Inhibition of lysosomal α -mannosidase by swainsonine, an indolizidine alkaloid isolated from *Swainsona canescens*

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An indolizidine alkaloid (swainsonine) was isolated from the plant *Swainsona canescens*. Swainsonine is a specific and potent inhibitor of α -mannosidase (EC 3.2.1.24) and when administered to animals produces a phenocopy of the genetically based lysosomal storage disease, mannosidosis. Evidence is presented to suggest that swainsonine is a reversible active-site-directed inhibitor of lysosomal α -mannosidase.

Swainsonine, an inhibitor of lysosomal α -mannosidase (EC 3.2.1.24), is a trihydroxylated indolizidine alkaloid contained in the plant *Swainsona canescens* (Colegate *et al.*, 1979) and is responsible for producing the disease process that is manifest in grazing livestock after prolonged ingestion of the plant. In all respects *Swainsona* toxicosis is a phenocopy of mannosidosis of humans and of Angus cattle (Dorling *et al.*, 1978). Swainsonine offers the possibility of inducing and terminating a lysosomal storage disease in any species of animal and thus should be a useful tool in helping to answer major questions related to the pathogenesis and reversibility of storage-mediated tissue injury *in vivo*. It also offers the possibility of studying the effects of storage on cell function in culture. It is therefore important that the specificity, reversibility and molecular mechanism of action of swainsonine be fully understood. The present study was carried out to help elucidate these parameters.

Materials and methods

Swainsonine (Fig. 1) was isolated from the plant *Swainsona canescens* (Benth.) A. Lee, by the method of Colegate *et al.* (1979).

Swainsonine

(8 α -indolizidine-1 α ,2 α ,8 β -triol; mol.wt. 173.1)

Fig. 1. Stereochemical structure of swainsonine

Inhibition of hydrolases

α -Mannosidase, α -glucosidase, β -galactosidase, hexosaminidase and β -glucuronidase activities were determined fluorimetrically by using the appropriate fluorogenic 4-methylumbelliferyl substrates (Koch–Light Laboratories, Colnbrook, Bucks., U.K.).

The reaction mixture consisted of 100 μ l of tissue extract, 100 μ l of water or aqueous inhibitor solution, plus 100 μ l of substrate in 0.2 M-citrate/phosphate buffer, pH 4.0, to give a substrate concentration in the incubation mixture of 5 mM. The mixture was incubated for 30 min at 37°C and the reaction was stopped by the addition of 2.0 ml of 0.5 M-glycine/NaOH buffer, pH 10.4. Fluorescence of liberated 4-methylumbelliferone was measured on a Farrand Mk I spectrofluorimeter with an excitation wavelength of 350 nm and an emission wavelength of 440 nm. The inhibitor solution contained 60 μ M-swainsonine in glass-distilled water.

The supernatant from a 1% homogenate of mouse liver in glass-distilled water centrifuged at 10000 g for 30 min was used as the tissue extract. The effect of swainsonine on α -mannosidase activity was assayed in similar extracts prepared from kidney, lymph node or liver of sheep, guinea pigs and rats and from liver of the lamprey eel (*Geotria australis*). The effect of swainsonine on the non-lysosomal α -mannosidase activity was assayed at pH 6.5 in an extract of mouse liver in which the lysosomal activity had been removed by passing the extract through concanavalin A–Sepharose 4B (Pharmacia fine chemicals) (Phillips *et al.*, 1976).

By using diluted samples of jack-bean (*Canavalia ensiformis*) α -mannosidase [Boehringer Mannheim (Australia) Ltd.] the above reaction mixture was appropriately modified to determine the effect of changing swainsonine concentration on α -mannosidase activity at pH 4.0 or the effect of

substrate or enzyme concentration on α -mannosidase activity in the presence of sufficient swainsonine to give approx. 60% inhibition at pH 4.0.

The reversibility of the inhibition of jack-bean α -mannosidase (pH 4.0) by swainsonine was tested by incubating 200 μ l of serial dilutions of an enzyme/swainsonine mixture with 100 μ l of 15 mM substrate in the pH 4.0 buffer described above. The undiluted mixture contained 50 nM-swainsonine which was sufficient to give approx. 60% inhibition of the enzyme preparation.

Determination of the pK_a of swainsonine

The pK_a was determined at the half-equivalence point after titration of an aqueous swainsonine solution (pH 8.9) against 0.01 M-HCl with a Radiometer Autoburette ABV 13 and TTT titrator linked to a Radiometer REC 61 Servograph and a PHM 64 pH meter.

Results and discussion

At a concentration of 20 μ M, swainsonine completely inhibited acid α -mannosidase (pH 4.0) from all mammalian tissues tested. It also completely inhibited acid α -mannosidase from the liver of the lamprey eel and the seeds of jack bean. On the other hand α -glucosidase, β -galactosidase, hexosaminidase and β -glucuronidase from mouse liver were not affected by 10 times the swainsonine concentration required for total inhibition of α -mannosidase at pH 4.0. In the presence of 20 μ M-swainsonine, the neutral form (pH 6.5) of α -mannosidase (in the absence of the acid form) was inhibited by approx. 60%.

Leaback (1968) suggested that a glycosyl ion intermediate is formed during hydrolysis of natural substrates by glycosidases and that inhibition of these enzymes by aldono-lactones is mediated by a similar cation. The pK_a of swainsonine is 7.4 and thus it would be fully ionized at pH 4.0 (the pH optimum of the lysosomal enzyme) and by using molecular models it can be seen that the relative positions of the cationic centre and the three hydroxy groups are similar to that of the hypothetical mannosyl ion. This may account for the apparent specificity of swainsonine for α -mannosidase.

Swainsonine will inhibit both acid and neutral α -mannosidases *in vitro*. But at the cellular level *in vivo* swainsonine will concentrate within lysosomes, since it would be almost fully ionized within the acid environment of this organelle and its outward passage across the lysosomal membrane would be greatly retarded. Within the lysosome it will have a major influence on acid α -mannosidase. Thus, in the affected animal, accumulation of oligosaccharides

and the production of a lysosomal storage disease would be the major pathological consequence of mannosidase inhibition. This does not exclude the possibility that, at very high doses, neutral α -mannosidase might be sufficiently affected to alter post-translational processing of glycoproteins.

On successive dilution of an acid α -mannosidase/swainsonine mixture, α -mannosidase specific activity increased (Fig. 2), indicating that the inhibitory effect of swainsonine was decreasing on dilution. This indicates that inhibition by swainsonine is reversible, which is supported by our observation that α -mannosidase inhibited by swainsonine can be reactivated by dialysis.

The reversible inhibition of acid α -mannosidase by swainsonine is quite complex. Fig. 3 presents results obtained by incubating jack-bean α -mannosidase with various concentrations of swainsonine. It shows a sigmoidal relationship between swainsonine concentration and α -mannosidase activity. Similar results were obtained whether the enzyme preparation was mouse liver supernatant, crude mouse liver α -mannosidase A or B prepared by the method of Phillips *et al.* (1974) or jack-bean α -mannosidase.

The jack-bean enzyme showed Michaelis-Menten behaviour with various substrate concentrations, whereas the partially inhibited enzyme deviated from this and exhibited parabolic curvature when results were presented by the method of Lineweaver & Burk (1934). The relationship between α -mannosidase

Fig. 2. Reversibility of α -mannosidase inhibition. Serial dilutions (200 μ l) of a mixture (1:1, v/v) of jack-bean α -mannosidase and water (●) or inhibitor solution (0.1 μ M) (■) were incubated with 15 mM-4-methylumbelliferyl α -D-mannopyranoside in pH 4.0 buffer (100 μ l). Activities (fluorescence units) were divided by relative enzyme concentration to give specific activities (arbitrary units).

Fig. 3. Effect of swainsonine concentration on α -mannosidase activity

Jack-bean α -mannosidase solution (100 μ l) was incubated with 100 μ l of 15 mM-4-methyl-umbelliferyl α -D-mannopyranoside in pH 4.0 buffer in the presence of 100 μ l of aqueous swainsonine solutions to give the required concentrations in the incubation media. Activities are direct fluorescence readings expressed as a percentage of an uninhibited assay.

activity and enzyme concentration was linear when the incubation mixture contained a constant substrate/inhibitor ratio.

A model that might explain certain aspects of the non-Michaelis-Menten behaviour of this enzyme describes the effect of active-site-directed effectors (Smith *et al.*, 1975). It relates to oligomeric enzymes that exist in solution in two forms in equilibrium. Phillips *et al.* (1974) presented evidence that acid

α -mannosidase does occur in two forms (A and B) and showed at least partial equilibrium between these two forms.

When considering the specificity of swainsonine and its inhibition of a wide range of acid α -mannosidases from mammals, primitive fish and plants, as well as its inhibition of both acid and neutral α -mannosidases, it seems more likely that swainsonine acts at the substrate site of the enzyme rather than at a highly conserved allosteric site. This suggestion is also supported by the steric similarities between swainsonine, mannose and the mannosyl ion. This leads to the conclusion that swainsonine is a specific reversible substrate site-directed inhibitor of α -mannosidase.

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