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Peptide generation from casein hydrolysis by immobilised porcine cathepsins

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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.
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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

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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.

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