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Glycosidic Enzymes of the Tropaeolum Majus

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GLYCOSIDIC ENZYMES OF THE *TROPAEOLUM MAJUS*

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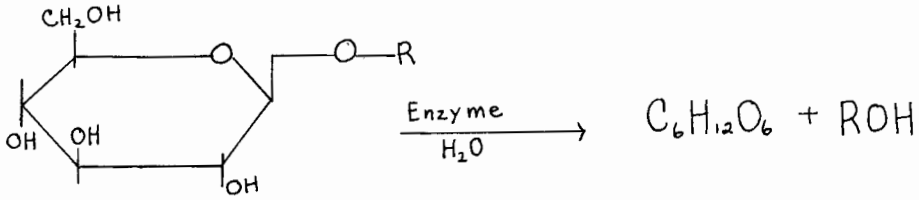
INTRODUCTION

A hydrolase is an enzyme that catalyzes a reaction which results in the addition of the elements of water and the loss of another group formerly bound to the site of addition. Glycoside hydrolases* specifically catalyze the hydrolysis of glycosides as represented by the following equation:



where R can be a carbohydrate, alkyl or aryl group. The enzymatic hydrolysis of O-glycosidic bonds is quite common in nature and involves such enzymes as amylases, cellulases, glucosidases, and invertases.

Many glycoside hydrolase systems are also included in the transferring enzymes. The reaction essentially involves a glycosyl transfer to a suitable acceptor molecule. The general mechanism for the enzymatic cleavage of glycosidic bonds is generally assumed to be a two step process involving an intermediate enzyme-glycosyl complex (1).

$$C\text{-}OR + EH \rightleftharpoons C\text{-}E + ROH$$

$$C\text{-}E + HOA \rightleftharpoons C\text{-}OA + EH$$

In this mechanism, C represents a carbohydrate residue

* The trivial names for the glycosidic enzymes will be used throughout this thesis. See appendix for nomenclature of enzymes.

(glycone portion), E the enzyme, -OA an acceptor (-OH in the case of water), and R may be another carbohydrate unit or a noncarbohydrate residue (aglycone portion).

Through the use of isotope labeling it has been shown that cleavage of the glycosidic bond takes place in the side of the bridging atom nearest to that part of the molecule for which the enzyme shows the greatest specificity (40). Similiar observations have been made with α- and β-amylases (24,43), yeast invertase (40), and β-glucosidases (5,63). In the case of sucrose hydrolysis by β-fructofuranosidase (invertase), the enzyme specificity is for the fructose portion of the molecule; thus, the bond cleaved is that between the bridge atom and the fructosyl moiety.

The range and type of specificity illustrated by the glycoside hydrolases varies considerable. An enzyme may have an absolute specificity for a portion of the molecule, in which no structural changes can be tolerated, or a relative specificity in which some changes in structure can occur. The glycone specificity is usually absolute. If a compound has a slight difference in the glycone, but is similar to the natural substrate, it generally acts as a competitive inhibitor in that although it may form the enzyme-substrate complex, it resists hydrolysis. It is common for a glycosidase to show product inhibition because

the products are similar in structure to the reactants (39).

Larger differences in glycone structure can cause complete loss of activity. A change from one epimer to another usually stops hydrolysis; for example, β-galactosidase does not catalyze the hydrolysis of α-galactosides (44). Methylation of any hydroxyl in the glycone portion of the substrate resulted in loss of activity for the β-galactosidase system (44,72). Ring size is also an important factor because β-galactofuranosides were not hydrolyzed by β-galactosidases (74).

The nature of the aglycone can vary from a carbohydrate residue to complex alkyl and aryl groups. The relative specificity for the aglycone is typical of glycoside hydrolases and has been demonstrated for alkaline and acid phosphatases (57,58), α- and β-glucosidases (23,69), invertases (51), β-galactosidases (44), and β-glucuronidases (69).

The discussion has centered around the hydrolysis of glycosides which contain the O-glycosidic bond. Compounds in which the oxygen of the glycosidic bond has been replaced by sulfur, thioglycosides, are also found in nature. The enzyme responsible for the hydrolysis is a thioglucoside glucohydrolase, which has been known since 1840 (4). Thioglucosidase has been reported in a variety of animal tissues (22), certain fungi (53), and higher plants (20).

In higher plants its occurance is usually associated with systems that contain mustard oil glycosides. These glycosides are found in the families of the Cruciferae, Resedaceae, Capparidaceae, and Tropaeolaceae.

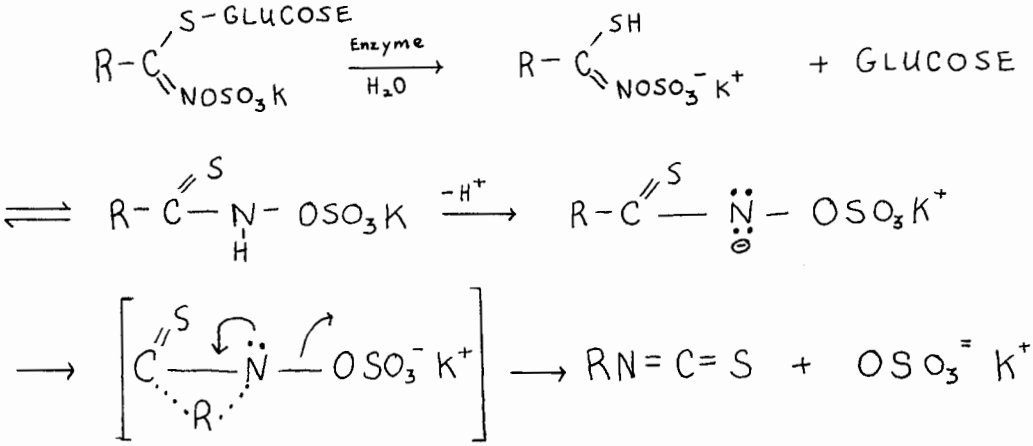
Thioglucosidase has been extensively studied in the mustard species of the Cruciferae family. It is not known whether the thioglucosidase shows absolute specificity for the sulfur linkage or if there is dual specificity allowing hydrolysis of both sulfur and oxygen linkages. Gaines and Goering (19) showed that the aglycone moiety had little effect on hydrolysis. However, the enzyme showed considerable specificity toward the glycone, resembling that observed for the β-glucosidase from almond emulsin. While it has been observed that almond emulsin β-glucosidase does not hydrolyze the mustard oil glucosides, Reese et al.(53). reported the hydrolysis of p-nitrophenyl glucoside, o-nitrophenyl galactoside, and several mustard oil glucosides by thioglucosidase systems. Tsuruo and Hata (68) showed dual activity with the thioglucosidase from yellow mustard. These systems appear to contain a single glycosidic enzyme responsible for the hydrolysis of the mustard oil glucosides and certain other β-linked glucosides.

Goodman et al.(20). prepared myrosinase (thioglucosidase of the mustard family) and showed that the enzyme was relatively non-specific not only with regard to the

aglycone moiety, but also to the glycone moiety. The 6-purine-β-D-thioglycosides of arabinose, galactose, mannose, and xylose were all hydrolyzed.

The reaction of the thioglucosidase with mustard oil glucosides may be represented as follows.

Ettlinger and Lundeen (11) visualized the enzymatic hydrolysis as the removal of D-glucose to yield a reactive intermediate which undergoes a Lossen rearrangement to give the observed products. Nagashima and Uchiyama (47) also have predicted this type of rearrangement. They employed as an analogy the mechanism of the rearrangement of hydroxamic acids proposed by Hurd and Bauer (31).



There has been some question as to the number of enzymes needed for the total hydrolysis of mustard oil glucosides. A thioglucosidase and sulfatase have been

reported by a number of workers (13,18,19,32,52,56); however, the need for the sulfatase is in doubt if the mechanism proceeds through a Lossen rearrangement. Nagashima and Uchiyama (47) supported this theory and felt that the liberation of sulfate was non-enzymatic. Reese et al. (53), on the basis of their work on the thioglucosidase of fungi, also agreed with this.

Gmelin and Virtanen have shown that there is more than one pathway for enzymatic breakdown. Thus, thiocyanate (21), isothiocyanate (20), and nitriles (60) have been reported as products under appropriate conditions. This raises some question as to whether there is a Lossen rearrangement in the hydrolysis mechanism.

While extensive experimentation has been carried out on the enzymes of the Crucifereae, little attention has been given to the enzymes of the Tropaeolaceae. A review of the literature of the Tropaeolaceae system shows that Gadamer (1899) (17) has isolated the mustard oil glucoside, glucotropaeolin,* and indicated that its hydrolysis by myrosinase yielded glucose, hydrogen sulfate ion, and benzyl isothiocyanate. Other workers (12,59) also have reported the occurance of glucotropaeolin in Tropaeolum majus. The occurance of the thioglucoside would suggest the presence

* Ettlinger and Lundeen synthesized glucotropaeolin and revised the mustard oil glucoside structure, that Gadamer had proposed, to the present day structure.

of a corresponding thioglucosidase. The purpose of this study is to investigate the hydrolytic enzymes of Tropaeolum majus. This particular plant is the only example of the Tropaeolum genus found in the northern hemisphere. It is the well-known Indian cress or common garden nasturtium.

EXPERIMENTAL

I. Methods

A. Chromatography

N,N-Diethylaminoethyl cellulose, Cell Ion DEAE (Nutritional Biochemicals Corporation), was prepared in 0.02 M barbital buffer, pH 8.6, and packed under gravity in a 2.2 cm diameter chromatography column. The column height was 25 cm. Protein samples up to 40 mg were applied to the column and eluted with buffer containing gradient NaCl up to 0.2 M. This procedure was followed in all subsquent chromatography methods. The flow rate was kept at about one ml per minute with three ml fractions being collected.

DEAE-Sephadex, A-50 (Sigma Chemical Company), was allowed to equilibrate in 0.02 M barbital buffer, pH 8.6 for 24 hours. After packing, the column (2.2 cm diameter x 18 cm high) was washed with 0.5 liters of buffer for stabilization. Protein samples of 40 mg were applied to the column. The elution flow rate was 0.4 ml per minute, and increased up to 1 ml per minute with increasing ionic strength. Fractions of three ml were collected.

Tetraethylaminoethyl cellulose, Cellex-T (Bio-Rad Laboratories, Richmond California), was dispersed in 0.02 M barbital buffer, pH 8.6 and packed as described above. The column was 2.2 cm in diameter and 18 cm high. Thirty mg

samples of protein were applied to the column and fractions were collected and tested for protein as before.

Calcium phosphate gel was prepared by the method of Keilin and Hartree (36). One ml of phosphate gel (0.131 grams per ml) was added to the enzyme solution, which had been dialyzed against distilled water, to give a gel-protein ratio of 2 to 1. The suspension, after setting 15 minutes at 4° C, was centrifuged. The gel pellet was eluted with 0.2 M NaCl.

B. Electrophoresis

Cellulose acetate electrophoresis was carried out on Sepraphore III strips with barbital buffer, pH 8.5, and ionic strength 0.075, or 0.05 tris-barbital buffer, pH 8.8. The electrophoresis was carried out for 2 hours with a potential gradient of 275 volts (27.5 volts per cm). After each run, the strips were stained with Ponceau S. The electrophoresis was performed in a Gelman's Deluxe Electrophoresis Chamber.

Starch gel electrophoresis was carried out in 0.02 M borate buffer, pH 8.2. The hydrolyzed starch was purchased from Sigma Chemical Company. A 15:100, weight to volume, ratio of starch to buffer was used in preparing the 3 cm x 11.5 cm x 0.5 cm gel blocks. Electrophoresis was carried out at 175 volts for 20 hours, the time being dependent upon the bromophenol blue marker which was applied at the origin.

The electrophoresis was stopped after the marker had traveled 7 cm. The bands were detected with amido black stain.

C. Assay procedures

The assay of protein was performed colorimetrically with Folin-Ciocalteu reagent (purchased from Scientific Products), according to the method of Lowry (42). Bovine serum albumin, Fraction V, (Sigma Chemical Company), was used as the standard. Qualitative protein determinations of column effluents were followed by absorption at 280 nm.

The assay for β-glucosidase activity was accomplished by following the hydrolysis of p-nitrophenyl-β-D-glucoside. The incubation mixture, 0.1 ml enzyme, 0.5 ml of 10^{-3} M substrate in buffer, and 2.4 ml of phosphate buffer, pH 6.8, was allowed to proceed for one hour at 37° C or until 5 to 35 micrograms of p-nitrophenol had been liberated. Since excessive heating caused decomposition of the substrate, the incubation mixture was placed in a boiling water bath for only 2 minutes to stop the hydrolysis. The absorption of the liberated p-nitrophenol was recorded at 420 nm. When necessary, any protein precipitate was removed by centrifugation. Enzyme and substrate blanks were compared concurrently.

The assay of reducing sugars liberated by enzymatic hydrolysis was accomplished by using the dinitrosalicylic method (DNS) (2). The conditions were the same as above,

except the total volume was reduced to 1 ml and substrate concentration was 10^{-2} M.

The thioglucosidase activity was also followed spectrophotometrically by the change in absorbance due to the disappearance of the substrate, sinigrin, at 227.5 nm, according to Schwimmer (61).

Enzyme activity was assayed in terms of micromoles of product (glucose or p-nitrophenol) liberated per hour per ml of enzyme.

D. Miscellaneous methods

All centrifugation was carried out for 20 minutes at 0° to 5° C at 25,000 x G using a Lourdes Beta-Fuge.

Dialysis was performed using a 100 to 1 ratio of water to enzyme solution at 4° C for 24 hours. 2-Mercapto-ethanol, 10^{-3} M was added to the water to minimize denaturation during the process.

Ascorbic acid was determined by the method of Tillman (37).

The activation energy was determined by diluting Fraction III to .52 mg of protein in the assay mixture. Enzyme concentration was kept constant. Substrate concentrations were 10^{-2} M so that the enzyme was at maximum velocity.

E. Michaelis constants

The Michaelis constants were calculated by the double-reciprocal plot of Lineweaver and Burk (41). Initial

velocities were determined from time-velocity curves at various substrate concentrations.

F. Ion inhibition

Ion inhibition of thioglucosidase activity was determined spectrophotometrically by following the disappearance of sinigrin. When this method could not be applied, the alternate method of assay described above was used. p-Chloromercuribenzoate, used as a sulfhydryl inhibitor, was dissolved in phosphate buffer by adding NaOH and back titrating to a pH of 6.9 with H_2SO_4. This pH was chosen because it was the lowest pH that would retain PCMB in solution at 2×10^{-3} M.

II. Materials

Tropaeolum majus seeds (Nasturtium Golden Gleam) were obtained from the Portland Seed Company, Portland Oregon. These seeds were fairly large, approximately 4-8 mm in diameter, and resembled a shriveled pea seed.

p-Nitrophenyl-β-D-glucoside, (pNPG), sinigrin, glycogen, glucosinalbin, amygdalin, α-Me-D-glucoside, and glucotropaeolin were purchased from California Corporation for Biochemical Research, Los Angeles, California. Amylose, sucrose, arbutin, isopropyl-β-D-cellobiose, D-melibiose, amylopectin and glucose were purchased from Sigma Chemical Company, St. Louis, Missouri. All other substrates were purchased from Nutritional Biochemicals Corporation, Cleveland,

Ohio. All substrates were of highest available grade.

2,4-Dinitrophenyl-S-glucoside, phenyl-S-glucoside, and p-nitrophenyl-S-glucoside were prepared by the method of Wagner and Nuhn (70).

All salts used in inhibition studies were analytical reagent grade.

Other compounds were purchased as follows: L-ascorbic acid from Matheson, Coleman and Bell, Cincinnati, Ohio; D-gluconic acid lactone and β-D-galactonolactone, Sigma Chemical Company; 2-mercaptoethanol, Aldrich Chemical Company, Milwaukee, Wisconsin; p-chloromercuribenzoate (PCMB) and L-cysteine, California Corporation for Biochemical Research; iodoacetic acid from Nutritional Biochemicals Corporation; and ethylenediaminetetraacetic acid (EDTA), J. T. Baker Company, Phillipsburg, New Jersey.

Tap distilled water was used for all procedures.

III. Isolation and purification of the enzymes

A. Fraction I

The finely-ground seeds of Tropaeolum majus were defatted with hexane using a soxlet extractor. The defatted seeds were allowed to incubate in a minimum amount of water for 24 hours to remove the natural substrates from the system. The extract was expressed through several layers of cheese-cloth and centrifuged to remove all insoluble materials. The resultant solution was the crude extract.

B. Fraction II

Solid ammonium sulfate (enzyme grade) was slowly added to the crude extract to bring the saturation to 80 percent. Magnetic stirring was used to prevent localized excesses of salt concentration. The solution was allowed to stand for 4 hours at 0° C and the protein was then centrifuged and suspended in a minimum amount of distilled water. This procedure removed nearly all of the enzyme activity from the crude extract and allowed the use of minimum volumes during further purification.

C. Fraction III

Fraction II was treated with solid ammonium sulfate to increase the saturation by 10 percent increments. The protein was centrifuged after each addition of ammonium sulfate and the centrifugate was suspended in a minimum amount of distilled water and dialyzed.

D. Fraction IV

A precipitate formed in Fraction III after standing 24 hours under refrigeration (4° C). This precipitate was collected and suspended in distilled water. This residue was only obtained when saturated ammonium sulfate solution rather than solid ammonium sulfate was used for the fractionation.

E. Fraction V and VI

The enzyme was further purified by chromatography on DEAE-cellulose (Fraction V) and DEAE-Sephadex (Fraction VI).

Protein from Fraction III was added to the chromatography column and eluted with gradient NaCl in barbital buffer, pH 8.6.

F. Crystalline protein

The protein in fractions 18 to 30 from the DEAE-Sephadex column was precipitated with ammonium sulfate. The protein was crystallized under these conditions and was collected and washed once with ice water.

G. Electrophoresis studies

Three to five microliters of Fraction III were subjected to electrophoresis on cellulose acetate strips as described elsewhere in the experimental section.

To test for enzyme activity, the cellulose acetate strips were divided longitudinally. One half-strip was then stained to show the position of the protein bands. Using this guide, the bands in the untreated strip could be approximately located, eluted, and tested for activity.

In starch gel electrophoresis, 10 microliters of Fraction III were applied to the prepared gel. To test for enzyme activity, the starch gel strips were sliced lengthwise into layers with a cheese cutter. One layer was stained to show the position of the protein bands. The other layer was cut up and the starch strips placed in test tubes overnight at 4° C with 1 ml of buffer to diffuse out the enzyme activity.

RESULTS AND DISCUSSION

I. Glycosidic Enzymes of the Tropaeolaceae

A. Purification and separation of the enzymes

The enzymes were easily obtained as a crude extract in water. It was found that the low oil content of the Tropaeolum seeds made it unnecessary to defat the samples prior to protein extraction.

In all experiments the glycosidic enzymes were located in the 50 - 70 percent saturation range (Fraction III), during ammonium sulfate fractionation. The activity of the β-glucosidase was essentially in the 70 percent fraction. Fraction III contained some precipitated protein which dissolved upon warming to room temperature.

When Fraction III was treated with saturated ammonium sulfate solution and allowed to stand under refrigeration (4° C), a protein precipitate appeared (Fraction IV). The solubility of this fraction was much greater at higher temperatures. This precipitate was found to possess mostly β-glucosidase and β-galactosidase activities and failed to hydrolyze the mustard oil glucosides. It also contained a system responsible for the hydrolysis of sucrose. When precipitation of Fraction IV was not desired, solid ammonium sulfate was used for further fractionation of the system.

Fractionation employing organic solvents proved

unsuccessful since the protein precipitated in a very gummy, nearly insoluble mass. Large quantities of water were needed to redissolve it, making further purification difficult.

Additional purification of the enzymes was attempted using ion exchange resins. The thioglucosidase was contained in the first protein peak eluted from the DEAE-cellulose and DEAE-Sephadex columns, indicating little adsorption (figures 1 and 2). The crystalline protein, isolated from the effluent of the DEAE-Sephadex column, contained a system responsible for the limited hydrolysis of the polysaccharides, amylopectin and glycogen.

The β-glucosidase was strongly adsorbed on the ion exchange columns with only a small percentage being eluted. A sodium chloride gradient up to 1.5 M would not elute the β-glucosidase. Helferich and Kleinschmidt (28), in their study of the β-glucosidase of almond emulsin, observed that at higher pH more β-glucosidase was adsorbed on DEAE-Sephadex. Some of the ion-exchange resin from the top of the column was incubated with substrate. The resulting β-glucosidase activity indicated that whereas the enzyme was bound to the resin, the active site was not affected. Elution of the enzyme from the adsorbents at lower pH was not tried because Fraction IV contained high activity and was easily obtained.

The use of calcium phosphate gel did not lead to

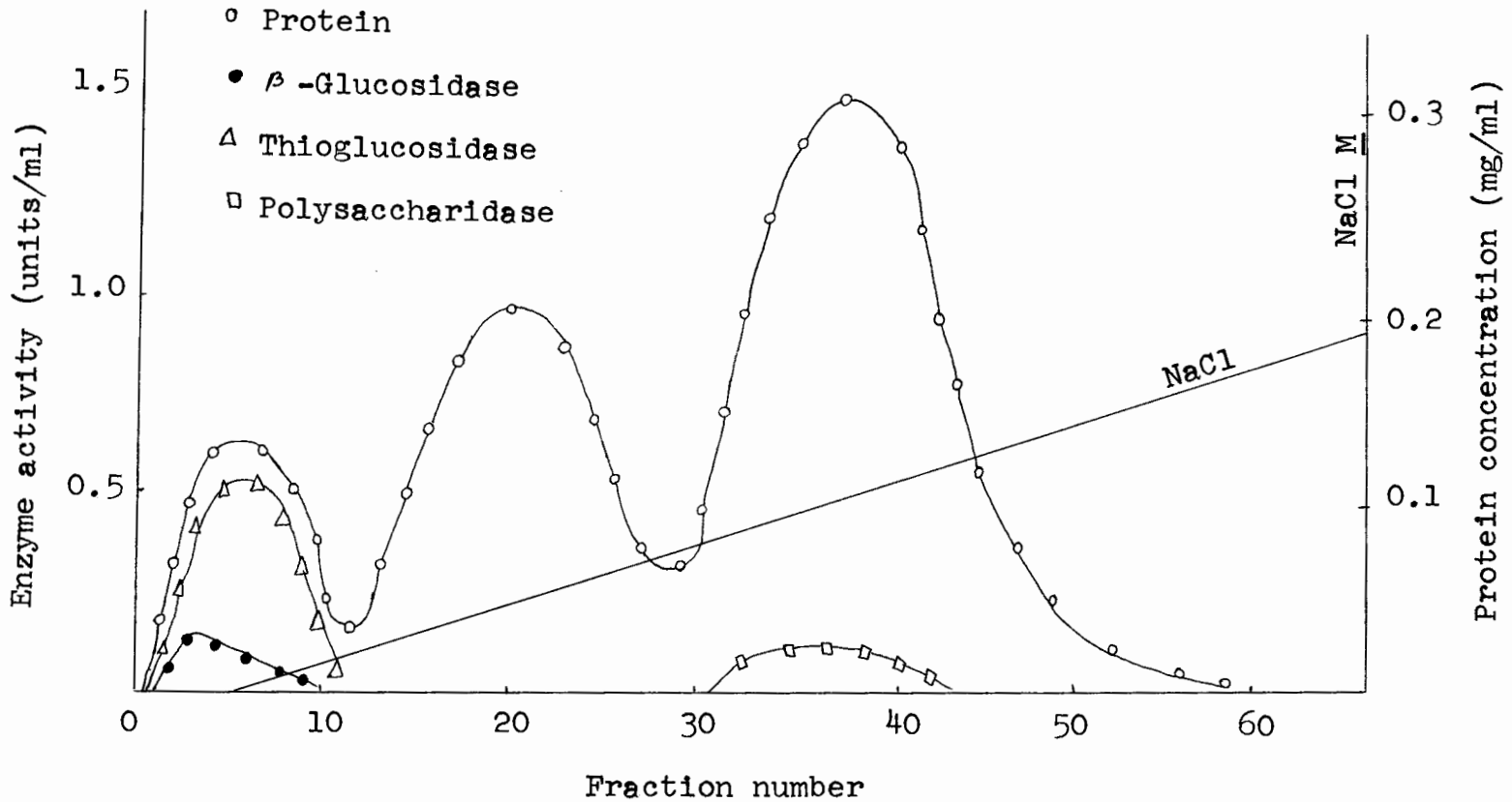


Figure 1. Chromatography of Fraction III on DEAE-cellulose. Column conditions and elution procedures are described in the text.

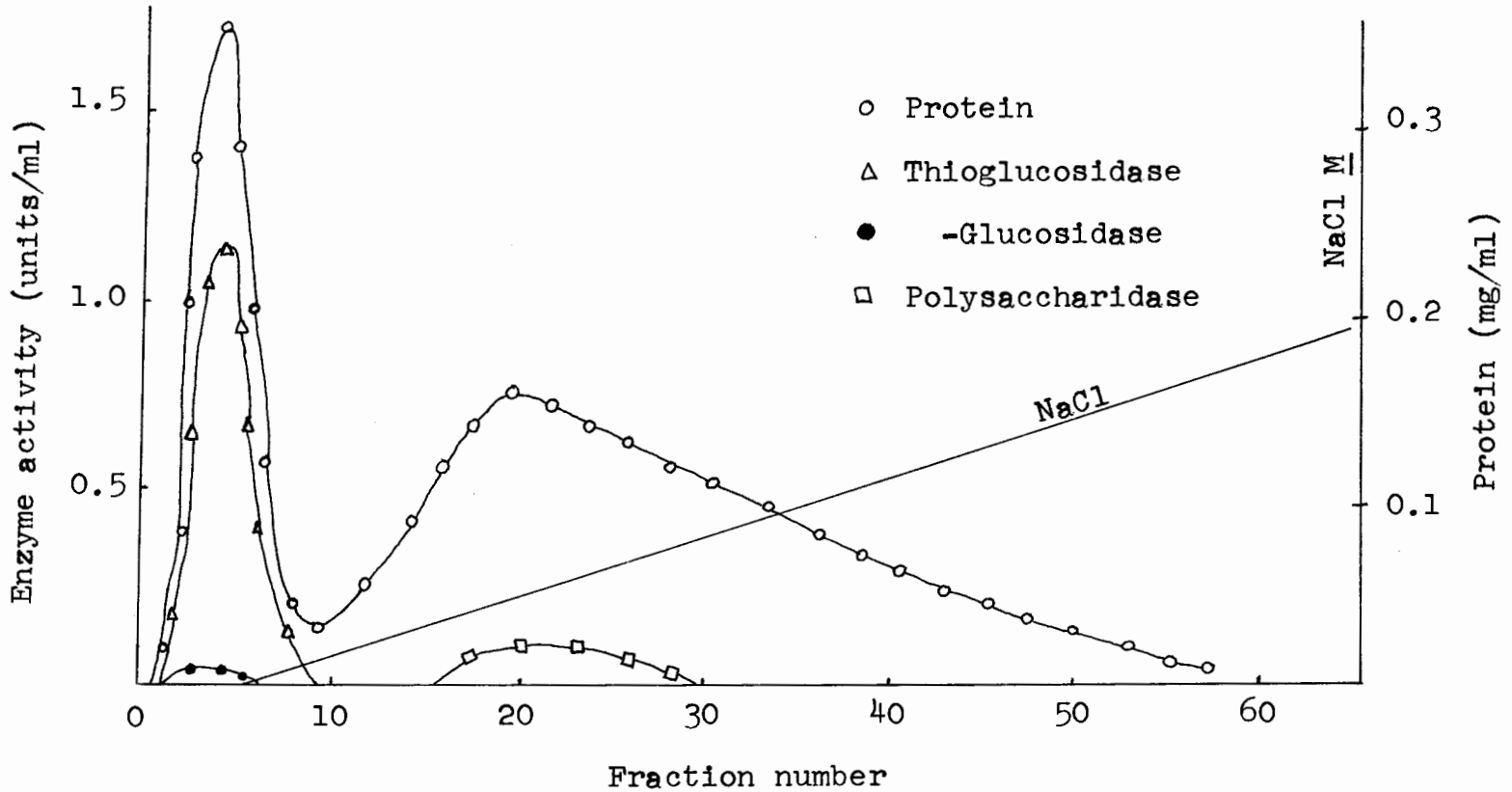


Figure 2. Chromatography of the ammonium sulfate fractionated extract on DEAE-Sephadex. Column conditions and elution procedures are described in the text.

extensive purification. The thioglucosidase was not adsorbed on the gel; however, the β-glucosidase was adsorbed and could not be eluted using 0.2 M NaCl.

Tsuruo et al. (64) had reported good separation of mustard thioglucosidase with TEAE-cellulose. Under the same conditions no separation could be obtained with the Tropaeolum system (figure 3).

The results of the purification of β-glucosidase, thioglucosidase, and β-galactosidase are reflected in their specific activities (Table I).

B. Electrophoresis studies

Electrophoresis of Fraction III on cellulose acetate gave 4 distinct protein bands, some much broader and more intense than others (figure 4). The direction of protein migration was toward the anode.

The locations of hydrolytic activity, using the band numbers assigned in figure 4, are seen in Table II. Overlap of the bands made absolute assessment of their activity difficult. There were two bands showing β-glucosidase activity, which could be interpreted to be a β-glucosidase and a β-galactosidase possessing dual activity. Protein possessing thioglucosidase activity was spread out over several bands.

Starch gel electrophoresis did not effect good separation. Amido black failed to stain low concentrations of

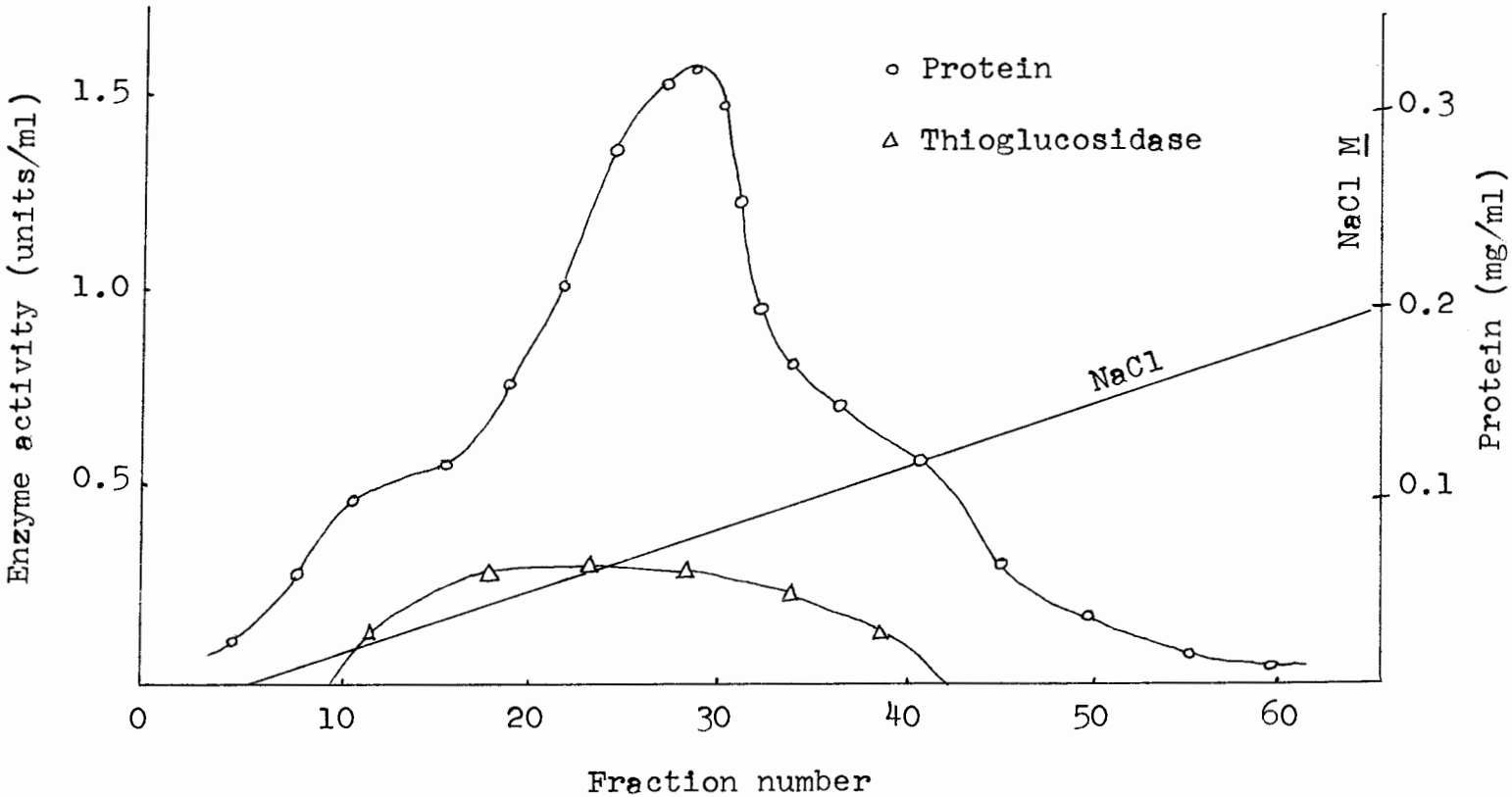


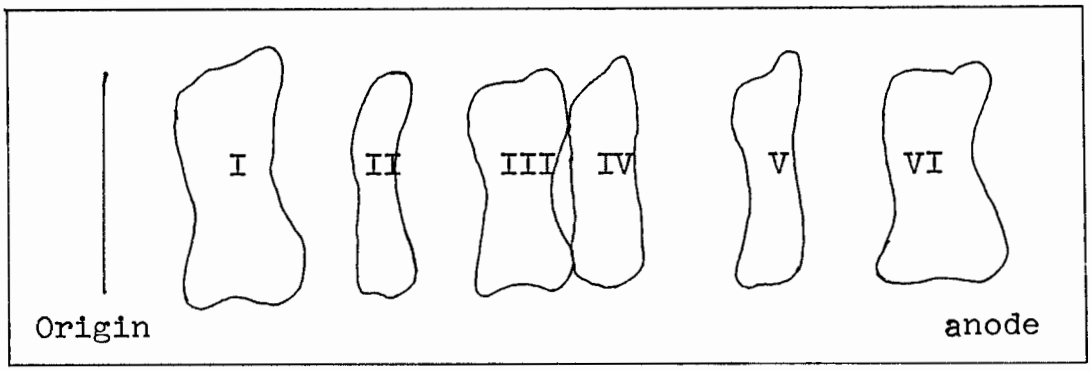
Figure 3. Chromatography of Fraction III on TEAE-cellulose. The conditions are described in the text.

TABLE I

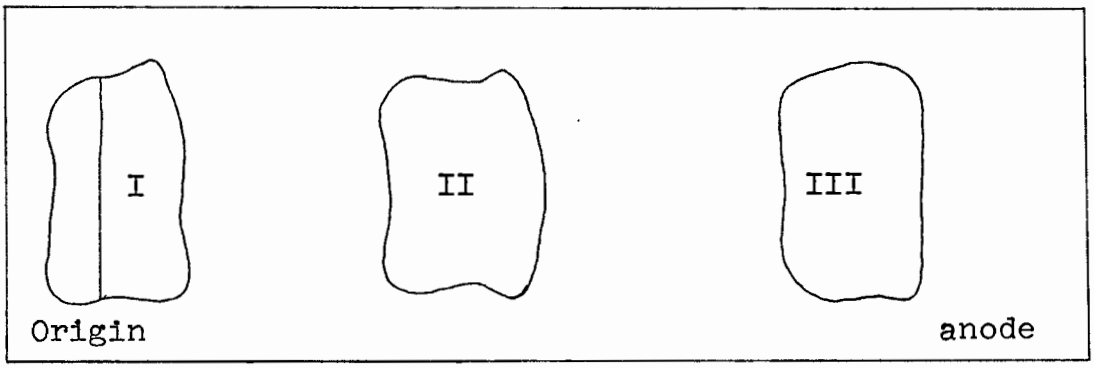
PURIFICATION OF β -GLUCOSIDASE, β-GALACTOSIDASE, THIOGLUCOSIDASE

Fraction	Ml	Protein mg/ml	Activity units/ml		Specific activity*
I	550	5.1	Glucosidase	0.82	0.16
H_2O extract			Galactosidase	0.41	0.08
			Thioglucosidase	1.3	0.25
II	150	19.	Glucosidase	5.7	0.30
80% salt			Galactosidase	2.5	0.13
precipitation			Thioglucosidase	4.5	0.24
III	58	13.	Glucosidase	4.8	0.38
50 to 70%			Galactosidase	2.0	0.16
saturation			Thioglucosidase	10.	0.80
IV	4	0.25	Glucosidase	14.4	57.0
precipitate of			Galactosidase	2.9	11.5
fraction III			Thioglucosidase	-	-
V	9	1.3	Glucosidase	-	-
DEAE-cellulose			Galactosidase	-	-
effluent			Thioglucosidase	11.0	8.40
VI	9	2.0	Glucosidase	-	-
sephadex			Galactosidase	-	-
effluent			Thioglucosidase	14.0	7.00

*units per ml of protein



Cellulose acetate strip



Starch gel strip

Figure 4. The electrophoresis was run as described in the text. The intensity of the bands stained with Ponceau S in the cellulose acetate was as follows: I - very, very light; II - very light; III - light; IV - dark; V - light; VI - very, very light. The intensities of the bands stained with amido black in the starch gel were all about the same.

TABLE II

CELLULOSE ACETATE AND STARCH GEL ELECTROPHORESIS

The band numbers are from Figure 4. Electrophoresis conditions are as described in the experimental part. The bands were eluted from the strips and tested for activity. Activity was described as the micromoles of product liberated per 24 hours per ml of enzyme.

Band	pNPG	Sinigrin	Sucrose	Glycogen
Cellulose acetate strip				
I	0.10	-	-	-
II	-	0.01	0.01	-
III	-	0.01	0.01	-
IV	-	0.01	0.01	0.16
V	-	-	-	-
VI	0.80	-	-	-
Starch gel strip				
I	0.10	0.16	0.16	-
II	0.10	0.16	0.16	0.08
III	0.50	0.16	0.16	0.12

protein, and increasing the sample size resulted in overloading the gel. Ponceau S could not be used with the starch gel because its removal from the gel was impossible and interfered with band identification.

C. Glycosidic enzymes

To observe the total glycoside hydrolase activity, it was necessary to incubate samples from the crude extract with various substrates (Table III). These results indicated several additional glycosidic enzymes other than the thioglucosidase known to be present in the system. The activities of Fractions IV, V, and the crystalline protein are seen in Table IV.

Samples of the crude extract, Fraction I, were adsorbed on a DEAE-cellulose column and eluted with an increasing sodium chloride gradient in 0.02 M barbital buffer, pH 8.6. The elution diagram is seen in figure 5. Similar experiments were performed after prolonged dialysis of the crude extract. These results, figure 6, show considerable shifting and spreading of the peaks. Also, the system responsible for the hydrolysis of sucrose was lost after this treatment.

It should be noted that the last protein eluted from the DEAE-cellulose column showed a small amount of thioglucosidase activity (figure 5). This suggested the possibility of an isozyme or even another enzyme; however, no attempt

TABLE III

GLYCOSIDASE ACTIVITY OF TROPAEOLUM MAJUS

Substrate	Hydrolytic activity[a]	Substrate	Hydrolytic activity
Amylopectin	+	α-Me-D-Glucoside	-
Glycogen	+	α-Me-D-Mannoside	-
Amylose	-	ϕ-β-D-Glucoside	+
Inulin	-	β-Me-D-Glucoside	+[b]
Sucrose	+	β-Me-D-Xyloside	-
Raffinose	+	Melibiose	-
Turanose	-	ϕ-β-D-Galactoside	+
Melezitose	-	p-NO_2-ϕ-β-D-Glucoside	+
Trehalose	+	p-NO_2-ϕ-β-D-Galactoside	+
Maltose	-	β-Me-D-Galactoside	-
Arbutin	+	o-NO_2-ϕ-β-D-Galactoside	+
Salicin	+	Sinigrin	+
Amygdalin	+	Glucosinalbin	+
Gentiobiose	+	Glucotropaeolin	+
Cellobiose	-	Isopr-β-D-Thiogalactoside	-
Lactose	-	β-Me-D-Thioglucoside	-

[a]Activity was noted when a measurable amount of product was observed.

[b]Hydrolytic activity on β-Me-D-Glucoside was negligible.

TABLE IV

HYDROLYTIC ACTIVITY OF PROTEIN FRACTIONS

FROM TROPAEOLUM MAJUS*

Substrate	Fraction IV	Fraction V	Crystalline protein
Amylopectin		0.08	0.08
Glycogen		0.29	0.29
Sucrose	0.71		
Raffinose	0.08		
Trehalose	0.06		
Arbutin	0.42		
Salicin	0.29		
Amygdalin	0.67		
Gentiobiose	0.12		
ϕ-β-D-Glucoside	0.40		
ϕ-β-D-Galactoside	0.08		
p-NO_2-ϕ-β-D-Glucoside	14.40		
p-NO_2-ϕ-β-D-Galactoside	2.90		
o-NO_2-ϕ-β-D-Galactoside	6.10		
Sinigrin		11.0	
Glucosinalbin		9.4	
Glucotropaeolin		5.5	

*Activity is expressed in enzyme units per ml protein.

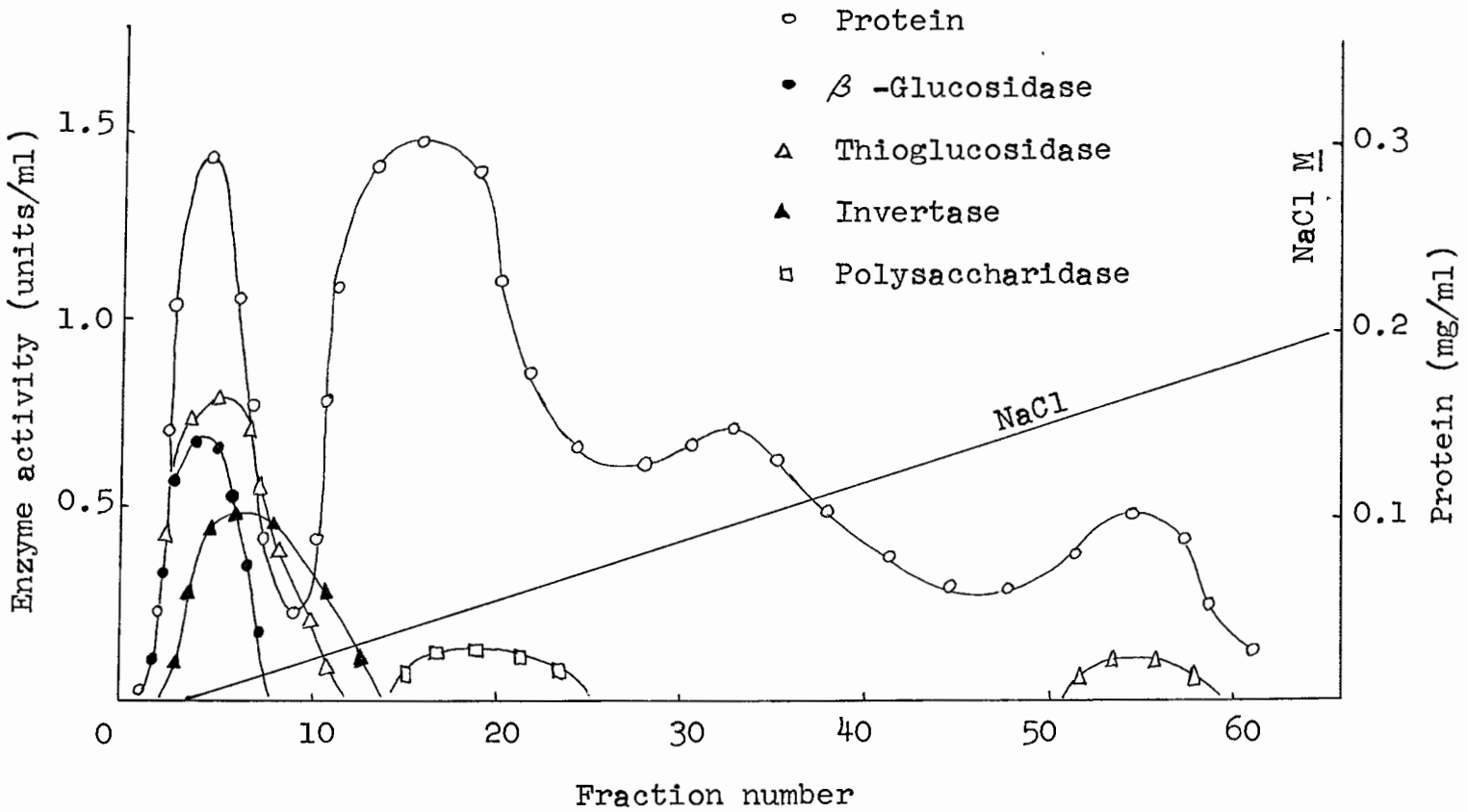


Figure 5. Chromatography of Tropaeolum crude extract on DEAE-cellulose. The conditions are described in the text.

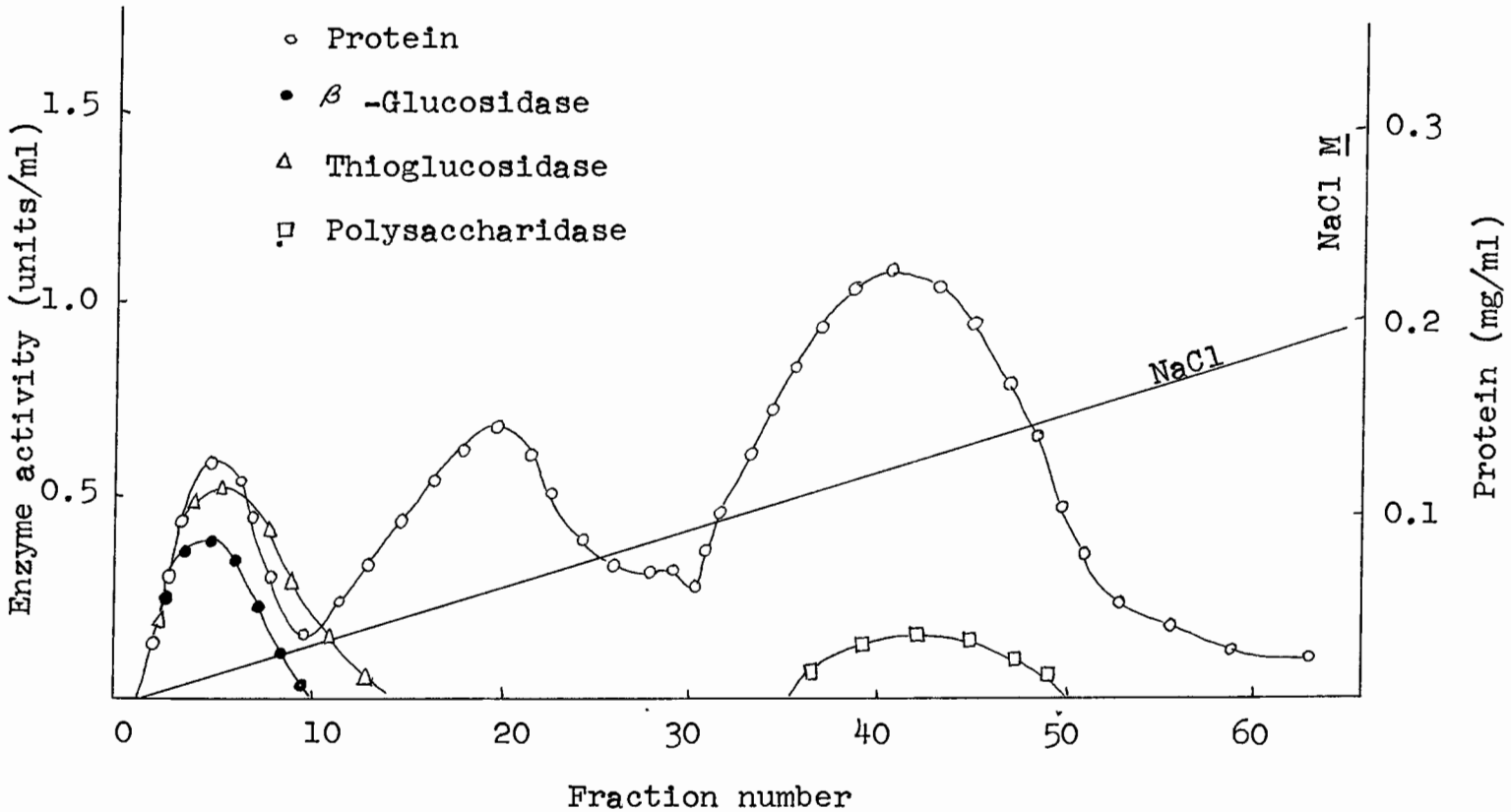


Figure 6. Chromatography of Tropaeolum crude extract on DEAE-cellulose after 36 hours of dialysis. Column conditions and elution procedures are described in the text.

was made to resolve this observation. Ettlinger et al. (10), in studying the thioglucosidase from yellow mustard, found two enzymes that catalyzed the same reaction. One of these enzymes was activated by L-ascorbic acid, although it was not an obligatory cofactor. The other enzyme was ascorbate independent and was noted as the "classical myrosinase." Tsuruo et al. (64), in their study of yellow mustard, also obtained two proteins which had thioglucosidase activity.

At least five glycosidic enzymes were obtained from the seeds of Tropaeolum majus, with the possibility of an additional system as is shown in Table III. The presence of a thioglucosidase was indicated by the known presence of the mustard oil glucoside, glucotropaeolin. This enzyme, which was purified about 40 fold, showed good activity toward sinigrin and glucosinalbin, but lacked hydrolytic activity toward alkyl-S-glucosides and O-glucosides. Earlier work has been reported indicating the hydrolysis of p-nitrophenyl glucoside and other β-glucosides by the thioglucosidase obtained from mustard seeds (22,53,60, 63). Separation of thioglucosidase from β-glucosidase activity in the Tropaeolum system raised some question as to the purity of the earlier preparations. However, this does not rule out the possibility of dual activity by the mustard seed thioglucosidase.

The β-glucosidase system, purified about 360 times,

contained β-galactosidase activity. This system catalyzed the hydrolysis of a number of naturally occuring β-glucosides and several substituted aryl glucosides. An attempt to crystallize the β-glucosidase resulted in impure, irregular aggregates.

The enzyme responsible for the hydrolysis of sucrose appears to be an invertase similar to that found in yeast (46). Characteristic invertase activity, e. g., greater hydrolysis of sucrose than raffinose, was experimentally observed. The possibility of an α-glucosidase may be ruled out by the lack of hydrolysis of maltose, turanose, melezitose and α-methyl -D-glucoside. The enzyme showed glycone specificity for an unsubstituted fructofuranosyl residue and does not hydrolyze inulin.

The hydrolysis of several β-galactosides suggested the possibility of a separate β-galactosidase, although it has been reported that certain β-glucosidases, having a nonconfigurational glycone specificity, catalyze the hydrolysis of β-galactosides. A change in the ratio of β-galactosidase to β-glucosidase activity during purification also suggests separate enzyme systems. For this system, substituted aryl galactosides were the best substrates, while only a small amount of phenyl-β-D-galactoside was hydrolyzed. Failure to hydrolyze lactose created some doubt of a separate β-galactosidase system. Several microbial β-galactosidase

systems show preferential hydrolysis for aryl galactosides, but also hydrolyze lactose (72). However, Doell and Kretchmer (8) demonstrated that rat and rabbit β-galactosidases contained two components; one of which hydrolyzed o-nitrophenyl-β-D-galactoside, but had little effect on lactose.

Characterization of the crystalline protein can not be determined at this time. The enzyme had moderate activity toward amylopectin and glycogen which might suggest an amylase, a phosphorylase, or an oligoglycosidase. The hydrolysis of amylose was attempted using the method of Bernfeld and Gurtler (3). Lack of hydrolysis of this substance after 24 hours would seem to rule out a β-amylase. Similarly, the low specific activity, measured by liberation of reducing sugar, would appear to eliminate dextrinizing enzymes such as α-amylase. Additional study of this system is necessary before any conclusions can be made.

A system was observed that hydrolyzed trehalose. The activity was weak in the crude extract and in Fraction III, indicating no purification. It would appear to be due to the presence of a trehalase. The trehalose was examined for homogeneity by paper chromatography and appeared to be free of other glucosides. This enzyme has been reported in many plant systems (23).

RESULTS AND DISCUSSION

II. The Thioglucosidase of *Tropaeolum majus*

A. Purification of the enzyme

The enzyme was isolated from seeds of *Tropaeolum majus* and purified by ammonium sulfate fractionation and column chromatography as described in the experimental section, summary of the results of purification is seen in Table 1.

Fraction III, when stored under refrigeration (4° C), retained constant specific activity up to 3 months. The addition of 2-mercaptoethanol helped keep the denaturation to a minimum.

The purified thioglucosidase was obtained from Fraction III by gradient elution from a DEAE-cellulose column. This enzyme preparation was diluted 20 to 1 for use in activity determinations. Exposure of the purified enzyme to room temperature for 30 minutes resulted in loss of one half of the activity. This increased lability may be due to the removal of inactive protein or polysaccharides which aid in stabilizing the enzyme.

The electrophoretic pattern of the purified thioglucosidase on both starch gel and cellulose acetate showed spreading of the protein zone as it moved toward the anode. Protein eluted from the support media showed activity due only

to the thioglucosidase.

The enzyme was subjected to heat inactivation studies at 65° C in 0.02 M phosphate buffer. The observed decrease in the activity followed first order kinetics, indicating the involvement of a single molecular species in the hydrolysis (figure 7).

B. Enzyme specificity

The function of the thioglucosidase in higher plants remains uncertain. The concentrations of the enzyme and the corresponding thioglucosides increase at seed maturation and vary considerably during other periods of plant development (35). Compounds of this type have been suggested for nitrogen storage, for possible defense mechanisms, or as possible end products in amino acid metabolism. The catabolism of these materials in the intact plant is unknown.

The specificity of the purified thioglucosidase was limited to hydrolysis of S-glycosidic bonds. These observations are contradictory to those reported for the thioglucosidase of the Cruciferae. Gaines and Goering (19), Reese et al. (53), and Tsuruo and Hata (68) observed, in addition to thioglucosidase activity, hydrolysis of O-glucosides. The mustard thioglucosidase has been purified 100 fold with no observed separation of thioglucosidase and β-glucosidase activity.

All mustard oil glucosides tested and several nitro-

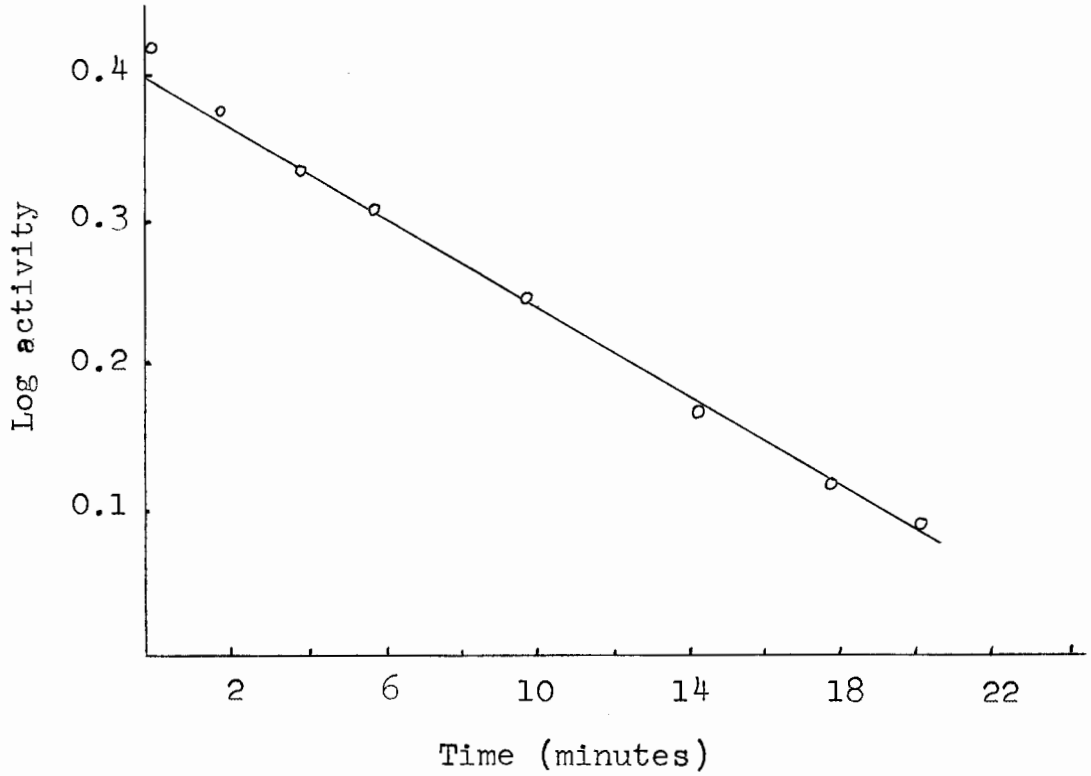


Figure 7. Heat inactivation of thioglucosidase at 64.5° C. The standard assay procedure was used as described in the text.

substituted aryl thioglucosides were hydrolyzed (Table V). Jermyn (34) reported very high blank values using p-nitrophenyl thioglucoside as a substrate and questioned the value of this material as a substrate. In this work, very little hydrolysis was observed in the control tubes, under the assay conditions, even after 24 hours. The 2,4-dinitrophenyl thioglucoside had a higher blank value, but, also appeared stable under the assay conditions.

C. Temperature and pH effects

The rate of enzymatic hydrolysis of sinigrin as a function of pH was studied in citrate, phosphate, and acetate buffers (figure 8). The optimum pH for the system was rather broad and nearly identical in all buffers, having a range from pH 6.5 to 7.5. The ratios of the maximum activities in acetate, phosphate and citrate buffers was 1:0.95:0.52 respectively. At pH values below 2.4 and above 9.9, the enzyme was irreversibly denatured.

The effect of temperature on the rate of hydrolysis of sinigrin was followed between 5° and 65° C (figure 9). The reactions were run in 0.002 M phosphate buffer, pH 6.8. A conventional Arrhenius plot of this data showed deviation from linearity above 52° C. The energy of activation within the temperature range studied was found to be 6.9 ± 0.4 kcal. per mole (figure 10). The procedure is as described in the experimental section.

TABLE V

THE ACTION OF THIOGLUCOSIDASE ON VARIOUS SUBSTRATES

The conditions of assay were as described in the text. The activity is in micromoles of product per hour per ml of enzyme.

Substrate	Concentration	Activity
Sinigrin	10^{-2}	11.0
Glucotropaeolin	10^{-2}	5.5
Glucosinalbin	10^{-2}	9.4
ϕ-β-D-thioglucoside	10^{-2}	-
p-NO_2-ϕ-β-D-thioglucoside	10^{-2}	1.3
Isopr-β-D-thiogalactoside	10^{-2}	-
ϕ-β-D-thiogalactoside	10^{-2}	-
2,4-dinitro-β-D-thioglucoside	10^{-2}	2.5
ϕ-β-D-thioglucoside with 10^{-3} M ascorbic acid	10^{-2}	-
p-NO_2-ϕ-β-D-thioglucoside with 10^{-3} M ascorbic acid	10^{-2}	4.1

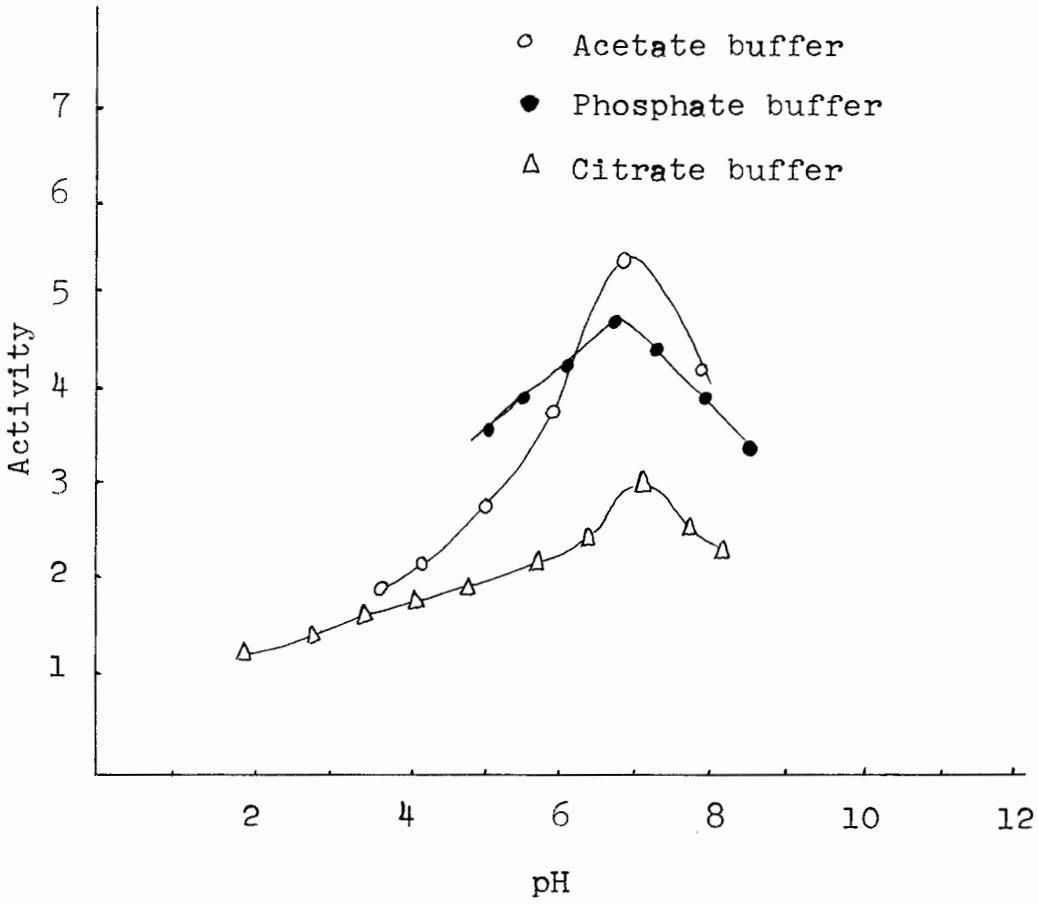


Figure 8. The effect of pH on thioglucosidase activity was determined at 37° C with 5 x 10^{-3} M substrate in the various 0.02 M buffers. Activity is in micromoles of product formed per hour per ml of enzyme.

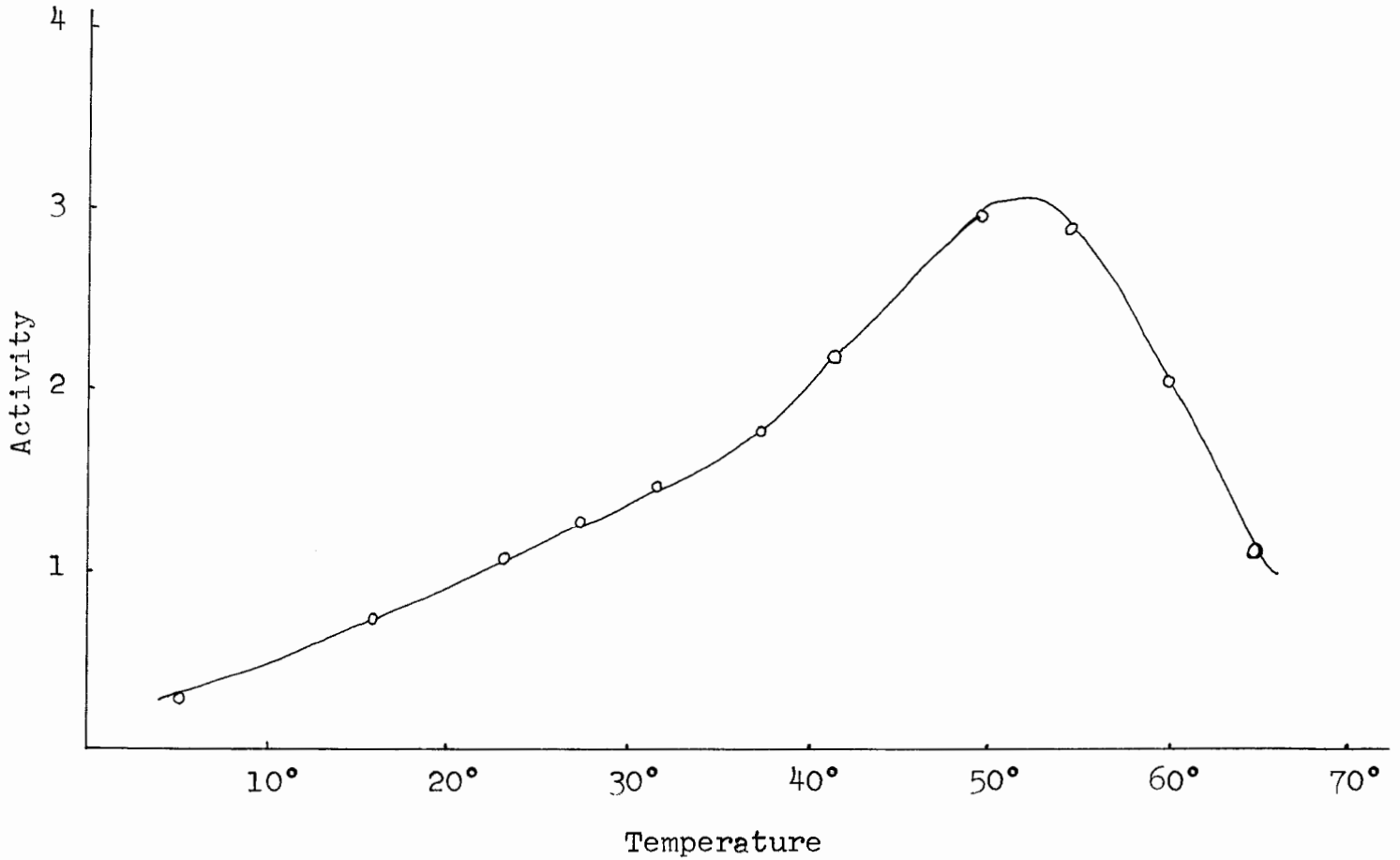


Figure 9. The effect of temperature on the rate of hydrolysis was determined using the standard assay mixture.

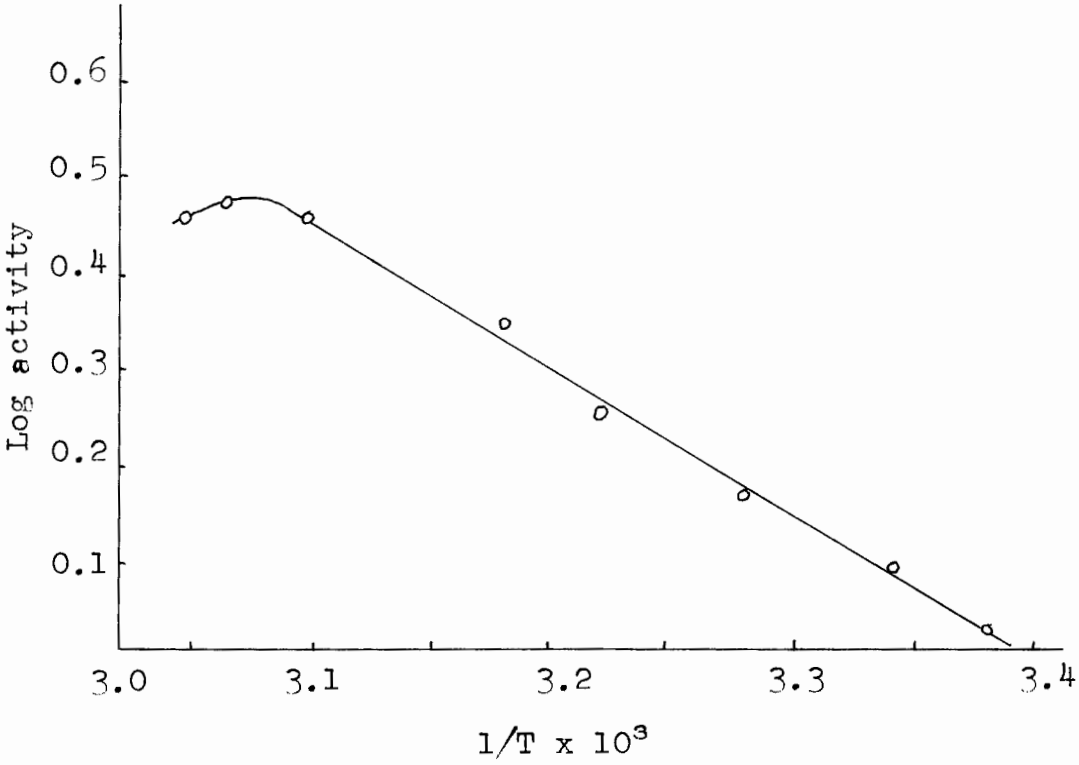


Figure 10. Arrhenius plot of sinigrin hydrolysis by thioglucosidase. T is in absolute temperature.

D. Activation by ascorbic acid

The activation of thioglucosidase by L-ascorbic acid was first reported by Nagashima and Uchiyama (48). They observed that L-ascorbic acid increased the rate of hydrolysis of sinigrin by 260 percent.

To explain this phenomena, Nagashima, Uchiyama, and Nishioka (49) suggested that the ascorbic acid was acting as a reducing agent, converting S-S bonds of the enzyme to S-H bonds, thereby increasing the activity of the enzyme. Ettlinger *et al.* (10) demonstrated that analogs of ascorbic acid which are nonreducing still activate the enzyme. Tsuruo and Hata (65) showed that the ascorbic acid was not oxidized or reduced in the hydrolysis of sinigrin. They also proposed that the site for ascorbic acid activation is different than the substrate site. The pH activity, pH stability, and temperature stability of the thioglucosidase were the same with or without ascorbic acid. Observed inhibition effects also appeared to be ascorbate independent. They concluded that the site and nature of the ascorbate activation of myrosinase may be difficult to deduce.

The activity of the crude extract was determined spectrophotometrically by observing the change in absorbance for the disappearance of sinigrin. It was noted that dialysis of the crude extract caused considerable loss of activity which could be restored by addition of 10^{-3} M

L-ascorbic acid (Table VI).

Analysis of the seeds of Tropaeolum majus indicated an ascorbic acid content of approximately 5 mg per 10 grams of dry seeds.

The activity was increased about four fold in the presence of ascorbic acid, using sinigrin as the substrate. The rate of hydrolysis of p-nitrophenyl-S-glucoside was also increased with ascorbate; however, there was no observed change in the activity toward phenyl-S-glucoside. It has been reported that the hydrolysis of β-O-glucosides and 2,4-dinitrophenyl-β-D-thioglucoside by mustard thioglucosidase is ascorbate independent (61,66).

For the most part, the presence of ascorbic acid did not alter the observed effects of added reagents at the concentrations tested. Sulfhydryl inhibition was essentially unchanged in the presence of ascorbate, thus implying the possibility of different substrate and activator sites. The most pronounced effect was noted in the presence of monovalent ion salts. These substances showed much greater inhibition in the presence of ascorbate.

Ascorbic acid reacts with DNS reagent, giving high blank values; therefore, all studies using ascorbic acid were performed by observing the change in absorbance during the disappearance of sinigrin.

TABLE VI

INFLUENCE OF L-ASCORBIC ACID ON THIOGLUCOSIDASE ACTIVITY

The activity was determined spectrophotometrically by observing the change in absorbancy for the disappearance of sinigrin, as described in the text. Activity is in micromoles of product formed per hour per ml of enzyme.

Fraction	Activity units/ml	mg protein/ml	Specific activity units/mg protein
I-undialyzed	20.8	15.0	1.39
I-dialyzed	1.5	5.1	0.29
I-dialyzed with 10^{-3} M ascorbic acid	6.8	5.1	1.33
III	12.5	13.0	0.96
III-with 10^{-3} M ascorbic acid	52.5	13.0	4.04

E. Inhibition of enzyme activity

The thioglucosidase was dialyzed against 10^{-3} M EDTA for 24 hours. This procedure did not change the specific activity of the enzyme. Since EDTA and sulfhydryl compounds have little effect on the enzyme, it is reasonable to assume that metal ions are not essential for enzyme activity. This is in agreement with the characteristics of glycosidic enzymes. Only α-amylase has been shown to be a metalloenzyme (26,50,62).

The effects of various salts on the hydrolysis of sinigrin are summarized in Table VIII. It is hard to determine the nature of the inhibition as all salts brought about a decrease in the activity. The effects of monovalent ions appear to be dependent upon the ionic strength, rather than on the individual ions. Di- and trivalent ions brought about varying effects, which may be more characteristic of the individual species. The heavy metal ions, Hg^{++} and Cu^{++}, resulted in strong inhibition. The addition of Ag^{+} formed an insoluble precipitate previously reported as silver sinigrate (15,37,73). Pb^{++} and Ba^{++} reacted with the substrate to form insoluble compounds and could not be used for ion inhibitors. Fe^{++} appeared to interact with the protein so values could not be obtained.

The reported work on ion inhibition of the mustard thioglucosidase is somewhat contradictory. Reese et al. (53)

TABLE VII

THE INFLUENCE OF VARIOUS SUBSTANCES ON THE β-THIOGLUCOSIDASE

The activity was determined using the standard assay procedure except acetate buffer, pH 6.0, was used. The activity was taken as 100 in acetate buffer, pH 6.0.

Substance	Concentration	Activity
Acetate buffer, pH 6.0	2×10^{-2} M	100
Citrate buffer, pH 6.0	2×10^{-2} M	52
Phosphate buffer, pH 6.0	2×10^{-2} M	95
L-ascorbic acid	10^{-3} M	414
p-NO_2-ϕ-β-D-glucoside	10^{-3} M	99
D-glucose	10^{-3} M	100
D-glucose	10^{-1} M	98
D-glucose with 10^{-3} M ascorbate	10^{-1} M	96
2-mercaptoethanol	10^{-3} M	102

showed inhibition with Ag^{+}, Mn^{++}, Ca^{++}, Fe^{++} at 10^{-3} M for both the fungal sinigrinase and myrosinase. Ions of Fe (III), Zn, Ca, Hg, and Mg had no effect. Nagashima and Ushiyama (48) reported inhibition of the thioglucosidase by 10^{-3} M Hg^{++}, Fe^{++}, Fe^{+++}, Cu^{++} and I_2 while most other mono- and divalent ions showed no effect. Tsuruo and Hata (66) noted that monovalent ions had a strong inhibitory effect with the ascorbate activated system, but showed little effect on the nonactivated enzyme. The effects of the monovalent ions were attributed to the ionic strength of the solution.

The instability of the enzyme in the presence of heavy metal ions led to an examination of sulfhydryl inhibitors. The enzyme was preincubated with the sulfhydryl inhibitor for 10 minutes. Substrate concentrations were kept low because of the competitive nature of the sulfhydryl reagent and the substrate for the -SH group. These results are presented in Table IX.

Inhibition by both sulfhydryl inhibitors was reversed by 10^{-2} M cysteine. There was actually activation with cysteine in the presence of PCMB. This could be due, in part, to partial denaturation by the inhibitor allowing the cysteine to activate newly exposed -SH groups. It was observed that at higher PCMB concentrations, more activation occured. There was little activation with just cysteine. It was also

TABLE VIII

ION INHIBITION OF THE THIOGLUCOSIDASE ACTIVITY

The reaction mixture was run in acetate buffer, pH 6.2, at 37° C. The sinigrin was added after 10 minutes of preincubation. Substrate concentration was 6×10^{-4} M.

Inhibitor	Concentration	Percent Inhibition
$HgCl_2$	10^{-3} M	73%
$Cu(Ac)_2$	10^{-3} M	53%
$Cd(Ac)_2$	10^{-3} M	8%
$Zn(NO_3)_2$	10^{-3} M	26%
$Mg(Ac)_2$	10^{-3} M	17%
$Al(NO_3)_3$	10^{-3} M	24%
$FeCl_3$	10^{-3} M	21%
LiCl	10^{-2} M	18%
LiCl	0.5 M	48%
NaCl	10^{-3} M	7%
NaCl	10^{-2} M	16%
NaCl	10^{-1} M	32%
NaCl	0.5 ionic strength	44%
NaBr	0.5 ionic strength	39%
NaI	0.5 ionic strength	40%
KNO_3	0.5 ionic strength	17%
$(NH_4)_2SO_4$	0.5 ionic strength	7%
NH_4Cl	0.5 ionic strength	35%
Na_2SO_4	0.5 ionic strength	4%

TABLE IX

SULFHYDRYL INHIBITION OF THE THIOGLUCOSIDASE

Inhibition was run with 6×10^{-4} M substrate concentration in phosphate buffer, pH 6.9, using the standard assay procedures.

Inhibitor	Concentration	Preincubation	Inhibition
PCMB	10^{-3} M	10 min	33%
PCMB	5×10^{-4} M	10 min	18%
PCMB	10^{-4} M	10 min	7%
PCMB with 10 fold substrate concentration	10^{-3} M	10 min	22%
Iodoacetate (IAA)	10^{-4} M	10 min	9%
IAA	10^{-3} M	10 min	23%
IAA	10^{-2} M	10 min	39%
IAA	10^{-3} M	0 min	21%
IAA	10^{-3} M	30 min	24%
PCMB with 10^{-2} M cysteine	10^{-3} M	10 min	165%
PCMB with 10^{-2} M cysteine	10^{-4} M	10 min	125%
IAA with 10^{-2} M cysteine	10^{-3} M	10 min	101%
IAA with 10^{-2} M cysteine	10^{-2} M	10 min	104%
Cysteine	10^{-2} M	10 min	105%

noted that with higher substrate concentrations, there was less enzyme inactivation, implying that there was competition between the substrate and sulfhydryl inhibitors for -SH groups.

As with the ion effects, there is some contradiction in the observations with sulfhydryl inhibitors. Reese et al. (53) reported that 1.4×10^{-3} M PCMB inhibited myrosinase. Iodoacetate and N-ethylmaleimide did not inhibit these systems and it was concluded that there was no sulfhydryl involvement. Nagashima and Uchiyama (48) reported strong inhibition of the myrosinase system by PCMB and reversal of the process by cysteine. Tsuruo and Hata (65) found strong inhibition of myrosinase by PCMB with and without ascorbate. This observed inhibition was not reversed with cysteine.

Glucose up to 10^{-1} M had little effect on the activity of the thioglucosidase with or without ascorbate. Also, O-glucosides and unhydrolyzed thioglucosides showed little effect. This seems to corraborate the work of Tsuruo and Hata (67); however, these authors reported inhibition by sugars at very high concentrations.

F. Enzyme kinetics

The Michaelis constant was determined by using the Lineweaver-Burk plot. The kinetic runs were followed continuously by observing the decrease in absorbancy resulting from the disappearance of sinigrin at 25° C, pH 6.8. The

disappearance of sinigrin followed pseudo-first order kinetics through 5 half-lives (figure 11). At high substrate and enzyme concentrations the absorbancy of the product, isothiocyanate, interfered with kinetic runs. The Michaelis constant was 1.6×10^{-4} M in the absence of L-ascorbic acid and 1.0×10^{-3} M in the presence of the ascorbate. As seen from figure 12, both Km and V_{max} increased when ascorbic acid was added to the enzyme.

While the thioglucosidase of Tropaeolum majus has not been previously characterized, much work has been done on the kinetics of the myrosinase system. Schwimmer (61) has reported a Km in the absence of ascorbic acid of 1.4×10^{-4} M, and Ettlinger et al. (10) with ascorbic acid, a value of 2.5×10^{-4} M, both K_ms at 25° C. Tsuruo et al. (64) have reported values at 37° C of 1.3×10^{-3} M and 1.8×10^{-4} M with and without ascorbate respectively.

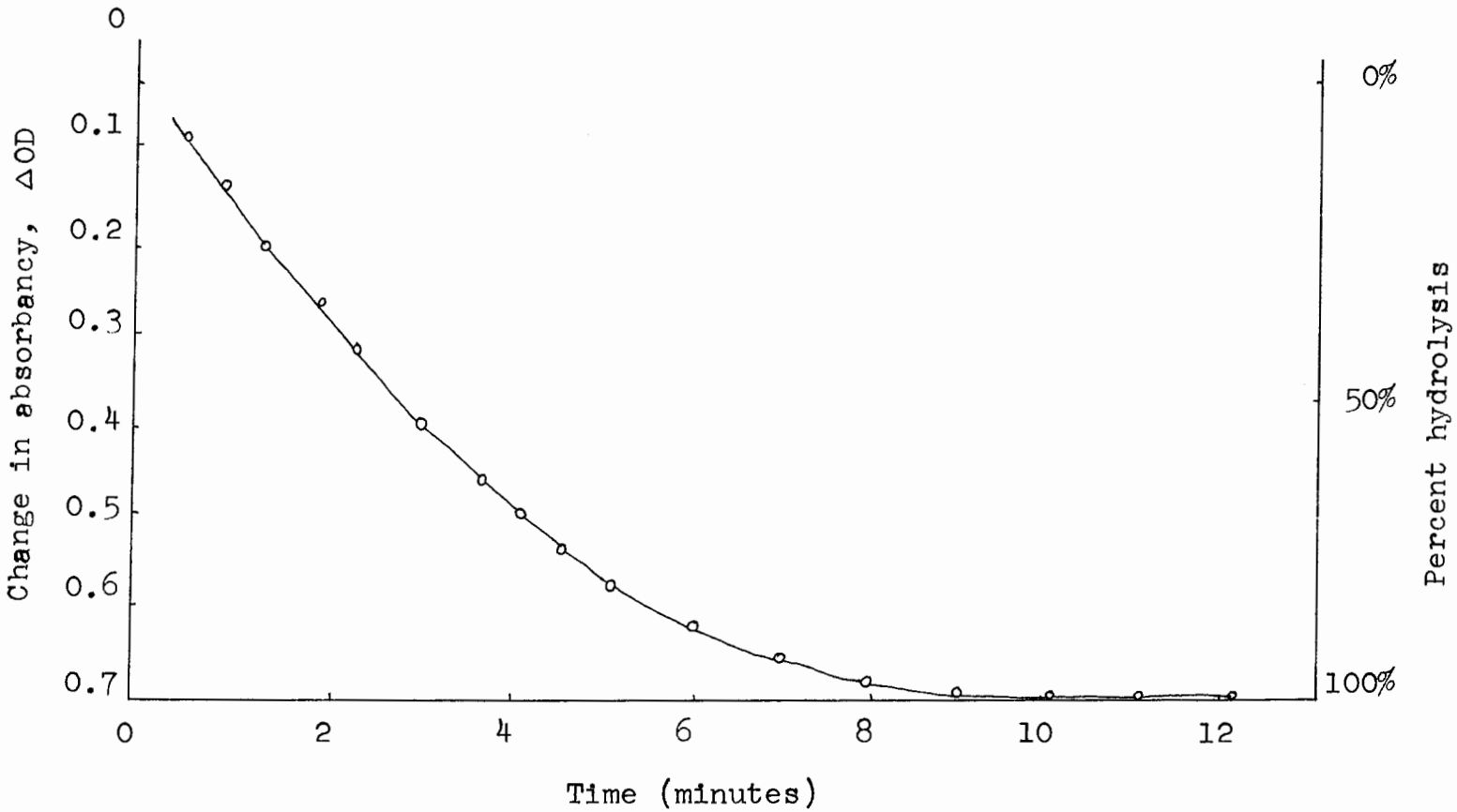


Figure 11. The absorbancy change with respect to time for the hydrolysis of sinigrin at 25° C by thioglucosidase. The initial reading was obtained 15 seconds after the start of the reaction. The light path at 227.5 nm was 1 mm. The assay mixture is described in the text.

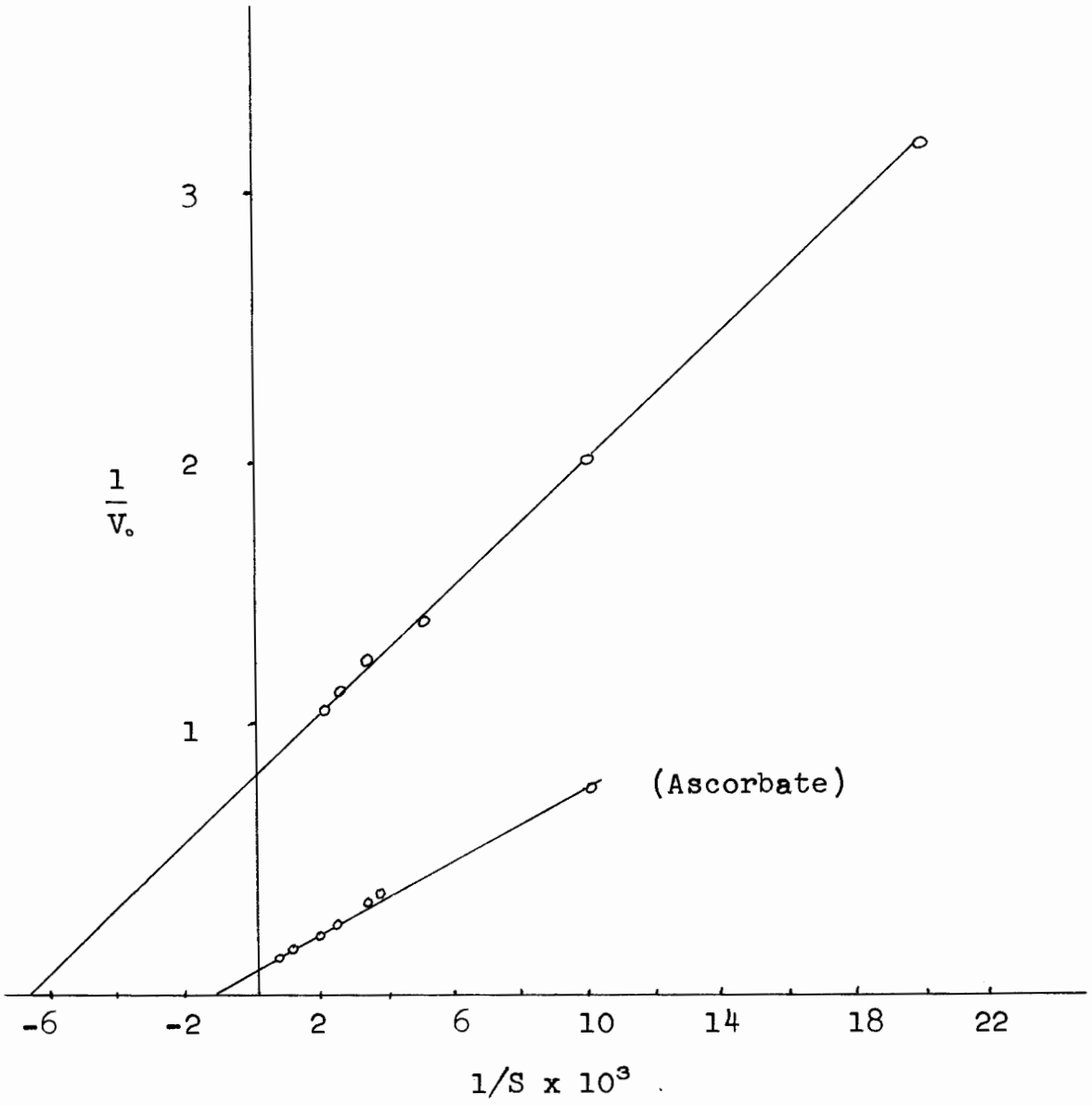


Figure 12. Lineweaver-Burk plot of the thioglucosidase with and without 10^{-3} M L-ascorbic acid using sinigrin as substrate at 25° C. The K_ms are 1.0 x 10^{-3} M and 1.6 x 10^{-4} M with and without the presence of ascorbic acid.

RESULTS AND DISCUSSION

III. The β-glucosidase of *Tropaeolum majus*

A. Purification

The enzyme was isolated from the seeds of *Tropaeolum majus* and purified by ammonium sulfate fractionation, as described in the experimental section. A summary of the purification results is given in Table 1.

The concentration of Fraction IV varied between 0.25 and 0.5 mg per ml of protein, being limited because of the insolubility of the protein. The enzyme showed high stability even at room temperature.

In both cellulose acetate and starch gel electrophoresis, the β-glucosidase moved in the direction of the anode. Two bands from the cellulose acetate showed β-glucosidase activity, but the small amount of protein present was not sufficient for further study with these fractions. A complete separation could not be carried out with the starch gel electrophoresis.

The homogeneity of the β-glucosidase was examined by heat inactivation studies. From the results it appears that the break in the denaturation curve is a result of two β-glucosidase systems (figure 13).

B. Temperature and pH effects

The effect of temperature on the rate of hydrolysis

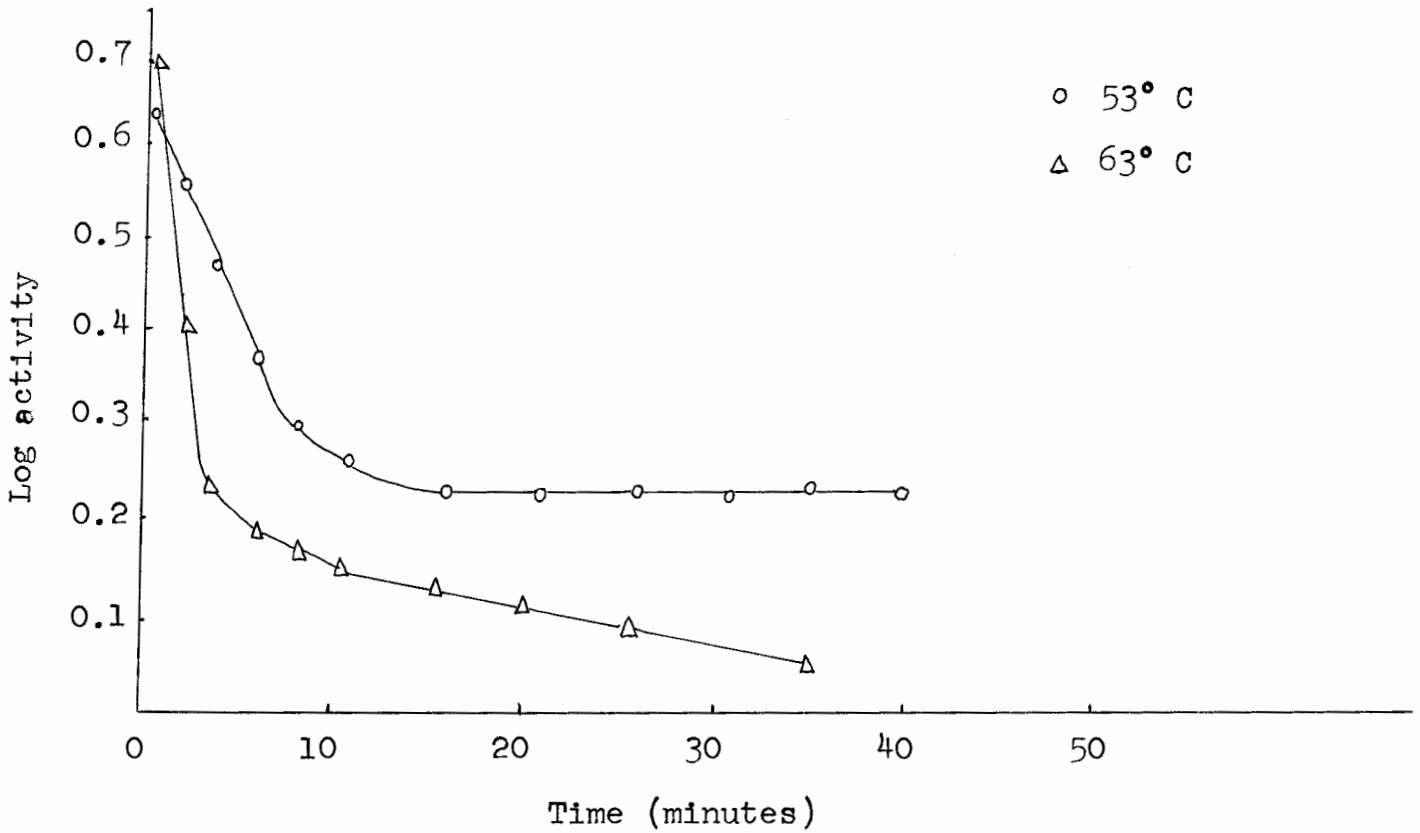


Figure 13. Heat inactivation of the β-glucosidase was determined using the assay procedure described in the text.

was studied in the temperature range of 5° to 70° C. The optimum temperature for β-glucosidase activity in Fraction III was 48° C as compared with a 60° C optimum temperature for Fraction IV. The latter fraction showed little β-galactosidase activity (figure 14).

The effects of pH studied in phosphate, and citrate, and acetate buffers, gave activity ratios of 2:3.1:4 respectively and an optimum range of pH 6.8 to 7.2. There was very little activity below pH 5 or above 8.5 (figure 15).

Certain observations on the Tropaeolum system suggested the possibility of two enzymes possessing β-glucosidase activity. Cellulose acetate electrophoresis showed two bands hydrolyzing pNPG. Optimum temperature and heat inactivation plots suggest the presence of two enzymes, one of which is more heat sensitive than the other.

The possibility of an additional enzyme or one with dual activity was also apparent from the variable hydrolysis of galactosides. During purification, the ratio of the activities for galactoside and glucoside hydrolysis did not remain constant. A different ratio for these activities was also observed from each Fraction IV obtained from various enzyme preparations.

If it is true that one enzyme fraction is more heat labile, then incubation above the observed optimum temperature should denature the enzyme, causing loss of activity.

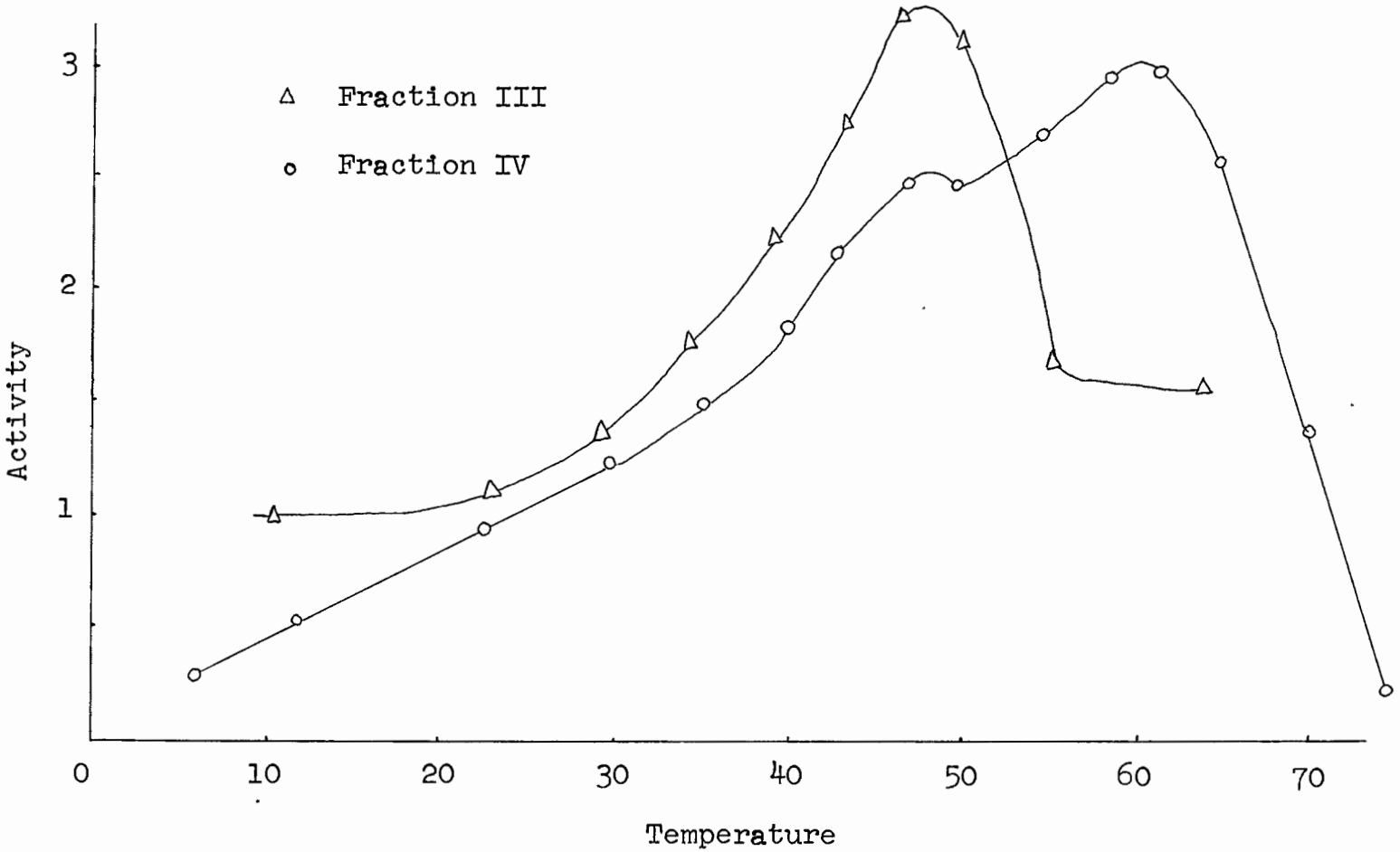


Figure 14. The effect of temperature on the rate of hydrolysis for the β-glucosidase system.

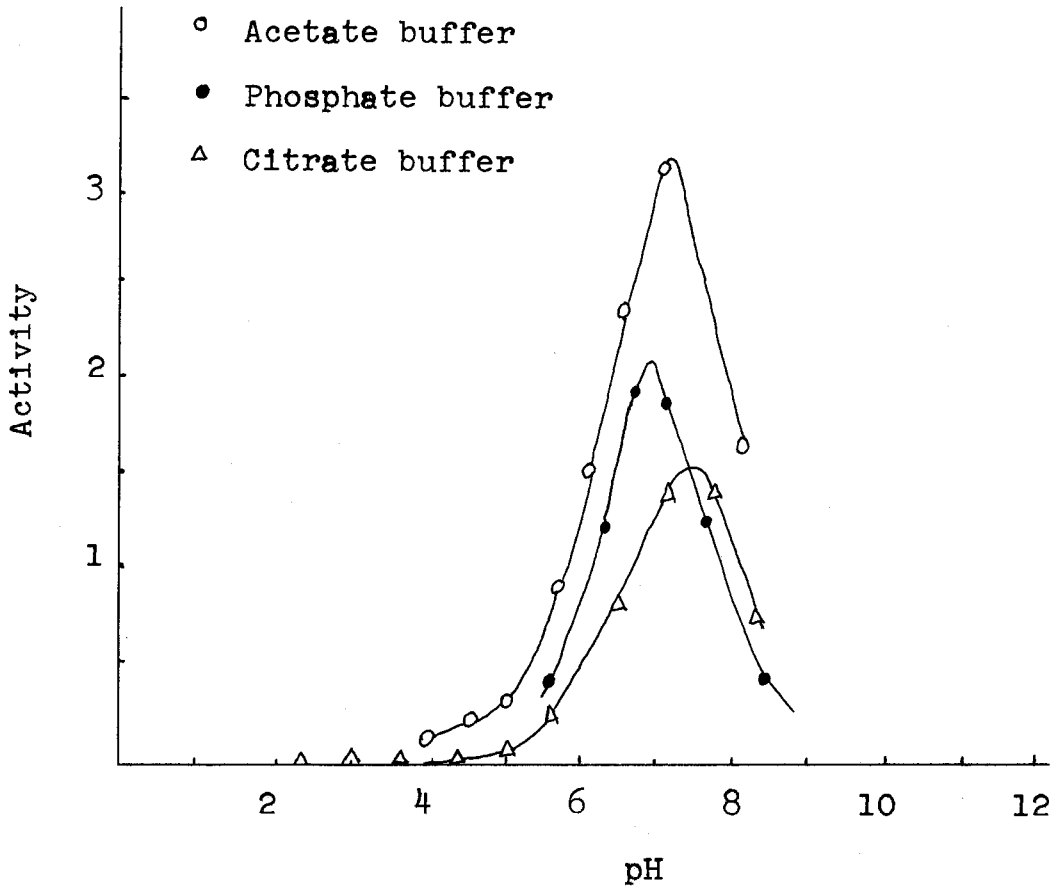


Figure 15. The effect of pH on β-glucosidase activity. Conditions are as described in the text.

Fraction III, which contained both β-glucosidase and β-galactosidase activity, was heated at 56° C for 15 minutes. This removed most of the galactosidase activity and nearly one half of the glucosidase activity. This observation may be explained if there are two enzymes present, one enzyme hydrolyzing only glucosides and the other a heat sensitive enzyme, hydrolyzing both galactosides and glucosides. Loss of activity also may have resulted from alteration of an active site in the protein structure, the galactosidase site being more heat sensitive than the glucosidase site. β-glucosidase free from β-galactosidase activity was obtained in a second precipitation from Fraction III at 0° C. The precipitated protein was heated at 56° C for 15 minutes without appreciable loss in activity. Thus, it appears that the system contains a β-glucosidase and an enzyme that is non-specific for carbon 4, showing both β-glucosidase and β-galactosidase activity.

The close association between the occurence of β-glucosidase activity and β-galactosidase activity has been observed in many systems. Whether these two activities are due to separate enzymes or a single enzyme has been a controversial subject for many years.

Horikoshi (30) demonstrated independent behavior of the two activities in Takadiastase. Heyworth and Walker (29) showed that the activity of almond emulsin β-glucosidase and

β-galactosidase occured at the same active site. Herferich (28) could not separate the β-glucosidase or β-galactosidase activity from almond emulsin after extensive purification. He reported identical ratios for the two activities during heat inactivation and purification studies. Rutenburg et al. (55) observed differences in the heat stability of the two enzymes and concluded that they have separate existence. The separation of a number of β-galactosidases on DEAE-cellulose was shown by Furth and Robinson (14). In a similiar study, β-galactosidase with no associated β-glucosidase activity and a component reacting with both substrates were isolated (54). Multiple forms of β-galactosidase have been reported from other sources (7,8).

C. Enzyme specificity

The β-glucosidase hydrolyzed a number of naturally occuring β-linked glucosides and several aromatic β-glucosides. Cellobiose and alkyl glucosides were not hydrolyzed, while substituted aryl-β-glucosides were the best substrates. In this respect, the β-glucosidase system appears to parallel the reported aryl-β-glucosidase system (33).

There seems to be a high glycone specificity for the β-glucosidase system. α-D-glucosides were not hydrolyzed and there was no hydrolysis of p-nitrophenyl-β-D-thioglucoside. These observations are consistent with the activities

of other β-glucosidase systems (58,69).

The β-glucosidase containing little β-galactosidase activity was uninhibited with galactose and phenyl-β-D-galactoside. Inhibition was observed with glucose, γ-glucono- and δ-galactonolactones (Table X).

D. Inhibition of enzyme activity

The β-glucosidase was dialyzed against 10^{-3} M EDTA for 24 hours with no loss of activity. The effects of various ions on the hydrolytic activity are seen in Table XI. The monovalent metal ions seemed to have no effect at low concentrations. At 0.5 M ion concentration, the β-glucosidase activity was decreased about the same for each ion. Heavy metal ions, and trivalent ions inhibited the activity in every case.

Duerksen and Halvorson (9), reporting on the β-glucosidase of yeast, found inhibition by ions of heavy metals, little inhibition by Mg^{++}, Ca^{++} and Mn^{++} at 10^{-3} M, and no effect by monovalent ions up to 0.1 M. Jermyn (33), in his study of β-glucosidase from fungi, showed that heavy metals inhibited the activity. Similiar observations have been reported for other systems (25,45,69).

Because the heavy metal ions inhibited the β-glucosidase so completely, the involvement of sulfhydryl groups was suggested. The enzyme was preincubated for 10 minutes with the sulfhydryl inhibitor. These results are

TABLE X

INHIBITION OF β-GLUCOSIDASE BY VARIOUS SUBSTANCES

Substance	Concentration	Inhibition
D-glucose	10^{-1} M	42%
D-galactose	10^{-1} M	0%
D-gluconolactone	10^{-3} M	64%
D-galactonolactone	10^{-3} M	24%
Phenyl-β-D-galactoside	10^{-1} M	0%
Acetate buffer	2×10^{-2} M	0%
Phosphate buffer	2×10^{-2} M	22%
Citrate buffer	2×10^{-2} M	49%

TABLE XI

ION INHIBITION OF β-GLUCOSIDASE ACTIVITY

The reaction was carried out in acetate buffer, pH 6.2, at 37° C. PNPG was added after a 10 minute pre-incubation period. Fraction IV was used. All cations were 10^{-3} M unless otherwise stated.

Inhibitor	Inhibition	Inhibitor	Inhibition
Ag^{+}	100%	Zn^{++}	45%
Hg^{++}	100%	Ba^{++}	9%
Hg^{+}	100%	Ca^{++}	8%
Pb^{++}	45%	Al^{+++}	64%
Cu^{++}	40%	Fe^{+++}	60%
Cd^{++}	40%	Mg^{++}	0%
Na^{+}	0%	Na^{+} 0.5 M	30%
NH_4^{+}	0%	NH_4^{+} 0.5 M	48%
Li^{+}	0%	Li^{+} 0.5 M	36%
K^{+}	0%	K^{+} 0.5 M	33%

seen in Table XII. For both inhibitors, the process was reversed by 10^{-3} M cysteine. At 10 times the substrate concentration, less inhibition was observed indicating competition between the substrate and the inhibitor for the sulfhydryl groups on the protein.

Duerksen and Halvorson (9) reported rearly complete inhibition at PCMB concentrations of 10^{-4} M and 29 percent inhibition with iodoacetate at 10^{-4} M. The activity of both inhibitors was reversed with cysteine.

E. Enzyme kinetics

The hydrolysis of pNPG by β-glucosidase showed typical Michaelis-Menton kinetics. The Michaelis constant (K_m) for pNPG at 37° C was 9.5×10^{-5} M as determined from a Lineweaver-Burk plot (figure 16).

This value is of the same order of magnitude as those reported for other β-glucosidase systems. β-glucosidases from yeast (19) and fungi (33) have reported K_m values (for pNPG) of 8.05×10^{-5} M and 4.5 to 5×10^{-5} M respectively.

TABLE XII

EFFECTS OF SULFHYDRYL AGENTS ON β-GLUCOSIDASE

Inhibition was carried out in 1.6 x 10^{-4} M pNPG, phosphate buffer pH 6.9, using Fraction IV. A 10 minute preincubation period was used.

Inhibitor	Concentration	Inhibition
PCMB	10^{-3} M	30%
PCMB	5 x 10^{-4} M	15%
PCMB	10^{-4} M	12%
PCMB with 10 fold substrate concentration	10^{-3} M	24%
Iodoacetate (IAA)	10^{-2} M	10%
IAA	10^{-3} M	6%
IAA	10^{-4} M	1%
Cysteine	10^{-3} M	2%
PCMB with 10^{-3} M cysteine	10^{-3} M	16%
PCMB with 10^{-3} M cysteine	10^{-4} M	2%
IAA with 10^{-3} M cysteine	10^{-2} M	2%
IAA with 10^{-3} M cysteine	10^{-3} M	1%

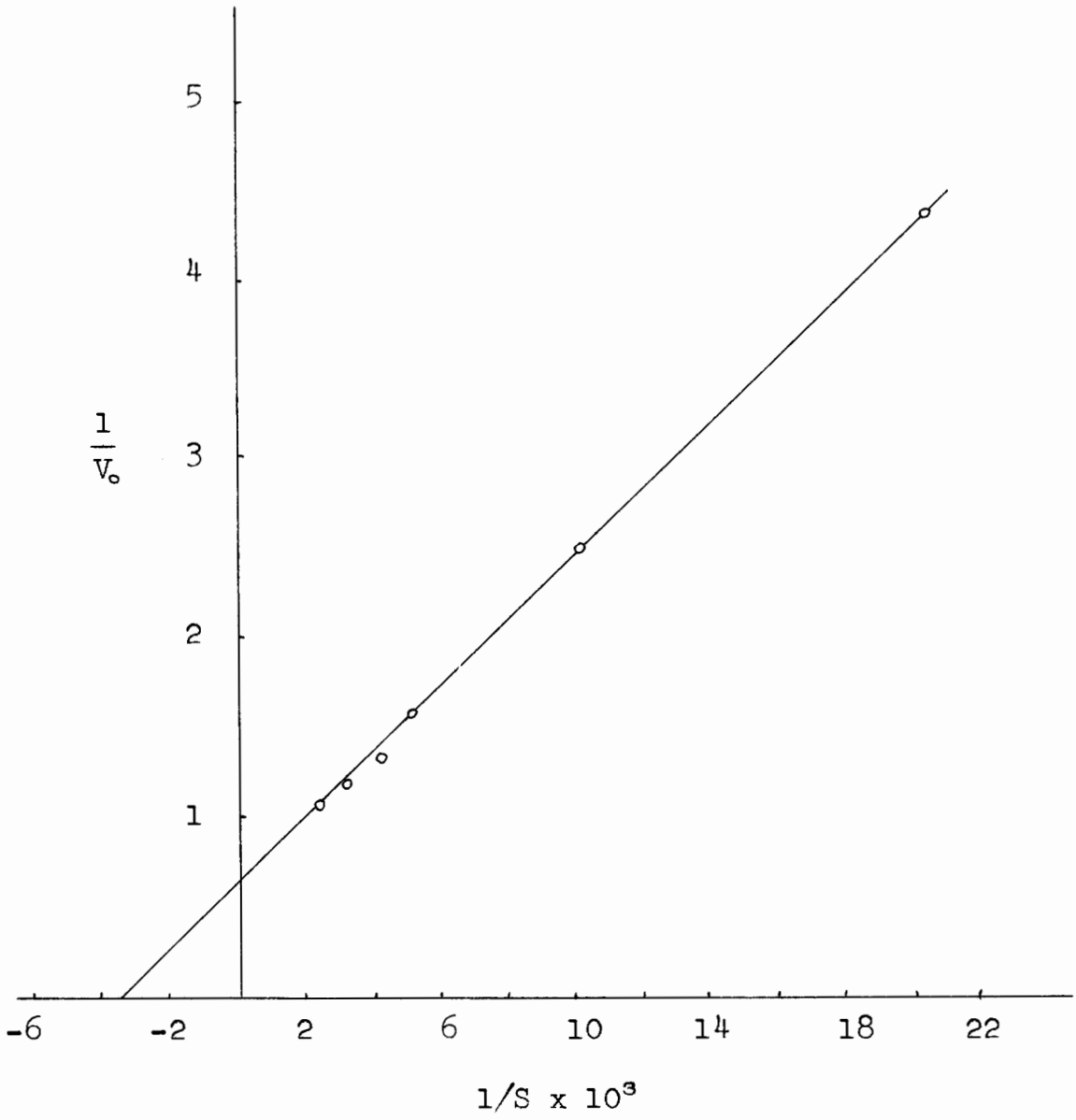


Figure 16. Lineweaver-Burk plot of the β-glucosidase using pNPG at 37° C. The K_m is 9.5×10^{-5} M.

SUMMARY

A number of glycosidic enzymes have been qualitatively identified in the seeds of Tropaeolum majus, the common nasturtium. Four of these systems, a thioglucosidase, a β-glucosidase, an invertase, and an enzyme causing partial hydrolysis of amylopectin and glycogen, have been isolated and partially purified. Hydrolytic activity also indicated the presence of an α-1,1-glucosidase and possibly the presence of a β-galactosidase, although the latter was not separated from β-glucosidase activity. The enzyme responsible for the hydrolysis of the polysaccharides was obtained as a crystalline protein, but the exact nature of the hydrolytic activity has not yet been determined.

The β-thioglucosidase from the seeds of Tropaeolum majus has been partially purified and characterized. The enzyme demonstrated a broad pH stability with an optimum in the range of pH 6.5 to 7.5. Temperature effects showed a deviation from linearity only above 52° C and gave an activation energy of 6.9 ± 0.4 kcal per mole. The enzyme was specific for the thioglucosidic bond and showed no hydrolysis of oxygen linked glucosides. Ascorbic acid brought about a four fold activation of the thioglucosidase, but was not a necessary cofactor. The K_m values for sinigrin hydrolysis in the presence and absence of ascorbate

were determined to be 1 x 10^{-3} M and 1.6 x 10^{-4} M respectively. The enzyme was inhibited by heavy metal ions and to a lesser degree by increasing concentrations of monovalent ion salts, particularly in the presence of ascorbic acid. The system also showed reversible inhibition by sulfhydryl reagents.

The β-glucosidase from the Tropaeolum system has been partially purified and characterized. Temperature effects showed two optimums depending upon the fraction used. Heat inactivation, electrophoresis studies, and purification ratios suggested two enzymes possessing β-glucosidase activity. One of these two enzymes seemed non-specific to carbon atom 4 by hydrolyzing β-galactosides. The β-glucosidase system was specific for the glucosidic bond and showed no hydrolysis of sulfur linked glucosides. The enzyme was inhibited by heavy metals and trivalent ions. Monovalent ions had little effect up to 0.5 M. The system showed inhibition by sulfhydryl inhibitors, which was reversed by the addition of cysteine. The K_m value for pNPG hydrolysis by the β-glucosidase was 9.5 x 10^{-5} M.

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APPENDIX

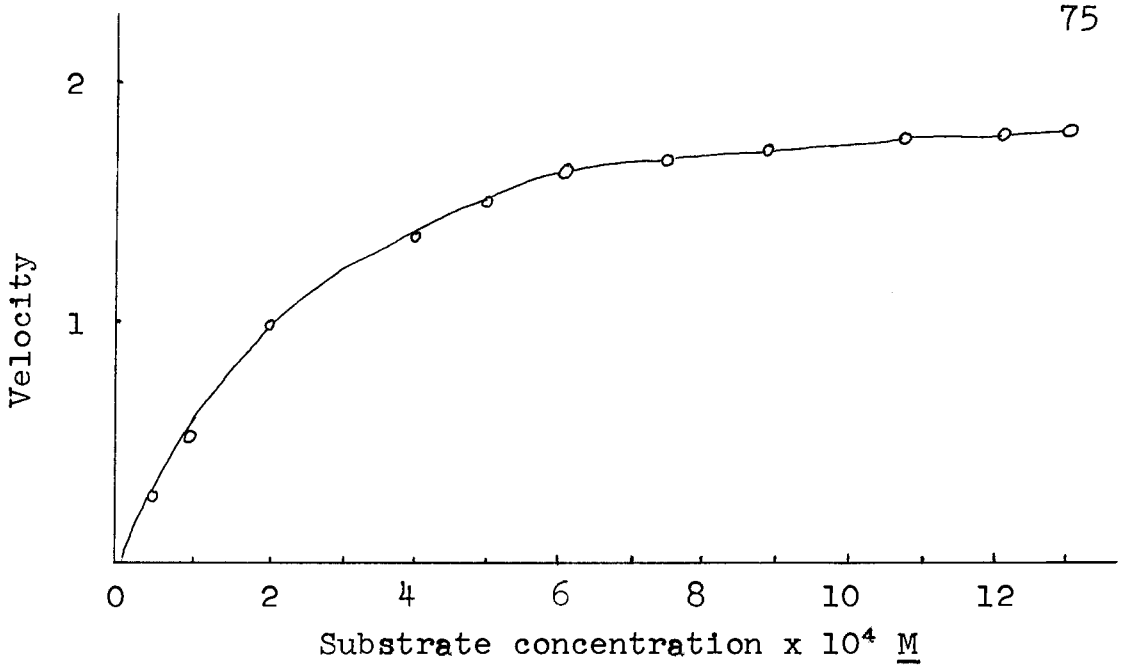

Figure 17. Hydrolysis of sinigrin by thioglucosidase at 25° C. The velocity is expressed as micromoles of glucose liberated per hour per ml enzyme.

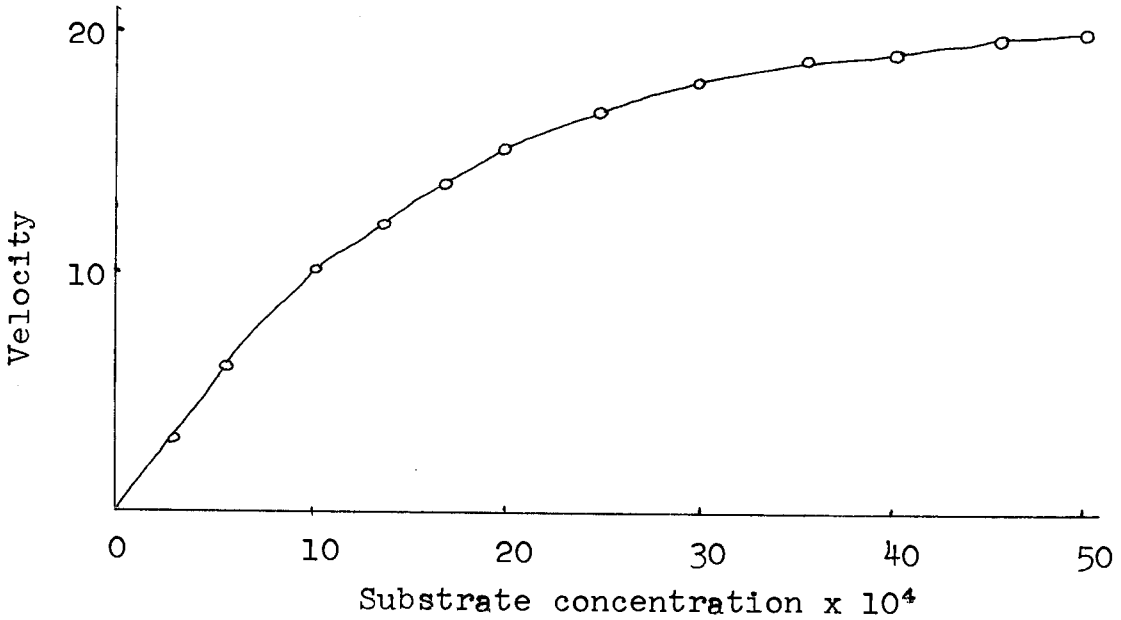

Figure 18. Hydrolysis of sinigrin by thioglucosidase with 10^{-3} M ascorbic acid at 25° C. The velocity is expressed as in the above figure.

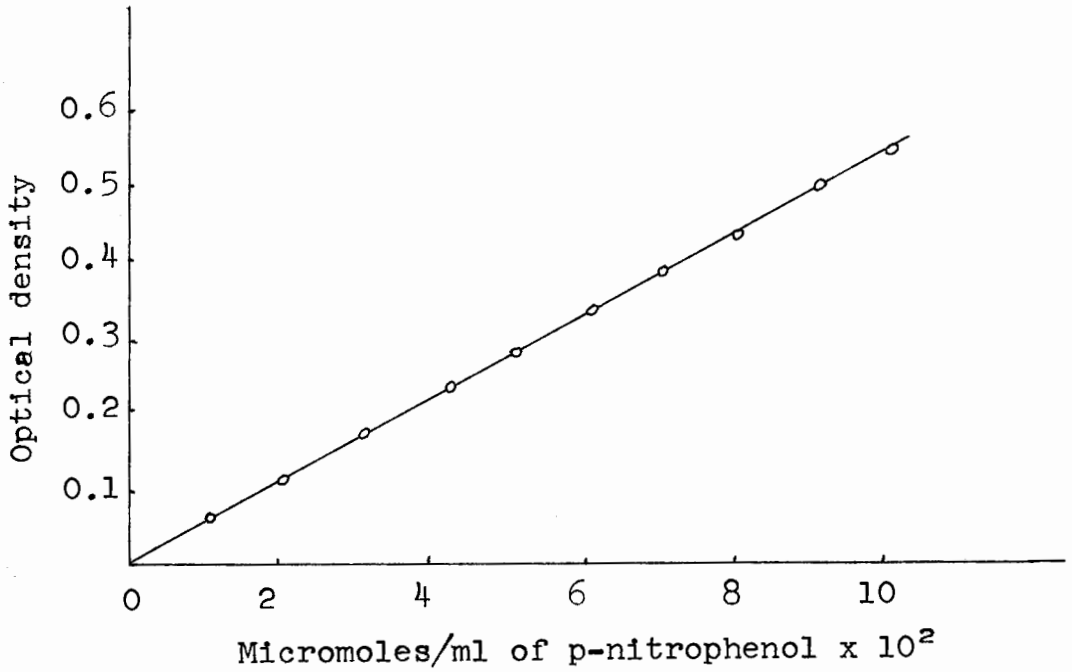

Figure 19. p-Nitrophenol standard curve (420 nm). The extinction coefficent was calculated as 5260.

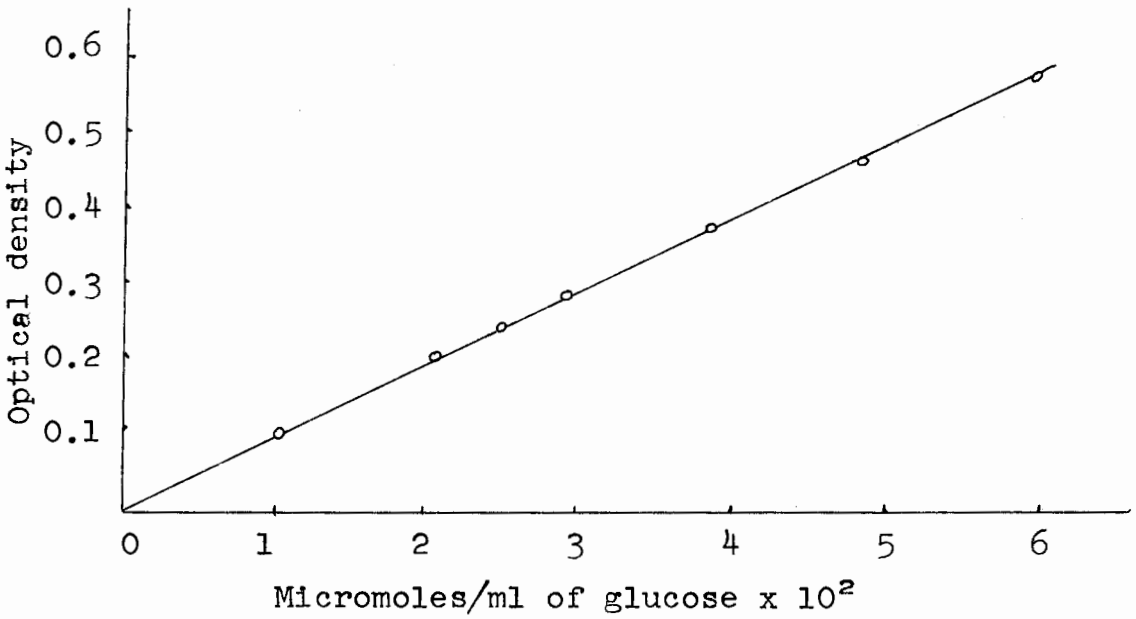

Figure 20. Glucose standard using DNS reagent (540 nm). Each time the reagent was prepared a curve was made.

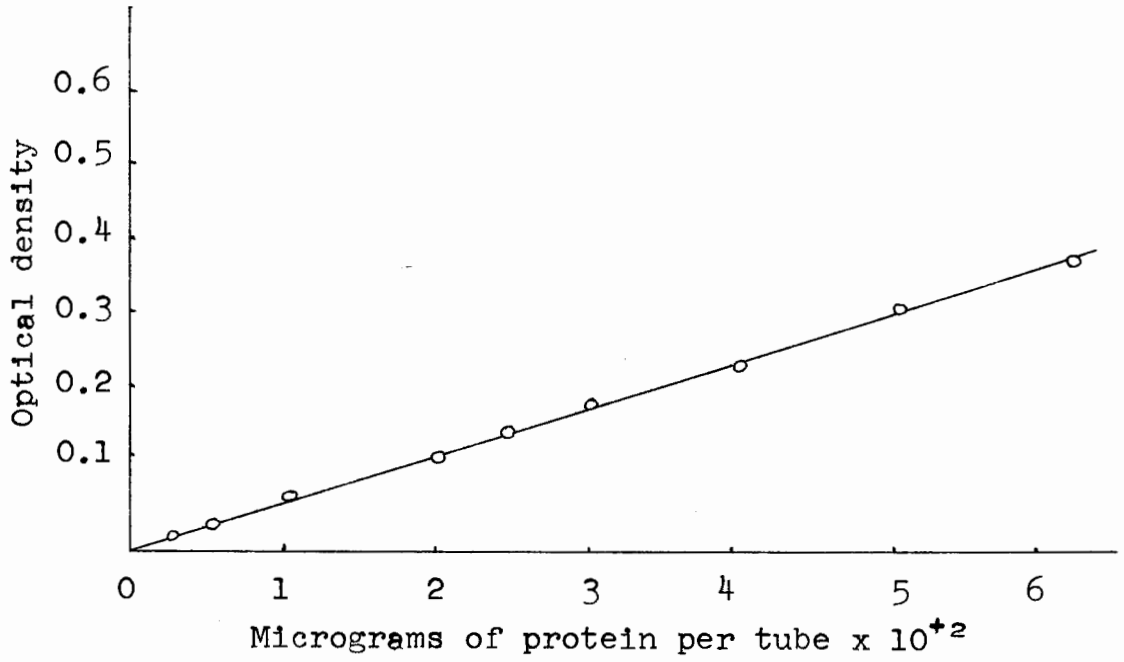

Figure 21. Protein standard using Folin-Ciocalteau reagent. (500 nm.)

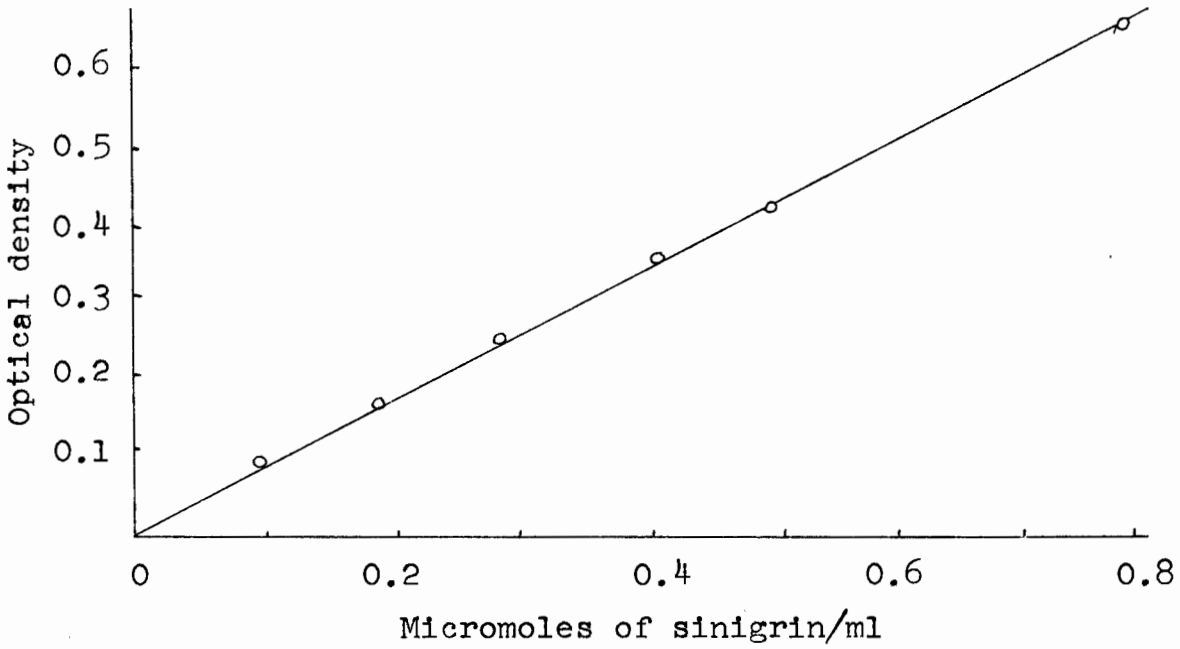

Figure 22. The absorption of sinigrin at 227.5 nm. The calculated extinction coefficent was 8010.

TABLE XIII

ENZYME NOMENCLATURE

Trivial Name	EC No.*	Systematic Name
α-amylase	3.2.1.1	α-1,4-glucan 4-glucanohydrolase
β-amylase	3.2.1.2	α-1,4-glucan maltohydrolase
Cellulase	3.2.1.4	β-1,4-glucan 4-glucanohydrolase
α-glucosidase	3.2.1.20	α-D-glucoside glucohydrolase
β-glucosidase	3.2.1.21	β-D-glucoside glucohydrolase
α-galactosidase	3.2.1.22	α-D-galactoside galactohydrolase
β-galactosidase	3.2.1.23	β-D-galactoside galactohydrolase
Invertase	3.2.1.26	β-D-fructofuranoside fructohydrolase
Trehalase	3.2.1.28	Trehalose 1-glucohydrolase
β-glucuronidase	3.2.1.31	β-D-glucuronide glucuronohydrolase
Thioglucosidase	3.2.3.1	Thioglucoside glucohydrolase

*Enzyme Commission number