Phytochemistry, 1980, Vol. 19, pp. 1349–1353. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0701-1349 $02.00/0

CHARACTERIZATION OF A β-GLUCOSIDASE FROM *GLYCINE MAX* WHICH HYDROLYSES CONIFERIN AND SYRINGIN

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(*Received* 31 *October* 1979)

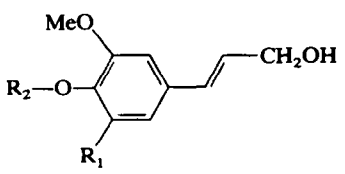
Key Word Index—*Glycine max*; Leguminosae; soybean; biosynthesis, lignin; β-glucosidases; coniferin; syringin.

Abstract—A β-glucosidase which rapidly hydrolyses the cinnamyl alcohol glucosides coniferin and syringin has been purified from cell cultures, hypocotyls and roots of *Glycine max*. Isoelectric focusing in a column separated the enzyme from several other β-glucosidases which were inactive against either substrate. Syringin and coniferin were the best substrates tested. Both exhibited identical V_{max} values, whereas the K_m of coniferin (0.6 mM) was twice that of syringin (0.3 mM). The widely used synthetic substrates 4-nitrophenyl-β-glucoside and 4-methyl-umbelliferyl-β-glucoside were poorly utilized. Glucono-1,5-lactone was an effective competitive inhibitor with a K_i of 0.01 mM. From the observed substrate specificity, a role in the lignification process of higher plants may be predicted for this β-glucosidase.

INTRODUCTION

The complex structure of lignin, one of the most abundant polymeric substances in higher plants, was elucidated in early investigations by Freudenberg and associates [1]. These studies showed that the lignin of gymnosperms is mainly composed of coniferyl alcohol units (Fig. 1) whereas that of dicotyledonous plants contains sinapyl alcohol monomers in addition to coniferyl alcohol. In more recent years nearly all of the enzymes involved in the biosynthesis of lignin have been characterized. These studies provide a clear picture of the synthesis of coniferyl and sinayl alcohol, the immediate monomeric precursors of lignin [2, 3]. The polymerization of these cinnamyl alcohols to form the lignin macromolecule is probably catalysed by peroxidase [4].

Several problems remain, however, in the field of lignification. One of these is the role of the cinnamyl alcohol glucosides coniferin and syringin (Fig. 1), which have been identified in several higher plants [5].



$R_1 = R_2 = H$	coniferyl alcohol
$R_1 = H$; R_2 = glucose	coniferin
R_1 = OMe; $R_2 = H$	sinapyl alcohol
R_1 = OMe; R_2 = glucose	syringin

Fig. 1. Structures of cinnamyl alcohols and cinnamyl alcohol glucosides.

In addition, a β-glucosidase activity has been demonstrated in lignifying tissues of various plants by means of histochemical staining with the chromogenic substrate indoxyl-β-glucoside [6, 7]. Some workers have therefore suggested that the cinnamyl alcohol glucosides are involved in lignification [1], but that view is not universally accepted (cf. [8]).

Very recently β-glucosidases specific for coniferin have been purified and characterized for the first time from the gymnosperm *Picea abies* [9] and from cell cultures of the angiosperm *Cicer arietinum* [10]. However, syringin was a poor substrate for this β-glucosidase in *C. arietinum*, despite the fact that the lignified fibers of dicotyledonous plants show a high content of sinapyl alcohol monomers in addition to coniferyl alcohol [11]. If syringin is involved in the lignification of dicotyledonous plants, there should exist in dicots a β-glucosidase with much greater activity towards syringin than the one demonstrated in *C. arietinum*. We report here the isolation and characterization of a β-glucosidase from *Glycine max* cell cultures and intact plants which shows a distinct specificity for both coniferin and syringin.

RESULTS

Purification of β-glucosidase

The proteins extracted from *G. max* cell suspension cultures 8 days after inoculation were precipitated by addition of solid $(NH_4)_2SO_4$ to 70% saturation, and then desalted on a Sephadex G-25 column. A first separation of β-glucosidase activities was then obtained by chromatography on a CM-Sephadex C-50 column (Fig. 2).

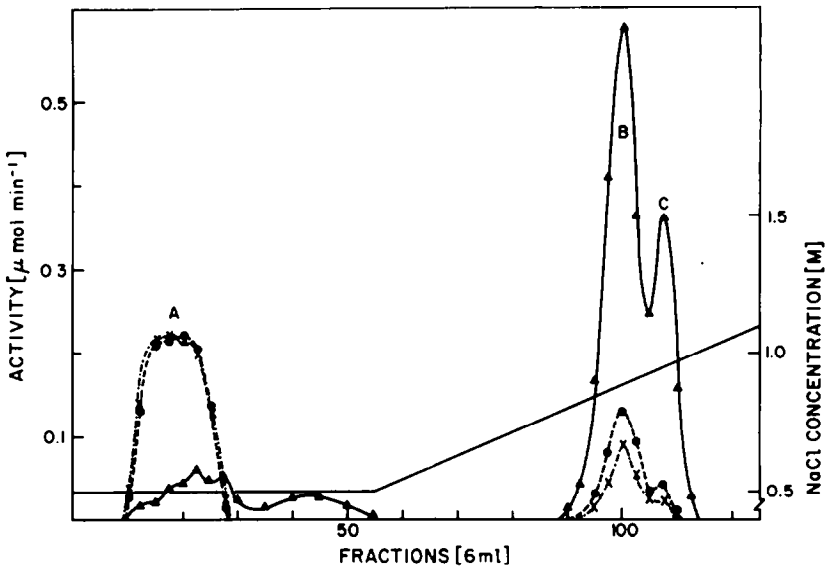


Fig. 2. Separation of β-glucosidases from *Glycine max* cell suspension cultures by chromatography on a CM Sephadex C-50 column. ●-----● Coniferin, X-·-·-X syringin, ▲———▲ 4-nitrophenyl-β-glucoside, ——— NaCl molarity.

Nearly all of the syringin-hydrolysing activity and most of that for coniferin were eluted in the non-binding fractions (peak A in Fig. 2), whereas the main part of the 4-nitrophenyl-β-glucoside-hydrolysing activity was bound and subsequently eluted by a NaCl gradient (peak B/C in Fig. 2). Some of the coniferin-hydrolysing activity and a small amount of syringin-hydrolysing activity were also eluted with the gradient, primarily in peak B. Preliminary results indicated that this activity might be similar to the β-glucosidase described for *C. arietinum* cell cultures [10].

Peak A of Fig. 2 was subsequently chromatographed on a DEAE 'Cellex' column to yield two fractions, peaks A_1 and A_2 (separation not shown). Most of the coniferin- and syringin-hydrolysing activity was eluted in the non-binding fraction (peak A_1 in Table 1), but a small part (peak A_2, Table 1) was bound and subsequently eluted with a linear sodium citrate–phosphate buffer gradient (0.02–0.5 M) at a concentration of 0.3 M. The non-binding peak A_1 was further chromatographed on a Sephadex G 200 column, and the coniferin- and syringin-hydrolysing activity eluted as a symmetrical peak with a MW of 40 000 (±10%). The activity for 4-nitrophenyl-β-glucoside behaved similarly, but the peak maximum appeared at slightly higher MW indicating that the β-glucosidase responsible for the hydrolysis of 4-nitrophenyl-β-glucoside is not identical to the enzyme which hydrolyses coniferin and syringin. A clear separation of the β-glucosidase hydrolysing syringin and coniferin from several other enzymes, active towards 4-nitrophenyl-β-glucoside, was finally achieved by preparative isoelectric focusing (Fig. 3).

Analytical isoelectric focusing of preparation A_1 (Fig. 3) revealed that several protein bands were still present, but only one of these showed β-glucosidase activity. Preparation A_1 could therefore be used for the subsequent determinations of the glucosidase activity. Peak A_2 from the DEAE-chromatography (Table 1) was not further purified since the low

Table 1. Scheme and data of purification of β-glucosidases from *Glycine max* cell suspension cultures

Purification step	Total activity (μmol/min)			Spec. activity (nmol/min/mg)			Protein (mg)	Yield* (%)	Purification* (-fold)
	Coniferin	Syringin	4-NP-β-glc	Coniferin	Syringin	4-NP-β-glc			
Crude extract	31.2	24.9	28.4	4.4	3.5	4.1	7012	100	1
0–70% $(NH_4)_2SO_4$ Sephadex G-25	5.7	4.5	7	9.1	7.1	11.3	625	18	2
CM-Sephadex (see Fig. 2)									
Peak A	4.9	3.2	1.6	26	17	8.8	186	16	11
Peak B/C	0.7	0.5	1.8	47	35	124	14	2	11
DEAE "Cellex"									
Peak A_1	2.6	1.8	1.3	26.4	18.3	13.3	99	8.3	6
Peak A_2	0.9	0.8	0.005	53	48	0.3	17	2.9	12
Sephadex G-200 (A_1)	1.7	1.0	0.8	66	39	30	27	5.5	15
Isoelectric focusing (see Fig. 3)									
Peak A_1	0.65	0.6	0.003	115	106	0.5	6	2.1	26

* Calculated for coniferin.

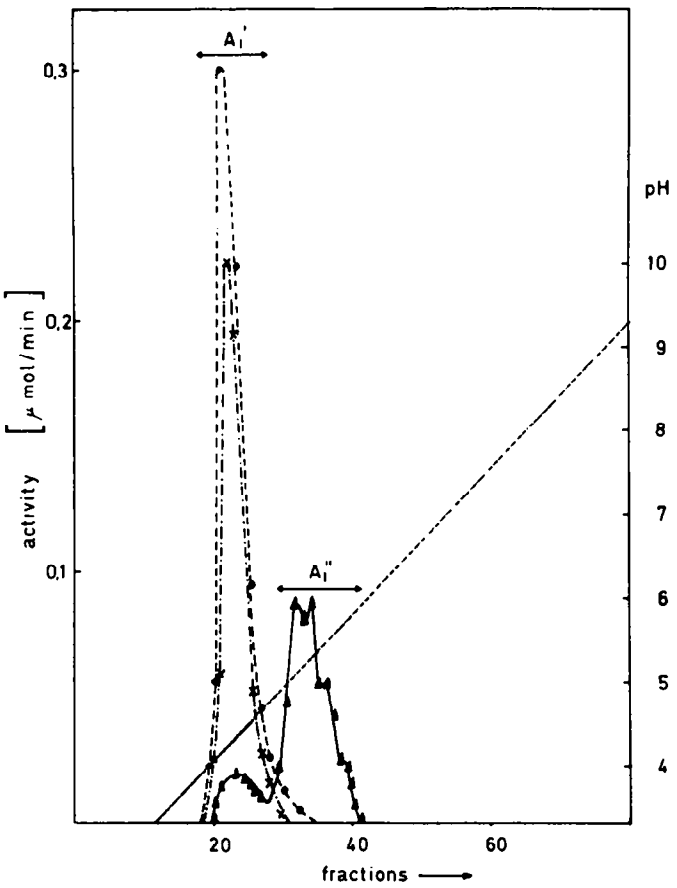


Fig. 3. Isoelectric focusing of the β-glucosidase fraction A_1 (see Table 1) from *Glycine max* cell suspension cultures in a 110 ml column with a pH gradient from 3.5 to 10. ●-----● Coniferin, X-·-·-X syringin, ▲——▲ 4-nitrophenyl-β-glucoside, ····· pH gradient.

amount of β-glucosidase activity made further separation difficult. Since the low activity towards 4-nitrophenyl-glucoside of preparation $A_{2'}$ when compared with activities towards coniferin and syringin suggested that no β-glucosidase other than the one hydrolysing coniferin and syringin was present, the substrate specificity of this glucosidase peak was determined at that purification stage.

Table 1 summarizes the separation procedures and the results obtained.

Characterization of β-glucosidase

The substrate specificity for the β-glucosidase peak $A_{1'}$ (Fig. 3) is presented in Table 2. This β-glucosidase clearly possesses high specificity for coniferin and syringin. Indeed, syringin exhibits a V_{max}/K_m value nearly twice that of coniferin. With one exception, the other synthetic or naturally-occurring glycosides tested were either poor substrates or inactive. Only ferulic acid-4-β-glucoside, whose structure is similar to that of coniferin, is hydrolysed at a reasonable rate, however, its K_m value is 3–4 times higher than that of coniferin.

Other properties of β-glucosidase $A_{1'}$ are as follows; its activity is maximal at pH 4.6–6 (50% of maximal activity is at pH 3.9 and 6.8), and the isoelectric pH is 4.2–4.4. MW determination by sucrose gradient centrifugation gave a MW of 45 000 (±10%). The activity was stable at 4° for several months. The enzyme was not inhibited by the following glucosidase inhibitors at the concentrations specified: bromcondurite A and bromcondurite B (0.5 mM respectively); conduritol B epoxide (1 mM); nojirimycin (3 mM). Glucono-1,5-lactone was a good inhibitor, the K_i value being 0.01 mM. β-Glucosidase $A_{1'}$ did not cross-react with antibodies prepared against coniferin-specific β-glucosidase from *C. arietinum* cell cultures [10].

Peak A_2 separated on DEAE 'Cellex' (cf. Table 1) showed essentially the same relative activity towards coniferin, syringin and 4-nitrophenyl-glucoside as the β-glucosidase $A_{1'}$, and the K_m values for coniferin and syringin were identical to those shown in Table 2. It is not yet clear whether the glucosidases $A_{1'}$ and A_2 are identical but behave differently for some unknown reason when chromatographed on DEAE, or whether they are different β-glucosidases present in the same plant and exhibit the same substrate specificity.

β-Glucosidase activities identical with fraction $A_{1'}$ have also been isolated from hypocotyls and roots of *G. max* seedlings.

DISCUSSION

Freudenberg suggested more than 20 years ago that the cinnamyl alcohol glucosides coniferin and syringin

Table 2. Substrate specificity of the β-glucosidase purified from *Glycine max* by isoelectric focusing (peak A_1 in Fig. 3)

Substrate	Relative activity (syringin = 100)	V_{max} (μmol/min/mg protein)	K_m (mM)
Syringin	100	0.115	0.3
Coniferin*	110	0.127	0.6
4-Coumaryl alcohol-4-β-glucoside*	7	—	—
Ferulic acid-4-β-glucoside*	51	0.135	2
4-Hydroxyacetophenone-4-β-glucoside (picein)	2	—	—
4-Hydroxy-3-methylacetophenone-4-β-glucoside (3-methylpicein)*	1.8	—	—
2-Hydroxybenzylalcohol-2-β-glucoside (salicin)	12.5	—	—
7-Hydroxy-4'-methoxyisoflavone-7-β-glucoside (formononetin-7-glucoside)[a]	0.5	—	—
4-Nitrophenyl-β-glucoside[b]	0.5	—	—
4-Methylumbelliferyl-β-glucoside*	0.4	—	—

Relative activity was measured at 1 mM substrate concentrations except: (a) 0.2 mM and (b) 2 mM.
* 5% (v/v) methoxyethanol was present in the assays for solubilization of the substrates.
— Not determined.

participate in the lignification process in higher plants [6]. However, the exact role of these glucosides was never fully established, and others [8] have questioned whether they participate in lignification. Recently, however, the metabolic turnover of coniferin has been demonstrated in spruce (*P. abies*) seedlings [12]. Moreover, β-glucosidases exhibiting good hydrolytic activity against coniferin have been detected and characterized in spruce hypocotyls [9] and chick pea (*C. arietinum*) cell cultures [10]. These results therefore supported a possible role for the glucosides in the lignification process.

On the other hand, the β-glucosidases described displayed only low hydrolytic activity against syringin, the aglycone of which (sinapyl alcohol) is an important component of the lignified fibers of dicotyledonous plants [11]. If syringin is to take part in lignification, a β-glucosidase with much higher activity against this substrate should be present in dicotyledonous plants. The enzyme described in this paper is the first β-glucosidase which shows high activity for syringin, for as noted, syringin and coniferin are by far the best of all substrates tested. The demonstration of this β-glucosidase in a dicotyledonous plant provides additional evidence for the involvement of the cinnamyl alcohol glucosides in lignification. The occurrence of this β-glucosidase in *G. max* cell cultures is in accord with the observations that these cultures contain lignin with sinapyl alcohol units [13] and show sinapic acid:coenzyme A ligase activity [14].

The physiological role of the cinnamyl alcohol glucosides is not yet known. It was suggested earlier that they are a storage form of lignin monomers in those plants where they occur (cf. [8]). Another reasonable hypothesis is that the cinnamyl alcohols are transported from the cytoplasm to the cell wall as glucosides. Since the glucosides, in contrast to the free alcohols, are not substrates for peroxidase, an irregular polymerization of the free alcohols at other places within the plant cell could be prevented. The hydrolysis of the glucosides by β-glucosidases in the cell wall would then liberate cinnamyl alcohols for polymerization by peroxidase at the right place.

The β-glucosidase described shows another remarkable feature. Coniferin and syringin which have simple aromatic aglycones elicit high activity, while synthetic glucosides with simple aromatic aglycones, e.g. 4-nitrophenyl-β-glucoside or 4-methylumbelliferyl-β-glucoside, are poor substrates. This fact again shows that the substrate specificities of glycosidases may be rather narrow and that they may not be detected unless the proper physiological substrates are used. On the other hand, this specificity casts doubt on the validity of conclusions about glycosidases which are only or mainly based on the use of readily available, synthetic glycosidic substrates.

EXPERIMENTAL

G. max cell suspension cultures were cultivated as described elsewhere [15]. Plants were grown from commercially available seeds in vermiculite in a 14 hr light–10 hr dark cycle at 24°. All chemicals were purchased from Merck and most of the glucosidic substrates were obtained from Sigma. DEAE 'Cellex' was obtained from Bio-Rad and all types of Sephadex from Pharmacia. Coniferin was kindly provided by Prof. K. Weinges, Chemisches Institut, Heidelberg; ferulic acid-*p*-glucoside was a gift from Dr. R. Tutschek, Botanisches Institut, Kiel; 4-coumaryl alcohol 4-β-glucoside was synthesized and kindly provided to us by Dr. S. Marcinowski, Biochemie der Pflanzen, Freiburg; formonetin 7-glucoside was a gift from Dr. A. Levai, Debrecin, Hungary. 3-Methylpicein, bromcondurite A and B, and conduritol B epoxide were kindly provided by Prof. G. Legler, Biochemisches Institut, Köln. Nojirimycin was a gift from Dr. E. Truscheit, Fa. Bayer, Wuppertal. Syringin was isolated from the bark of lilac stems as described by Freudenberg *et al.* [16].

Isolation and purification of β-glucosidases. Cells (1 kg fr. wt) were homogenized for 30 min in a mortar with

quartz sand and twice their wt of Na citrate–Pi buffer 0.2 M, pH 5, containing 0.5 M NaCl. The pellet from centrifugation at 20 000 g for 20 min was stirred with the same amount of buffer as before for another 1 hr and centrifuged again. The combined supernatants were subsequently purified by the following methods: 70% $(NH_4)_2SO_4$ precipitation, Sephadex G-25, CM Sephadex C-50, DEAE "Cellex", and Sephadex G-200 column chromatographies, and isoelectric focusing in a 110 ml LKB column (pH gradient 3.5–10). These purification steps and the 5–20% linear sucrose gradient centrifugation (marker: aldolase, MW 147 000) were performed as described previously [10, 17]. The same procedure was employed in the case of intact seedlings when 100 g of hypocotyls or 300 g of roots (fr. wt) were used as starting materials.

β-Glucosidase activity was monitored through all purification steps with the substrates 4-nitrophenyl-β-glucoside, coniferin and syringin. The substrate concns employed were 2 mM for the first substrate and 1 mM for the other two. In the standard assay, activity was measured by determining the *A* of the aglycone released at pH 5 in 0.05 M citrate–Pi buffer containing 0.05 M NaCl, in a total vol. of 0.5 ml at 30°. The other substrates used were measured according to essentially the same principle. Exact measurement procedures and quantitative calculations of reaction velocities of most substrates used were given earlier [10, 17]. The following wavelengths and ε values (M^{-1}/cm) were used: coniferyl alcohol, 325 nm, ε = 7022; sinapyl alcohol, 315 nm, ε = 11 200; ferulic acid, 350 nm, ε = 27 000; 4-coumaryl alcohol, 312 nm, ε = 8850; 4-hydroxyacetophenone and 4-hydroxy-3-methylacetophenone, 325 nm, ε = 25 600. K_m and V_{max} values were calculated by Lineweaver–Burk plots.

In inhibitor expts bromcondurite A and B and conduritol B epoxide were preincubated for 15 min with the glucosidase while nojirimycin and glucono-1,5-lactone were added to the assays before incubations. Both coniferin and syringin were used as substrates in these expts.

Antibodies against the coniferin-specific β-glucosidase from *C. arietinum* were raised in a rabbit by injection of a homogeneous prepn of that β-glucosidase (cf. [10]; Burmeister and Hösel, unpublished). The reaction of the β-glucosidase with the antibodies was tested by measurement of the glucosidase activity after preparations with different amounts of antiserum.

Protein was determined according to the method of Lowry.

Acknowledgements—Financial support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged. We thank Miss Borgmann for skilled technical assistance and Gerd Burmeister for doing the immunological experiments. We are further very grateful to Prof. Dr. Eric E. Conn and Steve Eklund, University of California, Davis, for valuable discussions during the preparation of the manuscript.

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