PARTIAL PURIFICATION AND IMMUNOLOGICAL ASPECTS OF CARBOXYLESTERASE FROM RAT LIVER MICROSONES

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Abstract—Carboxylesterase (CEase) was solubilized from rat liver microsomes by autolysis followed by cholate treatment and then purified by the combination of ammonium sulfate fractionation, gel filtration, chromatography on DEAE Sephadex A-50 and hydroxypatite and preparative Disc electrophoresis. The overall purification was 25-fold with a yield of 6% of the original enzyme activity. Analytical Disc electrophoresis of the final enzyme preparation showed a single band. However, SDS polyacrylamide gel electrophoresis revealed one main band of 93% and three other minor bands. To investigate the interaction between CEases of rat, monkey, pig and rabbit liver microsomes, rabbit antibody to the above enzyme preparation was prepared and immunological analyses, i.e., Ouchterlony's test and immunoelectrophoresis, were performed. In the comparative double diffusion test, the partial fusion of precipitation line between anti-rat CEase and the enzymes of other species was observed. In the second analysis, sharp arc precipitation lines also could be seen in all specimens and, furthermore, mobilities of each enzyme were different. These observations suggest that rat liver CEase seems to be immunologically related in part but not completely identical with the CEases of other species and the charge difference may exist in these specimens.

It is well known that liver microsomal carboxylesterase (CEase, EC 3.1.1.1) has a significant species difference in the activity, that is, CEase of guinea pig liver shows approximately 10-fold higher enzyme activity than that in rats (1). An explanation for such species differences in liver CEase has not been well documented. Papers from Krisch's laboratory demonstrated that there was no cross reactivity between anti-pig CEase and rat CEase (2). Kinetic parameters, such as Km and Vmax values of rat, rabbit and monkey liver microsomes were not significantly different (unpublished observations).

CEase hydrolyzes a variety of aromatic as well as aliphatic compounds (3). From the pharmacological and toxicological viewpoints, this enzyme plays an important role in the liver to metabolize various foreign compounds including drugs, such as phenacetin (4, 5), and various local anaesthetics (6-8). However, the endogenous substrate of liver CEase remains obscure. The present study deals with the immunological approach using anti-rat CEase to investigate the interaction between CEases of several animal species liver
MATERIALS AND METHODS

Chemicals: Sephadex G-75 and DEAE Sephadex A-50 were obtained from Pharmacia; hydroxyapatite from Nippon Chemical Co. Sodium cholate, sodium dodecyl sulfate (SDS), β-galactosidase, phosphorylase a, catalase, ovalbumin and carbonic anhydrase were from Sigma Chemical Co. Isocarboxazid (ISOC) was kindly donated by the Nippon Roche Research Center, Kamakura, Japan. Pig liver CEase was from Boehringer Co.

Preparative Disc electrophoresis: A 7.5% acrylamide gel of Davis (9), Tris-glycine system pH 8.6, was polymerized in the column (CD-50, Toyo Kagaku Sangyo Co., Ltd.). The length of the separation and spacer gel was 8 and 1 cm, respectively. The sample (10 mg of protein/5 ml of water containing 10% sucrose and 0.001% Bromophenol Blue) was layered on the spacer gel and overlayed with an electrode buffer. Electrophoresis was carried out at a constant voltage of 600 V (approx. 45 mA) until the Bromophenol Blue has migrated out of the gel (6 hr). The gel was then removed from the column and allowed to stand at −20°C for 15 hr. To determine the location of CEase, part of the gel was cut into a longitudinal strip (about 2 mm thickness) and stained in 0.25% Coomassie Blue. Gel slices containing CEase were homogenized in a Teflon-glass homogenizer and extracted with water. The extent of water extraction was about 80%. The water-extract was dialyzed against water and lyophilized.

Preparation of antibody: Antibody to CEase was obtained by the immunization of New Zealand white rabbits (2.5–3.0 kg) according to the following schedule. The highly purified CEase after preparative Disc electrophoresis was emulsified with an equal volume of Freund’s complete adjuvant. The antigen emulsion (1 ml/animal, containing 200 µg of enzyme protein) was injected in small volumes into the 2 foot pads and 4 intradermal sites. The injections were repeated thrice at 2 week-intervals. Serum was obtained from the carotid 9 days after the last injection, and treated with 50% saturation of ammonium sulfate. The precipitation was dissolved in a small volume of saline, dialyzed against water and lyophilized. The γ-globulin fraction thus obtained (redissolved in the one-fifth volume of saline to serum) was used as the antibody to CEase.

Immunodiffusion and immunoelectrophoresis: Ouchterlony plates containing 1.4% (w/v) agarose in phosphate buffered saline (0.15 M NaCl-0.01 M phosphate buffer, pH 7.4; PBS) were prepared and used for immunodiffusion studies. The plates were kept at room temperature in a humidity chamber. The precipitation lines could be seen after 24 hr and were maximally developed by 72 hr.

For immunoelectrophoresis, 1.4% agarose was prepared in barbital buffer (0.07 M, pH 8.6). Electrophoresis was carried out at 2 mA/cm for 3 hr at room temperature, and then troughs were filled with antibody followed by immunodiffusion.

Analytical methods

Enzyme assay: ISOC was used as the substrate throughout the purification. The enzyme activity was determined using the colorimetric assay based on conversion of ISOC.
to benzylhydrazine (BZH). The incubation mixture consisting of 0.5 ml ISOC (2 × 10^{-3} M) in a 0.2 M Tris-HCl buffer, pH 8.0, 0.1-0.5 ml enzyme preparation and sufficient water to make a final volume of 1.0 ml was incubated at 37°C for 30 min. The amount of BZH produced was measured according to the method described previously (10).

**Protein determination:** Protein was determined by the method of Lowry et al. (11), using crystalline bovine serum albumin as a standard.

**Electrophoresis:** Analytical Disc electrophoresis was performed using the Tris-glycine system of Davis (9) pH 8.6, at a 7.5% gel concentration. Electrophoresis in the presence of SDS was carried out by the method of Weber and Osborn (12). All gels were stained in 0.25% Coomassie Blue and destained electrically.

### RESULTS

#### Purification of CEase

Liver from male Wistar rats (250-300 g) were used as the enzyme source.

**Step 1. Separation of microsomal fraction:** A 100 g of livers was homogenized in 1.15% KCl at a final concentration of 10%. A Teflon-glass homogenizer was used, and the homogenate was separated into nuclear, mitochondrial, microsomal and supernatant fractions as described previously (13). The resultant microsomal pellet was suspended in 1.15% KCl (10 mg protein/ml). All subsequent steps were carried out at 4°C unless otherwise stated.

**Step 2. Solubilization:** The solubilization of CEase from rat liver microsomes was conducted by the combination of autolysis and cholate treatment. After incubation of microsomal suspension at 20°C for 15 hr, solubilization was done by addition of cholate to the enzyme suspension (0.2 mg/mg protein) followed by further incubation for 1 hr at 37°C. Incubation mixture was then centrifuged at 105,000 × g for 1 hr and the supernatant fraction was used as the solubilized enzyme source for further purification. By these procedures more than 95% of the total CEase was solubilized.

**Step 3. Ammonium sulfate fractionation and gel filtration:** The ammonium sulfate fractionation was performed as described previously (13). The active precipitation (40-65%) was then applied to a Sephadex G-75 column (2.6 × 50 cm) and eluted with 0.05 M Tris-HCl buffer, pH 8.0.

**Step 4. Chromatography on DEAE Sephadex A-50:** The enzyme preparation from step 3 was applied to a DEAE Sephadex A-50 column (2.6 × 50 cm) and chromatographed with the above buffer having a stepwise gradient of 0.1 and 0.2 M NaCl (600 ml each). The elution pattern consisted of two activity peaks; the first peak (D-1) was eluted in a 0.1 M NaCl and the second one (D-2) in a 0.2 M NaCl.

**Step 5. Hydroxyapatite chromatography:** Both eluates from the previous step were dialyzed against water and lyophilized. The dried materials were dissolved in a small volume of water, then applied to a hydroxyapatite column (2.0 × 6 cm), and eluted with a stepwise gradient of phosphate buffer, pH 8.0 (0.05 and 0.1 M, 80 ml each). When D-1 was used as the enzyme source, most of CEase was eluted with 0.05 M phosphate buffer (E-1).
However, in the case of D-2, the elution diagram showed two peaks of CEase as seen in Fig. 1 (E-2 and E-3).

Analytical Disc electrophoresis: The enzyme preparations from each purification step were subjected to polyacrylamide gel electrophoresis according to the method of Davis (9). As shown in Fig. 2, at least ten protein bands appeared in all enzyme preparations tested. Location of CEase upon the gel was detected by the following procedure, the gel was...

Fig. 1. Hydroxyapatite column chromatography of CEase. The eluate from a DEAE Sephadex A-50 column (D-2 fraction, 20 mg/10 ml) was applied to a hydroxyapatite column (2.0 x 6 cm), equilibrated with water and eluted with a stepwise gradient of 0.05 and 0.1 M phosphate buffer, pH 8.0. Aliquots of 4 ml were collected in each fraction.

Fig. 2. Analytical Disc electrophoresis on 7.5% acrylamide of each of the enzyme preparations. Methods of gel electrophoresis are described in Materials and Methods; 50–100 μg protein of each sample was applied. A; crude extracts, B; ammonium sulfate fraction, C; Sephadex G-75 filtrate, D-1 and D-2; eluate of 0.1 and 0.2 M NaCl on DEAE Sephadex A-50 column chromatography, E-1; eluate from the hydroxyapatite column chromatography of D-1, and E-2 and E-3; eluate of 0.05 and 0.1 M phosphate buffer from hydroxyapatite of D-2. Arrow indicates the location of CEase.
incubated for 3 hr at 37°C in 0.1 M phosphate buffer (pH 7.4) with 3 mM ISOC as the substrate, then placed in a tube containing both 3 ml of Ehrlich’s reagent and 1.5 ml of 5% metaphosphoric acid and the tube was kept for 30 min at room temperature. The enzyme was revealed by an orange band. In addition, on an SDS gel electrophoresis, we also observed a great number of protein bands in all enzyme preparations as well as in the analytical Disc electrophoresis.

Step 6. Preparative Disc electrophoresis: Analytical Disc electrophoresis indicated that the procedure by combination of ammonium sulfate fractionation, gel filtration and chromatography on DEAE Sephadex A-50 and hydroxyapatite did not give a suitable purity of CEase. Therefore, the enzyme preparation obtained at step 5 was subsequently subjected to preparative Disc electrophoresis to obtain a pure enzyme. Since E-1 fraction has the highest value of specific activity, this fraction was used as the starting material. As shown in Fig. 3, the higher purity was obtained by using preparative electrophoresis.

![Fig. 3. Preparative Disc electrophoresis. The samples eluted in 0.05 M phosphate buffer on the hydroxyapatite column chromatography of D-1 were loaded onto a 7.5% acrylamide gel, submitted to electrophoresis and part of gel was cut into a longitudinal strip and stained in 0.25% Coomassie Blue. (A) indicates the area to be extracted. (1); preparative Disc electrophoresis of the E-1 fraction and (2); analytical Disc electrophoresis of the water extract of (A).](image)

SDS polyacrylamide gel electrophoresis: Typical SDS gel profile and molecular weight of the purified enzyme preparation are shown in Fig. 4. Four protein bands (I-IV) were identified and the molecular weights of each band were 118,000±3,500, 97,000±3,000, 61,000±880 and 47,000±840, respectively. These figures indicated mean±S.E.M. from 7 experiments. To determine the ratio of each of the protein bands, gels were scanned by Gelman Densitometer-R scanner. The results indicated that the relative percentages of bands II and III were 7 and 93%, whereas, bands I and IV were not estimated by this system as there was only a trace amount of these substances.

Additionally, the overall purification was 25-fold with a yield of 6% of the original enzyme activity present in the liver microsomes. Table 1 summarizes the results of a typical purification procedure.

Immunological studies

Two types of immunological experiments, i.e., Ouchterlony’s test and immunoelectrophoresis, were carried out to determine the immunological relationship between rabbit
FIG. 4. Molecular weights of the protein bands observed upon electrophoresis in a 8.5% acrylamide-SDS gel system. The different protein bands are labeled I-IV. When standard proteins were employed, a linear function for the plots of the logarithm of the molecular weight of each protein and their relative mobilities allowed for estimation of the molecular weight of each of the protein bands. Inset indicates the SDS polyacrylamide gel electrophoresis of the purified enzyme preparation.

TABLE 1. Purification of CEase from rat liver microsomes

| Fraction                  | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Recovery (%) | Purification (fold) |
|---------------------------|--------------------|------------------------|------------------------------|--------------|---------------------|
| Microsomes                | 3240              | 133                    | 0.04                         | 100          | 1.0                 |
| Crude extracts            | 1670              | 133                    | 0.08                         | 103          | 2.0                 |
| Ammonium sulfate, 40-65%  | 320               | 77                     | 0.24                         | 58           | 6.0                 |
| Sephadex G-75 chromatography | 251            | 77                     | 0.31                         | 58           | 7.8                 |
| DEAE Sephadex A-50 chromatography, |               |                       |                              |              |                     |
| D-1                       | 36                | 28                     | 0.78                         | 21           | 19.5                |
| D-2                       | 25                | 13                     | 0.52                         | 10           | 13.0                |
| Hydroxyapatite chromatography, |             |                       |                              |              |                     |
| E-1                       | 24                | 20                     | 0.83                         | 15           | 20.8                |
| E-2                       | 9                 | 5                      | 0.56                         | 4            | 14.0                |
| E-3                       | 6                 | 3                      | 0.50                         | 2            | 12.5                |
| Preparative Disc electrophoresis | 8             | 8                      | 1.0                          | 6            | 25.0                |

Purification was carried out as described in the text. One unit of CEase was the amount of the enzyme that formed 1 μmole of BZH/30 min at 37 °C. Notations of D-1, D-2, E-1, E-2 and E-3 are shown in Fig. 2.
antibody to rat CEase and CEases of other animal species. CEases from the monkey and rabbit liver microsomes were the partially purified preparations by Sephadex G-75 and DEAE Sephadex A-50 column chromatography as described elsewhere. Results are shown in Figs. 5 and 6.

In the Ouchterlony's test, the presence of the precipitation line between antibody to rat CEase and the enzymes of other species, i.e., monkey and pig, was observed in 24 hr-developing. After 72 hr-developing, the cross reactivity between CEase of rabbit and anti-rat CEase was also revealed. Moreover, it was observed that monkey, pig and rabbit CEases gave a partial fusion. In the second analysis, sharp arc precipitation lines could be seen in all specimens and, furthermore, mobilities of each enzyme were different. This result indicates that the charge difference may exist in the liver CEases of these animals.

**DISCUSSION**

The purification steps of ammonium sulfate precipitation, gel filtration and fractionation
on DEAE Sephadex A-50 and hydroxyapatite chromatographies were essentially as reported by Arndt et al. (2) and Heymann et al. (14) who found that the enzyme preparation was homogeneous on SDS polyacrylamide gel electrophoresis. However, we did not obtain the same results and thus preparative Disc electrophoresis was used as a further purification step. On the extraction process of CEase from microsomal membranes, Arndt et al. (2) employed the combined autolysis and heat treatment and obtained the value of 77% solubilization, but the combination of autolysis and cholate treatment used herein gave approx. 100% extraction. The difference of SDS gel electrophoretic patterns in both studies may be due to the step of solubilization.

An analytical Disc electrophoresis of the final enzyme preparation showed a single band, and molecular weight was estimated to be about 180,000±2,900 by Sephadex G-200 gel filtration method as described previously (13). However, SDS gel electrophoresis revealed that this enzyme preparation contained 4 protein bands and the molecular weight of main band III (93%) was 61,000±880. For unspecific carboxylesterase (E1) from rat liver microsomes, it was found that this enzyme was a trimer and subunit weight of 61,500±1,400 (2). From these observations it is plausible that band III is a subunit of CEase. In some experiments, if a small volume of sample (about 30 µg) was applied, band I (118,000±3,500) was not detected. Thus, it can be assumed that band I may correspond to a dimeric association product. For the bands II and IV, we have no explanation at present.

From the results of immunological analyses, rat liver CEase seems to be immunologically related, at least in part, but not completely identical with the CEase of monkey, pig and rabbit, since it does not form the complete fusion of line with an antibody to rat CEase, however, CEases of these animal species gave a partial fusion in the comparative double diffusion test of Ouchterlony. This may be due to the small difference of charge of each CEase protein molecule of four animals. In a preliminary series, we found that K_m value in the rat, rabbit and monkey was 5.5×10^{-3}, 1.25×10^{-4} and 3.85×10^{-3} M; V_{max} (product/mg protein/30 min) was 6.2×10^{-3}, 2.68×10^{-4} and 1.25×10^{-4} M; optimum pH was 8.5–9.0, 8.0–8.5 and 8.5–9.0 and optimal incubation temperature was 60, 50 and 50°C, respectively. The minor differences in these enzymological parameters are in good agreement with the immunological relationship in these species.

To obtain more detailed information on differences in the mode of existence of CEase in liver microsomes of several animal species, immunohistochemical studies are now under way in our laboratories.

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