The isolation and purification of Brassica juncea myrosinase and a study of its glycoprotein nature
by Kenneth Nordahl Thompson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University

Abstract:
Myrosinase of high purity was isolated from seeds of yellow Oriental mustard,. Brassica juncea, by the following sequence of purification steps: (1) water extraction of the milled oil-free seeds, (2) ammdnium sulfate precipitation (40-80% saturated fraction recovered), (3) ethanol precipitation (50% fraction recovered), (4) step-wise elution from carboxymethylcellu-lose, and(5) gel filtration through Sephadex G-200. The enzyme-preparation, appeared to contain thioglucosidase and sulfatase activity when incubated with sinigrin solution at 3 7°C; however, separation of the two enzymes was not accomplished. A 250-fold purification of thioglucosidase and a 115-fold purification of sulfatase were obtained.

Myrosinase isolated in this work was estimated to have a molecular weight of 320,000 ± 15,000 by a gel filtration technique-and contained 19.2% carbohydrate. Analyses by paper and gas-liquid chromatography showed the carbohydrate to include residues of L-arabinose, L-fucose, D-xylose, D-mannose, D-galactose, D-glucose,-D-glucosamine, and N-acetyl-neuram-inic acid. Amino acid analysis revealed a high glutamic and aspartic acid content and a low percentage of sulfur-containing amino acids.

Digestion of myrosinase with a Streptomyces griseus protease and subsequent fractionation by a series of gel filtrations served to partially characterize the carbohydrate present. Major amounts of the carbohydrate appeared to be present as an associated heteropolysaccharide with an approximate molecular weight of 55,000. This polysaccharide contained L-arabinose, D-xylose, D-glucose, and D-galactose. Carbohydrate moities with molecular weights of approximately 1000 were indicated as present.. These contained D-xylose, L-fucose, D-mannose,. D-glucose, D-glucosamine , and N-acetyl-neuraminic acid; however, specific assignment of sugars to a particular moiety, or moieties to a particular enzyme, was not accomplished. The high carbohydrate content (85%) and the presence of serine, threonine, aspartic and glutamic acids in this oligosaccharide mixture suggested covalently-bound carbohydrate in myrosinase similar to that in known glycoproteins.

Treatment of myrosinase with 8 M urea solution followed by gel filtration appeared to free a large polysaccharide from most protein present. Studies with urea supported the results obtained from the protease digest.

THE ISOLATION AND PURIFICATION OF BRASSICA JUNCEA MYROSINASE AND A STUDY OF ITS GLYCOPROTEIN NATURE

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Chemistry

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March, 1966

ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Dr. K. J. Goering for his guidance and consideration during my years of graduate study.

I wish to acknowledge the financial assistance provided by the National Science Foundation research grant GB647 (a continuation of G7497), and by the Montana State University Chemistry Department and Chemistry Station.

I wish to thank the M. S. U. Chemistry Department staff and graduate students for their advice and assistance.

I am indebted to Dr. John Robbins of the University of Oregon for obtaining the sedimentation patterns contained in this thesis.

And last, but most of all, I wish to thank my parents for the help and encouragement which they have generously given.

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ABSTRACT

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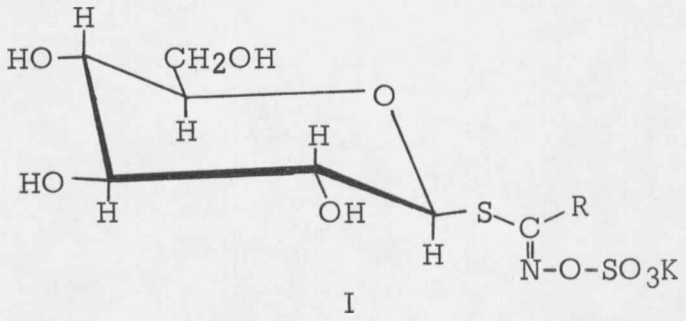
INTRODUCTION

Myrosinase is the enzyme system present in several members of Cruciferae, Tropaeolaceae, Capparidaceae and Resedaceae which is responsible for the breakdown of mustard oil glucosides (I) into glucose, inorganic sulfate and organic isothiocyanate (Gmelin and Virtanen, 1959).



I

Allyl isothiocyanate can be isolated from mustard seed, but observations over a century ago on black mustard revealed that this volatile mustard oil did not occur free in the intact seed (Boutron-Charland and Robiquet, 1831; Faure, 1831). These workers showed that isothiocyanate was liberated only when crushed seeds were suspended in water. In addition, they observed that if the crushed seeds were pretreated with protein-denaturing reagents, mustard oil liberation was prevented in the subsequent water suspension.

A glucoside isolated from black mustard seed provided evidence that glucose and inorganic sulfate were associated with the isothiocyanate in the seed (Bussy, 1840). Bussy discovered that a mixture of this glucoside

and a water-soluble protein extract from mustard seed caused the appearance of glucose, inorganic sulfate and allyl isothiocyanate. The glucoside was named as the salt of myronic acid and this later led to naming its hydrolyzing enzyme, myrosin. Today this glucoside is known as sinigrin (I, where R = CH_2=CH-CH_2-. As a result, the enzyme system has been variously referred to as myrosin, myrosinase or sinigrinase.

Initial studies on myrosinase suggested the presence of two enzymes, a thioglucosidase and a sulfatase (von Euler and Erickson, 1926). Sandberg and Holly (1932) confirmed these results and, shortly thereafter, actual separation of thioglucosidase activity from sulfatase activity was reported (Neuberg and Schoenebeck, 1933).

After two decades of apparent acceptance, the two-enzyme character of myrosinase was questioned (Ettlinger and Lundeen, 1956; 1957). One enzyme, a thioglucosidase, was postulated to act followed by a stabilizing non-enzymatic rearrangement that liberated sulfate and formed allyl isothiocyanate. Experimental data appeared later to support this premise (Nagashima and Uchiyama, 1959a-d).

New evidence for a two-enzyme system was obtained when methods different from those of Neuberg and Schoenebeck allowed separation of the two apparent enzyme activities in yellow Oriental mustard seed, Brassica juncea (Gaines and Goering, 1960; Gaines, 1960). When incubated individually with sinigrin, these enzymes released only one of the characteristic products, glucose or inorganic sulfate; however, if they were mixed together prior to incubation complete hydrolysis of sinigrin was achieved.

Each enzyme was shown to possess hydrolytic activity toward several synthetic substrates (Gaines, 1960; Gaines and Goering, 1960; 1962).

The disagreement between the one-enzyme and the two-enzyme theories is still not settled.

The thioglucosidase preparation of Gaines and Goering (1962) behaved generally as a β-glucosidase and in common with some other β-glucosidases (Helferich, 1943) was shown to contain carbohydrate (Gaines, 1960). Many glycosidases are associated with polysaccharides which seem to stabilize these systems. If for some reason this carbohydrate is eliminated during purification frequently the enzyme system becomes unstable. In most cases it is not known how these polysaccharides are attached to the protein moiety and it is conceivable that many of these systems are mixtures of polysaccharides and protein rather than specific molecular entities (Fisher and Stein, 1960).

Huotari (1962) obtained evidence for covalently-bonded carbohydrate in the thioglucosidase from yellow Oriental mustard seed. This carbohydrate appeared to be present as an oligosaccharide which was free of sialic acid and accounted for one percent of the thioglucosidase preparation. In the same work, galactose, arabinose, hexosamine and uronic acid were reported to be present in the carbohydrate moiety.

Proteins containing tightly-bound carbohydrate are numerous and have been known for many years. They are constituents of blood plasma, mucous, cell wall material, connective tissue and include some hormones, blood group substances, antibodies, as well as enzymes. Since these compounds are part of many vital processes in living things, their structure and

metabolism are of interest. Enzymes containing carbohydrate are of added interest since their carbohydrate moieties may serve in the enzymes' active sites. Helferich et al. (1938) suggested that the carbohydrate grouping of β-glucosidases may act as a "seeding" site necessary for enzyme activity. However, preliminary studies involving a mild periodate treatment of carbohydrate-containing enzymes seemed to indicate that the carbohydrate was not essential for enzyme activity (Pazur et al., 1963).

Prior to 1950, studies on proteins containing carbohydrate were primarily descriptive and have been adequately reviewed (Levene, 1926; Meyer, 1945; Stacey, 1946; Bettelheim-Jevons, 1958). These authors point out the confused nomenclature which exists in this field. Schmid (1964) has used the term "glycoproteins" to describe ". . . proteins, excluding nucleoproteins, that carry covalently-bound carbohydrate". Numerous glycoproteins have been discovered, but only three of these are known to have enzyme activity. They are Taka-amylase A (Tsugita and Akabori, 1959), acid deoxyribonuclease (Bernardi et al., 1965) and glucose oxidase (Pazur et al., 1965). The significant amount of carbohydrate that is apparently linked covalently in the enzyme myrosinase suggests that this system might be classified as a glycoprotein.

The purpose of this research was to further investigate the two-enzyme character of myrosinase and the nature of the carbohydrate-protein complex which is present.

MATERIALS AND METHODS

Assay for Myrosinase Activity

Enzymatic hydrolysis of sinigrin by myrosinase liberates glucose, inorganic sulfate and allyl isothiocyanate. Enzyme assays were made on incubation mixtures prepared by warming 0.2 ml of myrosinase solution with 0.5 ml of sinigrin solution (20 mg per ml) at 37°C. Solutions were in 0.05 M sodium citrate buffer, pH 6.1. Incubation times were varied to prevent total hydrolysis of substrate. The reaction was stopped with dinitrosalicylic acid solution when assaying thioglucosidase activity and with trichloroacetic acid solution when assaying sulfatase activity.

Sinigrin was obtained from Mann Research Laboratories, California Corporation for Biochemical Research, and Aldrich Chemical Company, Inc. Each sinigrin preparation was checked for purity by paper chromatography, along with a blank reaction containing assay reagents.

Glucose liberated by the water enzyme extract was determined by quantitative paper chromatography (Whistler and BeMiller, 1962) using n-butanol-pyridine-water (6:4:3, v/v) solvent and the phenol-sulfuric acid colorimetric procedure. The dinitrosalicylic acid method (Summer, 1925) was used to assay glucose at the other stages of enzyme purification. Sulfate was analyzed turbidimetrically as barium sulfate (Dodgson, 1961). Allyl isothiocyanate was not determined, although its odor was evident during every incubation in which glucose and sulfate were liberated.

Specific activity of myrosinase was calculated as previously done by Gaines and Goering (1962), wherein a unit of enzyme activity was defined

as that amount of enzyme which catalyzed the hydrolysis of 5 mg of substrate per hour at 37°C.

Protein Assays

Quantitative analysis for protein (Lowry et al., 1951) was accomplished using phenol reagent obtained from the Hartman-Leddon Company, Philadelphia, with egg albumin (California Corporation for Biochemical Research, B Grade, 5X recrystallized) as the standard. In dilute solution (<0.10 mg protein/ml), Kalckar's (1947) approximation provided a convenient and adequate protein determination.

Protein eluted from various chromatographic columns was followed by by optical density measurements at 280 mμ in either a Beckman Model DB or Model DK-2 spectrophotometer.

Carbohydrate Assay

The phenol-sulfuric acid method of Dubois et al. (1956) was used for quantitative analysis of carbohydrate in myrosinase. This method allowed the determination of carbohydrate without interference from protein (Keen and Opie, 1957). A composite sugar mixture containing galactose, mannose, arabinose, xylose, glucose, fucose and glucosamine was used to prepare a standardization curve for this technique. The reaction mixture was prepared by mixing 1 ml of solution containing carbohydrate (10-100 μg) with 1 ml of 5% redistilled phenol and then rapidly adding 5 ml of 95.0-98.0% sulfuric acid. After 10 minutes the solution was cooled for 30 minutes in running tap water. The optical density of the amber-colored solution was read at

490 mμ on a Beckman Model DB spectrophotometer. Carbohydrate eluted during column chromatography was followed by the same method.

Preparation of Ion-Exchange Celluloses

Diethylaminoethylcellulose (DEAE-cellulose) obtained from Eastman Organic Chemicals and carboxymethylcellulose (CM-cellulose) obtained from Sigma Chemical Company were both prepared for use in the recommended manner (Peterson and Sober, 1962) prior to equilibration in the desired buffer.

An ion-exchange cellulose column was packed by filling the glass tube to one-third its volume with starting buffer and then adding the slurry of equilibrated ion-exchange material. The column stopcock was opened and the column allowed to pack with occasional tapping to remove occluded air bubbles. Every column was equilibrated by allowing buffer to flow through it overnight at the rate used during chromatography. CM-cellulose was regenerated (Peterson and Sober, 1962) once; i.e., used two times, then discarded because its ion-exchange properties seemed to change with extended use. All elutions were run at room temperature.

Preparation of Gel Filtration Media

Two types of gel filtration material were used in this work: dextrans of various cross linkage (Sephadex) purchased from Pharmacia Fine Chemicals, Inc. and polyacrylamide (Bio-Gel P-300, 50-150 mesh) purchased from Bio-Rad Laboratories.

These materials were suspended in the eluting solvent to be used and were allowed to swell at least 3 hours in the case of gels with small pore

size (Sephadex G-15 and G-25) or at least 18 hours in the case of the gels with larger pore size. Sephadex preparations had to be "washed" free of fine particles to prevent plugging of their packed columns. "Washing" was accomplished in three repeated steps: (1) Suspension of Sephadex in the elution solvent, (2) a 30-minute settling of heavier particles, and (3) decantation of the supernatant solution which contained fine particles. Five or six repetitions of these steps were usually sufficient.

A column was packed by filling the glass tube to one-third its volume with eluting solvent and then adding a slurry of the gel to be used. After the gel had settled to a depth of 1-2 cm, the eluting solvent was allowed to flow. The formed column was equilibrated before use by passing solvent through it for at least 12 hours. All elutions were performed at room temperture.

Electrophoresis Procedures

A. Ionic nature of crude myrosinase

The ionic nature of the components in crude myrosinase was checked in a Gelman electrophoresis apparatus. This unit was equipped with Sepraphore III (microporous cellulose acetate) strips in sodium barbitol-acetate buffer, pH 8.2, and run at 100 volts and 4-5 milliamps per strip for two hours at room temperature. Approximately 25 μg of protein in 0.05 M sodium citrate buffer, pH 6.3, were applied per strip. Protein was detected on the undeveloped cellulose acetate strip with Amido Schwarz stain (100 mg Amido Schwarz per 100 ml 10% acetic acid).