INTRODUCTION

Leguminous plants (Leguminosae), of which 12,000 varieties are widely distributed in nature, rank with grasses (Gramineae) of 4,000 varieties as staple farm crops. In general, leguminous seeds contain protein at higher level than cereal grains in which starch predominates. Fortunately, root nodule bacteria (Rhizobium species) are so parasitic as to supply nitrogen for their host, that is, leguminous plants. Consequently, it is expected that cultivation of legumes leads to fertility of the soil. In addition, leguminous seeds lose gradually moisture during the course of ripening, so that the seeds are harvested as dry beans or peas fit for a long-term storage. In this way, leguminous seeds are important not only as calorie source but also as protein source.

The future food crisis is becoming more serious, because of overflowing and increasing population in Asian and African countries. The food shortage is chronic with some of these countries, in which many people are undernourished. The shortage of protein with good quality is a serious problem in other countries as well. Animal proteins are well-known to be of the higher nutritional value but more expensive than plant proteins. However, about one-third of the grain crops in the world is consumed to raise livestock (1). In the tropical and subtropical areas, many inhabitants are suffering from protein deficiency and 80% of the world population will reside there

at the end of this century (2). For this reason, it must be considered to take in plant protein as it is. The role of leguminous seeds in protein nutrition is much intrested in this connection. Among a number of legumes, those harvested in the tropical and subtropical areas are more conveniently considered as protein source, because a few crops are produced a year. Nevertheless, many of them are not fully available yet. It therefore is worthwhile to further investigate on these legumes from the food and nutritional standpoints.

Until recently, winged bean has been evaluated only as a minor crop in the tropical areas such as India, Southeast Asia and Papua New Guinea in spite of its high protein and oil contents comparable to those of soybean (3). Winged bean has much more nodules on the root than any other legumes including soybean, peanut, kidney bean and garden pea (4). Because of having so many nodules, in other words high nitrogen fixation capacity, winged bean has attracted much attention as a potent candidate for protein source (5). Furthermore, it is difficult to cultivate soybean in the tropics, while the tropical environment of high temperature and humidity is suited to the cultivation of winged bean. In such a sense, winged bean is now one of the most promising legumes.

Some of cereals and legumes contain more or less toxic substances or antinutritional factors, which are apt to injure human or livestock health. Many efforts have been put forth in elucidating

these factors in food. Much intersting among them are inhibitors of digestive enzyme. These inhibitors are widespread in plants including the major crops such as cereals and legumes (6). Especially, protease inhibitors have been extensively studied as to their structure, function, physiological action and nutritional value (7-10). There is, however, little information on amylase inhibitors, despite their latent importance. Taking into account the use of staple farm crops as calorie source rather than as protein source, the effect of amylase inhibitor on amylolysis should be clarified to utilize more effectively the crops or to improve hyperglycemia or corpulency.

With respect to naturally occurring proteinaceous α-amylase inhibitors, Kneen and Sandstedt reported in 1943 that water-soluble amylase inhibitors cccurred in wheat, rye and some of sorghum cultivars (11) and in 1945 Bowman referred to leguminous α-amylase inhibitor (12). Since then, proteinaceous α-amylase inhibitors have been found in a variety of cereals, legumes and other plants (13, 14). So far as α-amylase inhibitors in leguminous plant are concerned, some of them have been already isolated from kidney bean (Phaseolus vulgaris) (15-19), but little is known about their functional and structural features as well as bioavailability.

This doctor dissertation deals with the results of investigation on analysis of protein and other components in leguminos seeds for in relation to their effective utilization (Chapter I) and on characterization of newly isolated α-amylase inhibitors from

Phaseolus vulgaris seeds (Chapter Ⅱ).

Chapter Ⅰ

ANALYSIS OF COMPONENTS IN LEGUMINOUS SEEDS

Section 1

Amino Acid Composition of Protein Fractions from Leguminous Seeds

On the basis of their solubility, seed proteins were classified by Osborne (20) into albumins, globulins, glutelins and prolamins. The relative proportion of each in the seed determines the nutritional quality of the total seed protein because each fraction tends to have a different characteristic amino acid composition (21). The content of the sulfur-containing amino acids also has been reported to affect the nutritional value of legume protein (22,23).

In this section, protein fractions were extracted from leguminous seeds with four different solvents and thier amino acid compositions were determined to estimate the relative quality of the extractable protein.

MATERIALS AND METHODS

Materials

Twelve dried legume samples comprised of three varieties of adzuki beans (Phaseolus angularis (Willd.) Wight), kafae bean (Phaseolus angularis (Willd.) Wight), red kafae bean (Phaseolus angularis (Willd.) Wight), three varieties of lima beans (Phaseolus limensis Macf.), red lima bean (Phaseolus limensis Macf.), two

varieties of kidney beans (Phaseolus vulgaris L.) and field bean [Vicia faba L.], were imported from the countries shown in Table 1. All the legume seeds were ground in a pulverizer. The sea sand C (40-80 mesh), quartz sand (150-180 mesh) and Celite-545 used for the extraction of the protein fractions were purchased from Nacalai Tesque Inc. (Kyoto). All other reagents were of analytical grade.

Extraction of protein fractions

Continuous extraction of the protein fractions based on their differential solubilities in various solvents was carried out according to the method of Maes (24). Whole seed flour (1.5 g) first was ground with 1.5 g of celite in a mortar then 30 g of sea sand. This mixture was packed into a column (2 x 30 cm) with quartz sand and sea sand (Fig. 1). The protein fractions were extracted successively with four different solvents in the following order: 2% sodium chloride, 30% isopropyl alcohol, 4% lactic acid and 0.5% potassium hydroxide. For the elution from the column, 200 ml of the first three solvents and 300 ml of potassium hydroxide solution were used. The effluent fractions were monitored at a wavelength of 280 nm, and fractions with an absorbance of 0.20 or more were collected. The fraction extracted with 2% sodium chloride was separated by the different protein solubilities in water as follows. After dialysis against distilled water at 4℃ for 72 h, the fraction was centrifuged at 5,700 x g for 10 min. The supernatant formed was used as the water-soluble

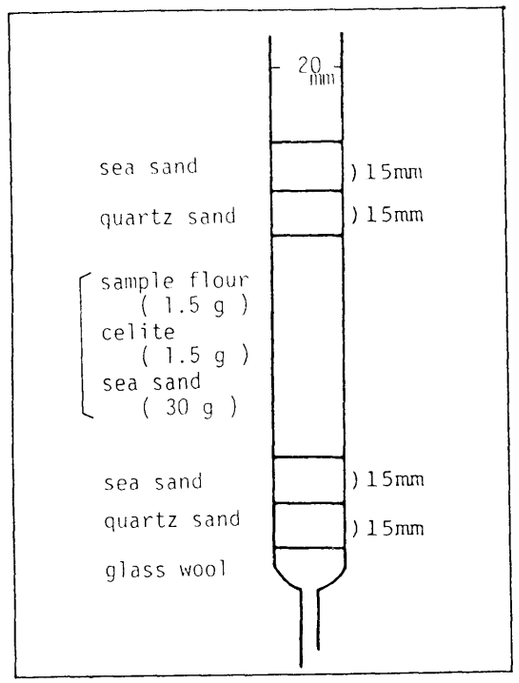

Fig. 1 *Column conditions for the extraction method of Maes. The sample flour was ground in a motar with celite and sea sand before being packed into the column.*

fraction (WS), and the precipitate was used as the water-insoluble fraction (WI). The other extracts were dialyzed against distilled water at 4℃ for 72 h. The fractions produced by these procedures were lyophilized.

Analytical method

The chemical composition of the whole seed flour and the protein extracts was determined by the mehtods of the Association of Official Analytical Chemists (25). The amino acid composition was determined with a Hitachi Model KLA-5 amino acid analyzer. Samples were hydrolyzed in 6 N HCl in vacuum-sealed tubes at 110℃ for 24 h.

Tryptophan was determined after alkali hydrolysis. All analyses were performed at least in duplicate.

RESULTS AND DISCUSSION

Chemical composition

Values for the chemical composition of the whole seed flour of the samples are shown in Table 1. The protein content in the sample

Table 1 *Chemical composition (dry basis) of the whole seed of sample legumes*

Legume	Protein[a] (%)	Fat (%)	Ash (%)	Moisture (%)
Adzuki bean (China)[b]	20.4	0.3	4.0	9.2
Adzuki bean (Thailand)	20.2	0.2	3.9	10.3
Adzuki bean (Taiwan)	22.3	0.2	3.3	12.7
Kafae bean (South Africa)	23.2	1.2	3.3	9.8
Red kafae bean (South Africa)	22.3	1.2	3.3	10.1
Lima bean (Burma)	19.6	0.8	3.2	10.7
Lima bean (South Africa)	19.8	1.2	4.1	10.8
Lima bean (Madagascar)	20.0	0.8	3.8	8.0
Red lima bean (Burma)	19.0	0.8	3.7	9.8
Kidney bean (Burma)	20.2	0.7	3.3	10.1
Kidney bean (Mexico)	20.4	1.1	3.9	9.7
Field bean (China)	27.7	1.0	3.2	11.5

[a] N x 6.25
[b] The legume samples were grown in the countries given in parentheses.

legumes ranged from 19.0-27.7%. The fat content of all the beans was generally less than 1.0%.

Extraction of protein fractions

The extraction profiles of the soluble protein fractions from seed flour are given in Table 2. All samples contained a high proportion (50-80%) of sodium chloride extract. Total recovery of pro-

tein in the extracts was more than 80% for all of the samples. Mitsunaga and Mitsuda (26) reported that the extraction method of Maes (24) gave a high recovery of protein from wheat grain. This sequential extraction is an effective method for the extraction of protein because it is convenient and gives high yield.

The sodium chloride extract was separatable into two fractions (WI and WS) based on its protein solubilities in water. WI was made up of globulins and WS of albumins. The ratio of the WI in the 2% sodium chloride extract is shown in Table 2. The residue of this WI is the WS content. A 2% sodium chloride solution was substituted for water as the first solvent of extraction because the raw legumes contained small amounts of inorganic salts which might have affected protein solubility. After lyophilization, the protein contents of WS and WI were determined. In all the samples, the content of WI was higher than that of WS.

Amino acid composition

The amino acid profiles of whole seed flour are shown in Table 3. High contents of lysine, leucine, aspartic acid and glutamic acid were found in all the legumes. Tha samples were generally low in the sulfur-containing amino acids. Evans and Boulter (27) showed that there is a different methionine content and a similar cystine con tent in peas and beans, whereas in this case, the same methionine content was found in lima bean and kidney bean varieties, respec-

Table 2 *Relative concentrations of the four soluble protein fractions from the seed flour of the sample legumes*[a]

Legume	2% Sodium chloride sol. (%)	WI[b] (%)	30% Isopropyl alcohol sol. (%)	4% Lactic acid sol. (%)	0.5% Potassium hydroxide sol. (%)	Total (%)
adzuki bean (China)	53.7	(85)	10.9	8.6	11.3	84.5
adzuki bean (Thailand)	51.2	(83)	9.5	8.4	9.9	79.0
adzuki bean (Taiwan)	57.5	(82)	10.6	8.8	8.5	85.4
kafae bean (South Africa)	56.9	(89)	14.2	2.4	9.8	83.3
red kafae bean (South Africa)	60.0	(72)	11.7	6.4	11.2	89.3
lima bean (Burma)	64.0	(94)	10.0	4.8	9.1	87.9
lima bean (South Africa)	74.0	(43)	10.2	2.8	7.0	94.0
lima bean (Madagascar)	81.4	(93)	6.7	3.9	7.1	99.1
red lima bean (Burma)	61.5	(79)	9.2	2.0	8.4	81.1
kidney bean (Burma)	68.9	(75)	8.2	3.6	10.4	91.1
kidney bean (Mexico)	60.2	(46)	9.8	3.6	11.8	85.4
field bean (China)	69.6	(54)	5.6	5.9	5.6	86.7

[a] All values are given in terms of Kjeldahl nitrogen x 6.25.
[b] WI content of the 2% sodium chloride extract

tively.

The essential amino acid patterns of the seed flours and the FAO/WHO (1973) provisional pattern (28) are shown in Table 4. All samples had more lysine, leucine and total aromatic amino acids than the FAO/WHO (1973) pattern, whereas the total sulfur-containing amino acids were less in all of the samples. Similar results have been reported for the amino acid composition of other legumes (22,29).

The amino acid composition of the main soluble fraction (Wl and WS) is shown in Table 5. Because it appears that much of the data does not differ significantly, the mean values for each of the beans listed in this Table are given there. The high lysine contents in the legumes may be attributed to the high globulin and low prolamin contents as described by Johnson and Lay (21).

Table 3 *Amino acid composition (g per 16g N) of the seed flour of the sample legumes*

Amino acid	Adzuki bean (China)	Adzuki bean (Thailand)	Adzuki bean (Taiwan)	Kafae bean (South Africa)	Red kafae bean (South Africa)	Lima bean (Burma)
Aspartic acid	11.4	12.9	11.5	10.8	9.9	11.2
Threonine	3.0	3.2	3.4	3.5	3.1	4.1
Serine	4.4	5.0	4.8	4.5	4.0	5.6
Glutamic acid	10.5	12.0	12.1	14.9	13.0	10.7
Proline	3.4	4.5	4.5	4.4	3.6	3.1
Glycine	3.8	4.2	3.6	3.7	3.4	3.6
Alanine	5.6	8.7	6.5	6.5	5.8	7.7
Valine	5.0	5.2	5.0	5.0	4.5	4.6
Cystine	1.0	0.6	1.1	0.7	1.1	0.8
Methionine	0.8	1.0	0.9	0.7	0.9	0.8
Isoleucine	4.1	4.5	3.4	3.9	3.6	4.6
Leucine	7.6	9.9	7.8	8.8	8.1	9.2
Tyrosine	2.5	3.0	2.2	2.8	2.5	3.1
Phenylalanine	4.4	6.7	4.3	4.7	4.3	4.9
Lysine	6.6	8.2	6.1	7.3	7.6	5.9
Histidine	2.0	3.0	2.0	2.2	2.2	2.1
Arginine	5.6	5.5	5.2	7.8	7.4	5.4
Tryptophan	0.7	1.0	1.2	0.8	1.3	1.1

Amino acid	Lima bean (South Africa)	Lima bean (Madagascar)	Red lima bean (Burma)	Kidney bean (Burma)	Kidney bean (Mexico)	Field bean (China)
Aspartic acid	10.9	11.5	10.9	10.4	10.0	9.9
Threonine	4.3	3.7	3.9	3.7	3.4	3.6
Serine	5.6	5.9	5.0	5.2	4.7	4.2
Glutamic acid	13.1	11.1	11.0	10.2	12.7	14.3
Proline	2.0	4.4	3.1	4.0	2.0	3.6
Glycine	4.0	3.7	3.9	3.7	4.0	4.0
Alanine	6.3	7.2	5.3	6.4	5.6	5.1
Valine	5.1	4.9	4.5	4.7	4.7	4.4
Cystine	1.3	1.0	0.6	1.0	1.0	0.7
Methionine	0.8	0.8	0.8	0.7	0.7	0.6
Isoleucine	4.0	4.0	3.7	4.2	3.7	4.2
Leucine	8.8	8.0	8.6	8.2	8.3	9.0
Tyrosine	3.0	2.8	2.4	3.0	2.7	2.5
Phenylalanine	4.8	4.9	4.0	4.7	4.4	3.8
Lysine	8.1	5.6	7.1	5.7	6.6	6.2
Histidine	2.5	2.2	2.1	2.2	2.0	1.8
Arginine	6.8	4.3	6.1	4.7	5.6	7.6
Tryptophan	1.3	1.8	0.5	0.9	0.6	1.0

Table 4 *Essential amino acid composition (mg per g N) of the seed flour of the sample legumes*

Legume	Lysine	Threo-nine	Total S. cont. A.A.	Valine	Iso-leucine	Leucine	Total aromatic A.A.	Trypto-phan
adzuki bean (China)	413	188	113	313	256	475	431	44
adzuki bean (Thailand)	513	200	100	325	281	619	606	63
adzuki bean (Taiwan)	381	213	125	313	213	488	406	75
kafae bean (South Africa)	456	219	88	313	244	550	469	50
red kafae bean (South Africa)	475	194	125	281	225	506	425	81
lima bean (Burma)	369	256	100	288	288	575	500	69
lima bean (South Africa)	506	269	131	319	250	550	488	81
lima bean (Madagascar)	350	231	113	306	250	500	481	113
red lima bean (Burma)	444	244	106	281	231	538	400	31
kidney bean (Burma)	356	231	106	294	263	513	481	56
kidney bean (Mexico)	413	213	106	294	231	519	444	38
field bean (China)	388	225	81	275	263	563	394	63
FAO/WHO (1973) pattern	340	250	220	310	250	440	380	60

Table 5 *Amino acid composition (g per 16g N) of the soluble fractions (WI and WI) of the sample legumes*[a]

	Adzuki beans		Kafae and red kafae beans		3 lima & red lima beans		2 Kidney beans		Field bean	
Amino acid	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS
Aspartic acid	11.0	11.3	11.2	10.7	11.7	12.8	12.4	12.6	11.3	12.6
Threonine	2.9	4.9	3.3	4.2	4.3	6.1	4.3	7.2	3.4	6.6
Serine	5.1	6.2	5.5	5.3	6.2	7.9	6.6	8.4	5.3	6.2
Glutamic acid	18.6	16.1	19.5	17.1	14.8	11.3	17.7	10.5	19.1	17.9
Proline	4.6	3.8	4.8	4.4	4.0	5.1	4.3	4.2	4.5	5.1
Glycine	3.0	4.1	3.1	4.1	3.9	3.5	4.1	3.7	4.5	5.3
Alanine	5.6	8.3	5.9	7.6	6.2	6.3	6.4	5.8	4.1	9.7
Valine	5.1	4.5	5.7	4.8	5.9	5.3	5.8	5.8	4.6	5.3
Cystine	1.0	1.9	0.8	0.9	0.6	1.6	1.1	2.2	1.3	1.9
Methionine	1.2	1.2	1.2	0.7	1.1	0.8	1.2	0.7	0.6	0.7
Isoleucine	4.2	3.8	4.6	4.2	5.6	4.4	3.9	4.6	4.1	3.9
Leucine	10.6	7.6	10.4	7.9	11.8	9.5	9.9	8.8	9.9	5.6
Tyrosine	3.2	3.2	3.3	3.3	4.1	4.0	3.9	4.3	3.2	3.7
Phenylalanine	6.2	4.0	6.4	5.6	6.2	5.9	5.5	5.7	4.0	2.9
Lysine	7.2	8.8	7.2	7.4	7.0	6.6	7.0	6.2	6.4	8.6
Histidine	2.2	1.9	2.3	1.9	2.5	2.1	2.0	2.4	1.9	2.7
Arginine	7.0	5.3	5.9	5.8	5.6	4.2	4.9	4.1	8.4	5.4
Tryptophan	0.7	0.5	0.7	1.2	0.9	1.3	1.1	1.5	0.6	0.6

[a] Values given are mean values for each of the samples listed.

Nutritional Components, Protease Inhibitor and Hemagglutinin in Leguminous Seeds

This section deals with nutrient composition of leguminous seeds, and their inhibitory activities against trypsin and chymotrypsin and hemagglutinating activity.

MATERIALS AND METHODS

Materials

Eight dried legume samples comprised of adzuki bean (Phaseolus angularis (Willd.) Wight), mung bean (Phaseolus aureus Roxb.), two varieties of lima beans (Phaseolus lunatus), two varieties of kidney beans (Phaseolus vulgaris L.) and two varieties of cow peas (Vigna sinensis Endel.), were imported from the countries shown in Table 6. The seeds were pulverized. α-N-benzoyl-DL-arginine-p-nitroanilide (BANA) and benzoyl-L-tyrosine-p-nitroanilide (BTNA) were from Protein Research Foundation (Osaka). Trypsin (2 x crystallized, from bovine pancreas) and α-chymotrypsin (3 x crystallized, from bovine pancreas) were from Sigma Chemical Co. Sepharose 4B attached covalently to Concanavalin A was from Pharmacia Fine Chemical Co.

Chemical analysis

Moisture, fat, ash and protein (N x 6.25) were determined by the standard method (25). Starch was determined by the method described by Osborne and Voogt (30). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined by the method of Van Soest (31). The amino acid composition was determined according to the procedure as described in Section 1. All analyses were performed at least in duplicate.

Extraction of protein fractions

Continuous extraction of the protein fractions from leguminous seeds and separation of WI and WS from saline extract were carried out as described in Section 1.

Assay of trypsin and chymotrypsin inhibitory activities

One and a half g of the seed flour were extracted with 20 ml of 2% NaCl at 4℃ overnight (with constant magnetic stirring) and centrifuged at 3,500 x g for 60 min. The residue after centrifugation was re-extracted with 20 ml of 2% NaCl at 4℃ for 2 h and centrifuged as above. The supernatant was combined and further centrifuged at 100,000 x g for 60 min. The resultant supernatant was used as the sample of protease inhibitory activity assay. Protease inhibitory activity was measured using BANA as substrate for trypsin and BTNA as substrate for chymotrypsin according to the method described by

Kanamori et al. (32).

Assay of hemagglutinating activity

Hemagglutinating activity was measured by a serial two-fold dilution method on microtiter plate using a 4% suspension of trypsinized human erythrocytes. Two g of the seed flour was extracted with 20 ml of phosphate-buffered saline (PBS; 10 mM phosphate buffer containing 0.15 M NaCl, pH 7.0) at 4℃ overnight and centrifuged at 6,000 x g for 30 min. The supernatant was recentrifuged at 100,000 x g for 30 min and then used as the test solution for the hemagglutinating activity assay. The agglutination was determined after incubation at 37℃ for 1 h. Isolation of hemagglutinating material by affinity chromatography on Concanavalin A bound Sepharose 4B was performed according to the method of Junqueira and Sgarbieri (33).

RESULTS AND DISCUSSION

Nutrient compositon

The proximate composition of leguminous seeds is shown in Table 6. The protein content ranged from 18.4-26.4%. The starch and fiber contents are shown in Table 7. Dietary fiber was considered more significant than crude fiber, because dietary fiber lowered the serum cholesterol levels in human (34). That is why the analysis of dietary fiber was performed. Table 8 indicates the amino acid profiles of the

Table 6 Proximate composition of tropical legume seeds.

Legume	Testa color	Protein[a] (%)	Fat (%)	Ash (%)	Moisture (%)
Adzuki bean (Burma)[b]	Yellow-brown	18.4	3.3	4.0	12.3
Mung bean (Thailand)	Pea green	23.4	1.8	3.3	11.3
Baby lima bean (U.S.A.)	White	21.0	1.8	3.9	13.6
Lima bean (South Africa)	White	24.1	4.4	4.4	13.8
Light red kidney bean (U.S.A.)	Light red	26.4	1.2	3.7	12.6
White kidney bean (South Africa)	White	19.8	1.2	4.1	10.8
Rose pea (Thailand)	Beige	22.6	2.3	3.6	12.1
Black pea (Thailand)	Black	23.8	1.5	3.1	12.0

a) $N \times 6.25$.
b) The legumes were grown in the countries given in parenthese.

Table 7 Starch and fiber content of tropical legume seeds.

Legume	Starch (%)	NDF (%)	ADF (%)
Adzuki bean	39	7.0	6.0
Mung bean	36	4.9	4.1
Baby lima bean	27	5.2	4.0
Lima bean	27	5.4	4.8
Light red kidney bean	26	5.1	4.2
White kidney bean	34	11.0	6.1
Rose pea	27	4.7	3.7
Black pea	31	6.7	5.3

seeds.

Fractional extraction of protein

The extraction profiles of the soluble protein fractions from seed flour are given in Table 9. All samples contained a high proportion (53.5-76.8%) of 2% NaCl extract. A 2% NaCl extract was separated

Table 8 Amino acid composition of tropical legume seeds.[a]

Amino acid	Adzuki bean	Mung bean	Baby lima bean	Lima bean	Light red kidney bean	White kidney bean	Rose pea	Black pea
Aspartic acid	10.4	10.7	9.4	11.3	10.2	10.9	11.0	11.0
Threonine	2.7	3.2	3.2	4.0	3.7	4.3	3.5	3.4
Serine	3.4	4.7	4.3	5.3	4.3	5.6	4.1	3.8
Glutamic acid	12.5	16.1	10.7	12.2	13.6	13.1	16.7	16.6
Proline	3.4	4.5	2.3	2.5	1.6	2.0	3.2	3.3
Glycine	3.5	4.4	3.0	3.6	3.3	4.0	4.1	3.9
Alanine	4.8	4.8	3.3	4.1	3.6	3.6	4.2	4.2
Valine	5.6	5.4	4.4	5.1	5.0	5.1	4.9	4.9
Cystine	0.6	0.4	0.5	0.5	0.6	0.8	0.4	0.6
Methionine	1.0	1.4	1.2	1.4	1.2	1.3	1.4	1.4
Isoleucine	3.6	4.5	4.2	4.5	4.4	4.0	4.1	4.2
Leucine	8.4	8.0	8.4	7.9	7.9	8.8	7.3	7.7
Tyrosine	2.5	2.7	2.5	2.7	2.3	3.0	2.5	2.6
Phenylalanine	5.7	5.4	4.2	4.8	4.4	4.8	5.0	6.1
Lysine	7.4	8.0	9.3	7.7	6.8	8.1	9.1	8.1
Histidine	3.3	2.8	5.0	2.7	2.9	2.5	3.5	3.5
Arginine	6.8	6.9	8.9	6.3	7.1	6.8	8.7	7.8
Tryptophan	1.2	1.2	1.8	1.7	1.5	1.3	1.4	1.5

a) Grams of amino acid per 16 g of nitrogen.

into two fractions (WI and WS) based on their protien solubilities in water. The essential amino acid composition of these main soluble fractions are shown in Table 10.

Antinutritional factors

Trypsin and chymotrypsin inhibitory activities of leguminous seeds are shown in Table 11. Both activities were found in the varieties of lima beans and kidney beans. In these cases, inhibitory activities against trypsin were higher than those against chymotrypsin.

Table 9 Relative concentrations of the four soluble protein fractions from tropical legume seeds.[a]

Legume	2% NaCl sol. (%)	WI[b] (%)	30% Isopropyl alcohol sol. (%)	4% Lactic acid sol. (%)	0.5% KOH sol. (%)	Total (%)
Adzuki bean	53.5	(75)[c]	11.3	8.2	9.9	82.9
Mung bean	65.2	(72)	10.2	3.7	8.7	87.8
Baby lima bean	76.8	(60)	10.1	4.0	7.6	98.5
Lima bean	75.1	(82)	9.5	5.0	8.3	97.9
Light red kidney bean	70.4	(50)	9.0	3.6	6.0	89.0
White kidney bean	74.0	(53)	10.2	3.0	7.0	94.2
Rose pea	59.3	(52)	12.5	4.6	6.2	82.6
Black pea	56.6	(55)	13.5	3.7	8.0	81.8

a) All values are given in terms of Kjeldahl nitrogen.
b) WI fraction from 2%NaCl extract.
c) WI content of 2%NaCl extract

Table 10 Essential amino acid composition of the protein fractions from 2% NaCl extract.[a]

Essential amino acid	Adzuki bean		Mung bean		Baby lima bean		Lima bean		Light red kidney bean		White kidney bean		Rose pea		Black pea		FAO/WHO (1973) pattern
	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS	WI	WS	
Lysine	450	514	444	438	513	406	675	431	435	388	513	613	438	513	450	363	340
Threonine	193	288	166	341	270	325	294	344	266	450	331	419	219	325	331	356	250
S-cont. A.A.[b]	94	131	100	163	100	131	106	144	125	131	88	181	100	156	131	169	220
Valine	315	284	363	388	406	338	413	394	363	363	331	300	338	356	388	419	310
Isoleucine	273	247	306	275	206	188	325	206	241	288	375	256	269	306	344	244	250
Leucine	655	484	466	463	518	475	700	538	619	547	788	575	525	475	406	450	440
Aromatic A.A.[c]	593	490	650	563	667	615	625	619	588	622	619	550	588	575	713	550	380
Tryptophan	63	75	63	76	63	113	63	94	75	88	44	81	50	88	63	94	60

a) Milligrams of amino acid per 1 g of nitrogen.
b) Methionine+Cystine.
c) Phenylalanine+Tyrosine.

Elkowicz and Sousulski (35) have been reported the similar results with respect to trypsin inhibitory activity. Table 11 also shows hemagglutinating activity in the seeds. Two lima beans and two kidney beans had the activity with human type A erythrocytes. Lectins (hemagglutinating materials) were isolated from these respective seeds by affinity chromatography on Concanavalin A bound Sepharose

Table 11 Trypsin, chymotrypsin inhibitory and hemagglutinating activities of tropical legume seeds.

Legume	TI activity[a] (unit/mg-protein)	CI activity[a] (unit/mg-protein)	Hemagglutinating activity[b] (titer/mg-protein)
Adzuki bean	—	—	—
Mung bean	—	—	—
Baby lima bean	330.0	131.1	406
Lima bean	140.7	70.8	340
Light red kidney bean	73.6	57.6	353
White kidney bean	151.0	82.1	764
Rose pea	—	—	—
Black pea	76.7	—	—

a) TI activity, trypsin inhibitory activity; CI activity, chymotrypsin inhibitory activity. One unit was the amount of inhibitor required for complete inhibition of 1 μg of the enzyme.
b) Hemagglutinating activity with human type A erythrocytes.

Table 12 Relative hemagglutinating activity with various erythrocytes of tropical legume seed lectins.[a]

Erythrocytes source	Baby lima bean	Lima bean	Light red kidney bean	White kidney bean
Human				
Type A	100	100 (12,000)[b]	100	100 (5,000)[b]
Type B	—	—	100	100 (1,250)
Type O	—	—	100	100 (1,250)
Type AB	100	100 (3,000)	100	100 (1,250)
Rat	—	—	200	200 (625)
Mouse	—	—	400	200 (3,125)

a) The activity with human type A erythrocytes was taken as 100.
b) The values were relative activities of partial purified lectins by affinity chromatography on concanavalin A bound Sepharose 4B.

4B. Relative hemagglutinating activities with four types of human

erythrocytes (A, B, O and AB) of isolated lectins are given in Table 12. Lectins from kidney beans were non-specific, while those from lima beans were specific for type A erythrocyte.

Section 3

Mineral and Phytic Acid Contents of Leguminous Seeds

Phytic acid and its derivatives are common constituents of plant tissue. Because of their ability to chelate metal ions such as calcium, magnesium, zinc, copper and iron to form insoluble complexes that are not readily absorbed from the intestinal tract, they have been held responsible for commonly observed interference that many plant souces of protein have on the availability of dietary minerals (36,37).

This section deals with the contents of phytic acid and mineral in leguminous seeds.

MATERIALS AND METHODS

Materials

Nineteen legume seeds tested in this section are summarized in Table 13. The seeds were ground into fine powder and then the powder was used as a sample for the following analyses.

Analytical methods

Moisture was determined by the standard method (25). Samples for the mireral analysis were prepared by ignition at 500-550℃. The

Table 13 Legumes.

Common name	Native source	Testa color	Botanical name
Adzuki bean	China	Yellow-brown	*Phaseolus angularis* (Willd.) Wight
Adzuki bean	Thailand	Red-brown	
Adzuki bean	Burma	Yellow-brown	
Adzuki bean	Taiwan	Red-brown	
Kafae bean	South Africa	Black-brown	
Red kafae bean	South Africa	Red-brown	
Lima bean	Burma	White	*Phaseolus lunatus*
Red lima bean	Burma	Red-brown	
Lima bean	Madagascar	White	
Lima bean	South Africa	White	
Baby lima bean	U.S.A.	White	
Kidney bean	Burma	Dark red	*Phaseolus vulgaris*
White kidney bean	South Africa	White	
Pinto bean	Mexico	Beige	
Light red kidney bean	U.S.A.	Light red	
Rose pea	Thailand	Beige	*Vigna sinensis* Endel.
Black pea	Thailand	Black	
Mung bean	Thailand	Pea green	*Phaseolus aureus* Roxb.
Broad bean	China	Pea green	*Vicia faba*

minerals except phosphorus were assayed using an atomic absorption spectrophotometer. Phosphorus was determined by Allen method (38).

Phytic acid was isolated by the method of Ogawa et al. (39) and determined as phytate-phosphorus by Allen method (38) after hydrolysis of the sample with 2 N HCl and perchloric acid, respectively. All analyses were carried out at least in duplicate.

RESULTS AND DISCUSSION

Results of the analyses are shown in Table 14. All samples

Table 14 Mineral and phytate phosphorus content of tropical legume seeds.

	(%) Moisture	(mg per 100 g of sample)									(% total P) Phytate P
		Mn	Zn	Fe	Mg	Ca	Total P	Phytate P			
Adzuki bean (China)	9.2	2.6	2.5	16.3	256	307	314	100			31.8
Adzuki bean (Thailand)	10.3	2.3	2.6	5.2	295	250	327	94			28.7
Adzuki bean (Burma)	12.3	1.7	2.7	14.9	260	265	336	78			23.2
Adzuki bean (Taiwan)	12.7	1.4	3.0	4.6	197	63	441	207			46.9
Kafae bean	9.8	1.5	3.1	5.1	230	60	402	155			38.6
Red kafae bean	10.1	1.9	2.9	5.7	224	66	366	119			32.5
Lima bean (Burma)	10.7	1.5	1.8	5.1	194	90	281	84			29.9
Red lima bean	9.8	1.6	2.8	6.2	246	65	488	175			35.9
Lima bean (Madagascar)	8.0	1.4	2.8	4.6	181	44	339	108			31.9
Lima bean (South Africa)	13.8	1.0	3.2	5.7	179	42	347	170			49.0
Baby lima bean	13.6	0.4	2.4	2.8	172	59	345	159			46.1
Kidney bean	10.1	1.5	2.3	5.4	200	60	322	130			40.4
White kidney bean	10.8	1.8	2.9	5.7	242	93	412	155			37.6
Pinto bean	9.7	1.5	3.0	5.9	219	134	419	169			40.3
Light red kidney bean	12.6	0.7	3.3	6.7	154	68	508	216			42.5
Rose pea	12.1	1.9	3.3	21.3	202	75	410	182			44.4
Black pea	12.0	1.7	4.0	4.2	197	87	371	159			42.9
Mung bean	11.3	1.1	2.9	3.1	188	91	383	166			43.3
Broad bean	11.5	1.0	3.4	4.4	174	100	523	228			43.6

The content of phytic acid can be estimated by multiplying the phytate phosphorus content by 3.55.

contained high phosphorus, magnesium and calcium. Phytic acid was expressed as phytate-phosphorus. Phytic acid content can be estimated by multiplying the amount of phytate-phosphorus by 3.55 based on the empirical formula $C_6P_6O_{24}H_{18}$. Phytate-phosphorus of some seeds represented a considerable percentage of the total phosphorus (40.3-49.0%).

Section 4

Chemical Composition of Winged Bean

As described in "INTRODUCTION", the winged bean (Psophocarpus tetragonolobus) has in recent years received considerable attention as a protein food source. In addition, this plant is unique among leguminous crops because every part of it (seeds, pods, leaves, flowers and roots) is edible and some parts have medicinal properties (40,41). In Japan, the winged bean varieties introduced from Indonesia and some countries have been cultivated in Okinawa prefecture since 1975.

This section deals with nutrient composition of the winged beans grown in the Okinawa prefecture of Japan and their trypsin and chymotrypsin inhibitory activities.

MATERIALS AND METHODS

Materials

The dry seeds of four varieties (WB 001,002,003 and 004), pods stalks and leaves were obtained from the Institute of Tropical Agriculture, Okinawa branch. The dry seeds were ground. Samples of immature, half-mature and mature pods, mature leaves and stalks were carefully washed and freeze-dried followed by pulverizing.

Analytical methods

Tannin was determined by the Folin-Denis method (42). All other methods are the same as described in Section 2 and 3. All analyses were performed at least in duplicate.

Extraction of protein fractions

Continuous extraction of the protein fractions from winged bean seeds and separation of WI and WS from 2% NaCl extract were carried out as mentioned in Section 1.

RESULTS AND DISCUSSION

Proximate composition

The proximate composition of dry seeds of the four varieties (WB 001, 002, 003 and 004) are shown in Table 15. These values are similar to those of soybeans and compare favourably with the other reports (43,44). The parts other than the seed of winged bean also are useful as a food resource but they have not been studied as much as the seed. The proximate composition of pods, stalks and leaves analyzed in this section is shown in Table 16. Data of pods were obtained from three samples of different extent of ripeness. The young pod is a very popular edible part.

Mineral composition

Table 15 Proximate composition (%) of winged bean seeds.

	Winged bean seeds			
	WB 001	WB 002	WB 003	WB 004
Moisture	8.5	8.3	9.7	8.0
Crude protein[a]	27.8	30.8	30.6	36.6
Crude fat	17.9	15.4	14.9	14.8
Ash	3.9	3.6	3.8	4.0
Carbohydrates				
Starch	10.0	9.9	8.5	7.9
NDF	14.6	13.2	14.3	12.3
ADF	13.5	12.4	12.7	11.6
Tannin[b]	0.77	0.87	0.89	0.97

[a] N × 6.25. [b] The values are expressed as tannic acid equivalents.

Table 16 Proximate composition of pods, stalk and leaf of winged bean.

	Parts of winged bean[a]				
	m-pod[b]	hm-pod[c]	im-pod[d]	Stalk	Leaf
Moisture (%)	55.0	85.7	91.2	79.8	76.3
(% of dry matter)					
Crude protein[e]	7.3	11.6	17.5	12.5	33.7
Crude fat	0.7	1.0	1.6	n.d.	5.0
Ash	8.5	7.5	5.3	5.6	8.4
NDF	65.9	52.7	27.7	—	—
ADF	45.9	34.2	16.2	—	—

[a] The samples were grown in Okinawa Prefecture. [b] Mature pod. [c] Half-mature pod. [d] Immature pod. [e] N × 6.25.

The mineral composition of all parts studied are shown in Table 17. The seeds contained high phosphorus, calcium and magnesium. Similar results except for iron content were reported (43,45,46). The iron content of the seeds was approximately half of the values of the above reports. The parts other than the seed had a similar content of mineral. Quite a high calcium content of the leaf was noted.

Table 17 Mineral composition of different parts of winged beans.

	Mn	Zn	Fe	Ca	Mg	P
Winged bean seeds			(mg per 100 g of whole seed)			
WB 001	8.5	2.8	5.2	297	366	492
WB 002	8.3	3.0	5.9	212	372	536
WB 003	6.7	3.1	4.9	102	338	550
WB 004	6.0	2.6	5.7	342	312	507
Other parts			(mg per 100 g of dry matter)			
Mature pod	2.7	0.6	5.1	156	131	156
Half-mature pod	5.6	1.4	9.1	299	177	226
Immature pod	11.3	1.3	8.9	454	264	265
Stalk	7.0	1.0	3.8	610	290	263
Leaf	16.5	1.6	13.5	1,551	469	311

Fractional extraction of protein

The extraction profiles of the soluble protein fractions from seed flour are given in Table 18. All samples contained a high proportion (60.2-77.6%) of 2% NaCl extract. A 2% NaCl extract was sep

Table 18 Relative concentrations of the four soluble protein fractions from winged bean seeds.[a]

		Winged bean seeds			
		WB 001	WB 002	WB 003	WB 004
2% NaCl sol.	(%)	60.2	71.2	77.6	68.4
WI[b]	(%)	(37)	(45)	(52)	(42)
WS[c]	(%)	(23)	(26)	(24)	(26)
30% isopropyl alcohol sol.	(%)	6.0	4.1	5.0	6.3
4% lactic acid sol.	(%)	6.8	5.2	1.6	4.5
0.5% KOH sol.	(%)	12.6	5.1	10.2	8.2
Total	(%)	85.6	85.6	94.4	87.4

[a] All values are given in terms of Kjeldahl nitrogen. [b] WI fraction from 2% NaCl extract. [c] WS fraction from 2% NaCl extract.

Table 19 Amino acid composition of winged bean flour and protein fractions (WI and WS).

	WB 001			WB 002			WB 003			WB 004		
	(g per 16 g N)											
	Flour	WI	WS	Flour	WI	WS	Flour	WI	WS	Flour	WI	WS
Aspartic acid	10.7	13.2	11.9	11.6	10.1	10.9	11.9	10.3	11.7	10.6	10.7	13.4
Threonine	4.0	4.1	4.6	2.8	3.0	4.1	2.9	3.9	4.5	4.2	3.4	4.9
Serine	4.8	5.4	5.3	3.7	4.4	4.8	4.0	4.6	6.6	3.7	5.7	5.5
Glutamic acid	13.3	18.9	15.3	16.0	16.0	12.7	16.5	15.0	12.0	14.0	18.1	12.0
Proline	2.9	6.6	6.5	2.3	5.4	5.7	2.2	6.6	6.0	3.1	6.4	5.8
Glycine	4.7	4.4	4.6	4.2	3.1	3.7	3.9	3.9	3.6	4.4	4.4	4.6
Alanine	5.7	6.3	7.6	5.5	5.9	6.9	5.8	7.3	5.0	5.2	6.4	6.1
Valine	5.3	4.3	5.7	5.1	4.0	5.0	5.2	4.6	5.7	5.0	4.3	5.3
Cystine	1.6	0.6	1.0	1.3	0.5	0.6	0.9	1.0	0.8	1.1	0.5	0.7
Methionine	0.7	0.8	1.0	0.8	0.7	0.9	0.9	1.0	0.7	0.8	0.8	0.9
Isoleucine	4.2	4.1	4.6	4.0	3.9	4.0	4.2	5.2	4.6	4.0	5.1	4.5
Leucine	8.7	10.5	8.3	8.3	8.6	7.0	8.7	11.6	7.5	8.6	11.8	7.2
Tyrosine	4.1	4.1	4.5	2.3	5.0	4.6	2.4	5.3	4.8	4.0	5.5	4.7
Phenylalanine	3.9	4.5	4.9	5.5	5.5	5.3	5.0	4.7	4.3	3.8	4.8	4.9
Lysine	7.7	8.7	7.8	7.6	11.8	8.8	8.0	9.3	8.1	7.5	8.5	8.0
Histidine	1.7	2.2	1.9	1.9	3.3	2.0	2.1	1.4	1.1	1.8	2.6	2.1
Arginine	4.5	5.2	4.7	4.8	8.1	5.0	5.1	5.3	6.0	5.2	5.5	4.1
Tryptophan	1.0	0.5	1.1	1.0	0.5	1.2	1.0	0.7	0.8	0.9	0.5	0.6

arated into two fractions (WI and WS) based on their protien solubilities in water. In all the samples, the amount of WI was 1.6-2.2 fold greater than that of WS. The amino acid profiles of seed flour and the main soluble fractions (WI and WS) are shown in Table 19. The seed flour of four varieties was generally high in lysine, leucine, aspartic acid and glutamic acid but low in the sulfur containing amino acids. Similar results were reported elsewhere (40,43, 46). The protein of winged bean can be utilized in supplementing cereal-based diets. The amino acid profiles of WI and WS are generally similar to that of seed flour except for a high content of proline in both soluble fractions.

Trypsin and chymotrypsin inhibitory activities

Trypsin and chymotrypsin inhibitory activities of the seeds are shown in Table 20. In each case, inhibitory activity against chymotrypsin was higher than that against trypsin. Similar results were reported (47,48). This observation may be due to the presence of

Table 20 Trypsin and chymotrypsin inhibitory activity in 2% NaCl extracts of winged beans.

Winged bean	Trypsin inhibitory activity (unit)[a]	Chymotrypsin inhibitory activity (unit)
WB 001	44.0	87.9
WB 002	40.8	86.4
WB 003	50.0	102.6
WB 004	46.0	109.6

[a] One unit was the amount of inhibitor required for complete inhibition of 1 mg of the enzyme.

trypsin inhibitor with chymotrypsin inhibitory activity (49) and chymotrypsin inhibitor without trypsin inhibitory activity (50) in winged bean seed.

Section 5

Preparation of Bean Milk (Aqueous Extract) from Winged Bean and Its Gelation Properties

In this section, preparation of bean milk from the seeds of winged bean and its gelation were attempted as an example of practical utilization of winged bean.

MATERIALS AND METHODS

Materials

The winged bean grown in Indonesia and soybean (var. Tsurunoko) were used here. Both seeds were ground before the experiment.

Analytical methods

Chemical composition of both seeds was determined by the standard methods (25).

Preparation of bean milk

Bean seeds were soaked overnight in three volumes (V/W) of distilled water at room temperature. Both hydrated seeds and water used for soaking were placed in a mixer and homogenized after adding two volumes of distilled water. The resulting slurry was boiled for 5 min

together with different volumes of boiling distilled water and then filtered through two-layers cheesecloth to yield two fractions; the bean milk and a residue consisting mainly of coarse fibrous material (okara). The volume and nitrogen content of bean milk were measured.

Effect of pH on nitrogen solubility

The aqueous extracts prepared with and without boiling for 5 min when the ratio of bean to water was 1 : 10 were used as heat-treated and no heat-treated bean milk, respectively. Each bean milk was adjusted to the desired pH by adding 0.1 M HCl or NaOH and stirred vigorously for 20 min, then centrifuged at 6,000 x g for 15 min. Nitrogen content of the supernatant was determined.

Gelation of bean milk

The bean milk (25 ml) was boiled at 95℃ with continuous stirring for 3 min followed by adding different concentrations of glucono-δ-lactone. Then the milk was poured into 50 ml vessel and stirred gently. After standing for 30 min at room temperature, it was observed to what extent the bean milk coagulated.

RESULTS AND DISCUSSION

Chemical composition

Results of the proximate analyses of winged bean and soybean

used in this experiment are shown in Table 21.

Table 21 Proximate composition of winged bean and soy bean

	winged bean (%)	soy bean (%)
protein (N x 6.25)	33.5	40.3
Lipid	16.8	18.7
Ash	3.8	4.8
Moisture	10.4	10.0

Ratio of water to bean for the preparation of bean milk

Values for nitrogen content of bean milk prepared using the various ratios of water to bean are shown in Table 22. The effective bean-water ratio used to extract bean milk was 1 : 6 for winged bean and 1 : 7 for soybean, because bean milk needed high protein concentration rather than high protein quantity. The yield of nitrogenous material extracted from winged bean in each case was lower than that from soybean. This is assumed to be due to the seed coat of winged bean which still remained after soaking overnight unlike that

Table 22 Effect of bean-water ratio in the preparation of bean milk on the extractability of nitrogen

Bean-water ratio (w/v)	winged bean		soy bean	
	N-content (mg/100ml)	Yield (%)[a]	N-content (mg/100ml)	Yield (%)[a]
1 : 5	292	15.0	434	20.9
1 : 6	533	43.8	798	58.9
1 : 7	519	45.5	821	61.8
1 : 8	446	50.3	721	67.7
1 : 10	376	52.0	621	68.5
1 : 20	177	52.1		

[a]Values are expressed as a percentage of the total N in bean milk to that in seed.

of soybean. The seed coat of winged bean prevented hydration of cotyledon (51).

Effect of pH on the nitrogen solubility of bean milk

Figure 2 shows nitrogen solubility of winged bean milk at different pH values. The bean-water ratio used to prepare bean milk was 1 : 10. Results obtained for bean milk are in agreement with those reported for defatted flour of winged bean (52,53). The minimum level of extraction was obtained at pH 4.5 which seemed to be an isoelectric point of winged bean protein (51,54). Although the extraction curve of heat-treated milk resemble that of no heat-treated one, overall level of extraction was less. This probably reflects increased protein denaturation due to the heat treatment. Figure 3 rep-

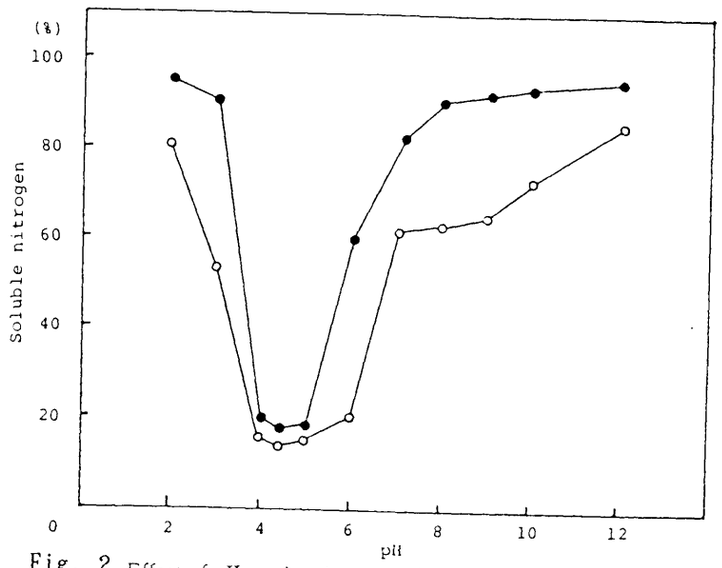

Fig. 2 Effect of pH on the nitrogen solubility of winged bean milk.
-●- no heat-treated; -○- heat-treated

resents nitrogen solubility of heat-treated soybean milk at different pH values. This extraction curve was quite similar to that of no heat-treated winged bean milk.

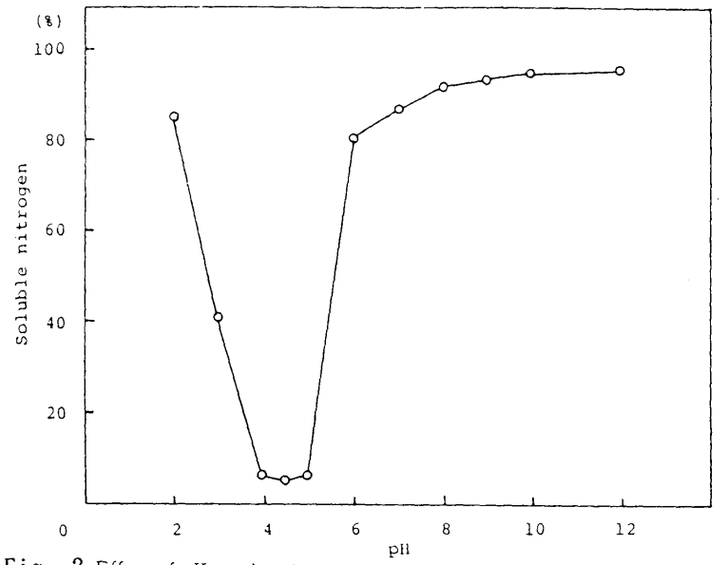

Fig. 3 Effect of pH on the nitrogen solubility of heat-treated soy bean milk

Gelation of bean milk

Gelation of winged bean milk as well as soybean milk was attempted using different concentrations of glucono-δ-lactone as coagulant and sensory evaluation of resulting bean curds was practiced. The bean-water ratios used for winged bean milk and soybean milk were 1 : 6 and 1 : 7, respectively. The coagulant concentrations ranged 0.1-0.8% were adopted by considering those which have been usually used for manufacturing of soybean curd (tofu). Solid curd was not formed from winged bean milk under the conditions tested. However addition of 0.5-0.8% glucono-δ-lactone led to the formation of

sticky and less cohesive curds. On the other hand, tofu was formed from soybean milk by adding at least 0.3% of the coagulant. The difference of gelation properties between winged bean milk and soybean milk is assumed to be due to their protein composition. Soybean protein contained both 7S and 11S globulins, while there was not 11S globulin in winged bean (55). 7S and 11S globulins were needed for the formation of tofu and the ratio between these components affected the texture of tofu (56). It seems difficult to produce solid curd like tofu from winged bean because of absence of 11S globulin.

Section 6

Antinutritional Factors of Winged Bean

This section deals with the results of an investigation on the antinutritional factors in the seeds of winged bean. Tannin and phytic acid contents were determined. Tannins may reduce protein quality by directly complexing with food proteins and may interfere with iron absorption (57). Phytic acid and its derivatives may decrease the availability of essential dietary minerals (37). Inhibitory activities against trypsin, chymotrypsin and α-amylase, and hemagglutinating activity were also analyzed.

MATERIALS AND METHODS

Materials

Mature winged bean seeds of four Papua New Guinea, four Indonesia and four Okinawa (Japan) varieties were used. The Okinawa varieties were experimentally cultivated in the Okinawa prefecture of Japan and supplied by the Okinawa Branch of the Tropical Agriculture Research Center. The seeds were ground into fine powder and defatted with n-hexane. The defatted powder was used as sample for analyses except for the measurement of tannin and phytic acid contents.

Analytical methods

Tannin content was determined by a vanillin-HCl method of Burns (58). Isolation of phytic acid and detemination of phytate-phosphorus and total phosphorus contents were carried out as described in Section 3. Phytic acid content was estimated by multiplying the amount of phytate-phosphorus by 3.55 based on the empirical formula $C_6P_6O_{24}H_{18}$.

The inhibitory activity against porcine pancreatic α-amylase was measured by the iodine staining method of Murao et al. (59) with modifications. Two g of defatted powder was extracted with 10 ml of distilled water for 2 h and centrifuged at 6,000 x g for 30 min. The supernatant was adjusted to pH 4 and heated at 70℃ for 15 min to inactivate any amylase present. The solution was clarified by centrifugation and neutralized, and then used for α-amylase inhibitor assay. Assays for trypsin and chymotrypsin inhibitory activities and hemagglutinating activity were measured by the methods described in Section 2. The effect of heat treatment on hemagglutinating activity was also investigated. The test solution for hemagglutinating activity assay was heated in a boiling water bath for 10 min. Immediately after cooling in ice, it was used in the agglutinating test.

Protein concentration was determined by the Lowry-Folin method (60) with bovine serum albumin as a standard.

All analyses were carried out on triplicate samples.

Tannin and phytic acid contents

The tannin contents of the seeds of twelve varieties of winged beans are shown in Table 23. A 5-fold variation in tannin content (1.35-6.75 mg/g of bean) was observed, while Tan et al. (61) reported a 25-fold variation (0.3-7.5 mg/g of bean). No correlation between seed coat color and tannin content was observed.

The phytic acid and total phosphorus contents are also shown in Table 23. Except for the values obtained from Indonesia varieties, these results are comparable with that of soybean seeds and higher than many other legumes (35). The phytate-phosphorus contributed a substantial portion of the total phosphorus (44.3-54.8 %).

Trypsin and chymotrypsin inhibitory activities

Trypsin and chymotrypsin inhibitory activities are shown in Table 24. In all winged bean specimens, inhibitory activity against chymotrypsin are about 2 fold higher than that against trypsin. It is known that tannins are nonspecific inhibitors of enzymes. However, de Luman and Salamat (62) reported that trypsin inhibitory activity, caused by the tannin, occupied only 1% of the total in winged beans. In this section, no correlation was found between tannin content and the activity.

Table 23 Tannin, Phytic Acid and Total Phosphorus of the Winged Bean

Variety	*Source*	*Seed coat color*	*Tannin*	*Phytic acid*	*Phytate-phosphorus*	*Total phosphorus*	*Phytate-phosphorus as % total phosphorus*
			(*mg/g of bean*)				
001	Okinawa (Japan)	Brown	1·93[a]	9·27[b]	2·61	5·38	48·5
002	Okinawa (Japan)	Brown	3·28	10·76	3·03	5·85	51·8
003	Okinawa (Japan)	Brown	1·95	10·37	2·92	6·09	47·9
004	Okinawa (Japan)	Brown	2·18	9·16	2·58	5·51	46·8
1014	Papua New Guinea	Brown	1·35	12·03	3·39	6·22	54·5
2826	Papua New Guinea	Brown	1·63	10·26	2·89	5·34	54·1
2891	Papua New Guinea	Brown	1·88	9·15	2·58	5·34	48·3
3154	Papua New Guinea	Brown	1·38	10·93	3·08	5·62	54·8
1	Indonesia	Brown	1·68	8·23	2·32	4·49	51·6
2	Indeonsia	Black	1·40	9·00	2·54	4·68	54·3
3	Indonesia	Brown	1·40	7·81	2·20	4·33	50·8
4	Indeonsia	Pale brown	6·75	7·77	2·19	4·94	44·3

[a] D-Catechin equivalents.
[b] Phytate-phosphorus × 3·55.

Table 24 Trypsin and Chymotrypsin Inhibitory Activities of the Winged Bean

Variety	*Trypsin inhibitory activity*	*Chymotrypsin inhibitory activity*
	(*units/mg protein*)[a]	
001	0·12	0·24
002	0·10	0·22
003	0·14	0·28
004	0·09	0·21
1014	0·12	0·22
2826	0·11	0·20
2891	0·12	0·23
3154	0·14	0·26
1	0·11	0·20
2	0·12	0·21
3	0·13	0·23
4	0·14	0·28

[a] One unit; the amount of inhibitor required for complete inhibition of 1 mg of enzyme.

α-Amylase inhibitory activity

Jaffe and Korte (63) examined α-amylase inhibitory activity in winged beans by the dinitrosalicylic acid method measuring reducing sugars liberated from starch used as a substrate during digestion with α-amylase, and found this activity in the seed of winged beans harvested in Papua New Guinea. In this section, the iodine staining method was adopted in the measurement of the activity because crude extract from winged bean seed contained significant amounts of reducing sugars which may interfere with the measurement of the activity by the dinitrosalicylic acid method. Consequently, α-amylase inhibitory activity could not be detected in any of the winged bean varieties used here.

Hemagglutinating activity

Table 25 shows hemagglutinating activity in winged bean seeds. Winged bean lectins agglutinated all types of trypsinized human

Table 25 Hemagglutinating Activity of the Winged Bean (titer/mg protein)

Variety	*Type of erythrocytes*		
	A	*B*	*O*
001	200	100	100
002	110	110	110
003	94	94	94
004	94	94	94
1014	97	97	97
2826	240	120	120
2891	490	490	490
3154	130	130	130
1	210	210	210
2	220	220	220
3	220	220	110
4	480	480	240

erythrocytes (A, B and O). In this section, the agglutination specificity for human erythrocyte types was classified into three patterns. Varieties 001 and 2826 showed that agglutination specificity for human erythrocytes decreased in the order, blood group A > B = O ; varieties 3 and 4, A = B >O, ; others, A = B = O. Turner and Liener (64) reported that soybean lectins have no apparent deleterious effects on animals while Higuchi et al. (65) showed that winged bean lectins are toxic to rats.

The effect of heating on hemagglutinating activity was also investigated to inactivate winged bean lectin. After heating the test solution in boiling water bath for 10 min, Assay for hemagglutinating activity was carried out using type A erythrocyte. The boiling

resulted in the disappearance of the activities from all winged bean varieties used here. Lectins in winged bean seed could be inactivated easily by 5 min of autocleave treatment (61). These results suggest that winged bean seed may be acceptable for a food source if cooked.

Chapter II

α-AMYLASE INHIBITOR FROM PHASEOLUS VULGARIS

Section 1

Occurrence of α-Amylase Inhibitor in Legumes and Its Activity Changes

This section deals with the occurrence of α-amylase inhibitor in the seeds of Phaseolus vulgaris as well as other leguminous varieties and the changes of α-amylase inhibitory activity in kidney beans during heating and germination.

MATERIALS AND METHODS

Materials

The leguminous seeds tested here were obtained from a market and a food importer. Porcine pancreatic α-amylase and bovine trypsin were purchased from Sigma Chemical Co. α-N-benzoyl-DL-arginine-p-nitroanilide (BANA) was from Protein Research Foundation (Osaka). Other chemicals of analytical grade were commercially available and used without further purification.

Extraction of α-amylase inhibitor and assay of its activity

Seeds were ground to pass through a 60-mesh sieve. The flour was extracted with five volumes (V/W) of distilled water for 2 h and centrifuged at 10,000 x g for 30 min. The supernatant was adjusted to

pH 4 and heated at 70℃ for 15 min to inactivate any amylase present. The solution was clarified by centrifugation and neutralized, and then used for inhibitory assay.

The activities of α-amylase and its inhibitor were measured by the iodine staining method. The reaction mixture containing an appropriate concentration of α-amylase, 0.5% soluble starch, 50 mM NaCl, 5 mM $CaCl_2$ and 0.01% ovalbumin in 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.9) was incubated at 37℃ for 10 min. The reaction was stopped by adding 2.5 ml of an acidic solution containing 0.08 M HCl and 0.4 M acetic acid to 1.5 ml of the reaction mixture. To its aliquot (0.4 ml), 5 ml of an aqueous solution containing 0.005% iodine and 0.05% potassium iodine was added to measure the absorbance at 660 nm. One unit of enzyme was defined as the amount of enzyme capable of producing 0.8 decrease in the absorbance. A definite amount of α-amylase that had been preincubated for 30 min together with the inhibitor solution was added to the assay medium and then the enzyme assay was carried out as stated above. The inhibitory activity was estimated from the difference in α-amylase activity between the absence and presence of the inhibitor. One inhibitor unit is defined as the amount of inhibitor that gives 50% inhibition of 1 unit of the α-amylase.

Heating and germination

Kidney beans (Phaseolus vulgaris var. Cranberry) were soaked in

four volumes of distilled water for 15 h at room temperature. Hydrated seeds were heated in boiling distilled water for 3-15 min and then cooled in an ice bath. The seeds were homogenized in a mortar with sea sand. Five volumes of distilled water was added to the homogenate followed by stirring for 2 h. After centrifugation, the supernatants obtained were used for thier α-amylase and trypsin inhibitory activities.

The kidney bean seeds soaked as stated above were germinated in the dark at 20-25℃ for 7 days while single layered in cotainers lined with several layers of wet cheesecloth. Samles for inhibitory activity measurements were prepared from the seeds of different stages of germination by the same method as above.

Assay of trypsin inhibitory activity

Trypsin inhibitory activity was determined using BANA as substrate by the method described in Chapter Ⅰ, Section 2. One inhibitor unit was the amount of inhibitor required for 50% inhibition of 20 μg of the trypsin.

Protein detemination

Protein was determined by the method of Lowry et al. (60) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

α-Amylase inhibitory activity in leguminous seeds

Table 26 shows the occurrence of α-amylase inhibitory activity in various legumes. The inhibitory acivities were found in all kidney bean cultivars examined but not in other leguminous seeds. These results are contrary to those for trypsin inhibitory activity which have been found in all of the same seeds as tested here (6).

Table 26 α-Amylase inhibitory activity in legume seeds.

Legumes	Scientific name	Inhibitory activity (unit/mg-protein)
Kidney beans (13 samples)	*Phaseolus vulgaris*	
White (South Africa)[a]		16.4
Great northern (USA)		31.9
Small white (Thailand)		5.3
Daifuku (Japan)		19.7
Light red (USA)		33.6
Dark red (USA)		28.9
Small red (USA)		23.9
Pink (USA)		35.5
Kintoki (Japan)		35.3
Pinto (Thailand)		33.0
Cranberry (USA)		56.0
Tora (Japan)		38.5
Uzura (Japan)		55.2
Lima beans (5 samples)	*Phaseolus lunatus*	ND[b]
Runner bean	*Phaseolus coccineus*	ND
Mung bean	*Phaseolus aureus*	ND
Adzuki beans (6 samples)	*Vigna angularis*	ND
Cow peas (2 samples)	*Vigna sinensis*	ND
Winged bean	*Psophocarpus tetragonolobus*	ND
Broad bean	*Vicia faba*	ND
Garden pea	*Pisum sativum*	ND
Lentil	*Lens esculenta*	ND
Chick pea	*Cicer arietinum*	ND
Soy bean	*Glycine max*	ND

[a] Kidney beans were grown in the countries given in parentheses.
[b] ND: Not detectable.

Change in α-amylase inhibitory activity during heating

Figure 4 represents the change in α-amylase inhibitory activity in kidney beans during heating. Cranberry bean was used as the sample because of its high inhibitory activity shown in Table 26. Soaking for 15 h led to 20% loss of the inhibitory activity and the activity disappeared completely after boiling treatment for 10 min. When the same treatment without presoking was performed, the activity was remained 50% of the initial activity even after 15 min heating. Dry heat treatment of the seeds was also carried out at 110℃ for 30 min. It did not cause any loss of the activity. These results suggested that α-amylase inhibitor in cranberry bean was heat stable under low moisture content, while it became heat labile under high moisture content. It was reported that the denaturation temperature of the proteins such as bovine skin collagen, ovalbumin and bovine hemoglobin increased greatly with decreasing the content of their absorbed water (66). The seeds without presoaking seemed to need higher denaturation temperature, that is, longer heat treatment to denaturate their protein than those soaked.

Changes in α-amylase and trypsin inhibitory activities during germination

Changes in α-amylase and trypsin inhibitory activities during germination up to 7 days are shown in Fig. 5. The level of α-amylase inhibitory activity in cranberry bean initially decreased and then gradually increased. It reached a maximum 4 days after germination. A similar change was observed for the trypsin inhibitory activity. Many other seeds have been examined for change in trypsin inhibitory activity during germination. Results similar to this were obtained from wheat (67) and black matpe (68). Mitsunaga et al. (67) indicated the plumules and radicles of wheat contained trypsin inhibitory activity. Contrary, Nagahiro (69) reported that α-amylase inhibitory activity in kidney beans occurred in the seeds but not in the germinated tissues.

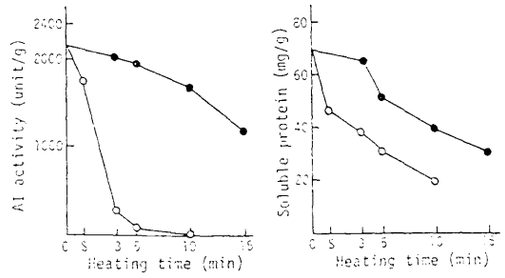

Fig. 4 Changes of AI activity and soluble protein content in kidney bean (cranberry) during heating at 100℃.

C, control (raw); S, after soaking (15 hr, room temperature); ○, heating after soaking; ●, heating without presoaking.

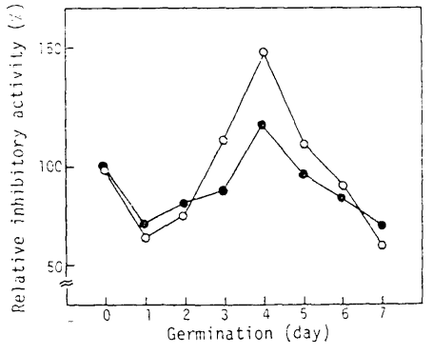

Fig. 5 Changes of AI and TI activities in kidney bean (cranberry) during germination.

○, AI activity; ●, TI activity.
Relative inhibitory activity was expressed as a percent to the specific activity (unit/mg-protein) in control (0 day of germination).

Section 2

Purification and Characterization of α-Amylase Inhibitors from Phaseolus vulgaris Seeds

Kidney beans (Phaseolus vulgaris) contain a large amount of amylase inhibitors (70). This section deals with the purification and the characterization of α-amylase inhibitors from two Phaseolus vulgaris cultivars, cranberry bean (Part A) and kintoki bean (Part B), respectively.

Part A

An α-Amylase Inhibitor from Cranberry Bean

MATERIALS AND METHODS

Materials

Cranberry beans were ground to pass through 60-mesh sieve. DEAE-cellulose was from Brown Co. Sephacryl S-200 was from Pharmacia Fine Chemical Co. Ampholytes used in an isoelectric focusing were from LKB. Other chemicals of analytical grade were commercially available and used without further purification.

Inhibitory assay

α-Amylase inhibitory activity was measured by the iodine staining method described in Section 1.

Isoelectric focusing

Isoelectric focusing was performed according to the method of Vesterberg and Svensson (71) with a carrier ampholyte pH 3−6. The electrophoresis was carried out at 4℃ for 72 h with 1 watt of electric power.

Other analytical methods

Protein was determined by the method of Lowry et al. (60). The carbohydrate content was determined by the phenol-sulfuric acid method of Dubois et al. (72) with mannose as the standard. Amino acids were determined by the method described in Chapter Ⅰ, Section 1. Cystine and methionine were assayed in a sample oxidized with performic acid by the method of Moore (73).

RESULTS AND DISCUSSION

Purification of CBAI

α-Amylase inhibitor was purified from cranberry bean to homogeneity by the procedure summarized in Table 27. Cranberry bean flour was extracted by being stirred for 2 h at room temperature with distilled water (1:5, W/V). After centrifugation (15,000 x g

Table 27 Purification of α-Amylase Inhibitor from Cranberry Bean

Step	Total act. (unit × 10^3)[a]	Total protein (mg)	Specific act. (unit/mg)	Recovery (%)
Extract	296	8300	35.7	100
Heat treatment	228	3600	63.3	77
Ethanol fraction	179	868	206	60
DEAE-cellulose	136	510	267	46
Sephacryl S-200	101	290	347	34

[a] One inhibitor unit is defined as the amount of inhibitor that gives 50% inhibition of a portion of α-amylase that produced a 0.8 decrease in the optical density at 660 nm for 10 min at 37°C.

for 60 min), the supernatant was adjusted to pH 4 with 1 M HCl, heated at 70°C for 15 min, and rapidly cooled in an ice bath. After centrifugation, the supernatant solution was neutralized with 1 M NaOH. 95% ethanol was added to the neutralized solution to make 40% ethanol at 4°C over a period of 30 min. The mixture was stirred for 3 h at 4°C and then centrifuged. The supernatant solution was adjusted to 80% ethanol. Again the mixture was stirred for 3 h at 4°C and centrifuged. The precipitate was dispersed in water, dialyzed against distilled water and lyophilized. The lyophilized material was dissolved in 20 mM phosphate buffer, pH 7.0, and put on a DEAE-cellulose column (2.6 x 40 cm) equilibrated with the same buffer. After elution of the unadsorbed material, the inhibitor was eluted with a linear gradient of 0 to 0.4 M NaCl in the same buffer (Fig. 6). The active fractions were pooled, dialyzed against water and lyophilized. The lyophilized material was dissolved in 30 mM phosphate buffer, pH 7.5, containing 0.2 M NaCl and then chromatographed on a Sephacryl S-200 column (2.6 x 100 cm) eluted with the same

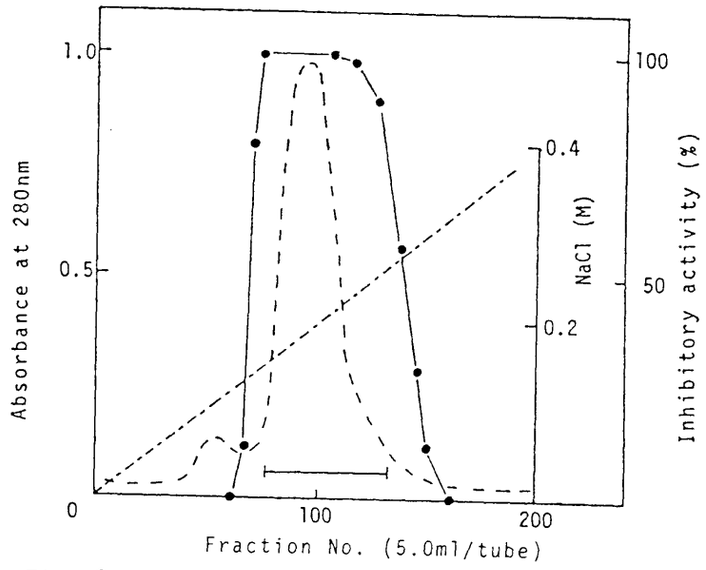

Fig. 6 DEAE-cellulose column chromatography of CBAI. The fractions represented by the bar were collected, dialyzed and lyophilized.
●, inhibitory activity; ....., absorbance at 280 nm; ---, NaCl concentration.

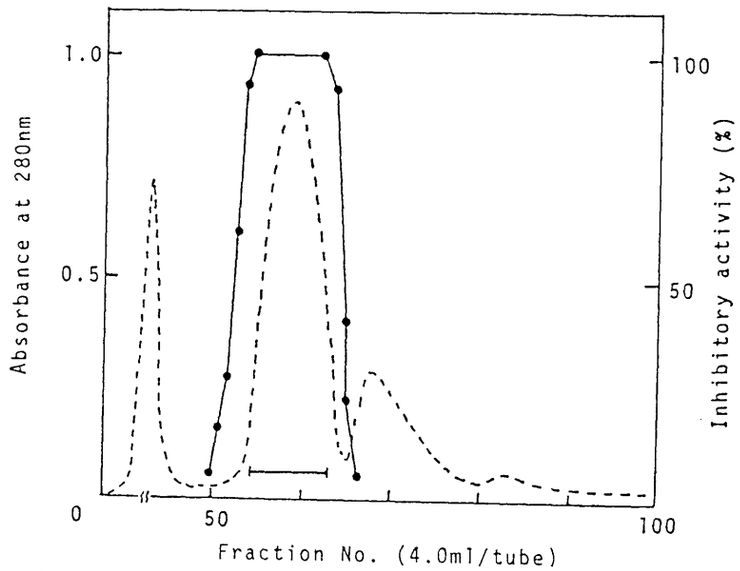

Fig. 7 Sephacryl S-200 column chromatography of CBAI. The fractions represented by the bar were collected, dialyzed and lyophilized.
●, inhibitory activity; ....., absorbance at 280 nm.

buffer (Fig. 7). Fractions with inhibitory activity were pooled, dialyzed against water and lyophilized; 290 mg of the purified inhibitor was obtained from 100 g of the flour. The yield was about 2-fold that from other kidney beans (15,16).

Chemical properties

The carbohydrate content was 14%. The apparent molecular weight of the inhibitor was 45,000 by gel filtration. Figure 8 shows the result of isoelectric focusing of the inhibitor. The isoelectric

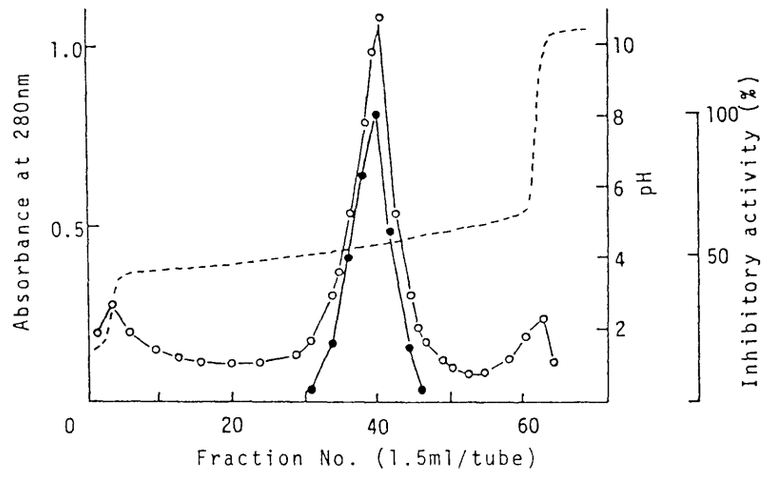

Fig. 8 Isoelectric focusing of CBAI.
●, inhibitory activity; ○, absorbance at 280nm; ....., pH.

point was found to be 4.68. The value was similar to the isoelectric point of 4.65 obtained from the red kidney bean α-amylase inhibitor (16) and that of 4.35 from the black kidney α-amylase inhibitor (19). The amino acid composition of the inhbitor is shown in Table 28. The inhibitor contained much aspartic acid, serine, valine, threonine, and glutamic acid, but it did not contain cystine. Inhibitors from other kidney beans (16,18) have little cystine. The red kidney bean inhibitor has no proline (16), but proline was found in this cranberry bean inhibitor.

Table 28 Amino Acid Composition of α-Amylase Inhibitor from Cranberry Bean

Amino acid	% of residues/mol[a]
Lysine	4.8
Histidine	1.4
Arginine	2.8
Aspartic acid	17.5
Threonine	9.0
Serine	14.1
Glutamic acid	7.6
Proline	2.3
Glycine	4.8
Alanine	5.9
Cystine	0
Valine	10.1
Methionine	0.8
Isoleucine	4.5
Leucine	5.1
Tyrosine	3.7
Phenylalanine	5.6
Tryptophan	nd[b]

[a] We calculated the values assuming a molecular wt. of 45,000 and carbohydrate content of 14%.
[b] Tryptophan was not assayed.
Samples were hydrolyzed with 6 N HCl at 110°C for 24 hr and their amino acids analyzed.

Ultraviolet absorption spectra

Figure 9 shows the ultraviolet (UV) absorption spectra of the inhibitor at various pHs. The absorption spectra were determined with a Hitachi recording spectrometer, model U-2000. The UV spectra in the pH range 5-8 were typical protein spectra, with a maximum at

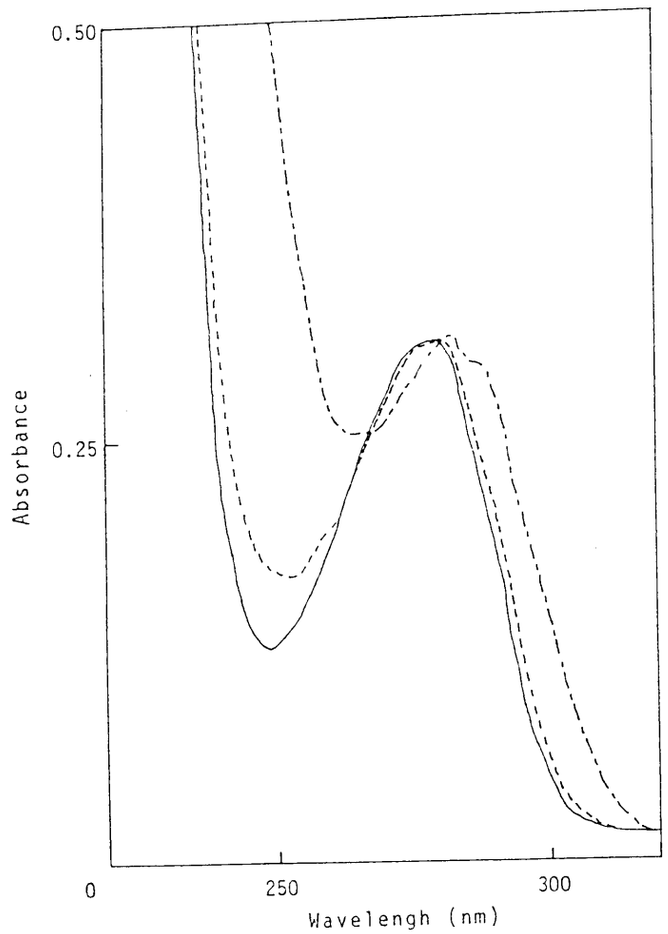

Fig. 9 UV absorption spectra of CBAI. Spectra were measured at various pHs with each 0.02% of CBAI solution in 10 mM buffer. The buffers used in this experiment were as follows; Na-acetate buffer for pH 5, $NaH_2PO_4-Na_2HPO_4$ buffer for pH 6, 7 and 8, $H_3BO_3 \cdot KCl-NaOH$ buffer for pH 10. For pH 12, 10 mM of NaOH solution was also used. ——, spectra at pH 5, 6, 7 and 8; ....., at pH 10; ---, at pH 12.

280 nm and a minimum at 250 nm, but those in the more alkaline range were shifted. Large change took place at pH 12, reflecting the ionization of tyrosine residues.

Absence of hemagglutinating activity

The hemagglutinating activity of the inhibitor was measured by serial 2-fold dilution method in microtiter plate using human erythrocytes. The aqueous extract from cranberry bean agglutinated human type A, B, O and AB erythrocytes, but the purified inhibitor did not have hemagglutinating activity.

Part B

An α-Amylase Inhibitor from Kintoki Bean

MATERIALS AND METHODS

Materials

Kintoki beans were ground to pass through 60-mesh sieve. All chemicals were the same as used in Part A.

Analytical methods

α-Amylase inhibitory activity was measured by the iodine staining method described in Section 1. Protein and carbohydrate were determined by the methods of Lowry et al. (60) and Dubois et al. (72), respectively. Amino acids were determined by the method described in Chapter I, Section 1. Cystine and methionine were assayed in a sample oxidized with performic acid by the method of Moore (73).

RESULTS AND DISCUSSION

α-Amylase inhibitor was purified from kintoki bean by water extraction, heat treatment and ethanol precipitation followed by DEAE-cellulose and Sephacryl S-200 column chromatography as described in Part A. Figure 10 shows the elution profile of chromatography on a DEAE-cellulose column (2.6 x 40 cm). Inhibitor was eluted with a linear salt concentration gradient. Figure 11 represents the elution profile of gel filtration on a Sephacryl S-200 column (2.6 x 95 cm). Fractions containing inhibitory activity were pooled, dialyzed against distilled water and lyophilized. The result of this purifica-

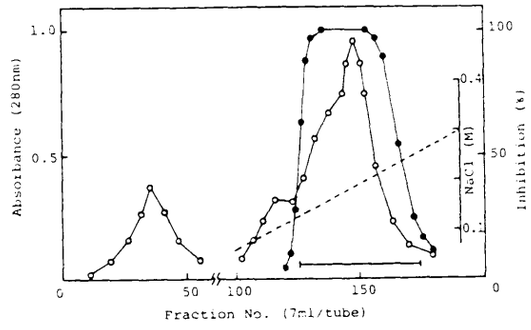

Fig. 10 Ion exchange chromatography of 40-80 % ethanol fraction on a DEAE-cellulose column (2.6 × 40 cm)

The fractions represented by the bar were collected, dialyzed and lyophilized. ○ absorbance at 280 nm, ● inhibition, ---- NaCl concentration.

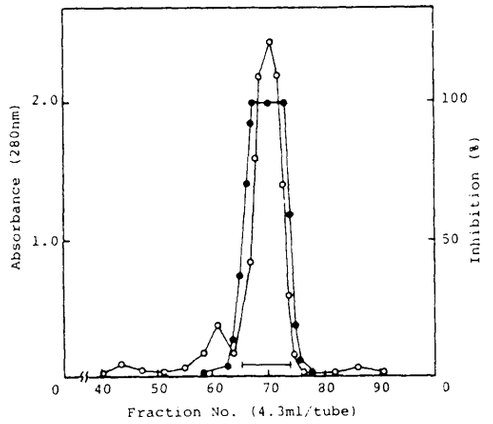

Fig. 11 Gel filtration of the inhibitor separated by DEAE-cellulose chromatography on a Sephacryl S-200 column (2.6 × 95 cm)

The fractions represented by the bar were collected. ○ absorbance at 280 nm, ● inhibition.

tion is summarized in Table 29.

Table 29 Purification of α-amylase inhibitor from Kintoki bean

Step	Total activity (IU)*	Protein (mg)	Specific activity (IU/mg)	Ratio	Yield (%)
Heat treatment	74,800	2,952	25.3	1.0	100
Ethanol fraction	65,900	370	178	7.0	88
DEAE-cellulose	39,600	186	213	8.4	53
Sephacryl S-200	31,400	110	285	11	42

* One inhibitor unit is defined as the amount of inhibitor required for 50 % inhibition of a portion of α-amylase that produced a 0.8 decrease in the absorbance at 660 nm for 10 min at 37℃.

The carbohydrate content of the inhibitor and its molecular weight by gel filtration were 15% and 45,000, respectively. The amino acid composition of the inhbitor is shown in Table 30. This amino composition was similar to that of cranberry bean α-amylase inhibitor.

Table 30 Amino acid composition of α-amylase inhibitor from Kintoki bean

Amino acid	% of residues/mol*
Lys	5.1
His	1.4
Arg	2.8
Asp	16.6
Thr	9.0
Ser	14.6
Glu	8.5
Pro	2.3
Gly	4.5
Ala	5.9
Cys	0
Val	9.6
Met	0.8
Ile	4.5
Leu	5.1
Tyr	3.7
Phe	5.6
Trp	n.d.**

* Based on a molecular weight of 45,000 and carbohydrate content of 15 %.
** Not determined.

Section 3

Specificity of Cranberry Bean α-Amylase Inhibitor and Its Formation of a Complex with α-Amylase

This section deals with the formation of a complex between Cranberry bean α-amylase inhibitor and porcine pancreatic α-amylase and the inhibition spectrum of the inhibitor against pancreatic α-amylase from various animals.

MATERIALS AND METHODS

Materials

An α-amylase inhibitor was purified from cranberry bean according to the method described in Section 2. The inhibitor is hereafter referred to as CBAI. Porcine pancreatic α-amylase (PPA), human salivary α-amylase and amylases from Aspergillus oryzae, Bacillus subtilis, barley malt and sweet potato were purchased from Sigma Chemical Co. Pancreas from pig, dog, cat, horse, sheep, cow, rabbit, guinea pig, rat and mouse were also obtained as acetone powder from Sigma Chemical Co., from which α-amylases were purified in this experiment by affinity chromatography with potato starch according to the method of Tsujisaka (74). Other chemicals of analytical grade were commercially available and used without further purifi-

cation.

Molar concentration

The molar concentrations of PPA and CBAI were calculated on the basis of their molecular weights of 54,000 (75) and 45,000, respectively.

Enzyme and inhibitory assay

The α-amylase and its inhibitory activity were measured by the iodine staining method described in Section 1.

pH Dependence of inhibition by CBAI

A unit of PPA was incubated at 37℃ for 30 min together with 1.2×10^{-8} M CBAI in various buffers composed of 10 mM sodium citrate (pH 4.0-5.5) or 10 mM sodium phosphate (pH 6.0-8.0) and 40 mM NaCl and 2 mM $CaCl_2$. The residual α-amylase activity after the indicated incubation period was measured as mentioned above, and the extent of inhibition was expressed in percentage as the difference between the activities in the presence and absence of CBAI under the assay conditions.

Gel filtration

A mixture of PPA at 1.8×10^{-5} M and CBAI at 9.2×10^{-6} M in 50 mM acetate buffer (pH 5.5) containing 50 mM NaCl and 5 mM $CaCl_2$ that

had been incubated at 37℃ for 60 min was applied to a Toyo-Pearl HW-55 column (2.6 x 95 cm) previously equilibrated with the same buffer, and the effluents were fractionated in each 4.0 ml. Aliquots (0.01 ml) of the fractions were assayed for their amylase and inhibitor activities.

Stoichiometry in formation of a complex

For the purpose of stoichiometric investigation on the formation of a complex between PPA and CBAI, a mixture of PPA at a fixed level (1.6×10^{-8} M) and CBAI at two fixed levels (6.5×10^{-9} M and 1.3×10^{-8} M) was incubated at pH 5.5 and 37℃ and its aliquots (1.0 ml) were withdrawn at stated intervals during incubation to measure the residual α-amylase activity. Subsequently, PPA (1.6×10^{-8} M) was incubated at pH 5.5 and 37℃ for 60 min together with CBAI at varied concentrations of 1.4×10^{-9} M to 1.4×10^{-8} M, and then, the residual α-amylase activity was likewise measured.

Reactivity of CBAI with various amylases

CBAI (1.2×10^{-7} M) was allowed to act on amylases from various sources such as animal, plant, fungus and bacteria in 50 mM acetate buffer (pH 5.5) or in 20 mM PIPES buffer (pH 6.9) at 37℃ for 30 min. The residual amylase activity was measured at pH 6.9 with α-amylases from animals and at pH 5.5 with amylases from other sources. The specificity of CBAI toward pancreatic α-amylases was investigated

using the acetate buffer (pH 5.5) and the PIPES buffer (pH 6.9), in which both incubation and activity measurement were carried out.

RESULTS AND DISCUSSION

Effect of preincubation temperature on inhibition by CBAI

A unit of PPA underwent 25% inhibition of the initial activity after incubation of 30 min at 25℃ and pH 6.9 in the presence of 6.2 x 10^{-8} M CBAI. The degree of inhibition by CBAI increased with increasing temperature, and the preincubation at 40℃ caused a 3 fold increase in inhibition relative to that at 25℃ (Fig. 12). In this

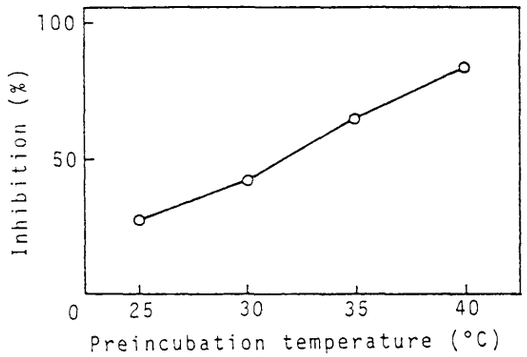

Fig. 12 Effect of preincubation temperature on the inhibition of PPA by CBAI. CBAI was incubated at pH 6.9 with PPA for 30 min at various temperatures ranging from 25-40°C. The extent of inhibition at different temperatures was calculated by setting α-amylase activity in each control incubation without CBAI at 100%.

connection, Marshall and Lauda (15) and Powers and Whitaker (76) have referred to the more rapid formation of a complex between PPA and

kidney bean α-amylase inhibitor at 25℃ than at 37℃, while Buonocore et al. (77) have found that there is a decrease in inhibition of chicken pancreatic α-amylase by wheat kernel inhibitors at above 30℃. Nevertheless incubation was carried out at 37℃ in this experiment.

Effect of pH on inhibition by CBAI

Figure 13 shows the effects of pH on inhibition of PPA by CBAI. CBAI exhibited the highest inhibitory effect on PPA at pH 5.5. The

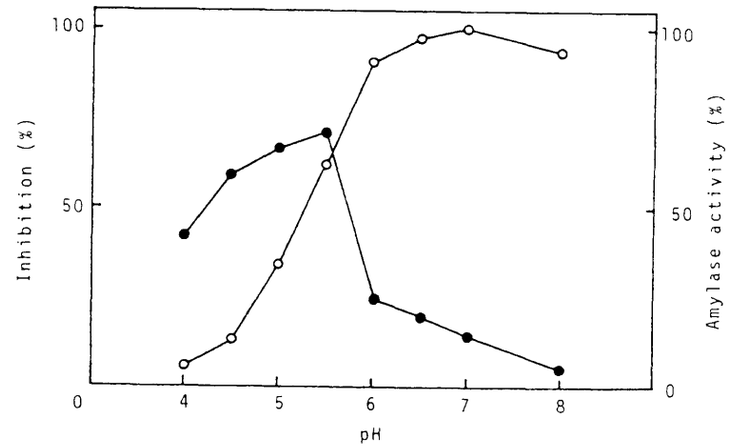

Fig. 13 Effects of pH on inhibition of PPA by CBAI. PPA was incubated at various pHs with CBAI for 30 min at 37°C. Controls were run without inhibitor at each pH simultaneously (○). The extent of inhibiton (●) at different pHs was calculated by setting α-amylase activity in each control incubation at 100%.

drop in inhibition below or above pH 5.5 was considered not due to denaturation of CBAI, because the inhibitor was stable over the pH ranges of 3 to 7 as described in the next section. The optimum pH of inhibition against PPA was in the range of 4.5 to 5.5 with α-amylase

inhibitors from many beans (15, 78), while wheat inhibitors were most inhibitory at pH 6.4-8.4 and pH 5.8-7.0 against human salivary and pancreatic α-amylases, respectively (79).

Formation of a complex with PPA

Figure 14 shows a typical gel filtration pattern of the CBAI and PPA mixture that had been previously incubated under the condi-

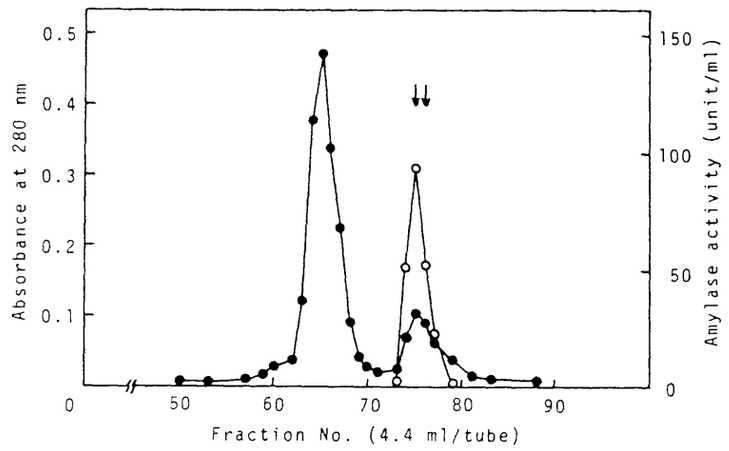

Fig. 14 Gel filtration of the mixture of PPA and CBAI. PPA and CBAI were incubated in 50 mM acetate buffer (pH 5.5) containing 50 mM NaCl and 5 mM $CaCl_2$ for 60 min at 37°C. The mixture was applied to a Toyo-Pearl HW-55 column (2.6ϕ × 95 cm) and then eluted with the same buffer. Left and right arrows indicate where free PPA and CBAI were eluted, respectively. Amylase activity (○), absorbance at 280 nm (●).

tions optimal for inhibition. In the presence of α-amylase excess to inhibitor, two peaks appeared on the chromatogram. Neither α-amylase nor inhibitor activities were detected in the first peak. Taking into account the elution volume, there is little doubt that the first peak component is responsible for a complex formed between PPA and

CBAI. Tanizaki and Lajalo (80) have reported that the complex between PPA and kidney bean α-amylase inhibitor is partially dissociative at acidic pH or above pH 6.5. On either apart side of the optimum pH, a similar dissociation may have occurred in the complex between PPA and CBAI. The second peak component was attributable mainly to PPA by activity measurement.

Stoichiometry in formation of the complex

Figure 15 shows the time course of inhibition of PPA at a fixed level by CBAI at two fixed levels. The complete inhibition took place

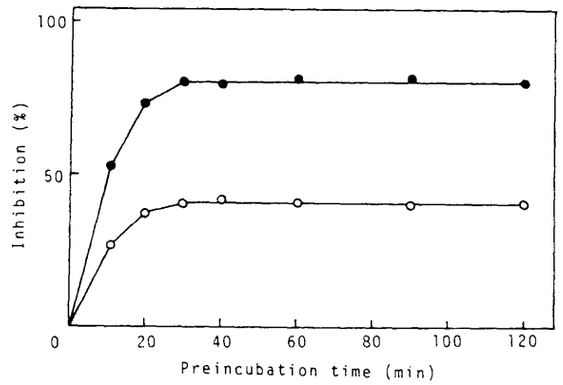

Fig. 15 Time course of inhibition of PPA by CBAI. PPA (1.6×10^{-8} M) was incubated with two different levels of CBAI at pH 5.5 and 37°C, respectively. After varying times of incubation, residual α-amylase activity was determined. 6.5×10^{-9} M of CBAI (○), 1.3×10^{-8} M of CBAI (●).

in 30 min at pH 5.5. However, the inhibition was not completed at pH 6.9 even after incubation of 6 h (data not shown). In this regard, it took 1 h at optimum pH for kidney bean inhibitors to completely inhibit PPA (15) but over 6 h at pH 6.9 for black kidney bean inhibitors to completely inhibit PPA (78). On the other hand, a Strepto-

myces inhibitor and PPA associated to form the complex almost completely within a few minutes (81). The ratio between the degrees of PPA inhibition by CBAI at two different levels suggests that a stoichiometric relationship is valid for their complex.

This relationship was also ascertained by using various concentrations of CBAI. As shown in Fig. 16, the residual activity of

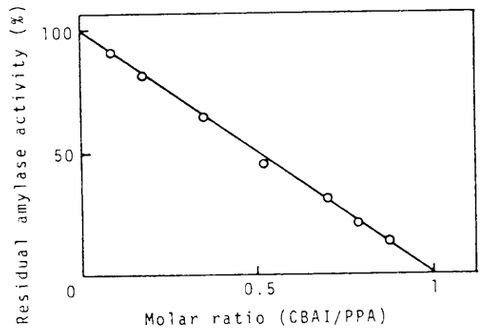

Fig. 16 Stoichiometry of inhibition of PPA by CBAI. PPA (1.6×10^{-8} M) was incubated at pH 5.5 and 37 C for 60 min with CBAI at varied concentrations of 1.4×10^{-9} M to 1.4×10^{-8} M, and then, the residual α-amylase activity was measured.

PPA decreased in inverse proportion to CBAI concentration and the molar ratio of CBAI to PPA to give a complete inhibition was obtained as 1:1 by extrapolation of the linear plot to the abscissa.

Specificity of CBAI on various amylases

Table 30 summarizes the inhibitory effects of CBAI on amylases from various sources. CBAI was as effective in inhibition against PPA as against human salivary α-amylase, while it had no inhibitory effect on amylases from plant, fungus and bacteria.

Table 31 compares the concentrations of CBAI to yield 50% inhibition against pancreatic α-amylases from mammals. Although mammalian pancreatic α-amylases corresponding to one unit were used at

Table 31 Inhibition of various amylases by CBAI.

Source of amylase	Inhibition (%)
α-Amylases	
Porcine pancreas	100
Human salivary	100
Barley malt	0
Aspergillus oryzae	0
Bacillis subtilis	0
β-Amylase	
Sweet potato	0
Amylase	
Cranberry bean[a]	0

[a]The extract from cranberry bean germinated for 7 days was used. Other enzymes were obtained from Sigma.

Table 32 Reactivity of CBAI with mammalian pancreatic α-amylases.

Source of pancreatic α-amylase[a]	Amount of CBAI (nM) required for 50% inhibition (pH 6.9)	(pH 5.5)
Pig	40	7.3
Dog	28	7.7
Cat	22	8.9
Horse	30	8.0
Sheep	250	6.7
Cow	>3,000	100
Rabbit	160	9.5
Guinea pig	180	12
Rat	>3,000	27
Mouse	150	8.4

[a]Preparations from pancreatic acetone powder were used as amylases.

either pH, the concentraion of CBAI to give 50% inhibition was much lower at pH 5.5 than at pH 6.9 for most of the enzymes. Under the physiological pH conditions, α-amylases from pig, dog, cat and horse were most effectively inhibited by CBAI, to which the enzymes from sheep, rabbit, guinea pig and mouse followed. Noteworthily the en-

zymes from cow and rat were little inhibited by CBAI even at a level of more than 3.0×10^{-6} M under the same conditions. There was a clear difference in optimum pH between the catalytic activity of pancreatic α-amylase and the inhibitory activity of its inhibitor. The concentrations of CBAI to give 50% inhibition against pancreatic α-amylases from cow and rat were 1.0×10^{-7} M and 2.7×10^{-8} M, respectively, at pH optimal for the inhibitory activity, pH 5.5. The reactivity of inhibitor with amylase have been hitherto evaluated at pH optimal for the amylase activity (81-83). Probably for this reason, multi-forms of wheat α-amylase inhibitors did not act on guinea pig pancreatic α-amylase (82), nor did microbial inhibitors on rabbit pancreatic α-amylase (81), nor did inhibitors from red gram, sorghum, pearl millet, sanwa millet and proso millet on horse pancreatic α-amylase (83). Such inhibitors may also become more inhibitory against α-amylases when assayed at weakly acidic pH.

CBAI even at sufficiently high concentrations showed little inhibition against pancreatic α-amylases from cow and rat at neutral pH, suggesting that CBAI would not always serve as inhibitor in the intestinal environments of these animals.

Section 4

Chemical Modification and Enzymatic Digestion of Cranberry Bean α-Amylase Inhibitor

As an example of practical application, may be cited the attempt to administer a purified preparation of α-amylase inhibitor from kidney bean to human subjects and thereby to lower postprandial glycaemia (84). Nevertheless, it is still obscure to what extent such inhibitors are resistant to proteolytic digestion in the gastrointestinal tract. In addition, there is not much information on pH or thermo-stability of these inhibitors or on their functional and structural features, except for a series of investigations on the inhibitors from grains (85, 86).

This section deals with the effects of incubation condition, chemical modification and protease treatment on the nature of CBAI.

MATERIALS AND METHODS

Materials

α-Amylases, bovine trypsin, bovine α-chymotrypsin and porcine pepsin were purchased from Sigma Chemical Co. 2,4,6-Trinitrobenzenesulfonic acid (TNBS), N-bromosuccinimide (NBS) and 1,2-cyclohexanedione (CHD) were from Nacalai Tesque Inc. (Kyoto). Other chemicals

commercially available were all of analytical grade, and were used without further purification.

Molar concentration

The molar concentration of CBAI was calculated by regarding of its molecular weight as 45,000. The molar absorption coefficients used to calculate the molar concentrations of proteases were as follows; 0.67 for bovine trypsin (87), 0.49 for bovine α-chymotrypsin (87) and 0.68 for porcine pepsin (88).

Inhibitory assay

The activities of α-amylase and its inhibitor were measured by the iodine staining method described in Section 1.

SDS-PAG electrophoresis

Electrophoresis was conducted at 8 mA/gel for 5 h in 10% polyacrylamide gel (PAG) using pH 7.2 phosphate buffer containing 0.1% sodium dodecyl sulfate (SDS) as described by Weber and Osborn (89). A pair of gels were individually stained with Coomassie brilliant blue to visualize protein band and with periodic acid-Schiff's reagent (PAS) to demonstrate glycoproteins.

Chemical modification of CBAI

CBAI was treated with TNBS according to the method of Haynes et al. (90), with CHD according to the method of Patthy and Smith (91), with NBS according to the method of Spande and Witkop (92), and with $NaBH_4$-formaldehyde according to Rice and Means (93). These modified inhibitors, after being well-dialyzed, were assayed for their inhibitory activities.

Protease treatment

A fixed level of CBAI was incubated at 37℃ together with two fixed levels of pepsin (pH 2.2), trypsin (pH 7.5) or chymotrypsin (pH 7.5), respectively. Aliquots (50 μl) withdrawn at half an hour intervals were either adjusted to neutral pH with PIPES buffer in case of pepsin digestion or heated at 70℃ for 10 min in case of trypsin or chymotrypsin digestion in order to inactivate the proteases.

RESULTS

Dissociation into subunits

The molecular weight of CBAI had already been proved to be approximately 45,000 by gel filtration on Sephacryl S-200. Then the purified preparation was applied to SDS-electrophoresis in 10% polyacrylamide gel. By protein staining with Coomassie brilliant blue (Fig. 17, A), CBAI has turned out to consist of three heterogeneous subunits; their molecular weights were 18,000 (a), 16,000 (b), and 11,000 (c), respectively, when compared with those of standards. A

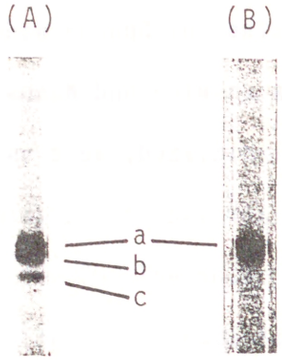

Fig. 17 A typical separation pattern of CBAI subunits by SDS-PAGE. Electrophoresis was carried out in the presence of 2-mercaptoethanol: (A), stained with Coomassie brilliant blue; (B), stained with PAS.

quite similar dissociation was observed irrespective of the presence or absence of 2-mercaptoethanol, because CBAI had no cysteinyl residues. Nothing but the subunit (a) with molecular weight of 18,000 was positive to sugar-staining with PAS (Fig. 17, B), although there was no evidence to show any role of the carbohydrate moiety in the inhibitory activity or in the resistance against enzymatic digestion.

Stability at various pH's and temperatures

When CBAI was allowed to stand in various buffers for a day at 37℃, its inhibitory activity decreased under alkaline conditions, although being almost unchanged at pH 3 to 7 (Fig. 18). The inhibitory activity of CBAI which had been heated for 20 min at pH 7 and 80℃, was more than half retained compared to its almost complete loss at pH 3 or 5 (Fig. 19). Thus, CBAI seemed to undergo little inactivation so long as physiological conditions are considered.

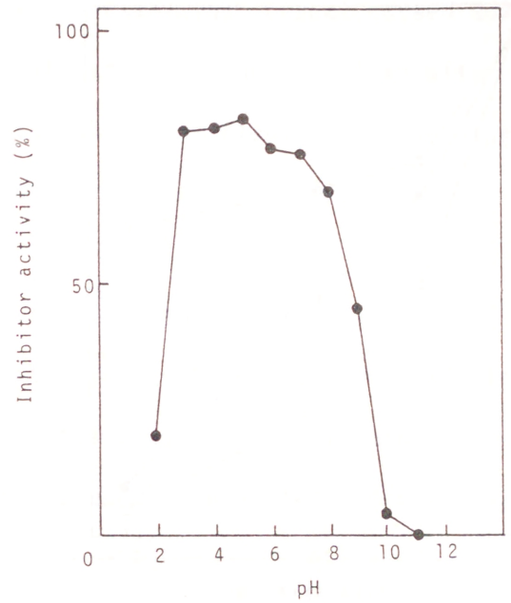

Fig. 18 Stability of CBAI at various pH's. Each solution containing CBAI (2.2×10^{-6} M) in 10 mM buffer was incubated at 37°C for 24 h. The buffers used in this experiment were as follows: Na-citrate-HCl buffer for pH 2 and 3; Na-acetate buffer for pH 4 and 5; NaH_2PO_4-Na_2HPO_4 buffer for pH 6, 7 and 8; H_3BO_3·KCl-NaOH buffer for pH 9 and 10; and Na_2HPO_4-NaOH buffer for pH 11. After a desired period, aliquots (50 μl) were withdrawn, adjusted to pH 6.9 with the PIPES buffer, and assayed for their residual inhibitory activity.

Effects of metal ions and reagents on CBAI

After respective incubations of CBAI (4.4 x 10^{-5} M) in metal salt solutions or reagent solutions at 37℃ for 2 and 24 h, each mixture was diluted 100-fold with water and the remaining α-amylase inhibitory activity in 0.1 ml aliquots was measured. The effects of metal ions and reagents on CBAI are shown in Table 33. The inhibitor was scarcely inactivated by 2 h incubation with the metal ions other than Hg ion. Respective 24 h incubations with 20 mM $CuSO_4$ and $HgCl_2$ resulted in 40% and 89% loss of the original activity. Incubation of

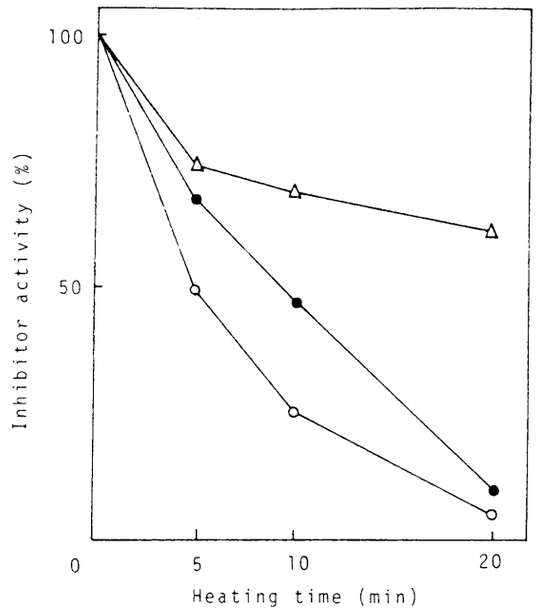

Fig. 19 Effects of heat treatment on stability of CBAI. The experimental conditions were the same as in Fig. 18, with the exception of heating at 80°C for indicated periods: ○, at pH 3; ●, at pH 5; △, at pH 7.

Table 33 Effects of metal ions and reagents on the action of CBAI

	% remaining inhibitory activity	
	2 h	24 h
None	100	100
$MnCl_2$	106	103
$CoCl_2$	92	94
$BaCl_2$	102	98
$ZnSO_4$	128	120
$CuSO_4$	106	60
$HgCl_2$	39	11
H_2O_2	76	44
$NaNO_2$	92	102
$Na_2S_2O_3$	99	112
2-Mercaptoethanol	104	92
EDTA	102	104
o-Phenanthroline	100	101

CBAI was incubated with 20 mM metal ions in water and with 20 mM reagents in 20 mM PIPES buffer, pH 6.9 at 37°C for 2 and 24 h.

CBAI with H_2O_2 as an oxidizing reagent caused 24% loss of the initial inhibitory activity after 2 h and 56% loss after 24 h. However, reducing reagents, chelating agents, and the other compounds tested had no effect on the activity of CBAI.

Inactivation by chemical modification

The inhibitory activity of CBAI was affected by chemical modification with various reagents. Treatments of the inhibitor with TNBS, CHD and NBS caused more or less remarked diminution in the

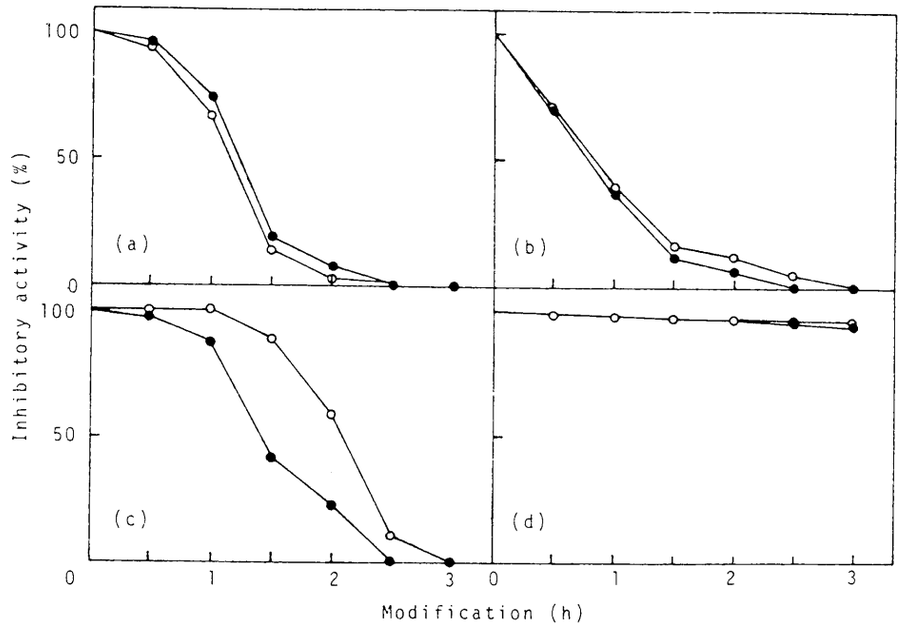

Fig. 20 Effects of chemical modification on inhibitory activity of CBAI. CBAI was treated with TNBS (a), CHD (b), NBS (c), and $NaBH_4$ + formaldehyde (d), according to the procedures previously described (90–93). Inhibitory activity was measured against PPA (○) and HSA (●).

activity, which was exhaustively lost within 3 h, while reductive alkylation of lysyl ε-amino groups with formaldehyde and $NaBH_4$ was

not responsible for inactivation during incubation for 3 h (Fig. 20). In addition, this treatment had no effect on the inhibitory activity even after the incubation period of more than 24 h (data not shown).

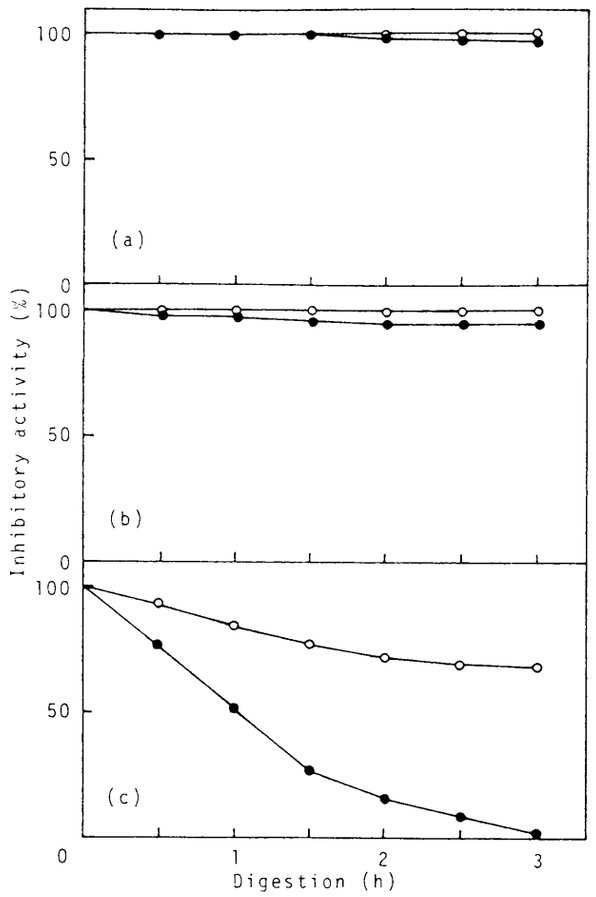

Fig. 21 Effects of *in vitro* digestion by pepsin, trypsin, and chymotrypsin on inhibitory activity of CBAI. Reaction mixtures containing CBAI at a fixed level (2.2×10^{-5} M) and protease at two fixed levels were incubated at 37°C for indicated periods. Open and closed circles represent concentrations of proteases used: (a), pepsin (○, 2.2×10^{-7} M; ●, 2.2×10^{-5} M); (b), trypsin (○, 2.2×10^{-4} M; ●, 2.2×10^{-3} M); (c), chymotrypsin (○, 2.2×10^{-6} M; ●, 2.2×10^{-5} M). Other experimental conditions are described in the text.

In vitro digestion by proteolytic enzymes

Changes in the inhibitory activity of CBAI by in vitro digestion were examined with proteolytic enzymes such as pepsin, trypsin and chymotrypsin (Fig. 21). The inhibitor was virtually resistant to the proteolysis by porcine pepsin of equimolar concentration over 3 h at 37℃ and pH 2.2. A similar resistance was observed for trypsin digestion even at 100-fold concentration of the enzyme, but not for chymotrypsin digestion. Digestion with trypsin or chymotrypsin was terminated by heating the reaction mixture for 10 min at 70℃ because the heat treatment itself led to no considerable decrease in the inhibitory activity. In contrast the inhibitor was so much attacked by bovine chymotrypsin of equimolar concentration as to lose completely the initial activity during incubation for 3 h. By chymotrypsin of one-tenth molar concentration, about three quaters of the inhibitor was left intact after 3 h.

DISCUSSION

There is great diversity in the number and molecular size of subunits concerning α-amylase inhibitors hitherto known. α-Amylase inhibitor from white kidney bean comprises four identical subunits with molecular weight of 11,000 (17), while the inhibitor from black kidney bean is composed of three different subunits (molecular weights of 17,500, 16,500, and 13,500) linked up with carbohydrate

chains (19). CBAI is distinguished from these inhibitors in subunit composition. The dissociation into subunits of CBAI containing neither sulfhydryl group nor disulfide bond has been never affected by the presence or absence of 2-mercaptoethanol as a reducing reagent, being dissimilar to the behavior in the inhibitors from rye (94) and sorghum (86). In general, the majority of proteinaceous α-amylase inhibitors are glycoproteins. As for CBAI, carbohydrate chains are exclusively localized in one of the three subunits. Taking into account the occurrence of non-glycoprotein α-amylase inhibitor in wheat (95), it seems unlikely that the carbohydrate moiety plays a crucial role in the interaction between CBAI and α-amylase. CBAI in solution was not only stable for a long time over the range of weakly acidic to neutral pH at ordinary temperature but also resistant to heat treatment at high temperature as compared with α-amylase inhibitors from other sources. Conversely the activities of both inhibitors from foxtail millet (85) and sorghum (86) decrease appreciably during the course of incubation at neutral pH and 70℃.

Andriolo et al. (96) have also demonstrated that phaseolamin, that is, an α-amylase inhibitor from white kidney bean is susceptible to chymotrypsin digestion, although being resistant to pepsin or trypsin digestion. Phaseolamin loses nearly all its activity within 2 h due to the action of chymotrypsin occurring in the 1 : 50 ratio of enzyme to inhibitor. On the other hand, Frels and Rupnow (78) have reported that α-amylase inhibitors from black kidney bean

are inactivated by trypsin of 100-fold molar concentration. Chemical modification of CBAI with TNBS caused a rapid loss of the inhibitory activity, indicating that amino groups were involved in inhibition of α-amylase. Nevertheless, reductive methylation of lysyl ε-amino groups with formaldehyde had no effect on the inhibitory activity. It can be presumed that the contradictory results obtained above are due to the following reason; while TNBS causes incorporation of bulky aromatic groups to lysine and N-terminal residues, formaldehyde converts these residues to dimethylated derivatives causing relatively small changes in the size of the residues. Iodoacetate did not inactivate CBAI (data not shown). As mentioned above, a short-term treatment of CBAI with CHD or NBS also resulted in a considerable loss of the activity. Wilcox and Whitaker (97) have reported that oxidation of tryptophan residue of red kidney bean α-amylase inhibitor with NBS led to loss of inhibitory activity. However, it is improper to emphasize that arginine as well as tryptophan are situated in the reactive site of CBAI or its vicinity because in some cases conformational changes may arise from modification of guanido groups or cleavage of indole ring. These structural problems under consideration remain to be further investigated.

The resistance to digestion in the enterogastric phase is presumably characteristic of α-amylase inhibitors including CBAI. As a matter of fact, the opportunity of CBAI coming into contact with stomach acids is considerably limited by interference of other food

ingredients or by gastric emptying. Thus, CBAI should be considered to pass through the stomach without significant loss of the activity. Unless inactivated by chymotrypsin within the intestinal lumen, CBAI is highly possible to react with pancreatic α-amylase to form a stable complex.

Section 5

Immunological Characterization of Cranberry Bean α-Amylase Inhibitor

This section deals with the inhibitor distribution in various bean cultivars as well as the variation in antigenicity during protease treatment in vitro, using the rabbit antiserum against CBAI.

MATERIALS AND METHODS

Materials

Leguminous seeds used in this experiment are as follows; 7 cultivars of Phaseolus vulgaris (great northern beans, small white kidney beans, light red kidney beans, kintoki beans, tora beans, uzura beans, and cranberry beans), 2 cultivars of Phaseolus lunatus (butter beans and baby lima beans), 2 cultivars of Vigna anglaris (bamboo beans and adzuki beans), and cultivars of Phaseolus coccineus (runner beans) and Psophocarpus tetragonolobus (winged beans). Kintoki, tora, uzura, and adzuki beans were purchased from a market and the others from a food importer. All chemicals commercially available were of analytical grade and were used without further purification.

Water extraction of pulverized beans

Flour ground from air-dried beans was passed through a 60-mesh sieve and stirred for 2 h at room temperature with five volumes of distilled water, followed by centrifugation (15,000 x g for 60 min). The resulting supernatant was heated at 70℃ for 15 min, centrifuged at same speed as above, and subjected to immunoassay in the manner described below.

Purification of CBAI

A highly purified preparation of CBAI was obtained from cranberry bean powder by means of (step 1) water extraction, (step 2) heat treatment, (step 3) ethanol precipitation, (step 4) ion-exchange chromatography with DEAE-cellulose and (step 5) gel filtration with Sephacryl S-200 as described in Section 2, Part A.

Immunization of a rabbit with CBAI

A New Zealand male rabbit weighing about 3 kg was immunized by subcutaneous injection with an emulsified W-in-O mixture of CBAI (5 mg), pH 7.2 phosphate buffer (1 ml) and Freund's complete adjuvant (1 ml). A booster shot of 2 mg of CBAI was given 2 weeks after the first injection, and a week later, the animal was bled to collect the serum.

Immunoassay

Double simple immunodiffusion for qualitative analysis was

carried out in flat agarose gel according to the method of Ouchterlony (98,99). Single radial immunodiffusion technique was employed for the quantification of antigen (100). In brief, 6 ml of prewarmed 1% agarose in phosphate buffer containing 0.5 ml of the antiserum was poured onto a glass plate (3 x 10.5 cm) and in a little while, 2 mm wide holes were punched in the gel on the plate and filled with 4 μl of the antigen solutions. The plate was then incubated in a humid atmosphere for a whole day, and the area of hole was determined by diameter measurement as a function of the antigen concentration. Quantification is based on the observation that the area of the hole has a linear relationship to the initial concentration of the antigen for a definite concentration of the antiserum. Precipitin lines in agarose gel after immunodiffusion were made visible by staining with Coomassie brilliant blue.

Inhibitory assay

α-Amylase inhibitory activity was measured by the iodine staining method as previously described.

Digestion by proteases

A definite concentration of CBAI (2.2×10^{-5} M) was treated at 37℃ with fixed concentrations of pepsin (pH 2.2, 2.2×10^{-4} M), trypsin (pH 7.5, 2.2×10^{-3} M) and chymotrypsin (pH 7.5, 2.2×10^{-4} M), respectively. Aliquots (50 μl) were taken out at 30 min inter-

vals. Then, pepsin was inactivated by neutralization with PIPES buffer, while trypsin and chymotrypsin were inactivated by heating at 70℃ for 10 min.

Molar concentration

The molar concentrations of CBAI and proteases used were determined as described in Section 4.

RESULTS AND DISCUSSION

Specificity inspection of antiserum

The rabbit antiserum raised against CBAI was tested for its immuno-reactivity with respective preparation at five purification steps. Immuno-precipitin reaction in agarose gel resulted in providing a single fused precipitin line without any spur as shown in Fig. 22. This implies that the antiserum contains no antibody against iso-inhibitors, if any, nor against contaminants possibly arisen from modification or denaturation of CBAI during purification process.

Limited occurrence of α-amylase inhibitor in leguminous seeds

Aqueous extracts from 13 kinds of bean cultivars were examined for both inhibitor effects on porcine pancreatic α-amylase and precipitin reactions with the rabbit anti-CBAI serum. Among various bean cultivars so far tested, only those of Phaseolus vulgaris were

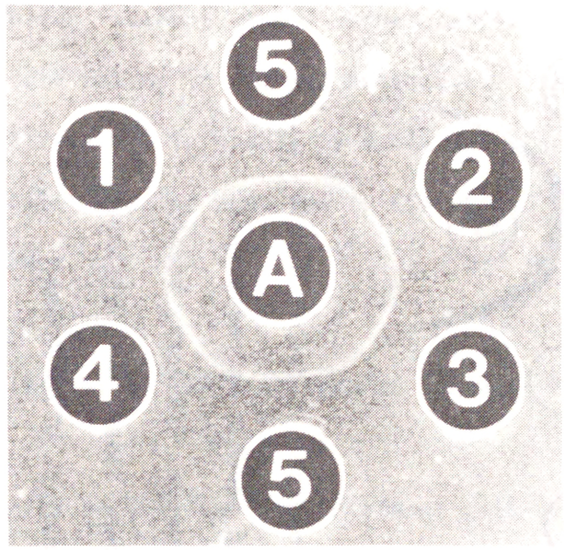

Fig. 22 Immuno-precipitin reaction in agarose gel by double simple diffusion between preparation at each purification and rabbit anti-CBAI serum. A, antiserum; 1～5, the corresponding preparation at step 1～5. A brief explanation of each step was given in the text.

found to be inhibitory to mammalian α-amylase and immuno-reactive with the antiserum as well. For this reason, the immuno-reactivity between the aqueous extracts from 7 kinds of Phaseolus vulgaris seeds and the anti-CBAI serum was depicted by a single line fused into CBAI in Fig. 23. Needless to say, the pre-immune serum gave no precipitin reaction with any aqueous extracts from beans.

These results indicate that such an inhibitor with common antigenicity is exclusively occurring in cultivars of the same kinds (Phaseolus vulgaris). In this connection, Pick and Wober (101) have reported that broad beans (Vicia faba) not having any inhibitor ef-

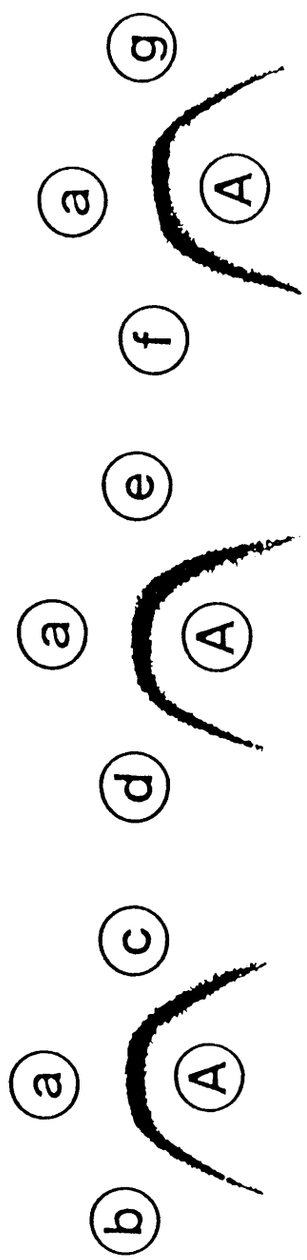

Fig. 23 Immuno-precipitin reaction in agarose gel by double simple diffusion between rabbit anti-CBAI serum and aqueous extracts from cultivars of Phaseolus vulgaris. A, antiserum; a, cranberry beans; b, great northern beans; c, small white kidney beans; d, light red kidney beans; e, kintoki beans; f, tora beans; g, uzura beans. Precipitin lines were stained with Coomassie brilliant blue.

fect on mammalian α-amylase are likewise inactive in immuno-reactivity with the rabbit antiserum raised against white kidney bean

α-amylase inhibitor. Although there is no remarkable difference in the inhibitor distribution in legumes between the inhibitor activity and immuno-reactivity, it is highly probable that functionally inactive but genetically similar proteins to CBAI would be widely distributed in legumes.

In vitro digestibility of CBAI; changes in inhibitor activity and antigenicity

The stability of CBAI against treatment with proteolytic enzymes such as pepsin, trypsin and chymotrypsin was assessed by measuring its time-dependent losses in inhibitor activity and antigenicity. CBAI was virtually resistant to pepsin digestion (37℃, 3 h) despite the enzyme concentration of 10-fold molar ratio to CBAI (Fig. 24a). A somewhat synchronized diminution in both inhibitor activity and antigenicity of CBAI was mainly due to its unavoidable denaturation when exposed to acidic pH (data not shown). A quite similar tendency was observed in case of trypsin digestion (Fig. 24b), in which the enzyme was a 100-fold as much as CBAI by molar concentration. In either case, a structural change might have been rather reflected in the loss of antigenicity than in that of inhibitor activity. In contrast to its resistance to digestion by pepsin or trypsin, CBAI was relatively sensitive to chymotrypsin digestion and its inhibitor activity was almost missing within 2 h in the presence of chymotrypsin a 10-fold as much as CBAI (Fig. 24c). The antigenicity of CBAI was

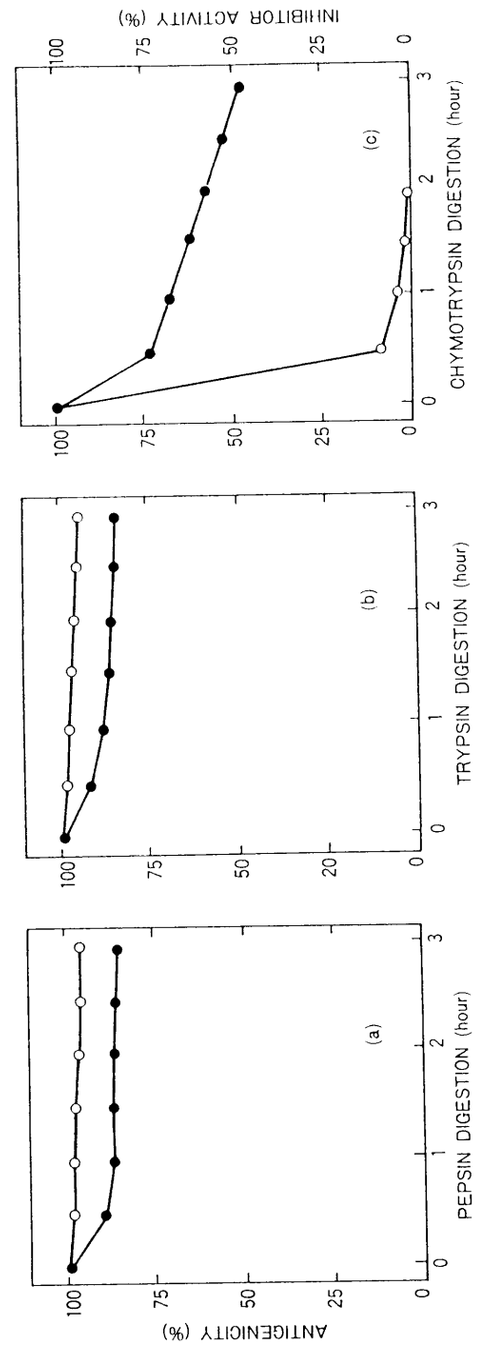

Fig. 24 Effects of digestion by pepsin (a), trypsin (b) or chymotrypsin (c) on both inhibitor activity and antigenicity of CBAI. Sampling from the reaction mixture containing fixed levels of CBAI (2.2×10^{-5} M) and pepsin (2.2×10^{-4} M), trypsin (2.2×10^{-3} M)) or chymotrypsin (2.2×10^{-4} M) was carried out at stated intervals, and the protease activity was terminated by pH adjustment or heat treatment. The assay procedures of α-amylase and the inhibitor (antigen) were the same as described in the text: ○, residual inhibitor activity; ●, residual antigenicity.

nevertheless kept by half even after complete disappearance of in-

hibitor activity. In order to avoid inactivation of CBAI by acidification, trypsin and chymotrypsin digestions were terminated by heating the reaction mixture for 10 min at 70℃, by which neither antigenicity nor inhibitor activity was affected. This difference in susceptibility to chymotrypsin digestion between the antigenicity and inhibitor activity is of much interest in considering the structure-activity (or antigenicity) correlation. It was revealed by SDS-polyacrylamide gel electrophoresis (data not shown) that chymotrypsin digestion did not produce minor fragments other than three subunits indicated in Section 4 for CBAI without chymotrypsin treatment. Even so a partial cleavage of the peptide bond is likely to have happened in the reactive site of CBAI or its vicinity. This may be the reason why the antigenicity does not necessarily corresponded to the inhibitor activity regarding their decrease rates.

Section 6

Intraluminal Movement of Cranberry Bean α-Amylase Inhibitor

Much interest has been arisen toward the use of an α-amylase inhibitor in therapy of diabetes mellitus or corpulency, because its oral administration is expected to prevent starch digestion and thereby to cause a great calorie cut. In this regard, Layer et al. (84) demonstrated that white kidney bean α-amylase inhibitor was effective in lowering postprandial glycemia in human subjects. Similar effects in rats were obtained with α-amylase inhibitor from black kidney beans (102). These observations gave apparent support to the possibility of practical application of the inhibitor, but did little information on to what extent it escapes from attack by proteases and acts on α-amylase in the lumen.

This section deals with the results of an investigation on the intraluminal behavior of CBAI containing [^{14}C]methylated CBAI after its gastric intubation from antigenic and radioactive viewpoints.

MATERIALS AND METHODS

Materials

Cholecystokinin octapeptide (CCK-8) was purchased from Sigma Chemical Co. [^{14}C]Formaldehyde (10 mCi/mol), [^{14}C]toluene (4.7 x 10^5 dpm/ml) and Protosol (a tissue solubilizer) were products of New England Nuclear. Clearsol for liquid scintilation and 3,5-dinitrosalicylic acid for amylase assay were from Nacalai Tesque Inc. (Kyoto). All other chemicals were of analytical grade commercially available.

Labeling of CBAI with radioisotope

Reductive methylation of CBAI with [^{14}C]HCHO and $NaBH_4$ was carried out according to Rice and Means (93); namely CBAI (9 mg) was dissolved in 2 ml of 0.2 M (pH 9) borate buffer, to which was added 10 μl of 40 mM [^{14}C]HCHO (4.6 mCi/mol) and 50 μl of 15 mM $NaBH_4$ five times at 30 sec intervals. The reaction mixture was allowed to stand for 6 h on ice-cold water and dialyzed several times against 500 ml of distilled water. These operations were repeated five times and the dialyzates were combined, followed by lyophilization.

Animal experiment

Male Wistar rats weighing about 160 g that had been fasted overnight, were given by gastric intubation one ml of a saline solution containing 2 mg of [^{14}C]-labeled CBAI and 8 mg of native CBAI, and at the same time, 100 μl of a saline solution containing 1 μg of CCK-8 was injected into the tail vein so as to stimulate pancreatic exocytosis. The rats were sacrificed at stated intervals to excise the gastrointestine, which was divided into stomach, three equal

small bowel segments (proximal, middle and distal parts) and caecum. Their corresponding intraluminal contents and washings were combined, individually lyophilized and stored in the refrigerator until use. Several rats receiving one ml of saline containing no CBAI were used for reference.

Enzyme and inhibitory assay

The activities of α-amylase and its inhibitor were estimated by a modification of the Bernfeld method (103) with 3,5-dinitrosalicylic acid as detecting reagent for reducible sugars. The assay medium contained 50 mM NaCl, 5 mM $CaCl_2$, 0.01% ovalbumin and an appropriate concentration of α-amylase in 20 mM PIPES buffer of pH 6.9. Reaction was initiated by adding 0.5 ml of 1% soluble starch to 1 ml of the assay medium, and after incubation at 37℃ for 3 min, terminated by heating its aliquot (1 ml) for 5 min in a boiling water bath together with 1 ml of 1% of 3,5-dinitrosalicylic acid in the same buffer as above. The reaction mixture was diluted with distilled water so favorably as to measure the absorbance at 540 nm. One unit of amylase activity was defined as the amount of enzyme producing a 1.0 increase in the optical density at 540 nm under the above conditions. The trypsin and chymotrypsin activities were colorimetrically determined as described in Chapter I, Section 2. Estimation of the pepsin activity was due to UV measurement of digested casein.

Immunoassay

Double immunodiffusion was done in the usual way (98,99), the center and peripheral wells in 2 mm thick agarose gel were charged with the same rabbit anti-CBAI serum as used in Section 5 and HCHO-treated and untreated CBAI samples, respectively. The gel after a day's diffusion was fully rinsed in phosphate buffer and stained with Coomassie brilliant blue. Quantification of the intraluminal CBAI content in each segments was due to single radial diffusion (100).

Radioactivity measurement

An aliquot of the intraluminal content (0.1 ml) was decolored by treatment at 50℃ for 30 min with an equal volume of hydrogen peroxide, and then dissolved in 0.3 ml of Protosol. After addition of 10 ml of Clearsol per vial, the radioactivity was measured with a Packard-Tricarb liquid scintillation counter. Corrections on quenching due to inadequate decolorization were made using [^{14}C]toluene as a standard.

Statistical analysis

It was evaluated on the basis of Student's t-test whether there is a significant difference between two means when F-test was significant ($P<0.05$) as a result of variance analysis.

RESULTS AND DISCUSSION

Justification of CBAI modification with [^{14}C]HCHO

CBAI was treated with [^{14}C]HCHO and $NaBH_4$ in order to obtain a radioactive probe. The efficiency of [^{14}C]methylation was however far low against expectation. CBAI with molecular weight of 45,000 contains 4 lysine residues per mole as described in Section 2, Part A so that at least 22 nmoles of [^{14}C]HCHO per mg of CBAI can be linked to the ε-amino group of lysine, being corresponding to 0.1 μCi (2.2×10^5 dpm). Actual counting of [^{14}C]HCHO-treated CBAI was approximately 1.5×10^4 dpm per mg of CBAI; implying that only partial lysine residues underwent chemical modification. Even if it were so, the treatment of CBAI with HCHO and $NaBH_4$ may possibly cause a change in its antigenicity. Figure 25 shows the result of double immunodiffusion in

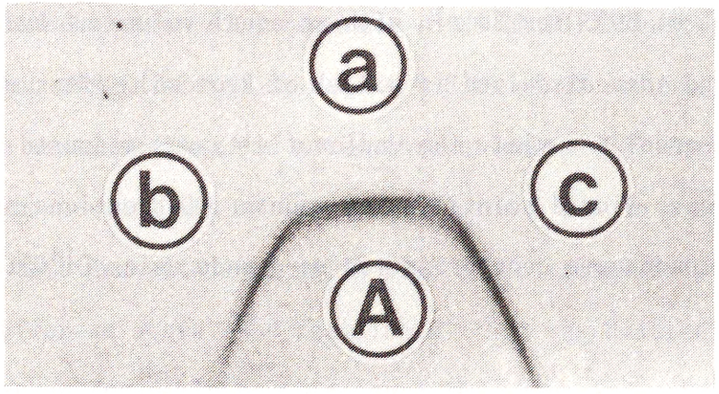

Fig. 25 Double immunodiffusion between rabbit anti-CBAI serum and CBAI with or without HCHO treatment. A, anti-CBAI serum; a, native CBAI; b, [^{14}C]HCHO-treated CBAI; c, CBAI treated with excess HCHO. A single fused precipitin line in agarose gel was made more visible by staining the plate with Coomassie brilliant blue.

agarose gel of the antiserum against CBAI samples with and without reductive methylation. Three precipitin lines were fused together with each other, and no spur was observed at each fusion point. For comparison, CBAI was treated with a large excess amount of cold HCHO. As a matter of course, the efficiency of CBAI methylation ought to have been markedly enhanced. Double immunodiffusion of the specific antiserum against more modified CBAI resulted in a single precipitin line without spur.

Subsequently it was examined whether the process of reductive methylation affects <u>in vitro</u> digestibility of CBAI. Figure 26 illustrates the result of CBAI digestion by chymotrypsin. Both HCHO-treated and untreated CBAI samples were unsusceptible to digestion by pepsin or trypsin (data not shown). On the contrary, the inhibitor activities of CBAI samples with and without chemical modification decreased gradually with prolonged chymotrypsin digestion, and largely disappeared at the first 30 min in the presence of chymotrypsin at a 10-fold molar concentration as much as CBAI. There was no difference in the loss of inhibitor activity between these modified and native samples. The decay curve for antigenicity sloped more gently than that for inhibitor activity. Even in this instance, differences between the two CBAI samples would be impossible. Such is the case in practice. The use of [^{14}C]HCHO-treated CBAI as a radioactive probe can be regarded as reasonable, because the reductive methylation of CBAI did not only affect its inhibitor activity but also did its

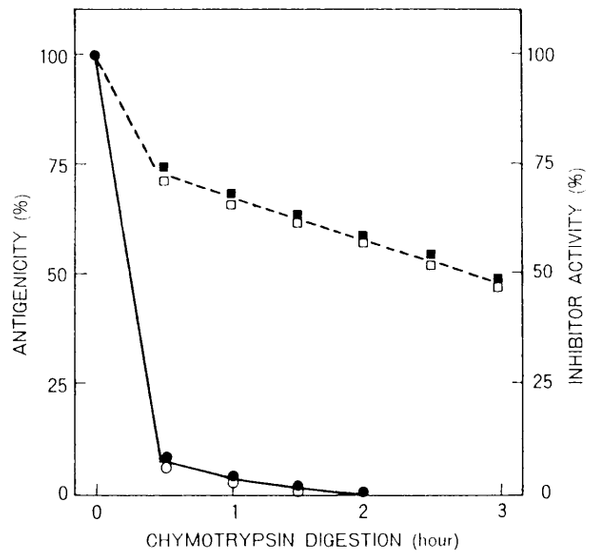

Fig. 26 Effect of HCHO treatment on susceptibility of CBAI to chymotrypsin digestion. Reaction mixture at 37℃ contained 2.2 x 10^{-5} M HCHO-treated or untreated CBAI and 2.2 x 10^{-4} M bovine chymotrypsin. Aliquots were withdrawn at stated intervals, followed by inhibitor activity and antigenicity measurements. The antigenicity was estimated as the area of the hole by single radial immunodiffusion. Inhibitor activity: ○, HCHO-treated CBAI; ●, native CBAI. Antigenicity: □, HCHO-treated CBAI; ■, native CBAI.

antigenicity.

Intraluminal movement of CBAI along gastrointestine

Figure 27 depicts the intraluminal inhibitor distribution in rat gastrointestine, which was represented by the proportion of radioactive CBAI in the respective segments at stated intervals after a dosage of 10 mg of CBAI containing 0.007 μCi of [^{14}C]methylated one. CBAI was thoroughly emptied of the stomach in 3 h. In this

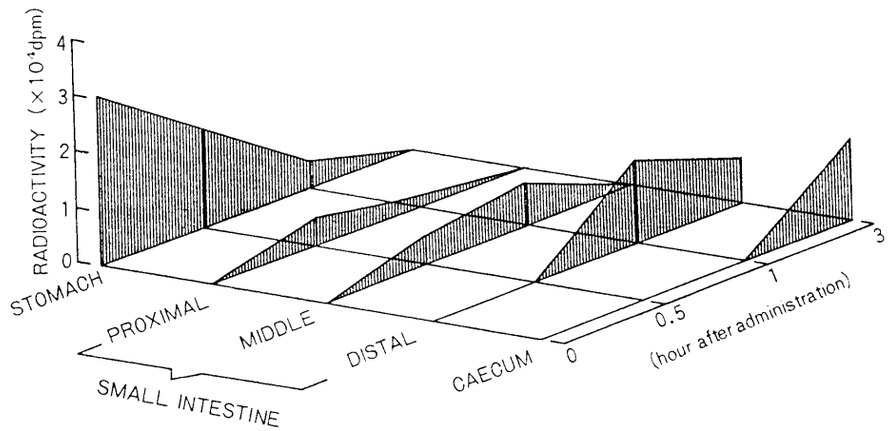

Fig. 27 Distribution of radioactive CBAI in gastrointestine at stated intervals after its administration. A mixture of cold CBAI and [^{14}C]labeled CBAI was administered by gastric intubation to rats, which received an intravenous injection CCK-8 at the same time. At each prescribed time, four rats were sacrificed and the digestive tract was excised to measure the residual radioactivity in its regions.

relation, gastric emptying seemed to be earlier than that ordinarily observed for rats given a solid chow. This is because CBAI was administered in dilute solution and moderate quantity. The radioactivity levels in the proximal and middle thirds of small intestine were relatively low at the first two time points (0.5 h and 1 h) after the CBAI administration, and were almost missing in 3 h. This fact suggests that chyme would have smoothly passed through these intralumens. The intraluminal radioactivity became maximal in 1 h in the distal third, although being pretty left in 3 h, and so did in 3 h in the caecum. Since the standard deviation from the average

recovery ranged from 12.5 to 36% (n=4), the amount of administered [^{14}C]CBAI can be regarded as almost quantitatively recovered at each measuring time.

Figure 28 summarizes the results of single radial immunodiffusion of CBAI remaining in the respective segments at indicated times.

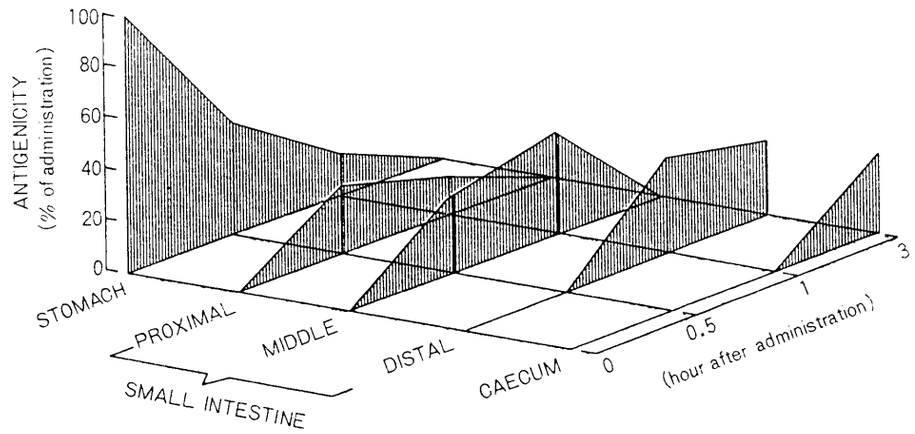

Fig. 28 Distribution of immuno-reactive CBAI in gastrointestine at stated intervals after its administration. The animals used in this assay were the same as in Fig. 27. The amount of immuno-reactive CBAI, i.e. the antigenicity level, remaining in each region at a prescribed time was estimated as above, and expressed in percentage of that in the stomach at zero time.

The residual CBAI levels estimated from this viewpoint were seemingly higher in the proximal and middle thirds and lower in caecum as compared with those simultaneously done on the basis of radioactivity measurement. Taking into account the experimental error (less than 10%) and standard deviation (ranged from 8 to 25%), however, a similar relation may safely be said to hold on the whole between the intraluminal CBAI movements judged by antigenicity and radioactivity.

CBAI does not undergo the action of pepsin itself, although its inhibitor activity being only a little inactivated when exposed to acidic pH below pH 2 as mentioned in Section 3. No significant loss was observed in the antigenicity of CBAI during its stay in the stomach. On the other hand, it was assumed from the in vitro digestion experiment that CBAI would be not sensitive to trypsin digestion but to chymotrypsin digestion. Even if its proteolysis should take place a little in the duodenojejunum, the amount of CBAI recovered from the small intestine which was obtained by radioactivity and antigenicity measurements, was almost equal to that sent out of the stomach at early stages after administration. In addition to such a quantitative recovery, the agreement between both radioactivity and antigenicity determinations at these measuring times suggests that CBAI would have been saved from intraluminal digestion. Interestingly, the average recovery of CBAI from the ileocaecum 3 h after its administration seemed to be less in antigenicity than in radioactivity. This difference can be fairly interpreted by considering that a partial cleavage of the peptide bond or a structural change leading to a decrease in antigenicity takes precedence over intestinal absorption leading to a decrease in radioactivity.

The amount of CBAI administration used in this experiment was too a little to stimulate pancreatic exocytosis. For the purpose of

raising secretion of the pancreatic juice, CCK-8 (1 μg) was injected into the tail vein. Prior to its injection, rats were forcedly given a saline solution containing 10 mg of CBAI. Twenty minutes later, the output of digestive enzymes began to increase and continued for a while. About that time, one-third to half of administered CBAI had been transferred from the stomach to the intestine, in which CBAI was in contact with secreted digestive enzymes. The results of radioactivity and antigenicity measurements implied that CBAI rid of digestion and absorption was transferred to the ileocaecum to some extent.

Section 7

In Vivo Action of Cranberry Bean α-Amylase Inhibitor

α-Amylase inhibitors from plant foods prevent more or less in vitro hydrolysis of starch by α-amylase (13) but there have been conflicting reports concerning whether they can effectively block α-amylase activity in vivo. For example, Plus and Keup (104) have described that wheat α-amylase inhibitor administered by stomach tube affects amylolysis in the gut of rats or dogs, and Macri et al. (105) have mentioned that successive feeding on the inhibitor leads to a decrease in chicken growth. On the other hand, Savaiano et al. (106) has reported that the intake of α-amylase inhibitor from red kidney bean does not disturb the growth of weanling rats. Conversely Lajolo et al. (107) have referred to the possibility that a hypoglycemic effect may be expected by feeding the inhibitor from black kidney bean.

This section deals with postprandial changes in intraluminal α-amylase activity as well as in plasma glucose and insulin concentrations in rats with CBAI loading.

MATERIALS AND METHODS

Materials

3,5-Dinitrosalicylic acid used for the assay of α-amylase activity was obtained from Nacalai Tesque Inc. (Kyoto). Casein, α-corn starch, vitamin mixture and mineral mixture were the products of Oriental Yeast Co. (Tokyo). Polyethylene glycol 4000 (PEG), an unabsorbable marker for measurement of gastric emptying, was from Wako Pure Chemical Co. (Osaka). All other chemicals commercially available were of analytical grade and used without further purification.

Animal experiments

Male rats of the Wistar strain weighing about 120 g were individually housed in a temperature (23±1℃)-controlled room with 12 h light / dark cycle. The rats that had been fed a 20% casein-based diet for 10 days under the meal-feeding conditions at AM 10:00 - 12:00, were given access to a 10% PEG-containing experimental diet punctually on the day of experiment. The composition of the experimental diet was as follows; casein (20%), α-corn starch (59%), oil mixture (5%), mineral mixture (5%), vitamin mixture (1%) and PEG (10%). CBAI was given at a dose of 10 mg per animal in 2 g diet. Each animal ate a fixed quantity of the diet (2 g) exhaustively within 20 min after the start of feeding. A pair of rats with and without CBAI loading were sacrificed at indicated intervals after feeding, by carotid amputation to collect the blood in a heparin-coated dish to excise the gut. The plasma was separated by centrifugation. The gut was divided into stomach and three intestinal (proximal, middle and

distal) segments with equal length, and their intraluminal leavings and washings were kept frozen at -20℃ until use.

Assays

Amylase activity was estimated using 3,5-dinitrosalicylic acid as described in Section 6. Trypsin and chymotrypsin activities were spectrometrically determined in the manner described in Chapter Ⅰ, Section 2. One unit of trypsin and chymotrypsin activities was defined as the amount of respective enzymes producing a 1.0 increase in the optical density at 410 nm for 10 min at 37℃. Measurement of PEG as an index of gastric emptying was according to the method of Malawer and Powell (108). Determinations of glucose and insulin concentrations in the plasma were carried out by the use of assay kits "Glucose B-Test" and "Insulin B-Test" from Wako Pure Chemical Co. (Osaka), respectively.

RESULTS

Re-evaluation of α-amylase inhibition by CBAI

We demonstrated in Section 3 that CBAI is not or little inhibitory to rat pancreatic α-amylase of which the activity measurement is due to the iodine-starch method. This time the assay was done by another procedure, that is, by the dinitrosalicylate method hitherto used widely (103). Figure 29 compares the differences

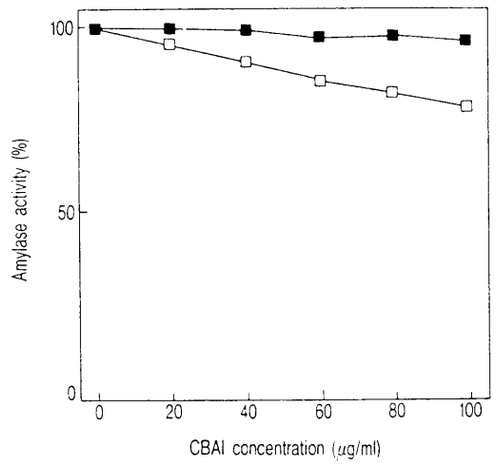

Fig. 29 *In vitro* inhibition of rat pancreatic α-amylase by CBAI at varied levels. The details of assay procedures are mentioned in the text. The 105,000 × *g* supernatant of the homogenate from rat pancreas was used as a source of α-amylase for enzyme assay. The amylase activity was measured according to either the iodine-starch method (■) or the dinitrosalicylate method (□), and expressed in % as the relative value to that of the control without CBAI (corresponding to 0.51 unit).

between the two methods in inhibitory effects of CBAI at varied concentrations on rat pancreatic α-amylase. The α-amylase activity decreased with increasing concentrations of CBAI, when assayed by the dinitrosalicylate method but not by the iodine-starch method. Needless to say, porcine pancreatic α-amylase was much more inhibited by CBAI than that from rat pancreas regardless of the analytical methods. Although being seemingly different in an amylolytic fashion from the porcine enzyme, it is probable that the rat enzyme undergoes in vivo inhibition by CBAI at a high concentration.

Gastric emptying

Figure 30 shows the rate of PEG disappearance from the stomach

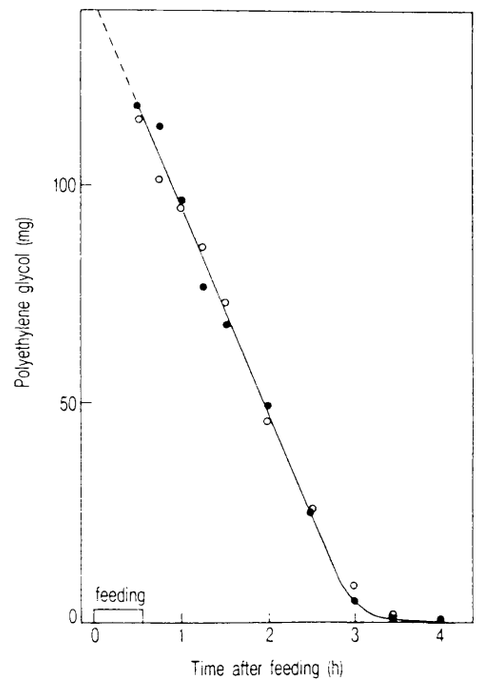

Fig. 30 Gastric emptying in rats given experimental diets with and without CBAI. A pair of rats with and without CBAI loading at a dose of 10 mg per animal were killed at indicated intervals after feeding to excise the gastrointestinal tracts. Their gastric leavings and washings were combined and then assayed for PEG, of which the disappearance from the stomach was regarded as gastric emptying. Closed (●) and open (○) circles represent the analytical data in rats with and without CBAI, respectively.

at various times after feeding. Irrespective of the absence or presence of CBAI, PEG was transferred from the stomach to the small intestine at a constant rate (PEG transit, approximately 40-45 mg/h), which had been reflected in the movement of chyme. Orally administered CBAI at a dose of 10 mg per animal had no effect on gastric emptying.

Intraluminal α-amylase activity

Figure 31 illustrates the intraluminal α-amylase activity

levels in the proximal, middle and distal thirds of the small intestine of rats fed PEG-containing experimental diets with and without CBAI, respectively. The amylase activities both in the proximal and

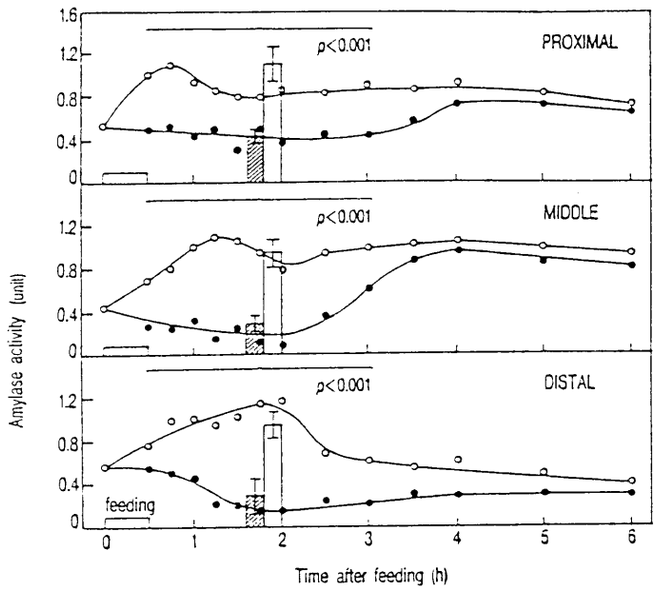

Fig. 31 Postprandial changes in intraluminal α-amylase activity in rats given experimental diets with and without CBAI. The same animals as in Fig. 30 were used for this assay. The small intestine was withdrawn at set times and immediately divided into the proximal, middle, and distal thirds, in which the residual α-amylase activity was measured in the usual way using 3,5-dinitrosalicylic acid. Heights and bars represent the means ± SD ($n=9$) for the data obtained between 30 and 180 min after the start of feeding: ▨, with CBAI; □, without CBAI. They were evaluated on the basis of Student's t-test as to whether there is a significant difference between two means.

middle segments were found at marginal levels within a few hours after the start of feeding in CBAI group. On the other hand, feeding the experimental diet without CBAI led to a gradual increase in the intraluminal amylase activity and the time showing a maximum activity varied with the proximal, middle and distal segments. With respect to proteolytic enzymes such as trypsin and chymotrypsin, there were not

significant differences in their intraluminal activities between both groups of rats fed the experimental diets with and without CBAI as shown in Fig. 32.

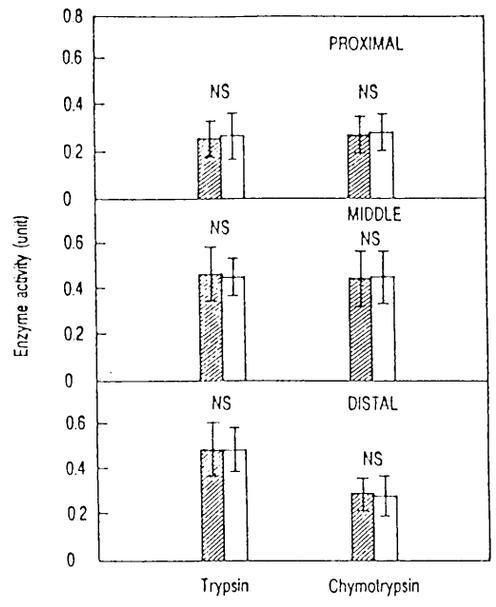

Fig. 32 Postprandial changes in trypsin and chymotrypsin activities in rats given experimental diets with and without CBAI. The experimental animals and conditions were the same as in Fig. 31. The trypsin and chymotrypsin activities were measured using α-*N*-benzoyl-DL-arginine-*p*-nitroanilide and benzoyl-L-tyrosine-*p*-nitroanilide, respectively. Heights and bars represent the means ± SD ($n=9$) for the data obtained between 30 and 180 min after the start of feeding. NS, not significantly.

Plasma glucose and insulin concentrations

Figure 33 shows postprandial changes of glucose concentrations in the plasma of rats fed the experimental diets with and without CBAI. In the control rats, the plasma glucose concentration was considerably raised by starch feeding, while such a raise was not the case for the rats with CBAI loading. This implies that amylolysis has

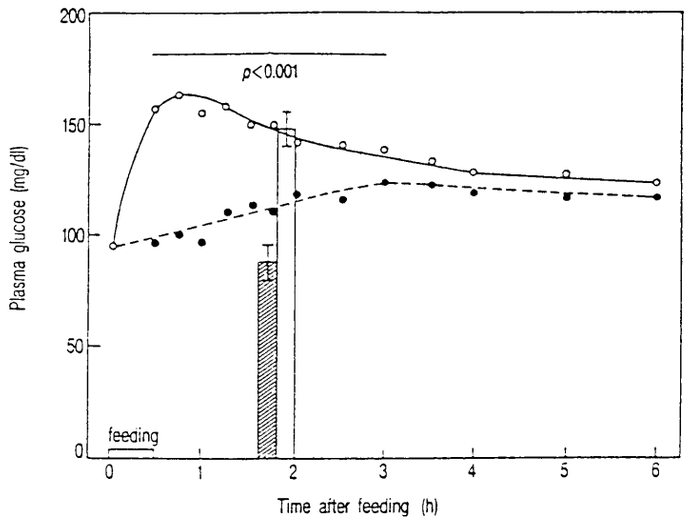

Fig. 33 Postprandial changes in plasma glucose concentration in rats given experimental diets with and without CBAI. The experimental animals and conditions were the same as in Fig. 32, except for the blood instead of the small intestine. The plasma glucose was estimated by means of an assay system consisting of glucose oxidase and peroxidase. Heights and bars represent the means ± SD ($n=9$) for the data obtained between 30 and 180 min after the start of feeding.

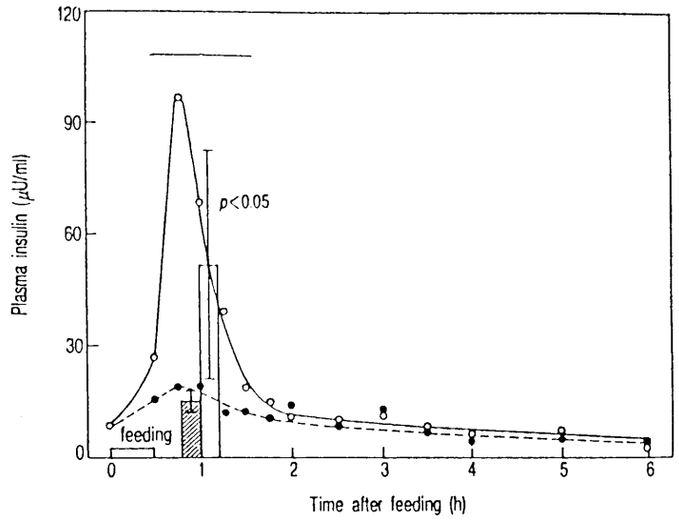

Fig. 34 Postprandial changes in plasma insulin concentration in rats given experimental diets with and without CBAI. The experimental animals and conditions were the same as in Fig. 33. The plasma insulin was determined using a commercial assay kit based on the principles of enzyme-immunoassay. Heights and bars represent the means ± SD ($n=5$) for the data obtained between 30 and 90 min after the start of feeding.

been blocked by CBAI ingestion. In this connection, both groups of rats with and without CBAI loading were examined for their postprandial changes of insulin concentrations in the plasma. Hyperglycaemia was smoothed and retarded in CBAI group. As shown in Fig. 34, the plasma insulin concentration in the latter was significantly higher ($p<0.05$) between 30 min and 90 min after the start of feeding than that in the former. In other words, insulin secretion from pancreatic islets was likewise depressed by CBAI administration at a level not leading to an additional increase in the blood sugar level.

DISCUSSION

It was ascertained in the in vitro experiment by the dinitrosalicylate method that rat pancreatic α-amylase underwent inhibition by CBAI and that its degree was intimately dose-dependent. When the amylase activity was assayed by the iodine-starch method, however, CBAI seemed not to function as an inhibitor of rat pancreatic α-amylase. The mechanism of α-amylase−catalyzed amylolysis has been generally accounted for by random cleavage of the α-1,4-glucoside linkage in starch and its accompanying release of reducible oligosaccharides. If rat pancreatic α-amylase can not catalyze so much

cleavage of long sugar chains into smaller fragments as other mammalian α-amylase do, it will be reasonable to consider that there is apparently no change in the reaction between iodine and starch even in the presence of CBAI. This explanation may be supported by the observation that reducible fragments are less liberated at higher concentrations of CBAI.

CBAI is unstable in the strongly acidic range below pH 2, although being quite resistant to peptic digestion itself as described in Section 3. CBAI given together with other components stays for a little while in the stomach, where it is mixed with gastric juice. CBAI is little exposed to the environment below pH 2 during stay in the stomach, because food ingredients such as protein, starch and others interfere with extreme acidification. Moreover chyme has been transferred to the small intestine within a few hours after ingestion. The amount of CBAI used in this experiment is at a level of 10 mg per animal weighing 200 g, which is compared to 3 g per person with body weight of 60 kg. This dose may be too much to be practically applied to therapy. However, the purpose of this section was to confirm whether or not CBAI could actually serve as a potent inhibitor of pancreatic α-amylase in the lumen of rat small intestine. For this reason, an excess amount of CBAI was administered to rats, taking account of a partial loss of the activity in gastric phase. The amount of CBAI administration was sufficient to depress some increase in the plasma glucose and insulin concentrations over a few

hours after feeding (Fig. 33 and 34). Similarly the intraluminal α-amylase activity was found to be effectively depressed by CBAI administration at the same level as above (Fig. 31). It thus can be concluded that CBAI at a sufficient level inhibits pancreatic α-amylase in the bowel tract and thereby exhibits a hypoglycemic effect. Similar effects of kidney bean α-amylase inhibitor have been obtained with normal and diabetic rats given a starchy meal by intubation (102). On the contrary, it have been reported that α-amylase inhibitor preparations derived from kidney beans have no effect on *in vivo* amylolysis when administered to humans (109,110). However, this observation can be explained in terms of their dose insufficient to inhibit pancreatic α-amylase, because the pancreas normally secretes a large excess of amylase required for hydrolysis of starch in a meal (111). Furthermore, contamination of amylase inhibitors with trypsin inhibitor also seems to be responsible for their inefficiency in human gut. In this regard, Menezes and Lajolo (102) have been reported that the contaminated trypsin inhibitor in α-amylase inhibitor preparations may lower the intraluminal trypsin level so as to stimulate the discharge of digestive enzymes from pancreas. As a matter of fact, CBAI preparation does not contain any trace amounts of trypsin inhibitors. Recently, Layer *et al*. (112) have described that administration of white kidney bean inhibitor with high antiamylase activity can virtually lead to inactivation of the intraluminal amylase activity in human.

Another explanation may be offered as to the differences among these observations in inhibitor efficiency; namely there is a diversity in specificity of leguminous α-amylase inhibitors for mammalian pancreatic α-amylases. Although CBAI was much more sensitive to pig or dog pancreatic α-amylase than to rat pancreatic α-amylase as described in Section 3, a similar diversity has been observed for action of red kidney bean α-amylase inhibitor on these pancreatic α-amylase (113).