



The carbohydrate associated with myrosinase
by Frances Huotari

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:

The purpose of this work was to investigate the carbohydrate associated with the thioglucosidase fraction of myrosinase.

The carbohydrate was separated by papain digestion of the enzyme followed by purification of the products. The resulting glycopeptide contains only 2 percent amino acids and appeared to be homogeneous in electrophoresis. Amino and carboxypeptidase digestions released amino acids which were not identified. Approximate composition (by weight) of the carbohydrate is 33 percent arabinose, 37 percent galactose, 20 percent uronic acid, and 23 percent amino sugar. Mild hydrolysis suggested that the pentose exists as an alpha-linked arabofuranose.

The carbohydrate portion of the protein is an oligosaccharide. Linkage to the protein was not established.

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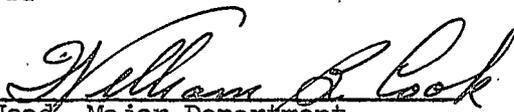
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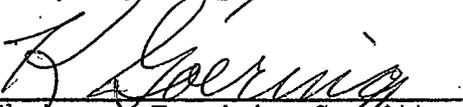
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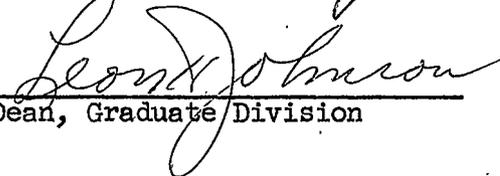
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ABSTRACT

The purpose of this work was to investigate the carbohydrate associated with the thioglucosidase fraction of myrosinase.

The carbohydrate was separated by papain digestion of the enzyme followed by purification of the products. The resulting glycopeptide contains only 2 percent amino acids and appeared to be homogeneous in electrophoresis. Amino and carboxypeptidase digestions released amino acids which were not identified. Approximate composition (by weight) of the carbohydrate is 33 percent arabinose, 37 percent galactose, 20 percent uronic acid, and 23 percent amino sugar. Mild hydrolysis suggested that the pentose exists as an alpha-linked arabofuranose.

The carbohydrate portion of the protein is an oligosaccharide. Linkage to the protein was not established.

INTRODUCTION

It has been clearly established that many proteins contain tightly bound carbohydrates. Several proteins of blood plasma, antibodies, and certain hormones have been shown to contain varying percentages of carbohydrate material. Protein-carbohydrate complexes are constituents of cell surfaces, components of connective tissue, and they are primarily responsible for the viscosity of mucous (1, 2).

Several enzymes have been "isolated" which contained varying amounts of carbohydrate. Since even highly purified β -glucosidases had been shown to contain a small percentage of carbohydrate, Helferich (3) suggested in 1937 that the carbohydrate might form the holding group of the enzyme. He compared adsorption of the glycoside sugar radical by the enzyme to crystallization on seed nuclei. Thus the bound carbohydrate could be responsible for enzyme specificity.

Since then various enzyme preparations have been found to contain carbohydrate.

In 1944 Bader (4) obtained a crystalline mucoprotein from horse serum with cholinesterase activity. However, the activity was much lower than other preparations of the same enzyme, so the purity is in doubt. In 1949 Surgenor (5) found cholinesterase activity in human plasma fractions along with at least two α_2 macropoteins. He did not suggest that the enzyme was a mucoprotein.

Fischer (6) found that a polymannan accompanied the purification of yeast invertase, He was able to separate carbohydrate from protein by adsorption on bentonite, although this resulted in relatively rapid denaturation of the enzyme. Fischer suggested that the carbohydrate

might serve to stabilize the invertase. A few years later Cifonelli and Smith (7) separated the invertase from accompanying mannan by adsorption on a charcoal column. Active invertase remained on the column and no activity could be found in the eluate.

Boser (8) detected mannose in crystalline yeast hexokinase. Commercial preparations contained more than 50 percent mannose, but paper electrophoresis separated the carbohydrate portion from one of the two protein bands which formed.

In 1954 Okasaki (9) purified saccharogenic amylase from Aspergillus oryzae. The enzyme was purified by adsorption on acid clay and activated carbon and contained glucose and xylose. Other workers have separated enzymes from carbohydrate impurities by similar methods; this suggests that the glucose and xylose may be part of the enzyme molecule.

The thioglucosidase fraction of myrosinase isolated by Gaines (10) also contained carbohydrate. Gaines was not able to separate the carbohydrate by electrophoresis or by tryptic digestion. The ratio of absorbance at two different wavelengths in the orcinol, sulfuric acid determination suggested that the carbohydrate was either galactose or a mixture of glucose and mannose.

The purpose of this thesis was to investigate the carbohydrate of myrosinase and to determine, if possible, its linkage to the protein.

METHODS AND MATERIALS

Sinigrin was purchased from California Biochemicals Corporation. Proteolytic enzymes were purchased from Nutritional Biochemicals Corporation. The enzymes were essentially free of carbohydrate as shown by the anthrone method (11).

For analysis of enzyme activity, 1.5 mg of substrate in 0.5 ml of 0.1 M citrate buffer, pH 6.2, and 0.002 M ascorbic acid was incubated for 1 hour at 37° C with 0.2 ml enzyme solution. Glucose was determined by the dinitrosalicylic acid method (12).

Protein analysis was done qualitatively by ultraviolet light absorption at 280 $m\mu$ and quantitatively by the method of Lowry (13) and by the biuret reaction. One percent CuSO_4 and 22 percent NaOH (1:3) were mixed immediately before use (0.25 percent CuSO_4 in 17 percent NaOH). Two ml each of reagent and protein solution were mixed, allowed to stand for ten minutes, and read at 560 $m\mu$ (14). Human serum obtained from the Red Cross was used as a standard. Amino acid analysis was done with the ninhydrin reagent of Moore and Stein (15). Protein-bound carbohydrate was determined colorimetrically by the modified anthrone method of Shetlar (11). Amino sugars were determined by the method of Lee and Montgomery (16) and uronic acids with carbazole according to Dische (17).

Substrate Purification

Two hundred grams of ground, defatted seed from Oriental yellow mustard, Brassica juncea, were boiled for 20 minutes with 2 liters of 75 percent acetone, decanted, and the supernatant concentrated to 400

ml in vacuo. The solution was then stirred with 200 ml Amberlite IR 4B (20 to 50 mesh) in the chloride form. The resin was washed thoroughly with water and packed into a column. The column was eluted with 1 liter distilled water adjusted to pH 3 with HCl followed by 1 liter of same adjusted to pH 2, and finally with 1 liter of 0.1 N KOH. Sinigrin appeared in the 0.1 N KOH eluates. Slightly colored solutions were decolorized by passing through a charcoal-celite column. The eluates were neutralized and evaporated. The crude sinigrin was recrystallized from 70 percent ethanol. The melting point of the product was 127-129° C.

Purification of Myrosinase

Thirty pounds of defatted, finely ground seed were mixed with enough water at 40° C to make a workable suspension. After 5 hours the mixture was filtered through a Sperry filter press. Twelve and a half pounds of $(\text{NH}_4)_2\text{SO}_4$ were added to raise the concentration to 25 percent saturation and the mixture was stirred for about an hour. It was then filtered with the aid of Super-cel and $(\text{NH}_4)_2\text{SO}_4$ was added to increase the concentration to 40 percent saturation. The mixture was stirred again and filtered with Super-cel. Washing the water easily removed the precipitated enzyme from the Super-cel. The 3 gallons of enzyme solution were dialyzed against cold running tap water, precipitated with an equal volume of 50 percent isopropyl alcohol and filtered with Super-cel. Washing with water again dissolved the crude enzyme from the filter aid. This enzyme was dialyzed and

frozen in small batches which were thawed immediately before final purification.

Sufficient DEAE cellulose was dispersed in 0.1 M citrate buffer, pH 7.0, to make a gravity packed column approximately 4.3 x 30 cm. It was washed by allowing the 0.1 M citrate buffer to drip through the column overnight and then rinsing with water. About 300 ml of enzyme solution was added to the column and eluted with 0.1 M citrate buffer, pH 6.2. This fractionation gave the two peaks reported by Gaines (10) which appear to be characteristic of the myrosinase system.

The thioglucosidase fraction (first peak) was again dialyzed, precipitated with alcohol and dried with alcohol and ether. This treatment denatured the enzyme, and the resulting dry powder was easily stored until needed for proteolytic digestion.

The DEAE column was regenerated by passing through an equal volume of 0.2 N NaOH. The column was then washed overnight with water and citrate buffer. After 1 or 2 regenerations considerable packing occurred so that it became necessary to re-pack the column.

The purity of the enzyme was examined by means of the phase rule (18) (Figures 1 and 2). The enzyme used in these measurements was obtained after the final dialysis before it was dried with alcohol and ether.

The following attempts were made to separate the carbohydrate from the protein by methods which would not break covalent bonds. A portion of the enzyme was dissolved in 1 N NaOH and ethanol added. Neither carbohydrate nor protein was precipitated at ethanol concentrations

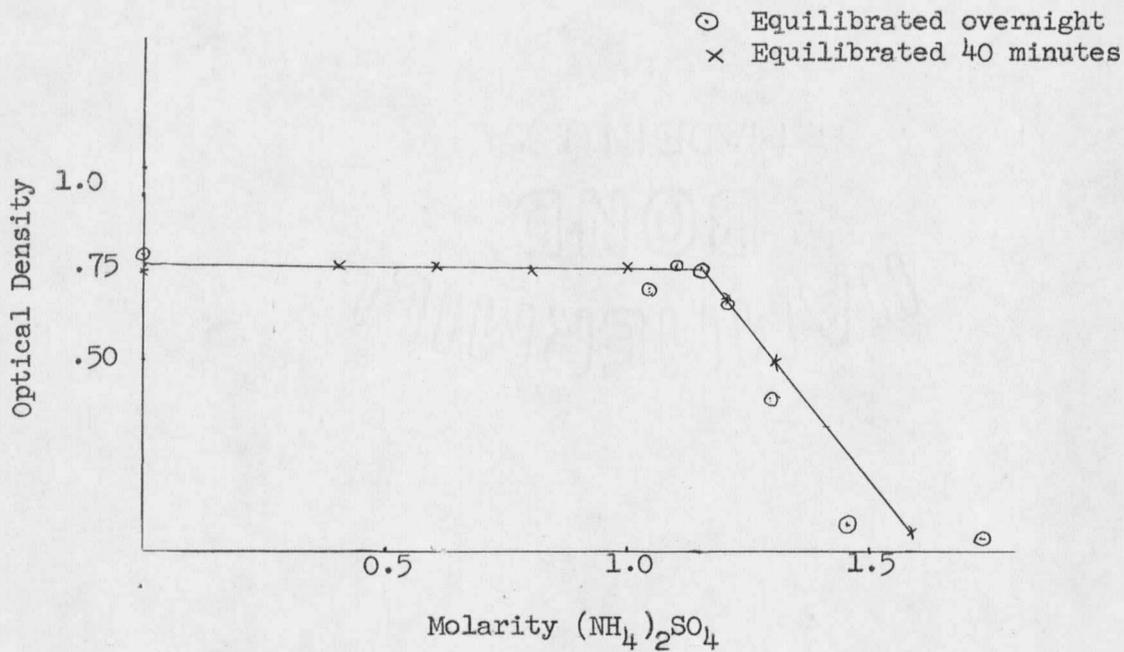


Figure 1. Solubility of β -thioglucosidase with increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ in Acetate buffer pH 5.5.

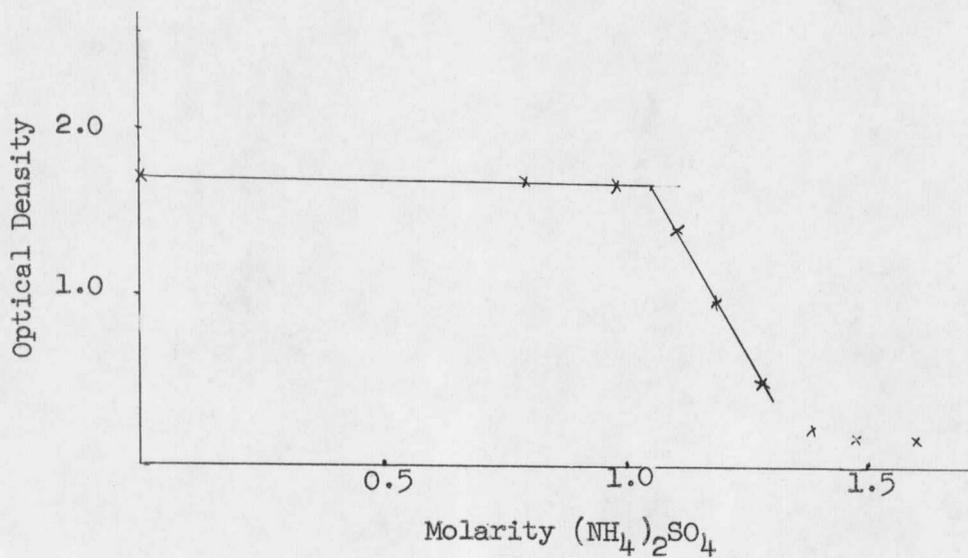


Figure 2. Solubility of β -thioglucosidase with increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ in Acetate buffer pH 5.5.

below that which would precipitate the sodium hydroxide.

In another case the enzyme was adsorbed on a charcoal-celite column and eluted with increasing concentrations of ethanol. Thorough elution with concentrations up to 95 percent ethanol failed to elute the carbohydrate fraction.

A partially hydrolyzed fraction from papain digestion was purified according to procedures which will be mentioned later. This partially digested glycopeptide was subjected to electrophoresis in a Spinco Model B paper electrophoresis apparatus. Good resolution was obtained with 0.05 M veronal buffer, pH 8.5, 6 ma, 150 volts for 16 1/2 hours. Peptides were detected with a reagent containing 0.025 percent ninhydrin (w/v) in 80 ml isopropyl alcohol, 10 ml glacial acetic acid, and 10 ml pyridine. The dried strips were dipped in the reagent and heated to 100° C for 5 min. Glycopeptides were detected on glass fiber strips¹ by a reagent composed of 50 mg α -naphthol, 2 ml concentrated sulfuric acid, and 48 ml absolute alcohol. The strips were dipped in the reagent, heated to 100° C for 5 min on a glass plate, and placed between strips of cellophane tape.

The results (Figure 3) show several peptide bands and several carbohydrate bands which appear to correspond to the peptides. However, the carbohydrate reagent reacted with some component or impurity in the glass fiber paper, making the results difficult to interpret.

¹The strips were cut from glass fiber filter paper obtained from H. Reeves and Co., 9 Bridewell Pl., Clifton, New Jersey.

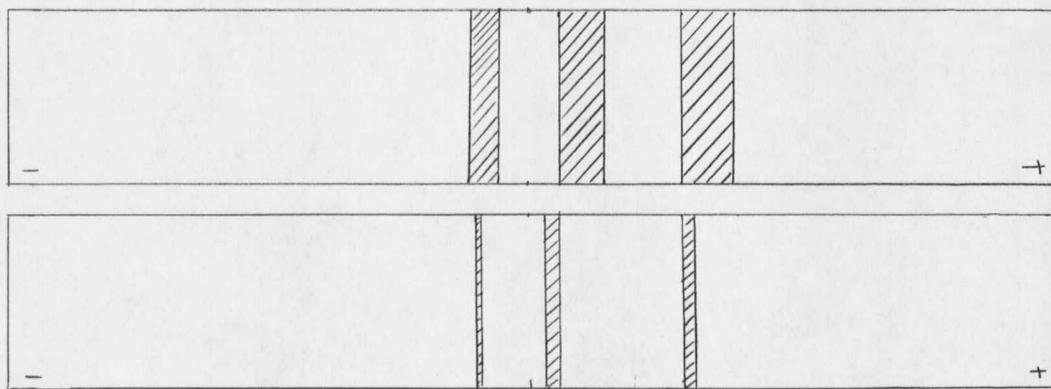


Figure 3. Electrophoresis patterns of partially purified glycopeptide. The top strip was stained with ninhydrin, the lower strip with α -naphthol in sulfuric acid. Electrophoresis was done in 0.05 M veronal buffer at pH 8.5, 6 milliamps, 150 volts for 16 $\frac{1}{2}$ hours.

Similar attempts were made with intact protein, but the carbohydrate forms 1 percent by weight of the enzyme and no identifiable separations could be attained.

Short pepsin digestions were made to try to separate the active site from the carbohydrate. Twenty minutes' digestion resulted in a gradual increase in thioglucosidase activity to a maximum followed by rapid loss of activity. Similar samples were hydrolyzed to the extent of maximum activity, neutralized, and dialyzed. Both activity and carbohydrate appeared in the dialyzate within a short time.

Isolation of the Carbohydrate Portion (19)

The thioglucosidase, 6.6 g, was suspended in 60 ml of water, boiled to insure that the enzyme was denatured, and treated with 3 ml of twice crystallized papain solution, 0.40 g disodium EDTA, and 200 mg cysteine. The pH was maintained at 6.5 by intermittent addition of 1 N LiOH. This mixture was incubated at 60° C and 1 ml aliquots of papain were added twice daily. The hydrolysis was continued for 96 hours. After digestion a small brownish precipitate remained which was removed by centrifugation. The clear solution was chilled to 0° C, passed through a 17 x 170 mm column of Dowex 50 X8 (100 to 200 mesh) in the H⁺ form, and eluted thoroughly with cold water. The glycopeptide solution was neutralized immediately with dilute NaOH. It was then concentrated in vacuo to 10 to 20 ml and the glycopeptide was precipitated by adding 9 volumes of absolute alcohol. The mixture was centrifuged and the supernatant discarded. This precipitation procedure was

repeated two or three times.

Purification data are shown in Table I.

The final product from ethanol precipitation will be referred to as the purified glycopeptide. The "partially hydrolyzed fraction" mentioned earlier was treated similarly except the papain hydrolysis was shortened to 48 hours.

The purified glycopeptide was hydrolyzed with HCl, 0.01 to 0.2 N, for 24 and 48 hours at 100° C. The hydrolyzed solutions were chromatographed with butanol, pyridine, water (6,4,3) and color developed with CD-1 (2 amino-biphenyl hydrogen oxalate) (20). Only galactose and arabinose were found present; they were identified by comparing with the same known sugars run simultaneously. (See Figure 4) The identification was not confirmed by using other solvent systems because of the limited supply of material.

Quantitative measurements of galactose and arabinose were made by hydrolyzing duplicate samples in 0.01 N HCl and Dowex 50 X8 (H⁺) at 100° C for 25 hours (21). The samples were decanted, evaporated, and developed in the same solvent system as above. One of the duplicates was sprayed with CD-1 and the other eluted and estimated using the phenol, sulfuric acid method (22).

Other samples of the purified glycopeptide were analyzed colorimetrically for uronic acids and amino sugars as indicated previously. Analysis by the direct Ehrlich reaction (23) showed no sialic acid. Lysozyme digestion gave no increase in reducing activity.

Table II illustrates approximate quantitative composition of the

