

Purification and Characterization of Hamster Hepatic Microsomal *N,O*-Acetyltransferase

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A microsomal *N,O*-acetyltransferase which activates carcinogenic arylacetohydroxamic acids was purified 75-fold from hamster liver sequentially by anion exchange column chromatography, chromatofocusing, gel filtration, and hydroxyapatite column chromatography. The purified enzyme, AT-2, was a glycoprotein with a molecular weight of 60000 and a pI value of 5.4. The N-terminal amino acid sequence of AT-2 was: Asp-Ser-Pro-Ser-Pro-Ile-Arg-Asn-Thr-His-Thr-Gly-Gln-Val-Arg-Gly-Leu-Val-His-Lys-. This sequence was highly homologous to that of the form 2 carboxylesterase of rabbit liver, but not to that of major hepatic microsomal carboxylesterases of hamster and other species. AT-2 catalyzed the hydrolysis of 4-nitrophenyl acetate and the *N,O*-acetyltransfer of *N*-hydroxy-2-acetylaminofluorene. Both enzyme activities were strongly inhibited by paraoxon, but not by iodoacetamide. These results demonstrate that this *N,O*-acetyltransferase is a member of carboxylesterase (EC 3.1.1.1).

Keywords acetyltransferase; carboxylesterase; carcinogenic arylamine; purification; hamster; hepatic metabolism; amino-terminal sequence; metabolic activation

Arylamines, such as 4-aminobiphenyl and 2-aminonaphthalene, have been associated with a high incidence of bladder cancer in those who have been employed in the manufacture or processing of these chemicals.¹⁾ Arylaceto-hydroxamic acids, formed from carcinogenic arylamines by *N*-acetylation and *N*-hydroxylation, are believed to be proximate carcinogens.²⁾ Arylhydroxamic acid *N,O*-acetyltransferase (AHAT), which transforms arylacetohydroxamic acids into the ultimate carcinogens, *N*-acetoxy-arylamines,³⁾ is present in many organs of various species and may be responsible for the carcinogenicity of arylamines.⁴⁻⁷⁾

A cytosolic AHAT, which is identical to cytosolic *N*-acetyltransferase and/or *O*-acetyltransferase, has previously been purified.⁸⁻¹⁰⁾ Although microsomal AHATs are widely distributed in the tissues of human and experimental animals,^{11,12)} little information is available with regard to their properties. We have recently partially purified two microsomal AHATs from dog liver, which lacks cytosolic AHAT activities.¹³⁾ Since hamsters have greater hepatic microsomal AHAT activities than other species and there are more AHAT activities in the microsomes than in the cytosol,¹²⁾ the microsomal AHATs in this species were investigated. In this paper, we report the purification and characterization of a microsomal AHAT of hamster liver. This is the first report of the N-terminal amino acid sequencing of a microsomal AHAT.

Materials and Methods

Materials Male Syrian golden hamsters were obtained from Shimizu Experimental Animal Co. (Kyoto, Japan). Sephacryl S-200, PBE 94, Sephadex G-50, concanavalin A-Sepharose, and Polybuffer 74 were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden) and DEAE-Toyopearl from Tosoh Co. (Tokyo, Japan). Bio-gel HT and a protein assay kit were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). *N*-Hydroxy-2-acetylaminofluorene (N-OH-AAF) was synthesized as previously reported.¹⁴⁾ All other reagents used were of reagent grade.

Assay of Enzyme Activities AHAT activity was determined by the fluorometric method developed for the assay of hydroxylamine sulfation and acetylation.^{15,16)} An enzyme preparation, in 245 μ l of 0.1 M Na-K phosphate buffer, pH 7.4, containing 1 mM dithiothreitol (DTT), was

incubated at 37 °C for 3 min. The enzyme reaction was started by adding 5 nmol N-OH-AAF dissolved in 5 μ l of EtOH and the mixture was incubated for an additional 15 min. A half ml of MeOH was then added and the mixture was centrifuged. The concentration of 2-aminofluorene formed from N-OH-AAF via *N*-acetoxy-2-aminofluorene in the supernatant was determined by the fluorescence intensity at 370 nm with an excitation at 282 nm. The blank consisted of the same reaction mixture without the substrate.

Hydrolysis activity toward 4-nitrophenyl acetate was determined colorimetrically by the method of Krisch.¹⁷⁾

Enzyme Purification The livers (50 g) removed from the hamsters (weighing 130–150 g) under ether anesthesia were perfused with 1.15% KCl and homogenized in 4 volumes of cold 50 mM Na-K phosphate buffer, pH 7.4, containing 1 mM DTT and 1 mM EDTA. Microsomes were prepared by differential centrifugation according to the method described previously,¹³⁾ and were suspended in the same buffer to achieve a concentration equivalent to 0.5 g wet liver per ml.

The microsomes were stirred in 0.25% Triton X-100 and the supernatant was dialyzed against 10 mM Bis-Tris buffer, pH 7.0, containing 1 mM DTT and 1 mM EDTA (buffer A). The dialysate was applied to a DEAE-Toyopearl column (2.0 \times 105 cm) equilibrated with buffer A. After being washed with 500 ml of buffer A, the column was eluted with a linear gradient of 0 to 0.3 M NaCl in buffer A (1200 ml). Fractions of 9.3 ml were collected.

Fractions (numbers 127–137) from the DEAE column (AT-2 in Fig. 1) were collected and dialyzed against 25 mM Bis-Tris buffer, pH 7.4 (buffer B). The dialysate was applied to a chromatofocusing gel PBE 94 column (1.0 \times 22 cm) equilibrated with buffer B. The column was eluted with an 8-fold diluted Polybuffer 74 (pH 4.0). The AHAT was eluted at pH 5.3–5.5. After being concentrated, the active fraction was chromatographed on a Sephacryl S-200 column (1.6 \times 100 cm) with 50 mM Na-K phosphate buffer, pH 7.4, containing 1 mM DTT, 1 mM EDTA, and 50 mM NaCl. The elution volume of the AHAT was 110–120 ml. This fraction was dialyzed against 10 mM Na-K phosphate buffer, pH 7.4, containing 1 mM DTT and 1 mM EDTA (buffer C) and applied to a Bio-gel HT hydroxyapatite column (1.0 \times 15 cm) equilibrated with buffer C. The column was eluted with a linear gradient of 10 to 200 mM phosphate in buffer C (100 ml). All the purification procedures were carried out at 4 °C.

Electrophoresis Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5% acrylamide gel plates according to the method of Laemmli.¹⁸⁾ Protein bands were visualized by silver staining.¹⁹⁾

Affinity Chromatography Concanavalin A affinity column chromatography was performed according to the method of Hosokawa *et al.*²⁰⁾

N-Terminal Amino Acid Sequencing Purified enzyme was desalted with a Sephadex G-50 column and sequenced with an Applied Biosystems model 473 A system.

Protein Determination Protein concentration was determined using a Bio-Rad protein assay kit.²¹⁾

Results and Discussion

Hamster hepatic microsomal AHATs were well separated into two fractions, AT-1 and AT-2, by DEAE column chromatography (Fig. 1). There was more AHAT activity in AT-2 than in AT-1. AT-2 was further purified by chromatofocusing, gel filtration, and then hydroxyapatite column chromatography. The results of a typical purification are shown in Table I. This enzyme was purified 75-fold in specific activity over the microsomes, and the recovery of the AHAT activity was 8.5%.

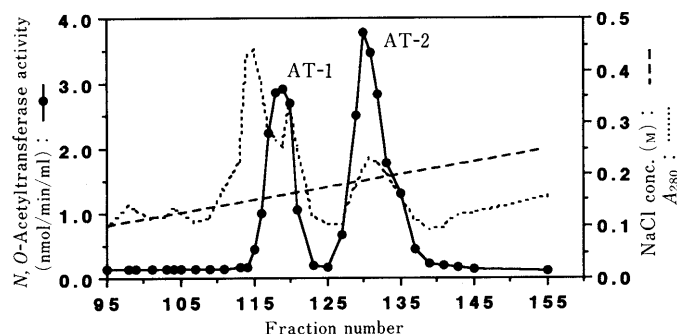


Fig. 1. Elution Profile of Hamster Hepatic Microsomal *N,O*-Acetyltransferase Activities on DEAE Ion Exchange Column Chromatography

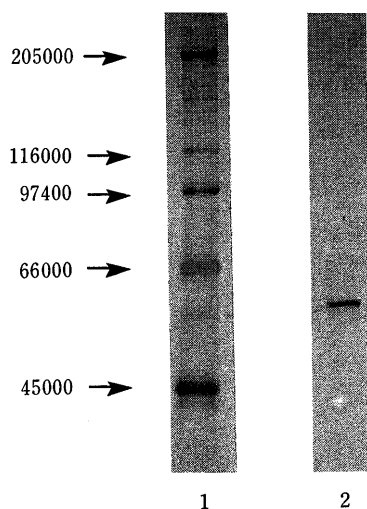


Fig. 2. SDS-PAGE of AT-2 Purified from Hamster Liver

Lane 1: molecular weight marker, rabbit muscle myosin (205000), *Escherichia coli* β -galactosidase (116000), rabbit muscle phosphorylase b (97400), bovine plasma albumin (66000), and ovalbumin (45000); lane 2: 0.2 μ g of purified AT-2.

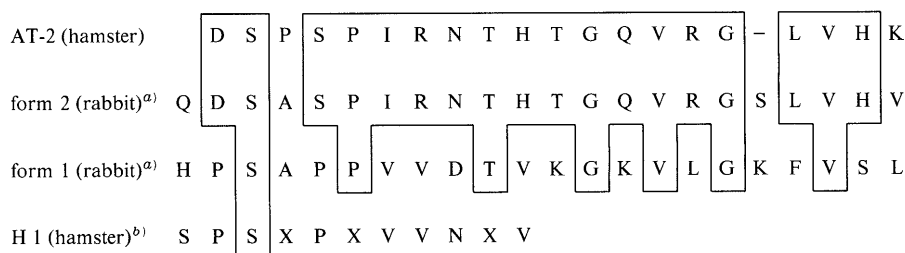


Fig. 3. Comparison of N-Terminal Amino Acid Sequence of the Hamster AT-2 with That of Rabbit and Hamster Carboxylesterases

Amino acid sequences are given in the conventional single letter code. In the sequence of AT-2, deletion is indicated by a dash. X indicates an amino acid not identified. The same sequences as that of AT-2 are shown by boxes. a) Data from ref. 22, b) data from ref. 20.

Chromatofocusing showed that the pI value of AT-2 was about 5.4. SDS-PAGE of AT-2 gave a single protein band with a molecular weight of 60000 (Fig. 2). The molecular weight of AT-2 was also determined to be about 60000 by gel filtration on the Sephacryl S-200, indicating that AT-2 exists as a monomer. AT-2 was not eluted from a concanavalin A-Sepharose column with high concentration of NaCl, but was efficiently eluted with α -methylmannoside (data not shown). This observation suggested that AT-2 is a glycoprotein.

The N-terminal amino acid sequence of AT-2 is shown in Fig. 3. The protein databases NBRF-PIR and SWISS-PROT were used to search for homologous protein sequences. The sequence of AT-2 was highly homologous only to that of the form 2 microsomal carboxylesterase of rabbit liver, which was recently reported by Ozols²²⁾ as a novel type of carboxylesterase. The sequence of AT-2 was different from the form 1, a major hepatic microsomal carboxylesterase in rabbits (Fig. 3). It was also different from carboxylesterase H1 of hamster hepatic microsomes, which was purified by Hosokawa *et al.*²⁰⁾

Purified AT-2 also catalyzed the hydrolysis of 4-nitrophenyl acetate, a representative substrate for carboxylesterases. Both AHAT activity and 4-nitrophenyl acetate-hydrolyzing activity of AT-2 were completely inhibited by 10 μ M paraoxon, an inhibitor of serine-type esterases.²³⁾ However, these activities were not inhibited by 50 μ M iodoacetamide, which strongly inhibited cytosolic AHAT.⁹⁾ This fact indicated that the hamster microsomal AHAT, AT-2, was apparently distinguishable from the cytosolic AHAT.

Carboxylesterases are known to be involved in the detoxication of xenobiotics and in the activation of ester and amide prodrugs.^{23,24)} The present study has identified two microsomal AHATs in hamster liver. Our results

TABLE I. Purification of the Microsomal *N,O*-Acetyltransferase AT-2 from Hamster Liver

Purification step	Protein (mg)	Specific activity (nmol/min/mg protein)	Purification fold	Recovery (%)
Microsomes	586	0.74	1.0	100
Supernatant	313	0.64	0.9	46
DEAE	11.1	16.0	22	41
Chromatofocusing	1.72	37.4	51	15
Sephacryl S-200	1.15	38.2	52	10
Hydroxyapatite	0.67	55.2	75	8.5

suggest that carboxylesterases may also be involved in the activation of carcinogenic arylamines. Purification and characterization of AT-1 are now in progress.

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