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β-GLUCOSIDASE WITH GIBBERELLIN A_8-2-*O*-GLUCOSIDE HYDROLYSING ACTIVITY FROM PODS OF RUNNER BEANS

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Key Word Index—*Phaseolus coccineus*; Leguminosae; runner bean; [^{3}H]gibberellin A_8-2-*O*-glucoside; β-glucosidase; localization.

Abstract—In enzyme extracts from immature fruits of *Phaseolus coccineus*, a β-glucosidase with high hydrolysing activity toward the endogenously occurring GA_8-2-*O*-β-D-glucopyranoside was detected and found to be predominantly localized in the pod in the form of a soluble enzyme. The GA_8-2-*O*-Glc hydrolase (pH-optimum: 5.0) could be separated from two other soluble β-glucosidases with unknown specificities and enriched 650-fold. Studying this activity during pod development a decrease in the later stages was observed. This enzyme may catalyse an important step of GA_8 glucose conjugate catabolism.

INTRODUCTION

The immature seeds of *Phaseolus* spp. are rich sources of many different GAs and in runner beans (*P. coccineus*) there are at least 15 GAs which either occur endogenously or are synthesized by cell free systems from immature seeds [1–3]. Besides the free GAs, glucose conjugates of GA_1, GA_8, GA_{17}, GA_{20} and GA_{28} are found in the same material [2, 4], from which GA_8-2-*O*-Glc, the main compound, was first isolated and characterized [5]. Neither the enzymic formation of GA_8-2-*O*-Glc *in vitro* [6, 7] nor its hydrolysis by enzyme extracts from immature fruits of *P. coccineus* [8] has been reported.

In our previous investigation of the enzymes from immature pods of *P. coccineus*, two different β-glucosidases were separated by DEAE-Sephadex A-50 chromatography but could not further be characterized for lack of suitable substrates [9]. The metabolism of [^{3}H]GA_1 in maturing runner been fruits gave rise to [^{3}H]GA_8-2-*O*-Glc [12] which was used as a substrate in enzymic studies.

The present paper reports on the detection, distribution and partial characterization of a β-glucosidase (EC 3.2.1.21) with GA_8-2-*O*-Glc hydrolysing activity from fruits of *P. coccineus*.

RESULTS AND DISCUSSION

[^{3}H]GA_8-2-*O*-Glc hydrolase activity was detected in enzyme extracts from runner beah fruits (Table 1). Most of the activity was found in the pods and exhibited a pH-optimum at pH 5.0 (half maximal activity at pH 3.9 and 5.9). The soluble enzyme fraction showed a *ca* 20-fold higher specificity quotient (see Experimental) than the membrane-associated fraction solubilized with 1 M NaCl (Table 1).

The separation of the soluble enzymes from pods on CM-Sephadex C-50 (Fig. 1A) led to three different β-glucosidase fractions (CM 1–CM 3) which were concentrated by ammonium sulphate precipitation and then tested for [^{3}H]GA_8-2-*O*-Glc hydrolase activity. The

Table 1. Distribution of [^{3}H]GA_8-2-*O*-Glc hydrolysing activity in immature fruits (25 cm long, field-grown) of *P. coccineus* (dialysed ammonium sulphate concentrates)

	[^{3}H]GA_8-2-*O*-Glc hydrolase			β-Glucosidase (pNP-β-Glc)	
Enzyme fraction	Hydrolysis rate (Bq/mg protein/hr)	Specific activity (pkat/mg protein)	Specificity quotient × 10^3	Activity (nkat/ml)	Specific activity (nkat/mg protein)
Buffer–1 M NaCl extracts of seeds without testa	0.27 ± 0.02*	0.001	0.004	1.21	0.184
Testa	0	0	0	0.001	0.018
Pods	67.9 ± 0.2	0.207	0.71	0.66	0.291
Pods					
Soluble enzymes	100.7 ± 21.1	0.307	7.32	0.108	0.042
Membrane-associated enzymes (solubilized)	37.2 ± 1.2	0.114	0.37	0.586	0.310

*Mean ± S.E.M (2).

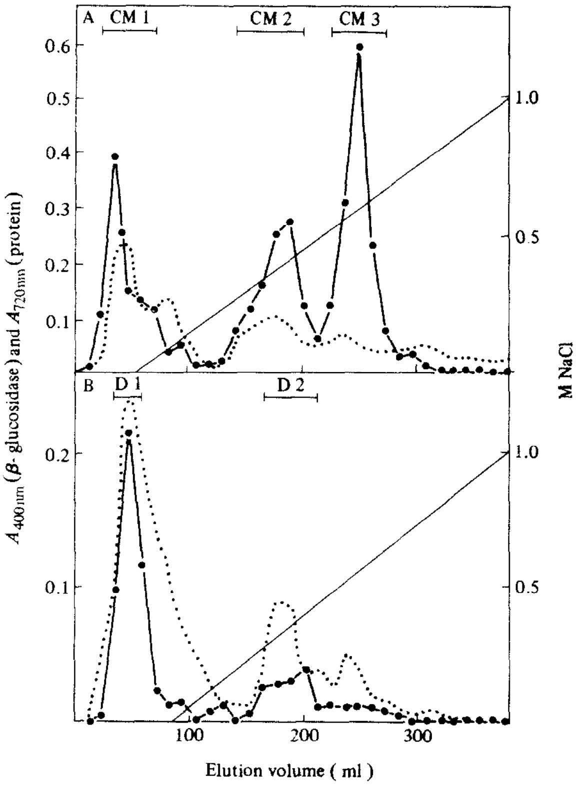

Fig. 1. Chromatography of soluble β-glucosidases from pods of immature *P. coccineus* fruits. A, CM-Sephadex C-50; B, DEAE-Sephadex A-50. ●——●, β-glucosidase; . . . ., protein; ——, NaCl gradient; Incubation time: A, 15,5 hr; B, 3 hr (pNP-β-Glc).

results (Table 2) revealed that nearly all of the activity was concentrated in fraction CM 1 which was not retained on the column. On DEAE-Sephadex A-50 (Fig. 1B) chromatography of the soluble enzymes the β-glucosidases, CM 2 and CM 3, left the column first (D 1) wheareas the β-glucosidase (D 2) with pronounced activity toward [^{3}H]GA$_8$-2-*O*-Glc was eluted with the NaCl gradient. The β-glucosidase fraction D 2 exhibited a 650-fold higher specific activity with [^{3}H]GA$_8$-2-*O*-Glc as substrate than the crude extract. The specific activity of the β-glucosidase fraction D 2 towards [H]GA$_8$-2-*O*-Glc was found to be one-fifth of that towards *p*-nitrophenyl-β-D-glucopyranoside (*pNP*-β-Glc), although the latter was used in 19-fold higher concentration.

Further evidence for the occurrence of a β-glucosidase with specificity towards GA$_8$-2-*O*-Glc was provided by the observation that this activity in the soluble crude extract was apparently inhibited by endogenously occurring compounds. Thus dialysis of the crude extract led to a *ca* 30-fold increase of the specific activity towards [^{3}H]GA$_8$-2-*O*-Glc whereas the specific activity towards *p*NP-β-Glc was raised only 2.5-fold (Table 2).

The membrane-associated enzymes after solubilization were separated in the same way. Only by prolongated incubation of the column eluates with *p*NP-β-Glc was a minor β-glucosidase fraction which showed [^{3}H]GA$_8$-2-*O*-Glc hydrolysing activity detectable whereas the major β-glucosidase fraction was almost inactive toward this substrate (data not shown). As the minor β-glucosidase had the same elution behaviour as the corresponding highly active soluble enzyme, it was presumed that this activity was due to cross-contamination of the membrane associated enzymes by traces of soluble ones or that only a small part of the [^{3}H]GA$_8$-2-*O*-Glc hydrolysing activity is membrane-associated.

As we were interested in the physiological function of the [^{3}H]GA$_8$-2-*O*-Glc hydrolyase during fruit development, the *p*NP-β-Glc and [^{3}H]GA$_8$-2-*O*-Glc hydrolysing activities of enzyme extracts from pods of immature fruits of different size (5–25 cm) and mature yellow turgorless fruits (designated 25T) were tested (Fig. 2). The *p*NP-β-Glc hydrolysing activity per pod increased during fruit development whereas the [^{3}H]GA$_8$-2-*O*-Glc hydrolase activity of the pods was found in two independent

Table 2. Purification, separation and partial characterization of soluble β-glucosidases from pods of immature *P. coccineus* fruits (25 cm long, field-grown)

	[^{3}H]GA$_8$-2-*O*-Glc hydrolase			β-Glucosidase (*p*NP-β-Glc)	
Enzyme fraction	Hydrolysis rate (Bq/mg protein/hr)	Specific activity (pkat/mg protein)	Specificity quotient × 10^3	Activity (nkat/ml)	Specific activity (nkat/mg protein)
Crude extract	0.66 ± 0.34*	0.002	0.06	0.051	0.031
Dialysed ammonium sulphate concentrate	108.8 ± 4.5	0.332	7.1	0.128	0.047
CM-Sephadex C-50 fraction					
CM 1	478.4 ± 0.3	1.460	66.4	0.033	0.022
CM 2	3.8 ± 1.2	0.012	0.2	0.052	0.057
CM 3	0	0	0	0.029	0.180
Crude extract	1.26 ± 0.32	0.004	0.5	0.014	0.007
Dialysed crude extract	38.2 ± 0.9	0.117	6.5	0.012	0.018
Dialysed ammonium sulphate concentrate	112.6 ± 2.8	0.344	12.7	0.090	0.027
DEAE-Sephadex A-50 fraction					
D 1	6.7 ± 0.6	0.020	0.4	0.159	0.048
D 2	851 ± 39	2.598	199.8	0.009	0.013

*Mean ± S.E.M. (2)

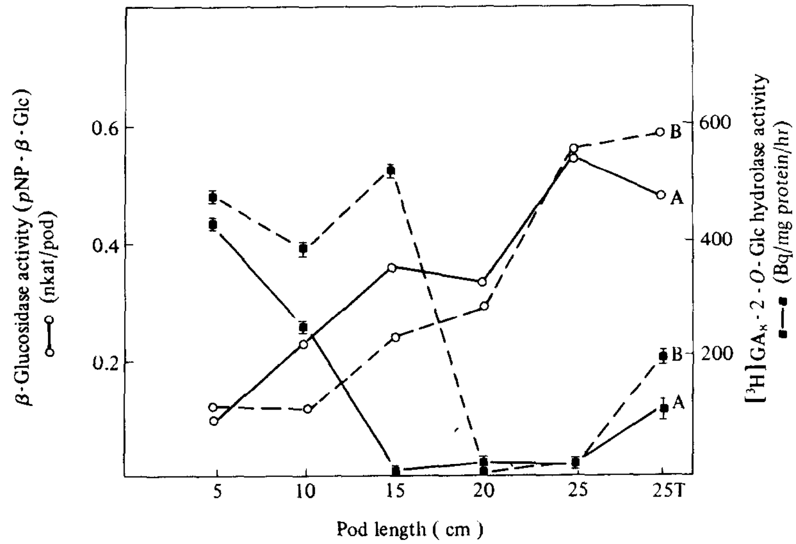

Fig. 2. Time courses of β-glucosidase and [^{3}H]GA_8-2-*O*-Glc hydrolase activities during fruit development. ——, Experiment 1; - - - -, Experiment 2.

experiments to be high at the beginning of the fruit development and relatively low in later stages (20 and 25 cm). When the fruit became yellow and turgorless (25T), a remarkable increase of the [^{3}H]GA_8-2-*O*-Glc hydrolase activity was observed.

Summarizing our results, it can be stated that a β-glucosidase with high activity toward the endogenously occurring GA_8-2-*O*-Glc was detected in immature fruits of runner beans for the first time. Furthermore, this enzymic activity was found to occur predominantly in the pods in the form of a soluble enzyme. The detection of the [^{3}H]GA_8-2-*O*-Glc hydrolysing activity in only one of the three soluble β-glucosidases (Fig. 1, Table 2), the relatively high rate of hydrolysis of the substrate and the 30-fold increase in its activity after dialysis of the soluble crude enzyme extract (Table 2) are strong indications of its specificity toward GA_8-2-*O*-Glc, although determination of kinetic constants and testing of substrate specificity was not possible for lack of other endogenously occurring GA-*O*-Glcs in radiolabelled form. The decrease of the [^{3}H]GA_8-2-*O*-Glc hydrolysing activity at later stages of fruit development (Fig. 2) may be correlated with the increase in GA glucosylating activity in the same tissue [6, 7].

On testing the glucosylating activity of different enzyme extracts from pods and testas with [^{3}H]GA_8 and [^{3}H]GA_3 in the presence of UDPG at pH 8.1 according to [6], only the soluble fraction of pods (50–80% ammonium sulphate precipitation) showed low [^{3}H]GA_3 glucosyltransferase activity whereas [^{3}H]GA_8 was not transformed (data not shown). The physiological function of the GA_8-2-*O*-Glc hydrolase cannot be to liberate a biologically active GA as GA_8 itself is a deactivated GA, but this enzyme may catalyse an important step of GA_8 glucose conjugate catabolism finally leading to the formation of a GA_8 catabolite.

EXPERIMENTAL

Plant material. Immature fruits (20–25 cm) from field-grown runner beans (*Phaseolus coccineus* L. cv 'Prizewinner') were harvested and stored at $-20°$. To study the time course of the changes in β-glucosidase activity during fruit development pods of different size (5–25 cm) were selected.

Preparation and separation of enzyme extracts. To investigate the distribution of the [^{3}H]GA_8-2-*O*-Glc hydrolysing activity in the fruits, the pods, testas, and embryonic tissues (mainly cotyledons) were separated and homogenized in McIlvaine buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 5.0, with addition of 1 M NaCl). After 1 hr the homogenates were centrifuged for 20 min at 20000 g. The supernatants were concentrated by $(NH_4)_2SO_4$ pptn (90% saturation) and after dissolving the ppt. in McIlvaine buffer (pH 5.0, 1 + 3 diluted) dialysed for 16 hr toward the same buffer. This procedure was also used in the time course experiment. For the separation of the soluble and the membrane-associated enzymes, pods were homogenized in McIlvaine buffer (pH 5.0, 1 + 3 diluted). After centrifugation (20 min, 20000 *g*), the particulate fraction was resuspended (× 3) with the same buffer and the washings added to the first supernatant. The combined supernatants were concentrated as described above.

For the solubilization of the membrane-associated enzymes, the particulate fraction was treated with McIlvain buffer (pH 5.0, 1 M NaCl) for 1 hr and the solubilized proteins concd as above. The dialysed enzyme fractions were directly used for chromatography on CM-Sephadex C-50 and DEAE-Sephadex A-50 columns (400 × 15 mm) developed with a linear gradient of NaCl (0–1 M) in McIlvain buffer (pH 5.0, 1 + 3 diluted). Fractions of 12 ml each were collected. All operations were carried out at 4°.

Determination of protein and enzyme activity. The determination of the protein content and the β-glucosidase activity using the chromogenic *p*NP-β-Glc (2 mM at pH 5.0) were performed as previously described [10, 11]. [^{3}H]GA_8-2-*O*-Glc (91 MBq/mMol, 666Bq/incubation) [12] was incubated with 70 μl enzyme soln (pH 5.0) at 37° for such a time that the hydrolysis did not exceed 20%. To stop the reaction the whole incubation mixture was strip-loaded (1 cm) on to a silica gel TLC plate (Kieselgel 60 G, Merck. 1 mm) which, after drying, was developed with the solvent system $CHCl_3$–MeOH–HOAc–H_2O (40:10:2:1). The hydrolysis of [^{3}H]GA_8-2-*O*-Glc was determined by measuring the radioactivity (scintillation counting) present at the R_f values coincident with authentic [^{3}H]GA_8 and

[^{3}H]GA_8-2-*O*-Glc. All data were corrected by measurements of control incubations carried out in the absence of added enzyme. The percentages of hydrolysis of [^{3}H]GA_8-2-*O*-Glc were converted into hydrolysis rates (liberated [^{3}H]GA_8 in Bq/mg protein $\times$ hr). By using the sp. radioact. of [^{3}H]GA_8-2-*O*-Glc the hydrolysis rates were converted into sp. act. (pkat/mg protein).

To compare the hydrolysis of [^{3}H]GA_8-2-*O*-Glc and *p*NP-β-Glc by the β-glucosidases we determined a specificity

$$\text{quotient: } \frac{\text{sp. act. ([}^{3}\text{H]GA}_8\text{-2-}O\text{-Glc)}}{\text{sp. act. (}p\text{NP-}\beta\text{-Glc)}}$$

The GA glucosylating activity was tested according to the method of ref. [6].

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REFERENCES

1. Sponsel, V. M. (1983) in *The Biochemistry and Physiology of Gibberellins* (Crozier, A., ed.) Vol. 1, p. 151. Praeger, New York.
2. Albone, K. S., Gaskin, P., MacMillan, J. and Sponsel, V. M. (1984) *Planta* **162**, 560.
3. Turnbull, C. G. N., Crozier, A., Schwenen, L. and Graebe, J. E. (1985) *Planta* **165**, 108.
4. Gaskin, P. and MacMillan, J. (1975) *Phytochemistry* **14**, 1575.
5. Schreiber, K., Weiland, J. and Sembdner, G. (1970) *Phytochemistry* **9**, 189.
6. Knöfel, H.-D., Schwarzkopf, E., Müller, P. and Sembdner, G. (1984) *J. Plant Growth Regul.* **3**, 127.
7. Sembdner, G., Knöfel, H.-D., Schwarzkopf, E. and Liebisch, H. W. (1985) *Biol. Plant.* **27**, 231.
8. Müller, P., Knöfel, H.-D., Liebisch, H.-W., Miersch, O. and Sembdner, G. (1978) *Biochem. Physiol. Pflanzen* **173**, 396.
9. Schliemann, W. (1980) *Biochem. Physiol. Pflanzen* **175**, 354.
10. Schliemann, W. (1982) *Biochem. Physiol. Pflanzen* **177**, 61.
11. Schliemann, W. (1984) *J. Plant Physiol.* **116**, 123.
12. Schliemann, W. (1987) *Biochem. Physiol. Pflanzen* **182**, 153.