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Glycosidases of Turnip Leaf Tissues

I. Physiochemical Properties of Myrosinase and Disaccharase Enzymes

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ABSTRACT

Four myrosinase (β-thioglucosidase EC. 3.2.3.1) and seven disaccharase (β-fructofuranosidase, EC. 3.2.1.26) isoenzymes were isolated from turnip leaves. The most active enzymes were isolated in pure form. Myrosinase and disaccharase mol wt was 62.0 × 10^3 and 69.5 × 10^3 dalton, respectively, on the basis of gel filtration on Sephadex G-200.

Myrosinase pH profile showed high activity between pH 5 and 7 with the optimum at pH 5.5. The purified enzyme was heat-stable for 60 min at 30°C with only loss of 24% of activity. Its activity is strongly inhibited (100%) by Pb^{2+}, Ba^{2+}, Cu^{2+}, and Ca^{2+} ions, and activated (70%) by EDTA at 0.04*M*. The pure enzyume failed to hydrolyze amylose, glycogen, lactose, maltose, and sucrose. The K_m and V_{max} values of myrosinase using sinigrin as specific substrate was 0.045 m*M* and 2.5 U, respectively.

The maximal activity of disaccharase enzyme was obtained at pH 4–5 and at 35–37°C. The enzyme was heat-stable at 30°C for 30 min with only 10% loss of its activity. Its activity is strongly activated (70–240%) by Ca^{2+}, Ba^{2+}, Cu^{2+}, and EDTA at 0.01*M*. The enzyme activity is specific to the disaccharide sucrose and failed to hydrolyze other disaccharides (maltose and lactose). The K_m *and* V_{max} of disaccharase were 0.123 m*M* and 3.33 U, respectively.

Index Entries: Turnip leaves; myrosinase (β-thioglucosidase); disaccharase (β-fructofuranosidase); Sephadex G-200.

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INTRODUCTION

Myrosinase (β-thioglucosidase) catalyzes the hydrolysis of sinigrin to glucose, sulfate, and a series of pungent sulfur- and nitrogen-containing compounds, such as isothiocyanates, thiocyanates, and so forth, depending on the reaction conditions used. The study of myrosinase has important applications for both the biological and technological aspects of the food and feed stuff industries, and for the assessment of the safety of Cruciferous materials in general (*1*).

In order to provide a deeper understanding of this enzyme, it is important that its structure and main physicochemical properties be characterized. As with any protein, the first step is to devise an efficient and complete procedure for the isolation of the pure enzyme. This was done in the previous study by Jwanny and El-Sayed (*2*).

Myrosinase is a glycoprotein that often exists in multiple forms. The number of isoenzymes present depends on plant species, genotype, and tissue type (*3*). It also depends on the technique used for isolating the enzyme, especially in the extraction step (*4*). Separation and characterization of myrosinase from mustard seeds have been reported (*5,6*). However, no information is available on the characterization of myrosinase from the green leaves of higher plants. MacGibbon and Allison (*7*) detected isoenzymes of myrosinase in leaves of *Brassica* species.

Snowden and Gaines (*8*) indicated the presence of a number of glycosidases, especially a β-fructofuranosidase from seeds of *Tropaeolum majus.* Disaccharase (β-fructofuranosidase) was also isolated and detected from grape leaves by Vasil'ena (*9*). Extraction and purification of disaccharase from yeasts were previously investigated by Thorsell (*10*), Nigoro and Hirano (*11*), and El-Sayed et al. (*12*).

Glycosidic enzymes of turnip leaf tissues were isolated and purified in the previous paper. The properties of the major β-thioglucosidase system (myrosinase) and that of disaccharase are identified and characterized in this article.

MATERIALS AND METHODS

A purified myrosinase and disaccharase enzymes from turnip leaves were prepared according to the method previously described (*2*). Briefly, this method included the following steps: ammonium sulfate precipitation, ion-exchange chromatography on DEAE-cellulose, hydroxylapatite adsorbent, and gel filtration on Sephadex G-200.

Enzyme Assay

Unless otherwise indicated, the standard reaction mixture contained 1.0% (w/v) of the substrates (sinigrin or sucrose) dissolved in 0.1*M* phosphate buffer, pH 6.0, and an appreciable amount of an enzyme source in a total final volume of 0.3 mL. The reaction mixture was incubated at 37°C in a water bath for 1 h. The reaction was stopped by boiling in a water bath for 10 min, and the liberated reducing sugar (glucose) in the reaction mixture was measured against a standard glucose by the method of Somogyi (*13*) and Nelson (*14*).

Protein Determination

The protein content was estimated by the method of Lowry et al. (*15*) using bovine serum albumim as standards. For the fractions collected from different columns, the method of Warburg and Christian (*16*) was used.

Preparation of Sinigrin

Sinigrin (thioglucoside) was prepared from the commercial black mustard powder as described by Thies et al. (*17*).

Optimal pH for Enzyme Activity

Acetate buffer, pH 3.5–5.5, 0.1*M* and 0.1*M* citrate-phosphate buffer, pH 6.0–7.5, were used for recording the pH profile.

Effect of Temperature on Enzyme Stability and Activity

Thermostability of the enzyme was tested by heating small enzyme aliquots for 15, 30, 45, and 60 min in a water bath at temperatures of 30, 40, 50, and 60°C. The heated samples were then assayed for enzyme activities at 37°C. Fresh nonheated aliquots of enzymes were assayed at different incubation temperatures ranging from 30 to 60°C. An arrhenius plot of sinigrin and sucrose hydrolysis by myrosinase and disaccharase, respectively, was examined. Velocity was plotted against the reciprocal of the absolute temperature. Activation energy (Ea) for the enzyme was calculated. In all of the above cases, the enzyme activity was expressed as a percentage of the control enzyme activity (100) under the assay conditions.

Effect of Various Additives

Pure enzymes were incubated for 1.0 h at 15°C in the presence of the respective cation (Ca^{2+}, Cu^{2+}, Ba^{2+}, and Pb^{2+}) and EDTA, and at the end of the incubation period, aliquots were withdrawn and assayed.

Michaelis Constant

The K_m values for enzymes were determined graphically by the method of Lineweaver and Burk (*18*). The substrate concentration used in this study ranged from 0.008 to 0.062 m*M*. The assay was conducted in 0.1*M* phosphate buffer, pH 6.0, at 37°C.

The molecular weight of the enzymes was determined by the method of Andrews (*19*), using gel filtration on Sephadex G-200 columns. The elution buffer was 0.01*M* phosphate buffer, pH 7.0. The column was calibrated with bovine serum albumin (69.5×10^3 daltons), trypsin (34×10^3 daltons), and pepsin (36×10^3 daltons). The elution volume (Ve) of each protein was estimated from an elution diagram by extraploting both sides of the protein peak to an apex. Determination of other gel-filtration parameters were according to Andrews (*19*), Flodin and Porath (*20*), Siegel and Monty (*21*), and Ackers (*22*).

UV Absorption Measurement

The absorbances of the enzymes preparation were measured in 0.01*M* phosphate buffer, pH 7.0.

Substrate Specificity

The enzymes activity was tested using various disaccharides (sucrose, maltose, and lactose), polysaccharides (glycogen and amylose), and glucosinolates (sinigrin) as substrates. The substrates were incorporated in the reaction mixture at a final concentration of 1.0% (w/v). The reaction tubes were incubated simultaneously with appropriate controls.

RESULTS AND DISCUSSION

Myrosinase and disaccharase enzymes were isolated and purified from turnip leaves as previously described (2). Purity and homogenity were judged by chromatography on Sephadex G-200 and polyacrylamide gel electrophoresis in which the enzyme showed a single protein band. The existence of myrosinases has been reported in some plants, such as *Cruciferae* seeds (23) and leaves (7). However, no details have been reported on the properties of these enzymes from leaves. We characterized a myrosinase and disaccharase from green leaves in this study. In a previous paper, Jwanny and El-Sayed (2) indicated that leaves of turnip (*Cruciferae* family) contain four myrosinase and seven disaccharase isoenzymes. They differ in their eluate number from DEAE cellulose and hydroxylapatite adsorpance. The major enzymes were isolated in pure form.

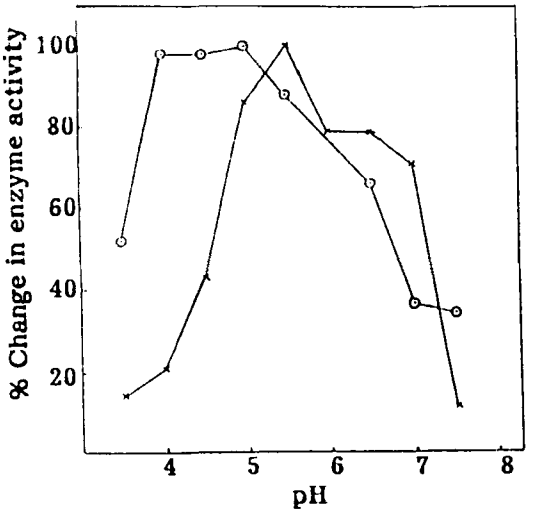

Fig. 1. Effect of pH on the enzyme activity. The buffers used were 0.1*M* acetate buffer (pH 3.5–5.5) and 0.1*M* citrate-phosphate buffer (pH 6.0–7.5). ⊙—⊙ Disaccharase and x—x myrosinase.

Enzyme Properties

Effect of pH on Enzyme Activities

Enzymes activities were measured at different buffer pHs ranging from pH 3.5 to 7.5. Optimal myrosinase activity was widespread between pH 5.0 and 7.0 with maximum at pH 5.5 (Fig. 1). It shows a steep decline in activity below pH 5.0 and above 7.0 with 57.1 and 89.3% loss of the activity at pH 4.5 and 7.5, respectively. Similarly, myrosinase enzymes from *Sinapis alba* seeds have optimal pH range between 4.5 and 7.8 (*24*), whereas maximal activity for disaccharase enzyme was observed between pH 4.0 and 5.0. This is in agreement with the maximal pH of disaccharase isolated from *Endomycopsis fibuligera* (*12*).

Thermostability of the Enzymes

Optimum temperature was measured using the conditions given for the enzyme assay procedure at various temperatures (30–60°C). The optimum incubation temperature of the purified myrosinase and disaccharase is 37°C (Fig. 2A). Disaccharase had a wide range of incubation temperatures (35–45°C). This is in agreement with the results of El-Sayed et al. (*12*). The results presented in Fig. 2A clearly show the protective effect of sucrose on the disaccharase enzyme, which fulfills the known phenomenon of Sandvick (*25*), who showed the effect of substrate on enzyme activity against heat denaturation. An Arrhenius plot (Fig. 2B) shows two

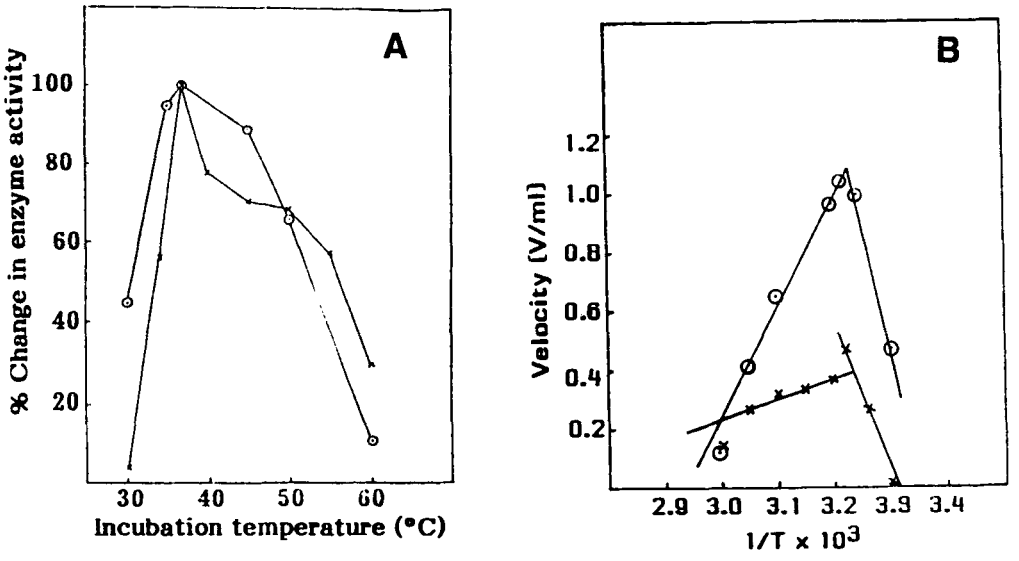

Fig. 2. **A.** Effect of temperature on the enzyme activity. ⊙—⊙ Disaccharase and x—x myrosinase. **B.** Arrhenius plot of sucrose ⊙—⊙ and sinigrin x—x.

distinct slopes, above and below 36.6°C, giving activation energies of about 37.8 kcal/mol and 21.4 kcal/mol for myrosinase and disaccharase, respectively. For disaccharase enzyme, a sudden drop in the plot after 36.6°C indicates enzyme inactivation, whereas for myrosinase enzyme, a gradual drop in the plot after 36.6°C was observed.

After incubating the enzyme solution for different times (15–60 min) at various temperatures, the residual activity was measured (Fig. 3A,B). Disaccharase enzyme tolerated preheating at 30 and 40°C, similar to the disaccharase obtained from yeast (*12*).

Myrosinase enzyme is somewhat more stable to heat than disaccharase enzyme. Its satisfactory heat stability suggests that it could be employed constructively in an industrial or agrochemical context.

Effect of Additives

Enzyme activities were tested in the presence of divalent cations at different concentrations ranging from 0.01 to 0.04*M* (Table 1). Myrosinase enzyme is strongly inhibited (88–98%) by Ca^{2+} and Cu^{2+}, whereas Pb^{2+} and Ba^{2+} lead to complete loss of enzyme activity. No metal ions activated the enzyme. It is activated (70%) by EDTA at 0.04*M* concentration. Thus, lack of stimulation by metals indicates that myrosinase enzyme does not require metals for activity like other myrosinase isolated from fungi (*26*). Disaccharase activity is strongly activated by metal ions, like Ca^{2+}, Cu^{2+}, Ba^{2+}, or by 0.01*M* EDTA (70–240%). On increasing the concentration of metal ions up to 0.04*M*, a decrease in the activity of the enzyme was noticed.

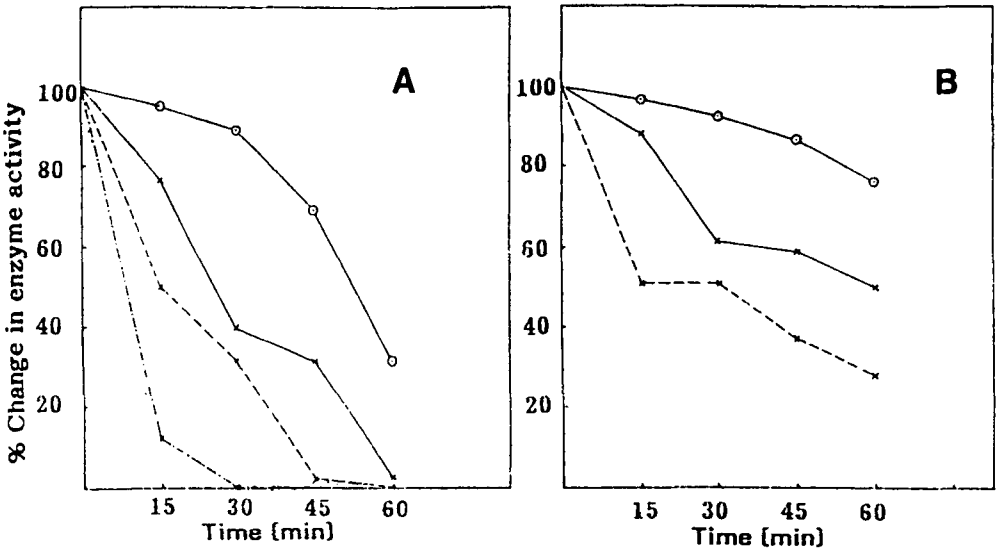

Fig. 3. Thermostability of the purified disaccharase (**A**) and myrosinase (**B**) enzymes at different temperatures (in absence of substrate). 30°C ⊙—⊙; 40°C x—x; 50°C x---x, and 60°C x-·-x.

Table 1
Effect of Additives on Myrosinase and Disaccharase Activities[a]

Additive	Concentration, *M*	Change in myrosinase activity, %	Change in disaccharase activity, %
Ca^{2+}	0.01	−38	+70
	0.02	−94	+30
	0.04	−98	−20
Cu^{2+}	0.01	−70	+200
	0.02	−80	+155
	0.04	−88	+20
Ba^{2+}	0.01	0.0	+185
	0.02	0.0	+145
	0.04	0.0	+10
EDTA	0.01	−20	+240
	0.02	−18	+140
	0.04	+70	+40

[a]The enzymes were preincubated in 0.1*M* phosphate buffer (pH 0.6) containing various additives (0.01–0.04*M*) for 1 h at 15°C. After preincubation, the activity was assayed by the standard procedure. Pb^{2+} led to almost complete loss of enzyme activities.

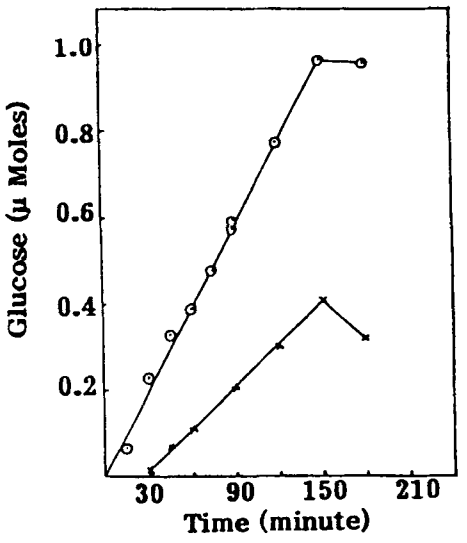

Fig. 4. Rate curves for the hydrolysis of sinigrin or sucrose with disaccharase ⊙—⊙ or myrosinase x—x, respectively.

The activities of the myrosinase and disaccharase were a linear function of incubation time up to 150 min beyond the time this inhibition occurred (Fig. 4). We concluded that increasing time led to excess products, which may inhibit the enzyme activity. Based on these results, all tests were conducted for 60 min to place them in the linear portion of the curve. Also, a linear function was found with enzyme concentration in reaction mixture using sinigrin or sucrose as substrates under the conditions mentioned above (Fig. 5). All tests were made employing the same enzyme and substrate concentrations as used before.

The K_m values of myrosinase and disaccharase using the specific substrate for each enzyme were 0.045 and 0.123 m*M*, respectively (Fig. 6A,B). The V_{max} values for myrosinase and disaccharase also were 2.5 and 3.33 U, respectively.

Ultraviolet absorbance profile of the enzymes at 0.01*M* phosphate buffer, pH 6.0, was shown in Fig. 7. No significant absorbance could be traced in the visible region indicating the absence of chromophore (27). The ratio of absorbance at 280 nm to that of 260 nm was 0.5 and 0.94 for myrosinase and disaccharase, respectively. Similarly, disaccharase from yeast had a ratio of 0.86 (*12*).

The estimated mol wt values of myrosinase and disaccharase by gel filtration on Sephadex G-200 were 62×10^3 and 69.5×10^3 dalton, respectively (Fig. 8). Disaccharase molecular weight from turnip leaves was similar to that reported for disaccharase isolated from yeast (*12*). On the contrary, myrosinase molecular weight is different from that isolated from *Cruciferae* seeds (4).

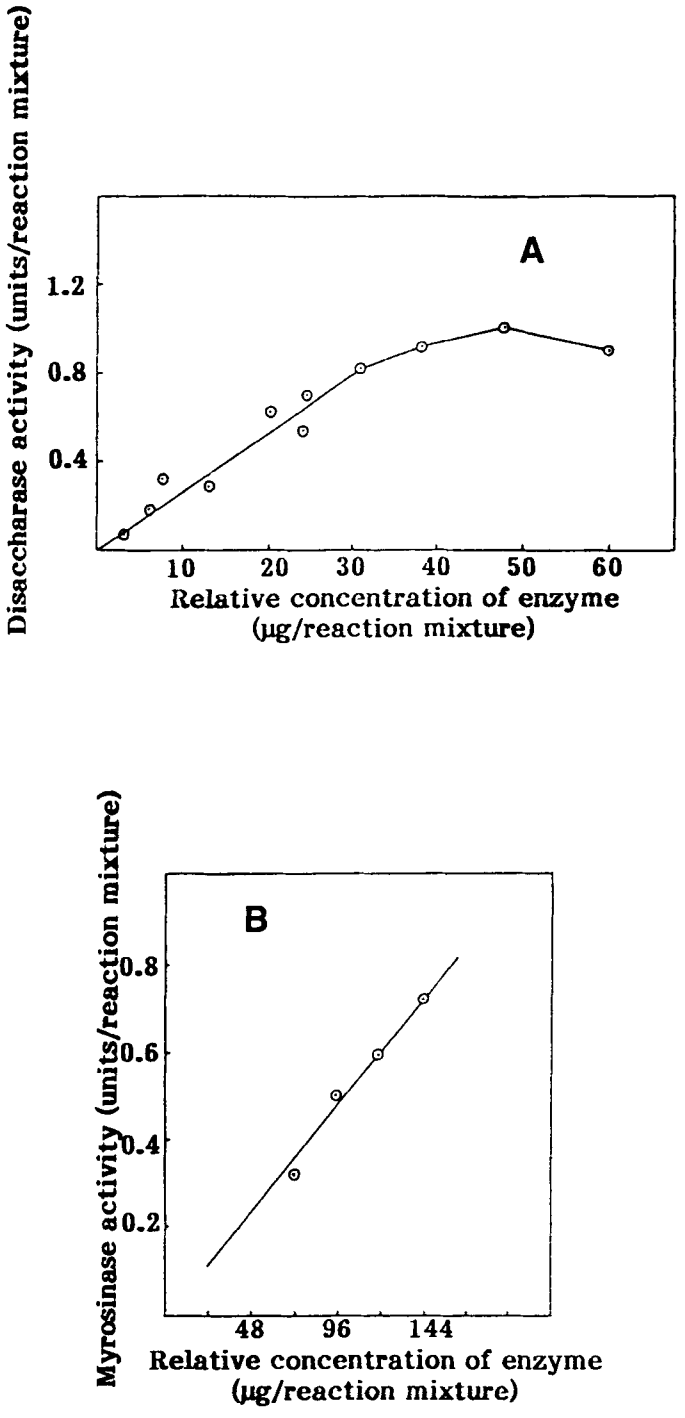

Fig. 5. Activity of various concentration of the purified disaccharase (**A**) and myrosinase (**B**) enzymes.

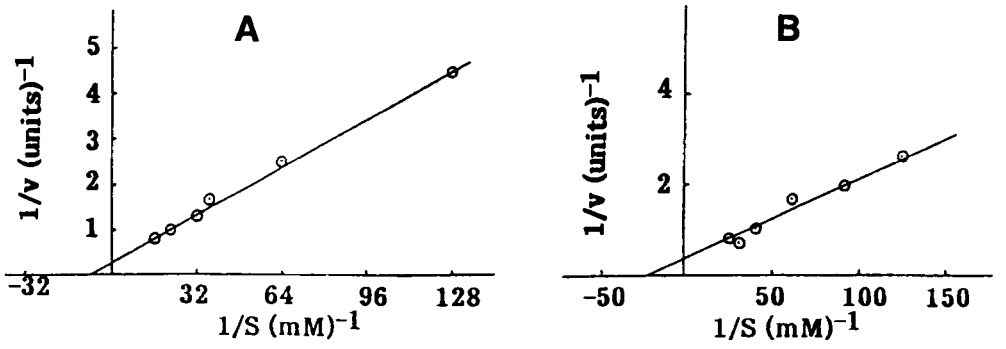

Fig. 6. Lineweaver-Burk plot for disaccharase (**A**) and myrosinase (**B**). The reciprocal of velocity (U) and substrate concentration (m*M*) is plotted above to determine K_m and V_{max}.

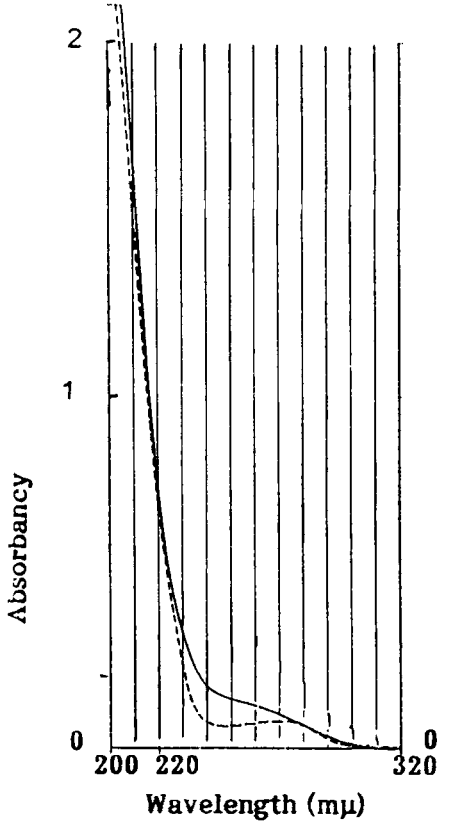

Fig. 7. Absorption spectrum of the purified disaccharase —— and myrosinase -----. Purified enzymes in 0.01*M* phosphate buffer (pH 7.0) were used.

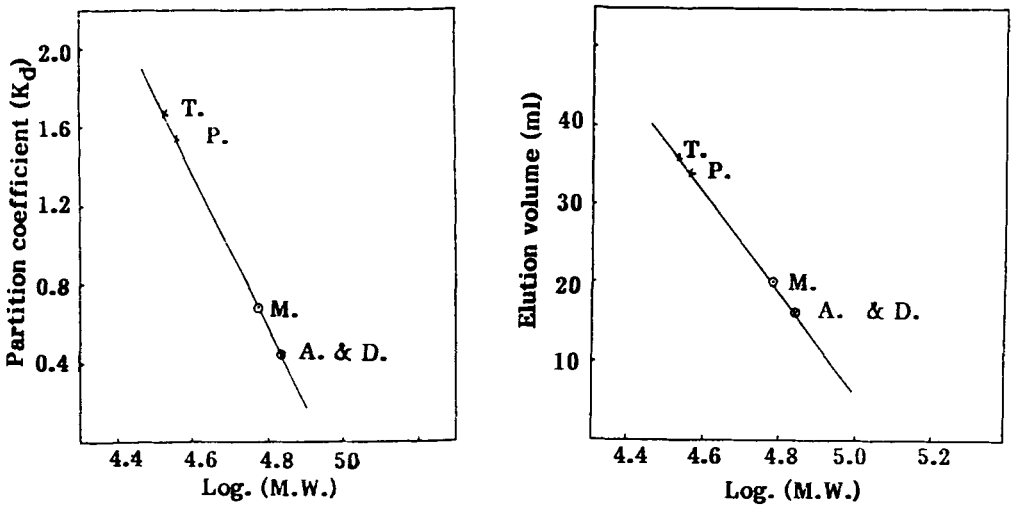

Fig. 8. Determination of molecular weight of leaf tissues disaccharase and myrosinase by gel filtration on Sephadex G-200 column (1.0 × 32 cm). Elution buffer was 0.01*M* phosphate buffer, pH 7.0. T: trypsin, P: pepsin, A: bovine serum albumin, D: disaccharase, and M: myrosinase.

In order to evaluate the specificity of the myrosinase, it appeared desirable to check for activity on various substrates other than sinigrin. The following compounds were checked as substrates for the enzyme and showed no activity: amylose, glycogen, lactose, maltose, and sucrose. From these data, it appears that the myrosinase is relatively free of all enzyme systems that show maltase, lactase, disaccharase, and amylase activity. At the same time, disaccharase hydrolyzed only sucrose and failed to hydrolyze the other substrates (maltose, lactose, amylose, glycogen, and sinigrin). It is probably specific for sucrose (aglycone specificity). Thus, it is fructofuranoside and specific for hydrolyzing α 1/2-glycosidic link.

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