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A chymosin-like extracellular acidic endoprotease from *Myxococcus xanthus* DK101

A potential new tool for protein engineering

Jean-Robert Carias, Joël Raingeaud°, Catherine Mazaud, Gilles Vachon°, Nathalie Lucas, Yves Cenatiempo°, Raymond Julien

Genius (Biotechnologie), Faculté des Sciences, 123 rue Albert Thomas, 87060 Limoges Cedex and °*Laboratoires de biochimie cellulaire et de biologie moléculaire, Faculté des Sciences, 40 avenue du Recteur Pineau, 86022 Poitiers Cedex, France*

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SDS-PAGE and N-terminal sequence analysis of hydrolysis products from 3 substrates containing a unique sensitive bond usually recognized by chymosin (κ-casein, a synthetic hexapeptide and a recombinant tripartite protein) revealed that a 45 kDa endoprotease of *Myxococcus xanthus* DK101 cleaved the same characteristic Phe-Met bond with high specificity. Such an enzyme, easy to obtain from culture supernatant and to use in acidic conditions, could be a new tool for protein engineering.

Myxobacteria; κ-Casein; Tripartite protein; Rennin; Protease; (*Myxococcus xanthus* DK101)

1. INTRODUCTION

Growth of Myxobacteria is stimulated by the presence of macromolecules in their environment and a characteristic feature of these bacteria is their ability to hydrolyse proteins with various extracellular enzymes [11]. Cleavage specificity of several proteases previously described [2-4], that exhibit an alkaline activity, has been rarely established. Only two enzymes that are specific for peptide bonds involving amino acids with non-polar side chains [2] and lysin residues have been characterized [3].

It is known that chymosin (EC 3.4.23.4) or pepsin (EC 3.4.23.3) cleave the single Phe^{105}-Met^{106} bond in κ-casein, this event triggering coagulation [5].

It has also been observed that *Myxococcus xanthus* DK101 supernatant can produce, in acidic conditions, milk and κ-casein clotting (Rosenberg, E., personal communication).

In this work, a partially purified acidic protease of *M. xanthus* has been tested on several peptidic substrates with an amino acid sequence identical or similar to that found in κ-casein around the Phe-Met bond. In order to determine the specificity of this enzyme, special attention was given to sequence analysis of the hydrolysis products from the following substrates: (i) κ-casein; (ii) a synthetic hexapeptide; and (iii) a tripartite protein obtained by gene fusion, all of them containing the known target site for chymosin.

Correspondence address: J.-R. Carias, Genius (Biotechnologie), Faculté des Sciences, 123 rue Albert Thomas, 87060 Limoges Cedex, France

Abbreviations: DABTH, 4-*N*,*N*-dimethylaminoazobenzene 4'-thiohydantoin; TLC, thin-layer chromatography; CTT, Casitone-Tris medium; Nle, Norleucine; IPTG, isopropyl β-D-thiogalactoside

2. MATERIALS AND METHODS

2.1. *Partial purification of endoprotease*

M. xanthus DK101 (variant of FB strain, isolated by Kuner and Kaiser [6]) was grown in CTT liquid medium ('Bactocasitone' Difco, Detroit, USA) at 30°C on an orbital agitator at 100 rpm. A 20-h old culture (10^8 bacteria/ml) was centrifuged and the supernatant filtered through a 0.22 μm filter (Millipore, Molsheim, France). All subsequent purification steps were carried out at 4°C. The supernatant (80 ml) was dialyzed overnight against piperazine buffer 0.02 M, pH 5.4 (Sigma, St. Louis, USA) and then applied to a column (2.4 × 6 cm) of DEAE-Trisacryl (IBF, Villeneuve La Garenne, France) equilibrated with the dialysis buffer. Endoprotease separation was performed according to [7] with a slow NaCl (Sigma) gradient from 0.1 to 0.5 M (2 × 100 ml), with a flow rate of 120 ml/h (5 ml fractions). Enzyme identification was performed by measuring clotting activity on κ-casein (Sigma).

2.2 *Determination of clotting activity*

A solution of 0.5% κ-casein in 0.01 M phosphate buffer, pH 6, containing 0.1 M NaCl, was digested by *Myxococcus* enzyme or pure recombinant chymosin (EC 3.4.23.4, Hansen's Laboratory, Milwaukee, USA). Enzyme solutions (20-50 μg in 100 μl) were incubated from 30 to 120 min at 30°C and the turbidity reading at 550 nm was recorded [8].

2.3. *Determination of endoprotease activity by the zymogram method*

On the one hand, an agarose gel, in 0.01 M phosphate buffer, NaCl 0.1 M, pH 6, was prepared with a solution containing 0.5% κ-casein,

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0.15% 'Pastagar' (Pasteur, Paris, France). The solution was heated to 100°C to be gelatinized. On the other hand, protein fractions were separated on 12.5% polyacrylamide gel. After electrophoresis, the polyacrylamide gel was horizontally applied on the agarose gel and incubated for 4 h at 30°C. $\varkappa$-Casein agarose gel was stained for 15 min with Coomassie blue and destained in a solution containing methanol, acetic acid and water (3:1:6, v/v/v). Hydrolyzed $\varkappa$-casein appeared as a non-stained band allowing location of protease activity [9].

2.4. *Electrophoresis and isoelectric focusing*

Enzyme molecular weight was determined with automatic SDS-PAGE (12.5% polyacrylamide, Pharmacia, Uppsala, Sweden) in the presence of Tris-HCl, (0.01 M, pH 8.0, 2.5% SDS, 5% β-mercaptoethanol (v/v). Isoelectric focusing (with the same system) was performed in gel with a pH range of 4–6.5. All gels were stained with the silver staining method according to [10].

2.5. *Synthesis and purification of a tripartite protein with a peptide linker substrate for chymosin*

A chimeric protein (fig.1a) containing at least one target site for chymosin (a $\varkappa$-casein heptapeptide, fig.1b) was purified from an overproducing *Escherichia coli* SE 5000 strain (*F⁻, ara D139, Δ lac 169, rpsL, thiA, recA*), previously transformed with the recombinant pGJ2 plasmid. Briefly, this plasmid is a pKK233-2 derivative (obtained from Pharmacia) harboring a Ptrc promoter and translational initiation signals, including an ATG codon immediately followed by an *Nco*I restriction site. *lacIq* and *lacZ* genes were inserted into pKK233-2 *Δ PvuII-EcoRI*, respectively upstream from Ptrc (*Eco*RI site) and downstream into the polylinker (*Pst*I and *Hin*dIII-filled in sites). The *Nco*I was used to clone in phase: a *PvuII-Hpa*I segment of IF2 gene [11] followed by a nucleotide sequence coding for a $\varkappa$-casein heptapeptide, recognized by chymosin [12], and further downstream *lacZ*. The cloned genes generate in the host cell a fusion protein of 180 kDa displaying β-galactosidase activity. The IF2 fragment is a 60 kDa peptide spanning the central and C-terminal regions of the factor. The double-stranded region coding for the $\varkappa$-casein heptapeptide was engineered by annealing two synthetic oligonucleotides. They were designed taking into account codon usage in *E. coli* as well as the presence of an *Hpa*I end (blunt end) on the IF2 gene and *Hin*dIII end in the polylinker in front of *lacZ*.

Cells were grown in LB medium [13] containing ampicillin. At the end of the logarithmic phase, synthesis of the hybrid protein was induced upon addition of 1 mM IPTG. β-Galactosidase activity was then monitored according to a previous method [13]. After maximal induction, bacteria were harvested and sonicated. The hybrid protein was purified from cell lysate by affinity chromatography [14]. The original method was modified as follows: in order to eliminate non-specific binding of cellular proteins, the cell lysate was first passed through a CH-Sepharose 4B column prior to the affinity column (para amino-phenyl-β-D-thiogalactoside Sepharose). Under those experimental conditions, more than 90% of the β-galactosidase activity was retained on the second column. After elution, proteins were precipitated with 70% ammonium sulfate, pelleted and kept at −70°C. They were later solubilized and extensively dialyzed against the appropriate buffer before enzymatic assay or SDS-PAGE.

2.6. *Analysis of hydrolysis products*

Hydrolysis products of $\varkappa$-casein (see section 2.2) were analyzed by SDS-PAGE (20% polyacrylamide) and their N-terminal sequence determined by the microsequencing method of Chang et al. [15] using Edman degradation. Identification of DABTH amino acids was obtained by TLC on micropolyamide sheets (Schleicher and Schuell, Dassel, FRG).

The action of the endoproteolytic enzyme of *M. xanthus* DK101 on a synthetic substrate specific for chymosin: Leu-Ser-Phe-Nle-Ala-Ile-OMe hexapeptide (Bachem, Bubendorf, Switzerland, [16]) was assayed as previously reported [17]. The reaction mixture containing 2 μg of endoprotease in 1 ml of hexapeptide 0.4 mM, pH 4.7, sodium acetate buffer 0.1 M was carried out at 30°C. The products of the enzymatic cleavage of the Phe-Nle bond were identified by TLC on silica gel (aluminium sheets silica gel 60, Merck, Darmstadt, FRG) treated with ninhydrine aerosol (Merck). The N-terminal sequence of peptides was determined according to [15].

Cleavage of the chimeric protein (fig.1a) by *M. xanthus* DK101 endoprotease preparation ([E]/[S] = 1/10, 30°C, phosphate buffer 0.01 M, pH 6, NaCl 0.1 M) was controlled by analysis of hydrolysis products on SDS-PAGE (12.5% polyacrylamide) and by N-terminal sequence determination according to [15].

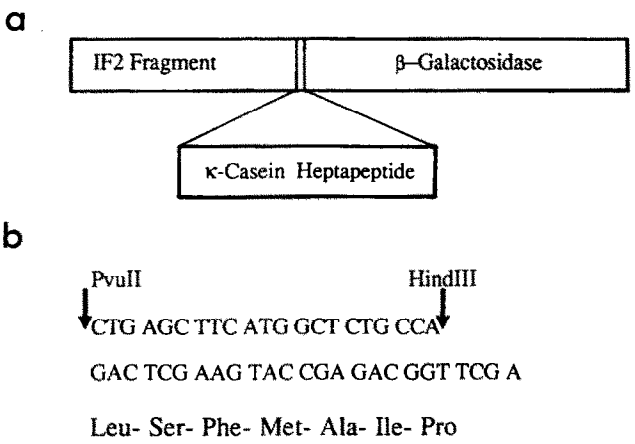

Fig.1. Schematic representation of the tripartite protein (a) and sequence of the double-stranded oligonucleotide, cloned in phase between the IF2 gene and *lacZ*, as well as its translation into an heptapeptide (b). See section 2 for details.

3. RESULTS

3.1. *Enzyme characterization*

The supernatant from *M. xanthus* DK101 culture was submitted to a DEAE-Trisacryl ion exchange chromatography. The presence of an acidic proteolytic activity was assayed in the eluate by monitoring $\varkappa$-casein clotting. The activity was recovered in a symmetric single peak eluted from the column by ca 0.2 M NaCl (fig.2). Zymogram analysis results of the pooled and concentrated fractions showed only one activity band (fig.2). Estimated protease molecular mass was 45 ± 5 kDa and its pH_i was close to 5.

3.2. *Enzyme specificity*

M. xanthus endoprotease or pure recombinant chymosin induced identical peptide products: one peptide (11 kDa) identified as para-$\varkappa$-casein and a second one (8 kDa) which is the glycomacropeptide [18] from $\varkappa$-casein (fig.3). N-terminal analysis of peptides resulting from the action of bacterial endoprotease as well as pure chymosin was performed. The two following N proximal amino acid sequences were found: Glu-Glu-Gln-Asn-Gln-Glu (identical to that of $\varkappa$-casein) and Met-Ala-Ile-Pro-Pro-Lys. This experiment demonstrated that the Phe^{105}-Met^{106} bond was effectively hydrolyzed. Using a synthetic hexapeptide as enzymatic substrate, similar analysis of tripeptides products confirmed that the Phe-Nle bond was specifically cleaved (not shown).

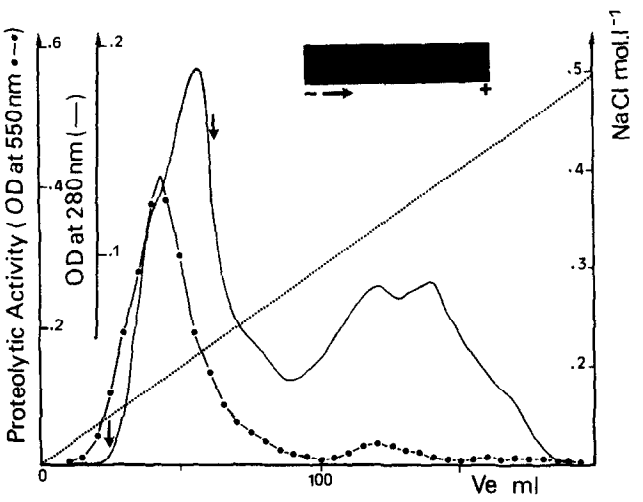

Fig.2. DEAE-Trisacryl ion exchange chromatography of dialyzed supernatant from *M. xanthus* DK101 culture. Insert: Zymogram analysis of pooled fractions (between vertical arrows) containing endoprotease activity.

Enzymatic cleavage of tripartite protein (180 kDa) by bacterial endoprotease or by pure recombinant chymosin, released (fig.4) a high-molecular-mass fragment (116 kDa) corresponding to β-galactosidase and 3 polypeptides which were identified, respectively, as an IF2 fragment (60 kDa) and its cleavage products ($\simeq$30 kDa). As described above with κ-casein and a synthetic hexapeptide, the amino terminal analysis of these products confirmed that the Phe-Met bond contained in the peptide linker was specifically cleaved. In latter experiments, another cleavage site whose nature needs further determination (see section 4) was recognized by both bacterial endoprotease and chymosin.

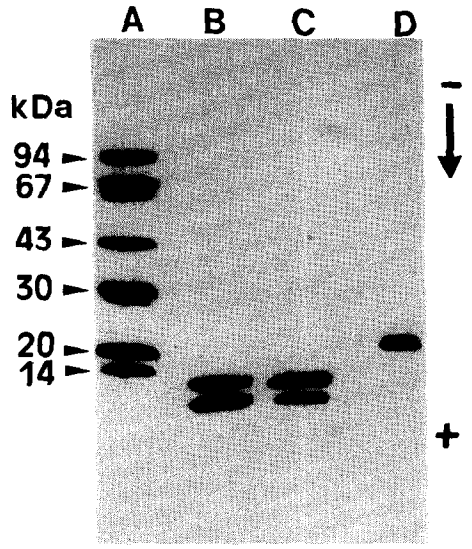

Fig.3. SDS-PAGE (20% polyacrylamide) analysis of κ-casein. Lanes loaded as follows: (A) molecular mass markers; (B) κ-casein after treatment by *M. xanthus* endoprotease; (C) κ-casein after treatment by pure recombinant chymosin; (D) κ-casein without treatment.

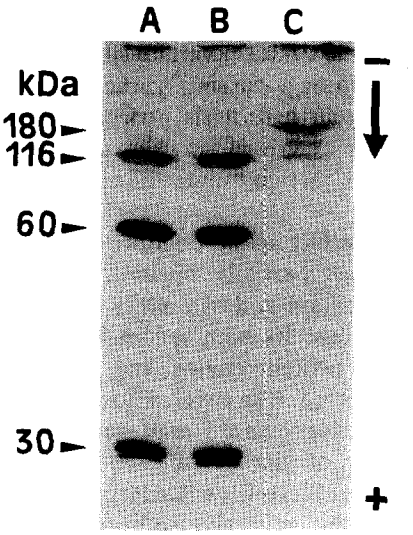

Fig.4. SDS-PAGE (12.5% polyacrylamide) analysis of tripartite protein. Lanes were loaded as follows: (A) tripartite protein after treatment (30 min) by *M. xanthus* endoprotease; (B) tripartite protein after treatment (120 min) by pure recombinant chymosin; (C) tripartite protein without treatment.

4. DISCUSSION

Extracellular endoprotease from *M. xanthus* described in the present report proved to be different from other activities found in *M. virescens* [2], *Myxobacter* AL1 [3], *Cytophaga* sp. [4], *Sorangium* [19], or *Arthrobacter globiformis* [20], that exhibited alcaline proteasic activities. A previous communication indicated that acidic endoprotease activities exhibiting different specificities were found in various Myxobacteria [21]. κ-Casein clotting obtained with an endoprotease from *M. xanthus* is the consequence of a specific cleavage of the Phe^{105}-Met^{106} bond which occurs naturally with chymosin from calf stomach. Hydrolysis of synthetic hexapeptide containing a Phe^{3}-Nle^{4} bond produced two tripeptides, confirming the high affinity of myxobacterial enzyme for one hexapeptide sequence similar to that present in κ-casein.

Finally, the pattern of hydrolysis products obtained with tripartite protein proved that partially purified endoprotease from *M. xanthus* DK101 has cleaved the Phe-Met bond into the heptapeptide linker inserted between β-galactosidase and IF2 fragments. Since β-galactosidase activity released after proteolysis of tripartite protein was maintained, as also observed when β-galactosidase alone was incubated with bacterial enzyme (not shown), the two other fragments ($\simeq$30 kDa, fig.4) obtained in this experiment could be due to internal IF2 cleavage. As previously described, chymosin can cleave secondarily the Glu-Tyr bond, with a lower affinity than the Phe-Met bond [22]. Since IF2 contains a Glu^{247}-Tyr^{248} link whose cleavage would generate two peptides of approximately 29 and 31 kDa,

the two bands observed in gel electrophoresis (fig.4) may correspond to this potential site of cleavage. The 3 results concerning $\varkappa$-casein, synthetic hexapeptide and tripartite protein hydrolysis proved that the bacterial endoprotease described in this report presents a 'chymosin-like' activity under our experimental conditions.

The *M. xanthus* acidic endoprotease is rapidly obtained, at least partially purified in one step from culture supernatant and can easily be used under mild acidic conditions. Its high specificity should allow its utilization in protein engineering when limited proteolysis is required. Moreover, knowledge of this newly described endoprotease may contribute to the understanding of the overall requirements of Myxobacterial cells. Since Myxobacteria display a large array of proteases to survive, other activities could be detected and their specificity determined by changing the target site for proteolytic enzymes inserted in chimeric proteins.

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