BBA 65660

STUDIES ON THE SOLUBILIZATION AND PURIFICATION OF RAT LIVER MICROSOMAL STEROID SULFATASE

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(Received May 29th, 1967)

SUMMARY

Digestion of rat liver microsomal preparations with heat-treated snake venom led to a mean 27% solubilization of the steroid sulfatase activity. Further purification by $(NH_4)_2SO_4$ precipitation followed by chromatography on DEAE-Sephadex A-25 afforded a 1.5-fold increase in the specific activity over that of untreated microsomes. The highest purification achieved with some preparations following chromatography on DEAE-Sephadex A-50 was 3-fold. The soluble preparation was associated with high molecular weight material (about 600 000, as judged by filtration on Sephadex G-200) and exhibited a tendency to form insoluble aggregates.

INTRODUCTION

Mammalian steroid sulfatase (sterol-sulfate sulfohydrolase, EC 3.1.6.2) exhibits interesting species and tissue distributions[1,2]. Thus, while the activity of the enzyme is high in rat liver, a relatively low activity is found in mouse and especially in guinea-pig liver; the high activity found in guinea-pig testis is contrasted by the relatively low activity in rat testis.

The question of whether there exist species or tissue variants of steroid sulfatase has been difficult to settle because of the insoluble (particulate) nature of this enzyme. The hepatic enzymatic activity is associated primarily with the microsomes[1,2] and attempts to solubilize the enzyme by various methods that employ sonic oscillation, organic solvents or bile salts have been unsuccessful (refs. 1 and 3 and unpublished observations).

The present report describes studies on the solubilization and further purification of steroid sulfatase from rat liver microsomal preparations using crude phospholipase A from snake venom.

MATERIALS AND METHODS

Sprague–Dawley type rats, 200–500 g, mostly males obtained from local suppliers, were used. Rat liver microsomes were prepared in 0.25 M sucrose by

centrifugation of the 15 000 × *g* (10 min) supernatant at 320 000 × *g* for 0.5–1.0 h as previously described[2,4]. The low-speed centrifugation was done in the Sorvall SS-1 Superspeed angle centrifuge, while centrifugation at higher speeds was done in the International Equipment Co. T60 ultracentrifuge. All reagents were of analytical grade. The source of snake venom phospholipase A was heat-treated extracts of lyophilized snake venom from the Miami Serpentarium Laboratories, Miami, Florida 33156.

Steroid sulfatase activity was determined using 7*a*-[^{3}H]dehydroisoandrosterone sulfate as substrate by the previously described procedure in 0.05–0.1 M Tris-chloride buffer (pH 7.4) at 37° (see ref. 2). The total incubation volume was 4.0 ml and 1.0-ml aliquots were removed at 2, 10 and 20 min. The substrate used had a specific activity of 0.8 mC/mmole and the concentration in the assay medium was 0.075 mM. ^{3}H was determined in an automatic Packard Tri-Carb liquid scintillation spectrometer (series 314E) set to an efficiency of 18.2%. The enzymatic activity is expressed as disint./min of free steroid released in 1 min (= 1 unit) under the conditions of the assay. Enzymatic activities were determined in the linear range of the activity–concentration curves, as described elsewhere[4], where a reasonable additivity (within ± 10%) obtains. Protein was determined by the method of LOWRY *et al.*[5].

Digestion with snake venom was done essentially according to IMAI AND SATO[6] using heat treated extracts of *Naja naja* or *Trimeresurus flavoviridis* venoms.

By soluble enzymatic activity is meant a preparation which does not sediment at 320 000 × *g* for 1 h.

EXPERIMENTAL AND RESULTS

Optimization of the snake venom digestion procedure

There was no significant difference in the yield of soluble enzymatic activity obtained following digestion with extracts from *Naja naja* or *Trimeresurus flavoviridis* venoms and most of the results reported were done with the latter. No significant difference was observed between the action of heat-treated venom (presumably containing only phospholipase A activity) and heat-untreated venoms, indicating that the proteinases of snake venom did not cause significant solubilization[6]. The results reported were done with heat-treated venom.

Typical results of the effect of pH on the stability of rat liver microsomal steroid sulfatase and the yield of soluble activity following digestion with snake venom is given in Table I. It appears that storage at pH 9 of venom-untreated microsomes caused a considerable loss in steroid sulfatase activity, while no significant change in activity was found at pH 8.3. Treatment with venom at pH 8.3 led to a smaller loss than treatment at pH 9, which may account for the higher yield of soluble activity obtained at pH 8.3. Optimal yields were obtained in the range of 8.3–8.5.

That the observed losses largely represented losses in enzymatic activity rather than the release of an inhibitor was determined from experiments with mixed digested and untreated microsomes. As may be seen from the 2 last rows of Table I, the mixed preparations at worst exhibited only a 15% lower activity than the combined activities of the preparations determined separately, which would not account for the observed lower activity after digestion.

TABLE I

EFFECT OF pH ON THE STABILITY AND YIELD OF SOLUBILIZATION OF RAT LIVER MICROSOMAL STEROID SULFATASE

Rat liver microsomes were suspended in 1.15% KCl to a tissue concn. of 1.8 g/ml. To 11-ml aliquots were added 4 ml of 0.5 M Tris-chloride buffer (at the specified pH) followed by 1 ml of a 1% venom extract (previously kept at 100° for 8 min in 0.05 M Tris chloride (pH 7.4)) ('venom treated') or by 1 ml of 0.05 M buffer alone ('untreated'). The suspensions were stored at 4° for 17 h and the pH adjusted to 7.4 with 1 M HCl. All fractions were brought up to 20 ml with distilled water, and aliquots of 1 ml taken for total sulfatase determination 'total'. The remainder was centrifuged at 320 000 × *g* and 1-ml aliquots taken for assay from the supernatants. 1-ml aliquots of untreated microsomes were mixed with either 1-ml aliquots of venom-treated microsomes (pH 8.3) or venom-treated microsomes (pH 9.0) and 1-ml aliquots of the mixtures assayed.

Conditions	*Sulfatase activity (units/ml)*	
	Total	*Soluble*
Venom treated (pH 9.0)	1000	570
Venom treated (pH 8.3)	1300	840
Venom treated (pH 7.4)	2000	0
Untreated (pH 9.0)	1600	0
Untreated (pH 8.3)	2150	0
Untreated (pH 7.4)	2100	0
Untreated (pH 7.4) + venom treated (pH 8.3)	1680	
Untreated (pH 7.4) + venom treated (pH 9.0)	1330	

TABLE II

YIELD OF SOLUBLE STEROID SULFATASE ACTIVITY OBTAINED FROM VARIOUS RAT LIVER MICROSOMAL PREPARATIONS

Digestion was done at pH 8.3 with heat-treated venom as described in the legend of Table I. With the exception of Prep. 4 (done with *Naja naja*), all preparations were done with *Trimeresurus flavoviridis*. Loss of activity was determined with the unfractionated digest after pH adjustment to 7.4.

Prep. No.	*Sex*	*Loss of activity due to venom treatment* (%)*	*Fraction soluble* (%)*
1	M	50	28
2	M	54	23
3	M	59	19
4	F	51	24
5	M	51	17
6	F	62	21
7	M	47	22
8	M	63	23
9	F	55	33
10	M	53	38
11	M	52	35
12	M	55	36

Abbreviations: M, male; F, female.
* Expressed as per cent of the original microsomal activity.

Varying the snake-venom concentrations of the extracts used from 0.3 to 3.0% did not significantly affect the yield and as a role 1% extracts as described by IMAI AND SATO[6] were used (see legend of Table I). Digestion with venom for 17 h (the time used by these investigators) also appeared to be optimal for the solubilization of steroid sulfatase. Digestion periods of 40 h led to approx. 50% loss of the soluble activity obtained after 17 h. Redigestion with venom of the insoluble activity (sedimenting at 320 000 × *g* after digestion) yielded only insignificant additional soluble enzyme.

Yields of soluble steroid sulfatase from various rat liver microsomal preparations

Table II summarizes the yields of soluble enzyme obtained with various rat liver microsomal preparations. This table also summarizes the observed losses of total enzymatic activity during digestion. There did not appear to be a significant sex difference in the per cent yield of soluble enzyme obtained, although a higher activity has been found in adult males than females[1,4].

TABLE III

PURIFICATION OF SOLUBLE STEROID SULFATASE ACTIVITY

Fraction	*Yield* (%)*	*Specific activity (units per mg protein)*
Microsomes	100	310
Venom digest	44	130
Venom-treated supernatant	22	180
$(NH_4)_2SO_4$, 20%	18	320
Dialysis	18	310
Chromatography on DEAE-Sephadex A25	10	450

* Expressed as per cent of the activity of the original microsomes.

Attempts at further purification and some properties of the soluble steroid sulfatase

Typical data of the purification of the soluble steroid sulfatase is summarized in Table III. As may be seen, the unfractionated soluble enzyme had a lower specific activity than the original microsomal enzyme, which is in line with the observed losses in activity during venom digestion. Most of the activity was precipitated with 20% $(NH_4)_2SO_4$ at which stage the specific activity in most cases (and as shown in Table III) increased to the untreated microsomal value. Further slight enrichment was sometimes obtained by a second $(NH_4)_2SO_4$ precipitation. In some cases the $(NH_4)_2SO_4$ precipitate did not completely dissolve in buffer and an additional centrifugation (at 20 000 × *g*) was required. Dialysis (in 0.05 M Tris chloride (pH 7.5)) of the soluble enzyme (after the $(NH_4)_2SO_4$ precipitation) for periods up to 72 h did not lead to a significant change in the specific activity. Chromatography on DEAE-Sephadex A-25 (on a 2 cm × 50 cm column in Tris-chloride (0.05 M, pH 7.4) buffer) with increasing NaCl concentrations led to a 1.5-fold increase of the specific activity; the enzyme emerged from the column with 0.5 M NaCl. However, this technique also led to a considerable loss of activity. A higher specific activity was achieved by

chromatography on DEAE-Sephadex A-50 (performed as described above, the steroid sulfatase emerging with 2 M NaCl). However, this procedure also led to an appreciable loss. The highest purification observed (which varied with the particular microsomal preparation used) was only 2–3-fold higher than that found in the original microsomes.

Chromatography on Sephadex G-100 or G-200 afforded only a slightly higher specific activity than that obtained by $(NH_4)_2SO_4$ precipitation. The sulfatase on G-200 emerged slightly earlier than thyroglobulin, indicating that the activity was associated with high molecular weight material probably >600 000.

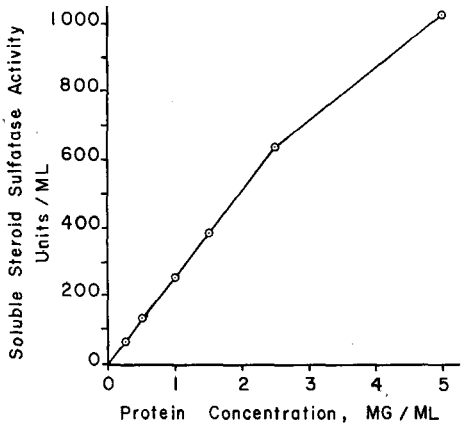

Fig. 1. Activity–concentration relationship of the soluble steroid sulfatase. The soluble preparation used was that obtained after the dialysis step (Table III).

A typical curve showing the relationship between activity and enzyme concentration with a soluble steroid sulfatase preparation is given in Fig. 1, from which it may be seen that the activity is linear with concentration only up to approx. 2 mg protein per ml. It is noteworthy that the linearity of the activity–concentration curve with the microsomal enzyme also broke down approximately above this concentration[4].

The soluble preparations exhibited a tendency to form enzymatically active insoluble aggregates spontaneously upon standing in the cold room without appreciable loss in total activity. The rate of aggregation varied from preparation to preparation, sometimes leading to more than 90% precipitation of the activity which could not be brought back into solution.

Acidification of soluble preparations led to appreciable clouding at pH 5.0, followed by complete clarification upon further acidification to pH 2.8. Acidification to pH 2.8 even for a few minutes at 0° caused more than 90% loss of activity. Increasing the pH from 5.0 back to 7.4 caused almost complete clarification, with only some opalescence remaining. This procedure did not lead to a significant change in activity. Precipitation with acetone (at −10°) abolished the solubility in buffer although 80% of the enzymatic activity was retained.

DISCUSSION

This report describes the preparation of a 'soluble' (not sedimented at 320 000 × *g* for 1 h) steroid sulfatase from rat liver microsomes, using heat-treated snake venom. By the chromatographic techniques used it was not possible to enrich the specific activity of the soluble preparations by more than 3-fold of that exhibited by the untreated microsomes. The activity seems to be associated with relatively high molecular weight aggregates (probably higher than 600 000) which apart from not sedimenting at the specified force and time, seem to resist further purification. The greatest difficulty encountered in the purification study was the tendency of the soluble activity to form insoluble aggregates which resisted further attempts at solubilization.

The question of the loss of enzymatic activity which followed (heat treated) snake-venom digestion is difficult to interpret. The possibility that the bulk of the phospholipids of microsomes may be required for activity can be excluded since it was possible to remove more than 90% of the phospholipids by solvent treatment and obtain preparations, which, although insoluble, retained 80% of their activity. However, the possibility that tightly bound specific phospholipids in small concentrations may play a role in the steroid sulfatase activity, cannot be eliminated at present since it was not possible to obtain active preparations entirely devoid of phospholipids.

ACKNOWLEDGEMENTS

This study was supported by Public Health Service Research Career Development Award 1-K3-CA-3949 from the National Cancer Institute and Research Grant AM-07206 from the National Institute of Arthritis and Metabolic Diseases. The technical assistance of C. F. McDONALD is gratefully acknowledged.

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