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DETECTION OF SOYBEAN GLYCOCONJUGATES ABLE TO INTERACT WITH SOYBEAN AGGLUTININ

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Abstract—Soybean seed glycoconjugates able to interact with soybean agglutinin (SBA) have been detected by their ability to inhibit SBA-induced haemagglutination. Preliminary characterization of the inhibitory factor(s) present in the seed cotyledons suggests it is a glycoprotein released into soluble form by homogenization. Its solubility properties, resistance to endogenous hydrolases and inability to bind to concavalin A distinguishes it from the major storage proteins of the seed.

INTRODUCTION

Seed lectins, particularly those of the legumes, have been characterized extensively [1]. In contrast, relatively little information is available concerning seed glycoconjugates that can interact with the lectins and may represent endogenous 'receptors' of physiological significance. In this report, the inhibition of haemagglutination is used as a semi-quantitative assay for 'receptors'. A similar approach has been applied previously for the detection of 'receptors' in erythrocyte cell membranes [2] and slime moulds [3]. The results suggest that soybeans contain glycoconjugates that have the ability to bind to soybean agglutinin.

RESULTS AND DISCUSSION

The assay was first standardized using purified soybean agglutinin (SBA) and a variety of defined glycoproteins, polysaccharides and monosaccharides (Table 1). Previously, inhibitors of the carbohydrate binding sites of SBA were determined using a spectrophotometric method [4] or in an assay based on lectin-induced precipitation of glycoconjugates [5]. The pattern of inhibition of haemagglutination by monosaccharide derivatives was similar to that observed in the previous studies. Removal of sialic acid from fetuin was found to greatly enhance the effectiveness of the glycoprotein as an inhibitor, presumably due to the exposure of sub-terminal galactosyl residues on the oligosaccharides [6]. Interestingly, galactomannans did not inhibit SBA-induced haemagglutination. This contrasts with recent observations in which the interaction of the polysaccharides with SBA was studied using a radioaffinity assay based on polyethylene glycol-induced precipitation of lectin-receptor complexes [7]. The association constant of SBA binding to galactomannan was found to be higher than that of lectin binding to fetuin or thyroglobulin [7]. It may be possible that the lack of inhibition of galactomannans in the haemagglutination assay, reflects steric hindrances between the oligomeric lectin, glycoconjugates on the erythrocyte surface and polysaccharides in solution.

Soybeans contain SBA predominantly within the cotyledons of the seed [8]. When levels of the lectin in extracts prepared from the cultivar Williams used in this study were compared using radioimmunoassay (RIA) and haemagglutination assay (HA), results shown in Table 2 were obtained. RIA estimates the total amount of lectin protein present in the tissues, whereas detection of lectin by the semi-quantitative HA is dependent on multivalent carbohydrate-binding activity. To calculate the amount of

Table 1. Inhibition of SBA-induced haemagglutination by defined glycoconjugates

Glycoconjugates	Minimum concentration of glycoconjugates (μg) and monosaccharides (mM) required to inhibit 4 HU of SBA
Fetuin	15.6
Asialofetuin	1.9
Thyroglobullin	5.8
Ovomucoid	>125
Mucin	>125
Galactomannan (Guar gum)	>25
Galactomannan (Locust bean)	>25
N-Acetyl-D-galactosamine	0.0234
pNP β-D-galactosaminide	0.0518
pNP α-D-galactoside	0.130
1-*O*-Methyl-α-D-galactoside	0.146
D-Galacturonic acid	0.146
1-*O*-Methyl-β-D-galactoside	0.198
pNP-α-D-galactoside	0.415
D-Lactose	0.781
γ-L-Galactonolactone	1.17
D-Galactosamine	2.34
D-Galactose	4.68

pNP = *p*-nitrophenol. Purified SBA gave a haemagglutination activity of 2560 HU/μg. 4 HU is equivalent to 1.5 ng SBA.

lectin present using HA, it must be assumed that SBA exhibits the same haemagglutination activity/mg lectin protein assayed as a pure protein or as a component within a seed extract. For cotyledons, axis and seed coat, estimations of lectin content by RIA were higher than those calculated from HA, suggesting a proportion of the endogenous SBA was inactive in the haemagglutination assay.

Prior to assaying the ability of soybean components to inhibit SBA-induced haemagglutination, all the endogenous lectin in the extracts was removed by passage through Sepharose-*N*-caproylgalactosamine [9]. The lectin-depleted extracts were then assayed for inhibitory activity towards a standardized amount of SBA. Results shown in Tables 2 and 3 are expressed as protein equivalents required to inhibit four haemagglutination units of SBA, i.e. 1.5 ng lectin which gave a titre of 4. It was found that axis and cotyledon extracts could be substantially diluted yet maintain their effectiveness as inhibitors of SBA. No activity was found in extracts prepared from seed coats.

It was possible that the inhibition was caused by seed components interacting with the erythrocyte surface rather than with the lectin. For example, soybean α-galactosidase can bind to erythrocytes under certain conditions and catalyse the hydrolysis of terminal galactosyl residues [10]. To investigate these possibilities, lectin-depleted extracts from cotyledons were incubated with an erythrocyte suspension and the red cells were then removed by centrifugation. The supernatant was found to contain the same inhibitory activity towards SBA and on resuspension the erythrocytes could again be agglutinated by SBA as before. In addition, as shown in Fig. 1, the amount of inhibitor required in the HA was dependent on the concentration of SBA present and could be expressed as a linear relationship on a log scale.

Table 2. Distribution of SBA in soybean seeds estimated by RIA and HA

	Amount of lectin estimated (μg SBA/g fr. wt)	
4 hr imbibed seeds	RIA	HA
Cotyledon	1010	650
Embryonic axis	244	18.9
Seed coat	4.8	0.48

Seeds were imbibed for 4 hr and used to prepare extracts as described in the Experimental section. Haemagglutination activity could be fully inhibited by 20 mM GalNAc.

Table 3. Ability of soybean extracts to inhibit SBA-induced haemagglutination

4 hr imbibed seeds	Protein content (mg/ml)	Amount of extract required to inhibit 4 HU of SBA	
		dilution	μg protein
Cotyledon	6.4	128	1.25
Embryonic axis	5.4	64	2.11
Seed coat	0.065	no inhibition	

Seeds were imbibed for 4 hr and extracts were prepared and depleted of endogenous lectin as described in the Experimental section.

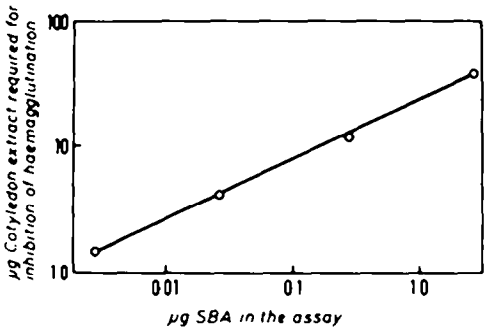

Fig. 1. Amount of cotyledon extract required to inhibit SBA. Haemagglutination activity was assayed using a constant volume of trypsin-treated erythrocytes. Serial dilutions of a cotyledon extract depleted of endogenous lectin were made in PBS. To each serial dilution, differing amounts of SBA were added in the range 1.5 ng–1.5 μg.

In summary, these results suggested that a component(s) of cotyledon and axis extracts had the ability to interact with SBA in such a way as to inhibit carbohydrate-binding activity of the lectin. When the lectin is present with these components such as in a seed extract, sufficient excess SBA would be present for the extract to express a positive haemagglutination activity irrespective of relative association constants between SBA and components of the seed and/or erythrocyte surface.

The inhibitory activity found in cotyledon extracts was further characterized as shown in Table 4. Results indicated that the factor(s) was recovered as a soluble component after centrifugation of the extract at 100 000 *g*, was retained within dialysis tubing and could be precipitated by 10% (w/v) TCA or excess cold acetone. Periodate oxidation abolished the inhibitory activity of the extract, indicating the involvement of carbohydrate. The factor(s) remained in solution at pH 4.0 under conditions that precipitate the majority of the soybean storage proteins glycinins and conglycinins [11]. Solubility and inhibitory activity was also maintained after one hour at 95°. Low levels of inactivation were caused by a 12 hr incubation at 30°, a treatment known to lead to extensive hydrolysis of cotyledon components by endogenous proteinases and glycosidases [12]. The factor(s) did not bind to concanavalin A (ConA) Sepharose, distinguishing it from mannosylated glycoproteins in soybeans such as the conglycinins [13]. It is probable that the glycoconjugate(s) in the seed extract responsible for inhibiting SBA-induced haemagglutination is a minor component with a high binding affinity for the lectin. As shown in Table 1, 1.9 μg of asialofetuin (molecular weight 45 K), equivalent to 0.79 μM of pure glycoprotein was required for the inhibition of 1.5 ng SBA, compared to 1.25 μg of total cotyledon protein. Other studies have indicated a K_d of 6.7×10^{-7} M for the direct interaction between asialofetuin and SBA [7].

The cotyledon extract was also found to contain inhibitory activity towards other lectins: ConA, wheat germ agglutinin (WGA) and peanut agglutinin (PNA).

Table 4. Characterization of the inhibitor(s)

Treatment of lectin depleted extract from cotyledons	Protein content (mg/ml)	Amount required to inhibit 4 HU of SBA	
		dilution	μg protein
None	6.4	128	1.25
Extract centrifuged at 100000 *g*, supernatant assayed	4.0	128/256	0.52
Extract made pH 4.0, centrifuged at 10,000 *g*, supernatant assayed	1.3	128	0.253
Extract dialysed vs. PBS, non-diffusible substances assayed	6.2	128/64	1.62
Extract made 10% (w/v) TCA, supernatant assayed after dialysis	0.110	no inhibition	—
Extract made 5 vols excess acetone (−20°) supernatant assayed after dialysis	0.010	no inhibition	—
Butanol extraction: (assayed after dialysis)			
aqueous phase	1.8	32	1.40
organic phase	0.010	no inhibition	—
Extract heated at 95° 1 hr, centrifuged at 10000 *g*, supernatant assayed	1.9	32	1.48
Extract incubated at 30° for 12 hr	5.12	32	4.02
Periodate oxidation	6.0	no inhibition	—
Extract applied to Con A-Sepharose, effluent assayed	5.1	256	0.498

Extracts were prepared, depleted of endogenous SBA and processed as described in the Experimental section.

The amount of cotyledon extract required for inhibition of 4 HU of these lectins (assayed with trypsin-treated erythrocytes) was 1.0–4.0 μg protein. This was a similar order of magnitude as for the inhibition of SBA. When the lectin-depleted extracts of soybean cotyledons were passed through ConA-Sepharose, only inhibitory activity towards ConA was removed. The factor(s) responsible for the inhibition of factor WGA and PNA was retained in the effluent. Inhibitory factor(s) specific for ConA and WGA was precipitated by treatment at pH 4.0, whereas that which inhibited PNA was soluble at this pH.

There is some evidence to suggest that at seed maturity, SBA is principally located within the protein bodies of the cotyledons and axes [14]. However, on homogenization, the relative fragility of the organelle membranes compared to that of the cell wall, leads to release of lumenal contents and recovery of the lectin and storage proteins in the soluble fraction. Although the inhibitor(s) of SBA was also found in the soluble fraction, it is not known whether its location *in situ* in the seed was cytoplasmic, within an organelle or within the cell wall. To determine the physiological significance of the inhibitor(s) as a potential receptor to SBA, its subcellular location must be established.

EXPERIMENTAL

Materials. Soybean seeds (*Glycine max* cv. Williams) were provided by the US Intsoy Program 1982 with a germination efficiency of 100%. SBA was prepared by affinity chromatography using Sepharose-*N*-caproylgalactosamine [9] and iodinated using chloramine T [7]. Glycoproteins: fetuin (type 3), thyroglobulin (Bovine, type 1) and polysaccharides were obtained from Sigma Chemical Co. Sialic acid was removed from fetuin by treatment with acid [15] to give asialofetuin. Goat anti-rabbit was from Behringer Institute Behringewerke, A.G. Marburg, West Germany. Galactomannans were used at 0.1% (w/v) concentration at which guar gum and locust bean galactomannan were soluble; if higher concentrations were used, viscous solutions resulted [16].

Haemagglutination assay. Rabbit erythrocytes were washed × 3 in PBS (10 mM KH_2PO_4/K_2HPO_4-145 mM NaCl, pH 7.4) and were treated with trypsin (1 mg/ml packed cells) for 30 min 35°. Cells were washed a further × 3 and resuspended in PBS at 2%. Lectin activity was assayed in microtitre plates where 25 μl of lectin were serially diluted with PBS. To each well 25 μl of the erythrocyte suspension was added and after 30 min at room temp. the haemagglutination titre was recorded. Inhibition of lectin activity was quantified by serially diluting the potential inhibitor with PBS and adding a standardized quantity of lectin.

Radioimmunoassay. Double-antibody complexes were formed by incubating rabbit anti-SBA with goat anti-rabbit serum, in the presence of 1 mg cytochrome *c* and RIA buffer [2% (w/v) Triton X-100, 0.2 M Gal, 0.5 M NaCl]. ^{125}I-SBA (3 pmol) and 1–200 pmol of unlabelled SBA were mixed with RIA buffer and added to the incubation. Incubations were mixed (Eppendorf shaker) for 2 hr at 4°. Complexes were recovered by centrifugation at 10000 *g* for 3 min (Eppendorf microfuge). Pellets were then washed twice with RIA buffer before determining radioactivity in a Rack Gamma (LKB) and construction of a standard curve using a spline function. For estimation of SBA in seed tissues, extracts were prepared by sonication in RIA buffer (1 g fr. wt./10 ml) and centrifuged at 10000 *g* prior to assaying.

Preparation of extracts for detection of inhibitory activity. Soybean seeds imbibed for 4 hr were dissected into cotyledons, embryonic axis and seed coats. The tissues were extracted in PBS (1 g fr. wt./10 ml) using a pestle and mortar, sonicated on ice (4 × 10 sec using a microprobe fitted to a Dawe Sonicator) and centrifuged for 5 min at 1000 *g* to provide a debris-free supernatant. SBA was removed by passage through the affinity matrix [9] at 4° in PBS. Protein was assayed using the Coomassie dye method [17].

Treatment at SBA-depleted extracts. Extracts were precipitated with either 10% (w/v) TCA for 20 min at 4°, or with 5 vols-20° cold Me_2CO for 20 min at 4°, or were titrated with 1 M citric acid to give a pH of 4.0. After each treatment, soluble material was recovered by centrifugation at 10000 *g* for 3 min and supernatants were dialysed against PBS before assaying for inhibition of haemagglutination. Alternatively, extracts were ultracentrifuged 100000 *g* for 3 hr at 4° before the supernatants were

assayed. For determination of lipid solubility, the lectin-depleted extracts were dialysed against H_2O and extracted with BuOH (1:1) on ice. Aqueous and organic phases were separated by centrifugation, dialysed against PBS and assayed. The stability of the inhibitory factor(s) was investigated by incubating the extract at 30° for 12 hr and assaying directly, or by heating at 95° for 1 hr and centrifugation at 10000 *g* before assaying the supernatant. For periodate oxidation, extracts were treated with 100 mM $NaIO_4$ for 18 hr at 4° [18] and dialysed against PBS before assaying.

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