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PURIFICATION AND CHARACTERIZATION OF GLUCOCORTICOID-INDUCIBLE STEROID ESTERASE IN RAT HEPATIC MICROSOMES

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Abstract—A steroid esterase hydrolysing methylprednisolone 21-hemisuccinate was purified from the hepatic microsomes of rats treated with dexamethasone, a potent inducer of the esterase. The enzyme was solubilized by Lubrol WX and purified up to 30-fold over the microsomal fraction by ammonium sulfate fractionation and successive chromatographies with gel permeation, DEAE-cellulose and hydroxylapatite. The steroid esterase thus purified showed a single band and a molecular mass of 58 kDa on SDS–polyacrylamide gel electrophoregram. The enzyme appears likely to exist as two interconvertible forms, which can be distinguished by pI values, pI 4.9 and 5.1. The enzyme was completely inhibited by organic phosphates, indicating that it can be classed as a carboxylesterase (EC 3.1.1.1). Both negatively charged and uncharged esters of several steroids (methylprednisolone, hydrocortisone, deoxycorticosterone and dehydrotestosterone) as well as various non-steroidal esters including 4-nitrophenyl esters were hydrolysed by the enzyme, but none of the amides were substrates. The enzyme showed higher activity with increasing lipophilicity of the substrates. It is noticeable that the optimum pH for charged esters was 5.5, whereas the highest activity was observed around pH 7–8 for uncharged esters. When methylprednisolone 21-hemisuccinate (one of the charged esters) was used as substrate, the K_m value was 2.8 mM and V_{max} was 59.3 μmol/mg protein for 1 min at the optimum pH of 5.5. Regarding the methyl ester of methylprednisolone 21-hemisuccinate, K_m and V_{max} values were 1.8 mM and 193 μmol/mg protein/min, respectively, at the optimum pH of 7.0. On the basis of these results, the enzyme is most likely a carboxylesterase.

Ester-type prodrugs are hydrolysed prior to exerting their pharmacological activities [1] and, in most cases, carboxylesterases (EC 3.1.1.1) categorized as serine hydrolase participate in such a reaction in mammals. A large number of isoenzymes have been purified from various tissues of mammals and characterized [2–5]. During the course of study on the metabolic fate of methylprednisolone 21-hemisuccinate (MP-hemisuccinate†), which is a water-soluble prodrug of MP and has been clinically used to immunosuppress or as an anti-shock drug at the time of surgery, we have found that the enzyme responsible for hydrolysis of this compound was markedly induced by the product, MP [6]. In addition, some kinds of glucocorticoids such as dexamethasone elevated the activity remarkably and the increase in esterase activity by glucocorticoid treatment correlated well with the potency of glucocorticoid action like antiinflammatory activity [6]. These results suggested that this enzyme possessed some physiological activities in addition to its activity as an esterase hydrolysing xenobiotic steroidal esters.

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† Abbreviations: MP, methylprednisolone; HC, hydrocortisone; DC, deoxycorticosterone; DT, dehydrotestosterone; BNPP, bis-4-nitrophenyl phosphate; DFP, diisopropyl fluorophosphate; PCMB, 4-chloromercuribenzoic acid.

As a part of the investigation of the physiological significance of this enzyme, we purified the enzyme from the liver of rats treated with dexamethasone. The object of this report is to describe the purification and properties of the enzyme.

MATERIALS AND METHODS

Materials

MP-hemisuccinate, methylprednisolone 21-acetate (MP-acetate), methylprednisolone 21-[8-methyl(2-sulfoethyl)amino]-8-oxooctanoate (MP-suleptanate), MP, hydrocortisone 21-acetate (HC-acetate), hydrocortisone 21-hemisuccinate (HC-hemisuccinate), hydrocortisone (HC) and fluoxymesterone were supplied from the Upjohn Co. (Kalamazoo, MI, U.S.A.). Methyl ester of MP-hemisuccinate (MP-methylsuccinate) was prepared in our laboratory with diazomethane in the usual way. Dehydrotestosterone 17β-acetate (DT-acetate), dehydrotestosterone 17β-propionate (DT-propionate), dehydrotestosterone 17β-hemisuccinate (DT-hemisuccinate), dehydrotestosterone 17β-hexahydrobenzoate (DT-hexahydrobenzoate), deoxycorticosterone 21-acetate (DC-acetate) and deoxycorticosterone 21-hemisuccinate (DC-hemisuccinate) were purchased from Steraloids Inc. (Wilton, NH, U.S.A.). 4-Nitrophenyl acetate, 4-nitrophenyl propionate, 4-nitrophenyl butyrate, 4-nitrophenyl valerate, 4-nitrophenyl caproate, 4-nitrophenyl caprylate, Lubrol WX, bovine serum

Table 1. Purification of the steroid esterase in hepatic microsomes of rats

Purification step	Total protein (mg)	Total activity* (U)	Yield (%)	Specific activity* (U/mg protein)	Purification factor
Microsomes	3400.0	1800	100.0	0.53	1.00
1. Solubilization, Lubrol WX	2520.0	1610	89.4	0.64	1.21
2. Ammonium sulfate fractionation, 40–70%	649.0	1490	82.8	2.30	4.34
3. Sephadex G-100	97.0	664	36.6	6.8	12.91
4. DEAE-cellulose	27.4	403	22.3	14.7	27.72
5. Hydroxylapatite	16.0	256	14.2	16.0	30.17

* One unit of esterase activity is defined as the quantity of enzyme hydrolysing 1 μmol of MP-hemisuccinate/min by the methods described in Materials and Methods.

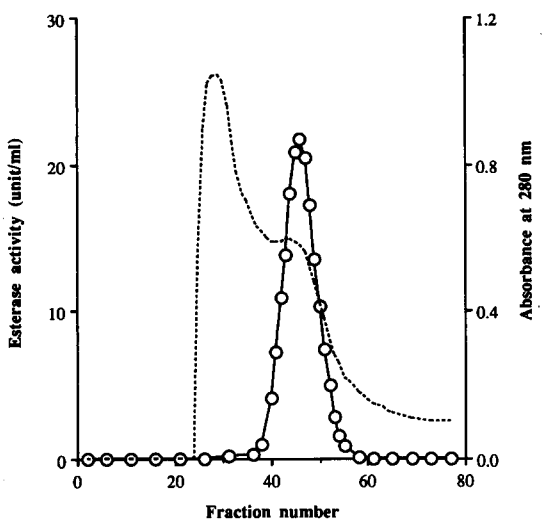

Fig. 1. Elution profile of the steroid esterase on gel permeation chromatography. Proteins obtained by ammonium sulfate fractionation were applied to a Sephadex G-100 column and eluted as described in Materials and Methods. Esterase activity (○) for MP-hemisuccinate was assayed in all fractions. Protein concentration (- - - -) was continuously monitored at 280 nm.

albumin, bis-4-nitrophenyl phosphate (BNPP), diisopropyl fluorophosphate (DFP) and 4-chloromercuribenzoic acid (PCMB) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Phenyl acetate, 1-naphthyl acetate, acetanilide, phenacetin, acetaminophen, 1-naphthylacetamide, 2-acetylaminofluorene and $HgCl_2$ were from Wako Pure Chemicals Ind. (Osaka, Japan). Sephadex G-25 and G-100 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), DEAE-cellulose (DE52) from Whatman BioSystems Ltd (Maidstone, U.K.), and hydroxylapatite (Bio-Gel HTP) from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other reagents used in the present study were of the highest analytical grade and were commercially obtained.

Male Sprague–Dawley rats (7 weeks old, 200–230 g) were purchased from Charles River Japan (Kanagawa, Japan).

Assay methods

For the standard assay of the esterase activity, the amount of MP formed from MP-hemisuccinate was determined by HPLC according to the procedure described in our previous report [6].

The reaction was linear with respect to time up to 15 min and the purified enzyme protein from 0 to 1 mg. The calibration curve was linear at the product concentrations from 2 to 200 nmol/mL.

Esterase activity for other steroidal esters (MP-acetate, MP-suleptanate, MP-methylsuccinate, HC-acetate, HC-caprylate, HC-hemisuccinate, DC-acetate, DC-hemisuccinate, DT-acetate, DT-propionate, DT-hexahydrobenzoate and DT-hemisuccinate) was determined by the same method as for MP-hemisuccinate. Retention times of HC, DC and DT, which were produced from the corresponding esters, were 4.1, 8.5 and 7.6 min, respectively, under the HPLC conditions previously described [6]. Esterase activity for these steroidal esters including MP-hemisuccinate was also examined at pH 8.0 in 50 mM Tris–HCl buffer according to the same procedure. Esterase activity for various 4-nitrophenyl esters was measured spectrophotometrically in 50 mM citrate–phosphate buffer, pH 5.5, or 50 mM Tris–HCl buffer, pH 8.0, each supplemented with Lubrol WX (1%, w/v) and glycerol (5%, v/v) by the method of Krisch [7]. The activity for 1-naphthyl acetate and phenyl acetate was determined in the above two buffers at pH 5.5 or 8.0 by the method of Johnston and Ashford [8]. Hydrolysis rates for acetanilide, acetaminophen, phenacetin, 1-naphthylacetamide and 2-acetylaminofluorene were determined by the diazo-coupling of the primary amino group of each product [9].

A unit of the esterase activity was defined as the quantity of enzyme catalysing the formation of 1 μmol of each product per min under the conditions of the assay.

Protein concentrations were monitored either by the method of Lowry *et al.* [10], in which bovine serum albumin was used as standard, or by measurement of absorbance at 280 nm.

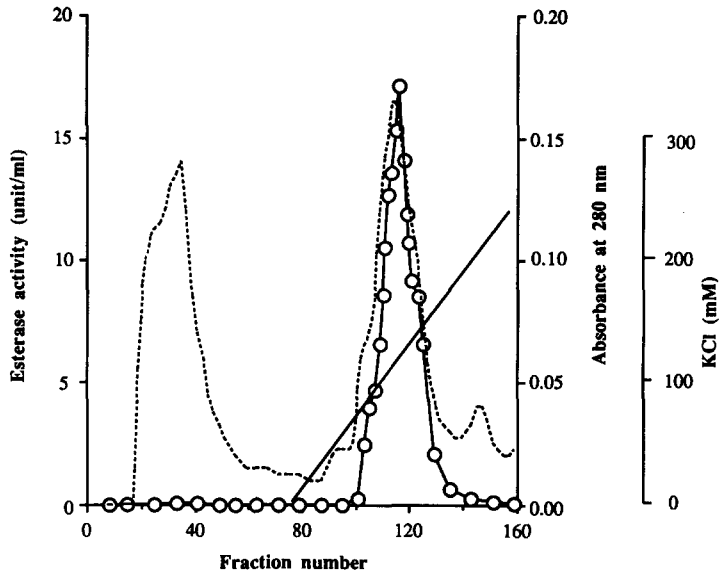

Fig. 2. Elution profile of the steroid esterase on DEAE-cellulose chromatography. Proteins (97 mg) obtained in active fractions (43–49) on gel permeation chromatography were applied to a DEAE-cellulose column and eluted as described in Materials and Methods. A linear gradient with KCl from 0 to 300 mM (——) was initiated at fraction 61. Esterase activity (○) for MP-hemisuccinate was measured in all fractions. Protein concentration (- - - -) was continuously monitored at 280 nm.

Purification of steroid esterase from rat liver

To obtain sufficient amounts of the steroid esterase for characterization, the enzyme was induced by dexamethasone prior to purification; eight male Sprague–Dawley rats were injected i.p. with dexamethasone suspended in sesame oil at a dose of 60 μmol/kg (2 mL/kg) four times at 24-hr intervals. Hepatic microsomes were prepared from rat liver (116 g) by a method similar to the one described in our previous report [6]. The microsomal suspension (340 mL) possessed about 70% of the total esterase activity for MP-hemisuccinate in the liver. The hepatic microsomal esterase was purified according to the following procedure by monitoring enzyme activity hydrolysing MP-hemisuccinate. All steps, unless otherwise noted, were performed at 0 to 4°. A typical example of purification of the steroid esterase was shown in Table 1.

Step 1. *Solubilization of microsomal esterase.* To the microsomal suspension (340 mL) was added an aqueous solution of Lubrol WX (8.5 mL, 200 mg/mL). The mixture was gently stirred at 22° for 1.5 hr, centrifuged at 105,000 g for 1 hr, and the supernatant (332 mL) collected. About 90% of the microsomal activity was solubilized by this method.

Step 2. *Ammonium sulfate fractionation.* To the supernatant (330 mL) obtained at Step 2, 80 g of powdered ammonium sulfate was added portionwise to give 40% saturation under stirring. The mixture was stirred for 90 min followed by centrifugation at 13,000 *g* for 30 min. To the supernatant (343 mL) was added 70 g of powdered ammonium sulfate to result in 70% saturation. After being stirred for 90 min, the suspension was centrifuged at 13,000 *g* to precipitate the active enzyme pellet. The pellet was redissolved in 50 mM potassium phosphate buffer, pH 7.0, to the volume of 21.5 mL.

Step 3. *Gel permeation chromatography.* The ammonium sulfate fraction (20 mL) was applied to a Sephadex G-100 column (25 × 840 mm) equilibrated with 50 mM potassium buffer, pH 7.0. Proteins were eluted with the same buffer at a flow rate of 0.8 mL/min and the eluate fractions of each 5 mL were collected. The esterase activity emerged in fractions 39–54 (Fig. 1). The active fractions from 43 to 49 were combined (35 mL).

Step 4. *DEAE-cellulose chromatography.* The enzyme solution obtained at Step 3 was applied to a DEAE-cellulose column (16 × 510 mm) previously equilibrated with 50 mM potassium phosphate buffer, pH 7.0. Proteins were eluted with 145 mL of the same buffer followed by 300 mL of the buffer containing a linear gradient concentration of 0–300 mM KCl at a flow rate of 0.3 mL/min. Eluate fractions of each 3 mL were collected and the esterase activity emerged in 103–137 fractions (Fig. 2). Fractions from 110 to 124 were combined (45 mL) and KCl was removed with Sephadex G-25 column (50 × 255 mm).

Step 5. *Hydroxylapatite chromatography.* The enzyme solution (90 mL) obtained at Step 3 was applied to a hydroxylapatite column (10 × 445 mm) previously equilibrated with 5 mM potassium phosphate buffer, pH 7.0. The column was first washed with 45 mL of the same buffer and the enzyme was eluted with 240 mL of the phosphate buffer with a linear gradient of 5–60 mM potassium phosphate buffer, pH 7.0, at a flow rate of 0.4 mL/min. Eluate fractions of each 3 mL were collected. The esterase activity for MP-hemisuccinate emerged in 77–98

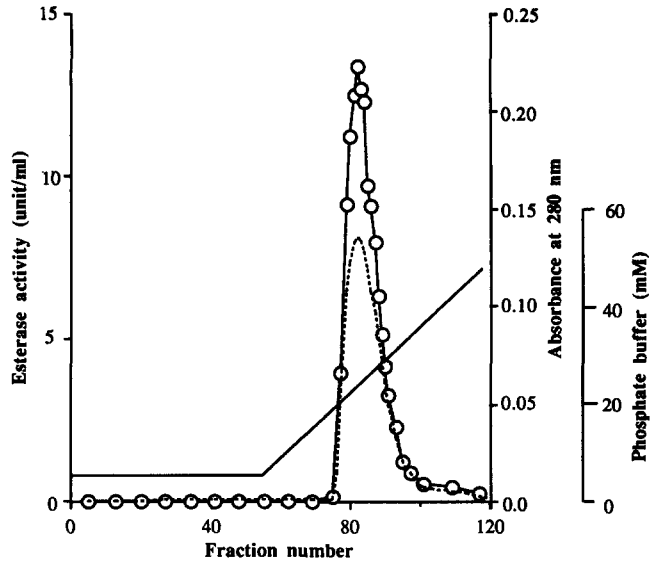

Fig. 3. Elution profile of the steroid esterase on hydroxylapatite chromatography. Proteins (27.4 mg) obtained in active fractions (110–124) of DEAE-cellulose chromatography were applied to a hydroxylapatite column, washed with 5 mM potassium phosphate buffer, pH 7.0, and eluted with a linear gradient of the buffer (——) from 5 to 60 mM. Esterase activity (○) for MP-hemisuccinate was assayed in all fractions. Protein concentration (- - - -) was continuously monitored at 280 nm.

fractions (Fig. 3). Fractions from 79 to 86 were combined (24 mL) and the enzyme solution was stored at 0° until use.

Analysis of amino acid composition

The amino acid composition of the purified esterase was determined by hydrolysing the enzyme with 6 N HCl at 170° for 1.25 hr and subsequent analysis with an automatic amino acid analyser (Model 420H, Applied Biosystems, Foster City, CA, U.S.A.).

Determination of molecular mass

The apparent molecular mass was determined by gel permeation chromatography with HPLC equipped with a TSK GEL column (G-3000 SWXL, 7.8 × 300 mm, Toso, Tokyo, Japan). The molecular mass standards (Gel Filtration Standard, Bio-Rad) used were: thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B_{12} (1.35 kDa). Proteins were eluted by 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM KCl at a flow rate of 0.8 mL/min and detected at 280 nm. The molecular mass was also determined by analytical polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) on 20% homogeneous polyacrylamide slab gel (40 × 40 mm, PhastGel Homogeneous 20, Pharmacia) with an automated electrophoresis system (Phast System, Pharmacia) according to the standard procedure provided by the manufacturer. The standard proteins (SDS–PAGE Standards, Low Range, Bio-Rad) used were: rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white albumin (42.7 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa). Proteins were stained with PhastGel Blue R (Pharmacia).

Isoelectric focusing

The isoelectric point of the enzyme (Step 5 enzyme) was determined by PAGE (PhastGel IEF, pH 4–6.5, Pharmacia) with an automated PAGE system (Phast System, Pharmacia) by the method described by the manufacturer. The following standard proteins (Low pI Calibration Kit, Pharmacia) were used for monitoring isoelectric points: glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20) and bovine carbonic anhydrase B (pI 5.85).

Kinetic analysis

Esterase activity for MP-hemisuccinate, MP-methylsuccinate and MP-acetate was measured at the respective optimum pH of 5.5, 7.0 and 8.0 over the concentrations ranging from 0.125 to 1 mM and the K_m and V_{max} were determined by double reciprocal plots.

RESULTS

Purification, molecular mass, amino acid composition, isoelectric point and stability

Treatment of rats with dexamethasone (60 μmol/kg, four times) elevated the esterase activity in the hepatic microsomes up to 65-fold in comparison with the basal level of 7.9 mU/mg protein. The induced steroid esterase was purified about 30-fold over the microsomes with a yield of about 14% of the esterase activity for MP-hemisuccinate according to the five-step procedure (Table 1). In a preliminary study,

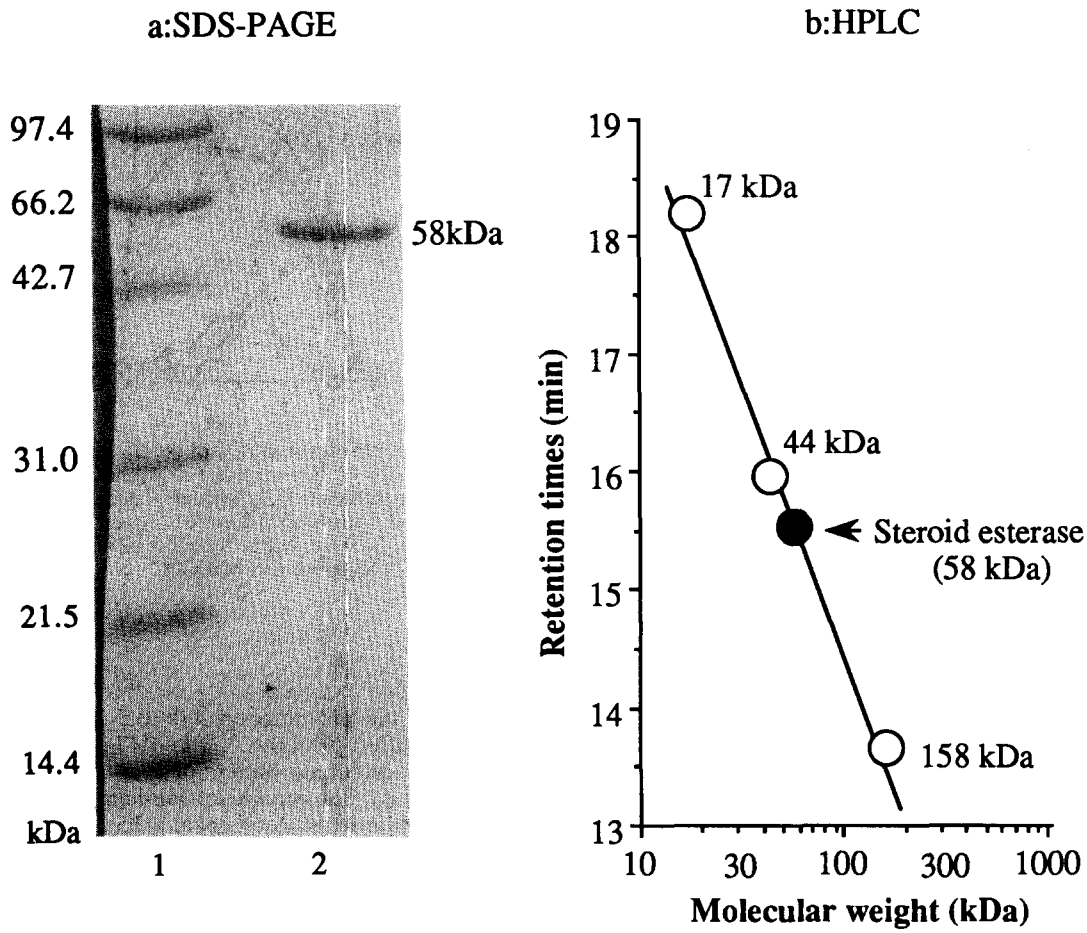

Fig. 4. Molecular mass of the steroid esterase. Apparent molecular mass of the purified enzyme was determined by comparison with the various standard proteins on SDS–PAGE (a), and gel permeation HPLC (b). (a) Standard proteins (lane 1) and the purified enzyme (lane 2) were applied to a PhastGel Homogeneous 20 containing SDS and electrophoresed. Proteins were stained with PhastGel Blue R. (b) The enzyme and standard proteins were chromatographed with HPLC equipped with a G-3000 SXWL gel column. Proteins were monitored at 280 nm.

we examined several kinds of detergents (Triton X-100, cholate, Lubrol PX and WX) to solubilize the esterase; more than 85% of the esterase activity for MP-hemisuccinate was solubilized from the rat hepatic microsomes only by Lubrol PX or WX at the concentration of 0.4% (w/v). Autolysis or treatment with phospholipase A_2 or trypsin resulted in the loss of esterase activity in microsomes. Based on this result, we used Lubrol WX for solubilization in this study. The enzyme at Step 5 showed a single band in the SDS–PAGE (Fig. 4a) and had a molecular mass of 58 kDa, which was identical to that obtained by HPLC gel permeation chromatography (Fig. 4b). These results indicate the steroid esterase is a monomeric protein.

The composition of amino acids except for cysteine and tryptophan (both amino acids could not be analysed by the present method) was determined for the purified enzyme (Table 2). A conspicuous feature of the enzyme is the existence of larger amounts of histidine and methionine and lower amounts of lysine, tyrosine and serine in comparison with other carboxylesterases [2, 11, 12]. The minimum molecular mass calculated from the data in Table 2 was about 47 kDa which accounted for 80% of the molecular mass of 58 kDa determined by SDS–PAGE and HPLC.

Unexpectedly, the isoelectric focusing of the purified steroid esterase gave two bands corresponding to pI 4.9 and 5.1 (Fig. 5a). When the gel corresponding to either of the two bands was excised from the plate and the enzyme in the gel was subsequently subjected to a second isoelectric focusing, two bands with pI values of 4.9 and 5.1 were observed again (data not shown). Incubation of the enzyme with DFP (0.1 mM) at room temperature for 15 min gave a single band with pI 4.9 in the isoelectric focusing (Fig. 5b).

During the storage of the purified esterase for 2 months at 0°, the esterase activity did not decrease to any significant extent, while about 30% of the activity was lost by freezing and thawing.

Table 2. Amino acid composition of the steroid esterase

Amino acid	Residues per mol
Ala	30.9 ± 0.6
Arg	15.8 ± 0.6
Asn/Asp	36.3 ± 1.0
Cys	ND
Gln/Glu	41.9 ± 0.7
Gly	28.7 ± 0.8
His	13.9 ± 0.4
Ile	15.6 ± 0.7
Leu	37.8 ± 2.1
Lys	15.5 ± 0.5
Met	14.9 ± 0.5
Phe	16.8 ± 0.8
Pro	27.4 ± 0.8
Ser	24.0 ± 0.6
Thr	19.1 ± 0.4
Try	ND
Tyr	9.0 ± 0.4
Val	27.0 ± 0.9

Values represent the means ± SEM from three determinations.
ND, not determined.

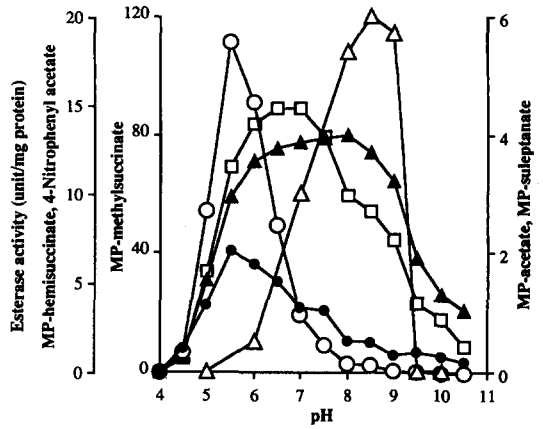

Fig. 6. Effect of pH on the activity of the steroid esterase. Esterase activities of the enzyme for MP-hemisuccinate (○), MP-methylsuccinate (□), MP-suleptanate (●), MP-acetate (▲) and 4-nitrophenyl acetate (△) were measured at various pH in three buffer systems as described in Materials and Methods.

Effect of pH

The effect of pH on the activity of the purified steroid esterase was examined with MP-hemisuccinate, MP-suleptanate, MP-methylsuccinate, MP-acetate and 4-nitrophenyl acetate (each 1 mM) over the pH range of 4.0–10.5 in citrate–phosphate buffer, pH 4.0–8.0, Tris–HCl buffer, pH 7.5–9.0, and carbonate buffer, pH 9.5–10.5 (each 50 mM) (Fig. 6). The activity for MP-hemisuccinate and MP-suleptanate was optimal at pH 5.5, whereas the enzyme showed persistently high esterase activity for MP-methylsuccinate and MP-acetate from pH

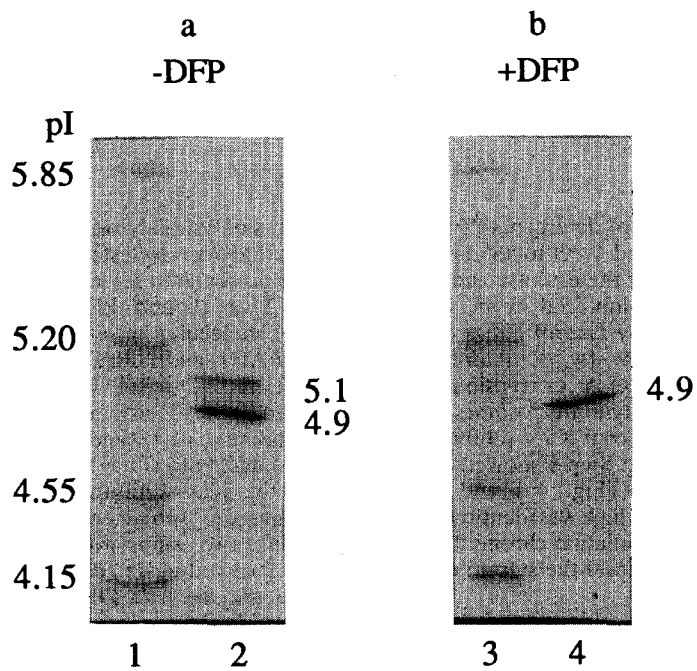

Fig. 5. Isoelectric focusing of the steroid esterase. The intact enzyme (a) or the enzyme treated with 0.1 mM DFP (b) was applied to a PhastGel IEF, pH 4.0–6.5, and subjected to an isoelectric focusing. Lanes 1 and 3, standard proteins; lane 2, intact enzyme; lane 4, enzyme treated with DFP.

Table 3. Substrate specificity of the steroid esterase

Substrate	Esterase activity (U/mg protein)	
	pH 5.5	pH 8.0
MP-acetate	3.04	4.66
MP-hemisuccinate	16.0	1.65
MP-methylsuccinate	36.2	46.4
MP-suleptanate	1.85	0.75
HC-acetate	3.79	4.88
HC-hemisuccinate	11.3	1.32
HC-caprylate	41.4	41.7
DC-acetate	2.72	4.68
DC-hemisuccinate	20.5	5.93
DT-acetate	<0.01	<0.01
DT-hemisuccinate	0.25	<0.01
DT-propionate	<0.01	0.22
DT-hexahydrobenzoate	0.72	0.84
4-Nitrophenyl acetate	1.00	21.8
4-Nitrophenyl propionate	2.14	46.5
4-Nitrophenyl butylate	2.26	53.3
4-Nitrophenyl valerate	2.39	50.6
4-Nitrophenyl caproate	3.03	57.4
4-Nitrophenyl caprylate	3.82	80.6
1-Naphthyl acetate	5.85	9.63
Phenyl acetate	10.1	17.9
Acetanilide	<0.01	<0.01
Acetaminophen	<0.01	<0.01
Phenacetin	<0.01	<0.01
2-Acetylaminofluorene	<0.01	<0.01
1-Naphthylacetamide	<0.01	<0.01

6.0 to 7.5 and from pH 6.0 to 9.0, respectively. Similar results were obtained for other steroidal ester substrates (Table 3); when the activity was examined at pH 5.5 and pH 8.0, the enzyme had somewhat higher activity for all the uncharged steroidal esters at pH 8.0 rather than pH 5.5, while the activity for the negatively charged esters was 3–25 times greater at pH 5.5 than that at pH 8.0. In contrast to steroidal esters, 4-nitrophenyl acetate showed a sharp pH dependency curve with an optimal pH of 8.5 (Fig. 6). Regarding other kinds of 4-nitrophenyl esters the esterase activity was about 20 times greater at pH 8.0 in comparison with pH 5.5 (Table 2).

Substrate specificity

The esterase activity for various steroidal esters, 4-nitrophenyl esters, phenyl acetate, 1-naphthyl acetate as well as several amides was examined at pH 5.5 and pH 8.0 (Table 2). The steroid esterase was found to be responsible for hydrolysis of all the ester-type substrates, whereas none of the amides examined in this study worked as substrate; the enzyme activity for each amide was less than 10 mU/mg protein.

When the steroidal esters with an identical acyl moiety were compared, the enzyme possessed a comparable activity for esters of methylprednisolone, hydrocortisone and deoxycorticosterone. On the other hand, the esterase activity for dehydrotestosterone esters was much lower than that for the esters of the above three steroids. The esterase showed the highest activity of about 0.8 U/mg protein for DT-hexahydrobenzoate, which was highly lipophilic among the dehydrotestosterone esters.

The enzyme showed higher activity for methyl ester of MP-hemisuccinate than the acid form as described in the "Kinetic analysis" section (Table 4); V_{max} of MP-methylsuccinate was approximately three times greater than that of MP-hemisuccinate, when each V_{max} was determined at the respective optimum pH. Interestingly, methyl ester moiety of MP-methylsuccinate was unlikely to be subjected to hydrolysis by the steroid esterase, because MP-hemisuccinate could not be detected in the incubation mixture of MP-methylsuccinate (detection limit: 2 nmol/mL of incubation mixture) in spite of the much lower catalytic rate of MP-hemisuccinate to methylprednisolone in comparison with that of MP-methylsuccinate at pH 7.0 (Fig. 6). On varying the carbon chain of the acyl group attached to the steroidal moiety, we found that the hydrolysing activity was greater for the esters having a longer carbon chain; the hydrolysing rates of HC-caprylate and DT-propionate were about 10 times and greater than 20 times higher, respectively, than that of corresponding acetate esters at pH 8.0 (Table 3). The similar result was observed for esters of 4-nitrophenol. Thus, the esterase activity was elevated with the increase in the number of carbon atoms on the acyl chain of esters of 4-nitrophenol (Table 3). These results suggest that the esterase has higher activity for the substrates with higher lipophilicity.

Table 4. Apparent kinetic constants of the steroid esterase

Substrate	pH†	K_m* (mM)	V_{max}* (nmol/mg protein/min)
MP-acetate	8.0	1.6	8.31
MP-hemisuccinate	5.5	2.8	59.3
MP-methylsuccinate	7.0	1.8	193

* Values were determined by double reciprocal plots of substrate concentration (0.125–1 mM) and enzyme activity for each substrate.
† Enzyme activity for substrate was measured at respective optimum pH.

Table 5. Effect of various reagents on the steroid esterase

Reagent	Concentration (mM)	Residual esterase activity* (%) MP-hemisuccinate (pH 5.5)	MP-acetate (pH 8.0)
None	—	100	100
DFP	1	3	6
BNPP	1	6	6
$HgCl_2$	10	5	115
PCMB	10	47	101

* All values are expressed as percentages relative to the esterase activities without above reagents, 16.0 and 4.22 U/mg protein for MP-hemisuccinate and MP-acetate, respectively.

This is the probable reason the enzyme showed much higher esterase activity for MP-methylsuccinate than MP-hemisuccinate.

Kinetic analysis

The steroid esterase activity at various concentrations of MP-hemisuccinate, MP-methylsuccinate and MP-acetate was examined under the standard incubation conditions at each optimum pH. The apparent K_m values for MP-hemisuccinate, MP-methylsuccinate and MP-acetate were 2.76, 1.80 and 1.57 mM with V_{max} of 59.3, 193 and 8.3 U/mg protein for 1 min, respectively, as determined from double reciprocal plots (Table 4).

Effect of various inhibitors

The purified enzyme (Step 5) was preincubated in 50 mM citrate–phosphate buffer, pH 5.5, at 37° for 5 min with two kinds of organic phosphates (DFP and BNPP), $HgCl_2$ or PCMB. After the preincubation, MP-hemisuccinate or MP-acetate (each 1 mM) was added to the reaction mixture and the enzyme activity was determined by the method described in the "Assay methods" section (Table 5). When organic phosphates (1 μM) were present in the reaction mixture, the activity was virtually negligible for the two substrates. Esterase activity for MP-hemisuccinate was inhibited completely by $HgCl_2$ (10 μM) and partially by PCMB (10 μM). On the contrary, the activity for MP-acetate was not affected by these sulfhydryl reagents. At present the reason these sulfhydryl reagents showed different inhibitory activity for MP-hemisuccinate and MP-acetate is not known.

DISCUSSION

In this study, the hepatic steroid esterase responsible for hydrolysis of MP-hemisuccinate, which had been induced up to 65-fold over the basal level of 7.9 mU/mg protein by i.p. administration of dexamethasone, was purified about 30 times over the microsomal fraction according to the conventional five-step procedure (Table 1). This esterase is probably a carboxylesterase (EC 3.1.1.1), because firstly, the enzyme activity was completely abolished by treatment with DFP and BNPP (Table 5), both of which exert inhibitory activity by a specific binding to a serine moiety at the active center of serine hydrolases [13], and secondly, only esters but not amides worked as substrate for the esterase.

It is notable that the enzyme purified in this study gave two bands corresponding to pI 4.9 and 5.1 on the isoelectric focusing electrophoregram (Fig. 5a), although the enzyme was found to be a monomeric protein. A possible explanation is the existence of two kinds of proteins with the same molecular mass but with different pI values in the solution at the final purification step. However, we consider that the two bands were associated with two interconvertible forms of a single enzyme, because when each band was subjected to the second isoelectric focusing, two bands again emerged at pI 4.9 and 5.1. Since the treatment of the purified

enzyme with DFP generated a single band in the isoelectric focusing (Fig. 5b), the active center of this enzyme is the probable contributory site to give the two enzyme forms.

The hydrolysis rate of steroidal esters possessing electrically uncharged acyl group was somewhat higher at pH 8.0 than at pH 5.5 (Table 3 and Fig. 6). In the case of esters of 4-nitrophenol the amount of the hydrolysed product, i.e. 4-nitrophenol, was about 20 times larger at pH 8.0 than at pH 5.5. These results suggest that physicochemical conditions—including stability, conformation, electric charge etc.—of the enzyme molecule are optimal at pH 8.0 to exert the catalytic activity. In contrast to the uncharged esters, when the negatively charged esters like MP-hemisuccinate (pK_a 5.4) and MP-suleptanate (pK_a 2.1) were used as substrates, the enzyme activity was optimum at pH 5.5 but much lower at pH 8.0 (Table 3 and Fig. 6). This difference in pH dependency between uncharged and negatively charged substrates is attributable to the change in the lipophilicity of negatively charged substrates at varied pH. Thus, around pH 8.0 numbers of negatively uncharged molecules, which showed higher affinity to the enzyme than the charged molecules, in the reaction mixture were smaller than at lower pH and the enzyme reaction was thus lowered.

Up to now, two groups of investigators purified carboxylesterases which had similar properties to the present enzyme. Mentlein *et al.* [11, 14] reported carboxylesterase, which was termed as ES-15A in the genetic nomenclature as revised by Van Zutphen [15], with pI 5.2 and a molecular mass of 58 kDa in the rat hepatic microsomes. Robbi and Beaufay [16] also purified esterase with pI 5.0 from microsomes of rat liver and concluded the enzyme was identical with ES-15A. In spite of the similarities with pI value and molecular mass, several differences were found between ES-15A and the enzyme purified in the present study: firstly, the N-terminal amino acid of ES-15A was determined to be glycine by Edman degradation [11], whereas the enzyme isolated here could not be analysed by this method probably due to the presence of an acyl moiety on the N-terminal amino acid (data not shown). Secondly, the esterase hydrolysing MP-hemisuccinate was likely to exist as two interconvertible forms with pI 4.9 and 5.1, in contrast ES-15A showed a single band corresponding to pI 5.2 on an isoelectric focusing [11]. Thirdly, ES-15A cleaved the amide bond of acetanilide [16], but the enzyme did not hydrolyse amides. The physiological significance of the steroid esterase is under investigation.

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