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The Immobilized Porcine Pancreatic Exopeptidases and Its Application in Casein Hydrolysates Debittering

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ABSTRACT

The practical application of exopeptidase has been limited by the high cost of the enzymes resulting from the low content of individual exopeptidase in the raw material. This can be overcome by the use of a combination of all the exopeptidases in the same enzyme source, as well as the use of the enzyme immobilization technology. A porcine pancreatic exopeptidase mixture was prepared by the ammonium sulfate precipitation at 35% saturation of the autolyzed pancreatic juice. The enzyme preparation was immobilized on thin shrimp chitin film by crosslinking with glutaraldehyde. The immobilized porcine pancreatic exopeptidases (IPPE) was effective in releasing the free amino acids from peptides. Of these amino acids, the concentrations of arginine, lysine, histidine, tyrosine, phenylalanine, leucine, and glutamine were increased much more than those of other amino acids. This indicated that both the porcine pancreatic exopeptidases preparation and the IPPE contained carboxypeptidase A, B, and aminopeptidase. The IPPE was also efficient in the decrease of the hydrophobicity of protein hydrolysates demonstrated by hydrophobic chromatographic analysis. This led to the application of the immobilized exopeptidases in protein hydrolysate debittering. The IPPE was able to remove the bitterness of the tryptic/chymotryptic casein hydrolysates.

Index Entries: Exopeptidase; immobilized enzyme; protein hydrolysates; casein; debittering.

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INTRODUCTION

Exopeptidases, including both carboxypeptidases and aminopeptidases, have many potential practical applications, such as protein synthesis (*1*), protein hydrolysis (*2*), and protein hydrolysate debittering (*3–7*). Exopeptidases exist in a variety of materials, but the content of each exopeptidase in these sources is not very high (*8–10*). Thus, it is difficult to use the exopeptidase for industrial purposes. Some raw materials contain several different kinds of exopeptidases. For example, porcine pancreas contains several carboxypeptidases and aminopeptidases with specificity for most types of amino acid residues (*11–12*). The individual exopeptidase content of porcine pancreatin is also very low. Nevertheless, the overall exopeptidase activity is high enough for practical industrial applications, if proper methods can be found to extract all the exopeptidases together and to immobilize the exopeptidases mixture on a support for potential industrial application.

One important application of the exopeptidases is to remove the bitterness of protein hydrolysates as stated above. This has been limited by the high cost of the exopeptidase as well as the possibly excessive hydrolysis of the peptides, which can lead to the high osmotic pressure of the protein hydrolysates (*13*). These limitations can be avoided by the use of enzyme immobilization technology. With the use of the immobilized exopeptidases, the enzyme cost can be reduced by the reuse of the enzymes. Furthermore, the degree of hydrolysis can be more easily controlled by adjusting the flow rate of the hydrolysates passing through the reactor.

The objectives of our research were to develop a method for the porcine pancreatic exopeptidases mixture extraction, to immobilize the enzyme mixture on a matrix of potential industrial application, and to study its possible application in casein hydrolysate debittering.

MATERIALS AND METHODS

Materials and Reagents

Trypsin and trypsin/chymotrypsin were prepared from fresh porcine pancreas according to the method of Van Melle et al. (*14*). *Bacillus subtilis* neutral protease (crystallized twice) and glutaraldehyde were the products of Shanghai Biochemicals Factory. *Aspergillus oryzae* protease was prepared by the method reported previously (*15*). Shrimp chitin was provided by Beijing Institute of Food Industry. Tryptone was a product of Oxoid. Other chemicals used were all analytical-grade reagents.

Preparation of Casein Hydrolysates

Casein hydrolysates were prepared by the methods described previviously (*15*). In order to generate intensive bitterness, the degree of hydrol-

ysis was controlled to be in the range of 40–60%. The endopeptidases used for the casein hydrolysates preparation were the immobilized trypsin (186 U/g), the immobilized trypsin/chymotrypsin (744 U/g), the immobilized *A. oryzae* protease (832 U/g), and the immobilized *B. subtilis* neutral protease (733 U/g).

Activity Determination of the Immobilized Exopeptidases

The specific activity of the prepared immobilized porcine pancreatic exopeptidases (IPPE) was determined by the following procedure. Ten grams of the freeze-dried IPPE were packed in a 16 × 250 mm jacketed glass chromatography column. The temperature of the column was maintained constant at 40°C. Tryptone solution (10 mg/mL in 0.02 mol/L phosphate buffer, pH 7.5) was used as the substrate. The substrate solution was passed through the column at a constant rate of 1 mL/min. The free amino acids content of the tryptone solution was determined with an improved 2,4,6-trinitrobenzenesulfonic acid method both before and after the treatment with immobilized exopeptidases (*16*). A mixture of amino acids, including lysine, arginine, histidine, glutamine, tyrosine, phenylalanine, leucine, valine, and isoleucine of 10 mg each, was used as the materials standard. One unit of exopeptidase activity was defined as the exopeptidase activity that releases 1 μg free amino acids in 1 mL of substrate under the condition described above.

Amino Acid Analysis

Free amino acid composition of protein hydrolysates was analyzed with a Beckman 121 MB amino acid analyzer.

Hydrophobic Chromatography of Casein Hydrolysates

Hydrophobic chromatography was performed on a Waters HPLC system with a C-18 column, 4 × 300 mm. The peptide was monitored by UV detector at 214 nm. Casein hydrolysates were first treated with 0.1% trifluoroacetic acid (TFA), then introduced into the column, and eluted with a linear gradient of acetonitrile from 0 to 100% in 0.1% TFA at a rate of 45% increase in 60 min. The flow rate was 0.8 mL/min.

Sensory Evaluation of Bitterness

Sensory tests were performed according to the method described by Adler-Nissen (*17*). Sixteen panelists were trained after passing the initial selection test and participated in the experiments. Ten ppm quinine hydrochloride solution were used as a standard bitter compound. The casein hydrolysate samples were tasted to test the bitterness.

Table 1
Effect of the Immobilized Porcine Pancreatic Treatment on the Free Amino Acid Ratio (%) of the Casein Hydrolysates and the Solubility (%) in 20% TCA of Casein Hydrolysates

Protease for casein hydrolysates preparation	Free amino acid ratio, %		Solubility in 20% TCA, %	
	Control	Treated sample	Control	Treated sample
Trypsin	1.25	6.74	34.10	65.26
Trypsin/chymotrypsin	0.75	6.43	22.19	46.44
B. subtilis neutral protease	1.44	12.44	51.64	61.77
A. oryzae protease	7.01	16.63	50.35	70.41

RESULTS AND DISCUSSION

Fresh porcine pancreas was used as the raw material for exopeptidase mixture preparation. To activate the exopeptidases in the pancreas, the pancreas was homogenized in the water (pancreas/water 1/10) and autolyzed at the room temperature. The exact conditions were pH 8.0 and 25°C for about 8 h. The pancreatic juice was separated from the autolyzed pancreas by filtration at 4°C. The juice then was subjected to ammonium sulfate precipitation at 35% saturation. The precipitate obtained was found to be rich in exopeptidase activity. After solubilization and dialysis of the precipitate in 0.02 mol/L phosphate buffer, pH 7.5, the porcine pancreatic exopeptidase preparation was ready for use without any further purification.

The thin shrimp chitin film was chosen as the enzyme matrix for preparing the IPPE because it has a good mechanical stability for industrial applications (*15*). The enzyme preparation (10–20 mg/mL) was mixed with thin shrimp chitin films at 4°C for 12–24 h to let the enzymes adsorb on the chitin matrix. Then, glutaraldehyde was added to make the final concentration to about 1–2%. The enzyme and shrimp chitin mixture was kept at 4°C for another 12–24 h to crosslink the enzyme on the chitin. Finally, the excessive free enzyme was removed by three times repeated washing with water. The immobilized exopeptidase prepared by this method had an SA of 8 U/g. It was found that the immobilized enzymes was stable in storage at 4°C for at least 6 mo and stable during continuous operation in the column reactor for 30 d without obvious loss of activity.

In order to determine the enzyme composition of the porcine pancreatic exopeptidase preparation, the free amino acid composition of casein hydrolysates before treatment with the IPPE was compared with that of casein hydrolysates after treatment with the IPPE. Casein hydrolysates (1–2%) were passed through a jacketed column (30 × 280 mm) packed with 20 g IPPE (dry wt) with an SA of 8 U/g at a rate of 5–10 mL/min. The temperature of the column was maintained at 45°C. The free amino acid ratio in the casein hydrolysates and the solubility in 20% trichloroacetatic acid both before and after the treatments with the IPPE are listed in Table 1.

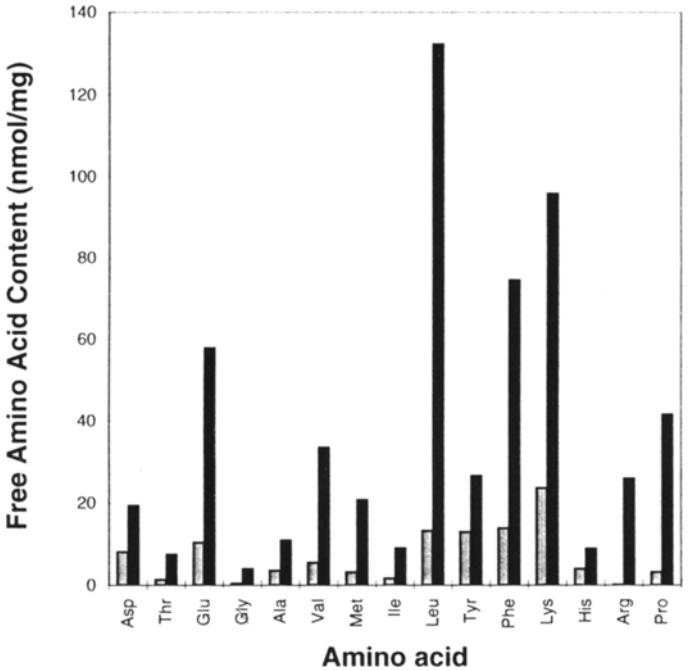

Fig. 1. The free amino acid composition (nmol/mg) changes of tryptic/chymotryptic casein hydrolysates resulting from the treatment with IPPE. Casein hydrolysates were prepared with trypsin/chymotrypsin, and the protein concentration and the degree of hydrolysis of casein hydrolysates before the IPPE treatment were 1.8% and 50%, respectively. The enzyme reactor for the IPPE treatment was packed with 20 g of IPPE (8 U/g). The reaction temperature was at 45°C. The flow rate was 5 mL/min. Control (▨); treated with IPPE (■).

It can be seen from Table 1 that the free amino acid ratio of the casein hydrolysates was increased greatly with the treatment of the IPPE. The free amino acid ratio increment was varied with the casein hydrolysates prepared with different endopeptidases. Similar phenomena were also observed in the casein hydrolysate solubility in 20% TCA. This indicated the possible existence of some endopeptidases activity in the immobilized exopeptidases.

Figure 1 presents the amino acid composition changes of the casein hydrolysates resulting from the treatment with the IPPE. The casein hydrolysates of Fig. 1 were originally prepared with trypsin/chymotrypsin. As shown in the figure, arginine, lysine, histidine, tyrosine, phenylalanine, and leucine were greatly increased compared to their original content in the casein hydrolysates. This indicated that carboxypeptidase A, B, and aminopeptidases activities existed in the IPPE. This also suggested that the exopeptidase preparation contained almost all the major exopeptidases of the porcine pancreas.

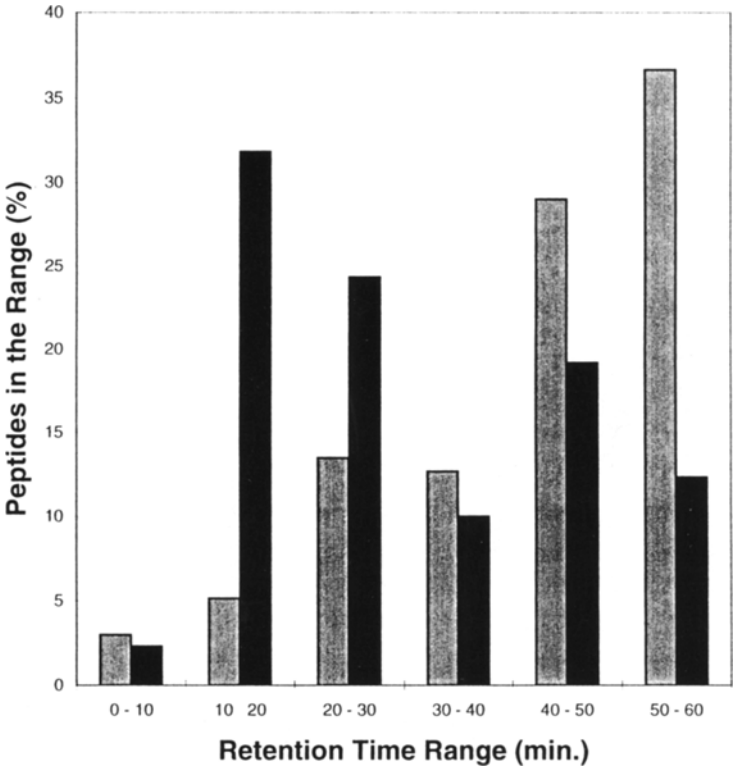

Fig. 2. The hydrophobicity changes of the tryptic casein hydrolysates after treatment with the IPPE. Casein hydrolysates were originally prepared with trypsin, and the concentration and the degree of hydrolysis of casein hydrolysates before treatment with IPPE were 1.5% and 46%, respectively. The enzyme reactor for the IPPE treatment was packed with 20 g of IPPE (8 U/g). The reaction temperature was at 45°C. The flow rate was 5 mL/min. Control (▨); treated with IPPE (■).

The hydrophobicity changes were also studied by chromatographic analysis of casein hydrolysates both before and after the treatment with IPPE. For ease of analysis, the hydrophobic chromatography of the casein hydrolysates was further treated by dividing the chromatogram into six equal retention time ranges. The peptides in each retention time range were added, and the total peptide content in each range was calculated. As shown in Fig. 2, the peptides tend to appear in the longer retention time ranges after treatment with the IPPE. This indicated that the IPPE treatment of the casein hydrolysates obviously decreased the hydrophobicity of the casein hydrolysates.

Since the bitterness of the peptides is directly related to its hydrophobicity, the treatment of the casein hydrolysates with the IPPE would lead to the removal or a decrease in the bitterness of the casein hydrolysates. It was found that the IPPE was effective in removing the bitterness of casein hydrolysates originally prepared by trypsin or trypsin/chymotrypsin.

Furthermore, the debittering efficiency of the IPPE was directly related to the flow rate of the casein hydrolysates in the column reactor. The slower the flow rate, the higher the debittering efficiency. However, for casein hydrolysates originally prepared by *B. subtilis* neutral protease or by *A. oryzae* protease, the debittering efficiency of the IPPE was not as high as for the casein hydrolysates prepared with trypsin or trypsin/chymotrypsin. This may be related to the peptide structures of casein hydrolysates. The peptide structure of casein hydrolysates is related to the specificity of enzyme used for the hydrolysate preparation.

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