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CHARACTERIZATION OF A MEMBRANE BOUND β-GLUCOSIDASE RESPONSIBLE FOR THE ACTIVATION OF OAT LEAF SAPONINS

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Key Word Index—*Avena sativa*; Gramineae; oat; β-glucosidase; membrane bound; saponins; antifungal; subcellular localization.

Abstract—Oat leaves contain a β-glucosidase (= avenacosidase) specific for the cleavage of the C-26 bound glucose moiety of the oat saponins avenacosides A and B. This transformation activates the fungitoxicities of the avenacosides. Evidence is presented that this enzyme is bound to the tonoplast membrane. The solubilized enzyme showed a pH optimum of 6.0–7.0, a temperature optimum around 40°, a molecular weight of 68 000 ± 3000 and a K_m of 183 (± 16) μM. The enzyme is inhibited by Hg^{2+} (10^{-2} M) but not by Cu^{2+} (10^{-2} M).

INTRODUCTION

Oat leaves and shoots contain two saponins of the spirostanol type [1–3]. These saponins (avenacosides A and B) are preformed chemical protectants, as they are present prior to infection of oat leaves with fungi [4]. The avenacosides contain a sugar chain at the C-3 position and, additionally, another glucose moiety at the C-26 position. Therefore, they are called bisdesmosidic saponins, which do not themselves possess antifungal activity. However, the avenacosides are transformed immediately to the antifungally active 26-desglucoavenacosides, if the cell is damaged [5–7]. Lüning and Schlösser [5] characterized a soluble β-glucosidase with a high specificity for the C-26 glucose moiety. However, these authors did not differentiate between the avenacosidase, specific for the cleavage of the C-26 glucose moiety, and other soluble β-glucosidases. Kesselmeier and Urban [8] produced evidence for a vacuolar localization of both the avenacosidase and the avenacosides in oat leaves. In the present investigation we report on the separation of this enzyme from other oat β-glucosidases and its localization, using isolated natural substrates.

RESULTS AND DISCUSSION

Many plant β-glucosidases with high substrate specificities have been described [9] and the existence of multiple forms of glucosidase is well documented [10]. The avenacosidase is a β-glucosidase with a high specificity for cleavage of the C-26 bound glucose moiety of the avenacosides, thereby producing the antifungal 26-desglucoavenacosides. Lüning and Schlösser [5] showed avenacosidase activity in 10 000 *g* supernatants of oat leaf homogenates. However they did not differentiate between avenacosidase and other β-glucosidases. As a first attempt to analyse oat β-glucosidases responsible for saponin activation, we separated a soluble enzyme fraction by homogenizing leaves in 0.2 M citrate buffer and by subsequent filtration and centrifugation (40 000 *g*). The supernatant (soluble enzymes) and the pellet (membrane-associated enzymes) were tested for their avenacoside activation activity (Table 1). The soluble β-glucosidases, obtained in the supernatant, were able to hydrolyse the C-26 bound glucose moiety. However, to a lesser extent they were also able to remove the sugars at the C-3 position, producing steroids other than the 26-desglucoavenaco-

Table 1. β-Glucosidase activity in the two enzyme fractions obtained from oat leaves against different substrates

Substrates	40 000 *g* membrane fraction (Avenacosidase) Products	40 000 *g* supernatant (soluble β-glucosidases)
4-Nitrophenyl-β-D-glucopyranoside	glucose + nitrophenol	glucose + nitrophenol
Hederacoside C	—	—
Cellobiose	—	glucose
Avenacoside A	26-desglucoavenacoside A	26-desglucoavenacoside A 3 unidentified steroids
Avenacoside B	26-desglucoavenacoside B	26-desglucoavenacoside B Avenacoside A 3 unidentified steroids

sides. Furthermore, they transformed avenacoside B to avenacoside A. In contrast, the sedimented β-glucosidase fraction showed a high specificity for the C-26 glucose, producing exclusively 26-desglucoavenacosides from their corresponding avenacosides. These results clearly show that the avenacosidase activity is localized within the membrane fraction, whereas the soluble fraction contains unspecific β-glucosidase activity. Analysing the specificity of the isolated enriched β-glucosidase fractions, we also used other substrates (Table 1). Both fractions hydrolyse 4-nitrophenyl-β-D-glucopyranoside, but do not work with hederacoside C, a triterpenoid saponin from ivy leaves. Only the soluble enzyme transforms cellobiose to glucose; therefore, a clear differentiation between the two enzyme fractions is possible.

For better characterization, we liberated the enzyme from the membranes by treatment with acetone. By this means we obtained a water-soluble form of avenacosidase. The pH-optimum of the solubilized enzyme ranges between pH 6.0 and 7.0 (50% activity at pH 4.8 and 8.0, respectively). The temperature optimum was around 40°. Gel electrophoretic analysis showed three protein peaks, two of which exhibited avenacosidase activity. A similar pattern was found separating the solubilized avenacosidase fraction on a calibrated Ultrogel column. Four protein peaks were found with two peaks showing avenacosidase activity (Fig. 1). From the elution volumes an M_r of 68 000 ± 3000 was estimated for the main peak. The K_m-values for the natural substrates, calculated by Lineweaver–Burk plots amount to 183 (±16) μM. The solubilized enzyme is sensitive to Hg^{2+} ions. A 50% inhibition was obtained at 5×10^{-4} M and a 100% inhibition was reached at 10^{-2} M concentration. Cu^{2+} ions did not show any inhibition of the enzyme up to 10^{-2} M. These results are similar to those reported for other β-glucosidases [11–15]. The restricted pH and temperature optima of the isolated enzyme are, however, strikingly different from the broad pH and temperature range of the avenacosidase reaction observed upon leaf homogenization [16]. These differences may be explained by protection of the avenacosidase in the membranes by surrounding lipid or protein molecules. Another explanation may be seen in the rapid transformation of the avenacosides: the transformation has to be completed before pH and temperature lead to conformational protein changes.

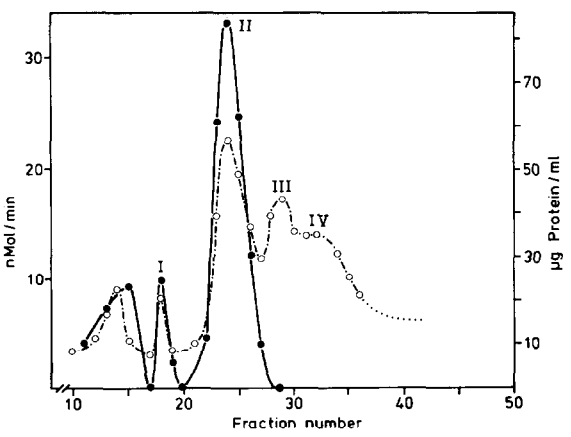

Fig. 1. Gel filtration of the avenacosidase fraction liberated from the 40 000 *g* sediment. O——O, Protein content (μg per ml); ●——●, avenacosidase activity (nmol 26-desglucoavenacosides produced per min).

To determine the subcellular localization of avenacosidase, leaves were homogenized in isotonic buffer systems to prevent damage of organelles. The 1000 *g* fractions contained intact plastids, the 40 000 *g* fraction intact mitochondria, whereas the 100 000 *g* fraction consists mainly of the microsomal membranes. The main activity is found in the 100 000 *g* sediment. This localization holds true for the total activity and for the specific activity. The high specific activity in the 100 000 *g* sediment shows that avenacosidase is a major enzyme of the microsomal fraction. This observation is consistent with results of Kesselmeier and Urban [8], who found that isolated oat vacuoles can transform the avenacosides, localized inside the vacuoles, to their corresponding 26-desglucoavenacosides upon disruption of the vacuoles. The present experiments show that avenacosidase is localized within or on the tonoplast membrane. Whether the remaining 20% of activity, found in the 100 000 *g* supernatant, is due to avenacosidase molecules solubilized during the homogenization and isolation procedure, or to enzyme entities bound to small membrane vesicles, or to the activity of other β-glucosidases, remains to be elucidated.

EXPERIMENTAL

Growth conditions. Oat seedlings were grown as described earlier [8].

Isolation and purification of avenacosides. Oat leaf extracts, obtained by boiling H_2O or boiling MeOH, were loaded onto a column filled with C-8 coated particles (RP-8; 30 μm; Merck, FRG). Column dimensions: 3.5 × 2.0 cm; loading volume: up to 100 ml. MeOH extracts were diluted with H_2O to MeOH-concns lower than 10% prior to loading, thus preventing the elution of saponins during the loading process. Separation was accomplished by a stepwise elution with MeCN–H_2O: 30 ml 20% MeCN containing sugars, phenolic compounds, 60–70 ml 35% containing saponins and 35 ml 100% MeCN containing chlorophylls, carotenoids and lipids. For isolation of 26-desglucoavenacosides, leaves were homogenized in water prior to MeOH extraction, leading to a complete transformation of the avenacosides to their corresponding 26-desglucoavenacosides. The collected fractions were analysed by HPLC and purified until a purity of 86–90% on a dry weight basis was reached.

Isolation of β-glucosidase fractions. Oat leaves (600 g fr. wt) were homogenized in a 0.2 M citrate buffer (pH 5.0). The homogenate was filtered through a nylon net (25 μm pore size) and centrifuged for 5 min at 1000 *g*. The 1000 *g* supernatant was again centrifuged for 20 min at 40 000 *g* to give a crude membrane fraction, which was tested for its avenacosidase activity by adding isolated avenacosides to the suspension and analysing the transformation products by HPLC. From the 40 000 *g* supernatant a soluble β-glucosidase fraction was precipitated by $(NH_4)_2SO_4$ according to ref. [17]. For further purification of the membrane bound β-glucosidase (avenacosidase) the 40 000 *g* fraction was homogenized with ice-cold Me_2CO, thus disintegrating the membrane structure. The Me_2CO suspension was centrifuged for 10 min at 40 000 *g* to give a protein sediment. Washing this Me_2CO sediment twice with citrate buffer (10 min, 40 000 *g*), a clear protein soln was obtained. From this fraction proteins were precipitated by adding $(NH_4)_2SO_4$ up to 70%. The protein precipitate was centrifuged at 40 000 *g* (20 min), dissolved in a few ml of 0.2 M citrate buffer (pH 6.5) and stored at 4°. For a better stabilization BSA was added up to 0.3% (w/v).

Protein determination. Protein was measured according to ref. [18].

Enzyme assays. If not specified, β-glucosidase activity was measured in 0.2 M citrate buffer, pH 6.5. Protein concns were adjusted to 6 μg per ml incubation assay in the case of solubilized avenacosidase and to 500 μg per ml for soluble β-glucosidases. The avenacoside concn was adjusted to 1.0 μmol per ml incubation assay.

Cellobiose concn was adjusted to 1 mg per ml incubation assay. After 2 hr incubation the reaction mixture was analysed by TLC on precoated silica gel plates (Macherey & Nagel, F.R.G.) using the solvent mixture *n*-BuOH–HOAc–H_2O (2:1:1). Cellobiose and the reaction product glucose were visualized by spraying the plate with 0.1% orcinol in 2 N H_2SO_4 and heating at 120° for 10 min.

The concn of Hederacoside C (Roth, F.R.G.) was adjusted to 750 μg per ml incubation assay. Samples were taken after 5, 30, 60 and 120 min and the reaction was stopped by adding MeOH up to 90% (v/v). After centrifugation at 40000 *g* for 20 min the supernatant was evaporated and the residue redissolved in 2 ml 50% MeOH and analysed by HPLC.

4-Nitrophenyl-β-D-glucopyranoside cleavage was measured according to ref. [19].

pH-optimum. For the determination of the pH-optimum of the isolated solubilized avenacosidase the following buffers were used: pH 4–7, 0.1 M citrate buffer; pH 5–8, 0.15 M phosphate buffer; pH 8–9, 0.2 M borate buffer. Enzyme concn was adjusted to 6 μg per ml and avenacoside concn to 400 nmol per ml incubation assay. After 2 min of incubation the reaction was stopped by adding MeOH up to 90%. Transformation products were measured by HPLC after preparation as described for Hederacoside C.

K_m*-assay.* The K_m-value was obtained by varying the avenacoside concentration between 30 and 600 μM. Incubation temperature was 25°. Samples were taken after 0.5, 1.0, 2.0 and 5.0 min. The reaction was stopped and the products were measured as described for Hederacoside C.

Temperature optimum. The reaction was started with the addition of the enzyme to the incubation assay. After 2 min the reaction was stopped and the products were measured as described for Hederacoside C.

M_r *Determination.* Solubilized avenacosidase was dissolved in 0.2 M citrate buffer and chromatographed on a Ultrogel ACA 44 (LKB)-column (98 × 1.6 cm). The column was calibrated with cytochrome *c*, myoglobin, chymotrypsinogen, ovalbumin, BSA and aldolase. Flow rate was adjusted to 10 ml per hr. Fraction volume was 2.7 ml. Each fraction was tested for protein content and avenacosidase activity: 50 μl eluate was mixed with 1 ml citrate buffer. The reaction was started by adding 1 μmol avenacosides dissolved in 80 μl of water. After 10 min the reaction was stopped and the products were measured as described for Hederacoside C.

Disc electrophoresis was performed according to ref. [20]. Gel slabs were divided into two halves, one of which was stained with Coumassie blue, the other fractionated. Each fraction was ground in 1 ml citrate buffer and the suspension tested for avenacosidase activity as described in the preceding chapter.

Inhibition of avenacosidase by Hg^{2+} and Cu^{2+}. $HgCl_2$ and $CuSO_4$-concns ranged between 10^{-2} and 10^{-6} M. After 2 min of preincubation the reaction was started by adding the substrate. After another 2 min the reaction was stopped and the products were measured as described for Hederacoside C.

HPLC-analysis of saponins. HPLC was performed according to ref. [21]. The detection at 200 nm allowed the analysis of avenacosides, 26-desglucoavenacosides and Hederacoside C.

Cell fractionation. Oat leaves were homogenized in an isotonic medium according to ref. [22]. The filtered homogenate was fractionated by centrifugation at 1000 *g* (5 min), 40000 *g* (20 min) and 100000 *g* (3 hr). Sediments 1–3 were carefully resuspended in a few ml citrate buffer and tested for avenacosidase activity. From the 100000 *g* supernatant β-glucosidases were precipitated by 70% saturation with $(NH_4)_2SO_4$ and the precipitated proteins redissolved in a few ml citrate buffer and tested for avenacosidase activity. 2.5 ml of each fraction were mixed with 0.5 ml H_2O containing 8.5 μmol avenacosides. From this incubation assay samples were taken after 0.5, 1.0, 2.0, 5.0 and 60 min and the reaction stopped and the products measured as described for Hederacoside C.

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