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Effect of amino acids on inhibition of lactate dehydogenase-X by gossypol

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Abbreviations: AA, amino acids; AGE, agarose gel electrophoresis; BSA, bovine serum albumin; EEO, electroendosmosis; GAA, gossypol acetic acid; LDH, lactate dehydrogenase; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Abstract

Gossypol acetic acid (GAA) has been shown to have male antifertility effects, but there are pronounced differences among animal species. In the search of endogenous effector molecules, which interfere with the functions of GAA, we have studied the *in vitro* effect of various amino acids on the inhibition of the purified LDH-X by GAA. Histidine, cysteine and glycine were shown to block the effect of GAA. The effects of these amino acids were concentration dependent. Histidine and glycine protection was found to be complex type in which both the Km and Vmax were decreased compared to control. Arginine, glutamic acid, phenylalanine and valine were found to be ineffective against the inhibitory action of GAA.

Keywords: amino acids, goat, gossypol acetic acid, LDH-X, testes.

Introduction

Gossypol is a yellow polyhydroxylated phenolic compound that occurs in roots and seeds of *Malvaceae*, a family of cotton. Three forms of this compound, gossypol, gossypol acetic acid (GAA) and gossypol formic acid (GFA) exist in nature (Qian, 1984). Several investigators, particularly in China, Austria and Brazil, have shown antifertility effects of all these forms in males (Qian, 1984; Morris *et al.,* 1986; Zhi Ping *et al.,* 1990). The antifertility properties of gossypol are associated specifically with the (-)-isomer (Morris *et al.,* 1986). There are clear differences among animal species in their sensitivity to the antifertility action of gossypol. Among the laboratory animals tested, hamsters seemed to be the most sensitive, followed by rats, monkeys and dogs in decreasing order, while rabbit and mice appeared to be insensitive (Wang *et al.,* 1979; Hahn *et al.,* 1981; Morris *et al.,* 1986). The sensitivity of GAA towards rat testes showed marked individual variations. Long-term treatment might cause complete atrophy of the semniferous epithelium in some of the animals and sterility is the likely consequence of this condition (Dai *et al.,* 1980; Qian, 1984).

Although the mechanism(s) of antifertility effects of GAA are not known, it has been suggested that it inhibits LDH (Gupta *et al.,* 1988). LDH-X is a sperm specific and used as an antigen to induce infertility in female mice (Singh *et al.,* 1994). It has been shown that immunization of male mice with LDH-X impaired fertilization (Mahi-Brown *et al.,* 1990). Similarly, LDH-X shows higher activity in patients showing oligospermia and genital tract infection (Virji, 1985). Thus it seems that LDH-X is more relevant to the reproductive functions as compared to other isoenzymes of LDH. LDH-X is one of the many regulatory enzymes involved in the metabolic processes needed for sperm motility and the absence of this isoenzyme may contribute infertility in men (Gavella and Cvitovic, 1985). The differential effects of GAA on various animal species suggest that certain endogenous molecules modulate the inhibitory effects of GAA on LDH. In our previous report (Javed and Waqar, 1995), we observed that the inhibition of LDH-5 by GAA in rat liver which was blocked by the presence of histidine in the reaction mixture. We therefore undertook this study to demonstrate that some other amino acids might also block the inhibitory action of GAA on LDH-X from goat testes which can be easily purified in large quantity.

Materials and Methods

Materials

GAA, NADH, amino acids, sodium pyruvate, and agarose type-1 low electroendosmosis (EEO) were from Sigma Chemical Company (St. Louis, USA). Colchicine-CH-Sepharose was a gift from Dr. Tomoji Kocha (Department of Hygienic Chemistry, Showa College of Pharmaceutical Sciences, Tokyo, Japan). All other chemicals were of analytical grade.

Purification of LDH-X

All purification procedures of LDH-X were performed at 4˚C, otherwise indicated. Fresh goat (*Capra hircus*) testes

were obtained from local slaughterhouse and transported to the laboratories on ice. These were frozen at -20˚C until use. Frozen testes were thawed and homogenized in 10 volumes of cold distilled water. After filtration through cheesecloth and centrifugation at 10,000 *g* for 30 min, the supernatant was heated for 10 minutes at 55˚C to destroy all LDH isoenzymes except LDH-X (Gupta *et al*., 1988). This was again centrifuged at 10,000 *g* to remove precipitated proteins. LDH-X from this supernatant was purified to homogeneity by ammonium sulfate fraction-ation, colchicine-CH sepharose column chromatography and again by heating at 65˚C for 30 min. The detailed methods have already been published elsewhere (Javed *at et*., 1995; Javed *et al.,* 1997). The LDH isoenzymes were separated by agarose gel electrophoresis (Javed *et al.,* 1995; Javed *et al.,* 1997) and visualized with a staining solution specific for LDH activity. The solution is freshly prepared in 20 mM Tris-HCl buffer (pH 8.5) contained 0.75 mM NAD^+, 94 mM lithium lactate, 0.37 mM nitro blue tetrazolium (NBT) and 0.008 mM phenazine methosulfate (PMS). After electrophoresis the gels were immersed in the staining solution at 37˚C in the dark for about 10 min. The stained gels were fixed in 5 % acetic acid and photographed.

Enzyme and protein assays

Enzyme activity was measured at 25˚C in a reaction mixture containing 50 mM potassium phosphate buffer, 0.18 mM NADH, 0.6 mM sodium pyruvate (pH 7.5) and appropriate amount of enzyme to obtain a measurable decrease in absorbance at 340 nm using Ames RA50 photometer. One unit of the enzyme was defined as the amount of enzyme that produced one mmole of NAD^+ per minute under our assay conditions (Javed *et al.,* 1995; Javed *et al.,* 1997). K_m and V_{max} values were calculated from initial velocity measurements, using Enzifitter (RJ Leather-barrow), a non-linear regression program that integrates the data into the Michaelis-Menton equation with simple weighting (Javed *et al.,* 1995; Javed *et al*., 1997). Proteins were determined by the method of Lowry *et al*. (1951) with BSA as standard.

For inhibition studies, 5 mM GAA was dissolved in ethanol. Three ml of this solution was incubated with about 0.3 units/ml enzyme in 50 mM phosphate buffer containing 0.6 mM pyruvate, pH 7.5 for about 20 min at 25˚C. Three ml ethanol served as control. The volume of the incubation mixture was 800 ml. The reaction was started with 200 ml of 0.9 mM NADH. Pyruvate was added in the incubation mixture because it has been shown (Gupta *et al.,* 1988) that it did not protect the inhibition of GAA on LDH. To observe the effect of amino acids on GAA inhibition, the enzyme (0.3 units/ml) was incubated with GAA in the presence or absence of different concentrations of amino acids. The volume of incubation mixture was again 800 ml. The pH of all amino acid solutions was adjusted to 7.5. The results are expressed as remaining activity (as %) compared to control (100%). The nature of protection by some amino acids (glycine and histidine) was determined by using Lineweaver-Burk plots of the data obtained in the presence of varied concentration of pyruvate or NADH. When the reaction was studied for different concentrations of NADH, then the reaction was started with 200 ml of desired concentration of NADH.

Results

Figure 1 confirms the purification of LDH-X from goat testes. It has already been confirmed by using SDS-PAGE that the colchicine-sepharose affinity

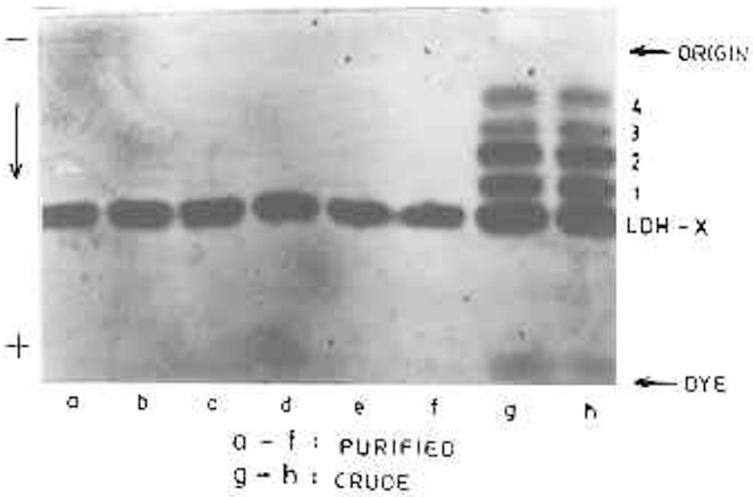


Figure 1. Agarose gel electorphoresis (AGE) of LDH from goat testes. Samples (2ml) were applied on 1% horizontal agarose gel and subjected to electrophoresis for 1 h at 4˚C. The LDH isoenzymes were detected as described in the 'Materials and Methods'. a-f, purified LDH-X; g-h, crude testes homogenate.

Table 1. Effect of amino acids on the action of GAA on LDH-X. The amino acid at desired concentration was incubated with LDH-X in the presence of 20 mM GAA, 0.18 mM NADH and 50 mM phosphate buffer (pH 7.5) and kept at room temperature for about 20 min. The control 'a' group was without GAA and amino acid but contained 3 ml ethanol, while the control 'b' contained only 20 mM GAA. The activity of the enzyme was performed as described in the 'Materials and Methods'. The values are shown in % as average of three separate determinations.

			Concentration of amino acids (mM) in the incubation mixture			
AAs	'a'	'b'	25	50	75	100
Gly	100	35	40	79	84	104
His	100	35	48	67	80	104
Cys	100	27	35	65	86	104
Phe	100	39	32	36	41	41
Val	100	44	43	39	43	46
Arg	100	43	50	43	44	49
Glu	100	43	42	40	40	49

chromatography completely purifies the total LDH (Kocha *et al.,* 1990; Javed *et al.,* 1995; Javed *et al.,* 1997). Figure 1 shows that the crude extract of testes showed five LDH isoenzymes (Lanes, g-h). The fast moving isoenzyme was considered as LDH-X (Camp and Sande, 1988) which has been shown to be heat resistant (Gupta *et al.,* 1988) while all other isoenzymes (LDH-1 to LDH-4) were heat-labile (lanes, a-f). The specific activity of the purified LDH-X was about 400 units/mg protein.

When LDH-X was preincubated with GAA in the presence of amino acids, histidine, cysteine and glycine, these amino acids strongly blocked the inhibitory effect of GAA. The protection was concentration dependent (Table 1). However, arginine, glutamic acid, phenylalanine, and valine had no protective effect, even at 100 mM (Table 1)

When we checked the nature of protection by some amino acids, glycine and histidine, both showed complex type of kinetics with GAA (Figures 2 and 3). At lower concentrations of pyruvate, glycine increased the initial velocity (*v*) but at higher concentration of pyruvate the glycine has decreased the V_{max} (43 mmol/min) as compared to control (91 mmol/min) and inhibited (Figure 2). Similarly, the histidine also increased the '*v*' at lower concentrations of NADH but again it decreased the

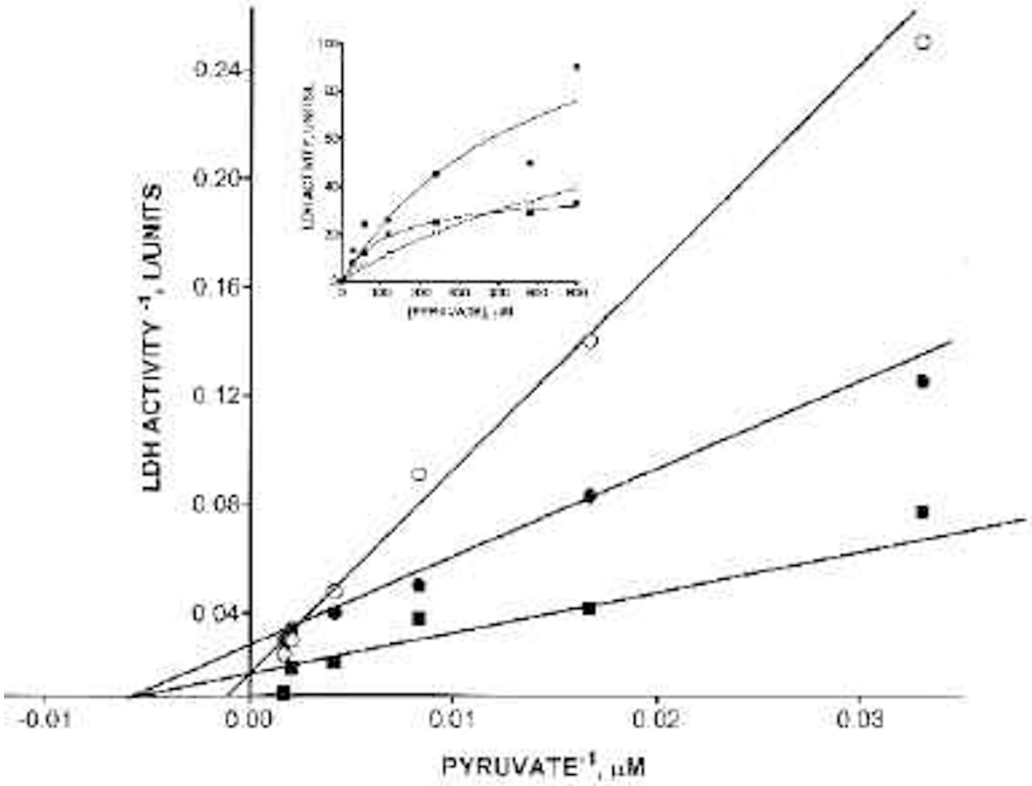
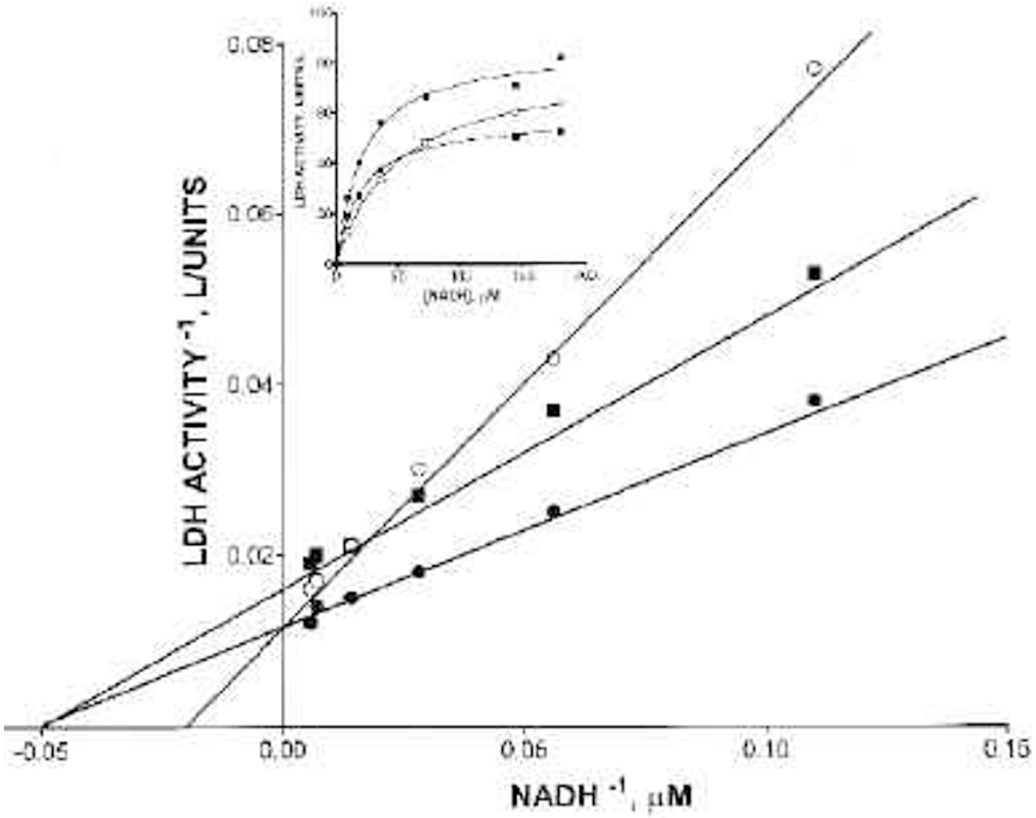


Figure 2. Lineweaver-Burk plots of LDH-X in the control (●-●), in the presence of GAA (■-■), and in both GAA and glycine (O-O). LDH-X activity was measured in the presence of 0.18 mM NADH as described in the 'Materials and Methods'. The values are average of three separate experiments. Inset: effect of pyruvate concentration on the activity of LDH. The symbols are identical as in the main figure.



Vmax (63 mmol/min) at higher concentration of NADH, compared to control (83 mmol/ min) (Figure 3).

Figure 3. Lineweaver-Burk plots of LDH-X in the control (●-●), in the presence of GAA (■-■) and in both GAA and histidine (O-O). The pyruvate concentration in the reaction mixture was 0.6 mM. The values are averages of three separate experiments. Inset: effect of NADH concentration on the activity of LDH. The symbols are identical as in the main figure.

Discussion

There are pronounced differences among animal species in their sensitivity to the action of GAA as a male antifertility agent (Qian, 1984; Morris *et al.,* 1986; Javed and Waqar, 1995). These differences may be due to some endogenous molecules that may block the antifertility effects of GAA in certain unresponsive species. In our previous work (Javed and Waqar, 1995), we have shown that the inhibitory effect of GAA on LDH-5 from rat liver was blocked by histidine. The present study also showed that the inhibitory action of GAA on LDH-X was also blocked by other amino acids (Table 1). However, at higher concentrations of substrates (pyruvate and NADH), the Vmax was also decreased in the presence of glycine or histidine in the reaction mixture, which indicates more inhibition, although Km was decreased. This indicates that at higher substrate concentrations, these amino acids might form some dead-end complex with the enzyme-GAA-substrate complex. In this way free concentration of both enzyme and GAA is decreased. Since the Km is decreased as compared to Km in the presence of GAA (Figures 2 and 3), this indicates that both glycine and histidine compete for the binding sites of GAA on LDH-X. As the Km of control group and Km in the presence of amino acids are almost same (Figures 2 and 3), the binding sites for these amino acids and pyruvate or NADH on LDH-X are mutually exclusive. Thus, it seems that glycine and histidine, somehow remove the GAA from binding sites on LDH-X and this site is then available for pyruvate. Morris *et al.* (1986) have shown that in the presence of human serum albumin or poly-L-lysine but not lysine, the human and hamster LDH-X were protected

from GAA. The protective role of human albumin was found to be due to its strong binding with GAA (Morris *et al.,* 1986). Thus free concentration of GAA was too much less in the reaction mixture compared to the added GAA. At present we are unable to explain why some amino acids protect the LDH-X from GAA inhibition while others do not. We do not know the binding behavior of GAA with individual amino acids that may affect the functions of GAA in the body.

It is also difficult for us to explain the physiological significance of these complex effects of amino acids on reversal effect against GAA inhibition on LDH-X. The contradictory effect of GAA on various animals as an antifertility compound may partly be due to the different intracellular concentrations of various amino acids in their testes. The effect of other amino acids and their detailed kinetic behavior with inhibitory action of GAA on LDH-X will be more useful and is in process.

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