PURIFICATION OF CATHEPSINS B AND L FROM BOVINE SPECIES AND PRODUCTION OF SPECIFIC ANTIBODIES

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Background

Endogenous muscle proteolytic enzymes are assumed to play a key role in post-mortem myofibrillar disruption , which is directly related with the development of tenderness in meat. Several evidence allow to conclude that tenderness is the most appreciated quality by consumers at the time to buy meat in the market. While fish flesh normally undergoes a rapid and adequate softening, in the case of mammals and poultry inconsistency and unpredictable variability in meat texture have been identified as two of the major problems that meat industry has to face. The cause of this variability is related with the biological complexity of skeletal muscle [Pette and Staron, 1990]. Calpains and cathepsins are largely the main proteolytic systems studied during years in relation with this problem, and in spite of that a lot of controversy still remains about the importance of each enzyme group in the development of meat texture during ageing [Ouali, 1999]. This controversy comes undoubtedly from the technical limitations we have actually to carry out an adequate quantification of these proteolytic enzymes in muscle crude extracts. Methods actually rely on the determination of their enzyme activities, but these are imprecise because there are no specific substrates for individual enzymes and so the previous separation of proteins is required, but this takes a lot of time. limiting the number of samples that can be analysed per day.

Objectives

With the aim to overcome the difficulties commented above, we thought of importance the necessity to develop alternative methods for a faster, more accurate and specific quantification of proteolytic enzymes without requiring a prior fractionation of muscle crude extracts. If the methods are also easy to use, this would allow us to analyse correctly a large number of samples per day and this could answer a lot of doubts about post-mortem meat biochemistry. We think immunochemistry could be a good alternative to achieve this goal. In this context, the objective of this work was to produce specific antibodies for a further immunochemical quantification of cathepsins B and L. Antipeptide antibodies have been raised against highly specific peptide regions of these enzymes. In addition, a purification protocol has been developed in order to obtain pure cathepsins B and L and certify the quality of antibodies in terms of specificity and cross-reactivity.

Methods

Production of antipeptide antibodies: Peptides containing specific sequences of bovine cathepsins B and L were synthesised and injected into rabbits. A first injection was followed by two boosters separated by 15 days, then antiserums were collected and tested in terms of affinity and cross-reaction to know if they recognise specifically cathepsin B and L, respectively, and if there is no recognition of other proteins in crude extracts.

Purification of cathepsins B and L: 500 grams of bovine kidney were utilised as a enzyme source to prepare a lysosomal-rich extract as described by Lardeaux et al. (1983). The extract was then fractionated with ammonium sulphate, collecting the precipitated protein between 30-70 % saturation. The pellet was redissolved in a minimum volume of 20 mM Bis-Tris buffer, pH 6.0, containing 200 mM NaCl, 2 mM β-mercaptoethanol (β-ME) and 1 mM EDTA and directly injected in a Sephacryl S-200 HR column (2.5 x 100 cm), previously equilibrated with the same buffer. Fractions containing maximal Z-Phe-Arg-AMC hydrolysing activity at pH 5.5 were pooled together and dialysed overnight against 20 mM Sodium Citrate, pH 4.8, containing 2 mM β-ME and 1 mM EDTA. Dialysed proteins were then injected in a POROS-HS cation exchange column (4.6 x 100 mm) and elution was carried out by a three step NaCl gradient as described in figure 1. Two main activity peaks against Z-Phe-Arg-AMC were identified, pooled separately and dialysed against Bis-Tris buffer, pH 6.5. Each pool was then injected in a DEAE-Sephacel column (1.2 x 7 cm) and proteins were eluted with a linear salt gradient from 0 to 500 mM NaCl in 40 ml, collecting in each case fractions with maximal Z-Phe-Arg-AMC hydrolysing activity and identifying the purified proteins as cathepsins B and L, respectively.

Immunoblotting procedures: Samples were first run on SDS-PAGE using 12 % gels, then proteins were transferred to PVDF membranes by electroblotting [Matsudaira, 1987] at 0.8 mA/cm^2. Proteins were developed with both rabbit anti-cathepsin B and anti-cathepsin L antipeptides, followed by alkaline phophatase-labeled goat anti-rabbit IgG and revealed with Lumi-Phos (Lumigen, USA).

Results and discussion

The purification procedure presented here has the objective to obtain appreciable amounts of highly purified cathepsins B and L, in order to test the quality of antipeptides generated against specific sequences of these enzymes. Figure 1 shows the step where cathepsins B and L can be well separated from each other. So, in cation exchange chromatography at pH 4.8 cathepsin B elutes at 188 mM NaCl, while cathepsin L eluted considerably later, at 480 mM NaCl, as reported by other authors [Mason et al., 1984; Dufour et al., 1987]. Western-Blot analysis of the purified proteins revealed with the generated antipeptides (figure 2) shows that they recognise well purified cathepsins B and L. Moreover, anti-cathepsin B and L antipeptides do not cross-react between them (fig. 2, lanes CB and CL). Bovine anti-cathepsin B antipeptide reveals two main bands for pure cathepsin B, at around 30 and 23 kDa (fig. 2A), corresponding to the native protein (light and heavy chain) and the heavy chain alone, respectively [Bradley and Whitaker, 1986]. No band is revealed for cathepsin B light chain, indicating that the peptide sequence recognised by the antibody is located in cathepsin B heavy chain. As for cathepsin B, bovine anti-cathepsin L antipeptide (fig. 2B) recognises specifically the native protein (32.5 kDa) and the heavy chain (25 kDa) of cathepsin L [Bando et al., 1986]. We can also see that antipeptides recognise specifically cathepsin B (fig. 2A, lane 1) or L (fig. 2B, lane 1) in a bovine muscle crude extract, confirming the high specificity of these antipeptide antibodies.

Conclusions

From the present results, we can conclude that the generated antipeptides against peptide sequences of cathepsin B and L seem to be adequate as capture antibodies to quantify separately these two cysteine peptidases in bovine muscle crude extracts by immunochemistry. The present purification scheme reveals to be a rapid and adequate way to obtain appreciable quantities of highly purified cathepsins B and L to be used as a source of antigens for immunological studies.

Pertinent literature
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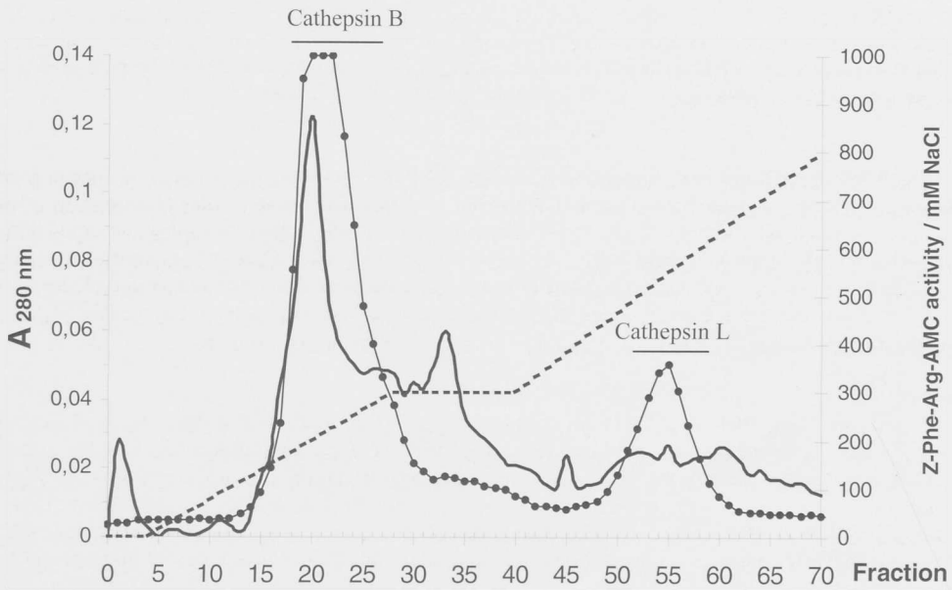

Figure 1: HPLC strong anion exchange chromatography on POROS-HS column (4.6 x 100 mm). Elution in 3-step gradient: a) from 0 to 300 mM NaCl in 24 ml; b) isocratic at 300 mM NaCl for 12 ml and c) from 300 to 800 mM NaCl in 30 ml. (—) $A_{280\ nm}$; (•) Z-Phe-Arg-AMC hydrolysing activity; (---) NaCl (mM).

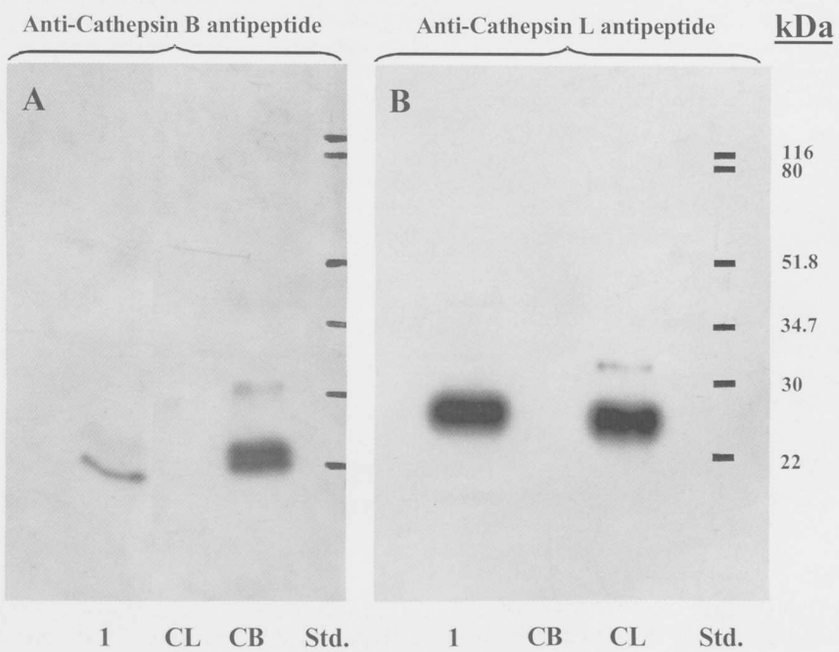

Figure 2: Immunoblot analysis to test the quality of generated antipeptides and that of the purified cathepsins: (1) bovine crude extract, (CB) purified cathepsin B, (CL) purified cathepsin L, and (Std.) protein standards. Membranes were developed with: (A) anti-cathepsin B and (B) anti-cathepsin L antipeptides.