Studies on the Mechanism of Castanospermine Inhibition of α- and β-Glucosidases

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Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine) is an indolizidine alkaloid that was isolated from the Australian plant, Castanospermum australe (1). We previously reported that this alkaloid was a potent inhibitor of almond emulsin β-glucosidase, as well as of fibroblast lysosomal α- and β-glucosidases (2). However, castanospermine did not inhibit a number of other glycosidases, including α-mannosidase, α- or β-galactosidases, β-N-acetylhexosaminidase, β-glucuronidase, or α-L-fucosidase. Castanospermine was also found to be a potent inhibitor of glycoprotein processing by virtue of the fact that it inhibits glucosidase I. Thus, when influenza virus was grown in canine kidney cells in the presence of the alkaloid, the viral hemagglutinin no longer had the normal complement of the complex types of oligosaccharides. Instead, more than 85% of the oligosaccharides were of the structures Glc\textsubscript{3}Man\textsubscript{7-9}GlcNAc\textsubscript{2} (3).

The purpose of the present study was to examine the kinetics of castanospermine inhibition on both α- and β-glucosidases, and to determine whether this inhibition was of the competitive type. Castanospermine proved to be a competitive inhibitor of amylglucosidase when tested at both pH 4.5 and at pH 6.0. However, with the β-glucosidase, competitive inhibition...
was observed at pH 6.5, while at pH 5.0 the inhibition was of the mixed type. An examination of the effect of pH on the alkaloid inhibition indicated that castanospermine was a better inhibitor at higher pH values. These data suggest that the unprotonated form of the alkaloid is a more effective inhibitor. Experiments with the N-oxide of castanospermine support this idea.

EXPERIMENTAL PROCEDURES

Materials. The α-glucosidase used in this study was amylglucosidase (EC 3.2.1.3), an exo-1,4-α-glucosidase, obtained from Sigma Chemical Company. Almond emulsion β-glucosidase and the various p-nitrophenyl-glycosides were also purchased from Sigma. Castanospermine was isolated in 0.3% yield from seeds of *C. australe* by extraction with water and methanol, and was purified by chromatography on Dowex 50 W-X8 (1). Seeds were obtained from H. G. Kershaw Limited, Terrey Hills, 2084 N.S.W., Australia.

Synthesis of castanospermine N-oxide. Castanospermine (0.5 g) in ethanol (5 ml) was treated with 30% hydrogen peroxide (1 ml) and the solution was stirred at room temperature for 24 h. The white crystalline precipitate which formed was filtered off and recrystallized from methanol as glistening white prisms, mp 202-203°C (decomp.), (0.34 g, 63%). The chemical ionization mass spectrum showed a strong molecular ion at m/z 190 (38%), 188 (36%), 172 (65%), 170 (42%), and 152 (30%), corresponding to loss of oxygen and sequential loss of three molecules of water.

Enzyme assays. The enzymatic activities of both amylglucosidase and β-glucosidase were determined colorimetrically using the appropriate p-nitrophenyl-glucoside as substrate (i.e., p-nitrophenyl-α-D-glucoside for amylglucosidase and p-nitrophenyl-β-D-glucoside for the β-glucosidase). The typical assay mixture for these enzymes contained 5 μmol of the indicated p-nitrophenyl-glucoside, 10 μmol of sodium acetate or sodium citrate buffer, pH 5.0, and either 10 μg of amylglucosidase or 1 μg of β-glucosidase, all in a final volume of 0.5 ml. In the case of amylglucosidase, the usual incubation for measurement of activity was 20 min at 37°C, whereas with the β-glucosidase it was 10 min at 37°C. At the end of the incubation, 2.5 ml of 0.4 M glycine buffer, pH 10.4, was added to each tube to stop the reaction and to develop the color due to the released p-nitrophenol. The p-nitrophenol was then measured at 410 nm in a spectrophotometer.

Various modifications of the standard assay mixture were made in order to test the effects of castanospermine and its N-oxide. Thus, in some cases, enzyme, buffer, and varying amounts of alkaloid were preincubated in a test tube at room temperature for 3 to 5 min, and the reaction was initiated by the addition of the substrate (i.e., p-nitrophenyl-glucoside). In these cases, controls were prepared without substrate to be certain that no color developed at 410 nm from enzyme and inhibitor alone. Experiments with castanospermine and either amylglucosidase or β-glucosidase were also done at a variety of pH values in order to determine whether pH had any effect on the inhibition. Although a variety of buffers was used to cover the pH ranges, in many cases 10 mM phosphate buffer was used at the appropriate pH values from 3 to 7. In other experiments, the substrate concentration was continuously raised at fixed concentrations of castanospermine in order to ascertain whether inhibition was of a competitive or noncompetitive nature.

All of the studies shown in this paper were done under conditions where product formation was proportional to time and to the amount of protein added.

pKₐ determination. The pKₐ of castanospermine was measured at the half-equivalence point by titration of a 0.01 M aqueous solution against 0.01 M aqueous hydrochloric acid using a Metrohm Model 636 titroprocessor and Model E635 Dosimat autoburette with a Model E147 combination glass microelectrode. The electrode was standardized against pH 4 and pH 7 buffers at 29°C and the pKₐ values were measured at the same temperature.

RESULTS

Castanospermine Inhibition of Amyloglucosidase

To test the effect of castanospermine on an α-glucosidase, we chose amylglucosidase, which is an exo-1,4-α-glucosidase. This enzyme was quite sensitive to inhibition by castanospermine, with 50% inhibition requiring about 0.5 to 1 μg/ml of alkaloid. The activity of the amylglucosidase towards the artificial substrate, p-nitrophenyl-α-D-glucoside, was proportional to enzyme concentration to about 150 μg of protein, and to time of incubation to about 90 min (data not shown). Since preliminary experiments had indicated that the extent of castanospermine inhibition was greatly dependent on the pH of the incubation mixture, we first examined the effects of alterations in pH on the inhibition. Figure 1 presents the results of this experiment. In Fig. 1A, amylglucosidase activity was measured at various pH values from 3.0 to 7.0, in the absence
and presence of castanospermine. The pH optimum for the uninhibited enzyme was about 4.0 to 4.5, and activity fell off sharply above pH 5.0. On the other hand, in the presence of alkaloid at 1 μg/ml, enzyme activity was optimum at pH values of 3.5 to 4.0, and activity declined rapidly above pH 5.0. The dotted line in Fig. 1A summarizes the actual percentage of inhibition (i.e., the amount of inhibition compared to each control value) at each pH value. The data demonstrates that castanospermine is a much better inhibitor at pH values of 6.0 to 6.5 than it is at the lower pH ranges. The decrease in inhibition observed at pH 7.0 may be artifactual since enzyme activity is very low even in the controls at this pH. In this particular experiment, the pH curves were all done using phosphate buffer, but essentially the same results were obtained with citrate buffer, acetate buffer, or citrate-phosphate buffer.

Figure 1B shows a castanospermine concentration curve to indicate the amount of inhibition of amyloglucosidase at pH 4.5 or at pH 6.0. Again, inhibition was much more pronounced at the higher pH values. Thus, 50% inhibition of the enzyme required only about 1 μg/ml of alkaloid when the reactions were done at pH 6.0, but at pH 4.5 it required almost 3 μg/ml. These data support the idea that castanospermine is a better inhibitor at higher pH values.

In order to determine whether castanospermine was acting as a competitive inhibitor, we examined the effect of substrate concentration on the inhibition of amyloglucosidase. Since the above studies indicated that the pH of the incubation medium affected the castanospermine inhibition, those studies were done at pH values of 4.5 and 6.0. For these experiments, four different concentrations of castanospermine, ranging from 0.25 to 2.5 μg/ml, were used. The data from these experiments were plotted according to the method of Lineweaver and Burk as shown in Fig. 2. Figure 2A shows the results at pH 4.5, while Fig. 2B shows the results at pH 6.0. As demonstrated above, the inhibition was more extensive at pH 6.0 than at 4.5. Nevertheless, in both cases, the castanospermine inhibition was clearly of a competitive nature with regard to p-nitrophenyl-α-D-glucoside. The data were also plotted by the method of Dixon (4), and again the inhibition was competitive (data not shown). The $K_i$ value for castanospermine
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FIG. 2. Effect of substrate concentration on castanospermine inhibition of amyloglucosidase. Reaction mixtures were as described in the text but contained various amounts of $p$-nitrophenyl-$\alpha$-D-glucoside. Castanospermine was added to certain tubes in amounts varying from 0.1 to 1 $\mu$g/ml. The data was plotted by the method of Lineweaver and Burk. (A) The incubation mixtures were run at pH 4.5; (B) the incubation mixtures were run at pH 6.0.

The effect of variations in the pH of the incubation medium on the castanospermine inhibition of the enzyme is shown in Fig. 3. The pH optimum for this enzyme in the uninhibited state was about 5.0, while in the presence of 15 $\mu$g/ml of castanospermine, the pH optimum was about 4.0 to 4.5 (Fig. 3A). As with the amyloglucosidase, when the percentage of inhibition was plotted as a function of pH (dotted line), the greatest inhibition was observed at pH 6.0 to 6.5. Figure 3B shows the concentration curve of castanospermine as an inhibitor of $\beta$-glucosidase at pH 5.0 (optimum pH for the uninhibited enzyme) and at pH 6.5 (optimum pH for inhibition). At pH 6.5, 50% inhibition of the enzyme required less than 5 $\mu$g/ml of alkaloid, but at pH 5.0 almost 20 $\mu$g/ml were necessary. These results are similar to those with the amyloglucosidase.

In our preliminary studies with castanospermine and $\beta$-glucosidase, we examined the nature of the inhibition by Lineweaver-Burk plots and found the inhibition to be of the mixed type (2). However, those experiments were done at pH 5.0. In view of the above results showing differences in inhibition as a function of pH, we reexamined the substrate concentration curves at pH 6.5. In this experiment, four different castanospermine concentrations were used, ranging from 1 to 10 $\mu$g/ml. The results of this experiment were plotted by the method of Lineweaver and Burk as shown in Fig. 4. Clearly, in this case castanospermine was a competitive inhibitor of the $\beta$-glucosidase with respect to $p$-nitrophenyl-$\beta$-glucoside at pH 6.5. The $K_i$ for castanospermine inhibition calculated at pH 5.0 was about 20 $\mu$M, whereas at pH 6.5 it was about 10 $\mu$M.

Inhibition of Glucosidases by Castanospermine N-Oxide

The pH studies suggested that the nitrogen in the ring was important in the
inhibition, and that the more active form of the alkaloid might be that in which the nitrogen was in the unprotonated form. We have determined the pK for castanospermine and found it to be 6.09. Thus, at pH 6.0, 50% of the alkaloid should be present in the unprotonated form. However, since it was not possible to assay the glucosidases above pH 6.5, we could not tell whether the inhibitory activity would continue to increase above the pK value.

Further evidence to suggest that the nitrogen in the ring is important in inhibition is shown by the study in Fig. 5. In this experiment, the N-oxide of castanospermine was tested as an inhibitor of amylglucosidase and β-glucosidase, and its activity was examined over the pH range from 4.0 to 7.0. The N-oxide was considerably less active than was castanospermine, as evidenced by the fact that 40 μg/ml only inhibited amylglucosidase 60–70%, whereas 600 μg/ml were required to cause 50% inhibition of β-glucosidase. As indicated by the pH curves with the N-oxide (Fig. 5), alterations in the pH of the incubation mixture had relatively small effects on the inhibition. Thus, maximum inhibition in these studies was observed at pH values near the optimum for the enzymes rather than at the higher pH values. The substrate concentration curve of p-nitrophenyl-β-D-glucoside was examined in the presence of several concentrations of the N-oxide with the β-glucosidase. The N-oxide also proved to be a competitive inhibitor, and Dixon plots indicated a $K_i$ of 0.76 mM (data not shown).
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FIG. 5. Effect of pH on the inhibition of amylglucosidase and \( \beta - \)glucosidase by castanospermine N-oxide. Incubations were as described in the text with the appropriate \( p - \)nitrophenylglycosides and the pH ranges shown in the figure. The N-oxide of castanospermine was added in the amounts shown (40 µg/ml for amylglucosidase and 600 µg/ml for \( \beta - \)glucosidase).

Reversibility of Castanospermine Inhibition

In a previous study with castanospermine, we found that, when \( \beta - \)glucosidase was mixed with an amount of castanospermine sufficient to inhibit all of the enzymatic activity, nearly all of this activity could be recovered when the enzyme mixture was serially diluted with buffer. This suggested that the inhibition by castanospermine was reversible. A similar experiment was done with amylglucosidase. Thus, enough castanospermine was added to a solution of amylglucosidase to completely inhibit this enzyme. This enzyme mixture was then subjected to serial dilution and each dilution was assayed for activity. A similar mixture of enzyme, but without castanospermine, served as a control and was diluted and assayed in the same way. The results of this experiment demonstrated that the activity could be almost completely recovered upon dilution (i.e., based on the specific activity in each diluted preparation), indicating that dilution of the inhibitor reversed its effects. We also found that castanospermine could be removed from either amylglucosidase or \( \beta - \)glucosidase by dialysis. Thus, when either of these enzymes were mixed with enough alkaloid to cause 50% inhibition and the mixture was placed in dialysis bags, almost all of the enzymatic activity could be recovered after dialysis for 4 h. Thus, the inhibition of these enzymes by castanospermine appears to be completely reversible.

DISCUSSION

A widely accepted theory to explain the mechanism of glycosidase action maintains that a general acid catalysis is promoted by the presence of an acidic group at the active site of the enzyme (5). Additional evidence for the presence of a negatively charged group at the active site was the finding that glycosidases were strongly inhibited by glucosylamines corresponding to the sugar specificity of the enzyme (6). In that study, the authors found that the basic or cationic form of the sugar, as conferred by the presence of an amine at the anomeric carbon, bound 220–4000 times more tightly to the active site than the neutral counterparts. The authors speculate that the increased affinity is the result of an interaction of the glycosidic amine with a negatively-charged group at the active site.

Using conduritol-\( \beta - \)epoxide, Legler deduced that the active site of almond \( \beta - \)glucosidase contains a carboxylate anion (7). In another study using \( N - \)benzyl-\( \beta - \)glucosyloseperidine, a pH-dependent inhibition of the \( \beta - \)glucosidase was observed, with maximum inhibition at pH 6.0–6.5 (8). Legler concluded that \( \beta - \)glucosidases have a carboxylate anion at the active site that protonates and stabilizes the binding of basic inhibitors.

In this paper, we found that castanospermine was a better inhibitor at higher pH for both almond \( \beta - \)glucosidase and amylglucosidase. Thus, it is attractive to postulate that the activity of castanospermine is affected by the ionization state of the indolizidine nitrogen, which would tend to be less charged at higher pH values. At the lower pH values, the nitrogen would be protonated and thus could be partially repelled by the presence of a protonated base-carboxylate ion pair, or could simply be unable to undergo protonation by the
active site. Using an \( N-\beta \)-glucosylpyridinium ion and \( N-\beta \)-glucosylimidazole, both of which are \( \beta \)-glucosyl derivatives bearing a permanent positive charge and are thus much weaker inhibitors than the neutral analogs, Legler concluded that the protonation of the inhibitor by \( \beta \)-glucosidases stabilizes the binding of basic inhibitors (8). Another possible explanation for the results reported here is that there is an ionizable group on both enzymes that has the same \( pK \), and this group, although not actually involved in catalysis, is close enough to the active site to hold the protonated castanospermine to the enzyme. An imidazole group would have a \( pK \) in that range.

Of course, interpretation of \( pH \) effects must be done with caution since a change in \( pH \) might also affect the ionization state of the active site. However, the observed effects do not appear to be caused by alterations in enzyme structure, since (i) the \( K_m \)'s of \( \beta \)-glucosidase and amylglucosidase were not affected by alterations in \( pH \); (ii) while the \( pH \) optimum for the \( \beta \)-glucosidase is different than that for amylglucosidase, both enzymes are more strongly inhibited by castanospermine at high \( pH \); and (iii) the \( pH \) effects were not seen with the N-oxide of castanospermine where the nitrogen has a permanent positive charge.

Castanospermine was found to be a competitive inhibitor of both amylglucosidase and almond \( \beta \)-glucosidase when these enzymes were assayed with the appropriate \( p \)-nitrophenyl-\( \beta \)-D-glucoside substrates. Although the absolute configuration of castanospermine has not been determined and the X-ray diffraction studies only defined the relative stereochemistry, the configuration of the hydroxyl groups are probably analogous to that of D-glucose. In addition, this configuration for castanospermine would also be similar to that for nojirimycin and deoxynojirimycin. These latter two compounds are also inhibitors of \( \alpha \)- and \( \beta \)-glucosidases (9). Interestingly enough, \( \beta \)-glucosidases are generally inhibited better by nojirimycin than by the deoxyderivative, whereas the reverse is true for the \( \alpha \)-glucosidases (10). Nevertheless, nojirimycin was also shown to be a competitive inhibitor of \( \beta \)-glucosidase when the enzyme was assayed with the \( p \)-nitrophenyl-\( \beta \)-D-glucoside (11).

Several other glucosidase inhibitors have recently been reported. These include conduritol-B-epoxide (7), bromoconduritols A and B (12), and acarbose (13, 14). Recently, 1,5-dideoxy-1,5-imino-D-mannitol was isolated from higher plants and shown to be a good inhibitor of \( \beta \)-glucosidase, \( \alpha \)-glucosidase, and insect trehalase. This compound also proved to be a competitive inhibitor of \( \beta \)-glucosidase (15). The structure of this compound resembles that of deoxynojirimycin except that it is the 2 epimer. This would suggest that the configuration of hydroxyls at carbons 3 and 4 and the presence of a nitrogen in the ring are necessary for glucosidase inhibition.

The fact that castanospermine is more effective at higher \( pH \) values probably helps to explain the fact that this alkaloid is a good inhibitor of glucosidase I, the neutral enzyme that is involved in glycoprotein processing (3). As a result of these studies, we also examined the effect of \( pH \) on the inhibition of jack bean \( \alpha \)-mannosidase by swainsonine (16, 17). This indolizidine alkaloid has been reported to have a \( pK \) of 7.4 and to be a better inhibitor of lysosomal \( \alpha \)-mannosidase at lower \( pH \) values (18). However, we found that swainsonine was a better inhibitor of jack bean \( \alpha \)-mannosidase at \( pH \) 5.5–6.0 than it was at \( pH \) 4.0–4.5. Again, this may help explain the finding that swainsonine is a potent inhibitor of the processing mannosidase II (19) and thus inhibits the normal processing of glycoproteins in various cell systems (20–25).

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REFERENCES


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