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**CONTINUOUS HYDROLYSIS OF β -CASEIN IN A MEMBRANE
REACTOR : PREPARATION OF A BIOACTIVE PEPTIDE**

S. Bouhallab*, D. Mollé and J. Léonil

*INRA, Laboratoire de recherches de technologie laitière,
65, rue de Saint-Brieuc, 35042 Rennes cedex, France.*

SUMMARY

Continuous Stirred Tank Membrane Reactor was used to investigate the continuous and selective extraction of a bioactive peptide β -CN (193-209) from bovine β -casein/chymosin hydrolysate. It was shown that the feasibility of the process depends on the nature and the area of ultrafiltration membrane used. With an inorganic (carbon-zirconia) membrane, high retention of the peptide constitutes a limit to the operation. However, when the reactor was equipped with a cellulosic type membrane, satisfactory transmission of the peptide was obtained. As shown by RP-HPLC and mass spectrometry analysis, only β -CN (193-209) permeates through the membrane.

INTRODUCTION

Continuous Stirred Tank Membrane Reactor (CSTMR) has been proposed for continuous hydrolysis of proteins in order to produce peptidic hydrolysates with improved nutritional and/or functional properties (Maubois *et al.*, 1980 ; Cheryan and Mehaia, 1986). Extensive degradation of proteins from various origins was achieved with this process by using enzymes with low specificity such as acidic protease (Iacobucci *et al.*, 1974), pronase (Deeslie and Cheryan, 1981), and alcalase (Bressolier *et al.*, 1988). The main objective of these studies was a complete hydrolysis of proteins into products small enough to permeate through the membrane.

In a previous work (Bouhallab *et al.*, 1992), we reported the first application of this process for continuous separation of bioactive peptides. From these data, it appears that continuous isolation of specific peptide(s) from reaction mixture requires 1- use of a highly specific enzyme ; 2- rapid release of the desired peptide ; 3- large molecular weight difference between bioactive peptides and others to promote selective separation ; 4- high transmission rate of bioactive molecule through the ultrafiltration (UF) membrane.

The present work extends these observations by showing the effect of a separation step on the performance of the process using another model : β -casein hydrolysis by chymosin (E.C. 3.4.23.4). Such a reaction leads to a mixture which essentially contains, regardless of the experimental conditions, peptides β -CN (193-209, Mr= 1881 Da and β -CN (1-192, Mr = 22000 Da) (Carles and Ribadeau-Dumas, 1984). Our aim was to study the continuous production of β -CN(193-209), an immunomodulatory peptide (Coste *et al.*, 1992).

MATERIALS AND METHODS

Preparation of β -casein was adapted from that described by Manson and Annan (1971) : after dissociation of β -casein at low temperature (4°C), microfiltration and ultrafiltration were used to isolate β -casein from depleted casein micelles. Chymosin (E.C. 3.4.23.4) was purified from rennet powder according to Garnot and Mollé (1982). HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). All other products were of analytical grade.

Hydrolysis of β -casein in batch reactor : β -casein (1 g/l, purity= 90%) was hydrolysed in distilled water by chymosin (molar ratio E/S = 1/2000) at 32°C, pH 6.2. The pH was maintained constant by addition of 0.05 M NaOH with a pH-stat. After 2 h, the reaction was stopped by heat inactivation of the enzyme (90°C, 15 min).

Continuous hydrolysis of β -casein in membrane reactor : the main system components of the reactor included a 1.5 l vat coupled to a membrane module via a volumetric pump (PCM 1.7 l 10 type, Vanves, France), a heat exchanger, a pH-stat, two pressure gauges, an electro-pneumatic valve (Masoneilan, Varipak type, Neuilly/Seine, France) and a peristaltic pump to feed fresh substrate solution. The volume of the system was maintained constant by matching the incoming feed flow rate to the permeate outflow. The loop recycling flow rate was 300 l/h. Two types of UF membranes were used: inorganic M5 membrane, nominal cut-off (MWCO) of 10 kDa and a 0.0226 m² filtering area (Tech-Sep, Miribel, France) and cellulosic type membrane, spiral-wound UF cartridge S10Y3, MWCO of 3 kDa, and a 0.95 m² filtering area (Amicon, Lexington, MA, USA).

Continuous hydrolysis experiments were conducted by first loading the reactor tank with 1.25 l of β -casein solution (1 g/l). After adjusting temperature (32°C) and pH (6.2) with 0.05 M NaOH, 0.9 mg of chymosin was added. The reactor pumps were immediately activated and the system regulated.

Experiments described here were carried out under four different conditions as follows : Experiment 1 : Continuous degradation of β -casein in the reactor equipped with zirconia (ZrO₂) membrane. Permeate flux = 11 l/h.m² ; residence time = reactor volume/permeate flow = 300 min. Transmembrane pressure (TP) increased progressively from 2 to 6 bars at the end of experiment (420 min). Experiment 2 : Ultrafiltration of batch hydrolysate in the presence of 1.3 M NaNO₃ for 4 h with permeate recycling. Experiment 3 : As experiment 2 but NaNO₃ was replaced by methanol 60% (v/v) in the hydrolysate. Experiment 4 : Continuous hydrolysis in the reactor equipped with cellulosic membrane. Permeate flux = 2 l/h.m² ; TP = 1 bar ; residence time = 37.5 min.

The transmission, Tr , of the peptide was calculated according to the equation :

$Tr \% = 100 \times C_p/C_r$ where C_p and C_r are respectively the concentrations of peptide in the retentate and in the permeate as determined by RP-HPLC analysis. For ZrO_2 membrane the experimental Tr was compared to the theoretical Tr according to the equation reported by Nau *et al.* (1993) : $Tr = 100 \times (1 - (1 - ((Mr/MWCO)^{1/3})^2)^2)$.

High Performance Liquid Chromatography (HPLC) : all separations were carried out on Vydac C18 column 218TP54 (Touzart & Maignon, Vitry/Seine, France). Operating conditions were : column temperature, 40°C ; flow rate, 1 ml/min ; solvent A : 20 mM sodium-phosphate pH 6.9; solvent B : 60% acetonitrile and 40% A. The elution was obtained by linear gradient from 0 to 80% solvent B in 45 min. The absorbance was recorded at 214 nm.

Identification of peptides : β -CN (193-209) was identified and quantified after acid hydrolysis by 6N HCl for 24 h at 110°C in a Pico-Tag station (Waters) and PITC derivatization of amino acids according to Bidlingmeyer *et al.* (1984). Electro-spray mass spectra was obtained on a Sciex API-I quadripole mass spectrometer (Thornhill, Ontario, Canada) equipped with atmospheric pressure ionization source. The samples, diluted in 80% aqueous acetonitrile and 0.1% (v/v) trifluoroacetic acid, were introduced at a flow rate of 5 μ l/min by a 22 infusion pump (Harvard Apparatus, MA, USA).

RESULTS AND DISCUSSION

The degradation of bovine β -casein by chymosin occurs in several distinct steps, the most susceptible bonds being successively Leu(192)-Tyr(193) and Ala(189)-Phe(190) (Pélicissier *et al.*, 1974 ; Carles and Ribadeau-Dumas, 1984). This reaction leads to the release of the C-terminal fragment β -CN (193-209), a bioactive peptide with an immunomodulating activity.

Experiments using ZrO_2 inorganic membrane :

In experiment 1, continuous hydrolysis of β -casein carried out with inorganic type membrane, resulted in highly conversion (90%) of the substrate. However, the bioactive peptide does not permeate through the UF membrane. Its transmission was only 5% at the steady state of the process. By applying the recently developed theory for the solute transmission through ZrO_2 membrane (Nau *et al.*, 1993) (see materials and methods), the expected Tr for β -CN (193-209) is 33%. Hence, some phenomena such as membrane fouling and/or electrostatic and hydrophobic interactions between peptide and membrane support might explain the difference between theoretical and experimental transmission. For this purpose, when β -casein was first hydrolysed and then ultrafiltered in the presence of either 1.3 M $NaNO_3$ to screen out the electrostatic interactions (experiment 2), or 60% methanol to screen out hydrophobic interactions (experiment 3), the β -CN (193-209) transmission was not notably improved (table 1). A more detailed investigation of the factors which affect β -CN (193-209) transport through ZrO_2 membrane is in progress.

Experiment using a cellulosic type membrane:

In order to investigate the influence of membrane nature on β -CN (193-209) transmission, the aforementioned inorganic membrane was replaced by a cellulosic one (experiment 4). The cut-off of 3 kDa was chosen, instead of 10 kDa, to achieve complete retention of substrate, enzyme and large other products. Figure 1 shows the RP-HPLC patterns of retentate and permeate of continuous process. At steady state, a good transmission,

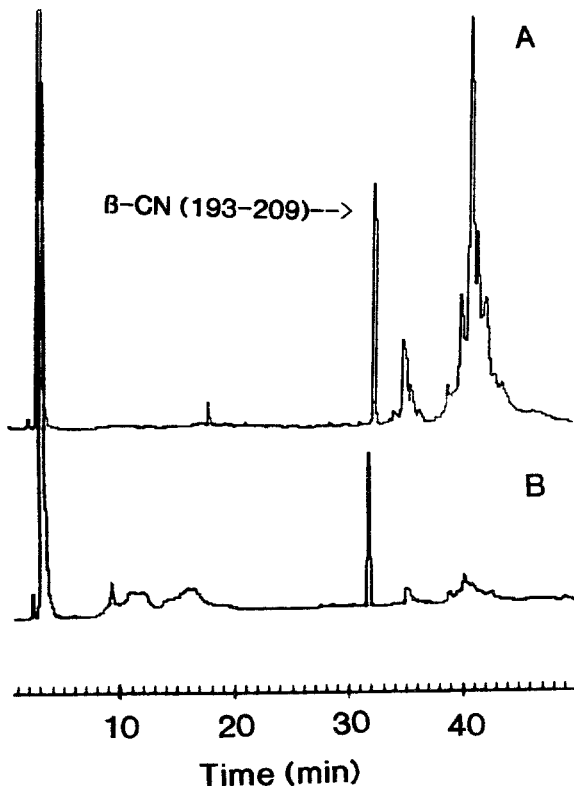


Figure 1: RP-HPLC elution patterns of retentate (A) and permeate (B) fractions after 180 min of the continuous process under the conditions of experiment 4.

i.e. 34%, was obtained (figure 2). Under these operating conditions, 40% of β -casein were transformed. Hence, the productivity of the process, after 420 min, was 160 mg of bioactive peptide in the permeate/ mg of enzyme. Figure 3 shows the electro-spray mass spectrometry analysis of the final permeate. On the other hand, no tripeptide Phe(190)-Leu-leu(192) was detected contrary to findings in permeates of experiments 1 to 3. This result might be due to the fact that its presence was too small to be detected or to the low operating residence time, assuming that the enzymatic cleavage of bond Leu(192)-Tyr(193) is much faster than that of bond Phe(189)-Leu(190) (Carles and Ribadeau-Dumas, 1984). Table 1 gives the transmission values of β -CN (193-209) obtained during the four experiments.

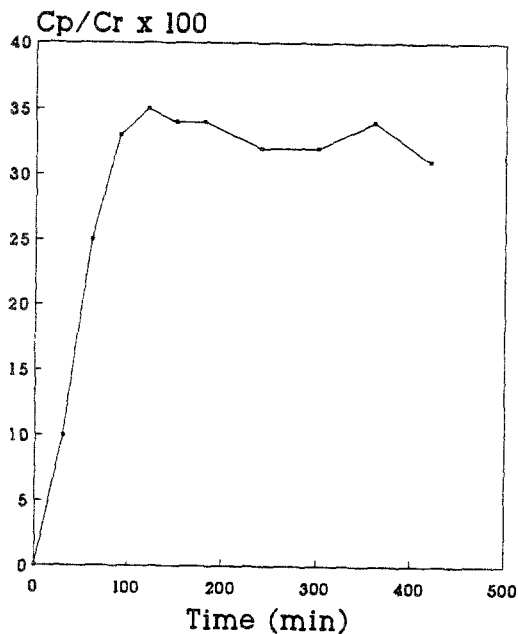


Table 1: Transmission rate of β -CN (193-209) under the four conditions studied.

Experiment	transmission (%)
Experiment 1	5
Experiment 2	5
Experiment 3	8
Experiment 4	34

Figure 2: Evolution of β -CN (193-209) in the permeate during the continuous process (experiment 4).

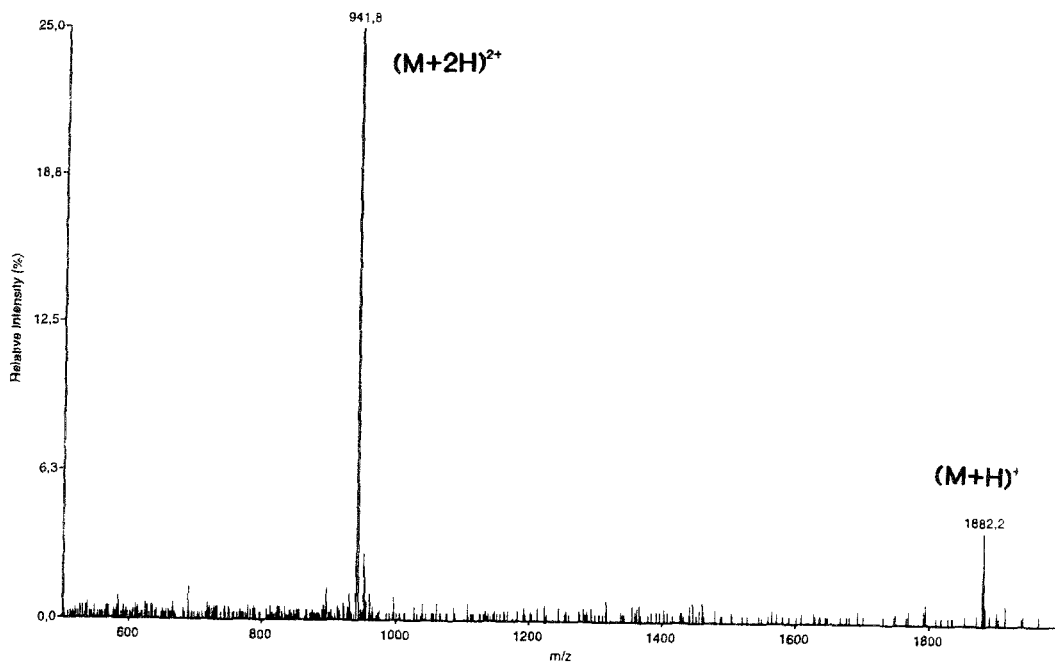


Figure 3: ES-mass spectra of the permeate (experiment 4). Calculated $M_r = 1881.26$ Da. Determined $M_r = 1881.39$ Da.

CONCLUSION

The most satisfactory peptidic transmission observed in the last experiment is probably related to the hydrophilic nature and high filtering area of the spiral-wound cellulosic membrane. In the case of the zirconia membrane, the low filtering area used impose operating conditions which increase concentration polarisation and enhance membrane fouling. These phenomena and others, i.e. hydrophobic and electrostatic interactions, lead to the high retention of β -CN (193-209). Studies on purified peptide behaviour should help us to understand the mechanisms which govern the β -CN (193-209) transmission through a mineral membrane.

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