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ANTI-GLYCOSYL ANTIBODIES: ANTIBODIES DIRECTED AGAINST THE CARBOHYDRATE MOIETIES OF A GLYCOPROTEIN*

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SUMMARY: Glucoamylase is an unusual type of glycoprotein containing twenty single mannose units and twenty-four oligosaccharide chains of mannose, glucose and galactose attached O-glycosidically to serine and threonine residues of the protein. The immunogenicity of glucoamylase has now been shown to reside in the oligosaccharide chains. Antibodies specific for the oligosaccharide moieties of glucoamylase have been isolated by affinity chromatography on an adsorbent of Sepharose with glucoamylase ligands. Such antibodies should prove useful for studying the mechanism and the locale of glycosylation of a nascent protein during the biosynthesis of the glycoprotein.

Glucoamylase (EC 3.2.1.2) is a novel type of glycoprotein containing single mannose residues and oligosaccharide chains of mannose, glucose and galactose attached O-glycosidically to serine and threonine residues of the protein moiety (1,2). The enzyme hydrolyzes starch and related compounds completely to glucose (3) and is used industrially in the manufacture of crystalline glucose (4) and high fructose-syrups (5). Recently a detailed structural analysis by methylation and alkaline β-elimination methods (6) has shown that the glucoamylase molecule contains twenty single mannose units and twenty-four oligosaccharide units of various combinations of mannose, glucose and galactose residues joined by α-(1,2), α-(1,3) or α-(1,6) linkages. This type of structure for a glycoprotein is unusual and has been substantiated by data from NMR measurements (7).

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Antibodies directed against glucoamylase would be useful for studies on biosynthetic pathways of a glycoprotein. Accordingly, experiments were undertaken to determine the nature of the immunogenicity of glucoamylase, to identify the immunodeterminant groups and, if possible, to isolate antibodies directed against the enzyme. Surprisingly, the immunogenicity of glucoamylase is more strongly associated with the carbohydrate portion of the molecule rather than the protein, even though the carbohydrate portion constitutes only 15% of the total enzyme (8). Further the immunogenicity is attributable to the oligosaccharide chains and not to the single mannose units attached to the protein. Antibodies directed against glucoamylase were isolated from serum of rabbits immunized with glucoamylase. These antibodies have been shown to be directed at oligosaccharide moieties of glucoamylase and should prove useful for studying the mechanism and the locale of glycosylation of the nascent polypeptide chain of the glucoamylase molecule.

METHODS

Glucoamylase - Glucoamylase was isolated from an enzyme preparation sold commercially under the trade name of DIAZYME, (Miles Laboratories, Elkhart, IN, 46514). DIAZYME is prepared from mutant strains of Aspergillus niger or Aspergillus foetidus (4). The isolation and purification of glucoamylase was achieved by fractionation with organic solvents and chromatography on DEAE-cellulose as described previously (4,8). Many strains of Aspergillus niger produce two isoenzymic forms of glucoamylase (8). The isoenzymic form with the faster electrophoretic mobility has been employed in the present studies. Result of gel electrophoresis showed that the purified enzyme migrated as a single band which stained with reagents which are specific for proteins (9) and with reagents specific for glycoproteins (10). Ultracentrifugation by the density gradient method (8) showed that the purified glucoamylase sedimented as one component of uniform molecular size.

A sample of 50 mg of the native glucoamylase was subjected to periodate oxidation by dissolving the enzyme in 10 ml of 0.02 M sodium periodate of pH 5 and allowing the oxidation to proceed at room temperature in the dark for 24 hr. The oxidized enzyme was analyzed for carbohydrate by the orcinol-sulfuric acid method (11) and for enzymatic activity by a coupled enzyme assay procedure (8). The results showed that approximately two-thirds of the carbohydrate residues of the enzyme were oxidized by periodate and that the specific activity of the oxidized enzyme was essentially the same as that for the native enzyme.

Immunological Methods - A solution of the antigen was prepared by dissolving 20 mg of the pure glucoamylase in 2 ml of sterile phosphate buffer of pH 7.2 in saline. This solution was then mixed with an equal volume of Freunds complete adjuvant and the mixture was used to immunize rabbits. A sample of 1 ml of the antigen solution was injected in the thigh muscle of the rabbit each week for three consecutive weeks. After an additional three week period, the animals were sacrificed and blood samples were collected. Antisera were prepared from the blood samples by standard immunological methods.

The antiserum obtained from each rabbit was tested for reactivity with the native and the periodate oxidized glucoamylase by the agar diffusion method. Quantitative precipitin tests were performed as described earlier (12) with the serum and the native and the oxidized glucoamylase.

Affinity Adsorbents - Two types of affinity adsorbents were prepared from cyanogen bromide activated Sepharose 4B and appropriate ligand derivatives. Mannosyl-Sepharose was prepared from 50 mg of p-aminophenyl α-D-mannoside (Cal Biochem-Behring Corp La Jolla, CA, 92112) and 4 gm cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ, 58854) utilizing a standard procedure (13). Glucoamylase-Sepharose was prepared from 25 mg of pure glucoamylase and 3 gm cyanogen bromide activated Sepharose 4B basically by the same procedure. In the preparation of the latter, the coupling reaction was conducted at pH 9 in 0.1 M carbonate buffer for 2 hrs with agitation in a shaker bath at 40°. The unreacted groups were blocked by reaction with ethanolamine and the adsorbents were poured into suitable sized columns. The columns were washed with 0.1 M sodium carbonate at pH 10, 0.1 M acetic acid of pH 2 and finally with 0.02 M phosphate buffer of pH 7 in saline.

RESULTS

Affinity Chromatography on Mannosyl-Sepharose - A sample of 2 ml of antiserum was introduced onto the column of mannosyl-Sepharose equilibrated with 0.02 M sodium phosphate buffer of pH 7 in saline. The column was washed with 150 ml of the phosphate buffer followed by 50 ml of 0.1 M mannose in phosphate buffer. The UV absorbance of the eluates was monitored with an ISCO analyzer. A photograph of the UV scan is reproduced in frame A of Fig. 1. It will be noted in the Fig. that UV absorbing material was eluted with the buffer solution but not with the mannose solution. The material which was eluted with the buffer was collected and dialyzed for 48 hr against distilled water. The dialyzed sample was then concentrated to the original volume by lyophilization. The concentrated sample, the original serum and the serum protein obtained from a glucoamylase-Sepharose column as described in the next section were examined for anti-glucoamylase antibodies by agar diffusion tests. The results showed that the original serum and the serum protein from the mannosyl-column yielded precipitin bands but the serum protein from the glucoamylase-Sepharose column did not. Apparently the antibodies had been adsorbed on the ligands of the latter adsorbent.

Affinity Chromatography on Glucoamylase-Sepharose - A sample of 2 ml of antiserum was subjected to affinity chromatography on a column of glucoamylase-Sepharose.

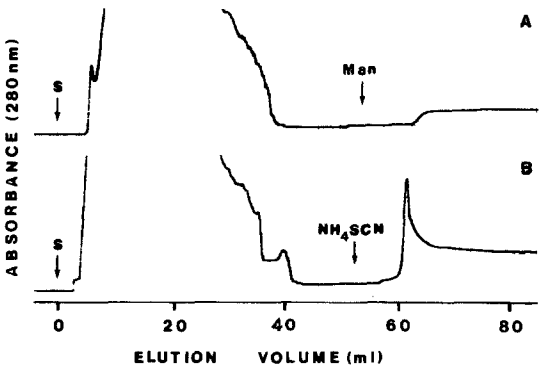

Fig. 1. Elution patterns for the immune serum from a mannosyl-Sepharose column (A) and a glucoamylase-Sepharose column (B): introduction of serum (S), elution with mannose (Man) and elution with ammonium thiocyanate (NH_4SCN).

After the introduction of the serum sample, the column was washed with 150 ml of 0.02 M sodium phosphate buffer of pH 7 in saline and then with 50 ml of 1 M ammonium thiocyanate in the phosphate buffer. The UV absorbance of the eluates was measured in an ISCO analyzer and the scan is reproduced in frame B of Fig. 1 It will be noted that UV absorbing substances were eluted both with the buffer and the ammonium thiocyanate. The eluates containing these substances were collected separately and the material which eluted with the buffer was concentrated to 2 ml by lyophilization while the material which eluted with the ammonium thiocyanate was concentrated to a final volume of 0.5 ml by ultrafiltration and dialysis. The latter sample was used in qualitative precipitin, agar diffusion and gel electrophoresis experiments. The former sample was used in agar diffusion experiments only.

DISCUSSION

The idea advanced several years ago (14) that carbohydrate residues of glucoamylase impart immunological specificity to the molecule has now been verified. The agar diffusion patterns reproduced in the photographs in Fig. 2 show that antiserum from an animal immunized with glucoamylase forms a precipitin band with the native glucoamylase but not with glucoamylase oxidized with periodate. A carbohydrate analysis on the native and oxidized enzyme showed

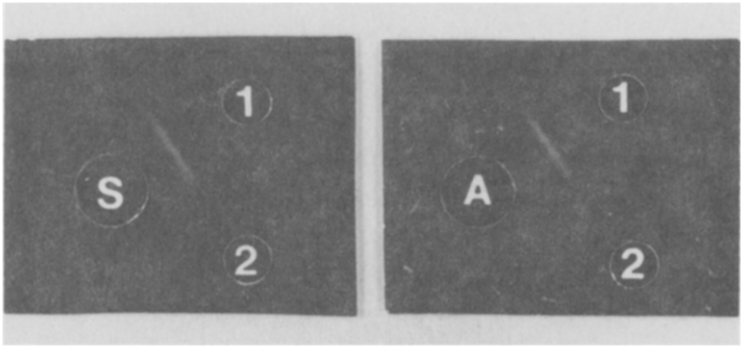

Fig. 2. Agar diffusion patterns of anti-glucoamylase serum (S) and of anti-glucoamylase antibodies (A) against glucoamylase (1) and oxidized glucoamylase (2).

that the oxidation had destroyed a high percentage of the carbohydrate residues of the enzyme. The antibodies isolated from the immune serum by affinity chromatography on glucoamylase-Sepharose behaved in an identical manner on agar diffusion. Data from quantitative precipitin experiments verified that a reaction did not occur between the oxidized enzyme and the antibodies. Accordingly it was concluded that the carbohydrate moieties of glucoamylase are the immunodeterminant groups of the enzyme. These groups react with the receptor substances on the surface of immunocytes and initiate the series of reactions leading to the synthesis of antibodies.

Based on recent studies (6), the structure of glucoamylase can be represented by the diagram in Fig. 3. In view of this type of structure and results obtained with bacterial carbohydrate antigens (12), it was anticipated that antibodies directed against the carbohydrate units of glucoamylase would be produced and such antibodies would be specific for terminal mannose residues. However, as shown in frame A of Fig. 1, antibodies in the immune serum were not adsorbed on mannosyl ligands of an adsorbent and anti-mannose antibodies were not present in such serum. Antibodies directed at glucoamylase could be isolated by affinity chromatography of the immune serum on glucoamylase-Sepharose. The elution of these antibodies was effected with ammonium thiocyanate and the elution pattern

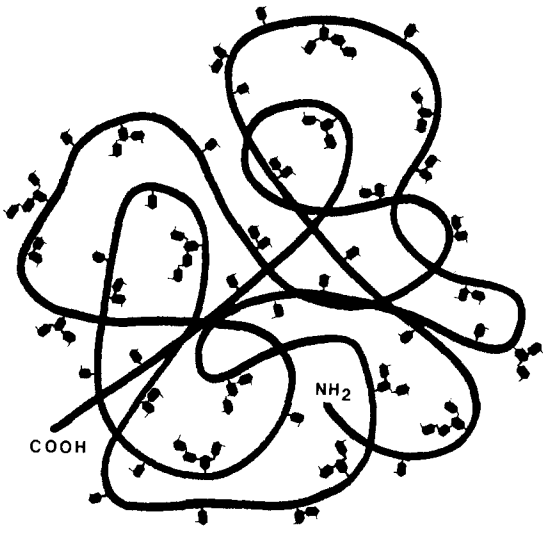

Fig. 3. Diagrammatic representation of the molecular structure of glucoamylase; solid line denotes the polypeptide chain with a carboxy terminus (COOH) and an amino terminus (NH_2), the single hexagons denote monosaccharide units and the groups of hexagons denote oligosaccharide chains.

for one experiment is shown in frame B of Fig. 1. The UV absorbing material which was eluted with the ammonium thiocyanate was collected and then dialyzed and concentrated by ultrafiltration. This material yields a precipitin band with the native glucoamylase but not the periodate oxidized glucoamylase as shown by the agar diffusion patterns in Fig. 2.

In recent years there has been much interest in the functions and biosynthetic pathways for the carbohydrate chains of glycoproteins. Biosynthetic pathways which involve the processing of the oligosaccharides of glycoproteins have been proposed (15). The processing of carbohydrate chains is quite feasible for glycoproteins with long carbohydrate chains. However, the processing of carbohydrate chains of glycoproteins with a structure such as glucoamylase is highly unlikely. Such chains consist of single or a few monosaccharide residues and are probably attached directly to the glycoproteins by glycosyl transferases of high specificity (16). Antibodies which are specific for the carbohydrate of a glycoprotein with short oligosaccharide chains should prove to be valuable

for investigating the glycosylation of the newly synthesized polypeptide chain of the glycoprotein.

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