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Isolation and Characterization of Pepsinogen from *Trimeresurus flavoviridis* (Habu Snake)

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Pepsinogen was isolated from the gastric mucosa of *Trimeresurus flavoviridis* (Habu snake) by DEAE-cellulose and DEAE-Sepharose ion-exchange chromatographies, and Sephacryl S-200 gel-chromatography. The yield calculated from the crude extract was 29% with 6.2-fold purification. The purified pepsinogen gave a single band on both native- and SDS-PAGE. As no other active enzyme was detected on the chromatographies, it was concluded that the Habu snake has one major pepsinogen. The molecular mass of the pepsinogen was estimated to be 38 kDa by SDS-PAGE. The sequence of the N-terminal 26 amino acid residues was determined and compared with those of other pepsinogens. The N-terminal structure of Habu snake pepsinogen was more homologous with those of mammalian pepsinogens C than those of mammalian pepsinogens A. The pepsinogen was rapidly converted to pepsin by way of an intermediate form induced by acidification. The optimum pH of Habu snake pepsin for bovine hemoglobin was 1.5–2.0, and it retained full activity at pH 6.2 and 30°C on incubation for 30 min. The optimum temperature for the snake pepsin was 50°C and it was stable at 40°C on incubation for 10 min. The proteolytic activity of the pepsin toward bovine hemoglobin was about two times higher than that of porcine pepsin A, however, the activity toward oxidized bovine insulin B-chain was lower than that of porcine pepsin A, and it did not hydrolyze oligopeptides. The specificity for oxidized bovine insulin B-chain of the pepsin was different from that of porcine pepsin A. Habu snake pepsin was inhibited by pepstatin A but not by serine, cysteine, or metallo protease inhibitors.

Key words: Habu snake, pepsin, pepsinogen, protease.

Many proteases have been isolated from snake venom and characterized (*1–5*). However, no snake proteases secreted from the digestive tract have been isolated to date. Snakes swallow animals without chewing and have enormous digestive capacities. Therefore, it is supposed that snakes have powerful digestive enzymes, especially in the stomach. It is well known that pepsin is responsible for the preliminary digestion of proteins. Pepsin is secreted as pepsinogen from the gastric mucosa of the gastric lumen and converted to the mature enzyme autocatalytically under acidic conditions (*6*). To date, pepsins and pepsinogens have been isolated and characterized from many mammals, however, among non-mammalian species, pepsinogens have only been isolated from birds (*7, 8*), fishes (*9–11*), frog (*12*), and turtle (*13*). The structural features and properties of non-mammalian pepsinogens have not been elucidated. Thus, it is important to isolate and characterize more pepsinogens and pepsins from non-mammalian species, especially reptiles and amphibians. Here, we isolated a pepsinogen from the Habu snake and investigated its properties.

MATERIALS AND METHODS

Materials—DEAE-cellulose was purchased from Whatman (Kent, England). DEAE-Sepharose and Sephacryl S-200 were products of Pharmacia Biotec (Uppsala, Sweden). Porcine pepsin was obtained from Sigma Chem. (USA). Bovine hemoglobin was purchased from Wako Pure Chem. (Osaka). *Trimeresurus flavoviridis* (Habu snake) were obtained on Amamiooshima, Kagoshima Prefecture.

Assay for Protease Activity—The amounts of active enzymes were determined by titration using dansyl-pepstatin (*14*). Pepsin activity was determined essentially by the method of Anson (*15*) with slight modification. An enzyme solution was added to 1 ml of a heat-denatured bovine hemoglobin solution (1.25% in 0.1 M citrate buffer, pH 2.0), followed by incubation for 10 min at 30°C. To this solution was added 2 ml of 5% trichloroacetic acid and then the whole mixture was incubated for 30 min. The resulting precipitate was filtered off with Advantec Toyo filter paper No. 5C and then the absorbance at 280 nm was measured. One unit of activity was defined as the amount of enzyme which

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Abbreviations: DFP, diisopropyl fluorophosphate, MIA, monoiodoacetic acid; PCMPS, *p*-chloromercuriphenylsulfonic acid; PMSF, phenylmethanesulfonylfluoride.

caused an increase of 1.0 in the absorbance at 280 nm per minute under the above assay conditions.

Determination of Protein—Protein concentrations were determined by the method of Lowry *et al.* (*16*) with bovine serum albumin as a standard.

Preparation of a Crude Extract—All purification procedures were performed at 4°C. The gastric mucosa (35 g) from the stomachs of 10 Habu snakes was cut into small pieces and then reduced to powder with 2 times the amount of dry ice using a mixer. To this powder was added 100 ml of 50 mM sodium phosphate buffer, pH 7.5 (buffer A), and then the mixture was stirred for 1 h. The mixture was centrifuged at 10,000 $\times g$ for 10 min and the resulting residue was reextracted with 70 ml of the same buffer and the supernatants were combined.

DEAE-Cellulose Chromatography—The crude extract (150 ml) was applied to a column (4.5 × 26 cm) of DEAE-cellulose equilibrated with buffer A and eluted with a linear gradient of NaCl (0 to 0.5 M) in a total volume of 1,000 ml of buffer A at the flow rate of 60 ml/h. Fractions of 11 ml were collected. The active fractions (Fr. 220–285) were combined and solid ammonium sulfate was added to 80% saturation. The resulting precipitate was collected by centrifugation, dissolved in 50 ml of buffer A and then dialyzed against buffer A.

DEAE-Sepharose Chromatography—The dialyzed solution was applied to a DEAE-Sepharose column (2 × 21 cm) equilibrated with buffer A and eluted with a linear gradient of NaCl (0 to 0.25 M) in a total volume of 1,000 ml of buffer A at the flow rate of 60 ml/h. Fractions of 10 ml were collected. The active fractions (Fr. 45–74) were combined and solid ammonium sulfate was added to 80% saturation. The resulting precipitate was collected by centrifugation, dissolved in 20 ml of buffer A and then dialyzed against buffer A.

Sephacryl S-200 Chromatography—A quarter of the dialyzed sample was concentrated with MILLIPORE IGC to 3 ml and then applied to a Sephacryl S-200 column (1.8 × 42 cm) equilibrated with buffer A. The protein was eluted with buffer A at the flow rate of 30 ml/h, fractions of 4.6 ml being collected. The active fractions (Fr. 32–35) were combined and solid ammonium sulfate was added to 80% saturation.

Polyacrylamide Gel Electrophoresis—SDS-PAGE under reducing conditions was performed according to the procedure of Laemmli (*17*) and the proteins were stained with Coomassie Brilliant Blue. The M_r markers were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonate dehydrogenase (30,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,000). Native PAGE was performed according to the procedure of Laemmli without SDS.

N-Terminal Sequencing Analysis—Automated Edman degradation of the purified pepsinogen was performed with an Applied Biosystems 477A protein sequencer. The phenylthiohydantoin derivatives were identified with an Applied Biosystems 120A analyzer.

Activation of Pepsinogen to Pepsin—Ten microliters of the pepsinogen solution (150 μM in buffer A) was mixed with 90 μl of 0.1 M citrate buffer, pH 2.0 (buffer B), followed by incubation at 0°C. Aliquots of 10 μl were removed at appropriate intervals, mixed with 40 μl of SDS-PAGE buffer and then subjected to SDS-PAGE.

Properties of Habu Snake Pepsin—The effects of pH and temperature on the activity and stability of Habu snake pepsin were determined with hemoglobin as a substrate. The assay conditions are given in the legend to Fig. 5.

Digestion of Bovine Insulin B-Chain—To 100 μl of a oxidized bovine insulin B-chain solution (1 mg/ml, 0.1 M HCl) was added 10 μl of the pepsin solution (0.5 μM). The mixture was incubated at 30°C. After 5 and 30 min, 50 μl of the solution was withdrawn, to which was added 10 μl of a 100 μM pepstatin solution in ethanol. The hydrolysis products were analyzed with an Applied Biosystems 477A protein sequencer and then 50 μl of the digested mixture was subjected to the HPLC assay [Applied Biosystems Model 150A; column, SPHERI-5 RP-18, 50 × 4.6 mm, Brownlee; linear gradient, from 0.1% TFA to 40% of 2-propanol/acetonitrile/water (49:21:30, v/v) containing 0.1% TFA for 30 min at the flow rate of 0.5 ml/min; detection at 220 nm]. The separated peptides were analyzed with the protein sequencer.

Digestion of Synthetic Peptides—To 100 μl of Gly-Gly-Phe-Phe-Gly and Gly-Gly-Leu-Phe-Gly solutions (1 mM, 0.1 M citrate buffer, pH 2.5) was added 5 μl of the pepsin solution (100 μM), followed by incubation at 30°C. After incubation for 30 min, 20 μl of the pepstatin solution (100 μM in ethanol) was added. The reaction mixtures were subjected to the HPLC assay as described above. The resulting products were identified in comparison with authentic peptides.

Effect of Inhibitors—Stock solutions of protease inhibitors were prepared in buffer B as follows: PMSF (2 mM), DFP (4 mM), PCMPS (2 mM), MIA (2 mM), EDTA (2 mM), and pepstatin A (0.04 mM). The enzyme solution (50 μl, 0.5 μM) was added to 50 μl of an inhibitor solution, followed by incubation at 30°C for 60 min, and then the remaining activity was measured by the assay method described above.

RESULTS AND DISCUSSION

The data obtained on purification of the pepsinogen are summarized in Table I. The yield calculated from the crude extract was 29% with 6.2-fold purification.

Pepsinogen was eluted as one major peak upon chromatography on DEAE-cellulose. As shown in Fig. 1, a small amount of activity was eluted in the pass through fractions, but this activity was completely adsorbed and eluted at the same elution volume as the adsorbed enzyme on rechromatography on DEAE-cellulose under the same conditions. It seems that the activity present in the pass through fraction was due to overflowing pepsinogen. Moreover, one active peak was eluted on DEAE-Sepharose and Sephacryl S-200 column chromatography, respectively (data not shown). Hirasawa *et al.* separated turtle pepsinogen into nine isoforms by Q-Sepharose column chromatography (*13*). Then, we subjected the DEAE-cellulose purified Habu snake pepsinogen to Q-Sepharose column chromatography under the same conditions as those described by Hirasawa *et al.*, although only one major active peak appeared (data not shown). To determine the purity of the pepsinogen, the purified pepsinogen was subjected to the HPLC assay with a mini Q column. The pepsinogen was not eluted as a clear peak because the adsorption of the pepsinogen to the column was too strong for the elution of clear peaks. The purified pepsinogen gave a single band on both native- and SDS-PAGE (Fig. 2, A and B). On the other hand, Hirasawa *et al.* showed that pepsinogen isoforms could be separated

by native-PAGE (*13*). The N-terminal amino acid sequence of the pepsinogen showed uniformity, indicating that it was isolated in a pure form. No other appreciable protease activity was detected on the chromatographies, indicating that the Habu snake has one major pepsinogen, and if other isoforms are present, their amounts must be small. This is in contrast with the multiple isoforms of pepsinogen found in many mammals, fishes, turtle, and frog (*6*, *9–11*, *13*, *18*).

The molecular mass of pepsinogen was estimated to be 38 kDa by SDS-PAGE (Fig. 2B). Other pepsinogens were reported to have molecular masses of about 40–43 kDa. It was reported that the molecular masses of some pepsinogens C estimated by SDS-PAGE were about 2–3 kDa smaller than those estimated from their amino acid compositions (*10*, *19–24*), so the molecular mass of the Habu snake pepsinogen should be about 40–41 kDa.

The N-terminal amino acid sequence of the enzyme was determined up to position 26 (Fig. 3). The sequence of the Habu snake pepsinogen was aligned with those of pepsinogens from other animals, the identity being as follows: human A 46% (*25*), porcine A 38% (*26*), chicken A 35% (*27*), turtle A 35% (*13*), human C 62% (*20*), monkey C 62% (*22*), frog C 58% (*18*), and rat C 46% (*24*). In particular, positions 14 and 16 of Habu snake pepsinogen are Glu and Met, in common with other pepsinogens C. On the other hand, positions 12, 21, and 22 of many pepsinogens A are Leu and position 24 is Asp, but these positions of Habu snake pepsinogen are Met, Val, Asn, and Pro, respectively. This indicates that Habu snake pepsinogen belongs to the pepsinogen C group.

Pepsinogen is rapidly activated at pH 2, and converted to pepsin within 15 s at 30°C (data not shown). Figure 4 shows the time course of the activation of the pepsinogen at 0°C. The pepsinogen was converted to pepsin through an intermediate form.

The optimum pH of the pepsin for heat-denatured bovine hemoglobin was 1.5–2.0 (Fig. 5A), as in the cases of other pepsins. However, the enzymatic activity of Habu snake pepsin was retained at a relatively high pH, for instance, 74% of the activity was retained at pH 3.5 compared to only 23% of the activity of porcine pepsin A (Fig. 5A). Similarly, bullfrog pepsin C retained about 90% of the activity at pH 3.5 (*18*).

As shown in Fig. 5B, Habu snake pepsin was stable at pH 6.2 for 30 min, while porcine pepsin A lost most of its activity under these conditions. It has been reported that non-mammalian pepsins were stable at pH 7.0 (*7*, *12*).

The optimal temperature for Habu snake pepsin was 50°C while that for porcine pepsin A was 60°C (Fig. 5C). Gildberg pointed out that the pepsins of cold-blooded animals with a low body temperature had low optimum temperatures compared with the pepsins of homoisothermal animals (*28*). As shown in Fig. 5D, the snake pepsin was unstable at high temperature.

The proteolytic activities of Habu snake pepsin and porcine pepsin were compared. It has been difficult to compare the activities of pepsins because the purities of the pepsins were different from each other. In the previous paper, we reported the measurement of active pepsin (*14*), the concentrations of active pepsins agreeding with the method used. Ten microliters of the pepsin solution (2.5 μM) was added to the bovine hemoglobin solution (pH 2.0), and then the proteolytic activities were measured. The increase in absorbance at 280 nm for porcine pepsin was 0.098, while that for the snake pepsin was 0.198. The proteolytic activity of

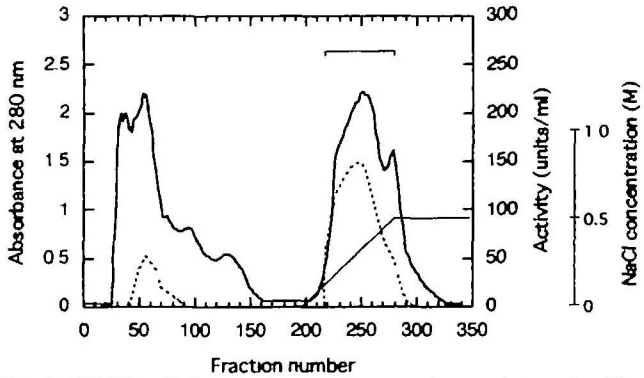

Fig. 1. **DEAE-cellulose anion exchange chromatography.** The crude extract (150 ml) was applied to a column (4.5 × 26 cm) of DEAE-cellulose equilibrated with buffer A and eluted with a linear gradient of NaCl (0 to 0.5 M) in a total volume of 1,000 ml of buffer A at the flow rate of 60 ml/h. Fractions of 11 ml were collected. Solid line, absorbance at 280 nm; dotted line, protease activity

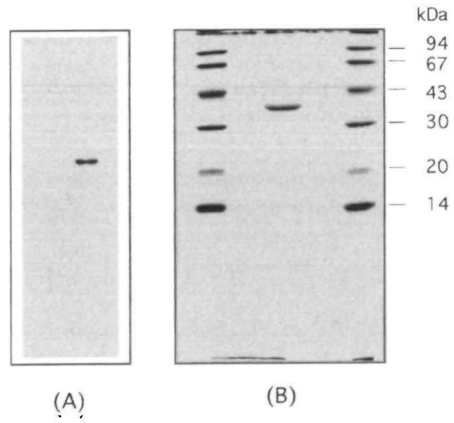

Fig 2. **Polyacrylamide gel electrophoresis.** SDS-PAGE was performed according to Laemmli, and the proteins were stained with Coomassie Brilliant Blue. Native PAGE was performed without SDS. (A) Native PAGE; (B) SDS-PAGE.

TABLE I **Purification of Habu snake pepsinogen.**

Purification step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Activity yield (%)	Purification (-fold)
Crude extract	126,000	2,400	53	100	1
DEAE-cellulose	76,450	770	99	61	1.9
DEAE-Sepharose	55,560	328	169	44	3.2
Sephacryl S-200	36,630	112	327	29	6.2

the snake pepsin was about two times higher than that of the porcine pepsin. It has been reported that pepsins C had higher activities than pepsins A (*21*, *23*, *29*), which agreed with in the case of other animal pepsinogens C. Furthermore, Norris and Mathies reported very high activity of fish pepsin (*30*).

To determine the substrate specificity of Habu snake pepsin, oxidized bovine insulin B-chain and synthetic oligopeptides were hydrolyzed with porcine and Habu snake pepsins. The hydrolysis products were analyzed by means of the HPLC assay and amino acid sequencing. As shown in Fig. 6, three peaks appeared after the incubation (incubation times: porcine pepsin, 5 min; Habu snake pepsin, 30 min). The amounts of hydrolysis products were little after an incubation time of 5 min for Habu snake pepsin digestion (data not shown). Peaks P-1 and H-1, P-2 and H-2, P-3 and H-3, and P-4 and H-4 were identified as a 1-14 fragment, a mixture of 1-15 and 16-30 fragments, a 15-30 fragment, and oxidized insulin B-chain, respectively. This shows that insulin B-chain was cleaved at Ala14-Leu15 and Leu15-Tyr16. Moreover, on amino acid sequencing analysis of the digested mixture with a protein sequencer, another cleavage site (Phe25-Tyr26) was identified in both enzymes. The cleavage sites of insulin B-chain with both the enzymes were the same but the hydrolysis rates were different. Habu snake pepsin cleaved Ala14-Leu15 faster than Leu15-Tyr16, but porcine pepsin A cleaved Leu15-Tyr16 faster than Ala14-Leu15 (Fig. 7) It has been reported that turtle pepsin, human pepsin A, human pepsin C, and shark pepsin cleaved Leu15-Tyr16 faster than Ala14-Leu15 (*13*, *11*). It is well known that pepsins prefer Phe, Tyr, and Leu at the P_1 position, so the Ala14-Leu15 bond did not agree with the normal pepsin specificity. Probably, the three-dimensional structure around this bond is preferred by Habu snake pepsin. The cleavage of Ala14-Leu15 and Leu15-Tyr16 must occur competitively because the exopeptidase activity of pepsin is weak, so if Ala14-

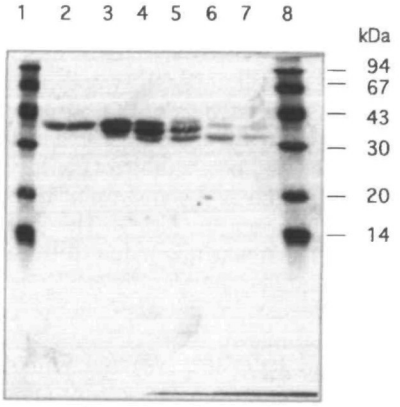

Fig 4. **Conversion of pepsinogen to pepsin.** Pepsinogen was incubated at pH 2 0 and 0°C, and aliquots were removed at appropriate intervals and subjected to SDS-PAGE Lanes 1 and 8, markers; lane 2, 0 s; lane 3, 15 s; lane 4, 30 s, lane 5, 1 min; lane 6, 5 min, lane 7, 10 min incubation.

Fig 3 **Comparison of the N-terminal amino acid sequence of Habu snake pepsinogen with those of various other pepsinogens.**

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
human A	I	·	Y	·	·	·	·	I	·	K	·	·	L	·	R	T	L	S	·	R	·	L	L	K	D	F	·
porcine A		L	V	·	·	·	·	V	·	K	·	·	L	·	Q	N	L	I	K	D	·	L	L	K	D	F	·
chicken A	S	I	H	R	·	·	·	·	K	G	·	·	L	·	K	Q	L	K	D	H	·	L	L	E	D	F	·
turtle A	L	V	T	·	·	·	·	Q	K	G	·	·	L	·	G	N	L	K	·	A	·	L	L	E	D	F	K
HABU snake		M	I	K	V	P	L	K	R	F	K	S	M	R	E	E	M	M	E	K	G	V	N	G	P	L	L
human C	A	V	V	·	·	·	·	·	K	·	·	·	I	·	·	T	·	K	·	·	·	L	L	·	D	F	·
monkey C	A	V	V	·	·	·	·	·	K	·	·	·	I	·	·	T	·	K	·	·	·	L	L	·	E	F	·
frog C		I	·	·	·	·	·	·	K	·	·	·	·	·	·	V	·	R	D	H	·	I	K	A	·	V	V
rat C	S	L	L	R	·	·	·	R	K	M	·	·	I	·	·	T	·	K	·	Q	·	·	L	K	D	F	·

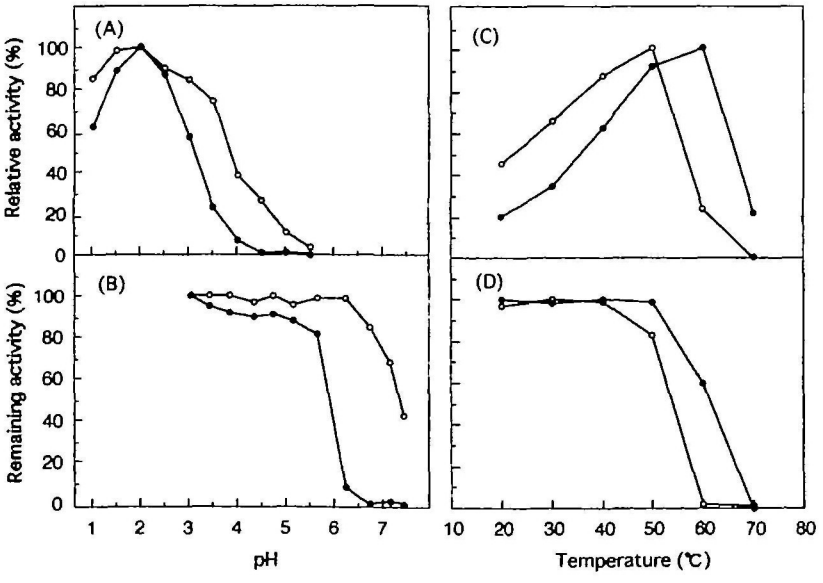

Fig 5 **Properties of Habu snake pepsin.** (A) Effect of pH on activity (B) pH stability (C) Effect of temperature on activity. (D) Temperature stability. (○) Habu snake pepsin, (●) porcine pepsin. The effect of pH on the activity of the protease toward bovine hemoglobin as a substrate was measured using 0.1 M citrate buffer (pH 1.5–5.0) pH stability was determined at the concentration of 2.5 μM in 0.1 M citrate buffer in the pH range of 2.0–8.0 for 30 min at 30°C. The effect of temperature on enzyme activity was determined in buffer B. Temperature stability was determined at the concentration of 2 5 μM in buffer B. Pepsin was heated for 10 min at 20–70°C, and then the remaining activity was measured

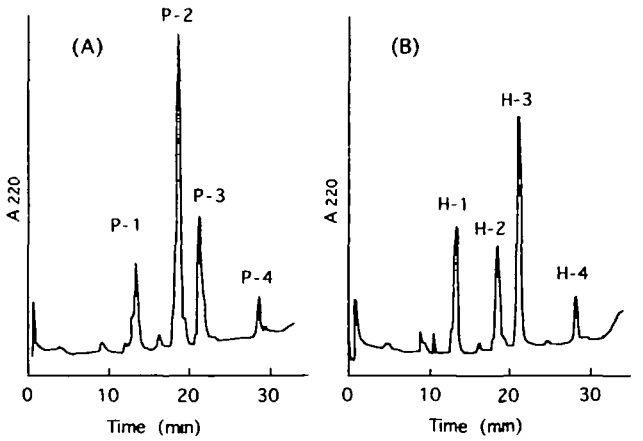

Fig. 6 **HPLC analysis of an insulin B-chain hydrolyzate.** Oxidized bovine insulin B-chain was incubated with porcine pepsin or Habu snake pepsin at 30°C. After the incubation, aliquots were withdrawn and then analyzed by the HPLC assay. (A) Porcine pepsin, incubation time of 5 min, (B) Habu snake pepsin, incubation time of 30 min.

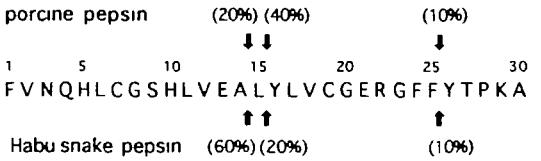

Fig 7 **Cleavage sites of insulin B-chain for porcine pepsin and Habu snake pepsin.** ↓ indicates a cleavage site and numerals in parentheses the rates of cleavage

Leu15 is cleaved initially, it is hard for the Leu15-Tyr16 bond to be cleaved. Furthermore, a 1-25 fragment of insulin B-chain was not detected even after 5 min incubation (data not shown). Then, the cleavage of Phe25-Tyr26 is followed by the cleavage of Ala14-Leu15 or Leu15-Tyr16. Moreover, insulin B-chain was cleaved about 5 times faster by porcine pepsin A than Habu snake pepsin (Fig. 6). In order to determine the specificities of the two pepsins for Phe and Leu, synthetic oligopeptides, Gly-Gly-Phe-Phe-Gly and Gly-Gly-Leu-Phe-Gly, were digested with both pepsins (*31*). The ratio of the hydrolysis rates for Gly-Gly-Phe-Phe-Gly and Gly-Gly-Leu-Phe-Gly was about 5:1 for porcine pepsin A (data not shown). However, these oligopeptides were not hydrolyzed by Habu snake pepsin even with a 10 times higher enzyme concentration (data not shown). It was concluded that Habu snake pepsin cleaved protein substrate such as hemoglobin faster than porcine pepsin A but the hydrolysis rate of polypeptide substrate was slower and did not cleave small oligopeptides. It was reported that fish pepsins had very high activity for hemoglobin but very low activity on small peptide substrates (*28*, *30*).

The effects of various compounds on the enzyme activity for bovine hemoglobin as a substrate were determined. Habu snake pepsin was completely inhibited by pepstatin A (0.02 mM), a typical carboxyl protease inhibitor, but not by inhibitors of serine, cysteine, or metallo proteases [PMSF (1 mM), DFP (2 mM), PCMPS (1 mM), MIA (1 mM), and EDTA (1 mM)].

As described above, Habu snake pepsinogen (pepsin) had typical properties of pepsinogens (pepsins) C, *i.e.* the amino acid sequence of the N-terminal, molecular mass estimated on SDS-PAGE and high activity toward hemoglobin, and ones of non-mammalian pepsins, *i.e.* the high activity toward hemoglobin, low activity toward small peptides, low optimum temperature, and pH stability.

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