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GLYCOSIDASES OF *TROPAEOLUM MAJUS*

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Abstract—A number of glycosidases have been qualitatively identified in the seeds of *Tropaeolum majus*, the common nasturtium. A thioglucosidase, a β-glucosidase, a fructofuranosidase, 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.

INTRODUCTION

WHILE enzymes catalyzing the hydrolysis of *O*-glycosidic bonds are widespread in nature, those involved in the cleavage of *S*-glycosidic bonds are much less frequent. In higher plants, the thioglucoside glucohydrolase* enzymes have been associated mainly with systems containing the mustard oil glucosides, and are found in the Cruciferae, Resedaceae, Capparidaceae and Tropaeolaceae. Most investigations have been concerned with the enzymes of the Cruciferae, particularly the mustard species, and these appear to be relatively free of associated glycosidic enzymes, although the dual activity reported in some instances might suggest some β-glucosidase activity.[1, 2] To date no *O*-glycosidase systems have been isolated from the thioglucosidase preparations.

In studying the thioglucosidase system of the Tropaeolaceae we observed glycosidase activities that could not be attributed to the thioglucosidase. These activities led to the isolation and partial purification of several glycosidic enzymes as reported in this paper.

RESULTS

Separation and Purification of the Enzymes

The aqueous extract of defatted seeds of *Tropaeolum majus* was incubated with various substrates in order to assess the total glycoside hydrolase activity (Table 1). These results indicated the presence of glycosidase activity other than that of the thioglucosidase known to be present in the system.

The crude extract, Fraction I, was adsorbed on a DEAE-cellulose column and eluted with 0·02 M barbital buffer, pH 8·6, with an increasing gradient of NaCl to 0·2 M. The elution diagram for the DEAE-cellulose column is shown in Fig. 1. All glycosidase activity appeared in the first 75 ml although protein elution continued over 60 fractions. Similar experiments

* For simplicity, the trivial names for the glycosidic enzymes have been used throughout this paper. Thioglucosidase is used in preference to the common name myrosinase.

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performed after prolonged dialysis of the crude extract gave comparable results, but showed considerable shifting and spreading of the peaks. Ammonium sulfate fractionation of the protein extract gave varying results depending on the nature of addition of the salt. The total protein was precipitated by the addition of solid ammonium sulfate to bring the saturation to 80 per cent (Fraction II).

TABLE 1. GLYCOSIDASE ACTIVITY OF *Tropaeolum majus*

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

* Activity was noted when a measurable amount of product was observed.
† Hydrolytic activity on β-Me-D-glucoside was negligible.

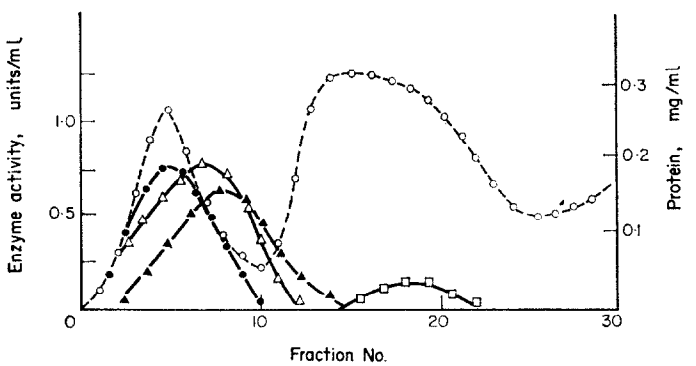

FIG. 1. CHROMATOGRAPHY OF THE CRUDE AQUEOUS EXTRACT (I) ON DEAE-CELLULOSE. THE CONDITIONS ARE DESCRIBED IN THE TEXT. ENZYME ACTIVITY IS IN μMOLES PRODUCT FORMED PER hr PER ml.
○ Protein. ● β-Glucosidase. △ β-Thioglucosidase. ▲ Fructofuranosidase. □ Polysaccharidase.

Fraction II, after dialysis, was treated with ammonium sulfate to increase the saturation by 10 per cent increments. In all experiments the glycosidases were located primarily in the 50–70 per cent saturation range. These fractions were combined for further purification (Fraction III). When Fraction III was allowed to stand overnight at 4°, a precipitate appeared. This precipitate, Fraction IV, contained essentially all of the β-glucosidase and β-galactosidase activity, but failed to hydrolyze the mustard oil substrates. It also contained the system responsible for the hydrolysis of sucrose.

To this point the ratio of glycoside hydrolase activity toward glucosidic and galactosidic substrates had remained relatively constant. The precipitated protein, Fraction IV, showed

a considerable increase in the specific activity toward both glucosides and galactosides, but resulted in a large change in the ratio of these activities.

In subsequent experiments it was found that the precipitated protein fraction could be obtained only when a saturated ammonium sulfate solution was used for the fractionation. The use of solid ammonium sulfate for the fractionation did not give rise to the protein precipitate, but did give a resultant fraction in the 50–70 per cent saturation range that contained much greater thioglucosidase activity. This fraction contained all glycoside hydrolase activity indicated in the crude extract.

Fraction III was subjected to calcium phosphate gel adsorption and to electrophoretic separation on cellulose acetate and starch gel as described in the Experimental section. Calcium phosphate adsorption did not improve the specific activity of the systems and hence was not applied in further purifications. Cellulose acetate electrophoresis gave four distinct zones, although they were very light in intensity after staining. All proteins migrated towards the anode. The β-glucosidase migrated most rapidly and was located primarily in the zone

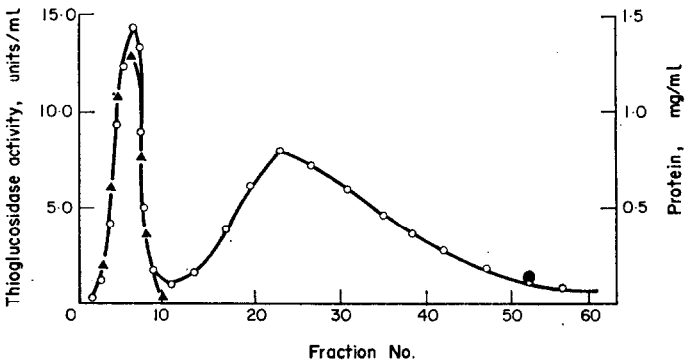

FIG. 2. CHROMATOGRAPHY OF FRACTION III ON DEAE-SEPHADEX. COLUMN CONDITIONS AND ELUTION PROCEDURES ARE DESCRIBED IN THE TEXT.

○ Protein. ▲ Thioglucosidase activity.

farthest from the origin. There was little separation of the remaining activities. Very little separation occurred during starch gel electrophoresis and only two zones were distinguishable. Again the β-glucosidase was concentrated in the most rapidly moving zone, while the remaining activities were spread throughout the two zones.

Adsorption of Fraction III on a DEAE-sephadex column gave a sharp separation of thioglucosidase activity (Fraction V) and a broad peak containing the remaining glycosidic enzymes. The elution diagram for this column is shown in Fig. 2.

The protein fractions, tubes 18–30, from the DEAE-sephadex column were combined and treated with solid ammonium sulfate until a slight sheen appeared in the solution. Under these conditions a crystalline protein was obtained. This material showed limited hydrolysis of the polysaccharides amylopectin and glycogen and possessed no hydrolytic activity toward the other attempted substrates. The activities of Fractions IV, V and the crystalline protein are summarized in Table 2. The resulting supernatant from the crystallization contained β-glucosidase, β-galactosidase and an invertase activity, but to a lesser extent than Fraction IV. A summary of β-glucosidase, β-galactosidase and thioglucosidase activities is given in Table 3.

TABLE 2. HYDROLYTIC ACTIVITY OF PROTEIN FRACTIONS FROM *Trapoeolum 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	0·08		
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		11·00	
Glucosinalbin		9·40	
Glucotropaeolin		5·50	

* Activity is expressed enzyme units per ml protein solution.

TABLE 3. PURIFICATION OF β-GLYCOSIDASE, β-GALACTOSIDASE AND β-THIOGLUCOSIDASE FROM *Tropaeolum majus*

Fraction	Volume (ml)	Protein (mg/ml)	Activity* (units/ml)		Specific activity (units/mg protein)
I. Crude extract	550	5·1	β-Glucosidase	0·82	0·16
			β-Galactosidase	0·41	0·08
			β-Thioglucosidase	1·26	0·25
II. 80% Salt precipitation	150	19·0	β-Glucosidase	5·7	0·30
			β-Galactosidase	2·5	0·13
			β-Thioglucosidase	4·50	0·24
III. 50–70% Salt fraction	58	13·0	β-Glucosidase	4·8	0·38
			β-Galactosidase	2·0	0·16
			β-Thioglucosidase	10·0	0·80
IV. Protein ppt. from Fraction III.	4	0·25	β-Glucosidase	14·4	57·0
			β-Galactosidase	2·9	11·5
			β-Thioglucosidase	—	—
V. DEAE-Sephadex effluent	9	1·3	β-Glucosidase	—	—
			β-Galactosidase	—	—
			β-Thioglucosidase	11·0	8·40

* Enzyme units are those defined in the experimental section.

DISCUSSION

The results summarized in Table 1 indicate at least four glycosidic enzymes, with the possibility of several additional systems. The *Tropaeolum* system contained a thioglucosidase as might be expected by the known presence of the mustard oil glucoside, glucotropaeolin.

This thioglucosidase, purified about 30-fold, also had good activity toward the mustard oil substrates sinigrin and glucosinalbin, but lacked hydrolytic activity toward alkyl-*S*-glycosides and *O*-glucosides. Several earlier papers have reported the hydrolysis of *p*-nitrophenyl-β-D-glucoside and other β-linked glucosides by the thioglucosidase systems from mustard.[1–3] Total lack of β-glucosidase activity by the purified *Tropaeolum* thioglucosidase raises some question as to the purity of the earlier preparations, but does not rule out the possibility of dual activity by the Cruciferae enzymes.

A β-glucosidase system was definitely indicated by the hydrolysis of a number of naturally occurring β-linked glucosides as well as several aromatic β-glucosides. The exact nature of this enzyme must await further study, but it appears to parallel the reported aryl-β-glucosidase systems[4,5] in that it does not hydrolyze cellobiose and shows only trace, if any, hydrolysis of alkyl glucosides. Certain glycone specificity may be indicated by lack of hydrolysis of β-methyl-D-xyloside; however, aromatic xylosides were not attempted as substrates at this time and may establish this lack of activity as aglycone specificity.

The enzyme responsible for the hydrolysis of sucrose appears to be a β-fructofuranosidase with a glycone specificity similar to that reported for yeast invertase.[6] The possibility of an α-glucosidase may be ruled out by lack of hydrolysis of maltose, turanose, melezitose and α-methyl-D-glucoside. The enzyme shows glycone specificity for an unsubstituted fructofuranosyl residue and does not hydrolyze inulin. These results appear to corroborate previous work on the invertase systems of higher plants.[7]

The crystalline protein obtained from the *Tropaeolum* system cannot at this time be positively identified as to activity. Hydrolysis of several polysaccharides was indicated, but the specific activity did not appear to be high enough to justify a classification. Lack of hydrolysis of amylose and limited hydrolysis of amylopectin, measured by increase in reducing sugar, indicates an endoglycosidase analogous to the *R* enzyme isolated from plants,[8] however, *R* enzyme has no appreciable activity on glycogen.[9]

Several additional glycoside hydrolase activities were indicated by the *Tropaeolum* system. The first, which was weak but repeatedly positive, involves the hydrolysis of trehalose. It would appear to be due to the presence of an α-1,1-glucosidase (trehalase). This enzyme has been reported in many plant systems.[10] The second activity was indicated by the hydrolysis of various β-galactoside substrates. The significant change in the ratio of β-glucosidase to β-galactosidase activity would appear to suggest that two enzymes are involved; however, as β-galactosidase activity could not be separated from β-glucosidase activity, it may be the result of nonconfigurational glycone specificity by the β-glucosidase rather than a separate system. Certain β-glucosidase systems have been reported to hydrolyze β-galactosides.[11] Substituted aromatic galactosides appear to be the best substrates, while phenyl-β-D-galactoside was hydrolyzed to a lesser degree. A major question concerning the possibility of a β-galactosidase is involved in the total absence of activity toward lactose. Several of the

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microbial β-galactosidase systems show preferential hydrolysis for aryl galactosides, but also hydrolyze lactose.[12] Further study is under way to resolve this observation.

EXPERIMENTAL

Methods and Materials

Tropaeolum majus seeds (Nasturtium Golden Gleem) were obtained from the Portland Seed Company, Portland, Oregon. All substrates were obtained from commercial sources. *N,N*-Diethylaminoethyl cellulose (Cell-Ion DEAE) was prepared in 0·02 M barbital buffer, pH 8·6. The column was washed repeatedly with buffer before sample application. After application of the protein samples, the columns were eluted with barbital buffer containing gradient concentrations of NaCl to 0·2 m. This elution procedure was followed in all chromatography experiments.

DEAE-Sephadex, A-50, was allowed to equilibrate in 0·02 M barbital buffer, pH 8·6, for 24 hr. The buffer was replaced by fresh buffer several times during the equilibration period. The column was allowed to gravity pack and was washed with 500 ml of buffer solution. The initial flow rate was 0·4 ml per min and increased to 1·0 ml per min as the ionic strength of the buffer was increased. In all chromatographic experiments three milliliter fractions were collected.

$Ca_3(PO_4)_2$ gel was prepared by the method of Keilin and Hartree.[13] 1 ml of phosphate gel (0·13 g per ml) was added to an enzyme solution to give a gel-protein ratio of 2:1. The suspension was allowed to stand at 4° for 15 min and then centrifuged. The gel pellet was eluted with gradient NaCl solution to 0·2 M.

Electrophoretic studies were carried out on cellulose acetate strips using 0·075 M barbital buffer, pH 8·5, or 0·05 M Tris-barbital buffer, pH 8·8. The potential gradient was 27·5 V per cm. Starch gel electrophoresis was carried out in 0·02 M borate buffer, pH 8·2, at 175 V for 20 hr. In all electrophoretic studies the enzymes activities were located by eluting the zone areas from unstained slices or strips of the support medium. Quantitative protein determinations were carried out by the method of Lowry[14] using a bovine serum albumin standard. Protein elutions were followed qualitatively by absorption at 280 nm.

Enzymatic assays were accomplished by measurement of the *p*-nitrophenol liberated or by the analysis of reducing sugars. For the *p*-nitrophenol assay, 0·1 ml of the enzyme preparation, 0·5 ml of 10^{-3} M substrate and 2·4 ml of phosphate buffer, pH 6·8, were allowed to incubate for 1 hr at 37°. The reaction was stopped by boiling the incubation mixture for 2 min on a water bath. The concentration of *p*-nitrophenol was measured at 420 nm. For substrates liberating reducing sugars, the incubation mixtures had a total volume of 1·0 ml and the reducing sugars were determined by the dinitrosalicylic acid method.[15] All reducing sugars were measured against a glucose standard. All dialysis procedures were carried out at 4° against water or buffer containing 10^{-3} M mercaptoethanol.

Enzyme activity is expressed as micromoles of product (reducing sugar or *p*-nitrophenol) liberated per hour per ml of enzyme solution under the assay conditions.

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