

Concanavalin A- and Wheat Germ Agglutinin-Conjugated Lectins as a Tool for the Identification of Multiple N-Glycosylation Sites in Heterologous Protein Expressed in Yeast

Rossana García,* Rosmari Rodríguez,* Raquel Montesino,* Vladimir Besada,†
Javier González,† and José A. Cremata*,¹

*GlycoLab, Bio Industry Division, and †Physico-Chemistry Division, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, Havana, Cuba

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We report here a methodology that allows the identification of glycosylation sites by a combination of protein enzymatic digestion, glycopeptide separation on a reverse-phase HPLC column, and further recognition in a dot-blot system using concanavalin A–horseradish peroxidase. Wheat germ agglutinin–horseradish peroxidase is used as the recognition system for peptides generated after proteolytic digestion of endoglycosidase H deglycosylated protein. Glycosylation sites were confirmed by automatic Edman degradation and fast atom bombardment mass spectrometry. This methodology was applied to a model glycoprotein, α -amylase from *Bacillus licheniformis*, which is unglycosylated in its natural host and appears highly glycosylated when expressed in the methylotrophic yeast *Pichia pastoris*. © 1995 Academic Press, Inc.

As the most common posttranslational modification of proteins, glycosylation has aroused a great interest in the last two decades. Reviews and monographs on whether or not carbohydrates are of fundamental value are frequently published (1–12).

It is well known that glycosylation plays a key role in the biological function of native and recombinant glycoproteins. Many theories have been advanced regarding the functions of oligosaccharides covalently attached to protein. On that subject, a complete and very interesting review was published recently by Varki (13) pointing out that oligosaccharides mediate specific recognition events and, in some cases, modu-

late biological processes, generating the functional diversity needed for further development and cellular differentiation as well as the interaction between cells and organisms.

Furthermore, recombinant proteins for therapeutic uses that appear glycosylated in nature must be glycosylated in order to display correct biological activity. This function depends also on the oligosaccharide pattern, i.e., oligomannoside, complex, or hybrid. For example, “*in vivo*” activity of recombinant EPO² is related not only to the ratio of di- and tetraantennary complex oligosaccharides but also to their sialic acid content (14).

At present, it is well known that the sequence Asn-Xaa-Thr/Ser (where Xaa may be any amino acid except proline) should be present to be recognized by the oligosaccharyltransferase in order to transfer an already assembled core oligosaccharide Glc₃Man₉GlcNAc₂ to a nascent protein when this tripeptide is exposed on the luminal side of the membrane of the endoplasmic reticulum. Many examples have been published in which this sequence motif is present as a potential glycosylation site but are not glycosylated at all. Thus, finding the glycosylation sites and the degree of glycosylation on a given recombinant glycoprotein is of fundamental interest, and it is also included as a regulatory require-

² Abbreviations used: EPO, erythropoietin; FAB, fast atom bombardment; RP, reverse phase; HPLC, high-performance liquid chromatography; PNGase F, peptide N-glycosidase F; LC, liquid chromatography; ES, electron spray; MS, mass spectrometry; ConA, concanavalin A; WGA, wheat germ agglutinin; HRP, horseradish peroxidase; Endo H, endoglycosidase H; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GlcNAc, N-acetylglucosamine; Asp-N, endoproteinase Asp-N; BSA, bovine serum albumin; TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed. Fax: 53 7 218070.

ment for its introduction, mainly as a therapeutic (15). Then, once the protein sequence has been verified, the specific residue to which an oligosaccharide is attached and the configuration of the linkage should be determined.

To fulfill these requirements, some methodologies have been published for locating glycosylation sites. One of those most extensively used was described initially by Carr *et al.* (16,17), with which it is also possible to determine the structural class, branching types, and molecular microheterogeneity of N-linked oligosaccharides at specific attachment sites. The crucial point resides in the comparison of the RP-HPLC chromatograms prior to and after deglycosylation with PNGase F, which allows identification of the glycopeptides from a proteolytic digest of the native glycoprotein. Recently, a more advanced approach appeared using liquid chromatography–electron spray mass spectrometry (LC-ESMS) and liquid chromatography–tandem MS (LC-ESMS/MS) for selective detection of N- and O-linked glycopeptides (18).

González *et al.* (19) published a new strategy to locate N-glycosylation sites in glycoproteins with a combination of PNGase F deglycosylation in $H_2^{18}O$ and collision-induced dissociation analysis in FAB-MS.

Other authors have included lectin recognition in a dot-blot system which facilitates localization of glycopeptides in a complex RP-HPLC chromatogram after enzymatic digestion of the glycoprotein (20,21).

In this report, we describe a simple methodology comparing enzymatic proteolytic digestion, fractionation on RP-HPLC for both native and deglycosylated forms of the glycoprotein and the specific recognition of glycopeptides by ConA–HRP and the GlcNAc-linked residue at the specific glycosylation site from Endo H digestion with WGA–HRP. Recognized fractions from native glycoprotein are further digested with Endo H and analyzed by FAB-MS or automatic Edman degradation. Positive fractions from deglycosylated glycoprotein are directly analyzed by FAB-MS. In this way, we were able to identify glycosylated asparagines at five of the potential glycosylation sites of the hybrid recombinant glycoprotein.

MATERIALS AND METHODS

Materials

Electrophoresis reagents were from Bio-Rad (electrophoresis grade). Concanavalin A–horseradish peroxidase, wheat germ agglutinin–horseradish peroxidase, and 4-chloro-1-naphthol were purchased from Sigma Chemical Co. (St. Louis, MO). Endo H, PNGase F, carboxypeptidase A, carboxypeptidase B, aminopeptidase M, and trypsin were obtained from Boehringer Mannheim (Indianapolis, IN). Hydrogen peroxide (30% solution) was from BDH Chemicals (U.K.). Nitrocellulose

BA 85 was purchased from Schleider & Schull (Germany). Acetonitrile (lichrosolv) was from Riedel-de-Haen (Germany) and trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL). All other reagents were of analytical grade. α -Amylase was purified from a *Pichia pastoris* MPA 3625 strain having an integrated α -amylase gene (22) supplied by the Center for Genetic Engineering and Biotechnology (La Habana, Cuba).

Methods

Polyacrylamide gel electrophoresis. SDS–PAGE was performed according to Laemmli (23). Gels were run employing a Mighty Small II SE 250 (Hoefer Scientific Instrument, San Francisco, CA).

Tryptic digestion. Protein was denatured by heating at 100°C in an SDS 0.01% solution for about 10 min. Tryptic digestion was performed at 37°C in 1% ammonium bicarbonate buffer, pH 8.0, for 4 h using an enzyme:substrate ratio of 1:50 (w/w).

Protein enzymatic deglycosylation. Denatured protein with 0.02% SDS was heated for 10 min at 100°C. Deglycosylation was carried out in 100 mM sodium citrate, pH 5.5, at 37°C for 16 h. To 1 ml protein solution (1 mg/ml), 20 mU of Endo H was added. After deglycosylation a precipitate is formed. By centrifugation at 8000 rpm for 10 min the protein was recovered and the supernatant discarded.

Peptide separation. The HPLC instrument used for peptide separation was from Pharmacia (Pharmacia LKB, Sweden). Tryptic peptides from native and deglycosylated proteins were fractionated in a 250 $\times$ 4.6-mm C_8 reverse-phase column (J. T. Baker, USA) at a flow rate of 0.8 ml/min with a linear gradient over 120 min from 5 to 65% acetonitrile containing 0.1% TFA.

Glycopeptide Recognition Using Dot-Blot Assay

Dot-blot assay with ConA–HRP. Peptides obtained on digestion of native protein were dried in a speed-vac, resuspended in water, and spotted in a prewetted nitrocellulose membrane with TBS buffer (50 mM Tris–HCl, 500 mM NaCl, pH 7.5). The spotted dry membrane was soaked with blocking solution (5% skim milk in TBS buffer) in a shaker for 1 h at 37°C to saturate the binding sites on the membrane. After this blocking treatment, the membrane was rinsed with TBS buffer and the solution was replaced by ConA–HRP conjugate solution (1:1000 dilution in 1.7 mM KH_2PO_4 , 3 mM NaH_2PO_4 , 2.6 mM KCl, 136 mM NaCl pH 7.5), the membrane was incubated with this solution for 1 h at 37°C. It was then rinsed three times (15 min each time) with 0.1% Tween 20 in TBS buffer, after which the membrane was soaked in substrate solution freshly prepared with 2 mg of 4-chloro-1-naphthol dissolved in

600 μ l of methanol, 10 ml TBS, and 10 μ l of hydrogen peroxide (30% solution) until the color developed. When the membrane was sufficiently stained, it was rinsed with distilled water to stop the reaction.

Dot-blot assay with WGA–HRP. Glycopeptides from deglycosylated protein were detected with WGA–HRP conjugate by using the same procedure for detection with ConA–HRP conjugate, but the blocking treatment was done with 0.1% Tween 20 in TBS buffer instead of skim milk and with longer rinsing time.

Enzymatic deglycosylation of glycopeptides. Dried glycopeptides were resuspended in 30 μ l of 100 mM sodium citrate, pH 5.5, and then 1 mU of Endo H was added and the glycopeptides were incubated for 16 h at 37°C.

Exopeptidase digestion. The glycopeptides were dissolved in 10 μ l of 1% NH_4HCO_3 buffer and 0.2 μ g of aminopeptidase M or a mixture of carboxypeptidases A and B (1:1, w/w) was added. The reaction mixture was allowed to stand at 37°C for 1 min, after which mass spectra were recorded.

Mass spectrometry. FAB-MS spectra were recorded with a JEOL JMS-HX110 instrument (Jeol Company, Japan) equipped with standard FAB ion source using a Xenon ionizing beam (4 kV). The matrix used was dithiothreitol:dithioerithrol:glycerol:thioglycerol (25 μ g:5 μ g:150 μ l:150 μ l). The ion source was at an accelerating potential of 10 kV. Mass data were acquired with a JEOL JMA-DA5000 data analysis system.

Sequence analyses. Automatic amino acid sequence analysis of peptides was performed using a Knauer Sequencer Model 810 equipped with a cross-flow reactor/loop reactor working with dual-phase-chemistry. Amino acid phenylthiohydantoin were analyzed by RP-HPLC using an on-line Knauer liquid chromatography system equipped with a photometric filter detector (269-nm filter) as recommended by the manufacturer.

RESULTS AND DISCUSSION

When purified α -amylase was subjected to SDS–PAGE (Fig. 1), it showed a molecular mass increase of 30 kDa due to glycosylation (as determined by the complete release of oligosaccharides by Endo H digestion). The recombinant enzyme is active (22), showing three times higher activity than the natural α -amylase (data not shown).

Fractionation on RP-HPLC of a comparative enzymatic proteolytic digestion with trypsin before and after Endo H digestion is shown in Figs. 2A and 2B, respectively. It was very difficult to identify possible glycopeptides from the relative shift in retention times because of the complexity of both chromatograms. However, by using conjugated lectins, it was possible to locate fractions that bind to ConA (Figs. 2A and 2C)

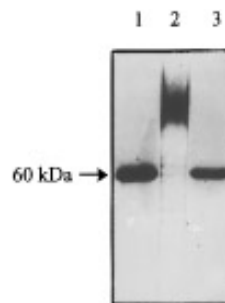


FIG. 1. Polyacrylamide gel electrophoresis of α -amylase from *Bacillus licheniformis* (lane 1) and α -amylase from *P. pastoris* before (lane 2) and after (lane 3) Endo H treatment (see Materials and Methods).

and WGA (Fig. 2B), suggesting the presence of glycopeptides bearing oligomannoside and GlcNAc residues, respectively. The proposed strategy to locate glycosylation sites is shown in Fig. 3.

Sequencing of ConA–HRP Binding Fractions

Some positive fractions recognized by ConA–HRP (11,15,16,18,24) (Fig. 2A) from native protein trypsin digestion were selected for glycosylation site determination because of their high abundance (Fig. 2C). Fractions 15, 18, and 24 were subjected to Endo H deglycosylation before analysis by FAB-MS. The results are shown in Table 1.

The deglycosylated peak 18 was assigned to two peptides of the protein structure by FAB-MS analysis (Fig. 4), i.e., $\text{L}_{25}\text{-K}_{47}$ (m/z 2761.3) and $\text{R}_{24}\text{-K}_{47}$ (m/z 2918.5), both plus 203 Da, corresponding to a GlcNAc residue that could be attached to an asparagine located in the sequon $\text{N}_{27}\text{-D-S}_{29}$. This result suggests that aspartic acid in position +1 does not affect glycosylation as has been claimed by Gavel and Heijne (24).

The mass spectrum of peak 24 (m/z 2852.7) matched $\text{E}_{255}\text{-K}_{276}$ plus one GlcNAc residue, suggesting that the sequon $\text{N}_{275}\text{-K-T}_{277}$ is also glycosylated. Fraction 15 was repurified after deglycosylation showing two well-resolved peaks (Fig. 5). The less retained peptide, which did not bind to ConA–HRP and WGA–HRP (m/z 1255.2), was assigned to peptide $\text{A}_{137}\text{-R}_{146}$ (without a potential glycosylation site). However, the second fraction (binding to WGA–HRP with m/z 2561 but not to ConA–HRP) matched the theoretical mass of peptide $\text{R}_{437}\text{-R}_{456}$ containing an GlcNAc unit, probably linked to N_{455} in the motif $\text{N}_{455}\text{-R-S}_{457}$.

Peaks 11 and 16 were analyzed by direct automatic Edman degradation. It was possible to identify on fraction 16 a peptide sequence T_{277} to R_{305} which includes the sequon $\text{N}_{280}\text{-H-S}_{282}$, detecting, on the fourth cycle, a small amount of D (instead of N) in comparison to the repetitive yield obtained in the other cycles. The

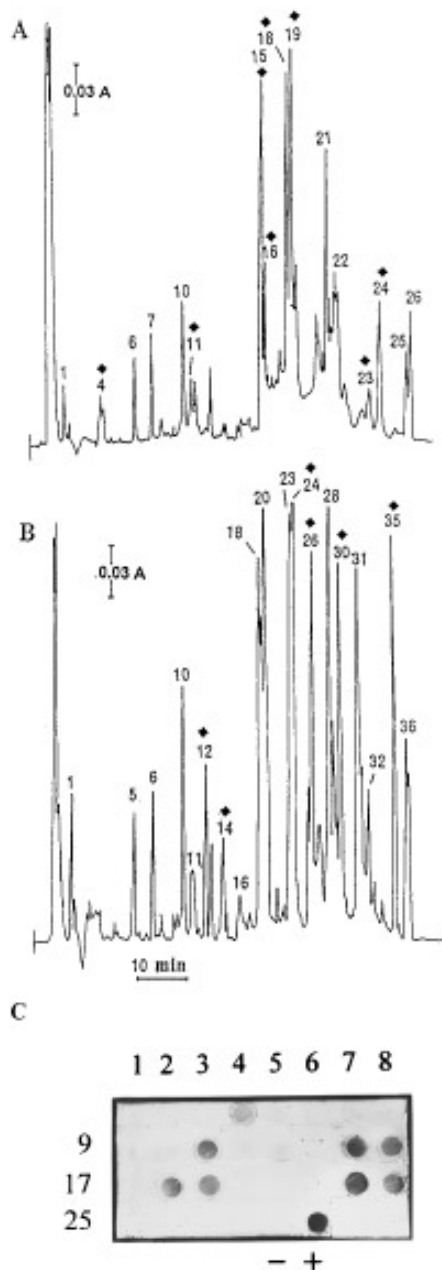


FIG. 2. Fractionation on C_8 RP-HPLC of a comparative enzymatic proteolytic digestion with trypsin before (A) and after (B) Endo H deglycosylation. Fractions indicated by $\blacklozenge$ on the chromatograms were bound to ConA-HRP (A) or WGA-HRP (B). (C) Affinodot-blot of fractions (A) recognized by ConA-HRP. Recombinant α -amylase and bovine serum albumin (spots marked by + and -) were used as positive and negative controls, respectively.

presence of D could be assigned to the cleavage of β -aspartylglycosylamine linkage of this glycopeptide due to the acid conditions characteristic of the Edman chemistry. In the case of fraction 11, the results reveal the presence of peptide Q_{443} - R_{456} with six amino acids less than fraction 15, but in both forms, glycosylated

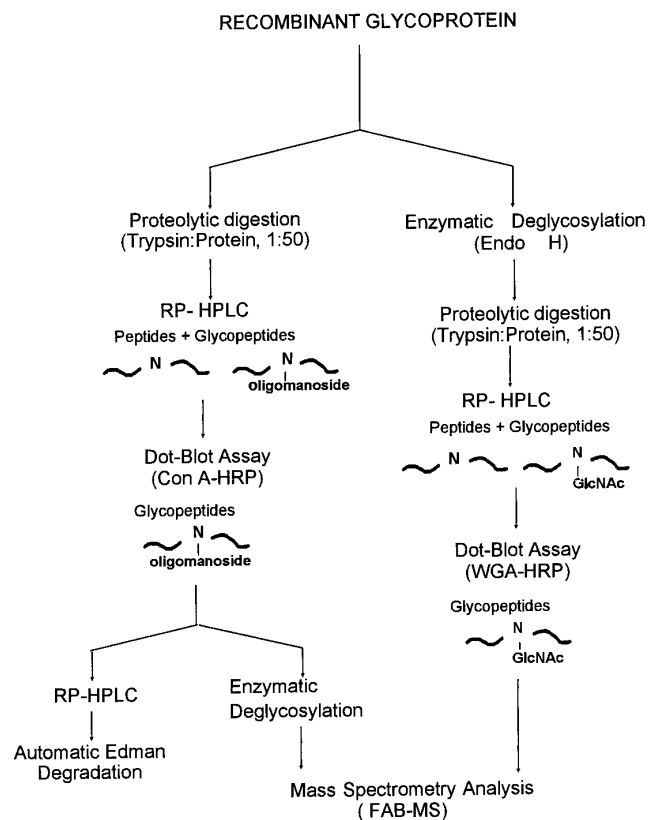


FIG. 3. Flow diagram of the proposed strategy.

and nonglycosylated, suggesting a partial glycosylation of N_{455} - R - S_{457} .

A similar result could be deduced from the methodology described by González *et al.* (19) employing PNGase F digestion in ^{18}O -labeled water on the same recombinant glycoprotein. They found glycosylation at N_{455} only after Asp N digestion of the Endo H deglycosylated protein, which could suggest that this residue is partially glycosylated and that the conversion frequency of N_{455} to D_{455} under this condition depends upon the level of glycosylation, thus affecting the proteolytic cleavage by Asp N at this position.

TABLE 1

Theoretical and Experimental FAB-MS Data Obtained after Endo H Deglycosylation of Glycopeptides Detected by ConA-HRP

Fraction no.	Experimental m/z	Experimental $m/z - 203$	Theoretical m/z	Assignment
15	1255.2	—	1255.6	A_{137} - R_{146}
	2561.9	2361.9	2362.1	R_{437} - R_{456}
18	2761.3	2558.3	2558.9	L_{25} - K_{47}
	2918.5	2715.5	2715.1	R_{24} - K_{47}
24	2852.7	2649.7	2649.9	E_{255} - K_{276}

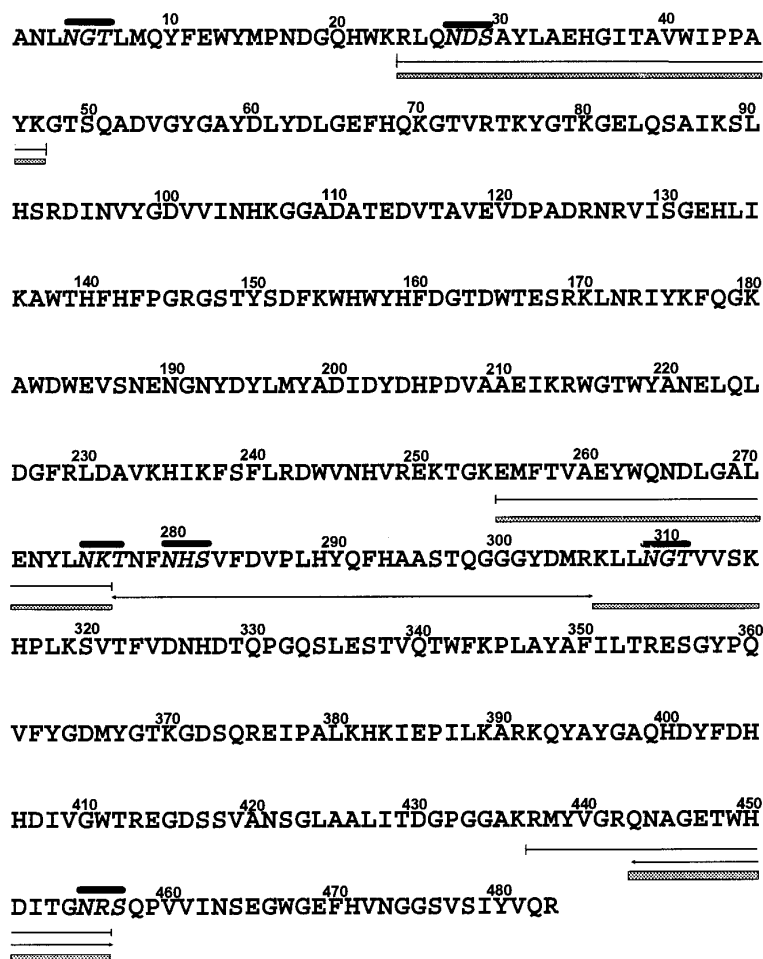


FIG. 4. α -Amylase amino acid sequence deduced from the nucleotide sequence (24) and verified by FAB-MS (J. González, unpublished work). Glycosylation sites are indicated by —. Glycopeptides recognized by ConA-HRP, Endo H deglycosylation, and FAB-MS (←→); glycopeptides recognized by ConA-HRP and automatic Edman degradation (|—|); glycopeptides recognized by WGA-HRP and FAB-MS (□).

Sequencing of WGA-HRP Binding Fractions

After protein deglycosylation and trypsin digestion, the peptide mixture was fractionated by RP-HPLC (Fig. 2B) and the fractions were analyzed in a dot-blot system using WGA-HRP conjugate. Unambiguous positive fractions 12, 14, 26, and 35 were submitted to direct FAB-MS characterization.

The glycosylation of N₂₇-D-S₂₉, which is included in peptides R₂₄-K₄₇ (m/z 2918.5) and L₂₅-K₄₇ (m/z 2761.9) from fraction 26, was confirmed, as suggested by the results shown in Table 1 (see fraction 18 recognized by ConA-HRP). Fraction 35 gave a unique mass signal (m/z 2852.6), which corresponds to peptide E₂₅₅-K₂₇₆ with an N-linked GlcNAc at N₂₇₅-K-T₂₇₇ that was also recognized by ConA-HRP in its native form (see fraction 24 of Table 1). Finally, fraction 12 was assigned to peptide Q₄₄₃-R₄₅₆ (m/z 1801.6), similar to that se-

quenced by Edman degradation from fraction 11 recognized by ConA-HRP.

From fraction 14, two new peptides, K₃₀₆-K₃₁₅ (m/z 1260.8) and L₃₀₇-K₃₁₅ (m/z 1133.3), were assigned after considering 203 Da that corresponds to a GlcNAc residue. These peptides were not identified by ConA-HRP. The sequence N₃₀₉-G-T₃₁₁ is included in these peptides.

Peptide Q₄₄₃-R₄₅₆ (fraction 12) was chosen for exopeptidase digestion taking into account that a glycosylation site was located toward the carboxy end. First, it was digested with carboxypeptidase A and B and analyzed by FAB-MS. In a similar way, aminopeptidase M digestion of peptide E₂₅₅-K₂₇₆ (fraction 35) was also analyzed by FAB-MS (data not shown).

FAB mass spectra of carboxypeptidase digestion of Q₄₄₃-R₄₅₆ showed an M⁺ and M⁺-Arg increase of 203 Da with respect to the theoretical mass, but M⁺-(Arg-Asn-

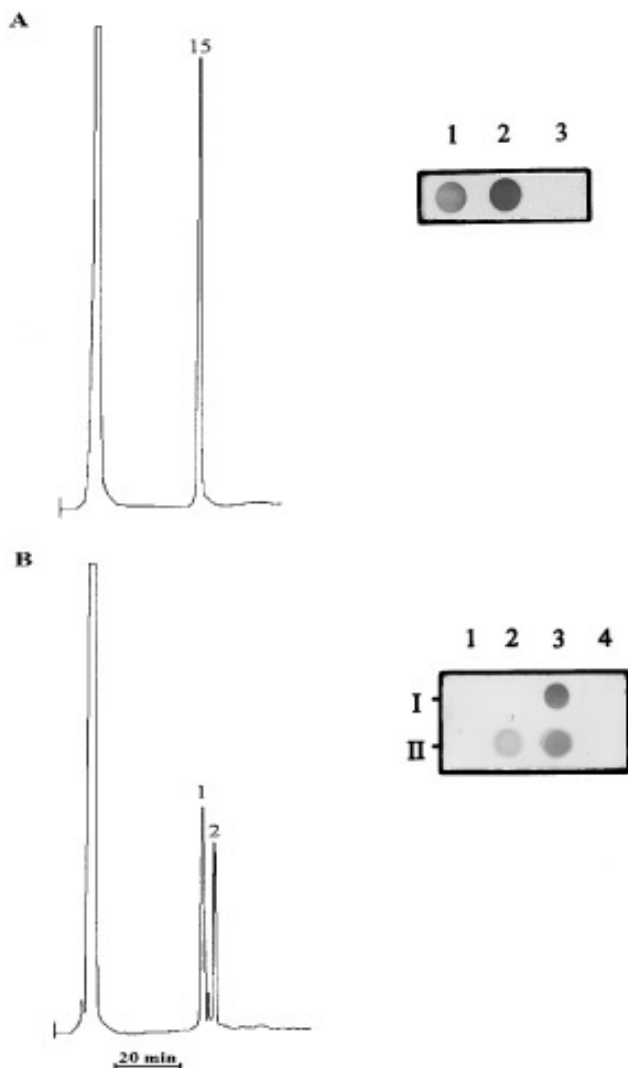


FIG. 5. C_8 RP-HPLC repurification of fraction 15 (positive to ConA-HRP, Figs. 2A and 2C) before (A) and after (B) Endo H treatment. The separated fractions were collected, spotted on nitrocellulose membrane, and stained using conjugated lectins as described under Materials and Methods. Dot-blot in A, ConA-HRP detection. Lane 1, fraction 15; lane 2, positive control (recombinant α -amylase); and lane 3, negative control (BSA). B, 1 and 2 correspond to RP-HPLC chromatogram of fraction 15 after Endo H deglycosylation. Dot-blot in B, (I) ConA-HRP detection using recombinant α -amylase as positive control (lane 3); (II) WGA-HRP detection using Endo H deglycosylated α -amylase as positive control (lane 3). BSA is negative control in both cases (lane 4).

Gly) and M^+ -(Arg-Asn-Gly-Thr-Ile) matched well with the unglycosylated peptide. Furthermore, aminopeptidase digestion of peptide E_{255} - K_{275} produce a set of peptide fragments all of which are 203 Da higher than the theoretical mass, which means that the GlcNAc attached to N_{275} is retained in every one of the newly generated peptides.

Table 2 summarizes the above results of using either

the ConA-HRP or the WGA-HRP recognition systems. Using ConA-HRP, glycosylation at N_{27} , N_{275} , and N_{455} was located by FAB-MS analysis, and N_{280} and N_{455} by automatic Edman degradation. Glycosylation at N_{27} , N_{275} , and N_{455} was also identified by WGA-HRP, except for the sequon N_{309} , that was located only by WGA-HRP. An increase of 1.5 to 2.5 min in retention time of the corresponding deglycosylated peptide was detected for fraction pairs 18/26 (sequon N_{27}), 24/35 (sequon N_{275}), and 11/12 (sequon N_{455}) due to a relatively increased peptide hydrophobicity once deglycosylated. The fact that these glycosylation sites were located by using either ConA or WGA undoubtedly confirms that they were totally or partially glycosylated.

The above results show that by following the strategy described in Fig. 3, it was easy to locate five potential glycosylation sites of the recombinant α -amylase expressed in *P. pastoris*, despite the fact that RP-HPLC chromatograms from the trypsin digestion of native and deglycosylated protein are both very complex. We did not analyze by either FAB-MS or automatic Edman degradation those HPLC fractions that showed weak signals in the dot-blot. This is the reason why glycosylation at N_4 is not reported here. However, in previous work, employing PNGase F digestion in $H_2^{18}O$ on the same recombinant glycoprotein, this site was reported as glycosylated.

We demonstrated that this strategy overcomes some difficulties that arise when a protein containing multiple glycosylation sites is studied. For example, trying to identify shifted peaks in one chromatogram with

TABLE 2

Comparison of the Main Characteristics of Glycopeptides Recognized by either ConA-HRP or WGA-HRP

Peak no. ConA ^a /WGA ^b	Retention time (min)	FAB-MS (<i>m/z</i>) ^c AED ^d	Assigned peptide	Sequon
18/26	51.5/54.0	2918.5/2918.5 2761.3/2761.9	R_{24} - K_{47} L_{25} - K_{47}	N27DS
24/35	68.5/70.0	2852.7/2852.6	E_{255} - K_{276}	N275KT
16/—	47.0/—	AED/—	T_{277} - R_{305}	N280HS
—/14	—/37.0	—/1260.8 —/1133.0	K_{306} - K_{315} L_{307} - K_{315}	N309GT
15/—	46.0/—	1255.2/— 2561.9/—	A_{137} - R_{146} R_{437} - R_{456}	— N455RS
11/12	31.5/33.0	AED/1801.6	Q_{443} - R_{456}	N455RS

^a ConA-HRP recognized glycopeptides after native protein trypsin digestion and further fractionation in C_8 RP-HPLC.

^b WGA recognized glycopeptides with an GlcNAc attached to it after Endo H protein deglycosylation, trypsin digestion, and C_8 RP-HPLC fractionation.

^c *m/z* comparison of deglycosylated glycopeptides identified by ConA-HRP or by direct FAB-MS recording of WGA-HRP recognized glycopeptides.

^d Peptides sequenced by automatic Edman degradation.

respect to another only by modification of the retention time (which has been postulated as indicative of a potential glycopeptide) would be a very hard task, and there could also be some glycosylation sites resistant to Endo H which would then be undetected by simple comparison of both chromatograms, since they would have the same retention time before and after the deglycosylation reaction.

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