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Isolation and Partial Characterization of *N*-Acetyl-D-Galactosamine-Binding Lectins from *Epiphragmophora trenquelleonis* Snail

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A human blood type A hemagglutinating activity was detected in albumin gland extracts of *Epiphragmophora trenquelleonis* snail separated by GalNAc-agarose affinity chromatography, of which two *N*-acetyl-D-galactosamine-binding lectins in the extracts were ETL1 was displaced from the affinity column with 1 mM GalNAc, and ETL2 with 20 mM GalNAc. Both lectins agglutinated specifically human blood type A and AB erythrocytes, but not type B and O erythrocytes. Gel filtration chromatography gave a native molecular weight of about 59 kDa for ETL1 and about 54 kDa for ETL2. On SDS-PAGE under nonreducing conditions, ETL1 showed two protein subunits of about 29 and 27 kDa, while ETL2 showed three protein subunits of about 27, 24, and 22 kDa. On SDS-PAGE under reducing conditions, both lectins showed four protein subunits of 17, 16, 12, and 11 kDa. By Western blot analyses developed with biotin-labeled lectins, *N*-linked oligosaccharides were detected in the 17- and 16-kDa protein subunits of ETL1 and ETL2, and in the 12-kDa protein subunit of ETL2. *O*-linked oligosaccharides were detected only in the 11-kDa protein subunit of ETL1 and ETL2. On isoelectric focusing both lectins exhibited microheterogeneity: ETL1 focused as three protein bands with pIs in the range of 5.6-6.0, while ETL2 focused as four protein bands with pIs in the range of 6.8-7.4. We suggest that native ETL1 and ETL2 are glycoprotein complexes with molecular weights of 59-54 kDa, composed of two 29-22-kDa nonreduced protein subunits held together by noncovalent hydrophobic interactions. Each of the nonreduced protein subunits seems to be composed of two 17-11-kDa reduced protein subunits held together by interchain disulfide linkages. The main differences between ETL1 and ETL2 could be due to different posttranslational modifications or to the relative contribution of one or more of their protein subunits.

Key words: albumin gland, *Epiphragmophora trenquelleonis* snail, invertebrate lectins, *N*-acetyl-D-galactosamine-binding lectins, snail.

Lectins are carbohydrate-binding proteins or glycoproteins of nonimmune origin, which are able to agglutinate cells and precipitate polysaccharides and glycoconjugates (*1, 2*). They have been described in plants, microorganisms, invertebrates, and vertebrates (*3*). Lectins have been used in human blood typing and in the characterization of the oligosaccharide structures of glycoconjugates present in many cell types (*4, 5*). In particular, *N*-acetyl-D-galactosamine-binding lectins, which agglutinate human blood type A erythrocytes, have been purified and characterized from plants such as *Dolichos biflorus* (*6, 7*) and snails such as *Helix pomatia* (*8, 9*), *Helix hortensis* (*10*), and *Otala lactea* (*11*). In this study we report the isolation and some biochemical properties of two *N*-acetyl-D-galactosamine-binding lectins from the albumin gland of *Epiphragmophora trenquelleonis* snail.

MATERIALS AND METHODS

Materials—GalNAc-agarose, peroxidase-labeled streptavidin, PVP, sugars, and trypsin were from Sigma (St. Louis, MO, USA). Isoelectric focusing and molecular weight standards, and SDS-PAGE reagents were from BioRad (Richmond, CA, USA). A, B, and H blood group-specific substances were from Athens Laboratories (Tampa, FL, USA). Biotin-labeled lectins were from Vector Laboratories (Burlingame, CA, USA). Centricon 10 M tubes were from Amicon (Danvers, MA, USA). Falcon microtiter U plates were from Becton Dickinson (Oxnard, CA, USA). All other reagents used were of analytical grade.

Lectin Isolation and Purification Procedures—(i) PBS extraction: E. trenquelleonis snails were harvested from Córdoba's hills (Argentina). Albumin glands were dissected and homogenized at 4°C in an Ultraturrax apparatus with 5 volumes of PBS (125 mM NaCl, 25 mM Na_2HPO_4/

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Abbreviations: Con A, concanavalin A agglutinin; DSA, *Datura stramonium* agglutinin; ETL1, *Epiphragmophora trenquelleonis* lectin 1; ETL2, *E. trenquelleonis* lectin 2; GalNAc, *N*-acetyl-D-galactosamine; JA, Jacalin agglutinin; LCA, *Lens culinaris* agglutinin; MAA, *Maackia amurensis* agglutinin; PBS, phosphate buffered saline; PNA, peanut agglutinin; PVP, polyvinylpirrolidone; SNA, *Sambucus nigra agglutinin*.

NaH_2PO_4, pH 7.4). The homogenate was centrifuged at 100,000×*g* for 1 h at 4°C and the supernatant (albumin gland extract) was stored at −20°C until use.

(ii) Affinity chromatography on GalNAc-agarose: Usually, the albumin gland extract (8.0 ml; 4.0 mg/ml of protein) was applied to a GalNAc-agarose column (0.7×4.0 cm) at a flow rate of 15 ml/h. The column was washed with PBS until no absorbance at 280 nm was detected in the effluent; and the adsorbed material was eluted with a step-by-step gradient of 1, 5, 20, and 100 mM GalNAc in PBS. Fractions with hemagglutinating activity were pooled, concentrated using Centricon 10 M and stored at −20°C until use. All procedures were carried out at 4°C.

Trypsin Treatment of Erythrocytes and Hemagglutination Assay—Human blood was collected in 3.8% (w/v) sodium citrate. A 3% (v/v) erythrocyte suspension was treated with 0.1% (w/v) trypsin for 15 min at 37°C, then erythrocytes were washed twice with PBS and suspended at concentrations of 4% (v/v) in PBS. Hemagglutination assays were done as previously described (*12*) using serial 2-fold dilutions of samples (25 μl) in microtiter U plates and both untreated and trypsin-treated human erythrocytes. To analyze the inhibitory effect of sugars and blood group-specific substances on hemagglutinating activity, saline was replaced by the corresponding inhibitor solutions. Titer was defined as the reciprocal of the highest dilution giving a visible hemagglutination. Specific activity was defined as the ratio of the titer to milligrams of protein (titer×mg^{-1}).

Gel Filtration Chromatography—The native molecular weight of *E. trenquelleonis* lectins was determined by gel filtration in a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with PBS containing 20 mM GalNAc to prevent any interaction between the lectins and the matrix. Chromatographic runs were done with a flow rate of 0.50 ml/min. The molecular weight was estimated by comparison of their availability coefficient (K_{av}) with those of molecular weight markers.

SDS-PAGE—Gel electrophoresis was done in a Mini Protein II apparatus (BioRad), as described by Laemmli (*13*) using a 15% separating polyacrylamide slab gel. Samples were heated for 3 min at 90°C, without or with the addition of 5% (v/v) mercaptoethanol. Electrophoresis was performed at 150 V for 1 h 30 min. Protein bands were stained with Coomassie Brilliant Blue R-250.

Western Blot Analyses with Biotin-Labeled Lectins—Western blot analyses using several biotin-labeled lectins were done essentially as described by Avellana-Adalid *et al.* (*14*). Briefly, after SDS-PAGE under reducing conditions, the separated proteins were transferred to nitrocellulose membrane in a semidry electroblotter (BioRad) at a constant voltage of 20 V for 15 min. Nitrocellulose membranes were blocked for 30 min with PBS containing 2% (w/v) PVP, and incubated for 1 h with PBS containing 50 μg/ml of the different biotin-labeled lectins. Then blots were washed with PBS containing 0.05% (v/v) Tween 20, and incubated for 1 h with PBS containing 5 μg/ml of peroxidase-labeled streptavidin. Finally, the peroxidase reaction product was developed in PBS containing 0.05% (w/v) 4-chloro-1-naphthol and 0.03% (v/v) hydrogen peroxide. To detect *N*-linked oligosaccharides we used as probe Con A and LCA, which recognize sequences containing α-linked mannose residues; and DSA, which recognizes *N*-acetyl-D-glucosamine and terminal *N*-acetyl-lactosamine residues. To detect *O*-linked oligosaccharides we used as probe JA and PNA, which recognize galactosyl (β1-3) *N*-acetyl-D-galactosamine residues. Also, we used MAA and SNA to detect sialic acid residues present in *N*- and *O*-linked oligosaccharides. Positive controls were done with several glycoproteins and biotin-labeled lectins. All procedures were carried out at room temperature.

Isoelectric Focusing—The isoelectric point of native *E. trenquelleonis* lectins was determined in a LKB isoelectric focusing apparatus (Pharmacia). A 1% (w/v) isoelectric focusing agarose gel containing 12% (w/v) sorbitol and 5% (v/v) ampholites selected to establish a pH gradient from 3 to 10 was prepared in a glass chamber (12.0×12.0×0.03 cm). Protein bands were stained with Coomassie Brilliant Blue R-250.

Protein Determination—Protein was estimated by the method of Bradford (*15*) using bovine serum albumin as standard.

RESULTS

Separation of Two N-Acetyl-D-Galactosamine-Binding Lectins from E. trenquelleonis Snail by Affinity Chromatography on GalNAc-Agarose—The albumin gland extract of *E. trenquelleonis* snail was applied to a GalNAc-agarose column as described above, and the adsorbed material was eluted with a step-by-step gradient of 1, 5, 20, and 100 mM GalNAc in PBS. A typical elution profile is illustrated in Fig. 1. When the elution buffer contained 1 and 20 mM GalNAc, two peaks with hemagglutinating activity and absorbance at 280 nm were detected. We were unable to detect any significant peak of hemagglutinating activity or absorbance at 280 nm when the elution buffer contained 5 and 100 mM GalNAc. As shown in Table I, ETL1 accounted for about a 20% of total hemagglutinating activity and was

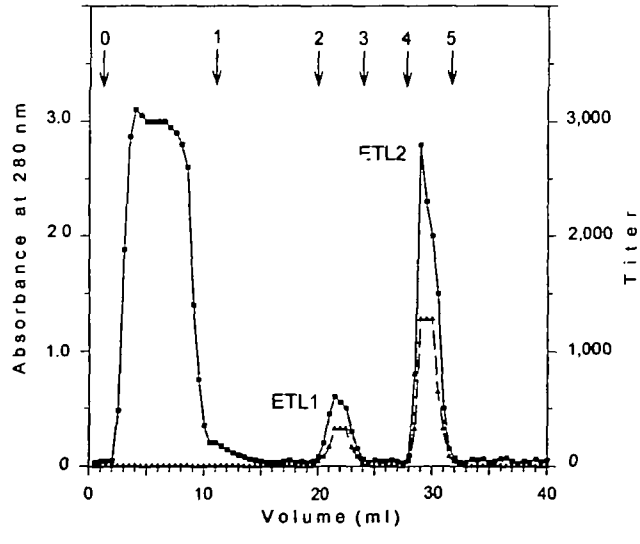

Fig. 1. **Affinity chromatography on GalNAc-agarose.** The albumin gland extract (8.0 ml; 4.0 mg/ml of protein) was applied to a column (0.7×4.0 cm) at 0. The arrows mark the fraction at which buffers were changed: arrow 1, PBS; arrows 2, 3, 4, and 5, PBS containing 1, 5, 20, and 100 mM GalNAc, respectively. Fractions of 0.5 ml were collected, dialyzed against PBS, and assayed for hemagglutinating activity (▲) and absorbance at 280 nm (■).

displaced from the affinity column with 1 mM GalNAc; and ETL2 accounted for about 75% of total hemagglutinating activity and was displaced from the affinity column with 20 mM GalNAc.

Human Blood Type Specificity—In hemagglutination assays, ETL1 and ETL2 agglutinated specifically human blood type A and AB erythrocytes, and their respective subtypes (A_1, A_2, A_1B, and A_2B). No significant hemagglutination was detected with human blood type B and O erythrocytes (Table II). In addition, we observed a remarkable increase in the hemagglutinating activity when erythrocytes were treated previously with trypsin; the human blood type specificity of these lectins, however, was not affected (Table II). As expected, GalNAc was the most potent sugar to inhibit the hemagglutinating activity of ETL1 and ETL2; and other sugars, such as galactose, fucose, *N*-acetyl-D-glucosamine, *etc.*, showed no significant inhibition. The hemagglutinating activity of ETL1 and ETL2 was also inhibited by the presence of A blood group-specific substance, whereas no significant inhibitory effect was detected with B or H blood group-specific substances (results not shown).

Gel Filtration Chromatography—As described in "MATERIALS AND METHODS," the native molecular weight of ETL1 and ETL2 was determined on Superose 12 HR 10/30. As shown in Fig. 2, ETL1 and ETL2 appeared as similar sharp single symmetrical peaks of about 59 ± 2 and 54 ± 2 kDa molecular weight respectively. It should be noted that we were unable to detect other fractions with positive hemagglutinating activity or absorbance at 280 nm, indicating that the presence of lower or higher molecular weight forms of ETL1 and ETL2 is unlikely.

SDS-PAGE—The protein band profiles of ETL1 and ETL2 under nonreducing and reducing conditions are shown in Fig. 3. ETL1 showed two different protein subunits of about 29 and 27 kDa (lane 2), while ETL2 showed mainly three protein subunits of about 27, 24, and 22 kDa (lane 3). When SDS-PAGE was done under reducing conditions, these nonreduced protein subunits of ETL1 and ETL2 disappeared; and ETL1 (lane 5) and ETL2 (lane 6) showed similar protein profiles of four protein subunits of about 17, 16, 12, and 11 kDa. Despite this similarity, the relative contributions of the reduced protein subunits in these lectins was different.

Analyses of the Presence of N- and O-Linked Oligosaccharides in ETL1 and ETL2—To investigate the presence of *N*- and *O*-linked oligosaccharides in ETL1 and ETL2, both lectins were subjected to SDS-PAGE under reducing conditions. The separated proteins were blotted as indicated in "MATERIALS AND METHODS," and incubated with several biotin-labeled lectins. To detect *N*-linked oligosaccharides we used as probe Con A, LCA, and DSA; while to detect *O*-linked oligosaccharides we used JA and PNA. In addition, MAA and SNA were used to detect the presence of sialic acid residues in the *N*- and *O*-linked oligosaccharide chains. As shown in Fig. 4, the 17- and 16-kDa protein subunits of ETL1 and ETL2, and the 12-kDa protein subunit of ETL2 reacted strongly with Con A and LCA, indicating the presence of α-linked mannose residues. In addition, the 17- and 16- kDa protein subunits of ETL1, and the 12-kDa protein subunits of ETL2 reacted weakly with DSA, suggesting a minor contribution of *N*-acetyl-D-glucosamine and terminal *N*-acetyl-lactosamine residues in the *N*-linked oligosaccharide chains. On the other hand, only the 11-kDa protein subunit of ETL1 and ETL2 reacted strongly with JA and PNA, indicating the presence of galactosyl (β1-3) *N*-acetyl-D-galactosamine residues. None of the reduced protein subunits of ETL1 and ETL2 reacted with MAA and SNA, indicating the absence of sialic acid residues in the *N*- and *O*-linked oligosaccharide chains. Finally, no significant reaction was detected between any of the biotin-labeled lectins used and the 12-kDa reduced protein subunit of ETL1, suggesting the absence of *N*- and *O*-linked oligosaccharide chains in this subunit. As a whole, our results indicate that ETL1 and ETL2 are glycoproteins; minor differences in the glycosylation profile among their protein subunits, however, have been detected.

Isoelectric Focusing—As shown in Fig. 5, the isoelectric point profiles of native ETL1 and ETL2 were markedly, different. Each lectin exhibited microheterogeneity in their pIs. ETL1 is an acidic protein and mainly focused as three protein bands of pIs 5.6, 5.8, and 6.0 (lane 1). ETL2, on the other hand, is a neutral protein and mainly focused as four

TABLE I. **Purification of two *N*-acetyl-D-galactosamine-binding lectins from *E. trenquelleonis* snail by affinity chromatography on GalNAc-agarose.**

Sample	Volume (ml)	Protein (mg/ml)	Titer[a]	Total activity	Specific activity[b]	Purification fold	Recovery (%)
Albumin gland extract	8.0	4.0	2,560	20,480	640	1.0	100
ETL1	3.0	0.4	1,280	3,840	3,200	5.0	18.8
ETL2	3.0	1.2	5,120	15,360	4,267	6.7	75.0

[a]Titer: the reciprocal of the highest dilution giving a visible hemagglutination. [b]Specific activity: titer $\times mg^{-1}$ protein.

TABLE II. **Human blood type specificity of ETL1 and ETL2.**

Human blood type	Titer[a]					
	Albumin gland extract		ETL1		ETL2	
	Untreated	Trypsin-treated	Untreated	Trypsin-treated	Untreated	Trypsin-treated
A_1	512	16,384	512	16,384	2,048	32,768
A_2	64	1,024	256	2,048	1,024	8,192
A_1B	128	4,096	512	4,096	2,048	16,384
A_2B	32	256	128	1,024	512	4,096
B	—	—	—	—	—	—
O	—	—	—	—	—	—

[a]Titer: the reciprocal of the highest dilution giving a visible hemagglutination.

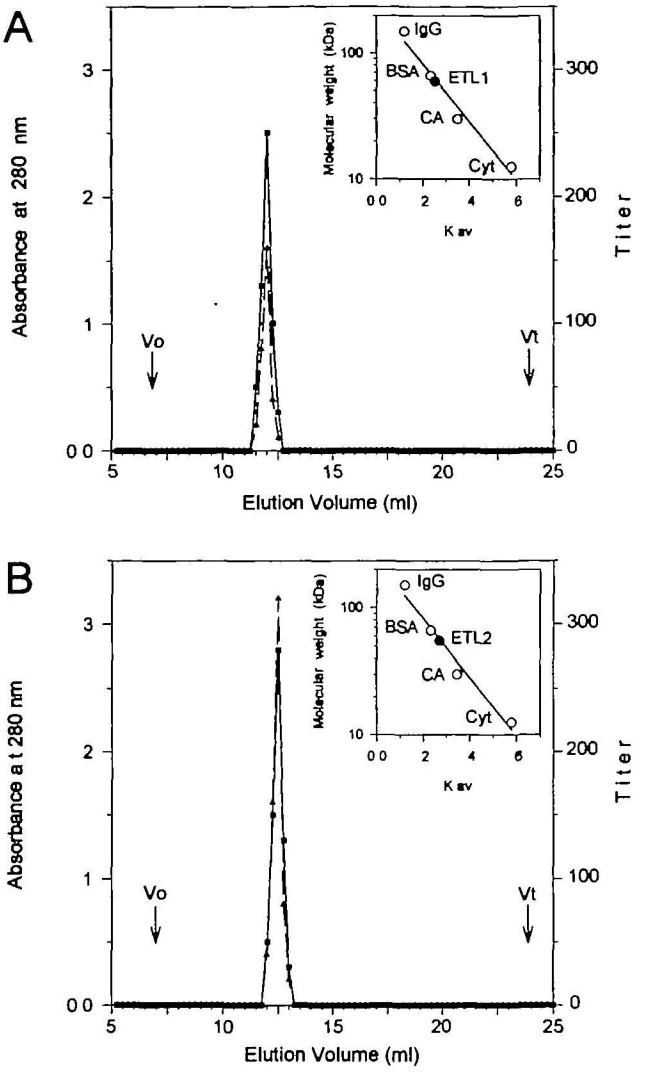

Fig 2 **Molecular weight determinations.** Gel filtration chromatography was done on a Superose 12 (HR 10/30) column. The column was equilibrated with PBS containing 20 mM GalNAc to prevent any interaction between the lectins and the matrix. Samples of 0.10 ml of ETL1 (A) and ETL2 (B) were subjected to chromatography at a flow rate of 0.50 ml/min Fractions of 0.25 ml were collected, dialyzed against PBS, and assayed for hemagglutinating activity (▲) and absorbance at 280 nm (■) Inset, determinations of M_r by gel filtration; and the molecular weight markers were human IgGs (IgG, 150 kDa), bovine serum albumin (BSA, 66 kDa), carbonic anhydrase (CA, 30 kDa), and cytochrome *c* (Cyt, 12 kDa). The filled circles show the positions of native ETL1 and ETL2

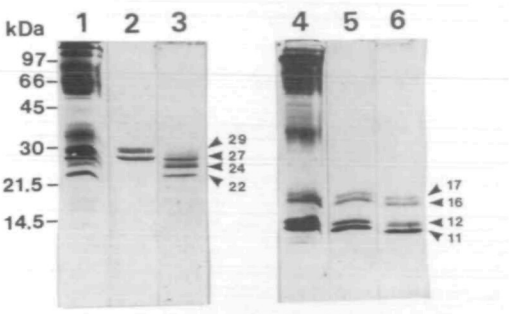

Fig. 3. **SDS-PAGE of ETL1 and ETL2.** Electrophoresis was performed on 15% separating polyacrylamide slab gel Samples were heated for 3 min at 90°C, without (lanes 1, 2, and 3) or with (lanes 4, 5, and 6) the addition of 5% (v/v) mercaptoethanol. Albumin gland extract (lanes 1 and 4, 100 μg); ETL1 (lanes 2 and 5; 15 μg); ETL2 (lanes 3 and 6; 15 μg) Standard proteins used were. phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14 5 kDa) Electrophoresis was performed at 150 V for 1 h 30 min and protein bands were stained with Coomassie Brilliant Blue R-250

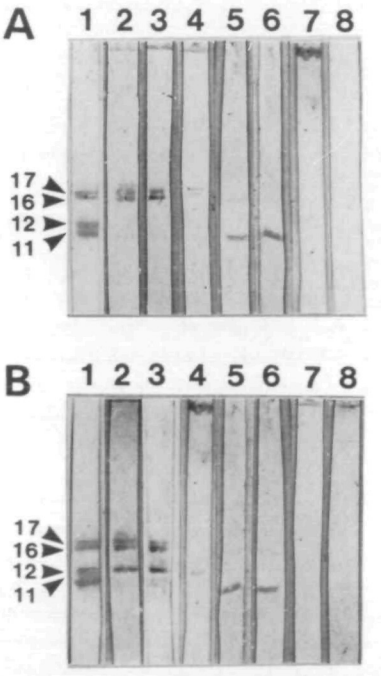

Fig 4. **Western blot analyses with biotin-labeled lectin.** ETL1 and ETL2 (15 μg) were subjected to SDS-PAGE under reducing conditions on 15% separating polyacrylamide slab gel, then transferred to nitrocellulose membrane Blots were stained with 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid, cut into strips (0.5×50 mm), and blocked with PBS containing PVP. As indicated under "MATERIALS AND METHODS," blots were incubated with 50 μg/ml of different biotin-labeled lectins. Then, blots were incubated with 5 μg/ml of peroxidase-labeled streptavidin Finally, the peroxidase reaction product was developed in PBS containing 4-chloro-1-naphthol and hydrogen peroxide. All incubation steps were performed for 1 h at room temperature. ETL1 (A) and ETL2 (B). Ponceau S (lane 1); Con A (lane 2); LCA (lane 3); DSA (lane 4); JA (lane 5); PNA (lane 6); MAA (lane 7), SNA (lane 8).

protein bands of pIs 6.8, 7.0, 7.2, and 7.4 (lane 2). To analyze the subunit composition of the native isoelectric focused proteins, each protein band was subjected to SDS-PAGE under nonreducing conditions. In all cases, the electrophoretic profiles of ETL1 and ETL2 nonreduced subunits were identical, as can be seen in Fig. 3 (lane 2 and 3) (result not shown).

DISCUSSION

The main finding of this work was the isolation of two *N*-acetyl-D-galactosamine-binding lectins (ETL1 and ETL2) from the albumin gland of *E. trenquelleonis* snail. The two lectins were separated by affinity chromatography

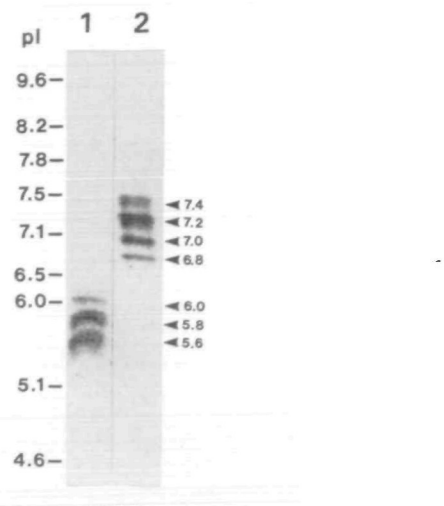

Fig 5. **Isoelectric focusing of ETL1 and ETL2.** Isoelectric focusing analysis was done in a 1% agarose gel with a pH gradient of 3 to 10. The anode electrolyte was L-aspartic acid (0.04 M) in distilled water and the cathode electrolyte was NaOH (1 M) in distilled water ETL1 (lane 1; 15 μg), and ETL2 (lane 2, 15 μg). The standard proteins for isoelectric point (pI) determination were: phycocyanin (pI 4.5), β-lactoglobulin B (pI 5.1), bovine carbonic anhydrase (pI 6.0), human carbonic anhydrase (pI 6 5), equine myoglobulin (pI 6.8, 7 0), human hemoglobulin A (pI 7.1), human hemoglobulin C (pI 7 5), lentil lectin (pIs 7.8, 8.0, 8.2) and cytochrome *c* (pI 9.6). Samples (10 μl) were placed on the gel near the anode in the wells of an applicator and the voltage was gradually increased over 1 h 45 min from 200 to 600 V. Protein bands were stained with Coomassie Brilliant Blue R-250.

on GalNAc-agarose using a step-by-step gradient elution. ETL1 and ETL2 both specifically agglutinated human blood type A and AB erythrocytes and their corresponding subtypes, but not type B and O erythrocytes. As expected, their hemagglutinating activities were inhibited only by GalNAc and by A blood group-specific substance.

The native molecular weight of both lectins as determined by gel filtration chromatography was very similar, about 59 kDa for ETL1 and 54 kDa for ETL2. On SDS-PAGE under nonreducing conditions, ETL1 was dissociated in two nonidentical subunits of 29 and 27 kDa, while ETL2 was dissociated in three nonidentical subunits of 27, 24, and 22 kDa. To our surprise, on SDS-PAGE under reducing conditions, the protein profiles of both lectins were similar. ETL1 and ETL2 were dissociated into four nonidentical subunits of 17, 16, 12, and 11 kDa. The relative contributions of the reduced protein subunits in ETL1 and ETL2 were different.

On the other hand, ETL1 and ETL2 are glycoproteins. The glycosylation pattern revealed the presence of *N*-linked oligosaccharides in the 17- and 16-kDa protein subunits of ETL1 and ETL2, as well as in the 12-kDa protein subunit of ETL2. *O*-linked oligosaccharides were detected only in the 11-kDa protein subunit of ETL1 and ETL2. Even though the glycosylation profiles of the protein subunits of both lectins were very similar, we detected a major difference in the absence of *N*- and *O*-linked oligosaccharide chains in the 12-kDa protein subunit of ETL1.

A significant difference between native ETL1 and ETL2 was their behavior or isoelectric focusing. Native ETL1 is an acidic protein that focused as three protein bands with pIs in the range of 5.6-6.0, while native ETL2 is a neutral protein that focused as four protein bands with pIs in the range of 6.8-7.4.

Concerning another snail lectin with *N*-acetyl-D-galactosamine specificity, native *Helix pomatia* lectin is a glycoprotein (*8*) with a molecular weight of about 80 kDa, which showed several isoforms with differents pIs (*16*). This lectin is composed of six identical subunits of 13 kDa, which are linked in pairs by interchain disulfide bonds to form three nonreduced subunits of 26 kDa. These are held together by noncovalent interactions to form an oligomeric structure of 80 kDa (*17*). Our results suggest that ETL1 and ETL2 are arranged in a similar oligomeric structure with molecular mass of 59-54 kDa, composed of two 29-22-kDa nonreduced subunits held together by noncovalent hydrophobic interactions. Each nonreduced subunit appeared to be composed of two 17-11-kDa reduced subunits held together by interchain disulfide linkages. Although it remains to be confirmed, we believe that main differences detected between ETL1 and ETL2 may be due to a particular posttranslational modification or to the different relative contribution of one or more of protein subunits. To know how different ETL1 and ETL2 are, the primary structure of each protein subunit will need to be determined. In addition, this kind of information will allow the comparison of their primary structure with those of *N*-acetyl-D-galactosamine-specific lectins from *H. pomatia Helix hortensis*, and other snails.

Finally, the *N*-acetyl-D-galactosamine-binding lectins present in plants (*Dolichos biflorus*) and snails (*H. pomatia, H. hortensis*, and *Otala lactea*) possess activity resembling monoclonal and polyclonal anti-human blood type A hemagglutinating activity. Thus, the *N*-acetyl-D-galactosamine-binding lectins from *E. trenquelleonis* snail may also be adapted as a reagent for routine human blood typing determinations.

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