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Proteolytic activity of four strains of *Pseudomonas* on crude extracts of contractile proteins

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Abstract

Hydrolytic activities of exogenous proteases obtained from *Pseudomonas* upon crude extracts of contractile proteins were studied. Four strains were used: C61 and C20, isolated from beef samples, and *P. fragi* (ATCC-4973) and *P. fluorescens* (NRRL-B-1244). Proteolytic activity was tested on crude extracts of actomyosin, myosin + actin + regulatory proteins, actin and high molecular weight proteins obtained from postrigor beef by differential precipitation according to their solubility in salt solutions of various ion strengths. Electrophoresis analysis showed considerable degradation of actomyosin in samples treated with a C61 enzymatic extract. Myosin degradation was similar for treatments with proteases obtained from C61 and *P. fragi*. Actin degradation was similar for all enzymatic extracts. The most active protease was a 46.8 kDa enzyme produced by C60. *P. fragi* produced two proteases of 49.2 kDa and 34.2 kDa, *P. fluorescens* one of 46.1 kDa, and C20 one of 49.2 kDa.

Keywords: *Pseudomonas*; Proteases; Myofibrillar proteins; Degradation

1. Introduction

Pseudomonas is the most predominant bacterial group in raw meat spoilage at refrigeration conditions. The role of proteolytic enzymes produced by psychrothrophs during spoilage of muscle foods is controversial. Although there are some reports supporting protein breakdown as a spoilage mechanism (Chung, 1968; Thompson, Naidu, & Pestka, 1985) other authors consider that non-protein, low molecular weight constituents of the muscle are precursors of spoilage metabolites (Gill, 1986; Lerke, Farber, & Adams, 1967).

Some authors reported the effect of pure cultures of pseudomonads on myofibrillar proteins. Tarrant, Jenkins, Pearson, and Dutson (1973) reported the degradation of the Z-line by a partially purified protease obtained from *P. fragi*. These authors concluded that the enzymatic extract was less active on sarcoplasmic than on myofibrillar proteins. The reduction of electrophoretic bands including beef myosin, actinin, actin and troponins T, I and C as well as tropomyosin when the meat was inoculated with a pure strain of *Pseudomonas* was studied by Dainty, Shaw, De Boer, and Scheps (1975). Shaw and Latty (1982) found few changes in these proteins after 18 days of storage at 5°C where mixed microflora was observed.

The objective of the present work was to study the proteolytic activity of two *Pseudomonas* strains isolated from beef, to compare it with the activity of two strains reported as protease producers (*P. fragi* ATCC-4973, *P. fluorescens* NRRL-B-1244) and to identify the molecular weight of proteases acting on crude extracts of actomyosin, myosin + actin + regulatory proteins, actin and high molecular weight proteins (titin, nebulin and filamin).

2. Materials and methods

2.1. Preparation of the crude protease

Four proteolytic strains of *Pseudomonas* isolated from beef were kindly supplied by Dr. Gabriela Rodríguez-Serrano, Food Science Section, Universidad Autónoma

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Metropolitana-Iztapalapa. The strains, labelled C20, C61, C68 and C79, plus two other obtained from culture collections and reported as protease producers (*P. fragi* ATCC-4973 and *P. fluorescens* NRRL-B-1244) were inoculated in gelatine (Mac, 1993) and casein media (Molin & Ternstrom, 1982) in Petri dishes and incubated during 48 h at 20°C. Strains producing the widest haloes in gelatin and casein media were selected (C20 and C61). The four strains were inoculated in trypticasein–soy broth (TSB) (Myhara & Skura, 1989) and incubated at 25°C for 24 h with continuous stirring. Bacteria propagation was carried out in TSB medium in baffled Erlenmeyer flasks added with 1% of the inocula (O.D. = 1, read at 660 nm) and incubated at 19°C for 48 h with continuous stirring (150 rpm). The cell fraction was centrifuged and the supernatant filtered through a 0.45 mm Millipore membrane. The protease extracts were precipitated with 65% ammonium sulphate at ca. 4°C and centrifuged. The supernatant was discarded, precipitate resuspended in a minimum volume of 50 mM Tris-HCl + 0.1 M KCl buffer, pH = 7.5, and dialysed against a 50 mM Tris-HCl + 0.6 M KCl buffer (Alanís, 1997).

2.2. Assay of protease activity

One percent casein broth was added with 2 mM $CaCl_2$ in 50 mM phosphate buffer, pH = 7.5. The substrate, plus the enzymatic extract, were incubated at 37°C for 1 h. The reaction was stopped by the addition of 5% trichloroacetic acid. The amount of hydrolysed casein was an indication of proteolytic activity, using a standard curve. One unit of enzymatic activity was defined as the amount of enzyme necessary to produce 1 mmol of tyrosine/min at 37°C in a reaction mixture of 2.5 ml (mm tyrosine/min ml), read at 280 nm (Kunitz, 1947).

2.3. Molecular weight determination of protease extracts

Molecular weight determination of protease extracts was carried out according to the method reported by Garcia Carreño, Dimes, and Haard (1993). The extracts were diluted 1:4 in a buffer containing SDS. Electrophoresis was carried in 12% polyacrylamide gels and 3% stacking gels at 15 mA per gel, 4°C in a Mini-Protean II slab cell, Bio-Rad (Richmond, CA). The gels were imbibed with 2% casein solution in 50 mM Tris-HCl buffer, pH = 7.5, rinsed with distilled water and stained with 0.1% Coomasie blue. Bioactive zones showed translucent bands on an opaque background. The gels were read in a Ultrascan XL densitometer, Pharmacia-LKB (Bromma, Sweden).

2.4. Preparation of crude protein extracts

Animals were slaughtered 18 h prior sampling. No pre-rigor handling was recorded. Fat and connective tissue were eliminated from postrigor *Biceps femoris* muscle. Lean meat was then minced in a domestic blender to obtain a paste. All operations were carried out at 4–6°C. Actomyosin extraction was carried out using a modification of the method described by Cofrades (1994), 700 g of meat were homogenised with 1500 ml of 50 mM sodium phosphate, the precipitate resuspended in 1500 ml 0.8 M NaCl + 50 mM sodium phosphate, pH = 7.5. The supernatant was carefully removed taking care not to disturb the protein precipitate. It was collected, centrifuged at 10,000 ***g*** for 15 min and resuspended in 50 mM sodium phosphate buffer + 0.8 M NaCl, pH = 7.5, the precipitate was discarded and the supernatant adjusted to a final 40% glycerol concentration. The suspension was stored at −20°C.

Myosin was extracted using the method described by Margossian and Lowey (1982); 700 g of meat were homogenised with 1500 ml of 0.15 M phosphate buffer + 0.3 M KCl, 20 mM EDTA, 5 mM $MgCl_2$ + 1 mM sodium pyrophosphate, the protein in the supernatant precipitated and resuspended in 50 mM potassium phosphate buffer + 1 M KCl + 25 mM EDTA, pH = 6.5 and dialysed during 24 h against 25 mM phosphate buffer + 0.6 M KCl + 10 mM EDTA and 1 mM DTT dithiothreitol, pH = 6.5. Actin from the dialysed protein fraction was precipitated at ca. 0.3 ionic strength. It was resuspended in a minimum volume of 10 mM potassium phosphate + 1 M KCl, pH = 6.5. In order to reduce the amount of contaminant actin the method described by Ebashi (1976) and modified by Martone, Busconi, Folco, Trucco, and Sánchez (1986) was followed. It is based on differential solubility of actomyosin and actin in β-mercaptoethanol and $MgCl_2$. Actomyosin and myosin fractions were added with 40% glycerol and stored at −20°C.

Actin was extracted using a modification of the method described by Pardee and Spudich (1982). It is based on the extraction of actin from meat previously dehydrated with acetone. Six hundred g of meat were extracted with 2000 ml 0.15 M sodium phosphate + 0.1 M KCl, pH = 6.5, centrifuged and the precipitate extracted with 0.05 M $NaHCO_3$. The residue was resuspended in EDTA, pH = 7.0. The precipitate was washed with cold distilled water and acetone, and filtered. The resulting residue was dried overnight at room temperature and stored at −20°C. The powder was extracted with 2 mM Tris-HCL + 0.5 mM sodium pyrophosphate, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$ + 0.005% sodium azide, pH = 8. The two combined supernatants were centrifuged and transferred to a beaker where actin polymerisation was carried out by adjusting concentration to 50 mM, Mg^{2+} to 2 mM and sodium pyrophosphate to 5 mM, adding the respective reagents up to the required concentrations in a known supernatant volume. Actin was then washed with 0.6 M KCl and centrifuged. The agglomerate was separated from the

tube and left overnight. G-actin was obtained in the supernatant.

2.5. Assay of protease activity on crude protein fractions

Protein fractions (actin, myosin + actin + regulatory proteins, actomyosin) were used as a substrate to determine the hydrolysis of enzymatic extracts. Myosin + actin + regulatory proteins as well as actomyosin fractions were diluted with 10 volumes of distilled water in order to precipitate the protein and eliminate glycerol. They were then centrifuged at 10,000 ***g*** for 10 min, the insoluble protein was resuspended in 50 mM Tris-HCl + 0.6 M KCl, pH = 7.5 and dialysed against the same buffer for 24 h. Actin was dialysed only against the buffer, the protein concentration of the three fractions were then analysed using the method described by Redinbaugh and Turley (1986).

Electrophoresis of actomyosin, myosin and actin extracts were carried out according to the method described by Laemmli (1970). Twelve percent acrylamide was used as separation gels and 4% as stacking gels in a Mini-Protean II slab cell. Sample concentration of 6 to 7 mg/ml and a mixture of SDS-gel markers (Sigma Chemical) of a molecular weight ranging between 30 and 200 kDa were applied. Each gel was later scanned using an Ultrascan XL densitometer (Pharmacia-LKB, Bromma, Sweden).

Activity of protease extracts from *P. fluorescens*, *P. fragi*, C61 and C20 on fractions of actin, actomyosin and myosin + actin + regulatory proteins was followed. A total volume of 0.9 ml of protein extract was placed in 6 ml polycarbonate test tubes and 0.1 ml of the enzymatic extract added to each one. Protein fractions with the enzyme extracts plus a blank of the protein fraction were incubated at 13°C for 48 h. Sampling was at 0, 4, 8, 12, 24, 36 and 48 h, taking 25 ml of the protein–enzyme extract mixture, diluting with 100 ml of a buffer containing SDS and β-mercaptoethanol, boiling for 5 min, and applied to a SDS–polyacrylamide gel. Electrophoresis was carried out applying a constant current of 200 V for 45 min. The gels were stained with 1% Coomasie blue for 30 min, developed with 40% ethanol + 10% acetic acid for 4 h and read in a densitometer. Ten microliters of 30 to 200 kDa standard (Sigma) were also applied to the gel.

To obtain the degradation patterns of high molecular weight proteins (> 200 kDa) such as nebulin, titin and filamin, enzymatic extracts from *P. fragi*, *P. fluorescens* and C61 were utilised. Experimental conditions and analytical methods were the same as those described in the previous paragraph.

3. Results and discussion

3.1. Selection and activity of Pseudomonas strains

Protease production by the strains isolated from beef as well as the two collection strains is shown in Table 1. Strains selected to produce enzymatic extracts, according to the halo diameter, were C61 and C20, besides the two collection strains used as a means of comparison. *P. fragi* ATCC-4973 did not show hydrolysis in casein medium but did show in gelatin. It is reported that this strain promotes structural changes in myofibrillar proteins of beef and pork (Bala, Marshall, Stringer, & Naumann, 1979; Tarrant et al., 1973). The activity of crude enzymatic extracts were 3.92 U/ml (mg tyramine/min ml) for *P. fragi* ATCC-4973, 2.83 for *P. flurescens* NRRL-B-1244, 5.1 for C61 and 3.52 for C20.

3.2. Composition of enzymatic extracts

The incubation period of the gels on casein was increased from 90 min, reported by García Carreño et al. (1993) to 2 h due to the fact that incubation for 90 min did not show clearly defined bioactive zones. Longer time periods reduced the band definition due to enzyme diffusion into the gel. Molecular weights were calculated by using a standard curve of log (molecular weight) vs R_f. The molecular weights obtained were 46.1 kDa for *P. fluorescens* NRRL-B-1244, 46.8 kDa for C61, 49.2 and 34.2 for *P. fragi* ATCC-4973, and 49.2 for

Table 1
Preliminary proteolysis test of *Pseudomonas* strains

Strain	Colony morphology	Gelatin medium	Casein medium
P. fragi ATCC-4973	Opaque	+[b]	Negligible
P. fluorescens NRRL-B-1244	Opaque[a]	+ + +	+ +
C61	Opaque[a]	+ +	+ + +
C79	Opaque	+ +	+
C68	White	Negligible	Negligible
NC20	Rough[a]	+ + +	+ + +

[a] Fluorescent in F medium.
[b] + 2–5 mm diameter halo; + + 6–10 mm diameter halo; + + + > 10 mm diameter halo.

C20 (Fig. 1). It has been reported that *P. fragi* ATCC-4973 produces a neutral metalloprotease, molecular weight between 40 and 50 kDa (Porzio & Pearson, 1975). However, our results showed the production of two bioactive zones probably due to the loss of an enzyme (MW = 34.2 kDa) during the purification steps in reports by other authors. This difference could be also due to the fact that our studies were carried out with crude extracts, not with purified enzymes. On the other hand, casein hydrolysis has not been applied by other authors to develop bioactive zones of these particular enzymes.

3.3. Extraction of protein fractions

Electrophoretic patters of contractile proteins are shown in Fig. 2. The concentration of the protein extracts were: actomyosin: 4.92 mg/ml; myosin: 2.90 mg/ml; actin: 2.64 mg/ml. Actomyosin showed a band of 200 kDa corresponding to heavy myosin, some bands

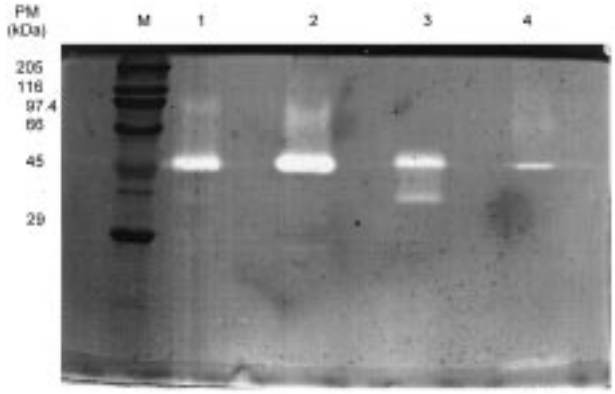

Fig. 1. SDS–mercaptoethanol polyacrylamide-casein gel electrophoresis of enzymatic extracts obtained from four strains of *Pseudomonas*: (M) Standards; (1) *P. fluorescens* NRRL-B-1244; (2) C61; (3) *P. fragi* ATCC-4973; (4) C20.

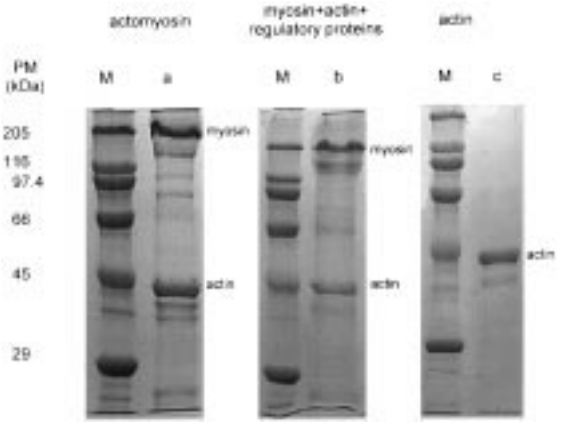

Fig. 2. Electrophoresis of protein extracts: (M) standards; (a), (b), (c), protein extracts.

of lower intensity were between 45 and 200 kDa, and between 30 and 45 kDa. These bands corresponded to regulatory proteins. At 45 kDa a band corresponding to actin was observed. The patter for myosin + actin + regulatory proteins showed two bands corresponding to myosin and actin. Actin in this pattern had a less intense band than in the actomyosin pattern probably due to the fact that actin in the second gel was a contaminant protein. The actin pattern showed a 45 kDa band corresponding to actin, and a second band corresponding to contaminant tropomyosin.

3.4. Hydrolysis of myofibrillar proteins

Figs. 3–5 show electrophoretic patterns and densitometry of actomyosin, myosin + actin + regultory protein, and actin at 0 to 48 h of hydrolysis by enzymatic extracts of *P. fragi* ATCC-4973, *P. fluorescens* NRRL-B-1244, C61 and C20.

Extensive protein degradation of actomyosin, actin and myosin as well as formation of polypeptides of 123 and 147 kDa were observed for treatments with the four enzymatic extracts. The amount of these polypeptides increased within the first 12 h of hydrolysis and later decreased probably due to further proteolysis of enzymatic extracts.

Fig. 3 shows a decrease in heavy myosin band probably due to degradation, polypeptides of high molecular weight were the result of this hydrolysis, as previously reported by Porzio and Pearson (1975). These authors found a neutral protease from *P. fragi* acting on myofibrillar proteins and rendering three fractions: heavy myosin, light myosin and premyosin with molecular weights from 80 to 175 kDa. The fact that polypeptides of high molecular weight were formed indicated that the cleavage sites of heavy myosin are susceptible to proteolysis by the studied enzymatic extracts.

Electrophoretic patterns of myosin + actin + regulatory proteins (Fig. 4) showed similar behaviour as those obtained for actomyosin. Degradation of myosin and actin by enzymatic extracts obtained from C61 was almost completed after 4 h of hydrolysis. Similarly to actomyosin fraction there was a formation of polypeptides of high molecular weight (124 to 151 kDa). Actin was also depleted in a larger extent by the extract obtained from C61 (Fig. 5).

Degradation patterns of high molecular weight proteins (> 200 kDa) by enzymatic extracts obtained from *P. fragi* ATCC-4973, *P. fluorescens* NRRL-B-1244 and C61 are shown in Fig. 6. Intensity of the titin band decrease whereas nebulin, shown as the second band in the gel, was completely depleted by all three enzymatic extracts. However, enzymes obtained from *P. fluorescens* were more active against this protein. At 4 h of hydrolysis titin band disappeared when treated with this extract, and at 8 h of hydrolysis when treated with

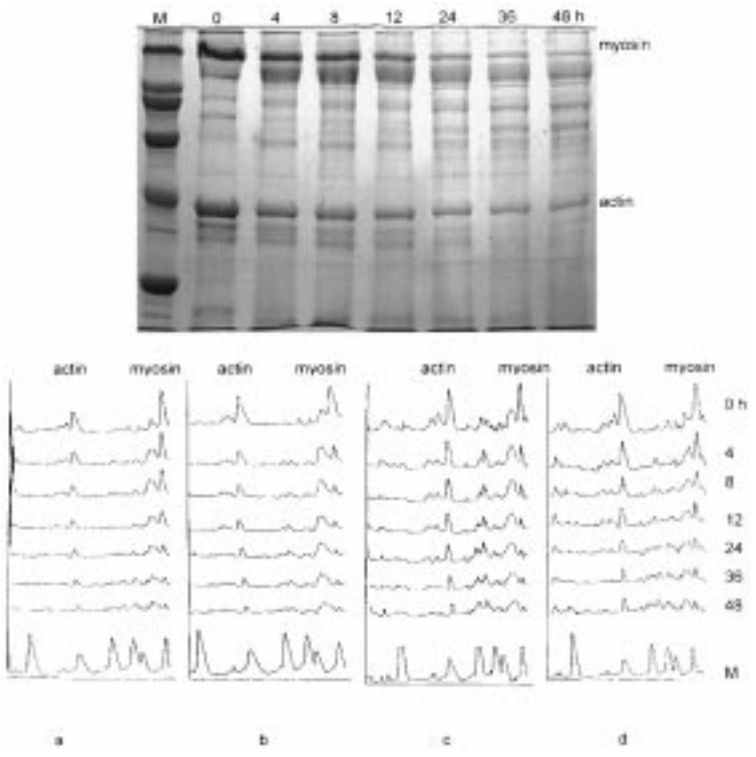

Fig. 3. Electrophoretic pattern and densitometry of actomyosin at 0 to 48 h of hydrolysis by enzymatic extracts of: (a) *P. fragi* ATCC-4973; (b) *P. fluorescens* NRRL-B-1244; (c) C61; (d) C20.

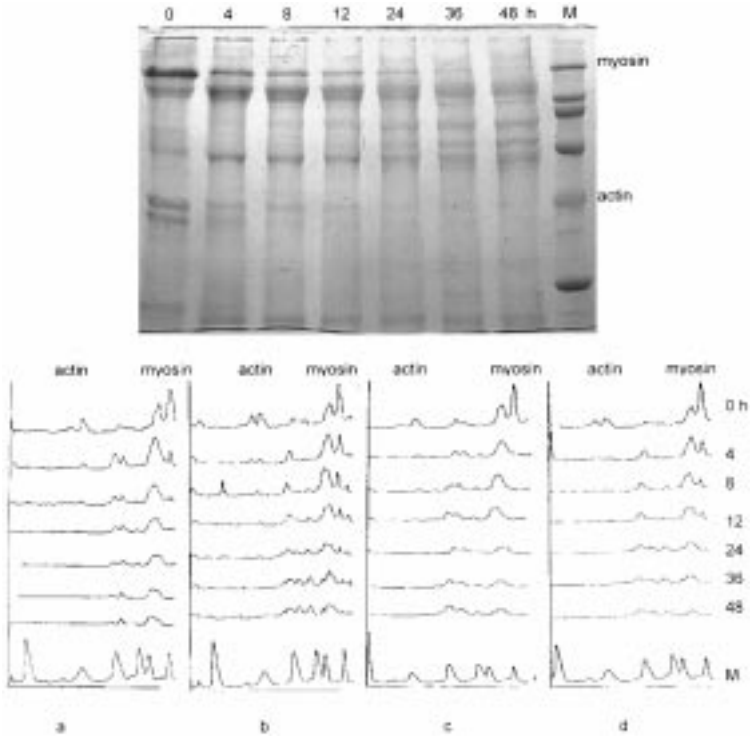

Fig. 4. Electrophoretic pattern and densitometry of myosin + actin + regulatory proteins at 0 to 48 h of hydrolysis by enzymatic extracts of: (a) *P. fragi* ATCC-4973; (b) *P. fluorescens* NRRL-B-1244; (c) C61; (d) C20.

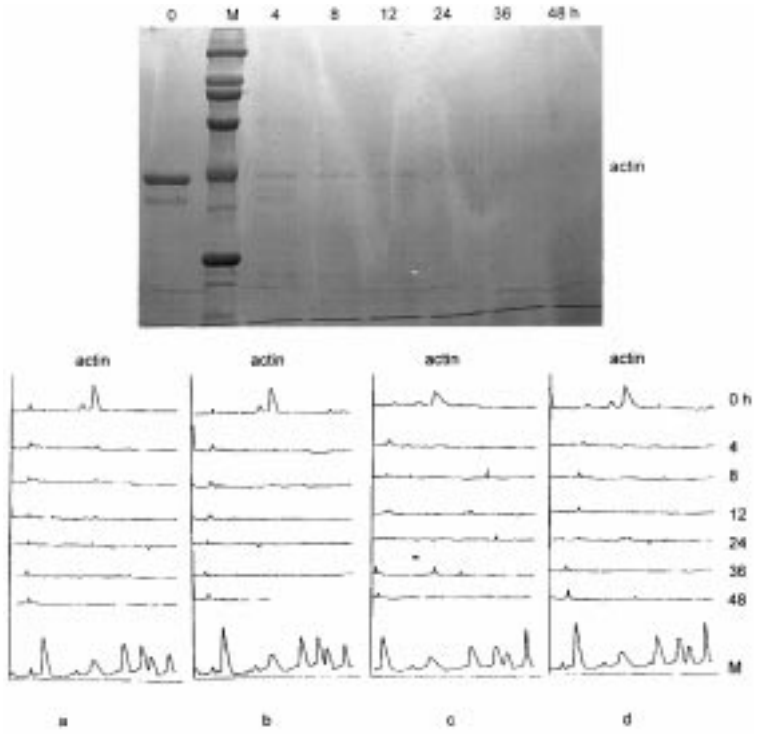

Fig. 5. Electrophoretic pattern and densitometry of actin at 0 to 48 h of hydrolysis by enzymatic extracts of: (a) *P. fragi* ATCC-4973; (b) *P. fluorescens* NRRL-B-1244; (c) C61; (d) C20.

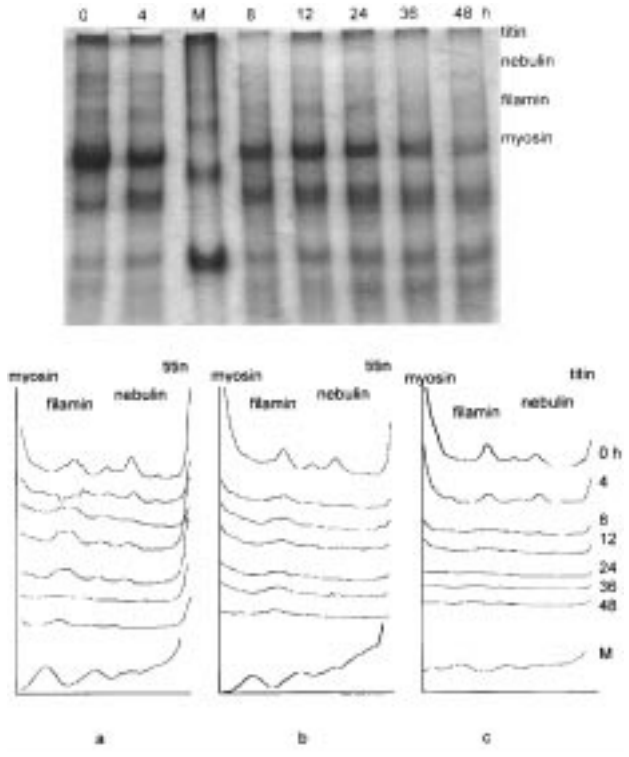

Fig. 6. Electrophoretic pattern and densitometry of high molecular weight proteins at 0 to 48 h of hydrolysis by enzymatic extracts of: (a) *P. fragi* ATCC-4973; (b) *P. fluorescens* NRRL-B-1244; (c) C61.

extracts obtained from *P. fragi* and C61. Filamin was more efficiently degraded by the extract obtained from C61, disappearing at 36 h of hydrolysis. Extracts from *P. fragi* and *P. fluorescens* had not the same proteolytic activity, showing light bands at 48 h of hydrolysis.

4. Conclusions

Of the four strains studied, *P. fluorescens* NRRL-B-1244 and strain C20, produced only one protease of 46.1, 49.2 and 46.8 kDa, respectively. However, *P. fragi* ATCC-4973, produced two proteases of 49.2 and 34.2 kDa, conversely as reported by other authors. Myosin showed particular susceptibility to cleavage by *Pseudomonas* proteases forming high molecular weight polypeptides. High molecular weight proteins were also degraded by enzymatic extracts.

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