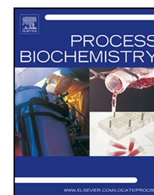
Process Biochemistry 50 (2015) 245–252

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Performance of coupled enzymatic hydrolysis and membrane separation bioreactor for antihypertensive peptides production from *Porphyra yezoensis* protein

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A R T I C L E I N F O

Article history:
Received 4 July 2014
Received in revised form 4 November 2014
Accepted 28 November 2014
Available online 18 December 2014

Keywords:
Porphyra yezoensis
Antihypertensive peptides
Enzymatic hydrolysis
Membrane bioreactor
Kinetic

A B S T R A C T

To develop more efficient methods for production of antihypertensive peptides from *Porphyra yezoensis* protein, a coupled enzymatic hydrolysis and membrane separation (CEH-MS) reactor system was studied and compared with the traditional enzymatic hydrolysis (EH) and offline membrane separation (MS) method. The CEH-MS reactor was operated in three modes: batch, continuous with water feeding, and continuous with substrate feeding. The operational factors of the CEH-MS reactor had significant effect on the protein conversion degree and their optimum values were found as enzyme concentration of 0.24 g/L, temperature of 50 °C, pH of 9.0, time of 60 min, pump speed of 300 rpm, and substrate concentration of 4.0 g/L. Compared to the traditional method, the protein conversion degree, yield of peptides, output of peptides per unit of enzyme, and antihypertensive activity of peptides for the batch operation of CEH-MS reactor were increased by 43.6%, 43.6%, 7.7%, and 3.9%, respectively. For the continuous operation with water feeding, these data were increased by 62.7%, 62.7%, 22.1%, and 4.4%, respectively. The output of peptides was increased by 216.9% for the continuous operation with substrate feeding. In general, the CEH-MS reactor was found to be more efficient than the traditional process in terms of high utilization rate of raw material and yield of peptides.

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1. Introduction

Porphyra yezoensis, an important ocean resource, is a potential source to produce peptides due to its high protein content of 25–50% [1,2]. The peptides extracted from red seaweeds contained cystine and serine which play a significant role in the treatment of hypertension patients [3,4]. At present, most of the researchers focus their work on the culture, breeding, and nutritional characteristic study of *P. yezoensis* [2,5–9]. However, little information is available on the production of antihypertensive peptides from *P. yezoensis* protein. Enzymatic hydrolysis (EH) method is commonly used for producing peptides as it is advantageous in terms of safety, production rate, and high efficiency [10,11]. The membrane separation (MS) technology, with the advantages of high selectivity and large permeate fluid flux, is frequently used for the separation of peptides based on their size [12–16]. Most often, the EH and MS techniques are operated separately and discontinuously, which results in a low yield of target peptides and a low conversion rate of substrate protein. Also, this operating mode could not make full use of the enzyme activity. In order to minimize the cost of peptide production and improve the operational efficiency, the combined use of EH and MS system was studied in this paper.

The coupled enzymatic hydrolysis and membrane separation (CEH-MS) reactor system should be able to fully utilize the benefits of biochemical engineering and membrane separation techniques to achieve effective separation of target product from the substrate. The CEH-MS reactor method may improve the conversion rate of substrate, utilization rate of enzyme, and yield of product by the integration of substrate hydrolysis, enzyme recovery and product separation into one process compared to the traditional EH and subsequent offline MS method [17]. Presently, the CEH-MS technology is used with two kinds of operational modes: batch and

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http://dx.doi.org/10.1016/j.procbio.2014.11.010
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continuous [18]. The batch CEH-MS mode is performed sequentially in an EH reactor and an online MS reactor. The continuous mode is performed by constantly feeding water or substrate solution from a recycle reactor and has the advantages of high efficiency, low labor cost, and possibility for full automation of the apparatus. Continuous feeding of substrate or water to the reactor is necessary to maintain a constant volume in the reactor by compensating the permeate fluid flux. However, little information is available about the practical operation and kinetics of CEH-MS technology for producing antihypertensive peptides from *P. yezoensis* protein.

The overall objective of this research was to develop more efficient methods with CEH-MS reactor system for the production of antihypertensive peptides from *P. yezoensis* protein. The specific objectives of this study were (1) to evaluate the effect of operational factors on the performance of CEH-MS reactor in batch operation, (2) to determine the optimum conditions and kinetic parameters of the CEH-MS reactor, and (3) to compare the performance of CEH-MS with that of the traditional EH and offline MS method for peptides production. The protein conversion degree, yield of peptides, output of peptides per unit of enzyme, and antihypertensive activity of peptides were determined to study the feasibility of CEH-MS reactor, which could provide both the theoretical and technological knowledge for industrial production of antihypertensive peptides.

2. Materials and methods

2.1. Materials and reagents

The dried *P. yezoensis* was obtained from Haian County Lanbo Industry Co., Ltd. (Nantong, Jiangsu, China). It was ground into powder using a hammer mill (FC-160, Shanghai Traditional Chinese Medicine Co., Ltd, Shanghai, China) to achieve the size of particles less than 40-mesh size. The powder samples were kept in a sealed plastic bag and stored at 4 °C until use. Alcalase 2.4LFG, with the activity of 2.670 U/g at the recommended reaction temperature of 50 °C and pH of 9.0, was purchased from Novozymes Co., Ltd. (Shanghai, China). Angiotensin converting enzyme (ACE) was extracted from the pig lung according to the reported method [19], and its activity was determined as 0.1 U/mL. The Hippuric acid (Hip) and Hippuryl-His-Leu acetate salt (HHL) purchased from Sigma–Aldrich Company (Shanghai, China) and the acetonitrile purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) were of HPLC grade. The other chemicals and solvents used for the experiments were of analytical grade.

2.2. Methods

2.2.1. Traditional EH and offline MS process

The traditional EH and subsequent offline MS processes were performed under the optimum conditions identified from our previous research [4] as described in the following steps. *P. yezoensis* powder was first mixed with water and then the soluble protein was pre-extracted under an alkaline pH of 9.0 and temperature of 50 °C. Subsequently, the protein solution was separated from the solid by centrifugation (Avanti J-25, Beckman Coulter Inc., Brea, CA, USA) at 10,000 rpm at ambient temperature (25 °C) for 10 min. Then the protein solution with substrate concentration of 3.0 g/L was allowed to react with 0.096 U Alcalase (i.e. enzyme concentration of 0.09 g/L) in a water bath (HH-6, Jintan Equipment Co., Ltd., Jiangsu, China) for 60 min. The temperature and pH of the reaction solution were kept constant at 50 °C and 9.0, respectively. After EH, the Alcalase in the hydrolysate was inactivated in a water bath at 100 °C for 10 min and then the hydrolysate was cooled to ambient temperature. The hydrolysate was used as the material for the subsequent offline MS, which is an ultrafiltration unit (UFU) consisting of an ultrafiltration device (Pellicon, Millipore Corporate, Billerica, MA, USA) equipped with a 3 kDa membrane (working area of 0.5 m^2). The antihypertensive peptides with molecular weight (MW) of less than 3 kDa in the hydrolysate were separated from other macromolecular components using the offline MS and collected as peptides for subsequent analysis.

2.2.2. Batch operation process of CEH-MS reactor

The schematic diagram of CEH-MS reactor set-up is displayed in Fig. 1. The batch CEH-MS reactor was operated without any feeding from the feeding reactor (FR), using the following procedure. First, the protein (C_{SL0}) was pre-extracted and separated using the method described in the Section 2.2.1. Then, Alcalase with enzyme concentration of 0.24 g/L was allowed to react with the protein solution having a substrate concentration of 6.0 g/L in a tank reactor (TR) at a temperature of 50 °C and pH of 9.0. At the same time, a recycle pump (RP) (BT00-300, Changzhou Chenghe Sanitary Arrangement Factory, Jiangsu, China) was operated at a speed of 200 rpm to achieve automatic recycling of liquid and a timer was started. The reaction solution was pumped through an online MS which had the same UFU as used for the offline MS. The peptides (C_P) with MW of less than 3 kDa in the permeate fluid were separated and collected for subsequent analysis. The enzyme (E), unhydrolyzed protein (C_{SL}) and other components (C_o) with MW of greater than 3 kDa left in the retentate were pumped into the TR again. When there was no outflow, the circulating system was stopped and the time taken for the process was recorded. The effect of processing time, temperature, pH, substrate concentration, enzyme concentration, and pump speed on the protein conversion degree was studied in the single-factor tests. Based on the single-factor test results, a $L_{18}(3^7)$ orthogonal experiment was performed to obtain the optimum conditions of batch CEH-MS reactor.

2.2.3. Continuous operation process of CEH-MS reactor with water feeding

The continuous operation of CEH-MS reactor with water feeding was performed by operating the CEH-MS reactor under the optimum conditions of batch CEH-MS (substrate concentration of 4.0 g/L, enzyme concentration of 0.24 g/L, temperature of 50 °C, pH of 9.0, and at a pump speed of 300 rpm) with constantly feeding water from the feeding reactor (FR) using a feeding pump (FP) at the speed of 300 rpm. The feeding pump (FP) was operated at the same speed of the recycle pump (RP) in order to maintain a constant effective volume (400 mL) in the tank reactor (TR). The circulating system and the timer were stopped when the concentration of peptides reached a very low value based on our observation. The peptides (C_P) in the permeate fluid were collected for subsequent analysis.

2.2.4. Continuous operation process of CEH-MS reactor with substrate feeding

The continuous operation of CEH-MS with substrate feeding was performed by operating the CEH-MS reactor under the optimum conditions of batch CEH-MS with constantly feeding protein (C_{SL1}) at the same concentration as that of initial protein (C_{SL0}) in the TR from the FR using the FP operated at 300 rpm. Similarly, the FP was operated at the same speed of the RP to maintain a constant volume (400 mL) in the TR. The circulating system and the timer were stopped when there was no outflow. The peptides (C_P) in the permeate fluid were collected for subsequent analysis.

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