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Glycosidases in Plant Tissues of Some *Brassicaceae*

Screening of Different Cruciferous Plants for Glycosidases Production

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ABSTRACT

Glycosidases namely myrosinase and β-amylase, have been isolated from *Brassicaceae*. These enzymes were identified and estimated by the rate of glucose and maltose formation from sinigrin (thioglucosinolate) and amylose (polysaccharides) hydrolysis, respectively. Their activities (U/g dry tissues) varied with the different species of the plant and with the different parts of their tissues. Generally, they were higher in the germinated seeds (3.3–8.0 times) than in powdered or defatted powdered dry seeds. The best amylase and myrosinase extracting solution for radish and white mustard germinated seeds was distilled water, and for turnip germinated seeds, it was 0.1*M* phosphate buffer, pH 6.0. In the light, the optimum germination temperature for amylase production or activation by radish and white mustard seeds was 25°C, and for turnip seeds, it was 30°C, whereas for myrosinase production or activation by radish and turnip, 25–27°C was the optimum temperature.

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The highest myrosinase activities in black mustard and radish defatted dry seeds were obtained by extraction with 1% NaCl at 27–30°C and distilled water at 25–27°C, after an incubation period of 4–6 h. Comparative studies indicated that fresh radish roots were the most potent amylase and myrosinase producers compared with radish leaves or roots, stems, and leaves of turnip and cabbage.

Amylase and myrosinase were partially purified from water extracts of fresh radish roots by optimum precipitation with ammonium sulfate (100%). Some physicochemical properties were studied.

Index Entries: Brassicaceae; cruciferae; radish roots; myrosinase (β-thioglucosidase); and β-amylase (1,4-α-D-glucan maltohydrolase).

INTRODUCTION

A number of glycosidases are widespread in higher plants. Myrosinase enzymes (β-thioglucosidase, EC 3.2.3.1) has been examined and detected in extracts of seeds, roots, petiols, and leaves from several Brassica species (*1–3*). Pihakaski and Pihakaski (*4*), Bones and Iversen (*5*), and Bones (*6*) followed its distribution and studied its occurrence in different plant organs in some Brassica species at different developmental stages.

β-amylase (1,4-α-D-glucan maltohydrolyase (EC 3.2.1.2) has been reported to occur in many plant species (*7–9*). Few investigators studied the isolation of β-amylase from Brassicaceae (*10*). Studies on its in vivo regulation have been largely confined to cereal seeds (*11,12*), whereas little information is available on Brassica seeds (*13,14*).

The aim of the present study was to identify a number of glycosidases from different parts of Brassicaceae plants under the most suitable conditions. This work also established a simple method for large-scale preparation and isolation of glycosidases, especially amylase and myrosinase, with substantial specific activities from aqueous extracts. Some of their properties were investigated.

MATERIALS AND METHODS

Preparation of Crude Enzymes

Different Brassica seeds were supplied by Vegetable Research Center, Dokki, Faculty of Science and Faculty of Pharmacy, University of Cairo. Seeds of *Sinapis alba* (white mustard), *Brassica nigra* (black mustard), *Brassica oleraceae* (cabbage), *Brassica napus* (rape), *Brassica campestris* (turnip), *Brassica juncea* (yellow mustard), and *Raphanus sativus* (radish) were treated in the following ways.

Seeds of different species were washed in sterile distilled water and germinated in Petri dishes on moist filter paper either in the dark or in white light (*5*), at different incubation temperatures (15–35°C) for different periods (1–10 d). Germinated seeds were used for preparing enzyme

samples as reported by Subbaramaiah and Sharma (*14*). The germinated seeds were homogenized in a mortar at 5°C, using extracting solutions (distilled water or 0.1*M* sodium-phosphate or citrate-phosphate buffer, pH 6.0, or 1% NaCl solution) at 4°C. The resulting homogenates were squeezed through three layers of gauze, and the crude filtrates obtained were centrifuged at 13,000*g* for 15 min. Each supernatant was dialyzed against distilled water to eliminate anthocyanins and other low-mol-wt substances present (< 12,000).

Dry seeds were crushed in a roller mill. One gram of the dry powder or defatted powder (with petroleum ether) was mixed with 10 mL of the extraction solution (distilled water, 0.1*M* citrate-phosphate, pH 6.0, 0.1*M* sodium-phosphate buffer, pH 6.0, or 1% NaCl) at different temperatures (15–30°C) for different periods (2–24 h). The insoluble materials were removed by centrifugation at 13,000*g* for 15 min at 4°C. The supernatants were dialyzed for 2 d against distilled water at 4°C to remove anthocyanins. During dialysis, the greater part of nonactive proteins precipitated and were easily removed by centrifugation.

The leaves, stems, and roots of turnip, radish, and cabbage were obtained from a local market. They were cut into small pieces and ground with distilled water and sea sand in a mortar at 5°C. The homogenate was filtered through cheesecloth and centrifuged at 13,000*g* at 4°C, and the supernatant was dialyzed against distilled water at 4°C for 2 d.

Preparation of Sinigrin

Sinigrin (thioglucosinolate) was prepared according to the method of Thies (*15*). It was isolated from *Brassica nigra* (black mustard) by extracting ground seeds with methanol, and then filtering and evaporating the methanol solution to dryness. The impure glucoside was passed through a DEAE-Sephadex G-25 column (10.0 × 0.8 cm) and eluted by potassium sulfate solution as potassium salt.

Determination of Myrosinase and β-amylase Activities

Enzymes activites were determined by incubating 0.2 mL enzyme solution with 0.1 mL sinigrin or amylose (1.0% w/v in 0.1*M* phosphate buffer, pH 6.0) for 1 h at 37°C. Formed glucose or maltose was determined by the methods of Somogyi (*16*) and Nelson (*17*).

Protein Content

This was determined by the method of Lowry et al. (*18*) using bovine serum albumin as standard.

Units

Enzymatic activities were expressed as micromoles of glucose or maltose liberated per hour under the assay conditions.

Purification of Amylase and Myrosinase from Radish Roots Tissues

Appropriate amounts of the precipitating agents (ethanol [30 or 60% saturation], acetone [30 or 60% saturation], and ammonium sulfate [30, 60, or 100% saturation]) were added to the crude enzyme solution at 5°C to increase the saturation of the solution by factor of 30% until no further precipitate appeared. The mixture was centrifuged after each addition of the precipitating agents, and the precipitated protein was collected, resuspended in distilled water, and dialyzed against distilled water for 24 h at 4°C. Each obtained fraction was checked for activity on different substrates (amylose, glycogen, and sinigrin).

RESULTS AND DISCUSSION

The aqueous extracts of different Brassicaceae plants were incubated with various substrates in order to assess the total glycoside hydrolase activity. They had higher amylase, myrosinase, and disaccharidase activities than maltase and pectinase. The lowest hydrolytic activity was found on soluble pectic acid and lactose. Thus, the results indicated the presence of at least amylase, disaccharidase, and myrosinase enzymes in appreciable amounts.

Our preliminary experiments showed that fresh leaves produced higher glycosidase activities than the frozen ones (16–40%), whereas root and stem samples gave nearly the same amount of glycosidase activities, either from fresh or frozen samples. At the same time, low activities in the undialyzed preparations were noticed, suggesting the presence of naturally existing inhibitors.

Preliminary tests indicated a small difference between the enzyme activities in the primary roots, stems, and leaves. This is in agreement with the results of Pihakaski and Pihakaski (*4*). However, Bones (*6*) found that the highest myrosinase activity was in the hypocotyls, and its lowest activity was in primary roots in 3-d-old seedlings of *Sinapis alba.*

Distribution of Amylase and Myrosinase Activities in Plant Tissues

Amylase and myrosinase activities were found in all the examined seeds and plants of Cruciferous family (Fig. 1 and Table 1). These results are in agreement with those found by Wilkinson et al. (*2*), Jwanny and El-Sayed (*3*), Subbaramaiah and Sharma (*14*), and Ettlinger and Kjaer (*19*). Figure 1 and Table 1 also showed that amylase and myrosinase activities varied not only with plant species, but also with different morphological parts of these tissues. Similarly, Bones (*6*) found that there were big differences in myrosinase activity among the plant species. The differ-

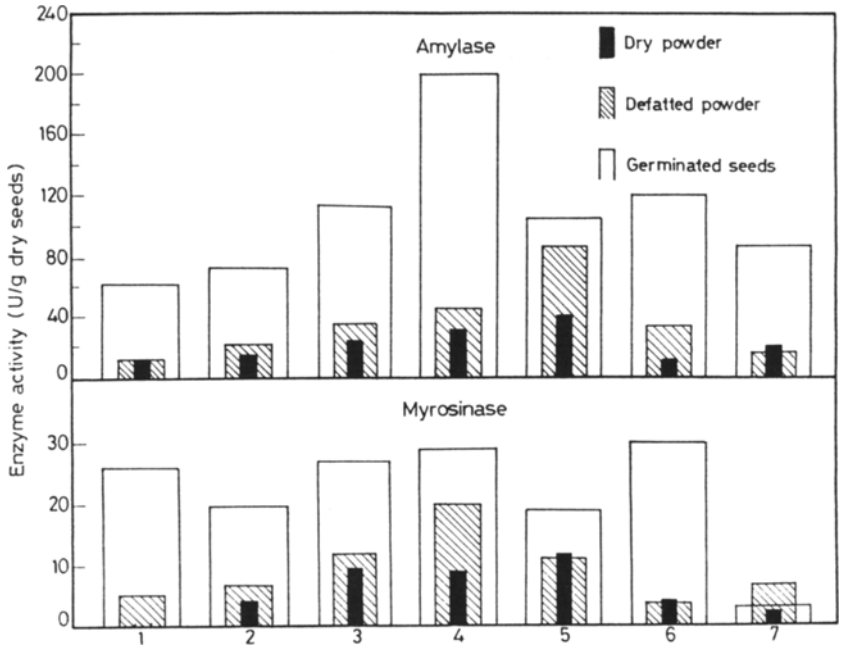

Fig. 1. Extraction of myrosinase and amylase from different *Brassicaceae* seeds using distilled water as extracting agent (yellow mustard 1, black mustard 2, white mustard 3, radish 4, cabbage 5, turnip 6, and rape 7).

Table 1
Isolation of Amylase and Myrosinase from Vegetative Brassicaceae Using Water as Extracting Agent

		Amylase activity		Myrosinase activity	
Plant	Part	U/g dry tissues	U/mg protein	U/g dry tissues	U/mg protein
Radish	Roots	185.0	4.33	35.6	0.83
	Leaves	112.2	1.44	12.7	0.16
Cabbage	Stems	108.3	3.18	4.4	0.13
	Leaves	113.4	7.09	4.9	0.31
Turnip	Roots	103.7	5.77	10.0	0.56
	Leaves	56.6	1.01	4.4	0.08

ences between the myrosinase enzymes obtained in this article and those described by Wilkinson et al. (2) indicated that there were also variations in diverse cultivars of the same plant species. Generally, higher enzymes activities were observed in the germinated seeds than in powdered or defatted powdered seeds.

Radish, turnip, cabbage, and white mustard seeds were the most potent amylase producers when germinated at 25–27°C for 5 d and then

extracted with distilled water. The enzyme activities on the fifth day of seedlings of radish, turnip, cabbage, and white mustard were about 6.25, 9.5, 2.5, and 4.6 times that of the original dry seeds, respectively.

Radish, turnip, and yellow and white mustard seeds were also the most powerful myrosinase producers when germinated at 25–27°C for 5 d in light and extracted with distilled water. The myrosinase activity was increased after germination by about 3.2 in radish, 5.0 in yellow mustard, 7.5 in turnip, and 2.8 times in white mustard than that of the original dry seeds. These results are in accordance with the results of Bones (*6*), who found that germinated white mustard, turnip, and radish seeds contained the highest myrosinase activity, especially in radish seeds.

The increase in myrosinase activity was higher in defatted powdered seeds when compared to that in powdered seeds of black mustard, radish, and rape (up to 2.2 times). The results in Table 1 also showed that roots of radish and turnip contained higher amylase activity than their leaves, whereas leaves of cabbage had higher activity than their stems. At the same time, the results showed that roots and leaves of radish contained higher myrosinase activity than leaves, roots, and stems of turnip and cabbage. Similarly, Bones (*6*) found that the highest myrosinase activity was in roots of mature plants and not in leaves. This increase in myrosinase activity in radish roots may be owing to the higher glucosinolate content in roots as compared to leaves, as we found (unpublished data). This indicates a relationship between the glucosinolate level and the myrosinase activity.

1-Amylase and Myrosinase Activities in the Germination Stage of Some Seeds

The results in Fig. 2 showed that amylase activity increased during germination when compared to that of the original dry seeds, and the best day was the sixth day of germination for turnip and radish seeds, and the fifth day for white mustard, with 8, 5.3 and 3.4 times increase, respectively, although myrosinase activity in turnip and radish seeds increased during germination up to the fifth and the sixth days (6.25 and 3.3 times), respectively, when compared to that of the original dry seeds. The enzyme activities declined with aging of the germinated seeds over 5-and 6-d-old (21–64%). This decline in activities was also investigated by Bones (*6*), Okamoto and Akazawa (*20*), and McGregor (*21*).

This increase of amylase and myrosinase during the germination process may be owing to the activation of latent form and/or the increase in the rate of *de novo* synthesis. Rowsell and Goad (*22*), Jacobsen and Varner (*23*), and Tronier and Ory (*24*) established that in cereal seeds, β-amylase exists in the latent form, being activated during the process of germination by the action of protease(s) secreted from the aleurone cells. On the other hand, Sharma and Schopfer (*13*) and Subbaramaiah and Sharma (*25*) indicated that the photo-regulated increase in β-amylase activity in mustard cotyledon exclusively resulted from an increase in the rate of *de*

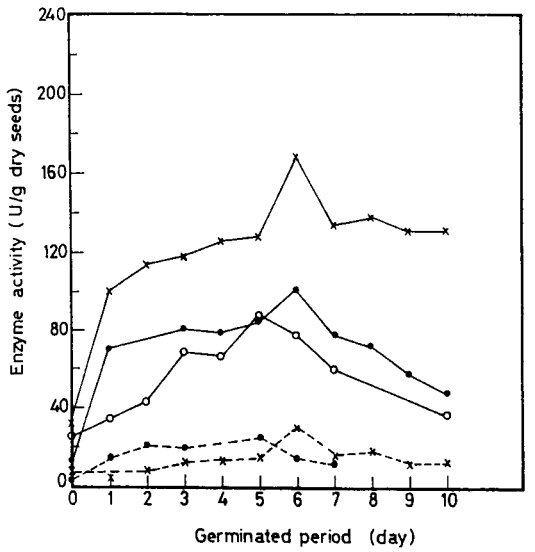

Fig. 2. Measurement of myrosinase (------) and amylase (——) with different germinated day from radish (×), turnip (●), and white mustard (○).

novo synthesis of β-amylase protein against a very low background rate of enzyme degradation.

Effect of Temperature, Light, and Extraction Solution on Amylase and Myrosinase Activities from Germinated Seeds

The results in Table 2 indicated that temperature and light are essential for amylase and myrosinase production during germination.

The maximum amylase activity produced during germination of radish and white mustard seeds was at 25°C, whereas in turnip seeds, it was at 30°C. On the other hand, the maximum production of myrosinase activity in turnip and radish seeds was obtained when germinated at 25–27°C. Similarly, Subbaramaiah and Sharma (*14,25*) reported an optimum germinating temperature to be 25°C in the light for β-amylase production in *Sinapis alba* (white mustard) seeds.

The decrease in production of amylase activity by turnip, radish, and white mustard during germination in the dark was 7.8, 18.6, and 38.7%, respectively, less than in corresponding germination in the light. At the same time, the myrosinase activity in germinated turnip and radish seeds increase in the light. It can be concluded that light acting via phytochrome initiates an increase in the β-amylase activity as confirmed previously (*25*). Thus, the factors light and temperature during germination showed important effects on the amylase and myrosinase production.

Extraction of amylase and myrosinase with 0.1*M* phosphate buffer, pH 6.0, from germinated turnip seeds increased by 1.4 and 1.6 times, respectively, over that extracted with water. However, germinated radish

Table 2
Extraction of Amylase and Myrosinase from Germinated Turnip, Radish, and White Mustard Seeds

	Amylase activity			Myrosinase activity	
	Turnip, U/g dry tissues	Radish, U/g dry tissues	White mustard, U/g dry tissues	Turnip, U/g dry tissues	Radish, U/g dry tissues
*Germinated in[a,b]					
Light	102	129	95	8.2	14.4
Dark	94	105	58	7.2	00.0
Extraction solution[b]					
1.0% NaCl	62	122	83	9.6	11.9
0.1*M* phosphate buffer, pH 6.0	139	77	29	13.9	9.0
0.1*M* citrate-phosphate buffer, pH 6.0	107	64	70	0.0	0.0
Germinated temperature (°C)[b]					
18	—	129	—	—	14.4
20	—	169	86	12.0	—
25	73	262	163	31.7	30.0
27	102	221	110	25.0	30.0
30	209	190	95	8.2	16.8

[a]Myrosinase and amylase were extracted with distilled water at 30°C, 5 d for turnip and white mustard and at 18°C, 6 d for radish seeds.
[b]No growth was noticed at 35°C.

seeds gave high amylase and myrosinase activities, and germinated white mustard seeds gave high amylase activity only when extraction was performed with distilled water. On the other hand, in both germinated seeds (turnip and radish), a complete inhibition in myrosinase activity was established when extraction was performed with 0.1*M* citrate-phosphate buffer, pH 6.0.

The above results (Fig. 2 and Table 2) established the optimum conditions for germination and extraction of amylase and myrosinase from Brassicaceae seeds. Thus, germination of seeds resulted in an approx 3.3- to 16.5-fold enhancement of the enzymes production compared to the original dry seeds.

Effect of Temperature, Extraction Solution, and Time on Myrosinase Activity from Defatted Powdered Seeds

The best solution of extraction and temperature for myrosinase activity from defatted powder of black mustard seeds was 1% NaCl at 27–30°C, whereas for powdered radish seeds, it was distilled water at 25–27°C

Table 3
Extraction of Myrosinase from Defatted Powder of Black Mustard and Radish Seeds

	Black mustard activity		Radish activity	
	U/g dry tissues	SA U/mg protein	U/g dry tissues	SA U/mg protein
Extracting solution[a]				
Distilled water	6.0	0.56	20.0	1.23
1% NaCl	11.9	0.64	18.2	2.37
0.1*M* phosphate buffer, pH 6.0	8.9	0.59	12.7	1.13
0.1*M* citrate-phosphate buffer, pH 6.0	8.1	0.42	10.8	0.99
Incubation temperature (°C)[b]				
25	6.7	0.27	20.0	1.23
27	11.9	0.64	21.3	1.93
30	12.6	0.55	9.0	0.63
37	7.5	0.34	7.3	0.86
40	7.7	0.45	4.3	0.26
Incubation time (h)[c]				
2	—	—	7.2	1.24
4	9.5	0.67	21.3	1.93
5	11.9	0.64	21.3	1.58
6	11.4	0.79	7.6	1.01
8	2.4	0.17	10.6	0.56
24	3.5	0.26	—	—

[a]Myrosinase was extracted from powdered seeds after incubation for 5 h at 25°C.
[b,c]Myrosinase was extracted with 1.0% NaCl for black mustard and with distilled water for radish seeds after incubation for 5 h ([b]) and at 27°C ([c]).

(Table 3). Differences in myrosinase activity when extracted by different solutions could be observed, and this may be owing to the relative solubility of the enzyme. Henderson and McEwen (*26*) and Palmieri et al. (*27*) extracted myrosinase enzymes from defatted powder of mustard seeds by distilled water, whereas Bjorkman and Lonnerdal (*28*) used 0.05*M* Na acetate buffer, pH 5.5.

Results in Table 3 showed the extraction of myrosinase from defatted black mustard and radish seeds at optimum conditions for different incubation periods. Five to 6 h of incubation was the most appropriate time for extracting the highest myrosinase activity from black mustard and 4–5 h for powdered radish seeds. Lower or higher periods afforded lower enzyme activities, presumably because of insufficient extracting time or loss of activity at longer periods. Thus, as expected, the type of extraction

Table 4
Purification of β-Amylase and Myrosinase from 1 kg Fresh Radish Roots

	β-Amylase				Myrosinase			
	Activity, U/kg, tissue	Yield, %	Specific activity, U/mg protein	Purity times	Activity, U/kg, tissue	Yield, %	Specific activity, U/mg protein	Purity times
Crude extract	11,052	100.0	4.20	1.0	2360	100	0.9	1.0
Ethanol								
30%	354	3.2	0.31	0.07	236	010	0.2	0.2
60%	1205	10.9	2.94	0.70	0.00	0.00	0.0	0.0
Acetone								
30%	1786	16.0	2.2	0.50	2148	91.0	2.7	3.0
60%	2266	20.5	5.74	1.37	0.00	0.00	0.0	0.0
Ammonium sulfate								
30%	884	8.0	2.8	0.67	1834	7.77	5.70	6.40
60%	3870	35.0	8.0	1.90	38	01.6	0.08	0.09
100%	2353	21.3	17.3	4.11	182	07.7	1.33	1.48
0.-100%	9835	89.0	11.2	2.70	2088	88.5	2.37	2.60

solution, different temperatures, and times affected the extractability and/or the activity of the isolated enzymes.

The above screening results could indicate that radish roots or germinated seeds were the most potent amylase and myrosinase producers. Therefore, radish roots were chosen for preparing partially purified amylase and myrosinase enzymes.

Purification of Amylase and Myrosinase from Radish Roots

Trials to precipitate the enzymes from solution with ethanol, acetone, or ammonium sulfate at 5°C are shown in Table 4. The ammonium sulfate (100%) gave a precipitate with satisfactory purification (about 2.7 and 2.6 times, 89.0 and 88.5% recovery) for amylase and myrosinase activities, respectively. Precipitation of the two enzymes with acetone (30% saturation) was possible, but caused 84% loss of amylase activity and only 9% loss of myrosinase activity. Attempts to purify the aqueous extract by acetone (60% saturation) and ethanol (30 and 60% saturation) failed because of great loss in activity (range from 80–100%). The above partially purified enzymes were used to investigate some of their properties.

Some Properties of Amylase and Myrosinase Enzymes

Activity of Different Substrates

The partially purified enzymes from radish roots had a good activity toward mustard oil thioglucosides (sinigrin) and other glucosinolates (glucocheirolin, progoitrin, glucocopparin, and glucosinalbin). These

results appear to be parallel to those reported by Bjorkman and Lonnerdal (*28*), who compared the activity of myrosinase from *Sinapis alba* for a number of glucosinolates. The partially purified enzymes also showed good activity toward different polysaccharides (amylose and glycogen) and disaccharides (sucrose).

Stability

The enzymes from radish roots were stored in distilled water at −20°C for several months without any appreciable loss of their activities, whereas when freeze-dried, myrosinase activity only decreased by about 51.8% of its activity.

pH Optima

The enzymes were active over the pH range from 3.5 to 6.5 using 0.1*M* acetate, citrate-phosphate, and sodium-phosphate buffer. Maximum activity was observed at pH 5.5 with amylose and pH 6.5 with sinigrin as substrates. Low myrosinase activity was found in 0.1*M* citrate-phosphate buffer (pH 5.5–6.5) with sinigrin as a substrate.

Type of Amylase

The enzyme activity toward amylose as a substrate was almost denatured when heated at 70°C for 5 min. It also had approx 49% of the maximal activity at pH 3.5.

Paper chromatography of the amylose hydrolyzates, by the extracted enzyme from radish roots, showed that maltose was the major hydrolysis product from amylose. During the entire incubation period, no evidence for the generation of glucose or higher maltodextrins was found. This indicated the presence of β-amylase in appreciable amounts and the absence of α-glucosidase activity. These results confirmed that the amylase activity is owing to the existence of β-form and complete absence of α-form. It is basically in agreement with the observations found by Okamoto and Akazawa (*20*) and Shi-Ching et al. (*29*).

Hydrolysis of sinigrin and amylose by the enzyme preparation was of a linear relationship with respect to their concentrations. As enzyme and substrate concentrations were increased, up to 7.04 and 0.74 mg protein and 0.15 mg sinigrin and 1.75% amylose/reaction mixture, the rate of the reaction diminished respectively.

Effect of Ascorbic Acid

Addition of various amounts of ascorbic acid (0.1–10 m*M*) at pH 6.5 (optimum pH) to a myrosinase-sinigrin mixture decreased the rate of hydrolysis. Lack of stimulation by ascorbic acid indicated that myrosinase enzymes did not require it for activity, as found by Wilkinson et al. (2) and Ettlinger et al. (*30*).

In Brassicaceae, some glycosidases (myrosinase and β-amylase) have been quantitatively identified and isolated from different plant organs (seeds, roots, stems, and leaves). The optimum conditions for extraction

of amylase and myrosinase from germinated and defatted powdered seeds were established. The results presented in this study show that radish roots and germinated seeds were the best convenient source for myrosinase and amylase. These enzymes have been isolated and partially purified from the most potent enzymes producer (radish roots). Further studies are under way to purify these enzymes. It is expected that extensive application of these enzymes will be possible in the clinical and industrial uses.

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