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SPECIFIC PHLORIDZIN GLUCOSIDASES FROM SEEDS AND LEAVES OF APPLE TREE

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Abstract—Five enzymatic fractions of β-glucosidase (EC 3.2.1.21) activity were found in stratified apple seeds. Differences in their substrate specificity towards some phenolic β-glucosides were stated. Some characteristics of the enzymes showing the highest and the lowest specificities towards phloridzin are reported.

β-Glucosidases of apple tree seedlings were fractionated. In spite of the markedly pronounced specificity of leaves glucosidases towards phloridzin those enzymes were found to be different from the analogous enzymes from the apple seeds.

INTRODUCTION

PHLORIDZIN is the main phenolic glucoside of apple tissues. Recently, this glucoside and other phenolic compounds were suggested to play a role in the regulation of growth and development of the apple tree. For example, Grochowska[1] demonstrated a correlation between the phloridzin level in leaves and the formation of flower buds; Hancock *et al.*[2] found phloridzin acted as a growth inhibitor and this finding was confirmed by Sarapuu,[4, 5] Kamiński and Pieniążek[5] showed that phloridzin synergically stimulates germination of apple seeds in the presence of gibberellins ahd cytokinins; and Noveroske *et al.*[6, 7] postulated that the products of enzyme catalysed phloridzin oxidation participate in the apple tree defence mechanisms against fungal infection.

Hydrolysis of the β-glucoside bond is the first of the possible reactions which occur during transformation of phloridzin, although Raa and Overeem[8] consider it possible that hydroxylation of the glycone moiety occurs simultaneously. The presence of glucosidase activity in crude enzyme preparations from apple leaves has been reported from several laboratories,[3, 6, 8, 9] and the specificity of apple leaves β-glucosidase towards phloridzin noted.[9]

The glucosidases of apple seeds have not been studied until now, but the quantitative changes in various phenolic compounds during secondary ripening of apple seeds[10] suggested that they contain such enzymes specific towards various endogenous substrates. This work was aimed at the characterization of these enzymes and comparison of the glucosidases specific towards phloridzin present in seeds and leaves of the apple tree.

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RESULTS AND DISCUSSION

Apple seeds contain approximately 20 per cent of lipids which, together with the protein represent their reserve material. They contain also relatively large amounts of phenolic

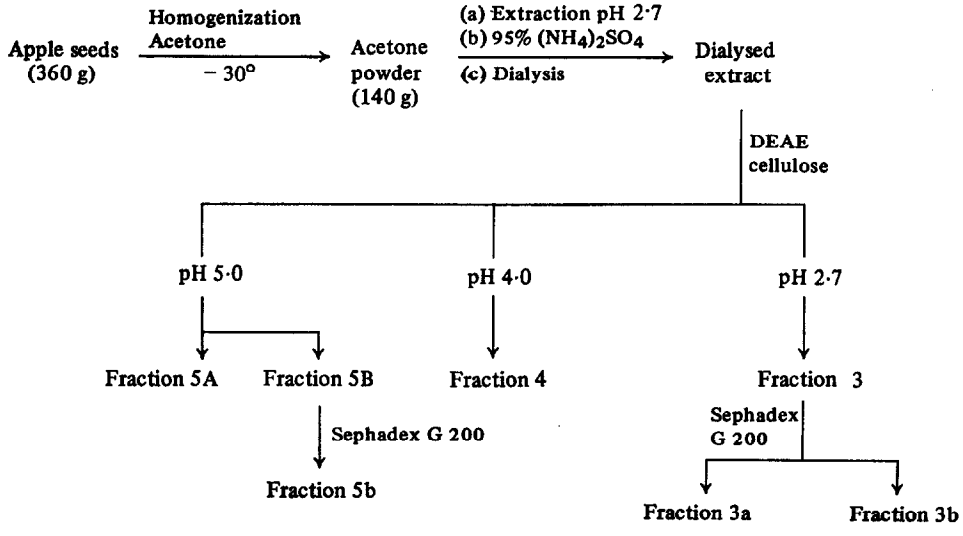

FIG. 1. FRACTIONATION OF APPLE SEED GLUCOSIDASES.

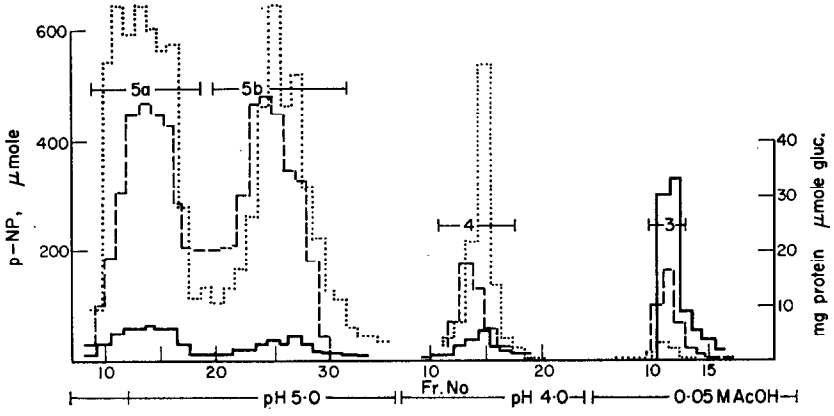

FIG. 2. SEPARATION OF STRATIFIED APPLE SEED GLUCOSIDASES BY DEAE–CELLULOSE CHROMATOGRAPHY.

The column was eluted consecutively with 0·05 M acetate buffers, pH 5·0, 4·0 and 0·05 M AcOH: —— activity towards phloridzin, μmoles of glucose. fraction/hr: ..... activity towards *p*-nitrophenyl β-glucoside, μmoles of *p*-nitrophenol/fraction/hr: ----- protein content, mg/fraction.

compounds and polysaccharides (up to 25 per cent of dry weight). The presence of these components has influenced the choice of methods applied for preparation and fractionation of the glucosidases from apple seeds. Figure 1 shows the scheme of preparation of enzymatic extract of apple seeds and of its fractionation.

Removal of lipids and part of phenolics by preparing an acetone powder made possible the subsequent use of aequous solutions. It was found that dilute acetic acid extracts contain

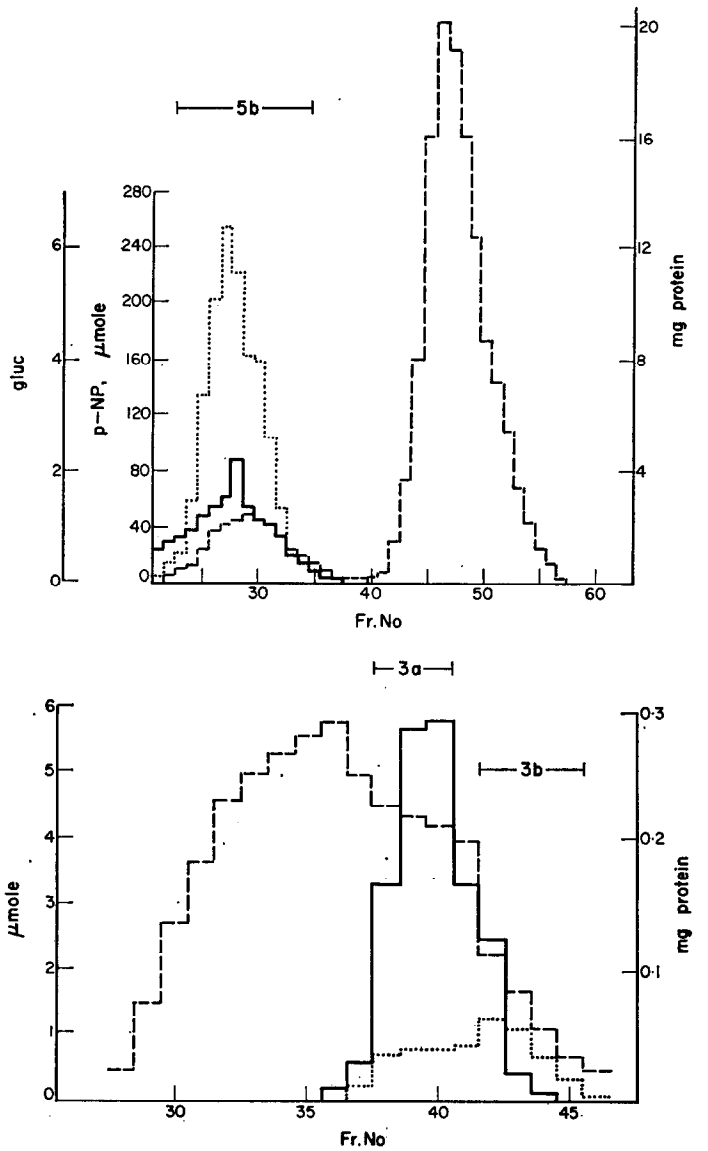

FIG. 3. SEPARATION OF FRACTION 5B (Fig. 3a) AND FRACTION 3 (Fig. 3b) BY SEPHADEX G-200 CHROMATOGRAPHY.

The columns were eluted with 0·05 M acetate buffer, pH 4·0. Activity towards: —— phloridzin, and ..... *p*-nitrophenyl β-glucoside as in Fig. 2: ---- protein content.

nearly the whole glucosidase activity from the acetone powder, whereas the reserve protein, which may constitute as much as 30 per cent of the seeds, was only slightly soluble under these conditions. Salting out and dialysis removed endogenous substrates of glucosidases and sugars which interfered with the determinations of enzyme activities.

Fractionation of the dialysed extract by DEAE–cellulose chromatography is shown in Fig. 2. Figures 3a and b show further fractionation of fractions 3 and 5B from the ion-exchange column by molecular sieving on Sephadex G-200.

The properties of the individual enzyme fractions are presented in Table 1. Since *p*-nitrophenyl β-glucoside is commonly used to determine the activity of non-specific β-glucosidases, the ratio of activities of the enzymes studied against phloridzin and *p*-nitrophenyl β-glucoside was taken as a criterion of purification. The data of Table 1, together with curves presented on Figs. 2 and 3, show that several different glucosidases are present in apple seeds.

Considerable losses (70 per cent) of total activity of the enzymes hydrolysing phloridzin accompany DEAE–cellulose chromatography. Nevertheless, this technique does give effective fractionation of the glucosidases. Two fractions 5A and 5B eluted from the column at pH 5 contain 37 per cent of the recovered total phloridzinase activity, but they do not

TABLE 1. FRACTIONATION OF APPLE SEED GLUCOSIDASES

Fraction*	Total protein content (mg)	Activity towards phloridzin: Total (μmoles/hr)	Activity towards phloridzin: Specific (μmoles/hrmg protein)	Ratio of activities towards phloridzin and *p*-nitrophenyl β-glucoside
Dialysed extract	850	493	0·57	0·02
Fraction 5A	176	42·2	0·24	0·007
Fraction 5B	212	26·4	0·12	0·008
Fraction 5b	26·9	27·7	1·03	0·009
Fraction 4	45·5	25·0	0·55	0·017
Fraction 3	30·9	88·1	2·85	0·80
Fraction 3a	0·56	15·6	27·8	7·56
Fraction 3b	0·75	15·0	20·0	1·82

* See Fig. 1.

contain the enzyme specific for phloridzin, as shown by the fact that their specific activity is lower than that of the dialysed extract towards this substrate. Further purification of fraction 5B on Sephadex G-200 gel (fraction 5A was not further purified because of its instability) gave a ten-fold increase in specific activity towards phloridzin. This treatment does not, however, cause any significant change in the ratio of the activities towards phloridzin and *p*-nitrophenyl glucoside (Table 1).

Among the fractions eluted from the DEAE–cellulose with more acidic buffers an enzymatic fraction most firmly bound to the support (fraction 3) is especially interesting. This fraction contains nearly 50 per cent of the recovered total phloridzinase activity. Its specific activity is over five-fold higher than that of the extract before chromatography, and its ratio of activities for phloridzin and *p*-nitrophenyl glucoside over forty-fold higher. Chromatographic separation of this fraction on Sephadex G-200 results in its further separation from both non-enzymatic protein (almost a ten-fold rise in specific activity) and other glucosidases (ten-fold increase of the ratio of activities for phloridzin and *p*-nitrophenyl glucoside in fraction 3a and two-fold in fraction 3b).

A comparison of the specific activities of the various enzyme fractions towards several

different phenolic glucosides provides further proof that apple seeds contain a number of enzymes with more or less distinct specificities for the aglycone moiety (Table 2).

The fraction most active for phloridzin (3a) shows the least activity with all the other glucosides tested. It appears that apple seeds also contain enzymes which catalyse the hydrolysis of both amygdalin, as suggested by Haisman *et al.*[11] and salicin. There are, however, distinct differences in the activities towards these two glucosides. Fraction 5b, which possesses the highest activity for all the substrates tested except phloridzin, is particularly interesting. Because of its high ability to catalyse the hydrolysis of *p*-nitrophenyl glucoside, this enzyme is considered to be a non-specific β-glucosidase.

The specificity of fraction 3 enzymes towards phloridzin and the non-specificity of those in fraction 5B was confirmed by the results of purification on Sephadex columns. The ratio of fraction 5B activities towards phloridzin and *p*-nitrophenyl glucoside does not change after chromatography on dextran gel and the low value of this ratio indicates a lack of specificity for phloridzin. The values of analogous ratios of activities for amygdalin and salicin against *p*-nitrophenyl glucoside indicate that the enzymes of fraction 5 are also non-specific for these natural substrates.

TABLE 2. SPECIFIC ACTIVITIES OF APPLE SEED GLUCOSIDASES TOWARDS SOME PHENOLIC GLUCOSIDES

Fraction	Substrate (specific activity (μmoles/hr mg protein))			
	Phloridzin	*p*-Nitrophenyl β-glucoside	Amygdalin	Salicin
5A	0·24	34·4	4·2	4·1
5b	1·03	112·5	16·8	8·7
4	0·55	32·2	10·8	8·8
3	2·85	3·5	9·0	1·6
3a	27·8	3·7	2·2	0
3b	20·0	11·1	22·4	0

On the other hand, purification of fraction 3 on Sephadex G-200 results in a ten-fold increase in the ratio of the phloridzin/*p*-nitrophenyl glucoside activities in fraction 3a and a simultaneous pronounced reduction in the analogous ratio for the other two natural glucosides. This demonstrates that the enzyme contained in fraction 3a has a high specificity for phloridzin. The Michaelis constants were determined for the enzymes in fractions 5b and 3a. The initial velocity was measured at 30°, pH 5·0, and the protein concentration was about 0·3 mg/ml. The K_m value obtained for the specific phloridzin glucosidase (3a) was $1{\cdot}4 \times 10^{-5}$ and that for the non-specific enzyme (5b) $5{\cdot}5 \times 10^{-3}$.

It should be noted that the specific phloridzin glucosidase appears in the last stages of stratification of the seeds while the non-specific glucosidase is probably present over the whole period of secondary ripening of the seed.

We decided to compare the specific phloridzin glucosidase from apple seeds with the glucosidases from the leaves of the same plant, since the latter enzymes had been reported earlier[9] to have a high specificity for phloridzin.

The apple leaves contain much less reserve protein than the seeds and apparently have a smaller number of different glucosidases. This meant that some steps, especially DEAE

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chromatography, in the separation procedure described earlier (Fig. 1) could be omitted. Separation of the dialysed extract by Sephadex G-200 gel chromatography (Fig. 4) gave three fractions with markedly distinct activities towards the phenolic glucosides studied (Table 3). The glucosidases from apple leaves did not hydrolyse salicin or amygdalin.

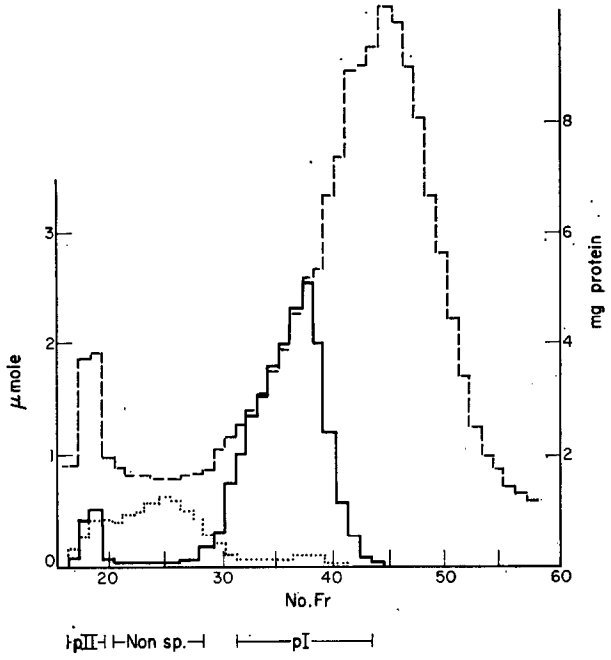

FIG. 4. SEPARATION OF APPLE LEAF GLUCOSIDASES BY SEPHADEX G-200 CHROMATOGRAPHY.

The column was eluted with 0·05 M acetate buffer, pH 4·0. Activity towards: —— phloridzin and ..... *p*-nitrophenyl glucoside as in Fig. 2: ---- protein content.

Two of the glucosidases from leaves show a pronounced specificity for phloridzin, but neither appears to be identical with the specific phloridzin glucosidase from stratified apple seeds. This is strongly indicated by considerable differences in the apparent molecular weights of these enzymes as determined by molecular sieving on Sephadex G-200 column calibrated with thyroglobulin, caeruloplasmin, human serum albumin, ovalbumin and cytochrome *c* (Table 4). Phloridzin glucosidase I from apple leaves, which represents 75 per cent of total glucosidase activity present in this tissue, shows a particularly high activity

TABLE 3. ACTIVITIES OF APPLE LEAF GLUCOSIDASES

Enzyme	Total activity (μmoles/hr 100 g leaves) towards		Ratio of activities towards phloridzin and *p*-nitrophenyl glucoside
	Phloridzin	*p*-Nitrophenyl β-glucoside	
Phloridzin glucosidase I	41·4	0·11	370
Phloridzin glucosidase II	2·4	1·6	1·5
Non-specific glucosidase	0·08	10·5	0·008

for phloridzin as against *p*-nitrophenyl glucoside (Table 3). Its molecular weight, however, is closest to that of enzyme 5b from the seeds which shows the least specificity.

The fact that in the seeds which are ready for germination or actually germinating the hydrolysis of phloridzin is catalysed by a different enzyme than that present in apple leaves may indicate that phloridzin plays different roles in the regulation of the processes of seed development and leaf growth. Phloridzin may stimulate seed development as suggested by Kamiński and Pieniążek,[5] but in leaves its role may be that of a growth inhibitor.[2, 12] The observations of Sarapuu[4] on the increase in phloridzin concentration in ageing leaves, together with our finding, confirmed by Raa and Overeem,[8] that the largest amounts of specific phloridzin glucosidase occur in young leaves, provide a support for the latter hypothesis.

TABLE 4. MOLECULAR WEIGHTS OF APPLE SEED AND LEAF GLUCOSIDASES

Material	Enzyme	$M \times 10^3$
Apple seeds	Specific phloridzin glucosidase (3a)	28
	Glucosidase 3b	22
	Non-specific glucosidase (5b)	39
Apple leaves	Phloridzin glucosidase I	41
	Phloridzin glucosidase II	270
	Non-specific glucosidase	108

EXPERIMENTAL

Material

Stratified seeds and leaves of apple seedlings, c.v Antonovka, were used throughout the experiments. The seeds, collected in 1966 and 1967, were obtained from the Experimental Station of the Institute of Pomology in Sinołęka.

The seeds were stratified for 90 days at 4° in sterile sand at 60% of full water capacity. The seeds were aerated and the water losses replaced at weekly intervals.

Apple seedlings were cultivated in soil under controlled temperature and light conditions. Day length, 12 hr, day temperature, 20°, night temperature, 15°. Light intensity, 5000 lux from fluorescent tubes. The leaves were harvested from 3-month-old seedlings, 15–20 cm high.

Analytical Methods

Protein was determined by the method of Lowry *et al.*[13] Relative protein concentration in eluates from chromatographic columns was estimated from absorbancy at 280 nm.

Glucosidase activity was determined by incubation of a fraction studied (0·05–0·5 ml) with 1 ml of 5 mM substrate solution in 0·05 M acetate buffer, pH 5·0. The incubations of samples, adjusted to 1·5 ml, were carried out at 30° for a defined period of time, depending on enzyme activity in the tested fraction (5–60 min). The amount of glucose liberated was determined by the method of Nelson.[14] Glucosidase activity towards *p*-nitrophenyl β-glucoside was determined by the method of Lederberg,[15] using 1 ml of 20 mM substrate solution in the same buffer. The amount of *p*-nitrophenol liberated was shown to be equivalent to the glucose estimated by Nelson's method.

Hydrolysis of amygdalin may liberate more than 1 molecule of reducing sugar, and some enzymes can also release hydrogen cyanide from this glucoside. These reactions can be a source of error in the assay of amygdalinase activity. Evaluation of such errors would require further studies on the structure of amygdalin degradation products. Our determinations of amygdalinase activity are thus of only relative value.

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Fractionation of Glucosidases from Apple Seeds

Stratified apple seeds (360 g) were homogenized in acetone (400 ml) at −30° using a Type 309 homogenizer (Unipan, Warszawa) for 5 min. The precipitate was filtered, and further homogenized 3 × with the same volume of cold acetone. The final precipitate (140 g) was dried in a vacuum desiccator over KOH and stored at 0°.

The acetone powder (140 g) was extracted at 0° with 3 × 280-ml portions of 0·05 M acetic acid with vigorous stirring for 60 min. The combined extracts were treated with solid $(NH_4)_2SO_4$ to 95% saturation, and precipitate formed centrifuged and washed 2 × with cold $(NH_4)_2SO_4$. The precipitate was then dissolved in 0·05 M acetate buffer, pH 5·0 (150 ml), and dialysed against 3 l. of 0·005 M buffer of the same pH at 5° for 24 hr. The dialysate, centrifuged to remove small amounts of precipitate formed, was freeze-concentrated to a final volume of 15 ml.

Twelve ml of this preparation were applied to a DEAE–cellulose column (320 × 17 mm) equilibrated with 0·05 M acetate buffer, pH 5·0. The column was washed consecutively with 0·05 M acetate buffer, pH 5·0 (250 ml); pH 4·0 (100 ml) and with 0·05 M acetic acid (100 ml). 5-ml fractions were collected with a Type 301 B (Unipan, Warszawa) fraction collector. Protein content and individual enzyme activities were determined in these fractions.

Selected fractions from the pH 5·0 and 0·05 M acetic acid eluates (Fig. 2) were combined and dialysed against water at 0° for 12 hr. The pH 5·0 fraction was centrifuged to separate small amounts of precipitate and freeze-concentrated to reduce the volume to 2 ml. The precipitate formed from the 0·05 M acetic acid fractions was dissolved in 2 ml 0·05 M acetate buffer, pH 4·0. These enzyme preparations were separated on a Sephadex G-200 column (730 × 17 mm) equilibrated with 0·05 M acetate buffer, pH 4·0, elution being carried out by the ascending technique using the same buffer. Three ml (3a and 3b) or 4-ml (5b) fractions were collected (Fig. 3).

Fractionation of Glucosidases of Apple Seedlings Leaves

Fresh apple leaves (44 g) were homogenized in 0·05 M acetate buffer, pH 5·0 (420 ml), at 0–5° for 5 min. The homogenate was filtered and the filtrate added dropwise to vigorously stirred acetone (1·4 l.) at −30°. After 30 min the precipitate formed was centrifuged, washed with 3 × 20-ml portions of cold acetone and dried in vaccum desiccator over KOH. Yield 600 mg.

The acetone powder (600 mg) was dissolved in 5 ml of 0·05 M acetate buffer, pH 5·0, and dialysed against water at 5° for 12 hr. The dialysate was freeze-dried to reduce volume to 2 ml and the concentrated preparation was subjected to molecular sieving on Sephadex G-200 gel as described above (Fig. 4).

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