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Original article

Continuous hydrolysis of caseinomacropeptide in a membrane reactor: kinetic study and gram-scale production of antithrombotic peptides

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Summary — A recycle ultrafiltration membrane reactor was used for continuous production of antithrombotic peptides from enzymatic digestion of caseinomacropeptide (CMP). The kinetic parameters of the continuous process were determined on hydrolysis of various substrate concentrations and compared to those of the batch reactor. On the basis of peptide liberation, enzymatic activity was 10-fold lower during the continuous process (according to k_{cat} values) while the K_m value was not modified. Taking into account these data, an experiment for bioactive peptide production from continuous hydrolysis of 34 g of CMP was carried out. After 6 h of continuous operation, permeate was concentrated by nanofiltration (volume concentration ratio = 12.5), freeze-dried, and the productivity as well as peptide yield determined. Although desired peptides were partially rejected by the ultrafiltration membrane during the continuous process, 2.65 g of these small molecules (500 to 780 Da) were obtained in the final powder.

caseinomacropeptide / hydrolysis / kinetic / antithrombotic peptide / production

Résumé — Hydrolyse continue du caséinomacropeptide en réacteur enzymatique à membrane : étude cinétique et production de peptides antithrombotiques. *Le réacteur enzymatique à membrane a été utilisé pour l'hydrolyse en régime continu du caséinomacropeptide (CMP) en vue de la production d'une famille de peptides de faible masse moléculaire, dotés d'activité antithrombotique. La transformation de diverses concentrations de substrat a permis de déterminer les paramètres cinétiques en régime continu et de les comparer à ceux obtenus en batch. Alors que la valeur de la constante de Michaelis-Menten (K_m) n'est pas modifiée, les valeurs des constantes catalytiques indiquent que l'activité de l'enzyme est 10 fois plus faible en réacteur à membrane. Utilisant une concentration en CMP proche du K_m, une expérience de production continue de peptides bioactifs à l'échelle du gramme a été réalisée. Après hydrolyse de 34 g de CMP, le perméat était concentré par nanofiltration (facteur de concentration volumique = 12,5) puis lyophilisé. La caractérisation du produit final obtenu indique que 2,65 g de peptides bioactifs étaient ainsi obtenus, soit un rendement de 53% par rapport à la quantité théorique attendue.*

caséinomacropeptide / hydrolyse / peptide antithrombotique / cinétique / production

INTRODUCTION

The primary function of dietary proteins is to supply the body adequately with essential amino-acids and organic nitrogen. Attention is now increasingly paid to food proteins, in particular milk proteins, which are recognized as a source of biologically active peptides (Fiat and Jollès, 1989; Maubois and Léonil, 1989). A first bioactive fraction from β-casein was identified in the late 70's as having morphine-like properties (Brantl *et al*, 1979). Since that time a great deal of research has been carried out and, up to day, about 12 active sequences have been identified in various casein molecules (α_S, β and κ caseins). Such bioactive peptides can be released by enzymatic proteolysis *in vitro* and *in vivo*. During an *in vitro* digestion process, the limiting step is the purification of the desired peptide from the reaction mixture.

A continuous recycle ultrafiltration (UF) membrane reactor, which combines enzymatic hydrolysis of substrate and simultaneous separation of the products, may be an attractive process for specific peptide separation. This bioreactor configuration has been widely used to produce hydrolysates with improved nutritional and/or functional properties (Maubois *et al*, 1979; Cheryan and Mehaia, 1986). In a previous work, we reported the first application of this continuous process for bioactive peptide preparation using caseinomacropeptide (CMP) / trypsin as model (Bouhallab *et al*, 1992). Small peptidic sequences derived from the N-terminal part of CMP have been identified as antithrombotic peptides which inhibited platelet aggregation and/or binding of fibrinogen to ADP treated blood platelets (Jollès *et al*, 1986; Maubois *et al*, 1991).

The continuous production of these small molecules in a membrane reactor was made possible because of the high molecular mass difference between these peptides and the other components in the system such as enzyme, substrate and complementary large fragments. In the present study, kinetic parameters of batch and continuous hydrolysis were compared. Productivity and peptide yield were determined on the basis of gram-scale preparation of these bioactive molecules.

MATERIALS AND METHODS

Reagents

Caseinomacropeptide (average M_r = 7500 Da) was prepared from sodium caseinate (Armor Protéines, St-Brice-en-Cogles, France) according to Brulé *et al* (1980). Its protein content, expressed on a dry basis, as total nitrogen (N) × 7.43 was 70%. The 7.43 factor was determined from amino acid composition of CMP. Trypsin (EC 3.4.21.4), M_r 23 500 Da, was from Novo industry (Copenhagen, Denmark). Trypsin activity was determined on the synthetic substrate N-tosyl-L-Arg-OMe. Acetonitrile, trifluoroacetic acid (TFA), as well as all other reagents used were analytical grade.

Batch hydrolysis of caseinomacropeptide

To investigate batch productivity, hydrolysis of CMP (187 μmol l^{-1}) was performed at 40°C in 10 mmol l^{-1} sodium phosphate buffer (pH 7.5). Digestion was started by trypsin addition (enzyme/substrate = 1/1560 (mol/mol)). After 3 h, the reaction was stopped by adjusting the solution to pH 2.2 with 5% TFA. To assess batch kinetic parameters from initial reaction rates, various substrate concentrations (from 47 to 933 μmol l^{-1}) were hydrolysed by addition of trypsin (0.2 μmol l^{-1}).

Continuous hydrolysis in a membrane reactor

Reactor system

The main components of the reactor included a 1.5 l vat coupled to a membrane module *via* a volumetric pump (PCM 1.7 I 10 type, Vanves, France), a heat exchanger, a pH-stat apparatus, two pressure gauges, an electropneumatic valve (Masoneillan, Varipak type, Neuilly-sur-Seine,

France), two flowmeters for retentate and permeate, and a peristaltic pump to feed fresh substrate solution. The volume (V) was maintained constant by matching the incoming feed flow rate to the permeate outflow (J).

Continuous experiments

Continuous experiments were conducted by first loading the reactor tank with the required substrate concentration. After adjusting temperature and pH, the enzyme solution was added. The pump connecting the tank to the membrane module was then activated and the system regulated. Experiments reported here were conducted at 40°C, reactor volume 1.25 l, tangential flow rate 2.95 m s^{-1}, permeate flow 0.25 or 0.5 l h^{-1}, pH maintained at 7.5 by continuous addition of 0.1 N NaOH, time duration 7 h. The influence of various substrate concentrations was studied with a single M5 inorganic membrane tube (TechSep, Miribel, France), which is a composite UF membrane of ZrO_2 filtering layer on a carbon support (6 mm id, 2.26 10^{-2} m^2 membrane area, molecular mass cut-off (MMCO) of 10 kDa). Before and after each run, the system was cleaned as described by Nau *et al* (1993). The water permeability of a cleaned membrane was 86 l h^{-1} m^{-2} bar^{-1} at 50°C. Caseinomacropeptide (M_r 7500 Da) at 1 g l^{-1} was completely rejected by this membrane at neutral pH. The same phenomenon has been reported by Tanimoto *et al* (1990).

For large-scale preparation of bioactive peptides, a high surface area membrane was used (Spiral-Wound UF Cartridge S10Y3, MMCO of 3 kDa, 0.96 m^2, Amicon, Lexington, MA, USA). In this last experiment, reactor volume and permeate flow were 1.9 l and 1.7 l h^{-1} respectively. Continuous hydrolysis was carried out during 6 h by addition of 1.6 μmol l^{-1} of trypsin at two times, *ie* 0 and 3 h. The permeate obtained (10.1 l) was concentrated to 0.82 l by an organic nanofiltration membrane R76A (Millipore) and then the retentate was freeze-dried. Operating conditions for the nanofiltration experiment were as follows: T = 40°C, pH 8, tangential flow rate = 0.6 m s^{-1}, transmembrane pressure (TP) = 10.7 bar (TP was controlled by gradual increase from 1 to 10.7 bar (1 bar min^{-1})).

Complete retention of trypsin in the presence of CMP by both UF membranes used was checked. No tryptic activity was detected in the permeates.

During all continuous experiments, samples from retentate and permeate were withdrawn, added to 5% TFA, and when needed concentrated by Speed-vac. Bioactive peptide liberation was quantified by RP-HPLC.

Analytical methods

Nitrogen content of the initial substrate and peptide powder were determined by the Kjeldahl procedure.

RP-HPLC analyses

Chromatographic analyses were carried out on an Ultrabase column C18 (4.5 × 250 mm; Shandon, Eragny, France). Operating conditions were as previously reported (Léonil and Mollé, 1990). Peptides were quantified from the linear relation between peak areas and injected amounts of individual peptides Met_{106}-Lys_{112} and Met_{106}-Lys_{111}. Bioactive peptides Lys_{112}-Lys_{116} and Asn_{113}-Lys_{116} were quantified only in the final powder.

Electro-spray mass analyses were carried out on a Sciex API-I quadripole mass spectrometer (Thornhill, Ontario, Canada) equipped with an atmospheric pressure ionisation source. The samples, diluted in 80% aqueous acetonitrile and 0.1% TFA, were introduced at 5 μl min^{-1} flow rate by a 22 infusion pump (Harvard apparatus, MA, USA)

RESULTS AND DISCUSSION

Batch experiments

CMP, a C-terminal part of κ-casein (Met_{106}-Val_{169}; average M_r = 7500 Da) is an heterogeneous mixture with respect to its different degrees of carbohydrate substitution. Its tryptic hydrolysis (fig 1) leads to the liberation of a family of four small antithrombotic peptides: Met_{106}-Lys_{112} (M_r = 785 Da); Met_{106}-Lys_{111} (656 Da); Lys_{112}-Lys_{116} (632 Da); Asn_{113}-Lys_{116} (503 Da); and the C-terminal complementary glycosylated macropeptide Thr_{117}-Val_{169} (average M_r =6230 Da). Using

TPCK-treated trypsin and on the basis of large fragments, *ie* Lys_{112}-Val_{169}, Asn_{113}-Val_{169} and Thr_{117}-Val_{169} quantification, Léonil and Mollé (1990) reported that among the three sensitive bonds, Lys_{111}-Lys_{112} and Lys_{112}-Asn_{113} were hydrolysed with the same kinetic parameters (K_m = 0.2 mmol l^{-1}; k_{cat} = 2.97 s^{-1}) and more rapidly than the Lys_{116}-Thr_{117} bond. In the present study, hydrolysis of both highly sensitive bonds was studied by monitoring the liberation of the two peptides Met_{106}-Lys_{111} and Met_{106}-Lys_{112}. The kinetic constants for the batch reactor were evaluated with various CMP concentrations. The Michaelis-Menten constants determined from the Lineweaver-Burk plot are reported in table I. The K_m value compares well with that reported for the same peptide bond hydrolysis on carbohydrate-free CMP followed by the liberation of large peptides (Léonil and Mollé, 1990). However, k_{cat} determined in the present work was 2-fold higher than that already reported. This discrepancy may be attributed to differences in specific activities of the two trypsin preparations.

Continuous reactor kinetics

The model used for the hydrolysis of CMP in the membrane reactor is based on the one developed by Deeslie and Cheryan (1981). However, conversion rate (X) is expressed on molar basis:

$$v = \frac{J \times So}{V}$$

$$X = \frac{\text{mmol } l^{-1} \text{ peptide in the permeate}}{\text{mmol } l^{-1} \text{ initial substrate concentration}} \quad (1)$$

where v = rate of reaction at steady-state of the process (mmol peptides l^{-1} min^{-1}); J = permeate flow (ml min^{-1}); X = substrate conversion (% of peptides produced); So = initial CMP concentration (mmol l^{-1}); V = reactor volume (ml).

The data obtained with the continuous reactor at various substrate concentrations are shown in figure 2. An apparent steady-state was reached after 90 min under these conditions, taking longer at higher substrate concentration as expected according to the reactor configuration (Mannheim and Cheryan, 1990). The steady-state values were used to calculate X for reaction rate determination (equation 1). The linearization plot for the continuous process is shown in figure 3 and the corresponding kinetic constants are given in table I. Whereas the K_m value was the same as the batch reactor, V_{max} and k_{cat} were 10-fold lower during the continuous system, indicating significant reduction in enzyme activity. Since the same enzyme concentration was used, these values mean that only 10% of the initial enzyme concentration was being utilized in the membrane reactor. Enzyme leakage through UF membrane and/or thermal inactivation are known to affect the catalyst during continuous process (Deeslie and Cheryan, 1982). However, complete rejection of the enzyme by the membrane during continuous hydrolysis of CMP was checked by incubation of concentrated permeates withdrawn at steady-state with synthetic substrate. Also, the activity reduction may be caused by some shear stress resulting from the pump, pressure valve and/or membrane module (Charm and Wong, 1981). However, kinetic parameters

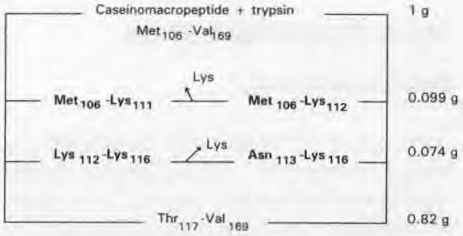

Fig 1. Action of trypsin on the three basic sites of caseinomacropeptide (CMP) and total amounts of bioactive peptides expected at 100% conversion of 1 g of CMP.
Action de la trypsine sur le caséinomacropeptide (CMP) et les quantités de peptides bioactifs attendues par hydrolyse de 1 g de CMP.

Table I. Kinetic parameters for the batch and continuous recycle membrane reactor.
Paramètres cinétiques de l'hydrolyse du caséinomacropeptide en batch et en réacteur enzymatique à membrane.

Kinetic constants	*Batch*	*Continuous UF reactor*
K_m (mmol l^{-1})	0.2	0.34
V_{max} (mmol l^{-1} min^{-1})	0.11	0.01
k_{cat} (s^{-1})	8.5	0.85
V_{max}/K_m (s^{-1})	32.3	1.92

determined in continuous reactor are only apparent values because, in the kinetic model used, rejection of product by the membrane was not taken into account. In this model, it was assumed that there were no mass transfer limitations. In fact, conversion rate (X) as expressed in the model, integrates enzymatic reaction as well as ultrafiltration behaviour of the products. This could lead to an underestimation of peptide concentration released at steady-state and consequently of the real activity of the enzyme. The average rejection coefficient of these products at steady-state, as determined by RP-HPLC, varied from 20 to 50% depending on the substrate concentration used.

The determined kinetic parameters were explored for large scale preparation of bioactive peptide fraction. The K_m value establishes an approximate value of substrate concentration to be used. A higher substrate concentration would cause a rapid decrease in performance due to fouling caused by the accumulation of high molecular mass products.

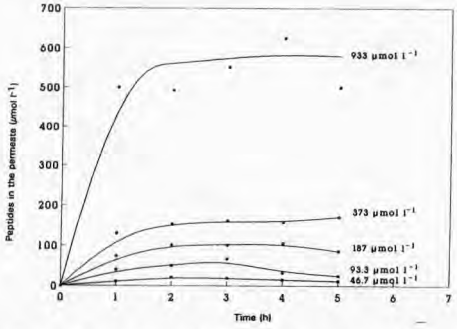

Fig 2. Evolution of products (peptides Met_{106}-Lys_{111} + Met_{106}-Lys_{112}) in the permeate of a membrane reactor at various substrate concentrations.
Hydrolyse continue du CMP en réacteur enzymatique à membrane. Évolution de la concentration des peptides Met_{106}-Lys_{111} + Met_{106}-Lys_{112}) dans le perméat en fonction de la concentration en substrat.

Production of bioactive peptides: application experiment

Continuous production of bioactive peptides from CMP at 373 µmol l^{-1} (total substrate treated = 34 g; enzyme, 2 × 1.6 µmol l^{-1}) was done according to the scheme presented in figure 4. The yield of bioactive peptides during various steps is shown in table II. During the continuous hydrolysis (step 1) 59% of the expected amount of the two peptides Met_{106}-Lys_{111} and Met_{106}-Lys_{112} was obtained in the permeate without the diafiltration step of the retentate. Mass spectroscopy analysis of the retentate at the end of the continuous hydrolysis (after 6 h) indicates that total conversion of the two bonds Lys_{111}-Lys_{112} and Lys_{112}-Asn_{113} was reached (no native CMP was detected). Hence, this relatively low

Table II. Yield of bioactive peptides during continuous process and concentration steps.
Évolution du rendement des peptides antithrombotiques lors des différentes étapes de préparation.

Peptides	*Theoretical amount*[a]	*Permeate step 1*		*Permeate step 2*		*Powder*	
	(g)	*(g)*	*Yield (%)*	*(g)*	*Yield (%)*[c]	*(g)*	*Yield (%)*[c]
Met_{106}-Lys_{111} + Met_{106}-Lys_{112}	2.8	1.65	59	1.53	93	1.47	89
Lys_{112}-Lys_{116} + Asn_{113}-Lys_{116}[b]	2.1	-		-		1.18	

Step 1: continuous hydrolysis in a membrane reactor; step 2: nanofiltration of permeate step 1; step 3: freeze-drying of retentate step 2. [a]Theoretical amount is equivalent to 100% hydrolysis at the three basic sites of CMP (see fig 1); [b]quantified only in the final powder; [c]yield of concentration steps.
Étape 1 : Hydrolyse continue en réacteur enzymatique à membrane ; étape 2 : nanofiltration du perméat de l'étape 1 ; étape 3 : lyophilisation du rétentat de l'étape 2. [a] Quantité théorique attendue après 100% d'hydrolyse du CMP total utilisé (voir fig 1) ; [b] quantifiés uniquement dans le produit fini ; [c] rendement de l'étape de concentration.

yield is due to rejection by the UF membrane. The average rejection coefficient (σ) was 63%, as determined from yield equation reported by Cheryan (1986)

:

$$1 - \frac{C_p V_p}{C_0 V_0} = (VCR)^{\sigma - 1}$$

where C_p and C_0 are peptide concentrations in the permeate and feed solution respectively; V_p and V_0 are volume of the permeate and total volume treated respectively; and VCR is volume concentration ratio. The downstream steps were carried out without major loss of the bioactive peptides. The good yield after nanofiltration (VCR = 12.5) indicates that this membrane technology is well suited for concentrating small peptides. The final product contained 2.65 g of bioactive peptides (including bioactive peptides derived from the hydrolysis of Lys_{116}-Thr_{117} bond). This represents 53% of the expected theoretical amount.

The productivity of the continuous process expressed as mass bioactive peptides obtained/mass enzyme was 18.5. Although a significant part of bioactive peptides was re-

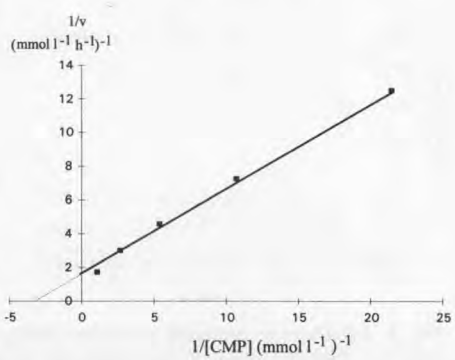

Fig 3. Lineweaver-Burk plot for CMP-trypsin hydrolysis in a membrane reactor.
Linéarisation selon la représentation de Lineweaver-Burk des données obtenues à l'état stationnaire lors de l'hydrolyse des différentes concentrations du CMP (fig 1).

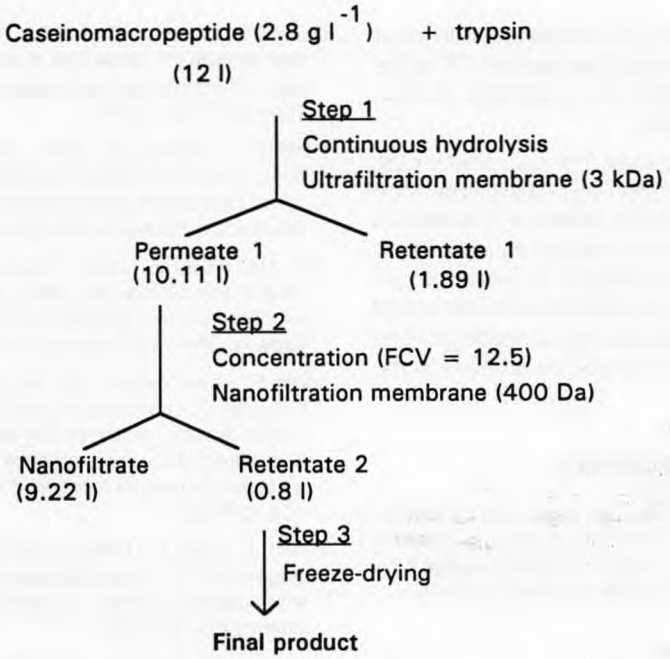

Fig 4. Continuous production and concentration steps of antithrombotic peptides.
Étapes de production continue et de concentration des peptides antithrombotiques.

jected by the UF membrane, the productivity of this process was 3-fold higher than that expected at 100% conversion (100% hydrolysis of the three bonds) in a batch system after only 5.5 reactor volume replacements. Performance of the continuous system would be greater taking into account that the batch process requires added steps such as enzyme inactivation and bioactive peptide separation from the reaction mixture.

The purity of bioactive peptides in the final product, expressed on molar basis reached 82%. However, on the basis of mass ratio, the purity decreased to about 50% due to the presence of 2.5 g of high molecular mass peptides. The transfer of this material through the UF-membrane, combined with the above-mentioned relatively high bioactive peptide rejection, may be explained by a change of membrane selectivity during the continuous hydrolysis step.

CONCLUSION

A continuous recycle membrane reactor combined with nanofiltration is a powerful process for large-scale production and concentration of bioactive peptides derived from food proteins. In this study, 2.65 g of small bioactive peptides (500 to 780 Da) were obtained from continuous tryptic hydrolysis of caseinomacropeptide (6 h) and nanofiltration of the permeate (1 h). However, the purity of the final product (mass of bioactive peptides / mass of total peptides) was not as high as expected due to the transmission of high molecular mass peptides (M_r > 6200 Da) through the ultrafiltration membrane (cut-off 3 kDa). Higher purity should be

obtained by using UF membrane with lower cut-off or by carrying out another UF of the permeate in order to decrease large molecular mass peptides.

At enzymatic level, the k_{cat} value for the continuous operation was 10-fold lower than for the batch process. However, this may not reflect the real enzymatic activity in the retentate side, since rejection of individual products was not included. A new kinetic model that incorporates rejection is needed for better estimation of kinetic parameters in the retentate side.

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