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Peptide generation from casein hydrolysis by immobilised porcine cathepsins

Fidel Toldrá *, Laura Molina, Sergio Vinuesa, M-Concepción Aristoy

Instituto de Agroquímica y Tecnología de Alimentos (C.S.I.C.), Apartado de Correos 73, P.O. Box 73, 46100 Burjassot (Valencia), Spain

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Abstract

A porcine kidney enzyme extract containing high levels of cathepsins B and L activities has been successfully immobilised onto porous glass beads. Cathepsins showed improved stability when immobilised, exhibiting a half-life of 97.5 h at 30 °C vs 5.6 h for the free enzyme in solution. Immobilised reactors were incubated at 30 °C and fed with a 0.2% casein solution at pH 6.0. κ-casein was rapidly hydrolysed and almost fully degraded at 24 h while the degradation of α-casein and β-casein was achieved in 24–48 h. Polypeptides of M.W. 11, 19.5 and 32 kDa were generated and further degraded, while others (18 and 21 kDa) remained undegraded. Hydrolysis was much higher than that observed with the free enzymes. A substantial number of peptides with cut-off < 10 kDa were generated, especially after 24 h. Most of them were of moderate to high hydrophobicity and only a few were polar and/or very small peptides (di- or tri-peptides). So, this type of reactor may be used for the production of certain peptides from casein hydrolysis with better yields than the free enzymes.

Keywords: Casein hydrolysis; Cathepsins; Immobilised cathepsins; Immobilised proteases; Peptides

1. Introduction

The need for protein hydrolysates with high hydrolysis degree and uniform peptide size requires the use of reactors with adequate control of hydrolysis rate. Furthermore, the use of soluble enzymes may be less efficient that acidic hydrolysis and enzymes remain with the products, catalysing further undesirable reactions. Better hydrolysis efficiency may be achieved with immobilised protease reactors.

Immobilisation has several advantages such as capability of reuse, non-leaching of the product, better control of hydrolysis rate, longer stability and reduced susceptibility to inhibitory effects. Different materials may be used as supports for enzyme immobilisation. One of the most used materials is porous glass. This material has several important advantages, namely the variety of available controlled shapes, sizes and characteristics, developed methodologies for covalent coupling of enzymes, stability against chemical substances and physical resistance to mechanical effects (Toldrá, Jansen, & Tsao, 1992; Weetall, 1993).

Different proteases, such as trypsin, chymotrypsin, papain, bromelain and many other enzymes, have been assayed in recent years for immobilisation on different available supports, such as glass, agarose gel particles, sliced shrimp chitin hull, calcium alginate gel particles, oxirane acrylic beads, alkyl or aryl sepharoses (Gauthier, Vuillemard, & Lizotte, 1991; Haque & Mozaffar, 1992; Hutchinson & Tunnicliffe, 2003; Lorenzen, Heitman, Martin, Baumeister, & Schlimme, 1998; Mohapatra & Hsu, 2000).

Proteolytic enzymes have shown good ability to degrade proteins, even though they are retained on a

* Corresponding author. Tel.: +34 96 3900022x2112; fax: +34 96 3636301.
E-mail address: ftoldra@iata.csic.es (F. Toldrá).

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support. However, no previous reports on the immobilisation of cathepsins are available, even though these enzymes are active against a wide range of proteins (Toldrá, 1998; Toldrá & Flores, 1998) and produce several tri- and di-peptides with taste properties, as in dry-cured ham (Sentandreu et al., 2003b) or even bioactive peptides when they act on myofibrillar proteins (Nishimura et al., 2002). In this way, a cathepsin-immobilised reactor could be useful for the production of peptides that, depending on the substrate and reaction conditions, could have taste characteristics, antimicrobial activity or specific physiological effects. Thus, the objective of this study was to immobilise porcine cathepsins onto porous glass beads for an efficient and continuous hydrolysis of casein and to improve the production of small peptides.

2. Materials and methods

2.1. Materials

Microreactors of 10 cm length × 1 cm diameter (Amersham, Uppsala, Sweden) were packed with 10.25 g of porous glass beads (Sigma, St. Louis, MO). These beads had 2 mm diameter with a mean controlled pore diameter of 500 Å. Glass beads were washed with 10% HNO_3 solution, rinsed with distilled water and dried at 75 °C before use.

2.2. Preparation of the enzyme extract

Pork kidney lysosomal cathepsins were used for the immobilisation. An extract enriched in lysosomes was prepared from pork kidney by following, with minor changes, the methodology described in Lardeux, Gouhot, and Forestier (1983) and Sentandreu, Aubry, and Ouali (2003a). Thus, 80 g of pork kidney, with no visible fat and free from connective tissue, was minced and homogenised, using a Polytron homogenizer (Kinematica, Switzerland), in four volumes of 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.25 M sucrose. The homogenate was then submitted to differential cold centrifugations, starting with 10 min at 1000*g* and again 10 min at 4000*g*. The supernatant was kept and cold centrifuged at 12 500*g* for 20 min and the pellet was collected and re-suspended in the minimum volume of 30 mM sodium phosphate buffer at pH 5.8, containing 0.01% Triton X-100. The re-suspended material was homogenised by using a mini-polytron (PT-1200, Kinematica), and then submitted to thermal shock by freezing. The frozen material was thawed at 25 °C, homogenised with Polytron, centrifuged at 15,000*g* for 20 min, and the supernatant (lysosomal extract, rich in cathepsins) collected.

2.3. Immobilisation method

The immobilisation procedure was based on previous works (Toldrá, Jansen, & Tsao, 1986). Reactors were operated in an up-flow mode and glass beads were activated with 3-aminopropyltriethoxysilane solution, pH 7.0, through re-circulation at 1 ml min^{-1} for 3.5 h at 75 °C. After extensive washing, a 2.5% w/v glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.0, was left in contact for 1 h under vacuum pressure and 1 h under atmospheric conditions at room temperature. Then the packing was extensively washed with distilled water, followed by 40 mM phosphate buffer, containing 0.4 mM EDTA, pH 6.0, and the reactor was ready for enzyme immobilisation.

The enzyme extract solution was fed into the reactor and re-circulated at 1 ml min^{-1} with a peristaltic pump (P-1, Amersham, Uppsala, Sweden) for 24 h at 20 °C. Protein concentration and enzyme activity were monitored in the free solution by removing small quantities of enzyme solution at periodic intervals. Once asymptotic conditions were achieved, the immobilization was considered as finished and the reactor was washed with 40 mM phosphate buffer containing 0.4 mM EDTA, pH 6.0, to remove any unbound enzyme.

Immobilised enzyme loadings were calculated on a material balance basis, considering the determinations of the enzyme concentration in the solution and in the washings. Protein concentration was determined by the bicinchochinic acid method at 562 nm using a multiplate reader (EL × 800, Biotek Instruments, Pierce, Rockford, NY, USA) and BSA as standard.

2.4. Assay of enzyme activity

The enzyme activity in free solution (fed-batch reactor) was measured by adding 100 μl of the extract (diluted 1:20 with reaction buffer containing the substrate) to 70 μl of reaction buffer. This buffer consisted of 40 mM phosphate buffer containing 0.4 mM EDTA and 10 mM cysteine, pH 6.0, with additional 50 mM N-CBZ-phenylalanine-arginine-7-amido-4-methylcoumarin as substrate (N-CBZ-Phe-Arg-AMC; Sigma, St. Louis, MO, USA). The reaction mixture was incubated at 37 °C, and continuously read up to 10 min. Fluorescence was measured in a multiscan fluorometer (Fluoroskan II, Thermo-Labsystems, Finland) using excitation and emission wavelengths of 355 and 460 nm, respectively. Three replicates were measured for each experimental point. One unit of enzyme activity (U) was defined as the release of 1 μmol of substrate ×1000 per hour at 37 °C. The protein concentration of the enzyme extract was 21.7 ± 1.8 mg protein ml^{-1} and the activity was 480 ± 35 U ml^{-1}. So, the specific activity was around 22.0 U mg^{-1}protein.

The immobilised enzyme activity was determined by passing the substrate in the reaction buffer, as described above, at 1 ml min^{-1}. Samples were collected at 5 min periodic intervals at the exit of the reactor and read for fluorescence under the same conditions as mentioned above. Immobilised enzyme activity was expressed as units of enzyme activity per gram of packed bed.

Kinetic constants, K_m and V_m, for the free enzyme were determined at 30 °C with the Michaelis–Menten equation. The apparent kinetic parameters $K'_{m\ ap}$ and $V'_{m\ ap}$, of the immobilised enzyme reactor were determined at 30 °C through the integrated form of the Michaelis Menten equation (Toldrá, Jansen, & Tsao, 1990)

$$S_0X = K'_{m\,ap}\ln(1-X) + V'_{m\,ap}t_r,$$

where S_0 is the inlet substrate concentration (μM), X is the conversion rate and t_r is the residence time which is VQ^{-1} where V is the reactor volume (ml) and Q is the flow rate (ml min^{-1}).

2.5. Determination of operational stability

Immobilised enzyme reactors were incubated at 30 and 40 °C and continuously fed with the reaction buffer containing the substrate. Free enzyme was incubated in a similar solution at 30 and 40 °C. In both cases, the activity was periodically assayed and the half-life determined.

2.6. Casein hydrolysis

Casein (sodium salt, Sigma) was dissolved (0.2% w/v) in the reaction buffer and hydrolysed in the immobilised enzyme reactor operating in recirculating mode and in fed-batch reactor (free enzyme). In both cases, the hydrolysis was assayed at 30 °C and monitored at periodic intervals by gel electrophoresis and RP-HPLC.

2.7. Gel electrophoresis of casein hydrolysates

The hydrolysis of casein was monitored by sodium dodecyl sulfate gel electrophoresis (SDS–PAGE) analysis (Laemmli, 1970), using 10% polyacrylamide gels, as described elsewhere (Flores, Moya, Aristoy & Toldrá, 2000). One hundred sample aliquots were mixed at 1:1 with a solution composed of 8 M urea, 2 M thiourea, 0.05 M tris, pH 6.8, 75 mM dithiothreitol, 3% (v/v) SDS and 0.05% bromophenol blue. The mixture was incubated at 100 °C for 4 min and kept at −20 °C until analysis. The amount of sample injected into gels was 10 μl. Gels were stained with Coomassie brilliant blue R-250 and destained overnight. Standard proteins from BioRad (Richmond, CA, USA) were simultaneously run for molecular weight identification.

2.8. RP-HPLC of casein hydrolysates

The hydrolysis of casein was also monitored by RP-HPLC. To this aim, 100 μl of sample was deproteinised by adding 250 μl of acetonitrile. After mixing and centrifuging (at 12 000g, for 5 min) in the cold (4 °C), 20 μl of supernatant were injected into the HPLC system.

The HPLC system consisted of a model 1050 (Agilent Technologies, Palo Alto, CA, USA) apparatus with a variable wavelength ultraviolet detector, an automatic injector and a column oven. Peptides were separated in a Symmetry C-18 (4.6 × 250 mm) column (Waters Corp., Milford, MA, USA) at 40 °C, by using a gradient between two solvents: 0.1% TFA in water (solvent A) and 0.085% TFA in water:acetonitrile, 40:60 v/v (solvent B). The flow rate was 1 ml/min and the gradient consisted of an initial 1% solvent B step for 5 min and then a linear gradient to 80% B in 60 min. After washing the column at 100% B for 10 min, initial conditions were reach and maintained for 15 min until a new injection. The detection was monitored at 214 nm.

3. Results and discussion

3.1. Reactor efficiency

The enriched porcine kidney enzyme extract, containing high levels of cathepsins B and L activities, was successfully immobilised onto porous glass beads. The immobilisation process was already monitored up to 24 h but full immobilisation was achieved within 3 h. The protein binding was 1.9 mg protein per gram support. The total immobilised activity was 190 ± 14 U with an activity of the immobilised cathepsins of 18.5 U/g support. The specific activity was 9.4 U/mg immobilised protein.

The apparent kinetic parameters, $K'_{m\ ap}$ and $V'_{m\ ap}$ were determined at 30 °C for the synthetic substrate. The values were 11.3 μM and 0.5 μM min^{-1}, respectively. These values differ from those for the free enzyme at the same temperature that were 108 μM for K_m and 1.6 μM min^{-1} for V_m. This difference has also been reported for the *S. griseus* protease immobilised on glass beads (Bliss & Hultin, 1977) and the decrease in the values of the kinetic constant was attributed to the substrate adsorption on the support, typical of glass (Messing, 1975; Weetall, 1993) and, thus, a relative increase of the substrate concentration in the microenvironment surrounding the immobilised enzyme.

3.2. Continuous operational stability of immobilised cathepsins

The immobilised enzyme reactors were assayed for stability at 30 and 40 °C (see Fig. 1A). Immobilised

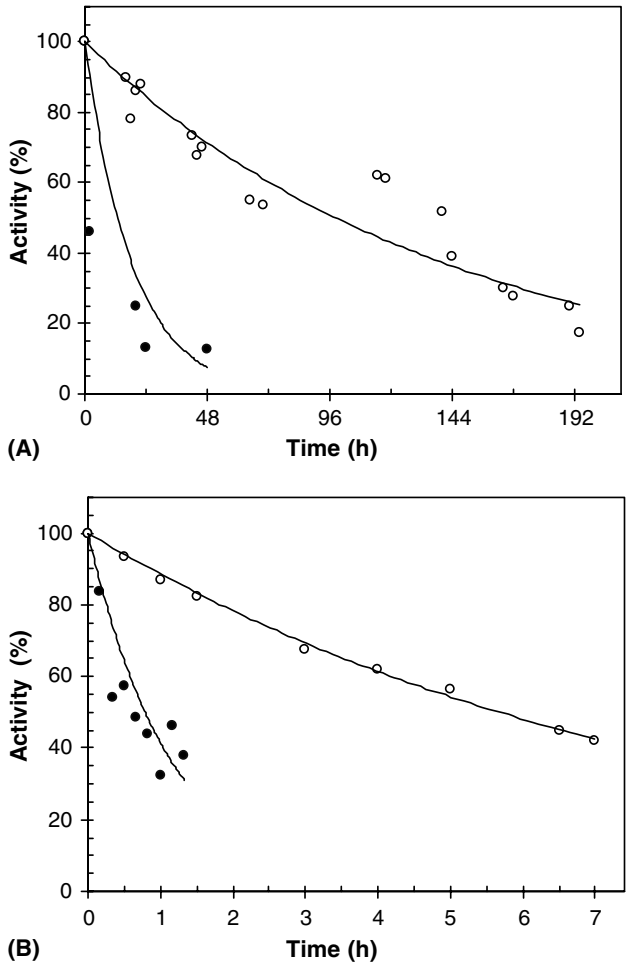

Fig. 1. Stability of free (A) and immobilised (B) cathepsins incubated at 30 °C (o) and 40 °C (•) in 40 mM phosphate buffer containing 0.4 mM EDTA, pH 6.0.

cathepsins showed better stability at 30 °C, exhibiting a half-life of 97.5 h, than at 40 °C, with a shorter half-life of 12.7 h. The higher activity at 40 °C is not compensated by the lower stability. Anyway, the system was considerably more stable than the free enzymes in solution that exhibited very short half lives, 5.6 h at 30 °C and 0.8 h at 40 °C (see Fig. 1B). The loss of activity was more rapid for the free enzyme than for the immobilised enzyme, as was also observed for other enzymes (Toldrá et al., 1986). The highest stability of immobilised cathepsins, with a half-life higher than 4 days at 30 °C, constitutes an additional important advantage, especially for potential industrial application. In fact, an enhanced stability of cathepsin B, immobilised in calcium alginate beads, in acidic as well as alkaline environments, in relation to the free enzyme for the hydrolysis of bovine serum albumin, has been reported (Kamboj, Raghav, & Singh, 1996).

3.3. Casein hydrolysis and peptides generation

The pH value was adjusted to 6.0 as this is the optimal pH for cathepsin activity. Casein was rapidly hydrolysed, as shown in Fig. 2. A substantial hydrolysis, especially of κ-casein, is already observed at 8 h (Fig. 2, lane B) and almost complete degradation achieved at 24 h (Fig. 2, lane C). Three clear fragments, of 11, 18 and 19.5 kDa, were generated after 8 h. The fragment of 18 kDa remained undegraded while the other two were further degraded and almost disappeared after 48 h. The degradation of α-casein and β-casein was achieved in 24–48 h with the generation of intermediate polypeptide bands of 21 and 23 kDa, and some other minor bands within the range 15–20 kDa (Fig. 2, lanes D and E) which remained up to 48 h, except that of 23 kDa, that was degraded. These results are similar to those obtained by Gallagher, Kanekanian, and Evans (1994) when using free bromelain and a protease from *B. subtilis* at higher temperatures. In our case, the electropherograms for the free enzyme assay are considerably more complex, due to the numerous bands of the enzyme extract (Fig. 3, lane A). Casein hydrolysis is also achieved in 24 h and some other bands are also degraded, although the electropherogram is not so clear as for the immobilised system (Fig. 3, lane C).

Peptide mappings for the immobilised cathepsins eluents are shown in Fig. 4 where a substantial increase in the number of generated peptides with cut-off <10 kDa, can be observed, especially after 24 h. As shown in the chromatogram corresponding to 48 h hydrolysis (Fig. 4D), the higher amount of the generated peptides starts the elution at around 17 min which corresponds to 8% of acetonitrile in the mobile phase (see dotted line in that Figure). This fact indicates that peptides are of moderate to high hydrophobicity, and there are very few, polar and/or very small peptides (di- or tri-peptides), which would appear earlier in the chromatogram. In any case, the generated peptides are homogeneously distributed along the chromatogram, from the first 17 min, indicating a high variety of hydrophobicity. The evolution of the total areas of all peaks follows a linear progressive increase through time of incubation as shown in Fig. 5. In the case of the free enzyme, several peptides were initially present due to the enzyme extract, although some of them were further hydrolysed and, in fact, disappeared after 24 h (see Fig. 6). However, an increase in new peptides can be observed, especially those eluting between 12 and 23 min of retention time.

The purpose of the enzymatic hydrolysis may change depending on the molecular size of the protein hydrolysate, those peptides with smaller MW being useful for the cosmetic industry and nutrition and those with higher MW for their emulsifying properties (Haque & Mozaffar, 1992). In another work, casein hydrolysis with a protease from *B. subtilis*, free in solution, gave a great variety of peptides with MW <10 kDa (Gallagher et al., 1994) which is somewhat similar to our findings. However, when these authors used a pineapple stem bromelain, a hydrophobic hydrolysate was obtained,

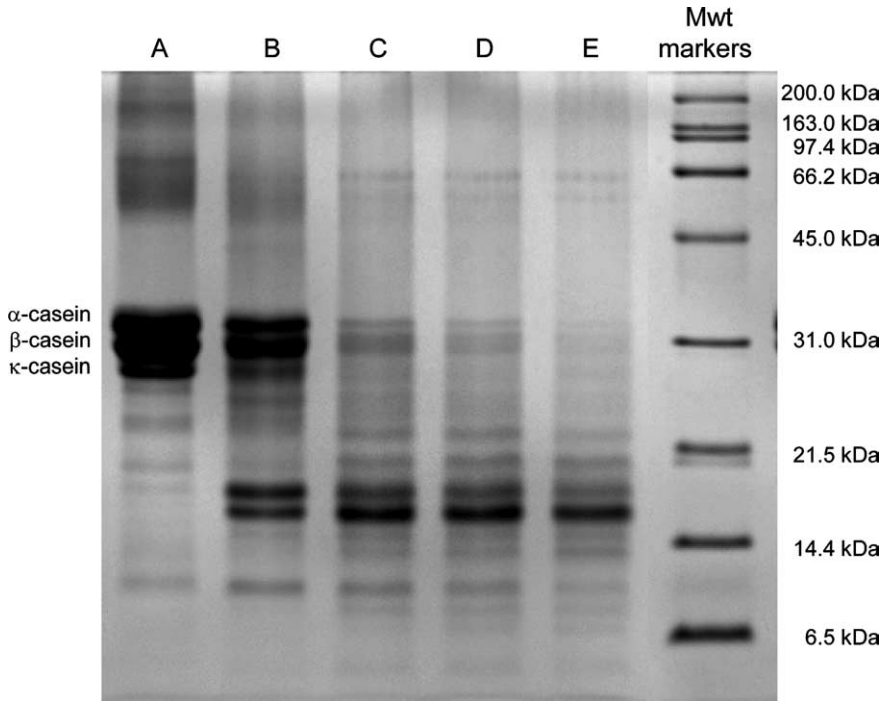

Fig. 2. Electropherograms of 0.2% casein hydrolysates by immobilised cathepsins for different times of incubation at 30 °C: 0 h (lane A); 8 h (lane B); 24 h (lane C); 32 h (lane D) and 48 h (lane E). Mwt: molecular weight.

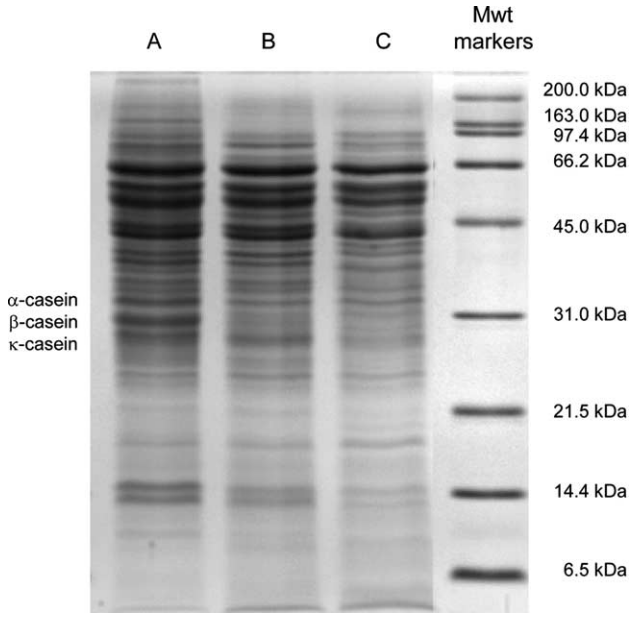

Fig. 3. Electropherograms of 0.2% casein hydrolysates by free cathepsins for different times of incubation at 30 °C: 0 h (lane A); 8 h (lane B); 24 h (lane C). Mwt: molecular weight.

containing a large number of peptides with bitter taste, having MW >10 kDa. Casein hydrolysis by other immobilised proteases, such as trypsin and papain, also gave extensive hydrolysis, except chymotrypsin that gave a slower hydrolysis and fewer peptides (Haque &

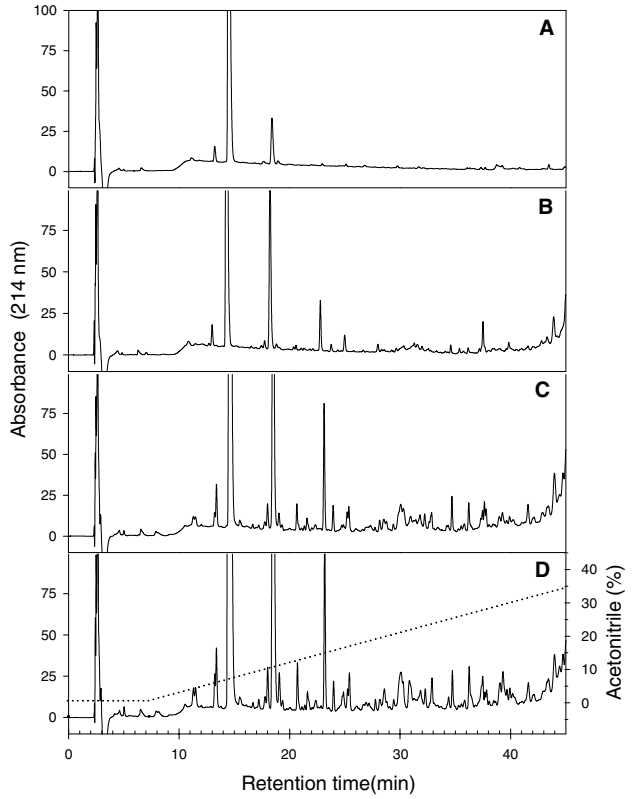

Fig. 4. Peptide mappings after RP-HPLC of immobilised cathepsins eluents with cut-off below 10 kDa after different times of incubation at 30 °C: 0 h (A); 8 h (B); 24 h (C) and 48 h (D). Dotted line represents the percentage of acetonitrile in mobile phase. Chromatographic conditions are described in text.

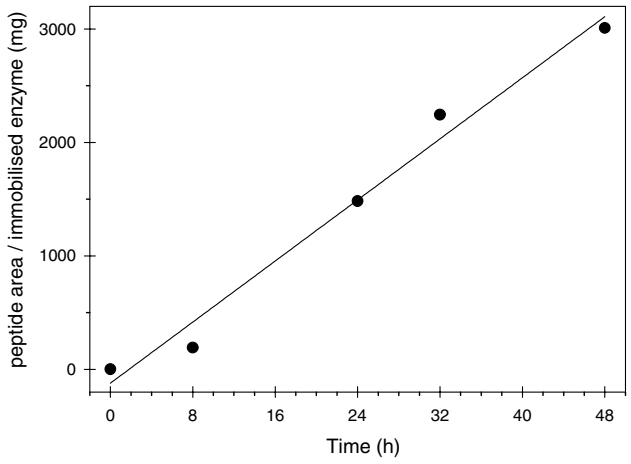

Fig. 5. Evolution of the total areas of all peptide peaks with cut-off below 10 kDa of immobilised cathepsins eluted after different times of incubation at 30 °C.

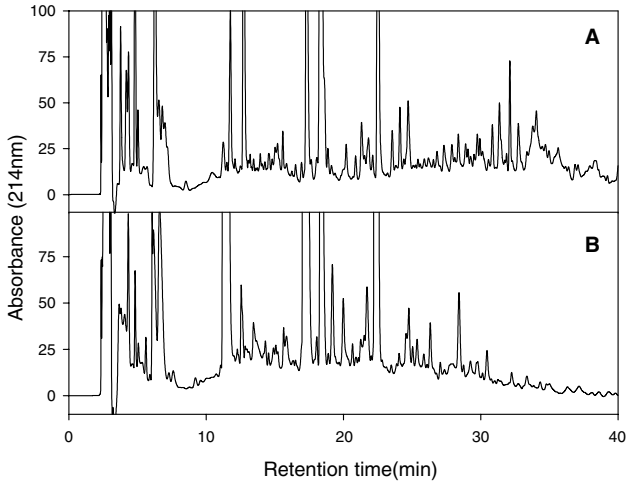

Fig. 6. Peptide mappings after RP-HPLC of free cathepsins eluents with cut-off below 10 kDa after different times of incubation at 30 °C: 0 h (A) and 24 h (B). Chromatographic conditions are described in text.

Mozaffar, 1992). A combined use of immobilised enzymes, such as trypsin, chymotrypsin and a protease from *A. oryzae*, efficiently hydrolysed casein but failed with other proteins, such as soybean protein and egg white protein, probably due to their higher MW (Ge, Bai, Yuan, & Zhang, 1996).

Porcine cathepsins are active against a wide range of meat proteins (Toldrá & Flores, 1998; Toldrá, 1998) and generate small peptides, some of them with taste characteristics (Sentandreu et al., 2003b) and others with physiological activity (Nishimura et al., 2002). In view of the efficiency of the immobilised cathepsin reactor when using casein as substrate, the next work will focus on the use of soluble meat proteins to produce peptides with either taste characteristics, antimicrobial activity or specific physiological effects.

4. Conclusions

Porcine cathepsins have been successfully immobilised on porous glass beads. The system showed a good efficiency against a typical specific substrate and high stability when incubated at 30 °C, in fact, 18 times more stable than the free enzymes. Casein was effectively hydrolysed by the immobilised enzymes and substantial numbers of smaller peptides were generated. This type of reactor may thus be used for the production of certain peptides from casein hydrolysis with better yields than the free enzymes.

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