PERGAMON

Toxicon 38 (2000) 1415–1427

TOXICON

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Purification of a novel antibacterial and haemagglutinating protein from the purple gland of the sea hare, *Aplysia dactylomela* Rang, 1828

Vânia M.M. Melo[a,*], Ana B.G. Duarte[a], Ana F.F.U. Carvalho[a], Elisângela A. Siebra[b], Ilka M. Vasconcelos[b]

[a]*Laboratório de Microbiologia e Imunologia, Departamento de Biologia, Universidade Federal do Ceará, Bloco 909, CEP 60451-970, Fortaleza-CE, Brazil*

[b]*Laboratório de Toxinas Vegetais, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Caixa Postal 6020, CEP 60451-970, Fortaleza-CE, Brazil*

Received 21 July 1999; accepted 18 October 1999

Abstract

Physicochemical characterisation and antibacterial and haemagglutinating properties of a new protein isolated from purple fluid of the *Aplysia dactylomela* are reported. The purification procedure consisted basically of ammonium sulphate fractionation, ion exchange, exclusion molecular and hydrophobic interaction chromatography. The highly purified protein, designated dactylomelin-P, is a single chain protein of 60,000 Da by SDS-polyacrylamide gel electrophoresis and 56,200 Da by gel filtration on calibrated Superose column at pH 7.5 and contains less than 0.05% of its weight in neutral carbohydrates. Dactylomelin-P has two biological activities, antibacterial and haemagglutinating. The antibacterial action is bacteriostatic but not bactericidal. The haemagglutinating activity is preferentially against rabbit erythrocytes. The glycoprotein fetuin was able to abolish the haemagglutinating activity but not the antibacterial one even when used at concentrations 10 fold higher. This is the first time that a chimeroprotein is described in the purple fluid of sea hares, which may be involved in the chemical defence mechanism of these organisms.

* Corresponding author. Fax: +85-287-2768.
E-mail address: vmmmelo@ufc.br (V.M.M. Melo).

0041-0101/00/$ - see front matter
PII: S0041-0101(99)00234-2

1. Introduction

Sea hares which are opistobranch molluscs have attracted the interest of many workers investigating the chemical compounds secreted by the purple gland or present in different tissues, possibly involved in the defence of these invertebrates. Some sea hare species have been shown to contain low molecular mass substances with antimicrobial (Rinehart et al., 1981; Ichida and Higa, 1986) and antitumor activities (Pettit et al., 1987; 1990) and also high molecular mass compounds such as the proteins aplysianins, julianins and dolabellanins from *Aplysia kurodai*, *Aplysia juliana* and *Dolabella auricularia*, respectively, with similar activities (Yamazaki, 1993). *Aplysia dactylomela* Rang, 1828 is a sea hare widespread along the Brazilian coast and also occurring in the intertidal zones from Southern Florida in the United States to Eastern India (Rios, 1985). This organism discharges a fluid from the purple gland when disturbed. This reaction suggests that this fluid contains bioactive factors which may act against potential enemies since the defence mechanisms of the sea hare differ from those of highly developed vertebrates (Cooper, 1980). Previous work has described the toxic, antimicrobial and haemagglutinating activities of the purple fluid of the *A. dactylomela* (Melo et al., 1998). This study aimed to isolate and characterise the protein of this fluid responsible for the antibacterial and haemagglutinating activities. The name dactylomelin-P is then proposed for this protein.

2. Materials and methods

2.1. General

Phenyl Sepharose, Sepharose-6B and Superose 12HR were obtained from Pharmacia Fine Chemical (Sweden). DEAE-cellulose, trypsin, fetuin, molecular weight markers and electrophoresis reagents were purchased from Sigma Chemical Company (USA). Agar Müller–Hinton and Müller–Hinton broth were from Difco Laboratories (USA) and tobramicin disks were from Cecon (Brazil). Other chemicals were of analytical grade.

2.2. Collection of the purple fluid

Specimens of *A. dactylomela* Rang, 1828 were collected at Pacheco Beach, Caucaia, State of Ceará, Brazil, in June and July. The purple fluid was obtained by irritating the hare, squeezing it gently for a few minutes outside the water. The secretion was collected into a sterile bottle and subsequently frozen at $-10^{\circ}C$ until used.

2.3. Protein determination

The protein content in the samples was carried out by the method described by Bradford (1976) using bovine serum albumin as standard.

2.4. Carbohydrate determination

Total neutral carbohydrate content of fractions was determined by the phenol-sulphuric acid method of Dubois et al. (1956).

2.5. Fast-protein liquid chromatography (FPLC)

A FPLC apparatus from Pharmacia-LKB was used with UV monitor for 280 nm. The samples were centrifuged at 14,000 *g* for 5 min before being introduced into the column. Gel filtration was performed in Superose 12HR 10/30 column equilibrated with 50 mM Tris–HCl, pH 7.5, at a constant flux of 0.5 ml min^{-1}. The column was calibrated with proteins of known molecular masses.

2.6. Polyacrylamide gel electrophoresis

Electrophoresis of protein fractions was carried out by the method of Laemmli (1970) in 12.5% polyacrylamide slab gels in 0.025 M Tris–HCl, 0.2 M glycine, pH 8.9, with or without 0.1% sodium dodecyl sulphate. The gels were stained with either Coomassie blue or silver nitrate (Oakley et al., 1980).

2.7. Molecular mass (Mr) determinations

Molecular masses were estimated after electrophoresis in 0.1% sodium dodecyl sulphate-polyacrylamide gels, according to Weber and Osborn (1969).

2.8. Antibacterial activity

Inhibition of bacterial growth by the samples obtained from purple fluid was determined as described by Bauer et al. (1966). Briefly, bacterial cultures were maintained in Müller–Hinton broth. Sterile swabs were immersed in the microbial suspensions (10^8 cells ml^{-1}) and evenly applied to Petri dishes containing Müller–Hinton agar. Sterile Whatman AA filter paper disks (6 mm in diameter) were fully imbibed with 30 μl of the samples and placed over the agar in the plates. Tobramicin disks were used as positive control. The plates were incubated overnight at 35°C and then examined for zones of growth inhibition around each disk. The bacteria used were the Gram positive *Staphylococcus aureus* (ATCC 6538) and the Gram negative *Escherichia coli* (ATCC 13863). To investigate whether the antibacterial action was bacteriostatic or bactericidal, dactylomelin samples were serially diluted in Müller–Hinton broth and incubated with cells of *S. aureus* for 18 h at 35°C. After this period, the minimum inhibitory concentration (MIC) was determined according to Collins et al. (1989) and the mechanism of growth inhibition evaluated by subculturing the cells in media without dactylomelin.

2.9. Erythrocyte agglutination

The haemagglutinating activity was assayed according to Vasconcelos et al. (1991). Serial 1:2 dilutions of the sample dialysed against 0.025 M Tris–HCl, pH 7.5 were mixed in small glass tubes with untreated or trypsin-treated rabbit red cell (2% suspension prepared in 0.15 M NaCl). The enzyme-treated cells were obtained by incubation of trypsin (0.1 mg) with 25 ml of the 2% suspension for 60 min at 4°C. After washing six times, a 2% suspension was prepared in 0.15 M NaCl. The degree of agglutination was monitored visually after the tubes had been left to stand at 37°C for 30 min and at room temperature for an additional 30 min. The results are reported as the minimal protein concentration required to produce visible agglutination.

2.10. Haemagglutination and antibacterial inhibition assays

The haemagglutination inhibition assay was done by following the ability of the glycoprotein fetuin to inhibit agglutination of rabbit erythrocytes. The sample was added to each tube at a minimum concentration required to produce visible agglutination. The lowest glycoprotein concentration giving full inhibition of agglutination was determined by two-fold serial dilution of solutions at 1 mg ml^{-1} initial concentration. Aiming to investigate whether the recognition of the carbohydrate moiety of the protein was a key step in the antibacterial activity, bacterial inhibition assays were also done with the protein incubated with fetuin in the same conditions described previously.

3. Results and discussion

The results now presented deal with the isolation and characterisation of a protein present in the purple fluid of *A. dactylomela* which is responsible for the antibacterial and haemagglutinating activities described previously (Melo et al., 1998).

3.1. Purification of antibacterial and haemagglutinating protein

The procedure described below and summarised in Fig. 1 leads to the purification of dactylomelin-P, in a reproducible manner.

3.1.1. Step A: fluid preparation

An aliquot of the fluid was dialysed (cut-off 12,000) thoroughly against distilled water during 24 h, at 4°C. The dialysed fluid was centrifuged at 21,000 *g* for 30 min, at 4°C. The supernatant was designated PREP.A.

3.1.2. Step B: ammonium sulphate fractionation

Solid ammonium sulphate was slowly added to PREP.A. The precipitated

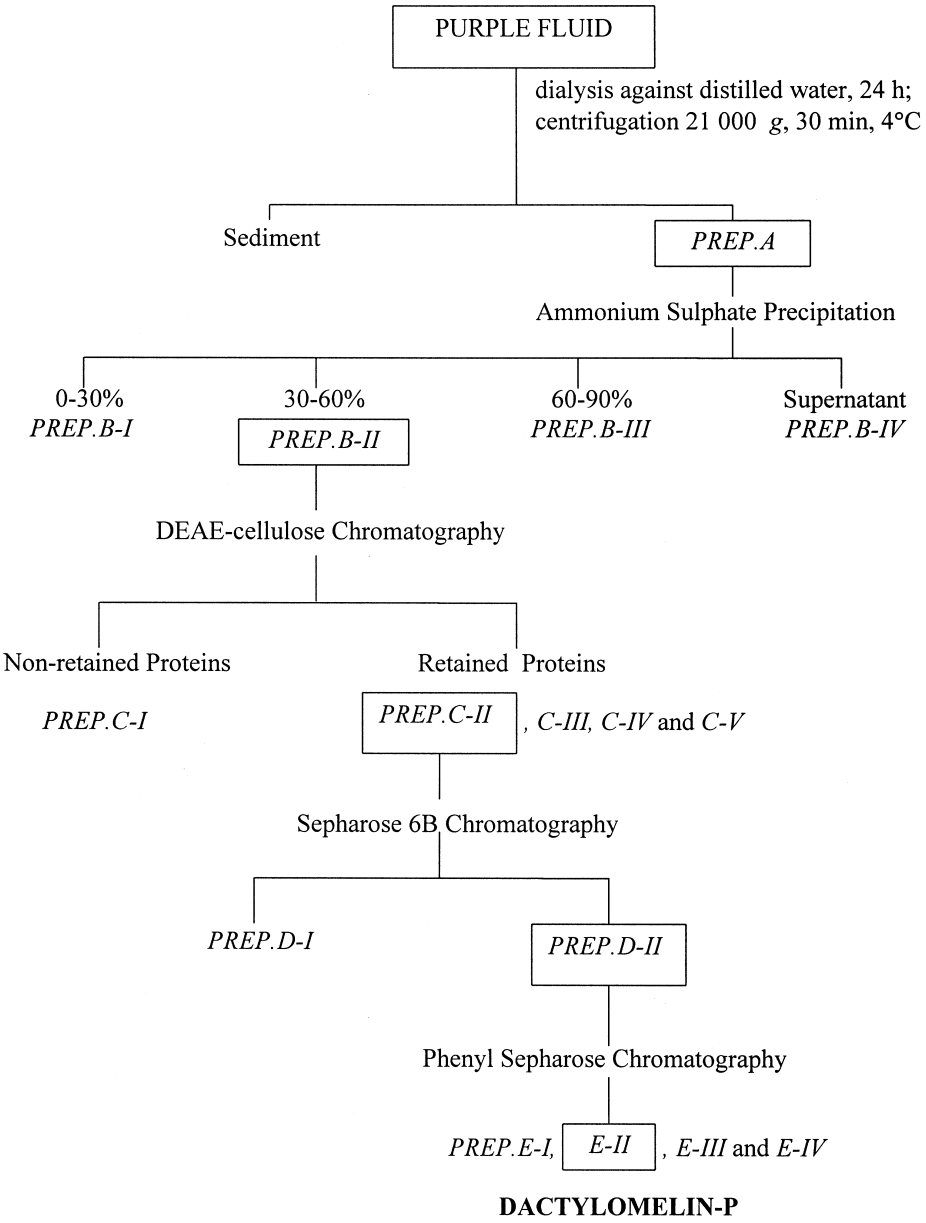

Fig. 1. Scheme of purification of dactylomelin-P.

formed at 30% saturation was separated by centrifugation at 21,000 *g* for 30 min. Additional salt saturation was used to make precipitates at 30–60 and 60–90% saturation. The precipitates obtained by centrifugation were dissolved in 0.05 M sodium acetate buffer, pH 6.0. The excess of salt in these fractions as well as the final supernatant was removed by exhaustive dialysis against the same buffer. The protein fractions corresponding to 0–30, 30–60 and 60–90% ammonium sulphate saturation as well as the final supernatant were designated PREP.B-I, B-II, B-III and B-IV, respectively. The distribution of the different biological activities among these fractions was studied. The antibacterial activity was found in all the fractions, except in PREP.B-IV. The PREP.B-II showed the highest activity against the tested Gram positive and Gram negative bacteria. The haemagglutinating activity, due to a lectin, was concentrated in the PREP.B-II. Thus, PREP.B-II was treated as follows.

3.1.3. Step C: DEAE-cellulose chromatography

Aliquots (100 mg each) of PREP.B-II in 0.05 M sodium acetate buffer, pH 6.0, were applied to a DEAE-cellulose column previously equilibrated with the same buffer. The column was percolated with the above buffer until complete removal of the non-retained proteins. Elution was done with the same buffer containing 0.15 and 1 M NaCl, 1 M NaOH and 1 M HCl. Fig. 2 shows that, after elution of the nonadsorbed proteins (PREP.C-I), two peaks (C-II and C-III) were eluted with the buffer containing 0.15 and 1 M NaCl, respectively. A fourth peak (C-IV) was obtained by eluting the column with 1 M NaOH and a fifth (C-V) with 1 M HCl. Assays for antibacterial and haemagglutinating activities have shown that PREP.C-II was toxic to *S. aureus* (selected target cell) and agglutinated rabbit erythrocytes. Active fractions corresponding to PREP.C-II were dialysed against distilled water and freeze-dried.

3.1.4. Step D: Sepharose 6B chromatography

Samples (22 mg) of PREP.C-II were applied into a Sepharose 6B column (1.7 × 41 cm) equilibrated in 0.05 M sodium acetate buffer, pH 6.0, containing 0,15 M NaCl. The column was percolated with the same buffer. As Fig. 3 shows, the proteins were washed out in two peaks (PREP.D-I and D-II). However, only D-II showed antibiotic and haemagglutinating activities. The fractions corresponding to PREP.D-II were pooled, dialysed and freeze-dried. Although this chromatographic process was not able to fractionate the proteins, it was very important for removing part of the pigments.

3.1.5. Step E: Phenyl Sepharose chromatography

A sample of PREP.D-II (20 mg) was dissolved in 0.05 M sodium acetate buffer, pH 6.0 and dialysed against the same buffer containing 1 M ammonium sulphate. After that, the sample was centrifuged at 21,000*g* for 10 min and the clear supernatant applied to a Phenyl Sepharose column (2 × 12 cm) equilibrated in the same buffer used for dialysis of PREP.D-II. The proteins were eluted in four peaks (Fig. 4). The first peak (PREP.E-I) was obtained by eluting the column with

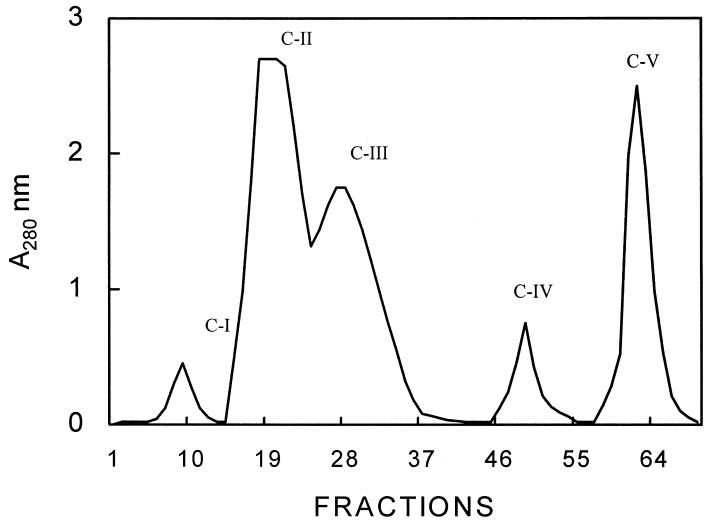

Fig. 2. DEAE-cellulose chromatography. Sample (100 mg) of PREP.B-II was applied to a column (1.6 × 28 cm) of DEAE-cellulose equilibrated with 0.05 M sodium acetate buffer, pH 6.0. The retained proteins were eluted at a flow rate of 40 ml h^{-1} with the same buffer containing 0.15 and 1 M NaCl, 1 M NaOH and 1 M HCl. Fractions (4.0 ml) were collected and monitored for protein content at 280 nm.

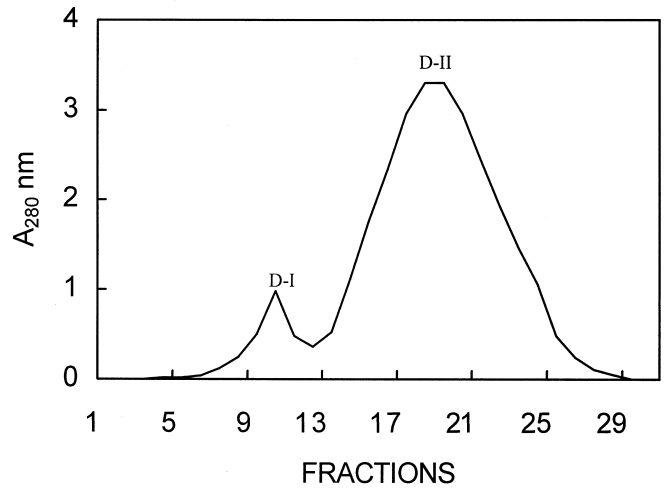

Fig. 3. Sepharose 6B chromatography. Sample (22 mg) of PREP.C-II was applied into a Sepharose 6B column (1.7 × 41 cm) equilibrated in 0.05 M sodium acetate buffer, pH 6.0, containing 0.15 M NaCl, at a flow rate of 15 ml h^{-1}. The column was percolated with the same buffer. Fractions (4.0 ml) were collected and monitored for protein content at 280 nm.

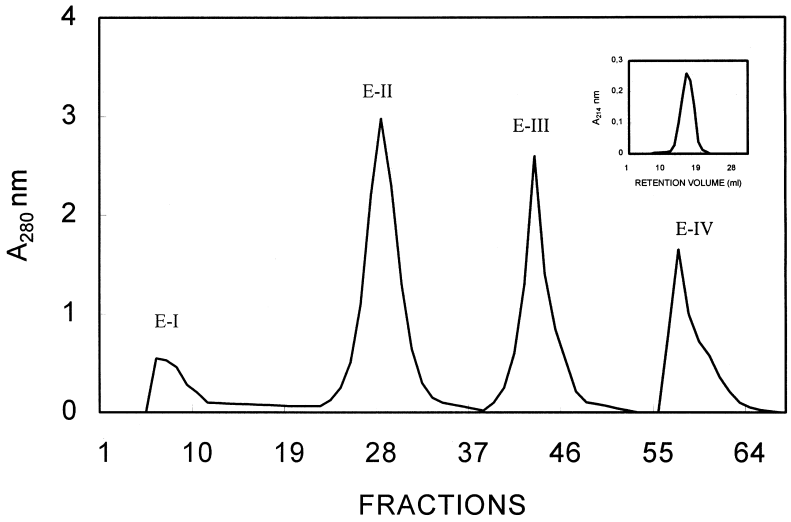

Fig. 4. Phenyl Sepharose chromatography. A sample of PREP.D-II (20 mg) was applied to a Phenyl Sepharose column (2 × 12 cm) equilibrated in 0.05 M sodium acetate buffer, pH 6.0, containing 1 M ammonium sulphate, at a flow rate of 40 ml h^{-1}. The proteins were eluted 0.05 M sodium acetate buffer plus 0.15 M NaCl, followed by 0.05 M sodium acetate buffer, water and 1 M NaOH. Fractions (2.8 ml) were collected and monitored for protein content at 280 nm. (Inset) Gel filtration of 100 μg of PREP.E-II on a calibrated Superose 12HR 10/30 column equilibrated in 50 mM Tris–HCl, pH 7.5, a constant flux of 0.5 ml min^{-1}.

0.05 M acetate buffer plus 0.15 M NaCl while the second one (PREP.E-II) with the same buffer without NaCl. The other peaks (PREP.E-III and E-IV) were eluted with water and 1 M NaOH, respectively. Assays for biological activities showed that only PREP.E-II was able to inhibit the bacterial activity and to agglutinate rabbit erythrocytes. This step was of extreme importance since an effective separation of the antibacterial and agglutinating protein was achieved.

Yamazaki et al. (1989) used a somewhat similar procedure for the purification of a citolytic factor from the purple fluid of *A. kurodai*. Their procedure consisted of two ionic exchange chromatographies and two gel filtrations. In this study, after several trials, we obtained better results by fractionating the fluid with ammonium sulphate followed by ionic exchange, molecular exclusion and hydrophobic interaction chromatographies. The simple procedure used for purification of the toxic and haemagglutinating protein allowed the isolation of dactylomelin-P in a stabilised and highly homogeneous state. The achieved purification index of the antibacterial and haemagglutinating activities were 58.3 and 1970.0 fold, respectively compared to the purple fluid (Table 1). As an alternative approach, dactylomelin-P can be obtained by applying the PREP.B-II on the fetuin Sepharose 4B column and eluting with 0.05 M glycine–HCl buffer, pH 2.2, containing 0.2 M NaCl (data not shown). Nevertheless, this approach was not selected because although the purification index of the antibiotic activity (61.4

Table 1
Purification of dactylomelin-P

Step[a]	Protein (mg 100 ml^{-1} purple fluid)[b]	Antibiotic activity		Haemagglutinating activy	
		µg[c]	purification index[d]	µg ml^{-1}[e]	purification index[d]
PREP.A (purple fluid)	1008 ± 6.5	350	1.0	39.4	1.0
PREP.B-II (30–60 % fraction)	840 ± 9.6	252	1.4	32.8	1.2
PREP.C-II (DEAE-cellulose)	185 ± 7.2	53	6.6	7.2	5.5
PREP.D-II (Sepharose 6B)	170 ± 4.2	48	7.3	3.3	11.9
PREP.E-II (Phenyl Sepharose)	29 ± 0.9	6	58.3	0.02	1970.0

[a] Purification steps are described under Results. These are mean ± S.D. of three similar runs.
[b] Indicates the total amount of protein recovered from 100 ml of purple fluid in each purification step.
[c] Antibiotic activity was expressed as the amount of protein in µg producing an inhibition zone with diameter around of 18 mm.
[d] Purification index was calculated as the ratio between the values of the antibiotic and haemagglutinating activities obtained at each purification step, respectively.
[e] Haemagglutinating activity was defined as the minimal protein concentration required to produce visible agglutination.

fold) had been the same than that obtained with the conventional techniques, the purification index of haemagglutinating activity (425.0 fold) was approximately 5 fold less than that obtained with the methodology described here. Besides, the use of fetuin Sepharose 4B column was not sufficient to remove the pigments which damage the column.

3.2. Characterisation of dactylomelin-P

The material obtained as PREP.E-II (dactylomelin-P) inhibited the growth of *S. aureus*, organism utilised for monitoring the activity (Table 1). As observed with the purple fluid (Melo et al., 1998), the PREP.E-II showed a bacteriostatic but not a bactericidal action, with a minimal inhibitory concentration of 15 μg ml^{-1}. Dactylomelin-P agglutinated rabbit erythrocytes, with the lowest protein concentration of 0.02 μg ml^{-1}. Antimicrobial proteins have been isolated from the purple fluid, albumen gland and eggs of sea hares of the genus *Aplysia* and *Dolabella* (Kisugi et al., 1987, 1989; Yamazaki et al., 1990; Iijima et al., 1994, 1995). The presence of bioactive proteins in the secretions of sea hares may be considered as indicative of their involvement in the chemical defence of these organisms. Also, agglutinins, inhibitable by D-galacturonic, have been described in *Aplysia* eggs and serum (Kamiya and Shimizu, 1981). In the present study, the haemagglutinating and antibacterial activities were shown to be due to the same protein.

The assay with the fetuin showed that the antibacterial activity was not inhibited by the glycoprotein, even when used at concentrations of 312 μg ml^{-1}. On the other hand, the haemagglutinating activity was abolished by fetuin at a minimal concentration of 31.2 μg ml^{-1}. As observed with the purple fluid, the haemagglutinating activity of the purified protein was not inhibited by glucose, mannose, galactose, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine or sialic acid when used at an initial concentration of 1 mg ml^{-1}. The fact that the antibacterial activity has not been inhibited by fetuin suggests that the recognition of the carbohydrate moiety is not a key step in this process. These results suggest that dactylomelin-P may be a chimerolectin which are proteins possessing a carbohydrate-binding domain tandemly arrayed with an unrelated domain, which has a well-defined biological activity that acts independently of the carbohydrate domain. Depending on the number of sugar-binding sites, chimerolectins behave as merolectins or hololectins (Peumans and Van Damme, 1995).

It is worthy mentioning that the activities of the dactylomelin-P, especially the antibacterial one, are quite stable since the protein conserved the same specific activities even after several months storage at 4°C.

3.3. Further physicochemical characterisation of dactylomelin-P

SDS-polyacrylamide gel electrophoresis (Fig. 5) of dactylomelin-P showed a band with molecular mass of 60,000 Da with pattern similar to those obtained from the crude and dialysed fluids. Gel filtration of dactylomelin-P on calibrated

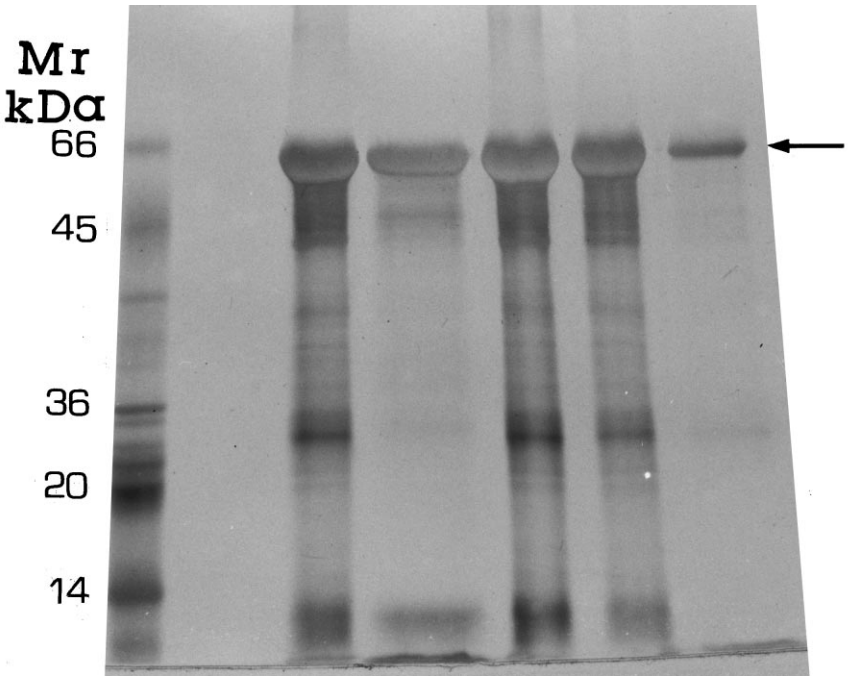

Fig. 5. SDS-polyacrylamide gel electrophoresis of the antibacterial and haemagglutinating fractions recovered from purple fluid of the *A. dactylomela*. The following materials were run: lane 1, standard proteins (bovine serum albumin, 67,000 Da; egg albumin, 45,000 Da; glyceraldehyde-3-phosphate dehydrogenase, 36,000 Da; carbonic anhydrase, 29,000 Da; trypsinogen, 24,000 Da; trypsin inhibitor, 20,100 Da and α-lactalbumin, 14,200 Da); lane 2, purple fluid; lane 3, 30–60% ammonium sulphate saturation; lane 4, PREP.C-II (antibacterial and haemagglutinating fraction retained by DEAE-cellulose); lane 5, PREP.D-II (antibacterial and haemagglutinating fraction obtained by Sepharose 6B); lane 6, PREP.E-II (dactylomelin-P, the bioactive fraction retained by Phenyl Sepharose).

Superose column gave rise to one peak with molecular mass of 56,200 Da (Fig. 4, inset). Native electrophoresis of this protein presented a single band revealing to be constituted by a single chain. It is not possible, however to establish that the dactylomelin-P is similar to aplisianin-P, with molecular mass of 60,000 Da and single chain, isolated by Yamazaki et al. (1990). Dactylomelin-P contains less than 0.05% of its weight in neutral carbohydrates. This result contrasted with that of the glycoprotein responsible for the antibacterial activity of the purple fluid from *A. kurodai*. Nevertheless, it is viable to speculate the possibility of homology between these two proteins since both come from the same secretion and the two organisms belong to the same genus.

In summary, we have shown that the purple fluid of the *A. dactylomela* contains a protein, dactylomelin-P, that is responsible for the antibacterial and the haemagglutinating activities. This study may support the involvement of this lectin in the chemical defence mechanism of the fluid as suggested earlier by Melo et al.

(1998). Thus, it seems important to gather more information concerning its physicochemical and biological properties.

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