PURIFICATION AND CHARACTERIZATION OF RAT LIVER TYROSINE AMINOTRANSFERASE

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1. Introduction

Several studies have been published describing the purification and properties of L-tyrosine-2-oxoglutarate aminotransferase (EC 2.6.1.5.). The purification was first attempted from the livers of L-tyrosine treated rats [1,2]. Later, a more extensive purification from the livers of rats previously treated with glucocorticoid hormones was reported [3–5].

In this paper, we describe a purification method for the soluble tyrosine aminotransferase (TAT) from livers of rats treated with the synthetic glucocorticoid: dexamethasone. The enzyme was purified by fractionation techniques utilizing heat treatment, chromatography on DEAE-cellulose and hydroxyapatite columns, gel-filtration on Sephadex G-200 and sedimentation in a sucrose gradient. During the whole purification procedure, the enzyme remained soluble since steps like precipitation by ammonium sulfate or concentration by freeze-drying were avoided. The overall purification factor was in the range of 3000–4000 with a recovery of 30–40% of the activity originally present in the crude extract. Several properties of the enzyme were studied.

2. Materials and methods

2.1. *Materials*

Dexamethasone, cortisol and corticosterone were generously donated by Roussel-Uclaf Co., Romainville, France. L-Tyrosine, α-ketoglutaric acid, pyridoxal-5′-phosphate and sucrose (for density-gradient ultracentrifugation) were purchased from Merck, dithiothreitol (Cleland's reagent) and phenylmethyl-sulfonyl fluoride (PMSF) from Sigma. DEAE-Cellulose (DE 52) and cellulose (CF 11) were obtained from Whatman. Hydroxyapatite (Biogel HTP) was purchased from Bio Rad Laboratories and Sephadex G-200 from Pharmacia. Dialysis tubing from Union Carbide Corporation was boiled twice for 20 min in 1% sodium carbonate and 10^{-4} M EDTA solution, rinsed and stored in distilled water at 4°C.

2.2. *Enzyme assay*

Tyrosine aminotransferase was assayed at 37°C by the method of Diamondstone [6]. One unit of activity represents the formation of *p*-OH-phenylpyruvate 1 μmol/min. Protein concentrations were measured by the method of Lowry et al. [7] with bovine serum albumin (Sigma) as the standard.

3. Results

3.1. *Stimulation and extraction of hepatic tyrosine aminotransferase*

Male Wistar rats, weighing 200–250 g each, were starved for 8 h preceding the intraperitoneal injection with the synthetic hormone dexamethasone given as a single dose of 10 mg in 0.5 ml 1% gelatin solution/100 g body w. (Gelatin was added in order to get a better suspension of the hormone and also to extend the hormonal effect.) The rats were decapitated 8–12 h after injection. In order to quickly chill the tissue and to remove the blood, the liver was immediately perfused via the vena cava with 20 ml ice-cold 0.9% NaCl, then with 20 ml phosphate buffer 1 (50 mM, pH 6.5, 0.5 mM α-ketoglutarate, 0.1 mM pyridoxal-5′-phosphate and 0.1 mM PMSF). This

Table 1
Hormonal stimulation of rat liver tyrosine aminotransferase

Hormone	Specific activity (munits/mg protein)
Control (0.9% NaCl)	15
Cortisol 5 mg	85
Corticosterone 10 mg	155
Dexamethasone 10 mg	320

Hormone dose is /100 g body weight. Each value is the mean for three animals (duplicate assays) measured 8 h after hormone treatment.

procedure took only 2–3 min. The following operations were carried out at 4°C. The liver was excised, cut into small parts and gently homogenized with 2 vol. phosphate buffer 1 in a glass Potter-Elvehjem homogenizer fitted with a motor-driven Teflon-pestle. The homogenates were centrifuged at 800 × *g* for 10 min to remove cell nuclei and the supernatant recentrifuged for 20 min at 10 000 × *g* to remove the mitochondrial fraction. The ribosomal pellet was discarded by a centrifugation at 105 000 × *g* for 2 h in a Beckman S30 rotor; the supernatant solution was collected and used as the enzyme source (crude extract).

Table 1 shows the differential stimulation by the synthetic hormone, dexamethasone, compared with the natural hormones, cortisol and corticosterone. A single injection of the synthetic hormone led to maximal values of TAT 8 h later and these levels remained relatively constant during a 12 h induction-cycle. However, Grossman and Mavrides [8] have shown that natural hormones must be given at hourly intervals throughout the experimental period, in order to reach and to sustain a 20–30-fold greater enzyme activity than the basal (non-induced) level. This phenomenon might be due to the fact that natural hormones are more rapidly metabolized than dexamethasone.

3.2. *Purification procedure*

Table 2 summarizes the procedure used. All operations were performed at 4°C unless otherwise stated and the buffers used contained 0.1 mM PMSF.

3.2.1. Step 1

The crude extract from 40 rats (in 250 ml batches) was placed in a water bath at 70°C and was stirred constantly until the temperature reached 65°C. After 5 min at 65°C, the extract was cooled in an ice-brine bath. The precipitate was removed by centrifugation at 12 000 × *g* for 15 min. The supernatant solution was collected and dialysed twice for 45 min against 10 vol. of phosphate buffer 2 (25 mM, pH 7.6, 0.1 mM pyridoxal-5′-phosphate, 0.5 mM α-keto-glutarate, 1 mM dithiothreitol).

3.2.2. Step 2

The extract was next applied to a DEAE-cellulose column developed as described in fig.1a. The enzyme fractions eluted by increasing ionic strength (0.24–0.34 M KCl) were combined and dialyzed against phosphate buffer 2.

3.2.3. Step 3

The enzyme solution was then chromatographed on hydroxyapatite as shown in fig.1b. The active fractions eluted by the phosphate gradient were collected and concentrated to 1–2 ml by dialysis under reduced pressure against phosphate buffer 2.

Table 2
Purification scheme for tyrosine aminotransferase

Step	Stage	Volume (ml)	Total protein (mg)	Activity (units/ml)	Total activity units	Specific activity (units/mg protein)	Yield (%)	Purification (*n*-fold)
	Crude extract	750	18 700	8.5	6350	0.34	100	
1	Heat treatment	720	3950	8.6	6150	1.38	97	4
2	DEAE-Cellulose	130	157	41.2	5350	32	84	94
3	Hydroxyapatite	80	25.4	52.5	4200	165	66	485
4	Sephadex G-200	40	5.5	78.7	3150	572	49	1680
5	Sucrose gradient	10	2	240	2400	1200	37	3500

The results above mentioned are taken from a typical preparation starting with the livers of 40 rats.

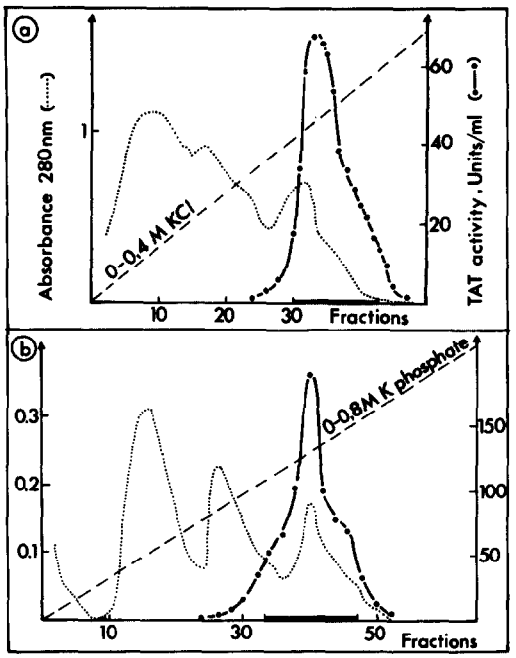

Fig.1(a). Chromatography on DEAE-cellulose. The enzyme solution (1 vol.) was applied to a column (5 × 10 cm) of packed cellulose (one-fifth volume) equilibrated with the dialysis buffer. The column was then washed with buffer 2 (one-third volume). Chromatography was carried out at a flow-rate of 1 ml/min by the use of 1 vol. of a linear gradient from 0–0.4 M KCl in phosphate buffer 2. Fractions of 10 ml were collected. Fig.1(b). Chromatography on hydroxyapatite. Hydroxyapatite was packed together with 10% (w/w) of cellulose in a column (2.3 × 15 cm) and equilibrated in phosphate buffer 2. The enzyme solution was applied; after adequate washing with the buffer, the column was developed with a potassium phosphate gradient from 25–800 mM (total vol. 800 ml in 6 ml fractions). The column was eluted at the rate of 0.5 ml/min. Fractions were assayed for absorbance at 280 nm (·····) and TAT activity (•——•).

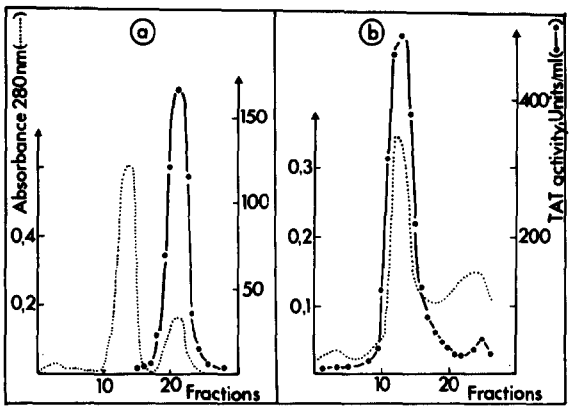

Fig.2(a). Gel-filtration on Sephadex G-200. The column (2.2 × 60 cm) was eluted with buffer 1 at a flow-rate of 22 ml/h. Fractions of 5 ml were collected and analyzed. Fig.2(b). Sedimentation profile of TAT in a sucrose gradient. The enzyme solution (0.5–1 ml) was layered on a 11 ml linear sucrose gradient (5–20%) in phosphate buffer 2 and centrifuged at 40 000 rpm in a Beckman SW41 rotor for 40 h. The bottoms of the tubes were pierced and 0.5 ml fractions were collected and analyzed.

3.2.4. Step 4

The concentrated solution was applied to a Sephadex G-200 column. The elution profile presented in fig.2a shows that the second peak which does not occur with the void volume of the column included all of the enzyme activity. The fractions containing enzyme activity were pooled and concentrated under reduced pressure to a volume of 1–2 ml.

3.2.5. Step 5

The extract was finally sedimented in a sucrose gradient where enzyme activity appeared to be associated only with the fast sedimenting peak (fig.2b). The enzyme appears homogeneous at this stage of purification (table 2); the specific activity reaches a value between 950 and 1300 units/mg protein depending on the preparation.

The purified enzyme was concentrated by dialysis under reduced pressure against phosphate buffer 2 containing 50% (v/v) glycerol. It could be stored at −20°C for several months without appreciable loss of activity.

3.3. *Properties of the enzyme*

The purified enzyme was shown to be homogeneous by the criteria of disc-gel electrophoresis and ultracentrifugation. Polyacrylamide gel electrophoresis of tyrosine aminotransferase showed a single component as revealed with Coomassie Blue. Staining with the specific reagent [10] gave a positive reaction at the same position on the gel (fig.3a,b). A single protein band was obtained by electrophoresis in the presence of sodium dodecyl sulfate (fig.3c). A molecular weight of 52 000 was determined when its position was compared to known standards (ovalbumin 43 000, bovine serum albumin dimer 136 000, chymotrypsin

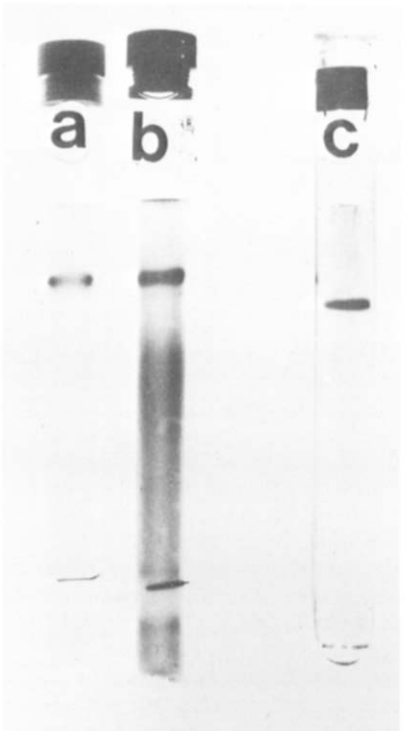

Fig.3. Polyacrylamide gel electrophoresis. The purified enzyme preparation was electrophoresed according to Reisfeld and Small [9]. Samples of 20 μg and 0.01 μg were applied, respectively, for (a) staining with Coomassie Blue and (b) specific staining with *p*-iodonitrotetrazolium violet, phenazine methosulfate, monoiodotyrosine [10]. (c) Sodium dodecyl sulfate–gel electrophoresis was performed according to the technique of Shapiro [11] using a 20 μg sample.

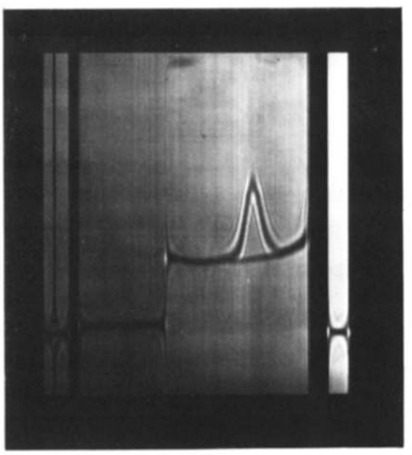

Fig.4. Schlieren sedimentation pattern of tyrosine aminotransferase. A sample of 3.5 mg/ml of enzyme in phosphate buffer (50 mM, pH 7.6) was sedimented at 50 740 rev./min using a synthetic boundary-cell. The picture was taken 32 min after reaching full speed (Bar angle: 40°).

23 000). Analytical ultracentrifugation showed a single fairly symmetrical peak (fig.4) with a sedimentation coefficient $S^0_{20,w}$ 5.84. Assuming a partial specific volume $\bar{v}$ of 0.735 calculated from the amino acid composition [5], the molecular weight given by the high speed equilibrium method [12] led to the value of 104 500.

After electrofocusing in a 110 ml LKB 8100-1 column with Ampholine pH-ranges of pH 3–10 or pH 3.5–6 in a sucrose gradient, a single form was observed which had an isoelectric point of 4.3 (measured at 4°C). Enzyme activity and a positive reaction with the specific staining reagent were found in this same eluant fraction.

As numerous enzymes from animal source contain glucidic moieties, tyrosine aminotransferase was submitted to gas-chromatography in order to detect possible glucidic residues. The enzyme was first dialyzed for three days against distilled water in order to remove all sucrose and glycerol. It is interesting to emphasize that this treatment did not alter the enzymatic activity. No measurable amounts (less than 0.01 μg) of fucose, mannose, galactose, *N*-acetyl-glucose, *N*-acetyl-galactose, nor sialic acid were present in a 175 μg sample of the purified enzyme.

4. Discussion

The purification procedure of tyrosine aminotransferase described in this paper yields a pure enzyme after a limited number of purification steps and represents a significant improvement over those previously reported [1–5].

The perfusion of the liver followed by gentle homogenisation in the presence of phenylmethylsulfonyl fluoride prevented massive losses in enzyme activity, probably by avoiding large increase in proteolytic activity [13,14] under destruction of the lysozomes [15,16]. Concentration by salting out the enzyme with ammonium sulfate has been discarded as large activity losses occurred [2]. Loss of activity

was minimal in all steps and transaminase activity was fractionated fairly sharply on DEAE-cellulose as well as on hydroxyapatite and Sephadex G-200. As the enzyme loses activity rapidly on storage at −20°C [2], freezing has been prevented by the addition of glycerol in the last step. However, after several months of storage, minor components appeared on gel electrophoresis which represent aggregates of the principal form of TAT very similar to those observed by Valeriote et al. [5].

The purified tyrosine aminotransferase was found to have an $s^{\circ}_{20,w}$ value and a molecular weight very close to those measured previously [5], and an isoelectric point of 4.3. It does not seem to be a glycoprotein. A more extensive characterization of the purified enzyme will be published elsewhere.

In a comparison of liver from various species, Canellakis and Cohen [17] found that dog and rabbit liver showed maximal activity and represent therefore a good source of the enzyme. We found that the non-induced level of TAT in liver from control rabbits was about 4–5-fold higher than the basal activity in rat liver. This result might be due to the fact that the rabbits were not adrenalectomized, especially as dexamethasone treatment stimulated only a minute increase of enzyme synthesis over an induction-cycle of 12 h. Thus, livers from dexamethasone induced rats were an obvious choice as starting material in purification, since a single injection of the synthetic hormone increases the specific activity of homogenates over 20-fold and enriches available preparation to the fullest extent possible.

The purification reported here allowed us to reach our initial aim of isolating a single molecular species in amounts sufficient to elicit an anti-tyrosine aminotransferase antibody which is now available for immunochemical work. The purification should also help in the elucidation of the complex mechanism responsible for protein degradation, regulated by various environmental means [18], in mammalian cells.

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