Tryptic hydrolysis of k-caseinomacropeptide: Control of the enzymatic reaction in a continuous membrane reactor

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Tryptic hydrolysis of κ-caseinomacropeptide: Control of the enzymatic reaction in a continuous membrane reactor

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The kinetics of the tryptic release of bioactive peptides from caseinomacropeptide was investigated in both batch and continuous mode in an enzymatic membrane reactor. The hydrolysis of the three susceptible bonds, $Lys_{111}–Lys_{112}$, $Lys_{112}–Asn_{113}$, and $Lys_{116}–Thr_{117}$, was monitored by quantitative determination of the released products. A kinetic study in the batch system showed that the overall catalytic process follows a sequential mechanism where the $Lys_{116}–Thr_{117}$ bond was only cleaved on the intermediary products resulting from the cleavage of the $Lys_{111}–Lys_{112}$ and $Lys_{112}–Asn_{113}$ bonds. When the reaction was performed in the continuous enzymatic membrane reactor, it was found that the enzyme preference toward the $Lys_{116}–Thr_{117}$ bond depended on the relative concentrations of both the caseinomacropeptide and the intermediary products accumulated at steady state. Such concentrations were controlled by the enzyme and substrate concentrations and the substrate feeding flow rate; hence, by control of the operating parameters and with the understanding of the reaction mechanism, the enzyme action toward various peptidic bonds can be oriented in the continuous mode, offering the possibility of better control of the type of product recovered in the reactor output. © 1998 Elsevier Science Inc.

Introduction

Enzymatic hydrolysis of proteins is an attractive means of obtaining hydrolysates with improved nutritional and functional properties.[1,2] In the last decade, proteins and their hydrolytic products have become recognized as functional foods since they constitute a source of several biologically active peptides.[3] Concurrent with research aimed at establishing the use of these components in emerging functional foods, the design and development of process for their continuous production has increasingly received considerable attention by the food industry.

The enzymatic membrane reactor (EMR) which integrates enzymatic hydrolysis, product separation, and catalyst recovery into a single operation is an attractive configuration for this purpose. It has been widely applied to total conversion of food proteins of various origins in order to produce hydrolysates with improved nutritional and/or functional properties.[4–7] In the last few years, the use of EMR for the continuous production of specific peptidic sequences, i.e., bioactive peptides, has been reported.[8,9] This new approach requires i) highly specific enzymes; ii) better control of the enzymatic reaction; and iii) high selective membranes to separate the desired peptides from the reaction mixture.[8]

Until now, the continuous extraction of bioactive peptides in EMR was mainly applied to milk proteins since they contain peptide sequences implicated in biological functions such as the biotransfer of minerals, opiate activity, immunomodulation, antihypertension, and antithrombosis.[10] In particular, the antithrombotic activity of a family of small peptides derived from κ-caseinomacropeptide (CMP) has been reported by Jollès *et al.*[11] CMP is the C-terminal part of κ-casein (sequence 106–169). It contains three specific cleavage sites by trypsin, $Lys_{111}–Lys_{112}$, $Lys_{112}–Asn_{113}$, and $Lys_{116}–Thr_{117}$, located in the N-terminal part of the molecule. The complete action of trypsin on the three bonds results in the release of a large peptide (Mw > 6,000 g mol^{-1}) and a family of small antithrombotic peptides

Met - Ala - Ile - Pro$_2$- Lys ┊ Lys ┊ Asn - Gln - Asp - Lys ┊ Thr - Glu - ....- Val

106   111 ┊ 112 ┊ 113   116 ┊ 117   169
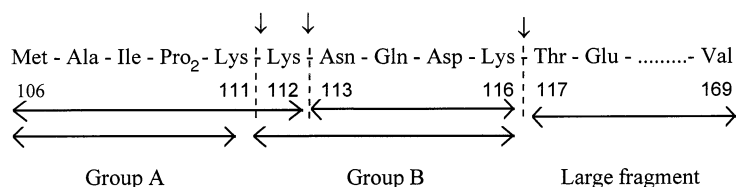
Group A   Group B   Large fragment

Figure 1 Amino acid sequence of the N-terminal part of caseinomacropeptide. Arrows indicate the tryptic cleavage sites

($Mw < 700$ g mol^{-1}). In a previous paper, we demonstrated the feasibility of continuous production of these bioactive peptides in an ultrafiltration EMR.[12] We concluded that optimization of the continuous process needs the control of enzymatic release of desired products as well as their transfer through the membrane which means acquiring knowledge of the mechanisms involved in both operations. The separation aspect as well as the mechanism involved in peptide transmission through a nanofiltration membrane have been further investigated;[13] hence, the purpose of the present work was to study the mechanism of enzymatic hydrolysis of the three lysyl bonds of CMP by trypsin in both batch and continuous EMR. The rates of tryptic cleavage at various substrate and enzyme concentrations were determined from individual peptide quantification at steady state. The mechanism of hydrolysis as well as the influence of continuous mode on the enzymatic cleavage of the three bonds is then discussed.

Materials and methods

Reagents

Caseinomacropeptide used as substrate was prepared from sodium caseinate (Armor Protéines, St. Brice-en-Coglès, France) according to Brulé *et al.*[14] with a purity of 80%. Porcine pancreatic trypsin (EC 3.4.21.4) was obtained from NOVO Industry (Copenhagen, Denmark). Its proteolytic activity was 159 units mg^{-1} as determined on the synthetic substrate *p*-tosyl-L-arginine methyl ester (Sigma Chemical, St. Louis, MO). Isopropanol (Normapur, Prolabo, Paris, France), trifluoroacetic acid (TFA; Pierce Chemical, Rockford, IL) together with all other chemicals used were analytical reagent grade.

Tryptic hydrolysis of CMP

The time-course hydrolysis of the three lysyl bonds of CMP was monitored by quantitative determination of the four small peptides

released, group A (106–112 and 106–111) resulting from the cleavage of Lys$_{111}$–Lys$_{112}$ and Lys$_{112}$–Asn$_{113}$ bonds, and group B (113–116 and 112–116) resulting from the cleavage of Lys$_{116}$–Thr$_{117}$ bond (*Figure 1*).

Batch experiments

Batch hydrolysis was performed under stirring at 40°C in a 10 mM sodium phosphate buffer pH 7.5. The reaction was started by the addition of trypsin. At selected times, aliquots were taken and the reaction stopped by adjusting the solution to pH 2 with 5% (v/v) TFA. Kinetic parameters were determined from the initial rates obtained at substrate concentrations ranged from 0.05–1.07 $\times$ 10^{-3} mol l^{-1} using trypsin at 0.26 $\times$ 10^{-6} mol^{-1}.

Continuous experiments in EMR

Figure 2 shows the scheme of the membrane reactor system. The main components included a 1.5-l vessel coupled to a membrane module via a volumetric screw pump (PCM 1.71 10 type, Vanves, France), a heat exchanger, two pressure gauges, a pH-stat apparatus, two flow meters for retentate and permeate, and an electropneumatic valve (Masoneillan, Varipak type, Neuilly-sur-Seine, France). The reactor was equipped with an inorganic nanofiltration membrane, Kerasep Solgel (Tech-Sep, Miribel, France) which completely retains the enzyme and the substrate.[15] It consisted of a ZrO$_2$ layer over a mineral tubular support (19 channels; 2.5 mm inner diameter; 0.85 m long; 0.127 m^2 filtering area). All data were collected through an analog to a digital convector multichannel analyzer (μMac 1060, Analog Devices, Norwood, MA) and registered by an IBM PS 2 microcomputer (Vendôme, France).

Continuous hydrolysis experiments were conducted by first loading the reactor tank with 1.25 l of substrate solution at the required concentration. After adjusting the temperature and pH to optimal values, the enzyme solution was added. The operating conditions of all runs were: T = 40°C; pH 8; tangential velocity = 0.9 m s^{-1}; reactor volume = 1.25 l; permeate flow rate = substrate feeding flow rate = 0.5 l h^{-1} or 1.5 l h^{-1}. Depending on the
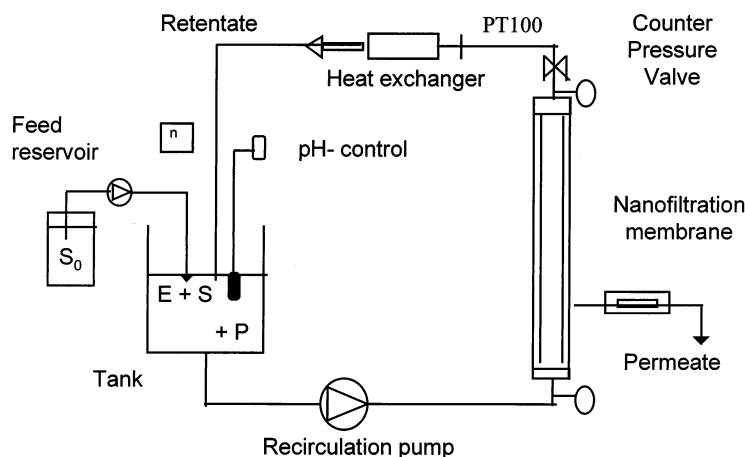
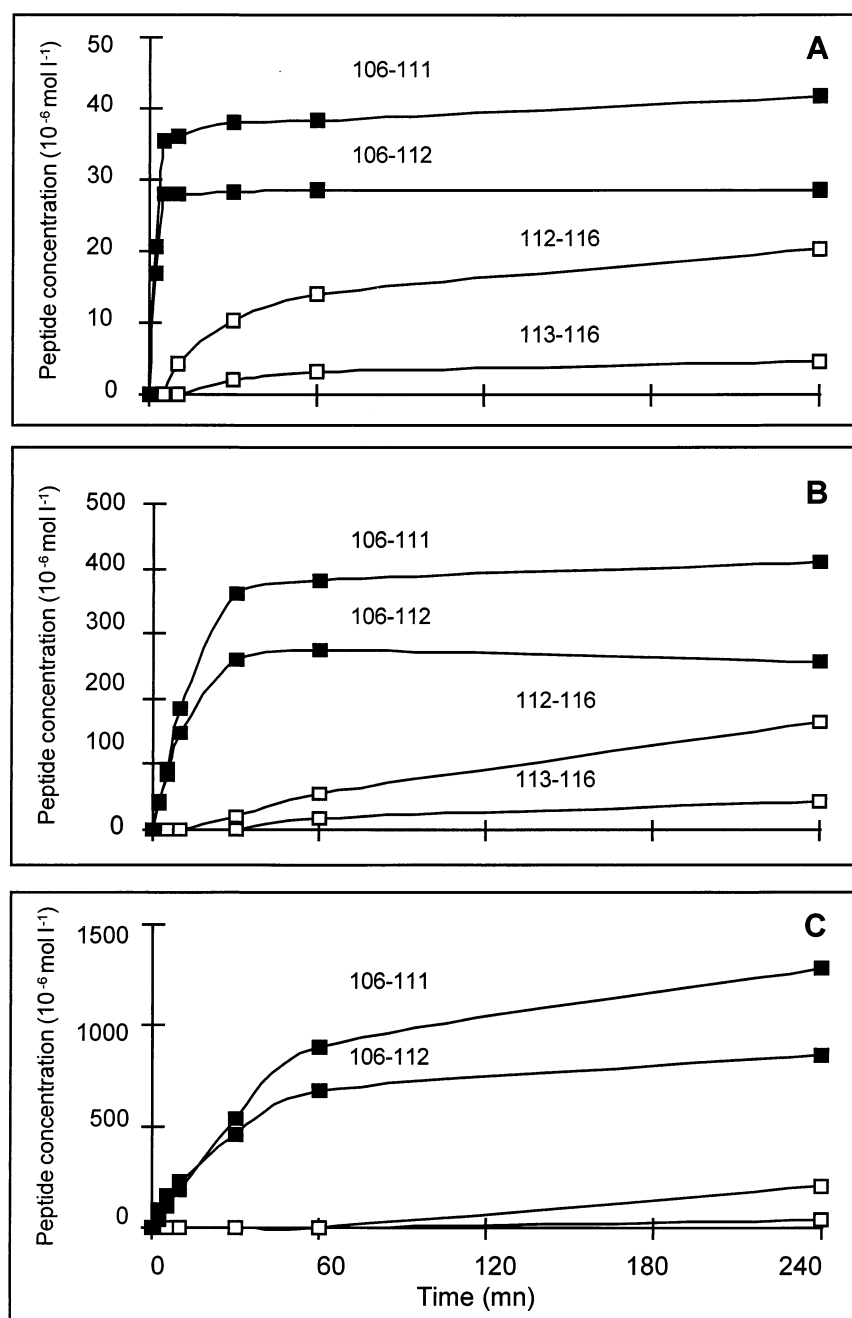**Figure 2** Schematic diagram of the continuous enzymatic membrane reactor

Figure 3 Time evolution of batch hydrolysis of CMP at various concentrations. (A) 0.11×10^{-3} mol l^{-1}; (B) 1.07×10^{-3} mol l^{-1}; (C) 4.26×10^{-3} mol l^{-1}. Enzyme concentration: 0.26×10^{-6} mol l^{-1}

substrate concentration used in the feed solution, the transmembrane pressure applied to maintain a constant permeate flow rate varied from $0.5–2 \times 10^5$ Pa. The reactor volume was kept constant by a continuous feed of substrate solution. Samples from permeate and retentate were taken at various intervals during the continuous process, and the reaction was stopped by dilution in 5% (v/v) TFA prior to RP-HPLC analysis.

Before and after each run, the system was cleaned with the following solutions at 60°C, with a transmembrane pressure of 3×10^5 Pa and tangential velocity of 0.9 m s^{-1} for 40 min: i) NaOH, 0.2 mol l^{-1} + NaOCl containing 0.25 g l^{-1} active Cl$_2$; ii) distilled water rinsing until neutrality; iii) HNO$_3$ 0.06 mol l^{-1} and iv) distilled water rinsing until neutrality.

Analytical methods

Samples of tryptic hydrolysate were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) on a Hypercarb column (4.6 i.d $\times$ 100 mm, Life Sciences, Cergy Pontoise, France) equilibrated in solvent A (0.11% v/v TFA in water). Elution was performed at 50°C at a flow rate of 0.6 ml min^{-1} with solvent B (0.09% v/v TFA in isopropanol: water, 90:10 v/v). The following gradient was applied: 2.6–3.5% solvent B from 0–20 min, 3.5–8.9% from 20–26 min, and 8.9–13.8% from 26–48 min. The absorbance was monitored at 214 nm.

Quantitative analysis of the peptides was performed using their amino acid composition following acid hydrolysis with 6 N HCl

Table 1 Tryptic hydrolysis of caseinomacropeptide: Kinetic parameters of the three lysyl bonds

	Tryptic cleavage bonds		
	Lys_{111}–Lys_{112}[a]	Lys_{112}–Asn_{113}[a]	Lys_{116}–Lys_{117}[b]
Km (10^{-3} mol l^{-1})	0.17	0.23	0.09
V_{max} (10^{-6} mol l^{-1} min^{-1})	26.75	27.85	3.63
V_{max}/K_m (min^{-1})	0.16	0.10	0.04

[a]Determined on CMP (sequence 106–169)
[b]Determined on intermediary fragments: (sequences 112–169 and 113–169)

for 24 h at 110°C in a Pico-Tag station (Waters, Milford, MA) and a PITC derivatization as described by Bidlingmeyer *et al.*.[16] The data shown are representative of results obtained in three separate experiments (n = 3, coefficient of variation < 5%).

Results

Batch hydrolysis

Batch hydrolysis was performed at a constant enzyme concentration of 0.26×10^{-6} mol l^{-1} and the various substrate concentrations ranged from 0.05–4.26×10^{-3} mol l^{-1}.

Figure 3 shows the time course hydrolysis as determined from RP-HPLC profiles at three different substrate concentrations. For the substrate concentration of 0.11×10^{-3} mol l^{-1} (*Figure 3A*), peptides of group A were rapidly released and reached maximal concentrations after 10 min of hydrolysis. Peptides of group B were released after a delay time of 10 min and their rate of appearance was lower compared to that of group A. When substrate concentration increased, the concentration of both group A and group B increased proportionally; however, the required time for the maximal release of group A as well as the delay time for the appearance of group B increased from 10 min at low CMP concentrations to 30 min or 240 min, respectively, at CMP concentrations of 1.07×10^{-3} mol l^{-1} (*Figure 3B*) and 4.26×10^{-3} mol l^{-1} (*Figure 3C*). It is interesting to note that the appearance of peptides of group B seemed to be dependent on the rate of appearance of those of group A since the initial delay time for group B was always correlated with the time for complete release of group A. This observation together with the fact that the 106–116 fragment was never detected suggested a sequential hydrolysis mechanism in which the substrate for the cleavage of the Lys_{116}–Thr_{117} bond (release of peptides of group B) is not the whole CMP, but rather the fragments subsequent to hydrolysis of the Lys_{111}–Lys_{112} and Lys_{112}–Asn_{113} bonds (release of peptides of group A). Accordingly, the kinetic constants of each reaction were calculated by the determination of the initial rates of appearance of each group in relation to its respective substrate, the CMP for group A and the intermediary fragments for group B. For this last peptide group, a prehydrolysis was first performed at a CMP concentration of 4.26×10^{-3} mol l^{-1} for 1 h in order to obtain their precursor substrates. After enzyme inactivation and dilutions, fresh enzyme was added and the initial rate of appearance was then measured.

The kinetic parameters determined from the Lineweaver-Burk plot are shown in *Table 1*. Identical K_m and V_{max} values were found for the splitting of Lys_{111}–Lys_{112} and Lys_{112}–Asn_{113} bonds, indicating that both bonds were cleaved at similar rates. The V_{max} value for the Lys_{116}–Thr_{117} bond was lower than that found for the two adjacent bonds, Lys_{111}–Lys_{112} and Lys_{112}–Asn_{113}; however, taking into account that the K_m value was slightly lower, the enzyme specificity, expressed as V_{max}/K_m ratio, was similar for the three bonds.

Continuous hydrolysis in the EMR

In order to study the effect of the continuous mode on the enzymatic cleavage of the three bonds, continuous hydrolysis in EMR was performed at various substrate and enzyme concentrations and substrate feeding flow rates.

The effect of substrate concentration was studied in the range 0.05–2.13×10^{-3} mol l^{-1} at an enzyme concentration of 1.04×10^{-6} mol l^{-1} and a permeate flow rate of 0.5 l h^{-1}. *Figure 4* shows the evolution of peptide concentrations in the permeate side of the continuous EMR at two different substrate concentrations. At a low substrate concentration (0.11×10^{-3} mol l^{-1}), an apparent steady state was reached after 1 h for peptides of group A, taking longer for peptides of group B (*Figure 4A*). An initial delay time was observed for group B since they were not detected in the permeate during the first 30 min of the continuous operation. Among peptides of group A, the concentration of peptide 106–112 in the permeate was lower than that of peptide 106–111. This was attributed to the low transmission of the former peptide, 106–112, through the membrane since the concentration of both peptides was the same in the retentate side. A tenfold increase in substrate concentration (*Figure 4B*) resulted in a proportional increase of peptides of group A released at steady state. Surprisingly, peptides of group B were not detected either in the permeate or in the retentate even after 7 h of continuous hydrolysis. A further increase in substrate concentrations led to the same result, revealing that above CMP concentrations of 1.07×10^{-3} mol l^{-1}, the hydrolysis of the Lys_{116}–Thr_{117} bond did not occur. According to the sequential mechanism stated previously, the enzyme action would be completely displaced toward the Lys_{111}–Lys_{112} and Lys_{112}–Asn_{113} bonds and as a consequence, only peptides of group A were generated. Such a result appeared to fit with the continuous mode of operation in EMR, in particular with the continuous feed of substrate which would indefinitely extend the delay time for the hydrolysis of the Lys_{116}–Thr_{117} bond observed in the
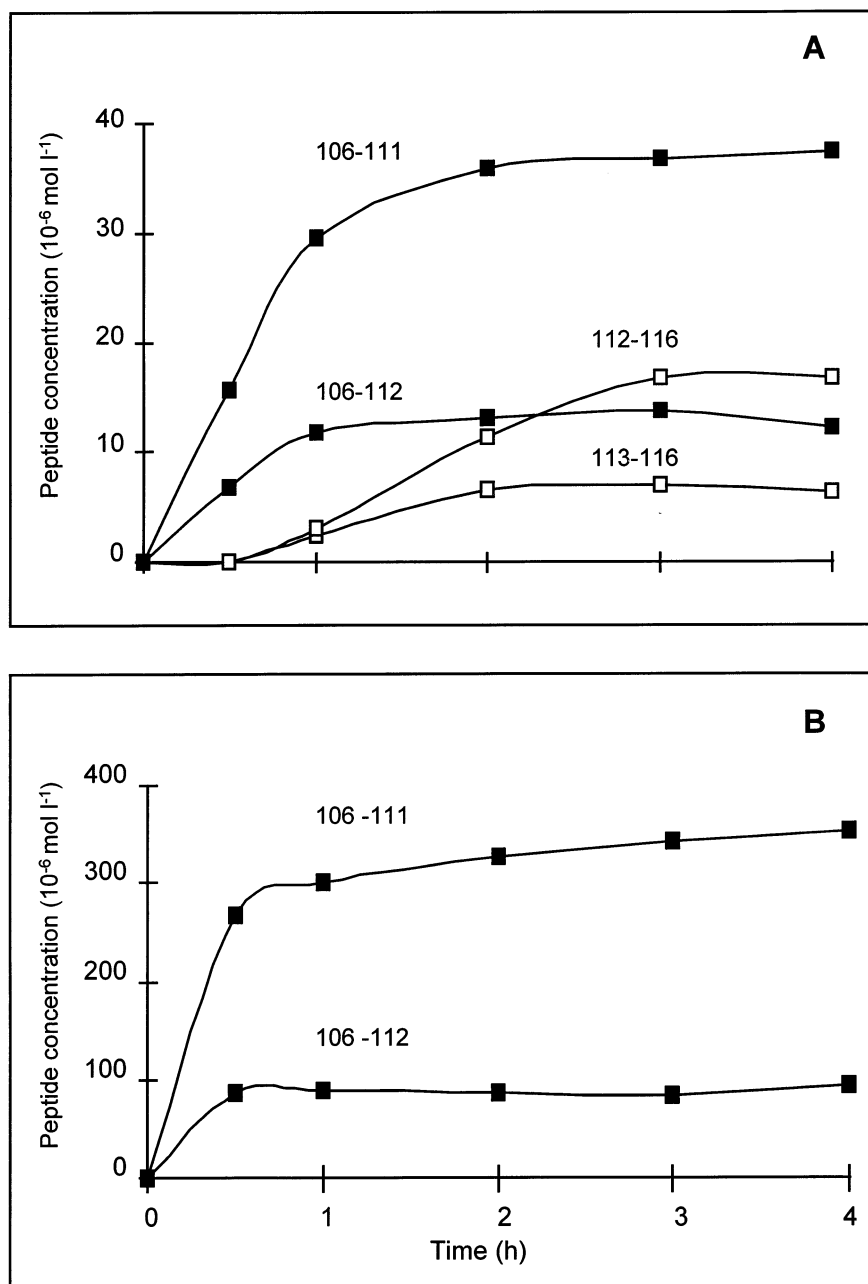
Figure 4 Production of peptides of group A (■) and peptides of group B (□) in the permeate during continuous hydrolysis in the enzymatic membrane reactor for two substrate concentrations. (A) 0.11×10^{-3} mol l^{-1} and (B) 1.07×10^{-3} mol l^{-1}. Enzyme concentration: 1.04×10^{-6} mol l^{-1}. Permeate flow rate: 0.5 l h^{-1}

batch system; moreover, apart from the substrate feed concentration, the hydrolysis of the three bonds was also affected by the feeding flow rate. When hydrolysis was performed under the same conditions as those reported in *Figure 4B*, but with a threefold higher flow rate, peptides of group B were released (*Figure 6*; next section).

The influence of enzyme concentration on the selective hydrolysis of the three bonds and the consequent effect on permeate composition was studied by varying trypsin concentration from $0.26-10.4 \times 10^{-6}$ mol l^{-1} at a constant substrate concentration of 0.53×10^{-3} mol l^{-1} and a permeate flow rate of 0.5 l h^{-1} (*Figure 5*). At low enzyme concentrations (*Figure 5A*), a steady state for peptides of group A was reached after 30 min of continuous operation while this time was needed to detect

the appearance of group B in the permeate. The distribution profile of concentrations at steady state, $106–111 > 112–116 > 106–112 > 113–116$, was similar to that obtained at low substrate concentrations. A strong increase in enzyme concentration (*Figure 5B*) affected principally the release of peptides of group B for which the delay time of appearance was reduced and their concentrations in the permeate strongly increased. As far as peptides of group A are concerned, a subsequent hydrolysis of the C-terminal lysyl residue of peptide $106–112$ could explain, in part, its lower concentration under these experimental conditions. As a consequence, the distribution of individual concentrations at steady state was significantly changed: $106–111 > 112–116 > 113–116 > 106–112$.
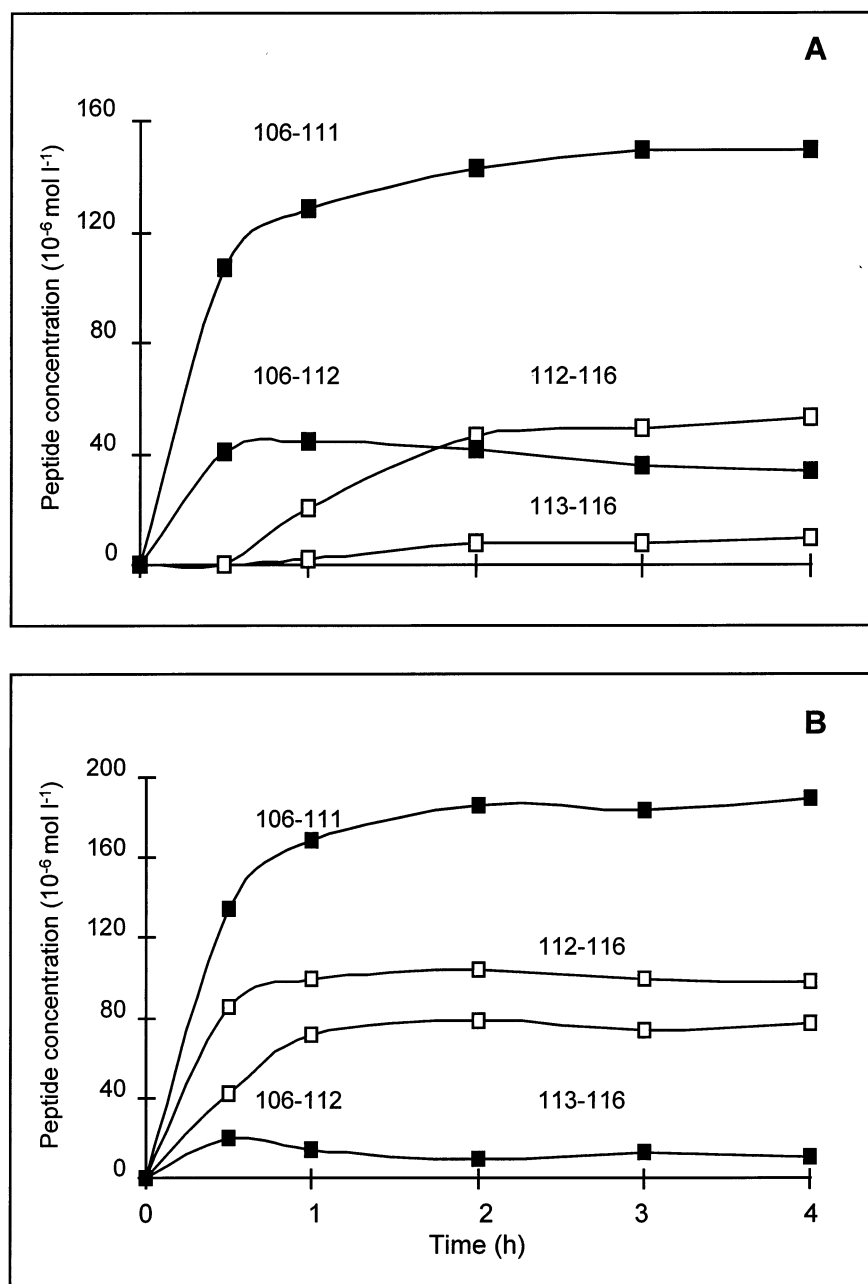
Figure 5 Production of peptides of group A (■) and peptides of group B (□) in the permeate during continuous hydrolysis in the enzymatic membrane reactor as a function of the enzyme concentration. (A) 0.26×10^{-6} mol l^{-1} and (B) 10.4×10^{-6} mol l^{-1}. Substrate concentration: 0.53×10^{-3} mol l^{-1}. Permeate flow rate: 0.5 l h^{-1}

Discussion

In the present study, the time course-hydrolysis of the three lysyl bonds of CMP, $Lys_{111}–Lys_{112}$, $Lys_{112}–Asn_{113}$, and $Lys_{116}–Thr_{117}$ was monitored in both continuous and batch experiments. From these experiments, we concluded that trypsin acts on CMP according to a sequential mechanism. The overall reaction scheme can be described as follows:

$$E + S \xrightarrow[\text{Group A}]{(1)} S_1 + S_2 \xrightarrow[\text{Group B}]{(2)} LF$$
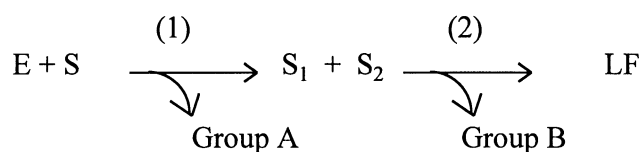
where S = CMP (106–169); S_1 and S_2 = intermediary products (112–169 and 113–169); LF = large fragment (117–169); group A = (106–112 and 106–111); group B = (113–116 and 112–116).

The first step of the reaction involves the parallel hydrolysis of the two adjacent lysyl bonds, $Lys_{111}–Lys_{112}$ and $Lys_{112}–Asn_{113}$, according to the results previously reported by Léonil *et al.*[17] This reaction leads to the liberation of group A and the accumulation of new substrates, S_1 and S_2, which are further hydrolyzed at the $Lys_{116}–Thr_{117}$ bond in subsequent reaction, generating peptides of group B and the large final fragment. The fact that $Lys_{116}–Thr_{117}$ is only cleaved on the intermediary fragments, S_1 and S_2, indicates that this bond is not accessible to the enzyme on the whole CMP. This can be explained by
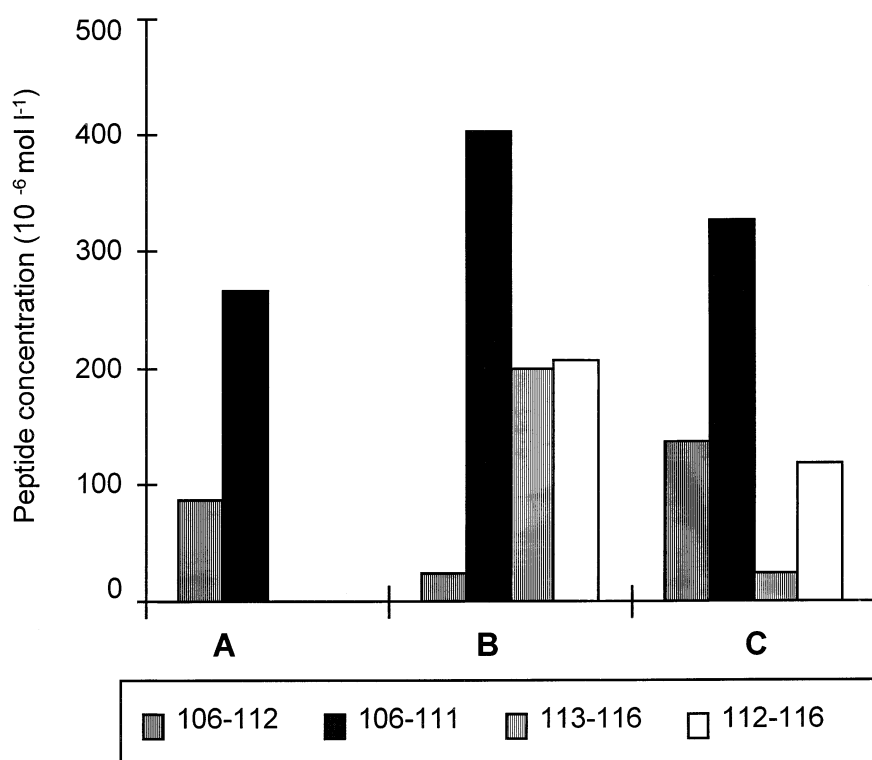
Figure 6 Effect of enzyme concentration (E) and substrate feeding flow rate (J) on permeate composition at the steady state of continuous hydrolysis in the membrane reactor. (A) $E = 1.04 \times 10^{-6}$ mol l^{-1}; $J = 0.5$ l h^{-1}. (B) $E = 10.4 \times 10^{-6}$ mol l^{-1}; $J = 0.5$ l h^{-1} (C): $E = 1.04 \times 10^{-6}$ mol l^{-1}; $J = 1.5$ l h^{-1}

the fact that cleavage of the first two bonds induces a new conformation state with an exposed Lys_{116}–Lys_{117} susceptible site as has been described for other protein/protease couples.[18]

The single consecutive reactions are generally described by an unfavorable specificity constant, K_{cat}/K_m, for the second reaction.[19,20] In the case of CMP/trypsin, the specificity constants of the three sensitive bonds measured on S for the first two bonds and on S_1 and S_2 for the third one are similar, thereby, indicating that the occurrence of the second reaction is not limited by unfavorable interaction constants of trypsin with the Lys_{116}–Thr_{117} bond. Although the delay time for the occurrence of the second reaction depends on the accumulation of intermediary products, the finding that, at a given enzyme concentration, this delay time increases as CMP concentration increases suggests that cleavage of the Lys_{116}–Thr_{117} bond depends not only on the absolute $(S_1 + S_2)$ concentration accumulated but also on the $S/(S_1 + S_2)$ concentration ratio. Interesting information about the importance of these relative concentrations was derived from experiments conducted in continuous mode where S and $(S_1 + S_2)$ were simultaneously present at the steady state of the process. In these conditions, the cleavage of the Lys_{116}–Thr_{117} bond is favoured by an excess of $(S_1 + S_2)$ compared to S. These favorable conditions were achieved more rapidly when the initial substrate concentration S was low. In contrast, operating in the EMR at substrate concentrations higher than 1.07×10^3 mol l^{-1}, the high ratio $S/(S_1 + S_2)$ was maintained constant by continuously feeding S; therefore, the enzyme preference toward the Lys_{111}–Lys_{112} and Lys_{112}–Asn_{113} bonds is maintained and the reaction occurs as if delay time for the

release of group B was indefinitely extended. As a consequence, only peptides of group A are obtained (*Figure 6A*); however, at the same substrate concentration, this steady-state equilibrium can be modified by increasing either the enzyme concentration (*Figure 6B*) or the reactor feeding flow rate (*Figure 6C*). With high enzyme concentrations, the rate of the first reaction is strongly increased, thereby, leading to a rapid accumulation of $(S_1 + S_2)$ and thus, a rapid consecutive conversion in group B. As a consequence, the final products of each step, group A and group B, are obtained in the first few min of the continuous operation. Accordingly, with enzyme concentrations high enough to completely hydrolyze the intermediary fragments, the same concentration of both groups of peptides would be reached. In the same way, feeding the substrate S at a higher flow rate rapidly generates a situation where $S \gg K_m$. Under these conditions, the reaction rate for the first step becomes insensitive to changes in S concomitant with the accumulation of $(S_1 + S_2)$ that allows for a rapid release of the peptides of group B.

Conclusions

The results obtained in batch as well as continuous hydrolysis in EMR allowed us to establish the mechanism of tryptic hydrolysis of the three bonds of caseinomacropeptide. The overall hydrolytic process occurs via two consecutive reactions where the hydrolysis of the third bond is controlled by the accumulation of the intermediary products. Such accumulation depends on enzyme concentration, substrate concentration, and substrate feeding flow rate;

thus, by means of these parameters, the extent of the sequential reactions can be controlled by preventing or enhancing the concentration of intermediary fragments at the steady state of the continuous EMR. The results reported here show that EMR allowed not only for a better control of product composition, but also constituted a powerful means of studying the mechanism of protein digestion by proteolytic enzymes which opens up new fields in applied enzymology.

Acknowledgements

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