TOXIC INHIBITION OF SOME DEHYDROGENASES BY METHYL STERCULATE – A NATURAL OCCURRING SUBSTANCE IN COTTON SEEDS

Y. Malevski and M. W. Montgomery[1]

Instituto Venezolano de Investigaciones Tecnológicas e Industriales (INVESTI), Apartado 1697, Carmelitas Caracas, Venezuela

SUMMARY

According to the present study, crude preparations of rainbow trout liver and rabbit muscle lactate dehydrogenase (LDH,EC 1.1.1.27) were not inhibited by methyl sterculate and oleate, while trout liver glucose-6-phosphate dehydrogenase (G6PDH,EC 1.1.1.49) was activated by these esters. Methyl sterculate inhibited purified preparations of trout liver, rabbit muscle, and bovine heart LDH in contrast to methyl oleate which inhibited only a portion of the activity of purified rabbit muscle LDH and had no appreciable effect on the activities of the other purified LDH preparations. Trout liver LDH preperations were not inhibited by *p*-Chloromercuribenzoate (*p*CMB), while rabbit muscle and bovine heart LDH were sensitive to the presence of this inhibitor. Trout liver G6PDH was activated at the lower concentrations of *p*CMB. These data suggest that the reduction of the activities of liver dehydrogenases in the rainbow trout fed a diet containing methyl sterculate was not due to inhibition of these dehydrogenases by this cyclic fatty acid.

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1. Present address: Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331, USA. Technical paper No. 3609, Oregon Agricultural Experiment Station.

INTRODUCTION

Sterculic acid is the major cyclopropenoid fatty acid (CPFA) found to occur naturally in the oils of plants of the order Malvaceae. This order includes the economically important cotton plant. Ingestion of CPFA has been implicated in causing toxicological effects in several species of animals (1, 2) and in being a cocarcinogen for aflatoxin and 2-acetylaminofluorene in the rainbow trout (2).

Results from studies on the mechanism for the biological activity of CPFA suggest that this reacts with cellular sulfhydryl groups on proteins and within membranes (3, 4). The double bond of the cyclopropene ring of CPFA has been shown to form addition products with mercaptans (5). Ory and Altschul (6) reported that lipase which contains sulfhydryls at the active site, was inhibited by sterculate. Methyl sterculate was found to inhibit the fatty acid desaturase system (7, 8). Raju and Reiser (7) suggested that the mechanism of inhibition of desaturase was non-competitive, and was due to the reaction of the cyclopropene ring with the sulfhydryl groups on the enzyme. Allen *et al.* (8), however, concluded that the inhibition was competitive and irreversible and reacted with the sulfhydryl groups of the active site of the appropriate enzyme. In contrast to these observations James *et al.* (9) reported that glutathione did not protect or reverse the inhibition of the desaturase system by sterculate and suggested that the latter affected the steps leading to desaturation.

Recently, reduced activities of several liver dehydrogenases involved in carbohydrate metabolism (10) and glucose-6-phosphatase (4) have been reported in rainbow trout fed 200 ppm of CPFA.

Since the mechanism for the reduction of the activities of these enzymes is not known, the present study was undertaken to determine, *in vitro*, the effect of methyl sterculate on trout liver lactate dehydrogenase (LDH, EC 1.1.1.27) and glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49).

MATERIALS AND METHODS

Frozen trout livers or fresh rabbit muscle were diluted 1:4 (w/v) with 0.01 M tris-citrate (pH 7.0) and homogenized for 10 sec with a Tissumizer (Teckmar Co., Cincinnati, Ohio). The

homogenates were centrifuged for 10 min at 30,000 g. Supernatants were diluted 1:50 (v/v) with 0.01 M tris-citrate (pH 7.0) and used as sources for LDH and G6PDH. These supernatants are referred to as "crude" preparations.

To purify trout liver LDH, 85 ml of the above supernatant was made 0.5 saturated with ammonium sulfate, stirred for 15 min, and centrifuged for 10 min at 30,000 g. The precipitate was discarded and the supernatant (89 ml) was made 70% saturated with ammonium sulfate. After centrifugation at 30,000 g for 10 min, the precipitate was dissolved in 10 ml of 0.05 M potassium phosphate (pH 7.6). This solution was dialyzed against three changes of 0.005 M potassium phosphate (pH 7.6) for 24 hr. The dialyzed solution was applied to a DEAE-cellulose column (2.5 x 45 cm), prepared according to the manufacturer's instructions (Whatman Technical Bulletin IE 2), and equilibrated with 0.005 M potassium phosphate at pH 7.6. After eluting the initial protein fraction, a linear gradient to 0.2 N NaCl in 0.005 M phosphate (pH 7.6) was started. The eluent was continuously monitored at 280 mm and 10 ml fractions were collected. The three most active fractions were pooled, lyophilized and used in these studies. Crystalline rabbit muscle LDH and bovine heart LDH were obtained from Sigma Chemical Co., St. Louis, Mo.

Activities of LDH and G6PDH were determined spectrophotometrically by observing the change in cofactor (NADH or NADP) concentration at 340 nm (10). The concentration of the enzyme preparations was adjusted to provide a change in absorbance of 0.1 to 0.4 per min. To determine the effect of the inhibitors, 0.1 ml of the inhibitor was added to 0.5 ml of enzyme solution. After 5 min of incubation at 25°C, 2.5 ml of a solution containing the buffer, substrate and cofactor were added, and the change in absorbance was followed for 3 min at 25°C. One unit of LDH activity was defined as the amount of enzyme which, under the given conditions of assay, oxidizes 1 µmole of NADH per min. One unit of G6PDH activity was that amount of enzyme which reduces 1 µmole of NADP per min (11).

Methyl sterculate was prepared as described by Pawlowski *et al.* (12) and methyl oleate, a primary standard, was obtained from Hormel Institute, Austin, Minn. The *p*-Chloromercuribenzoic acid (*p*CMB) was obtained from K & K Laboratories, Hollywood, Ca. Solutions of methyl sterculate and methyl oleate were prepared in propylene glycol, while aqueous solutions of *p*-CMB were used.

RESULTS AND DISCUSSION

Results of the purification of trout liver LDH are presented in Table 1 and Fig. 1. Ammonium sulfate fractionation increased the specific activity 2.5-fold. Chromatography on DEAE-cellulose resulted in a further increase in specific activity to a purification of 125-fold in fraction 26. The elution profile (Fig. 1) shows one peak of LDH activity. Polyacrylamide-gel electrophoresis of the preparation (not shown) by the method of Petropakis *et al.* (13) revealed one band of LDH activity (14) and one protein component with a relative mobility corresponding to the LDH band. Purified rabbit muscle LDH demonstrated the characteristic five LDH isozymes (15).

Methyl sterculate did not inhibit crude preparations of trout liver LDH and G6PDH or rabbit muscle LDH (Table 2). Purified preparation of trout liver LDH and bovine heart LDH were only partially inhibited by methyl sterculate, while purified rabbit muscle LDH was completely inhibited (Table 2). These results indicate that methyl sterculate did not inhibit LDH activity in the crude extracts and that purified trout liver and bovine heart LDH were not sensitive to methyl sterculate as purified rabbit LDH was.

Pande and Mead (16) presented evidence demonstrating that the inhibition of stearyl coenzyme A desaturase by sterculate was non-specific and due to the detergent nature of the free fatty acids. To determine if a similar mechanism was responsible for the inhibition of LDH noted in Table 2, the effect of methyl oleate on activities of the LDH preparations was studied. The results (Table 2) reveal that crude extracts of trout liver and rabbit muscle LDH as well as purified preparations of trout liver and bovine heart LDH were not appreciably inhibited by methyl oleate. The data, however, indicate that 1 mM methyl oleate inhibited 31% of the activity of purified rabbit muscle LDH. Therefore, only a small portion of the inhibition of the LDH preparations by methyl sterculate could be due to hydrophobic interaction between the enzyme and the methyl sterculate.

Trout liver G6PDH was activated to the same extent by methyl sterculate and methyl oleate (Table 2). These data suggest hydrophobic interactions between these lipids and the enzyme may have been responsible for the activation.

Data presented in Table 3 show trout liver LDH was not appreciably inhibited by *p*CMB, whereas rabbit muscle and bovine

TABLE 1

PURIFICATION OF RAINBOW TROUT LIVER LDH

Fraction	Vol. (ml)	Activity[a] (units/ml)	Protein (mg/ml)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Liver extract	85	7.4	31	0.24		
50-70% saturated $(NH_4)_2 SO_4$ ppt	20	25.7	42	0.61	81	2.5
DEAE-cellulose						
Fraction No. 25	10	0.2	0.63	0.35	0.4	1.4
26	10	20.5	0.68	30.1	40.3	125.0
27	10	21.9	0.96	22.8	43.0	95.0
28	10	5.1	0.89	5.7	10.0	24.0

[a] One unit of activity represents the oxidation of one μmole of NADH per minute under the conditions described in the text.

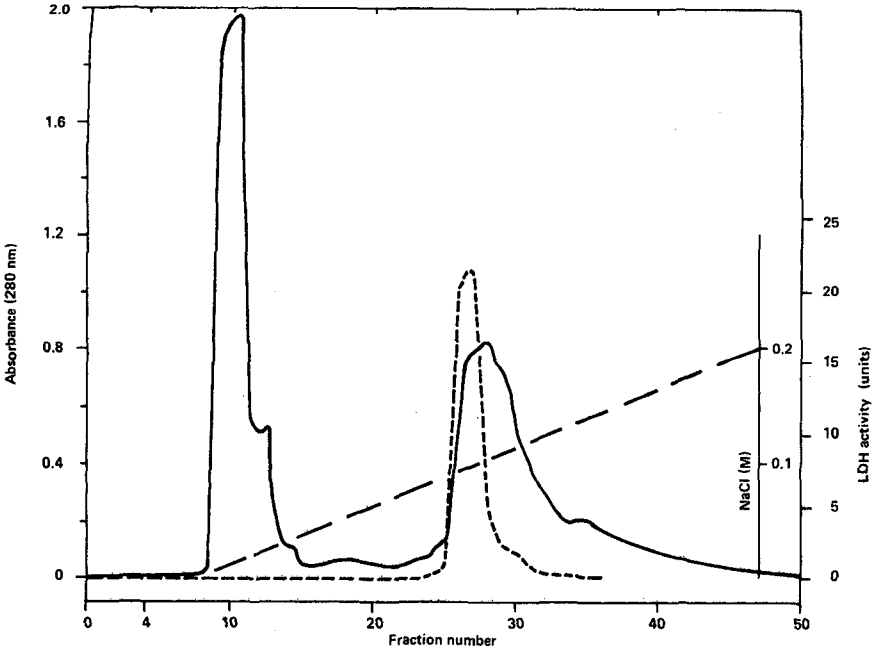

FIGURE 1

Elution profile of trout liver LDH. (Absorbance at 280 nm ——— ; LDH activitiy – – – ; NaCl concentration — — —. See text for details

TABLE 2

EFFECT OF METHYL STERCULATE AND METHYL OLEATE ON ACTIVITY OF LDH AND G6PDH

Inhibitor concentration (μ M)	Trout liver LDH		Rabbit muscle LDH		Bovine heart LDH	Trout liver G6PDH
	Crude	Purified	Crude	Purified	Purified	Crude
	Per cent inhibition					
Methyl sterculate						
0.1	0	0	0	6	4	−7[a]
1	0	0	0	7	5	4
10	0	3	0	64	7	2
100	−6[a]	11	0	100	7	−27[a]
1000	−6[a]	21	0	100	31	−33[a]
Methyl oleate						
0.1	0	0	0	0	−2[a]	−3[a]
1	0	3	0	0	2	−3[a]
10	0	3	0	6	−2[a]	−3[a]
100	−3[a]	7	0	18	0	−27[a]
1000	−3[a]	5	0	31	6	−36[a]

[a] Negative values indicate activation.

heart LDH were inhibited. Since *p*CMB reacts slowly with LDH (17), trout liver LDH was preincubated with 2.7×10^{-4} M *p*CMB for various lengths of time before determination of activity. Only 6% inhibition of the trout liver LDH activity occurred after 75 min when the experiment was terminated.

Raju and Reiser (7) suggested CPFA inhibited fatty acid desaturase by reacting with essential sulfhydryl groups. The similarity of the inhibition patterns of methyl sterculate (Table 2) and *p*CMB (Table 3) suggests sterculate my have reacted with the essential sulfhydryl groups of rabbit muscle and bovine heart LDH. The lack of inhibition of trout liver LDH by *p*CMB, however, indicates that either trout liver LDH does not possess an essential sulfhydryl group, or that the sulfhydryl group did not react with the inhibitor. This observation may account for the lower sensitivity of trout liver LDH to methyl sterculate.

Rainbow trout fed low levels of CPFA had reduced LDH and G6PDH activities and specific activities (10). Although the results of the current study showed a low level of inhibition of purified trout liver LDH by methyl sterculate, this cyclic fatty acid did not inhibit crude trout liver LDH and activated G6PDH. The reduction of dehydrogenase activity levels in livers of CPFA fed trout, therefore, does not appear to have been due to inhibition of the enzymes by CPFA. Reduced protein synthesis (17), inhibition by a metabolite of CPFA, or changes in membrane properties (3, 4) may explain the reduction of activities of the dehydrogenases in livers of CPFA fed trout.

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RESUMEN

INHIBICION TOXICA DE ALGUNAS DEHIDROGENASAS POR ESTERCULATO DE METILO, SUSTANCIA QUE SE ENCUENTRA EN LAS SEMILLAS DE ALGODON

El estudio de que aquí se da cuenta reveló que las preparaciones crudas de lactato dehidrogenasa (LDH, EC 1.1.1.27) provenientes del hígado de la

TABLE 3

EFFECT OF *p*CMB ON ACTIVITY OF LDH AND G6PDH

*p*CMB concentration (μ M)	Trout liver LDH		Rabbit muscle LDH	Bovine heart LDH	Trout liver G6PDH
	Crude	Purified	Purified	Purified	Crude
	Per cent inhibition				
0.01	1	0	14	−5[a]	0
0.1	0	0	23	−10[a]	2
1	2	4	28	−4[a]	−3[a]
10	0	4	48	18	−17[a]
100	6	8	100	24	0

[a] Negative values indicate activation.

trucha arcoiris y del músculo de conejo, no fueron inhibidas por el esterculato de metilo ni por el oleato de metilo; en cambio, la glucosa-6-fosfato dehidrogenasa (G6PDH, EC 1.1.1.49) de hígado de trucha sí fue activada por estos dos ésteres. El esterculato de metilo produjo inhibición sobre preparaciones purificadas de LDH de hígado de trucha, músculo de conejo, y corazón de bovino; en contraste, el metiloleato que inhibió solamente parte de la actividad de LDH de músculo de conejo purificado no tuvo efecto apreciable sobre las otras preparaciones purificadas de esta enzima. El *p*-cloromercurio benzoato (*p*CMB) no inhibió las preparaciones de LDH proveniente de hígado de trucha, mientras que las LDH provenientes de músculo de conejo y corazón de bovino se mostraron sensibles a la presencia del inhibidor.

La G6PDH del hígado de trucha fue activada a bajas concentraciones de *p*CMB. Estos resultados sugieren que la reducción de las actividades de las dehidrogenasas del hígado de las truchas arcoiris, alimentadas con una dieta que contiene esterculato de metilo no se debe a la inhibición de estas dehidrogenasas por el ácido graso cíclico.

BIBLIOGRAPHY

1. Phelps, R. A., F. S. Shenstone, A. R. Kemmerer & R. J. Evans. A review of cyclopropenoid compounds: biological effects of some derivatives. **Poultry Sci., 44**: 358-394, 1965.
2. Lee, D. J., J. H. Wales, J. L. Ayres & R. O. Sinnhuber. Synergism between cyclopropenoid fatty acids and chemical carcinogens in rainbow trout *(Salmo gairdneri)*. **Cancer Res., 28**: 2312-2318, 1968.
3. Nixon, J. E., T. A. Eisele, J. W. Wales & R. O. Sinnhuber. Effect of subacute toxic levels of dietary cyclopropenoid fatty acid composition in the rat. **Lipids, 9**: 314-321, 1974.
4. Scarpelli, D. G., D. J. Lee, R. O. Sinnhuber & M. Chiga. Cytoplasmic alterations of hepatocytes in rainbow trout *(Salmo gairdneri)* induced by cyclopropenoid fatty acids. **Cancer Res., 34**: 2984-2990, 1974.
5. Kircher, H. W. The addition of mercaptans to methyl sterculate and sterculene: an hypothesis concerning the nature of the biological activity exhibited by cyclopropene derivative. **J. Am. Oil Chem. Soc., 41**: 4-8, 1964.
6. Ory, R. L. & A. M. Altschul. Cyclopropenyl compounds as sulfhydryl reagents. **Biochem. Biophys. Res. Comm., 17**: 12-16, 1964.
7. Raju, P. K. & R. Reiser. Inhibition of fatty acyl desaturase by cyclopropene fatty acids. **J. Biol. Chem., 242**: 379-384, 1967.
8. Allen, E., A. R. Johnson, A. C. Fogerty, J. A. Pearson & F. S. Shenstone. Inhibition by cyclopropene fatty acids of the desaturation of stearic acid in hen liver. **Lipids, 2**: 419-423, 1967.

9. James, A. T., P. Harris & J. Bezard. The inhibition of unsaturated fatty acid biosynthesis in plants by sterculic acid. **Eur. J. Biochem., 3**: 318-325, 1968.
10. Taylor, S. L., M. W. Montogomery & D. J. Lee. Liver dehydrogenase levels in rainbow trout *(Salmo gairdneri)* fed cyclopropenoid fatty acids and aflatoxin B_1. **J. Lipid Res., 14**: 643-646, 1973.
11. Noltmann, E. A. & S. A. Kuby. D-Glucose 6-phosphate and 6-phosphogluconate dehydrogenases. In: **The Enzymes**. P. O. Boyer, H. Lardy and K. Myrback, (Eds.). New York, Academic Press, 1963, p. 223-242.
12. Pawlowski, N. E., D. J. Lee & R. O. Sinnhuber. Synthesis of 1,2 dialkylcyclopropenes, methyl malvalate and methyl sterculate. **J. Org. Chem., 37**: 3245-3248, 1972.
13. Petropakis, H. J., M. W. Montgomery, W. D. Davidson & A. F. Anglemier. Separation of bovine sacroplasmic proteins by vertical gel electrophoresis. **Can. Inst. Food Technol. J., 2**: 108-111, 1969.
14. Williscroft, S. N. & H. Tsuyuki. Lactate dehydrogenase systems of rainbow trout - evidence for polymorphism in liver and addition: ' subunits in gills. **J. Fish. Res. Bd. Canada, 27**: 1563-1567, 1970.
15. Wieland, T. & G. Peleiderer. Isozymes and heteroenzymes. **Angew. Chemie, 1**: 169-224, 1962.
16. Pande, S. V. & J. F. Mead. Inhibition of the stearyl coenzyme A desaturase system by sterculate. **J. Biol. Chem., 245**: 1856-1861, 1970.
17. Takenaka, Y. & G.W. Schwert. Lactic dehydrogenase. III. Mechanism of the reaction. **J. Biol. Chem., 223**: 157-170, 1956.
18. Malevski, Y., M. W. Montgomery & R. O. Sinnhuber. Liver composition and protein synthesis in rainbow trout *(Salmo gairdneri)* fed cyclopropenoid fatty acids. **Fed. Proc., 32(3)**: 277, 1973.