PHARMACOLOGICAL STUDIES ON CARBAPENEM ANTIBIOTICS

II. ISOLATION OF A PS-5-INACTIVATING FACTOR FROM THE RAT KIDNEY

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A factor responsible for the *in vivo* metabolism of PS-5 was isolated from the microsomal fraction of the rat kidney. This factor, which did not attack penicillins and cephalosporins, was enzymologically identified with particle-bound renal dipeptidase. Under the action of this factor, PS-5 was inactivated to give three products designated PS-5D I, PS-5D II and PS-5D III.

In the preceding paper, we have reported that PS-5,[1] a carbapenem antibiotic, was rapidly metabolized in the kidney, resulting in the very low blood level and urinary recovery of PS-5.[2] To elucidate the detailed mechanism of *in vivo* metabolism of carbapenem compounds, we have attempted to isolate some factor involved in the PS-5 inactivation from the rat kidney.

The present paper describes the localized presence of a PS-5-inactivating factor in the microsomal fraction of the rat kidney. By action of this factor that was enzymologically identified with particle-bound renal dipeptidase,[3] PS-5 was inactivated to yield three breakdown products.

Materials and Methods

Materials

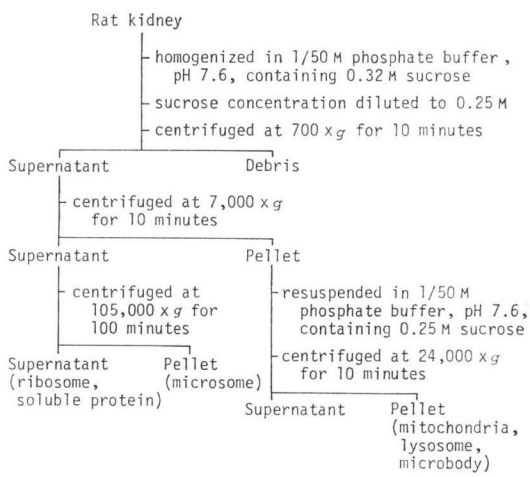
PS-5 sodium salt was prepared in our laboratories as described in a previous paper.[1]

Glycyldehydrophenylalanine was prepared by the method of PRICE *et al.*[4] Other peptide substrates, inhibitor compounds and reagents were purchased from commercial sources.

Subcellular Fractionation of the Rat Kidney

According to the differential centrifugation method of HOGEBOOM,[6] the rat kidney was fractionated into subcellular components. Fresh rat kidneys (4.4 g) were cut into small pieces with scissors and then ground in a Teflon homogenizer in 22 ml of 1/50 M phosphate buffer, pH 7.6, containing 0.32 M sucrose. After the sucrose concentration was reduced to 0.25 M with the phosphate buffer, the rat kidney homogenate was subjected to differential centrifugations under refrigeration at the indicated centrifugal forces in a Sakuma refrigerated centrifuge 35 (700 $\times g$ and 7,000 $\times g$) and in a Hitachi ultracentrifuge 65P. For easy comparison, each fraction was suspended or diluted, if necessary, to give the same volume (37 ml) (Chart 1).

Chart 1. Subcellular fractionation of the rat kidney.



Isolation of the PS-5-inactivating Enzyme from the Microsomal Fraction of the Rat Kidney

The microsomal fraction of the rat kidney collected from 12 g of fresh rat kidneys was treated with 400 μg/ml of trypsin at 0°C for 16 hours and then centrifuged at 0°C for 60 minutes at 100,000×g. The pellet was recovered and suspended in 20 ml of 20 mM tris-HCl buffer, pH 7.6, containing 0.4% Triton X-100. After stirring at 0°C for 30 minutes, the solution was centrifuged at 0°C for 60 minutes at 105,000×g. The supernatant solution (20 ml) was charged on a DEAE-Sephadex A-50 column (Cl^- form; 15×100 mm) which had been equilibrated with the said buffer containing 0.4% Triton X-100. A linear concentration gradient of sodium chloride in the said buffer containing 0.4% Triton X-100 from 0 to 0.5 M was employed for elution. Fractions which showed a PS-5-inactivating activity were combined and concentrated to 5 ml with Carbowax 4000. The enzyme concentrate was successively purified on Sephadex G-150 (25×900 mm) and Sephadex G-200 (25×900 mm) columns to give a partially purified solution of the PS-5-inactivating enzyme (5 ml).

Susceptibility Tests of Conventional β-Lactam Compounds to the Partially Purified PS-5-inactivating Enzyme

Reaction mixtures consisting of 0.1 ml of β-lactam solutions (10 mg/ml), 0.2 ml of 1/5 M phosphate buffer, pH 7.6, and 0.2 ml of the partially purified PS-5-inactivating enzyme were incubated under mild agitation at 37°C for 60 minutes. After heat-treatment at 100°C for 15 seconds, the substrates and possible metabolites were analyzed by high voltage paper electrophoresis followed by visualization with ninhydrin, iodine chloroplatinate and Ehrlich reagents or by bioautography with *Comamonas terrigena* B-996.

Preparative-scale Production of PS-5 Metabolites (PS-5D I, PS-5D II and PS-5D III)

For chemical characterization of PS-5 metabolites, 100 mg of PS-5 sodium salt in 20 ml of 1/10 M phosphate buffer, pH 7.6, was mixed with 20 ml of the partially purified enzyme solution and the mixture was incubated under gentle shaking at 37°C for 60 minutes. The reaction mixture was diluted to 80 ml with cold water and charged on a column (11×900 mm) of QAE-Sephadex A-25(Cl^- form). By using a linear concentration gradient of sodium chloride in 1/40 M phosphate buffer, pH 7.6, from 0 to 0.3 M, three metabolites were recovered from the column. They were named PS-5D I, PS-5D II and PS-5D III depending on the extent of closeness to the origin on a high voltage paper electrophoretogram. Desalting with Bio-Gel P2 (11×900 mm) followed by freeze-drying yielded these breakdown products in powder form.

Assay Methods

1) PS-5-inactivation Assay: Two tenths milliliter of a solution to be assayed for the PS-5-inactivating activity was added to 0.2 ml of 40 μg/ml PS-5 in 1/20 M phosphate buffer, pH 7.6, and the mixture was incubated at 37°C for 30 minutes under gentle shaking. After heat-treatment at 100°C for 15 seconds, the amount of unchanged PS-5 remaining in the reaction mixture was disc-assayed as the antimicrobial activity against *Comamonas terrigena* B-996[1].

2) Inhibitor Assay: The protective effect of an inhibitor compound on the PS-5-inactivation by renal enzyme was examined by incubating 0.1 ml of an 80 μg/ml solution of PS-5, 0.2 ml of the fresh rat kidney homogenate and 0.1 ml of 1/5 M tris-HCl buffer, pH 7.6, containing none, 10^{-5} M (final concentration) or 10^{-3} M of the inhibitor compound. Under routinely used assay conditions, each inhibitor was shown to have no growth-inhibitory (or antimicrobial) effect on *Comamonas terrigena* B-996. After incubation at 37°C for 30 minutes, the antimicrobial potency of the reaction mixture was bioassayed by the disc-agar diffusion method as described in a previous paper.[1] The protective effect of an inhibitor was judged from the difference in the amount of unchanged PS-5 between the control reaction mixture (without inhibitor) and the test one (with inhibitor).

3) High Performance Liquid Chromatography: PS-5 was analyzed with a solvent system of 1/100 M ammonium phosphate buffer, pH 7.6, containing 15% methanol under the same conditions as specified for PS-6 and PS-7.[5]

4) High Voltage Paper Electrophoresis: Assay samples were applied to Toyo filter paper No. 51 and were subjected to electrophoresis in Veronal buffer, pH 8.6 (I=0.05), at 75 V/cm for 20 minutes at 0~4°C (electrophorator HV 3000A; Savant Instruments Inc., U. S. A.).

5) Peptidase Assay: One milliliter of a 5 mg/ml solution of substrate in 1/50 M tris-HCl buffer, pH 8.0, was added to 1 ml of the PS-5-inactivating factor. Under incubation at 37°C with mild agitation, 100 μl of the reaction mixture was collected at 0, 5, 10, 20, 30 and 60 minutes and was immediately boiled at 100°C for 5 minutes. Products in the reaction mixture were analyzed by high voltage paper electrophoresis followed by visualization with ninhydrin.

Results

Location of a PS-5-inactivating Factor in the Subcellular Fractions of the Rat Kidney

According to the established subcellular fractionation method, the fresh rat kidney was homogenized and fractionated. Table 1 shows the comparative distribution of the PS-5-inactivating activity in various subcellular fractions.

It is apparent from Table 1 that the PS-5-inactivating factor is exclusively localized in the 105,000$\times g$ pellet or the microsomal fraction of the kidney (refer to Chart 1).

Characterization of the Renal PS-5-inactivating Factor

On the assumption that this factor might be an enzyme, we have studied fundamental properties of the rat kidney homogenate. The results summarized in Table 2 indicate that the PS-5-inactivating factor is non-dialyzable; thermo-labile and precipitable with ammonium sulfate and acetone, suggesting its enzymatic nature.

For further characterization, we have checked the susceptibility of the PS-5-inactivating factor to typical enzyme inhibitors. At a final concentration of 10^{-3} M, AgCl, $CuSO_4$, iodoacetamide, *p*-chloromercuribenzoate, oxidized glutathione and potassium cyanide showed no

Table 1. Comparative PS-5-inactivating activities of the subcellular fractions of the rat kidney.

Fraction	Relative activity* (%)
Whole homogenate	76.0
700$\times g$ supernatant	74.4
pellet	40.0
7,000$\times g$ supernatant	74.4
pellet	20.0
24,000$\times g$ supernatant	0.0
pellet	20.0
105,000$\times g$ supernatant	0.0
pellet	60.0
Control (With buffer only)	0.0

* Expressed in the % amount of inactivated PS-5.

Table 2. Enzymatic properties of the PS-5-inactivating factor of the rat kidney.

	Relative PS-5-inactivating activity* (%)
Homogenate (700$\times g$ supernatant)	76
Dialyzed homogenate	75
Heat-denatured homogenate (100°C, 15 seconds)	0
Ammonium sulfate fraction (80% saturation; precipitate)	75
Acetone fraction (80%; precipitate)	70
Control (with buffer only)	0

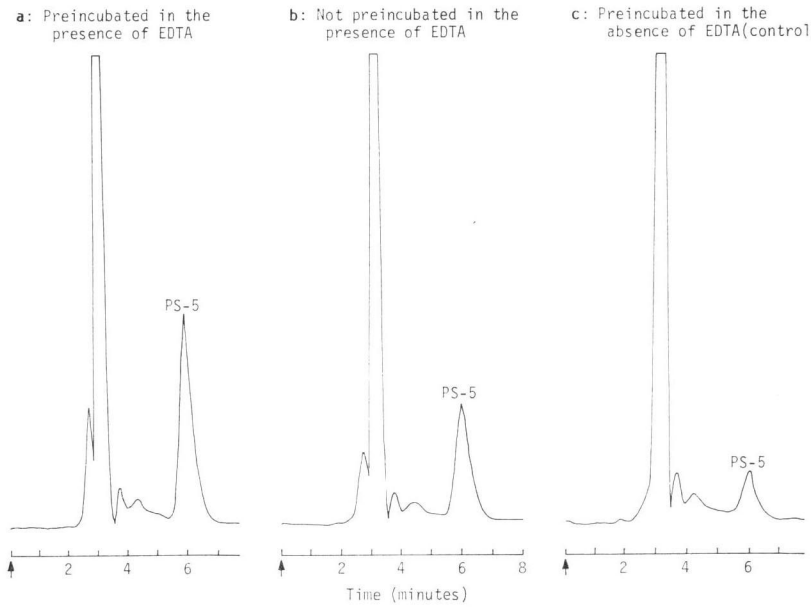
* Expressed in the % amount of inactivated PS-5.

Table 3. Influences of various chelating agents on the renal PS-5-inactivating factor.

Chelator	Percent inhibition*	
	10^{-3} M	10^{-5} M
Sodium citrate	0%	0%
Thiourea	0	0
Sodium salicylate	0	0
Diphenylthiocarbazone	24.4	0
EDTA	55.6	24.4
1,10-Phenanthroline	48.8	6.7
Control (−chelator)	0	
Blank (−factor)		

* % Inhibition (= % protection)
=[(sample−control)/(blank−control)]×100
(calculated on the basis of the amounts of unchanged PS-5 in reaction mixtures).

Fig. 1. Protective effect of EDTA on the PS-5-inactivation by the renal factor.



a : The renal microsomal fraction (0.2 ml) was mixed with 0.1 ml of 1/10 M tris-HCl buffer, pH 7.6, containing 4×10^{-5} M EDTA and the mixture was preincubated at 37°C for 5 minutes. An 80 μg/ml PS-5 solution (0.1 ml) was added and incubated at 37°C for 30 minutes under gentle shaking.

b : Except that the preincubation of the renal microsomal factor with EDTA was omitted, same as in **a**.

c : Except that EDTA was removed from the buffer, same as **a**.

H.P.L.C. conditions:

Column: μBondapak C_{18} (4×300 mm).
Solvent system: 1/100 M ammonium phosphate buffer, pH 7.6, containing 15% methanol.
Injection volume: 100 μl.
Flow rate: 0.84 ml/minute.
Detection: UV absorbance at 300 nm.

inhibition at all. Only ethylenediaminetetraacetate (EDTA), a metal-chelating compound, inhibited the PS-5-inactivating activity of the renal microsomal factor by 25% at 10^{-5} M and by 55% at 10^{-3} M. In other words, EDTA protected PS-5 from inactivation by the renal microsomal factor. In Table 3, the influences of various chelating agents on the PS-5-inactivating factor are summarized.

EDTA is again the strongest inhibitor, followed by 1,10-phenanthroline and diphenylthiocarbazone. As 1,10-phenanthroline is known to inhibit zinc-containing enzymes,[7] the PS-5-inactivating factor was assumed to be a metalloenzyme.

The protective effect of EDTA on the PS-5-inactivation by the microsomal factor was also confirmed by high performance liquid chromatography (Fig. 1).

The significant protective effect of EDTA is clearly understandable in Figs. 1b and 1c. In addition, when the renal microsomal factor was preincubated with EDTA, the peak area of PS-5 is markedly increased (Fig. 1a).

It is important to note here that the amount of unchanged PS-5 measured by high performance liquid chromatography satisfactorily agreed with the bioassay data using *Comamonas terrigena* B-996. In

Table 4. Substrate profile of the PS-5-inactivating factor of rat kidney.

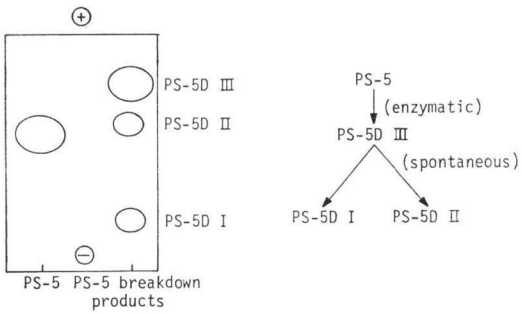
Positive	Dipeptides (Gly-phe, Ala-ala)
	Dehydrodipeptide (Gly-dehydrophe)
Negative	Tripeptide (Gly-gly-leu)
	N-Acylamino acids (*N*-Acetylgly, *N*-Acetylphe, *N*-Formylleu)
	N-Acyldipeptides (*N*-Acetylgly-phe, *N*-Acetylgly-dehydrophe)
	Dipeptide esters (Gly-phe methyl ester, Gly-dehydrophe methyl ester)

Table 5. Physicochemical properties of breakdown products of PS-5.

Reagent	PS-5D I	PS-5D II	PS-5D III
Ninhydrin	Yellow	Yellow	Yellow
Iodine chloroplatinate	+	(+)*	+
Ehrlich	Purple	Red	Purple
UV absorption (Max.)		308 nm	End abs.

* Rapidly turns negative.

Fig. 2. Analysis of PS-5 breakdown products by high voltage paper electrophoresis.



Conditions for H.V.P.E.: 75 V/cm for 30 minutes in Veronal buffer, pH 8.6 (I=0.05).
Visualized with Ehrlich reagent.

other words, unlike cephalothin that is converted *in vivo* and *in vitro* to a less antimicrobial metabolite by esterase,[8] PS-5 was metabolized to products which had no antimicrobial activity against the bioassay organism at all.

Based on the above-described experimental findings, enzymes with possibly identical or similar properties were surveyed in the literature. Finally renal dipeptidase[8] was chosen as one of probable candidates for detailed identification studies. Table 4 presents the substrate profile of the renal PS-5-inactivating factor to amino acid derivatives and peptides. It is obvious from Table 4 that the microsomal fraction of rat kidney hydrolyzes only dipeptides with the free amino and carboxyl terminals, whereas tripeptide, *N*-acylamino acids, *N*-acyldipeptides and dipeptide esters are insusceptible. Furthermore, dehydrodipeptides such as glycyldehydrophenylalanine were attacked by the renal microsomal factor, indicating its probable identity with renal dipeptidase.[9]

Isolation of Breakdown Products of PS-5 by Renal Dipeptidase

As the PS-5-inactivating factor of rat kidney was found to be an enzyme, we have attempted to solubilize the responsible enzyme from the microsomal fraction. According to the purification procedure explained in the Materials and Methods section, a partially purified preparation of renal dipeptidase was obtained.

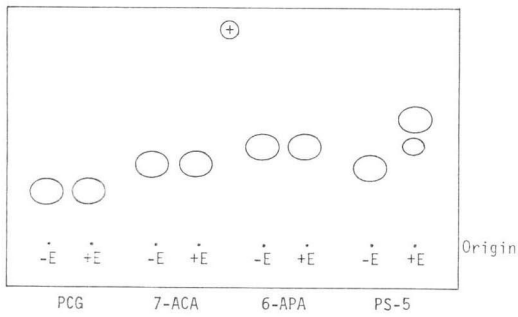
After 100 mg of PS-5 was incubated with the renal enzyme at 37°C for 60 minutes, three breakdown products of PS-5 were isolated by column chromatography. They were designated PS-5D I (very minor), PS-5D II (minor) and PS-5D III (major) corresponding to the distances of movement on a high voltage paper electrophoretogram (Fig. 2). In the subsequent chemical work, PS-5D I and PS-5D II were found to form spontaneously from PS-5D III.

The color reactions and UV absorption data of these metabolites are shown in Table 5.

Insusceptibility of the Conventional β-Lactam Compounds to Rat Renal Dipeptidase

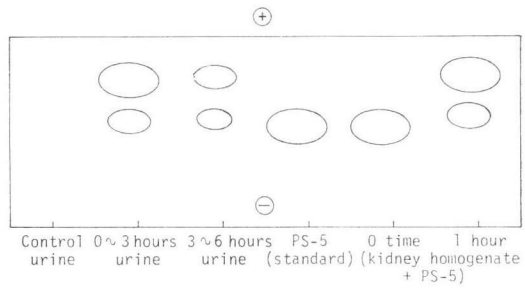
As β-lactamase-like enzymes are reported to exist in animals,[10,11] and as carbapenem compounds were susceptible to some types of β-lactamase,[15] it was interesting to examine if the renal PS-5-inactivat-

Fig. 3. Susceptibility of conventional β-lactams to the PS-5-inactivating enzyme.



E=PS-5-inactivating enzyme.
PCG=benzylpenicillin.
7-ACA=7-aminocephalosporanic acid.
6-APA=6-aminopenicillanic acid.
Visualized with iodine chloroplatinate, Ehrlich reagent and ninhydrin.

Fig. 4. H.V.P.E. analysis of the urines collected from a mouse receiving a single dose of 4 mg PS-5, s.c..



Visualized with Ehrlich reagent.

ing enzyme also acted on conventional β-lactam compounds such as penicillins and cephalosporins.

Results in Fig. 3 clearly show that PS-5 (and other carbapenem compounds such as thienamycin[13]) is the sole substrate for renal dipeptidase, while benzylpenicillin, 6-aminopenicillanic acid and 7-aminocephalosporanic acid are resistant.

Identification of PS-5D III with the *In Vivo* Metabolite of PS-5

The experimental results described so far were obtained by using the renal PS-5-inactivating enzyme of rat kidney *in vitro*. Therefore it was necessary to examine if the *in vitro* metabolic pattern summarized in Fig. 2 was also seen *in vivo* and in other animal species.

When the fresh kidney homogenates of mouse and pig were incubated with PS-5, the same high voltage electrophoretograms as is seen in Fig. 2 were observed. Thus it seems acceptable to think that PS-5D III is the primary metabolite of PS-5 in most animal species.

Very similar metabolic patterns of PS-5 were also obtained in urine and blood samples after a single injection of PS-5 to mice and rats. As an example of such analyses, Fig. 4 shows the product analysis of the urine samples collected from a mouse receiving a single subcutaneous injection of 4 mg of PS-5.

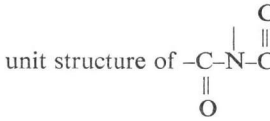
As for the *in vitro* inactivation of PS-5 by renal dipeptidase of rat, PS-5D III was the major metabolite of PS-5 together with a small amount of PS-5D II. As PS-5D III was clearly located even in the 3~6 hours urine sample, it seemed to be fairly stable in urine.

Based on these findings, it is reasonable to consider that in most animal species PS-5 is metabolized by renal dipeptidase to give PS-5D III which is spontaneously converted to PS-5D I and PS-5D II.

Discussion

The PS-5-inactivating factor partially purified from the microsomal fraction of rat kidney was concluded to be very probably identical with renal dipeptidase,[2] although carbapenem compounds are unreasonable substrates for this enzyme.

Renal dipeptidase was first isolated as an amino peptidase from the swine kidney by ROBINSON *et al.*[12] Then, using a crystalline preparation, CAMPBELL *et al.* intensively characterized the enzyme to show that it is a unique dipeptidase which can also act on unsaturated dipeptides such as glycyldehydrophenylalanine.[9]

Scientists of the Merck Co. have recently reported that carbapenem compounds such as thienamycin, *N*-formimidoylthienamycin, epithienamycins and PS-5 were inactivated by dehydropeptidase-I.[13] Since dipeptidase is defined to act selectively on dipeptides having both the free amino and the free carboxyl group,[9] it is unexpected that carbapenem antibiotics having no free α-amino group are susceptible to renal dipeptidase. Furthermore the normal peptide bond is absent in the molecule of the carbapenem family of compounds. In spite of these unexplainable observations, a possible and sole site of action by dipeptidase is the unit structure of  As expected, conventional penicillins and cephalosporins were completely resistant to very high concentrations of the renal PS-5-inactivating enzyme of rat. Thus the susceptibility to renal dipeptidase seems to be characteristic of the carbapenem skeleton.

Compared with PS-5D III, PS-5D I and PS-5D II are difficult to isolate because of their chemical instability. As PS-5D II possessed a UV absorption maximum at 308 nm, it seems to differ from a degradation product of thienamycin.[14] The chemical structures of the latter two metabolites are under investigation.

The chemical structure of PS-5D III will be elucidated in the subsequent paper.

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