Volume 246, number 1,2, 171–176 FEB 06966 March 1989

Selective cleavage at lysine of the 50 kDa-20 kDa connector loop segment of skeletal myosin S-1 by endoproteinase Arg-C

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Received 4 January 1989

The reaction of endoproteinase Arg-C on the skeletal myosin head heavy chain was investigated through characterization of peptides and amino acid sequence analysis. The protease splits exclusively the 50 kDa–20 kDa junction at the lysine cluster spanning residues 639–641 and does not affect any other protease-sensitive region of the entire myosin heavy chain. The sensitivity of the cleavage to actin and nucleotide binding makes this protease a very specific conformational probe of S-1. The nicked S-1 derivative, containing an intact NH_2-terminal 75 kDa fragment, may serve as a tool for gaining further insights into the domain structure and function of the myosin head.

Myosin head; Heavy chain structure; Connector segment; Protease Arg-C

1. INTRODUCTION

The interaction of the myosin head (S-1) with F-actin and ATP is essential for force production during energy transduction in muscle [1]. Because the 95 kDa heavy chain of the head contains the actin- and nucleotide-binding sites, knowledge of its substructure and functioning is required in order to provide a complete description of the molecular mechanism of muscle contraction. Proteolytic dissection of skeletal chymotryptic S-1 led to the first suggestion as to the existence of domain-like regions in the heavy chain, represented by the three major 25, 50 and 20 kDa tryptic fragments [2–5]. These are connected by two protease-vulnerable segments forming flexible loops at the surface of the protein [6,7]. F-Actin binding involves sites on the 50 and 20 kDa fragments as well as the lysine clusters within the connector segment between these two COOH-terminal fragments [2,8,9]. The binding of ATP, which occurs on portions of the 25 and 50 kDa peptides [10], is believed to promote relative movements of these actin domains affecting the actin-myosin interactions; and vice versa, the attachment of actin modulates the conformation of the ATPase domains [11]. Thus, inter-domain motions in S-1 should determine the large-scale motions of the myosin heads during muscle activity [12,13]. Recently, direct visualization of the domain structure of S-1 was approached by electron microscopy, showing the head to contain a major cleft that divides the protein into a large, distal domain and two smaller regions linked to the head-tail junction of myosin; the mass of the former domain may accommodate the NH_2-terminal 75 kDa heavy chain segment with the 50 kDa–20 kDa junction possibly very close to this cleft [14,15].

Here, we illustrate the particular feature of the 50 kDa–20 kDa loop region as being the only region of S-1 or myosin that is susceptible to cleavage by endoproteinase Arg-C which severs the intact 75 kDa domain from the rest of the heavy chain. Moreover, the unexpected observation was made that the site of cleavage by the protease is located not at arginyl residues but only at the cationic, actin-sensitive lysine cluster between

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

Lys-636 and Lys-641 of the loop. The novel (75 kDa–21 kDa) split S-1 produced may serve for further characterization of the domain architecture and functions in the myosin head.

2. MATERIALS AND METHODS

Subfragment-1 was isolated and purified after chymotryptic digestion of rabbit skeletal muscle myosin as in [16]. F-Actin was prepared as in [17]. S-1 and F-actin were labeled with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) according to [2].

Digestion of S-1 (2–5 mg/ml) with endoproteinase Arg-C from mouse submaxillary gland (Boehringer Mannheim) was performed at 25°C in 50 mM Tris-HCl (pH 8.0) using 5–10 U protease/mg S-1. The reaction was monitored by gel electrophoresis over the time interval 0–120 min. Digestion of S-1 with staphylococcal protease or trypsin was carried out as in [3]. Electrophoresis on 5–18% polyacrylamide slabs in SDS was according to [2]. Fluorescent bands were located by illumination with a long-wavelength ultraviolet light before staining with Coomassie blue. Densitometric scanning of the gels was performed with a Shimadzu model CS-930 high-resolution gel scanner.

Actin-activated Mg^{2+}-ATPase activity of S-1 was assayed as in [2].

Chemical crosslinking between F-actin and Arg-C nicked S-1 was conducted in the presence of 10 mM 1-ethyl-3-(3-dimethylamino)propyl carbodiimide (EDC) in 50 mM Mes buffer, pH 6.5 [8].

For localization of the Arg-C cleavage site, a 120 min proteolytic digest of S-1 was first purified on a PD 10 column (Pharmacia) eluted with 0.25% NH_4HCO_3 (pH 8.0). An aliquot of the protein fraction (1 mg) was adjusted to pH 2.5 with 1 M TFA and then submitted to reverse-phase HPLC using a Beckman model 332 B gradient liquid chromatograph and a 4.6 × 75 mm Ultrapore C3 column. The COOH-terminal 21 kDa fragment was isolated using two successive linear gradient solutions consisting respectively of 0–30% acetonitrile in 0.1% TFA (10 min elution) and 30–60% acetonitrile in 0.1% TFA (60 min elution). Automated Edman degradation of the peptide was performed on an Applied Biosystems 470 A sequenator connected to a PTH HPLC analyzer (model 120 A) and operated as in [18]. The peptide was sequenced up to the 18th residue.

3. RESULTS AND DISCUSSION

3.1. *Nicking of the S-1 heavy chain with Arg-C protease*

The limited digestion of native S-1 with the so-called specific Arg-C protease [19,20] was initially intended to cleave the heavy chain at arginyl bonds

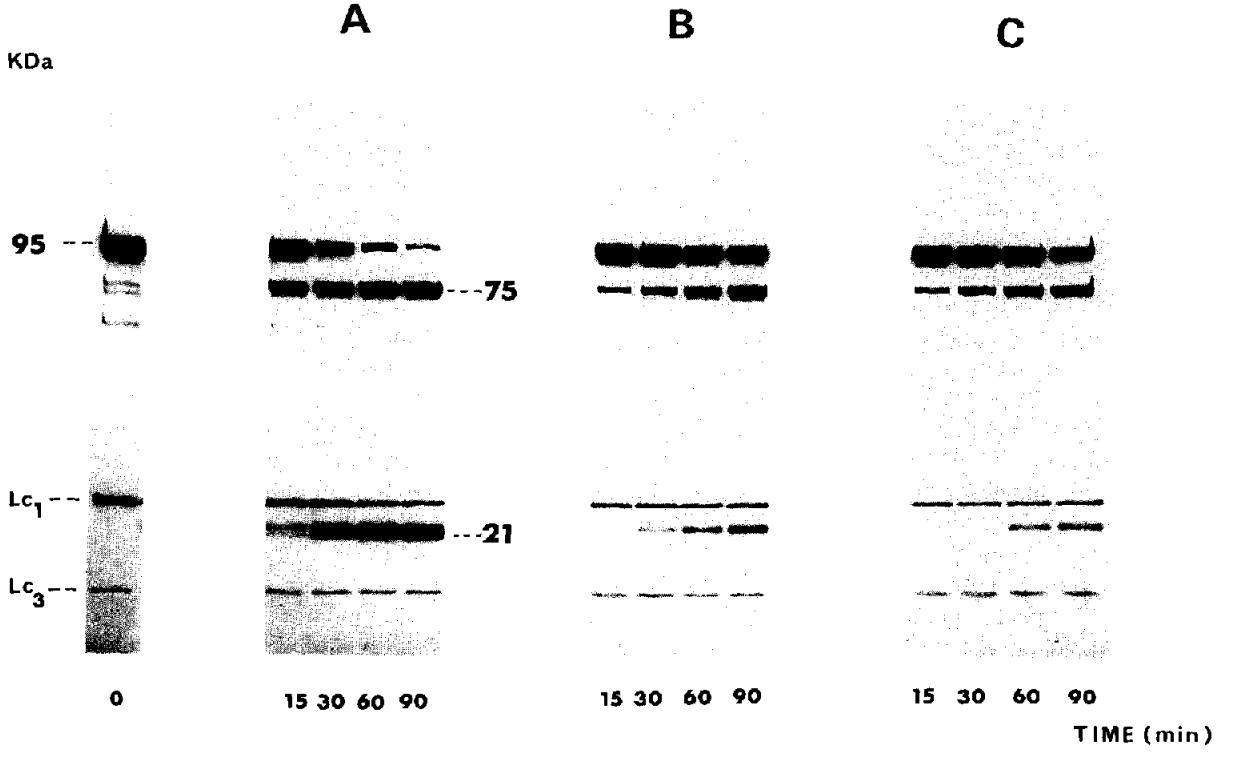


Fig.1. Time course of nicking of skeletal S-1 heavy chain by Arg-C protease. Conditions: S-1 (2 mg/ml) in 50 mM Tris-HCl, pH 8.0, 25°C; 10 U protease/mg S-1. Digestion was in the absence (A) and presence of 5 mM Mg-ATP (B) or Mg-ADP (C).

flanking the two linker regions, such as Arg-190 and Arg-652 [21]. As illustrated in fig.1A, the 95 kDa heavy chain was readily and almost completely cut by the protease into only two fragments of 75 and 21 kDa, respectively. The 21 kDa species was first released as a doublet; its accumulation after prolonged digestion occurred concurrently with a decrease in the accompanying slower band, suggesting the latter product to be a precursor of the stable 21 kDa peptide. Light chains 1 and 3 remained unaffected by the protease. The rate and extent of the cleavage reaction were very sensitive to the binding of S-1 to nucleotides or F-actin. Addition of Mg^{2+}-ATP or Mg^{2+}-ADP did not promote proteolysis at new sites but strongly decreased the rate of breakdown of the heavy chain (fig.1B,C). In particular, the protease did not split the Arg-23 peptide bond that is readily attacked by trypsin in the presence of ATP [22]. A quantitative analysis of the time course of fragmentation in the absence and presence of 5 mM Mg^{2+}-ATP is presented in fig.2. After 120 min digestion, 50% of the heavy chain was still intact in the presence of the nucleotide whereas nearly 90% of the protein was degraded in its absence. On the other hand, fig.2 shows that the binding of S-1 to F-actin leads to total protection of the heavy chain against Arg-C protease. Furthermore, the data in fig.3 indicate that scission of the heavy chain by the protease correlates with extensive inhibition of the actin-activated Mg^{2+}-ATPase of S-1. This inhibitory effect is similar to that exerted by trypsin upon cleavage by this protease of the lysine-rich peptide stretch, GKKGGKKKG, located in the 50 kDa–20 kDa junction [6] and contrasts with the lack of influence of the *S. aureus* protease which cuts this connector at a Glu residue preceding this sequence [6].

3.2. *Localization of the Arg-C clip site on the S-1 heavy chain*

The positioning of the 75 and 21 kDa peptides was first assessed by chemical crosslinking between

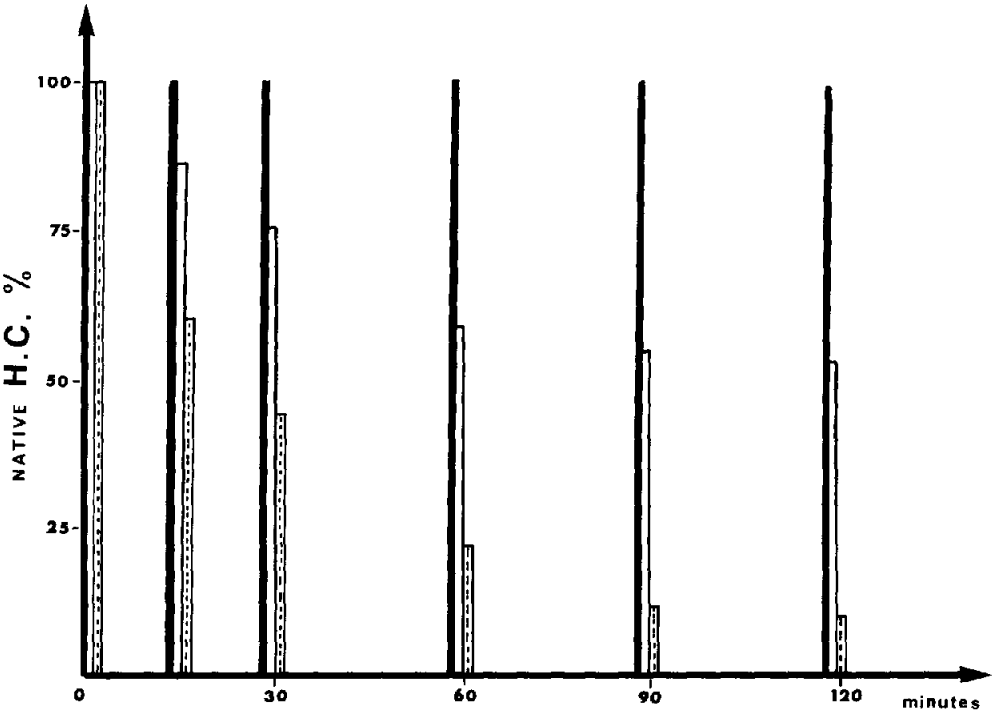


Fig.2. Influence of ATP and F-actin on susceptibility of S-1 to cleavage by Arg-C protease. The intensity of the residual 95 kDa heavy chain band present on electrophoretic gels was measured by densitometry after digestion of S-1 in the presence of 5 mM Mg-ATP (unfilled bars), 2-fold molar excess of F-actin (filled bars) and in the absence of ligands (bars with broken line).

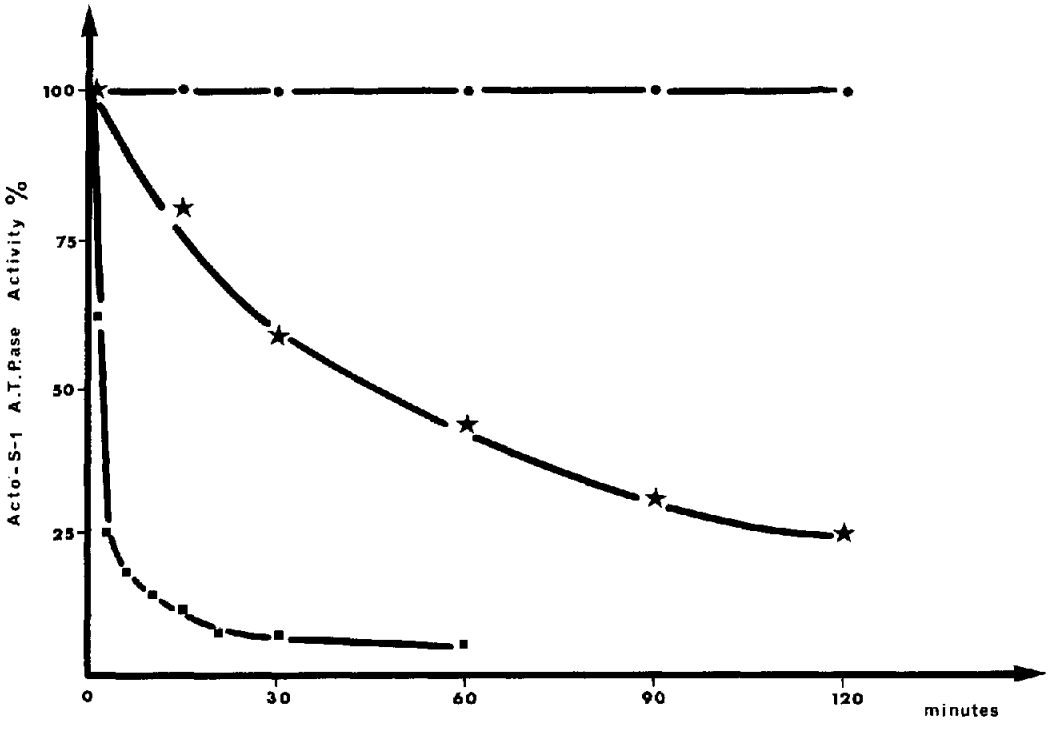


Fig.3. Arg-C induced inhibition of the actin-stimulated Mg^{2+}-ATPase of S-1. The effect of this protease (★) is compared to that resulting from digestion of S-1 by trypsin (■) or *S. aureus* V8 protease (●).

actin and nicked S-1, using 1,5-IAEDANS-labeled proteins. As shown in fig.4A and B, actin was crosslinked to either fragment forming acto-75 kDa and acto-20 kDa adducts. When S-1 labeled on SH_1 (Cys-707) was employed, the fluorescence was imparted to the 21 kDa fragment and to the acto-21 kDa adduct. This indicates unambiguously that the 75 and 21 kDa peptides represent respectively the NH_2- and COOH-terminal moieties of the heavy chain. Precise localization of the cleavage site was achieved by microsequencing of the NH_2-terminal part of the isolated 21 kDa fragment. The data indicated the presence of 2 peptides; the major one (75%) begins at KGSSF and the other at KKGSSF. As illustrated in table 1, Arg-C nicking occurred mainly on the lysyl bonds at positions 639 and 640 of the 50 kDa–20 kDa junction and was adjacent to the tryptic site on Lys-641 [6,23].

Recent structural studies have shown the Arg-C protease to be capable of splitting Lys-Arg [24] and Lys-Ala bonds [25], so that the specificity of the enzyme appears less stringent than reported in [19,20]. Polylysine was found to be refractory to the protease [20] but our work reveals the possible extensive hydrolysis of a lysine cluster in a protein. However, Arg-C did not act on the trypsin-sensitive lysine cluster of residues 204–206 present in the 25 kDa–50 kDa connector, nor did it cut the head-tail junction of myosin which was broken down into only 75 and 150 kDa fragments [26]. Thus, the 21 kDa peptide must contain the intact COOH-terminal portion of the S-1 heavy chain. As shown in fig.5, the latter region could indeed be excised by further digestion with trypsin which converts the 21 kDa peptide into the usual tryptic 20 kDa entity.

In conclusion, the Arg-C protease is a highly specific conformational probe of the skeletal 50 kDa–20 kDa loop. The strong cationic environment of this region involved in electrostatic actin-S-1 interactions [11] might have been the determinant of the activity of the enzyme on S-1. The new nicked S-1 and myosin derivatives are useful for analyzing the functions of the intact 75 kDa domain [26,27].

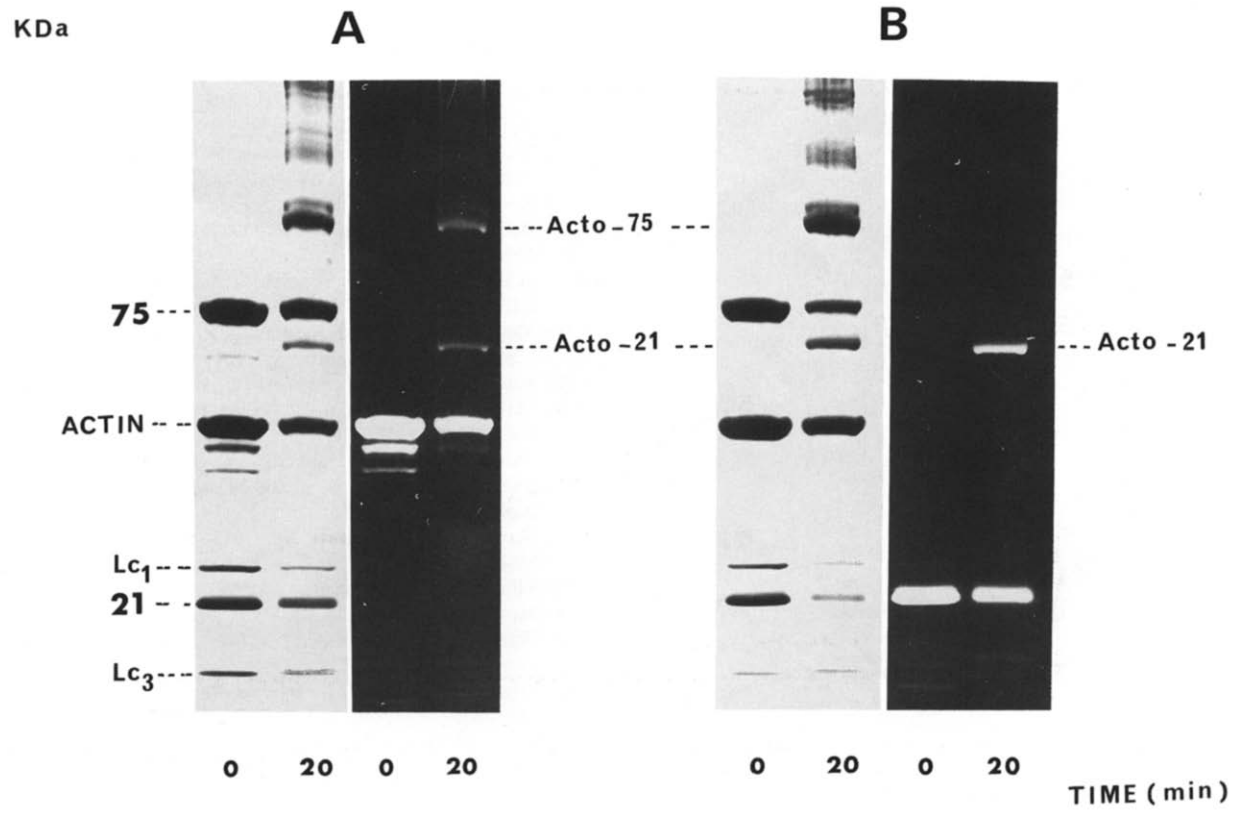
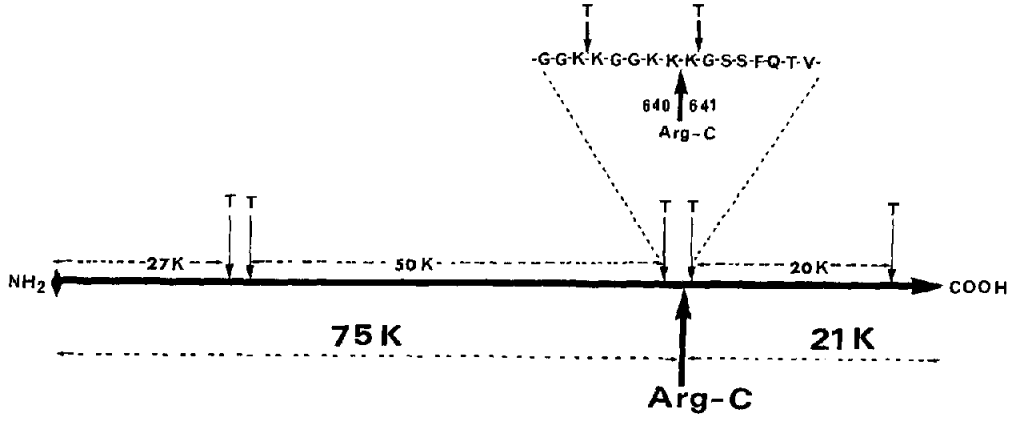


Fig.4. Covalent crosslinking of actin to the Arg-C produced 75 kDa and 21 kDa fragments of the S-1 heavy chain. Complexes of AEDANS–F-actin–(75–21 kDa)–S-1 (A) and AEDANS–(75–21 kDa)–S-1–F-actin (B) were crosslinked for 20 min in the presence of carbodiimide. The crosslinked products were identified by gel electrophoresis.

Table 1

Localization of the cleavage sites of endoproteinase Arg-C within the 50–20 kDa connector of rabbit skeletal S-1 heavy chain (T, tryptic cleavage sites)



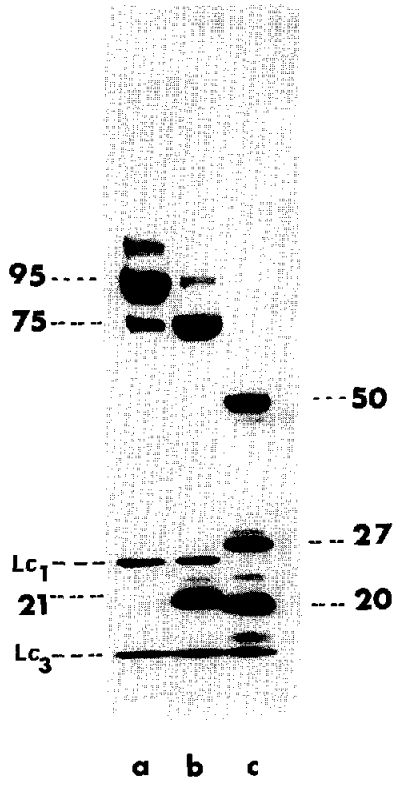


Fig.5. Successive digestions of S-1 with Arg-C protease and trypsin. Native S-1 (a) was first subjected to treatment with Arg-C, for 2 h at 25°C and pH 8.0 (b). The digest was then incubated with trypsin (enzyme/S-1, 1:100, w/w) for 30 min at 25°C (c). The 75 kDa part was cut by trypsin at the 27–50 kDa junction whereas the 21 kDa segment was truncated at the C-terminus generating the final 20 kDa peptide. For further details, see table 1.

Acknowledgements: This research was supported by grants from Centre National de la Recherche Scientifique and Institut National de la Santé et de la Recherche Médicale. We thank Dr M. Elzinga for access to unpublished sequences and E. Audemard for help in peptide isolation.

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