Isolation and characterization of chicken thymic electrolectin

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(Received 10 June 1984/Accepted 30 October 1984)

1. We have detected the presence of a β -D-galactoside-binding lectin (electrolectin) in extracts of the thymus of adult chickens. 2. This lectin was purified by affinity chromatography on a lactosyl-Sepharose column to yield 1.4 mg of pure protein from 230 g of thymus. 3. The chicken thymic electrolectin (CTE) has an M_r of 15 300 when analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and of 30 000 when analysed by gel filtration. 4. The amino acid composition of CTE is similar to that of other electrolectins purified from human and rat lung. 5. CTE cross-reacts immunologically, but is not identical, with electrolectins from electric-eel electric organ and from chick-embryo pectoral muscle. 6. CTE agglutinates chicken thymocytes but does not appear to promote their mitosis.

Electrolectins are bivalent β -D-galactoside-binding lectins of animal origin (Teichberg, 1978). Their presence is revealed by their ability to agglutinate trypsinized rabbit erythrocytes (Teichberg *et al.*, 1975). The name electrolectin originates from the fact that the first animal lectin described was isolated from the electric organ of the electric eel *Electrophorus electricus*. They have been found in teleosts, amphibia, avians and mammals in many organs, including skeletal muscle, heart, brain, liver, intestine, thymus, spleen and skin (Teichberg, 1978; Barondes, 1981), and at very early stages of embryonic development (Cook *et al.*, 1979). Their tissue concentration is often, but not always, ontogenically regulated, reaching a maximum at a given state of development and then drastically decreasing (Kobiler & Barondes, 1977; Simpson *et al.*, 1977; Levi & Teichberg, 1984). In the thymus and spleen of mouse (Levi & Teichberg, 1984), calf (Barak-Briles *et al.*, 1979) and chicken, the electrolectin titre remains high through adulthood. Although many electrolectins have similar physicochemical properties and cross-react immunologically (Barak-Briles *et al.*, 1979; Childs & Feizi, 1979; Levi & Teichberg, 1982), different organs from the same species may contain electrolectins similar in sugar-specificity but structurally and antigenically distinct (Beyer *et al.*, 1980).

Following our observations that the pure electric-eel electrolectin has a prophylactic and therapeutic effect on the experimental model of the

autoimmune disease myasthenia gravis (Levi *et al.*, 1983), we have focused our attention to the role of electrolectins in the immune system. The presence of electrolectin in the thymus and its selective binding to mouse immature thymocytes has brought up the suggestion of a role of this protein in the development of the immune response (Levi & Teichberg, 1983).

In the present paper, we report the purification and partial characterization of electrolectin from chicken thymus and describe its structural and antigenic relation to other electrolectins. We also present the results of experiments aiming at the elucidation of the biological role of thymic electrolectin.

Materials and methods

Materials

Thymuses were excised from white Leghorn chickens aged 2–3 months obtained from a local slaughterhouse. Sepharose 4B was from Pharmacia, Uppsala, Sweden, and Bio-Gel P100 from Bio-Rad Laboratories, Richmond, CA, U.S.A. Thiodigalactoside and concanavalin A were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Tissue-culture media were from Bio-Lab, Jerusalem, Israel.

Assay of electrolectin activity

The presence of electrolectin was revealed by its ability to agglutinate trypsinized rabbit erythrocytes. The method followed was essentially that described by Teichberg (1978). A quantitative

Abbreviation used: CTE, chicken thymic electrolectin.

agglutination assay was performed in microtitre plates as described by Levi & Teichberg (1981). Lactose (10mM) was systematically added in control wells and only the lactose-blockable agglutinating activity was attributed to electrolectin.

Purification of CTE

The CTE was purified by affinity chromatography on a lactosyl-Sepharose column prepared as previously described (Levi & Teichberg, 1981). The chicken thymuses (230g) were carefully cleaned from fat and blood vessels and homogenized for 5 min in an Omni-Mixer table homogenizer in 230ml of buffer A (composition 0.124M-NaCl, 5mM-KCl, 1.24mM-KH₂PO₄, 26mM-Tris base and 14mM-2-mercaptoethanol, pH7.3) supplemented with 10 μ M-phenylmethanesulphonyl fluoride. The homogenate was centrifuged in a Sorvall RC-5 centrifuge [GSA rotor, 11000rev./min (19600g) for 60min] at 4°C. The supernatant (S1) (340ml) was collected and centrifuged for 1 h in a Beckman LB 70 ultracentrifuge [Ti35 rotor, 35000rev./min (100000g) for 1 h at 4°C]. The supernatant (S2) (270ml) was collected and left for 48 h at 4°C in order to allow the precipitation of material that otherwise would block the affinity column. Fraction S2 was submitted to a further centrifugation at 100000g (Ti35 rotor, 35000rev./min) for 1 h at 4°C to obtain a supernatant S3 (250ml), which, after filtration on glass-wool, was directly applied to a 30ml lactosyl-Sepharose column. The column was then extensively washed with buffer A at a flow rate of 1 ml/min until no fluorescence at 330nm could be detected when exciting the effluent at 285nm. Then 0.1M-lactose in buffer A was applied to the column at a flow rate of 1 ml/min, and 2ml fractions were collected. The elution of CTE was followed by measuring the fluorescence of each fraction at 330nm (excitation 285nm). For the determination of the specific activity, the protein concentration was measured at each step of the purification by the Fluram (fluorescamine) method of Udenfriend *et al.* (1972), with, as a reference, standard solutions of bovine serum albumin in buffer A.

Electrophoretic analysis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in continuous 15% acrylamide slab gels (Laemmli, 1970). Protein zones were detected by staining with Coomassie Blue R.

Spectroscopic methods

Fluorescence measurements were carried out with a Perkin-Elmer MPF-3L fluorimeter equipped with an Omni-Scribe recorder. All

fluorescence measurements were performed in 1 cm quartz cuvettes (Hellma) in a thermostatically controlled cell jacket. All solutions were routinely passed through a Millipore filter (HAWP 0.45). The absorbance spectrum was measured with a double-beam Cary 219 spectrophotometer.

Amino acid analysis

Protein samples were dialysed extensively against water and freeze-dried. They were then hydrolysed in 6M-HCl for 12 h at 110°C. The amino acid composition was determined with a Dionex D-502 automatic amino acid analyser. Samples for cysteine determination were hydrolysed in 6M-HCl containing 0.2M-dimethyl sulphoxide at 110°C for 18 h (Spencer & Wold, 1969).

Immunization

Rabbits (New Zealand White) received a primary immunization with 50 μ g of the purified electrolectin emulsified in complete Freund adjuvant. A secondary immunization was administered 2 weeks later by using the same procedure. The rabbits were bled 4 weeks after the secondary immunization. The antisera, divided into small portions, were kept frozen at -20°C.

Solid-phase radioimmunoassay

The ability of each antiserum to recognize different electrolectins was determined by a solid-phase radioimmunoassay with ¹²⁵I-labelled Protein A as previously described (Levi & Teichberg, 1982).

Mitogenicity of CTE

Single-cell suspensions of thymocytes were obtained by forcing the minced thymuses through a fine metallic mesh. Cells were cultured at 37°C, under 5% CO₂ in air, in RPMI 1640 medium supplemented with 2mM-L-glutamine, 50 units of penicillin/ml, 50 units of streptomycin/ml, 2% (v/v) foetal-calf serum and 4 μ M-2-mercaptoethanol. Cells were plated in microtitre plates (Falcon 3040) at a density of 10⁶ cells/well and cultured for 48 h or 72 h. At 16 h before harvesting of the cells, the medium was supplemented with 50 μ l of the culture medium containing 1 μ Ci of [³H]thymidine (Nuclear Research Center, Negev, Israel). The cells were then filtered through a glass-fibre paper (GF/C, Whatman) and washed six times with 0.9% NaCl in an automatic harvesting machine. The filters were dried and the radioactivity was measured. The capacity of electrolectins to induce suppressor activity on chicken thymocytes was assayed as described by Rich & Rich (1975). CTE was added, in final concentrations ranging from 100 to 0.2 μ g/ml, to a

suspension of chicken thymocytes (10^7 cells/ml) in supplemented RPMI 1640 medium. Control cultures were kept in the absence of CTE. The cells (suppressors) were cultured for 48 h at 37°C, under 5% CO₂ in air and then irradiated (2000 R). These cells (0.2×10^6 or 0.4×10^6) were then co-cultured with 0.5×10^6 freshly prepared chicken thymocytes (responders) in microtitre plates in 200 µl of supplemented RPMI 1640 medium in the presence or in the absence of 2 µg of concanavalin A/ml. The rate of thymidine incorporation was measured as described above, after 72 h of culture. The suppressor activity was estimated by the following formula:

$$\text{Suppression (\%)} = [1 - (A - B)/(C - D)] \times 100$$

where *A* is c.p.m. of (responders + suppressors) with concanavalin A, *B* is c.p.m. of (responders + suppressors) without concanavalin A, *C* is c.p.m. of responders with concanavalin A and *D* is c.p.m. of responders without concanavalin A.

Results and discussion

Saccharide specificity of CTE

When a suspension of trypsinized rabbit erythrocytes is mixed with the high-speed supernatant (S3) of a chicken thymus homogenate, haemagglutination occurs within 5 min. A number of saccharides were tested for their ability to inhibit this haemagglutinating activity. The saccharide concentration needed to inhibit 50% of the lectin activity is 0.2 mM for thiodigalactoside, 0.4 mM for lactose and 50 mM for galactose. The following saccharides at concentrations up to 50 mM were found to be ineffective in blocking the haemagglutinating activity: *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-mannose, D-glucose, D-fucose, L-fucose, sucrose, melibiose, maltose and cellobiose. From these results, it appears that the sugar specificity of the thymic lectin is closely similar to that of the known electrolectins, including those from the electric-eel electric organ (Levi & Teichberg, 1981), chicken intestine, liver and

embryonic muscle (Beyer *et al.*, 1980), rat and human lung (Powell, 1980), calf heart and lung (De Waard *et al.*, 1976) and mouse tissues (Levi & Teichberg, 1984).

Isolation of CTE

The CTE is readily adsorbed on a lactosyl-Sepharose column and can be eluted by 0.1 M-lactose. It emerges from the column as a symmetric peak as detected by its fluorescence emission at 330 nm on excitation at 285 nm. Table 1 summarizes the results of the evaluation of the activity of CTE at the various purification steps. The affinity chromatography allowed a total purification of 1675-fold with respect to the homogenate.

Determination of *M_r*

Electrophoresis in the presence of sodium dodecyl sulphate was carried out on dialysed and freeze-dried samples of CTE. As shown in Fig. 1, a sharp protein band could be detected at an apparent *M_r* 15 300 together with a faint band at an apparent *M_r* 17 000.

The *M_r* of the subunit of CTE is in the same range of mass of those found for other electrolectins, which, in general, vary from 12 000 for calf heart lectin (Barak-Briles *et al.*, 1979) to 16 500 for electric-eel electrolectin (Levi & Teichberg, 1981).

To evaluate the *M_r* of the native form of CTE, we passed it through a calibrated column of Sephadex G-100 in the presence of 0.3 M-lactose in order to prevent any interaction between the lectin and the matrix. After elution, the fractions were dialysed against buffer A and the haemagglutinating activity present in each fraction was measured. As shown in Fig. 2, the main peak of electrolectin activity emerged from the gel-filtration column in a position corresponding to *M_r* 30 000 ± 3000. It thus appears that CTE, like other known electrolectins, has a dimeric structure.

Spectroscopic properties of CTE

CTE has a u.v.-absorption spectrum characterized by a maximum at 284 nm, shoulders at 290,

Table 1. Purification of CTE

The preparation was from 230 g of fresh tissue as starting material. For experimental details see the Materials and methods section. Activity is expressed in terms of agglutinating units as defined by Levi & Teichberg (1981).

Fraction	Total protein (mg)	$10^{-4} \times$ Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Recovery of activity (%)
Homogenate	4240	34.5	81	1.0	100
Supernatant S3	2040	19	94	1.2	55
Affinity chromatograph, lactose eluent	1.4	10	135 700	1675	55

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of CTE

Electrophoresis in 15% polyacrylamide gel was performed in the presence of 0.1% sodium dodecyl sulphate as described by Laemmli (1970). Markers are: a, ovalbumin, M_r 43000; b, carbonic anhydrase, M_r 30000; c, trypsin inhibitor, M_r 20100; d, α -lactalbumin, M_r 14500. The arrows represent the positions of the main and minor (broken arrow) components of purified CTE.

272 and 265 nm and a minimum at 262 nm. When excited at 285 nm, CTE fluoresces with a maximum at 332 nm and displays the typical emission spectrum of a tryptophan in a hydrophobic environment. The addition of lactose causes no change in the intensity or in the position of the emission peak; in this respect, CTE is different from electric-eel electrolectin, to which the addition of lactose produces changes in the fluorescence spectrum (Teichberg *et al.*, 1975; Levi & Teichberg, 1981).

Amino acid analysis

The amino acid composition of CTE is shown in Table 2. Cysteine was determined as cysteic acid after oxidation with performic acid. The tryptophan content of CTE was not determined, but from the spectroscopic properties of this protein (see above) it is possible to deduce that it possesses at least one tryptophan residue.

Using the method of Metzger *et al.* (1968), we compared the amino acid composition of CTE with that of the known electrolectins and obtained an estimation of the extent of similarity of these lectins. In this method, one calculates a difference index (D.I.) between two proteins; D.I. is 50 times the sum of the differences of the molar fraction of each amino acid. If the value of this index is below 10, the two proteins compared are considered to be very similar. A value of D.I. above 30 indicates that the two proteins are totally unrelated. On comparing CTE with the lectins from human and

Fig. 2. Gel filtration of CTE on Sephadex G-100

The column (1.2 cm $\times$ 30 cm bed volume) was equilibrated in buffer A containing 0.3 M-lactose to prevent interactions of the lectin with the matrix. Fractions (1 ml) were collected, and dialysed against buffer A, and the haemagglutinating activity was measured. V_e , Elution volume; V_0 , void volume; V_i , elution volume of low- M_r species. Calibration markers are: 1, cytochrome *c*, M_r 12400; 2, α -lactoglobulin, M_r 18400; 3, ovalbumin, M_r 45000; 4, bovine serum albumin, M_r 66000.

Table 2. *Amino acid composition of CTE*

The means for three determinations are given.
Abbreviation: N.D., not determined.

Amino acid	Composition (residues/monomer)	
	Found	Nearest integer
Lys	4.8 ± 0.2	5
His	4.1 ± 0.1	4
Arg	3.1 ± 0.1	3
Asx	10.2 ± 0.3	10
Thr	5.9 ± 0.2	6
Ser	17.3 ± 0.6	17
Glx	18.3 ± 0.5	18
Pro	5.8 ± 0.2	6
Gly	20.6 ± 0.5	21
Ala	9.4 ± 0.2	9
Cys	2.1 ± 0.2	2
Val	6.0 ± 0.1	6
Met*	1.0 ± 0.1	1
Ile	3.1 ± 0.1	3
Leu	5.9 ± 0.1	6
Tyr	1.3 ± 0.1	1
Phe	4.5 ± 0.2	5
Trp	N.D.	N.D.

* Determined as methionine sulphone after performic acid oxidation.

rat lung (Powell, 1980), D.I. values of 5 and 8 were found respectively, thus indicating the strong similarity of these proteins. When CTE was compared with electric-eel electrolectin (Levi & Teichberg, 1981) and with chicken heart lectin (Barak-Briles *et al.*, 1979), the D.I. values were 21 and 23 respectively. D.I. values higher than 30 were obtained when CTE was compared with the foetal-calf muscle lectin (Montelione *et al.*, 1981) and the lectins from calf heart and calf spleen (Barak-Briles *et al.*, 1979).

As for most other electrolectins, the maintenance of the agglutinating activity of CTE was found to be dependent on the presence of 2-mercaptoethanol. This requirement can probably be attributed to the presence in CTE of a sensitive cysteine residue rather than to the oxidation of a tryptophan into an oxindole residue as was found for electric-eel electrolectin (Levi & Teichberg, 1981). With CTE, in contrast with electric-eel electrolectin, no change in the fluorescence emission at 330nm could be observed upon exposure of the protein to oxidizing conditions.

Addition of EDTA or EGTA (50mM) to buffered CTE solutions did not alter the activity of the protein, indicating that the agglutinating properties of this electrolectin, as with all other known electrolectins, are not metal-ion-dependent.

Immunological cross-reactivity of CTE

By using a solid-phase radioimmunoassay, we have studied the antigenic relations between CTE and two other electrolectins purified respectively from the electric organ of the electric eel and from chick-embryo pectoral muscle. Wells of microtitre plates were coated with one of the pure lectins and then incubated with different dilutions of rabbit antisera directed against each of the three electrolectins. Control wells contained antiserum raised against bovine serum albumin. The amount of bound antibody was measured by using ^{125}I -Protein A. The degree of cross-reactivity could be estimated from the amount of radioactivity specifically bound when all the available binding sites were saturated (the plateau of the binding curve). The results of this series of experiments are shown in Fig. 3.

As can be seen, the chick-embryo pectoral muscle electrolectin and CTE are recognized to a similar extent by all the three antisera (Figs. 3a and 3c), whereas the electric-eel electrolectin is recognized only partially by antibodies directed against the chick pectoral muscle electrolectin (65% recognition) and to CTE (20% recognition) (Fig. 3b). These results indicate that these three electrolectins share some common antigenic determinants but are not identical. When considered together with the results obtained by comparing the amino acid compositions of electric-eel electrolectin and CTE, these findings suggest that some antigenic determinants have been conserved through evolution, although most of the amino acid sequences of these proteins might be very different.

These results and the previously established data that electrolectins from different species are structurally and antigenically related (Childs & Feizi, 1979; Barak-Briles *et al.*, 1979; Levi & Teichberg, 1982), as well as the finding that different organs of the same animal contain structurally unrelated electrolectins with identical sugar-specificity (Beyer & Barondes, 1982), lead us to the hypothesis that families of electrolectin genes could be under the control of different promoters. The latter would control the expression of β -D-galactoside-binding lectins in different organs so that electrolectins would be expressed only as specific stages of development.

Binding of CTE to chicken thymocytes

When CTE is incubated at a concentration of 100 $\mu\text{g}/\text{ml}$ with a suspension of chicken thymocytes, it promotes a massive agglutination. No agglutination is observed in the presence of 10mM-lactose or for CTE concentrations below 50 $\mu\text{g}/\text{ml}$. These results therefore indicate that chicken

Fig. 3. Antigenic cross-reactivity between CTE, chick-embryo pectoral muscle electrolectin and electric-eel electrolectin

The wells of microtitre plates were coated respectively with chick-embryo pectoral muscle electrolectin (a), electric-eel electrolectin (b) and CTE (c). Immune antisera against each of the three electrolectins were then added to each of the plates and the amount of bound antibodies was measured with ^{125}I -Protein A. Δ , Anti-CTE serum; $\square$, anti-(chick pectoral-muscle electrolectin) serum; $\circ$, anti-(electric-eel electrolectin) serum. In control wells, anti-(bovine serum albumin) serum was added ($\bullet$).

thymocytes do possess CTE-binding sites on their membrane. When tested for its mitogenicity on chicken thymocytes, CTE was found to be devoid of such an activity in the range of concentrations from $200\mu\text{g/ml}$ to $0.2\mu\text{g/ml}$. In parallel experiments, concanavalin A produced a very marked mitogenic effect. When thymocytes (10^7 cells/ml) were cultured for 48 h with CTE in the range of concentrations from $200\mu\text{g/ml}$ to $0.2\mu\text{g/ml}$, irradiated (2000R) and tested for their ability to suppress the mitogenic response of responder thymocytes to concanavalin A, no significant

differences were observed between treated and non-treated cells.

In conclusion, we have isolated and characterized the lactose-binding lectin from the chicken thymus; this lectin is antigenically and structurally related to other β -D-galactoside-binding lectins of animal origin; the fact that binding sites for this lectin are present on chicken thymocytes suggests that these cells could be the target of this protein. The chicken thymic lectin is not a mitogen for chicken thymocytes and does not stimulate their maturation into suppressor cells. The precise role of this lectin in the thymus remains now to be established.

This work was supported by grants from the Muscular Dystrophy Association of America and the Minerva Foundation. V. I. T. holds the Louis and Florence Katz-Cohen professorial chair in neuropharmacology.

References

- Barak-Briles, E., Gregory, W., Fletcher, P. & Kornfeld, S. (1979) *J. Cell Biol.* **81**, 528–537
- Barondes, S. H. (1981) *Annu. Rev. Biochem.* **50**, 207–231
- Beyer, E. C. & Barondes, S. H. (1982) *J. Cell Biol.* **92**, 23–27
- Beyer, E. C., Zweig, S. E. & Barondes, S. H. (1980) *J. Biol. Chem.* **255**, 4236–4239
- Childs, R. A. & Feizi, T. (1979) *Biochem. J.* **183**, 755–758
- Cook, G. M. W., Zalik, S. E., Milos, N. & Scott, V. (1979) *J. Cell Sci.* **38**, 293–304
- De Waard, A., Hickman, S. & Kornfeld, S. (1976) *J. Biol. Chem.* **251**, 7581–7587
- Kobiler, D. & Barondes, S. H. (1977) *Dev. Biol.* **60**, 326–330
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Levi, G. & Teichberg, V. I. (1981) *J. Biol. Chem.* **256**, 5735–5740
- Levi, G. & Teichberg, V. I. (1982) *FEBS Lett.* **148**, 145–148
- Levi, G. & Teichberg, V. I. (1983) *Immunol. Lett.* **7**, 35–39
- Levi, G. & Teichberg, V. I. (1984) *Biochem. Biophys. Res. Commun.* **119**, 801–806
- Levi, G., Tarrab-Hazdai, R. & Teichberg, V. I. (1983) *Eur. J. Immunol.* **13**, 500–507
- Metzger, H., Shapiro, M. B., Marzmann, J. E. & Vinton, J. E. (1968) *Nature (London)* **219**, 1166–1168
- Montelione, G. T., Callahan, S. & Podleski, T. R. (1981) *Biochim. Biophys. Acta* **670**, 110–123
- Powell, J. T. (1980) *Biochem. J.* **187**, 123–129
- Rich, R. R. & Rich, S. S. (1975) *J. Immunol.* **114**, 1112–1118
- Simpson, D. L., Thorne, D. R. & Loh, H. H. (1977) *Nature (London)* **266**, 367–369
- Spencer, R. L. & Wold, F. (1969) *Anal. Biochem.* **32**, 185–190
- Teichberg, V. I. (1978) *Methods Enzymol.* **50**, 291–302
- Teichberg, V. I., Silman, I., Beitsh, D. D. & Resheff, G. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1383–1387
- Udenfriend, S., Stein, S., Bohlen, P., Diarmann, W., Leimgruber, W. & Weigle, M. (1972) *Science* **178**, 871–874