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BINDING OF HYDROLASES FROM WHEAT ALEURONE TO CONCANAVALIN A- AND WHEAT-GERM AGGLUTININ-SEPHAROSE

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; aleurone layers; concanavalin A; Con A-Sepharose; glycosidases; hydrolases; wheat-germ agglutinin; WGA-Sepharose; wheat grains.

Abstract—We have studied the ability of hydrolases (acid phosphatase and glycosidases) from the aleurone layers of resting wheat grains to interact with Con A- and WGA-Sepharose as a way to examine their glycoprotein nature. Aliquots (6–85% depending on the enzyme) of all the enzymes interacted with Con A-Sepharose. The major part of α-mannosidase activity (85%) was present in this form. Aliquots (2–20% depending on the enzyme) of the following four enzymes, β-galactosidase, α-mannosidase, β-*N*-acetylglucosaminidase and acid phosphatase, interacted with WGA-Sepharose. All the enzymes were also found in forms which were unable to interact with either lectin. No forms of hydrolases interacting with both lectins were found in the crude extract. The specific activities of most of the enzymes recovered from the lectin-Sepharose gels were greater than those measured in the crude extract. In particular, the highest specific activities were found for β-*N*-acetylglucosaminidase and β-galactosidase recovered from WGA-Sepharose. Different lectin-binding forms of hydrolases were compared with respect to pH optimum and stability under various conditions (heat and guanidine hydrochloride treatments). The lectin-binding pattern of the hydrolases released in the incubation medium by the aleurone layers was similar to that reported above for the enzymes extracted from these tissues, suggesting that none of the hydrolase forms found in the aleurone layers is selectively released during incubation of these tissues.

INTRODUCTION

Because of their ability to interact with specific sugars, either free or glycosidically linked, the plant lectins, such as concanavalin A (Con A) and wheat-germ agglutinin (WGA), covalently attached to a Sepharose support, have been widely used to isolate and characterize animal glycoproteins, including many lysosomal hydrolases [1, 2]. Recently, the glycoprotein nature of glycosidases from mature seeds of mung bean has been examined by their interaction with Con A-Sepharose [3].

The purpose of the present investigation was to study the ability of hydrolases extracted from the aleurone layers of resting wheat grains to interact with Con A- and WGA-Sepharose as a way to examine the glycoprotein nature of these enzymes. It is well known that the aleurone layers of resting cereal grains contain a variety of hydrolases which are secreted into the starchy endosperm during germination [4]. Although extensively studied as far as their relationship to gibberellic acid is concerned [5–7], these enzymes have been poorly characterized and little is known about their molecular properties, such as glycoprotein nature and the structure of the oligosaccharide moiety. However, it is of interest to elucidate such molecular aspects since the carbohydrate moieties of these enzymes could be involved in the secretion process [8] and/or in their targeting to specific sites, as demonstrated for mammalian lysosomal glycoprotein hydrolases [9, 10].

RESULTS AND DISCUSSION

Fractionation of the hydrolases by sequential incubations of the crude extract with Con A- and WGA-Sepharose

As expected, the crude extract from the aleurone layers of resting wheat grains exhibited acid phosphatase and glycosidase activities (Table 1). In addition to hydrolases, whose occurrence in these tissues has already been reported [4–7], we detected β-mannosidase activity, giving, to our knowledge, the first experimental evidence for the presence of this enzyme in this plant tissue.

Sequential incubations of the crude extract with Con A- and WGA-Sepharose allowed us to obtain three fractions of hydrolases showing the following lectin-binding characteristics: ability to interact with Con A (fraction I), ability to interact with WGA but not Con A (fraction II), inability to interact with either lectin (fraction III). The hydrolases of fraction I interacted with Con A, but not with WGA; in fact, no binding of hydrolases to WGA was observed when the eluate from Con A-Sepharose was incubated with WGA-Sepharose. Therefore, none of the hydrolases considered was found in forms interacting with both lectins.

Each enzyme was present in the crude extract in multiple forms showing different lectin-binding characteristics (Table 1). Aliquots of all the enzymes (6–85% depending on the enzyme) appeared to be glycoproteins containing structures recognizable by Con A. The largest part of α-mannosidase (85%) was present in this form. Studies on structural determinants for the binding of the *N*-glycoside type glycopeptides to Con A-Sepharose have indicated that at least two non-substituted or 2-*O*-substituted α-mannosyl residues are required [11, 12]; for that, the high mannose-type glycopeptide and, to a lesser extent, the complex-type glycopeptides, possessing two peripheral branches of sialic acid → galactose → *N*-acetylglucosamine (the so-called 'biantennary' glycopeptides) are bound by Con A, whereas the complex-type glycopeptides possessing three of the above peripheral branches ('triantennary' glycopeptides) are not [11, 12].

Table 1. Fractions of hydrolases separated by sequential incubations of the crude extract with Con A- and WGA-Sepharose*

Enzyme	Crude extract	Fraction I		Fraction II		Fraction III	
	Total activity (units)	Activity bound to Con A		Activity bound to WGA		Activity WGA-unbound	
		(units)	(% of crude extract)	(units)	(% of crude extract)	(units)	(% of crude extract)
α-Galactosidase (EC 3.2.1.22)	955	55	5.8	0	—	900	94.2
β-Galactosidase (EC 3.2.1.23)	153	46	30.5	31	20.2	75	49.2
α-Glucosidase (EC 3.2.1.20)	36	21	57.0	0	—	15	43
β-Glucosidase (EC 3.2.1.21)	171	86	50.4	0	—	85	49.5
α-Mannosidase (EC 3.2.1.24)	160	136	84.9	2	1.4	21	13.6
β-Mannosidase (EC 3.2.1.25)	125	10	8.5	0	—	114	91.5
β-N-Acetylglucosaminidase (EC 3.2.1.30)	351	154	43.8	47	13.4	150	42.7
Acid phosphatase (EC 3.1.3.2)	3816	289	7.6	77	2.0	3448	90.4

* Details of the fractionation procedure are given in the Experimental. Parallel incubation of the crude extract with albumin-Sepharose was used as a control for non-specific adsorption and for enzyme degradation during incubation. The extent of binding to the lectin-Sepharose gels was calculated from the difference between the values of hydrolase activities of the washings from the columns of albumin-Sepharose and lectin-Sepharose.

Aliquots (2–20% depending on the enzyme) of the following enzymes, β-galactosidase, α-mannosidase, β-*N*-acetylglucosaminidase and acid phosphatase, interacted with WGA-Sepharose. This interaction was highly specific, since the following sugars were unable to replace *N*-acetyl-D-glucosamine (GlcNAc) in eluting the hydrolases bound to WGA-Sepharose: D-glucose, D-mannose, L-fucose, D-galactose. In addition, the WGA-binding fraction inhibited the hemagglutinating activity of WGA (Carratù *et al.*, manuscript in preparation). It has been reported that the primary structural determinants for binding to WGA-Sepharose are the *N,N'*-diacetylchitobiose moiety and a GlcNAc residue linked to C-4 of the core β-linked mannose residue, as found in hybrid-type oligosaccharides [13]. Thus, such structural determinants could be the sites of interaction for this hydrolase fraction with WGA-Sepharose.

All the enzymes occurred also in forms which were unable to interact with either lectin. Such forms, therefore, may either be non-glycoproteins or contain carbohydrate moieties unreactive with either lectin.

Experiments indicating that the heterogeneity of hydrolases is not caused by the action of glycosidases during the fractionation procedure

Removal of sugar units from the oligosaccharide moiety of a glycoprotein might cause either the loss (if the removal alters the structural determinants for the binding) or the gain (if additional sugars reducing the lectin affinity are removed) of the ability to interact with a given lectin [14, 15]. Then, the varying lectin-binding forms of each enzyme might originate from a single glycosylated form, during the fractionation procedure, because of the removal of sugar units by glycosidases. In the experimental conditions we adopted for fractionating the crude extract (operations carried out at 4°, presence of 5×10^{-3} M $HgCl_2$ in the extraction buffer), the glycosidase activities which might act on the oligosaccharide moiety of glycoproteins [16] were, if present, completely inhibited. In fact, when aliquots (100 μl) of crude extract were added with each (0.6 μmol) of the *p*-nitrophenyl-glycosides (α-D- and β-D-mannopyranoside, α-L- and β-D-fucopyranoside, α-D- and β-D-galactopyranoside, *N*-acetyl-β-D-glucosaminide, *N*-acetyl-β-D-galactosaminide, α-D- and β-D-glucopyranoside) and incubated for 6 hr at 4°, no release of *p*-nitrophenol from these substrates was observed. No *endo*-β-*N*-acetylglucosaminidase or sialidase activities were detected under the same experimental conditions by using [^{3}H]-Dansyl-Asn $(GlcNAc)_4$ $(mannose)_6$ and mucin respectively as substrates [17, 18]. Thus, it is likely that the heterogeneity of lectin-binding forms of hydrolases occurring in the crude extract from aleurone layers reflects the *in vivo* situation rather than being caused *in vitro* during fractionation.

Extent of purification of the hydrolases eluted from lectin-Sepharose gels

0.4 M Methylmannoside and 0.2 M *N*-acetyl-D-glucosamine (GlcNAc) eluted, to an appreciable extent, the hydrolases bound to Con A- and WGA-Sepharose, respectively (Table 2). The specific activities of most of the enzymes recovered from the lectin-Sepharose gels were greater than those measured in the crude extract. It is noteworthy that the highest specific activities were found for β-*N*-acetylglucosaminidase and β-galactosidase recovered from WGA-Sepharose. Thus, affinity chromatography on lectin-Sepharose may be a useful tool in the purification of these enzymes.

pH *Optimum and stability of different forms of hydrolases*

As shown in Table 3, all the enzymes considered exhibited an acid pH optimum. No difference of pH optimum was observed between the different fractions of each enzyme.

It has been shown that the carbohydrate moiety of a glycoenzyme may have a stabilizing effect on the enzymatic activity under a variety of conditions [19, 20]. Since the heterogeneity of lectin-binding forms of each enzyme observed in this study has to reflect differences in the composition and/or structure of the carbohydrate moieties of these enzymes, it seemed interesting to compare the different fractions of the same enzyme with respect to heat stability and to treatment with a denaturating agent such as guanidine hydrochloride. The data, reported in Table 3, show that most differences were slight, with two possible exceptions. Firstly, β-galactosidase of fraction III appeared to be more stable to heat treatment than that of the other two fractions, and secondly, although all three forms of acid phosphatase were equally susceptible to denaturation on incubation in 4 M guanidine hydrochloride, the recovery of the enzyme activity in fractions I and II was greater than in fraction III when the concentration of the denaturant was reduced by dilution. Although a more detailed analysis is required, it is possible that these differences are related to variations in the carbohydrate structures of the enzymes.

Lectin-binding characteristics of hydrolases released by the aleurone layers during incubation

As expected [4–7], the aleurone layers released hydrolases in the medium during incubation (Table 4). Sequential fractionations of these enzymes with Con A- and WGA-Sepharose gave a pattern similar to that obtained with the hydrolases extracted from the aleurone layers (Table 1), except for β-galactosidase and α-glucosidase, which interacted with the lectin-Sepharose gels to a lesser extent. However, since the forms of β-galactosidase from the aleurone layers capable of interacting with the lectin-Sepharose gels were found to be less stable to heat than the others (Table 3), inactivation of these forms might occur during incubation. Thus, the pattern of hydrolases found in the incubation medium of the aleurone layers suggests that none of the forms of each enzyme was selectively released during incubation.

Conclusion

This study has demonstrated that, as observed for lysosomal hydrolases [1] and glycosidases from mature seeds of mung bean [3], hydrolases (acid phosphatase and glycosidases) from the aleurone layers of resting wheat grains occur in glycosylated forms recognizable by lectins. The structural sugar determinants for the binding to Con A are more common and/or more accessible to this lectin than those for the binding to WGA. In fact, Con A-Sepharose was found to interact with a larger number of hydrolases and to a greater extent than WGA-Sepharose.

For each enzyme, multiple forms having different

Table 2. Elution of hydrolases from the lectin-Sepharose gels*

Enzymes	Crude extract	Con A-Sepharose				WGA-Sepharose			
		Activity recovered				Activity recovered			
	Sp. act. (units/mg)	Total act. (units)	(% bound)	Sp. act. (units/mg)	Purification (-fold)	Total act. (units)	(% bound)	Sp. act. (units/mg)	Purification (-fold)
α-Galactosidase	4.15	44	79	3.9	0.9	0	—	—	—
β-Galactosidase	0.66	35	76	3.1	4.7	27.8	89	23.16	35.09
α-Glucosidase	0.16	15	72	1.3	8.3	0	—	—	—
β-Glucosidase	0.74	50	58	4.4	6.0	0	—	—	—
α-Mannosidase	0.69	103	75	9.1	13.2	1.6	72	1.33	1.92
β-Mannosidase	0.54	8	84	0.7	1.3	0	—	—	—
β-*N*-Acetylglucosaminidase	1.52	105	68	9.3	6.1	42.5	90	35.41	23.29
Acid phosphatase	16.59	231	79	20.4	1.2	63.7	81	53.08	3.20
Total protein (mg)	230			11.3				1.2	

*The hydrolases bound to the columns of Con A- and WGA-Sepharose were eluted respectively with 0.4 M methylmannoside and 0.2 M GlcNAc in 50 mM citrate buffer (pH 6.2) as reported in Experimental.

Table 3. pH Optima and stability of various lectin-binding forms of hydrolases*

Property	Acid phosphatase			α-Mannosidase			β-Galactosidase			β-*N*-Acetylglucosaminidase		
	Fr. I	Fr. II	Fr. III	Fr. I	Fr. II	Fr. III	Fr. I	Fr. II	Fr. III	Fr. I	Fr. II	Fr. III
pH optimum	4.6–5.4	4.6–5.4	4.6–5.4	4.6	4.6	4.8	4.5	4.5	4.5	5.2	5.2	5.2
Heat stability (relative activity %)												
40° for 1 hr	95	90	98	100	100	100	76	76	100	100	100	100
50° for 15 min	52	54	62	100	100	100	14	13	36	78	75	75
60° for 15 min	5	5	12	73	70	76	0	0	25	10	11	14
Guanidine HCl treatment (relative activity %)												
Denaturation	15	10	14	0	0	0	0	0	0	5	5	5
Renaturation	74	69	25	18	16	10	10	13	12	43	43	34

*The pH optimum was determined in the pH range 4–5.8, using citrate buffer. To determine the heat stability, aliquots of the different fractions were pre-incubated without substrate at the indicated temperatures and times and then assayed at 37° under standard conditions. Denaturation by guanidine hydrochloride was studied by incubating aliquots of the different fractions, concentrated 20-fold, in the presence of 4 M guanidine hydrochloride, at 4° for 30 min. After this time, the samples were diluted 20-fold with 50 mM citrate buffer (pH 5) containing (for denaturation assay) or without (for renaturation assay) 4 M guanidine hydrochloride and the enzyme activities were assayed under standard assay conditions. The final concentration of guanidine hydrochloride in the reaction mixtures for the denaturation assay was 4 M.

Table 4. Binding to lectin-Sepharose gels of hydrolases released by the aleurone layers in the incubation medium*

Enzyme	Total activity in the medium (units)	Activity bound to Con A-Sepharose (% of total act.)	Activity bound to WGA-Sepharose (% of total act.)	Activity WGA-unbound (% of total act.)
α-Galactosidase	30	4.5	0	95
β-Galactosidase	7	7.2	1.5	91
α-Glucosidase	3	16.4	0	83
β-Glucosidase	24	42.3	0	57
α-Mannosidase	8	66.7	0.7	32
β-Mannosidase	6	5.4	0	94
β-*N*-Acetylglucosaminidase	17	34.3	11.3	54
Acid phosphatase	355	6.5	1.8	91

*1 g of aleurone layers was incubated under aseptic conditions in 3 ml of buffer A at 20° for 72 hr with agitation. After this time, the medium was collected, centrifuged at 12000 *g* to remove the precipitate, dialysed overnight against the same buffer, and then processed as described for the crude extract of aleurone layers (see legend to Table 1). The ratio medium/lectin-Sepharose was 2:1.

lectin-binding characteristics were found to occur in the crude extract. This heterogeneity seems to reflect the *in vivo* situation rather than changes caused *in vitro* during the fractionation procedure by the action of glycosidases on the oligosaccharide moiety of the enzymes.

A similar lectin-binding pattern was observed for the hydrolases released from the aleurone layers during incubation, suggesting that none of the forms of each enzyme was selectively released during incubation.

Although the interaction of WGA with animal hydrolases has been frequently reported [1], this is, to our knowledge, the first report dealing with the binding of WGA to hydrolases extracted from the same organ where the lectin occurs.

EXPERIMENTAL

WGA and all other organic chemicals were purchased from Sigma. Cyanogen bromide-activated Sepharose, WGA-Sepharose 6B (5 mg/ml sedim. gel), Con A-Sepharose 4B (10 mg/ml sedim. gel) and the materials for gel filtration were purchased from Pharmacia. Bovine serum albumin was coupled to Sepharose 6B according to the instructions of the manufacturer. Grains of wheat (*Triticum aestivum* L. cv Libellula) were obtained from the Consorzio Agrario (Mantova, Italy).

Plant material. Aleurone layers were prepared under aseptic conditions according to ref. [21]. Dry de-embryonated grains were sterilized for 20 min in 1% Na hypochlorite, rinsed several times in sterile H_2O and incubated on moistened filter paper in Petri dishes for 3 days at 25°. After this time, the aleurone layers with attached coats were easily removed from the starchy portion of the endosperm.

Enzyme assays. The respective *p*-nitrophenyl derivatives were used as substrates for measuring the activity of the following acid hydrolases: acid phosphatase, α- and β-galactosidase, α- and β-mannosidase, α- and β-glucosidase and β-*N*-acetylglucosaminidase. Unless stated otherwise, assay mixtures contained, in addition to the enzyme, 1.2 μmol substrate, 250 μg Triton X-100 and 10 μmol citrate buffer (pH 5) in a total vol. of 0.2 ml. After 30 min incubation at 37°, the reaction was stopped by the addition of 0.7 ml 0.2 M Na_2CO_3 and A_{420} was determined [22]. In all cases, 1 unit is defined as the amount of enzyme that hydrolyses 1 μmol of substrate per 30 min at 37°. Specific activity is expressed as units per mg of protein.

Fractionation of the crude extract by sequential incubations with Con A- and WGA-Sepharose. All operations were carried out at 4°. Usually, 6 g of tissue was extracted in a glass homogenizer with 60 ml 50 mM citrate buffer (pH 6.2) containing 0.2 M NaCl and 5 $\times 10^{-3}$ M $HgCl_2$ (buffer A). $HgCl_2$ was added to the extraction buffer to inhibit the glycosidase activities during the fractionation procedure. The $HgCl_2$ inhibition was reversible since the enzyme activities were recovered by removing this salt by dialysis. The homogenate was centrifuged at 12000 *g* for 1 hr and the supernatant, after dialysis against buffer A (crude extract, total vol. 60 ml), was fractionated by sequential incubations with Con A- and WGA-Sepharose as described below. The crude extract was first incubated with 30 ml bed vol. of Con A-Sepharose which had been equilibrated previously with buffer A. The suspension was kept under agitation for 4 hr and then transferred within a column to pack the gel. The column was washed until no hydrolase activities were detected in the washings and the washings were concd to 60 ml by ultrafiltration with a PM 10 membrane filter (Amicon). Two fractions were obtained: the Con A-bound fraction (fraction I) and the unbound one. Under these experimental conditions, the binding capacity of the lectin-Sepharose gel was not exceeded; in fact, when the unbound fraction was re-incubated with Con A-Sepharose, no binding of hydrolases to the lectin was observed. The unbound fraction was then incubated with 30 ml bed vol. of WGA-Sepharose which had been equilibrated with buffer A. The suspension was kept under agitation for 1 hr and then processed as described for Con A-Sepharose. In this case too, the binding capacity of the lectin-Sepharose gel was not exceeded. A WGA-bound fraction (fraction II) and a unbound one (fraction III) were obtained. Hydrolases bound to the columns of Con A- and WGA-Sepharose were eluted with 0.4 M methylmannoside and 0.2 M GlcNAc in buffer A, respectively. Before assaying the hydrolase activities, the unbound fraction and the eluates from the lectin-Sepharose gels were dialysed overnight against 50 mM citrate buffer (pH 6) to remove $HgCl_2$ and the eluting sugars, and centrifuged at 10000 *g* for 30 min to remove the ppt. formed during dialysis. When the eluate from the column of Con A-Sepharose was incubated, after dialysis against buffer A, with WGA-Sepharose and processed as described above, no binding of hydrolases to the lectin-Sepharose gel was observed. This demonstrates that no hydrolases capable of interacting with both lectins occurred in the crude extract.

Protein determination. The method of ref. [23] was used with bovine serum albumin as standard.

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