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Amino Acid Sequence and Molecular Characterization of a D-Galactoside-Specific Lectin Purified from Sea Urchin (*Anthocidaris crassispina*) Eggs†

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ABSTRACT: The complete amino acid sequence of a 11.5-kDa subunit of D-galactoside binding lectin purified from sea urchin (*Anthocidaris crassispina*) eggs is presented. The 105-residue sequence of the subunit was determined by analysis of the intact S-carbamoylmethylated protein and peptides generated by digestion with *Achromobacter* protease I or *Staphylococcus aureus* V8 protease. The lectin exists as a disulfide-linked homodimer of two subunits; the dimeric form is essential for hemagglutination activity. However, the monomeric form obtained by partial reduction retains the carbohydrate binding capacity. Neither Ca^{2+} nor SH reagent is essential for hemagglutination or carbohydrate binding. The sequence has no similarity to that of any known protein and apparently represents a new type of galactoside binding lectin.

Endogenous animal lectins are widely distributed in extracellular matrix, cell membranes, cytoplasm, and nuclei (Barondes, 1984; Hubert et al., 1989). Although their physiological functions are not well understood, there is evidence that they play an important role in molecular recognition by interacting with sugar chains of glycoproteins or glycolipids (Sharon & Lis, 1989).

On the basis of our knowledge of primary structures of animal lectins purified from various species and tissue types, these lectins can be classified into two groups depending on sequence similarity and common characteristics such as sugar binding specificity, presence of a cysteinyl residue in the active site, and ion requirements (Drickamer, 1988). Recently, human endothelial leukocyte adhesion molecule 1 (ELAM-1) and the homing receptor of lymphocyte and rat cartilage proteoglycan core protein were shown by cDNA cloning to share a similar sequence with lectins (Bevilacqua et al., 1989; Siegelman et al., 1989; Doege et al., 1987). Expressed cDNA derived from the lectin domain of rat cartilage proteoglycan core protein, in fact, showed affinity to a specific carbohydrate (Halberg et al., 1988). The bifunctional characteristics of these "new" lectins have been revealed through structural analysis, i.e., amino acid sequence analysis, cDNA cloning, or immunochemical studies.

Two distinct lectins from the sea urchin *Anthocidaris crassispina* have been reported. One was purified from coelomic fluid and named echinoidin (Giga et al., 1985). On the basis of its amino acid sequence, echinoidin was shown to belong to a group of Ca^{2+}-dependent vertebrate lectins. Interestingly, it has an RGD sequence which is a cell binding signal common in cell adhesion proteins such as fibronectin, von Willebrand factor, and laminin (Giga & Ikai, 1987). The other was a D-galactoside binding lectin purified from sea urchin eggs (Sasaki & Aketa, 1981). The function of sea urchin egg lectin (SUEL)[1] is not clearly understood; however, a dramatic change in distribution of the lectin from cytoplasm to cortex after fertilization was observed by immunohistochemical study using anti-lectin polyclonal antibody (IgG) (Sasaki & Aketa, 1981). This finding suggests that SUEL may have an important role in the activation of eggs (triggered by fertilization), or in their subsequent differentiation.

To increase our knowledge concerning possible functions of SUEL, we determined its amino acid sequence and analyzed some other biochemical properties of this lectin.

Materials and Methods

Preparation of SUEL. A. crassispina eggs were obtained by coelomic injection of 0.5 M KCl and dejellied by acid seawater. Lipids were removed by treatment with cold acetone. Acetone powder of unfertilized eggs was used as the starting material for purification of D-galactoside binding lectin by the method of Sasaki and Aketa (1981). Briefly, the acetone powder was mixed with TBS (150 mM NaCl/20 mM Tris-HCl, pH 7.5) containing 2 mM EDTA and sonicated at 0 °C. Supernatant after centrifugation (27000*g*, 30 min) was applied to a column (10 × 100 mm) of TDG (Sigma)-conjugated Sepharose 6B (Pharmacia) at 4 °C. After extensive washing of the column with TBS, SUEL was eluted with TBS containing 100 mM lactose and dialyzed against TBS or 100 mM NH_4HCO_3. In general, 2–3 mg of SUEL was purified from 10 g of acetone powder.

Assay for Hemagglutination Activity. Hemagglutination activity was assayed by serial 2-fold dilution in microtiter V-plates using trypsinized and fixed rabbit erythrocyte cells, and expressed as titer.

SDS-PAGE and IEF. SDS-PAGE (15% separating gel) was performed according to the method of Laemmli (1970). The gel was stained by either CBB R-250 or silver staining (Wray et al., 1981). IEF was performed at 4 °C with 1-mm-thick slab gels (5 × 7 cm) containing 7% polyacrylamide, 8 M urea, 2% Triton X-100, 10% glycerol, and 2% Biolyte 3/10 (Bio-Rad).

Preparation of Monomeric SUEL. SUEL was dissolved in 100 mM NH_4HCO_3 containing 2.5 mM 2-ME and incubated for 6 h at 25 °C under a N_2 stream. Aliquots were taken at intervals for testing of hemagglutination activity. Conversion

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[1] Abbreviations: CAM, carbamoylmethyl; CBB, Coomassie brilliant blue; Da, dalton(s); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; 2-ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate, SUEL, sea urchin egg lectin; TBS, Tris-buffered saline; TDG, thiodigalactoside; TFA, trifluoroacetic acid.

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of dimer to monomer was monitored by SDS–PAGE. SUEL treated with 5 mM 2-ME for 12 h at 4 °C was applied to a TDG–Sepharose column. The column was washed with 100 mM NH_4HCO_3 containing 5 mM 2-ME and eluted with the same buffer containing 100 mM lactose. Proteins in the pass-through and eluate fractions were concentrated in vacuo to remove 2-ME and monitored by SDS–PAGE.

Preparation of CAM-SUEL. In a typical preparation, 20 nmol of SUEL was reduced with 65 mM dithiothreitol in 600 mM Tris, 0.6 mM EDTA, and 6 M guanidine hydrochloride (pH 8.5) at 37 °C for 4 h and S-carbamoylmethylated with iodoacetamide (140 mM) for 30 min at 25 °C.

Enzymatic Digestion. One nanomole of CAM-SUEL was dissolved in 1 mL of 100 mM Tris-HCl buffer (pH 9.0) containing 4 M urea and digested with *Achromobacter* protease I (a gift from Dr. T. Masaki, Ibaraki University, Japan), which specifically cleaves lysyl bonds (Masaki et al., 1981), at a molar ratio of 1:300 at 37 °C for 6 h. One nanomole of CAM-SUEL was digested with *Staphylococcus aureus* V8 protease (Miles) in 0.5 mL of 100 mM NH_4HCO_3, pH 8.0, at a molar ratio of 5:100 at 37 °C for 20 h.

Each digest was separated by reversed-phase HPLC on a column of Cosmosil 5C18 (C18) (4.6 × 150 mm; Nacalai Tesque, Inc., Kyoto, Japan) or Cosmosil 5C8 (C8) (4.6 × 100 mm; Nacalai Tesque, Inc.), using a gradient of acetonitrile into dilute aqueous TFA (Mahoney & Hermodson, 1980).

Amino Acid and Sequence Analysis. Amino acid compositions were determined by using a Hitachi L8500 amino acid analyzer after hydrolysis in 6 N HCl containing 0.5% phenol at 110 °C for 20 h. Amino acid sequences were determined by using an Applied Biosystems 470A protein sequencer connected to a 120A PTH analyzer.

Sequence homology was searched in the protein sequence database of the National Biomedical Research Foundation on a VAX 3600 computer using the WORDSEARCH program (Wilbur & Lipman, 1983) (version 6.0, April 1989). The alignment procedure used the SEGMENT program (Smith & Waterman, 1981). Hydropathy analysis was carried out with a Vax 3600 computer using the method of Kyte and Doolittle (1982).

Results

Molecular Characterization of SUEL. SUEL was easily purified by TDG–Sepharose 6B affinity chromatography. The apparent molecular mass of SUEL was 11.5 and 23 kDa under reducing and nonreducing conditions, respectively (Figure 1). These values were almost identical with those obtained by Sasaki and Aketa (1981), although they used the SDS–PAGE method of Weber and Osborn (1969). IEF of SUEL gave p*I* values of 5.3–5.4 (data not shown).

Since most animal lectins so far isolated require Ca^{2+} or SH reagent for their activity, effects of these reagents on SUEL were studied. Hemagglutination activity of SUEL was independent of Ca^{2+} concentration, and 100 mM EDTA had no inhibitory effect on hemagglutination activity or binding to TDG–Sepharose 6B (data not shown). Addition of 2-ME did not increase SUEL-induced hemagglutination; rather, it gradually decreased this activity. Therefore, neither Ca^{2+} nor SH reagent is essential for the hemagglutination or TDG binding activity of SUEL.

Effects of Reduction of SUEL on Hemagglutination and TDG Binding. Hemagglutination activity of SUEL was decreased to 50% of initial activity after incubation with 2.5 mM 2-ME for 3 h, and was essentially lost after 6 h (Figure 2A). To study the effect of reduction on carbohydrate binding, we applied SUEL reduced with 2-ME for 12 h to a TDG–Sepharose 6B column in the presence of 5 mM 2-ME. The column was eluted with 100 mM NH_4HCO_3 containing 5 mM 2-ME, and then with the same buffer containing 100 mM lactose. Aliquots of the pass-through fraction and lactose eluate were analyzed by SDS–PAGE under nonreducing conditions. Over 90% of the reduced monomeric SUEL was bound to the column and recovered in the lactose eluate (Figure 2B).

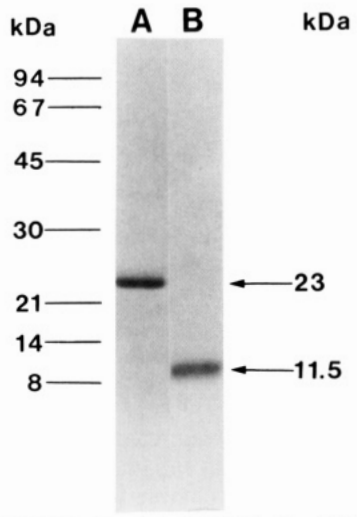

FIGURE 1: SDS–PAGE of purified SUEL before (A) and after (B) reduction with 150 mM 2-ME. The molecular mass of SUEL was estimated to be 11.5 and 23 kDa before and after reduction, respectively. Numbers on the left indicate the molecular mass of markers: phosphorylase *b* (94 kDa); bovine serum albumin (67 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (21 kDa); α-lactalbumin (14 kDa); horse heart myoglobin fragment (8 kDa).

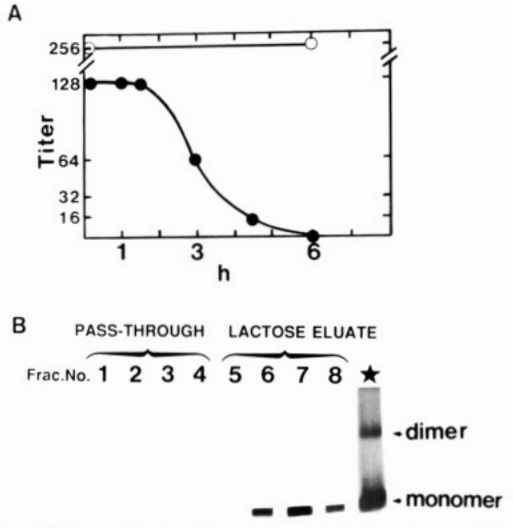

FIGURE 2: Effects of reduction on hemagglutination activity and TDG–Sepharose binding of SUEL. (A) Purified SUEL was treated with (●) or without (○) 2.5 mM 2-ME for 0–6 h at room temperature, and hemagglutination activity was assayed. (B) SUEL treated with 5 mM 2-ME for 12 h at 4 °C was applied to a TDG–Sepharose column (10 × 30 mm) in the presence of 5 mM 2-ME and eluted with 100 mM lactose. Aliquots of each fraction were analyzed by SDS–PAGE under nonreducing condition. Bands 1–4 represent pass-through fractions, bands 5–8 are lactose-eluted fractions, and the asterisk is a mixture of dimeric and monomeric SUEL used as a molecular marker. The gel was treated by silver staining.

These results suggest that the disulfide-bonded dimeric form of SUEL is essential for hemagglutination whereas the reduced monomeric form retains TDG binding capacity.

Generation of Peptides. A digest of the CAM protein (1.0 nmol) with *Achromobacter* protease I was separated by re-

Table I: Amino Acid Composition of SUEL and Peptides Used for Sequence Analysis[a]

fragment	K1	K2	K3	K4	K5	K6	K7	K8	whole	E8	E10
residue no.	1–9	10	11–39	40–53	54–56	57–73	74–94	95–105	CAM-protein	43–67	72–105
CAMCys[b]	0.8 (1)		1.7 (2)	1.2 (1)		1.5 (2)	1.6 (2)	0.6 (1)	7.4 (9)	0.8 (2)	2.3 (3)
Asp/Asn			2.0 (2)		(1)	2.0 (2)	2.0 (2)		7.7 (7)	1.5 (2)	2.8 (2)
Thr			1.0 (1)	1.3 (1)			1.8 (2)	0.9 (1)	4.9 (5)	0.7 (1)	2.5 (3)
Ser	1.2 (1)		2.8 (3)			3.3 (4)	3.6 (4)	1.0 (1)	12.0 (13)	2.0 (3)	4.0 (5)
Glu/Gln	2.2 (2)		4.1 (4)	1.8 (1)		3.8 (4)			11.7 (11)	2.1 (3)	
Pro			0.8 (1)	0.8 (1)			1.9 (2)		4.0 (4)	(1)	1.8 (2)
Gly			3.3 (3)	4.3 (3)		1.3 (1)	2.1 (2)		13.6 (9)	2.0 (2)	3.3 (3)
Ala			1.0 (1)	1.5 (1)			1.9 (2)	1.0 (1)	5.2 (5)	0.8 (1)	2.7 (3)
Val	1.0 (1)		1.4 (2)	1.5 (1)		1.4 (2)	1.9 (2)	1.0 (1)	7.5 (9)	1.5 (3)	2.7 (3)
Met									0.4 (0)		
Ile			3.0 (4)					1.0 (1)	5.3 (5)		1.6 (1)
Leu	1.9 (2)		1.0 (1)	1.7 (1)			1.0 (1)	1.8 (2)	6.8 (7)	1.0 (1)	2.6 (3)
Tyr			1.0 (2)					1.4 (2)	2.8 (4)		1.2 (2)
Phe	1.0 (1)			2.9 (2)			1.0 (1)	0.9 (1)	4.7 (5)	1.1 (2)	1.7 (2)
His									0.0 (0)		
Lys	1.0 (1)	(1)	1.0 (1)	1.0 (1)	(1)	1.0 (1)	1.0 (1)		7.0 (7)	1.3 (2)	2.0 (2)
Arg			1.9 (2)	1.9 (1)	(1)	1.0 (1)			4.6 (6)	1.2 (2)	
Trp[c]	ND[c] (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)	ND (0)
total residues	9	1	29	14	3	17	21	11	105	25	34

[a] Results are expressed as residues per molecule by analysis of 20-h acid hydrolysate or, in parentheses, from the sequence. [b] *S*-(Carbamoylmethyl)cysteine, analyzed as *S*-(carboxymethyl)cysteine. [c] Trp was not determined (ND).

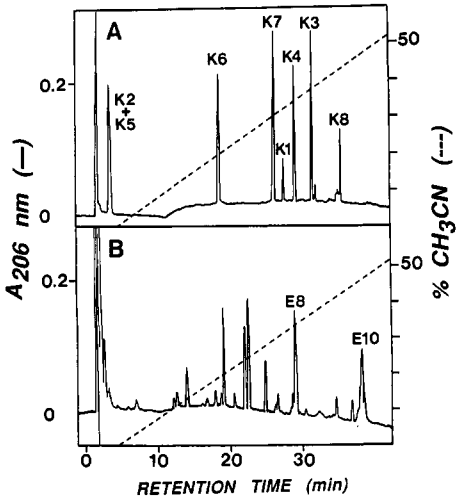

FIGURE 3: Elution profile of peptides generated by enzymatic digestion of the CAM protein. (A) HPLC profile of a *Achromobacter* protease I digest on a Cosmosil C18 column. (B) HPLC profile of *S. aureus* V8 protease digest on a Cosmosil C8 column. Peptides were eluted by a gradient of acetonitrile into dilute aqueous TFA.

versed-phase HPLC on a C18 column (Figure 3A). Eight major peptides, designated K1–K8, were isolated and subjected to amino acid analysis (Table I) and sequence determination.

A digest of the CAM protein (3.0 nmol) with *S. aureus* V8 protease was separated in a similar manner (Figure 3B). Two peptides, designated E8 and E10, were found to align with K peptides K4–K8.

Sequence Analysis. Sequence analysis of 300 pmol of intact CAM-SUEL yielded the N-terminal sequence from Glu-1 to Asn-54, except for residues 51 and 52. This region of the protein (residues 1–53) included peptides K1–K4, as verified by amino acid composition analysis (Table I). The sequences of peptides K5–K8 were determined to the C-terminus (Figure 4). The sequence of peptides E8 (residues 43–67) and E10 (residues 72–105), isolated from a digest with *S. aureus* V8 protease, provided the alignment of peptides K4–K6 and K6–K8, respectively (Figure 4). The complete amino acid sequence of SUEL, thus determined, is shown at the top of Figure 4. These sequence data indicate that SUEL is composed of 105 amino acid residues, with a molecular mass calculated to be 11 375 Da, which is in good agreement with the value obtained by SDS–PAGE (11.5 kDa). No His, Met, or Trp residues are present. The protein contains many acidic residues (Asp and Glu), resulting in a low isoelectric point (p*I* 5.3–5.4). When SUEL was treated with iodoacetamide without reduction, no CAM-Cys was detected by amino acid analysis (data not shown), indicating that all Cys residues form inter- and/or intrasubunit disulfide bonds. All Asn, Ser, and Thr residues were detected as their PTH derivatives during sequencing, indicating that SUEL contains no carbohydrate. The hydropathic nature of SUEL, predicted from the amino acid sequence by the method of Kyte and Doolittle (1982), suggests that SUEL does not contain any uninterrupted, uncharged segment of length sufficient to serve as a membrane binding domain, signal anchor, or any such, as shown in Figure 5.

Discussion

Cytosolic galactoside binding lectins and their isoforms are widely distributed in many animal tissues (Barondes, 1984).

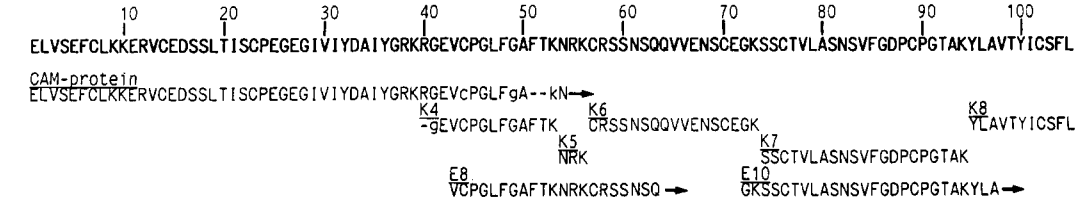

FIGURE 4: Detailed summary of sequence determination of SUEL. The proven sequences of specific peptides are given in one-letter code (Table I) below the summary sequence (boldface type). CAM-protein, sequence of intact CAM-SUEL by sequencer. K and E, peptides generated by cleavage of the CAM-protein at lysyl and glutamyl bonds, respectively. Peptide sequences in upper case letters were proven by Edman degradation, and those in lower case letters indicate tentative identification, or that deduced from amino acid compositions. Those not identified are shown by dashes or by arrows, which indicate a long unidentified sequence.

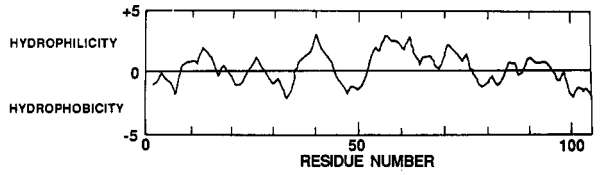

FIGURE 5: Hydropathic nature of SUEL.

Most of them are dimeric or multimeric and present common features such as the presence of a free cysteinyl residue, subunit molecular mass of about 14 kDa, and sequence similarity in the carbohydrate recognition domain (Drickamer, 1988). SUEL is a soluble galactoside binding lectin which exists as a homodimer (subunit molecular mass 11.5 kDa) and presents no significant sequence similarity to any other known protein. In contrast to most animal lectins, SUEL activity is not dependent on the presence of Ca^{2+} or SH reagent. Its unique amino acid sequence and other biochemical characteristics suggest that SUEL represents a novel type of galactoside binding lectin.

There have been few reports on lectins from eggs (particularly mammalian eggs), because of difficulty in obtaining sufficient material. Sakakibara et al. (1976) purified a Ca^{2+}-independent 17-kDa lectin from *Rana catesbeiana* eggs which agglutinates human type A erythrocytes. This hemagglutination was inhibited by *N*-acetyl-D-galactosamine, lactose, and melibiose. It is not known whether this frog egg lectin shares similar properties with SUEL. Perhaps D-galactoside binding lectins homologous to SUEL are widespread or ubiquitous in animal eggs. We are currently attempting to identify SUEL-like lectins in other animal eggs by affinity purification and by Western blotting using anti-SUEL IgG. A lectin termed echinoidin was obtained from *A. crassispina* coelomic fluid (see the introduction). However, SUEL was not obtained from coelomic fluid by affinity chromatography (data not shown).

Immunohistochemical studies have shown that SUEL is abundant in the cytoplasm of unfertilized eggs and shifts to the peripheral region after fertilization (Sasaki & Aketa, 1981). Changes in the distribution pattern of SUEL during ontogeny should be studied by similar techniques, using specific antibodies. Like SUEL, most vertebrate D-galactoside binding lectins are found in cytoplasm, but some have been detected in extracellular matrix or in the nucleus (Barondes, 1984; Carding et al., 1985). An extracellular matrix protein termed "echinonectin", which is a homodimer of a 110-kDa subunit and shows high attachment activity for dissociated embryonic cells, was purified from sea urchin (*Lytechinus variegatus*) embryos (Alliegro et al., 1988). Recently, Veno et al. (1990) identified echinonectin as a lectin which specifically binds galactose or fucose polymer. Although the functional role of echinonectin is not clear, the cell adhesion and carbohydrate binding properties of this protein may play an important role in molecular interaction between protein and protein, or protein and carbohydrate, in extracellular matrix. Several lectins including SUEL, echinoidin, and echinonectin are suggested to play an important role during sea urchin development. Study of changes in SUEL distribution patterns subsequent to early embryonic cell division may be important for elucidating the functional role of this lectin. Although the monomeric form of SUEL has equal carbohydrate binding capability, only the dimeric form is found in eggs, suggesting some unknown physiological function. Experiments are underway to determine whether microinjection of monomeric SUEL into eggs disrupts early development. Identification of the endogenous ligand of SUEL is clearly important for understanding its functional role. To this end, coupling of affinity columns with SUEL should provide a useful tool.

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