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Preparation of casein phosphopeptides using a novel continuous process of combining an enzymatic membrane reactor with anion-exchange chromatography

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ABSTRACT

A novel process for continuous preparation of casein phosphopeptides (CPPs) was developed using an enzymatic membrane reactor (EMR) and anion-exchange chromatography (AEC). Alcalase was selected to hydrolyse casein (with an initial concentration of 6% (w/w)) at pH 8.5 and temperature 50 °C in an enzymatic membrane reactor. CPPs were then separated from the enzymatic hydrolysates through a weakly basic macroporous anion-exchanger. The yields of CPPs and phosphorus from casein were 8.11%, 15.66% and 21.41%, and 19.10%, 52.70% and 90.01% for EMR fitted with 3 kDa, 5 kDa and 10 kDa membranes, respectively. The yield and quality of CPPs improved with increase in the nominal molecular weight cut-off (NMWCO) of the membrane. However, use of a high NMWCO membrane resulted in substantial enzyme leakage in the permeate.

1. Introduction

Currently, there is growing interest in functional foods that may be beneficial to health. Among the abundant functional substances in food, bioactive peptides exhibit many physiological activities including antihypertensive, antibacterial and mineral binding effects (Laparra et al., 2008). Casein phosphopeptides (CPPs) are bioactive peptides that are released by enzyme-mediated proteolysis of casein (García-Nebot et al., 2010). The phosphate groups of CPPs are considered as mineral carriers with the potential to improve mineral absorption (Cosentino et al., 2010; FitzGerald, 1998). Furthermore, it is demonstrated that CPPs may directly affect osteoblast-like cell growth, calcium uptake and ultimately calcium deposition in the extracellular matrix (Tulipano et al., 2010). Recently, CPPs have been proposed as potential dietary antioxidants in new product development due to their excellent antioxidant effects (Laparra et al., 2008). Therefore, CPPs would play an important role as bioactive ingredients in nutraceuticals, functional foods as well as pharmaceutical applications.

Conventionally, tryptic release of CPPs from casein and isoelectric precipitation of residual casein were followed by scalable methods such as selective precipitation with a classical batch reactor, giving low yields and low purity products (Gao et al., 2008). In this process, the enzyme was used only once followed by inactivation at the end of the reaction. This type of bioreactor presented series of disadvantages such as difficulty in continuous operation, considerable loss of enzymes and substrates, high cost and low efficiency (Rios et al., 2004). However, using an ultrafiltration (UF) enzymatic membrane reactor (EMR) could greatly reduce or even eliminate the problems existing in the classical batch reactor. EMR is a specific mode for continuous reaction and simultaneous separation of products from hydrolysates (Cheison et al., 2006a). It can also continuously reduce antigenicity (Prieto et al., 2007) and separate enzymes from end products with a selective membrane (Rios et al., 2004).

Previously, CPPs have been prepared with little focus on scalable methods in view of creating an economical method for a high yield of pure CPPs. Traditionally, the separation and purification of CPPs were conducted by selective precipitation using $CaCl_2$ or acid and ethanol (Ellegard et al., 1999). High cost and low efficiency limited the industrial-scale application. In particular, the introduction of new salts and removing of ethanol make this procedure more tedious and higher costs. Kim et al. (2007) isolated CPPs from the hydrolysed sodium caseinate by aggregating with $CaCl_2$ at different pH followed by precipitation of aggregates using ethanol. The yield of CPPs under the optimal conditions was about 13% with a low purity (around 5% of phosphorus content of CPPs). Zhu and FitzGerald (2010, 2012) employed calcium aggregation, ethanol precipitation and gallium immobilized metal affinity chromatography to enrich the CPPs. However, the lack of metal affinity chromatography for large-scale production of CPPs is a major hindrance. Recently, ion exchange membrane chromatography has emerged as a promising technique for the enrichment of peptide fractions from protein

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hydrolysates. Welderufael and Jauregi (2010), Welderufael et al. (2012) developed an integrative process with six steps including resin adsorption, microfiltration, hydrolysis and membrane separation resin adsorption and microfiltration, which could integrate the selective separation of β-lactoglobulin-and casein-derived angiotensin converting enzyme (ACE) inhibitory peptides from rennet whey and their hydrolysis. Different from the integrative process developed by Welderufael and Jauregi (2010) and Welderufael et al. (2012), an integrative process which consists of continuous hydrolysis reaction and simultaneous separation (EMR) and enrichment by anion-exchange chromatography (AEC) was employed to prepare CPPs from alcalase casein hydrolysates in the present study. The different target peptides with different structures, molecular sizes, physiological activities and separation characterizations require different preparation and separation process such as using different hydrolytic enzymes, different types of resin and membrane. In this study, caseins are hydrolysed into CPPs and non-phosphorylated peptides (CNPPs) during the enzymatic hydrolysis process of CPPs. In comparison with CNPPs, CPPs possess a higher amount of PO_3^{2-} with negative charge. The abundant of PO_3^{2-} in CPPs allow these compounds to bind with an anion-exchange resin leading to the separation of CPPs from CNPPs. This provides possibilities for enriching CPPs with a low molecular mass, and can be easily up-scaled. Combined with EMR, the use of AEC for continuous reaction and simultaneous separation and purification of CPPs could facilitate to ameliorate the problems associated with production of CPPs. Based on these facts, the aim of this research is to establish a continuous method for the preparation and isolation of high quality CPPs using a combination of EMR and AEC.

2. Materials and methods

2.1. Materials

The UF cell, using tangential flow filtration (TFF) membrane, consisted of a Pellicon® 2 "Mini" Filter PLBC (Generated cellulose) cassette – Catalogue No. P2PLBCC01 (size 0.1 m^2, 3 kDa), Pellicon® 2 "Mini" Filter PLCCC (Generated cellulose) cassette – Catalogue No. P2C005C01 (size 0.1 m^2, 5 kDa), Pellicon® 2 "Mini" Filter PLCGC (Generated cellulose) cassette – Catalogue No. P2C010C01 (size 0.1 m^2, 10 kDa), respectively. During use, the cassette was placed in a Millipore Pellicon™-2 Mini Holder Catalogue No. XX42PMini. A Millipore pump Catalogue No. XX80EL005 was used. All this equipment was purchased from Millipore Corporation, Bedford, MA, USA.

Casein isolate (Kjeldahl N × 6.38, ca. 91% w/w protein) was obtained from Gansu Hualing Casein Co., Ltd. Alcalase® 2.4 FG was purchased from Novozymes® A/S (Tianjin, China). Macroporous Weakly Basic Anion-exchanger D303 was kindly provided by Shanghai Huazhen Sci. & Tech. Co., Ltd. All the chemicals and reagents were of analytical or guaranteed grade.

2.2. Casein isolate hydrolysis

The experiments were performed using a set up illustrated in Fig. 1. Hydrolysis conditions were determined according to the preliminary experiments. Casein (6% (w/v)) was hydrolysed with Alcalase 2.4 FG (initial reaction volume 300 mL, ratio of Alcalase concentration to substrate concentration, E/S = 0.06 mL/g casein) at 50 °C and pH 8.5 in a 500 mL stirred jacketed tank reactor with a thermostatic setup. The pH of the reaction was kept constant by adjustment with 2 mol/L NaOH. The hydrolysates were pumped continuously to the TFF unit from which products small enough to pass through the membrane were collected as permeate. Large polypeptides, enzyme and native protein were recycled back to the hydrolysis tank.

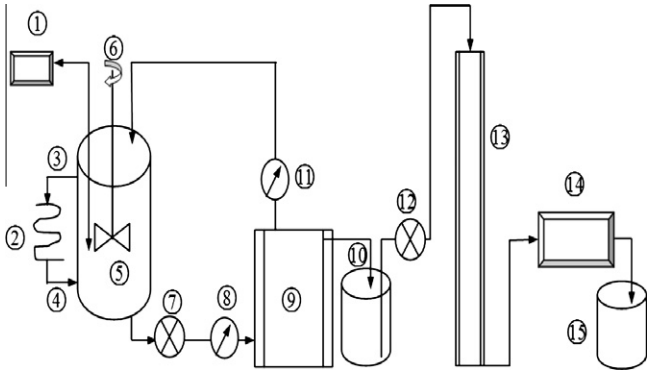

Fig. 1. The EMR in operation in our process with the Pellicon 2 cassette ultrafiltrtion membrane incorporated into the reaction chamber. (Legend: 1. pH-metre; 2. Thermostatic water heater; 3. Cool water from reactor; 4. Heating water in; 5. Jacketed tank reactor; 6. Stirrer; 7. Peristaltic pump; 8. Feed pressure gauge; 9. Pellicon 2 cassette assembly; 10. Permeate collector; 11. Retentate pressure gauge; 12. Peristaltic pump for chromatography; 13. Chromatography column containing styrene resin D303; 14. UV detection; and 15. Eluent collector).

Table 1
Two different running model designs for three membranes with different molecule cut-off.[a]

NMWCO (kDa)	Constant retentate pressure (0 bar)		Constant retentate pressure (0.5 bar)	
	Recirculation flow rate (mL/min)	Constant J_i[a] (mL/min)	Recirculation flow rate (mL/min)	J_i[a] (mL/min)
3	360	10	105	10
5	310	10	105	16
10	70	10	105	75

[a] Initial permeate flow rate.

The initial water permeate flow rate (J_i) was set with deionized water equilibrated at the working retentate temperature according to the method of Cheison et al. (2007) with few modifications. An outlet valve on the return line was used to regulate the retentate pressure. Casein was dissolved in deionized water and the temperature and pH stabilized. Enzyme hydrolysis was carried out for 5 min before the pump was started, to reduce membrane fouling caused by large molecular weight protein. The conditions of UF were carried out according to the summary of experimental design in Table 1. First, the valve was unscrewed on return line maximum and the initial flow rate (J_i) was adjusted to 10 mL/min by regulating the recirculation rate. Secondly, the retentate pressure was controlled at 0.5 bar by adjusting the retentate pressure control valve and the recirculation rate was 105 mL/min. These settings were also found to be acceptable during the screening stage.

During hydrolysis, a specified volume of 3% fresh substrate solution in each experimental run was prepared. Each time 25 mL of permeate was collected, 3% fresh casein solution was added to keep the reactor concentration and volume constant. The supplementing substrate ran out within the set time. Dosing with deionized water continued the reaction in order to maximise the yield of hydrolysates during the 3 h hydrolysis. At the end of the reaction, the reflux outlet was completely opened to drain and clean the membrane with 100 mL deionized water. Cleaning also recovered as much residual reaction as possible.

2.3. Selection of the molecular weight cut-off of the membrane

The effect of different nominal molecular weight cut-off (NMWCO) UF membrane (3 kDa, 5 kDa, 10 kDa) on separation