Purification and Characterization of a Galactan-Reactive Agglutinin from the Clam *Tridacna maxima* (Röding) and a Study of its Combining Site

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1. A β -galactosyl-binding lectin was purified from the haemolymph of the clam *Tridacna maxima* by affinity chromatography using poly(1-vinyl-2-pyrrolidone) larch galactan, D-galactosamine coupled to epoxy-activated Sepharose or acid-treated Sepharose. Elution with N-acetyl-D-galactosamine or lactose displaced the bound lectin, which appeared homogeneous by sedimentation analysis. On immunoelectrophoresis at pH 8.6 and against rabbit antisera to crude *T. maxima* haemolymph, the lectin gave one precipitin arc in the α -region. 2. On alkaline polyacrylamide disc gels, one lightly stained band and a broad diffuse band were seen close to the cathode. Isoelectric focusing in solution revealed two peaks of pI 4.05 and 4.25 and a shoulder, pI 4.0, whereas at least three bands close together (pI 3.9-4.3) were seen after electrofocusing in gel. 3. The agglutinin is a glycoprotein with a mol.wt. of $470\,300 \pm 20\,000$. Amino acid analysis revealed no methionine and a significant amount of half-cystine residues. 4. *Tridacna* lectin is a metalloprotein requiring Ca^{2+} for its haemagglutinating and precipitating activities. 5. In haemagglutination studies the agglutinin exhibited a broad pH optimum (4.8-10.6). 6. Polysaccharides and glycoproteins with terminal non-reducing β -D-galactosyl residues reacted with the lectin to form precipitates both in gel and in solution. Inhibition experiments showed that N-acetyl-D-galactosamine was the best inhibitor of the agglutinin combining sites, followed by *p*-nitrophenyl β -D-galactoside, methyl β -D-galactoside, D-galactosamine and 6-O- β -D-galactopyranosyl-D-galactopyranose. On a molar basis, N-acetyl-D-galactosamine was 20-fold more active than D-galactose and nearly 10-fold more inhibitory than D-galactosamine. 7. Circular-dichroism studies showed that the lectin contains a relatively high proportion of β -structure. 8. Mercaptoethanol treatment of the agglutinin followed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed subunits with approx. mol.wts. of 10000, 20000 and 40000.

Lectin-like proteins that react with carbohydrate structures and that show precipitating and/or haemagglutinating properties have been found in a number of invertebrate species (Cushing *et al.*, 1963; Acton & Weinheimer, 1974; Gold & Balding, 1975). Interest in invertebrate agglutinins or lectins (Sharon & Lis, 1972) has been sustained for two main reasons. Although invertebrates lack the adaptive immune mechanisms found in vertebrates, the capacity to recognize and distinguish foreignness is well developed throughout the Invertebrata (Hillemann, 1974). The specificity shown by some

invertebrate agglutinins (McKay *et al.*, 1969) and their capacity to bind to foreign cells (Tripp, 1966), has led to speculation that the agglutinins have a role in defence of the host (Acton & Weinheimer, 1974). Burnet (1968) has suggested that the pseudo-immunological capacities of invertebrate agglutinins may have provided the basis from which the vertebrate antibody system evolved. A second major stimulus for interest in invertebrate agglutinins has been the relatively recent recognition of the usefulness of lectins in research and their wide application to many areas of the biomedical sciences (Lis & Sharon, 1977). Lectins mediate a wide range of biological effects (Nicolson, 1974) and, as a consequence of their ability to bind to both free and cell-bound carbohydrate structures, plant and invertebrate agglutinins are being increasingly used as affinity-

Abbreviation used: SDS, sodium dodecyl sulphate.

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chromatography support media for the purification of polysaccharides, peptidoglycans and glycoproteins (Lloyd, 1970; Adair & Kornfeld, 1974; Bridgen *et al.*, 1976; Ross *et al.*, 1976).

Clams of the family Tridacnidae are specialized bivalves that inhabit the shallower waters of coral reefs. *Tridacna maxima* (Röding), the most ubiquitous species of the family, is distributed widely in the Pacific and Indian Oceans within an area ranging from East Africa to Eastern Polynesia (Rosewater, 1965). Haemolymph from *T. maxima* contains a Ca^{2+} -dependent protein, tridacnin, that strongly agglutinates some animal erythrocytes and that precipitates with a number of polysaccharides and glycopeptides (Baldo & Uhlenbruck, 1975a,b; Uhlenbruck *et al.*, 1975). Although tridacnin has been isolated (Baldo & Uhlenbruck, 1975c), characterization of the purified agglutinin and detailed combining-site studies were not carried out.

In the present study we describe three different methods for the purification of tridacnin, together with quantitative precipitin-inhibition studies designed to explore the specificity and size of the lectin-combining sites. Results of electrophoretic, molecular-weight, amino acid- and carbohydrate-composition, circular-dichroism and isoelectric-focusing studies are also presented and, from investigations of the subunit composition of the protein, a tentative model is proposed for the structure of the agglutinin.

Experimental

Materials

Sephacrose 2B, 4B and 6B and epoxy-activated Sepharose 6B were from Pharmacia, Uppsala, Sweden. L-Leucine *N*-carboxyanhydride (lot no. LE 45) was obtained from Miles-Yeda, Rehovoth, Israel. Acrylamide was from Eastman Organic Chemicals, Rochester, U.S.A. and *NN'*-methylene-bisacrylamide, *NNN'*-tetramethylethylenediamine, sodium dodecyl sulphate and ninhydrin from Sigma Chemical Co., MO, U.S.A. Agar (Noble) and Freund's complete adjuvant were obtained from Difco Laboratories, Detroit, MI, U.S.A. Ampholines pH 3.5–5.0 and pH 3.5–10 were supplied by LKB-Produkter A.B., Bromma, Sweden.

Cytochrome *c* (horse heart) and ribonuclease were from Schwartz-Mann, Orangeburg, NY, U.S.A., pepsin (from pig stomach mucosa; three-times-crystallized) from Koch-Light Laboratories, Colnbrook, Bucks., U.K., lysozyme (hen's egg white) and bovine serum albumin from Sigma Chemical Co. and β -galactosidase from Worthington Biochemical Corp., Freehold, NJ, U.S.A.

The disaccharide 6-*O*- β -D-galactopyranosyl-D-galactopyranose was synthesized as described below

under 'Methods'. Methyl α -D-galactopyranoside monohydrate, methyl β -D-galactopyranoside and 6-*O*-methyl D-galactopyranose were purchased from Koch-Light. All other monosaccharides, oligosaccharides and glycosides were from Sigma. Polysaccharides and glycopeptides were prepared or obtained as previously described (Baldo & Uhlenbruck, 1973, 1975b; Uhlenbruck *et al.*, 1975; Eichmann *et al.*, 1976; Baldo *et al.*, 1977).

Human erythrocytes were supplied by the Red Cross Blood Transfusion Service, Perth, Western Australia, Australia. Erythrocytes from other species were obtained fresh as needed by bleeding animals directly into Alsever's solution.

Methods

Haemolymph. Clams obtained from Israel were opened by immersion in CO_2 -treated sea water and haemolymph was collected after careful dissection of the clams with a knife. Before being used in purification experiments, haemolymph was dialysed extensively against water and freeze-dried.

Purification of tridacnin. Tridacnin was isolated from previously dialysed and freeze-dried haemolymph by affinity chromatography using the following support media and procedures.

1. Polyleucyl-arabinogalactan. Larch arabinogalactan (Serva, Heidelberg, Germany) was dissolved in 0.07M- NaHCO_3 at 4°C and co-polymerized with an equal quantity of the *N*-carboxyanhydride of L-leucine (Tsuyuki *et al.*, 1956; Kaplan & Kabat, 1966). The precipitate was recovered by centrifugation at 30000g for 40min and washed twice with 0.07M- NaHCO_3 and twice with water. For column chromatography, five parts of polyleucyl-arabinogalactan was mixed with two parts of Sepharose 4B in 0.9% NaCl containing 0.01M- Ca^{2+} (saline/ Ca^{2+}), left at 4°C for 24h and then poured into small columns (1cm $\times$ 10cm). After washing with saline/ Ca^{2+} , *T. maxima* haemolymph extract (10–20mg in 1ml of saline/ Ca^{2+}) was added to the column and eluted with the same vehicle. Fractions were collected and examined for A_{280} and for haemagglutinating activity against human blood-group-O erythrocytes. Bound agglutinins were eluted from the column by adding 0.025M-*N*-acetyl-D-galactosamine in saline/ Ca^{2+} . Fractions showing haemagglutinating activity and/or absorbance at 280nm were pooled, dialysed against frequent changes of water, concentrated by ultrafiltration and freeze-dried.

2. Acid-treated Sepharose. Sepharose 2B, 4B or 6B was treated with 0.2M-HCl at 50°C for 2h as described by Ersson *et al.* (1973). The acid-treated beads were packed into a column, washed with saline/ Ca^{2+} and used as an affinity support medium for the preparation of tridacnin. Elution of bound lectin was effected with 0.1M-lactose or 0.025M-

N-acetyl-D-galactosamine, and recovery was carried out as described above.

3. D-Galactosamine hydrochloride coupled to epoxy-activated Sepharose. Application of this method is based on the work of Sundberg & Porath (1974). Epoxy-activated Sepharose 6B (lot no. 5084; 3g) was swollen in water for 30min and then washed on a sintered-glass filter with 350ml of water. D-Galactosamine hydrochloride (100mg) was dissolved in 5ml of water and the pH adjusted to 13 by the addition of 2.5M-NaOH. The alkaline sugar solution was added to the washed epoxy-activated Sepharose and the suspension gently shaken in a 30°C water bath for 20h. After centrifugation the supernatant was retained and freeze-dried while the coupled Sepharose was washed successively with water, 0.1M-borate buffer, pH8, and 0.1M-acetate buffer, pH4. The amount of ligand bound was estimated to be approx. 5 μ equiv./ml of sedimented gel. Excess free activated groups were blocked by the addition of 10ml of 1M-ethanolamine, pH9, and leaving the mixture at room temperature for 4h. Before use in affinity-chromatography experiments the D-galactosamine-Sepharose conjugate was washed extensively with water followed by saline/Ca²⁺. Adsorption, elution and recovery of tridacnin was carried out as described above.

Immunological methods. Haemagglutination and haemagglutination-inhibition methods using 2% erythrocyte suspensions and Perspex haemagglutination trays have been described (Baldo & Boettcher, 1970; Baldo, 1972). Solutions of sugars, glycosides, polysaccharides and glycoproteins used in inhibition experiments were prepared by the use of techniques applicable to quantitative analytical procedures.

Immunodiffusion in gel was done in Petri dishes with 1.5% agar containing 0.9% NaCl, 0.2% NaN₃ and 0.001M-Ca²⁺. Immuno-electrophoresis was carried out by using the procedures of Scheidegger (1955) as described by Baldo (1973). Gels containing 1% agar, 0.2% azide and 0.001M-Ca²⁺ were made up in 0.05M-barbital buffer, pH8.6. Immunodiffusion and immuno-electrophoresis gels were washed with saline/Ca²⁺ before staining with Amido Black.

Antisera to tridacnin were produced in New Zealand White rabbits as previously described (Baldo & Uhlenbruck, 1975c).

Quantitative precipitin and precipitation-inhibition methods (Kabat, 1961) were carried out on a micro scale as previously described (Baldo & Uhlenbruck, 1975a). Nitrogen in the washed precipitates was determined by using ninhydrin (Schiffman *et al.*, 1964).

Polyacrylamide-gel disc electrophoresis. Electrophoresis was performed in 5 and 7% (w/v) acrylamide-gel columns (10cm $\times$ 0.5cm) at pH8.9 and 5mA/gel by using the materials and procedures described by Davis & Ornstein (1968). Polymerization of the gels

was initiated with ammonium persulphate. Electrophoresis was continued until the Bromophenol Blue dye marker had reached within approx. 0.5cm of the ends of the gels. Gels were fixed in 7% acetic acid and stained with Coomassie Brilliant Blue or Amido Black.

SDS/polyacrylamide-gel electrophoresis. This was carried out by the method of Weber & Osborn (1969) with 10% (w/v) polyacrylamide gels in 0.1M-sodium phosphate buffer containing 0.1% SDS. Cytochrome *c* (mol.wt. 11700), ribonuclease (13700), lysozyme (14300), β -lactoglobulin (18400), pepsin (35000), bovine serum albumin (68000) and β -galactosidase (130000) were used as molecular-weight standards. The protein standards were treated as described by Weber & Osborn (1969). Tridacnin (2mg/ml) was dissolved in 0.5ml of 0.01M-sodium phosphate buffer, pH7, containing 1% SDS and 0.5M-2-mercaptoethanol. The solution was incubated at 37°C for 2½h and then dialysed overnight against 500ml of 0.1M-sodium phosphate buffer, pH7, containing 0.1% SDS and 0.1% 2-mercaptoethanol. Electrophoresis was performed at a constant current of 8mA/gel. Proteins were stained with Coomassie Brilliant Blue.

Isoelectric focusing. Isoelectric focusing in free solution was carried out as described by Vesterberg & Svensson (1966) and as set out in the LKB 8100 Ampholine Electrofocusing Equipment Instruction Manual. A 110ml Ampholine column LKB 8101 was used, together with a final concentration of 2% carrier ampholytes (pH range 3.5–5.0). Tridacnin was mixed with the light solution and the sucrose density gradient prepared with the aid of a two-chamber gradient mixer (Pharmacia). Electrofocusing was carried out at 4°C for 6 days at an initial voltage of 300V and a current of 2mA. The final current was 1mA at 300V. Fractions were collected with the aid of an LKB 7000 Ultrarac fraction collector and were each examined for *A*₂₈₀ and pH. Pooled fractions were dialysed against water and freeze-dried.

Electrofocusing in gel was carried out as described by Eder (1972) with 4.6% (w/v) acrylamide and 3% ampholytes (pH3–10). A current of 1mA/tube and a maximum of 400V for 7h was used with H₂SO₄ (0.05M) and NaOH (0.03M) electrolyte solutions. Gels were washed repeatedly with 12.5% trichloroacetic acid before staining with Coomassie Brilliant Blue.

Amino acid analysis. Hydrolyses were carried out in 6M-HCl/1% phenol *in vacuo* for 24, 48 and 72h in duplicate, and corrections were applied for serine and threonine. Analyses were made with a Beckman model 120B amino acid analyser with sodium citrate elution for the short (pH5.28, 0.35M) and long (pH3.28, 0.2M; pH4.25, 0.2M) columns. Half-cystine was determined after performic acid oxidation by the method of Moore (1963). Tryptophan was not

determined, but was estimated from the absorption coefficient.

Carbohydrate content. The procedure used was the same as that described by Newman *et al.* (1976). Individual hexoses and hexosamines were determined after hydrolysis by g.l.c. Lectin (1 mg) was hydrolysed in 3M-HCl for 4 h at 100°C, erythritol (30 µg) was added as internal standard and the solution neutralized by the addition of Ag₂CO₃. After centrifugation and washing with water, the mixture was freeze-dried, re-acetylated with acetic anhydride and then trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane. Portions were chromatographed on a Hewlett-Packard 7620A Research Chromatograph on columns of coiled glass packed either with 3.1% silicone gum rubber SE-30 on Gas-Chrom Q (80–100 mesh) or with 3% OV-17 on Gas-Chrom Q (Serva Ltd.) with a temperature program from 125 to 230°C (2°C/min) with a N₂ flow rate of 45 ml/min. Monosaccharide amounts were calculated by measuring peak heights.

Absorption coefficient. Duplicate samples of tridacnin were exhaustively dialysed against Tris/HCl buffer, pH 7.0 (0.05M-Tris/0.10M-KCl). The protein concentration was determined with a Brice Phoenix differential refractometer by using a value of 1.8×10^{-3} dl/g for the specific refractive increment. The absorption of the same solution was measured at 280 nm with a Zeiss PMQII spectrophotometer.

Molecular weight. Sedimentation experiments were carried out in a Spinco model E analytical ultracentrifuge. Sedimentation coefficients were corrected for solvent viscosity and temperature in the usual way (Svedberg & Pedersen, 1940). Sedimentation-equilibrium experiments were conducted by the meniscus-depletion method of Yphantis (1964), Kel F polymer oil being used as the inert base fluid in the standard six-channel cell. The time for the attainment of equilibrium was shortened by using the overspeed technique of Hexner *et al.* (1961). The final angular velocity was 17150 rev./min and the concentration distributions were independent of time after 8 h. The partial specific volume of the protein was assumed to be 0.73 ml/g. The buffer used was Tris/HCl, pH 7.5 (0.05M-Tris/0.1M-KCl).

Circular dichroism. Measurements were made with a Jasco circular dichrometer modified from a J5 to the equivalent of J20 configuration. Cell path lengths were 0.1 mm in the far u.v. and 10 mm in the near u.v. The far u.v. spectrum was compared with a set of theoretical spectra computed by the method of Saxena & Wetlaufer (1971).

Synthesis of 6-O-β-D-galactopyranosyl-D-galactose. 1,2:3,4-Di-O-isopropylidene-α-D-galactose (520 mg), HgBr₂ (30 mg), yellow HgO (480 mg) (Schroeder & Green, 1966) and powdered CaSO₄ (1 g) were stirred in dichloromethane (6 ml, 0.5 h). Tetra-O-acetyl-α-D-galactopyranosyl bromide (910 mg) in dichloro-

methane (4 ml) was added dropwise with stirring (1 h), stirring continued (2 h), and the mixture filtered. The filtrate was then washed with KI solution (20%), dried (anhydrous Na₂SO₄) and evaporated to yield a foam that was chromatographed on silica gel [diethyl ether/hexane, 1:1 (v/v) as eluent] to give 6-O-(tetra-O-acetyl-β-D-galactopyranosyl)-1,2:3,4-di-O-isopropylidene-α-D-galactose as a foam (870 mg; 74%), $[\alpha]_D^{20} -44^\circ$ [*c* 0.9 in chloroform; literature value (Kochetkov *et al.*, 1967) -47°].

This disaccharide derivative (590 mg) was dissolved in methanol (1 ml), water (0.5 ml) and triethylamine (0.5 ml) and kept (15 h, 0°C). Evaporation yielded the impure 6-O-β-D-galactopyranosyl-1,2:3,4-di-O-isopropylidene-α-D-galactose as a foam (400 mg).

This foam (400 mg) and H₂SO₄ (Kochetkov *et al.*, 1967) (2 ml of 0.1M) were stirred (80°C, 1.5 h), the solution neutralized with BaCO₃ and filtered. Evaporation yielded 6-O-β-D-galactopyranosyl-D-galactose, which crystallized as a white solid (300 mg, 88%), m.p. 120–122°C [methanol/acetone; literature value (Curtis & Jones, 1965) 120–121°C, methanol/butanol], $[\alpha]_D^{29} 29^\circ$ [*c* 0.8 in water; literature value (Curtis & Jones, 1965) 29°].

Results

Purification of tridacnin

Purification of tridacnin was accomplished in essentially two steps: dialysis and freeze-drying of the clam haemolymph and chromatography on an affinity column. Polyleucyl-arabinogalactan, D-galactosamine coupled to epoxy-activated Sepharose and acid-treated Sepharose all proved to be excellent affinity support media for the isolation of the lectin. In each case, *N*-acetyl-D-galactosamine (0.025M) or lactose (0.1M) proved effective in eluting bound agglutinin from the columns. Yields of purified lectin from the different affinity columns were: polyleucyl-arabinogalactan, 26–30%; D-galactosamine coupled to epoxy-activated Sepharose, 26–28%; and acid-treated Sepharose, 21–25%. Higher yields could be obtained from the latter support medium by continuing the elution with sugar after the column had been left overnight in the presence of the sugar. Sepharose that had not been treated with acid proved to be a poor affinity support medium for the isolation of the agglutinin. Yields were low and variable (2.5–10%) depending on the batch and type of Sepharose used. In a typical experiment in which both untreated and acid-treated Sepharose 6B were examined, previously dialysed and freeze-dried haemolymph (10 mg) was added to 1 cm × 24 cm columns of the different support media. The yield of purified agglutinin from the acid-treated Sepharose was 2.4 mg. With untreated Sepharose 6B, however, most of the haemagglutinating activity

Fig. 1. Polyacrylamide-gel disc electrophoresis using *Tridacna maxima* haemolymph extract and tridacnin purified by affinity chromatography on acid-treated Sepharose

(a) Dialysed and freeze-dried *T. maxima* haemolymph (52 µg, 7% gel). (b) Purified tridacnin (34 µg, 7% gel). (c) Purified tridacnin (30 µg, 5% gel). All gels were at pH 8.9. Both (a) and (b) were stained with Amido Black; (c) was stained with Coomassie Brilliant Blue.

passed unimpeded through the column and only 0.3 mg of agglutinin was recovered.

Polyacrylamide-gel disc electrophoresis

In 7% (w/v) polyacrylamide gels at pH 8.9, the isolated agglutinin preparation failed to migrate into the gel and was detected as a heavily stained area on top of the column (Fig. 1b). In 5% acrylamide gels at pH 8.9, however, some migration did occur, and two bands, one broad and diffuse and the other slower and more discrete, were observed (Fig. 1c). Previously dialysed and freeze-dried haemolymph showed at least 13 bands distributed throughout the length of a 7% gel (Fig. 1a).

Immunological examination of the isolated agglutinin

Haemagglutinin studies. The purified agglutinin strongly agglutinated erythrocytes from a number of different vertebrates including man, rabbit, guinea pig, rat, chicken and pigeon. With human blood-group-O erythrocytes, agglutination was observed at a minimum concentration of 0.15–0.3 µg/ml. No agglutination was seen with horse erythrocytes, and sheep and mouse erythrocytes reacted weakly. No haemagglutination occurred in the absence of Ca²⁺.

The effect of pH on tridacnin-induced agglutination of human blood-group-O erythrocytes was

Fig. 2. Effect of pH on the agglutination of human blood-group-O erythrocytes by tridacnin

Haemagglutination was carried out using tridacnin (0.31 mg/ml) in the presence of Ca²⁺ and in the buffers set out in the Results section.

Table 1. Polysaccharides and glycopeptides that precipitate with tridacnin

Galactans from:

- Bovine lung
- Helix pomatia* (roman snail)
- Larix laricina* (larch)
- Lolium multiflorum* (rye grass)
- Lymnaea stagnalis* (fresh-water snail)
- Pomacea urceus* (snail)
- Triticum vulgare* (wheat)
- Pneumococcus* type XIV polysaccharide
- Pig H-blood-group substance
- Human H-blood-group substance
- Bovine erythrocyte mucoid
- Pig amnion mucoid 4.7% AcNeu
- Pig amnion mucoid 6% AcNeu
- Torulopsis groppengresseri* (yeast) galactomannan

investigated over the pH range 3.7–10.6 by using the following buffers: 0.01 M-sodium acetate/acetic acid, pH 3.7–5.4, 0.01 M-sodium cacodylate/HCl, pH 6.0–7.0, 0.01 M-Tris/HCl, pH 7.5–8.8 and 0.01 M-glycine/NaOH, pH 9.0–10.6. All buffers were made up in saline/Ca²⁺. At pH values below 4.0, no haemagglutination occurred, but, as the pH was increased, activity rapidly increased to reach a maximum at pH 4.8. This maximum was maintained over the entire range tested up to pH 10.6 (Fig. 2).

Precipitation studies. Tridacnin precipitated in gels and in solution with a number of galactans and some other polysaccharides and glycopeptides containing terminal D-galactose residues (Table 1). Precipitation occurred only in the presence of Ca²⁺. Removal of Ca²⁺ with EDTA or citrate prevented the formation of precipitates or dissolved them once formed. Polysaccharides and glycopeptides containing terminal

Fig. 3. *Precipitation of some galactans by tridacnin and examination of supernatants*

(a) Immunodiffusion in agar gel. The centre well contained 3.2 mg of tridacnin/ml. Peripheral wells: 1 and 6, purified locust-bean gum (1 mg/ml); 2 and 5, larch galactan (1 mg/ml); 3 and 4, purified guar gum (1 mg/ml). (b) Quantitative precipitin curves of tridacnin (80 μg/tube) with larch (●) and bovine lung (○) galactans. The total volume was 125 μl. (c) Haemagglutination titres against human blood-group-O erythrocytes found in quantitative precipitin supernatants after reaction of tridacnin with the galactans set out in (a) above. Symbols as in (a). Haemagglutination titre in control tubes (0.9% NaCl with 0.01 M-Ca²⁺ and tridacnin only) = 2¹³.

β-D-galactosyl units, for example larch and bovine lung galactans, reacted strongly with the lectin, but biopolymers with terminal α-D-galactosyl residues, for example purified guar and locust-bean gums, did not (Fig. 3a).

Precipitin examinations in saline/Ca²⁺ were carried out to obtain a quantitative assessment of the reactions between the galactans and tridacnin. Both larch and bovine lung galactans reacted readily in solution, producing significant precipitation with as little as 1–5 μg of galactan. With lung galactan, maximum precipitation occurred with more than 60 μg of galactan, but with larch galactan the equivalence point was reached with < 30 μg of material (Fig. 3b). These findings were reflected in the results obtained with supernatants from each precipitin tube. After precipitation with larch galactan, only tube 1 (containing 5 μg of galactan) had haemagglutinins in the supernatant. A more gradual decrease in haemagglutinating activity was seen in supernatants from the tubes containing up to 26 μg of bovine lung galactan (Fig. 3c).

The effect of increasing salt concentration on the reaction between tridacnin and larch galactan was investigated by using the quantitative precipitin technique. In the presence of Ca²⁺ (0.01 M) and using the optimum proportions of tridacnin and larch galactan previously determined in quantitative precipitin studies (see Fig. 3b), no significant differences were found in the amount of precipitate that formed in the presence of from 0.02- to 1.5 M-NaCl.

Immunoelectrophoresis. A single precipitin arc in the α-region resulted when dialysed and freeze-dried *T. maxima* haemolymph (5 mg/ml) was subjected to electrophoresis in 1% agar at pH 8.6 and subsequently allowed to diffuse against larch galactan. When two different rabbit antisera to the dialysed haemolymph were used after electrophoresis, five to seven precipitin arcs were seen. Single precipitin arcs in the α-region were observed when tridacnin purified by affinity chromatography was used in immunoelectrophoresis studies with larch galactan and the rabbit antisera (Fig. 4).

Fig. 4. *Immunoelectrophoretic examination of immuno-adsorbent-purified tridacnin*

The well contained 1.8 mg of tridacnin/ml and the troughs contained: top, larch galactan (1 mg/ml); bottom, rabbit anti-(*Tridacna maxima* haemolymph) (bleed 2, undiluted).

Fig. 5. Inhibition by monosaccharides, galactosides and oligosaccharides of precipitation of larch galactan by tridacnin. Tridacnin (80 μ g) was used with 13.1 μ g of larch galactan/tube in a total volume of 135 μ l. Key to symbols:

① ● <i>N</i> -Acetyl-D-galactosamine	⑨ ■ 2-Deoxy-D-galactose	⑰ ▼ D-Mannose
② ○ <i>p</i> -Nitrophenyl β -D-galactoside	⑩ ⊖ <i>p</i> -Nitrophenyl α -D-galactoside	⑱ ▲ L-Rhamnose
③ ● Methyl β -D-galactoside	⑪ ● Methyl α -D-galactoside	⑲ ■ 6- <i>O</i> -Methyl-D-galactose
④ ● D-Galactosamine hydrochloride	⑫ ◇ Melibiose	⑳ ◆ <i>N</i> -Acetyl-D-mannosamine
⑤ ⊗ 6- <i>O</i> - β -D-Galactopyranosyl-D-galactopyranose	⑬ ◆ Stachyose	㉑ ▽ D-Arabinose
⑥ ● Lactose	⑭ ◆ Raffinose	㉒ □ L-Fucose
⑦ □ D-Talose	⑮ ■ D-Fucose	㉓ + D-Glucosamine hydrochloride
⑧ ○ D-Galactose	⑯ △ L-Arabinose	㉔ * D-Glucose

Immunochemical specificity. Inhibition of the tridacnin-larch galactan precipitin reaction was investigated using monosaccharides, methyl and *p*-nitrophenyl glycosides and some di- and oligosaccharides (Fig. 5). An examination of the inhibitor concentrations required for 50% inhibition of this reaction showed that, of the compounds tested, *N*-acetyl-D-galactosamine was clearly the best inhibitor. On a molar basis, this monosaccharide was approx. 20 times as inhibitory as D-galactose and 9–10 times as inhibitory as D-galactosamine and 6-*O*- β -D-galactopyranosyl-D-galactopyranose. *p*-Nitrophenyl β -D-galactoside was twice as inhibitory as methyl β -D-galactoside and both β -galactosides were markedly better inhibitors than their corresponding α -anomers. Melibiose, raffinose and

stachyose, compounds that each contain terminal α -linked D-galactose, produced 50% inhibition of precipitation at concentrations of 2.8, 4.4 and 5.2 μ mol respectively. Inhibitory concentrations of this order were also obtained with the monosaccharides D-fucose and L-arabinose, both of which have a D-galactose-like configuration. The 50%-inhibitory concentrations of the compounds tested are shown in Table 2.

Physical and chemical analyses

Molecular weight. Tridacnin, prepared by affinity chromatography on acid-treated Sepharose, sedimented as a symmetrical boundary with a sedimentation coefficient ($s_{20,w}$) of 15.0S (concn. 3.5mg/ml).

Table 2. Inhibition of tridacnin-larch galactan precipitin reaction by monosaccharides, galactosides and oligosaccharides

Data obtained from Fig. 6. For conditions of assay, see under 'Methods'.

Inhibitor	Concn. required for 50% inhibition ($\mu\text{mol}/135\ \mu\text{l}$)
N-Acetyl-D-galactosamine	0.046
p-Nitrophenyl β -D-galactoside	0.22
Methyl β -D-galactoside	0.41
D-Galactosamine hydrochloride	0.43
6-O- β -D-Galactopyranosyl-D-galactose	0.46
Lactose	0.70
D-Talose	0.77
D-Galactose	0.94
2-Deoxy-D-galactose	2.05
Melibiose	2.8
D-Fucose	4.3
L-Arabinose	4.3
Raffinose	4.4
Stachyose	5.2
D-Mannose	7.4
L-Rhamnose	8.4
6-O-Methyl-D-galactose	10.8

A small amount (approx. 8%) of fast-moving material (22.1S) was present, but this could be removed by passage through a Sepharose 4B column (90cm $\times$ 1.5 cm). A sedimentation-equilibrium experiment was carried out on tridacnin immediately after it had been eluted from the Sepharose 4B column. Plots of $\log(\text{fringe displacement})$ versus $(\text{radial distance})^2$ were linear, and the mol.wt. was $470\ 300 \pm 20\ 000$ (average for two equilibrium runs, six solutions, in the concentration range 0.09–0.31 mg/ml).

Absorption coefficient. The value of A_{280} was 24.4 ± 0.4 . This value was used for all future physical measurements.

Circular dichroism. The far-u.v. spectrum (Fig. 6) is characterized by a weak minimum at 215 nm indicative of a low α -helix content and a relatively high proportion of β -structure. The experimental data in Fig. 6 are compared with a theoretical spectrum simulated for a molecule containing 10% α -helix, 40% β -structure and 50% random structure by the method of Saxena & Wetlaufer (1971). The near-u.v. spectrum is dominated by a maximum at 265 nm characteristic of tyrosine and/or phenylalanine contributions. The minima of 290 nm and 298 nm are characteristic of the 1L_b and 1L_a transitions of tryptophan respectively (Strickland *et al.*, 1969), although the 290 nm band may also have a contribution from tyrosine.

Amino acid composition. The results of amino acid analyses are summarized in Table 3. We draw attention to the very low methionine content and the significant amount of cyst(e)ine.

Fig. 6. Circular-dichroic spectra of purified tridacnin in the far (a) and near (b) u.v.

Ellipticities $[\theta]$ are expressed on a mean-residue-weight basis. The broken line is the spectrum computed for a molecule containing 10% α -helix, 40% β -structure and 50% 'random' structure by the method of Saxena and Wetlaufer (1971).

Table 3. Amino acid composition of tridacnin. Experimental details are given under 'Methods'. Tryptophan was determined from the absorption coefficient assuming exposure of all tryptophan residues to the solvent.

Amino acid	Composition (mol/100 mol of recovered amino acids)
Lys	8.3
His	5.8
Arg	2.1
$\frac{1}{2}$ Cys	5.0
Asp	12.2
Thr	7.0
Ser	2.6
Glu	12.3
Pro	1.1
Gly	12.1
Ala	9.1
Val	4.5
Met	< 0.1
Ile	2.4
Leu	6.3
Tyr	3.3
Phe	6.0
Trp	5.0

Carbohydrate content. Analyses showed that tridacnin contains L-fucose, D-galactose, D-glucose, D-mannose, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine, plus two unidentified sugars, giving a total carbohydrate content of at least 7% (Table 4).

Isoelectric focusing. When tridacnin was focused in a sucrose gradient in the pH range 3.5–5.0, the absorbance profile of the fractions obtained from the column showed two clearly defined peaks at pH 4.25 and 4.05 and a shoulder at pH 4.0 (Fig. 7a). Isoelectric focusing in gels revealed at least three bands at the anode end of the gel in the pH range 3.9–4.3 (Fig. 7b).

Table 4. *Monosaccharide composition of tridacnin*
Values are an average for two batches. Variation was less than 5% of each value. Sugar analyses were carried out as described under 'Methods'. Calculations of the amounts of monosaccharide (mol/mol of glycoprotein) were based on a glycoprotein mol.wt. of 470300.

Mono-saccharide	Monosaccharide content of glycoprotein		
	(mg/100mg)	(μ mol/100mg)	(mol/mol)
L-Fucose	2.88	17.50	82.3
D-Galactose	1.00	5.52	26.0
D-Glucose	0.87	4.83	22.7
D-Mannose	0.91	5.05	23.7
N-Acetyl-D-galactosamine	0.61	2.75	12.9
N-Acetyl-D-glucosamine	0.66	2.98	14.0

Fig. 7. *Isoelectric focusing of tridacnin*
(a) In solution: electrofocusing was carried out at 4°C for 6 days as described under 'Methods'. Tridacnin (5.1mg) was used with 2% Ampholine (carrier ampholytes, pH3.5–5.0). Fractions (3.5ml) were collected. The solutions used were: cathode solution, ethanolamine 0.4ml, water 14ml, sucrose 12g; anode solution, phosphoric acid 0.1ml, water 10ml; dense solution, Ampholine (pH3.5–5.0) 40% (w/v) 3.6ml, water to 42ml; sucrose 28g dissolved in dense solution; 'light' solution, Ampholine (pH3.5–5.0) 40% (w/v) 1.4ml, water to 60ml. Tridacnin was dissolved in the light solution. (b) Isoelectric focusing of tridacnin (40 μ g) in 4.6% polyacrylamide gel containing 3% ampholytes, pH3–10. The gel was stained with Coomassie Brilliant Blue. For other details see under 'Methods'.

Fig. 8. *Polyacrylamide-gel disc electrophoresis using reduced and alkylated tridacnin*
(a) Tridacnin (120 μ g, 5% gel); (b) tridacnin treated with 2-mercaptoethanol followed by iodoacetamide (130 μ g, 7% gel). For details see under 'Methods'. Gels (pH8.9) were stained with Amido Black.

Subunit study. In preliminary studies aimed at dissociating the tridacnin molecule, the purified lectin was treated with the chelating agent EDTA and with 2-mercaptoethanol. Although tridacnin requires Ca^{2+} for its haemagglutinating and precipitating actions, no change in the number or position of the bands was seen when EDTA-treated lectin was examined on polyacrylamide disc gels at pH8.9. After 2-mercaptoethanol treatment, however, marked changes were observed. Tridacnin (200 μ g) was reacted with 2-mercaptoethanol (0.5M) for 30min and then dialysed, first against 200ml of 0.02M-iodoacetamide for 5h and then against 2 litres of water for 15h. Tridacnin treated in this way gave three and occasionally four bands on 5 or 7% acrylamide gels at pH8.9 (Fig. 8). After reduction with 0.5M-2-mercaptoethanol in the presence of 1% SDS, tridacnin produced three bands on 10% SDS/polyacrylamide gels: two lightly stained bands of mol.wt. 10000–12000 and 40000–42000 and a heavily stained band of mol.wt. 19000.

Discussion

Tridacnin, the agglutinin from *T. maxima* haemolymph, can be easily isolated by simple one-step

affinity-chromatography methods using any one of three different support media. With each method, yields of 25% from dialysed haemolymph are readily obtained. For most of our studies we have used tridacnin isolated by reaction with acid-treated Sepharose. Sepharose (agarose) is a linear polymer consisting of alternate units of D-galactose and 3,6-anhydro-L-galactose. The reaction that readily occurs between tridacnin and acid-treated Sepharose, and the weak reactivity observed with the untreated polymer, suggests that acid treatment of the cross-linked gel exposes an increased number of D-galactose units (hydrolysis of the 3,6-anhydro linkages) that are then available for reaction with the agglutinin [as found by Ersson *et al.* (1973) for *Crotalaria juncea* (sunn-hemp) seed lectin]. This method is simpler and less expensive than using epoxy-activated Sepharose or L-leucine *N*-carboxyanhydride as a polymerizing agent for galactans.

The presence of more than one band after electrophoretic examination of tridacnin on polyacrylamide-gel columns, and the small amount of material with sedimentation coefficient of 22.1S detected in the analytical ultracentrifuge, suggested that the purified tridacnin contained some aggregated material. The appearance of more than one component after isoelectric focusing, both in gel and in free solution, indicates that tridacnin contains 'isoelectins'. This result is similar to findings with some other lectins (Lis *et al.*, 1966; Hayes & Goldstein, 1974; Pereira & Kabat, 1974).

All so-called anti-galactan reagents from plants, micro-organisms, invertebrates and vertebrates combine with D-galactose and with some macromolecules containing terminal, non-reducing D-galactose, but, when considering the exact specificities of the combining sites, the D-galactose-binding agglutinins may be divided into two groups (Baldo, 1977): (1) those in which D-galactose is a better inhibitor of the agglutinin binding sites than *N*-acetyl-D-galactosamine; (2) those in which the reverse is true.

Although tridacnin precipitates with a number of galactans, and the lectin has been described as having anti-galactan specificity (Baldo & Uhlenbruck, 1975*d*; Eichmann *et al.*, 1976; Baldo *et al.*, 1977), *N*-acetyl-D-galactosamine is a better inhibitor of the combining sites than D-galactose. The clear inhibitory superiority of *N*-acetyl-D-galactosamine over D-galactose and D-galactosamine suggests that the acetamido group on C-2 of *N*-acetyl-D-galactosamine is an important contact structure involved in the binding between the sugar and the lectin combining site. Hammarström *et al.* (1977) have shown that the carbonyl oxygen of the acetamido group of *N*-acetyl-D-galactosamine is an important contact group in the reactions between *N*-acetyl-D-galactosamine and the *Helix pomatia* (snail) and soya-bean agglutinin combining sites.

A second feature of the specificity of the tridacnin combining sites that emerges from our inhibition studies is the clear β -anomeric specificity shown by the lectin. Tridacnin is a powerful haemagglutinin and precipitin and, because of its β -anomeric specificity for sugars with a D-galactosyl configuration, the lectin is useful in both the free and matrix-bound state for a variety of studies by immunologists, biochemists and cell biologists. Terminal, non-reducing and sub-terminal β -D-galactosyl units occur widely in Nature on free and cell-bound polysaccharides, peptidoglycans and glycoproteins (Kennedy, 1973; Pazur & Aronson, 1973; Montreuil, 1975; Kornfeld & Kornfeld, 1976). When sub-terminal, D-galactose is often masked by terminal *N*-acetylneuraminic acid (Montreuil, 1975) and in such cases the macromolecules do not react with tridacnin. Exposure of the β -D-galactosyl units with the aid of neuraminidase, however, frequently confers tridacnin-reactivity on the macromolecules (Uhlenbruck *et al.*, 1977, 1978).

Carbohydrate analyses showed that tridacnin, in common with many other plant and invertebrate lectins, is a glycoprotein. Another feature shared with a number of other carbohydrate-binding proteins from plants, invertebrates and vertebrates is the presence of β -structure as the major ordered structure of the *T. maxima* agglutinin [Jirgensons *et al.*, 1970; Harisdangkul *et al.*, 1972; see Lonngren *et al.* (1976) for other references].

After reduction of tridacnin with 2-mercaptoethanol, and polyacrylamide-gel electrophoresis in the presence of SDS, subunits with approx. mol.wts. of 10000, 20000 and 40000 were seen. From analytical-ultracentrifuge studies, tridacnin was found to have a mol.wt. of $470\,300 \pm 20\,000$, but, as the molecule is a glycoprotein, estimates of the molecular weights of the subunits may be incorrect (Reid *et al.*, 1972; Segrest & Jackson, 1972). Even so, it is tempting to speculate that the tridacnin molecule may consist of 12 units each of mol.wt. 40000, and that each unit is divisible into two 20000- or four 10000-mol.wt. subunits. Although this model is convenient for summarizing our present knowledge of the structure of tridacnin, a more precise model must await the isolation and characterization of each of the subunits and data on the number of combining sites on the agglutinin molecule.

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