19. Caldwell, P.R.B., Seegal, B.C., & Hsu, K.C.: Angiotensin-converting enzyme: vasocular endotherial localization. Science, 191:1050-1051, 1976.

20. Bruneval, P., Hinglais, N., Alhene-Gelas, F. et al.: Angiotensin I converting enzyme in human intestine and kidney. Histochemistry, 85:73-80, 1986.

21. Erdos, E.G. & Skidgel, R.A.: The unusual substrate specificity and the distribution of human angiotensin I converting enzyme. Hypertension, 8(Sup I):34-37, 1986.

22. Johnson, A.R., Skidgel, R.A., Gafford, J.T. et al.: Enzymes in placental microvilli: angiotensin I converting enzyme, angiotensin-

ase A, carboxypeptidase, and neutral endopeptidase("enkephalinase"). Peptides, 5:789-796, 1984.

23. Yoshioka, M., Erickson, R.H., & Woodley, J.F.: Role of rat intestinal brush-border membrane angiotensin-converting enzyme in dietary protein digestion. Am. J. Physiol., 253:G781-G786, 1987.

24. Ward, P.E., Shridan, M.A., Hammon, K.J. et al.: Angiotensin I converting enzyme (Kininase II) of the brush border of human and swine intestine. Biochem. Pharmacol., 29:1525-1529, 1980.

25. Stewart, T.S., Weare, J.A. & Erdos, E.G.: Purification and characterization of human converting enzyme (Kininase II). Peptides, 2: 145-152, 1981.

26. Cushman, D.W. & Cheung, H.S.: Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol., 20:1637-1648, 1971.

27. Cushman, D.W. & Cheung, H.S.: Concentration of angiotensin-converting enzyme in tissues of the rat. Biochim. Biophys. Acta, 250:261-265, 1971.

28. Hazato, T. & Kase, R.:Isolation of angiotensin-converting enzyme inhibitor from porcine plasma. Biochem. Biophys. Res. Commun., 139:52-55, 1986.

29. 羽里忠彦, 西村欣也, 竹林幸弘 他：ウシ髄液に存在する内因性ACE阻害物質の単離精製に関する研究. 日本薬学会第110年会, 講演要旨集3:59, 1990.

30. 河村幸男：食品タンパク質由来の抗血圧上昇性ペプチド；大豆とイワシで高血圧は防止できるか．化学と生物，27：766-768，1989.

31. Friedland, J., Silverstein, E., Drooker, M., et al.: Human lung angiotensin-converting enzyme: Purification and antibody preparation. J. Clin. Invest., 67: 1151-1160, 1981.

32. Ehlers, M.R.W., Maeder, D.L. & Kirsh, R.E.: Rapid affinity chromatographic purification of human lung and kidney angiotensin-converting enzyme with the novel N-carboxyalkyl dipeptide inhibitor N-[1(S)-carboxy-5-aminopentyl]glycylglycine. Biochim. Biophys. Acta, 883:361-372, 1986.

33. Nishimura,K., Yoshida, N., Hiwada, K. et al.: Purification of angiotensin-converting enzyme from human lung. Biochim. Biophys. Acta, 483:398-408, 1977.

34. Lanzillo, J.J., Stevens, J., & Dasarathy, Y.,: Angiotensin-converting enzyme from human tissues: physicochemical, catalytic, and immunological properties. J. Biol. Chem., 260:14938-14944, 1985.

35. Takada, Y., Hiwada, K. & Kokubu, T.: Isolation and charactrization of angiotensin-converting enzyme from human kidney. J. Biochem., 90:1309-1319, 1981.

36. Weare, L.A., Gafford, J.T., Lu, H.S. et al.: Purification of human kidney angiotensin I converting enzyme using reverse-immunoadsorp-

tion chromatography.
Anal. Biochem., 123:310-319, 1982.

37. Lieberman, J.: Elevation of serum angiotensin-converting enzyme(ACE) level in sarcoidosis. Am. J. Med., 59:365-372, 1975.

38. Yokoyama, M., Hiwada, K., Kokubu, T., et al.: Angiotensin-converting enzyme in human prostate. Clin. Chim. Acta, 100:253-258, 1980.

39. Harris, R.B., Ohlsson, J.T. & Wilson, I.B.: Purification of human serum angiotensin I-converting enzyme by affinity chromatography. Anal. Biochem., 111:227-234, 1981.

40. Oza, N.B., Lanzillo, J.J., & Ryan, J.W.: Resistance of angiotensin I converting enzyme to hydrolysis by

serine proteases. Biochem. Pharmacol., 31:1443-1445, 1982.

41. Kase, R., Sekine, R., Katayama, T., et al.: Hydrolysis of neo-kyotorphin (Thr-Ser-Lys-Tyr-Arg) and [Met]enkephalin-Arg^{6}-Phe^{7} by angiotensin-converting enzyme from monkey brain. Biochem. Pharmacol., 35:4499-4503, 1986.

42. Erdos, E.G. & Skidgel, R.A.: The angiotensin I-converting enzyme. Lab. Invest., 56:345-348, 1987.

43. Thiele, E.A., Strittmatter, S. & Snyder, S.H.: Subsatance K and substance P as possible endogenous substrate of angiotensin-converting enzyme in the brain. Biochem. Biophys. Res. Commun., 128:317-324, 1985.

44. Yoshioka, M., Erickson, R. & Kim, Y. S.: Digestion and assimilation of proline-containing peptides by rat intestinal brush boredr membrane carboxypeptidases: role of the combined action of angiotensin-converting enzyme and carboxypeptidase P. J. Clin. Invest., 81: 1090-1095, 1988.

2

学位論文

ヒト小腸アンギオテンシン変換酵素の精製およびその性状について

早狩　誠

弘前大学医学部法医学教室

（指導：村上　利教授）

附図　16枚

附表　2枚

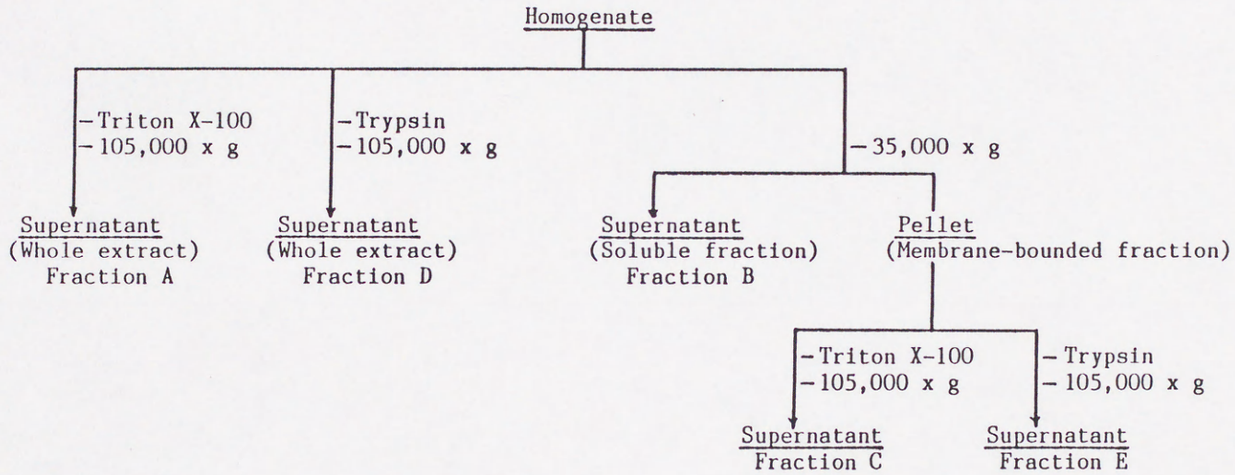

Fig. 1. Preparation procedure of human intestinal subcellular distributuion of ACE.

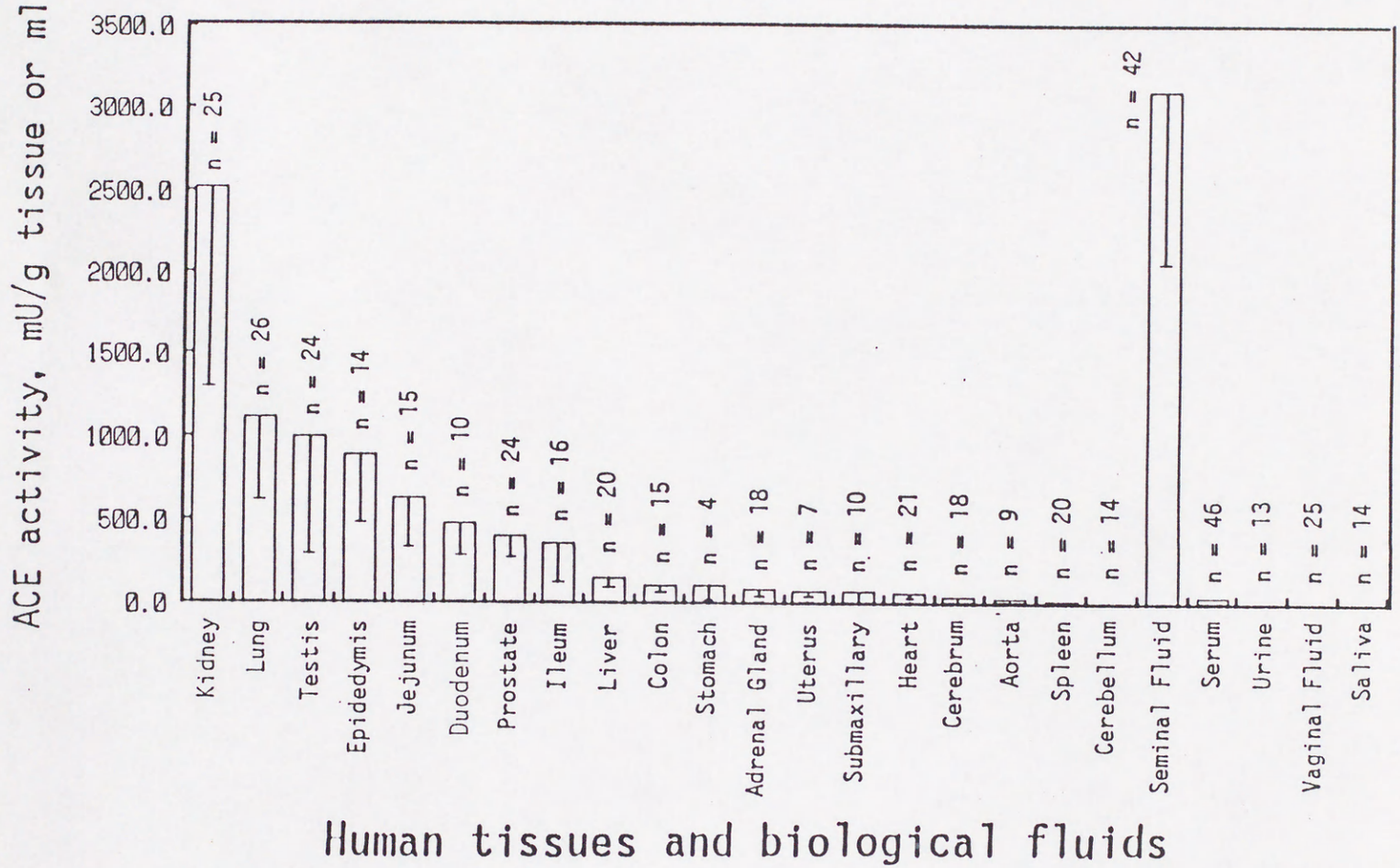

Fig. 2. Angiotensin-converting enzyme activity in postmortem human tissues and bilogical fluids.
Results are expressed in mU/g tissue or mU/ml and obtained with both "Experimental Methods".
The vertical bars indicate the range found(n = number of samples studies).

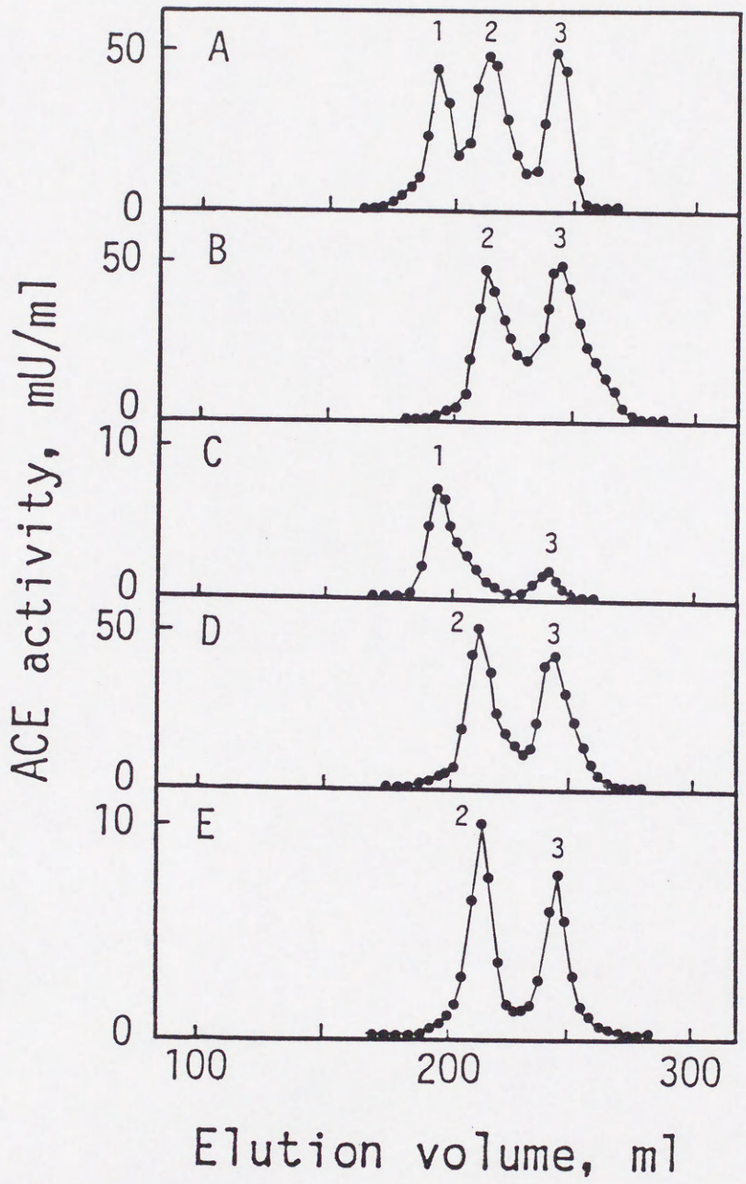

Fig. 3. Sephacryl S-300 HR gel chromatographic behaviour of ACE.
A: extract of intestinal whole homogenate treated with Triton X-100, B: soluble fraction of intestinal homogenate, C: extract of membrane-bounded fraction treated with Triton X-100, D: extract of intestinal whole homogenate treated with trypsin, E: membrane-bounded fraction treated with trypsin.

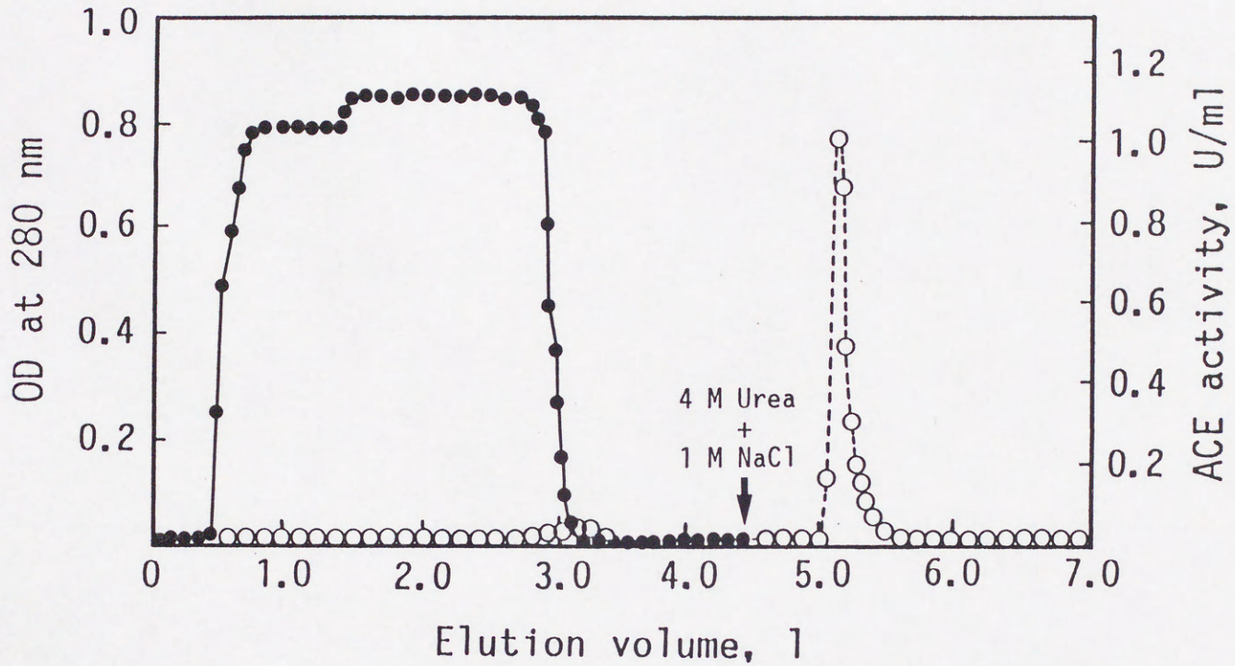

Fig. 4. Lisinopril-linked Sepharose 6B column chromatography of human intestinal extract.

The sample fraction(1,050 ml) from DEAE-cellulose chromatography was applied on a column of lisinopril-linked Sepharose 6B(4.6 x 60 cm) equilibrated with 20 mM potassium phosphate buffer pH 8.3, containing 0.3 M NaCl, 0.05% Triton X-100 and 0.02% NaN_3. ACE was eluted with 500 ml of 4.0 M urea containing 1.0 M NaCl in water at the flow rate of 60 ml/h. Each fraction volume was 20 ml. ACE activity was assayed with HHL as substrate. ●---●, absorbance at 280 nm, ○---○, enzyme activity.

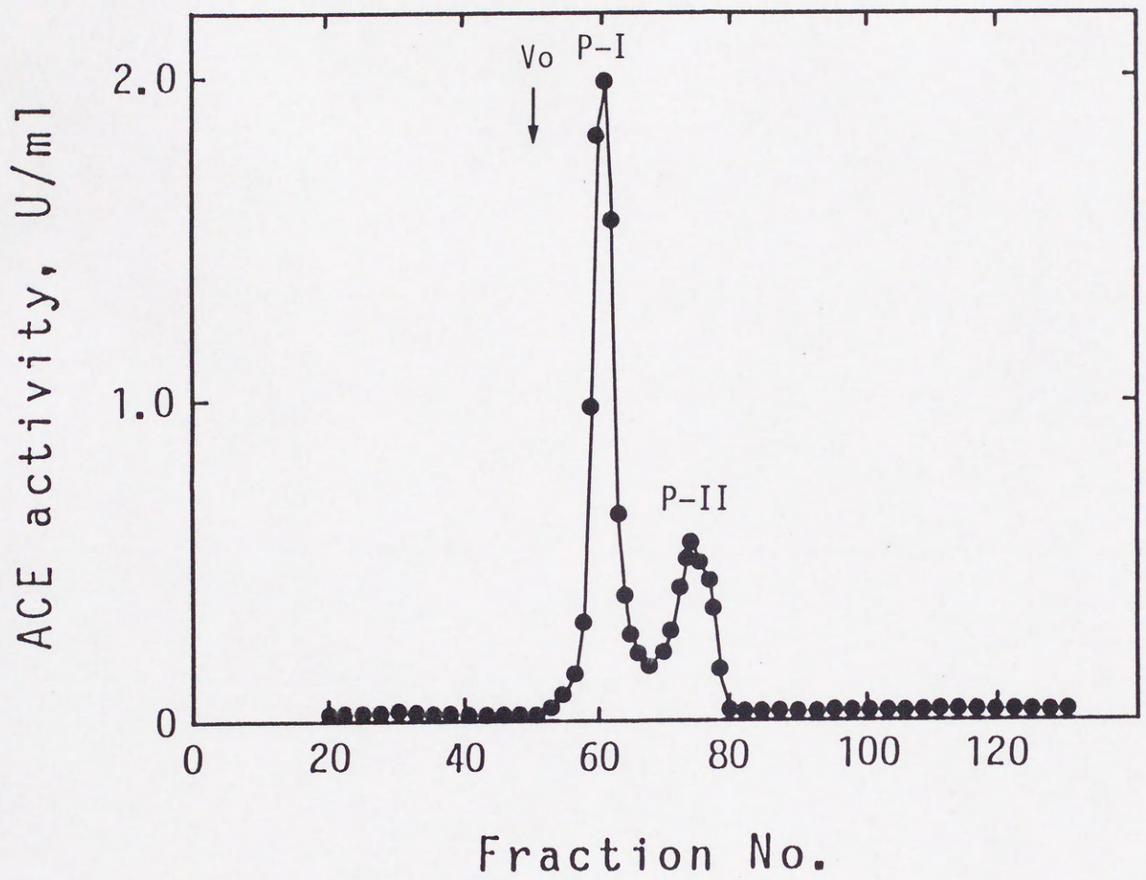

Fig. 5. Sephacryl S-300 HR column chromatomraphy of human intestinal ACE.

Pooled and concentrated ACE fraction(0.714 mg protein), which was eluted from lisinopril-linked Sepharose 6B column was chromatographed on a column of Sephacryl S-300 HR(2.6 x 100 cm) equilibrated with the same buffer described in Fig. 4. Each fraction volume was 4 ml, and the flow rate was 12 ml/h. ACE activity was assyed with HHL as substrate. Fraction I(P-I) and fraction II(P-II) were pooled and used as enzyme source. ●---●, enzyme activity.

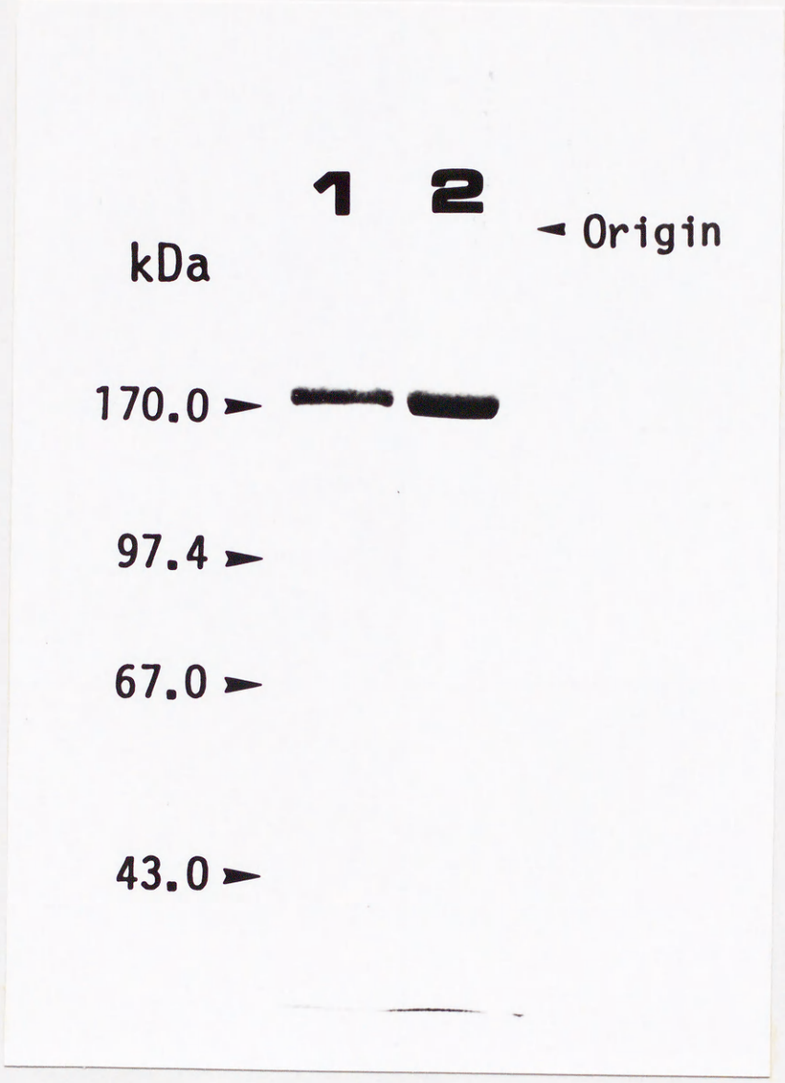

Fig. 6. SDS-Polyacrylamido gel electrophoresis of purified human intestinal and kidney ACE.

Both samples were incubated at 60 ℃ for 15 min in 1% SDS and 1% 2-mercaptoethanol, and then SDS-polyacrylamide gel electrophoresis was performed in 8.0% acrylamide gel using discontinuous buffer system [15]; proteins were stained with Coomassie Brilliant Blue. Lane 1 and 2: 10 ug of purified human intestinal and kidney ACE, respectively.

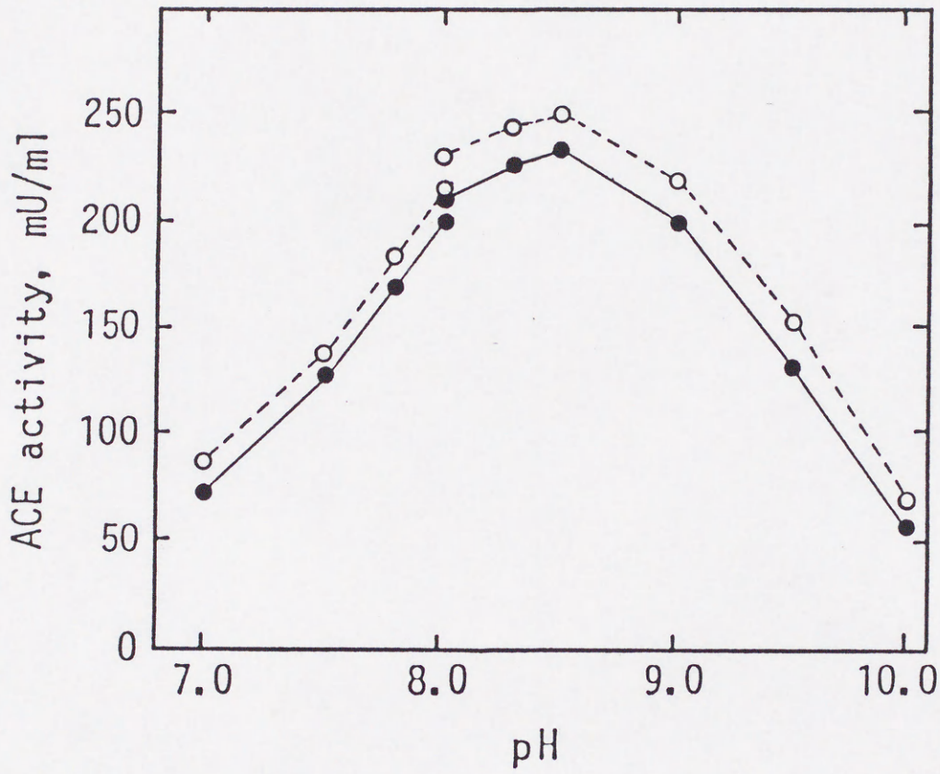

Fig. 7. Effects of pH on the activity of human intestinal ACE.

ACE activities were assayed decribed in "Experimental Methods". Each of the following buffers was adjusted to yield the indicated pH in the presece of 0.8 M NaCl:80 mM potassium phosphate; pH 7.0,7.5, 7.8 and 8.0,100 mM sodium borate; pH 7.8, 8.0, 8.3, 8.5, 9.0, 9.5 and 10.0. ●---●; purified human intestinal ACE(P-I:10.4 mU), ○---○; partially purified human intestinal ACE(P-II:9.1 mU).

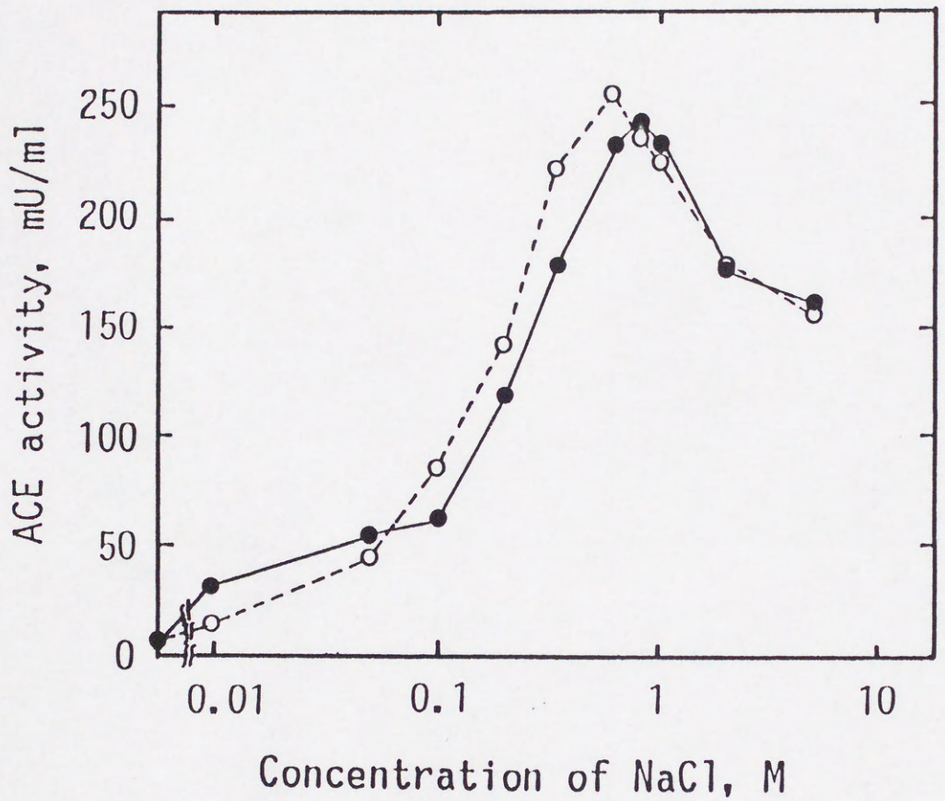

Fig. 8. Effects of NaCl concentration on the activities of human intestinal ACE.

ACE(P-I & II) were dialyzed against a salt-free buffer. ACE activities were assayed under the standard conditions described in "Experimental Methods" except for the addition of NaCl. HHL hydrolysis(5.0 mM in 0.1 M sodium borate buffer, pH 8.3) was estimated over the range 0-5 M NaCl. ●---●; purified human intestinal ACE(P-I: 10.4 mU), ○---○; partially purified human intestinal ACE(P-II:9.1 mU).

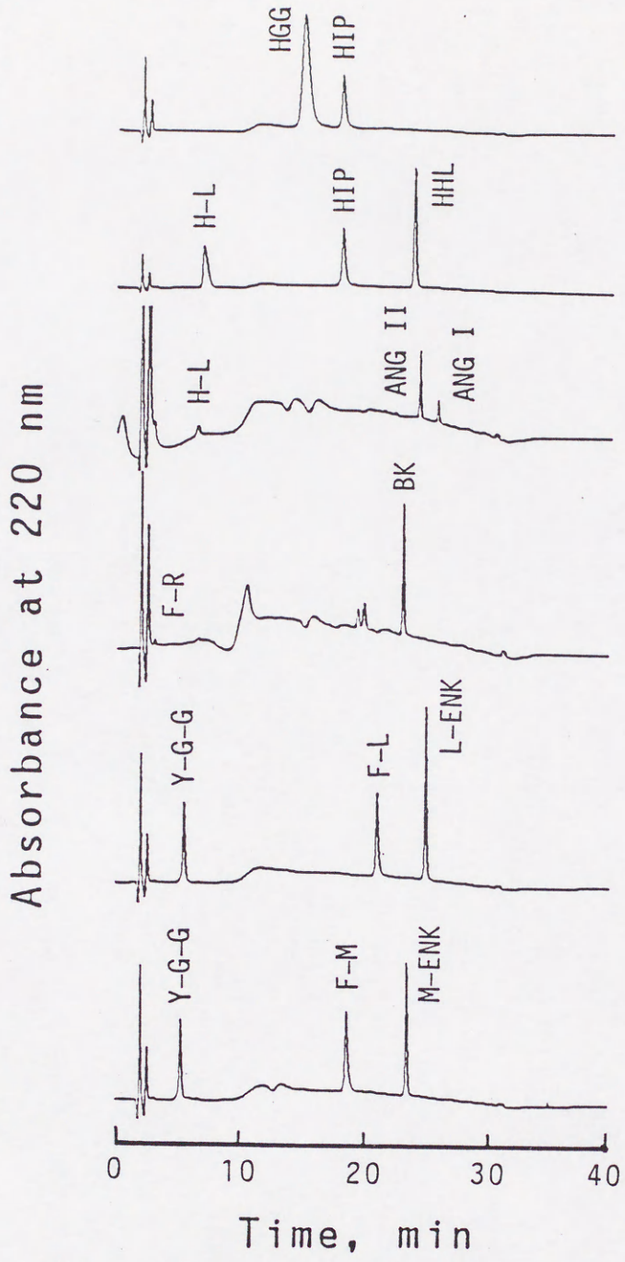

Fig. 9. HPLC analysis of the products of cleavage of artificial and endogeneous substrates by human intestinal ACE.

ACE(P-I, 10.4 mU) was incubated at 37 C with substrates for 20 or 90 min, respectively, in the presence of 0.8 mM NaCl. Products were separated by HPLC using an ODP-50 C_{18} column(4.6 x 150 mm) which had been equilibrated with 2% acetonitril in 0.05% trifluoroacetic acid. Gradient elution using 2-40% acetonitril in 0.05% trifluoroacetic acid was performed at a flow rate of 1.0 ml/min at 40 C. Peaks were identified by coelution with standards or by amino acids analysis. HIP:Bz-Gly, HGG:Bz-Gly-Gly-Gly, HHL:Bz-Gly-His-Leu, H-L:His-Leu, ANG I:angiotensin I, ANG II:angiotensin II, BK:bradykinin, F-R: Phe-Arg, L-ENK:Leu^5-enkephlin, , F-L:Phe-Leu, Y-G-G:Tyr-Gly-Gly, M-ENK:Met^5-enkephlin, F-M:Phe-Met.

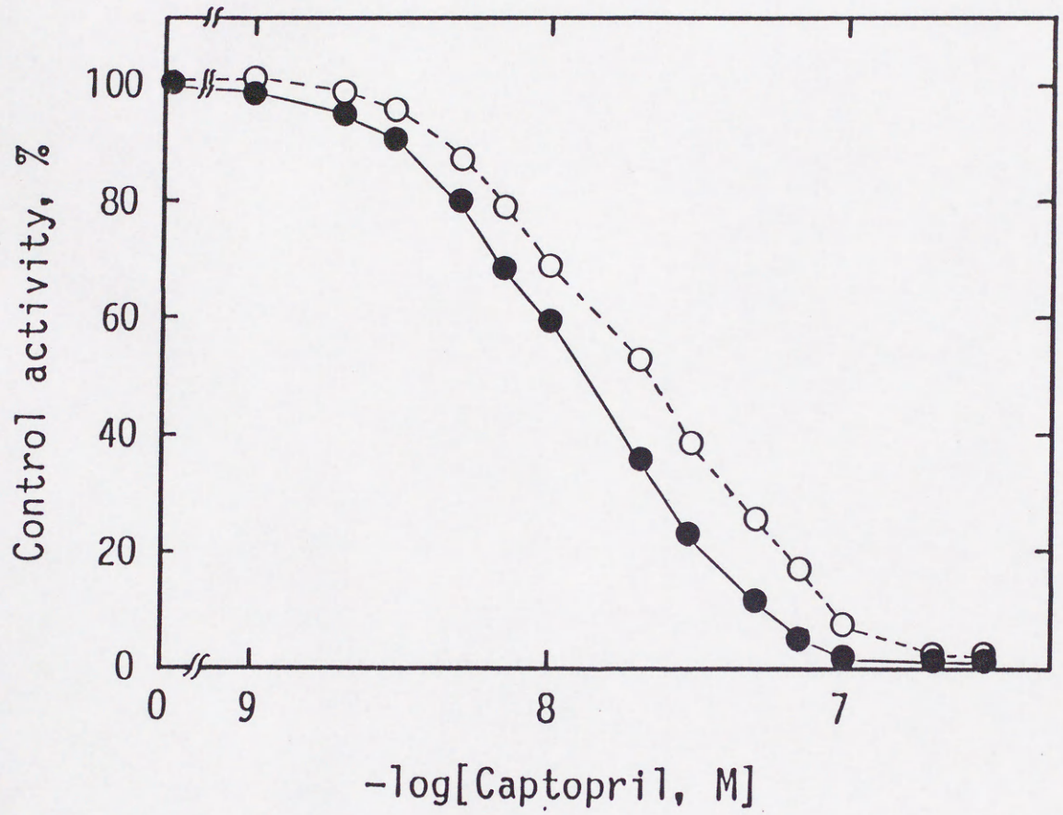

Fig. 10. Inhibition of human intestinal ACE activity by captopril.

ACE(P-I:10.4 mU, P-II:9.1 mU) were pre-incubated for 10 min at 37 C with captopril and then HHL hydrolyzing activity was assayed in 100 mM sodium-borate buffer, pH 8.3, as described in "Experimetal Methods". The concentrations of captopril described in this figure show the final concentrations. The data represent the percentage of the control activity. The IC_{50} = 13 nM captopril for P-I(●---●) and 20 nM for P-II(○---○).

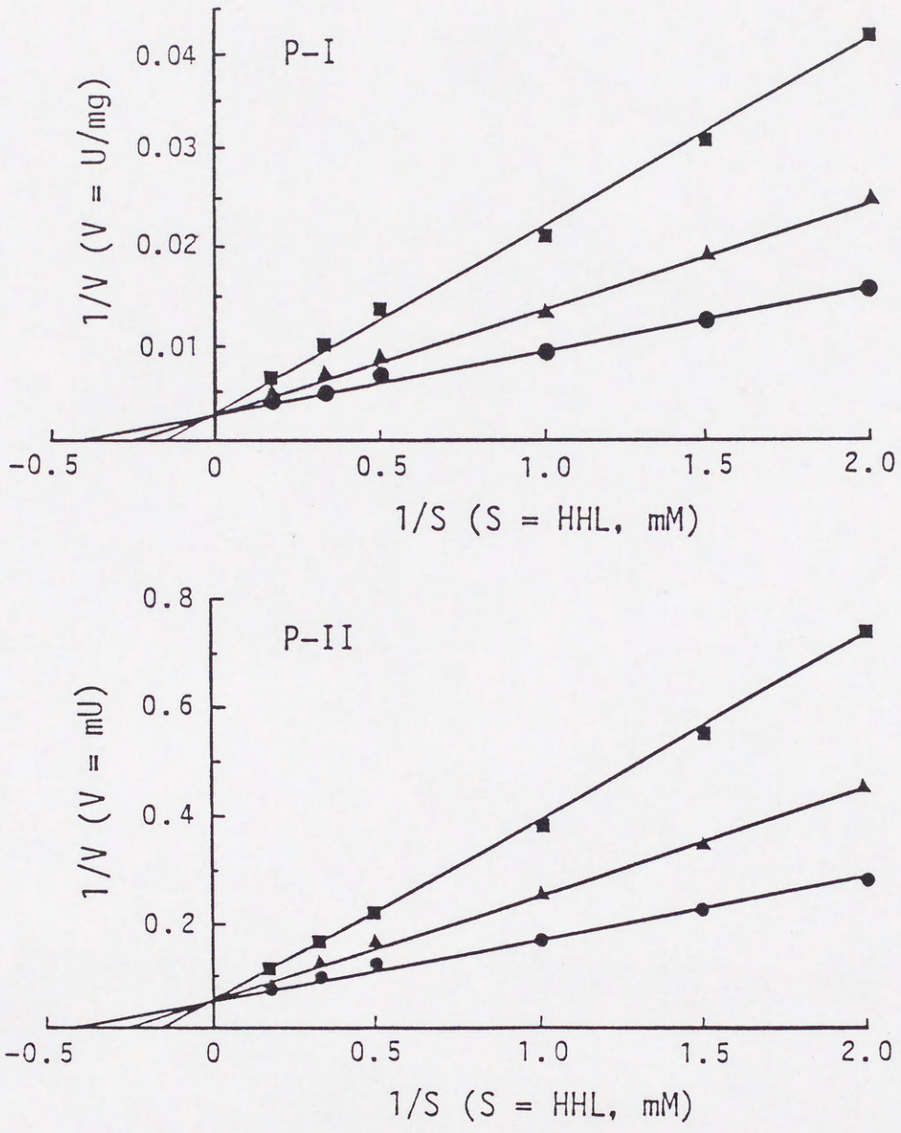

Fig. 11. Lineweaver-Burk analysis of captopril inhibition of human intestinal ACE.

Purified human intestinal ACE(P-I:10.4 mU = 66 ng) and partially purified human intestinal ACE(P-II:9.1 mU) were incubated with 0.5, 0.67, 1.0, 2.0, 3.0 and 5.0 mM HHL in the presence of 5 nM(▲---▲) and 10 nM(■---■) captopril for 20 min at 37 C.

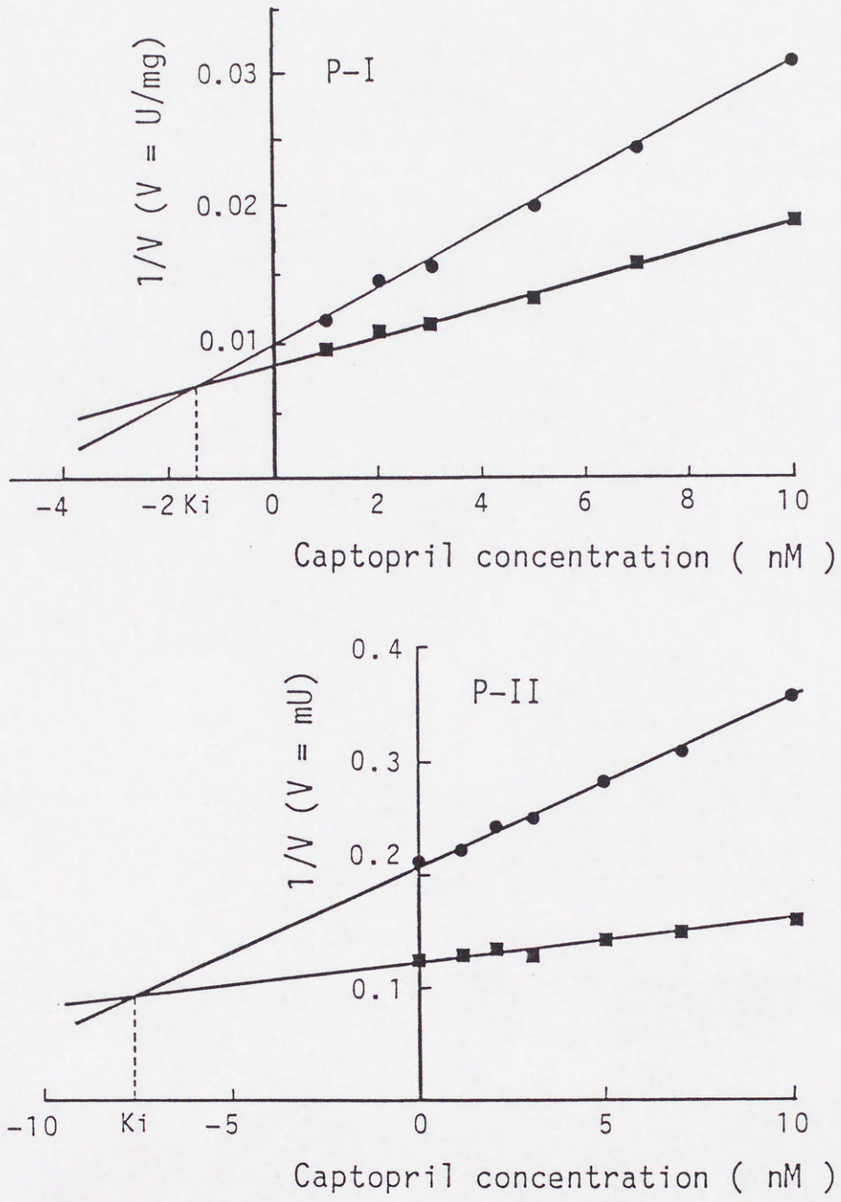

Fig. 12. Dixon plot, demonstrating the competitive inhibition of human intestinal ACE by captopril.

Inhibition of ACE activities(P-I:10.4 mU = 66 ng, P-II;9.1 mU) were determined over a range of inhibitor concentrations from 1.0 nM to 10 nM at two substrate concentrations of 0.67 mM(●---●) and 1.0 mM(■---■) HHL for P-I, or 0.67 mM(■---■) and 2.0 mM(●---●) HHL for P-II. Ki = 2.8 nM for P-I and 6.7 nM for P-II, indicated by the intersection of the dashed line with the abscissa.

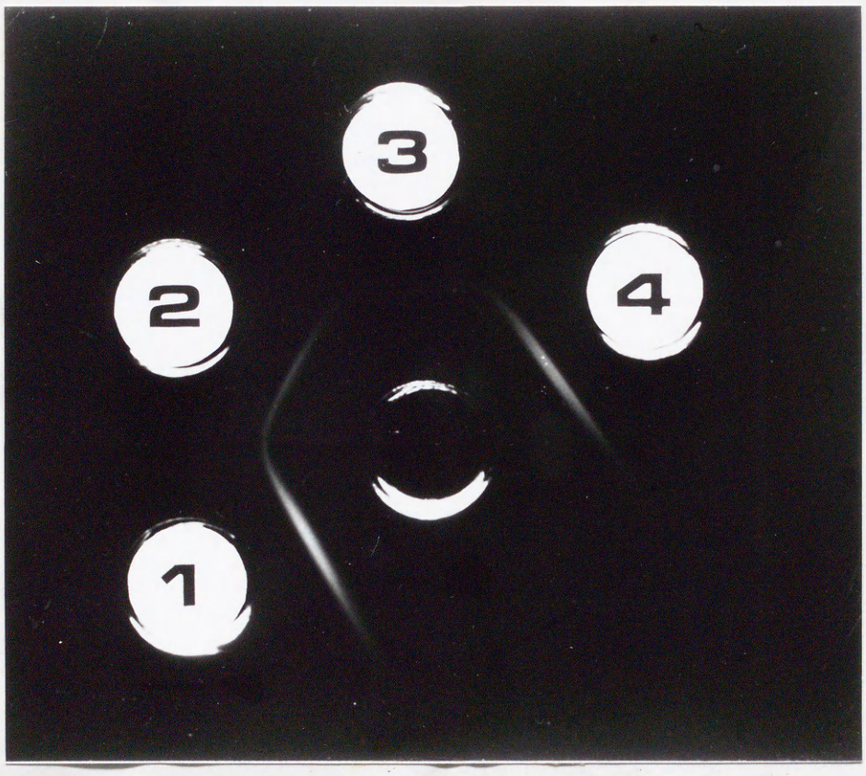

Fig. 13. Double immunodiffusion analysis of human ACE.

Center well contained antibody to human kidney ACE(20 ul). Outer wells contained purified and partially purified ACE. Wells 1,4: human kidney enzyme, 10 ug; well 2: purified human intesinal enzyme(P-I), 10 ug; well 3: partially purified human intestinal enzyme(P-II), 20 ug.

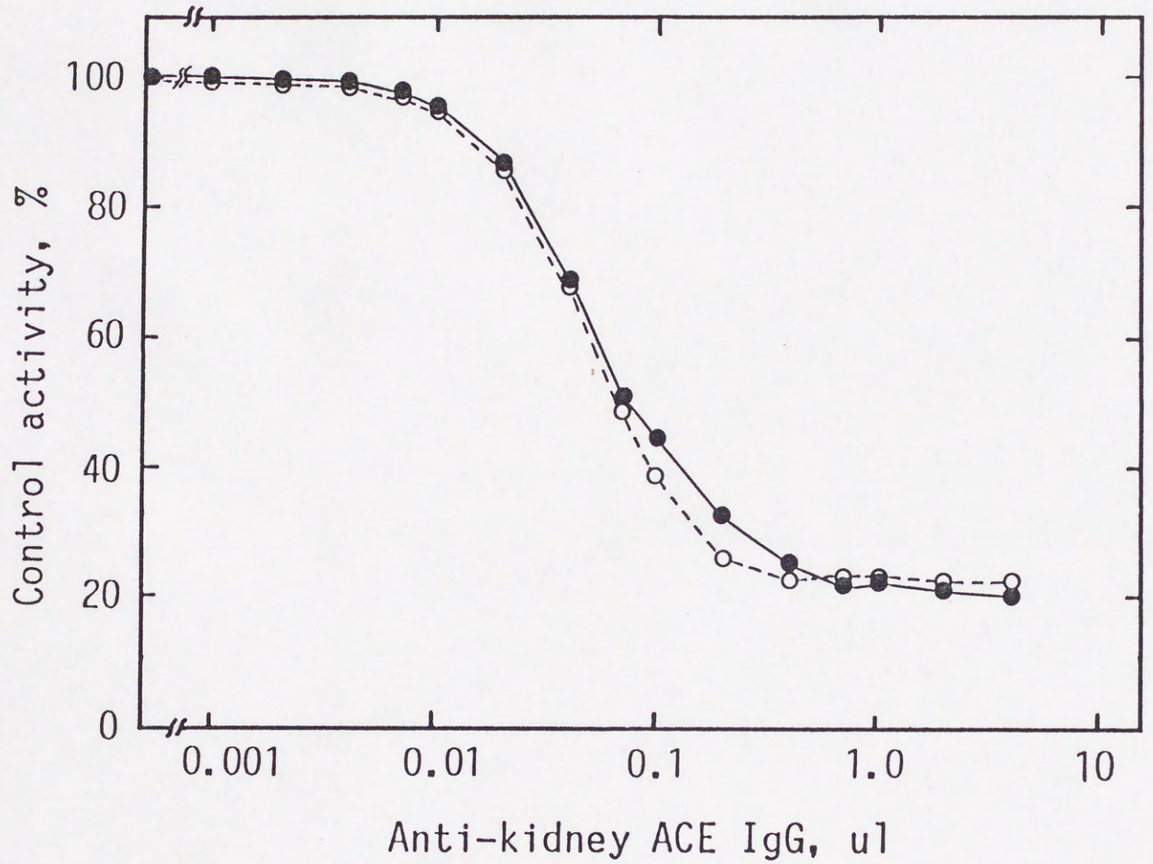

Fig. 14. Titration of purified and partially purified ACE activity with anti-human kidney ACE IgG.
ACE(P-I & II;100 ul = 25 mU) were incubated with 100 ul of serially diluted solution of anti-human kidney ACE IgG(0.66 mg/ml) for 20 hour at 4 C. After centrifugation at 13,000 g for 20 min at 4 C, supernatants(100 ul) were assayed of the remaining ACE activities for 50 min at 37 C.

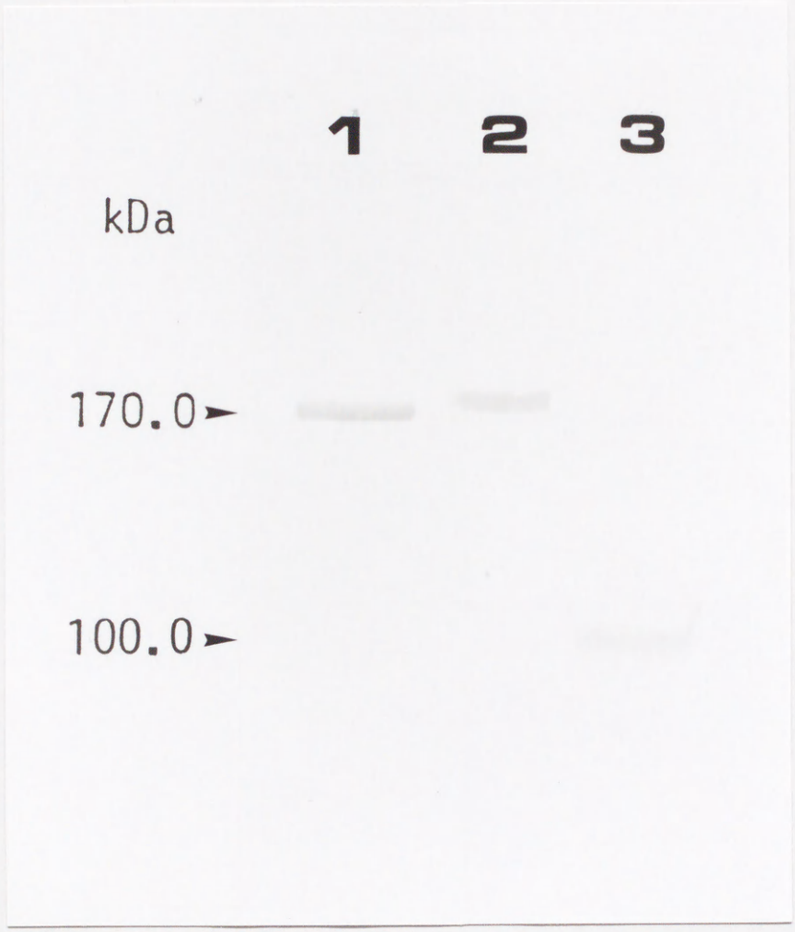

Fig. 15. Immunofixation of purified and partially purified human ACE transferred to nitrocellulose-membrane by Western-blooting procedure after SDS-PAGE.

Samples are as follows:lane 1; pure human kidney enzyme(500 ng), lane 2; pure human intestinal enzyme(P-I:500 ng), lane 3; partially purified human intestinal enzyme(P-II:170 mU).

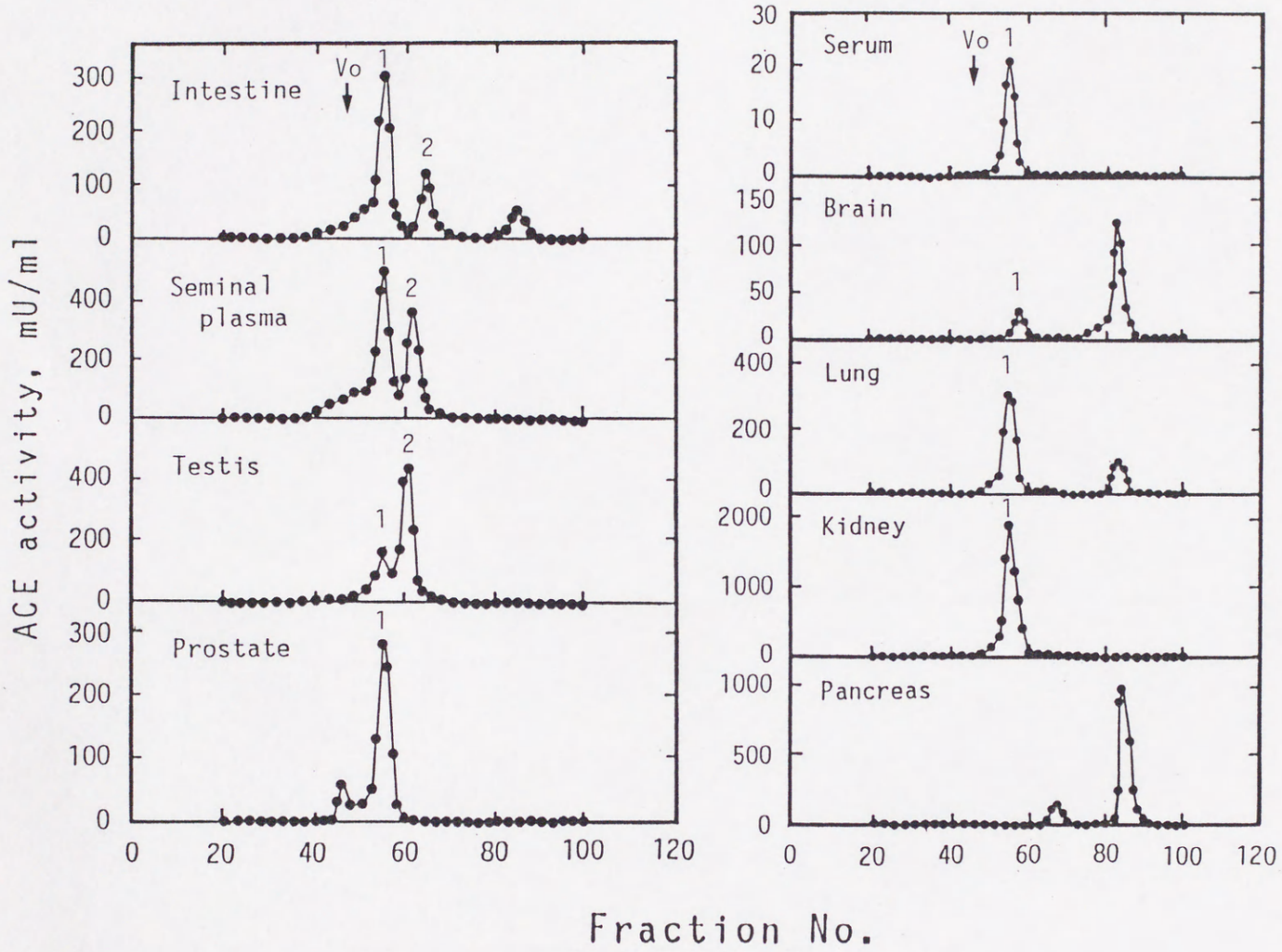

Fig. 16. Sephacryl S-300 HR chromatographic behavior of ACE in human tissues and biological fluids.
Samples(brain, lung, kidney and testis) were extracted by treatment with trypsin, and others were not treated with trypsin, as described in "Experimental Methods". The column was eluted at the flow rate of 13 ml/h. Each fraction volume was 4.3 ml and enzyme activities were assayed. Peak 1 and 2 showed ACE.

Table 1.

RELATIVE SOLUBILITY OF ANGIOTENSIN-CONVERTING ENZYME IN VARIOUS HUMAN TISSUES

Tissue	ACE activity, mU/g tissue		Soluble activity (%)
	Whole homogenate	Supernatant	
Cerebrum	36.1	5.6	15.5
Cerebellum	18.7	0.8	4.3
Lung	941.3	150.9	16.0
Heart	73.1	14.4	19.7
Liver	191.6	24.1	12.6
Spleen	10.3	5.2	50.5
Kidney	1747.2	72.3	4.1
Stomach	63.5	43.2	68.0
Small intestine*	594.4	32.2	5.4
Duodenum	630.6	574.5	91.1
Jejunum	773.2	760.8	98.4
Ileum	395.2	329.5	83.4
Colon	202.7	192.4	94.9
Prostate	287.0	206.4	71.9
Testis	794.4	17.4	2.2
Epididymis	1284.9	385.0	30.0
Uterus	158.9	67.4	42.4
Aorta	26.8	11.0	41.0

*: Freshly prepared sample

Table 2.

PURIFICATION OF HUMAN INTESTINAL ANGIOTENSIN-CONVERTING ENZYME

Purification steps	Total protein (mg)*	Total activity (U)**	Specific activity (U/mg)	Purification (-fold)	Yield (%)
35,000 g Supernatant	4033	249.0	0.062	1.0	100
Ammonium sulphate fractionation	1931	248.6	0.129	2.1	100
DEAE-Cellulose	1321	211.2	0.161	2.6	85
Lisinopril-linked Sepharose 6B	0.714	67.3	94.293	1520.0	27
Sephacryl S-300 HR					
P-I	0.314	49.1	156.369	(2522.1)	20
P-II	0.165	5.3	32.121		2

*:Protein determined by the methods described in "Experimental Methods".
**:One units is one μmole HHL hydrolyzed per min at 37 C under standard assay conditions.