

Glycan Research on Barley, Maize, Oats, and Sorghum Grain α -Amylases: Comparison with Rice α -Amylase

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α -Amylases from germinated maize, oats, rice, and sorghum were isolated by glycogen precipitation and hydrophobic interaction chromatography. Several methods were used for the detection of glycoproteins, including barley α -amylase isozymes purified as previously described and using the rice α -amylase as a positive control for glycosylation. Affinoblotting using concanavalin A, immunoblotting using a xylose-specific serum which reacts with complex N-linked glycans, and endo- β -N-acetylglucosaminidase H treatment of amylases gave negative results for maize, oats, sorghum, and barley. However, after deglycosylation with trifluoromethanesulfonic acid, the molecular weight of one maize α -amylase constituent was clearly decreased. The same result was obtained after β -elimination in mild conditions. Together these results indicated probable O-linked glycosylation of one maize α -amylase when barley, oats, and sorghum α -amylases did not appear to be glycosylated. Chemical deglycosylation of rice α -amylase resulted in the production of two polypeptides with different molecular weights.

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Secreted proteins are synthesized by polysomes bound to the endoplasmic reticulum and pass through the Golgi complex. Most proteins which are sorted in the Golgi are glycoproteins but a few examples of nonglycoproteins which accumulate in vacuoles and protein bodies have been found in plants (3). However, demonstration of the nonglycosylation of a plant-secreted protein is not actually available.

The glycosylation of the main rice α -amylase and the crucial role of the Golgi complex in the intracellular traffic of the enzyme are well established ((4) for review). Two pathways of rice amylase secretion by the scutellum

have been distinguished (5). The two corresponding forms differ by their sensitivity to endo- β -N-acetylglucosaminidase, and thus by their glycan content. On the contrary, only puzzling observations are available concerning the presence of oligosaccharide moieties in seed α -amylases from other cereals. Positive staining reactions for carbohydrate were obtained, by different ways, with α -amylase purified from barley (6, 7) and sorghum (8) or secreted by aleurone layers of barley (9). Carbohydrate analyses indicated a low amount of sugar and it was concluded that secreted α -amylases were heterogeneous with respect to glycosylation (9). Tunicamycin inhibited the secretion of α -amylase in barley by 60 to 80% (10) and a possible glycosylation site was found in sequences corresponding to the high-pI enzyme (11). On the other hand, unpublished results of Jacobsen *et al.*, cited in Ref. (12), indicated that the bulk of barley α -amylase is probably unglycosylated. Negative results were obtained by these authors for incorporation of radioactive sugars, Con A binding, and endo H hydrolysis.

α -Amylases of maize, oats, and sorghum seeds were characterized and shown to bear common epitopes with high-pI and low-pI isoenzymes of barley (13). The experiments reported here aimed at determining if the purified α -amylases of barley, maize, oats, and sorghum are glycoproteins. Affinity for concanavalin A, immunodetection by antibodies specific for glycans modified in the Golgi, and enzymatic and chemical removal of oligosaccharides were used for this glycan research on purified proteins, including rice α -amylase as a positive control. We present evidence that barley, oats, and sorghum α -amylases are nonglycosylated. One maize α -amylase is glycosylated, like the rice enzyme, but their glycosylation modes appear different.

MATERIALS AND METHODS

Purification of α -amylases. α -Amylases were extracted from 6-day-germinated grains of maize (*Zea mays* L., cv Dea), barley (*Hordeum vulgare* L., cv Menuet), sorghum (*Sorghum bicolor* L., cv Oasis),

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rice (*Oryza sativa* L., cv Cigalon), and oats (*Avena sativa* L., cv Avalanche) after removing the roots and shootlets. For barley, separate isoenzyme groups were prepared as previously described (1). For the other cereals, grains were homogenized in a mortar in the presence of 50 mM Na-malate, 50 mM NaCl, 2 mM CaCl_2 , pH 5.2 (5 ml/g). After standing for 30 min, the extracts were ethanol and glycogen precipitated according to Schramm and Loyter (14). The precipitate was resuspended in extraction buffer and incubated for 1 h at 37°C to digest the glycogen. The preparation was dialyzed with 50 mM Na-acetate, 1 mM CaCl_2 , pH 5.0, and then 25% saturated with $(\text{NH}_4)_2\text{SO}_4$ at 20°C. The preparation was chromatographed on a 1 × 8-cm column of octyl-Sepharose CL-4B (Pharmacia), using a decreasing linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (25–0%) and an increasing linear gradient of ethylene glycol (0–30%), with a flow rate of 0.5 ml/min. Fractions displaying α -amylase activity were pooled, dialyzed against the Na-acetate buffer and acetone precipitated.

Protein determination and α -amylase assay. The protein content in crude extracts and purified α -amylases was determined by microtitration using Pierce BCA protein reagent, as described by Sorensen and Brodbeck (15).

α -Amylase was assayed with Enzyline reagent (BioMérieux) as previously described (16).

Carbohydrate staining. Strong alkali hydrolysis, TLC, and subsequent orcinol staining were performed according to Argade *et al.* (17), with 130 μg of each purified α -amylase.

Enzymatic and chemical deglycosylation. Enzymatic removal of the high mannose glycans with endo- β -N-acetylglucosaminidase H was performed according to Trimble and Maley (18). Deglycosylation with trifluoromethanesulfonic acid was carried out according to Edge *et al.* (19).

β -elimination in mild conditions was carried out for 50 min at 50°C with 0.05 N NaOH.

Gel electrophoresis, blotting, and detection methods. Proteins were separated by SDS-PAGE,² using the method of King and Laemmli (20) with 12% gels and a ratio of acrylamide to bisacrylamide of 200:1.

After endo H and TFMS experiments, SDS-gels were loaded with protein amounts allowing only the visualization, after Coomassie staining, of the major α -amylase of maize, oats, sorghum, and rice. This avoided difficulties of interpretation due to the presence of minor α -amylase components.

Before Con A/peroxidase staining or immunodetection, proteins were transferred from SDS-gels to nitrocellulose paper as described by Towbin *et al.* (21). N-linked oligosaccharides were characterized by affino blotting with Con A/peroxidase as described by Hawkes (22) but with gelatin as a blocking agent. Immunodetection of α -amylase after blotting was achieved with an equimixture of the barley α -amylase 1 and α -amylase 2 antisera previously used (23). It was recently shown (13) that these anti-barley sera recognized the different α -amylase isoforms of maize, oats, and sorghum. Modified N-linked glycans containing xylose were detected with a specific antiserum (2).

RESULTS AND DISCUSSION

Purification of Maize, Oats, Rice, and Sorghum α -Amylases

α -Amylases were purified by a two step procedure: glycogen precipitation and hydrophobic interaction chro-

matography on octyl-Sepharose. Elution diagrams on octyl-Sepharose (Fig. 1) were found similar for all cereals. α -Amylases were eluted between 6.5 and 9.5% ammonium sulfate (and 22 to 19% ethylene glycol) and thus seemed to present similar hydrophobicities. The purification schedule is reported in Table I for maize.

The purification was mainly achieved by glycogen precipitation (purification factor: 43) the second step allowing a further twofold enrichment. By SDS-PAGE (Fig. 2, left part), one minor contaminant band was visible in the glycogen precipitate (arrow) but undetected in the octyl-Sepharose fraction. From its molecular weight and glycogen binding, it can be assumed to be β -amylase. Starting from the same weight of seeds, protein amounts of the purified fractions were 65, 28, and 21% of the maize α -amylase amount for sorghum, rice, and oats respectively.

A precedent study (13) had shown that crude extracts of maize, oats, and sorghum contained two to three antigens with a very major one in each case. Rice contained also a major antigen and two minor ones (24). After glycogen precipitation and octyl-Sepharose, corresponding forms were detected by SDS-PAGE and Coomassie staining for each cereal. No contaminant band was visible on gels loaded with 10 μg protein, for each purified fraction. Identification of the purified fraction with α -amylases by western blotting is illustrated in Fig. 2 (right part). The validity of these barley α -amylase antibodies for characterization of other cereal isoforms was previously described (13). After SDS-PAGE of a crude extract, anti- α -amylase antibodies allowed the detection of several bands. The diagram obtained is identical to the one obtained for the purified fraction (Fig. 2, left part).

Thus, a fair degree of purification was obtained, for the enzymes of the four cereals, with this two-step method, which is simpler than the procedures used before for rice (25) and sorghum (8) α -amylases. A better yield was obtained by this procedure in comparison to the method of Silvanovich and Hill (26) using affinity chromatography after glycogen precipitation.

Carbohydrate Staining

After TLC of alkali-treated α -amylases and orcinol treatment, no clear staining was observed, even for the glycosylated rice α -amylase. This result can be explained by the low carbohydrate content in the rice enzyme (5). For this reason and also because purified α -amylases can be contaminated by strongly bound carbohydrates, direct analysis of the carbohydrate content was ruled out.

Affinity for Concanavalin A

Con A/peroxidase affino blotting after SDS-PAGE of the purified α -amylases was used to detect N-linked oligosaccharides which bind to Con A. The results indi-

² Abbreviations used: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; endo H, endo β -N-acetylglucosaminidase H; TFMS, trifluoromethanesulfonic acid; TLC, thin layer chromatography.

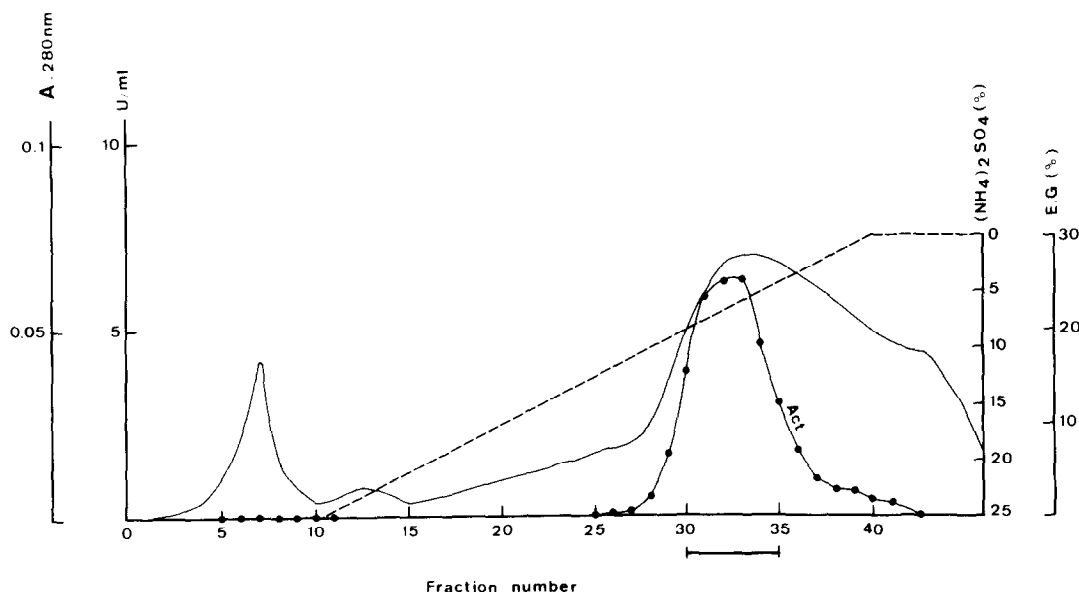


FIG. 1. Octyl-Sepharose elution profile of maize α -amylases purified from 10 g germinating seeds, with decreasing gradient of $(\text{NH}_4)_2\text{SO}_4$ and simultaneous increasing gradient of ethylene glycol (E.G.) (---). α -Amylase activity (Act) was determined with the Enzyline micro-assay.

cated that maize, oats, sorghum, and barley α -amylases are not Con A-binding glycoproteins (Fig. 3). A peroxidase staining was obtained only for rice α -amylase, as expected (Fig. 3). Not only high-mannose oligosaccharides sensitive to endo H, but also some complex side chains resistant to endo H were shown to interact with Con A (27). However, the differential affinity of Con A for glycoproteins is known, and the presence of high mannose oligosaccharides which poorly bind to Con A cannot be completely ruled out.

Sensitivity to Endo H

Endo H is known to specifically remove the high-mannose N-linked oligosaccharides. After enzymatic digestion of the different purified and denaturated α -amylases, products were analyzed by SDS-PAGE. A

modification of molecular weight was noted for rice major α -amylase (Fig. 4). In agreement with Mitsui *et al.* (5), digestion of the purified enzyme by endo H yielded two reaction products (Fig. 4, arrows), one resistant (R) and one susceptible (S) to the glycosidase action. These two products differed in molecular weight by about 2000 Da. No modification of molecular weight was noted for the other α -amylases (Fig. 4). These results, in conjunction with the experiments of Con A binding indicated the absence of high mannose N-linked oligosaccharides, in α -amylases of barley, and major maize, oats, and sorghum enzymes.

Use of a Xylose-Specific Antiserum

Applications of antibodies which bind primarily to the xylose β , 1 $\rightarrow$ 2 mannose structure commonly found in the complex glycans of plant glycoproteins, were recently described (2). Since the glycoprotein-modifying xylosyltransferase is located in the Golgi complex (28), a positive reaction with these antibodies would indicate that transport of the protein occurred via the Golgi. This antiserum was used here in western blotting after SDS-PAGE of the purified fractions (Fig. 5). As for affinity blotting, peroxidase staining was obtained only for rice α -amylase. This result is consistent with that of Mitsui *et al.* (5) who showed the presence of galactose, fucose, and xylose in the purified enzyme preparation by high-performance liquid chromatographic analysis. α -Amylases of barley, maize, oats, and sorghum are devoid of xylose β , 1 $\rightarrow$ 2 mannose structure and thus probably of complex glycans. The structure Man_3 (Xyl) (Glc

TABLE I
Purification of α -Amylase from 10 g
Germinating Maize Seeds

	Total proteins (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	111.5	160	1.43	100
Glycogen precipitate	2.06	126	61	79
Octyl-Sepharose	0.54	59	110	37

Note. Proteins were determined with the Pierce BCA microassay, and activity with the α -amylase Enzyline test.

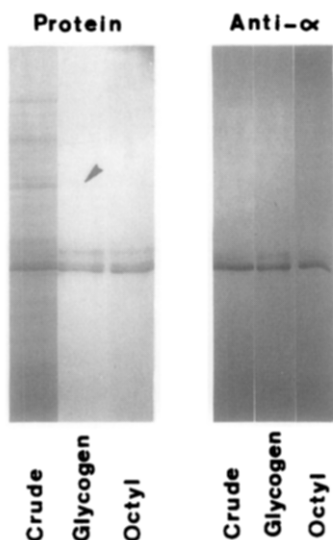


FIG. 2. Purification of maize α -amylases. Crude extract, glycogen preparation, and octyl-Sepharose fraction were analyzed by SDS-PAGE. Proteins were stained with Coomassie blue (left part) or electrotransferred onto nitrocellulose paper (right part). Anti-barley α -amylase antibodies were used for the immunodetection on nitrocellulose. With Coomassie staining, a contaminant protein is visible after glycogen precipitation (arrow).

$\text{N}(\text{Ac})_2$ Fuc, containing mannose, xylose, *N*-acetylglucosamine, and fucose has been found in several plant glycoproteins such as phaseolin, laccase, bromelain, Erythrina cristagalli, and Sophora japonica lectins (see references in (2)). However, the number of glycan structures precisely known till now is limited, and the presence of modified side chains which do not contain xylose cannot be excluded.

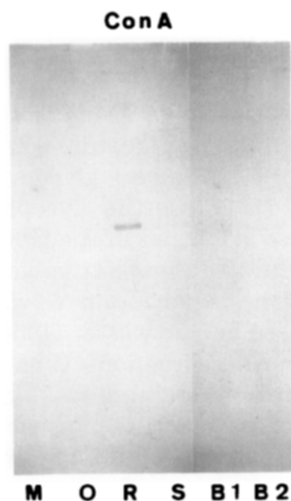


FIG. 3. Affinoblotting after SDS-PAGE using concanavalin A and peroxidase staining on purified α -amylases from maize (M), oats (O), rice (R), sorghum (S), and isoenzyme groups 1 and 2 from barley (B1 and B2). Each lane was loaded with the same amount of protein.

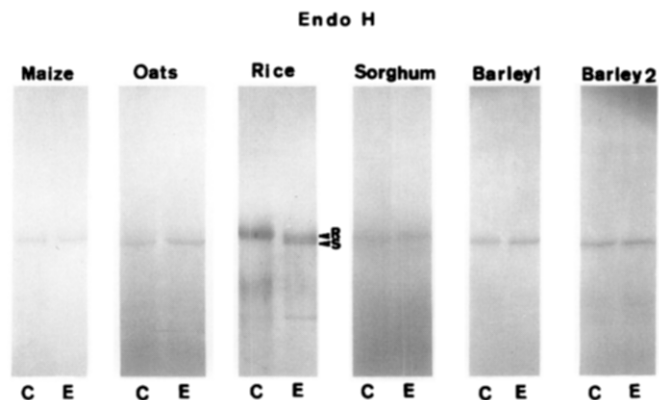


FIG. 4. SDS-PAGE analysis of the purified α -amylases from different cereals, after endo-glucosaminidase H digestion (E) in comparison with the untreated enzymes (control, C).

Deglycosylation by Trifluoromethanesulfonic Acid and β -Elimination

This chemical was shown to efficiently remove glycans without peptide backbone cleavage (19). By SDS-PAGE analysis after TFMS treatment of purified α -amylases, no clear modification of molecular weight in comparison with the untreated control could be evidenced for barley, oats, and sorghum enzymes (Fig. 6). In the case of rice, two reaction products were observed, called 1 and 2 (Fig. 6). Their molecular weights differed from the control by about 2000 and 4000 Da, respectively. The molecular weight of polypeptide 1 is similar to the molecular weight of the endo H-sensitive α -amylase (Fig. 4) and it is possible that this form contained only high-mannose



FIG. 5. Immunodetection with anti-xylose antibodies after blotting on purified α -amylases from maize (M), oats (O), rice (R), sorghum (S), and isoenzyme groups 1 and 2 from barley (B1 and B2). Each lane was loaded with the same amount of protein.

glycans. In this case, the endo H resistant α -amylase would correspond to the TFMS product 2 with a modified glycan content higher than the high-mannose content of the other form. But it is also possible that the endo H-sensitive form contains modified glycans. In this case, the two α -amylases would be modified further by the TFMS treatment in comparison to the endo H deglycosylation. The presence of identical polypeptide chains in S and R type molecules was investigated recently by Mitsui and Akazawa (29) who could not detect major difference between the two polypeptide chains, by peptide mapping after protease digestion analysis. A clear difference of molecular weight is found here between the two polypeptide chains obtained after complete deglycosylation, indicating deeper co- and post-translational modification differences between the two forms of rice α -amylase. In the case of maize (Fig. 6) two polypeptide chains were also obtained after TFMS treatment. One form had a decreased molecular weight in comparison to the control, the other is unchanged. This result indicates a polymorphism of this main α -amylase, with one glycosylated and one nonglycosylated form. The same pattern was obtained by β -elimination under mild conditions, which are inadequate to cleave N-glucosidic linkages (Fig. 7). Thus, and because negative results were obtained with Con A, xylose-specific antibodies, and endo H experiments, it is likely that one form of maize α -amylase is glycosylated via O-glycosyl bonds. This result is coherent with the efficient cleavage of O-glycosyl bonds by TFMS demonstrated by Edge *et al.* (19). Further studies are needed to confirm by other ways and to determine the O-linked glycan content of this maize α -amylase, reported here for the first time.

CONCLUSION

By the comparison of four methods of glycoprotein detection, it can be concluded that the two isoenzyme

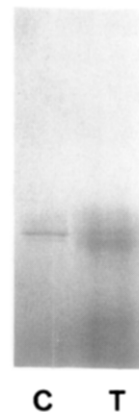


FIG. 7. SDS-PAGE analysis of the purified maize α -amylase, after mild β -elimination treatment (T) in comparison with the untreated enzyme (control, C).

groups of barley and the main component of oats and sorghum α -amylases are nonglycoproteins. The secretion of barley α -amylase from aleurone cells is well established and thus this is a first example of a plant-secreted protein which is not glycosylated. It is possible that the carbohydrates detected by several authors in the α -amylases purified from barley and sorghum were bound by affinity with the enzymes. O-linked glycosylation of a maize α -amylase form is shown. Thus, among the cereal α -amylases, the bulk of the protein can be glycosylated as in rice, not glycosylated as in barley, oats, and sorghum or heterogeneous as in maize with glycosylated and nonglycosylated forms. However, the possibility that some minor isoenzyme group(s) are glycoproteins cannot be excluded. Many questions have now to be resolved, concerning the structure of the glycan moiety as well as the intracellular transport of this maize α -amylase.

TFMS

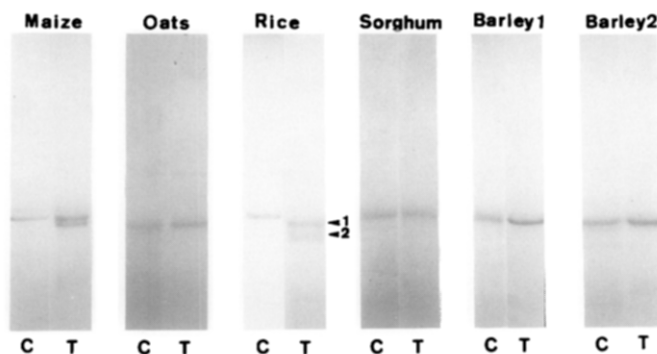


FIG. 6. SDS-PAGE analysis of the purified α -amylases from different cereals, after chemical deglycosylation with trifluoromethanesulfonic acid (T) in comparison with the untreated enzymes (control, C).

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