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TYROSINE AMINOTRANSFERASE FROM RAT LIVER, A PURIFICATION IN THREE STEPS

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SUMMARY

Tyrosine aminotransferase from rat liver was isolated by a three step purification method involving affinity chromatography, CM-50 chromatography and G-200 gel filtration. In order to synthesize the affinity gel, the coenzyme pyridoxamine-5'-phosphate was coupled via a spacer group to a sepharose matrix. The enzyme preparation showed a single band in SDS-acrylamide gel electrophoresis and contains three multiple enzyme forms. A molecular weight of 50,000 of the tyrosine aminotransferase subunit was estimated.

INTRODUCTION

Tyrosine aminotransferase (TAT) from rat liver (EC 2.6.1.5) is a well known system for investigations on regulatory mechanisms and hormone actions in higher organisms (1-4). As we are interested in specific TAT antibodies as a tool for further investigations on this field we have been working on a fast and simple purification procedure for TAT. Two recently published procedures on TAT isolation involve up to seven purification steps (5,6). We propose now a three step purification method which is based on affinity chromatography, CM-50 chromatography and G-200 gel filtration.

MATERIAL and METHODS

Materials: AH-Sepharose-4B, CM-50 and G-200 Sephadex were purchased from Pharmacia (Freiburg i. Br., West-Germany), hydro-

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cortisone acetate, L-tryptophane, pyridoxamine-5'-phosphoric acid hydrochloride, pyridoxal-5'-phosphate, succinic anhydride, N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride and gum arabic fine powder were obtained from Merck (Darmstadt, West-Germany), spermine x 4 HCl, bovine serum albumin, phenylmethyl-sulfonyl fluoride, cycloheximide by Serva (Heidelberg, West-Germany) and α-ketoglutaric acid, pyruvate kinase from rabbit, aldolase from rabbit were purchased from Boehringer (Mannheim, West-Germany).

Buffers: Tris buffer: 50 mM Tris-HCl, 25 mM KCl, 5 mM $MgCl_2$ (pH 7.6). Elution buffer: 50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, 30 mM ß-mercaptoethanol, 0.5 M NaCl, 10 mM pyridoxal-5'-phosphate, 0.15 M sucrose, 3 mM spermine (pH 10). Dialysis buffer: 50 mM sodium phosphate, 1 mM EDTA, 1 mM ß-mercaptoethanol, 2.5 mM α-ketoglutarate (pH 6.5).

Enzyme assay: Tyrosine aminotransferase was assayed by a modified method of DIAMONDSTONE described by GRANNER and TOMKINS (7). One unit of enzyme activity catalyses the formation of one µM p-hydroxyphenylpyruvate per minute at 37° C. Protein was measured by the method of LOWRY et al. (8) with bovine serum albumin as a standard in order to estimate the specific activity.

Ultrafiltration: Enzyme solutions were concentrated by ultrafiltration through a Diaflo XM 50 (Amicon, Witten, West-Germany) membrane under a nitrogen pressure of 1 - 3 atm.

Preparation of the affinity gel: For the synthesis of succinyl aminohexylsepharose 15 g of swollen and washed AH-Sepharose-4B were suspended in 50 ml distilled water with 5.5 g succinic anhydride. The suspension was gently stirred for 5 hours at pH 6 and 4° C and kept in the cold overnight. The gel was washed with 500 ml icecold distilled water. Pyridoxamine-5'-phosphoric acid was coupled to the gel according to MILLER et al. (9) with a second addition of 1 g N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimid hydrochloride after one hour of reaction. For regeneration the gel was washed first with 100 ml 0.1 M NaOH, then with 500 ml distilled water. The gel was then adjusted to pH 7.6 with Tris buffer.

RESULTS

Purification procedure: A typical purification scheme is given in table 1. All procedures were carried out at 4° C.

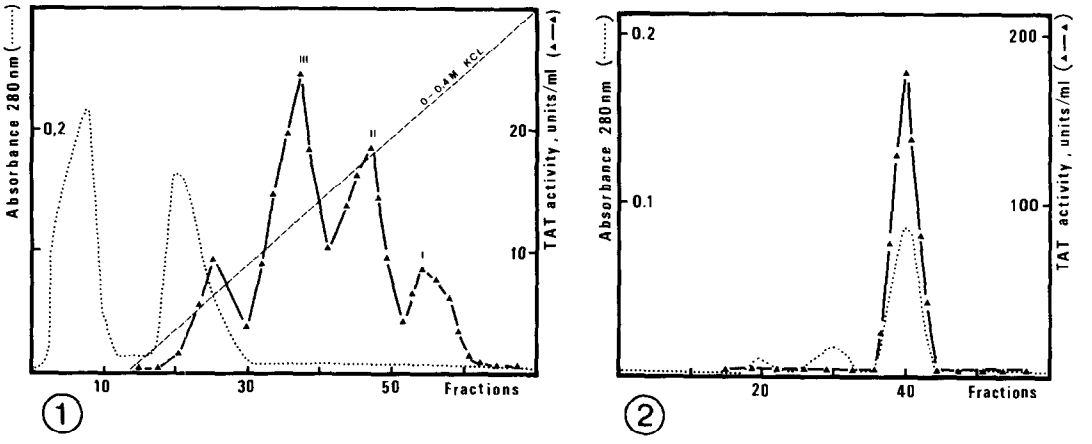
Stimulation of the hepatic tyrosine aminotransferase: In order to increase the enzymatic activity 25 male Wistar rats were pretreated intraperitoneally with 100 mg hydrocortisone acetate, 500 mg L-tryptophane and 350 µg cycloheximide per kg, 16 and 19 hours later with 100 mg hydrocortisone acetate and

Table 1: Purification scheme of rat liver tyrosine aminotransferase.

Step	Stage	Volume (ml)	Total protein (mg)	Total activity units	Specific activity units/mg protein	Yield %	Purification n-fold
	100000 g supernatant	555	22200	7112	0.32	100	-
1	Affinity chromatography	60	63	5202	83	73	258
2	CM-50 chromatography	222	7.3	2900	396	41	1238
3	G-200 filtration	17	2.9	1900	655	27	2047

250 mg L-tryptophane per kg. The substances were suspended in 1 % gum arabic fine powder. The livers were removed after 6 hours. In these livers TAT activity had reached 15 - 20 times the activity level of the untreated animals.

Step 1: The sliced livers were suspended in a solution of 0.1 M KCl, 1 mM EDTA, 1 mM phenylmethyl-sulfonyl fluoride up to a final volume of 390 ml. This suspension was blended for one minute with an Ultra Turrax and then centrifugated in a Beckman 35 rotor at 35,000 rpm for one hour. If this procedure was repeated with the pellet 10 - 20 % more of the enzyme activity could be obtained. The supernatants were applied overnight to the affinity column (5 cm x 2.5 cm). After washing the gel with 500 ml Tris buffer, the enzyme was eluted with 200 ml elution buffer, concentrated up to 50 - 60 ml by ultrafiltration and dialysed against 8 l dialysis buffer for 48 hours. 7112 enzyme units (100 %) were applied to the affinity column, 5898 units (83 %) were still bound after washing with Tris buffer and 5200 units (73 %) could be eluted. There



Fis. 1: Chromatography on CM-50 Sephadex. The gel (2.6 x 25 cm) was equilibrated with dialysis buffer and the chromatography was carried out at a flow rate of 0.45 ml/minute. A linear gradient of 500 ml (0 - 0.4 M KCl in dialysis buffer) was applied. Fractions of 10 ml were collected.

Fig. 2: Gel filtration on Sephadex G-200 was carried out at a flow rate of 0.2 ml/minute in dialysis buffer. Fractions of 50 drops were collected.

is no loss of enzyme activity during the dialysis step.

Step 2: The dialysed enzyme preparation was chromatographed on CM-50 Sephadex (Fig. 1). The TAT activity appeared in three peaks representing the three multiple forms of the enzyme as described by JOHNSON et al. (10). Fractions with TAT activity were pooled and concentrated by ultrafiltration to 1 - 2 ml. 4109 enzyme units were bound when 5200 units were applied to the column, 2900 were pooled and 784 units appeared in the non-pooled fractions.

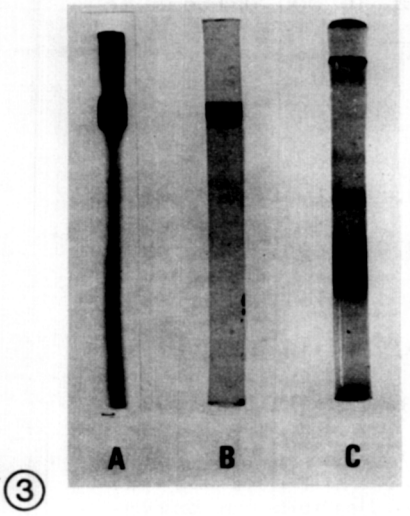
Step 3: The concentrated enzyme preparation was applied to Sephadex G-200 with a recovery of 70 % (Fig. 2). Normally the specific activity of the eluated TAT was 600 - 700 U/mg protein. The enzyme was stored at -20° C in dialysis buffer.

Enzyme properties: The purified enzyme shows a single band in

polyacrylamide gel electrophoresis (Fig. 3a) and in SDS-acrylamide electrophoresis (Fig. 3b) with a molecular weight of 50,000 Daltons of the enzyme subunit (Fig. 4). Three enzymatically active bands were detected in acrylamide gel electrophoresis (Fig. 3c). Sometimes a small additional band appeared. These findings are in good agreement with observations already reported (13).

DISCUSSION

Tyrosine aminotransferase from rat liver is purified by a three step procedure. The idea of purifying TAT by means of affinity chromatography was originally described by MILLER et al. (9). According to their report, a long spacergroup between matrix and coenzyme is necessary for a satisfactory binding of the enzyme. We started our synthesis with a sepharose gel which contained already a spacer, therefore synthesis became easier. The affinity gel described here has a binding capacity of 120 enzyme units per ml gel. The specific activity of the purified enzyme (600 - 700 units/mg) is lower than the one reported for other purification procedures (5,6). Neither sedimentation of TAT in a sucrose gradient after gel filtration on G-200 as reported by BELARBI et al. (5), nor DEAE Sepharose CL-6B chromatography as an additional purification step leads to a further increase of the specific activity. As our enzyme protein shows one single band in polyacrylamide gel electrophoresis and SDS gel electrophoresis (Fig. 3a, 3b), inactivation might be the reason for the low specific activity. On the other side the good recoveries of each purification step makes the required inactivation of 50 % to yield the reported values of 1,200 - 1,400 units/mg



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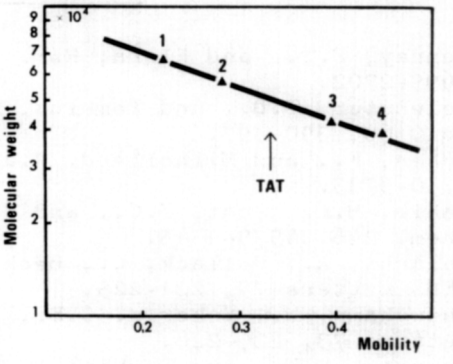

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Fig. 3: a) Polyacrylamide gel electrophoresis according to REISFELD and SMALL (16). b) SDS gel electrophoresis in a 10 % gel according to WEBER and OSBORN (11). c) Disk electrophoresis of TAT according to MILLER et al. (9). TAT activity in the gel was localized corresponding a method described by VALERIOTE et al. (12). Samples of 40 µg (a,b) and 1 µg (c) were applied.

Fig. 4: Determination of the molecular weight of the TAT subunit by SDS gel chromatography according to WEBER and OSBORN (11). The following marker proteins were used: (1) bovine serum albumin 68,000), (2) pyruvate kinase from rabbit (57,000), (3) tryptophan pyrrolase from rat liver (43,000), and (4) aldolase from rabbit (38,500).

unlikely. Three and sometimes four enzymatically active so called multiple forms (I-III) of TAT could be separated by CM-50 Sephadex chromatography (Fig. 1) or disc polyacrylamide gel electrophoresis (Fig. 3c) at every purification step. In order to prevent the interconversion of form III into form II and I phenylmethyl sulfonyl fluoride is present during step 1 (15). The three multiple forms from the purified enzyme did not show any significant difference in specific activity. LEE et al. (14) and BELARBI et al. (5) found a molecular weight of 50,000 and 52,000 Daltons respectively

of the TAT subunit. Our estimated value of 50,000 Daltons is in good agreement with their findings.

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