Biochemical Pharmacology, Vol. 36, No. 14, pp. 2381–2385, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

CASTANOSPERMINE: AN APPARENT TIGHT-BINDING INHIBITOR OF HEPATIC LYSOSOMAL ALPHA-GLUCOSIDASE

BRENT R. ELLMERS, BARRY L. RHINEHART and KEITH M. ROBINSON*
Merrell Dow Research Institute, Indianapolis, IN 46268-0470, U.S.A.

(*Received* 27 *October* 1986; *accepted* 23 *December* 1986)

Abstract—Castanospermine is a potent inhibitor of rat hepatic lysosomal α-glucosidase *in vitro*. The alkaloid showed time-dependent inhibition with an IC_{50} of 5×10^{-6} M without preincubation and 1×10^{-7} M with 1 hr of preincubation. Inhibition appeared competitive without preincubation but noncompetitive after preincubation. The time-dependent inactivation of the enzyme followed pseudo-first-order kinetics with an inactivation constant of 1.2×10^3 M^{-1} sec^{-1}. The apparent irreversibility of enzyme inhibition by castanospermine is postulated to be by tight-binding inhibition.

Lysosomal α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) is an exoglucosidase that has both α-1,4- and α-1,6-glucosidase activities. This enzyme plays an important role in glycogen disposal by catalyzing the hydrolysis of lysosomal glycogen [1]. Absence of this enzyme, as occurs in Pompe's disease, results in lysosomal glycogen accumulation in most cell types [2].

Castanospermine (CS†; 1,6,7,8-tetrahydroxyoctahydroindolizine, Fig. 1) is an alkaloid found in the seeds of the Australian tree *Castanospermum australe* [3, 4]. This alkaloid inhibits glucosidases from a variety of plant and animal sources [5–8]. However, limited information is available on the kinetics of enzyme inactivation. CS has been reported to demonstrate a reversible, competitive inhibition with amyloglucosidase and with almond β-glucosidase [5] and a noncompetitive inhibition with Caco-2 cell sucrase [6].

Administration of CS to rats markedly reduces liver lysosomal α-glucosidase activity, causing excessive deposition of glycogen in hepatocyte lysosomes [7]. Recovery of lysosomal α-glucosidase activity requires several days, suggesting that either enzyme synthesis is inhibited or enzyme inhibition is not readily reversible. In the present study, we have investigated CS inhibition of liver lysosomal α-glucosidase *in vitro* in an effort to explain the effects on liver lysosomal α-glucosidase activity observed *in vivo*.

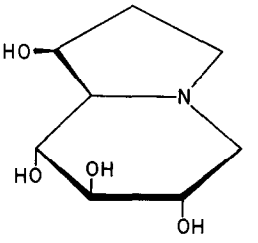

Fig. 1. Structure of castanospermine (1,6,7,8-tetrahydroxyoctahydroindolizine).

METHODS

CS was isolated from seeds of *Castanospermum australe* by extraction with 2-propanol and was purified to homogeneity by chromatography on Dowex 50-X8 and Dowex 1-X8 ion-exchange resins [8]. *p*-Nitrophenyl-α-D-glucopyranoside was purchased from the Sigma Chemical Co., St. Louis, MO. All other chemicals were from commercial sources. Lysosomal α-glucosidase was isolated from livers of male Sprague–Dawley rats (Harlan Industries Ltd., Indianapolis, IN) by the procedure of Dissous *et al.* through the ammonium sulfate precipitation step [9], and then stored at −20°. The enzyme was characterized by its ability to hydrolyze maltose to glucose and by its pH optimum. Free glucose was determined by a glucose dehydrogenase method (Seragen Diagnostics, Indianapolis, IN). The specific activity of the lysosomal α-glucosidase preparations used in this study ranged from 20 to 40 mUnits/mg protein with *p*-nitrophenylglucoside as substrate. One unit of enzyme is the amount necessary to hydrolyze 1 μmol substrate per min. Protein was determined by the method of Lowry *et al.* [10].

In the studies reported here, enzyme activity was determined with *p*-nitrophenyl-α-D-glucoside in a final volume of 0.6 ml of 0.1 M sodium acetate, 25 mM KCl, pH 4.2. All reactions were incubated at 37° for 30 min unless otherwise stated. Reactions were terminated by heating at 90°. Insoluble protein was removed by centrifugation. One milliliter of 0.1 M Na_2CO_3 was added to the supernatant fraction,

* All correspondence should be sent to: Keith M. Robinson, Ph.D., Merrell Dow Pharmaceuticals Inc., 2110 E. Galbraith, Cincinnati, OH 45215.

† Abbreviations: CS, castanospermine; IC_{5u}, inhibitor concentration that reduces enzyme activity 50%; and K_{is}, dissociation constant of enzyme–inhibitor complex.

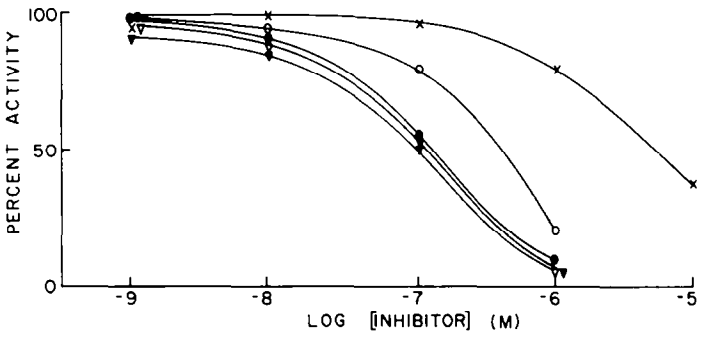

Fig. 2. Castanospermine inhibition of lysosomal α-glucosidase. Enzyme and inhibitor were preincubated for 0 (×), 30 (○), 60 (●), 90 (▽), or 120 (▼) min at 37° before adding *p*-nitrophenyl-α-D-glucoside. Values are presented as a percent of the enzyme activity assayed with no castanospermine and after the appropriate period of preincubation. This activity was 55–66 nmol product/hr/assay tube. Substrate concentration was 5 mM. Lines were visually fit to the data.

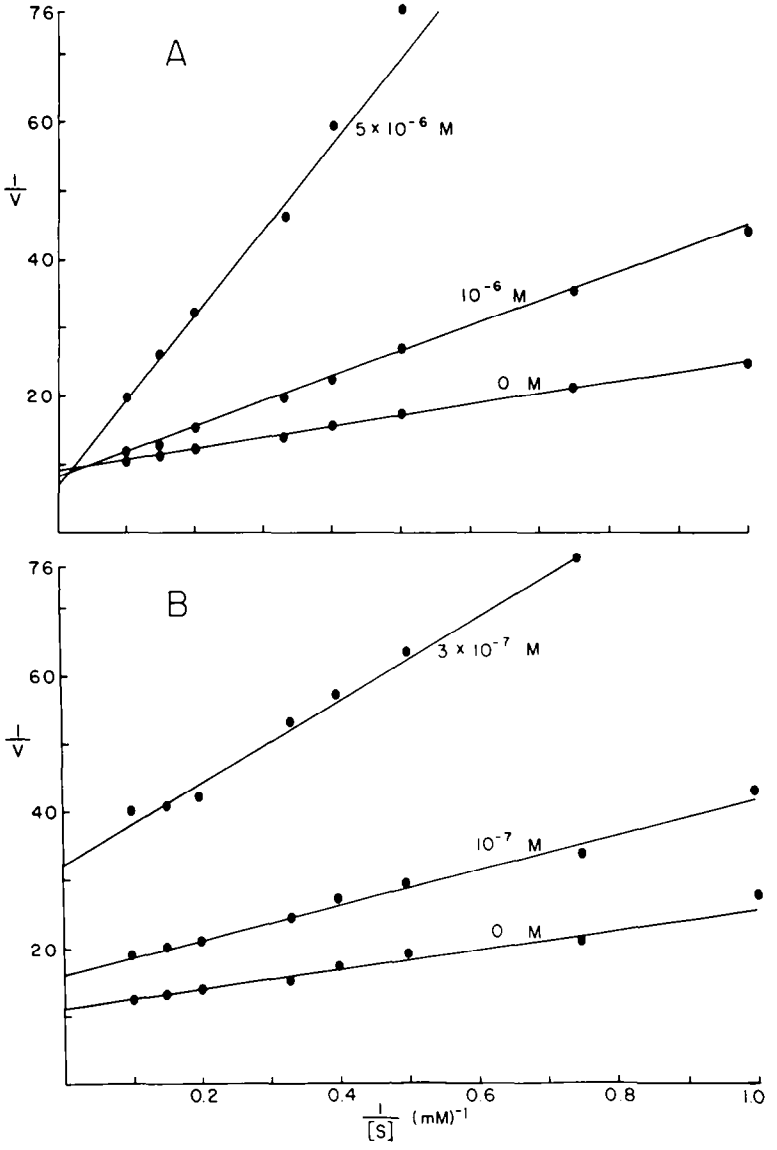

Fig. 3. Double-reciprocal plots of enzyme activity in the presence of castanospermine without (A) and with (B) 60 min of preincubation at 37°. *p*-Nitrophenyl-α-D-glucoside concentrations were 1.0, 1.3, 2.0, 2.5, 3.3, 5.0, 6.7 and 10 mM. Castanospermine concentrations are shown in the figure. Velocity (V) is expressed in nmol substrate hydrolyzed per min. Data were analyzed using the computer program of Cleland [13].

and liberated *p*-nitrophenol was determined by absorption at 410 nm. Enzyme activity was proportional to protein concentration and time of incubation.

In preincubation experiments, the enzyme was mixed with inhibitor, incubated at 37°, and the reaction started by substrate addition. Otherwise, the reactions were started by enzyme addition.

RESULTS

Partially purified rat liver lysosomal α-1,4-glucosidase showed a pH optimum of 4.2 and a K_m for maltose of 3.8 mM, values that agree closely with those of Jeffrey *et al.* [11, 12]. The K_m for *p*-nitrophenylglucoside was 1.9 mM. CS inhibited lysosomal α-glucosidase with an IC_{50} of 5×10^{-6} M (Fig. 2). Preincubation of enzyme with inhibitor decreased the IC_{50} to 1×10^{-7} M. This time-dependent inhibition required approximately 60 min at 37° to reach a maximum.

The type of inhibition was examined by determining enzyme activity at various substrate and inhibitor concentrations. Without preincubation, CS behaved as a competitive inhibitor of lysosomal α-glucosidase with an apparent K_{is} of 0.9×10^{-6} M (Fig. 3A). Competitive inhibition suggests that CS interacts at the active site of the enzyme. However, when incubated with enzyme for 60 min before adding substrate, CS behaved as an apparent noncompetitive inhibitor with a K_{is} of 1.2×10^{-7} M (Fig. 3B). Apparent noncompetitive inhibition under these conditions suggests a very slow dissociation of the enzyme–inhibitor complex [14].

To further investigate the CS inhibition of lysosomal α-glucosidase, a reaction progress curve was determined as described by Morrison [15]. In the absence of CS, the reaction followed first-order kinetics. In the presence of CS but without preincubation, the reaction rate for the first 30 min was almost the same as without CS but slowed as the reaction continued, again demonstrating a very slow interaction between enzyme and inhibitor (Fig. 4A).

In an effort to examine reversal of enzyme inhibition, the enzyme was preincubated for 60 min with the same concentration of inhibitor, dialyzed overnight to remove excess inhibitor, and incubated with substrate (Fig. 4B). No recovery of enzyme activity was observed as the reaction proceeded, demonstrating as does Fig. 3B that substrate does not readily displace the inhibitor. Tris, another inhibitor of α-glucosidase, was used in an effort to displace CS from the enzyme. The enzyme was incubated for 60 min with 10^{-6} M CS, dialyzed 24 hr with 0.1 M Tris acetate, pH 4.0, and then dialyzed overnight against the sodium acetate buffer to displace the Tris. No recovery of enzyme activity was observed in the CS-treated preparation, whereas the control preparation retained 65% of the original activity. In the next experiment we attempted to reverse the inhibition by treating the enzyme–inhibitor complex to a more exhaustive dialysis. After incubation of enzyme with 10^{-6} M inhibitor for 1 hr, the mixture was dialyzed for 6 days against four changes of 3 liters of the acetate buffer. Prior to dialysis, the enzyme–CS preparation had 15% of the activity in a similar preparation without inhibitor. After dialysis, the activity of the enzyme–CS preparation remained

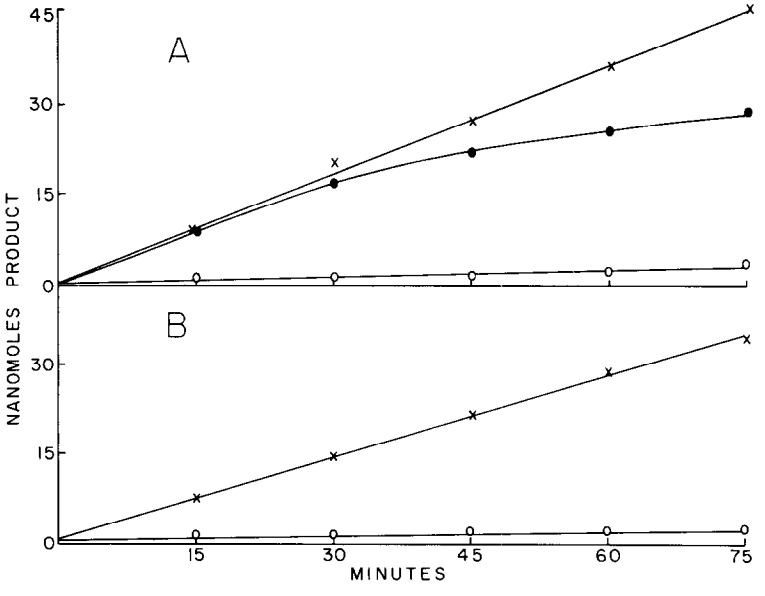

Fig. 4. Reaction progress curves. (A) No inhibitor (×), 1×10^{-6} M castanospermine without preincubation (●), or 1×10^{-6} M castanospermine preincubated for 60 min at 4° (○). (B) No inhibitor and dialyzed 24 hr against acetate buffer (×), 1×10^{-6} M castanospermine preincubated for 60 min at 4° and then dialyzed for 24 hr against four changes of 1 liter of acetate buffer to remove unbound inhibitor (○). *p*-Nitrophenyl-α-D-glucoside concentration was 5 mM. For each experimental condition, reactions were started by substrate addition. Aliquots were taken at 0, 15, 30, 45, 60 and 75 min, and the amount of product was determined.

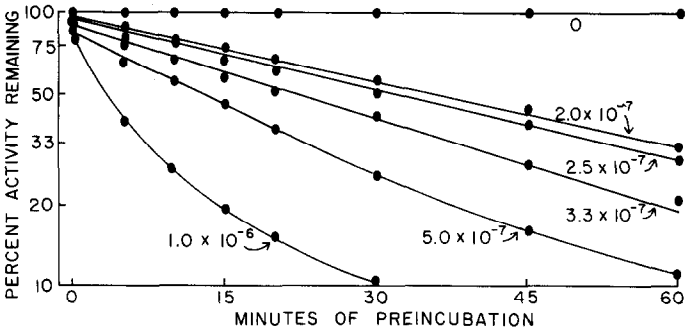

Fig. 5. Time-dependent inactivation of lysosomal α-glucosidase by castanospermine. Enzyme and inhibitor were preincubated for 5, 10, 15, 20, 30, 45, or 60 min at 37° before the addition of 5 mM *p*-nitrophenyl-α-D-glucoside. Values are presented as a percent of the enzyme activity assayed with no castanospermine and after the appropriate period of preincubation. This activity was 28–30 nmol product/hr/assay tube. Lines were visually fit to the data.

15% of the control preparation; the control retained 93% of its original activity. These results suggested that CS acts as an irreversible inhibitor when allowed sufficient time to interact with the enzyme.

To determine the kinetics of enzyme inactivation, enzyme and CS were incubated at various inhibitor concentrations, and enzyme was determined as a function of time. Inactivation of the enzyme by CS followed pseudo-first-order kinetics (Fig. 5). A plot was constructed of $T_{\frac{1}{2}}$ (time required for 50% inactivation) versus inhibitor concentration (Fig. 6). From these data the first-order inactivation constant ($K = 0.693/\text{slope}$) was calculated to be $1.2 \times 10^3\ M^{-1}\ sec^{-1}$.

DISCUSSION

The present results show that CS is a potent inhibitor of rat hepatic lysosomal α-glucosidase *in vitro*. The inhibition was time dependent (Figs. 2 and 4A), indicating slow interaction between inhibitor and enzyme. This slow interaction probably occurred at the active site of the enzyme (Fig. 3A), and once it occurred the inhibitor apparently bound very tightly. Attempts to reverse the inhibition by dialysis and by substrate displacement of inhibitor were unsuccessful (Figs. 3B and 4B). The inability to recover enzyme activity after CS treatment implies a possible covalent modification of the enzyme. However, with our current understanding of the mechanism of action of glucosidases [16], the structure of CS does not favor a covalent interaction. Alternatively, both are consistent with a strong noncovalent interaction. Strong noncovalent interactions between enzymes and inhibitors have been described by Williams and Morrison [17] and by Cha [18]. These inhibitors are called tight-binding inhibitors. The distinction between tight-binding and irreversible inhibitors is generally based upon reversibility of inhibition. Without direct evidence demonstrating reversibility of inhibition, we can only postulate that CS is a very tight-binding inhibitor of lysosomal α-glucosidase.

Tight-binding inhibition of an enzyme could result from the formation of a stable transition-state analog at the active site of the enzyme [16]. The protonated tertiary amine of CS could substitute for the carbonium ion generated in the transition-state of the substrate and interact with the carboxylate residue believed to be present at the active site of the enzyme [19]. The interaction between the amine and the carboxylate residue together with other binding forces such as hydrogen-bonding, van der Waals

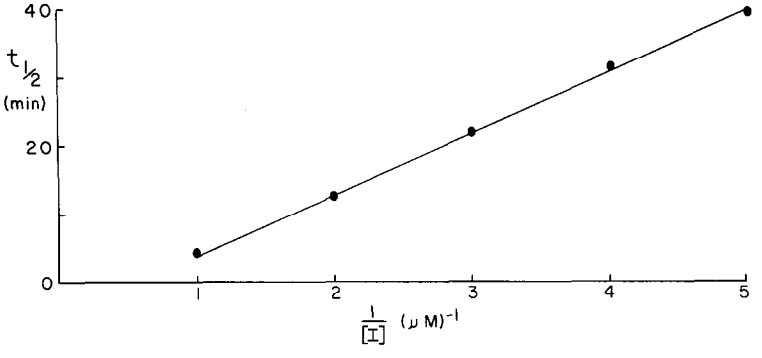

Fig. 6. Plot of time required for 50% inactivation versus reciprocal of inhibitor concentration. Values for $T_{\frac{1}{2}}$ were obtained from the curves in Fig. 5 by visually interpolating the 50% activity for each line.

forces and, possibly, conformational changes could stabilize the enzyme–inhibitor complex to the extent that dissociation of the complex would be very slow and inhibition would appear irreversible. Tight binding of CS to an α-glucosidase is consistent with the observation that CS is a time-dependent inhibitor of sucrase isolated from Caco-2 cells and shows an apparent noncompetitive inhibition when preincubated with the enzyme [6]. Tight binding of CS can also explain its prolonged action on lysosomal α-glucosidase observed *in vivo* [7].

In conclusion, our data indicate that castanospermine is a tight-binding inhibitor of lysosomal α-glucosidase which behaves like an irreversible inhibitor. We suggest that the loss of lysosomal α-glucosidase activity in CS-treated rats is the result of tight-binding inhibition of the enzyme by CS.

Note added in proof: J. P. Chambers and A. D. Elbein, *Enzyme* **35**, 53 (1986) have recently demonstrated a similar potency of inhibition for CS with the human enzyme.

REFERENCES

1. H. G. Hers and T. de Barsy, in *Lysosomes and Storage Diseases* (Eds. H. G. Hers and F. Van Hoof), pp. 197–216. Academic Press, New York (1973).
2. R. R. Howell and J. C. Williams, in *Metabolic Basis of Inherited Diseases* (Eds. J. B. Stunbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein and M. S. Brown), pp. 141–66. McGraw-Hill, New York (1983).
3. R. C. Bernotas and B. Ganem, *Tetrahedron Lett.* **25**, 165 (1984).
4. L. D. Hohenschutz, E. A. Bell, P. J. Jewess, D. P. Leworthy, R. J. Pryce, E. Arnold and J. Clardy, *Phytochemistry* **20**, 811 (1981).
5. R. Saul, R. J. Molyneux and A. D. Elbein, *Archs. Biochem. Biophys.* **230**, 668 (1984).
6. G. Trugnan, M. Rousset and A. Zweibaum, *Fedn Eur. Biochem. Soc. Lett.* **195**, 28 (1986).
7. R. Saul, J. J. Ghidoni, R. J. Molyneux and A. D. Elbein, *Proc. natn. Acad. Sci. U.S.A.* **82**, 93 (1985).
8. R. Saul, J. P. Chambers, R. J. Molyneux and A. D. Elbein, *Archs. Biochem. Biophys.* **221**, 593 (1983).
9. C. Dissous, J. F. Ansart, A. Cheron and J. Krembel, *Analyt. Biochem.* **116**, 35 (1981).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. P. L. Jeffrey, D. H. Brown and B. I. Brown, *Biochemistry* **9**, 1403 (1970).
12. P. L. Jeffrey, D. H. Brown and B. I. Brown, *Biochemistry* **9**, 1416 (1970).
13. W. W. Cleland, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan) Vol. 63, pp. 103—38. Academic Press, New York (1979).
14. S. Cha, R. P. Agarwal and R. E. Parks, *Biochem. Pharmac.* **24**, 2187 (1975).
15. J. F. Morrison, *Trends biochem. Sci.* **7**, 102 (1982).
16. P. LaLegerie, G. Legler and J. M. Yon, *Biochimie* **64**, 977 (1982).
17. J. W. Williams and J. F. Morrison, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 63, pp. 437–67. Academic Press, New York (1979).
18. S. Cha, *Biochem. Pharmac.* **24**, 2177 (1975).
19. H. Matsui, M. Sasaki, E. Takemasa, T. Kaneta and S. Chiba, *J. Biochem. Tokyo.* **96**, 993 (1984).