Purification and Properties of Rat Liver Microsomal Esterases*

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SUMMARY

Several rat liver microsomal esterases have been isolated. One of these was purified to homogeneity and its physical and catalytic properties were investigated. The enzyme is composed of two subunits of equal molecular weight. Each 70,000 molecular weight subunit appears to have an active site. Evidence is presented which suggests that catalysis involves the formation of an acyl-enzyme intermediate and requires the presence of an unprotonated residue with a pK of about 6.0.

This report describes a study of the physical and catalytic properties of a purified rat liver microsomal esterase. The purpose of this investigation was to provide a well characterized enzyme with which to study the fluoride inhibition of an enzyme which does not require metal ions for catalysis. A subsequent report (1) describes the fluoride inhibition of this enzyme. The physical and catalytic properties are in general similar to those of the well characterized esterases from beef and pig liver.

The multiplicity of carboxyl esterases in mammalian tissues has been well established (2). In liver, esterase activity is found primarily in the microsomal fraction (2). There have been several recent reports regarding attempts to purify rat liver esterases. Those of Arndt and Krisch (3) and Akao and Omura (4) appeared after our purification method had been established, but during an investigation of catalytic properties and fluoride inhibition of the purified esterase. Hyasce and Tappel (5) employed a mechanical disruption technique to solubilize a microsomal esterase which was fractionated further by precipitation and chromatographic procedures. Their investigation was primarily concerned with substrate specificity, and little data were presented regarding the homogeneity of the preparation or its physical and catalytic properties. Ljungquist and Augustsson (6) resolved small quantities of two esterases which had been solubilized by treatment with phospholipase A. Arndt and Krisch (3) separated five different esterases from acetone powders of rat liver. At least one of these was homogeneous, but was characterized principally by its chromatographic properties and molecular weight, about 170,000. Most recently, Akao and Omura (4) reported the purification of an acetylcholinesterase from rat liver that had a subunit molecular weight of 60,000 to 65,000.

The physiological role of the microsomal esterases is not understood because they are active toward carboxylic acid esters of short chain fatty acids (2). In vivo, some liver and adipose tissue esterases have been shown to be interchangeable with lipase activity (7–11) i.e., active toward esters of long chain fatty acids, but their role as lipolytic enzymes has not been demonstrated in vivo (2). Rather than lipolytic activity, the esterases may function primarily in the metabolism of foreign compounds (2).

MATERIALS AND METHODS

Reagents—Proteins used for standardization and reagents for esterase and protein stains were obtained from Sigma Chemical Co. Ethyl butyrate and reagents for acrylamide gels were products of Eastman. Phenyl butyrate and diethyl p-nitrophenyl phosphate were obtained from K and K Laboratories and Baker, respectively. Other reagents were the best grade available and were used without further purification. Solutions were prepared in distilled water which had been passed through a Barnstead deionizer. Ethyl butyrate was distilled before use, and a-naphthyl acetate was recrystallized from ethanol. Phenyl butyrate was applied to a silicic acid column in hexane and eluted with 3.5% (v/v) ether in hexane to remove free phenol. No phenol was detected by spectral analysis of the purified ester, and complete hydrolysis indicated at least 98% purity.

Protein Assay—Protein concentrations were routinely determined by a modification (12) of the Lowry method. The biuret method was used in determination of an extinction coefficient (ε1% = 12.8) used to calculate kinetic constants and the equivalent weight. Results from the two colorimetric methods differed by about 10%. The protein standard was crystalline bovine serum albumin.

Esterase Activity. During the purification procedure, esterase activity was determined by one of two methods. Portions of the major protein fractions (Table I) were added to stirred 10-ml reaction mixtures containing 1.5 mM phenyl butyrate and 3.0 mM KNO3. Hydrolysis rates were measured at pH 8.0, 25°C with a Brinkman photostat equipped with a 0.2-ml buret containing 2 to 10 mM NaOH. All solutions were prepared in boiled water and the reaction vessel was continuously flushed with nitrogen. Phenyl butyrate solutions, up to 2.4 mM, were prepared by rapid stirring at 40°C for 20 min. When modifications of this method were used to measure esterase activity at relatively high or low pH values, appropriate corrections were made for nonenzymatic
Hydrolysis, for the ionization of phenol, and for the incomplete ionization of butyric acid.

The enzyme purification of individual fractions from column chromatography was measured at 25° by addition of 5 to 20 μl of appropriately diluted sample to 1.0 ml of 1.7 ml phenyl butyrate in 50 mm Tris chloride, pH 8.0. After 2 to 10 min, hydrolysis was halted by the addition of 0.2 ml of 35 mg per ml of sodium dodecyl sulfate and the phenol concentration was measured by its absorbance at 270 nm.

Enzyme activity was also measured in buffered solutions by the use of a Varian model 635 recording spectrophotometer equipped with a thermostated multiple sample compartment. Phenol was detected at 270 nm using ε = 1480 M⁻¹ cm⁻¹.

Electrophoresis—Polyacrylamide disc gel electrophoresis was performed at constant current with Caneco equipment using the Tris-glycine system. Viscosity (15). Films were mounted after staining with naphthol blue black in 7% (v/v) acetic acid. Esterase activity was detected by slight modification of the method of Holmes and Masters (14), using α-naphthyl acetate as the substrate. Electrophoresis in the presence of sodium dodecyl sulfate was performed using the 5% gels described by Dunker and Rueckert (15). Gels were stained with Coomassie blue, destained by diffusion, and accored in Z% acetic acid.

Electrophoresis results of hydroxylapatite column chromatography were performed at 0-4°C. The homogenate was not washed as described under “Materials and Methods,” the recovery was only 45%. The same observation was made during preliminary investigations when p-nitrophenyl propionate was used as the substrate. Triton X-100 effectively solubilized the esterase activity at pH 7.4. Subsequent adjustment of the pH to 5.0 allowed insoluble material to be removed by low speed centrifugation. The acetone treatment of the solubilized protein served to remove the detergent, concentrate the protein, and provide additional purification.

Representative results of hydroxylapatite column chromatography of the solubilized esterases are shown in Fig. 1. The major and minor enzyme fractions were designated esterase A and esterase B, respectively. The relative amounts of the two fractions were similar for each of several trials of this method. Fig. 2 shows the results of DEAE-Sephadex column chromatography of esterase A. A single major protein fraction was eluted which contained all of the esterase activity. Although significant additional purification was not achieved, this step removed small amounts of contaminating proteins (including red pigments and esterase B) and demonstrated the purity of the esterase A fraction.

When the esterase B fraction from the hydroxylapatite step was chromatographed on DEAE-Sephadex columns, two esterase fractions were resolved (Fig. 3). The first esterase fraction, esterase B₁, was the subject of further investigation, described below. Esterase B₁ was eluted by the starting buffer, but the second fraction, esterase B₂, was eluted by the NaCl gradient. Upon rechromatography of esterase B₁, it was eluted an initially observed and no other esterase was eluted by the gradient. The second enzyme fraction, B₂, was not investigated in detail, however, its elution properties and electrophoretic mobility were.

**Table I**

| Fraction               | Volume | Protein | Specific activity | Yield |
|------------------------|--------|---------|------------------|-------|
|                        | ml     | mg      | units/mg         | %     |
| Homogenate             | 400    | 29,700  | 2.2              | 100   |
| Microsomes             | 299    | 4,310   | 11               | 60    |
| Triton X-100, pH 5    | 391    | 440     | 50               | 33    |
| Acetone treatment      | 35     | 163     | 110              | 20    |
| Hydroxylapatite        |        |         |                  |       |
| Esterase A             | 22     | 36      | 340              | 19    |
| Esterase B             | 14     | 31      | 99               | 4.6   |
| DEAE-Sephadex          |        |         |                  |       |
| Esterase A             | 13     | 29      | 310              | 16    |
| Esterase B₁            | 13     | 7       | 140              | 1.4   |
similar to that for esterase A. Investigation of the fluoride inhibition of the esterase B fraction before and after resolution on DEAE-Sephadex also suggested that the second fraction (B2) was esterase A. This purification step was valuable for the resolution of the two esterase B subfractions, but sometimes resulted in the loss of up to 40% of the total esterase activity.

Both esterase A and esterase B were stable for several months when stored at 2°C. Esterase A precipitated slightly during storage at a concentration of 2 mg per ml, but no decrease in specific activity was detected.

**Purity and Metal Ion Content of Esterase A**—Disc gel electrophoresis of esterase A in the Tris-glycine system (Fig. 4) indicated a high degree of purity. Electrophoresis in the presence of sodium dodecyl sulfate (Fig. 5) also demonstrated the purity of the preparation. A very minor band was detected in this system with a mobility corresponding to a molecular weight twice that of the principal band. When 40 μg of protein was treated with alkaline urea and electrophoresis was carried out at pH 2.7 in the presence of urea (16), one major and two to four very faint minor bands were detected (not shown) by the sensitive Coomassie blue stain. The $A_{405}:A_{280}$ ratio of esterase A solutions was always 1.70 to 1.78. Values of $A_{405}:A_{410}$ were always greater than 80 and were as high as 250 which indicates a reasonable separation from heme containing proteins (17).

Neutron activation analysis of 15 mg of exhaustively dialyzed esterase A did not reveal the presence of any significant quantity of metal ions. Both EDTA and 1,10-phenanthroline had no effect on enzyme activity at pH 7 to 7.5 either during extensive dialysis or during catalysis. This observation was important since we wished to investigate fluoride inhibition of an enzyme that did not contain nor require the presence of added metal ions.

**Molecular and Equivalent Weights of Esterase A**—The molecular weight was estimated to be 135,000 to 149,000 by gel filtration on Sephacryl G-200 as shown in Fig. 6. The subunit molecular weight, 66,000 to 72,000, was determined by disc gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 7). The equivalent weight, about 73,000, was estimated from the enzyme-dependent amount of p-nitrophenol released from diethyl p-nitrophenyl phosphate (19, 20). The reaction at pH 8.0, 25°C, was complete in 10 to 15 s and did not proceed further during the subsequent 30 min. Pretreatment of the esterase with diisopropylfluorophosphate prevented the release of p-nitrophenol. The relative values of the molecular, subunit, and equivalent weights are 2:1:1, respectively. The molecular and equivalent weights of esterase A are summarized in Table II.
Catalytic Properties of Esterase A—The stoichiometric reaction of esterase A with diethyl p-nitrophenyl phosphate suggested that the enzyme was a serine hydrolase. This was further indicated by the observation that the enzyme catalyzed the methanolysis of phenyl butyrate (Fig. 8). These results support the conclusion that catalysis proceeds by the formation of an acyl-enzyme intermediate, presumably a serine ester.

The pH dependence of esterase A activity is shown in Fig. 9. The $K_m$ (0.2 to 0.25 mM) remained essentially constant over the pH range, while the $V_{max}$ depended on the ionization state of an enzyme residue with a $pK$ of about 6.0. Reciprocal plot analysis revealed the $K_m$ and $V_{max}$ values to be $0.2$ and $0.25$ mM, respectively.

**TABLE II**

| Property measured | Method                        | Value | Relative value |
|-------------------|-------------------------------|-------|----------------|
| Native mol wt     | Gel filtration, Sephadex G-200 | 135,000-140,000 ± 10% | 2               |
| Subunit mol wt    | Sodium dodecyl sulfate gel electrophoresis | 66,000-72,000 ± 5% | 1               |
| Equivalent wt     | Active site titration         | 73,000 ± 8% | 1               |

*The first value was determined from the calibration curve (Fig. 6), and the second value was determined from the slope of the calibration curve and the observed elution volume relative to the serum albumin dimer. The error indicated is based on the probable uncertainty of the calibration curve.

The first value was determined from the calibration curve (Fig. 7), and the second value from the slope of the calibration curve and the observed mobility relative to serum albumin. The indicated error is the range of values of multiple measurements in three separate experiments.

The value is the average of four experimental values, and the indicated error is the range of the experimental values. The uncertainty of the protein concentration is not included in the estimated error.
Estherase A. All gels were stained for protein.

Electrophoresis was carried out as described in the legend to Fig. 4. Two different preparations I and II of esterase B were shown, along with esterase A for comparison. The samples were, from left to right, esterase B-I; a mixture of esterase B-I and esterase A; esterase B-II; and a mixture of esterase B-II and esterase A. All gels were stained for protein.

The properties of esterase A are shown in Fig. 10. The hydrolysis of ethyl butyrate was accompanied by substrate inhibition at concentrations greater than 0.6 mm (Fig. 10). Extrapolation of the linear portion of the curve indicated that $V_{\text{max}}$ was 770 units per mg and $K_m$ was 0.9 mm.

Properties of Esterase B.—Electrophoresis of the esterase B-I fraction in the Tris-glycine system indicated that the fraction contained two or three electrophoretic species (Fig. 11) which were distinct from esterase A. Each protein band had esterase activity, but the most intense esterase stain was associated with the slowest minor protein component. Electrophoresis of esterase B-I preparations in the presence of sodium dodecyl sulfate revealed a single protein band with the same mobility as esterase A (Fig. 5). Thus, in the presence of sodium dodecyl sulfate both esterase A and the esterase B-I proteins had the same electrophoretic mobility, but were distinguished in the non-denaturing Tris-glycine system. Therefore, the subunit molecular weight of esterase A and the esterase B-I proteins must be similar. Estimates of the molecular weight of esterase B-I by gel filtration were inconclusive.

An analysis of the pH dependence of the enzyme activity of the esterase B-I fraction revealed a more complicated relationship than observed for esterase A. The presence of more than one esterase species in the fraction probably contributed to the complexity of the pH dependence. The esterase B-I fraction could be distinguished from esterase A by the observation that it did not appreciably catalyze methanolysis of phenyl butyrate, and ethyl butyrate hydrolysis was not accompanied by substrate inhibition even at 9 mm ethyl butyrate ($K_m = 15$ mm).

Discussion

This report describes some definitive physical and catalytic properties of a purified rat liver microsomal esterase. Attempts to isolate distinct esterase species from rat liver have been made only in recent years and sufficient descriptive data are not available to allow conclusive comparison of the various enzymes isolated and assayed by a variety of techniques. The esterases described by Akao and Omura (4) and Ljungquist and Augustsson (6) are similar in some respects to the esterases described in this report.

The microsomal fraction of liver homogenates was found to contain 70% of the esterase activity. The smaller recoveries of esterase in this fraction reported by others (6, 21) may have resulted from lack of washing, or incomplete washing of the initial low speed pellet (22). When microsomal suspensions containing Triton X-100 (0.35 mg per ml) were centrifuged at 105,000 x g for 60 min, 80% of the esterase activity and 20% of the protein were recovered in the supernatant fraction. Similarly, Akao and Omura (4) found that rat liver microsomal esterase was readily solubilized by nonionic detergents, but not by sodium chloride solutions.

The esterase A preparation appeared to be essentially homogeneous when protein stains were used in three electrophoretic systems, including two that involve protein denaturation. However, in the Tris-glycine system, the very sensitive esterase stain sometimes revealed several very minor bands and shorter and less mobility than the principal band. Some of these minor bands had the same mobility as that of the two to three esterase species in the esterase B-I preparation. The high degree of purity of esterase A was also indicated by its elution from DEAE-Sephadex with essentially constant specific activity. In the catalytic studies, the esterase concentrations were in the range 0.02 to 0.2 mg per ml so that any very minor esterase contaminants would be expected to have no significant effect on the observed properties of esterase A.

The purified carbamoyl esterases from pig and beef liver are reversibly dissociable into active subunits (2). Most analyses have indicated a dimeric structure, but recent work by Heymann et al. (23) and Aune (24) has suggested that the native beef and pig liver esterases may be trimeric structures. The results presented here for esterase A from rat liver parallel the earlier reports for the pig and beef liver esterases. Certainly more precise techniques could be applied, but the data presented here support the conclusion that the enzyme consists of two subunits of equal molecular weight, and that the native enzyme contains two active sites. Although each subunit probably has an active site, no unequivocal evidence is available to support this conclusion.

It has been well established that the catalytic mechanism of the pig and beef liver esterases involves the formation of an enzyme-bound acyl-serine intermediate (2). Esterase A reacts stoichiometrically with diethyl p-nitrophenyl phosphate and readily catalyzes the methanolysis of phenyl butyrate. These two lines of evidence support the conclusion that esterase A is a serine hydrolase. If the criteria described by Greenzaid and Jencks (25) are applied to the data describing methanolysis catalyzed by esterase A (Fig. 8), the results indicate that in the absence of methanol, acylation and decylation occur at comparable rates. If decylation were the rate limiting step in catalysis, the $V_{\text{max}}$ for ethyl- and phenyl butyrate hydrolysis
should be the same. The $V_{\text{max}}$ for these two substrates at 25°C was 770 and 440 units per mg, respectively, which suggests that decacylation is not entirely rate limiting and supports the conclusion made from the data regarding methanolysis. These results, however, are not considered precise enough to be the basis for any definite conclusions regarding rate limiting reaction because measurements of $V_{\text{max}}$ for ethyl butyrate hydrolysis were made at substrate concentrations no more than 0.6 $K_m$ due to substrate inhibition.

Although the role of a serine residue at the active site of pig and beef liver esterases has been established, only very recently has evidence been reported (1) that a histidine residue participates in catalysis (26). The data presented here clearly indicate that the catalytic activity for the rat liver esterase A fraction is histidine. Although the data are not definitive, this represents the first attempt to explain the pH dependence of rat liver esterase activity, and is an approach not previously reported for the well characterized beef and pig liver enzymes.

Disc gel electrophoresis of the esterase B₁ fraction in the Tris-glycine system revealed the presence of two or three principal proteins, each with esterase activity. It was usually noted that the most intense esterase stain corresponded to one of the least abundant proteins. When the same esterase B₁ fraction was subjected to disc gel electrophoresis in the presence of sodium dodecyl sulfate, a single protein band was observed which co-migrated with the esterase A subunit. Although the data may suggest that esterase A and the esterase B₁ fraction are related as dimer and monomer, respectively, no direct evidence is available to support this conclusion. In fact their catalytic properties are quite distinct. Esterase B₁ did not readily catalyze the methanolysis of phenyl butyrate, it did not undergo substrate inhibition by ethyl butyrate, and its inhibition by fluoride was not time dependent (1).

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