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Purification and properties of a new psychrophilic metalloprotease (Fpp2) in the fish pathogen *Flavobacterium psychrophilum*

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Abstract

To go further into the characterization of the proteolysis exocellular system of the salmonid pathogen *Flavobacterium psychrophilum*, the purification and characterization of a novel protease designated Fpp2 (*F. psychrophilum* protease 2) was undertaken. A protease (Fpp2) hydrolyzing azocasein was purified. The Fpp2 can be defined as a metalloprotease, it had an estimated molecular mass of 62 kDa with calcium playing an important role in the thermostability of the enzyme. Proteolytic activity was optimal at pH 6.0–7.0 and 24°C and activation energy for the hydrolysis of azocasein was determined to be 5.4 kcal mol^{-1}, being inactive at temperatures above 42°C. All these results are characteristic of 'cold adapted enzymes'. Fpp2 proved to be a broad range hydrolytic enzyme because in optimal conditions it was able to hydrolyze matrix and muscular proteins. It can be concluded that the Fpp1, a previously characterized 55 kDa metalloprotease, and the Fpp2 protease were produced under different physiological conditions and were immunologically as well as biochemically different.

Keywords: Metalloprotease; Psychrophilic; Fish; Pathogen; *Flavobacterium psychrophilum*

1. Introduction

Flavobacterium psychrophilum is a Gram-negative, psychrophilic bacterium which is responsible for the salmonid disease known as 'cold water disease' (CWD). The microorganism is widespread, causes important worldwide economic losses in salmonid aquaculture and outbreaks have occurred at water temperatures between 12 and 14°C. In spite of this, there are few studies regarding their physiology, genetics and biochemistry. The bacterium is unable to utilize any carbohydrate [1,2] and presents a fastidious and slow growth even in a complex medium [3]. Similarly, most of the microorganism's pathogenic mechanisms are practically unknown and no commercial vaccine has been developed for the prevention of this disease.

Different extracellular activities seem to be involved in the virulence of this bacterium [2]. Among these, it was evident that proteolytic activity is related, at present, in an undetermined way to the pathogenic process [4]. Thus, proteolytic analysis using gel substrate electrophoresis showed that a large number of different proteases were present in culture supernatants of *F. psychrophilum* and a correlation has been established between the protease profile on gel substrate electrophoresis and virulence with different strains [4]. Additionally, Madsen and Dalsgaard [5] also show a relation between elastase production and pathogenicity, while Ostland et al. [6] confirm the implication of a zinc-dependent proteolytic activity on the erosion of external tissue. More recently, Secades et al. [7] have identified, purified and characterized the Fpp1 protease from *F. psychrophilum*, a metalloprotease induced specifically by calcium. Following the same line, there is a study which correlates the proteolytic activity of different strains of *F. psychrophilum* with the incubation temperature [8].

The role of bacterial proteases produced by pathogenic microorganisms is uncertain. It seems that its main role should be to provide nutrients for bacteria growth, but at the same time, possibly as an indirect effect, this process could facilitate the alteration and erosion of host tissue contributing to colonization and invasion. Thus, enzymes which degrade connective and muscular tissues such as

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gelatinases, elastases and collagenases, may play an important role in pathogenesis. Different results have been obtained in studies carried out with bacterial proteases from fish pathogens. Indirect experiments showed the participation of proteolytic enzymes on pathogenesis [9–12]. Through genetic approaches using the truncation of protease encoding genes, it has been shown that proteolytic enzymes are involved in the virulence of fish pathogen bacteria, although not as a norm. Thus, the virulence of *Aeromonas salmonicida* and *Aeromonas hydrophila* carrying deletions in the *aspA* and *ahpA* genes, respectively, encoded for serine proteases, was the same as the respective parental strains [13,14]. By contrast, an elastase and the *yrp1* metalloprotease mutated strains of *A. hydrophila* [15] and *Yersinia ruckeri* [16], respectively, showed the implication of these proteins in the pathogenesis of these bacteria.

In order to investigate further into the complex exocellular proteolytic system of this bacterium, we identified, purified and characterized a second psychrophilic metalloprotease (Fpp2), produced, in contrast to Fpp1 [7], in the absence of calcium. The Fpp2 enzyme was thermal stabilized by calcium, and had a broad specificity for degrading extracellular matrix components and muscle proteins.

2. Materials and methods

2.1. Culture conditions and protease purification

The *F. psychrophilum* 1947^{T} strain was used in this work. The microorganism was routinely cultured on nutrient broth (NB) (5 g of peptone from gelatin per l, 3 g of beef extract per l) or NB agar (NA) (Pronadisa) at 12 or 15°C, respectively. Growth curves of *F. psychrophilum* were obtained by monitoring the culture OD_{525} by using a Perkin-Elmer spectrophotometer at different incubation times.

5 ml portions of a stationary-phase culture of *F. psychrophilum* were used to inoculate 2 l Erlenmeyer flasks containing 500 ml of NB medium pH 6.0. After 96 h of incubation at 12°C and 250 rpm, cells were harvested by centrifugation (23 500 × *g* for 30 min at 4°C) and the supernatant was used as the starting source for protease purification. All steps were carried out at 4°C.

2.1.1. Ammonium sulfate precipitation

First, $CaCl_2$ was added to 2 l of supernatant at 10 mM final concentration in order to stabilize the protease. Then, 780 g of ammonium sulfate were slowly added to 2 l of supernatant to achieve 60% saturation. After 2 h at 4°C and with continuous stirring, the precipitate was recovered by centrifugation (30 000 × *g* for 45 min), dissolved in 25 mM Tris–HCl pH 7.6 containing 10 mM $CaCl_2$ (Tris-calcium buffer) and dialyzed twice (for 16 h the first time and 6 h the second) against the same buffer. After dialysis the total volume was adjusted to 50 ml by adding Tris-calcium buffer.

2.1.2. Ion-exchange chromatography

The bulk of the dialyzed material (50 ml) was added to a slurry containing 25 ml of DE52-cellulose (Whatman) previously equilibrated with Tris-calcium buffer. After 2 h of gentle stirring at 4°C, the unbound protein was separated from the gel by centrifugation (30 000 × *g* for 5 min) and the gel was washed twice with Tris-calcium buffer. Then, the absorbed protein was eluted with Tris-calcium buffer containing 0.5 M NaCl. The eluted material was dialyzed twice against Tris-calcium buffer and the recovered volume (80 ml) was applied at a flow rate of 0.5 ml min^{-1} to a DE52-cellulose column (1.0 by 15 cm) previously equilibrated with Tris-calcium buffer. The column was then washed with 200 ml of the same buffer and the bound proteins were eluted with an 80 ml linear gradient of NaCl ranging from 0 to 0.5 M at a flow rate of 0.5 ml min^{-1}. Fractions (1.5 ml) were collected and aliquots were assayed for azocasein hydrolysis. Positive fractions (range, 10–30 fractions) were pooled together (total volume, 30 ml) and after dialysis for 16 h against 4 l of Tris-calcium buffer, ammonium sulfate of a 3.5 M ammonium sulfate solution in Tris-calcium buffer was added to achieve 1.2 M final concentration.

2.1.3. Hydrophobic chromatography

The protein solution was loaded onto a 1 ml butyl-Sepharose Hi-Trap column (Amersham) at a flow rate of 1 ml min^{-1}. The column was washed with 15 ml of Tris-calcium buffer containing 1.2 M ammonium sulfate and the bound protein eluted in a 30 ml linear gradient of ammonium sulfate ranging from 1.2 to 0 M. Fractions (1.1 ml) were collected and five to 14 active fractions (total volume, 10 ml) were pooled. The protein solution was concentrated and washed with Tris-calcium buffer by filtering it through a Centricon 4M-30 filter (Amicon).

2.1.4. Fast protein liquid chromatography (FPLC) gel filtration chromatography

Next, 0.1 ml of the concentrated protein solution (total volume, 0.3 ml) was loaded at a flow rate of 0.2 ml min^{-1} onto a Superdex 75 column (Pharmacia) previously equilibrated with Tris-calcium buffer containing 150 mM NaCl. Fractions (0.5 ml) were collected and analyzed for proteolytic activity. Protein standards were subsequently injected into the column in order to estimate the molecular mass of the protease. A total volume of 1 ml of purified protein was obtained and aliquots of it were stored at −80°C. For some experiments, fractions of the purified Fpp2 protease were filtered through a Centricon 4M-30 filter with 25 mM Tris–HCl (pH 7.6) in order to completely eliminate the calcium in the sample. The enzyme obtained in this way was designated as calcium-free Fpp2.

2.2. Assay of proteolytic activity and protein content

Proteolytic activity was assayed at 30°C by using azocasein (Sigma) as a substrate, as described by Secades et al. [7] using 25 mM PIPES, pH 6.5 containing $CaCl_2$ (final concentration, 5 mM) as reaction buffer. One unit of enzyme activity was defined as the amount which yielded an increase in A_{420} of 0.01 in 2 h under the assay conditions used. Elastinolytic activity was assayed in the same conditions previously described for the proteolytic activity assay but using elastin-Congo red (Sigma) (5 mg ml^{-1}) as a substrate. After the incubation, the insoluble material was removed by centrifugation at $12\,000 \times g$ for 5 min and the OD of the supernatant was measured at 495 nm.

The protein content of the sample was estimated by the Lowry et al. [17] method by using bovine serum albumin as the standard.

2.3. N-terminal sequence determination

The purified Fpp2 metalloprotease was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [18] and electroblotted onto an Immobilon-P transfer membrane according to the method of LeGendre and Matsudaira [19]. The stained band was cut out and subjected to N-terminal sequencing on an Applied Biosystems 475 protein sequencer.

2.4. Characterization of the Fpp2 enzymatic activity

The effect of pH on the Fpp2 protease activity was tested with different buffers at pH values from 4.4 to 11 in the assay system. The buffers employed were the following: for pH range 4.4–5.0, 25 mM acetate; for pH range 5.5–6.0, 25 mM MES; for pH range 6.5–7.0, 25 mM PIPES; for pH range 7.0–7.5, 25 mM MOPS; for pH range 8.0–9.0, 25 mM Tris–HCl; and for pH range 9.5–11, 25 mM CAPS.

The purified enzyme was incubated both in the absence and presence of 5 mM $CaCl_2$ at 4, 9, 14, 19, 24, 29, 33, 37, 42 and 48°C for 2 h in 25 mM PIPES (pH 6.5) containing 1% azocasein as substrate. The enzyme activity was determined as previously described [7]. The activation energy (E_a) was estimated from the slope ($-E_a/R$) of Arrhenius plots of ln k ($k = 100 \times$ enzyme units (U)) against the reciprocal of the temperature (in Kelvin). The effect of different ions on protease activity was determined by using the Fpp2 calcium-free enzyme in the presence of different ions. For the inhibition studies, the calcium-free Fpp2 was preincubated with different compounds for 10 min on ice in 25 mM PIPES (pH 6.5) before the proteolytic activity was measured under standard conditions. Metal-depleted Fpp2 for the reconstitution experiments was obtained by 5 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) treatment of the calcium-free Fpp2. The thermal stability of Fpp2 was evaluated by measuring the residual activity as a function of time when the calcium-free Fpp2 was incubated at 40°C in 25 mM PIPES pH 6.5 buffer both alone and in the presence of 5 mM $CaCl_2$ and $SrCl_2$.

Immunodetection experiments were performed as previously described [7] using antiserum (1:500) raised against Fpp1 and anti-rabbit immunoglobulin-alkaline phosphatase antibodies (1:10 000; Sigma).

3. Results

3.1. Purification of Fpp2 from the supernatant of F. psychrophilum

To facilitate the Fpp2 purification the bacterium was grown in NB at pH 6 and 12°C. Under these conditions, the Fpp1 protease was not produced and, at the same time, these conditions are optimum for protease production as was previously shown [7]. The Fpp2 purification process from the supernatant of stationary-phase cultures of *F. psychrophilum* is shown in Table 1. The enzymatic solution, after the ammonium sulfate precipitation step, was first treated in a batch form with the anion-exchange DE52. This step was important in order to remove most of a yellow material present in the protein solution which interferes with the purification process. The enzyme adsorbed on the resin was eluted and then loaded onto the same resin in a column. Hydrophobic interaction chromatography on butyl-Sepharose produced an increase (about six-fold) in the specific activity of the enzyme. Finally, the Fpp2 was purified to homogeneity by chromatography on a Superdex 75 FPLC column. The apparent molecular mass of the Fpp2 was 62 kDa by SDS–PAGE (Fig. 1A) and molecular exclusion chromatography (data not shown). The sequence of the first N-terminal amino acids

Table 1
Purification of Fpp2 from culture supernatant of *F. psychrophilum*

Purification step	Total protein (mg)	Total activity (U)	Sp. Act. (U mg^{-1})	Purification (cult)	Yield (%)
Culture supernatant	4 207	331 279	78.74	1	100
Ammonium sulfate	86.8	278 ,475	3 208	40.74	84.06
DE52 cellulose (batch)	19.97	104 560	5 236	66.49	31.56
DE52 cellulose (gradient)	8.62	58 327	6 766	85.93	17.6
Butyl Sepharose (gradient)	0.34	12 892	37 917	481.55	3.89
Superdex 75 (FPLC gel filtration)	0.18	11 755	65 305	829.38	3.54

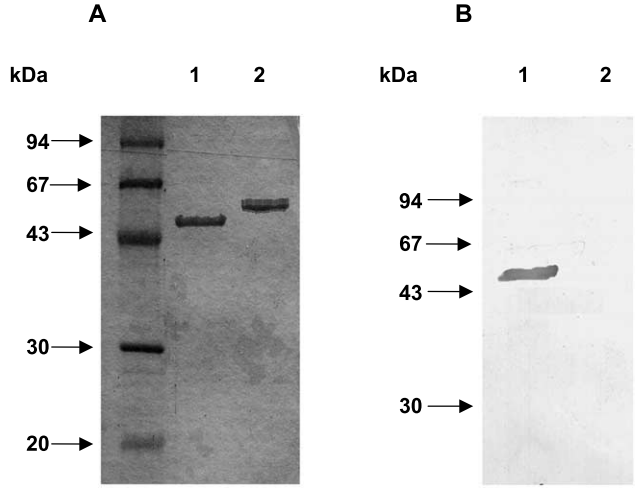

Fig. 1. SDS–12.5% PAGE of purified Fpp2 protease produced by *F. psychrophilum*. A: Protein (3.1 μg) pooled at the FPLC filtration chromatography (Superdex 75) purification step was gel loaded and, after electrophoresis, stained with Coomassie brilliant blue R-250. Lanes 1 and 2, purified 55 (Fpp1) [7] and 62 kDa (Fpp2) proteins, respectively. B: Immunoblot of a similar SDS–12.5% PAGE of purified Fpp1 (lane 1) and Fpp2 (lane 2) proteases probed with antibodies (1:500) raised against the Fpp1 (55 kDa) protease [7]. Protein molecular mass markers (expressed in kDa) are indicated on the left.

of the Fpp2 was TVYNIPV. On the other hand, experiments using antibodies raised against the Fpp1 metalloprotease showed that both enzymes are not immunologically related as can be observed in Fig. 1B. Several attempts were carried out in order to raise antibodies against the Fpp2 protease but the obtained sera showed a high background and low specificity.

3.2. Biochemical properties of Fpp2

The pH measurements showed optimal activity of the Fpp2 protease at pH 6.5 (Fig. 2). However, the enzyme presented a broad range of pH for azocasein hydrolysis with approximately 53% of the maximum level at pH 5.5 and 9 (Fig. 2).

The effects of different potential inhibitors, as well as

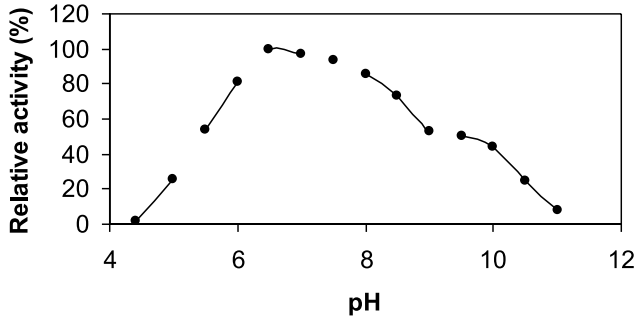

Fig. 2. Effect of pH on the activity of the Fpp2 protease. The purified enzyme (2 μg) was incubated for 2 h at 37°C in 600 μl portions of the buffers described in Section 2 containing 5 mM $CaCl_2$ and 1% azocasein as substrate. The value obtained at pH 6.5 was defined as 100%. The relative activities are averages based on two independent experiments.

cations, on the activity of the protease are summarized in Table 2. The enzyme was drastically inhibited by chelating agents such as ethylenediamine tetraacetic acid (EDTA), EGTA and 1,10-phenanthroline, but not by zincov. In contrast, inhibitors of serine proteases such as phenylmethylsulfonyl fluoride (PMSF) had no effect on the activity. An increase in proteolytic activity was observed when calcium or strontium was present in the reaction (Table 2). Also, the reconstitution experiments carried out to define the nature of the metal ion involved in the active site of the metalloprotease showed, after EGTA treatment, that the enzymatic activity could be restored to 78 and 106% of the original value in the presence of 10 mM of $CaCl_2$ and $SrCl_2$, respectively (Table 2).

The incubation temperature influence on the Fpp2 proteolytic activity is shown in Fig. 3A. The enzyme presented a maximum activity at 24°C, being active at temperatures ranging from 4 to 37°C with approximately 45 and 16.5% activity at 12 and 37°C, respectively. The presence of 5 mM $CaCl_2$ in the reaction produced a shift in the optimal temperature of the Fpp2 enzyme from 20–24°C to 33–37°C. Although, a similar proteolytic activity was found at temperatures below 20°C for both forms of the enzyme as the temperature was increased, a divergent Fpp2 activ-

Table 2
Effect of different compounds on the Fpp2 caseinolytic activity

Compound(s) (conc [mM])	Caseinolytic activity (%)
None	100
$CaCl_2$ (0.1)	122.4
$CaCl_2$ (0.5)	148.2
$CaCl_2$ (1)	158.5
$CaCl_2$ (5)	145.2
$CaCl_2$ (10)	90
$MgCl_2$ (1)	104.1
$MgCl_2$ (5)	75.3
$BaCl_2$ (1)	107.6
$BaCl_2$ (5)	89.3
$ZnCl_2$ (0.005)	78.5
$ZnCl_2$ (0.01)	79.8
$ZnCl_2$ (0.1)	54.2
$FeCl_2$ (0.005)	88.2
$FeCl_2$ (0.01)	93.1
$FeCl_2$ (0.1)	90.73
$SrCl_2$ (1)	141.2
$SrCl_2$ (5)	151.2
PMSF (1)	101
PMSF (5)	99.3
1,10-Phenanthroline (1)	21.7
1,10-Phenanthroline (5)	3.88
Dithiothreitol (1)	61.8
Dithiothreitol (5)	34.3
EDTA (1)	27.3
EDTA (5)	2.9
EGTA (1)	23.7
EGTA (5)	3.1
Zincov (1)	78
EGTA (5)+$CaCl_2$ (10)	78.2
EGTA (5)+$SrCl_2$ (10)	106.8

Values are averages of three determinations.

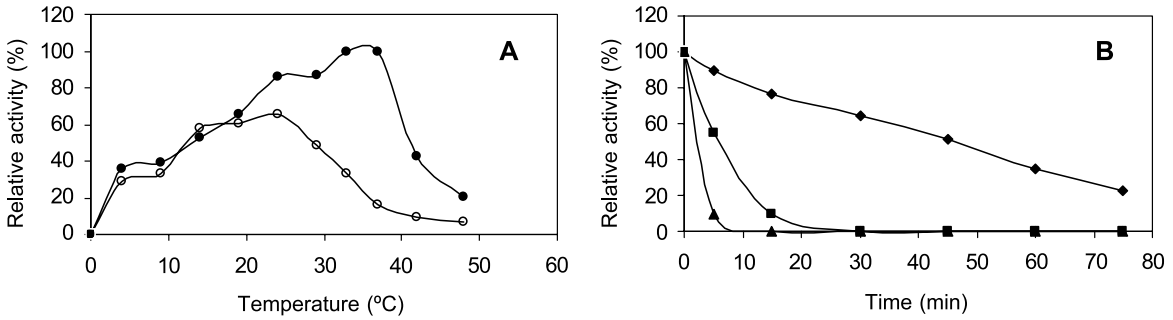

Fig. 3. Effect of temperature in the activity of the Fpp2 protease. A: The enzyme (2 μg) was incubated in 600 μl of PIPES buffer pH 6.5 and 1% azocasein for 2 h at various temperatures and caseinolytic activity determined as described in Section 2. Symbols: (○) calcium-free Fpp2; (●) calcium-enriched enzyme (5 mM $CaCl_2$). B: Thermal stability of Fpp2 protease (▲) calcium-free Fpp2 and in the presence of (♦) calcium (5 mM $CaCl_2$) or (■) strontium (5 mM $SrCl_2$). The enzyme (2 μg) was incubated in 100 μl portions of 25 mM PIPES (pH 6.5) at 40°C for different times. Then, the remaining activity was measured by adding 500 μl of 1% azocasein solution to the same buffer. The reaction was carried out as described in Section 2. The relative activities are averages based on two independent experiments.

ity profile was obtained (Fig. 3A). Therefore, at 37°C the maximum level of Fpp2 activity was obtained when $CaCl_2$ was present in the reaction and only 16.6% proteolytic activity remained at this temperature when the enzyme was assayed. As can be deduced from the Arrhenius plot, the enzyme became unstable at about 38.5°C and the estimated E_a for the hydrolysis of azocasein in the range from 4 to 37°C was 5.4 kcal mol^{-1}. Thermal stability studies indicated that total proteolytic activity was lost after Fpp2 incubation for 5 min at 40°C (Fig. 3B). However, in the presence of 5 mM of $CaCl_2$ or $SrCl_2$, 95 and 58% of the original activity were present after 5 min of incubation, respectively. Moreover, the thermal stability of the enzyme increased in the presence of calcium with 35% activity remaining after 60 min of incubation (Fig. 3B).

Matrix proteins such as fibrinogen, fibronectin, laminin, and the muscle proteins, actin and myosin, were degraded by the Fpp2 enzyme (Fig. 4A). Type IV collagen was degraded but type II collagen was only slightly hydrolyzed. In contrast, type I collagen was refractory to degradation (Fig. 4B). The enzyme lacked elastolytic activity when assayed with elastin Congo red as a substrate.

4. Discussion

Although Fpp2 is an extracellular protein, its purification process proved to be complex. The most relevant step was the hydrophobic chromatography on butyl-Sepharose where a six-fold purification was obtained. At the end of the process about 180 μg of pure Fpp2 were routinely purified from 2 l of culture supernatant with an approximate 3.5% yield. According to the SWALL database an internal sequence of the methylglyoxal synthase of *Thermotoga maritime* (GenBank accession number AE000512) is identical to the N-terminal of the Fpp2 protein. The metalloprotease nature of the Fpp2 enzyme is confirmed by the effect of chelating agents and reconstitution experiments. Thus, the metal-depleted enzyme through EGTA treatment and subsequent assay in the presence of Ca^{2+} or Sr^{2+}, restored the activity of the enzyme. Both ions also produced a similar increase in the activity of the enzyme. However, the effect of those ions in the thermal stability of the protease was different with the Ca^{2+} being much more efficient. So, whereas the activation of the enzyme could be obtained by Ca^{2+} and Sr^{2+}, the thermal stabilization was

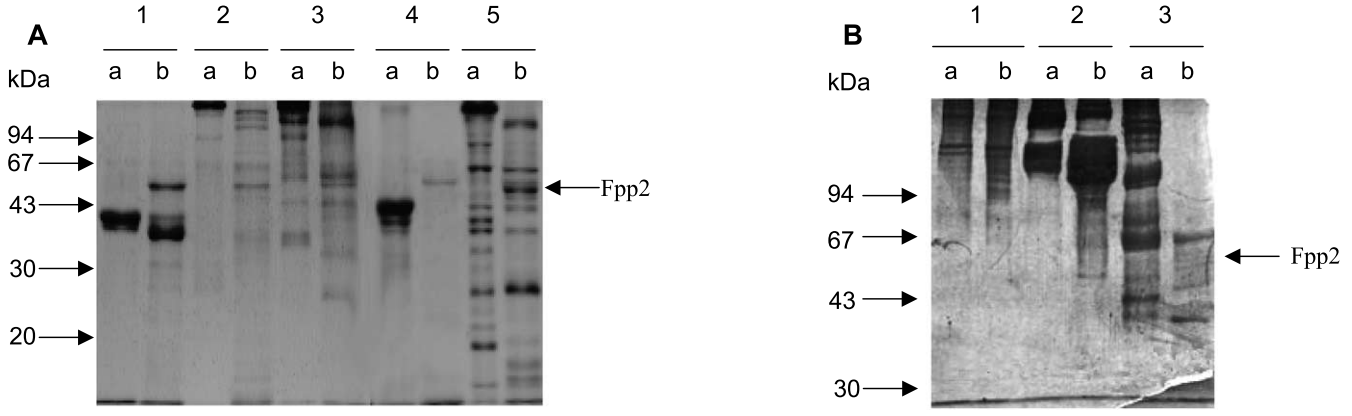

Fig. 4. Extracellular matrix and muscular protein degradation by Fpp2 protease. The substrates (12 μg) were incubated in the absence (lanes a) or in the presence (lanes b) of Fpp2 enzyme (1 μg) at 12°C for 16 h. Then, reactions were terminated by the addition of EGTA (10 mM final concentration) and reaction products separated on SDS–10% PAGE gels. A: Lanes 1, fibrinogen; lanes 2, fibronectin; lanes 3, laminin; lanes 4, actin; lanes 5, myosin. B: Lanes 1, type I collagen; lanes 2, type II collagen; lanes 3, type IV collagen. Molecular mass markers in kDa are indicated on the left. The position of Fpp2 in lanes b is indicated on the right.

Ca^{2+} dependent. Ca^{2+} ions are required for the activity and/or thermal stability of different metalloproteases, among which the most widely studied are probably the subtilisin family of proteases [20,21].

The Fpp2 protease digests components of the extracellular matrix including laminin, fibronectin, etc., and also muscle proteins (Fig. 4). It can be speculated that the absence of substrate specificity, in terms of the broad spectrum of matrix and muscle proteins hydrolyzed by Fpp2, probably brings about the usefulness of this for both nutrient and pathogenic functions. The pattern of protein degradation is similar to the matrix metalloproteases described in eukaryotic organisms and associated with tumor progression in cancer [22]. Thus, the Fpp2 protease could be involved in facilitating the colonization and invasion of the host by degrading different tissues and, at the same time, using the generated peptides as a nutrient.

A comparison between the Fpp1 and Fpp2 enzymes indicates that they presented some similarities and some differences. Both enzymes (as metalloproteases) are activated by Ca^{2+} and Sr^{2+} ions and responded to the same specific inhibitors. In contrast, they showed some differences in the temperature and pH profiles of activity. Thermal stability of both enzymes was increased by calcium ions, although the Fpp2 enzyme was more thermostable than the Fpp1 one. Thus, after 60 min of incubation at 40°C in the presence of 5 mM $CaCl_2$, 35% remaining activity was found in the Fpp2 enzyme whereas no activity at all remained under those same conditions in the Fpp1 case [7]. However, Fpp1 and Fpp2 optimum temperatures, temperature profiles and a low E_a, defining the psychrophilic biochemical behavior of the enzymes [23], were similar. On the other hand, although the optimum pH for both enzymes was around 6.5, the pH range was broad in the Fpp2 enzyme when compared to the Fpp1 one [7]. Finally, the substrate range of both proteases, in relation to the matrix and muscle protein, was similar.

From a physiological point of view, the most relevant difference between both enzymes was the production of the Fpp1 protease dependent on the presence of calcium in the culture medium [7]. In contrast, the Fpp2 was produced in the absence of this cation. Based on all the particular characteristics and differences of these two enzymes, a different role of these two proteases in the bacterium could be speculated, but to date, the data do not allow us to go further. So, we will continue our research line working in the exocellular proteolytic system of this bacterium in order to define specific functions of these two proteases and these of probable new ones yet to be discovered.

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