ENHANCEMENT OF HEPATIC MICROSOMAL ANILINE HYDROXYLATION BY HABU SNAKE (*Trimeresurus flavoviridis*) VENOM FRACTIONS

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Abstract—Enhancement of aniline hydroxylation by Habu snake venom was studied in vitro. The fraction which was isolated from Habu venom through column chromatography caused the enhancement of aniline hydroxylation of hepatic microsomes obtained from untreated rats. This fraction was heat stable and free from phospholipase activity. Enhancement of aniline hydroxylation was not clear in crude Habu venom or the unheated fraction, but appeared after heating of the fraction. This fraction enhanced aniline hydroxylation at low concentrations, followed by inhibition at high concentrations, but inhibited aminopyrine N-demethylation dose-dependently. Aniline hydroxylation was enhanced by the heated fraction at more than 1 mM aniline, and the enhancement was rather decreased at more than 10 mM aniline. The degree of the enhancement by the heated fraction increased with increasing pH.

The activity of hepatic microsomal drug-metabolizing enzymes varies not only by endogenous conditions such as age (1) or sex (2), but also by exogenous factors such as drug injection (3) or X-irradiation (4). We previously reported that Habu snake (*Trimeresurus flavoviridis*) venom inhibits the drug-metabolizing enzymes under both in vitro and in vivo conditions, and we have isolated the drug-metabolizing enzyme inhibitor from the venom (5). This inhibitor is heat stable and is separated from phospholipase which is known to convert cytochrome P-450, the terminal oxidase of the drug-metabolizing enzyme system, to cytochrome P-420 (6). In studying the isolation of the inhibitor from the venom, we noticed that the aniline hydroxylation of microsomes was rather enhanced by a fraction of the inhibitor which eluted early through the ion exchange column. So we conducted further

fractionation of Habu venom to clarify the enhancement of aniline hydroxylation by the venom.

Materials and Methods

Materials: Habu venom used in this study was a pool of lyophilized venom taken from specimens of Habu (*Trimeresurus flavoviridis*) collected on Okinawa Island in 1978. Male Wistar rats weighing 150 to 300 g were used. Nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate were purchased from the Sigma Chemical Co., St. Louis. Glucose-6-phosphate dehydrogenase was from the Oriental Yeast Co., Tokyo. Sephadex G-100 (particle size 40–120 μ) was provided by Pharmacia Fine Chemicals, Uppsala. Amberlite CG50 (type II) was purchased from the Rohm & Hass Co., Philadelphia. *p*-Tosyl-L-arginine methyl ester (TAME) hydrochloride was obtained from

Nakarai Chemicals Ltd., Kyoto. Acetylacetone and aniline were purified by distillation, and *p*-aminophenol was purified by sublimation. All other chemicals were of analytical reagent grade.

Isolation of the stimulator of aniline hydroxylation from Habu venom: The second fraction obtained from gel filtration on Sephadex G-100, as presented in the previous report (5), was applied to a column of Amberlite CG50 and eluted with two steps of a linear gradient (0 to 0.2 M NaCl and 0.2 to 0.3 M NaCl) in 0.005 M borate-HCl buffer (pH 8.3). The fraction which enhanced aniline hydroxylation was heated at 60°C for 5 min and was rechromatographed on the Amberlite CG50 column using the same conditions, except that the gradient was 0 to 0.3M NaCl. Protein content of the eluent was measured by UV absorption at 280 nm.

Enzyme assays: Hepatic microsomes of rats were prepared as described in the previous report (5). The reaction mixture for the assay of aniline hydroxylation and aminopyrine N-demethylation contained microsomes (0.5 to 0.7 mg of protein), 80 mM Tris-HCl buffer (pH 7.4), the NADPH generating system (0.33 mM NADP, 8 mM glucose-6-phosphate, 6 mM MgCl₂, and 0.2 units of glucose-6-phosphate dehydrogenase), and the desired concentration of substrates in a final volume of 1.0 ml. When necessary, Habu venom fractions which were eluted through column chromatography were added to the mixture. The control contained elution buffer instead of the venom fraction. The mixture was incubated in a shaking water bath for 20 min at 37°C under air. The activity of the hydroxylation of aniline and the demethylation of aminopyrine were determined by measuring the formation of *p*-aminophenol and formaldehyde using the method of Brodie and Axelrod (7) as modified by Imai et al. (8) and the method of Nash (9), respectively. Proteinase, phospholipase and

arginine ester hydrolase activities were measured by the same methods as presented in the previous report (5). Protein contents of microsomes and Habu venom fractions were determined by the method of Lowry et al. (10), using bovine serum albumin as the reference protein. All assays and experiments were at least in duplicate.

Results

Isolation from Habu venom of a stimulator of hepatic microsomal aniline hydroxylation:

Figure 1 shows the chromatographic profile of the Sephadex G-100-2 fraction on the Amberlite CG-50 column. Fourteen fractions were obtained and designated as CG50-1, CG50-2, and so on, in the order of elution. Protein content and various activities of each fraction after heating at 60°C for 5 min were determined and shown in Table 1. Both CG50-2 and CG50-10 enhanced aniline hydroxylation, but inhibited aminopyrine N-demethylation. When 0.1 ml of the heated fraction was added to the incubation mixture, aniline hydroxylation was increased to 122% of the control by CG50-2 and to 117% by CG50-10, and aminopyrine N-demethylation was decreased to 66% by CG50-2 and to 63% by CG50-10. Since CG50-11 was shown to be an inhibitor of drug-metabolizing enzymes, CG50-10 agreed with the previous observation that an early eluted fraction of the inhibitor caused the increase in aniline hydroxylation, rather than inhibition. So first we examined the further fractionation of CG50-10 as an aniline hydroxylation stimulator. CG50-10 had very high proteinase activity which was lost by heating and contained about 27% of a heat stable component. Heated CG50-10 showed slight phospholipase activity and low arginine ester hydrolase activity. A pool of CG50-10 and other fractions corresponding to CG50-10 obtained from a previous study [designated as CG-50-2 in previous report (5)] were

Fig. 1. Chromatography on Amberlite CG50 of venom fraction II (G-100-2) from Sephadex G-100. The column (1.6×32 cm) was equilibrated with 0.005 M borate-HCl buffer (pH 8.3). Lyophilized G-100-2 (300 mg) was dissolved in 5 ml of the same buffer and applied to the column. Two steps of linear gradient elution were carried out: the first gradient was 200 ml of the buffer in the mixing vessel and buffer of the same volume containing 0.2M NaCl in the reservoir, and second step was 250 ml of the buffer containing 0.2M NaCl in the mixing vessel and 0.3M NaCl in the reservoir.

Table 1. Effect of each fraction on aniline hydroxylation or aminopyrine N-demethylation and some enzyme activities

CG50 fraction	Nonheated				Heated			
	Protein* (μ g/ml)	Proteinase (OD280nm/mg)	Protein content* (μ g/ml)	(%)	AP** (%)	AN** (%)	Lipase# (min)	Hydrolase## (TAME nmole/mg)
1	310	0.05	305	98.3	42.3	77.7	20	98.1
2	505	—	475	94.1	66.9	122.2	8	22.9
3	455	—	430	94.5	42.7	82.2	20	9.9
4	370	0.14	315	85.1	38.0	71.1	>20	13.4
5	393	0.09	387	98.4	29.9	62.2	>20	28.5
6	272	0.79	253	93.0	49.0	93.3	20	42.0
7	387	3.06	350	90.4	42.7	71.1	20	128.0
8	355	4.17	280	78.8	57.3	95.5	9	256.3
9	305	5.62	100	32.7	55.4	100.0	8	65.8
10	393	5.33	110	27.9	63.7	117.1	3	44.2
11	600	2.78	263	43.8	64.9	95.5	5	13.7
12	387	5.60	180	46.5	52.2	93.3	9	16.9
13	280	7.28	160	57.1	54.1	84.4	15	19.2
14	367	1.01	315	85.8	39.4	60.0	>20	11.8

* Protein content of each fraction was determined by the method of Lowry et al. (10). In heat treatment each fraction was heated at 60°C for 5 min, and the supernatant obtained after centrifugation was used for the determination of protein content.

** Activity of aniline hydroxylation (AN) and aminopyrine N-demethylation (AP) was determined under the same conditions described in Materials and Methods using 1 mM substrate. The activity is represented as the percentage of the control when 0.1 ml of heated fractions was added to the incubation mixture.

Phospholipase activity represents coagulation time of egg yolk in the presence of 0.1 ml of each heated fraction, as described in Materials and Methods.

Arginine ester hydrolase activity is expressed as the amount of hydrolyzed substrate (TAME, nmole) per mg of each fraction.

heated at 60°C for 5 min and were rechromatographed. The results are indicated in Fig. 2. The effect of each fraction on aniline hydroxylation and aminopyrine N-demethylation is shown in Fig. 3. The eluate corresponding to tube numbers 80 to 84 caused enhancement

of the hydroxylation, but inhibition of the demethylation; and the eluate of tubes 86 to 100 inhibited both the hydroxylation and the demethylation. Thus, the former fraction and the latter fraction were designated as the S-fraction and I-fraction, respectively. The S-

Fig. 2. Rechromatography of venom fraction X (CG50-10) on Amberlite CG50. A pool of CG50-10 and other fractions obtained from a previous study which corresponded to CG50-10 (5) was dissolved in 0.005 M borate-HCl buffer (pH 8.3) and heated at 60°C for 5 min. The supernatant of heated CG50-10 (36 mg protein) was applied to the Amberlite CG50 column (1.6×30 cm) and eluted under the same conditions as Fig. 1 except a one step linear gradient of 0 to 0.3 M NaCl was used.

Fig. 3. Effect of each fraction on aniline hydroxylation and aminopyrine N-demethylation. Eluate (0.1 ml) obtained from column chromatography as shown in Fig. 2 was added to the incubation mixture, and the activities of aniline hydroxylation and aminopyrine N-demethylation were determined using 1 mM substrate as described in Materials and Methods. Activity was represented as percentage of the control. □ : aminopyrine N-demethylation, ▨ : aniline hydroxylation.

Table 2. Protein recovery of each fraction

Fraction	Protein	
	(mg)	(%)
G-100-2	630.000	100.00
CG50-10	9.387	1.49
Heated CG50-10	2.205	0.35
S-fraction	0.047	0.007

Protein content of each fraction was determined by the method of Lowry et al. (10).

Fig. 4. Effect of substrate concentration on the enhancement of aniline hydroxylation by heated CG50-10. Heated CG50-10 (8 μ g) was added to the incubation mixture and incubated at 37°C for 20 min. Aniline hydroxylation activity was determined as described in Materials and Methods. ●: control, ○: heated CG50-10.

fraction was free from phospholipase activity, and the recovery of protein content was 0.007% of G-100-2 fraction as summarized in Table 2.

Stimulatory action of isolated fractions on aniline hydroxylation: Figure 4 shows the effect of heated CG50-10 on aniline hydroxylation using various concentrations of aniline. The hydroxylation was increased to about 125% of the control in the concentrations more than 1 mM of aniline and showed little increase at low or high concentrations. Effect of pH on the enhancement of aniline hydroxylation by heated CG50-10 is presented in Fig. 5. The enhancement of the hydroxylation increased as the pH of the incubation mixture was increased and was more clear than at pH 7. Dose dependence

Fig. 5. Effect of pH on the enhancement of aniline hydroxylation by heated CG50-10. Conditions were the same as described in Fig. 4, except that 1 mM aniline and 80 mM Na,K-phosphate buffer at differing pH values were used. Enzyme activity represents the percentage of aniline hydroxylation of the control in the presence of heated CG50-10.

of each fraction on aniline hydroxylation and aminopyrine N-demethylation is shown in Figs. 6 and 7. Aniline hydroxylation was enhanced at low concentrations of less than 9 μ g by S-fraction, but was inhibited at more than 10 μ g. Heated CG50-10 also showed the enhancement of the hydroxylation at low concentrations, but the inhibition at high concentrations was not observed. The I-fraction inhibited aniline hydroxylation dose dependently. Aminopyrine N-demethylation was decreased by all fractions. The demethylation activity, when 10 μ g of each fraction was added to the incubation mixture, was reduced to 85% of the control by the S-fraction, 66% by heated CG50-10, and 61%

Fig. 6. Relationship between the amounts of venom fractions and aniline hydroxylation activity. Conditions were the same as described in Materials and Methods, except for the use of 1 mM aniline. ■: heated CG50-10, ○: S-fraction, ★: I-fraction.

Fig. 7. Relationship between the amounts of venom fractions and aminopyrine N-demethylation activity. Conditions were the same as described in Fig. 6, except 1 mM aminopyrine was used. ■: heated CG50-10, ○: S-fraction, ★: I-fraction.

by the I-fraction.

Discussion

Enhancement by a Habu venom fraction of aniline hydroxylation in hepatic microsomes was elucidated in this paper. Two fractions, CG50-2 and CG50-10, enhanced the hydroxylation, and CG50-10 was the first to be used for further fractionation. The

enhancement of aniline hydroxylation appeared clearly when using heated CG50-10 and was very low in unheated CG50-10 (data not shown). CG50-10 contained high proteinase activity which was lost by heating. It is known that the proteinase trypsin causes the conversion of cytochrome P-450 to its inactive form, cytochrome P-420 (11); therefore, it is assumed that the enhancement of aniline hydroxylation of unheated CG50-10 may be disturbed due to proteinase and may appear after the loss of proteinase activity. Habu venom contained phospholipase (12) and arginine ester hydrolase (13), which are known as heat stable enzymes, but CG50-10 and the S-fraction were separated from these enzymes. Recently, we obtained evidence that the heat stable component involved as the drug-metabolizing inhibitor from Habu venom causes the contraction of guinea pig ileum and prolongation of blood coagulation time (unpublished observations). Further study is necessary to determine whether or not the S-fraction can cause such biological actions.

On the rechromatography of CG50-10, the fraction which caused aniline hydroxylation enhancement, designated as the S-fraction, was found to be distinct from the component which inhibited both aniline hydroxylation and aminopyrine N-demethylation. This S-fraction showed the enhancement of the hydroxylation at lower concentrations, followed by inhibition at higher concentrations. The enhancement of the hydroxylation by the S-fraction was about 1.4 times that of the control at maximum; however, we observed that the S-fraction caused greater enhancement of the hydroxylation in microsomes obtained from phenobarbital treated rats (unpublished observations). Therefore, it seems likely that the S-fraction is a stimulator of aniline hydroxylation in Habu venom. The protein recovery of the S-fraction, however, is very small, and further

study for the isolation of the stimulator from the venom is necessary.

Heated CG50–10 enhanced aniline hydroxylation at more than 1 mM aniline; but at low concentrations of aniline, the CG50–10 showed less enhancement of the hydroxylation, without inhibition. This is different from the enhancement of aniline hydroxylation by cyanide which inhibited the hydroxylation at low concentrations of aniline and enhanced only at high concentrations (14). The enhancement of aniline hydroxylation by heated CG50–10 was found to be dependent upon the pH of the reaction mixture, with more enhancement being observed at higher pH values. This effect of pH was similar to the enhancement of aniline hydroxylation by ethyl isocyanide (15) and by acetone (16). However, it was reported that ethyl isocyanide and acetone produced their effects on aniline hydroxylation in a different manner. Kinetic studies are in progress to account for the mechanism of the enhancement of aniline hydroxylation by the Habu venom fraction.

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