PURIFICATION OF TRIDACNIN, A NOVEL ANTI-β-(1-6)-DIGALACTOBIOSE PRECIPITIN FROM THE HAEMOLYMPH OF *TRIDACNA MAXIMA* (RÖDING)

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1. Introduction

Haemolymph from the elongate clam, *Tridacna maxima* (Röding) agglutinates human erythrocytes and precipitates strongly with a number of galactans and some polysaccharides and glycopeptides [1–3]. Inhibition experiments have shown that the haemagglutinin and precipitin activity is inhibited by 2-acetamido-2-deoxy-D-galactopyranoside and by other sugars having a D-galactosido configuration [1–3]. Precipitation, haemagglutination (HA), absorption, inhibition and immunoelectrophoretic experiments in the presence of CA^{++} indicate that a single precipitin, named Tridacnin, is present in the haemolymph which posesses both the HA and precipitating activities [1–4].

The anti-galactan activity of the *T. maxima* agglutinin shows anomeric specificity since β-lactose and *p*-nitrophenyl β-D-galactopyranoside were good inhibitors but *p*-nitrophenyl α-D-galactopyranoside and oligosaccharides containing α-linked D-galactopyranosyl residues either did not inhibit or were comparatively weak inhibitors of *Tridacna*-induced HA and precipitation ([1,2]; Baldo and Uhlenbruck, in preparation). Meanwhile, we hace obtained convincing evidence that Tridacnin reacts with β-O-D-galactopyranosyl-(1-6)-D-galactose structures present in most galactans, and thus behaves in a very similar way to certain mouse myeloma IgA proteins [4].

In this paper we describe a method for purifying the *Tridacnin* using an affinity chromatography method and present the results of electrophoretic and isoelectric focussing studies on the isolated material.

2. Materials and methods

T. maxima haemolymph was obtained from dissected clams, dialysed against distilled water and lyophilized. *Lymnaea stagnalis* galactan was prepared according to the method of Horstmann and Geldmacher-Mallinckrodt [5]. Calcium chloride, reagent grade, was obtained from British Drug Houses.

2.1. *Preparation of immunoadsorbent*

Larch arabinogalactan from Serva Laboratories, Heidelberg, FRG was copolymerized with an equal quantity of the *N*-carboxyanhydride of L-leucine (Miles-Yeda, Rehovoth, Israel, lot no LE 45) at 4°C in a vehicle of 0.07 M $NaHCO_3$ [6,7]. The insoluble product was sedimented by centrifugation at 17 000 rev/min for 40 min and washed twice with 0.07 M $NaHCO_3$ and twice with distilled water.

2.2. *Affinity chromatography*

Polyleucyl-arabinogalactan (PL-AG) was mixed with Sepharose 4B (Pharmacia, Uppsala, Sweden) in the ratio 5:2, suspended in physiological saline containing 0.01 M Ca^{++} (PS-Ca^{++}) and allowed to stand for 24 hr. The mixture was then poured into a small column and washed extensively with PS-Ca^{++}. *Tridacna* extract dissolved in PS-Ca^{++} was added to the column and eluted with PS-Ca^{++}. Fractions were collected and examined for absorbance at 280 nm and for HA activity against human group O erythrocytes. Specific elution of the haemagglutinin was effected by adding 0.025 M *N*-acetyl-D-galactosamine in PS-Ca^{++} to the

column. Fractions showing absorbance at 280 nm and/or HA activity against human group O erythrocytes were pooled, dialysed extensively against frequent changes of distilled water, concentrated by ultrafiltration against distilled water and lyophilized.

2.3. *Immunological methods*

HA and immunoelectrophoresis (IEP) were carried out as previously described [9,10]. Antiserum to *T. maxima* haemolymph was produced in rabbits. An initial injection of 5 mg of previously dialysed and lyophilized haemolymph emulsified with 0.5 ml Difco Freund's complete adjuvant was injected intramuscularly (IM) into each hind leg of a New Zealand White rabbit (day 1). A second IM injection identical with the first was given on day 23. Subcutaneous injections of 5 mg haemolymph in PS were given at multiple sites on days 16, 44 and 51 and the animal was bled on days 21 (B1) and 58 (B2).

2.4. *Disc gel electrophoresis*

This performed in 5% and 7% polyacrylamide gels using the procedures described by Davis and Ornstein [8]. Electrophoresis was carried out using a current of 2.5 or 5 mA/tube and until the bromophenol blue dye marker had reached within approximately 0.5 cm of the ends of the gels. Gels were either fixed in 12½% trichloracetic acid or 7% acetic acid, stained with amido black or 0.05% Coomassie brilliant blue and destained in 7% acetic acid.

2.5. *Isoelectric focusing (IEF) in gel*

Electrofocusing was carried out as described by Eder [11] using gels consisting of 4.6% acrylamide and 3% ampholytes pH range 3–10. Separations were carried out at 4°C with a current of 1 mA/tube and a maximum voltage of 400 V for 7 hr. H_2SO_4 (0.05 M) and NaOH (0.03 M) were used as electrolyte solutions. Gels were washed extensively with 12½% trichloracetic acid [11] before staining with Coomassie blue and destaining in a solution containing methanol 25% and acetic acid 10%.

3. Results and discussion

3.1. *Isolation of Tridacnin*

After adding *T. maxima* haemolymph to PL-AG columns and washing with PS-Ca^{++}, *N*-acetyl-D-galactosamine solution (0.025 M) proved effective in eluting material which absorbed at 280 nm and which showed potent HA activity against human group O erythrocytes. When sufficient PL-AG was used, no HA activity was detected in the first peak off the column (fig.1). At least 30% of the material applied to the columns in such experiments was recovered in the fractions eluted with *N*-acetyl-D-galactosamine. The isolated Tridacnin agglutinated a 2% suspension of human group O erythrocytes at a minimum concentration of 0.32 μg/ml.

3.2. *Immunodiffusion and IEP examination of isolated Tridacnin*

Purity of the material isolated by *N*-acetyl-D-galactosamine elution was examined in gel diffusion and IEP experiments using rabbit anti-*T. maxima* haemolymph sera. The isolated Tridacnin gave only one band in gel

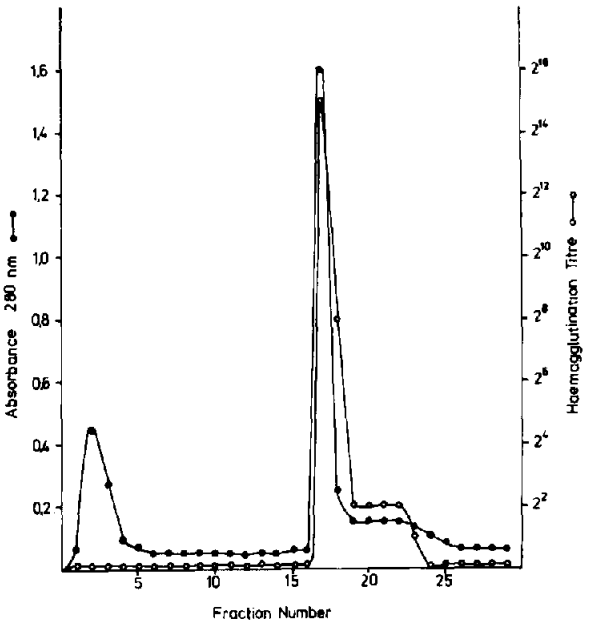

Fig.1. Isolation of Tridacnin from a polyleucyl-arabinogalactan column (1 ×10 cm). Lyophilized *Tridacna maxima* (Röding) haemolymph (15 mg) was applied to the column which was washed with saline containing 0.1 M Ca^{++} and then eluted (beginning at tube 16) with 0.025 M *N*-acetyl-D-galactosamine in saline–Ca^{++}. Flow rate 0.5 ml/hr at 4°C. Each fraction (4 ml) was examined for (•) protein by measuring the absorbance at 280 nm and for its (○) haemagglutinating activity against human group O erythrocytes.

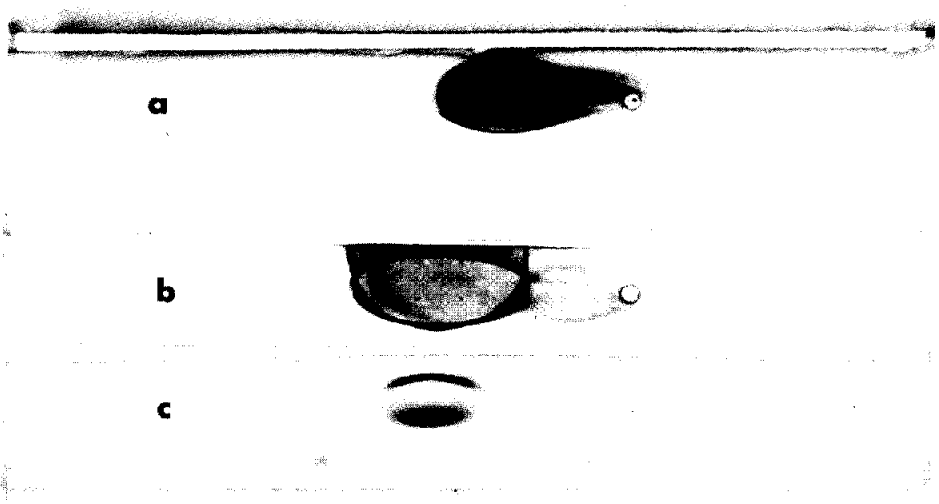

Fig.2. Immunelectrophoretic examination of *Tridacna maxima* haemolymph and immunoadsorbent-purified *Tridacnin*. (a) Well: *T. maxima* haemolymph 15 mg/ml. Troughs: Top. Rabbit anti-*T. maxima* haemolymph B1, undiluted. Bottom. *Lymnaea stagnalis* galactogen 1 mg/ml. (b and c). Wells: Top. *T. maxima* haemolymph 25 mg/ml. Bottom. Immunoadsorbent-purified *Tridacnin* 1.33 mg/ml. Troughs: Top. Rabbit anti-*T. maxima* haemolymph B2, diluted 1–2. Middle. Anti-*T. maxima* haemolymph B2, diluted 1–4. Bottom. Anti-*T. maxima* haemolymph B2, undiluted.

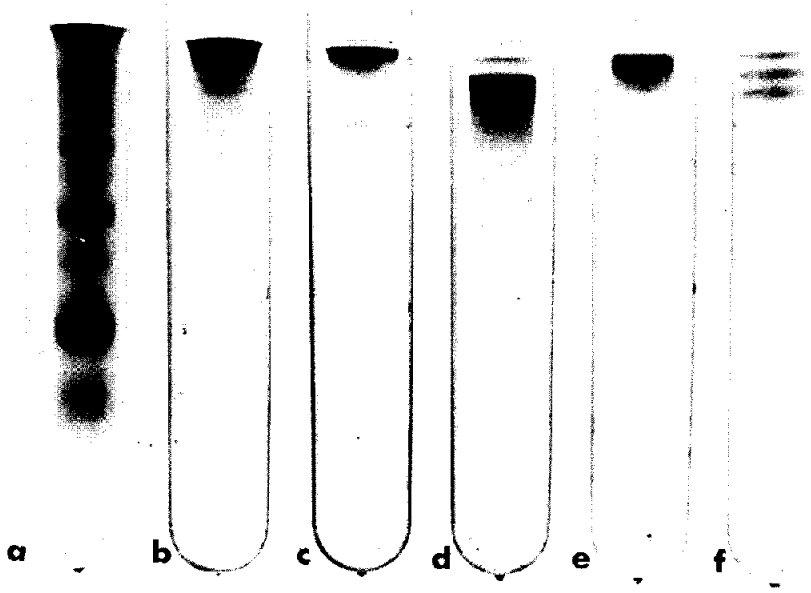

Fig.3. Polyacrylamide disc gel electrophoresis pH 8.9 (tubes a–e) and gel isoelectric focusing patterns (tube f) obtained with *Tridacna maxima* haemolymph and immunoadsorbent-purified *Tridacnin*. (a) *T. maxima* haemolymph 128 μg, 7% gel. (b) *Tridacnin* 49 μg, 7% gel. (c) *Tridacnin* 103 μg, 7% gel. (d and e) *Tridacnin* 34 μg, 5% gel. f. *Tridacnin* 20 μg, 4.6% acrylamide, 3% ampholytes pH 3–10. Gels a, b, d, and f were stained with Coomassie blue, gels c and e with amido black.

diffusion while the crude extract revealed at least 5 or 6 bands. The precipitin line obtained with the purified Tridacnin fused completely with only one of the precipitin lines present in front of the wells containing unfractionated haemolymph. On IEP at pH 8.3 a single arc was observed in the α-region when whole haemolymph was used with *L. stagnalis* galactan (fig.2a) and when the immunoadsorbent-purified component was used with rabbit anti-*T. maxima* haemolymph (fig.2c). At least 5 lines were visible when haemolymph was examined with the same antiserum B2 (fig.2b). Undiluted antiserum B1 reacted with the Tridacnin present in haemolymph but this antiserum produced only one other precipitin arc (fig.2a).

3.3. *Gel electrophoresis and gel IEF*

Disc gel electrophoresis in 7% acrylamide gels at pH 8.9 showed that the purified Tridacnin hardly moved into the gel. Protein staining revealed a heavily stained area at the top of the gel with a very lightly stained diffuse band approx. 1–1.5 cm from the top of the gel (fig.3b,c). When gels were stained with Coomassie blue, two thin bands close together were visible near the top of the gel (fig.3b). When 5% gels were used the stained materials migrated a little further into the gels revealing one narrow band and one broad, heavily stained area (fig.3d,e). The latter probably consists of more than one band, very close together. By contrast, whole haemolymph showed at least 10 bands spaced at intervals from the anode to the cathode end of the gel (fig.3a). The IEF experiments with affinity chromatography-purified Tridacnin clearly demonstrated 3 bands at the anode end of the gel in the pH range 3.9–4.3 (fig.3f).

3.4. *Conclusions*

Gel IEF and gel electrophoresis at pH 8.9 demonstrated that more than one component was present in the immunoadsorbent-purified Tridacnin preparation. This result is similar to findings of Hayes and Goldstein [12] and Pereira and Kabat [13] with the *Bandeiraea simplicifolia* and *Lotus tetragonolobus* lectins respectively. Other investigators have reported similar findings with purified lectins (see [12] for references) and possible reasons for this have been discussed by Hayes and Goldstein [12] and Hammarström and Kabat [14]. Preparative IEF experiments in free solution are now in progress so that the isoelectric point of each component can be established with greater accuracy and so that we can isolate sufficient of each of the 3 *Tridacna* components for further specificity and physico-chemical examinations.

The large volumes of haemolymph which can be obtained from individual clams, the high yields of Tridacnin which can be isolated and its ability to react with a large number of saccharide-containing macromolecules [1–4], makes Tridacnin a valuable reagent for use in a variety of preparative, analytical and cellular studies. The specificity of the Tridacnin for some natural galactans, glycopeptides and polysaccharides [1,3,4] indicates that it will find wide use in the immobilized form as an immunoadsorbent. In addition, we found (Balso and Uhlenbruck, in preparation) that Tridacnin agglutinates certain tumor cells and different sorts of lymphocytes, so that it can be used as an excellent marker.

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