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Proteolysis of bovine F-actin by cathepsin B

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Abstract

The proteolytic specificity of cathepsin B on bovine F-actin was investigated. Actin (0.5 mg/ml) was incubated with cathepsin B (1.65 U/ml) for 6 h at 37°C and samples were taken periodically for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). During incubation, actin was hydrolysed with the simultaneous appearance of three peptides detectable by SDS–PAGE with molecular masses of 35, 33, and 29 kDa. These peptides were electroblotted from SDS–PAGE gels onto polyvinylidene difluoride membranes and their N-terminal sequence determined by Edman degradation. Principal cleavage sites of cathepsin B activity on actin were identified at Met_{49}–Gly_{50}, Thr_{68}–Leu_{69} and Leu_{107}–Thr_{108}. Reverse-phase high performance liquid chromatography (RP-HPLC) was performed on 2% trichloroacetic acid-soluble fractions of the 6 h hydrolysate. Thirteen peptides separated by RP-HPLC were collected and identified from their N-terminal sequence and, in some cases, from their mass (as determined by mass spectrometry). Cleavage sites were identified at: Gly_{22}–Phe_{23}, Ala_{24}–Gly_{25}, Arg_{30}–Ala_{31}, Lys_{70}–Tyr_{71}, His_{75}–Gly_{76}, Gly_{76}–Ile_{77}, Thr_{79}–Asn_{80}, Lys_{86}–Ile_{87}, Phe_{92}–Tyr_{93}, Arg_{97}–Val_{98}, Thr_{105}–Leu_{106}, Thr_{251}–Ile_{252}, Ala_{321}–Leu_{322}, Leu_{322}–Ala_{323}, Ile_{329}–Lys_{330}, Lys_{330}–Ile_{331}, and Glu_{363}–Tyr_{364}. The results of this study showed that actin was degraded extensively by cathepsin B with the majority of the peptides released arising from the N- and C-termini of the protein.

Keywords: Actin; Cathepsin B; Proteolysis specificity

1. Introduction

Cathepsins are one of the major groups of proteolytic enzymes in muscle. Up to 20 cathepsins have been identified in muscle, four of which (cathepsins B, D, H and L) are considered important in the degradation of muscle proteins post mortem (Zeece, Woods, Keen, & Reville, 1992). The cathepsins exhibit exo- and/or endo-peptidase activity and all have an acidic pH optimum. Studies using model systems have indicated that myofibrillar proteins are susceptible to hydrolysis by the cathepsins. Matsukura, Okitani, Nishimuro, and Kato (1981) showed that cathepsin L degraded myosin heavy chain, α-actinin, actin, troponin T, and troponin I at pH 5.0. Matsumota, Okitani, Kitamura, and Kato (1983) reported that cathepsin D hydrolysed myosin heavy chain, α-actinin, tropomyosin, troponins T and I at pH 3.0. Noda, Isogai, Hayashi, and Katunuma (1981) found that cathepsin B hydrolysed myosin, troponin, tropomyosin, and actin at pH 6.0. In addition cathepsins B and D have been shown to hydrolyse myosin and actin at pH 5.0 (Schwartz & Bird, 1977).

Cathepsin B is a cysteine proteinase with a molecular mass of between 24 and 27 kDa (Barrett, 1977), and a pH optimum of 6.0 (Barrett & Kirschke, 1981; Keilová, 1971). Although much work has been performed on the activity of cathepsin B on myofibrillar proteins, little information is available on the cleavage specificity of this enzyme on muscle proteins. Schwartz and Bird (1977) reported that cathepsin B hydrolysed actin at pH 5.0 to yield a polypeptide of approximately 35 kDa. In contrast, Noda et al. (1981) concluded that actin was degraded slowly by cathepsin B but no degradation products were detected by SDS–PAGE. 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 this protein.

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2. Materials and methods

2.1. Hydrolysis of bovine F-actin by cathepsin B

Actin was isolated from the *sternomandibularis* muscle of a freshly slaughtered steer according to the method of Pardee and Spudich (1982). The actin solution was dialysed against incubation buffer (50 mM Na acetate buffer, pH 5.5, containing 12.5 mM NaCl, 1.5 mM disodium ethylenediaminetetraacetic acid, 1.5 mM dithiothreitol and 7.7 mM sodium azide) overnight. The protein concentration of the extract was determined by the method of Lowry, Rosenbrough, Farr, and Randall (1951), using bovine serum albumin as a standard. Bovine cathepsin B (E.C. 3.4.22.1.), containing 28 units/mg protein was obtained from Sigma Chemical Co. (St. Louis, MO; 1 unit hydrolyses 1 μmole of Nα-carboxybenzyloxylysine-*p*-nitrophenyl ester per min at pH 5.0 and 25°C). The enzyme was solubilised in incubation buffer, and stored at −20°C until required. The enzyme (1.65 U) was preincubated in the incubation buffer for 5 min at 37°C prior to addition of protein (diluted to a final concentration of 0.5 mg/ml). The action of cathepsin B on actin was studied over a period of 6 h at 37°C. Samples were taken after 0 and 30 min, 2, 4, and 6 h for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970), in gels containing 12.5% T (acrylamide plus bisacrylamide), 4% C (bisacrylamide as a percentage of T). After electrophoresis, the gels were stained using Coomassie Brilliant Blue R-250 (0.1%) in fixative (40% methanol, 10% acetic acid). Destaining was performed using 40% methanol and 10% acetic acid. Following addition of SDS–PAGE sample buffer, aliquots for analysis were heated at 100°C for 5 min prior to electrophoresis. A control was prepared by heating the enzyme at 100°C for 15 min prior to addition of protein. The molecular weights of the hydrolysis products were estimated by reference to the relative mobilities of standard proteins (bovine serum albumin, 66 kDa; chicken egg ovalbumin, 45 kDa; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine erythrocyte carbonic anhydrase, 29 kDa; bovine pancreas trypsinogen, 20 kDa; soybean-trypsin inhibitor, 20 kDa).

2.2. Electroblotting

Peptides, which stained on SDS-PAGE gels, were isolated by electroblotting using a mini Trans-Blott™ electrophoretic transfer cell (Bio-Rad, Richmond, CA). Electroblotting was performed at 90 V (300 mA) for 12 min in transfer buffer (10 mM-3-[cyclohexylamino]-1-propanesulphonic acid, pH 11 in 10% methanol), onto a polyvinylidenedifluoride membrane, pore size 0.22 μm (ProBlott™, Applied Biosystems Inc., Foster city, CA). Membranes were stained for 5 min using Coomassie Brilliant Blue R-250 in 50% methanol, 11% acetic acid and subsequently destained in 50% methanol. The electroblotts were stored at −18°C until N-terminal sequencing was performed.

2.3. 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 hydrolysates. RP-HPLC was performed on a Shimadzu liquid chromatograph (Kyoto, Japan) consisting of a model LC-9A pump, SIL-9A autosampler and a SPD-6A UV spectrophotometric detector. HPLC was performed using a Nucleosil C_8 column (300 Å, 5 μm, 250×4.6 mm). Elution was by means of a gradient of solvent A [0.1% trifluoroacetic acid (TFA) in H_2O] and solvent B (0.1% TFA in acetonitrile, Aldrich Chemical Co., Milwaukee, WI) formed as follows: 100% A for 5 min, 0–40% B at a rate of 0.73% B per min followed by 4% B per min for 5 min. The concentration of B was maintained at 60% for 3 min and then increased at a rate of 8.75% B per min for 4 min. Flow rate was 0.75 ml/min and detection was at 214 nm. Peptides were isolated by RP-HPLC from hydrolysates containing a protein concentration of 1.5 mg/ml and enzyme concentration of 4.95 U/ml incubated at 37°C for 6 h. Hydrolysates containing 0.5 mg/ml protein and 1.95 U/ml cathepsin B were prepared at 0, 0.5, 2, 4, and 6 h for peptide profiling by RP-HPLC. TCA to a final concentration of 2% was added to the hydrolysates which were then centrifuged at maximum speed for 5 min in a Sanyo (UK) MSE Eppendorf centrifuge and the supernatant was retained. The centrifugation step was repeated twice followed by filtration through a 0.45 μm filter, SRP15 (Sartorius, Germany) prior to injection of a 100 μl sample volume. Peptides were collected and freeze-dried and identified by N-terminal sequencing and mass spectrometry.

2.4. Identification of peptides

Peptides were sequenced at the National Food Biotechnology Centre, University College, Cork, Ireland, by Edman degradation on an automated pulsed liquid-phase protein–peptide sequencer (Applied Biosystems Inc., Foster City, CA; model 477A). Liberated amino acids were detected as their phenylthiohydantoin derivatives by means of a model 120A analyser (Applied Biosystems Inc.).

Mass spectrometric analyses were performed at the Department of Biochemistry, Faculty of Medicine and Health Sciences, Queens Medical Centre, Nottingham,

UK. Mass spectrometry was carried out on the 2% TCA-soluble peptides isolated by RP-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).

3. Results and discussion

3.1. SDS–PAGE

SDS–PAGE electrophoretogram of bovine F-actin incubated with cathepsin B for 6 h showed almost complete degradation of the protein, with the appearance of three degradation products with molecular masses (estimated by reference to molecular weight standards) of 35, 33 and 29 kDa, respectively (Fig. 1). Hydrolysis of actin was rapid with degradation products being evident after 30 min incubation. The intensity of the peptide bands did not increase from 2 to 6 h, even though the actin continued to be hydrolysed, perhaps because these degradation products were subsequently hydrolysed by the enzyme. The control containing heat-inactivated enzyme showed no actin degradation after 6 h of incubation, thus indicating the absence of indigenous proteinase activity in the actin preparation. Schwartz and Bird (1977) reported that a single polypeptide of approximate molecular weight of 35 kDa was released from bovine F-actin by cathepsin B at pH 5.0. The results of this study also showed a degradation product of 35 kDa; however two further polypeptides of masses of 29 and 33 kDa were also observed. The three high molecular weight peptides observed in this study were produced from cleavage towards the N-terminus of the protein molecule (Fig. 3). Noda et al. (1981) reported that actin was degraded slowly by cathepsin B at pH 6.0, but no hydrolysis products were detected by SDS–PAGE gels. A fourth band of approximately 23 kDa was also evident on the gel (Fig. 1). The N-terminal sequence of this band was determined and commenced at Val_{48} in the cathepsin B sequence. Katunuma and Kominami (1983) reported that cathepsin B is cleaved by limited proteolysis including autolysis between Asn_{47} and Val_{48} to form two polypeptide chains, the larger of which was visible by SDS–PAGE.

At present there is little information available on the cleavage specificity of indigenous proteinases on muscle proteins. The cleavage sites which resulted in the production of the N-termini of the peptides detectable by SDS–PAGE were identified at Met_{49}–Gly_{50}, Thr_{68}–Leu_{69} and Leu_{107}–Thr_{108} (Table 1). The identities of the C-termini of the three peptides were not determined; however, considering the cleavage sites and their approximate molecular masses it is likely that peptides A, B and C extend to or close to the C-terminus of the protein molecule.

3.2. RP-HPLC

The RP-HPLC elution profile of the 2% TCA-soluble fraction of a 6 h hydrolysate of actin by cathepsin B is shown in Fig. 2. Preliminary experiments were necessary to select an elution gradient that gave adequate resolution of the peptides in the hydrolysate. However, a number of single peaks, when isolated and sequenced, were found to contain more than one peptide. The N-termini of 13 of the peptides isolated by RP-HPLC were

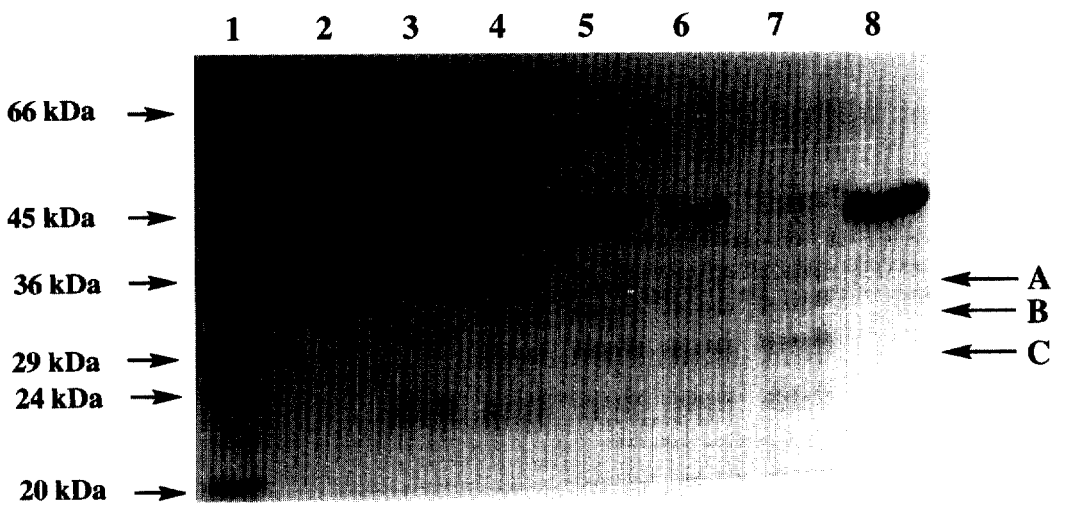

Fig. 1. F-actin (0.5 mg/ml) was incubated with cathepsin B (1.65 U/ml) at 37°C for 6 h in a 50 mM Na acetate buffer, pH 5.5 containing 12.5 mM NaCl, 1.5 mM EDTA, 1.5 mM diothiothreitol and 7.7 mM sodium azide. A substrate protein load of 5.4 μg was applied to the gel. Lane 1, molecular weight standards ranging from 66 to 20 kDa; Lane 2, control at 0 min. Lanes 3–7, hydrolysate taken at 0, 0.5, 2, 4, and 6 h; Lane 8, heat-inactivated control at 6 h. A, B and C indicate hydrolysis products.

identified (Table 2). The C-termini of seven of these peptides were identified by mass spectrometry. In general the masses of the peptides determined experimentally agreed closely with the theoretical masses of the corresponding peptides. The C-termini of peptides 2–5, 10 and 11 (Table 2) were not identified by mass analysis; these peptides may be relatively short as their N-terminal sequences were weak after the second cycle of Edman degradation. The majority of the peptides identified were produced from the N-terminus of the actin molecule (Fig. 3). The release of small peptides from the N-terminus is likely due to the fact that the major peptides were released from this area of the protein during the first 30 min of incubation, thus liberating protein fragments which would be more readily hydrolysed by the enzyme.

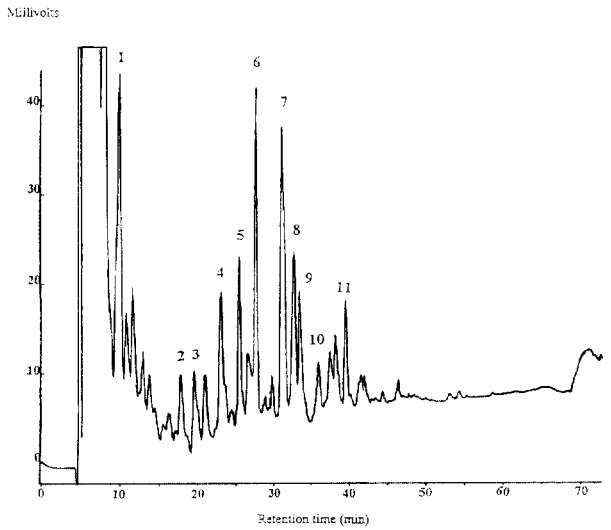

Fig. 2. Reversed-phase HPLC (C_8) chromatogram of 2% TCA-soluble peptides released from bovine F-actin (1.5 mg/ml) by cathepsin B (4.95 U/ml) incubated in 50 mM Na acetate buffer, pH 5.5 at 37°C for 6 h.

Cathepsin B had a broad specificity and cleaved actin at 20 sites: Met_{49}–Gly_{50}, Thr_{68}–Leu_{69}, Leu_{107}–Thr_{108}, Gly_{22}–Phe_{23}, Ala_{24}–Gly_{25}, Arg_{30}–Ala_{31}, Lys_{70}–Tyr_{71}, His_{75}–Gly_{76}, Gly_{76}–Ile_{77}, Thr_{79}–Asn_{80}, Lys_{86}–Ile_{87}, Phe_{92}–Tyr_{93}, Arg_{97}–Val_{98}, Thr_{105}–Leu_{106}, Thr_{251}–Ile_{252}, Ala_{321}–Leu_{322}, Leu_{322}–Ala_{323}, Ile_{329}–Lys_{330}, Lys_{330}–Ile_{331}, and Glu_{363}–Tyr_{364}. These results are consistent with those of Keilova (1971) who reported that cathepsin B cleaved various types of bonds in the oxidized B chain

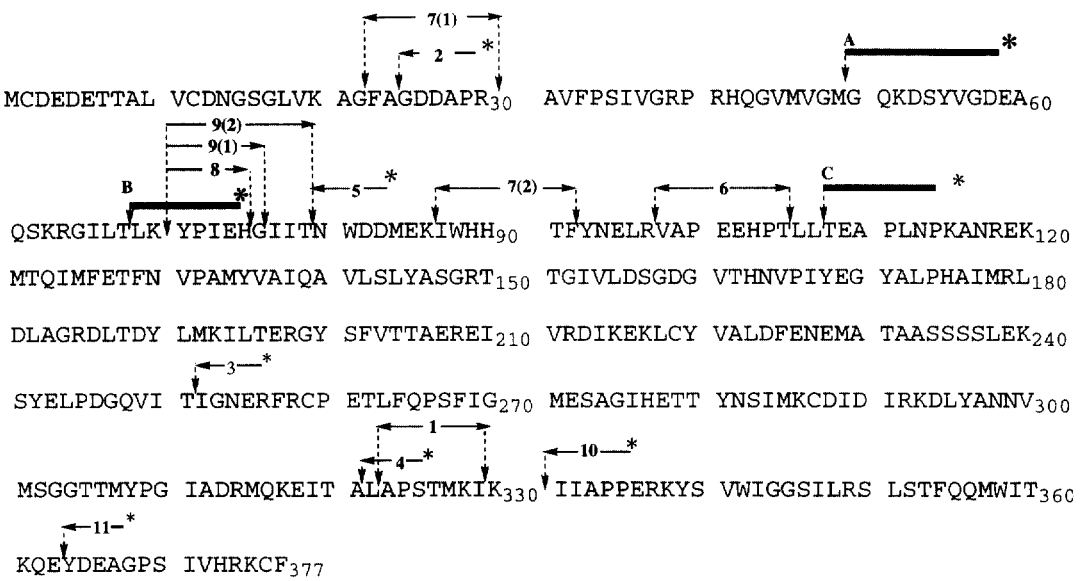

Fig. 3. Primary structure of bovine α actin showing the position of the peptides produced by hydrolysis with cathepsin B at pH 5.5. Letters represent the peptides identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Fig. 1). Numbers correspond to peptides isolated by reversed phased-HPLC (Table 2). Regions of the peptides that were sequenced are indicated by —/— Cleavage sites are indicated by ↓. * Incomplete sequences.

Table 1
Identity of peptides detectable by sodium dodecyl sulphate-polyacrylamtide gel electrophoresis produced from bovine F-actin by cathepsin B

Peptide	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.

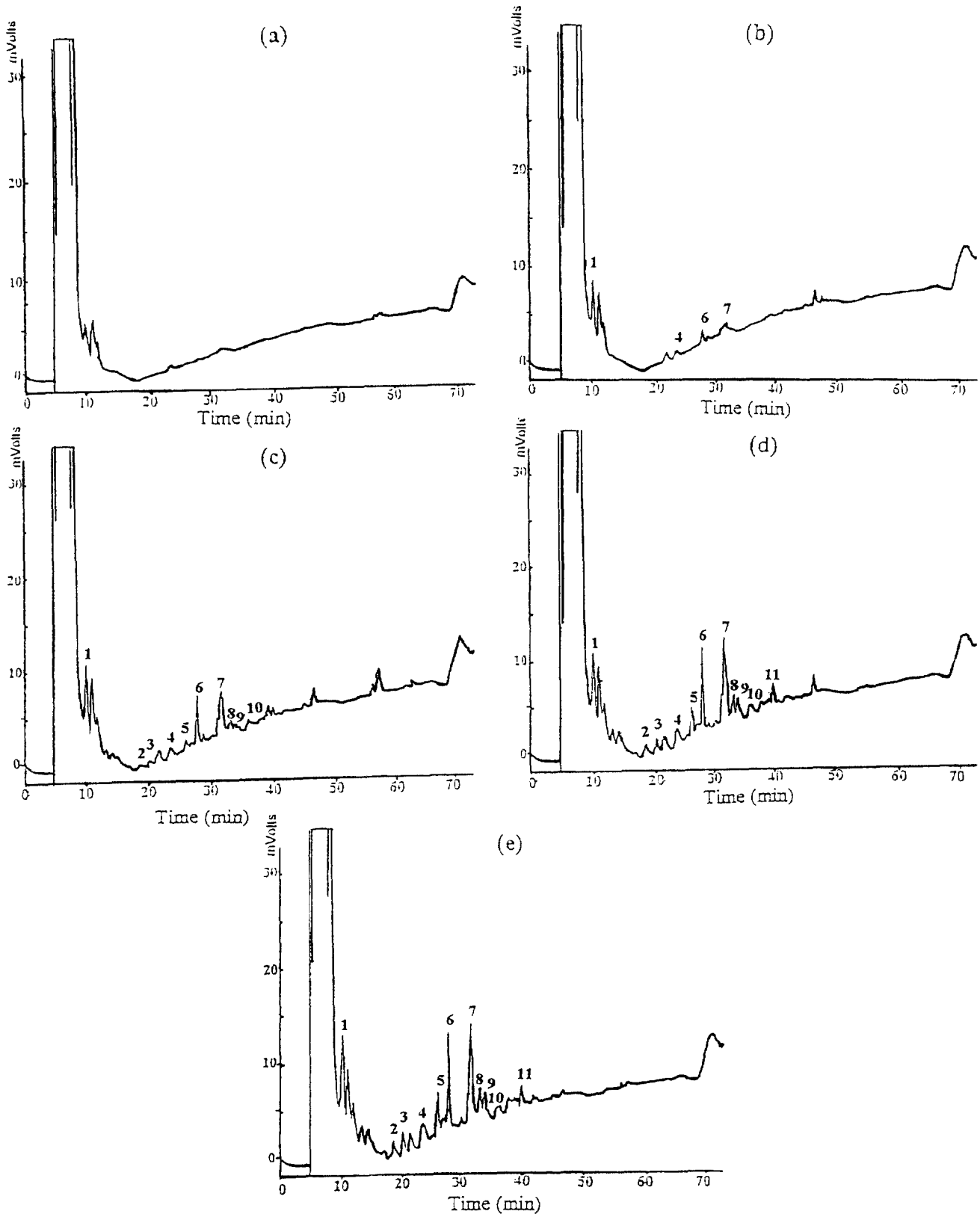

Fig. 4. Reversed-phase HPLC (C_8) elution profiles of 2% TCA-soluble peptides produced from bovine F-actin (0.5 mg/ml) by cathepsin B (1.65 U/ml) in 50 mM Na acetate buffer, pH 5.5 at 37°C for 0, 0.5, 2, 4, and 6 h (a, b, c, d and e).

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–Glu–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_{364}....	Glu_{363}–Tyr_{364}	*

* 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.

of insulin including the following Phe_1–Val_2, Asn_3–Gln_4, His_5–Leu_6, Gly_8–Ser_9, Glu_{13}–Ala_{14}, Ala_{14}–Leu_{15}, Leu_{15}–Tyr_{16}, Leu_{17}–Val_{18}, Gly_{20}–Glu_{21}, Arg_{22}–Gly_{23}, Gly_2–Phe_{24}, and Tyr_{26}–Thr_{27}. Both studies show that many bonds cleaved by cathepsin B involve hydrophobic residues. Changes in RP-HPLC elution profiles of the 2% TCA-soluble peptides released from actin by cathepsin B over time are shown in Fig. 4. The profiles show an increase in the concentration of most of the peptides released over the 6 h time period. A peptide with a retention time of approximately 46 min was produced, after 30 min, but was subsequently degraded between 4 and 6 h (Fig. 4). A peptide with retention time of 57 min present in the 2 h hydrolysate was degraded by 4 h (Fig. 4(d)).

The peptides produced as a result of myofibrillar protein degradation in fermented meats have an important influence on flavour. High levels of small peptides impart a spicy taste while low concentrations of small peptides give rise to beefy and sweet sausages. Hydrophilic peptides are associated with desirable flavour, while hydrophobic peptides impart off flavours to the product (Verplaetse, 1994). The peptides released from actin by cathepsin B in this study were resolved in the hydrophilic and intermediate regions of the HPLC profiles (0 to 40 min). Verplaetse, DeBosschere, and Demeyer (1989) showed that a 30% reduction in the actin concentration occurred during the ripening of fermented sausages, which was believed to be primarily due to the activities of cathepsins B and D. The peptides released from myofibrillar proteins during the ripening of fermented sausages have not been identified to date and therefore it is not known if the peptides identified in this study play a role in the flavour development of fermented meat products.

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