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COMPOSITION OF α-AMYLASE SECRETED BY ALEURONE LAYERS OF GRAINS OF HIMALAYA BARLEY

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Key Word Index—*Hordeum vulgare*; Gramineae; amino acid analysis; glycoprotein; α-amylase; seeds; secretion; gibberellic acid; enzyme purification.

Abstract—α-Amylases secreted by the aleurone layer of whole barley grains were relatively rich in histidine and relatively poor in glutamate/glutamine and serine when compared to other eukaryotic proteins. The secreted α-amylases had an estimated 0.5 residues each of glucose, mannose and *N*-acetylglucosamine per molecule of protein (MW 41 400 daltons), and gave positive staining reactions for carbohydrate on sodium dodecylsulfate polyacrylamide gels. Because the average α-amylase molecule had less than one sugar residue per enzyme molecule, it was concluded that secreted α-amylases were heterogeneous with respect to glycosylation. A second protein co-purified with α-amylase, but the amino acid composition of this protein was different from that of barley or wheat α-amylase. This protein was composed of two 21 500 dalton polypeptides. No significant amounts of L-leucine (^{14}C-U) were incorporated into this second protein in isolated aleurone tissue during incubation with gibberellic acid, perhaps because much of it was already present in the starchy endosperm at the time of hormone addition.

INTRODUCTION

The aleurone cells of intact germinating barley grains secrete α-amylase into the starchy endosperm, or into the bathing medium if isolated aleurone tissue is incubated with gibberellic acid [1]. Once α-amylase synthesis has begun, about 60 min are required for the synthesis and secretion of each molecule of enzyme [2]. Many secreted enzymes are glycoproteins [3, 4] and α-amylase is possibly one of these. A limited number of glycoproteins have been purified and characterized from higher plants including several phytohemagglutinins [5], some plant cell wall glycopeptides [6], an allergenicity factor from ragweed pollen [4], storage proteins such as vicilin and legumin [7] and a few enzymes including bromelain, ascorbic acid oxidase [4] and horseradish peroxidase [8]. In general, studies on the glycosylation of enzymes have been limited by the amounts of enzyme protein which can be purified and freed of non-covalently bound carbohydrate. One study concerning the glycosylation of barley α-amylase has already been made [9]; a number of sugars co-purified with α-amylase, but the molar ratio of each of these to the α-amylase polypeptide was not determined.

This paper deals with both the amino acid and carbohydrate compositions of the α-amylase secreted by the aleurone cells of grains of Himalaya barley. Since these cells secrete four isozymes of α-amylase [10] the composition reported here represents the average composition of the total enzyme.

RESULTS

α-Amylase was extracted from the bathing solution and starchy endosperm of Himalaya barley grains which had been incubated with 10 μM gibberellic acid for 4.5 days. The enzyme was partially purified then fractionated using ammonium sulfate. About 95% of the α-amylase precipitated at an ammonium sulfate concentration between 20 and 40% of saturation and the rest precipitated between 50 and 60% of saturation.

The 20 to 40% ammonium sulfate fraction was further characterized as follows. The enzyme solution was analyzed by electrophoresis on SDS-polyacrylamide gels showing the presence of two protein bands (Fig. 1). The first of these, corresponding to a MW of 41 400 daltons, was α-amylase and represented 80% of the protein on the gel. Protein in this band incorporated radioactivity when seeds without embryos were incubated with 1 μM GA and leucine (^{14}C-U) after 3 days of imbibition on sand without GA. This α-amylase was known to be composed of at least four isozymic variants which could be clearly separated by agar gel electrophoresis [10]; these isozymes could be resolved into two active α-amylase peaks by preparative isoelectric focusing. The position of the second band, designated the band-2 protein, corresponded to a MW of 21 500 daltons. The band-2 protein was not noticeably labeled with leucine (^{14}C-U) during GA treatment, and the amount of this protein was decreased if the starchy endosperm was removed before the isolated aleurone tissue was transferred to a medium containing GA. However, although the band-2 protein apparently was not synthesized during GA treatment, it had many properties in common with α-amylase which prevented the separation of the two proteins except by SDS-polyacrylamide gel electrophoresis. For example band-2 protein co-precipitated with α-amylase during the glycogen binding step, it co-precipitated with the bulk of the α-amylase during ammonium sulfate precipitation, it had the same approximate isoelectric point as the major α-amylase peak, and it eluted very slightly

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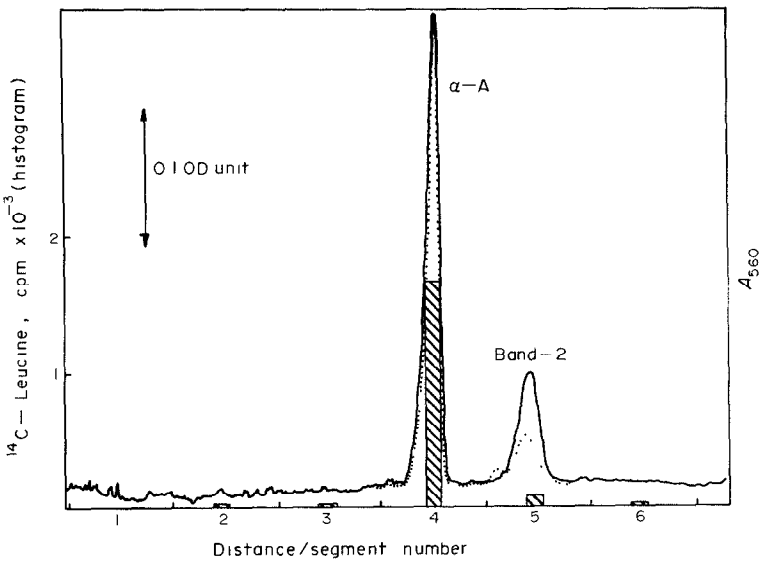

Fig. 1. Purity of the α-amylase after precipitation by glycogen. SDS-polyacrylamide gels were stained with Coomassie Brilliant Blue R250, then were scanned at 560 nm. Proteins secreted by barley grains solid line; from isolated aleurone tissue dotted line. The histogram represents the radioactivity associated with 1 cm sections of the gel which contained the α-amylase from isolated aleurone tissue.

ahead of α-amylase on Bio-Gel P-60, indicating that its native form was a dipeptide of approximately the same MW as α-amylase. Because band-2 protein is probably involved in starch hydrolysis in the Himalaya barley grains (since it binds to glycogen) and because it co-purified with the α-amylase, its chemical composition was determined along with the composition of barley α-amylase.

Amino acid composition

The amino acid compositions of the α-amylase band and the contaminating band-2 protein were determined by excising the respective areas of the SDS-polyacrylamide gels, eluting the protein from the gel pieces, and carrying out an amino acid analysis of the acid hydrolyzed protein (Table 1). Only tryptophan and cysteine could not be determined by this procedure. The number of residues of each amino acid per molecule of α-amylase was calculated without correcting for the losses of these amino acids; the number of the amino acid residues of band-2 protein was similarly determined, considering it as a dipeptide. The amino acid composition of wheat α-amylase [11] is shown for comparison.

A comparison of the proteins (Table 2) shows that barley α-amylase was reasonably similar to wheat α-amylase, although the former was comparatively lower in glutamate/glutamine, while the composition of both α-amylases were substantially different from the band-2 protein. Barley α-amylase was also compared to a 'standard' protein composition which had been arrived at by pooling data on the amino acid compositions of a number of proteins [12]. The barley α-amylase had 2.2 times the histidine content and 1.7 times the tyrosine content of this average protein composition, while having only 0.6 times the serine and glutamate plus glutamine contents.

Glycosylation

SDS-polyacrylamide gels containing α-amylase and the band-2 protein were stained for carbohydrate by two methods: the periodic acid-Schiff reaction and a procedure [23] whereby the periodate oxidation product was further reacted with dansylhydrazine

Table 1. Amino acid compositions of barley α-amylase, band-2 protein and wheat α-amylase

Amino acid	Number of residues per molecule		
	Barley α-amylase*	Band-2 protein (two peptides)†	wheat α-amylase‡
Ala	33.2	35.0	33.0
Gly	46.2	43 8	42.0
Val	22.3	21.6	24 8
Thr	16.0	19 4	20.4
Ser	13.9	24.0	20.4
Leu	25.3	26.4	27.4
Ilu	17.2	11.4	19 9
Pro	19.6	33.8	26 1
Met	6.5	3 4	6.2
Asp	49.1	39.6	41 5
Phe	16.8	16.6	14 3
Glu	26.5	29.8	41 0
Lys	22.2	16.8	18.8
Tyr	14 3	16 4	12.7
Arg	16.7	26.8	17.3
His	16.2	22.2	12.8
Cys	—§	—§	2 2
Trp	—§	—§	11.0

*Main band, Fig 1 The number of residues was calculated based on a MW of 41400 daltons, an estimated Trp content of 4.75% (weight %) and an estimated Cys content of 1.04%. Such are the Trp and Cys contents of wheat α-amylase.

†Calculated for two polypeptides, each with a MW of 21 500 daltons.

‡From Tkachuk and Kruger [11], based on a MW of 43300 daltons.

§Not determined.

Table 2. Comparison of the amino acid compositions of barley α-amylase, wheat α-amylase, the band-2 protein, and an 'average' protein molecule

Amino acid	Ratio †			
	Barley α-A / Wheat α-A‡	Barley α-A / Band-2 prot	Wheat α-A‡ / Band-2 prot	Barley α-A / avg protein§
Ala	1 05	0 99	0 93	0 96
Gly	1 13	1 09	0.95	1 01
Val	0 93	1 07	1.13	1 03
Thr	0 86	0 85	1 04	0 98
Ser	0 70*	0 60**	0 84	0 63**
Leu	0 97	1 00	1.03	1 18
Ilu	0.81	1.57**	1 74**	0 80
Pro	0 76**	0.60**	0 76*	0 85
Met	1 13	1 98**	1 81**	1 06
Asp	1 22	1 29	1 04	1 38*
Phe	1 25	1 05	0 85	1.29
Glu	0 67**	0.92	1 36*	0 57**
Lys	1 25	1 37*	1 11	1 07
Tyr	0 94	0 90	0 79	1 74**
Arg	1 02	0 64**	0 66**	0 96
His	1 15	0 76**	0 59**	2.17**

†Number of amino acid residues per molecule (Table 1) were normalized to the same protein molecular weight.

‡From Tkachuk and Kruger [11].

§From Krampitz and Fox [12].

**Double asterisks indicate the amino acids for which the mole% of that amino acid in protein was half again as great as the mole% of that amino acid in the second protein

With the periodate-Schiff reaction positive reaction products appeared at the positions of the two protein bands, indicating that sugar residues were covalently attached to the peptides. The peak height for α-amylase (72 units) was 0.46 A_{560} units and for the band-2 protein was 0.14 A_{560} units. The staining reaction was slow, however; while ovalbumin (4% carbohydrate) became colored within 24 hr of incubation in the final de-staining solution, several days were required for α-amylase and the band-2 protein to develop a color. With the dansylhydrazine procedure, again both α-amylase and the band-2 protein were stained, the positive reaction product being detectable under long-wavelength UV light of 350 nm. Gels containing α-amylase and band-2 protein, ovalbumin, or lysozyme which had been variously reacted with periodate and dansylhydrazine are shown in Fig. 2. Control gels were only treated with dansylhydrazine (no periodate oxidation step) so that the degree to which the dansylhydrazine became non-specifically associated with the protein moiety could be determined. Both α-amylase and the band-2 protein gave strongly positive reactions. As few as 10 units of α-amylase could be detected on the basis of this reaction. α-Amylase gave a stronger reaction than did ovalbumin on a weight basis. The non-carbohydrate containing proteins bovine serum albumin and chymotrypsinogen (100 μg protein per gel) showed no carbohydrate staining reactions. While the dansyl-hydrazine-coupling reaction showed that α-amylase and the band-2 proteins were glycosylated, it must also be acknowledged that lysozyme, an enzyme devoid of carbohydrate also gave a positive test.

Therefore, to verify that α-amylase was a glycoprotein, the α-amylase was purified and the sugars attached to the polypeptide were directly identified and quantitated (Table 3). Since SDS and β-mercaptoethanol converted the protein to more-or-less linear molecules,

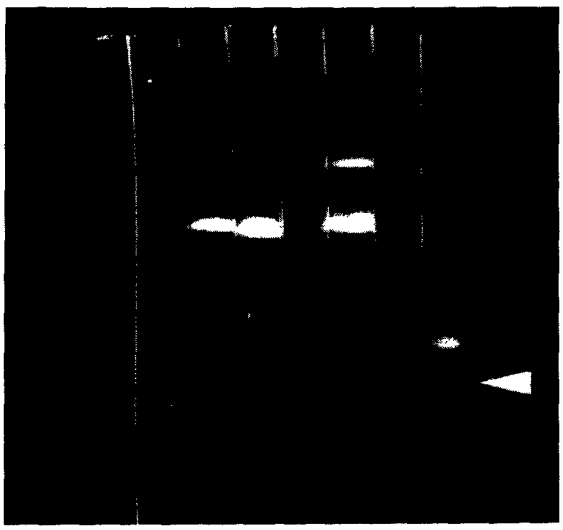

Fig. 2 Staining of protein by the periodate-dansylhydrazine reaction. SDS-Polyacrylamide gels were stained for carbohydrate [23] and were photographed under 350 nm light. Left to right: (1) to (4), α-amylase plus the band-2 protein at 100 μg, 10 μg, 35 μg and 100 μg, respectively, of protein per gel; (5) and (6) ovalbumin with three concentrations of protein added sequentially on each gel such that the lower band has 100 μg, the middle band 35 μg, and the upper band 10 μg; (7) and (8) lysozyme, lower band 100 μg, middle band 35 μg, and upper band, 10 μg. The arrow marks the tracker dye position. Control gels (1), (5) and (7) have not been oxidized by periodate; gels (2), (3), (4), (6) and (8) have undergone the complete reaction sequence.

Table 3. Carbohydrate composition of barley α-amylase and the band-2 protein

Protein	$(NH_4)_2SO_4$ fraction	Method of analysis*	Residues per polypeptide: Mannose	Glucose	*N*-Acetylglucosamine
α-Amylase	20 to 40%	A	0 43	—	0
α-Amylase	20 to 40%	B	0 49	0 54	0 45
α-Amylase	50 to 60%	C	0 47	0 24	0 17
Band-2	20 to 40%	A	0	—	0 06
Band-2	20 to 40%	B	0	0 65	0 25

Sugars were identified and quantified as their TMSi-methyl-glycosides using gas chromatography [18]. The number of protein molecules was calculated from the numbers of individual amino acids in an acid hydrolysate of an identical sample and the molecular weight of the polypeptide as determined by SDS-polyacrylamide gel electrophoresis [19].

*A. *In situ* hydrolysis in the polyacrylamide gels using 2N TFA.

B. Protein eluted from gels by electrophoresis then treated with MeOH–HCl.

C. Protein dialyzed against 10 mM glycine-HCl, pH 3, and hydrolysed in MeOH–HCl.

†Values could not be determined due to background levels of glucose in the gels.

I assumed that only the carbohydrate which was covalently attached to the polypeptide could enter the gel during electrophoresis. The sugars which were cleaved from the polypeptide were converted to the trimethylsilylated derivatives of their methyl glycosides and were identified by GLC.

The sugars were removed while the glycoprotein was fixed in the gel (Table 3, line 1) or the glycoprotein

was first removed from excised pieces of the gel by a second period of electrophoresis (Table 3, line 2). In both cases about 0.5 residues of mannose were detected per molecule of α-amylase. In the first method glucose present throughout the gel interfered with glucose determination, and no glucosamine was detected. In the second method, which provided more information, each α-amylase molecule was found to have on the average approximately 0.5 residues of glucose and 0.5 residues of glucosamine in addition to the mannosyl residues. To arrive at these figures, the number of α-amylase molecules present in the sample had to be determined in parallel experiments by direct quantitation of the number of residues of each amino acid which were present in an acid hydrolysate of the protein. The minor α-amylase fraction which had precipitated by ammonium sulfate at 50 to 60% of saturation was also analyzed. It had contained only a trace of band-2 protein and was tested directly for carbohydrate without interposition of an electrophoresis step. This α-amylase fraction also contained mannose (0.5 residues) but lesser amounts of glucose and glucosamine were detected.

The band-2 protein (Table 3, lines 4 and 5) lacked mannose, but contained about 0.6 residues of glucose and 0.25 residues of glucosamine per polypeptide of 21 500 daltons.

DISCUSSION

Four isozymes of α-amylase are synthesized and secreted by the aleurone tissue of Himalaya barley [10] and these segregate into two groups based on their immunoprecipitation characteristics [13]. The syntheses of all of these isozymes are stimulated by GA, and they are all labeled when incubated with radioactive amino acids in the presence of the hormone [10]. Utilization of techniques designed for the purification of α-amylase from barley grains resulted in the co-purification of two polypeptides from this cultivar. The major polypeptide had a MW of 41 400 daltons and was the only polypeptide of the two that became labeled to any great extent with leucine (^{14}C-U) during GA-treatment of isolated aleurone tissue. The four isozymes of α-amylase co-migrate during electrophoresis on SDS-polyacrylamide gels and hence must have the same or closely similar molecular weights.

The minor polypeptide, band-2 protein, was only labeled to a small extent during GA-treatment and since its ratio to α-amylase decreased when the starchy endosperm was removed before the isolated aleurones were placed into medium containing GA, it appeared that it was probably not present in the aleurone cells at the time of the addition of hormone. At the very least, this protein must be released before α-amylase synthesis begins because no proteins containing polypeptides of less than 40 000 daltons are released by aleurone layers during α-amylase synthesis [14]. It was unlikely that the band-2 protein was a true α-amylase because its amino acid composition was quite different from that of either barley or wheat α-amylase and because no α-amylases described have yet been found to consist of more than one polypeptide [15].

Yomo and Varner [16] reported that the α-amylase of Himalaya barley had a 3-fold higher concentration of tryptophan residues than were present in the rest of the barley proteins. Barley α-amylase also has a 2.2-fold greater histidine content and a 1.7-fold greater tyrosine content than might be expected for a eukaryotic protein [12] (cf. Table 2).

Carbohydrate analysis of barley α-amylase has shown that the α-amylase molecules are at least partially glycosylated but not to very great extent. α-Amylase which was subjected to electrophoresis of SDS-polyacrylamide gels could subsequently be stained by the periodic acid–Schiff reaction and by the periodic acid–dansylhydrazine reaction, both of which test for the presence of sugar residues. The eluted α-amylase was found to contain approximately 0.5 residues each of mannose, glucose and *N*-acetyl glucosamine per molecule which amounts to a maximum carbohydrate content of 0.7%, by weight, for barley α-amylase. Mitchell [9] estimated that barley α-amylase contained about 3% carbohydrate consisting of undetermined amounts of glucose, galactose, mannose, xylose, fucose and glucosamine. However, he took no special precaution to ensure that traces of carbohydrate which may have been attached non-covalently to the enzyme were removed prior to the analysis. Wheat α-amylase has been reported to be carbohydrate-free [11] while that of sorghum had 2% carbohydrate [15]. A periodic acid–Schiff staining reaction of barley α-amylase has been described elsewhere [17].

The heterogeneity of glycosylation of the barley α-amylase iso enzymes is implied by the fact that there are fewer residues of each sugar than there are molecules of α-amylase. Why should secreted α-amylase isoenzymes be heterogeneous in their carbohydrate contents? One possibility would be that certain isozymes were glycosylated during or after translation while others were not. Alternatively, all of the isozymes may have been more fully glycosylated during their synthesis, but they may have been variously de-glycosylated following their secretion, either by the proteolytic removal of a small glycopeptide or by the action of carbohydrases in the external milieu.

EXPERIMENTAL

Incubation of grains for α-amylase purification. Barley seeds, harvested in 1969, (*Hordeum vulgare* L cv Himalaya) were obtained from the Agronomy Club at Washington State University, Pullman, Washington, USA. 160 g grains were surface sterilized in 1% NaOCl, and incubated with shaking in the dark for 4.5 days in 11.10 mM Na succinate buffer (pH 5.3), 20 mM $CaCl_2$, 0.1% chloramphenicol, and 10 μM GA.

Purification of α-amylase After incubation the soln was collected by decantation and the grains further shaken for 30 min with 1 mM Na acetate buffer, pH 4.8, with 200 mM $CaCl_2$. This was added to the original soln The grains were then pressed with a pestle to remove the starchy endosperm from the aleurone layer The aleurone layers, embryos and extruded starch were added to the total extract and M $CaCl_2$ was added to give a final concn of 200 mM This extract was incubated for 2 hr at 22°, filtered and centrifuged at 5900 *g* for 20 min. The supernatant contained about 200 000 units of α-amylase activity assayed by the method of ref [20].

The supernatant was adjusted to pH 8 with 0.1 M Tris base and centrifuged at 5900 *g* for 30 min at 0°. The supernatant was heated to 70°, centrifuged at 5900 *g* for 30 min, yielding 150 000 units of α-amylase in the supernatant. Cold EtOH was added to this supernatant to 40%, and the soln stored at 0° for 18 hr, centrifuged at 6900 *g* for 30 min and the pellet discarded. Deproteinized oyster glycogen (1.6%) [21] was

added to the cold supernatant in the proportion of 1 μl per 7 units of α-amylase. After 18 min stirring, the soln was centrifuged at 5900 *g* for 30 min and the pellet suspended in 5 mM Tris–HCl, pH 8, and 5 mM $CaCl_2$ and incubated for 2 hr at 22°. The insoluble ppt. was removed by centrifugation and the α-amylase–glycogen complex re-ppd with 30%, then 20% EtOH. The complex was then resuspended in Tris/calcium buffer and ppd at 0° with solid $(NH_4)_2SO_4$ at 20, 40, 50, 60 and 80% satn. Each $(NH_4)_2SO_4$ ppt. was dialysed against Tris/calcium buffer for 40 hr at 7°.

Preparation of ^{14}C-labeled α-amylase from aleurone layers. Aleurone layers (200) were separated from barley grains from which embryos had been removed and imbibed under sterile conditions on sand for 3 days [22]. The aleurone layers were incubated in 20 mM Na succinate buffer (pH 5.3), 0.2 M $CaCl_2$, 0.1% chloramphenicol, 1 μM GA, and 6.2 μCi/ml L-leucine (^{14}C-U, 286 mCi/mmol) at 25° for 24 hr. The medium was decanted, diluted to 50 ml with 0.2 M $CaCl_2$ and the pH adjusted to 8 with 0.1 M Tris base. The soln was heated to 70° for 20 min, centrifuged at 13000 *g* and the ppt. discarded. Cold EtOH was added to a final concn of 35% and after 1 hr the soln was centrifuged at 13000 *g*. One ml of 1.6% glycogen was added to the supernatant, the glycogen–amylase complex collected by centrifugation and resuspended in 1 ml of 5 mM Tris–HCl buffer (pH 8) with 5 mM $CaCl_2$. 1500 units of α-amylase were recovered.

Polyacrylamide gel electrophoresis. Proteins were separated electrophoretically by SDS-polyacrylamide gel electrophoresis [19]. Protein was eluted from the gel segments electrophoretically. The pieces were placed in a gel tube closed by dialysis tubing (purified by boiling in Na_2CO_3 and EDTA) and buffer added. Current was passed through the tube for sufficient time to remove the protein into the buffer. Gels were stained for protein using Coomassie Brilliant Blue R250 and scanned at 560 nm. Radioactivity was determined in 1 cm dried gel pieces by combustion using a Packard Tri-Carb Sample Oxidizer (Model 306).

Staining of gels for carbohydrate The SDS-polyacrylamide gels were stained for carbohydrate by either the periodate–Schiff reaction [19], or the periodate dansylhydrazine method [23].

Amino acid analysis. Protein, eluted electrophoretically from preparative SDS-polyacrylamide gels, was ppt'd with TCA to a final concn of 20%, centrifuged and the ppt. rinsed with EtOH (× 5). The final pellet was dried under N_2 and incubated in constant boiling HCl at 107° for 18 hr. The dried residue was resuspended 0.01 N HCl, and analysed using a Technicon system (Technicon Corporation, Tarrytown, NY, USA) as modified in ref. [6]. 3 to 4 determinations were made for each protein analyzed. Tryptophan was destroyed by this procedure and yields of cysteine were not reliable

Carbohydrate analysis. Sugars in glycoproteins were identified and quantitated by GLC as in ref. [18] with suitable standards. *ca* 1 mg of α-amylase and 0.24 mg of band-2 protein were used per determination. The glycoproteins were in dry MeOH containing 1.5 M HCl at 80° for 12 hr, cooled, neutralized with $AgNO_3$, and acetylated with Ac_2O [24]. After centrifugation the supernatant fraction, and 2 × MeOH washes of the ppt. were re-centrifuged at 13300 *g* for 20 min and the clear supernatant soln evapd at 45° under N_2 and dried in *vacuo* (P_2O_5). The trimethylsilylating reagent, 25 μl of Py, trimethylchlorosilane, and hexamethyldisilazane (5:1:1) was added and after 45 min the samples were analyzed on a Perkin–Elmer 900 gas chromatograph. The glycoproteins could also be hydrolysed *in situ* on the gels. The gels were fixed for 48 hr in 50% MeOH containing 5% MeCOOH and portions of the gel containing the ppd protein were excised and mixed with 2 vol. of 3 N trifluoroacetic acid (TFA) and an internal standard of mannitol. The whole was incubated at 121° for 60 min, cooled and the aq. phase dried under N_2 at 45°. The residue was dried *in vacuo* and the monosaccharides then treated as described above [24].

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