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3-HYDROXY-3-METHYLGLUTARYL COA REDUCTASE OF HEVEA LATEX : THE OCCURRENCE OF A HEAT-STABLE ACTIVATOR IN THE C-SERUM

Ramli B. Mohd. Isa and A. B. Sipat[1]

Department of Biochemistry and Microbiology,
Faculty of Science and Environmental Studies,
Universiti Pertanian Malaysia,
Serdang, Selangor,
Malaysia.

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The effect of the C-serum (the cytosol) on the activity of 3-hydroxy-3-methylglutaryl CoA reductase in the latex of Hevea brasiliensis was investigated. Depending on the clone from which the latex was obtained, the C-serum was found to depress or activate or have little effect on the enzyme activity. Boiling the C-serum however, resulted in a consistent activation effect in all the clones examined. Optimal activation was obtained with 20 µl boiled C-serum. Dialysis or EDTA (40 mM) treatment of the boiled C-serum did not diminish the activation effect. Although not essential, dithiothreitol complemented the activation effect of the boiled C-serum and the optimal concentration was 10 mM. Trypsin digestion of the boiled C-serum resulted in the complete loss of the activation effect. The activator in the boiled C-serum was salted out by ammonium sulphate at 25 - 100% saturation. Hevein had no effect on reductase activity.

INTRODUCTION

The latex of Hevea brasiliensis is a cytoplasmic suspension of numerous rubber particles and two other distinct organelles, viz., the lutoids and the Frey-Wyssling complexes (1). Centrifugation of the latex at 42,000 g results in its separation into three main fractions, and these are the rubber layer containing rubber particles, the clear C-serum which corresponds to the cytosol and the sedimented bottom fraction consisting largely of lutoids as well as a smaller number of Frey-Wyssling complexes (2). The rubber itself is a polymer of isoprene with MW ranging from 10^5 - 4 x 10^6. One of the enzymes in the pathway of rubber biosynthesis from acetate is HMG CoA[2] reductase (3-hydroxy-3-methylglutaryl CoA reductase (NADPH), EC 1.1.1.34). This enzyme is found in the bottom fraction of latex obtained from mature trees of H. brasiliensis and it is membrane-bound (3). While the C-serum has neglible reductase activity, its effect on the membrane-bound enzyme has not been studied. In view of the findings that the rat liver microsomal

1. To whom correspondence should be addressed
2. Abbreviations: HMG CoA, 3-hydroxy-3-methylglutaryl CoA; HMG acid, 3-hydroxy-3-methylglutaric acid.

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reductase is profoundly affected by factors present in the cytosol (4), this aspect of the latex enzyme was therefore investigated. Knowledge of the interaction between the reductase and the C-serum is essential in understanding the role of this enzyme in the regulation of rubber biosynthesis.

MATERIALS AND METHODS

Chemicals - CoA (the Lithium salt), NADPH, dithiothreitol, HMG acid and trypsin (porcine pancreas - Type II) were purchased from Sigma Chemical Co., while [3-^{14}C]HMG acid was from New England Nuclear, U.S.A. DL-[3-^{14}C]HMG CoA was prepared as described by Goldfarb and Pitot (5) using excess [3-^{14}C] HMG acid anhydride. The excess [3-^{14}C]HMG acid was removed by column chromatography (6). Hevein (the pH 5 eluate) was a gift from Dr. S.J. Tata of the R.R.I.M. Experimental Station Sungei Buloh.

Collection and Fractionation of Latex - The fresh latex was collected and fractionated as described elsewhere (3). The fractions used in the present study were the bottom fraction and the C-serum. The bottom fraction was resuspended in Buffer A (pH 7.1) consisting of 0.1 M triethanolamine-HCl and 20 mM dithiothreitol and this is referred to as the bottom fraction suspension (BFS). The washed bottom fraction suspension (WBFS) was prepared by sonicating (for 30 sec) a suspension of the bottom fraction in Buffer B (pH 7.1) containing 5 mM triethanolamine-HCl and 2 mM dithiothreitol and this was then centrifuged at 103,000 g for 1 hr. The 103,000 g pellet obtained was resuspended in Buffer A (WBFS). Alternatively, this pellet was resuspended in Buffer B containing 1% (w/v) Triton X-100 in the ratio of 1:3 (pellet/v). This suspension was shaken on a vortex mixer for 1 min, allowed to stand on ice for 15 min and then centrifuged at 103,000 g for 1 hr. The resulting 103,000 g supernatant contained typically about 50% of the reductase activity with the remaining still being membrane-bound. All operations were carried out at 0 - 4°C.

Assay of HMG CoA reductase activity - The assay procedure for the enzyme activity in the BFS, WBFS and for the solubilised enzyme was essentially as described by Shapiro et al. (7). The reaction mixture contained : enzyme protein, 0.2 - 0.8 mg; NADPH, 0.46 µmol; DL-HMG CoA, 36 nmol (444 dpm/nmol) and Buffer A in a final volume of 0.15 ml.

The incubation was carried out at 30°C for 30 min. The isolation and the measurement of mevalonate is described elsewhere (3).

Protein Assay - Protein was determined by the method of Lowry et al. (8) after precipitating with trichloroacetic acid (10%, w/v) and using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Activation effect of the C-serum - The effect of the C-serum on reductase activity was examined in three different preparations of the enzyme. The BFS contained not only the membrane-bound reductase but also various hydrolytic enzymes and other proteins released upon rupturing the organelles in the bottom fraction. These soluble proteins are removed by washing and thus the membrane-bound reductase in the WBFS was relatively cleaner. The detergent soluble fraction contained the solubilised reductase according to the criterium of non-sedimentation even at 103,000 g (9). As shown in Table 1, the C-serum has an activation effect on reductase activity irrespective of whether the enzyme is soluble or membrane-bound. Boiling the C-serum did not

Table 1. Effect of C-serum on reductase activity

Enzyme source	Addition	Specific Activity (nmol mevalonate formed/ 30 min/mg protein)	Relative Activity %
BFS	none	11.47 ± 0.47	100
	C-serum	15.67 ± 0.61	137
	boiled C-serum	15.40 ± 0.44	134
WBFS	none	38.65 ± 1.31	100
	C-serum	52.62 ± 7.57	136
	boiled C-serum	57.61 ± 1.19	149
Soluble Fraction	none	6.05 ± 0.43	100
	C-serum	8.24 ± 0.60	136
	boiled C-serum	8.85 ± 2.21	146

The preparation of the BFS, WBFS and Soluble Fraction, and the assay of reductase activity, were as described in Materials and Methods. The boiled C-serum was the supernatant obtained after centrifuging (3000 g, 15 min) C-serum which had been boiled for about 20 min. The amount of C-serum or boiled C-serum present in each reaction mixture was 40 µl and this contained approximately 480 µg or 40 µg protein respectively. Each value in the Table is the mean, ± S.D., of triplicate measurements.

diminish the activation effect. A comparison of the activation effect of the boiled and deproteinised (by centrifugation at 3000 g) C-serum against boiled but not deproteinised C-serum showed that the former (henceforth referred to as the boiled C-serum) was more effective. Thus in the ensuing studies, the boiled C-serum and the WBFS were used, the latter since it also contained a higher reductase activity compared to the soluble enzyme preparation. The boiling treatment also removed the bulk of the protein in the C-serum so that about 0.9 - 1.1 mg/ml boiled C-serum remained from the original concentration of approximately 12 mg protein/ml C-serum.

The effect of the C-serum from other clones of H. brasiliensis on the reductase in the WBFS from RRIM 600 (U.P.M.) was also investigated. The results in Table 2 show that the effect of the C-serum from each of these clones was variable. Clone PB 86 produced the highest activation while clone RRIM 501 showed an inhibition instead. The activation produced by the C-serum from GT 1 and Tjir 1 was minimal. The boiled C-serum from all these clones however, produced an activation effect and the extent of activation obtained was remarkably similar as compared to the variability observed with

Table 2. Effect of the C-serum from various clones on reductase activity in WBFS from RRIM 600 (U.P.M.)

Addition		Specific Activity (nmol mevalonate formed/ 30 min/mg protein)	Relative Activity %
None		5.16 ± 0.78	100
C-serum	Clone		
	RRIM 600 - U.P.M.	8.37 ± 0.49	163
	RRIM 600	8.08 ± 1.28	157
	RRIM 501	3.74 ± 1.10	73
	RRIM 701	6.37 ± 0.45	124
	GT 1	5.63 ± 0.82	109
	Tjir 1	5.85 ± 0.17	113
	PB 86	9.51 ± 0.35	185
boiled	RRIM 600 - U.P.M.	9.25 ± 1.07	179
C-serum	RRIM 600	9.54 ± 0.65	185
	RRIM 501	10.22 ± 0.77	198
	RRIM 701	9.35 ± 1.05	181
	GT 1	9.07 ± 0.94	176
	Tjir 1	9.50 ± 0.17	182
	PB 86	9.31 ± 0.42	181

The C-serum of each of the above clones (except for RRIM 600 - U.P.M.) was prepared in the R.R.I.M. Sungei Buloh Laboratory and transported to U.P.M. These preparations were stored frozen overnight before being tested for their activation effect on the reductase in WBFS prepared from RRIM 600 (U.P.M.) as described in Materials and Methods. The amount of C-serum or boiled C-serum used in each assay was 60 µl. Each value in the Table is the mean, ± S.D., of triplicate measurements.

the respective C-serum. These results indicate that the C-serum contains a heat-stable activator of the reductase but its effect however, appears to be dependent upon a factor which is heat-labile. The net effect observed with the C-serum from the various clones is thus the balance between this activator and its heat-labile modulator.

An examination of the concentration effect of the boiled C-serum showed that maximum activation was obtained with 20 µl under the assay conditions described in Materials and Methods. Dialysis of the boiled C-serum against distilled water (using Visking 8/32 tubing) did not diminish the activation effect and neither did treatment of the dialysed or the undialysed boiled C-serum with EDTA (40 mM).

Effect of dithiothreitol - Preliminary experiments established that the reductase in BFS and WBFS requires about 2 mM and 20 mM dithiothreitol respectively for optimal activity. The C-serum itself is known to contain

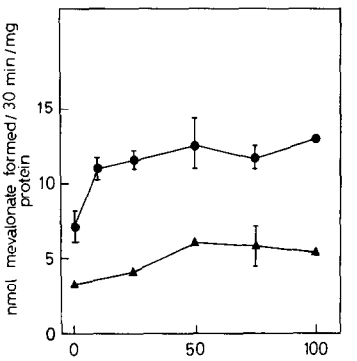

Fig. 1 Effect of dithiothreitol on the activation of the reductase in WBFS by boiled C-serum.

Dithiothreitol was omitted from the appropriate buffers during the preparation of the WBFS. Reductase activity was then assayed without (—▲—▲—) or with (—●—●—) boiled C-serum (25 µl) in the presence of the above concentrations of dithiothreitol. Details of the experimental procedures are given in Materials and Methods. Each point on the curve represents the mean, ± S.D., of triplicate measurements.

various thiols (10). In order to determine whether the activation effect of the boiled C-serum was due to the presence of these endogenous thiols, an experiment was performed in which the reductase activity in the presence of a fixed amount of boiled C-serum (25 µl) was assayed with varying concentration of dithiothreitol. The results in Fig. 1 show a biphasic effect in which initially the activation was dependent on the amount of dithiothreitol but above a concentration of 10 mM, the activation obtained was maximal. The boiled C-serum thus does not function as a simple protective agent for sulphydryl groups of the reductase, since one would then expect both curves in Fig. 1 to coincide at high dithiothreitol levels. These results also indicate that the boiled C-serum requires free sulphydryl groups for optimal activation effect.

Effect of trypsin digestion of the boiled C-serum - The activator in the boiled C-serum is apparently not a metal ion or metal-dependent nor is it a low molecular weight compound. Another major component present in the boiled C-serum is a heat-stable protein. To see whether this protein was involved in the activation effect, the boiled C-serum was therefore treated with trypsin. As shown in Table 3, the efficacy of the boiled C-serum as an activator decreased considerably after 30 min incubation with trypsin, and after incubation for 90 min, the activation effect was no longer observed. The activator thus appears to be a polypeptide.

The occurrence of a heat-stable protein in latex is not uncommon. Hevein, a protein of MW of about 10,000, is heat-stable but it is present mainly in the 'B-serum' which is the liquid obtained by repeated freeze-

Table 3. Effect of trypsin-treated boiled C-serum on reductase activity in WBFS.

Addition	Specific Activity (nmoles mevalonate formed/ 30 min/mg protein)	Relative Activity %
None	3.97 ± 0.80	100
Boiled C-serum	8.00 ± 0.18	201
Trypsin-treated boiled C-serum (30 min)	5.26 ± 0.88	132
Trypsin-treated boiled C-serum (90 min)	3.58 ± 0.12	90
Boiled C-serum treated with denatured trypsin (90 min)	7.60 ± 0.84	191
Boiled C-serum plus distilled water (90 min)	7.54 ± 0.31	190

1.0 ml of boiled C-serum (see legend to Table 1) was incubated at 30°C with either trypsin (1 mg in 20 µl Buffer A) or denatured trypsin (by boiling ; 1 mg in 20 µl Buffer A) or distilled water (20 µl). The incubation time ranged up to 90 min and the reaction was terminated by boiling. Each of the thus treated boiled C-serum (55 µl) was then assayed for its activation effect on reductase activity as described in Materials and Methods. Each value in the Table is the mean, ± S.D., of triplicate measurements.

thawing of the bottom fraction (11). The activator however, is not hevein since the latter when tested at a concentration of 1.1 mg(dry wt)/ml water showed no activation effect (an activity of 15.13 ± 1.30 was obtained in comparison to 14.84 ± 0.47 (in units of µmol mevalonate formed/30 min/mg protein) in the absence of hevein).

Ammonium Sulphate Fractionation of the boiled C-serum - Since the above results indicate that a heat-stable protein is involved, an attempt was made to precipitate the activator using ammonium sulphate. It was found that most of the activator was salted out within the range of 25 - 100% saturation. Very little activator was present in the supernatant fraction after 100% saturation. Not all the material precipitated by 0 - 100% ammonium sulphate saturation will redissolve in distilled water. There usually remained an insoluble greyish white residue. This residue, when resuspended in distilled water by sonication (3 min), had a slight activation effect on the reductase in the WBFS. The bulk of the activator was water-soluble.

CONCLUSION

The activity of HMG CoA reductase is known to be affected by various modulators which range from low molecular weight compounds to proteins (12). Most of these modulators exert an inhibitory effect on the enzyme except for a protein in the cytosol of the rat liver (4). The latter is now known to be a protein phosphatase which acts by dephosphorylating the inactive phosphorylated form of the reductase (13).

The present study demonstrates the occurrence of a heat-stable activator of the reductase in the C-serum (the cytosol) of Hevea latex. This activator appears to be a non-enzymic protein and it is not hevein. Further work is currently being carried out to purify and characterise this protein and to determine its mechanism of action.

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