Salicortin: a repeat-attack new-mechanism-based *Agrobacterium faecalis* β-glucosidase inhibitor

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Salicortin, a natural product abundant in most members of the Salicaceae family, is a mechanism-based inactivator of *Agrobacterium faecalis* β-glucosidase. Inactivation is delayed in the presence of competitive inhibitors, thereby demonstrating the requirement for an enzyme-bound salicortin before inactivation. Product studies suggest that inactivation proceeds via a quinone methide intermediate formed by the fragmentation of the aglycone of salicortin while it is bound to the enzyme. Tryptic digest and HPLC/MS studies confirm the role of quinone methide attack and also show that the enzyme undergoes multiple modifications. In addition, when the inactivation was run in the presence of a mutant inactive form of the enzyme, HPLC/MS analyses clearly showed no modification of the mutant enzyme, demonstrating that the quinone methide does not exist in free solution and suggesting that inactivation is active-site directed.

INTRODUCTION

Increased interest in inhibitors of glycosidases has arisen over the past decade. One driving force behind this surge of interest and activity has been the value of inhibitors in elucidating the mechanism(s) of action of glycosidases [1–6]. Competitive inhibitors have been used in determining the intermolecular forces and steric constraints between the protein and substrate, conformational changes that occur during the rate-limiting step, and the overall energetics of enzymic action, while irreversible active-site-directed inactivators have found great utility in labelling active-site peptides for amino acid sequencing.

There are only a handful of irreversible active-site-directed inactivators of β-glucosidases. Among the best known examples are conduritol epoxide [7], glucosyl methyltriazene [8], the aziridine triol derivative of piperidine [9], conduritol aziridine [10], β-D-glucosyl isothiocyanate [11] and cyclophellitol [12]. Each of these substances, however, has structural attributes that decrease their specificity towards β-glucosidases. The conduritol derivatives, for instance, lack a hydroxymethyl group and hence are analogues of xylose, not glucose. They also contain a symmetry element that makes them equally suitable as both α- and β-glycoside analogues. These problems have recently been overcome by the isolation of cyclophellitol, one of the most specific active-site-directed inactivators of β-glucosidase so far found [12]. Nevertheless, all of these inactivators, including cyclophellitol, are highly reactive substances that are activated by simple protonation and hence might not be suitable for drug design.

The *o*- and *p*-difluoromethylaryl-β-D-glucosides **I-II** [13] and 2-deoxy-2-fluoro-D-glycosyl fluorides with good leaving groups such as **III** [14], however, form reactive species only after cleavage of the glucose moiety and hence are highly specific inactivators of enzymes possessing β-D-glucosidase activity (Figure 1). Here we report studies on another mechanism-based inactivator of β-D-glucosidase, salicortin, whose mode of inactivation seems to be unusual.

MATERIALS AND METHODS

Materials

Agrobacterium faecalis β-glucosidase was purified and assayed as described previously [6]. Salicortin was isolated from *Populus balsamifera* twigs [15]. Sodium phosphate and SDS/polyacrylamide gel were obtained from Fisher Scientific Company. The buffer employed for all experiments was 50 mM sodium phosphate buffer adjusted to pH 7.0 with HCl. All other reagents were from Sigma.

Inactivation kinetics

All kinetic studies were performed by monitoring the UV/visible absorbance of enzyme/substrate solutions in a thermostatically controlled (37 °C) Hewlett Packard Diode Array spectrophotometer (Model 8452A). Cells of 1 cm path length were employed for all experiments. The wavelengths used were 400 and 368 nm for *p*-nitrophenyl β-D-glucopyranoside and 4-methylumbelliferyl β-D-glucopyranoside respectively. General time-dependent inactivation experiments were performed by incubating β-glucosidase in buffer in the presence of salicortin at 37 °C. Residual enzyme activity was determined at time intervals by removal of a 10 μl aliquot of the salicortin/enzyme mixture, addition of this sample to 1.5 ml of buffered substrate in a 1 cm cuvette and measurement of aglycone release.

Protection against inactivation

A 7.66 mM deoxynojirimycin solution (657 μl) was added to 5 ml of enzyme solution (1.35 μg/ml) and incubated for 3 min. Then 200 μl of buffered salicortin solution (25 mM) was added to this mixture. Residual enzyme activity was determined by the removal of 100 μl aliquots at appropriate intervals and assayed for the rate of hydrolysis by using 2.5 mM *p*-nitrophenyl β-D-glucopyranoside (2.0 ml). A control was run under the same conditions with 657 μl of buffer in place of the deoxynojirimycin solution.

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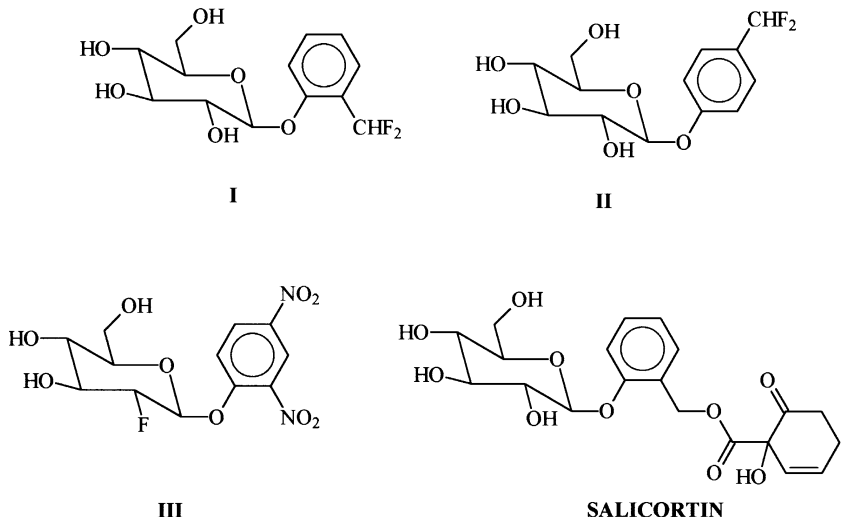


Figure 1 Structure of salicortin and irreversible inactivators of β-glucosidases that are activated on cleavage of the glycosidic bond

HPLC/MS study

β-Glucosidase (10 μl, 17.5 mg/ml) was added to 18 μl of buffer and 7 μl of 25 mM buffered salicortin solution. A sample (1 μl) of this mixture was diluted to 1000 μl with buffer. Aliquots (10 μl) of this diluted enzyme/salicortin solution were withdrawn at appropriate intervals and assayed with buffered 0.5 mM *p*-nitrophenyl β-D-glucopyranoside. After 17 min 20 s, when the rate of hydrolysis was stable, 10 μl of the diluted enzyme/salicortin solution was injected on to a 1 mm × 50 mm reverse-phase column (Michrom Bioresources) for HPLC/MS analysis.

Trypsin digest

Monitoring by SDS/PAGE [12% (w/v) gel], we found that undenatured enzymes resisted tryptic digestion. To resolve this problem, the enzyme solution was denatured by heating for 2 min at 85 °C before trypsin digestion. β-Glucosidase (200 μg in 100 μl of buffer solution) was treated with salicortin (100 μl, 2.5 mM in buffer) and incubated for 1 h (room temperature, 23 °C), by which time the enzyme was 38% inactivated as assayed with *p*-nitrophenyl glucoside. Excess salicortin and non-protein substances were removed by ultrafiltration. The resulting enzyme solutions were subjected to tryptic digestion at room temperature with an enzyme-to-trypsin ratio of 100:1 (w/w). Proteolysis was monitored by the removal of aliquots at appropriate intervals followed by SDS/PAGE. Cleavage was complete within 6 h.

A sample (20 μl) of this tryptic digest solution was separated by reverse-phase HPLC with an Alltech C_{18} (5 μm, 4.6 mm × 250 mm) column attached to a Beckman (Model 126) with System Gold software and interfaced with a photodiode-array detector (Model 168). A binary mobile-phase system was used: solvent A, 0.1% (v/v) trifluoroacetic acid in water; solvent B, 0.085% (v/v) trifluoroacetic acid in acetonitrile/water (7:3, v/v). A linear gradient of 0–70% B was run over 110 min at a flow rate of 0.5 ml/min. Peptides and phenol groups were detected by absorbance at 215 and 280 nm respectively. A control chromatogram was run with enzyme subjected to identical conditions except for the salicortin treatment.

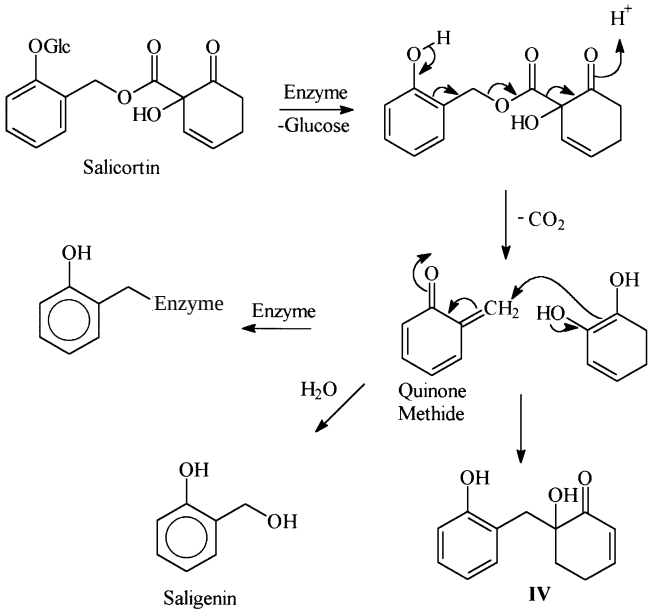
In the study of the stoichiometry of incorporation, three different salicortin concentrations (0.078, 0.313 and 0.625 mM) were used. Enzyme inactivation, ultrafiltration, proteolysis and reverse-phase HPLC separation were performed as described above except that 10 μl of resorcinol standard (1.86 mg/ml) was also added as an internal standard.

RESULTS AND DISCUSSION

Salicortin is an aryl glucoside found in most species of *Salix* and *Populus*, often in amounts exceeding 5% of the dry weight of the plant part [16]. It has been shown to be a powerful 'natural insecticide' against a variety of insect species [17–22], and several of its products from enzymic hydrolysis (esterases) are natural antifeedants to snowshoe hares [23]. In our continuing studies on the ecological relevance of salicortin, we decided to examine the fate of salicortin after enzymic hydrolysis of the glucosidic linkage. We found that the treatment of salicortin with almond β-glucosidase (Sigma) resulted in a decrease in enzyme activity, with the concomitant formation of a fragmentation–recombination product **IV** that we envisage being formed as shown in Scheme 1 [24].

These early observations were consistent with a simple model. We proposed that most of the aglycone fragments into *o*-quinone methide and an enol while still bound in the enzyme's hydrophobic pocket and that these compounds rapidly recombine to form **IV**. A small amount of *o*-quinone methide, however, is captured by water to yield saligenin, a minor product of the reaction. Similarly, a small amount of quinone methide is captured by nucleophilic sites at or near the active site of the enzyme, resulting in loss of activity. This model is bolstered by the report [13] that *o*- and *p*-quinone methides generated within the active site of β-glucosidases on the hydrolysis of difluoromethyl aryl glucosides (I and II) lead to inactivation of the enzyme.

Initial kinetic studies to test this model yielded unexpected results, which we thought might reflect problems with the commercial enzyme. Almond β-glucosidase contains at least two isoenzymes with different kinetic properties [25], and purification is difficult. In addition, almond β-glucosidase is a large glycoprotein (135 kDa) whose amino acid sequence has not been determined. For these reasons we chose to continue our investigations with *A. faecalis* β-glucosidase. This enzyme has been



Scheme 1 Fate of salicortin on enzymic hydrolyses of the glycosidic bond

cloned and expressed in *Escherichia coli* [26], is easily purified and has been characterized in detail [6]. Characterization of this enzyme revealed that it is dimeric, with a monomer molecular mass of 50 kDa, arising from 458 amino acid residues. It has relatively relaxed substrate specificity and is quite non-specific with regard to the aglycone moiety.

Inactivation kinetics

Incubation of *A. faecalis* β-glucosidase with salicortin resulted in a time-dependent loss of activity (Figures 2 and 3). Enzymic activity could not be restored after the removal of non-protein material by repeated rinsing and ultrafiltrations of the deactivated enzyme. Therefore it is unlikely that deactivation is the result of

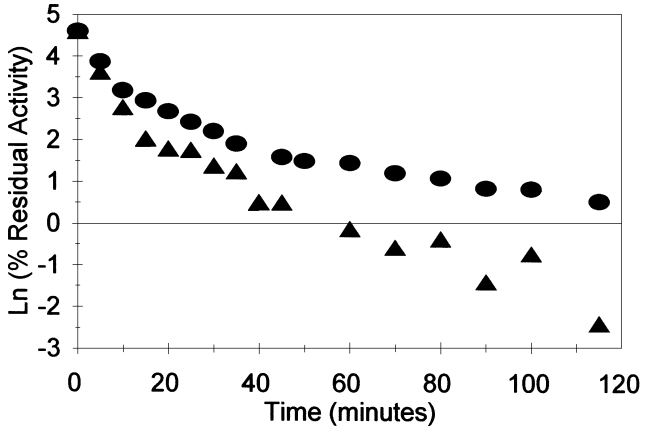


Figure 2 Deactivation of *A. faecalis* β-glucosidase by salicortin assayed with different substrates

Enzyme (0.1 mg in 1.0 ml buffer) was treated with 10 mg of salicortin. Aliquots were taken at timed intervals and added to solutions of substrates [either *p*-nitrophenyl β-D-glucoside (●) or 4-methylumbelliferyl β-D-glucoside (▲)] and the rate of hydrolysis was followed by UV absorption to determine residual enzyme activity.

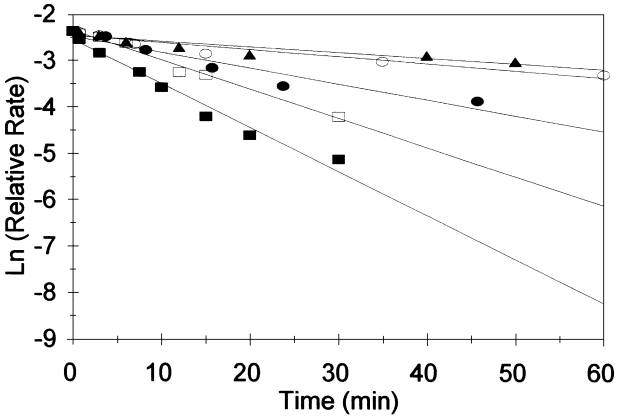


Figure 3 First-order inactivation of β-glucosidase by salicortin

Each assay contained 0.2 mg BSA and 0.664 μg of *A. faecalis* β-glucosidase in 200 μl of 50 mM sodium phosphate, pH 7.0. Initial concentrations of salicortin were 20 (■), 5 (□), 2.5 (●), 1.0 (○) and 0.5 (▲) mM. A control (results not shown) was run with no added salicortin. The observed values of k_i and K_i were 0.14 min^{-1} and 5.2 mM respectively.

the formation of some strong competitive inhibitor during the hydrolysis. Furthermore the time dependence of inactivation is substrate-dependent (Figure 2). This observation cannot be explained by a single attack leading to inactive enzyme. Instead the modified enzyme must retain some activity that is determined, in part, by the choice of assay substrate.

Some measure of the inactivation parameters was obtained by monitoring inactivation as a function of time with several different concentrations of salicortin. *p*-Nitrophenyl glucoside was used as assay substrate. Apparently pseudo-first-order kinetic behaviour was seen, as shown in Figure 3. A replot of the pseudo-first-order rate constants so obtained against the concentration of salicortin revealed saturation kinetics from which kinetic parameters for inactivation, $k_i = 0.14\ min^{-1}$ and $K_i = 5.2$ mM were estimated, as described previously [10].

Competitive inhibition

Incubation of β-glucosidase with salicortin (0.85 mM) in the presence of competitive inhibitor deoxynojirimycin ($K_i = 34\ \mu M$) results in a decrease in the rate of inactivation (Figure 4) and supports our earlier model that salicortin is accepted as a substrate by the enzyme before deactivation.

Electrospray ionization MS study

Measurement of the mass spectrum of the partly inactivated enzyme by electrospray ionization MS revealed that multiple labelling was indeed occurring, as shown in Figure 5. A series of peaks due to β-glucosidase (molecular mass 51 200 Da) labelled successively with two, three, four and five units of quinone methide is clearly seen, with possibly more at higher mass. The average mass difference (104 Da) corresponds well, within error, to the mass of the quinone methide moiety (107 Da). At longer incubation times with salicortin the mass spectrum became too complex to deconvolute, as yet more labels were added. This experiment therefore clearly shows multiple labelling occurring with this reagent, though it does not necessarily indicate that the inactivation is active-site-directed. The possibility exists that inactivation arises from the production of reactive products that

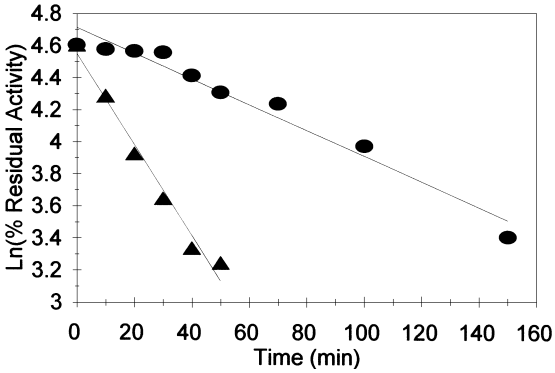


Figure 4 Time-dependent inactivation of *A. faecalis* β-glucosidase by salicortin with (●) and without (▲) the addition of the strong competitive inhibitor deoxynojirimycin ($K_i = 34\ \mu M$) at 0.89 mM

Both reactions were otherwise run under identical conditions. The reaction was followed by adding aliquots at timed intervals to a *p*-nitrophenyl β-D-glucoside solution, pH 7.0, and following the release of *p*-nitrophenolate spectrophotometrically at 400 nm.

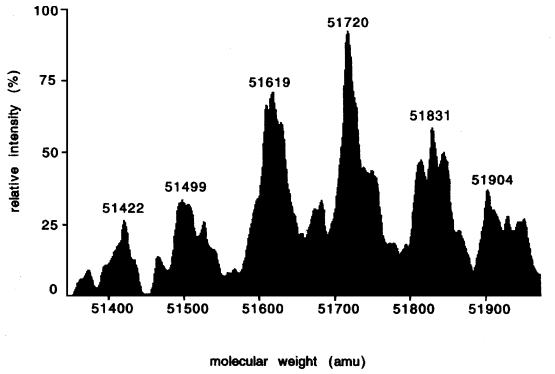


Figure 5 Reconstructed electrospray ionization MS of *A. faecalis* β-glucosidase inactivated for 6.2 min with salicortin (5 mM)

are released into solution after the enzymic hydrolysis of salicortin and subsequently captured by other enzyme molecules. To probe this, a pair of additional experiments was performed. First, salicortin was incubated with a completely inactive mutant of β-glucosidase (Glu-358 → Ala) from which the essential active-site nucleophile had been removed. Mass spectrometric analysis revealed no modification of the enzyme, confirming that active enzyme is required to generate the quinone methide. In a separate experiment a mixture (1:10) of wild-type β-glucosidase and the Glu-358 → Ala mutant was incubated with salicortin and samples were analysed by electrospray ionization MS and by assay of activity. Although the activity decreased in the normal manner, indicating the generation of quinone methide, no modification of Glu-358 → Ala β-glucosidase was observed by electrospray ionization MS. Thus none of the generated quinone methide (or its aglycone precursor) survived transfer in aqueous solution from one enzyme molecule to another and deactivation must be active-site directed.

Trypsin digest

To gain further insight into the active-site modifications, modified and unmodified enzymes were freed of excess salicortin by ultrafiltration, then subjected to trypsin digestion; the resultant peptides were analysed by reverse-phase HPLC. Except for two additional peaks associated with the modified enzyme, the chromatograms were identical. One of these peaks proved to be saligenin on the basis of a comparison of its UV spectrum and retention times with authentic material. The other peak was not identified but its UV spectrum was very similar to that of saligenin and had a λ_{max} of 280 nm, suggesting a similar chromophore to that of saligenin. Because the trace amounts of side products had been removed earlier by repeated rinsing and ultrafiltrations (confirmed by HPLC analysis of the modified protein), both of those substances must have been products of the trypsin digest. Furthermore the facile formation of saligenin by trypsin treatment suggests that it was initially bound to the modified enzyme via either an amide or an ester linkage.

An internal standard (resorcinol) was used to confirm that the amount of saligenin released from the trypsin digest of modified enzyme was consistent with a multiple-attack mechanism of inactivation. We determined that when the enzyme's activity was decreased to 62% of its original value by salicortin, an average of four saligenin molecules were released per molecule of enzyme. This is in rough agreement with the electrospray ionization MS results reported above. These results confirm that the enzyme has undergone sequential modifications by *o*-quinone methide and that most of these modified forms of the enzyme retain some activity with the substrates employed.

Conclusion

Salicortin is a new suicide inhibitor of *A. faecalis* β-glucosidase. The aglycone produced by enzymic hydrolysis rapidly fragments to *o*-quinone methide, which is subsequently captured by nucleophiles at or near the active site of the enzyme. This leads to the formation of modified enzymes that have a decreased activity and an altered substrate specificity. These altered enzymes can still accept salicortin as a substrate, leading to an iterative series of modified enzymes. Presumably a stage is reached at which salicortin is no longer a suitable substrate and hence no further modifications are observed. Although we have not ascertained the number of possible modifications, direct evidence exists for more than five sequential attacks by quinone methide on the enzyme.

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