

## Molecular association of lectin and $\beta$ -glucosidase in corn coleoptile

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### Abstract

Corn coleoptile lectin is present with  $\beta$ -glucosidase (EC. 3.2.1.2.1) in a single tightly bound molecular association complex (88.7 kDa). SDS-PAGE of the molecular complex dissociates into two main components. Of these, at a concentration of 75%, the corn coleoptile  $\beta$ -glucosidase (60 kDa) is identified by enzymatic activity, with two 16-amino acid tryptic peptides displaying close homology with the primary structure of the enzyme. In separate experiments, we isolated homogenous monomeric enzyme of corn coleoptile. This allowed us to conclude that lectin properties like erythrocyte agglutination, found in the (88.7 kDa) molecular complex, is not due to the  $\beta$ -glucosidase bound in it. Another protein (30 kDa) dissociated from the same SDS-PAGE gels rendered several tryptic peptides, including a 20-amino acid sequence V(L)GP(Q)W(A)GGSGGSPVDITAEPQR closely homologous to the putative  $\beta$ -glucosidase aggregating factor (BGAF) precursor described recently. Tryptic peptide SAFTE(A)WN(V)ELK(V) was also present in the BGAF precursor. KFHEQR peptide was not present in BGAF precursor or any other protein sequence examined. Tryptic peptide TYGPFGA showed good homology with the BGAF precursor protein, FEGLYLFHTPLGSGAN peptide displayed identity with the BGAF precursor sequence. Thus, the 30 kDa protein does not appear to be identical to BGAF, but is rather a similar molecule which could be endowed with the lectin properties of the 88.7 kDa molecular complex. © 2004 Elsevier B.V. All rights reserved.

**Keywords:** Corn coleoptile lectin;  $\beta$ -Glucosidase;  $\beta$ -Glucosidase molecular association; Galactose lectin; *Zea mays*

### 1. Introduction

Several authors have already identified and studied lectin in corn coleoptile [1,2] and in corn grain [3,4]. A recent paper described studies done on purified lectin in which it was isolated by cold acetone precipitation followed by lactose-sepharose affinity chromatography. This work indicates that the purified lectin shows preferential affinity for galactose, either free or in glycoconjugates, as well as hemagglutination capacity to ABO human groups and red cells of various animals. In non-dissociating gel electrophoresis, the lectin is

present as a single broad band with a molecular mass of 88.7 kDa [5].

In this article, we present results that demonstrate that purified corn coleoptile lectin shows strong  $\beta$ -glucosidase activity in the same affinity chromatography samples displaying agglutination. When examined in SDS-PAGE with mercaptoethanol [6], purified lectin dissociates into two main bands [7]. We observed enzymatic activity and carried out sequence analysis of two amino acid peptides (18 amino acids each) displaying 100% homology with published enzyme sequences. The results show that the band with a molecular mass of 60 kDa is  $\beta$ -glucosidase [8–10], (EMBL/GenBank/DDBJ database accession number P49235). The second, more anodic band (30 kDa), produced tryptic peptides with useful sequences, several of them similar to the  $\beta$ -glucosidase aggregating factor (BGAF) precursor protein sequence, which was recently described [11,12].

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These results identify the lectin, described previously as purified corn coleoptile lectin [2,5], as a molecular complex containing  $\beta$ -glucosidase in close association with other proteins (peptides) that appear endowed with lectin properties. On the other hand, homogeneous monomeric  $\beta$ -glucosidase from corn coleoptile, isolated in separate experiments, showed no hemagglutinating activity.

This investigation places the lectin–enzyme complex in the field of corn physiology. Contrary to the lectin, the tightly associated  $\beta$ -glucosidase, controlled by gene ZM-p60.1 [13], is known to have a clear role in the liberation of active growth factors and various metabolites [14–16], and may also participate in defense mechanisms in corn [17,18]. It appears that the galactose specificity of the lectin and the glucoytic specificity of the enzyme, tightly bound in the same (88.7 kDa) molecular complex, are not fortuitous events.

## 2. Material and methods

Purified corn coleoptile lectin was prepared using 500 g wet/weight coleoptiles from Berentsen 58-302, certified by the Mexican Department of Agriculture (Secretaria de Agricultura de México). The corn germinated for 5 days in covered trays at 23 °C. As described before [5], the crude homogenate was acetone-precipitated in the cold (4 °C), dissolved in 70 ml 0.2 M citrate buffer at pH 6.4, and affinity-separated through a Sepharose 6B-lactose (Sigma Co., USA) 1.5×20-cm column inside a cold cabinet at 4 °C.

Tubes 5–15 (Fig. 1) were pooled (25 ml), dialyzed overnight against 50 mM sodium acetate pH 4.6, and the precipitate recovered by centrifugation at 7500×g and 4 °C. It was then washed three times with 6 ml each of 50 mM sodium acetate pH 4.6 solution before being dissolved in 3 ml of 0.2 M citrate pH 6.4 cold buffer. A second precipitation and set of washings rendered a preparation displaying two main bands only.

The affinity purified lectin was tested in 96 conic well plates for hemagglutination activity with human freshly obtained type O red cells. The titer was estimated using the last dilution that produced clear agglutination of 2% washed human red cells. In the same affinity-recovered samples,  $\beta$ -glucosidase activity was assayed using *p*-nitrophenyl  $\beta$ -glucopyranoside (Sigma) as substrate following the technique in Ref. [8]. One unit of enzyme activity was equal to 1  $\mu$ M of nitrophenol/hour at 23 °C room temperature.

SDS-PAGE [6] of the molecular complex (88.7 kDa) was stained with Coomassie blue and the bands present were analyzed by densitometry. A Helena Junior 24 (France) densitometer at 580 nm was used to obtain the relative concentration of the bands separated from the molecular complex.

Another sample of the molecular complex (88.7 kDa) was electrophoresed to PVDF membranes, according to the method described in Refs. [19,20]. The membrane was stained with Coomassie blue and the 60 kDa band was cut out and sent to HHMI Biopolymer Facility/W.M. Keck

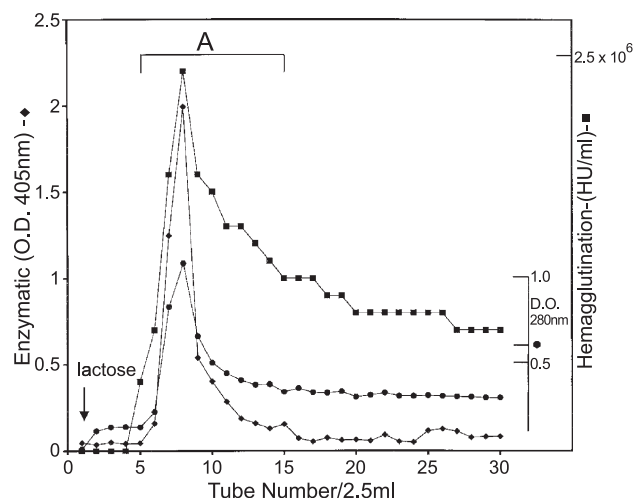


Fig. 1. Affinity purification of corn coleoptile lectin and  $\beta$ -glucosidase. Corn coleoptile extract after acetone precipitation was dissolved in 0.2 M citrate pH 6.4 buffer and poured onto a (2×20) Sepharose-lactosyl column. It was then washed exhaustively with the same buffer and eluted using 0.2 M lactose in the cold at 4 °C, as shown above. The fraction eluted in tubes 5–15 were pooled and dialyzed against 50 mM sodium acetate pH 4.6 overnight in the cold room. The precipitate was recovered by centrifugation at 4 °C and centrifuged at 7000 rpm, then dissolved in 3 ml citrate buffer pH 6.4 and tested for hemagglutination activity in the presence of 2% human erythrocytes type O in PBS.  $\beta$ -Glucosidase activity was tested using *p*-nitrophenyl  $\beta$ -D-glucopyranoside as substrate. Lactose (0.15 M) was used for elution as shown in the figure.

Biotechnology Research Laboratory at Yale University, New Haven, USA, for sequencing using Edman degradation chemistry. The amino-terminal and the Edman degradation were performed using the Applied Biosystems model 470A automated sequencer and manufacturer's procedures.

The proteins were then subjected to 26 cycles of N-terminal sequencing to obtain an internal amino-terminal sequencing. The 60 kDa protein from SDS-PAGE was digested with 1  $\mu$ g of trypsin for 6 h at 37 °C. The peptides produced by digestion were separated from HPLC and a peptide with a predicted mass of 1824 Da was used with the Edman degradation method to obtain the internal amino-terminal sequence.

The second band in the gel (30 kDa) was dissected and sequenced as described earlier. Tryptic peptide No. 25 displayed a short sequence showing no homology with known sequences. Peptide No. 68 consisted of a peptide mixture in small amounts that could not be used for sequencing. Peptide No. 96 displayed a 10-amino acid sequence and peptide No. 89 revealed a 20-amino acid sequence. Peptide No. 119 rendered a 7-amino acid peptide present in the BGAF precursor protein sequence, as well as a 16-amino acid sequence closely similar to the same BGAF precursor protein. These sequences were compared with published protein primary structures by means of protein databanks (GenBank AAFT1261).

In separate experiments, a homogeneous sample of corn coleoptile  $\beta$ -glucosidase was prepared according to the technique described in Ref. [8]. Briefly, the corn coleoptile extract prepared in 50 mM sodium acetate pH 4.6 was placed

in a small, 1 × 5 cm Bio-Rad (USA) cationic column, and the β-glucosidase was isolated using a 5.1–6.4 pH, 50 mM sodium acetate stepwise gradient. The preparation displayed β-glucosidase activity. It was then examined in SDS-PAGE electrophoresis for the presence of a single neat component with a molecular mass of 60 kDa, corresponding to the corn coleoptile homogenous monomeric β-glucosidase [8].

To determine if the molecular complex identified by affinity chromatography could be discerned in whole homogenates, avoiding the acetone precipitation, we set up a gel filtration column (2.5 × 60 cm) with Sepharose 6B and 1 M sodium chloride as the eluant. A sample of 5 ml of fresh corn coleoptile homogenate clarified by centrifugation was separated into 5.5 ml samples for agglutinin activity and enzymatic activity assays. Commercial (Sigma) proteins were employed for molecular weight standardization of the elution volume of the column [21]. The protein in the samples was estimated using the Coomassie blue binding test and bovine serum albumin as standards [22].

### 3. Results

Fig. 1 presents the results of the Sepharose-lactosyl affinity column (1.5 × 20 cm) of the acetone-precipitated sample of corn coleoptile extract. It is evident that the hemagglutination activity peak and the β-glucosidase activity peak eluted out at the same position: when approximately 200 ml of 0.15 M lactose buffer had passed through the column.

Results in 4%, 7.5%, 10% and 12% separate acrylamide gels (against proteins of known molecular mass as controls) revealed that a sample from the chromatography pool (tubes

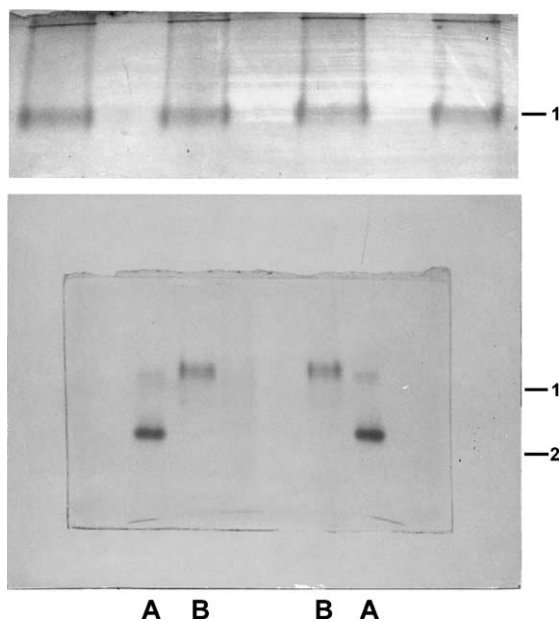


Fig. 2. Native-PAGE (pH 8.3) of the corn coleoptile lectins and β-glucosidase isolated in affinity chromatography as association complex. The complex is present as a single diffuse band of multiple identical samples. (1 and B) Molecular complex. (2 and A) Bovine serum albumin.

Table 1

Lectin and β-glucosidase activities in the molecular association complex		
	Hemagglutination (units/mg)	Enzymatic (units/mg)
Corn coleoptile molecular association complex (88 kDa)	45,000	160
Corn coleoptile homogenous β-glucosidase monomer	2	200

Hemagglutination specific activity and β-glucosidase specific activity of the molecular complex isolated and purified from Sepharose-lactosyl affinity chromatography (as shown in Fig. 1) compared with β-glucosidase activity exclusive in the (60 kDa) enzyme monomer, from separate experiments (Fig. 5).

5–15) in 0.2 M citrate buffer pH 6.4 displayed a single broad band with a molecular mass of 88.7 kDa. This was identical to the work using purified corn coleoptile lectin reported in previous publications [2,5] (Fig. 2). The pool sample revealed a protein content of 4.3 mg using a Coomassie blue binding test (Bradford). The hemagglutination activity was  $20 \times 10^6$  units, almost 75% of the hemagglutination activity of the whole preparation. The same sample displayed a β-glucosidase activity of 160 units/mg/protein (Table 1).

When the molecular complex precipitate was analyzed in SDS-PAGE by the Laemmli procedure [6], two main bands were consistently present in the Coomassie blue-stained gel. The estimated molecular mass values were 60 kDa for the more anionic band and 30 kDa for the second band (Fig. 3).

The blue-stained gel was examined in a densitometer and the relative band concentration obtained as shown in (Fig. 4). The first band (75%) was cut from the gel and tryptic digestion was used to obtain sequences from the complete 60 kDa protein and from a 1824 kDa polypeptide. The results were IGVQMLSPSEIPQRDWFP and LAGSYNMLGL-

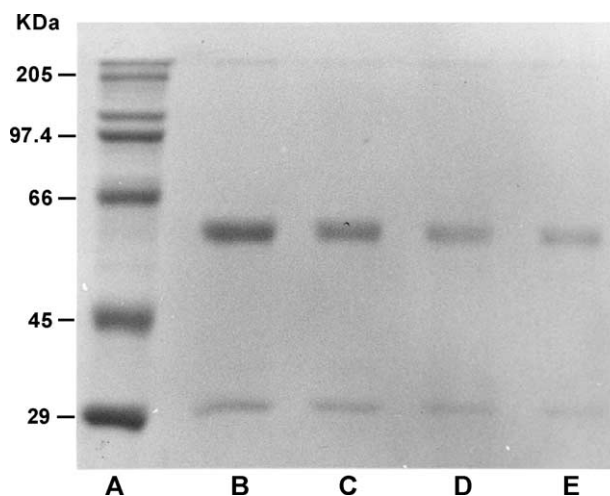


Fig. 3. SDS-PAGE of affinity-purified corn coleoptile lectin and β-glucosidase molecular complex. Two dissociated protein bands are present from the single protein in PAGE (Fig. 2) One of the 60 kDa bands corresponding to monomeric β-glucosidase and a 30 kDa protein from the hypothetical lectin. The molecular weight markers are: myosin (205 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), oboalbumin (45 kDa) and anhydrase (29 kDa). (Line A) Molecular markers. (Lines B to E) Molecular complex concentrations: (B) 10 μg, (C) 5 μg, (D) 3 μg, (E) 2 μg.

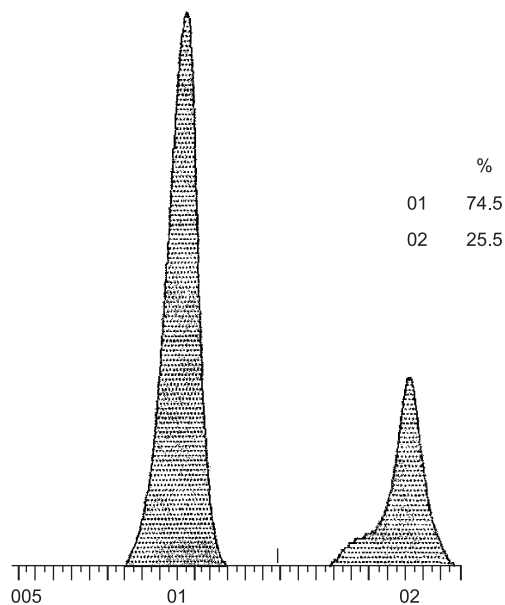


Fig. 4. Densitometric analysis of Coomassie blue stained SDS-PAGE protein bands, using a Helena Junior densitometer at 590 nm. The first protein band (60 kDa) comprised 75% of the sample, corresponds to corn coleoptile  $\beta$ -glucosidase (see Fig. 5). Protein band two appeared to be 25% of the total, which could be endowed with the lectin activity.

NYYTTSR, respectively (Table 2). A computer search in protein databases (EMBL/GenBank/DBJ database, accession number P49235) showed that both N-terminal sequences matched perfectly with various glucosidases from *Zea mays*, including  $\beta$ -glucosidase chloroplast precursor [8,9].

The more anionic band (25%) was separated from the gel and processed like the first band. Several tryptic peptides

were obtained (Table 2). Peptide No. 96 gave a sequence SAFTE(A)WN(V)ELK(V) and peptide No. 89 a sequence V(L)GP(Q)W(A)GGSGGSPVDITAEPQR. The first sequence was compared with the signal peptide sequence, a sequence from a recently described primary structure of putative aggregating factor (BGAF) [12]. This structure binds  $\beta$ -glucosidase in corn coleoptile preparations from “null” varieties of corn described previously (Essen and Blanchard) [11]. The results showed 90% statistically non-significant homology (Yale Center).

The second sequence was homologous to sequence 161–180 of the same BGAF, including an 11-amino acid sequence also present in the primary structure of Jacalin, a plant  $\alpha$ -galactose specific lectin [23]. A third tryptic peptide from the same band (30 kDa) was sequenced as KFHEQR, but no homology was found in either  $\beta$ -glucosidase or in the BGAF precursor protein primary structure (Table 2). Two more sequences were obtained from tryptic peptides: TYGPFGA (peptide 119 A), homologous to BGAF mature protein, and FEGLYLFHTPLGFGAN (peptide 119 B), homologous to peptide signal, containing a third peptide not present in BGAF precursor protein (Table 2).

We then proceeded to test for lectin properties in corn coleoptile  $\beta$ -glucosidase (identified as part of the 88.7 kDa molecular complex). A homogeneous sample of corn coleoptile  $\beta$ -glucosidase was isolated in separate experiments, according to the technique described in Ref. [8]. The sample was very active (200 units/mg protein) against *p*-nitrophenyl  $\beta$ -glucopyranoside, and a single band of 60 kDa was obtained in SDS-PAGE (Fig. 5). The sample presented negligible agglutination using human type O erythrocytes (Table 1).

Table 2  
Molecular complex tryptic peptide sequences from SDS-PAGE (Fig. 3)

(Band1) (60 kDa)	a.a. sequences from tryptic peptides	Observations
Peptide 1	62                      79 IGVQMLSPSEIPQRDWFP	100% homology corn $\beta$ -glucosidase ①
Peptide 2	375                      390 LAGSYNMLGLNYTTSR	
(Band 2) 30 kDa	a.a. sequences from tryptic peptides	Observations
Peptide 25	KFHEQR	no-homology
Peptide 68	(no sequence)	peptide mixture (MALDI-TOF)
Peptide 89	160                      180 V(L)GP(Q)W(A)GGSGGSPVDITAEPQR	95% homology with BGAF promoter ②
Peptide 96	10                      19 SAFTEWNELK	90% homology with BGAF peptide signal ②
Peptide 119 (A)	258    264 TYGPFGA	100% homology with BGAF mature protein ② (sequence underlined)
Peptide 119 (B)	20                      36 FEGLYLFHTPLGSGAN	81% homology with BGAF mature protein ②

①EMBL/GEN BANK/DBJ, P49235

②GEN BANK.AAFT1261

Sequence composition of tryptic peptides from SDS-PAGE protein bands comprising the molecular complex. Protein band 1, peptides homologous with  $\beta$ -glucosidase primary structure. Peptides in band 2, postulated to correspond to a 30 kDa protein with lectin activity, associated with the enzyme in the original molecular complex.

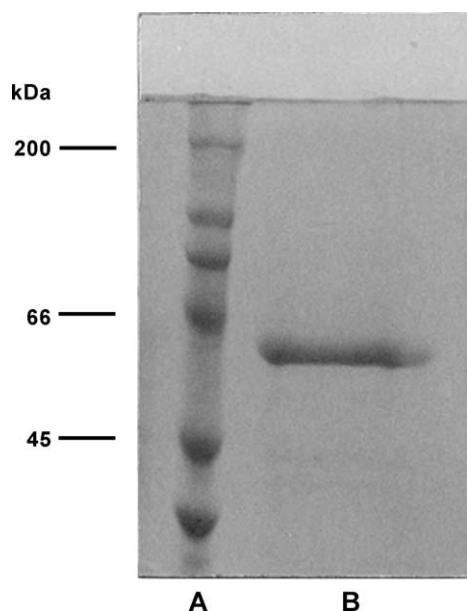


Fig. 5. SDS-PAGE of isolated and purified corn coleoptile β-glucosidase (60 kDa) monomer as a single protein band from separate experiment. Molecular weight markers are: myosin (200 kDa), bovine serum albumin (66 kDa), ovoalbumin (45 kDa). (A) Molecular markers. (B) Corn coleoptile β-glucosidase.

These results suggest that the lectin activity is not due to the 60 kDa enzyme firmly integrated in the molecular complex. Rather, it appears that the molecular complex lectin capacity resides in the 30 kDa protein disclosed in the dissociated gel, and/or is an inherent property of the whole molecular complex itself.

Finally, experiments using gel filtration chromatography of whole corn coleoptile homogenate in 1 M NaCl allowed us to demonstrate the presence of a single peak containing both agglutination and enzymatic activities. Extrapolation of the elution volume of the collected single peak against elution volumes of proteins with known molecular weights gave us a molecular weight value of 88 kDa, very similar to the value obtained for the molecular complex isolated by the usual affinity purification procedure (Fig. 1).

#### 4. Discussion

In this paper, we present results that confirm the isolation and purification of a corn coleoptile lectin described previously in this laboratory [2,5]. We also present results that demonstrate that the purified corn coleoptile lectin is actually an 88.7 kDa molecular association complex containing the 60 kDa β-glucosidase of corn coleoptile, as well as a 30 kDa protein apparently endowed with the hemagglutination capacity that the molecular complex exhibits. The molecular complex (88 kDa) was also evidenced in gel filtration experiments of whole corn coleoptile homogenates (Fig. 6).

These results suggest that the lectin–enzyme complex of high molecular weight is present and active in the growing corn coleoptile. This is probably due to the fact that β-glucosidase is an essential enzyme that, by means of splitting β-glucosyl linkages, releases various corn metabolites [14], hydroxamic acid [15], active agents that protect corn from European cork borer, such as 2:4 dihydroxy-7-methoxy-1–4 benzoxazin [16], secondary plant metabolites that protect it from aphid–plant interaction [17], as well as other defense chemicals, including a number of hydroxamic acids [18]. β-Glucosidase is also active in the release of novel phytohormones [24].

As we have shown in earlier publications, the purified corn coleoptile lectin (identified in this paper as the molecular complex) appears to be present not only in corn, but also in teosinte *Zea diploperennis*. This presence further underscores the importance of β-glucosidase for all species of the *Zea* genus [5].

As for the binding forces and the mechanism that produce such a strong association between a lectin and β-glucosidase in corn coleoptile, we can only speculate. At this time, we suspect that binding links and a mechanism similar to the ones postulated by Esen and Blanchard [11] for BGAF are present in this new case. When we consider the sequence similarities found between the 30 kDa protein and BGAF amino acid sequence, it is tempting to assume that a similar molecular organization mechanism is found in both molecular complexes. However, with BGAF, the determining difference resides in the activity of a lectin displaying clear galactose hemagglutination specificity in close molecular equilibrium with β-glucosidase, even in whole tissue homogenates that one can assume are close to a natural environment.

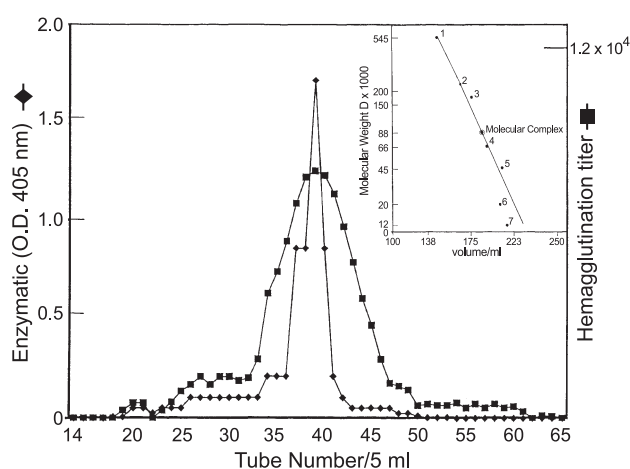


Fig. 6. Gel filtration experiment in a column (2.5×60 cm), with Sepharose 6 B developed with 1 M NaCl. 10 ml whole 0.1 M citrate sodium, pH 6.4 corn coleoptile homogenate was applied to the column and 5 ml fractions collected. Molecular weight markers. (Sigma): 1—Urease (545 kDa); 2—β-amylase (200 kDa); 3—γ-globulins rabbit (150 kDa); 4—serum albumin bovine (66 kDa); 5—ovoalbumin (45 kDa); 6—trypsin inhibitor (20 kDa); 7—cytochrome *c* (12.4 kDa). Inset: calibration curve indicating an 88.7 kDa molecular weight for the dual peak of the molecular complex.

Esen and Blanchard [11] attribute this protein a chaperone role in BGAF association, one that protects the enzyme from external proteolysis inside the plant cell. We cannot discount a similar role in the molecular association described here; their antigen–antibody model appears valid, too. In spite of this, a further role for the lectin in the 88.7 kDa corn coleoptile molecular complex remains to be disclosed, as the agglutination with galactose specificity found appears to be a significant finding.

Isolation experiments now underway will help resolve the issue of the existence of lectin activity in the molecular complex. A possibility is that the 30 kDa protein is active by itself. More likely, however, is that we will find that the high hemagglutination titers consistently present in the coleoptile preparations are the result of the supra-molecular organization of the complex described. In that case, a reciprocal chaperone effect would exist between the active proteins involved.

At the present time, the nature of the structural properties and binding forces that participate in the organization of the molecular complex is puzzling. It appears clear that the molecular enzyme–lectin complex is not due to an extraction-purification artifact, since it is present with the same molecular weight (88.7 kDa) and hemagglutination and enzyme activity in fresh crude coleoptile homogenates as in the complex isolated by affinity chromatography. Thus, despite being faced with a true molecular organization with a clear role in plant physiology and development, we cannot at this time advance any idea about the surface complement that reinforces and stabilizes the molecular complex itself.

The molecular complex with active  $\beta$ -glucosidase displays greater activity approximately 7 days after germination *in vitro* [5]. This suggests that the complex and the enzyme within it are important at that critical moment in corn development when the availability of corn promoters and defensive factors are most needed. It is perhaps for the same reason that a molecular form of enzyme bound to a galactose-specific lectin becomes the appropriate structure to perform this essential role in the tender, 7-day-old plantlet. Further experiments are in progress in this direction.

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