

Further characterization of the cold agglutinin from the snail *Achatina fulica*

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The cold agglutinin from the albumin gland of the snail *Achatina fulica* was purified to homogeneity by using sheep gastric mucin–Sepharose 4B as affinity column followed by gel filtration on Bio-Gel P-300. The homogeneity was checked by alkaline gel electrophoresis, immunodiffusion and immunoelectrophoresis. The purified cold agglutinin is a glycoprotein of native M_r 220000 consisting of three non-covalently bound subunits of M_r 84000, 74000 and 62000 and having a pI value of 4.5. The predominant amino acids are aspartic acid and glutamic acid (or amides) and serine, which account for 39% of the residues. About 3% of the residues are half-cystine. The lectin is a glycoprotein with about 30.7% carbohydrate, the most abundant sugars being galactose, *N*-acetylgalactosamine and *N*-acetylglucosamine. Mannose, xylose and fucose are also present. The inhibition of agglutination of human umbilical-cord erythrocytes by the cold agglutinin is specific for methyl β -D-galactoside and also for glycolipids present on cord erythrocytes. The c.d. data show only negative ellipticity values in the far-u.v. region for the protein at various concentrations and temperatures and also in the presence of the hapten lactose (at different concentrations), indicating the presence of a random-coil conformation in the agglutinin that varies according to temperature.

INTRODUCTION

Invertebrates are rich sources of various agglutinins, which are carbohydrate-binding proteins (Yeaton, 1981). These have been found in various organs. Of these organs the albumin glands of gastropods have been recognized as a rich source of agglutinins, as at least 134 species of snail have been shown to have agglutinin in this gland (Gold & Balding, 1975). One of the most widely studied of the snail agglutinins is that from the albumin gland of *Helix pomatia*, which preferentially agglutinates human A erythrocytes (Hammarström & Kabat, 1969). We have reported the presence in the albumin gland of the snail *Achatina fulica* of a cold agglutinin that is a lectin (Sarkar *et al.*, 1984). This contrasts with cold agglutinins that are human IgM antibodies having specificity towards blood groups I, i and Pr antigens (Roelcke, 1974). In a previous paper (Sarkar *et al.*, 1984) we reported the purification and partial characterization of the cold agglutinin, which has a specificity for *N*-acetyl-lactosamine groups. The present paper describes the purification of the cold agglutinin by use of a new affinity matrix and further characterization of the agglutinin.

MATERIALS AND METHODS

Materials

All the chemicals were obtained from Sigma Chemical Co. and were of the highest purity available. The glycolipids were a gift from Dr. S. Kundu (Albert Einstein College of Medicine, New York, NY, U.S.A.). *Achatina fulica* snails were obtained from local markets in Calcutta. Sheep gastric mucin, a glycoprotein with blood-group-Ii activity (Wood *et al.*, 1980), was isolated

and purified from sheep stomach lining (Bendich *et al.*, 1946), obtained from the slaughterhouse in Calcutta. An affinity column was prepared by coupling sheep gastric mucin (50 mg) with CNBr-activated Sepharose 4B (20 ml) (Cuatrecasas & Anfinsen, 1971). The amount of sheep gastric mucin coupled (39 mg) was determined by weighing the freeze-dried unbound mucin after exhaustive dialysis against distilled water. After coupling, the gel was washed with phosphate-buffered saline (10 mM-sodium phosphate buffer, pH 7.2, containing 150 mM-NaCl) and kept at 4 °C.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (globulin-free; Sigma Chemical Co. lot 61F-9335) as a standard. The absorbance of agglutinin solution at 280 nm was also measured as a convenient method for estimating protein concentration. $A_{280}^{1\%}$ of purified cold agglutinin was found to be approx. 13.4.

Haemagglutination

Haemagglutination was performed at 10 °C with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, VA, U.S.A.) with 25 μ l loops and 25 μ l of a 2% (v/v) rabbit erythrocyte or 2% (v/v) human umbilical-cord erythrocyte suspension. Haemagglutination titre was determined 1 h after incubation at 10 °C. Inhibitions of haemagglutininations were performed by incubating agglutinin (20 μ g) with serial dilutions of each sugar and glycolipid for 15 min and then mixing the solutions with equal volumes of erythrocytes. The solutions of glycolipids were made by sonicating the glycolipids with a 1:1 (v/v) mixture of phosphate-buffered saline and 10% (w/v) egg phosphatidylcholine.

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Isolation and purification of the cold agglutinin

The cold agglutinin was isolated from the albumin gland of the snail by the procedure described previously (Sarkar *et al.*, 1984). The clear yellowish-green layer having haemagglutinating activity was passed through a sheep gastric mucin-Sephadex 4B column equilibrated with phosphate-buffered saline at 10 °C. After a washing, the bound protein was eluted at 37 °C with phosphate-buffered saline as described previously (Sarkar *et al.*, 1984). The eluate, after concentration, was passed through a Bio-Gel P-300 column (40 cm × 1.5 cm) equilibrated with the same buffer for further purification. Fractions (1.2 ml) were collected and monitored by measurement of absorbance at 280 nm and by haemagglutination assays with rabbit erythrocytes.

Electrophoresis

Disc gel electrophoresis of the purified cold agglutinin was carried out in 7.5% polyacrylamide gels (Davis, 1964) at pH 8.9.

Analytical slab gel electrophoresis was carried out in SDS/10% polyacrylamide as separating gel (pH 8.8) and SDS/3% polyacrylamide as stacking gel (pH 6.8) respectively (Laemmli & Favre, 1973). Dissociation and reduction of the proteins were performed by heating at 100 °C for 10 min in 0.1% SDS with or without 0.1% (v/v) 2-mercaptoethanol or by incubation for 24 h at 37 °C in 8 M-urea containing 1% SDS with or without 0.1% 2-mercaptoethanol. The dissociation, reduction and alkylation with iodoacetamide were performed in accordance with Weber *et al.* (1972). The final solution was used for SDS/polyacrylamide-gel electrophoresis.

Analytical isoelectric focusing was performed in LKB slab-gel isoelectric-focusing apparatus with 5% polyacrylamide containing 2% carrier Pharmalyte (pH 3–10) (Rufo *et al.*, 1982). The gel was fixed in 10% (w/v) trichloroacetic acid containing 5% (w/v) sulphosalicylic acid for 1 h and then washed three times with 10% (w/v) trichloroacetic acid in order to remove excess sulphosalicylic acid. Staining and destaining was then done according to the manufacturer's instructions. The marker proteins used were myoglobin (pI 7.35 and 6.85), trypsin inhibitor (pI 4.55) and α -chymotrypsinogen (pI 9.3).

Gel filtration

About 1.8 mg of protein was passed through a column (50 cm × 2.5 cm) of Sephadex G-200 equilibrated with phosphate-buffered saline containing 5 mM-galactose. The void volume (V_0) of the column was 53 ml. The column was calibrated with thyroglobulin, ferritin, catalase and fructose-bisphosphate aldolase as markers.

Immunodiffusion and immunoelectrophoresis

An antiserum to a crude extract of albumin gland of *Achatina fulica* snails was prepared in rabbits by first injecting a small amount of crude extract subcutaneously on 3 successive days to avoid killing the animal if any toxic substances were present. This was followed by regular injections of the extract with Freund's complete adjuvant into the foot-pads of each rabbit (Kabat *et al.*, 1947). Each rabbit was then injected subcutaneously without adjuvants three times in 1 week with 0.2 ml of diluted crude extract (1:10 dilution) and then given one

injection per week for 3 weeks into the foot-pads with about 3.5 mg of crude lectin extract with Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). They were finally bled after 1 week by cardiac puncture. The serum was tested by immunodiffusion (Ouchterlony, 1948) with 1.5% agar in 0.9% NaCl and by immunoelectrophoresis with 1.5% agar in 50 mM-sodium barbital buffer, pH 8.3 (Graber & Williams, 1953).

Carbohydrate analysis

Two different g.l.c. methods were used for the analysis of neutral sugars, and the amino acid analyser was used for the amino sugars.

The alditol acetate method was carried out as follows. The agglutinin (3 mg) was hydrolysed with 2 M-trifluoroacetic acid in a sealed tube for 2 h at 120 °C (Albersheim *et al.*, 1967). *myo*-Inositol was added as internal standard, and after hydrolysis the acid was removed under vacuum. Trace amounts of acid were removed by co-distillation with water by evaporation *in vacuo*, and the hydrolysate was reduced with NaBH₄ and acetylated with acetic anhydride/pyridine (1:1, v/v) (Sloneker, 1972). G.l.c. was carried out in a metal column (2 m × 6 mm internal diam.) containing 3% ECNSS-M on Gas-Chrom Q in a Hewlett-Packard 5730A gas chromatograph fitted with flame ionization detector.

The trimethylsilyl derivative method was carried out in accordance with the procedure of Chambers & Clamp (1971). The agglutinin (0.5 mg) was methanolysed in 500 μ l of 1 M-HCl in methanol under N₂ at 85 °C for 4 h, with mannitol as internal standard. The methanolysate was then dried under a stream of N₂, and the methyl glycosides were converted into the trimethylsilyl

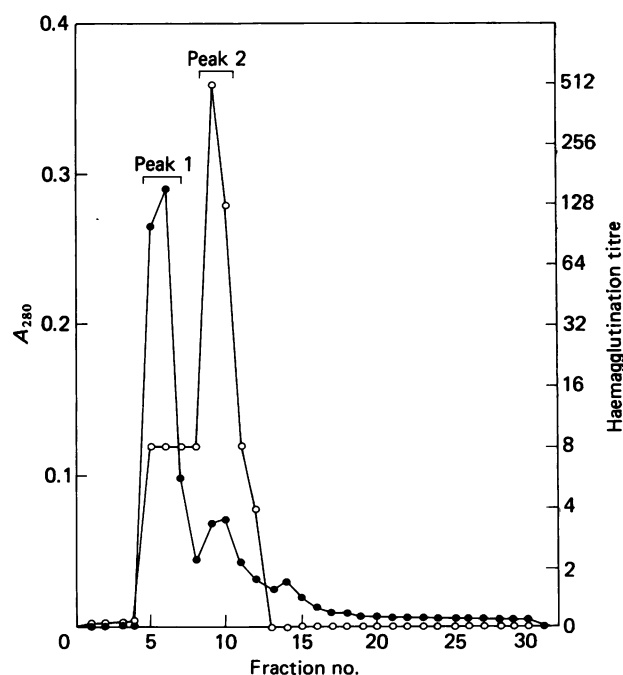


Fig. 1. Purification of *Achatina fulica* cold agglutinin by gel filtration on a Bio-Gel P-300 column

A portion (about 4 mg of protein) of affinity-isolated agglutinin was applied to the column. Fractions collected were assayed for A_{280} (●) and haemagglutinating activities towards rabbit erythrocytes (○). For full experimental details see the text.

Table 1. Purification of cold agglutinin from *Achatina fulica*

Details of the purification procedure are given in the Materials and methods section. The reciprocal of the highest dilution of the agglutinin that produced visible haemagglutination was taken as the titre. The specific activity is defined as the total haemagglutination titre or units per mg of protein. Total activity (units) is the titre \times total volume (ml). Abbreviation: SGM, sheep gastric mucin.

Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude albumin-gland extract	220	154000	1760	0.114	100	1
SGM-Sepharose 4B affinity-column eluate	30	15	960	64	55	576
Bio-Gel P-300 eluate (peak 2)*	7.4	1.8	947	526	53.8	4597

* The fractions 9 and 10 showing high haemagglutinating activity indicated by the peak 2 bar on Fig. 1 were pooled from four batches of gel filtration (4 mg of protein applied in three successive batches and 3 mg in last batch), then concentrated to 7.4 ml and checked for activity and protein.

derivatives by the addition of the silylating reagent (trimethylchlorosilane/hexamethyl disilazane/pyridine, 1:3:9, by vol.) and incubation for 10 min at 25 °C. The trimethylsilyl derivatives were separated with N₂ by a capillary g.l.c. system on a WCOT column (25 m \times 0.32 mm internal diam.) of fused silica with a coating of CP-SILB (Chrompack; Middeburg, The Netherlands). The gas chromatograph was a Varian model 3300 apparatus fitted with flame ionization detection, and the peaks were integrated by an attached Varian 4290 integrator.

Amino sugars were determined on the amino acid analyser after hydrolysis of the glycoprotein at 100 °C for 24 h in 3 M-toluene-4-sulphonic acid under N₂. This procedure gives recoveries in excess of 90% (Allen & Neuberger, 1975).

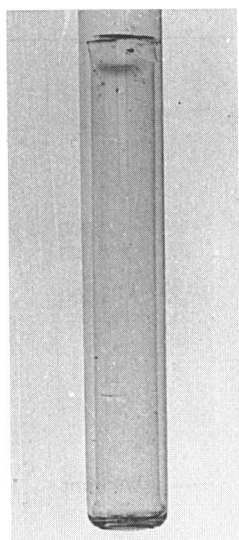


Fig. 2. Disc-gel electrophoresis of purified *Achatina fulica* cold agglutinin

Electrophoresis was carried out in 7.5% polyacrylamide gel. Mobility was from top to bottom. The gel was stained with Coomassie Brilliant Blue. For full experimental details see the text.

Amino acid analysis

Amino acids were analysed on a Locarte amino acid analyser by reaction with ninhydrin after hydrolysis in 3 M-toluene-4-sulphonic acid under N₂ for 24 h at 110 °C, with *p*-fluorophenylalanine as an internal standard. Half-cystine values were obtained from samples that had been previously oxidized with performic acid (Hirs, 1967). For further details of the analytical systems that were used see Allen *et al.* (1976). Tryptophan was determined spectrophotometrically (Edelhoch, 1967).

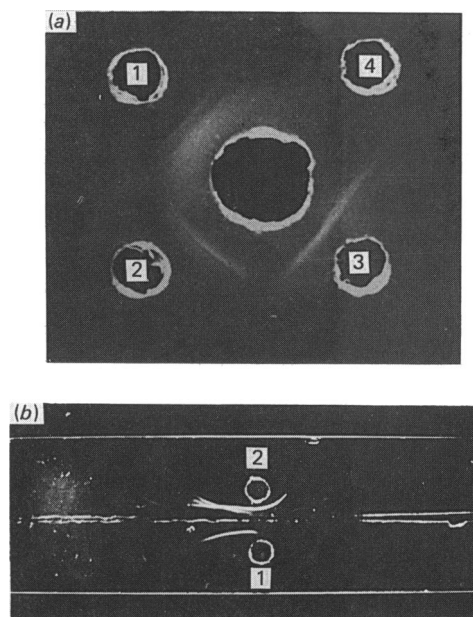


Fig. 3. (a) Immunodiffusion and (b) immunoelectrophoretic pattern of purified *Achatina fulica* cold agglutinin

(a) Immunodiffusion. The centre well contained rabbit antiserum to crude extract of the albumin gland. Well 1, crude extract; well 2, affinity eluate; well 3, peak 2; well 4, phosphate-buffered saline. (b) Immunoelectrophoresis. Well 1, peak 2; well 2, crude extract. For full experimental details see the text.

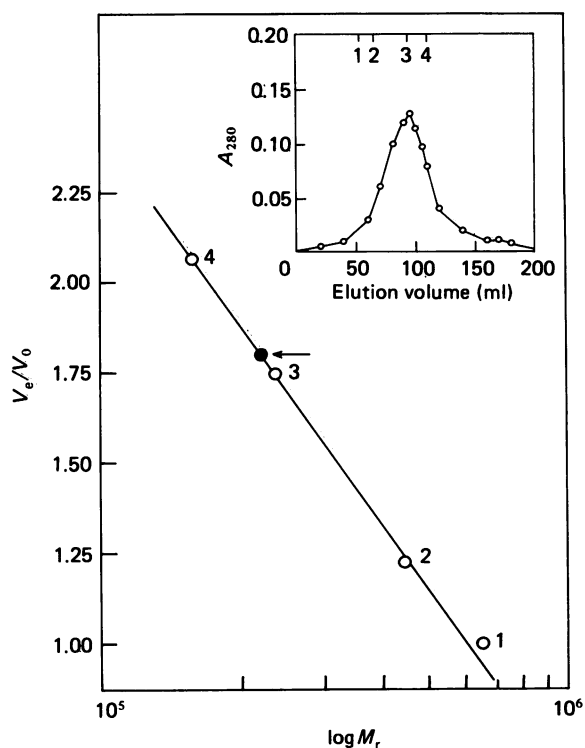


Fig. 4. Determination of the M_r of purified *Achatina fulica* cold agglutinin by gel filtration on a Sephadex G-200 column equilibrated with phosphate-buffered saline containing 5 mM-galactose

The arrow indicates the position of agglutinin. Inset gives the elution profile of the agglutinin. Points represent thyroglobulin (1), ferritin (2), catalase (3) and fructose-bisphosphate aldolase (4).

C.d. studies

C.d. spectra of the cold agglutinin were measured on a JASCO-J-20A recording spectropolarimeter with a cell of 1 ml capacity and 2 mm pathlength. These data are

expressed in terms of mean residue ellipticities $[\theta]$ in degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ by using the relation:

$$[\theta] = \frac{M \cdot \theta}{10 C \cdot l}$$

where M is the mean residue weight taken as 110, θ is the observed ellipticity in degrees, C is the concentration in mol of residue per litre and l is the pathlength in dm. All recordings were performed in phosphate-buffered saline at 25 °C unless otherwise stated. The c.d. spectra were recorded at three different concentrations of protein (0.4, 0.2 and 0.1 mg/ml), and additionally at 10 °C at 0.1 mg of protein/ml. The sugar-agglutinin interactions were studied with lactose at three different concentrations (23.3, 46.6 and 69.9 μM) at 10 °C. The effect of addition of lactose (69.9 μM) to the protein at 25 °C was also recorded. The errors due to noise were ± 2 degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$.

RESULTS

The cold agglutinin from the albumin gland of the snail *Achatina fulica* bound at 10 °C to an affinity column of sheep gastric mucin-Sepharose 4B (1.95 mg of mucin bound/ml of Sepharose 4B). The agglutinin could be eluted by washing the column with phosphate-buffered saline at 37 °C. The affinity-isolated active material was further purified by gel filtration on a Bio-Gel P-300 column, which yielded two major peaks (Fig. 1). Since peak 1 showed a lower agglutinating titre than did peak 2, the material from this second peak was investigated further. The yield of this protein was 1.8 mg from 275 g of albumin gland with approx. 5000-fold purification (Table 1), and it was shown to be homogeneous by alkaline polyacrylamide-gel electrophoresis (Fig. 2). The purified agglutinin was then tested by immunodiffusion and immunoelectrophoresis with rabbit antiserum to crude extract. As shown in Figs. 3(a) and 3(b), the purified agglutinin showed a single band by the Ouchterlony method and a single band moving towards

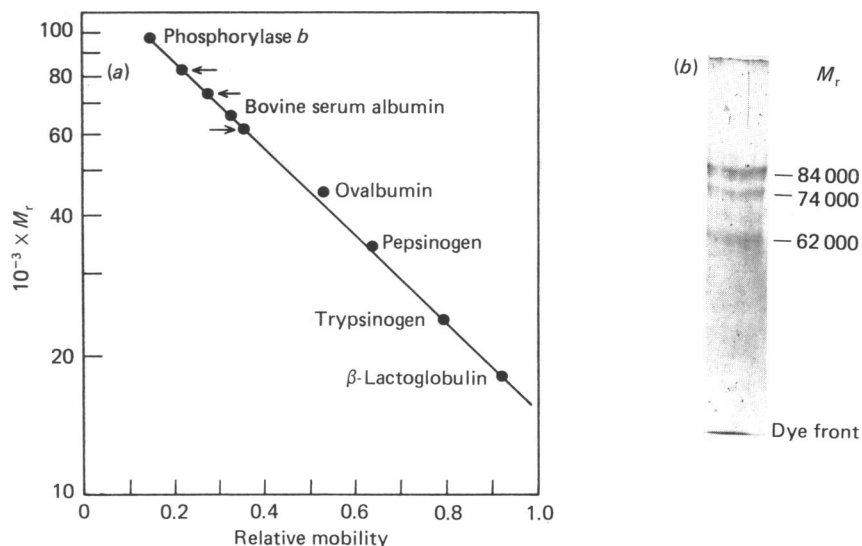


Fig. 5. Determination of the M_r of purified *Achatina fulica* cold agglutinin subunits by SDS/polyacrylamide-gel electrophoresis with or without 2-mercaptoethanol

For experimental details see the text. In (a) the mobilities of subunits of the protein are indicated by arrows. (b) shows the SDS/polyacrylamide-gel-electrophoretic pattern of the cold agglutinin.

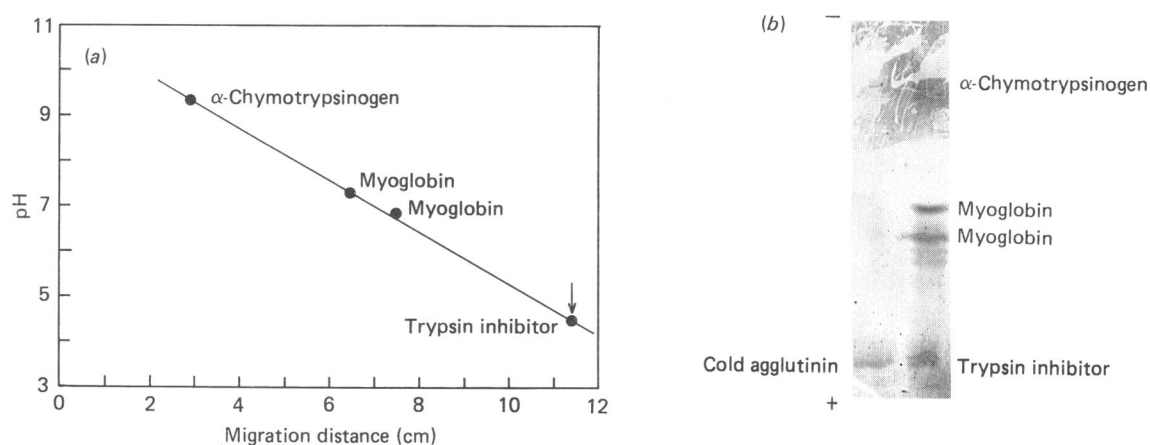


Fig. 6. Determination of the pI of purified *Achatina fulica* cold agglutinin by isoelectric focusing in a pH gradient of 3–10

For experimental details see the text. In (a) the arrow indicates the position of the cold agglutinin; (b) shows the isoelectric-focusing-gel pattern.

Table 2. Amino acid and carbohydrate compositions of the purified *Achatina fulica* cold agglutinin

The values were calculated as molar proportions present in the glycoprotein of M_r 220 000. The values, given to the nearest integer, were derived from an average of three hydrolyses in 3 M-toluene-*p*-sulphonic acid at 110 °C, except for threonine and serine which were extrapolated to zero time, valine and isoleucine, for which the 72 h values were taken, and for cysteine and tryptophan (see footnotes). For further details see the Materials and methods section.

	Composition (mol of residue/mol)
Amino acid	
Aspartic acid	199
Threonine	86
Serine	173
Glutamic acid	184
Proline	112
Glycine	114
Alanine	107
Half-cystine*	43
Valine	53
Methionine	1
Isoleucine	54
Leucine	85
Tyrosine	36
Phenylalanine	59
Histidine	15
Lysine	60
Tryptophan†	4
Arginine	43
Carbohydrate	
D-Galactose‡	176
N-Acetyl-D-glucosamine§	81
N-Acetyl-D-galactosamine§	81
D-Mannose‡	22
D-Xylose‡	10
D-Fucose‡	7

* Determined separately as cysteic acid after hydrolysis of a performic acid-oxidized sample (Hirs, 1967).

† Determined by a spectrophotometric method (Edelhoch, 1967).

‡ Determined by g.l.c. after acid hydrolysis (Albersheim *et al.*, 1967; Chambers & Clamp, 1971).

§ Determined with an amino acid analyser (Allen & Neuberger, 1975).

the anode in immunoelectrophoresis; the antiserum gave two bands with crude extract. From the gel-filtration pattern on a Sephadex G-200 column compared with standards (see the Materials and methods section) the native M_r of the cold agglutinin was found to be 220 000 (Fig. 4). On SDS/polyacrylamide-gel electrophoresis, the purified protein, after being boiled with 1% SDS for 10 min with or without reduction with 2-mercaptoethanol, dissociated into subunits of M_r 84 000, 74 000 and 62 000 (Fig. 5). Furthermore, the agglutinin reduced with 2-mercaptoethanol in 8 M-urea containing 1% SDS or with dithiothreitol in the presence of 6 M-guanidinium chloride and subsequently alkylated with iodoacetic acid gave the same pattern on SDS/polyacrylamide-gel electrophoresis. Isoelectric focusing of the purified protein showed a single band at pH 4.5 (Fig. 6).

The amino acid and carbohydrate compositions of the lectin are given in Table 2. Aspartic acid and glutamic acid together with their amides and serine are the most abundant amino acids, together accounting for 39% of the residues. Half-cystine or cysteine accounts for 3% of the residues, which would allow a considerable degree of disulphide bonding. The agglutinin is heavily glycosylated, with 30.7% (w/w) carbohydrate. Galactose is the most abundant sugar and was detected by both the g.l.c. methods that were used; the smaller amounts of mannose, xylose and fucose were only detected by the sensitive capillary g.l.c. of the trimethylsilyl derivatives.

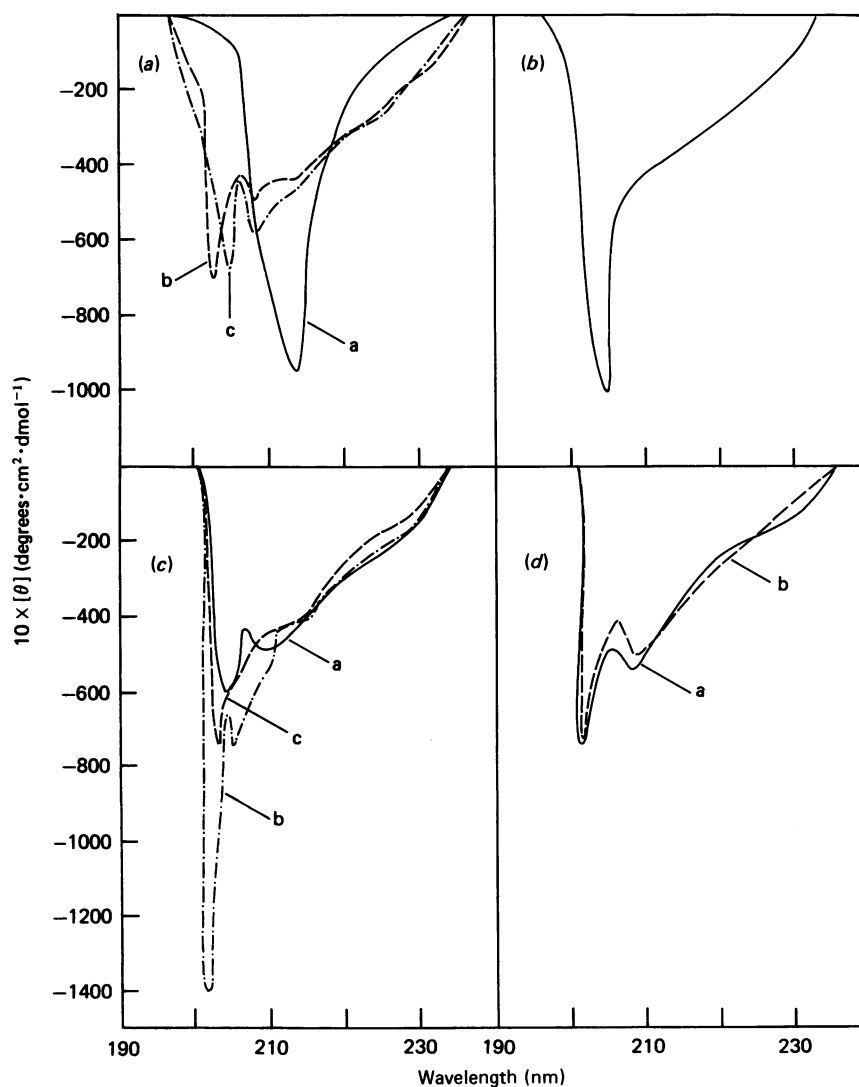
Inhibition of haemagglutination of human umbilical-cord erythrocytes by the purified cold agglutinin was studied with D-galactose and its derivatives, oligosaccharides and some glycolipids of human origin. The results are given in Table 3. The agglutinin is specific for methyl β -D-galactoside, as also reported earlier with rabbit erythrocytes. Among the glycolipids, paragloboside, which has a β -D-galactose residue at the non-reducing end, is the best inhibitor among the mono- and oligo-saccharides tested. This was about twice as active as lactosylceramide and 5 times as active as methyl β -D-galactoside and lactose. The globoside, which has an N-acetyl- β -D-galactose residue at the non-reducing terminal, is also as active as paragloboside. Inhibition with N-acetyl-lactosamine was not tested because of non-availability.

The c.d. spectra of purified cold agglutinin at different concentrations, at different temperatures and in the

Table 3. Inhibition of haemagglutination by purified *Achatina fulica* cold agglutinin by various sugars and glycolipids

Portions (25 μ l) of purified cold agglutinin (20 μ g) were incubated with serially diluted sugars and glycolipids (25 μ l) for 15 min at 10 °C, and then equal volumes (50 μ l) of 2% (v/v) human umbilical-cord erythrocytes were added. The values represent the minimum concentrations (in mg/ml) of sugars and glycolipids required for complete inhibition of agglutination (in 0.1 ml). For details see the Materials and methods section.

Sugar or glycolipid	Quantity giving complete inhibition (mg/ml)
D-Galactose	5.0
Methyl α -D-galactopyranoside	20.0
Methyl β -D-galactopyranoside	0.625
Melibiose (DGal α 1 \rightarrow 6DGlc)	10.0
Lactose (DGal β 1 \rightarrow 4DGlc)	0.625
Globoside (DGalNAc β 1 \rightarrow 3DGal α 1 \rightarrow 4DGal β 1 \rightarrow 4DGlc-Cer)	0.125
Paragloboside (DGal β 1 \rightarrow 4DGlcNAc β 1 \rightarrow 3DGal β 1 \rightarrow 4DGlc-Cer)	0.125
Lactosylceramide (DGal β 1 \rightarrow 4DGlc-Cer)	0.125
Globotriosylceramide (DGal α 1 \rightarrow 4DGal β 1 \rightarrow 4DGlc-Cer)	Tested up to 2 mg

**Fig. 7. C.d. spectrum of purified *Achatina fulica* cold agglutinin in various conditions**

(a) At various concentrations of the agglutinin: 0.4 mg/ml, 0.2 mg/ml and 0.1 mg/ml in phosphate-buffered saline at 25 °C (spectra a, b and c respectively). (b) At a protein concentration of 0.1 mg/ml at 10 °C. (c) Addition of lactose at three concentrations (23.3, 46.6 and 69.9 μ M) to cold agglutinin at 0.1 mg/ml at 10 °C (spectra a, b and c respectively). (d) Addition of lactose (69.9 μ M) at a protein concentration of 0.1 mg/ml at 25 °C (spectrum a); spectrum b is that of the protein at 0.1 mg/ml alone at 25 °C.

presence of hapten inhibitors are presented in Fig. 7. The protein at a concentration of 0.4 mg/ml at 25 °C exhibits a trough at 213 nm of ellipticity value, $-9500 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ (Fig. 7a, spectrum a), but the pattern is changed on dilution of the protein. At the concentration of 0.2 mg/ml the protein exhibits two troughs at 208 and 202 nm, and at 0.1 mg/ml one at 204 nm, with the amplitude of ellipticity bands fluctuating slightly (Fig. 7a, spectra b and c). At 10 °C, only one trough at 205 nm is seen, with an increased ellipticity value of $-10000 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ for the cold agglutinin at a concentration of 0.1 mg/ml (Fig. 7b).

Addition of the hapten inhibitor lactose at different concentrations (23.3, 46.6 and 69.9 μM) causes some interesting changes in the c.d. spectra of the cold agglutinin (0.1 mg/ml) at 10 °C (Fig. 7c). With 23.3 μM -lactose two troughs are seen at 208 and 204 nm instead of one at 205 nm for the free protein (Fig. 7c, spectrum a). With 46.6 μM -lactose also two troughs are seen, at 205 and 202 nm (Fig. 7c, spectrum b), but with 69.9 μM -lactose only a single band like that of free protein is seen but with a shift to 203 nm and a reduced ellipticity value of $-7390 \text{ degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ (Fig. 7c, spectrum c). In contrast, at 25 °C the presence of 69.9 μM -lactose causes very little change in the c.d. spectra of the cold agglutinin (0.1 mg/ml), as shown in Fig. 7(d). The glycoprotein showed no significant c.d. spectrum in the aromatic region (250–310 nm).

DISCUSSION

In the work described in the present paper the cold agglutinin from the albumin gland of the snail *Achatina fulica* is purified to homogeneity by a new procedure. Instead of asialofetuin, sheep gastric mucin (having *N*-acetyl-lactosamine at the non-reducing end) coupled with Sepharose 4B is used as an affinity matrix followed by gel filtration on Bio-Gel P-300 rather than ion-exchange chromatography. On gel filtration, the affinity-purified sample separates into two major peaks. The agglutinin is recovered from peak 2 (Fig. 1). The new purification procedure gave almost the same degree of purification and recovery of the protein (Table 1) as reported previously (Sarkar *et al.*, 1984) and provides a relatively simple and inexpensive method for the isolation of the protein. The protein of peak 1 of Fig. 1 was not further studied in the present work. It may be a polymer of cold agglutinin isolated from peak 2 or a different protein.

The purified cold agglutinin is demonstrated to be homogeneous by a variety of techniques. It is a glycoprotein of native M_r 220000. The molecule is composed of three non-covalently bound non-identical subunits. The 21 disulphide bonds as obtained from about 43 mol of half-cystine (from amino acid analysis) are not involved in subunit interactions. The low pI (4.5) is in agreement with the data from the amino acid analysis if one assumes that the values for aspartate and glutamate are due to the free acids rather than their amides in the intact protein. The agglutinin has a very high carbohydrate content, with galactose as the most abundant sugar; the presence of fucose and xylose in the carbohydrate structures may mean that this glycoprotein resembles the *Helix pomatia* α -haemocyanin (Van Kuik *et al.*, 1985), which has these sugars attached to an Asn-GlcNAc₂Man₃ core structure. As far as we are aware

this is the first report of a snail lectin that contains fucose and xylose.

In our previous paper (Sarkar *et al.*, 1984) the specificity of the agglutinin was already reported to be β -D-galactose-specific, and the most effective inhibitor was *N*-acetyl-lactosamine. In the present work we have used human umbilical-cord erythrocytes instead of rabbit erythrocytes for inhibition studies. With these cells we found that methyl β -D-galactoside was a potent inhibitor. Certain glycolipids were also found to be inhibitors. Globoside and paragloboside, having *N*-acetyl- β -D-galactose and β -D-galactose respectively at the non-reducing end, are the most potent inhibitors, whereas globotriosylceramide, having α -D-galactose at the non-reducing end, is inactive. This result is in good agreement with the earlier report (Karol *et al.*, 1980) that umbilical-cord erythrocytes contain large amounts of paragloboside and globoside but no globotriosylceramide. It is surprising that lactosylceramide is a better inhibitor than lactose. This is probably because of the micellar structure of the glycolipid, which results in the presentation of lactose moieties as a multiple number of agglutinin-binding sites (Fukuda, 1985). Thus the present cold agglutinin is specific not only for *N*-acetyl-lactosamine but also for the glycolipids present on the umbilical-cord erythrocytes.

The c.d. spectrum of the cold agglutinin exhibits a negative ellipticity value in the far-u.v. region. As it is in this region of the spectrum where the peptide chromophore absorbs, the data indicate the presence of mainly random coil in the agglutinin. The spectrum shows no evidence for α -helix content or any appreciable amount of β -sheet in the cold agglutinin. Usually lectins or agglutinins contain a large amount of β -structure (Pflumm *et al.*, 1971; Pèrè *et al.*, 1975; Lonngreu *et al.*, 1976), exceptions being wheat-germ agglutinin, which has only 10% β -structure (Thomas *et al.*, 1977), and potato lectin, which shows no evidence of this structure (Van Holst *et al.*, 1986).

The cold agglutinin shows a change in conformation at 10 °C from that at 25 °C, though both have spectra that could be interpreted as showing the random-coil structure with negative bands in the far-u.v. region. The c.d. spectra also indicate that the conformation of the protein changes in the presence of its inhibitor lactose at 10 °C (Figs. 7b and 7c), but that there is no such change at 25 °C (Fig. 7d). These spectra indicate strongly that the cold agglutinin changes its conformation when the temperature is changed from 25 °C to 10 °C and that the conformation acquired at 10 °C is essential for the binding of the sugar residues.

This unique cold agglutinin is not only important for its cold-agglutinating property, but also due to its specificity towards human umbilical-cord erythrocytes, on which, other than paragloboside and globoside, an oligosaccharide of *N*-acetyl-lactosamine, known as i antigen, is expressed (Marsh, 1961). The cold agglutinin of human origin, specific for Ii antigens, has been reported to be a powerful reagent in studies of erythrocyte differentiation and maturation, and in detecting antigenic changes in neoplastic epithelial cells and erythrocytes and leucocytes associated with haematological disorders (Feizi, 1981). The present cold agglutinin, having similarity with cold agglutinin of human origin, could replace the use of anti-I and anti-i cold agglutinin.

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