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An Endogenous Proteinacious Inhibitor for *S*-Adenosyl-L-methionine-dependent Transmethylation Reactions; Identification of *S*-Adenosylhomocysteine as an Integral Part

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A proteinacious inhibitor with a molecular weight of 1,600 Da which inhibits S-adenosyl-L-methionine-dependent transmethylation reactions was purified from porcine liver to homogeneity by procedures including boiling, Sephadex G-25 column chromatography and repeated HPLC. Employing both Nuclear Magnetic Resonance (NMR) and Fast Atom Bombardment-Mass (FAB-Mass) spectroscopy, S-adenosylhomocysteine was conclusively identified as an integral part of the inhibitor. The purified S-adenosylhomocysteine was competitive with S-adenosyl-L-methionine with *Ki* value of 6.3×10^{-6} M towards protein methylase II.

Key words : S-adenosylhomocysteine, Methylation, Proteinacious inhibitor

INTRODUCTION

Structure and function of many biological compounds are modulated by transmethylation reaction, and the biological methylation is one of the most important ubiquitously occurring reactions. The methyl donor for these transmethylation reactions is almost exclusively S-adenosyl-L-methionine (AdoMet), and several review books on this subject have been published (Shapiro and Schlenk, 1965; Salvatore *et al.*, 1977; Usdin *et al.*, 1979; Usdin *et al.*, 1982; Borchardt *et al.*, 1986). Because of its importance in the regulation of function and metabolism of a vast array of compounds, numerous attempts have been made to regulate the intracellular concentration of AdoMet, thereby modulating cellular activity. Among the biologically occurring endogenous inhibitors, S-adenosyl-L-homocysteine (AdoHcy), one of the products of AdoMet-dependent transmethylation reaction, has been the most prominent (Lawrence and Robert-Gero, 1990).

In addition to AdoMet, however, there have been a few reports on the existence of proteinacious inhibitors. Earlier, Kim and Paik identified in rat liver particulate fractions an inhibitor for protein methylase II (AdoMet: protein-carboxyl *O*-methyltransferase; EC 2.1.1.24) (Kim and Paik, 1971). The inhibitor was non-dialyzable, thermolabile and partly inactivated by tryptic digestion. Later, Chiva and Mato resolved two proteinacious factors in rat liver cytosol which suppressed phosphatidylethanolamine methyltransferase activity (EC 2.1.1.17) (Chiva and Mato, 1984). These inhibitors with apparent molecular weights (Mr) of 3,200 and 1,600 Da were resistant to the action of DNase, RNase, but partly inactivated by subtilisin. Amino acid analysis of the highly purified inhibitor with 1,600 Da revealed very high glycine content, ranging from 16 to 51% of the total amino acids (Hong *et al.*, 1986; Park *et al.*, 1993a; Park *et al.*, 1993b). Since all the preparations analyzed were highly purified as judged by repeated HPLC analysis, the above widely variable results on the glycine content might suggest either unusually unstable nature of the inhibitor and/or there might be a concomitant factor which give rise to the formation of glycine on acid-hydrolysis. It has been known that purine bases gave rise to the formation of glycine during acid-hydrolysis (Lindsay *et al.*, 1962). In the present paper, we purified the inhibitor of 1,600 Da from porcine liver, and the presence of AdoHcy as an integral part of the inhibitor was conclusively established by NMR and FAB-Mass spectrometric analysis.

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MATERIALS AND METHODS

Materials

S-Adenosyl-L-[methyl-^{14}C]methionine (specific activity,

54 mCi/mmol in H_2SO_4) was purchased from Amersham Life Science, the United Kingdom. Histone (type II-AS from calf thymus; a mixture of various histone fractions), bovine serum albumin, Sephadex G-25 (exclusion limit, 5,000 Da), S-adenosyl-L-homocysteine, acetonitrile, and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co., U.S.A. The rest of the chemicals were from various commercial sources and of the highest grade available.

Preparation and assay method for protein methylase II

For testing the inhibitory activity of an endogenous inhibitor for AdoMet-dependent transmethylation reaction, protein methylase II was chosen[1]. Therefore, protein methylase II was partially purified from porcine liver according to the method described previously (Kim, 1984). Briefly, freshly obtained porcine liver was homogenized in 4 volumes of 0.25 M sucrose solution containing 5 mM EDTA, 2.4 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF), and the whole homogenate was centrifuged at 105,000 g for 1 hr. The supernatant obtained was then treated with finely powdered ammonium sulfate at 50% saturation and the precipitate formed was dialyzed for 12 hrs against 20 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 2.4 mM 2-mercaptoethanol. The enzyme preparation remaining inside the bag was concentrated by lyophilization and was stored at -70 until use. The preparation had a specific activity of 7 picomoles of methyl transferred/min/mg protein.

An assay for protein methylase II activity was carried out as described (Kim, 1984). The incubation mixture containing 0.05 M citrate-phosphate buffer (pH 6.0), 0.6 mg of histone suspension, appropriate amounts of protein methylase II and the inhibitor in a total volume of 0.09 ml was preincubated at 37 for 5 min and was further incubated for 15 min after the addition of 0.01 ml of 25 μM Ado[methyl-^{14}C] Met (one picomole had 100 dpm). At the end of incubation, 0.1 ml of 0.125 M sodium-borate buffer (pH 11.0) was added to the incubation mixture, and the mixture was further incubated for 5 min at 37°C. One ml of isoamyl alcohol was added to the mixture, the mixture was vortexed, and 0.7 ml of the supernatant after centrifugation was counted for radioactivity by liquid scintillation counter (Pharmacia LKB, Sweden). Protein concentration was determined by the method of Bradford (Bradford, 1976), employing bovine serum albumin as the standard.

Purification of an endogenous inhibitor

Forty grams of freshly obtained porcine liver were cut into small pieces and was homogenized in 4 volumes of 1 mM phosphate buffer (pH 7.4) by Bio-homogenizer for 2 min at 1,200 rpm. The whole homogenate was passed through a double layer of cheese-cloth and was centrifuged at 12,500 g for 20 min at 4°C. Unless otherwise indicated, the following procedures were carried out at 4°C. The supernatant was further centrifuged at 105,000 g for 1 hr. The supernatant obtained was then heated at 100 for 15 min and the coagulated protein was removed by centrifugation at 15,000 g for 20 min. The supernatant was lyophilized. The lyophilized material was dissolved in 5.0 ml of water and the suspension was loaded onto Sephadex G-25 column (exclusion limit 5,000; 1.4 cm (i.d.)×120 cm) which had been equilibrated with 1 mM phosphate buffer (pH 7.4). The column was eluted with the above phosphate buffer at a flow rate of 30 ml/hr (Fig. 1). Seven and half ml of each fractions were collected, and subsequently 0.5 ml portion of each fraction was concentrated by lyophilization (during the early stage of purification, the amount of inhibitor was too small to examine without concentration). Inhibitory activity was assayed individually toward protein methylase II. Fractions containing the inhibitory activity (fractions under the peak C in Fig. 1) were pooled and lyophilized. The lyophilized sample above was then dissolved in 0.5 ml of 0.1% trifluoroacetic acid (TFA)

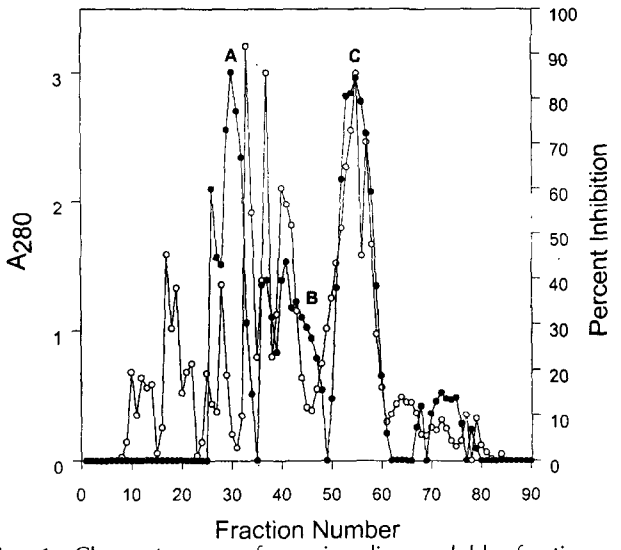

Fig. 1. Chromatogram of porcine liver soluble fraction on Sephadex G-25 column. Forty g of freshly obtained porcine liver was homogenized in 1.0 mM phosphate buffer (pH 7.4 and the whole homogenate was centrifuged at 105,000 g fo 1 hr. The supernatant was heated at 100 for 15 min and the precipitate was removed by centrifugation. The clear supernatan was lyophilized and dissolved in 5.0 ml of water. The suspensior was applied onto Sephadex G-25 column (1.4 cm (i.d.)×12(cm) and the column was eluted with 1 mM phopshate buffer 7.5 ml each fractions were collected with a flow rate of 3(ml/hr, and 0.5 ml aliquot from each fraction was lyophilized and reconstituted to an appropriate volume for inhibitor assay Protein methylase II was employed. Open circle represent A_{280} and closed circle for inhibitory activity. The rest of the experimental procedures is described under Methods.

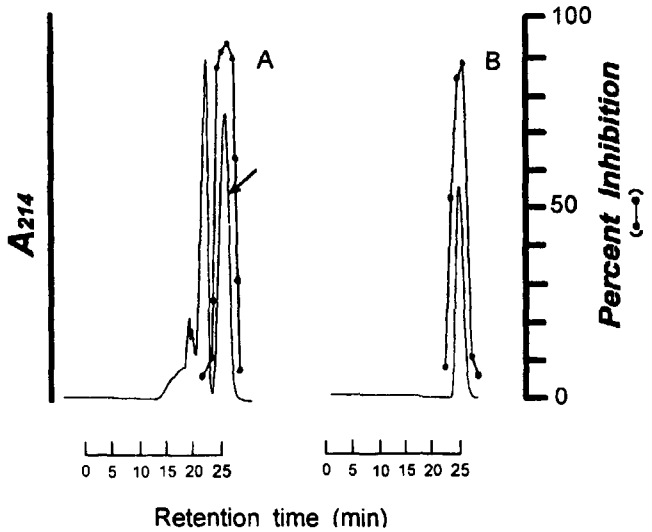

Fig. 2. Chromatography of partially purified inhibitor on ODS C_{18} column. Peak C sample in Fig. 1 was lyophilized and was further purified on ODS C_{18} column (2.0 cm (i.d.)×25 cm). Mobile phase was 5% acetonitrile in 0.1% TFA and flow rate was 5.0 ml/min. 1.5 ml each fractions were collected and each fraction was lyophilized separately. Lyophilized samples were reconstituted to 1.0 ml and 0.03 ml aliquots were assayed for inhibitory activity. Fig. 2B represents rechromatography of the fractions which had the inhibitory activity in Fig. 2A. The straight line represents A_{214} and closed circle for the inhibitory activity.

and the sample was further purified by preparative HPLC (ODS C_{18} column 2.0 cm, (i.d.)×25 cm) equilibrated with 0.1 % TFA. The column was first eluted with 0.1% TFA in water for 1 hr followed by the elution with 0.1% TFA in 5% acetonitrile. The flow rate was 5.0 ml/min and 1.5 ml of each fractions were collected and lyophilized. Thirty ml aliquots were assayed for inhibitory activity. The fractions containing the inhibitory activity were pooled (Fig. 2A) and the pooled sample was lyophilized. The above HPLC-purified sample was dissolved in 0.5 ml of 0.1% TFA, and chromatographic purification of the inhibitor was repeated on HPLC by the identical method described above (Fig. 2B). Finally, the entire sample under the A_{214} peak was lyophilized, dissolved in 1.0 ml of water, and was stored at -70°C.

Structural identification of the inhibitor by NMR spectroscopy and FAB-Mass spectrometry

NMR spectroscopy (Bruker AM-300 and Bruker AMX-500, Germany) and FAB-Mass spectrometry (VG70-VSEQ, U.K.) were employed for structural determination of the purified inhibitor.

RESULTS

Purification of the inhibitor

When porcine liver soluble fraction treated at 100°C for 15 min was applied onto Sephadex G-25 column (exclusion limit, 5,000 Da), there were numerous A_{280} absorbance peaks, however, there appeared three major peaks with the inhibitory activity for protein methylase II (Fig. 1). These peaks at fraction numbers 30, 41 and 55 were designated as peak A, B, and C, respectively. Peaks A and C in Fig. 1 corresponded to peak I and II in rat liver soluble fraction, respectively, observed by Chiva and Mato (Chiva and Mato, 1984). The apparent molecular weights of the peak I and II were 3,200 and 1,600 Da, respectively. Possible significance of an extra peak (B) in the porcine liver in addition to two peaks in the rat liver soluble cytosol is not clear at present. The material under peak C in Fig. 1 was further purified by repeated HPLC procedures (Fig. 2). Fig. 2A shows the first HPLC run and 2B the second. As shown in Fig. 2B, the inhibitor appeared to be pure. Approximately 1.4 mg of the inhibitor was obtained from 1 kg of fresh porcine liver.

Approximation of molecular weight of the purified inhibitor

Approximate molecular weight of the inhibitor was estimated employing Sephadex G-25 column chromatography. As shown in Fig. 3, the molecular size of the inhibitor was far less than bradykinin whose molecular weight was 1,060 Da. It should be noted here that the apparent molecular weight of the inhibitor in crude state (Fig. 1) was about 1,600 Da, suggesting that the inhibitor may be dissociated from the rest of the molecule during purification process.

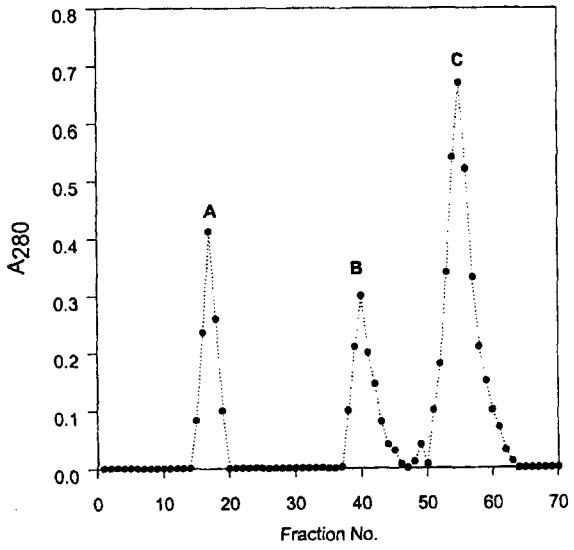

Fig. 3. Chromatogram of purified inhibitor on Sephadex G-25 column. HPLC-purified inhibitor, bovine serum albumin (peak A, 66,000 Da), and bradykinin (peak B, 1060.2 Da) were applied onto Sephadex G-25 column (0.6 cm (i.d.)×100 cm) and the column was eluted with 1mM phosphate buffer (pH 7.4) at a flow rate of 30 ml/hr. Each fraction contained 4.0 ml.

Identification of the purified inhibitor with S-adenosyl-L-homocysteine by NMR spectroscopy and FAB-Mass spectrometry

The purified inhibitor was found to be identical with S-adenosyl-L-homocysteine (AdoHcy) as determined by both ^{1}H-NMR (D_2O) and ^{13}C-NMR (D_2O) analysis (Fig. 4). The data of ^{1}H-NMR (D_2O) and ^{13}C-NMR (D_2O) were as follows.

^{1}H-NMR (300 MHz, D_2O) δ : 2.14 (2H, m, homocysteine-2''), 2.71 (2H, t, J=7.7 Hz, homocysteine-1''), 3.0 (1H, dd, J=14.2, 6.5 Hz, ribose-5'), 3.09 (1H, dd, J=14.2, 4.7 Hz, ribose-5'), 3.83 (1H, ribose-4'), 4.37 (1H, ribose-3'), 4.45 (1H, ribose-2'), 6.11 (1H, d, J=5.1 Hz, ribose-1'), 8.31 (1H, s, adenine-1), 8.41 (1H, s, adenine-2)

^{13}C-NMR (125.78 MHz, D_2O) δ : 30.4 (homocysteine-2''), 36.1 (homocysteine-1''), 51.5 (ribose-5'), 56.4 (homocysteine-3''), 75.0 (ribose-2'), 75.9 (ribose-3'), 86.1 (ribose-4'), 90.3 (ribose-1'), 121.6 (adenine), 151.6 (adenine), 157.5 (adenine), 143.2 (adenine-1), 154.3 (adenine-2), 176.6 (homocysteine COOH).

FAB-Mass spectrometric analysis showed positive FAB-MS : m/z 385 $[M+H]^+$. The above analytical data confirmed conclusively that one of the inhibitors in porcine liver contained AdoHcy.

Inhibition kinetics

Fig. 5 illustrates the effect of concentration of the inhibitor on protein methylase II activity. From the curve, IC_{50} (inhibitor concentration to suppress the enzyme activity by 50%) was estimated to be 8.7×10^{-6} M. Lineweaver-Burk plot of the inhibitor activity at different concentrations with AdoMet as a substrate was shown in Fig. 6. The mode of inhibition was competitive with Ki value of 6.3×10^{-6} M which was quite close to the IC_{50} observed in Fig. 5.

DISCUSSION

The function and structure of many biologically important compounds are modified by *in vivo* methylation (Chiang *et al.*, 1996). Practically in every cases, S-adenosyl-L-methionine (AdoMet) serves as the methyl donor for transmethylation reaction. Therefore, numerous attempts have been made to modulate the effect of AdoMet as the biological methyl donor. This includes derivatization of AdoMet structure, and one of the derivatives, S-adenosyl-L-homocysteine (AdoHcy), has been recognized as one of the most potent competitive inhibitors for AdoMet-dependent transmethylation reactions (Lawrence and Robert-Gero, 1990). Two proteinacious peaks were separated in the rat liver cytosol which inhibited phosphatidylethanolamine methyltransferase (Chiva and Mato, 1984). The both inhibitors were resistant to the action of trypsin or papain, except subtilisin which inactivated the activity by 50%. These results suggested that the inhibitors had proteinacious characteristics, however, it might be atypical protein. As demonstrated previously, the glycine content on the amino acid composition of the purified inhibitor of Mr 1,600 represented widely varying values from 16 to 51% (Hong *et al.*, 1986; Park *et al.*, 1993a; Park *et al.*, 1993b). These unusually variable values suggested that the inhibitor molecule was very unstable or a possibility that glycine might have arisen from an inherent compound during acid-hydrolysis. There has been an earlier report that purine-containing compounds gave rise to the formation of glycine on acid-hydrolysis (Lindsay *et*

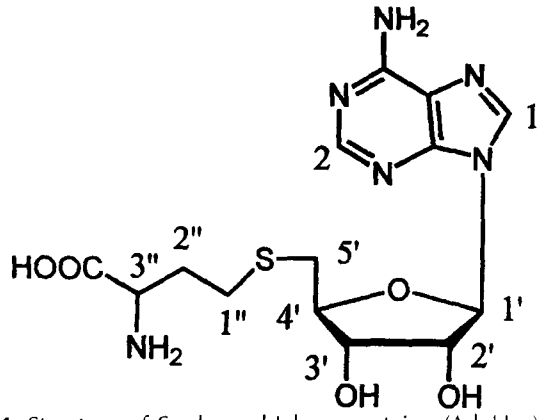

Fig. 4. Structure of S-adenosyl-L-homocysteine (AdoHcy).

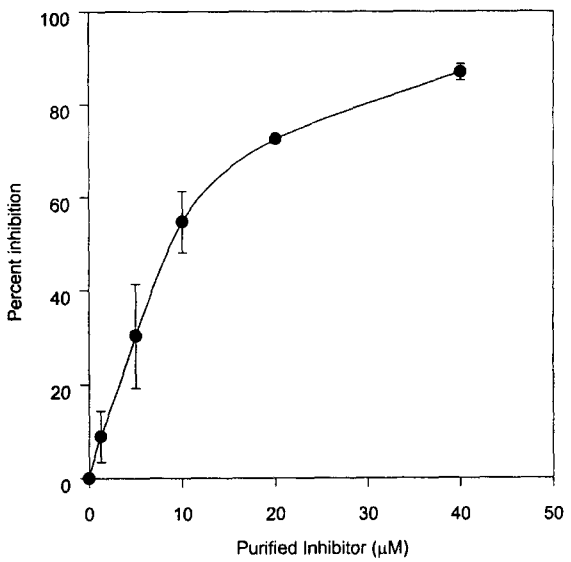

Fig. 5. Relationship between the concentration of the purified inhibitor and its inhibitory effect on protein methylase II. The incubation mixture containing 0.05 M citrate-phosphate buffer (pH 6.0), 0.6 mg of histone suspension, 0.2 mg of protein methylase II and increasing amounts of the inhibitor in a total volume of 0.09 ml was preincubated at 37 for 5 min and was further incubated for 15 min after the addition of 0.01 ml of 25 μM Ado[methyl-^{14}C] Met (one picomole had 100 dpm). Detailed conditions and procedures are described under Methods.

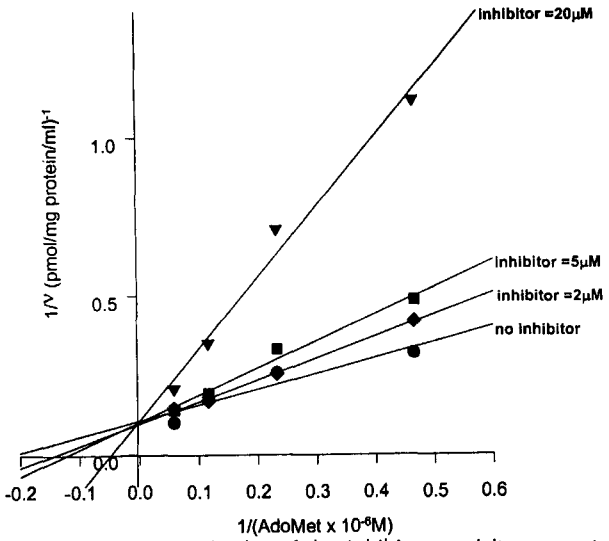

Fig. 6. Lineweaver-Burk plot of the inhibitory activity on protein methylase II. The assay was performed by varying the concentration of the purified inhibitor ($0\sim20\times10^{-6}$ M) and AdoMet ($2.15\sim17.2\times10^{-6}$ M). The *Ki* value was 6.3×10^{-6} M. Detailed conditions are described under Methods.

al., 1962). We had purified to identify the proteinacious inhibitor from porcine liver as described (Park *et al.*, 1993b), however, failed to sequence the inhibitor due to the absence of N-terminus. Therefore, we have improved the purification process to acquire the highly pure inhibitor with slight modification.

In the present study, S-adenosylhomocysteine (AdoHcy) was identified as a component of one of the inhibitors in soluble fraction of the porcine liver. The identification was confirmed by both NMR and FAB-Mass spectrometric analysis and size exclusion column chromatography. In addition, the presently purified compound had a competitive inhibition with S-adenosyl-L-methionine (AdoMet) with *Ki* value of 6.28×10^{-6} M (Fig. 6). This value falls in the range of *Ki* values (range of $2\sim10\times10^{-6}$ M), values being dependent on the substrate proteins and source of protein methylase II (Lawrence and Robert-Gero, 1990).

Biological significance of conjugation of AdoHcy with a proteinacious component *in vivo* is not clear at present. It is of interest to note that the inhibitor with proteinacious component still attached showed a non-competitive inhibition with AdoMet with *Ki* value of 1.9×10^{-8} M, two magnitudes lower than the *Ki* value of free AdoHcy (Park *et al.*, 1993a). It is, therefore, postulated that the existence of conjugated AdoHcy *in vivo* enhances the inhibitory efficiency with a non-competitive inhibitory mode of action. Alternatively, the existence in the conjugated form might protect AdoHcy from intracellular degradation or facilitate the transport across various organellar membranes.

FOOTNOTES

Footnote 1. Not only protein methylase II, but also the following transmethylases were inhibited by the inhibitor : Phenylethanolamine (EC 2.1.1.28), catechol O-methyltransferase (EC 2.1.1.6), thiol S-methyltransferase (EC 2.1.1.9), DNA (EC 2.1.1.37), RNA methylase, AdoMet : protein-arginine N-methyltransferase (protein methylase I; EC 2.1.1.23), AdoMet : protein-lysine N-methyltransferase (protein methylase III; EC 2.1.1.43), and phosphatidyl-ethanolamine methyltransferase (EC 2.1.1.17). [see Ref. (Hong *et al.*, 1986)].

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