LOW MOLECULAR MASS PROTEASE: EVIDENCE FOR A NEW FAMILY OF PROTEOLYTIC ENZYMES

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1. Introduction

We have isolated from decapode crustacea a proteolytic enzyme with the remarkably low M_r value of 11 000 [1]. This low M_r protease is synthesized in the hepatopancreas and secreted into the stomach-like cardia where it serves digestive processes. We have investigated the immunological properties of the low M_r protease in the species *Astacus fluviatilis, Astacus leptodactylus* (crayfish) as well as in *Carcinus maenas* (crab) [2,3]. The enzyme occurs in multiple and closely related forms varying from species to species [4].

Proteolytic enzymes with similar properties have been noticed in other crustacea, e.g., *Orconectes virilis* [5], *Homarus americanus* [6] and *Penaeus setiferus* [7]. However, the distribution of low M_r protease in other invertebrate species is still unknown, and in vertebrate animals this enzyme is apparently lacking completely. The amino acid composition, length of the protein chain, and other observations suggested that the low M_r protease might be a member of a hitherto unknown family of proteolytic enzymes [8]. This finding has been confirmed by the results of chemical modification, cleavage specificity toward peptide substrates, and partial amino-terminal sequence analysis of the enzyme.

2. Materials and methods

Digestive juice (~500 ml) was collected from the cardia of living crayfish *(Astacus fluviatilis)* as in [9]. The dark-brown crude extract contained ~1 mg enzyme/ml. After centrifugation, 100 ml extract was applied to a DEAE-Sephacel column (4 × 45 cm, 560 ml bed vol.) equilibrated with Tris–HCl buffer 0.01 M (pH 8.0). The enzyme was eluted by applying a NaCl gradient (0.2–2.0 M). Further purification was achieved by gel filtration through Sephadex G-50 (see fig.1). The repetition of this procedure resulted in a product apparently free of any protein contaminant. Antisera against pure low M_r protease were produced in New Zealand rabbits by repeated intramuscular injection of 500 μg enzyme in complete Freund's adjuvant. Amino-terminal sequence analysis was performed with a solid-phase peptide sequencer (LKB model 4020). Low M_r protease (2 mg) was dissolved in 0.4 M dimethylallylamine–trifluoracetate (pH 9.5), denatured by the addition of 6 M guanidine–HCl and attached to DITC-3 aminopropylglass as in [10].

3. Results and discussion

Fractionation of the digestive juice of *A. fluviatilis* by anion-exchange chromatography resulted in the elution pattern of fig.1a. By use of specific substrates, carboxypeptidase B [11] and 3 multiple forms of *A. fluviatilis* trypsin [2] can be distinguished from the single peak of low M_r protease. Upon gel filtration this peak easily separates from overlapping material which also includes tryptic activity (fig.1b). The whole purification procedure is considerably facilitated by the fact that the low M_r protease is distinguished from most of the accompanying proteins by a rather low isoelectric point (pH ~ 4.0) and its small size.

Fig.2a demonstrates that the pure enzyme preparation, as judged by disk electrophoresis, consists of 2 multiple forms. When subjected to 2-dimensional Laurell-immunoelectrophoresis only one precipitation

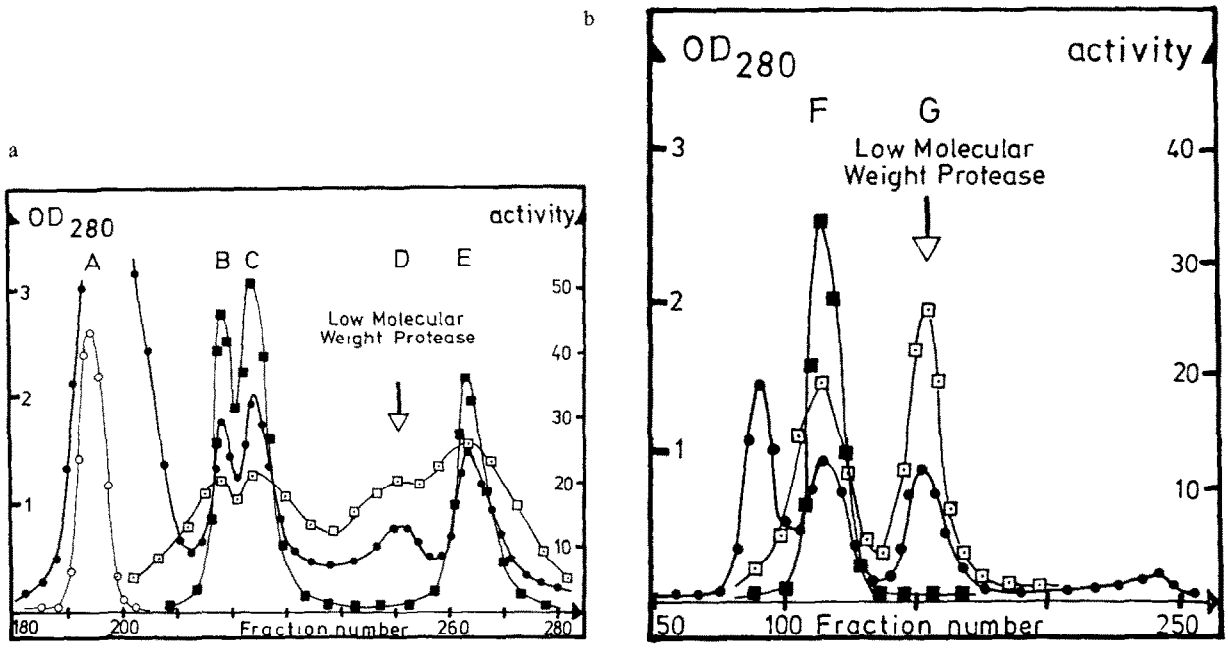

Fig.1.(a) Anion exchange chromatography of 100 ml digestive juice of *Astacus fluviatilis*. Column dimensions: 4 × 45 cm DEAE–Sephacel in 0.01 M Tris–HCl (pH 8.0), 560 ml bed vol. NaCl gradient 0.2–2.0 M. (A) Crayfish carboxypeptidase; (B,C,E) crayfish trypsin, multiple forms; (D) Low M_r protease. (b) Gel-filtration of low M_r protease (peak D, fig.1a). Three connected columns 1.8 × 95 cm, bed vol. 300 ml each. Sephadex G-50, 0.01 M Tris–HCl (pH 8.0) + 0.5 M NaCl; (F) Crayfish trypsin; (G) Low M_r protease; (•) A_{280}; (○) hydrolysis of hippuryl-L-arginine (pH 7.6); (■) hydrolysis of BzArgOEt (pH 8.0); (□) caseinolytic activity (pH 8.0).

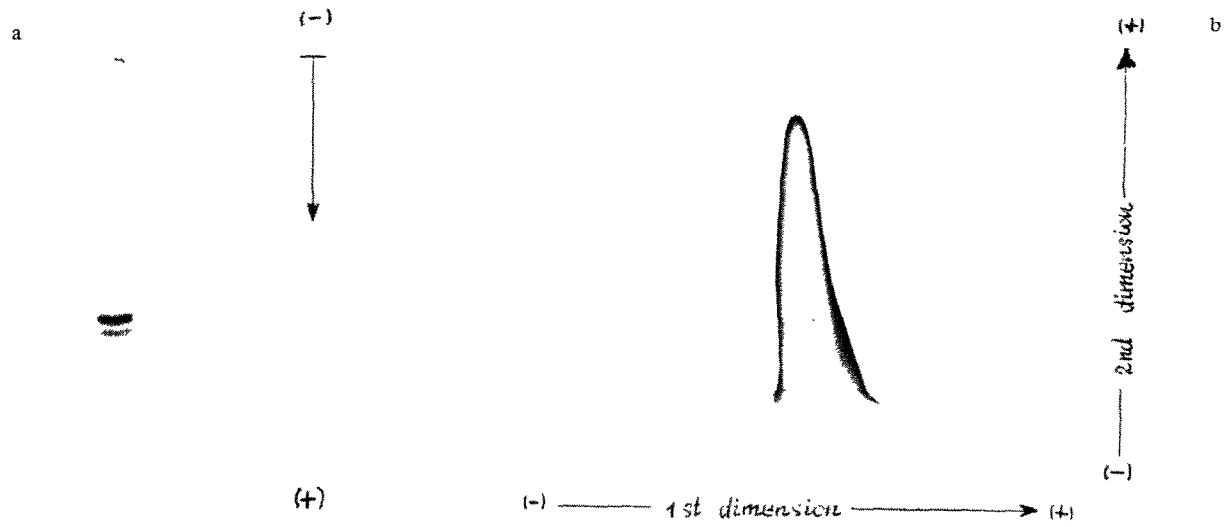

Fig.2.(a) Disk electrophoresis [12] of the purified low M_r protease (200 μg). The two closely related bands are multiple forms of the low M_r protease and are both caseinolytically active. Polyacrylamide (15%), tubes 6 × 130 mm, Tris–glycine buffer (pH 8.6), 6 h at 250 V and 3 mA/gel slab; staining, amido black. (b) Laurell immunoelectrophoresis [13] of 40 μg low M_r protease; 8% antiserum gel; 1st dimension, 250 V and 16 mA/50 min; 2nd dimension, 100 V and 10 mA/14 h; staining, amido black.

peak could be detected thus demonstrating that the two bands are immunologically identical (fig.2b). A high degree of homogeneity was also inferred from the very low background during automated Edman degradation of the enzyme.

The low M_r protease does not represent an active fragment of crayfish trypsin or carboxypeptidase since there is no immunological cross reaction between any of these enzymes. This possibility also can be excluded on the basis of the unique cleavage specificity and the partial amino acid sequence of the enzyme. The low M_r protease is clearly an endopeptidase hydrolyzing very effectively casein and other protein and peptide substrates. However, the enzyme is inactive toward any of the common ester substrates specific for trypsin or chymotrypsin, such as α-*N*-benzoyl-L-arginine ethyl ester, α-*N*-benzoyl-L-arginine β-naphthylamide, *N*-acety-L-tyrosine ethyl ester, and *N*-acetyl-L-phenylalanine β-naphthyl ester. This is in accordance with the observation that none of the protein inhibitors of trypsin and chymotrypsin, including ovomucoid, soya bean and lima bean trypsin inhibitor, inactivate this enzyme. On the contrary, native soybean trypsin inhibitor is degraded by the low M_r protease. For these reasons no affinity chromatography procedure for a single step purification of the enzyme could be developed so far. However, Suc–Ala–Ala–Ala–*p*-nitroanilide is a good substrate for the low M_r protease and a sensitive colorimetric assay can be based on this amidase activity of the enzyme [14].

Table 1 shows the points of cleavage of various peptides by the low M_r protease. These have been unambiguously identified as noted.

The enzyme seems to liberate peptides with short aliphatic amino-terminal side chains, preferentially those of alanine, glycine, threonine, and serine. This finding was confirmed by Dr. H. Ponstingl of the Deutsches Krebsforschungszentrum, Heidelberg, who applied the low M_r protease to the fragmentation of intact tubulin and recovered >12 Ala–X–Y peptides [15]. The model peptide Leu–Trp–Met–Arg–Phe–Ala, designed to cover the specificity requirements of most proteolytic enzymes (e.g., leucine amino peptidase, carboxypeptidase, trypsin and chymotrypsin) is not hydrolyzed by the low M_r protease. Likewise, the collagenase substrate Gly–Pro–Gly–Gly–Pro–Ala is not split and this is true also of a number of other di- and tripeptides of various composition. These properties make the enzyme a potentially useful tool for protein sequence analysis. Contrary to most other proteolytic enzymes, the specificity of the low M_r protease is directed by the amino acid residue providing the amino part of the susceptible peptide bond. The character of the amino acid at the carboxyl side of the hydrolyzed peptide bond does not seem to have any influence on the cleavage specificity. Preliminary side chain modification experiments showed that under conditions which inactivate crayfish as well as bovine trypsin, the activity of the low M_r protease is retained. These observations together indicate that the mechanism of action of the low M_r protease is different from any characterized so far. Additional support to this conclusion comes from the elucidation of the partial amino-terminal sequence of the

Table 1
Cleavage specificity of the low M_r protease[a]

Substrate	Point of cleavage
Insulin A-chain, oxidized	– Cys – Cys ↓ Ala – Ser – (6 7 8 9)
Insulin B-chain,[b] oxidized	– Leu – Cys ↓ Gly – Ser – (6 7 8 9) – Cys – Gly ↓ Ser – His – (7 8 9 10) – Glu – Ala ↓ Leu – Tyr – (13 14 15 16) – Phe – Tyr ↓ Thr – Pro – (25 26 27 28)
Mellitin	– Ile – Gly ↓ Ala – Val – (2 3 4 5) – Val – Leu ↓ Thr – Thr – (8 9 10 11)
Glucagon[b]	– Thr – Phe ↓ Thr – Ser – (5 6 7 8) – Phe – Thr ↓ Ser – Asp – (6 7 8 9) – Lys – Tyr ↓ Leu – Asp – (12 13 14 15) – Leu – Asp ↓ Ser – Arg – (14 15 16 17) – Arg – Arg ↓ Ala – Gln – (17 18 19 20)
δ-Sleep inducing peptide	– Ala – Gly ↓ Gly – Asp – (2 3 4 5)

[a] After cleavage, peptides were separated and subsequently identified by amino acid analysis

[b] In insulin B-chain (pos. 7–9) and in glucagon (pos. 6–8) two adjacent bonds are susceptible to the action of low M_r protease, but cleavage of one bond seems to preclude cleavage of the other

Table 2
Partial amino-terminal sequence of the low M_r protease

X[a] –	Ala –	Ile –	Leu –	Gly –	Asp –	Glu –	Tyr –	Leu –	Trp –
1	2	3	4	5	6	7	8	9	10
X[a] –	Gly –	Gly –	Val –	Ile –	Pro –	Tyr –	Thr[a] –	Phe –	
11	12	13	14	15	16	17	18	19	
Ala –	Gly –								
20	21								

[a] In position 18 the recovery was low; positions 1 and 11 were not identified

The sequence is based on 3 independent determinations

low M_r protease (table 2). The sequence shows no similarity with the conservative amino-terminal sequence Ile–Val–Gly–Gly– characteristic for serine proteases, thus excluding the possibility that the low M_r protease is a member of this protein family. Moreover, that part of the primary structure of the low M_r protease presented here, which includes ~20% of the complete structure, does not reveal any resemblance to other known protein sequences. It is likely, therefore, that the low M_r protease is a member of a hitherto unknown protein family. This enzyme might thus offer the opportunity to study a catalytic mechanism 'invented' and developed independently of those classified. The remarkably low M_r of this protease, which as far as we know represents the lowest M_r found for any enzyme, might be of interest for studies of the minimum architectural prerequisites of a polypeptide chain to act as an enzyme.

Acknowledgements

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