Communications to the Editor

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PARTIAL PURIFICATION OF THE ENZYME OBTAINED FROM THE RAT KIDNEY MICROSOMAL MEMBRANE WITH SUCCINYL TRIALANINE p-NITROANILIDE-HYDROLYZING ACTIVITY AND THE ONE SIMILAR TO SIGNAL PEPTIDASE

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An endopeptidase was obtained from the rat kidney microsomal membrane through its treatment with 1 M - KCl, Brij 35 and Nonidet P-40, and subsequently by DEAE-Sephadex chromatography and Sepharose CL-6B gel filtration, in which process the enzyme was subjected to partial purification by means of the substrate succinyl trialanine p-nitroanilide. We found the enzyme cleaves the substrate into succinyl dialanine and alanine p-nitroanilide. Further, the post-translational processing experiments have revealed the presence of an activity in the partially purified enzyme to cleave the pre-human placental lactogen (pre-hPL) into its mature form (hPL).

KEYWORDS —— rat kidney; membrane-bound endopeptidase; succinyl trialanine p-nitroanilide; signal peptidase; post-translational processing; human placental lactogen

The precursors of secreted polypeptides contain an amino-terminal signal sequence that is not present in the final product.[1] In eukaryotic cells, the cleavage of signal peptides is a co-translational reaction that occurs early to the polypeptide chain in the assembly.[2] It is catalyzed by the signal peptidase in microsomal membranes.[3] Several groups of workers have described the post-translational cleavage of signal peptidase in the presence of detergent-solubilized membrane.[4 a-d] Since it has been suggested that an enzyme obtained from dog pancreatic microsomes which hydrolyzes succinyl dialanine phenylalanine 7-amino-4-methyl-coumarine (Suc $(Ala)_2$ Phe-AMC) may be a signal peptidase,[5] we have partially purified an enzyme taken from rat kidney microsomal membrane which hydrolyzes succinyl trialanine p-nitroanilide (Suc $(Ala)_3$ -pNA), one of the synthesized analogous substrates to the hydrophobic region of signal sequence, and have found that this enzyme has an activity to cleave the pre-human placental lactogen (pre-hPL) into its mature form (hPL).

Male Wistar rats (about 200 g) were starved for 18 - 20 h prior to sacrifice. The kidney (83.4 g) was quickly excised after saline perfusion and homogeneized

in a Teflon homogenizer with 8 volumes of 0.25 M sucrose. The homogenate was centrifuged for 5 min at 8,000 x g. Microsomes were prepared by centrifuging the supernatant fraction for 60 min at 100,000 x g. The pellet was homogenized in a buffer containing 10 mM Tris-HCl (pH 8.0) and 1 M KCl, and centrifuged as described above. The pellet was then homogenized in a buffer containing 10 mM Tri-HCl (pH 8.0), 0.1 M NaCl and 0.5% Brij 35, and centrifuged as described above. The pellet thus obtained was solubilized in a buffer containing 10 mM Tris-HCl (pH 8.0), 1% Nonidet P-40 and 25 mM NaCl, and centrifuged as described above. The supernatant solution was applied to a column of DEAE-Sephadex previously equilibrated with buffer 1 (10 mM Tris-HCl, pH 8.0 and 0.05% Nonidet P-40) containing 25 mM NaCl. The enzyme was eluted by using buffer 1 with a linear gradient of 25 - 325 mM NaCl, and the fractions of eluate containing the activity which hydrolyzes the Suc $(Ala)_3$-pNA were combined. After the solution was concentrated by ultrafiltration, the enzyme solution (28.2 mg protein) was applied to a column of Sepharose CL-6B equilibrated with buffer 1 containing 25 mM NaCl. The enzyme was eluted in the same buffer, and the active fractional portions were pooled and used. The recoveries of protein and enzyme activity are consecutively given in tabular form (see below).

TABLE I. Purification of Suc $(Ala)_3$-pNA-Hydrolyzing Enzyme Obtained from the Rat Kidney Microsomes

Step	Protein (mg)	Enzyme activity (Total units)	Specific activity (u/mg x 10^3)	Yield (%)
1. Microsomes	1860	17.4	9.35	100
2. KCl-washed pellet	1310	23.2	17.7	133
3. Brij treated pellet	834	19.4	23.3	111
4. Nonidet extract	384	17.2	44.8	101
5. DEAE-Sephadex A-50	28.2	9.2	319	53
6. Sepharose CL-6B	10.3	5.8	563	33

As is shown in Fig. 1, only Ala-pNA was detected in the absence of leucine aminopeptidase (LAP), while pNA was detected in the presence of the LAP. This indicates the enzyme cleaves the substrate into succinyl dialanine and Ala-pNA. Recently, similar membrane-bound endopeptidase have been purified from porcine kidney[6] and human kidney[7] by using Suc $(Ala)_2$ Phe-AMC and Suc $(Ala)_3$-pNA, respectively, as their substrates. However, the activity of a signal peptidase has been left untouched. In the cource of our study of rat kidney membrane-bound protease which hydrolyzes Suc $(Ala)_3$-pNA, we have been much interested in this point.

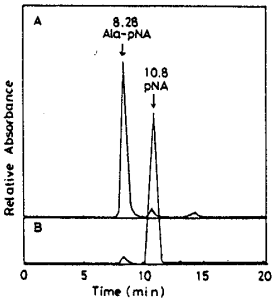

Fig. 1. Identification of Enzymatic Digestion Product of the Substrate Suc $(Ala)_3$-pNA by Means of High-Performance Liquid Chromatography

The value of each peak indicates the retention time for the compound. The reaction mixture was incubated with leucine aminopeptidase (B) and without it (A).
pNA; p-nitroaniline.

The enzyme obtained here had an activity to cleave pre-hPL into hPL. And further, both the pre- and mature forms of hPL had the same electrophoretical mobilities as the results obtained by the experiment using deoxycholate (DOC)-extracted enzyme of dog pancreatic membranes (Fig. 2). The DOC extract of dog pancreatic microsomal membrane had been used as a good source of signal peptidase.[4a, 5a]

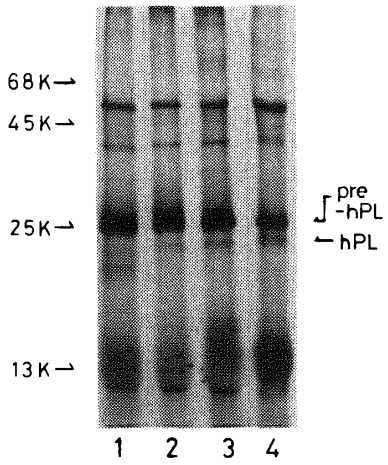

Fig. 2. Identification of Signal Peptidase Activity by Fluorography

Assay of signal peptidase activity was performed as is described in EXPERIMENTAL. Lane 1, 20 μl of buffer 1 containing 25 mM NaCl (experiment without the enzyme preparation); lanes 2 and 3, 10 μg and 20 μg of the enzyme preparations (in the same buffer system as lane 1), respectively; lane 4, 400 μg of protein (the amount is sufficient for the maximum cleavage; about 20% in the post-translational assay system) obtained from dog pancreatic microsomes (in 20 μl of 0.5%(w/v) sodium deoxycholate).

Position of mature hPL was identified with the protein staining of authentic hPL (obtained from National Biological Standard Board, London, England). The numerical indications on the left hand of the gel represent the apparent molecular weight of protein standard. Markers used: Bovine Serum Albumin (68K), Ovalbumin (45K), Chymotrypsinogen (25K) and Cytochrome c (13K).

In the presence of chymostatin and o-phenanthroline, which are known to be potent inhibitors of signal peptidase,[4c, 5a] not only was the cleavage of pre-hPL into hPL prevented, but the Suc $(Ala)_3$-pNA hydrolyzing activity was also inhibited. 0.15 mM and 1.5 mM of chymostatin and 0.5 mM and 2.0 mM of o-phenanthroline inhibited Suc $(Ala)_3$-pNA hydrolysis to 42.5% and 90% and to 40% and 83%, respectively. On the other hand, the enzymatic activity was not inhibited by phenyl methane sulfonyl fluoride, antipain, bestatin, elastatinal, leupeptin or pepstatine. These unusual results may indicate that the enzyme is a new type of protease hitherto not known. Although the enzyme was partially purified, several results found in this study suggested that the Suc $(Ala)_3$-pNA hydrolyzing enzyme might be a signal peptidase. On the other hand, surprisingly, further purification of the enzyme results in loss of the signal peptidase activity, though the activity of Suc $(Ala)_3$-pNA hydrolysis still remains. It has been reported that phospholipid is required for the signal peptidase activity.[8] However, no effect was observed by the addition of phospholipids to the further purified enzyme in the signal peptidase assay system, the other factor(s) seemed to be necessary. Experiments to elucidate the relationships of activities between the signal peptidase and the Suc $(Ala)_3$-pNA hydrolysis, including the factor(s), are now in progress.

EXPERIMENTAL

Enzyme Assay — The activity which hydrolyzes Suc $(Ala)_3$-pNA (product of the Protein Research Foundation, Minoh, Japan) was assayed by the following two methods. (1) In purification procedure, the enzyme activity was measured by an indirect-

coupled enzyme assay. The reaction mixture (0.25 ml) contains 1 mM Suc $(Ala)_3$-pNA, 4% N-methyl-2-pyrrolidone, 44 mM Tris-HCl (pH 8.0), 1.32 units of leucine aminopeptidase and the enzyme. The incubation was terminated by adding the solution containing 25% ethanol, 0.12 N HCl and 1.59 mM p-(dimethylamino) cinnamaldehyde to be coupled with p-nitroaniline. The coupled product was measured at 565 nm. One unit was defined to be the amount of enzyme which gives 1 μmol of the coupled product per min. (2) Concerning identification of the hydrolyzed products, the reaction mixture for the assay of protease activity (0.25 ml) was incubated at 25°C for 60 min and then the reaction products were extracted by employing the volume of ethylacetate equal to that of the reaction mixture. The resulting organic phase was evaporated to dryness under reduced pressure. The residues were dissolved in 125 μl buffer (5 mM phosphate buffer of pH 3.9, containing 50% methanol). The aliquot (5 μl) was analyzed by using the same buffer according to the Shimadzu HPLC system consisting of a TSK-GEL LS-410A ODS SIL (4.0 ID x 300 mm) column (Toyo Soda). The flow rate was 0.5 ml/min and the 340 nm absorbance was measured.

Assay of Signal Peptidase Activity —— Protein synthesis was performed as described previously,[9] except that the reaction mixture contained 0.1 mCi of [^{35}S] methionine (1107 Ci/mmol, obtained from New England Nuclear) and 0.5 μg human placental poly(A) having RNA instead of [^{14}C] leucine and globin mRNA. The reaction mixture (25 μl) for the post-translational assay contained 5 μl of a translation mixture having completed pre-hPL (hot trichloroacetic acid insoluble radioactivity: 30,000 cpm) and 20 μl of the enzyme solution. After incubation at 25°C for 90 min, the sample for SDS-polyacrylamide gel electrophoresis was prepared as shown previously,[10] and the electrophoresis and fluorography were performed based on the methods of Laemmli[11] and Laskey et al.,[12] respectively.

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