0273-2289/91/3003—0325$02.60

Inhibitors of Xylose Reductase from the Yeast *Pichia stipitis*

STEVEN R. WEBB AND HUNG LEE*

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

Received October 13, 1990; Accepted January 8, 1991

ABSTRACT

Several compounds were examined for their inhibitory effects on xylose reductase from the yeast *Pichia stipitis* NRC 2548. Mercuric chloride, cupric chloride, menadione sodium bisulfite, and sodium bisulfite inhibited enzyme activity in a sigmoidal dose-dependent manner, whereas quercetin and rutin were observed to have nonsigmoidal dose-response curves. Diphenylhydantoin, hydantoin, and valproic acid had no effect on xylose reductase activity. Mercuric chloride was the most potent inhibitor tested, with an IC_{50} (the concentration that inhibited enzyme activity by 50%) of $4.7 \times 10^{-6} M$. Three distinct inhibition patterns were observed amongst selected inhibitors. Mercuric chloride and quercetin were noncompetitive inhibitors of xylose reductase with respect to substrate and cofactor. Sodium bisulfite was an uncompetitive inhibitor with respect to substrate and cofactor, whereas menadione sodium bisulfite was a competitive inhibitor with respect to substrate, but noncompetitive to the cofactor.

Index Entries: Aldose reductase; inhibition; xylose reductase; D-xylose fermentation; yeast.

INTRODUCTION

The aldopentose D-xylose is second only to D-glucose in natural abundance and represents a major portion of all sugars in plant biomass (*1*). Significant quantities of D-xylose are found in the environment as agricultural and forestry residues and in some effluents of pulp and paper mills

*Author to whom all correspondence and reprint requests should be addressed.

(*1*). There is considerable interest in microorganisms that can utilize these wastes as substrates for bioconversion to industrially important liquid fuels and chemicals. The discovery of D-xylose-fermenting yeasts in the early 1980s (*2,3*) has enhanced the viability of a biomass conversion scheme.

D-Xylose metabolism in yeasts proceeds initially via the D-xylose-inducible enzyme system (*4*). The first cytoplasmic enzyme, NAD(P)H-dependent xylose reductase, reduces D-xylose to xylitol, which is then oxidized by the NAD^+-dependent xylitol dehydrogenase to D-xylulose. As the first enzyme in the pathway, xylose reductase plays a regulatory role in D-xylose metabolism. The activity of this enzyme has been shown to control the rate of D-xylose utilization. For example, mutant strains of *Pachysolen tannophilus* and *Pichia stipitis* deficient in NADPH-dependent xylose reductase displayed significantly reduced growth rates on D-xylose when compared to wild-type strains (*5,6*). In addition, the ability of certain yeasts, such as *P. tannophilus* (*7*), *Candida shehatae* (*8*), and *P. stipitis* (*9*), to ferment D-xylose anaerobically has been suggested to result from the dual NADH or NADPH cofactor requirement of their xylose reductases (*10*).

Although xylose reductases have been purified from several yeasts and some of their properties characterized, little is known about their catalytic mechanism or nature of the active site. Some researchers have classified yeast xylose reductases as members of aldose reductases (alditol: $NAD(P)^+$ 1-oxidoreductase, E.C. 1.1.1.21) (*7,9*), enzymes that are extensively distributed in mammalian systems (*11*). The activity of these enzymes, which reduces D-glucose to sorbitol, has been implicated in serious human diabetic complications (*11,12*) and in Duchenne muscular dystrophy (*13*). Therefore, there is considerable interest in properties of mammalian aldose reductases, with the objective of developing more specific and potent inhibitors for the pharmacological management of these complications (*11*).

The relationship between yeast xylose reductase and mammalian aldose reductases is of interest from an evolutionary viewpoint. This study identified some chemical inhibitors and determined their inhibition patterns on xylose reductase from the yeast *P. stipitis* NRC 2548. Some of these compounds are also potent inhibitors of aldose reductases from mammalian sources.

MATERIALS AND METHODS

Chemicals

Quercetin (3,3′,4′,5,7-pentahydroxylflavone), rutin (quercetin 3β-D-rutinoside; 95% purity), menadione sodium bisulfite (2-methyl-1,4-naphthoquinone sodium bisulfite; 95% purity), diphenylhydantoin (5,5-diphenylhydantoin), hydantoin (2,4-imidazolidinedione), and valproic acid

(2-propylpentanoic acid) were obtained from Sigma Chemical Company, St. Louis, Mo. Sodium bisulfite (sodium metabisulfite) was obtained from Fisher Scientific, Toronto, Ontario, Canada. All other chemicals were of reagent grade and were obtained commercially.

Microorganism

Pichia stipitis NRC 2548 was kindly supplied by R. K. Latta, National Research Council Culture Collection, Ottawa, Ontario, Canada. It was maintained on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, Mich, USA) at 4°C and subcultured prior to growth in a liquid medium.

Preparation of Inocula

A single colony of *P. stipitis* grown at 30°C for 48 h on a PDA plate was transferred to a 125-mL Erlenmeyer flask containing 20 mL of 0.67% (w/v) yeast nitrogen base (YNB, Difco) without amino acids and 2% (w/v) glycerol (*14*). The YNB without amino acids and the glycerol were sterilized separately by filtration through a 0.45 μm-pore-size membrane. The culture was kept at 30°C for 48 h and shaken at 200 rpm on a New Brunswick gyratory incubator shaker.

Preparation of Cell-Free Extract

Two 250-mL flasks, each containing 100 mL of 0.67% (w/v) YNB without amino acids and 4% (w/v) D-xylose, were set up. Each flask was inoculated with 1 mL of inoculum and the cultures were incubated as described above. The YNB without amino acids and the D-xylose were sterilized separately by filtration, as above. After 24 h, the cells were harvested by centrifugation at 13,000*g* for 10 min using a Sorvall refrigerated centrifuge (GSA rotor). The cell pellet was washed in ice-cold 250 m*M* potassium phosphate buffer (pH 7.0) and centrifuged as above, after which the pellet was stored at −10°C until analysis.

The cell pellet was thawed and resuspended in sufficient ice-cold 250 m*M* potassium phosphate buffer (pH 7.0) containing 5 m*M* EDTA to form a thin paste. About 1.5 g of alumina was added and the suspension was subjected to 12–15 bursts of sonication (30 s separated by 30 s of cooling in an ice-water bath) using a Fisher Model 300 Dismembrator equipped with a microprobe at the maximum power. After sonication, cell debris and fragments were removed by centrifugation in a Sorvall refrigerated centrifuge at 13,000*g* for 10 min (SS34 rotor). The supernatant was further clarified by centrifugation at 30,000*g* for 1 h (*15*).

Assay of Xylose Reductase Activity

Xylose reductase activity was measured spectrophotometrically using a Philips model PU8620 spectrophotometer at 23°C. The reaction mixture

(1 mL) contained 250 m*M* potassium phosphate buffer (pH 7.0), 0.1 m*M* NADPH, and 100 m*M* D-xylose or 50 m*M* DL-glyceraldehyde. The reaction was initiated by adding 16–17 μg of protein, which was determined according to the method of Bradford with bovine serum albumin as standard (*16*). The activity was measured by the initial rate of decrease in absorbency at 340 nm.

Effect of Enzyme Inhibitors

The following compounds were tested for their effect on xylose reductase activity: quercetin, rutin, diphenylhydantoin, hydantoin, menadione bisulfite, sodium bisulfite, valproic acid, mercuric chloride, and cupric chloride. Concentrated solutions of quercetin, rutin, diphenylhydantoin, and hydantoin were prepared by dissolving these compounds in absolute ethanol. When added to the reaction mixture, concentration of ethanol was 5% (v/v) and, in these experiments, enzyme activity in the presence of 5% (v/v) ethanol served as controls. Menadione sodium bisulfite, sodium bisulfite, valproic acid, mercuric chloride, and cupric chloride were dissolved in deionized water. The effect of inhibitors was determined by including in the reaction mixture the test compound at specified concentrations. Enzyme activities were determined at several fixed concentrations of quercetin, menadione sodium bisulfite, sodium bisulfite, and mercuric chloride at various substrate (D-xylose or DL-glyceraldehyde) or cofactor (NADPH) concentrations.

Data Analysis

Substrate-dependency data were presented as Lineweaver-Burke plots. The lines were drawn by least-squares linear regression. The apparent kinetic parameters (V_{max}, K_m, K_i, K_{iE}, K_{iES} [E = enzyme; ES = enzyme substrate complex]) were calculated according to Engel (*17*) and are expressed as mean ± SEM. All experiments were repeated two to five times. In each instance, similar enzyme-inhibition trends were observed.

RESULTS AND DISCUSSION

Previous studies have shown that *P. stipitis* contains a single xylose reductase protein that can use either NADPH or NADH as the cofactor (*9,18*). This was confirmed by Hagedorn and Ciriacy (*6*), who also showed that xylose reductase activity arises from a single gene in this yeast. However, analysis of revertants of *P. stipitis* mutants deficient in xylose reductase revealed the existence of two genes encoding this enzyme, one of which was apparently cryptic (*6*). Xylose reductase of *P. stipitis* is composed of two subunits with a total molecular mass of 64 kDa (*6,9,18*). The enzyme is active with various aldose substrates, including DL-glyceralde-

hyde (K_m, 18 m*M*; V_{max}, 51.8 μmol/min/mg) and D-xylose (K_m, 42 m*M*; V_{max}, 23.2 μmol/min/mg) (*9*).

In the present study, xylose reductase activity in cell-free extract was stable for over 1 mo when stored frozen at −10°C. The activity was also found to be higher on DL-glyceraldehyde than on D-xylose, in agreement with previous studies (*9,18*). Moreover, the enzyme had considerably higher affinity for DL-glyceraldehyde (K_m, 3.84±0.47 m*M*) than for D-xylose (K_m, 39.05±1.64 m*M*). Substrate-dependence studies revealed that the enzyme displayed Michaelis-Menten kinetics for D-xylose, DL-glyceraldehyde, and NADPH. Although the functional enzyme is a dimer (*9,18*), there was no evidence of allosteric interaction between the two subunits.

The flavanoids quercetin and rutin are mammalian aldose reductase inhibitors (*19,20*), and both were found to inhibit yeast xylose reductase activity in a nonsigmoidal fashion (Fig. 1A). The extent of inhibition increased with increasing inhibitor concentrations. The maximum flavanoid concentrations investigated were $10^{-4}M$, because at higher levels both quercetin and rutin formed yellow precipitates.

Quercetin was more inhibitory than rutin at all the concentrations tested, although they are structurally similar compounds. The lower potency of rutin may be related to the presence of the disaccharide rutinose at the 3-OH position. In a study of the inhibitory potency of quercetin and its derivates on rat lens aldose reducatase, Varma and Kinoshita (*19*) found that the presence of carbohydrate groups at the 3-OH position led to reduced inhibition of enzyme activity. Similarly, Nazarova et al. (*21*) reported that quercetin was a more potent inhibitor of NAD-dependent glyceraldehyde phosphate dehydrogenase complex than rutin. The inhibitory effect of quercetin on aldose reductases varies considerably with the enzyme source. For example, at $10^{-4}M$, quercetin inhibited yeast xylose reductase activity by about 40% (this study), whereas rat lens aldose reductase was completely inhibited (*19*). In another study, the concentration of quercetin that inhibits rabbit lens aldose reductase activity by 50% (IC_{50}) was reported to be about $1\times10^{-5}M$ (*20*). Variability in potency of quercetin between these studies may be attributed to differences in the sources of enzymes, types of substrates, or the purity of enzyme used.

Mercuric and cupric chlorides both inhibited yeast xylose reductase activity in a dose-dependent manner. The patterns of inhibition were sigmoidal with apparent IC_{50} of $4.7\times10^{-6}\pm3.8\times10^{-7}M$ and $5.2\times10^{-5}\pm 3.5\times10^{-6}M$ for mercuric and cupric chlorides, respectively (Fig. 1B). Purified xylose reductase from *P. stipitis* was previously shown to be readily inactivated by mercuric chloride or the sulfhydryl reagent *p*-chloromercuribenzoate (*9*). Similarly, *p*-hydroxymercuribenzoate was reported to be a potent inhibitor of human erythrocyte aldose reductase (*12*). This sigmoidal dose–response pattern of inhibition differed from those displayed by quercetin and rutin. This pattern also suggests that the metal compounds interacted with specific components of the enzyme to effect inactivation. A

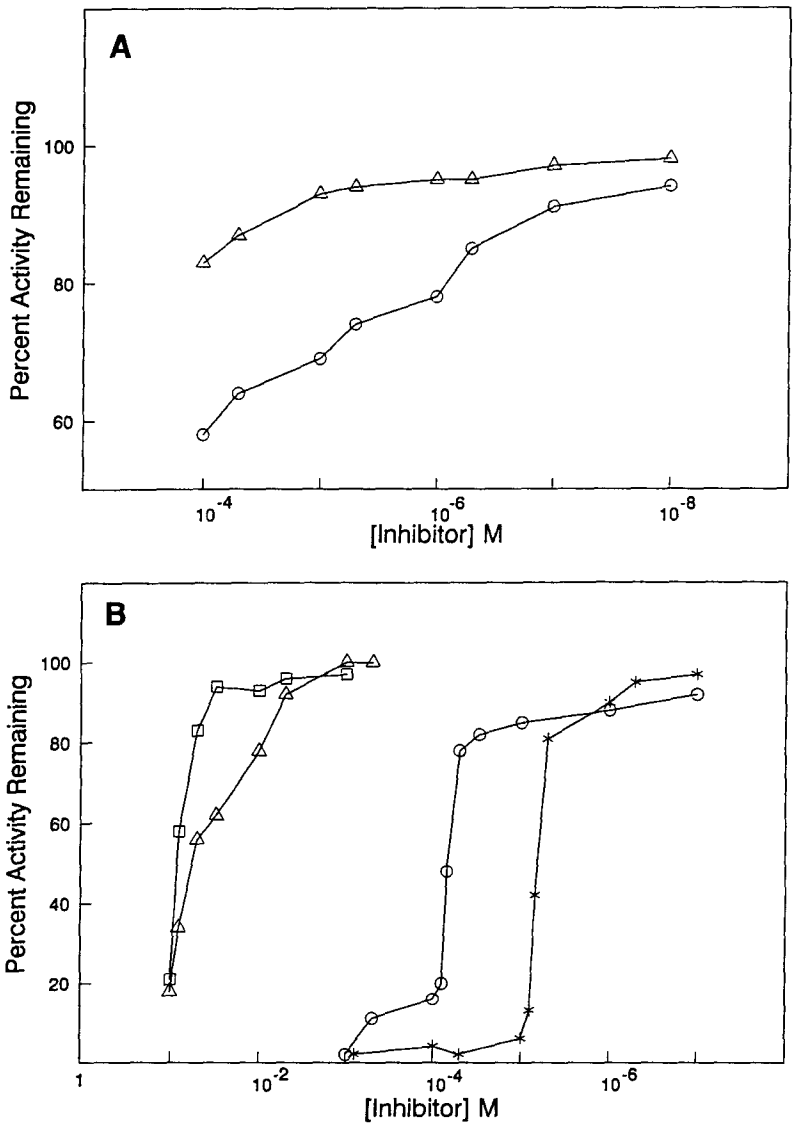

Fig. 1. Dose-response curves of various inhibitors on *P. stipitis* xylose reductase activity. The figure shown is a representative plot chosen from three independent experiments. Panel **A:** quercetin (○) and rutin (△). Panel **B:** menadione sodium bisulfite (□), sodium bisulfite (△), cupric chloride (○), and mercuric chloride (*).

recent study has implicated cysteine residues as being critical to the binding of cofactor to human placental and kidney aldose reductases (22). It is likely that there are analogous sulfhydryl groups in the yeast xylose reductase. Further studies with more specific inhibitors are required to provide a more definitive answer on the nature of the active and binding sites of yeast xylose reductase.

Similar sigmoidal dose–response inhibition patterns were observed for sodium bisulfite and menadoine sodium bisulfite, a water-soluble derivative of vitamin K (Fig. 1B). However, they were considerably less potent than the metals with IC_{50} values of $6.5\times10^{-2}\pm4.0\times10^{-3}M$ and $8.1\times10^{-2}\pm1.2\times10^{-3}M$ for sodium bisulfite and menadione sodium bisulfite, respectively. Although diphenylhydantoin and hydantoin are effective inhibitors of mammalian aldose reductases (*13*), they had no effect on the yeast enzyme at concentrations up to $5\times10^{-3}M$ (data not shown). Similarly, valproic acid, an inhibitor of mammalian aldehyde reductase (*23*), did not affect yeast xylose reductase activity at concentrations as high as 2 m*M* (data not shown).

Selected compounds were tested at varying concentrations to determine their mode of inhibition on the yeast xylose reductase. Three distinct categories were identified. Category (I) inhibitors were noncompetitive to DL-glyceraldehyde, D-xylose, and NADPH. Category (II) inhibitors were noncompetitive to NADPH, but competitive to DL-glyceraldehyde and D-xylose, and Category (III) inhibitors were uncompetitive with respect to both substrates and cofactor.

Mercuric chloride and quercetin belong to catery (I) (Figs. 2 and 3). Mercuric chloride, the most potent inhibitor tested, inhibited xylose reductase activity noncompetitively with respect to D-xylose (K_i $6.7\times10^{-6}\pm3.0\times10^{-6}M$) and NADPH ($K_i$ of $3.6\times10^{-6}\pm1.6\times10^{-6}M$) (Fig. 2A,B). However, with DL-glyceraldehyde, the metal showed a mixed pattern of inhibition (Fig. 2C), with apparent K_{iES} of $5.9\times10^{-5}\pm2.7\times10^{-5}M$ and K_{iE} of $5.2\times10^{-6}\pm2.1\times10^{-6}M$. Quercetin was less inhibitory than mercuric chloride, with apparent K_i values of $8.0\times10^{-5}\pm1.4\times10^{-5}M$, $6.5\times10^{-4}\pm3.2\times10^{-4}M$ and $6.9\times10^{-5}\pm3.6\times10^{-5}M$ for DL-glyceraldehyde, D-xylose, and NADPH, respectively. Quercetin has previously been shown to be a noncompetitive inhibitor of other mammalian aldose reductases (*24*) as well as glutathione *s*-transferases (*25*).

Menadione sodium bisulfite is a category (II) inhibitor. This compound competitively inhibited yeast xylose reductase with respect to DL-glyceraldehyde (K_i $1.9\times10^{-2}\pm6.5\times10^{-3}M$) and D-xylose ($K_i$ $6.4\times10^{-2}\pm6.5\times10^{-3}M$) (Fig. 4A,B). These results are in agreement with a previous study that found menadione sodium bisulfite to be a competitive inhibitor of chicken aldose reductase (*13*). This compound showed a mixed type of inhibition with respect to NADPH (Fig. 4C), with apparent K_{iE} and K_{iES} of $2.5\times10^{-2}\pm9.4\times10^{-3}M$ and $6.1\times10^{-2}\pm7.8\times10^{-3}M$, respectively.

Sodium bisulfite is a category (III) inhibitor with respect to both substrates and cofactor (Fig. 5). The apparent K_i values of $3.9\times10^{-2}\pm1.4\times10^{-2}M$ and $3.5\times10^{-2}\pm1.0\times10^{-2}M$ for D-xylose and DL-glyceraldehyde were similar to those found with menadione sodium bisulfite. An apparent K_i of $8.2\times10^{-2}\pm7.8\times10^{-3}M$ was observed for NADPH.

Xylose reductase from yeasts has been grouped with the aldose reductase family of enzymes based on similarities in substrate specificity (*7*,

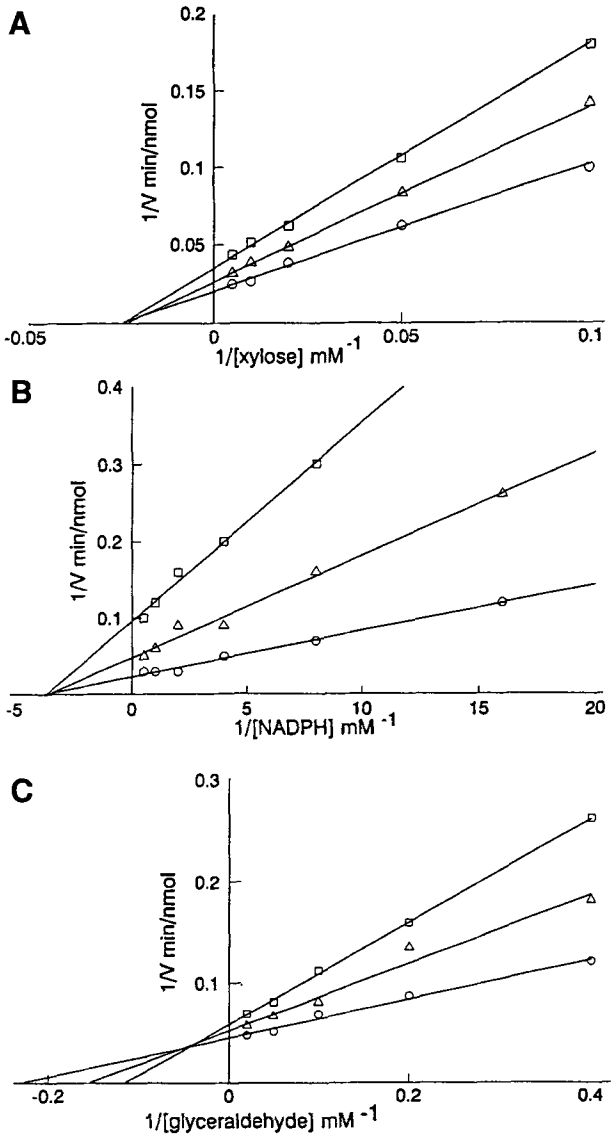

Fig. 2. Lineweaver-Burke plots of the inhibition of *P. stipitis* xylose reductase by various concentrations of mercuric chloride. Symbols: (○) control; (△) $3\times10^{-5}M$; (□) $5\times10^{-5}M$. Figures shown are representative plots chosen from six independent experiments.

9,18). However, several other enzyme families have overlapping substrate specificities, including the aldehyde reductase (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.2) and carbonyl reductase (secondary alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.18) (*26*). These enzymes have been suggested to comprise the aldo-keto reductase superfamily based on substrate specificity, physicochemical properties, and amino acid composition and sequence

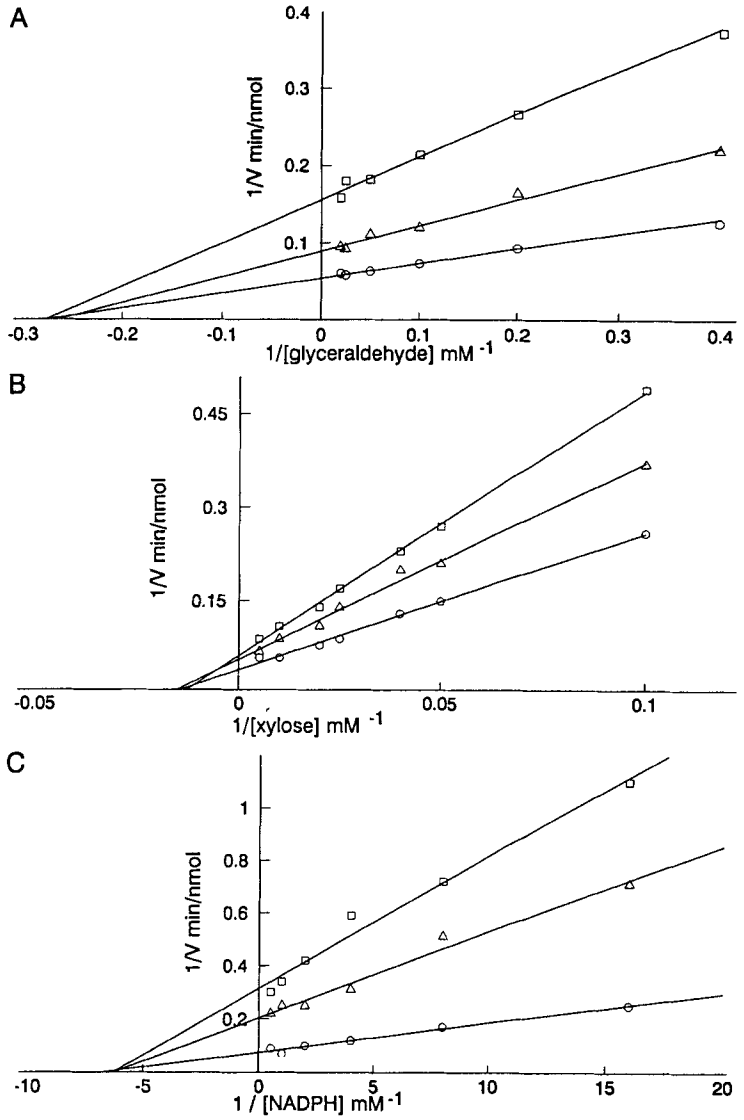

Fig. 3. Lineweaver-Burke plots of the inhibition of *P. stipitis* xylose reductase by various concentrations of quercetin. Symbols: (○) control; (△) $5 \times 10^{-5} M$; (□) $1 \times 10^{-4} M$. Figures shown are representative plots chosen from four independent experiments.

(*26*). The response of these aldo-keto reductases to different chemical inhibitors varies considerably. For instance, in this study yeast xylose reductase was not inhibited by valproic acid. This supports its grouping as an aldose reductase, because mammalian aldose reductases are not substantially inhibited by valproic acid, which is an inhibitor of aldehyde reductases (*23*). In this study quercetin displayed a noncompetitive type of

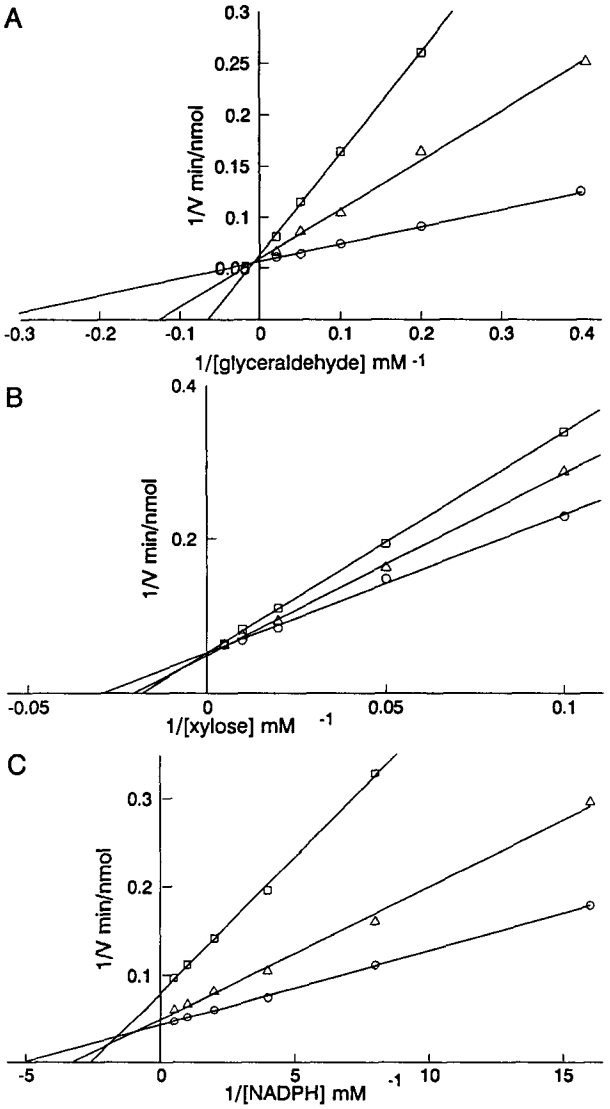

Fig. 4. Lineweaver-Burke plots of the inhibition of *P. stipitis* xylose reductase by various concentrations of menadione sodium bisulfite. Symbols (○) control; (△) $3.0\times10^{-2}M$; (□) $5.0\times10^{-2}M$. Figures shown are representative plots chosen from three independent experiments.

inhibition. Similarly, quercetin was recently found to be a noncompetitive inhibitor of aldose reductase, but an uncompetitive inhibitor of aldehyde reductase (22). The inhibition patterns observed for yeast xylose reductase supports the view that this enzyme is indeed a member of the aldose reductase family.

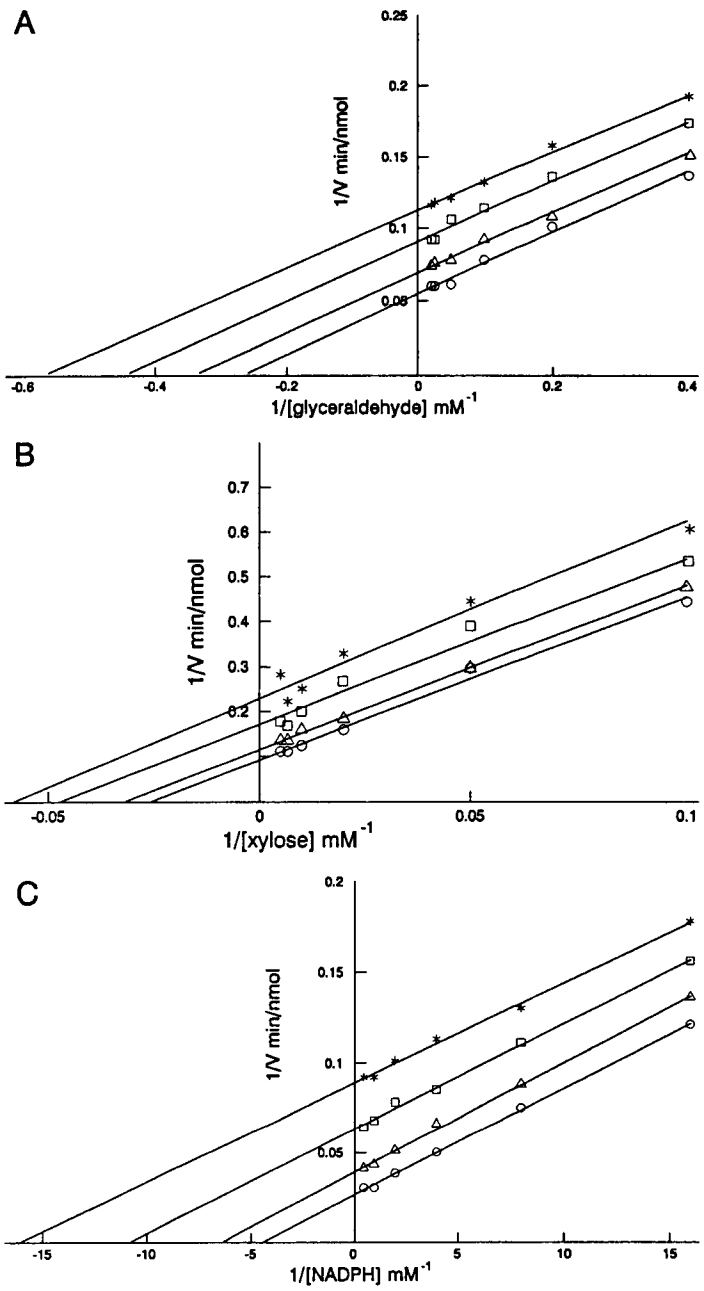

Fig. 5. Lineweaver-Burke plots of the inhibition of yeast *P. stipitis* reductase by various concentrations of sodium bisulfite. Symbols (○) control; (△) $1.0\times10^{-2}M$; (□) $3.0\times10^{-2}M$; (*) $5.0\times10^{-2}M$. Figures shown are representative plots chosen from three independent experiments.

ACKNOWLEDGMENTS

This research was supported by the Natural Sciences and Engineering Research Council of Canada. We thank D. Hamilton for help with graphic work.

REFERENCES

1. Biely, P. (1985), *Trends Biotechnol.* **3,** 286–290.
2. Schneider, H., Wang, P. Y., Chan, Y. K., and Maleszka, R. (1981), *Biotechnol. Lett.* **3,** 89–92.
3. Slininger, P. J., Bothast, R. J., van Cauwenberge, J. E., and Kurtzmann, C. P. (1982), *Biotechnol. Bioeng.* **24,** 371–384.
4. Webb, S. R. and Lee, H. (1990), *Biotechnol. Adv.* **8,** 685–697.
5. Schneider, H., Lee, H., Barbosa, M., de F. S., Kubicek, C. P., and James, A. P. (1989), *Appl. Environ. Microbiol.* **55,** 2877–2881.
6. Hagedorn, J. and Ciriacy, M. (1989), *Curr. Genet.* **16,** 27–33.
7. Bolen, P. L., Roth, K. A., and Freer, S. N. (1986), *Appl. Environ. Microbiol.* **52,** 660–664.
8. Ho, N. W. Y., Lin, F. P., Huang, S., Andrews, P. C., and Tsao, G. T. (1990), *Enzyme Microb. Technol.* **12,** 33–39.
9. Verduyn, C., Van Kleef, R., Frank, J., Schreuder, H., Van Dijken, J. P., and Scheffers, W. A. (1985), *Biochem. J.* **226,** 669–677.
10. Bruinenburg, P. M., de Bot, P. H. M., van Dijken, J. P., and Scheffers, W. A. (1984), *Appl. Microbiol. Biotechnol.* **19,** 256–260.
11. Kador, P. F., Robinson, Jr., W. G., and Kinoshita, J. H. (1985), *Annu. Rev. Pharmacol. Toxicol.* **25,** 691–714.
12. Das, B. and Srivastava, S. K. (1985), *Arch. Biochem. Biophys.* **238,** 670–679.
13. Murphy, D. G. and Davidson, W. S. (1985), *Biochem. Pharmacol.* **34,** 2961–2965.
14. Bicho, P. A., Runnals, P. L., Cunningham, J. D., and Lee, H. (1988), *Appl. Environ. Microbiol.* **54,** 50–54.
15. Lee, H., James, A. P., Zahab, D. M., Mahmourides, G., Maleszka, R., and Schneider, H. (1986), *Appl. Environ. Microbiol.* **51,** 1252–1258.
16. Bradford, M., (1976), *Anal. Biochem.* **72,** 248–254.
17. Engel, P. C. (1977), *Enzyme Kinetics: the Steady-State Approach,* Chapman Hall, London.
18. Rizzi, M., Erlemann, P., Bui-Thanh, N., and Dellweg, H. (1988), *Appl. Microbiol. Biotechnol.* **29,** 148–154.
19. Varma, S. D. and Kinoshita, J. H. (1976), *Biochem. Pharmacol.* **25,** 2505–2513.
20. Nakai, N., Fujii, Y., Kobashi, K., and Nomura, K. (1985), *Arch. Biochem. Biophys.* **239,** 491–496.
21. Nazarova, G. N., Muzafarov, E. N., and Lyubimov, V. Yu. (1989), *Biochemistry* **54,** Part 2: 680–684.
22. Bhatnagar, A., Liu, S., Das, B., Ansari, N. H., and Srivastava, S. K. (1990), *Biochem. Pharmacol.* **39,** 1115–1124.

23. Terubayashi, H., Sato, S., Nishimura, C., Kador, P. F., and Kinoshita, J. H. (1989), *Kidney Int.* **36,** 843–851.
24. Bhatnagar, A., Lui, S., Das, B. and Srivastava, S. K. (1989), *Mol. Pharmacol.* **36,** 825–830.
25. Frohlich, D. R., Burris, T. E., and Brindley, W. A. (1989), *Comp. Biochem. Physiol.* **94B,** 661–665.
26. Bohren, K. M., Bullock, B., Wermuth, B., and Gabbay, K. H. (1989), *J. Biol. Chem.* **264,** 9547–9551.