Table V—Correlation Coefficients of Bioavailability Parameters Between Humans and Beagles

Beagle	Human	r
$C_{0.5}$[a]	C_1	0.789
	C_3	0.505
C_1	C_1	0.678
	C_3	0.448
C_{max}	C_{max}	0.388
t_{max}	t_{max}	0.711
AUC_{24}	$AUC_{47.5}$	0.306

[a] C_t shows the plasma or serum concentration at time t.

in beagles contrary to its lower absorption in humans. Considering the insignificant effects of the volume of water on the bioavailability, the ultramicrosize formulation discrepancy may be attributed to the physiological differences in the GI tract. None of the *in vitro* dissolution methods indicated such a superiority of the ultramicrosize formulation in beagles. These findings suggest that this formulation may disintegrate into the original ultramicrosize particulate state of the drug and allow rapid dissolution in the GI tract in beagles beyond the expectation from the *in vitro* dissolution findings.

The t_{max} values in beagles for different formulations were smaller than those in humans (Fig. 4). This suggests rapid transition of the drug to the absorption site, namely, fast gastric emptying of the drug in beagles. The good bioavailability of the ultramicrosize formulation may be related to the rapid gastric emptying of the drug which leads to dissolution of the drug in the small intestinal tract.

Formulation D provided higher C_{max} and plasma levels at earlier sampling times than Formulation C as observed in the human test. The *in vitro* dissolution rate of the drug from Formulation D was enhanced over that from Formulation C by pretreatment with plastic beads. These findings suggest that in beagles and humans there is a strong intens deaggregation action on the particles or aggregates of the drug dur their transition into the GI tract.

Although there was no significant difference in AUC_∞ values amc the formulations in humans, the AUC_{24} (considered as AUC_∞) of F mulations C and D were significantly lower than that of Formulatio: in beagles. This suggests their incomplete dissolution during passe through the GI tract and also suggests the short absorption site and fast transition of the drug in the GI tract in beagles as previously sho for the bioavailability of diazepam in beagles (3).

Although the *in vivo* findings of the ultramicrosize formulation beagles did not agree with those in humans, the bioavailabilities of t microsize formulations showed good agreement. Considering this, beag may serve as a useful animal model for bioavailability studies of cert griseofulvin tablet formulations, but not ultramicrosize ones.

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NOTES

Antibradykinin Active Material in *Aloe saponaria*

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Abstract □ A material having antibradykinin activity on isolated guinea pig ileum was partially purified from the nondialysate of the pulp of *Aloe saponaria* by repetition of gel chromatography using a hydrophilic polyvinyl gel and dextran gels. From the results of amino acid and carbohydrate analyses, the antibradykinin-active material was estimated to be a glycoprotein. It was found that this material catalyzes the hydrolysis of bradykinin at pH 7.4. The results of peptide analysis using reversed-phase high-performance liquid chromatography coupled with amino acid analysis indicate that this glycoprotein cleaves the Gly[4]-Phe[5] and Pro[7]-Phe[8] bonds of the bradykinin molecule.

Keyphrases □ Antibradykinin—active material in *Aloe saponaria*, guinea pig ileum, glycoprotein, high-performance liquid chromatography □ Glycoprotein—antibradykinin active material in *Aloe saponaria*, high-performance liquid chromatography, guinea pig ileum □ *Aloe saponaria*—antibradykinin active material, glycoprotein, high-performance liquid chromatography, guinea pig ileum

Cardiac stimulant action of the constituents in the dialysate of the pulp from *Aloe saponaria*[1] on isolated cardiac muscles has been reported (1). Antibradykinin activity of the nondialysate of the pulp has been examine here to obtain pharmacological evidence for its anti-in flammatory action (2). In this report, the results of partia purification of material having antibradykinin activit from *A. saponaria* on isolated guinea pig ileum and it proteolytic property against bradykinin are presented.

[1] *Aloe saponaria* is also known as white spotted *aloe* or soap *aloe*.

EXPERIMENTAL

Materials—The following materials were purchased from supplier dextran gel[2,3], hydrophilic polyvinyl gel[4], dialysis membrane[5], synthet bradykinin[6], and bromelain[7]. The gel filtrations were performed at roor temperature at a flow rate of 21 ml/hr using a microtube pump[8].

Methods of Analysis—Protein and carbohydrate contents in sample

[2] Sephadex G-100, Pharmacia Fine Chemicals, Uppsala, Sweden.
[3] Sephadex G-25, Pharmacia Fine Chemicals, Uppsala, Sweden.
[4] Toyopearl HW 40, Toyo Soda Mfg., Co. Ltd., Tokyo, Japan.
[5] Visking tube, Visking Co., Union Carbide Corp.
[6] The Protein Research Foundation, Osaka, Japan.
[7] Nakarai Chemical Co., Ltd., Kyoto, Japan.
[8] Tokyo Riakkikai Co., Ltd., Tokyo, Japan.

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were determined colorimetrically by a previously reported method (3) and the phenol–sulfuric acid method (4), respectively. Elution of materials through gel filtration was monitored by absorbance of effluents at 260 nm[9]. Paper electrophoresis[10] was carried out with a buffer solution of pyridine–acetic acid–water (1:10:489, pH 3.5) at 600 V for 2 hr. Paper chromatography was performed by developing the paper[10] with the solvent of butanol–pyridine–water (6:4:3). Peptide separation was accomplished by reversed-phase[11] high-performance liquid chromatography (HPLC)[12]. Peptides were applied to a column (4 × 250 mm), and the column was eluted with a linear gradient of ethanol concentration from 1% (40 ml) to 50% (40 ml), both containing 0.1% concentrated HCl at a flow rate of 0.4 ml/min (5). Peptide elution was monitored by absorbances of effluents at 210 and 260 nm[13]. The elution volume of bradykinin was 48 mL. For amino acid analysis the sample was hydrolyzed in 6 *N* HCl at 110° for 20 hr in evacuated sealed tubes. At the end, the hydrolysates were evaporated to dryness, dissolved in 0.02 *N* HCl, and analyzed with an amino acid analyzer[14]. Ultracentrifugation was performed on an analytical ultracentrifuge with an optical system[15] in a single sector at 20° and 56,000 rpm.

Assay Method for Antibradykinin—Antibradykinin activity was estimated by the biological assay on the guinea pig ileum by a previously described method (6). Briefly, a strip from 2 to 3 cm of guinea pig ileum was suspended in 10 ml of magnesium ion-free Tyrode solution which was oxygenated with oxygen–carbon dioxide (95:5) in an organ bath at 30°. The contractile responses of various concentrations of bradykinin were measured with a mechanoelectric transducer[16] equipped with a recorder[17] for 45 sec after injection. At the beginning of the assay, responses to a series of four doses of bradykinin (usually 25, 50, 75, and 100 ng dissolved in 1 ml of 10 m*M* phosphate buffer at pH 7.4) were checked, and each of them was repeated two or three times to obtain a standard dose–response curve. For measurement of anti-bradykinin activity, 0.2 ml of bradykinin solution (1 μg/ml) was incubated with 0.2 ml of a sample for 5 min at 30°, and the contractile response by 0.2 ml of this mixture (100 ng bradykinin equivalent) was measured. When the activity of a sample was so potent that >70% of bradykinin was inactivated, the sample was diluted with the same buffer to give at least 30 ng of bradykinin remaining after 5 min of incubation.

Extraction and Purification of Antibradykinin Active Material from *A. saponaria*—*Water Extraction*—The fresh leaves of *A. saponaria*[18] were harvested from the green house of the herbal garden of Kyushu University in September or December, 1979. The leaves (7 kg) were cut in half and colorless gelatinous pulp was separated carefully by scraping the green cortical layer containing yellow phenolics. The homogenized pulp (3 kg) was centrifuged at 10,000 rpm for 30 min. The supernatant was dialyzed with cellulose tubing[5] against distilled water for 48 hr. The nondialyzable fraction was lyophilized to yield colorless, soft crude Extract A (obtained from the leaves harvested in September, 5.8 g) or B (obtained from the leaves harvested in December, 7.0 g). No considerable difference between crude Extracts A and B in antibradykinin activity was observed (Table I).

Ammonium Sulfate Precipitation—Ammonium sulfate was added to a solution of crude Extract A (5.8 g) in distilled water (200 ml) to make 35% saturation, and after standing overnight it was centrifuged at 10,000 rpm for 30 min. The precipitate was dissolved in distilled water, dialyzed against distilled water for 48 hr, and then lyophilized to yield ammonium sulfate fraction (1.2 g).

Gel Filtration on Hydrophilic Polyvinyl Gel and Dextran Gels—Ammonium sulfate fraction (0.1 g) dissolved in 0.3 *M* NaCl (6 ml) was applied to a column of hydrophilic polyvinyl gel[4] (20 × 690 mm), and the column was eluted with the same solution. The eluate (108 ml) was dialyzed against distilled water followed by lyophilization to give polyvinyl gel fraction (50 mg). The fraction (50 mg) was subjected to filtration through a column of dextran gel[2] (20 × 790 mm) using 0.3 *M* NaCl as a solvent, and the eluate from the column was fractionated to three portions. Each portion was dialyzed against distilled water followed by lyophilization to yield gel filtration Fractions A (20 mg), B (10 mg), and C (negligible amount). Fraction B (120 mg) was further chromatographed through a column of dextran gel[3] (16 × 880 mm) using distilled water as a solvent followed by lyophilization to give Fraction D (26.3 mg).

[9] Hitachi 200-10 spectrophotometer.
[10] Toyo filter paper No 50, Toyo Roshi, Co. Ltd., Tokyo, Japan.
[11] Lichrosorb RP-8, 5 μm, Merck, Darmstadt, West Germany.
[12] Hitachi 635A liquid chromatograph.
[13] Hitachi 635M liquid chromatograph detector.
[14] Hitachi 835 amino acid analyser.
[15] Schlieren, Spinco Model E, Beckman Instrument Inc.
[16] FD-Pick up SB-L-TH, Nihon Koden, Tokyo, Japan.
[17] VP-651B, National Co., Ltd., Tokyo, Japan.
[18] A voucher specimen is available for inspection at Higashiyama Botanical Garden, Nagoya, Japan.

Table I—Antibradykinin Activity of Fractions on Isolated Guinea Pig Ileum

Sample	Protein, %	Carbohydrate, %	Unit/g[a]	Ratio
Crude Extract A	1.7	66[b]	6.34	1.00
Crude Extract B	1.9	63	8.94	1.41
Ammonium sulfate precipitate	2.4	68	27.9	4.40
Gel filtration Fraction A	—	72	6.0	0.94
Gel filtration Fraction B	7.2	63	85.4	13.47
Bromelain			1114.0	175.70

[a] One kininase unit was defined as the amount of enzyme preparation that could degrade 1 μg of bradykinin for 1 min, at 30°, pH 7.4. For statistical purposes 3–5 guinea pigs were used for each assay. [b] The measured content of carbohydrate and protein does not account for 100% of the material. This may reflect the presence of water, since a polymer is extremely hygroscopic.

RESULTS AND DISCUSSION

Extraction and Concentration of Antibradykinin Active Material from *A. saponaria*—The pulp of *A. saponaria* leaves was extracted with water and the extract was dialyzed against distilled water. The nondialyzable fraction (crude extract) showed antibradykinin activity when assayed on the inhibition of the contractile response on isolated guinea pig ileum. As can be seen in Table I, no considerable difference in antibradykinin activity was observed between crude Extracts A and B, which were obtained from the leaves harvested in September and December, respectively. Crude Extract A was precipitated with ammonium sulfate at 35% saturation. The precipitate gained 4.4 times the increased specific activity compared with crude Extract A (Table I). Several attempts to increase the specific activity by means of dextran gel chromatographies were unsuccessful, because the material was too viscous to obtain a practical flow rate. Thus, the chromatography over hydrophilic polyvinyl gel[4] was performed first to remove viscous material from the antibradykinin active material, which was then chromatographed on dextran gel[2] to give three fractions (A–C). As shown in Table I, Fraction A having low specific activity is composed of only carbohydrate, while Fraction B with 13.5 times higher specific activity than crude Extract A is composed of carbohydrate and a low content of protein. Fraction C was not analyzed because of its low yield. To remove contaminated salts completely, Fraction B was chromatographed over dextran gel[3] using distilled water as an eluting solvent to yield Fraction D. Further purification was not continued because of shortage of the sample (total yield, 0.003%). As a positive control, bromelain containing ~2% carbohydrate was employed for the assay and extremely potent antibradykinin activity was observed (7).

Characterization of Antibradykinin Active Material—To clarify the nature of the antibradykinin active material, the pH-dependency of antibradykinin activity and the thermal stability were studied using crude Extract A. Each solution of crude Extract A (10 mg) in 0.067 *M* phosphate buffer (1.0 ml) at various pH values (4.5, 5.0, 6.0, 7.0, 8.0, and 8.5) was kept standing at 30° for 1 hr.

After the dialysis, followed by lyophilization, antibradykinin activity of crude Extract A at each pH was estimated and expressed as percent of the activity at pH 7.4. The results showed that crude Extract A is comparably active at pH 5.0–7.0, but <20% of antibradykinin activity was observed at pH 4.5, 8.0, or 8.5. Antibradykinin activity was completely lost when crude Extract A was treated at 90° for 10 min. These results suggest that the antibradykinin active material may be a protein or a glycoprotein having enzymatic activity rather than a small molecule such as norepinephrine (8).

To ascertain this speculation, the purest material obtained here, Fraction D, was subjected to an ultracentrifugation experiment, uv spectral absorption measurement, amino acid analysis, and carbohydrate analysis. Fraction D in 0.3 *M* NaCl was analyzed by ultracentrifugation with an optical system[15] for determination of the sedimentation coefficient. Since Fraction D (10 mg/ml of 0.3 *M* NaCl) indicated a single peak having $S_{20,w}$ = 0.79 *S*, it appeared to be homogeneous in size. The UV absorption spectrum of Fraction D in 0.3 *M* NaCl indicated the presence of an absorption band with a maximum at 260 nm. On acid hydrolysis of Fraction D, only D-mannose was observed as sugar moiety on paper chromatography, and Asp, Thr, Ser, Glu, Gly, Ala, Val, Ile, Leu, Phe, Lys,

Arg, and Pro as amino acid moieties were observed on amino acid analysis. All of these results support the theory that the antibradykinin active material is a protein or a glycoprotein.

Action of the Antibradykinin Active Material Against Bradykinin Molecule—Bradykinin (0.5 mg) in 10 m*M* phosphate buffer (0.7 ml, pH 7.4) was incubated with 1.0 mg of Fraction D at 30° for 2 hr. The lyophilized mixture was subjected to paper electrophoresis with acetate–pyridine buffer (pH 3.5) to afford two ninhydrin positive spots of fast and slow moving ones compared with that of bradykinin which migrates towards the cathode. These two spots were not observed in the blank experiments without bradykinin or Fraction D.

The fast moving spot was eluted from unstained paper strips and the extract was subjected to amino acid analysis. The fast moving spot was confirmed to be a peptide composed of Arg, Pro, and Gly in the ratio of 1:2:1, which was consistent with the residues 1–4 (Arg^1-Pro^2-Pro^3-Gly^4) of bradykinin, indicating that Fraction D cleaved the bond between Gly^4 and Phe^5 in bradykinin and, therefore, had a kininase activity. The slow moving spot, however, was not clearly separated from the spot due to bradykinin on paper electrophoresis.

The incubation mixture after lyophilization was subjected to HPLC on a reversed-phase column. Three major peaks were observed at an elution volume of 6, 38, or 48 ml in HPLC, and each peak was collected and subjected to amino acid analysis to afford the following amino acid composition, respectively: Arg, Pro, Gly (1:2:1); Arg, Pro, Gly, Phe, Ser (1:3:1:1:1); and Arg, Pro, Gly, Phe, Ser (2:3:1:2:1).

Each composition was consistent with the structures Arg^1-Pro^2-Pro^3-Gly^4, Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7, and Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9 (bradykinin), respectively. All of these results indicate that the antibradykinin active material in *A. saponaria* has a kininase activity and cleaves the peptide bonds at *N*-terminuses of two phenylalanine residues in bradykinin.

As a kininase from plant origin, bromelain, papain, and ficin are known to cleave Gly^4-Phe^5 and Phe^5-Ser^6 bonds of the bradykinin molecule, while both shimejikininase (9) from mushroom, *Tricholoma congluobatum*, and kinonase AI (10) from microbes, *Streptomyces* species, cleave Gly^4-Phe^5 and Pro^7-Phe^8 bonds of the bradykinin molecule. Thus, the action of the glycoprotein obtained here is similar to that of shimejikininase and kinonase AI.

As one of the pharmacological evidences for anti-inflammatory activity of *A. saponaria*, the presence of an antibradykinin active glycoprotein was confirmed here. Further study on the anti-inflammation effect is in progress.

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