Bioactive peptide production by hydrolysis of porcine blood proteins in a continuous enzymatic membrane reactor

Jen-Ting Wei and Been-Huang Chiang*

Abstract

BACKGROUND: During slaughter a hog produces approximately 3 L of blood. However, only a small proportion of porcine blood is currently used in food, feed or fertiliser, most of it being treated as waste and discarded. In this study the possibility of hydrolysing porcine blood proteins by enzyme in a membrane reactor for the production of bioactive peptides was investigated. Red blood corpuscles, blood plasma and defibrinated blood plasma were hydrolysed by various proteases, and the hydrolysates were evaluated for bioactive properties.

RESULTS: The hydrolysate produced by hydrolysing red blood corpuscles with a mixture of trypsin, chymotrypsin and thermolysin had the highest angiotensin I-converting enzyme (ACE)-inhibitory activity (IC50 = 0.58 mg mL−1) and scavenging effect on α,α'-diphenyl-β-picrylhydrazyl (DPPH) (65%) after 6 and 10 h of hydrolysis respectively. When the hydrolysis was carried out in an enzymatic membrane reactor with an enzyme/substrate ratio of 1 : 5 and a residence time of 100 min, the process reached steady state in 2 h. The ACE-inhibitory activity of the product during the steady state process was 86% and its scavenging effect on DPPH was 54%. The membrane process also decolourised the enzyme-hydrolysed product, thus improving the appearance of the product.

CONCLUSION: This study demonstrated that hydrolysates of porcine blood possess antihypertensive and antioxidant activities. Using red blood corpuscles as the substrate, the hydrolysis could be carried out in a membrane reactor with a mixture of proteases to produce bioactive peptides continuously. Therefore processing of porcine blood in an enzymatic membrane reactor is a potential method for producing a health-promoting product.

Keywords: porcine blood; enzymatic hydrolysis; bioactive peptides; membrane reactor

INTRODUCTION

Functional foods have attracted much attention recently because of the concept that ‘foods should possess health-promoting quality’. In this regard, ‘bioactive peptides’ produced from animal and plant sources have been widely investigated. Among them, peptides derived from bovine milk proteins are the most extensively studied food-derived bioactive peptides. Other peptides derived from animal proteins, such as collagen, hen’s egg and fish, and peptides derived from plant proteins, including soybean, wheat and corn, have also been investigated. Bioactive peptides from different animal and plant proteins have been shown to exhibit a variety of functional properties, including antibacterial, opioid, angiotensin I-converting enzyme-inhibitory, immunomodulatory, antioxidant and antitumour activities. However, very few reports concerning bioactive peptides derived from porcine blood can be found in the literature, apart from a recent study on angiotensin I-converting enzyme-inhibitory bioactive peptides from porcine haemoglobin.

Pork is the main animal food consumed in East Asia. During slaughter a hog produces approximately 3 L of blood. Porcine blood contains 170–180 g kg−1 protein and is thus a good source of protein for food. However, only a small proportion of porcine blood is currently used in food, feed or fertiliser, most of it being treated as waste and discarded. Both to eliminate a sizable pollution hazard and to prevent the loss of a valuable protein source, it is necessary to develop a procedure that will permit the utilisation of animal blood on a large scale. Based on these considerations, we tried to investigate the possibility of hydrolysing porcine blood proteins by enzyme for the production of bioactive peptides.

Enzymatic hydrolysis can be carried out in batch, immobilised or membrane reactors. The membrane reactor is advantageous over the other reactors because it can be operated at steady state for a long time and saves a significant amount of enzyme usage during the process. The membrane reactor separates the hydrolysed products from the enzyme continuously, so products with uniform molecular sizes can be obtained. The enzymatic membrane reactor has proven to be a useful tool for producing whey protein hydrolysates with low allergenicity and antioxidant activities.

* Correspondence to: Been-Huang Chiang, Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan. E-mail: bhchiang@ntu.edu.tw

Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan
activity.31 The objective of the present study was to investigate the feasibility of manufacturing bioactive peptides by enzymatic hydrolysis of porcine blood proteins in a membrane reactor.

MATERIALS AND METHODS

Chemicals

Angiotensin I-converting enzyme (ACE), angiotensin II (molecular weight (MW) 1002.2 Da), aprotinin (MW 6500 Da), bacitracin (MW 1450 Da), boric acid, butylated hydroxytoluene (BHT), α,α′-diphenyl-β-picrylhydrazyl (DPPH), disodium tetraborate decahydrate, dithiothreitol 99% (DTT), hipppuryl-L-histidyl-L-leucine (HHL), Leu-Gly, β-mercaptoethanol, o-phenaldialdehyde 99% (OPA), sodium chloride (NaCl), sodium citrate, sodium phosphate dibasic, sodium phosphate monobasic, 5-sulfosalicylic acid (SSA), sodium tetraborate, sodium dodecyl sulfate (SDS) and α-tocopherol were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). All other chemicals were of reagent grade or purer.

Blood samples and separation of defibrinated plasma

Whole porcine blood was collected at the time of slaughter from a local slaughterhouse (ChungMei Co. Ltd, Taoyuan, Taiwan). Sodium citrate solution was immediately added to the blood to a final concentration of 5 g L\(^{-1}\) as an anticoagulant. The plasma fraction was separated by centrifugation at 6000 \(\times\) g for 15 min and stored at \(-20^\circ\)C until further use. Defibrinated plasma was separated from the plasma by cold precipitation.13 The red blood corpuscles, plasma and defibrinated plasma were freeze-dried and the resultant powders were used for peptide production by enzymatic hydrolysis.

Enzymatic hydrolysis

The substrate solutions used for proteolysis contained 10 g L\(^{-1}\) red blood corpuscles, plasma and defibrinated plasma in 0.1 mol L\(^{-1}\) phosphate buffer (pH 7.4). The proteases used were Alcalase (Novo Nordisk, Bagsvaerd, Denmark), Flavourzyme (Novo Nordisk) and crude preparations of trypsin (Biocon, Nagoya, Japan), chymotrypsin (Sigma) and thermolysin (Sigma). During a preliminary study the individual enzymes and various combinations thereof were tested, and only those that could hydrolyse porcine blood to a reasonable extent were chosen for further study. The reaction temperature and pH respectively were adjusted to 38 °C and 7 for trypsin, 35 °C and 7.5 for the mixture of trypsin, thermolysin and chymotrypsin, 55 °C and 7.5 for Alcalase and 55 °C and 7 for the mixture of Alcalase and Flavourzyme. It should be noted that the means of optimal pH and temperature were used for the enzyme mixtures employed in this study, because the optimal conditions of these enzymes were not far apart. A 2 mL aliquot of 5 g L\(^{-1}\) protease solution was added to 50 mL of substrate (enzyme/substrate ratio 1:50) and the enzymatic reaction was allowed to proceed for 24 h. Samples were withdrawn at regular intervals from each proteolytic mixture and heated in boiling water for 15 min to inactivate the proteases. The hydrolysates were then centrifuged at 23 400 \(\times\) g for 15 min and the resultant supernatants were stored at 4 °C until further use.

Analysis of carnosine and anserine

A 1.2 mL aliquot of protein hydrolysate was mixed with 0.3 mL of 100 g L\(^{-1}\) SSA at 4 °C for 30 min and then centrifuged at 6000 \(\times\) g for 10 min at 4 °C to precipitate the protein. The supernatant thus obtained was filtered (0.45 μm filter) and the sample was analysed in a high-performance amino acid analyser (Beckman 6300, Fullerton, CA, USA).

Scavenging effect on DPPH radical

The scavenging effect of the hydrolysate on the DPPH radical was estimated using a modification of the method of Blois.33 The hydrolysate (1 mL) was added to a methanolic solution of DPPH radical (75 μmol L\(^{-1}\), 4 mL). The mixture was shaken vigorously and left in the dark at room temperature for 60 min, after which the absorbance was measured at 517 nm. The DPPH-scavenging effect (%) was calculated as [(OD\(_{517}\) control – OD\(_{517}\) sample)/OD\(_{517}\) control] × 100, where OD is optical density. The controls used were α-tocopherol and BHT.

Assay of ACE-inhibitory activity

The ACE-inhibitory activity was assayed by the method of Cushman and Cheung34 as modified by Maruyama and Suzuki.35 Each 380 μL assay mixture contained the following components: 100 mmol L\(^{-1}\) sodium borate buffer (pH 8.3), 300 mmol L\(^{-1}\) sodium chloride, 5 mmol L\(^{-1}\) HHL, 8 μL of ACE and 30 μL of hydrolysate. The reaction was performed at 37 °C for 30 min. The hippuric acid liberated was extracted with ethyl acetate and measured spectrophotometrically (228 nm). The IC\(_{50}\) value was defined as the concentration of peptide (mg protein mL\(^{-1}\)) required to inhibit 50% of ACE activity and was determined by regression analysis of ACE inhibition (%) versus peptide concentration.

Assay of peptide concentration

The peptide concentration was assayed by the method of Church et al.36 The OPA reagent was made by combining the following solutions and diluting to a final volume of 50 mL with water: 25 mL of 100 mmol L\(^{-1}\) sodium tetraborate, 2.5 mL of 200 g L\(^{-1}\) SDS, 1 mL of 40 g L\(^{-1}\) OPA (in methanol) and 100 μL of β-mercaptoethanol. This reagent must be freshly prepared for use. A 50 μL aliquot of hydrolysate was added directly to 2 mL of OPA reagent in a 5 mL quartz cuvette. The solution was mixed briefly by inversion and incubated for 2 min at 25 °C, after which the absorbance at 340 nm was measured in a Hitachi 1100 spectrophotometer (Tokyo, Japan). A standard curve was constructed using 0.2–1 mg mL\(^{-1}\) Leu-Gly solutions.

Distribution of molecular weight

Hydrolsates were fractionated by gel filtration on a Sephadex G-25 column (1.5 cm × 90 cm; Pharmacia, Uppsala, Sweden) and eluted with 0.01 mol L\(^{-1}\) NaCl at a flow rate of 30 mL h\(^{-1}\). Each 2 mL fraction was collected and the absorbance at 220 nm was determined.

Membrane reactor operation

A hollow fibre ultrafiltration (UF) membrane module (A/G Technology Polysulfone Membrane UFP-3-c-4MA, Massachusetts, US) with 3000 Da molecular weight cut-off and 0.15 mmol L\(^{-1}\) was used to concentrate the hydrolysate. A peristaltic pump was installed to form the membrane reactor. A peristaltic pump was installed to circulate the solution through the system. A constant temperature of 35 °C was maintained by continuously circulating warm water through the jacket of the glass tank. A schematic diagram of the system is shown in Fig. 1. During operation a solution of 500 mL of 10 g L\(^{-1}\)
red blood corpuscles in 0.1 mol L\(^{-1}\) phosphate buffer (pH 7.5) was charged to the reaction tank along with an appropriate amount of enzyme mixture containing trypsin, chymotrypsin and thermolysin (1:1:1 w/w/w). The process was operated at a constant permeation rate of 5 mL min\(^{-1}\); thus a residence time of 100 min was allowed for the enzyme reaction to proceed in the reaction vessel. The transmembrane pressure was initially set at 10 kPa but was increased gradually in order to compensate for the increased resistance due to membrane fouling. The mixture in the reaction tank was pumped through the membrane module and the resulting permeate was diverted to a collection vessel, while the retentate was returned to the reaction tank for further reaction. The substrate solution (red blood corpuscle solution) was fed continuously to the reaction tank from the substrate tank at a rate equal to the permeation rate in order to maintain a constant volume of the mixture in the reactor.

The independent variable investigated in this study was the enzyme/substrate (E/S) ratio (1:5, 1:10 and 1:100). The performance of the enzymatic process was evaluated by estimating the ACE-inhibitory activity and DPPH-scavenging effect of the permeate.

**RESULTS AND DISCUSSION**

**ACE-inhibitory activity**

Several frequently used commercial proteases, including trypsin, chymotrypsin, thermolysin, Alcalase and Flavourzyme, were used to hydrolyse the proteins of porcine blood. Table 1 shows the ACE-inhibitory activity of the porcine blood hydrolysates. Results indicated that the reaction time for the hydrolysate to reach its maximum activity varied with different substrates and enzymes. The hydrolysate of blood plasma hydrolysed by the single enzyme trypsin possessed weak ACE-inhibitory activity. The hydrolysate produced by hydrolysing red blood corpuscles by multiple enzymes exhibited the highest ACE-inhibitory activity (inhibition 89%, \(I_{50} = 0.58 \text{ mg mL}^{-1}\)) after 6 h of reaction. It has been reported that ACE inhibitors are usually short-chain peptides having 2–12 amino acids.\(^{37}\) When the degree of hydrolysis was higher, the average length of peptide chain was shorter. Therefore, as the hydrolysis time increased, shorter peptides possessing stronger ACE-inhibitory activity might have been produced. The molecular weight distributions of the hydrolysates of porcine red blood corpuscles after 2 and 10 h of reaction are shown in Fig. 2.

As the hydrolysis time increased from 2 to 10 h, the major peptides produced had molecular weights ranging from 1002 to 1450 Da, composed of 9–13 amino acids.\(^{37}\) When the degree of hydrolysis was higher, the average length of peptide chain was shorter. Therefore, as the hydrolysis time increased, shorter peptides possessing stronger ACE-inhibitory activity might have been produced. The molecular weight distributions of the hydrolysates of porcine red blood corpuscles after 2 and 10 h of reaction are shown in Fig. 2.

**Scavenging effect on DPPH radical**

The proton radical-scavenging action is known as an important mechanism of antioxidation. DPPH was used to determine the proton radical-scavenging action of the porcine blood hydrolysates because it possesses a free proton radical and shows a characteristic absorption at 517 nm. Table 2 shows the scavenging
as potent intracellular pH buffers, and their antioxidant many physiological functions in animals. They are recognised were also estimated. These histidine-containing dipeptides have levelled off. 

The DPPH radical-scavenging activity increased with hydrolysis time in the case of the hydrolysate produced by the combination of trypsin, chymotrypsin and thermolysin. The hydrolysate produced by hydrolysing red blood corpuscles with trypsin, chymotrypsin and thermolysin showed the highest scavenging effect (65%) on DPPH after 10 h of hydrolysis. However, when the hydrolysis time was increased further, the DPPH radical-scavenging activity levelled off.

The anserine and carnosine contents of the hydrolysates were also estimated. These histidine-containing dipeptides have many physiological functions in animals. They are recognised as potent intracellular pH buffers, and their antioxidant activities have attracted considerable attention because of their effective prevention of oxidation of foods and diseases such as eye disease related to lifestyle. A proposed mechanism of antioxidant activities of carnosine and anserine is ascribed to the inhibition of free radical reaction. In addition, the biological roles of these two dipeptides have been postulated in the control of enzyme activities and neurotransmitter function. In this study, anserine was found to be absent in all hydrolysates. However, carnosine was found in the hydrolysates that had a better scavenging effect on DPPH (Table 3). It is worthy of note that the carnosine content in the hydrolysate produced by hydrolysis of red blood corpuscles with the combination of trypsin, chymotrypsin and thermolysin also reached its highest level after 10 h of hydrolysis, the same as the antioxidant activity of the hydrolysate. We suspect that carnosine is one of the peptides contributing to the antioxidant activity of the hydrolysate.

**Performance of continuous membrane reactor**

A UF membrane with 3000 Da molecular weight cut-off was used for the membrane reactor. This membrane, theoretically, could allow peptides consisting of less than 22 amino acids (assuming that the average MW of amino acids is 137 Da) to pass through and be collected in the permeate. Figure 3 shows the effect of E/S ratio on the ACE-inhibitory activity of the permeate. The ACE-inhibitory activity of the permeate increased as the E/S ratio increased. However, when the E/S ratio was 1 : 5, the ACE-inhibitory activity in the permeate reached a value higher than 80% within 2 h, suggesting that higher amount of enzyme in the membrane reactor could expedite the process to reach steady state.

The DPPH-scavenging activity of the permeate from the membrane reactor operated at an E/S ratio of 1 : 5 was also investigated. Similarly, the permeate with the highest antioxidant activity could be obtained within 2 h of operation (Fig. 4). This result further suggested that the process reached steady state within 2 h when sufficient enzyme existed in the system. The DPPH-scavenging activity of the permeate produced from the membrane reactor at steady state was about 54% (Fig. 4).
order to determine the role of carnosine in the hydrolysate in terms of antioxidant activity, we prepared a standard solution of carnosine at a concentration identical to that in the hydrolysate (157.3 μg mL\(^{-1}\)) and analysed its DPPH-scavenging activity. We found that carnosine at this low concentration had no antioxidant activity, and significant DPPH-scavenging activity could only be detected at a much higher concentration such as 10 mg mL\(^{-1}\), as shown in Fig. 4. Therefore other low-molecular-weight peptides must have contributed to the antioxidant activity of the permeate.

It is worthy of note that the red blood corpuscle solution in the reactor vessel was dark red in colour, but the permeate collected from the membrane reactor was clear and slightly yellowish, similar to clarified chicken broth. This result was expected, because haemoglobin with MW larger than 60 kDa could not pass through the UF membrane used in this study. Incidentally, the pleasant appearance of this enzyme hydrolysate from the membrane reactor makes the product more useful for food applications.

**Stability of enzymatic membrane reactor**

It is important for industrial processing that the reactor can be operated at steady state for a long time. For an enzymatic membrane reactor, after prolonged use, the enzyme may become denatured or the membrane may be fouled during operation. The stability of the permeate flux as well as the amount of hydrolysed product produced during operation can reveal the stability and performance of the system. It was found that the system could be operated for at least 6 h and still maintained a constant permeate flux (Fig. 5), suggesting that membrane fouling was under proper control during the process. It appeared that the hydrolytic activity of the proteases might have removed possible foulants, namely blood proteins, from the surface of the membrane, thus maintaining the permeation rate during the process.

The peptide content in the permeate reached a plateau in approximately 2 h of operation, which was in agreement with the
ACE-inhibitory and DPPH-scavenging activities of the permeate. Thereafter the peptide content of the permeate was maintained at 2.3 mg mL⁻¹. These results demonstrated that the membrane reactor could produce a product with constant composition and bioactivity continuously.

CONCLUSIONS
This study showed that the hydrolysates of porcine blood possessed antihypertensive and antioxidant activities. Studies are in progress to determine other pharmacological activities of the hydrolysates of porcine blood and identify the sequence of bioactive peptides. It can be concluded that hydrolysing the red blood corpuscles in a membrane reactor with a mixture of proteases could produce bioactive peptides continuously, and this is a potentially feasible method for producing a health-promoting product. Besides the commonly known benefits of enzymatic membrane reactor processing, such as high productivity, continuous long-term operation and homogeneous products, using an enzymatic membrane reactor to process porcine blood has another advantage. The unpleasant dark red colour of the blood is removed during the process, thus yielding a product with a pleasant golden yellowish appearance, which makes the product more useful for food applications.

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REFERENCES
