. Results and Discussions

Bacillus licheniformis protease and papain were immobilized by entrapping in silica gels and in calcium alginate (according to the method II.3). The results obtained for the immobilization of enzymes are presented in Table 1.

Table 1. Proteolytic enzymes entrapped in porous gels

Enzyme			Proteolytic activity U/mL, U/mg
Alcalase	Free		1044.10
	Imobilized	TMOS	2.90
		TEOS	5.48
		Alginate	11.34
Papain	Free		0.40
	Imobilized	TMOS	0.21
		TEOS	0.25

The best results, in terms of enzymatic activity, were obtained for Alcalase. Free Alcalase has a much higher proteolytic activity than papain. For both enzymes, the enzyme activity was determined by the Anson method, and casein was used as the substrate.

The activity of the microbial protease entrapped in silica gels is lower compared to that obtained in the case of enzymes entrapped in alginate gel, results that were presented by other authors [14]. The silica gels containing the immobilized enzyme were used in the research in the form of dry powder. The drying caused the pores to shrink. The three-dimensional matrix with small pores caused large diffusion limitations. Our results agree with the results obtained by other authors [9], [11], [13]. The alginate gel with the entrapped enzyme, on the other hand, was used in the form of wet pearls and thus explains the higher enzyme activity. The disadvantages of silica gels are balanced by their advantages, which are very important when using enzymes in immobilized form, the most important being mechanical stability and resistance to microbial attack [9], [11], [13], [20].

Hydrolysis of casein with free and immobilized proteases

Hydrolysis of casein was carried out with Alcalase and papain in free and immobilized form (according to the method II.3). The number of tyrosine units resulting after 24 hours of reaction was determined (Table 2).

Table 2. Hydrolysis of casein with free and immobilized proteases

Enzyme			TU
Alcalase	Free		4844.26
		TMOS	2708.82

	Immobilized	TEOS	3373.53
		Alginate	3570.72
Papain	Free		4.98
	Immobilized	TMOS	1.05
		TEOS	1.76

The same amount of casein and the same number of total enzyme units were used in each hydrolysis reaction. It is observed that hydrolysis of casein was achieved much more efficiently with Alcalase. Regardless of the form used, free or immobilized, Alcalase resulted in obtaining a greater number of tyrosine units after 24 hours of hydrolysis.

The kinetics of the casein hydrolysis reaction with Alcalase and papain

In order to study the hydrolysis in the initial moments of the reaction, the kinetics of casein hydrolysis with Alcalase and papain were followed in the first two hours of the reaction (Fig. 1). A continuous increase in the concentration of tyrosine is observed, a more pronounced increase in the case of Alcalase. Considering that the same amount of casein was used in both hydrolyses, the difference in the tyrosine concentration obtained after the same reaction time is explained by the higher proteolytic activity of Alcalase than papain.

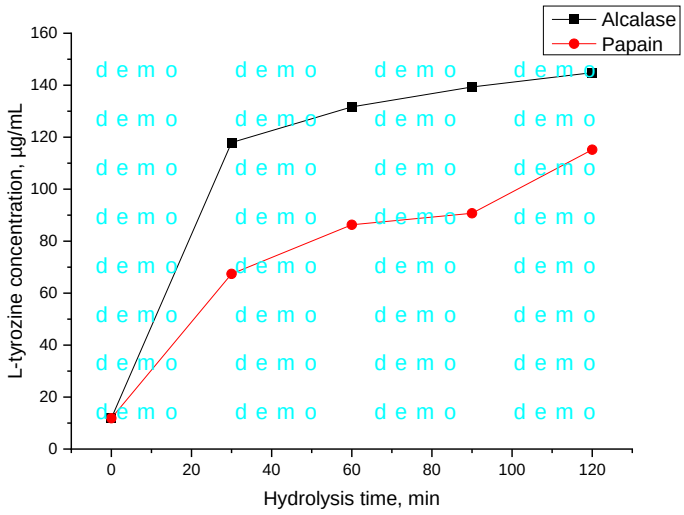

Fig. 1. The kinetics of the casein hydrolysis reaction with Alcalase and papain

The enzymatic hydrolysis of casein with the two proteolytic enzymes was followed quantitatively for 72 hours. The protein content (Lowry method), L-tyrosine concentration (extinction measurement at 280 nm) and amino acid concentration (ninhydrin method) in the hydrolysates were determined (Fig. 2, 3 and 4). In Fig. 2 it can be seen that the decrease in protein content is more pronounced, for the same time period, when Alcalase was used for casein hydrolysis. It agrees with the increase in L-tyrosine and amino acid concentrations in the reaction catalysed by the microbial enzyme (Fig. 3 and 4).

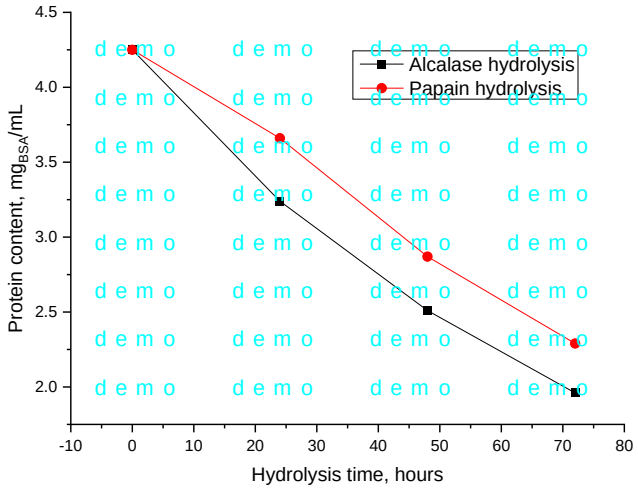

Fig. 2. The protein content of the enzymatic hydrolysis of casein with Alcalase and papain

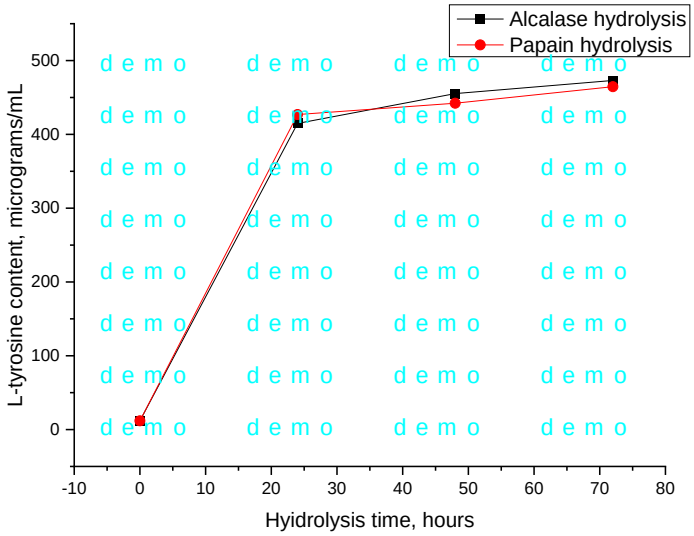

Fig. 3. The L-tyrosine content of casein enzymatic hydrolysis products with Alcalase and papain

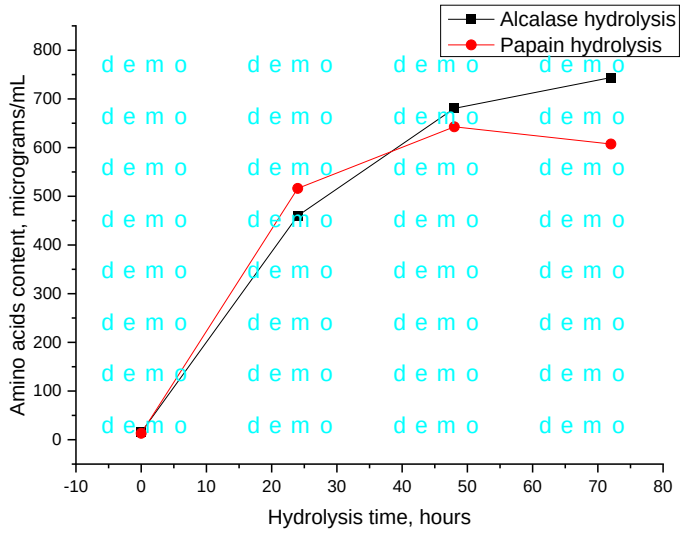

Figure 4. The amino acids content of the enzymatic hydrolysis of casein with Alcalase and papain

Qualitative separation of amino acids by thin layer chromatography

In order to analyse the casein hydrolysis reaction with the two proteolytic enzymes, in addition to the previously presented quantitative methods, a qualitative method, thin layer chromatography, was also proceeded. 10 amino acid standards were used (D,L-aspartic acid, L-glutamine, D,L-valine, D,L-leucine, D,L-phenylalanine, L-tyrosine, L-methionine, L-lysine, D,L-arginine, D,L-glycine) and R_f retention factors were calculated for each of them (Table 3).

Table 3. R_f values of amino acid standards

Amino acid	**Asp**	**Gln**	**Val**	**Leu**	**Phe**	**Tyr**	**Met**	**Lys**	**Arg**	**Gly**
R_f	**0.15**	**0.15**	**0.42**	**0.52**	**0.57**	**0.53**	**0.45**	**0.10**	**0.15**	**0.24**

The chromatographic migration of the amino acid standards, but also of the hydrolysis products after 24 hours, 48 hours, 5 and 6 days of enzymatic hydrolysis of casein with Alcalase and papain was studied.

In order to chromatographically characterize the casein substrate subjected to hydrolysis, in parallel with the amino acid standards and the hydrolysis products, a casein sample with a concentration equal to the concentration used in the hydrolysis reaction was also applied to the chromatographic plates. On none of the chromatograms, no coloured spots appeared in the casein sample, so casein does not contain hydrolysis products that would give ninhydrin coloration.

Although the chromatographic method used was qualitative (the samples were applied to plates with uncalibrated capillaries), the chromatograms showed that as the hydrolysis time increased, the spots had a more intense colour and were more clearly outlined, which indicates the increase in the concentration of amino acids resulting from the enzymatic hydrolysis of casein. This method highlights, once again, the higher efficiency of Alcalase compared to that of papain.

Following the TLC analysis, the presence of intense bands corresponding to phenylalanine, tyrosine, methionine was observed; less intense, differently coloured bands were also observed, corresponding in terms of migration distance and Rf retention factors to the amino acids lysine and arginine, but these could also be peptide residues resulting from incomplete hydrolysis.

4. Conclusions

Both, spectrophotometric methods and thin layer chromatography show hydrolysis products when using the proteolytic enzymes, Alcalase and papain.

Even if better results were obtained for Alcalase, it can be said that casein hydrolysis can be efficiently achieved with both proteolytic enzymes.

Entrapment of enzymes does not destroy the activity of Alcalase and papain and allows their use in immobilized form as stable biocatalysts in protein hydrolysis reactions.

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