0273-2289/94/4901—0023$06.40

Glycosidases of Turnip Leaf Tissues

II. Isolation, Purification, and Some Physiochemical Characterization

ETIDAL W. JWANNY* AND SANAA T. EL-SAYED

Biochemistry Department, National Research Center, Tahrir St., Dokki, Cairo, Egypt

Received November 24, 1993; Accepted January 24, 1994

ABSTRACT

A number of glycosidase enzymes have been isolated and identified in healthy fresh leaves of turnip. Myrosinase (β-thioglucosidase, EC. 3.2.3.1), disaccharase (β-fructofuranosidase, EC. 3.2.1.26), and β-amylase (α-1,4-glucan maltohydrolase, EC 3.2.1.2) have been isolated and purified in powder form. The purification methods were salting out with ammonium sulfate, DEAE-cellulose column chromatography, hydroxylapatite batch chromatography, and gel filtration through Sephadex G-200. Four isoenzymes of myrosinase enzyme could be isolated. The most active one was purified (131.3 times) and found to have an SA of 19.7 U/mg. An enzyme causing hydrolysis of amylose and glycogen has been isolated and partially purified. It had an SA of 22.6 U/mg and 49.1-fold purification. Seven isoenzymes of disaccharase were isolated, but only one was purified (C) with SA of 1448.5 U/mg and 1316.8-fold purification.

The yield of the purified myrosinase and disaccharase enzymes was 3.68 and 0.5 mg, respectively, from 100 gm dry wt of turnip leaves. Confirmation of purity with disk electrophoresis was performed. A single sharp band was obtained for each pure enzyme by disk electrophoresis. The chromatographic analysis of the hydrolytic end product of the β-amylase indicated the presence of maltose. This results confirm the β-form of amylase enzyme.

Index Entries: Turnip leaves; *Cruciferae* family; myrosinase (β-thioglucosidase); disaccharase (β-D-fructofuranosidase); β-amylase (1,4-α-D-glucan maltohydrolase); DEAE-cellulose; hydroxylapatite; and Sephadex G-200.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Since 1985, we have been giving high priority to research on new proteins sources. Jwanny et al. (*1*) studied protein production as byproducts from sugar beet leaves for human use. It was of interest to isolate enzymes from leaves of different plants. Turnip leaves are always thrown out or burned because of the fact that they are not used by Egyptians as food or feed stock.

The turnip is one of the *Cruciferae* family, and most investigations have been concerned with the myrosinase enzyme of the *Cruciferae*, particularly the mustard species. These appear to be relatively free of associated glycosidic enzymes, although the dual activity reported in some instances might suggest some β-glucosidase activity (*2*). Snowden and Gaines (*3*) studied the isolation and partial purification of several glycosidic enzymes of the *Tropaeolum majus*. Chapman et al. (*4*) isolated a β-amylase from leaves of *Vicia faba*. Jwanny and El-Sayed (*5*) studies the isolation and characterization of β-amylase from beet-root leaves.

The lack of information on the properties of glycosidases from leaf tissues prompted us to isolate and examine the enzymes from turnip leaves. The present article describes the purification of glycosidase enzymes from turnip leaf tissues, and reports some of its physical and chemical properties.

MATERIALS AND METHODS

Preparation of Crude Enzymes

Green healthy, fresh turnip leaves were collected from fields in the early morning. They were homogenized in a Brown mixer, using either distilled water or 1.0% (w/v) NaCl solution at 4°C. The resulting homogenate was squeezed through three layers of cheesecloth, and then filtered through Whatman No. 1 paper and a clay (Hyalo Super-Gel) layer to separate the remaining green particles left in the filtrates. The filtrates were dialyzed against distilled water for 48 h at 4°C and then centrifuged at 3000 rpm for 15 min at 5°C.

Preparation of Sinigrin

Sinigrin (thioglucoside) was prepared from the commercial black mustard powder as described by Thies et al. (*6*).

Enzyme Assays

Phosphate buffer pH 6.0, 0.1*M* containing 1.0% (w/v) amylose, sucrose, or sinigrin as substrate was used. The reaction mixture contain-

ing 0.1 mL of each substrate and 0.2 mL of the enzyme solution was incubated at 37°C for 1 h. The reaction was stopped by boiling for 10 min. Enzymatic activities were determined by measuring the liberated reducing sugar (maltose or glucose) by the method of Somogyi (*7*) and Nelson (*8*).

Enzyme Unit

One unit of enzyme activity is defined as the amount of enzyme that will catalyze the formation of 1 μmol of reducing sugar (maltose or glucose)/h under the standard assay condition, and specific activity is expressed as U/mg of protein.

Protein Analysis

Protein content was determined by the method of Lowry et al. (*9*) using bovine serum albumin as a standard. For the fractions collected from different columns, protein content was measured spectrophotometrically (*10*).

Substrate Specificity

Substrate specificity of the crude extract solution was determined using amylose (soluble starch), glycogen, sucrose, sinigrin, lactose, and maltose as substrate. Each of the above substrates was dissolved in 0.1*M* phosphate buffer, pH 6.0, at a concentration of 1.0% (w/v).

Purification of the Enzymes

The enzymes were partially purified by three separate methods, ethanol (*11*), acetone (*12*), and ammonium sulfate fractionation from aqueous extracts of the homogenized leaves. Appropriate amounts of solid ammonium sulfate were added to the crude enzyme solution at 4°C to increase the saturation of the solution by factors of 30% until no further material appeared to precipitate. The mixture was centrifuged after each addition of ammonium sulfate and the precipitated protein was collected, resuspended in distilled water, and dialyzed against distilled water for 24 h at 4°C. Each fraction obtained by salt precipitation was checked for activity on different substrates (amylose, glucogen, maltose, lactose, sucrose, and sinigrin).

N,N-Diethylaminoethyl Cellulose Column Chromatography

DEAE-cellulose was activated and dispersed in 0.01*M* citrate-phosphate buffer, pH 6.0. The DEAE-cellulose column (1.2 × 32 cm) was washed repeatedly with buffer before use. The enzyme was eluted with the same citrate-phosphate buffer containing gradient concentrations of NaCl 0.1 to 0.5*M* (*13*).

Hydroxylapatite Batch Chromatography

Hydroxylapatite media was prepared by the method of Tiselius et al. (*14*). Hydroxylapatite was added to an enzyme solution in 0.0001*M* phosphate buffer, pH 7.0, in a ratio 2:1, the suspension was allowed to stand at 4°C for 15 min and then was centrifuged. The hydroxylapatite was washed several times with small portions of buffer and then eluted stepwise with gradient phosphate buffer with different molarities ranging from 0.0001 to .05*M*.

Gel Filtration

A glass column (1.2 × 35 cm) loaded with Sephadex G-200 was used. The column was equilibrated with 0.01*M* phosphate buffer, pH 7.0. The concentrated enzyme was applied to the column, and the protein was eluted from the column with the same buffer at a flow rate of 25 mL/h.

Disk Electrophoresis

Polyacrylamide gel electrophoresis was performed by the procedure of Davis (*15*) with Tris-HCl buffer, pH 8.9. Electrophoresis was carried out at 2 mA/g for 3 h, using a concentration of 7.5% polyacrylamide in the running gel until the bromophenol blue tracking dye migrated to the end of the lower gel. Proteins were stained with amido black.

Paper Chromatography

An aliquot of the purified β-amylase was incubated at 37°C with 1.0% amylose dissolved in 0.1*M* acetate buffer, pH 5.0. The products were examined by paper chromatography. Descending chromatography was run using pyridin:*n*-butanol:water (9:15:6) for 24 h, dried, and then diped in aniline phthalate solution (*16*). Sugars were visualized as orange-colored spots, after heating the chromatograms at 110°C for 10 min.

RESULTS AND DISCUSSION

Purification of the Enzymes

Much of the enzyme activities were lost by precipitation of the 0.5% NaCl extract or from frozen turnip leaves. Therefore, a water extract from healthy green turnip leaves was fractionated by the salting-out procedure with ammonium sulfate at 4°C. Precipitation with alcohol or acetone caused considerable loss in activities in turnip leaves aqueous extract. This result was in accordance with those results of Jorgensen and Jorgensen (*17*). The protein fraction obtained at 30% ammonium sulfate saturation was incubated after dialysis with various substrates in order to assess the total glycoside hydrolase activity. Results indicated that this fraction

hydrolyzed each of the following substrates: amylose, glycogen, sucrose, and sinigrin, but did not digest maltose or lactose. These results indicated the presence of glycosidase activities other than that of thioglucosidase known to be present in the *Cruciferae* (*18*). These activities led to the isolation and purification of several glycosidic enzymes (disaccharase, β-amylase, together with myrosinase).

DEAE-Cellulose Column Chromatography

The dialyzed enzyme solution of 30% ammonium sulfate precipitation was applied to a DEAE-cellulose column (1.2 × 32 cm), which had been equilibrated with 0.01*M* citrate-phosphate buffer, pH 6.0. Elution was carried out with the same buffer after stepwise increases in the concentration of NaCl to 0.5*M*. Active fractions A, B, and C were obtained from DEAE-cellulose chromatography. Fraction A was passed through the column without adsorption in 0.01*M* citrate-phosphate buffer and was found to have enzyme activities as disaccharase, myrosinase, and β-amylase with SA of 10.5, 2.4, and 3.7 U/mg, respectively. Fractions B and C were adsorbed to the exchanger. Elutions of these fractions was carried out by 0.2 and 0.5*M* NaCl concentration, respectively. The disaccharase SA found in fractions B and C was 12.7 and 68.2 U/mg, respectively.

Sephadex G-200 Gel Filtration

Since fraction C showed the highest disaccharase specific activity (68.2 U/mg), it was further subjected to Sephadex G-200 gel filtration. Figure 1 shows two protein fractions. The disaccharase activity was associated with the first protein fraction. This step showed about 1316.8-fold disaccharase purification.

Hydroxylapatite Batch Chromatography

Fraction A was subjected to further purification using hydroxylapatite and different molarities of phosphate buffer (0.0001–0.5*M*), pH 7.0. Seven eluates (1–7) were obtained (Fig. 2). Eluates 5 and 6 showed only disaccharase activity, but failed to hydrolyze amylose or sinigrin. Eluates 1 and 2 have only myrosinase activity, although the other three eluates (3, 4, and 7) exhibited activities toward the sucrose, amylose, and sinigrin substrates.

The hydroxylapatite batch chromatography showed a mean recovery of 70.7% for four myrosinase and 46.6% for five disaccharase isoenzymes, which separated at different molarities of phosphate buffer. To our knowledge, only MacGibbon and Allison (*19*) detected the presence of myrosinase in plant leaves of the order *Rhoeadales*. They also demonstrated the presence of glucosinolases activity as isoenzymes ranging from a single to four or more in some species. Our results are in accordance to their findings.

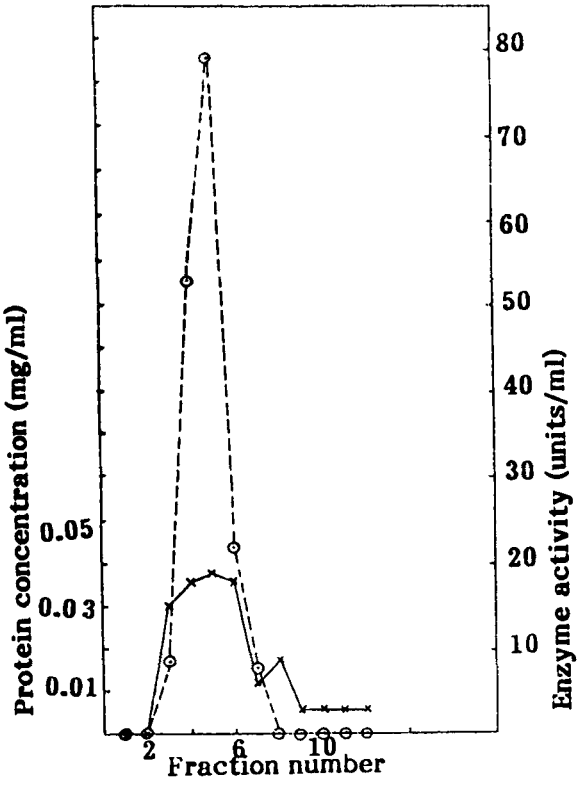

Fig. 1. Chromatography of the turnip disaccharase enzyme on Sephadex G-200 column. Elution was made with 0.01*M* phosphate buffer, pH 7.0. The fractions were monitored for protein x—x and for disaccharase activity ⊙...⊙.

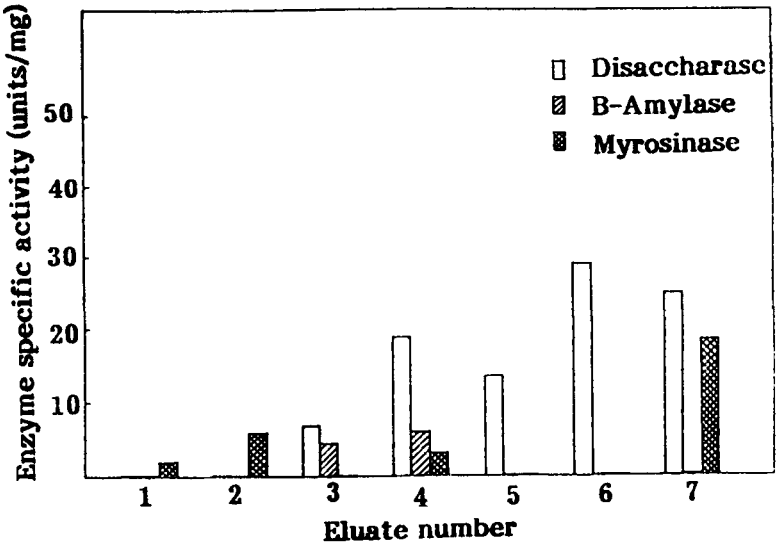

Fig. 2. Chromatography of the turnip glycosidases on hydroxylapatite in 0.0001*M* phosphate buffer, pH 7.0. A linear gradient from 0.0001 to 0.5*M* phosphate buffer was applied for elution of the protein.

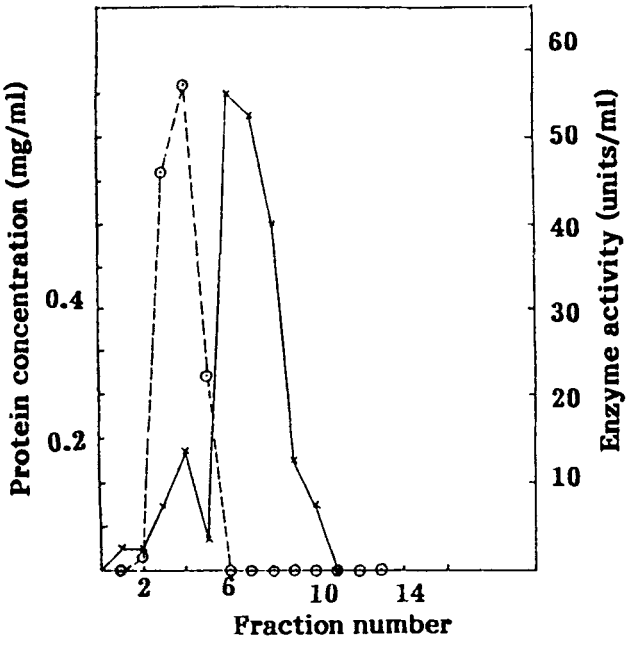

Fig. 3. Chromatography of the turnip disaccharase enzyme on Sephadex G-200 column. Elution was made with 0.01*M* phosphate buffer, pH 7.0. The fractions were monitored for protein x—x and for disaccharase activity O...O.

Eluate (6) with disaccharase having the highest SA of 28.8 U/mg was run on Sephadex G-200 column. Two peaks were obtained with the activity shown in the first peak only (Fig. 3). A summary of the disaccharase purification is presented in Table 1.

The eluate (2) with the highest myrosinase SA (6.0 U/mg) was further purified using Sephadex G-200 column. The first peak was found to have myrosinase activity of 19.7 U/mg (Fig. 4 and Table 1) and showed a 131.3-fold increase in specific activity.

Eluates 3 and 4 contained β-amylase activity with SA of 4.4 and 5.7 U/mg, respectively. They also contained the system responsible for the hydrolysis of sucrose, but failed to hydrolyze sinigrin, lactose, or maltose. This eluate (3) was further applied to Sephadex G-200, and a partially purified preparation was obtained in the first protein peak (Fig. 5 and Table 1). It had two activities toward amylose and sucrose.

From the above results, it can be concluded that turnip leaf tissues contain disaccharase, β-amylase, and myrosinase enzymes, which could be isolated and purified. Glycosidase enzymes have been detected by Vasiléna (*18*), by MacGibbon and Allison (*19*), and Spencer and Weston (*20*), which have been isolated and purified by Chapman et al. (4) and by Jwanny and El-Sayed (*5*) from the leaves of some plants.

Table 1
Purification of Turnip Leaf Disaccharase, Myrosinase, and β-amylase

	Total protein, mg	Total activity, U	purity, U/mg	Purification fold	Yield, %
Disaccharase activity					
Crude extract	1292	1438	1.1	—	100
$(NH_4)_2SO_4$ precipitation	91	918	10.1	9.2	63.8
DEAE-cellulose					
Fraction A	57.2	599	10.5	9.5	41.7
Fraction B	21.2	270.6	12.8	62.0	18.8
Fraction C	2.79	190.3	68.2	62.0	13.2
Sephadex G-200					
for fraction C	0.13	188.3	1448.5	1316.8	13.1
Hydroxylapatite for					
fraction A					
Eluate (3)	10.8	76.3	7.1	6.5	5.3
Eluate (4)	13.1	234.3	17.9	16.3	16.3
Eluate (5)	5.5	72.8	13.2	12.0	6.1
Eluate (6)	5.4	155.7	28.8	26.2	10.8
Eluate (7)	2.4	59.5	24.8	22.5	4.1
Sephadex G-200					
for eluate (6)	0.49	141.1	288.0	261.8	9.4
Myrosinase activity					
Crude extract	1292	198	0.15	—	100
$(NH_4)_2SO_4$ precipitation	91	141	1.5	10.0	71.2
DEAE-cellulose					
Fraction A	57.2	140	2.40	16.0	70.7
Hydroxylapatite for					
fraction A					
Eluate (1)	10.4	22.9	2.2	14.7	11.6
Eluate (2)	6.0	36.1	6.0	40.0	18.2
Eluate (4)	13.1	36.7	2.8	18.7	18.6
Eluate (7)	2.4	44.3	18.6	123.3	22.4
Sephadex G-200					
for eluate (2)	0.92	18.1	19.7	131.3	9.1
β-amylase activity					
Crude extract	1292	600	0.46	—	100
$(NH_4)_2SO_4$ precipitation	91	273	3.0	6.5	46.6
DEAE-cellulose					
Fraction A	57.2	213.6	3.7	8.0	35.6
Hydroxylapatite for					
fraction A					
Eluate (3)	10.8	47.6	4.4	9.6	7.9
Eluate (4)	13.1	74.7	5.7	12.4	12.5
Sephadex G-200					
for eluate (3)	1.4	31.6	22.6	49.1	5.3

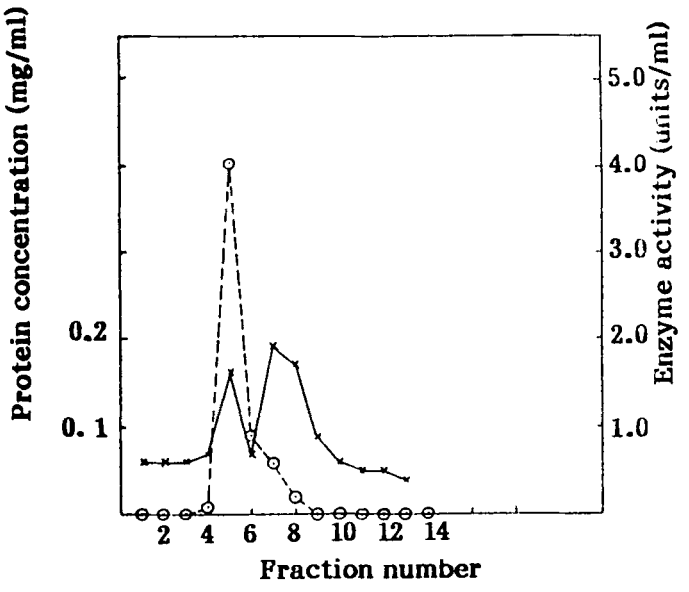

Fig. 4. Chromatography of the turnip myrosinase enzyme on Sephadex G-200 column. Elution was made with 0.01*M* phosphate buffer, pH 7.0. The fractions were monitored for protein x—x and for myrosinase activity ⊙...⊙.

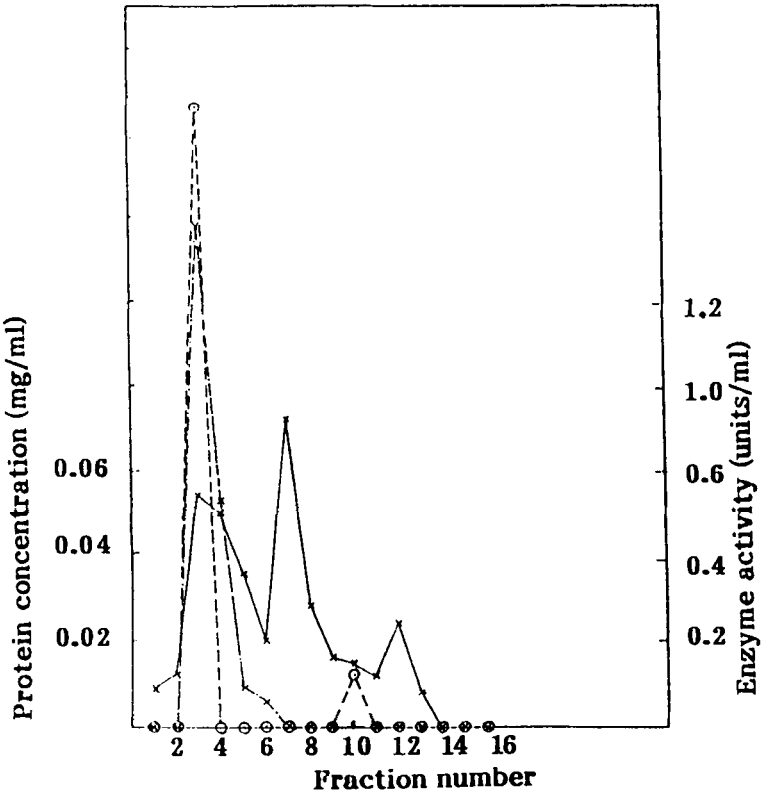

Fig. 5. Chromatography of the turnip β-amylase and disaccharase enzymes on Sephadex G-200 column. Elution was made with 0.01*M* phosphate buffer, pH 7.0. The fractions were monitored for protein x—x, for β-amylase activity x-·-·-x, and for disaccharase activity ⊙...⊙.

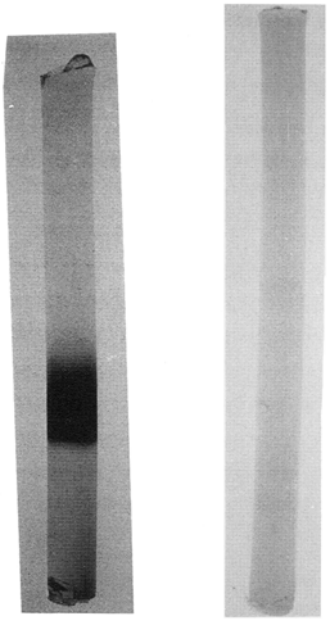

Fig. 6. Polyacrylamide gel electrophoresis patterns of the purified turnip disaccharase and myrosinase enzyme. Electrophoresis was carried out at 2 m*M* gel for 3 h in Tris-HCl buffer, pH 8.9. Twenty micrograms of the pure enzyme were applied to the gel. The protein staining with amido black. (**A**) disaccharase; (**B**) myrosinase.

Some Properties of the Purified Turnip Leaves' Glycosidic Enzymes

Homogeneity was shown in Figs. 1, 3, and 4 using Sephadex G-200. Only one peak was obtained for each enzyme (disaccharase and myrosinase). Furthermore, disk electrophoresis analysis was carried out for each purified enzyme. Figure 6 confirmed the homogeneity of each enzyme, since a single sharp band was shown for each enzyme.

The myrosinase enzyme, purified about 131.3-fold, had good activity toward mustard oil substrate sinigrin, but lacked hydrolytic activity toward *O*-glucosides. This result appears to parallel the reported results of Snowden and Gaines (*3*), but contradicts the earlier paper of Gaines and Goering (*2*), who reported the hydrolysis of β-linked glucosides by the thioglucosidase systems from mustard seeds.

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 (*21*). These results appear to corroborate previous work on the invertase systems of higher plants (*3*).

The paper chromatographic analysis of the amylose hydrolyzate by turnip β-amylase revealed that only maltose is produced as a detectable product. During the entire incubation period, no evidence for the generation of glucose or higher M dextrins was obtained. The absence of α-glucosidase activities in the enzyme preparation was substantiated by paper chromatographic experiments. It was basically in agreement with those found by Thoma et al. (*22*), Shi-Ching et al. (*23*), and Okamoto and Akazawa (*24*). The possibility of an α-glucosidase also may be ruled out by lack of hydrolysis of maltose.

The substrate specificity of turnip β-amylase is akin to that of other β-amylases (*5,25,26*). They hydrolyze amylose and glycogen, but did not digest maltose. They do not catalyze the hydrolysis of maltose (*27*).

The enzymes are extremely stable in solution and can be kept at −4°C for months without any appreciable loss of activity. Its excellent aqueous storage characteristics suggest that it could be employed constructively in an industrial or agrochemical context.

REFERENCES

1. Jwanny, E. W., Montanari, L., and Fantozzi, P. (1993), *Bioresource Technol.* **43,** 67.
2. Gaines, R. D. and Goering, K. J. (1962), *Arch. Biochem. Biophys.* **96,** 13.
3. Snowden, D. R. and Gaines, R. D. (1969), *Phytochemistry* **8,** 1649.
4. Chapman, G. W., Jr., Pallas, J. E., Jr., and Mendicino, J. (1972), *Biochem. Biophys. Acta* **276,** 491.
5. Jwanny, E. W. and El-Sayed, S. T. (1993), in Proceeding of the 13th Egyptian Chemical Conference, April 10–13 (9th Arab Chemical Conference).
6. Thiés, W. (1988), *Fat Sci. Technol.* **90,** 311.
7. Somogyi, M. (1952), *J. Biol. Chem.* **195,** 19.
8. Nelson, N. (1944), *J. Biol. Chem.* **153,** 375.
9. Lowry, O. L., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **193,** 265.
10. Layne, E. (1957), in *Methods in Enzymology,* Colowick, S. P. and Kaplan, N. O., eds., vol. 111, Academic, New York, p. 147.
11. Wrede, F. (1941), in *Die Methoden der Fermentforschung,* Bamann E. and Myrback, K., eds., vol. 2., Thieme, Leipzig, p. 835.
12. Schwimmer, S. (1961), *Acta Chem. Scand.* **15,** 535.
13. Peterson, E. A. and Soben, H. A. (1956), *J. Am. Chem. Soc.* **78,** 751.
14. Tiselius, A., Jenten, S. H., and Levin, O. (1956), *Arch. Biochem. Biophys.* **65,** 132.
15. Davis, B. J. (1964), *Ann. NY Acad. Sci.* **121,** 404.
16. Wilson, C. M. (1959), *Anal. Chem.* **131,** 1199.
17. Jorgensen, B. B. and Jorgensen, O. B. (1963), *Acta Chem. Scand.* **17,** 1765.
18. Vasiléna, Z. V. (1956), *Lenia* 133.
19. MacGibbon, D. B. and Allison, R. M. (1970), *Phytochemistry* **9,** 541.

20. Spencer, R. and Weston, T. J. (1966), *Tobacco* **163,** 28.
21. Myrback, K. (1960), in *The Enzyme,* Boyer, P. D., Lardy, H., and Myrbach, K., eds., vol. 4, Academic, New York, p. 384.
22. Thoma, J. A., Sprodlin, J. E., and Dygert, S. (1971), *The Enzymes,* Boyer, P. D., ed., Academic, New York, p. 115.
23. Shi-Ching, H., Jong-Ching, S., and Hsien-Yi, S. (1977), Chung-Kuo Nung Yeh Hua Hsuch Hui Chin, 15, 3. cf. C. A. 87(19), 150405 w (1977).
24. Okamoto, K., and Akazawa, T. (1978), *Agric. Biol. Chem.* **42,** 1379.
25. Green Wood, C. T. and Milne, E. A. (1968), *Adv. Carbohydr. Chem.* **23,** 281.
26. Subbaramaiah, K. and Sharma, R. (1990), *Phytochemistry* **29,** 1417.
27. Abdullah, M. and French, D. (1966), *Nature* **210,** 200.