# PROTEOLYSIS OF BOVINE F-ACTIN BY CATHEPSIN B

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## INTRODUCTION

Lysosomal cathepsins are one of the major groups of proteolytic enzymes present in muscle. Studies using model systems have indicated which myofibrillar proteins are most susceptible to degradation by the cathepsins (Matsumoto *et al.*, 1983, and Matsukura *et al.*, 1981). Actin degradation by the cathepsins has been reported to occur during the ripening of fermented sausages (Verplaetse 1994). Little information is available on the cleavage specificity of the cathepsins on muscle proteins.

The objectives of this study were to investigate the proteolysis of bovine F-actin by cathepsin B and to determine the specificity of its action on F-actin.

## MATERIALS AND METHODS

### Hydrolysis of bovine F - actin by cathepsin B

Bovine F-actin(0.5mg/ml) was incubated with bovine cathepsin B(1.65U/ml) at pH 5.5 for a period of 6 h at 37$^0$C. Samples were taken periodically for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemlii (1970).

### Electroblotting

Peptides which stained on SDS-PAGE gels were isolated by electroblotting onto a polyvinylidenedifluoride membrane, pore size 0.22μm using a mini Trans - Blott$^{TM}$ electrophoretic transfer cell (Bio-Rad, Richmond, CA94804, USA) and their N-terminal sequence determined.

### Reversed phase high performance liquid chromatography

Reversed phase high performance liquid chromatography (RP-HPLC) was performed on the 2% trichloroacetic acid (TCA) soluble fraction of the 6 h hydrolysate. RP-HPLC was performed using a Nucleosil column, ($C_8$ 300A°, 250 x 4.6 mm). Elution was by means of a gradient using solvent A (0.1% trifluoroacetic acid (TFA) in $H_2O$. and solvent B (0.1% TFA in acetonitrile) as follows: 100% A for 5 min, 0 - 40% B at a rate of 0.73%/min followed by an increase of B by 4%/min for 5 min. The concentration of B was kept constant at 60% for 3 min and then increased at a rate of 8.75%/min for four min. Flow rate was 0.75 ml/min and detection was at 214nm.

### Identification of peptides.

N - terminal sequence analysis.

Peptides were sequenced by Edman degradation on an automated pulsed liquid-phase protein - peptide sequencer (Applied Biosystems Inc., Foster city, CA 94404, USA; model 477A). Liberated amino acids were detected as their phenylthiohydantoin derivatives by means of a model 120A analyser (Applied Biosystems Inc.).

Mass Spectrometry.

Mass spectrometry was carried out on the 2% TCA soluble peptides isolated by HPLC using a plasma desorption time of flight mass spectrometer. Identification of peptides by mass was based on partial sequence data combined with mass searches using the GPMAW program ( Lighthouse Data, DK - 5250 Odense SV, Denmark).

## RESULTS AND DISCUSSION

SDS-PAGE electrophoretograms of bovine F-actin incubated with cathepsin B showed almost complete degradation of the protein, with the simultaneous appearance of three degradation products with molecular masses estimated by reference to molecular weight standards of 29, 33 and 35 kDa, respectively (Fig.1). Hydrolysis of actin was rapid with degradation products being evident after 30 min incubation. The three high molecular weight peptides were produced from cleavage towards the N-terminus of the protein molecule. The cleavage sites which resulted in the production of the N-termini of the high molecular weight peptides were identified (Table 1).

The RP-HPLC elution profile of the 2% TCA soluble fraction of a 6 h cathepsin B hydrolyzate of actin is shown in Fig. 2. The N-termini of thirteen of the peptides isolated by HPLC were identified (Table 2). The C-termini of seven of these peptides was identified by mass spectrometry. The release of many of the small peptides from the N-terminus is likely due to the fact that the large peptides were released from this area of the protein after 30 min of incubation, thus liberating fragments which maybe further hydrolysed by the enzyme (Fig 3). Cathepsin B had a broad cleavage specificity on actin, cleaving many types of bonds including Met - Gly, Thr - Leu, Gly - Phe, Lys - Tyr, Lys - Ile, Thr - Asn, Arg - Val, Thr - Ile, Met - Tyr, Ala - Leu, Glu - Tyr, Arg - Ala, His - Gly, Phe - Tyr. The bonds most susceptible to hydrolysis by cathepsin B in this study contained hydrophobic amino acid residues on the carboxyl side of the bond cleaved.

### Conclusions

The proteolytic specificity of cathepsin B on bovine F-actin in vitro has been determined. The majority of the peptides detected were released from the N and C-termini of the protein molecule.

### References

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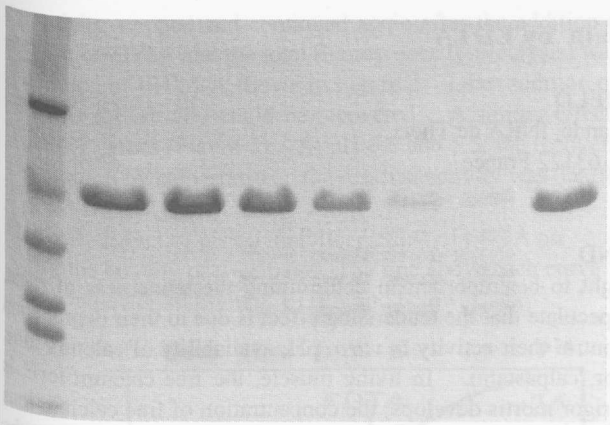


Figure 1: F-actin(0.5mg/ml) was incubated with cathepsin B (1.65U/ml) at 37 °C for 6 h in 50mM Na acetate buffer, pH 5.5 containing 12.5 mM NaCl, 1.5 mM EDTA, 1.5 mM Diothioreitol and 0.05% Sodium azide. A substrate protein load of 5.4 ug was applied to the gel. A 12.5% gel with a 4% stacking gel was used. Lane 1 contains molecular weight standards ranging from 66-20 KDa. Lane 2 is the control at 0 time. Lanes 3 - 7 contain hydrolysate samples taken at 0, 0.5, 2, 4, and 6 h. Lane 8 is the control at 6 h.

Table 1: Identity of peptides detectable by SDS-polyacrylamide gel electrophoresis produced from bovine F-actin by cathepsin B at pH 5.5.

| | N-Terminal sequence | Identity | N-Terminal Cleavage site |
|---|---|---|---|
| A | Gly-Gln-Lys-Asp-Ser-Tyr-Val-Gly-Asp | $H_2N$-$Gly_{50}$-* | $Met_{49}$ - $Gly_{50}$ |
| B | Leu-Lys-Tyr-Pro-Ile-Glu- | $H_2N$-$Leu_{69}$-* | $Thr_{68}$ - $Leu_{69}$ |
| C | Thr-Glu-Ala-Pro-Leu-Asn-Pro- | $H_2N$-$Thr_{108}$-* | $Leu_{107}$ - $Thr_{108}$ |

* Incomplete sequences

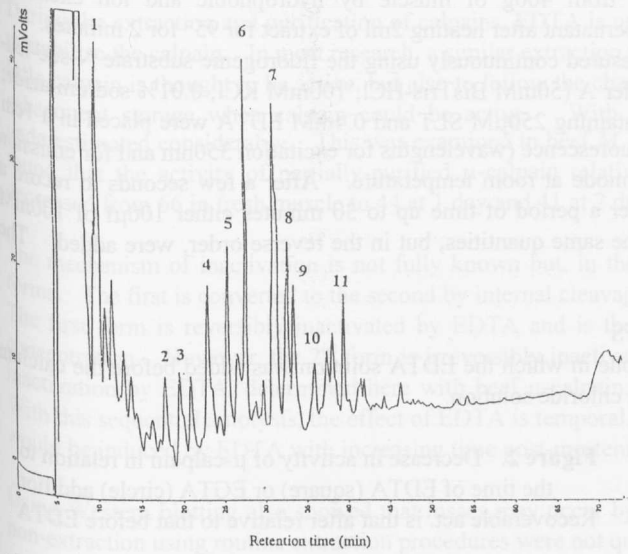


Figure 2 : RP - HPLC chromatogram of 2% TCA soluble peptides released from bovine f - actin by cathepsin B at pH 5.5.

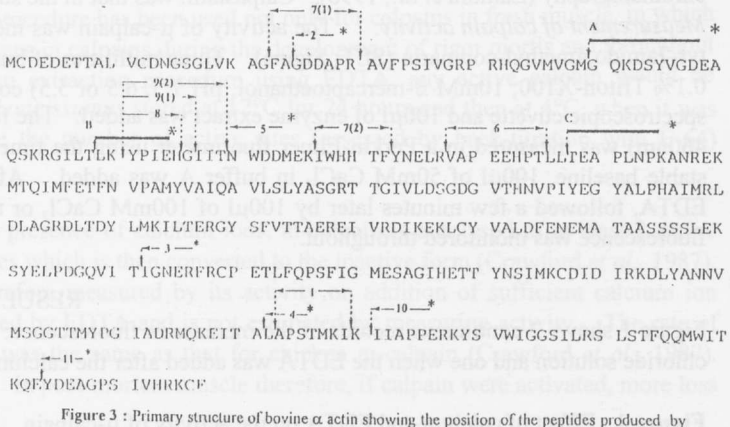


Figure 3 : Primary structure of bovine α actin showing the position of the peptides produced by hydrolysis with cathepsin B at pH 5.5. Letters represent the peptides identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Regions of the peptides that were sequenced are indicated by ——— / ——— and numbers correspond to peptides isolated by RP-HPLC. Cleavage sites are represented by ——► Astericks indicate incomplete sequences.

Table 2 : Identity of the 2% TCA soluble peptides produced from bovine f - actin by cathepsin B at pH 5.5.

| HPLC peak no. | Sequence identity | Experimental mass (Da) | Theoretical mass (Da) | Peptide identity | N-Terminal cleavage site | C-Terminal cleavage site |
|---|---|---|---|---|---|---|
| 1 | $H_2N$-Ala-Pro-Ser-Thr-Met-Lys-Ile-COOH | 793.5 | 746.93 | $Ala_{323}$ - $Ile_{329}$ | $Leu_{322}$ - $Ala_{323}$ | $Ile_{329}$ - $Lys_{330}$ |
| 2 | $H_2N$-Gly-Asp-Asp-Ala-Pro..... | - | - | $Gly_{25}$..... | $Ala_{24}$ - $Gly_{25}$ | * |
| 3 | $H_2N$-Ile-Gly-Asn-Gly-Arg..... | - | - | $Ile_{252}$..... | $Thr_{251}$ - $Ile_{252}$ | * |
| 4 | $H_2N$-Leu-Ala-Pro-Ser..... | - | - | $Leu_{322}$..... | $Ala_{321}$ - $Leu_{322}$ | * |
| 5 | $H_2N$-Asn-Trp-Asp-Asp..... | - | - | $Asn_{80}$..... | $Thr_{79}$ - $Asn_{80}$ | * |
| 6 | $H_2N$-Val-Ala-Pro-Glu-Glu-His-Pro-Thr-COOH | 880.5 | 878.94 | $Val_{98}$ - $Thr_{105}$ | $Arg_{97}$ - $Val_{98}$ | $Thr_{105}$ - $Leu_{106}$ |
| 7(1) | $H_2N$-Phe-Ala-Gly-Asp-Asp-Ala-Pro-Arg-COOH | 847.2 | 847.88 | $Phe_{23}$ - $Arg_{30}$ | $Gly_{22}$ - $Phe_{23}$ | $Arg_{30}$ - $Ala_{31}$ |
| 7(2) | $H_2N$-Ile-Trp-His-His-Thr-Phe-COOH | 847.2 | 839.94 | $Ile_{87}$ - $Phe_{92}$ | $Lys_{86}$ - $Ile_{87}$ | $Phe_{92}$ - $Tyr_{93}$ |
| 8 | $H_2N$-Tyr-Pro-Ile-Glu-His-COOH | 672.8 | 672.72 | $Tyr_{71}$ - $His_{75}$ | $Lys_{70}$ - $Tyr_{71}$ | $His_{75}$ - $Gly_{76}$ |
| 9(1) | $H_2N$-Tyr-Pro-Ile-Glu-His-Gly-COOH | 737.7 | 729.77 | $Tyr_{71}$ - $Gly_{76}$ | $Lys_{70}$ - $Tyr_{71}$ | $Gly_{76}$ - $Ile_{77}$ |
| 9(2) | $H_2N$-Tyr-Pro-Ile-Glu-His-Gly-Ile-Ile-Thr-COOH | 1070.4 | 1057.2 | $Tyr_{71}$ - $Thr_{79}$ | $Lys_{70}$ - $Tyr_{71}$ | $Thr_{79}$ - $Asn_{80}$ |
| 10 | $H_2N$-Ile-Ile-Ala-Pro-Pro..... | - | - | $Ile_{331}$..... | $Lys_{330}$ - $Ile_{331}$ | * |
| 11 | $H_2N$-Tyr-Asp-Glu-Ala..... | - | - | $Tyr_{164}$..... | $Gly_{161}$ - $Tyr_{164}$ | * |

* C-Terminal cleavage sites not identified by mass spectrometry.
..... C-Terminus of peptides not identified by mass spectrometry.
- Experimental mass not determined by mass spectrometry.