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SHORT COMMUNICATION

# PARTIAL PURIFICATION AND CHARACTERIZATION OF A FLAVONOID-3-β-D-GLUCOSIDASE FROM PETALS OF *IMPATIENS BALSAMINA**

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**Abstract**—A flavonoid-3-β-D-glucosidase preparation from petals of *Impatiens balsamina* was partially purified and characterized. The glucosidase showed a strict requirement for an aromatic aglycone and a single β-linked glycoside. Two pH optima were found at 3·5 and 5·6. When the preparation was incubated with pelargonidin-3-monoglucoside, hydrolysis of the glucoside was followed by a spontaneous decomposition of the aglucone nucleus. Both glucosidase and galactosidase activities were detected.

## INTRODUCTION

DURING a series of earlier studies on the differentiation of pigmentation in flowers of *Impatiens balsamina*, the existence of flavonoid-β-D-glucosidase activity was demonstrated in both the colored and white genotypes.[1,2] In the present paper, we report the partial purification and characterization of a flavonoid-β-glucosidase isolated from the petals of *I. balsamina* although similar activity can be detected in other portions of the plant.[3]

## RESULTS AND DISCUSSION

### *Properties of the Flavonoid-3-β-D-glucosidase Preparations*

(1) Acetone powders yielded extracts with high activity which could be chromatographed and further purified on DEAE-Sephadex (A-50). The preparations were stable during extraction and were unaffected by the presence of large amounts of endogenous phenolic compounds, which can greatly affect the isolation of active enzymes from many plant sources.[4–6] Extracts of the enzyme could be chromatographed or allowed to stand at room

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temperature with little or no loss of activity. Chromatography revealed several different 280-nm absorbing fractions (protein) but the bulk of the glucosidase activity was located in a single peak beginning at approximately 0·25 M NaCl. The preparations were unaffected by repeated freezing and thawing. Ionic strengths of up to 0·4 M NaCl in 0·1 M acetate buffer (pH 7·6), repeated dialysis, or concentrations of EDTA up to 0·1 M did not affect the stability or activity of the preparations.

(2) pH activity. There are two definite peaks at pH 3·5 and 5·6. Activity cannot be detected below pH 2·5 or above 8·5. It remains to be determined whether the loss of activity below pH 2·5 and above 8·5 is reversible but it is likely that at least part of the decrease in activity below 3·5 may be attributed to denaturation.

*Decolorization of Pelargonidin-3-monoglucoside*

Cell-free hydrolysis experiments with pelargonidin-3-monoglucoside have revealed a remarkable similarity between these preparations and those from *Aspergillus* previously described by Huang.[7–9] Huang reported a slight lag in color loss during the first 10 min followed by a rapid loss of color until a residual level of absorbance remained constant. It can be seen in Fig. 1 that our preparations also show a slight lag in loss of absorbance for the first 10 min followed by a rapid decline until approximately 25–30 per cent of the original absorbance remains. Chromatographic analysis of 100-$\mu$l aliquots of the 3 $\mu$M reaction mixture revealed that during the first 40 min both substrate and aglucone can be observed. There is a progressive loss of substrate which finally becomes indetectable. The aglucone accumulates apparently until a maximal level is reached. This probably accounts for the 20–30 per cent of residual absorbancy which is observed in each reaction mixture.

*Hydrolysis of a Variety of Glycosides*

The specificity of this preparation has been examined on the basis of general group specificities (Table 1). There is apparently a strict requirement for an aromatic ring system attached to the sugar moiety, since little or no activity was observed with substrates lacking this group. The preparations were also specific for the $\beta$-linkage, but showed activity toward both glucose and galactose residues; consistently it was observed that there was a 5–7 to 1 ratio of glucosidase to galactosidase.

*Activity of Preparations against other Flavonoid Glucosides*

Besides the purified pelargonidin-3-monoglucoside tested above, we attempted to detect glucosidase activity using other flavonoid glucosides. In these studies, rates were not analyzed due to a lack of available substrate. The reaction mixtures were analyzed chromatographically according to the methods of Hagen.[10] The determining factor of these studies was whether a decrease in substrate and the appearance of aglucone could be detected. Consistently, it was observed that only substrates which had a single glucoside at the 3-position were hydrolyzed (kaempferol, malvidin, and pelargonidin-3-monoglucosides). A substitution at the 5-position as well as the 3-position or a 3-diglucoside were not susceptible to hydrolysis (pelargonidin-3,5-diglucoside and kaempferol-3-diglucoside). Harborne and Sherratt[11] (see also Harborne[12]) have examined the specificity of the fungal anthocyanase and found this preparation

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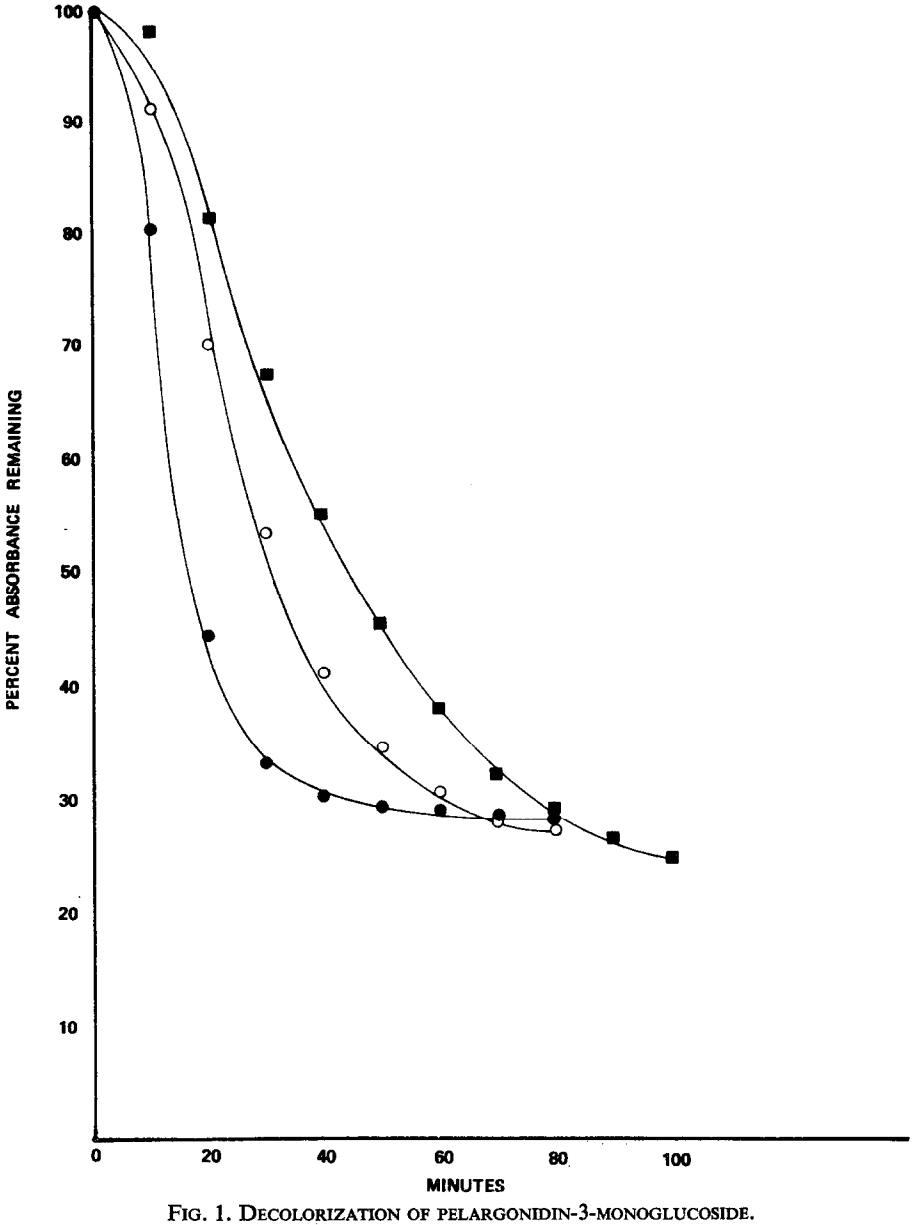


FIG. 1. DECOLORIZATION OF PELARGONIDIN-3-MONOGLUCOSIDE.

○ 1 μM substrate;
● 2 μM substrate;
■ 3 μM substrate.
(See Experimental for reaction mixture.)

most active toward anthocyanindin-3-glucosides, 3-diglucosides, 3-galactosides, and 3,5-diglucosides. Lesser activity was shown toward 3-rhamnoglucosides, and acylated-3,5-diglycosides. Thus it appears that our preparations possess activity similar to that of *Apsergillus* but exhibit a more narrow specificity range.

TABLE 1. RATES OF HYDROLYSIS OF VARIOUS GLYCOSIDES

| Substrate* | Hydrolysis ($\mu$M/min/ml) |
|---|---|
| Salicin† | 0·077 |
| Arbutin† | 0·111 |
| *p*-Nitrophenyl-$\beta$-D-glucopyranoside | 0·180 |
| *p*-Nitrophenyl-$\alpha$-D-glucopyranoside | 0·001 |
| *p*-Nitrophenyl-$\beta$-D-galactopyranoside | 0·044 |
| *p*-Nitrophenyl-$\alpha$-D-galactopyranoside | 0·001 |
| $\beta$-Methyl glucoside† | 0·002 |
| $\alpha$-Methyl glucoside† | 0·001 |
| Cellobiose† | 0·002 |
| Pelargonidin-3-monoglucoside‡ | 0·077 |

* 0·9 ml of 5 mM substrate in 0·025 M citrate–phosphate buffer, pH 5·5; 0·1 ml of enzyme; 25°.

† Released glucose was determined by the Somogyi–Nelson method after 30 min.

‡ Values calculated from linear portion of Fig. 1. See Experimental for reaction mixture.

From the data presented in this paper and the evidence presented by Hagen[1] and Miles and Hagen,[13] it appears evident that the aryl-$\beta$-D-glucosidase activity of balsam petals is only hydrolytic. Because *in vitro* glucose transfer reactions, which are a normal part of endogenous pigment biosynthesis, are actually enhanced by the inhibition of the glucosidase activity,[13] it seems apparent that this hydrolytic activity is either inhibited or in some way separated from the 3-monoglucosides and other key intermediates involved in the biosynthesis of more highly elaborated anthocyanin and flavonol derivatives. The character of this activity suggests that it may be compartmentalized during normal development.

## EXPERIMENTAL

### *Enzyme Preparation*

Mature fully open flower petals of the white genotype (11hhpp)[10] were homogenized in cold acetone, filtered, and the residue air-dried at room temperature. 1 g of the acetone powder was homogenized in the cold with 150 ml of 0·01 M phosphate buffer, pH 7·6. The homogenate was dialyzed against phosphate buffer for 18 hr then centrifuged at 30,000 × *g* for 20 min. The pellet was extracted twice more with 150 ml of buffer after which the supernatants were combined. The resulting supernatant was dialyzed twice against 4 l. of 0·005 M Tris-HCl buffer, pH 7·6, and slurried into a DEAE-Sephadex (A-50) column. The column was washed with buffer before the sample was applied. After the entire sample was applied the column was washed with buffer before starting a 600 ml gradient elution with sodium chloride in the Tris-HCl buffer, pH 7·6. The gradient was from zero to 0·4 M NaCl; the flow rate was 0·5 ml per min and 5-ml fractions were collected. Enzyme activity was assayed in every third tube by the *p*-nitrophenyl-$\beta$-D-glucoside reaction described below and absorbance at 280 nm was measured as an index of protein concentration.

### *Enzyme Assay*

For the Sephadex chromatography and the pH studies the preparations were assayed with *p*-nitrophenyl-$\beta$-D-glucoside. A 0·1 ml aliquot of the enzyme was incubated with 0·1 ml of 0·01 M substrate and 0·8 ml of citrate-phosphate buffer, pH 5·5, at 25° for 20 min. The reaction was terminated by the addition of 2 ml of 1 M $Na_2CO_3$ and the liberated *p*-nitrophenol was read at 400 nm in a Beckman DU spectrophotometer.

### *Substrate Preparation*

Pelargonidin-3-monoglucoside was prepared from fresh strawberries; the flavonoid glucosides were purified from the appropriate genotype of *Impatiens balsamina*;[14] by the method described by Mansell and Hagen.[2] All other substrates were purchased as reagent grade chemicals.

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*Decolorization of Pelargonidin-3-monoglucoside*

Purified substrate was first dissolved in 0·01 per cent HCl and then diluted to the desired concentration with 0·025 M citrate–phosphate buffer, pH 3·4. The enzyme preparation was also dialyzed against this buffer. The reaction mixtures contained 1·0 ml of substrate, 0·5 ml of enzyme, and 2·5 ml of buffer. The final pH was 3·4 and the reactions were carried on at room temperature in a Beckman DB-G recording spectrophotometer. The starting OD of each reaction mixture at 497 nm was: 1 $\mu$M − 0·274; 2 $\mu$M − 0·429; and 3 $\mu$M − 0·638. The hydrolysis rate from Table 1 was calculated from $r = \Delta C/[(\text{ENZ})\Delta t]$ where $C$ was determined spectrophotometerically.

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