p-Nitrophenyl butyrate hydrolyzing activity of hormone-sensitive lipase from bovine adipose tissue

T. Tsujita,[1] **H. Ninomiya, and H. Okuda**

Department of Medical Biochemistry, School of Medicine, Ehime University, Shigenobu, Ehime 791-02 Japan

Abstract The "esterase" activity of hormone-sensitive lipase (HSL) was studied using water-soluble p-nitrophenyl butyrate (PNPB) as a substrate. Bovine adipose tissue HSL was purified to near homogeneity by precipitation at pH 5.0, followed by chromatography on DEAE-cellulose, phenyl-Sepharose, and high performance ion-exchange columns on Mono Q and Mono S. The purified preparation hydrolyzed emulsified triolein and cholesteryl oleate (CO), and water-soluble PNPB. In the two last steps of purification, the elution profile of the CO-hydrolyzing activity coincided with that of PNPB-hydrolyzing activity. The HSL was adsorbed to heparin-Sepharose and the CO- and PNPB-hydrolyzing activities were eluted together in the same peak. Diisopropylfluorophosphate (DFP) strongly inhibited the HSL activities and the inhibition profiles of the triolein-; CO-, and PNPB-hydrolyzing activities were essentially identical. Only one polypeptide of M_r 84,000 in partial purified HSL fraction was labeled by affinity labeling with [^{3}H]DFP. On digestion of the enzyme with trypsin, the triolein- and CO-hydrolyzing activities were lost more rapidly than the PNPB-hydrolyzing activity. Phosphorylation increased the triolein-hydrolyzing activity to 40% more than that of the control, but did not affect the CO- and PNPB-hydrolyzing activities.—**Tsujita, T., H. Ninomiya and H. Okuda.** p-Nitrophenyl butyrate hydrolyzing activity of hormone-sensitive lipase from bovine adipose tissue. *J. Lipid Res.* 1989. **30:** 997–1004.

Supplementary key words water-soluble substrate • cholesteryl oleate • triolein

The rate-limiting step in mobilization of fat from fat cells is its hydrolysis to free fatty acid and glycerol. At least two types of triglyceride lipase (lipoprotein lipase and hormone-sensitive lipase) are present in fat cells. HSL is the key enzyme regulating fatty acid mobilization from fat cells (1), but its mechanism of action is not well understood. Some lipolytic enzymes are known to show differences in specificity that are related to the physicochemical nature of the substrate rather than to its chemical configuration (2, 3). For instance, pancreatic lipase shows considerably higher activity against insoluble emulsified substrates than against soluble ones, whereas liver esterase acts preferentially on monomeric or water-soluble substrates (4). HSL shows less substrate specificity than pancreatic lipase, readily acting on triacylglycerols, diacylglycerols, monoacylglycerols, and cholesteryl esters (1, 5). In the present investigation, we found that HSL also catalyzed the hydrolysis of water-soluble esters, which are useful in studies on the molecular dynamics of lipase, and we examined the relation between the lipase and esterase activities of purified HSL using triolein, cholesteryl oleate, and water-soluble PNPB as substrates.

MATERIALS AND METHODS

Materials

Enzyme substrates and reagents were obtained from the following sources: [^{3}H]triolein (tri[9,10-^{3}H]oleoylglycerol), cholesteryl [1-^{14}C]oleate, and [^{3}H]DFP from Amersham Japan (Tokyo, Japan); triolein, PNPB, DFP, Triton N-101, trypsin, soybean trypsin inhibitor, and the catalytic subunit of cyclic AMP-dependent protein kinase from Sigma (St. Louis, MO); cholesteryl oleate from Funakoshi Kogyo Co. (Tokyo, Japan); DEAE-cellulose (DE-52) from Whatman Ltd. (UK); phenyl-Sepharose and heparin-Sepharose from Pharmacia Fine Chemicals (Uppsala, Sweden) and Emulgen 120 ($C_{12}H_{25}O(CH_2CH_2O)_{13}H$) from Kao Chemicals (Tokyo, Japan). ApoC-II was kindly provided by Dr. K. Shirai and Dr. Y. Saito (Chiba University, Japan). Bovine serum albumin was obtained from Wako Pure Chemical Industries (Osaka, Japan) and

---

Abbreviations: HSL, hormone-sensitive lipase; PNPB, p-nitrophenyl butyrate; CO, cholesteryl oleate; DFP, diisopropylfluorophosphate; FPLC, fast-protein liquid chromatography.

[1]To whom reprint requests should be addressed at: Department of Medical Biochemistry, School of Medicine, Ehime University, Shitsukawa, Shigenobu, Onsen-gun, Ehime 791-02, Japan.

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 19, 2012

was extracted by the method of Chen (6) to remove free fatty acid.

Methods

Purification of hormone-sensitive lipase. HSL was purified from bovine perirectal adipose tissue by the procedures of Cordle, Colbran, and Yeaman (7), and Nilsson and Belfrage (8) with some modifications. The fresh adipose tissue (1.5 kg) was homogenized for 30 sec at 10–15°C in a Waring Blender with 2 volumes of buffer solution consisting of 0.25 M sucrose, 1 mM EDTA, 0.1 mM benzamidine, 1 mg/l leupeptin, 1 mg/l pepstatin, 0.1% (v/v) 2-mercaptoethanol, and 20 mM Tris, pH 7.4. The homogenate was strained through cheesecloth and centrifuged at 13,000 *g* for 15 min, and the fluid under the fat layer was filtered through glass wool and adjusted to pH 5.0. The resulting precipitate was collected by centrifugation at 13,000 *g* for 15 min and resuspended in a total volume of 500 ml in 10 mM K_2HPO_4/KH_2PO_4 (pH 7.4) containing 1 mM EDTA, 0.1 mM benzamidine, 0.1% 2-mercaptoethanol, 1 mg/l leupeptin, and 1 mg/l pepstatin (buffer A). The enzyme was partially solubilized by the addition of Triton N-101 (0.2%, v/v) and NaCl (10 mM) and sonicated for 10 min at 10–15°C in a model UR-2000 Sonicator (Tomy Seiko Co. Ltd., Tokyo, Japan) at a setting of 6. The mixture was then loaded onto DEAE-cellulose (6 × 10 cm) pre-equilibrated with buffer A supplemented with 0.2% Triton N-101. The column was washed with 300 ml of buffer A containing 0.2% Emulgen 120 and then the enzyme was eluted with buffer A containing 0.2% Emulgen 120 and 100 mM NaCl. Solid NaCl and glycerol were added to final concentrations of 1 M and 15%, respectively, and the mixture was resonicated (5 min at 4°C at setting 6). The enzyme solution (160 ml) was then applied to a phenyl-Sepharose column (2 × 11 cm) pre-equilibrated with 5 mM K_2HPO_4/KH_2PO_4 (pH 7.4) containing 1 M NaCl, 0.1 mM benzamidine, 1 mM dithiothreitol, and 0.2% Emulgen 120. The column was washed with the equilibration buffer and the enzyme was then eluted with 5 mM K_2-HPO_4/KH_2PO_4 (pH 7.4) containing 50% glycerol, 0.2% Emulgen 120, 1 mM dithiothreitol, 0.1 mM benzamidine, 1 mg/l leupeptin, and 1 mg/l pepstatin. The fractions containing enzyme were pooled and concentrated with DEAE-cellulose. The concentrated enzyme was dialyzed against 20 mM Tris (pH 7.4) containing 20% glcyerol, 1 mM dithiothreitol, 0.2% Emulgen 120, 0.1 mM benzamidine, 1 mg/l leupeptin, and 1 mg/l pepstatin for 8 h at 4°C. Half the dialyzed enzyme preparation was applied to a Mono Q column (0.5 × 5 cm, The FPLC System, Pharmacia, Uppsala, Sweden); flow rate of 1.0 ml/min at 20–25°C. The column was washed with equilibration buffer and the enzyme was eluted with a linear gradient of 0–0.5 M NaCl. The fractions with lipase activity were pooled, dialyzed, and applied to a Mono S column (0.5 × 5 cm) by the method of Nilsson and Belfrage (8). The column was washed with equilibration buffer and the enzyme was eluted with a linear gradient of 50 mM K_2HPO_4/KH_2PO_4 (pH 6.35) to 300 mM K_2HPO_4/KH_2-PO_4 (pH 7.4). Fractions with enzyme activity were pooled and dialyzed against 10 mM Tris (pH 7.0) containing 1 mM dithiothreitol, 0.2% Emulgen 120, and 50% glycerol. The enzyme solution was stored at −70°C.

Heparin-Sepharose column chromatography. Partially purified HSL (from phenyl-Sepharose) was applied to a heparin-Sepharose column (1.2 × 8 cm) equilibrated with 5 mM K_2HPO_4/KH_2PO_4 (pH 7.4) containing 30% glycerol, 1 mM dithiothreitol, and 0.2% Emulgen 120. The column was washed with equilibrated buffer and the enzyme was eluted with a linear gradient of 0–0.5 M NaCl.

Enzyme assay. PNPB-hydrolyzing activity was determined by measuring the rate of release of *p*-nitrophenol (absorbance at 400 nm at 25°C) by a modification of the procedure described previously (9). The assay mixture consisted of 1.0 ml of 50 mM sodium phosphate buffer (pH 7.25) containing 0.5 µmol of PNPB, 1% (v/v) acetonitrile, 10% glycerol, 0.1 mmol NaCl, and 20 µl of enzyme solution. Released *p*-nitrophenols were determined continuously by monitoring the increase in the absorbance at 400 nm using incubation mixture without enzyme as a blank. Triolein-and CO-hydrolyzing activities were measured by the method of Khoo et al. (10) with [^{3}H]triolein and [^{14}C]CO. Incubations were carried out at pH 6.8 and 37°C, and free oleic acid was extracted and determined by the method of Belfrage and Vaughan (11).

Trypsin digestion of hormone-sensitive lipase. Aliquots of 160 µl of lipase solution (from Mono Q) at a protein concentration of 75 µg/ml in 50 mM Tris, 1.5 mM dithiothreitol, 31 mM $CaCl_2$ (pH 7.4), containing 30% glycerol were digested with various amounts of trypsin (40 µl) for 15 min at 37°C. Then 40 µl of trypsin inhibitor (100 µg/ml) was added and the remaining enzyme activity was assayed.

Inhibition studies. Aliquots of 180 µl of lipase solution (from Mono Q, protein concentration, 40 µg/ml) in 10 mM K_2HPO_4/KH_2PO_4, 1 mM dithiothreitol containing 30% glycerol were incubated with various amounts of inhibitor (20 µl) for 30 min at 37°C and the remaining enzyme activity was assayed.

[^{3}H]DFP-labeling of hormone-sensitive lipase. Enzyme (0.33 mg/0.5 ml, after phenyl-Sepharose) was incubated with [^{3}H]DFP as previously described (9). The protein was precipitated by addition of 1 volume of 20% ice-cold trichloroacetic acid and extracted with diethyl ether–ethanol 3:1 (v/v).

Other procedures. SDS-gel electrophoresis was performed in 8% gel under reducing conditions as described by Laemmli (12). Silver staining was carried out with a Bio-Rad silver stain kit (Bio-Rad Laboratories, Richmond, CA). Fluorography of [^{3}H]DFP-labeled enzyme was per-

Downloaded from www.jlr.org by guest, on June 19, 2012

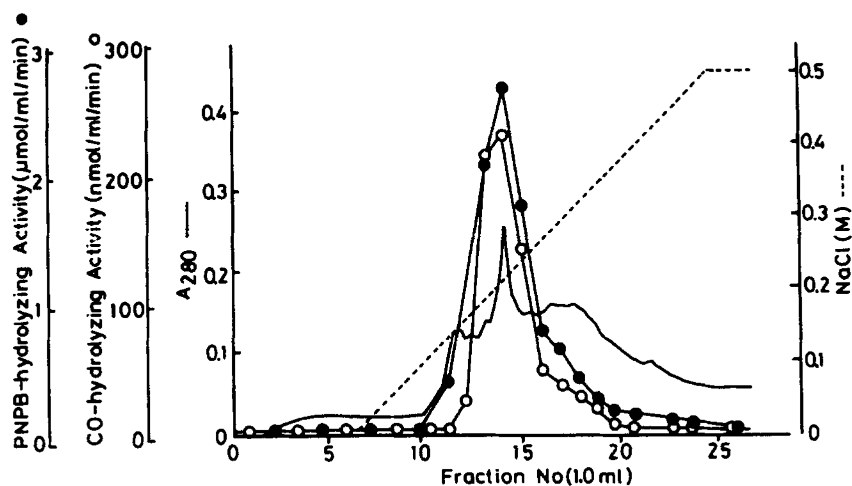

Fig. 1. Mono Q FPLC of bovine adipose tissue HSL. Approximately 3.1 mg enzyme protein was applied to the column. The column was washed with buffer and developed with a linear gradient of 0–0.5 M NaCl. Fractions of 1.0 ml were collected and NaCl concentration (----), A_{280} (—), and CO- (-O-) and PNPB- (-●-) hydrolyzing activities were measured.

formed by the method of Chamberlain (13). Relative protein concentration was determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

RESULTS

The CO-hydrolyzing activity of adipose tissue homogenate was precipitated completely at pH 5.0. This CO-hydrolyzing activity of HSL was adsorbed to a column of phenyl-Sepharose, whereas 90% of the PNPB-hydrolyzing activity was not. The HSL also was adsorbed to a Mono Q column and the CO- and PNPB-hydrolyzing activities were eluted together in the same peak, as shown in **Fig. 1.** The active fractions were pooled, dialyzed, and applied to a Mono S column. The CO- and PNPB-hydrolyzing activities were recovered in a single peak (**Fig. 2**).

The steps of enzyme purification and the yields of enzyme at each step are summarized in **Table 1**. At the final step of purification, the specific activities for CO and PNPB were 3.8 and 22 μmol/mg protein per min, respectively. The purified enzyme gave one major band on SDS-

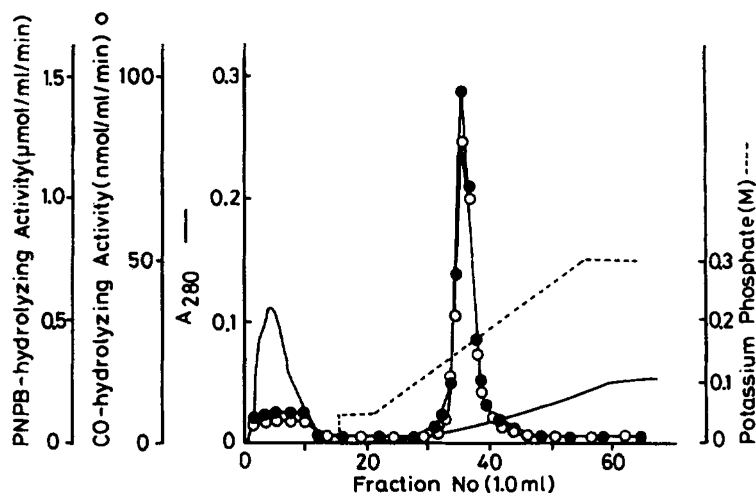

Fig. 2. Mono S FPLC of bovine adipose tissue HSL. Approximately 250 μg enzyme protein was applied to the column. The column was washed with buffer and the enzyme was eluted with a linear gradient of potassium phosphate. Fractions of 1.0 ml were collected and the potassium phosphate, concentration (----), A_{280} (—), and CO- (-O-) and PNPB- (-●-) hydrolyzing activities were measured.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

TABLE 1. Purification of hormone-sensitive lipase from bovine adipose tissue

	Protein	Activity: Cholesteryl Oleate Total	Cholesteryl Oleate Specific	PNPB Total	PNPB Specific
	mg	*µmol/min*	*µmol/mg/min*	*µmol/min*	*µmol/mg/min*
13K Sup	1648	11.5	0.007	214	0.13
pH 5.0 ppt	410	11.1	0.027	135	0.34
DE-52	133	6.67	0.05	53.2	0.40
Phenyl-Sepharose	6.3	2.71	0.43	6.00	0.95
Mono Q[a]	0.51	2.95	2.5	5.87	11.5
Mono S[a]	0.14	0.53	3.8	3.08	22.0

[a]Combined enzyme from two identical chromatographies of the initial batch.

polyacrylamide gel electrophoresis from which its molecular weight was estimated to be 84,000 (**Fig. 3**).

The HSL (after phenyl-Sepharose) was adsorbed to a heparin-Sepharose column and CO- and PNPB-hydrolyzing activities were eluted in the same peak with about 0.15 M NaCl (**Fig. 4**). No further PNPB-hydrolyzing activity was eluted from the heparin-Sepharose column by 2.0 M NaCl (data not shown). The triolein-hydrolyzing activity (at pH 8.5) of the partially purified enzyme fraction (after phenyl-Sepharose) was not activated by apoC-II (data not shown).

Results of HSL-catalyzed hydrolysis of PNPB are shown in **Fig. 5A.** The release of *p*-nitrophenol was linear with HSL concentrations of up to 0.5 µg/ml. Fig. 5B shows the enzymatic activity as a function of the PNPB concentration. The K_m value was estimated to be 164 ± 27 µM. The purified enzyme hydrolyzed *p*-nitrophenyl esters with various acyl chain lengths. After purification of the enzyme on Mono Q, the specific activities of the enzyme for *p*-nitrophenyl acetate, butyrate, laurate, and palmitate were 4.7, 9.8, 0.8, and 0.9 µmol/mg protein per min, respectively.

DFP inhibited the triolein-, CO-, and PNPB-hydrolyzing activities of HSL. The profiles of inhibition of the activities with the three substrates were essentially identical (**Fig. 6**). DFP at 12 µM inhibited the activities by about 50% after 30 min preincubation at 37°C. When partially purified HSL fraction (after phenyl-Sepharose) was incubated with [^{3}H]DFP, only one polypeptide of M_r 84,000 was ^{3}H-labeled (Fig. 3, **Fig. 7**).

Concentrations of NaF in the mM order inhibited the PNPB-hydrolyzing activity (20 mM NaF caused 50% inhibition) (**Fig. 8**). NaF at 20 mM also inhibited the triolein- (70%) and CO- (60%) hydrolyzing activities (data not shown). The three activities were also inhibited about 70% by 100 µM $HgCl_2$ (data not shown). Nonionic detergents are strong inhibitors of HSL. Emulgen 120 caused concentration-dependent inhibitions of the triolein- and CO-hydrolyzing activities, but did not inhibit PNPB-hydrolyzing activity (**Fig. 9**). Emulgen 120 at 0.1% inhibited 70% of the triolein-hydrolyzing activity and 95% of the CO-hydrolyzing activity, but only 20% of the PNPB-hydrolyzing activity.

On treatment of purified HSL with trypsin for 15 min at 37°C, the CO-hydrolyzing activity decreased markedly with increase in the trypsin concentration, whereas the PNPB-hydrolyzing activity decreased only gradually (**Fig. 10**). Thus the ratio of PNPB- to CO-hydrolyzing activity of HSL increased from 4.1 to 16.4 on treatment with trypsin at 10 µg/ml.

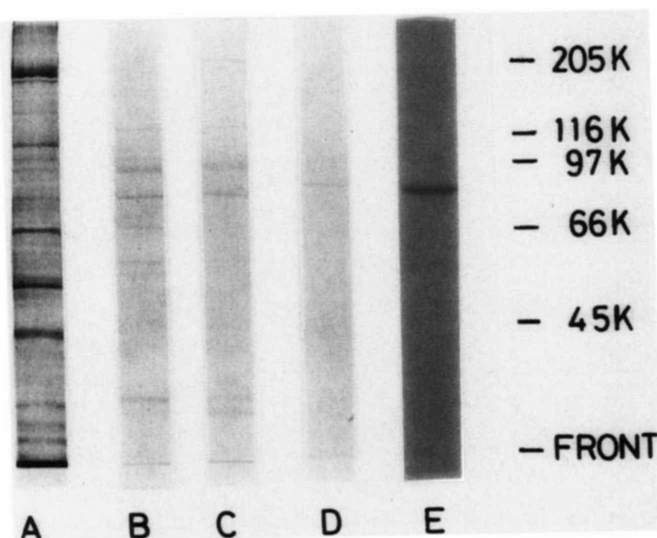

Fig. 3. SDS-polyacrylamide gel electrophoresis of HSL. Protein obtained from (A) DEAE-cellulose (approximately 40 µg protein), (B) phenyl-Sepharose (approximately 30 µg protein), (C)Mono Q FPLC (approximately 5 µg protein), and (D) Mono S FPLC (approximately 1.5 µg protein). (E) Fluorograph of enzyme obtained from phenyl-Sepharose (approximately 70 µg protein) following incubation with [^{3}H]DFP. Electrophoresis was on an 8% gel at a constant current in a Tris-buffer system (pH 8.3). The numbers refer to the M_r of reference proteins.

Downloaded from www.jlr.org by guest, on June 19, 2012
ASBMB JOURNAL OF LIPID RESEARCH

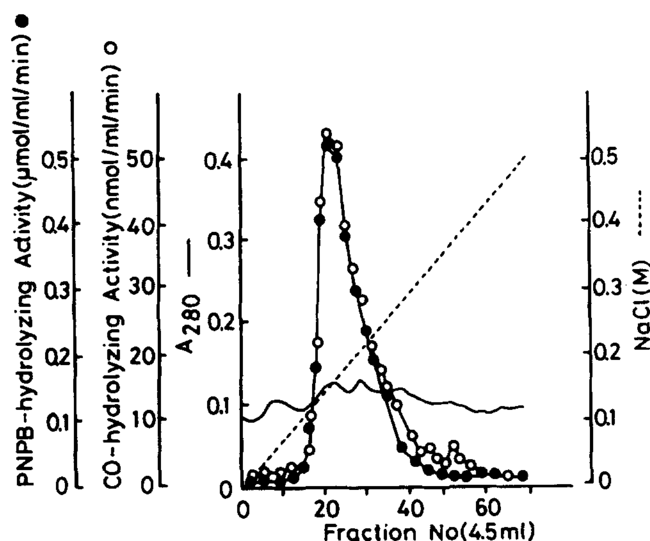

Fig. 4. Heparin-Sepharose column chromatography of bovine adipose tissue HSL. Approximately 2 mg enzyme protein (after phenyl-Sepharose) was applied to the column. The column was washed with the buffer and the enzyme was eluted with a linear gradient of 0–0.5 M NaCl. Fractions of 4.5 ml were collected and NaCl concentration (----), A_{280} (—), and CO- (-○-) and PNPB - (-●-)hydrolyzing activities were measured.

On incubation with ATP-Mg^{2+} and the catalytic subunit of cyclic AMP-dependent protein kinase, the triolein-hydrolyzing activity of purified HSL (Mono Q fraction) was activated 40% (**Table 2**) but the CO- and PNPB-hydrolyzing activities were not affected.

DISCUSSION

The purified HSL showed low substrate specificity, acting on triolein, CO, and water-soluble PNPB at relative rates of 1:1.8:6.5, respectively. The elution pattern of the CO-hydrolyzing activity coincided with that of PNPB-hydrolyzing activity in the two last purification steps and heparin-Sepharose chromatography. Furthermore, the DFP-inhibition profiles of the three substrate-hydrolyzing activities were identical and only one polypeptide of M_r

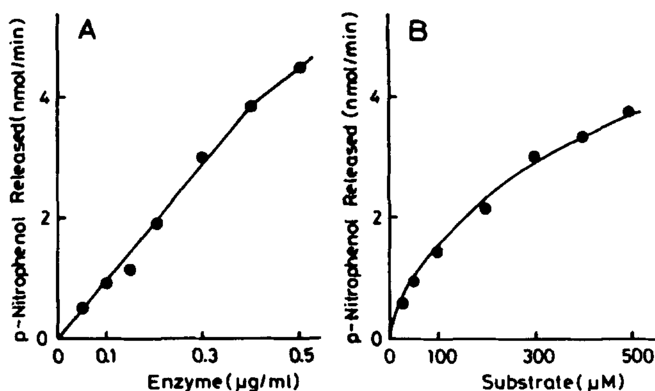

Fig. 5. HSL-catalyzed hydrolysis of PNPB. (A) Effect of HSL concentration. The incubation mixtures contained 0.5 mM PNPB, 1% (v/v) acetonitrile, and the indicated amount of HSL in a final volume of 1.0 ml of standard buffer containing 50 mM sodium phosphate, 10% glycerol, and 0.1 M NaCl. (B) Effect of PNPB concentration. Incubation mixtures contained the indicated amount of PNPB, 1%, (v/v) acetonitrile, and HSL(0.4 μg/ml) in a final volume of 1.0 ml of standard buffer.

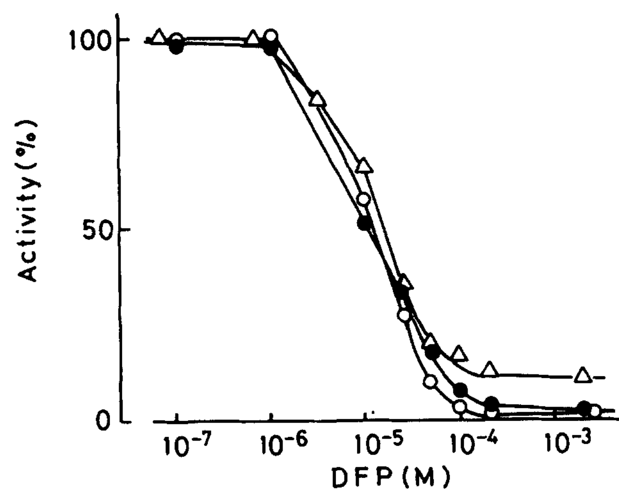

Fig. 6. Inhibition by DFP of HSL activities. Enzyme was incubated with various amounts of DFP as described under Materials and Methods. Remaining CO- (○), triolein- (△) and PNPB- (●) hydrolyzing activities of HSL were measured.

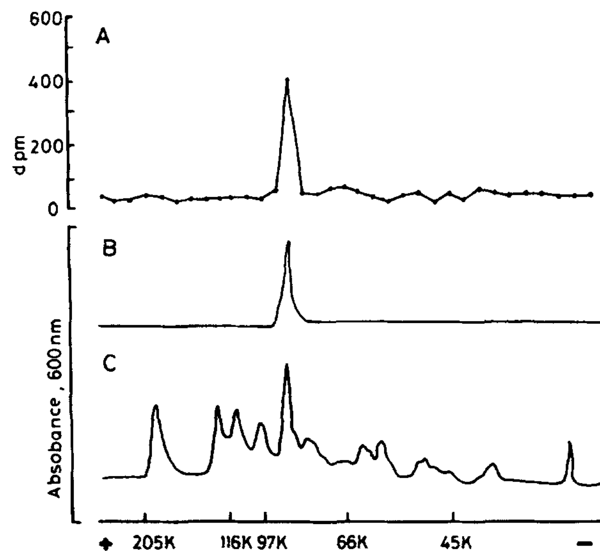

Fig. 7. Scanning patterns from SDS-polyacrylamide gel electrophoresis of HSL. HSL fraction from phenyl-Sepharose was incubated with [^{3}H]DFP and analyzed by SDS-polyacrylamide gel electrophoresis (approximately 70 μg protein). (A) The radioactivity was determined by liquid scintillation counting after the gels had been cut into 2-mm-thick slices and soaked with ACS-II (Amersham Japan, Tokyo, Japan) overnight. (B) Scanning pattern after fluorography. (C) Scanning pattern after the protein was stained with Coomassie brilliant blue.

Downloaded from www.jlr.org by guest, on June 19, 2012

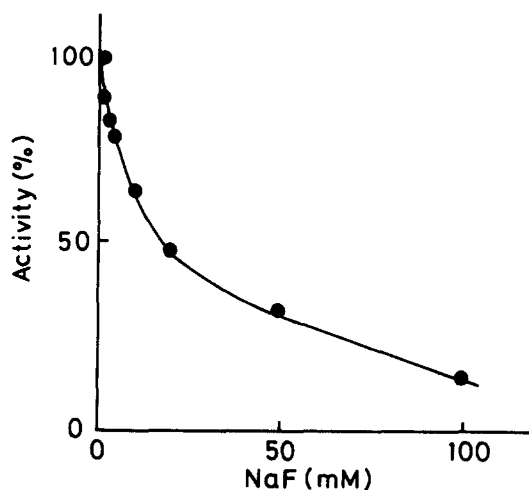

Fig. 8. Effect of NaF concentration on the HSL-catalyzed hydrolysis of PNPB. Experimental conditions were as described under Materials and Methods.

84,000 was affinity-labeled with [^{3}H]DFP. These findings showed that HSL from bovine adipose tissue acted on water-soluble PNPB. Its Michaelis constant (K_m) for water-soluble PNPB was 0.16 mM, which was one-third of that of bovine milk lipoprotein lipase (14). At least two types of water-soluble PNPB-hydrolyzing enzymes other than HSL are present in fat cells: (lipoprotein lipase and carboxylesterase) (14,15). The results of heparin-Sepharose profile and effect of apoC-II suggest that the HSL fraction (after phenyl-Sepharose) does not contain lipoprotein lipase. The result of DFP-labeling of HSL also suggests that this enzyme fraction is free of carboxylesterase.

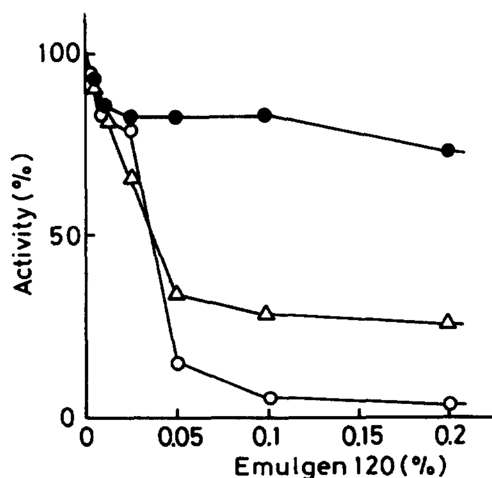

Fig. 9. Effect of Emulgen 120 on HSL activities. Enzyme was incubated with various amounts of Emulgen 120 as described under Materials and Methods. Remaining CO- (○), triolein- (△), and PNPB- (●) hydrolyzing activities of HSL were measured.

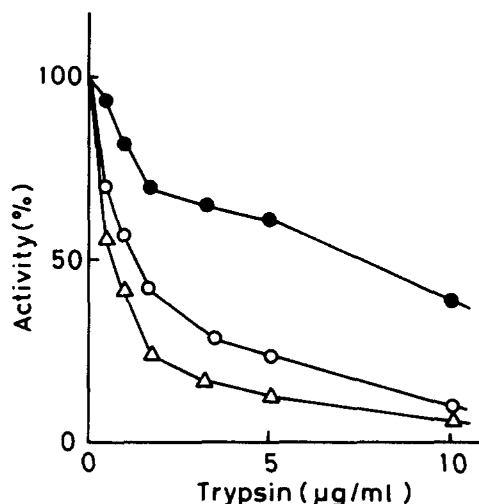

Fig. 10. Proteolytic digestion of HSL with trypsin. HSL was digested with various amounts of trypsin. After incubation for 15 min at 37°C, trypsin inhibitor was added and CO- (○), triolein- (△), and PNPB- (●) hydrolyzing activities were measured.

In the two last purification steps, the ratio of the activities and the percentage recoveries of the two activities were different: the ratio of PNPB- to CO-hydrolyzing activity of the enzyme increased during purification. These results indicate that purification of HSL resulted in a change in its catalytic properties; its activity on emulsified substrates such as CO decreased markedly, but its activity on water-soluble substrates such as PNPB decreased only slightly. This phenomenon is similar to the finding of Okuda and Fujii (16) that after acetone treatment the Ediol-hydrolyzing activity of rat adipose tissue lipase was reduced but the water soluble methyl butyrate-hydrolyzing activity was not. They postulated that the lipid-protein complex of lipase determined the substrate specificity. In adipocytes, HSL might be bound to mem-

TABLE 2. Effect of phosphorylation on HSL activities

Substrates	ATP-Protein Kinase	Activity
		μmol/mg/min
Cholesteryl oleate	+	1.85 ± 0.06[a]
	−	1.88 ± 0.07[a]
Triolein	+	1.31 ± 0.09[a]
	−	0.90 ± 0.04[a]
PNPB	+	6.80 ± 0.19[b]
	−	6.71 ± 0.19[b]

Aliquots of the Mono Q fraction (14 μg/ml) were incubated at 37°C for 15 min with 10 mM Mg^{2+}, 1 mM dithiothreitol, 0.1 mM ATP, and 45 U/ml cyclic AMP-dependent protein kinase active subunit. ATP and protein kinase were both omitted from controls and CO-, triolein-, and PNPB-hydrolyzing activities were measured as described under Materials and Methods.
[a]Oleic acid released.
[b]*p*-Nitrophenol released.

Downloaded from www.jlr.org by guest, on June 19, 2012

brane (endoplasmic reticulum). Therefore, some membrane component (such as phospholipid or lipoprotein) may be necessary for its high activity on emulsified substrate.

Mild treatment with trypsin also caused change in the catalytic properties of HSL, having more effect on the activity of HSL on triolein than on its activity on PNPB. Similar results have been obtained with lipoprotein lipase (17) and hepatic triglyceride lipase (18, 19). After trypsin cleavage, lipases retain virtually full activities against water-soluble substrates, but their activities against emulsions of long-chain triacylglycerols are greatly reduced. These findings suggest that lipases have an interface recognition site and catalytic site, and that the catalytic site may not be damaged by proteolytic digestion and so may not change the activity with water-soluble substrates. Why is the activity on emulsified long-chain triacylglycerol sensitive to proteolysis? Two possibilities may be considered. *1*) A recognition site may be destroyed by proteolytic digestion and the binding-affinity for substrate may be decreased. Shirai, Saito, and Yoshida (19) reported that after trypsin digestion hepatic triglyceride lipase could not be adsorbed to dipalmitoylphosphatidylcholine vesicles. *2*) The proteolytic digestion may change the stability of the enzyme at the substrate-water interface. Some enzymes are denaturated at lipid-water interface (9). Bengtsson and Olivecrona (17) reported that tryptic-digested lipoprotein lipase was almost completely adsorbed to emulsion droplets, but that its activity was nonetheless very low. We postulate that HSL also has a recognition site and a catalytic site and that the catalytic site is not destroyed by proteolytic digestion like that of lipoprotein lipase and hepatic triglyceride lipase. However, the PNPB-hydrolyzing activity of HSL was decreased by trypsin treatment. HSL is strongly associated with tissue lipid, and the lipid-protein complex fraction (pH 5.0 precipitate fraction) is quite stable. Nevertheless, solubilized HSL is not stable and proteolytic digestion may make HSL more unstable. Therefore, the PNPB-hydrolyzing activity was decreased by trypsin treatment.

Inhibitors that act directly on the catalytic site, such as DFP and $HgCl_2$, inhibited the activities on both emulsified and water-soluble substrates. However, Emulgen 120 markedly inhibited the activity on emulsified substrates but not on water-soluble substrate. This suggests that Emulgen 120 may interfere with the adsorption of HSL to the substrate-water interface. Phosphorylation may also change the affinity of HSL to a substrate-water interface, but may not cause direct activation of the catalytic site.

The rate-limiting step in mobilization of fat stored in fat cells is hydrolysis to free fatty acids and glycerol. The first step in this hydrolytic process is thought to be catalyzed by HSL, the activity of which is regulated by a variety of hormones. The hormonal regulation of the enzyme is proposed to be mediated by cAMP-dependent phosphorylation (1, 20). Hormones stimulate adenylate cyclase in the fat cell membrane and increase the cyclic AMP content of the cells. This increased level of cyclic AMP then stimulates protein kinase activity, which in turn activates HSL, and the activated HSL catalyzes hydrolysis of fat. In contrast to this well established theory on hormone-induced lipolysis, Okuda et al. (21, 22) demonstrated the existence of another mechanism in which the hormone did not act on lipase but on endogenous fat as substrate. The site of lipolysis in the fat cell is the interface between the endogenous lipid droplet and cytoplasmic water-phase. More generally, lipolysis is subject to regulation by the physical properties of the substrate-water interface such as the charge, lipid composition, and lipid-packing density (23). Therefore, if hormone changes the interfacial properties of endogenous lipid droplets, the lipolytic activity in fat cells might be changed (21). Irrespective of whether the hormone acts on lipase or the endogenous substrate, the affinity of HSL to endogenous lipid droplets may be important in hormonal regulation of lipolysis. Therefore, the properties of the recognition site of HSL are important for understanding the mechanism of lipolysis. In this study we demonstrated the presence of water-soluble PNPB-hydrolyzing activity of HSL and showed that this activity depends only on the catalytic site. Water-soluble substrates are useful in studies on the molecular dynamics of lipase and in conjunction with emulsified substrates are useful in determining the properties of the recognition site.

We thank Mr. K. Kameda (University of Ehime) for carrying out the FPLC analyses.

Manuscript received 20 September 1988 and in revised form 19 January 1989.

REFERENCES

1. Belfrage, P., G. Fredrikson, P. Strålfors, and H. Tornqvist. 1984. Adipose tissue lipases. *In* Lipases. B. Borgström and H. L. Brockman, editors. Elsevier, Amsterdam. 365-416.
2. Desnuelle, P., and P. Savary. 1963. Specificities of lipases. *J. Lipid Res.* **4:** 369-384.
3. Morgan, R. G. H., J. Barrowman, H. Filiper-Wender, and B. Borgström. 1968. The lipolytic enzyme of rat pancreatic juice. *Biochim. Biophys. Acta.* **30:** 355-366.
4. Sarda, L., and P. Desnuelle. 1958. Action de la lipase pancréatique sur les esters en émulsion. *Biochim. Biophys. Acta.* **30:** 513-521.
5. Bergland, L., J. C. Khoo, D. Jensen, and D. Steinberg. 1980. Resolution of hormone-sensitive triglyceride/diglyceride lipase from monoglyceride lipase of chicken adipose tissue. *J. Biol. Chem.* **255:** 5420-5428.
6. Chen, R. F. 1967. Removal of fatty acid from serum albumin by charcoal treatment. *J. Biol. Chem.* **242:** 173-181.
7. Cordle, S. R., R. J. Colbran, and S. J. Yeaman. 1986. Hor-

Downloaded from www.jlr.org by guest, on June 19, 2012

mone-sensitive lipase from bovine adipose tissue. *Biochim. Biophys. Acta.* **887**: 51-57.
8. Nilsson, S., and P. Belfrage. 1986. Purification of hormone-sensitive lipase by high-performance ion exchange chromatography. *Anal. Biochem.* **158**: 399-407.
9. Tsujita, T., and H. L. Brockman. 1986. Regulation of carboxylester lipase adsorption to surfaces. 1. Chemical specificity. *Biochemistry.* **26**: 8423-8429.
10. Khoo, J. C., D. Steinberg, J. J. Huang, and P. R. Vagelos. 1976. Triglyceride, diglyceride, monoglyceride, and cholesterol ester hydrolysis in chicken adipose tissue activated by adenosine 3′ 5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **251**: 2882-2890.
11. Belfrage, P., and M. Vaughan. 1969. Simple liquid-liquid partition system for isolation of labeled oleic acid from mixtures with glycerides. *J. Lipid Res.* **10**: 341-344.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **224**: 680-685.
13. Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gel with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**: 132-135.
14. Shirai, K., and R. L. Jackson. 1982. Lipoprotein lipase-catalyzed hydrolysis of *p*-nitrophenyl butyrate. *J. Biol. Chem.* **257**: 1253-1258.
15. Tsujita, T., H. Okuda, and N. Yamasaki. 1982. Purification and some properties of carboxylesterase of rat adipose tissue. *Biochim. Biophys. Acta.* **715**: 181-188.
16. Okuda, H., and S. Fujii. 1967. Lipase and esterase in adipose tissue and liver. *J. Biochem. (Tokyo).* **61**: 313-319.
17. Bengtsson, G., and T. Olivecrona. 1981. Lipoprotein lipase: modification of its kinetic properties by mild tryptic digestion. *Eur. J. Biochem.* **113**: 547-554.
18. Tsujita, T., A. Nakagawa, K. Shirai, Y. Saito, and H. Okuda. 1984. Methyl butyrate-hydrolyzing activity of hepatic triglyceride lipase from rat post-heparin plasma. *J. Biol. Chem.* **259**: 11215-11220.
19. Shirai, K., Y. Saito, and S. Yoshida. 1984. Post-heparin plasma hepatic triacylglycerol lipase-catalyzed tributyrin hydrolysis: effect of trypsin treatment. *Biochim. Biophys. Acta.* **795**: 9-14.
20. Khoo, J. C., and D. Steinberg. 1983. Hormone-sensitive lipase of adipose tissue. *In* The Enzymes. Vol. 16. P. D. Boyer, editor. Academic Press, New York. 183-204.
21. Okuda, H., T. Tsujita, M. Sumida, Y. Takahashi, D. Shimizu, and S. Fujii. 1983. Role of endogenous lipid droplets of fat cells in epinephrine-induced lipolysis. *J. Biochem. (Tokyo).* **93**: 575-582.
22. Okuda, H., T. Tsujita, and M. Kinutani. 1986. Studies on a protein kinase inhibitor-insensitive, phospholipase C-sensitive pathway of lipolysis in rat adipocytes. *Pharmacol. Res. Commun.* **18**: 877-893.
23. Brockman, H. L. 1984. General features of lipolysis: reaction scheme, interfacial structure and experimental approaches. *In* Lipases. B. Borgström and H. L. Brockman, editors, Elsevier, Amsterdam. 185-204.

ASBMB JOURNAL OF LIPID RESEARCH JLR
Downloaded from www.jlr.org by guest, on June 19, 2012