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Procathepsin D cannot autoactivate to cathepsin D at acid pH

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The amino acid sequence of the propart of bovine procathepsin D was determined at the protein level. Incubation of the isolated procathepsin D at pH 3.5–5.0 for 30–120 min leads to a 2 kDa reduction in its molecular mass, as seen by SDS-PAGE. The activation product is pseudocathepsin D and is the result of a proteolytic cleavage between LeuP26 and IleP27 in the propart. Incubation at pH 5.0 for 20 h of either procathepsin D or pseudocathepsin D results in both cases in approximately equal amounts of pseudocathepsin D and a further processed intermediate, nine amino acids shorter than pseudocathepsin D. No reaction products corresponding to cathepsin D with a mature amino terminus were observed, showing that autoproteolysis alone cannot generate the mature form found in the lysosomes.

Aspartic proteinase; Proenzyme activation; Sequence analysis; Procathepsin D

1. INTRODUCTION

Cathepsin D is an intracellular aspartic proteinase found in the lysosomes of mammalian cells [1]. Mature porcine cathepsin D consists of 339 residues [2]. Like other mammalian aspartic proteinases cathepsin D is synthesized as a proenzyme with an amino terminal propart, which in porcine procathepsin D contains 44 residues [3,4]. The amino acid sequence of procathepsin D is well conserved among species and exhibits a high degree of similarity with other aspartic proteinases, e.g. pepsinogen and prochymosin [5–7].

The processing of procathepsin D to the mature enzyme involves conversion of the 46–53 kDa precursor to a 44–47 kDa form, presumably in prelysosomal compartments [3,4,8]. Depending on the species, this single-chain molecule may be cleaved by a cysteine proteinase in the lysosomes into a two-chained form, a 15 kDa light chain derived from the amino terminus, and a 30 kDa carboxy-terminus-derived heavy chain, by excision of 2–7 residues [9,10].

Because of the rapid intracellular processing it has been difficult to obtain procathepsin D for activation studies. However, a small fraction of procathepsin D is secreted from cultured cells, and appears in the medium [3], and the amount of procathepsin D is elevated in media from cultured human breast cancer cells [11]. Experiments with procathepsin D recovered from cell culture media [12–14], and also with procathepsin D from cardiac tissue extract [15] showed that the proenzyme undergoes a pH-dependent reduction in molecular mass of 1–2 kDa, which could be inhibited by pepstatin. The molecular mass of the product was larger than that of mature single-chained cathepsin D, suggesting the formation of an activation intermediate. The product was observed to be proteolytically active [12,13], thereby demonstrating an autocatalytic activation of procathepsin D [12,13,15], which may be intramolecular [14].

To obtain larger quantities of procathepsin D for activation studies a human cDNA was cloned and expressed in *Escherichia coli* [16]. This non-glycosylated procathepsin D required acid pH for refolding, and underwent a simultaneous cleavage, yielding a stable, proteolytically active intermediate [17]. The cleavage had occurred between LeuP26 and IleP27 of the propart [17]. The intermediate was termed pseudocathepsin D by analogy with the activation intermediate of pepsinogen.

Bovine milk contains a proteinase with proteolytic activity at acid pH [18]. In this paper we report the isolation of procathepsin D from bovine milk, the amino acid sequence of the propart, and the investigation of the structural changes occurring when this proenzyme is incubated at acid pH.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine hemoglobin, bovine spleen cathepsin D and Coomassie brilliant blue R were obtained from Sigma. Pepstatin was from Bachem, *N,N*-dicyclohexylcarbodiimide was from Pierce and *N*-hydroxysuccinimide was from Aldrich. DEAE Sepharose fast flow and EAH (aminohexyl) Sepharose 4B were from Pharmacia. Pepstatin was coupled to EAH Sepharose 4B according to [19]. ProBlott PVDF membranes

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Abbreviations. DTE, dithioerythritol; PVDF, polyvinylidene difluoride.

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and reagents for sequence analysis were from Applied Biosystems. Fresh bovine milk was obtained from MD Foods Research and Development Center, Brabrand, Denmark.

2.2. *Enzyme assay*

The assay of proteolytic activity at acid pH was modified from the methods described in [20,21]. The substrate solution was prepared by mixing one part of 5% w/v aqueous solution of bovine hemoglobin and three parts of 0.25 M HCOONa, pH 3.2, and then preincubated at 37°C for 30 min. Pepstatin was dissolved in CH_3OH, and diluted before use. Test tubes contained 50 μl enzyme solution, 50 μl 0.25 M HCOONa, pH 3.2, containing 0.04% v/v CH_3OH and 200 μl preincubated substrate. Final pH was 3.4. Controls were prepared in parallel, but contained 1 μM pepstatin in addition. After incubation at 37°C for 17 h the reaction was stopped by addition of 200 μl cold 17.5% w/v trichloroacetic acid. The precipitated mixture was allowed to stand for 20 min at 4°C, and then centrifuged in an Eppendorf 5415 C centrifuge at 10,000 × *g* for 15 min at 4°C. The liberated peptides in the supernatant were measured by reading the absorbance at 280 nm. Duplicates were carried out for both test and control assays. One unit of enzyme activity was defined as the net absorbance value of 1.0 of the test assay supernatant in excess of the control.

2.3. *Purification of procathepsin D*

All steps were carried out at 4°C. The milk (6 litres) was skimmed by centrifugation at 6,500 × *g* for 10 min in an IEC DPR 6000 centrifuge. The skim milk fraction was acidified to pH 3.5 on ice by addition of 1 M H_2SO_4, and centrifuged at 6,500 × *g* for 10 min. Solid ammonium sulphate was added to the acid whey supernatant to 400 g/l of original volume. After centrifugation at 6,500 × *g* for 15 min, the precipitated proteins were dissolved in 250 ml 0.02 M CH_3COONH_4, pH 5.5, and dialysed overnight against 20 l of the same buffer. DEAE Sepharose fast flow resin was equilibrated in 0.02 M CH_3COONH_4, pH 5.5, and packed in a column (5 × 51 cm). The dialysate, usually about 650 ml, was centrifuged and loaded onto the column. When the proteins had been loaded, the column was washed with approximately two bed volumes of the equilibration buffer to elute non-bound proteins. The remaining proteins were washed out by isocratic elution with 2.0 M CH_3COONH_4, pH 5.5, until the absorbance at 280 nm returned to baseline. The flow rate was 120 ml/h. By enzyme assays, the breakthrough fractions were seen to contain aspartic proteinase activity. Active fractions were pooled and concentrated 2–3 times by ultrafiltration on Amicon (PM 10 membranes). NaCl was added to the concentrated sample to 0.5 M, and the sample was then mixed with 15 ml of the Pepstatinyl-Sepharose gel, which had previously been equilibrated in 0.05 M CH_3COONa, 0.5 M NaCl, pH 3.5. The pH of the suspension was adjusted to 3.5 by addition of 6 M HCl. For batch adsorption, the suspension was rotated for 30 min after which most of the unbound material was removed by filtering on a Büchner funnel without allowing the column material to dry out. The gel slurry was then packed in a column (1 × 20 cm) and washed with 0.05 M CH_3COONa, 0.5 M NaCl, pH 3.5, at a flow-rate of 15 ml/h, until the absorbance at 280 nm returned to baseline. This was followed by a wash with 2–3 bed volumes of 0.05 M CH_3COONa, 0.5 M NaCl, pH 5.3. Bound protein was eluted with 0.05 M Tris-HCl, 0.2 M NaCl, pH 8.5. Following activity assays, the active fractions were pooled, concentrated by ultrafiltration on Amicon to less than 1 ml, and stored at −18°C. For activation studies this solution of purified procathepsin D was acidified to pH 3.5 or pH 5.0 by addition of 5% HCOOH.

2.4. *SDS-PAGE and electroblotting*

Discontinuous SDS-PAGE was carried out in 10–20% gradient gels layered with a 4% stacking gel [22]. The gel was stained in 0.25% w/v Coomassie brilliant blue R in ethanol/acetic acid/water (5:1:4, by volume), and destained in ethanol/acetic acid/water (5:1:4, by volume), followed by 10% acetic acid. For electroblotting, proteins were transferred from unstained SDS-PAGE gels to a PVDF ProBlott membrane [23]. The PVDF membrane was stained for 1 min with 0.1% w/v Coomassie brilliant blue R in 40% methanol/1% acetic acid and destained in 50% methanol.

2.5. *Amino acid and sequence analysis*

Protein content was determined by amino acid analysis [24] after hydrolysis in sealed, evacuated tubes containing 50 μl 6 M HCl, 0.05% phenol and 5 μl thioglycollic acid at 110°C for 16 h.

Amino terminal sequence analyses were performed on a model 477A pulsed liquid sequencer connected on-line to a model 120A PTH derivative analyzer HPLC from Applied Biosystems. The sequencer was equipped with a cross-flow reaction cartridge from Applied Biosystems.

3. RESULTS

3.1. *Purification of bovine procathepsin D*

Procathepsin D was concentrated from acid whey by precipitation with ammonium sulphate. The precipitated proteins were dissolved and applied to a DEAE Sepharose fast flow column at pH 5.5. Under these conditions, the aspartic proteinase could be separated from the majority of the whey proteins, i.e. bovine serum albumin, α-lactalbumin and β-lactoglobulin. Activity assays showed that the proteinase appeared in the breakthrough fractions, of which lactoferrin and lactoperoxidase were the major components. The proteinase was purified from this mixture by Pepstatinyl-Sepharose affinity chromatography (Fig. 1). The proteinase was adsorbed to the gel at pH 3.5. It has been reported that porcine procathepsin D binds pepstatinyl Sepharose at pH 3.5, but not at pH 5.3 [14]. An elution at pH 5.3 was therefore included after adsorption of the proteins to the column (Fig. 1), but no aspartic proteinase activity was found to elute with the pH 5.3 buffer. Instead, bound proteinase was eluted by raising the pH to 8.5, and by measuring the absorbance at 280 nm two small peaks were seen. Enzyme assays showed that the aspartic proteinase activity followed the first peak. The

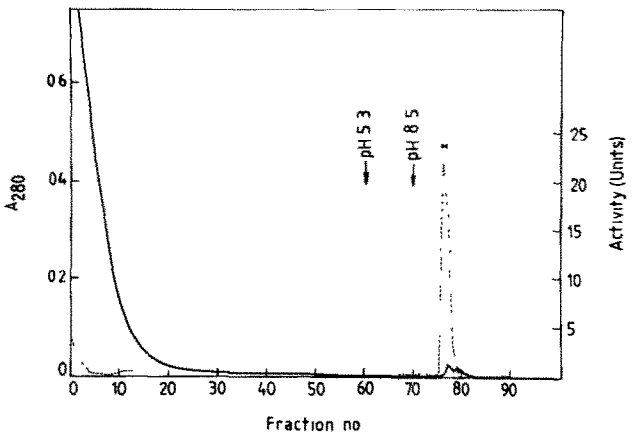

Fig. 1. Purification of procathepsin D from other milk proteins by pepstatinyl Sepharose 4B. Proteins were adsorbed to the affinity resin at pH 3.5. Arrows indicate shifts in the pH of the elution buffer. Fractions were pooled as indicated by the horizontal bar. A_{280} (—); activity (---).

corresponding fractions were subsequently pooled, as indicated in Fig. 1. The purified proteinase was concentrated by ultrafiltration and analysed by SDS-PAGE, revealing a band with a M_r of 45,000 (Fig. 2, lanes 1 and 2).

By electroblotting and sequence analysis of this 45 kDa band, the amino terminus of the protein was found to be valine. We identified 24 of the 27 amino terminal residues unambiguously, while 3 positions appeared heterogenous (Fig. 3). The obtained sequence, corresponding to P1–P26 of the presumed propart, showed a high degree of similarity with the amino-terminal sequence of procathepsin D from other species [5,25–27], suggesting that the purified protein was bovine procathepsin D. This suggestion was further supported by rocket immunoelectrophoresis, where the purified proteinase cross-reacted with antibodies raised in rabbits against bovine spleen cathepsin D (results not shown).

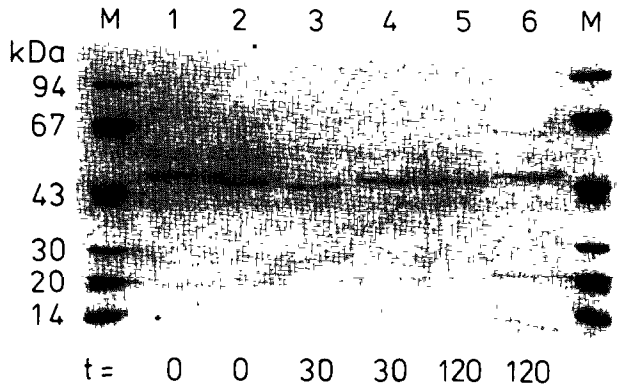

Fig. 2. Autoproteolysis of procathepsin D at pH 3.5. Purified bovine procathepsin D was analysed by SDS-PAGE (10–20%) before and after acid incubation. Lanes 1 and 2: purified procathepsin D before acid activation, corresponding to $t = 0$; lanes 3 and 4: procathepsin D after 30 min of acid activation; lanes 5 and 6: procathepsin D after 120 min of acid activation; M denotes molecular mass marker. The marker and lane 1 are reduced by DTE. Procathepsin D in lanes 4 and 6 was incubated in the presence of 5 μM pepstatin.

3.2. *Acid activation of procathepsin D*

The acid activation of the proenzyme at pH 3.5 was studied in a 2 μM solution of procathepsin D after incubation at 37°C. Samples were drawn at $t = 0$, $t = 30$ and $t = 120$ min, and analysed by SDS-PAGE and sequence analysis after electroblotting. From lanes 2 and 3 (Fig. 2) it is apparent that after incubation at 37°C for 30 min, the molecular mass of procathepsin D has decreased by approximately 2 kDa to an M_r of 43 kDa. Sequence analysis of the band corresponding to Fig. 2, lane 3 revealed that a new amino terminus had been generated, starting at position IleP27. This form corresponds to the intermediate, pseudocathepsin D. By sequence analysis, the 22 amino terminal residues of bovine pseudocathepsin D were identified, covering the rest of the propart (IleP27–GlnP1) and the 4 first residues of the mature bovine cathepsin D (Gly1-Pro2-Ile3-Pro4) [28].

Prolonged acid activation (120 min) at pH 3.5 did not lead to further amino-terminal processing of pseudocathepsin D, as revealed by sequence analysis of the 43 kDa band corresponding to Fig. 2, lane 5. The sequence, covering 10 steps, was identical to that seen after 30 min of incubation. This demonstrates that pseudocathepsin D is quite stable. Pepstatin inhibits the proteolysis, as no decrease in molecular mass occurred in lanes 4 and 6, Fig. 2. Furthermore, the sample represented in Fig. 2, lane 4 was electroblotted and 15 cycles of sequence analysis were performed. The obtained amino terminus was confirmed to be identical with the proenzyme, starting at position ValP1. The activation of the 45 kDa procathepsin D at pH 5.0 was also investigated. In these experiments the procathepsin D concentration was raised to 7 μM. Samples were analysed by SDS-PAGE after 30 min, 120 min, and 20 h (results

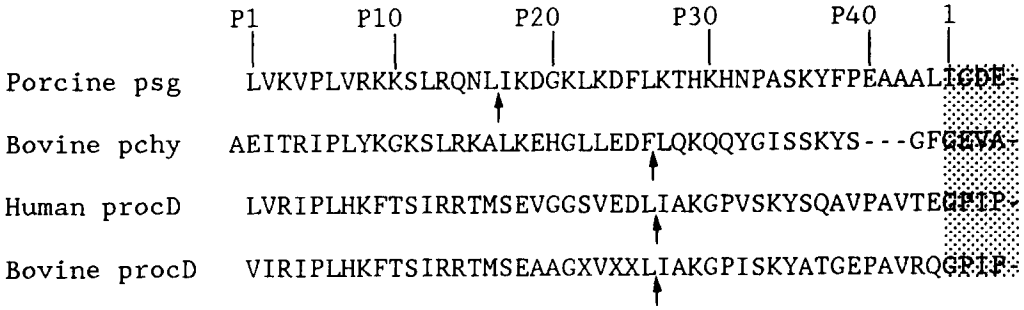

Fig. 3. Alignment of the propart of bovine procathepsin D with other aspartic proteinase prosequences, and comparison of cleavage sites. Psg, pch and procD designate pepsinogen, prochymosin and procathepsin D, respectively. The pepsinogen propart has been numbered P1 P44. The arrows indicate the cleavage sites generating the pseudoforms. Residue 1 corresponds to the amino terminus of mature enzyme. Residue 1 of human cathepsin D was inferred by homology to porcine cathepsin D [19]. X indicates residues in bovine procathepsin D, which could not be unambiguously assigned. In the indicated positions, the following PTH derivatives were seen: P22, Gly and Pro; P24, Thr, Gly, Pro and Glu; P25, Asp, Thr and Glu. References: porcine pepsinogen [29,34,35]; bovine prochymosin [31]; human procathepsin D [5,17,18]; bovine cathepsin D [28].

not shown). In every case the 45 kDa band had been converted into a 43 kDa form. By electroblotting and 10 steps of sequencing it was shown that after incubation at pH 5.0 for 120 min procathepsin D had been converted into pseudocathepsin D with the amino terminus IleP27. The sample incubated at pH 5.0 for 20 h contained approximately equal amounts of pseudocathepsin D and a further processed form with the amino terminus Ala-Thr-Gly-Glu-Pro-Ala-Val, corresponding to an amino terminus at position AlaP36. This shows that after very prolonged incubation at pH 5.0 a cleavage between TyrP35 and AlaP36 can occur. An equivalent series of experiments in which the proenzyme was preincubated at pH 3.5 and 37°C for 30 min, yielding pseudocathepsin D, before the pH 5.0 incubation, was also performed, with identical result. No sequence corresponding to the amino terminus of mature bovine cathepsin D (Gly1) was observed at any time. It therefore appears that native, bovine procathepsin D, like human recombinant pseudocathepsin D [17], is unable to autoactivate to mature cathepsin D.

4. DISCUSSION

Procathepsin D was purified from bovine milk, and its activation studied. After incubation at pH 3.5 the mobility in SDS-PAGE of the purified proenzyme had increased, corresponding to a loss of about 2 kDa. By sequencing the activation product the proteolytic cleavage was defined to have occurred between LeuP26 and IleP27, yielding pseudocathepsin D. The cleavage was inhibited by pepstatin, and was therefore caused by an aspartic proteinase. The high degree of purity of procathepsin D in this study suggests that the cleavage is the result of autoproteolysis, but we cannot totally exclude the possibility that it has been catalysed by a very small amount of another aspartic proteinase. This proteolytic cleavage site, yielding pseudocathepsin D, is identical with the autoproteolytic cleavage occurring when human recombinant procathepsin D is refolded in acid [17]. The pseudocathepsin D may correspond to the proteolytically active intermediates formed when procathepsin D from cell culture media is exposed to acid pH [12,13].

When bovine prochymosin is activated at pH below 2.5 the initial cleavage site occurs between PheP27 and LeuP28 (prochymosin numbering), while the initial cleavage site in porcine pepsinogen is different, occurring between LeuP16-IleP17 (Fig. 3) [29,30]. Incubation of prochymosin at pH below 2.5 yielded pseudochymosin only, while generation of mature chymosin required prolonged incubation of pseudochymosin or prochymosin at pH 4.2–5.5 [31]. The separate proteolysis of pseudocathepsin D and procathepsin D at pH 5.0 was therefore investigated. In both cases, the final product after incubation for 120 min was pseudocathepsin D, while after 20 h of exposure a mixture of pseudocathepsin D and a further processed form with amino terminal AlaP36 appeared. Generation of mature cathepsin D was not observed. These findings support the idea that another proteinase is involved in the intracellular generation of cathepsin D with mature amino terminus [15,17,32].

Since maturation of lysosomal enzymes occurs after segregation from the secretory pathway [8], the presence of the precursor form of cathepsin D in milk suggests a secretion, and not a release from damaged cells. It is not known whether procathepsin D has a physiological role in milk, e.g. after ingestion by the neonate, or whether it has escaped the intracellular sorting, perhaps as a result of the enormous protein secretion from the mammary gland during lactation. Procathepsin D may, after acid activation at pH 3.5–5.0, be able to degrade caseins, or other milk proteins. The acid proteinase partly purified from bovine milk [18], which could be identical to procathepsin D, was able to degrade caseins [33].

The availability of intact procathepsin D from bovine milk opens for future experiments on the transport and activation of this otherwise intracellular and short-lived aspartic proteinase zymogen.

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