J. Pesticide Sci. **16**, 179–188 (1991)

Original Article

Characterization of the Ecochemical in Kidney Beans Which Inhibits the Larval Growth of the Azuki Bean Weevil

Bongotrat SMITANOND,* Vinai PITIYONT,* Kanju OHSAWA,**
Hiroshi HONDA and Izuru YAMAMOTO

***Department of Agricultural Chemistry, NODAI Research Institute, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156, Japan*

(Received September 17, 1990)

An inhibitor of larval growth of the azuki bean weevil was isolated from kidney beans. The growth inhibitor was a glycosylprotein with a molecular weight of *ca.* 48,000 and an isoelectric point of 4.46, which had several subunits and moieties sensitive or insensitive to endo-*N*-acetyl-β-D-glucosaminidase H. Its carbohydrate content was 15.2%, based on 20 mannose, 1 fucose, 2 xylose and 15 *N*-Ac-glucosamine residues per one mole (8.5% by the phenol-sulfuric acid method). It inhibited α-amylases of animal origins, but not those of plant and microbial origins. It had no trypsin inhibitory and lectin activities. Both carbohydrate and native protein moieties seemed to be required for growth and α-amylase inhibition. The overall results indicated that the growth inhibitor was identical with or similar to the α-amylase inhibitors previously isolated from kidney beans.

INTRODUCTION

An ecochemical present in kidney beans (*Phaseolus vulgaris*) which inhibits the larval growth of the azuki bean weevil (*Callosobruchus chinensis*) has been isolated as a glycoprotein and partially identified by Ishimoto & Kitamura[1,2] and Smitanond *et al.*[3] There are certain discrepancies in the molecular weight between the two groups. Both have demonstrated that the inhibitor is an α-amylase inhibitor which has no trypsin inhibitory and lectin (hemagglutinin) activities (see also Tanaka *et al.*[4]). On the other hand, Janzen *et al.*[5] have claimed that the growth inhibition of *Callosobruchus maculatus* in *P. vulgaris* is due to the presence of lectin, and Gatehouse *et al.*[6] subsequently claim to have confirmed the toxicity of the seed lectins of *P. vulgaris* towards developing larvae. Also, without any regard to larval growth inhibition, several workers (Marshall & Lauda,[7] Powers & Whitaker,[8,9] Pick & Wober,[10] Frels & Rupnow,[11] Wilcox & Whitaker,[12] Lajolo & Filho,[13] Moreno *et al.*[14]) have isolated α-amylase inhibitors as glycoproteins from kidney beans and characterized them. Therefore, comparison should be made to see whether or not their α-amylase inhibitors are identical with our larval growth inhibitor. The present investigation is concerned with a further study on the properties of the isolated growth inhibitor in order to provide some baseline data for extending our ongoing study toward the genetic breeding of weevil-resistant beans. In the present paper, our larval growth inhibitor is called the growth inhibitor in order to differentiate it from the α-amylase inhibitor.

* Present address: Division of Agricultural Toxic Substances, Department of Agriculture, Bangkok, Thailand.

MATERIALS AND METHODS

1. Isolation of the Growth Inhibitor

Extraction from red kidney bean flour, and successive chromatography on Sepharose 6B and Sephadex G-75 were described in a previous paper.[3] The active (growth inhibition) fraction B (20 mg, 2.9 g-eq.) obtained from a Sephadex G-75 column was dissolved in 0.02 M phosphate buffer (pH 7 containing 25% ammonium sulfate) and dialyzed against the buffer. The dialyzed solution was applied on a 1.0×22 cm butyl Toyopearl 650 M column previously packed and equilibrated with the above buffer. The column was washed with the buffer until the absorbance at 280 nm of the eluate approached zero, then with 150 ml of 0.02 M phosphate buffer (pH 7, containing a reverse linear concentration gradient of ammonium sulfate, 25 to 0%). Aliquots of 2.5 ml were collected at a flow rate of 36 ml/hr and were measured for α-amylase inhibitory activity.

2. Assay of Hemagglutination Activity

Erythrocytes were prepared from rabbit blood, measured by a serial dilution of the growth inhibitor and compared with standard lectin as in a conventional assay.

3. Optimum Conditions for α-Amylase Inhibition and Stability

3.1 Porcine pancreatic α-amylase

3.1.1 Effects of preincubation time and temperature on inhibitory activity

The growth inhibitor (3 μg) was preincubated with α-amylase (1 unit) in 0.42 ml of 0.02 M potassium phosphate-0.05 M NaCl-1 mM $CaCl_2$, pH 6.9 at 25 and 37°C. Aliquots (140 μl) were withdrawn at intervals of 5 to 30 min and assayed for remaining α-amylase activity.

3.1.2 Effect of pH on inhibitory activity

α-Amylase (1 unit) was incubated with the growth inhibitor (0.5 μg) in digests (140 μl) different in pH at 30°C for 10 min. The buffers were 0.04 M sodium acetate (pH 4.3–5.6) and 0.04 M potassium phosphate (pH 6.0–6.9). Ionic strength was adjusted to 0.04 M with KCl. After 10 min, the remaining α-amylase activity was determined.

3.1.3 Effect of pH on stability of the growth inhibitor

The inhibitors (each 1 μg) were incubated at various pH's in the above buffers for 4 hr at 30°C, then assayed for inhibitory activity at 30°C for 10 min against α-amylase (1 unit).

3.1.4 Thermal stability of the growth inhibitor

The inhibitors (each 1 μg) in 70 μl of 0.04 M acetate buffer–0.04 M $CaCl_2$, pH 5.4 were treated for 15 min at a temperature of 30–100°C and cooled for 2 hr. Assay for inhibitory activity was performed at 30°C for 10 min against α-amylase (1 unit).

3.2 α-Amylase of larval C. chinensis

α-Amylase preparations of *C. chinensis* were prepared from fresh whole body homogenates of late-third instar larvae.[3]

3.2.1 Effects of preincubation time and pH on α-amylase inhibitory activity

The growth inhibitor (1 μg) was preincubated with larval α-amylase (1 unit) at various pH's and times at 30°C. The buffers were 0.04 M sodium acetate (pH 4.5–5.0) and 0.04 M potassium phosphate (pH 6.0–6.9). Ionic strength was adjusted to 0.1 M with KCl. Assay for remaining α-amylase activity was performed at intervals ranging from 10 to 60 min.

3.2.2 Effect of ionic strength on inhibitory activity

The larval α-amylase (1 unit) was incubated with the growth inhibitor (1 μg) at various ionic strengths (various concentrations of KCl) for 30 min at 30°C, pH 5.4 (0.04 M sodium acetate) and remaining α-amylase activity was determined.

4. Inhibition by the Growth Inhibitor of α-Amylases of Different Origins

The growth inhibitor (1 μg) was incubated with α-amylase each from human saliva, barley malt, *Bacillus subtilis* and *Aspergillus oryzae* in digests (140 μl) at pH 5.4 (0.04 M sodium acetate-0.01 M $CaCl_2$). After 10 min at 30°C, remaining α-amylase activity was determined.

5. Determination of Carbohydrates

The content of total carbohydrates in the growth inhibitor was determined by a conventional phenol-sulfuric acid method.[15]

Carbohydrate composition was determined by two gas chromatographic methods with alditol acetate and trimethyl silyl derivatives and subsequently confirmed by conventional GC-MS.

6. Periodate Oxidation[8]

The growth inhibitor (0.6 mg) in 0.7 ml of 0.3 M sodium acetate (pH 4.5) was oxidized with 0.1 ml of 0.2 M sodium metaperiodate at 4°C. Aliquots (0.05 ml) were removed at intervals into 0.1 ml of 50% ethylene glycol to terminate the oxidation and measured for α-amylase inhibitory activity.

7. Alkaline Hydrolysis

Samples (20 mg×2) were dissolved in 0.05 N NaOH in screw-cap test tubes, adjusted to pH 10 and an equal volume of freshly prepared cold 0.05 M NaOH-1 M $NaBH_4$ was added. The solutions were incubated at 45°C for 16 hr. The first tube was cooled and neutralized with a few drops of 50% acetic acid to pH 6 and subjected to the Biogel P-4 column (100×1.5 cm) which had been previously equilibrated with 1% acetic acid and eluted with the same buffer. The second tube, after cooling, was supplemented with an equal volume of 2 M NaOH-1 M $NaBH_4$ and heated at 100°C for 6 hr. The reaction mixture, after cooling and adjusting to pH 6 with a few drops of 50% acetic acid, was subjected to the Biogel P-4 column and eluted with the same buffer. Aliquots of 3 ml were collected, and the carbohydrate-containing fractions were measured by the phenol-sulfuric acid method (absorbance at 484 nm) and the peptides by absorbance at 230 nm.

8. Pronase Digestion

The growth inhibitor (20 mg) was dissolved in 0.5 ml of 0.015 M $CaCl_2$ containing 0.2% sodium azide. Pronase E (*Streptomyces griseus*, Merck, FRG) (0.15 mg) was added and the mixture was incubated at 40°C for 24 hr with its pH maintained at 8.5 with 0.1 M NaOH. After 24 hr, the proteolytic digest was supplemented with more pronase (0.1 mg) and the incubation was continued for 48 hr at the same pH 8.5. The cooled solution was passed through the Biogel P-4 column (80×1.5 cm) which had been well equilibrated with 1% acetic acid[16] and eluted with the same buffer. Aliquots of 2 ml were collected and analyzed as described in section *7*. The reaction mixture was lyophilized and tested for inhibition of α-amylase and larval growth.

9. Endo-N-acetyl-β-D-glucosaminidase H (Endo-H) Treatment

The growth inhibitor (1 mg) was incubated in acetate buffer (0.02 M, pH 5.3 containing 0.2% SDS) and in 0.05 ml PMSF (6 mg in 95% ethanol) for 1 min at 50°C in order to denature the inhibitor.[17-19] Then 0.5 units of Endo-H was added and the mixture was incubated at 30°C for 10 hr. The digest was applied to the Biogel P-4 column (100×1.5 cm) and analyzed as described in section *7*.

10. Chromatography of the Glyco Chains

The glycopeptides or glycans resulting from section *7* or *9* (2 mg in 0.5 ml of equilibration buffer) were further fractionated on the Concanavalin A-Sepharose 4B column (5×1 cm) which had been previously equilibrated with 5 mM acetate buffer (pH 5.2) containing 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$ at a flow rate of 9 ml/hr at room temperature. They were eluted in three buffers[16] while monitored with phenol-sulfuric acid: the equilibration buffer (5 column volume), 0.01 M methyl α-D-glucoside (3 column volume) and 0.3 M methyl α-D-glucoside (5 column volume) in the equilibration buffer (all containing 0.02% sodium azide).

All the other methods used in the present work were described in the previous paper.[3]

RESULTS AND DISCUSSION

Our results on the growth inhibitor are compared with those obtained by others on α-amylase inhibitor from kidney beans (Table 1).

1. Isolation of the Growth Inhibitor

Chromatography on a hydrophobic butyl-Toyopearl 650 column of the active fraction obtained by Sepharose 6B and Sephadex G-75 chromatography gave a sharp peak (Fig. 1) with a single band on PAGE, which had no hemagglutination activity. Recovery and level of purification are shown in Table 2. The

Table 1 Comparison of the properties of the growth inhibitor and α-amylase inhibitors described by different workers.

	Marshall & Lauda, 1975[7]	Powers & Whitaker, 1977[8]	Pick & Wober, 1978[10]	Wilcox & Whitaker, 1984[12]	Frels & Rupnow, 1984[11]	Lajolo & Filho, 1985[13]	Moreno *et al.*, 1990[14]	Smitanond *et al.*, 1990[3]
Variety	White (Great northern)	Red	White	Red	Black	Black	cv. (Greensleeves)	Red (Taishokintoki)
Molecular weight	45,000–50,000	43,000–49,000	43,000	45,000	I: 49,000 II: 47,000	49,000–56,000	—	48,000
Isoelectric point	—	4.50–4.65	5.2	—	I: 4.93 II: 4.86	4.35	—	4.46
Carbohydrate content	13%[a]	8.6%[a]	15%[a]	13%	I: 7.5%[a] II: 11.9%[a]	14.5%[a]	—	15.2% (8.5[a])
Monosaccharide	—	—	—	25 Mannose 1 Fucose 2 Xylose 17 *N*-Ac-glucosamine	Mannose	Mannose Galactose Xylose Glucosamine		20 Mannose 1 Fucose 2 Xylose 15 *N*-Ac-glucosamine
Subunit		d″, e″: 28,000–32,000[b] a″: 15,000–17,000[b] b″: 12,000–15,000[b] c″: 11,000–12,000[b]	a: 18,000 or 11,000		Ic: 32,000–34,000[b] IIc: 32,000–33,000[c] IId: 26,500–28,000 Ia: 15,000–17,000[b] IIa: 15,500–17,000[b] Ib: 13,500–14,000[c] IIb: 13,000–13,500[c]	d′: 32,000[b] a′: 17,500[b] b′: 16,000[b] c′: 13,500[b]	p‴: 32,000 doublet a‴: (18,500)[b] b‴: (17,500)[b] c‴: (16,400)[b] e‴: (14,400)[c]	d: 32,000[d] e: 19,000[d] a: 18,000[d]–18,500[b] b: 17,000–17,500[b] f: 16,500[d] c: 14,400–15,000[c]

[a] By phenol-sulfuric acid.

[b] Positive for the presence of carbohydrates; [c] negative; [d] ambiguous.
Underline indicates major bands; parenthesis indicates estimation by us.

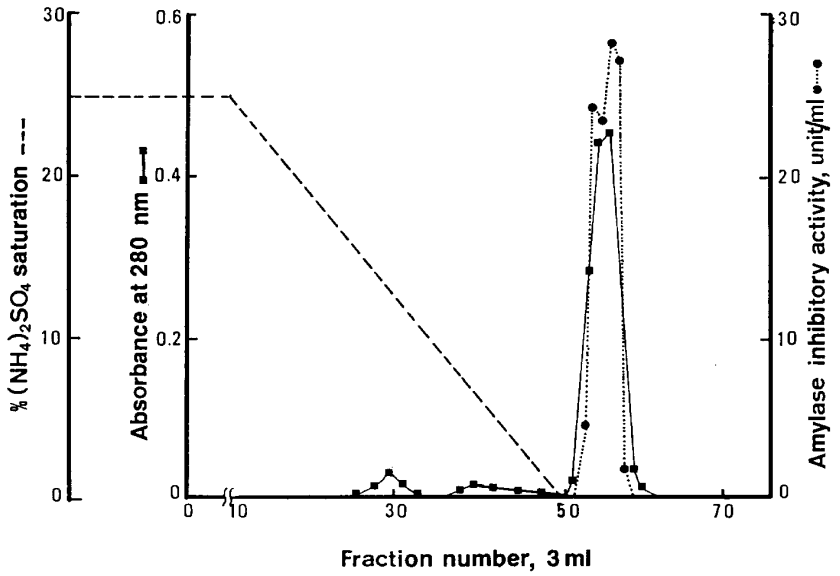


Fig. 1 Butyl Toyopearl–650M hydrophobic chromatography of Fr. B eluted with 0.02 M phosphate buffer (pH 7.0), 25–0% $(NH_4)_2SO_4$.

Table 2 Purification of the growth inhibitor from kidney beans.

Step	Yield		Protein	Total activity	Specific activity	Recovery	Purification
	Weight (g)	%	(mg)	(unit)	(unit)	(%)	(fold)
Bean flour	40.00	100.00					
Crude extract	7.03	17.50	203.0	3,766	18.6	100.0	1.0
Sepharose 6B	1.90	4.76	24.0	2,467	102.0	65.5	8.2
Sephadex G-75	0.27	0.61	5.2	1,096	210.7	29.0	11.3
Butyl Toyopearl–650 M	0.12	0.31	1.8	608	388.0	16.0	18.2

yield of the purified larval growth inhibitor was 0.3%. The value was generally in agreement with the ones obtained by other workers (0.4–0.5%,[7] 0.15%[10]). Only Frels & Rupnow[11] have reported two distinct α-amylase inhibitors from black bean (*P. vulgaris*), suggesting that a labile inhibitor has been lost by others[7,8] heat treatment in the isolation procedures. Our purification procedures involved no heat treatment, but only a single growth inhibitor was obtained.

2. *Optimum Conditions for α-Amylase Inhibitory Activity of the Growth Inhibitor and Stability*

When porcine pancreatic α-amylase was incubated with the growth inhibitor at pH 6.9, it took 10 and 20 min to reach maximum inhibition at 25 and 37°C, respectively and the extent of maximum inhibition was 60% at 37°C, whereas only 20% at 25°C. The optimum pH during incubation (30°C for 10 min) was found to be pH 5.4. Therefore, incubation conditions of α-amylase with the growth inhibitor were standardized at pH 5.4 and 30°C for 10 min, if not otherwise mentioned. pH and temperature affected the stability of the growth inhibitor. At pH 5.6 there was almost no change in the inhibitory activity after 4 hr, but at pH 4.3, 5.0, 5.4, 6.0, 6.4 and 6.9, the inhibitory activity was lost by 30.6, 12.5, 5.3, 25.5, 26.4 and 37.5%, respectively. Treatment of the growth inhibitor at 37°C for 15 min had no effect on its stability. At higher temperatures of 40, 50, 60, 70, 80, 90 and 100°C, the inhibitory activity decreased by 12.3, 48.2, 62.2, 60.8, 90.9, 91.7 and 100%, respectively. At 100°C, 5 min was enough to completely destroy the inhibitory activity.

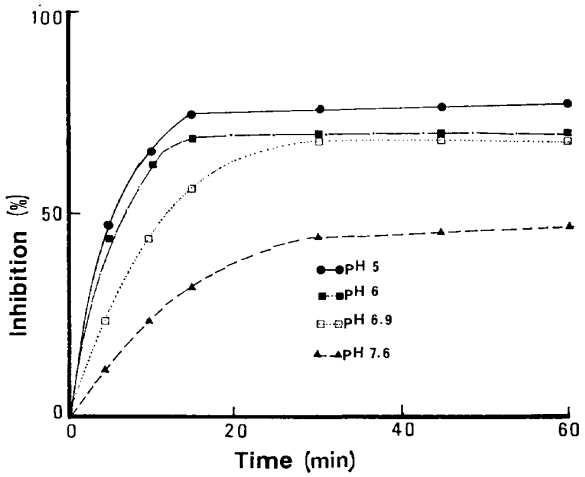


Fig. 2 Effects of pH and preincubation time on the extent of inhibition of *C. chinensis* α-amylase by the growth inhibitor.

The growth inhibitor inhibited the α-amylase from larval *C. chinensis* to a lower extent as compared with the pancreatic α-amylase under incubation conditions of pH 5.4, 30°C and 10 min as reported earlier.[3] Both pH and ionic strength were found to be involved in the extent of the inhibition. The higher the pH, the lower the inhibition (Fig. 2), whereas the higher the ionic strength, the higher the inhibition (inhibition of 10.5, 25.2, 28.6, 34.3 and 42.9% at an ionic strength of 0.04, 0.08, 0.10, 0.12 and 0.15, respectively).

3. *Inhibition of α-Amylases of Various Origins by the Growth Inhibitor*

The human salivary, porcine pancreatic and weevil α-amylases were inhibited by the isolated inhibitor to significant extents (95, 98 and 60%, respectively), whereas α-amylases of plant (barley malt) and microbial (*Aspergillus oryzae* and *Bacillus subtilis*) origins were not affected by the inhibitor in agreement with others' data.[7-11,13,14]

4. *Carbohydrate Content of the Growth Inhibitor*

The carbohydrate content was 8.5% by the phenol-sulfuric acid method. Other groups (Table 1) gave a value ranging between 8.6 and 19.4%. Although it provides a convenient method, the value obtained is not accurate. Therefore, the individual carbohydrates were quantitated by gas chromatography to give 20 mannose, 15 *N*-Ac-glucosamine, 1 fucose and 2 xylose residues per one mole of the growth inhibitor, from which the carbohydrate content was calculated to be 15.2%. Frels & Rupnow[11] noted only mannose by thin layer chromatography. Lajolo & Filho[13] identified mannose, galactose, xylose and glucosamine qualitatively by paper chromatography. Our identification must be more precise in view of the techniques we used. However, with similar techniques, Wilcox & Whitaker[12] obtained a value of 13% based on 25 mannose, 17 *N*-Ac-glucosamine, 1 fucose and 2 xylose per one mole of the α-amylase inhibitor.

5. *Molecular Weight*

The apparent molecular weight (*Mr*) was reported earlier by us[3] as 48,000 by gel filtration chromatography on a calibrated Sephadex G-75 column, which is generally in agreement with those obtained by others (Table 1).

6. *Subunits*

SDS-PAGE (Fig. 3) resulted in two major bands (both positive for carbohydrate staining) having *Mr* of 18,000–18,500 and 17,000–17,500 and a minor band (negative for carbohydrates) having 14,400–15,000 as well as three faint bands of 19,000, 16,500 and 32,000 (am-

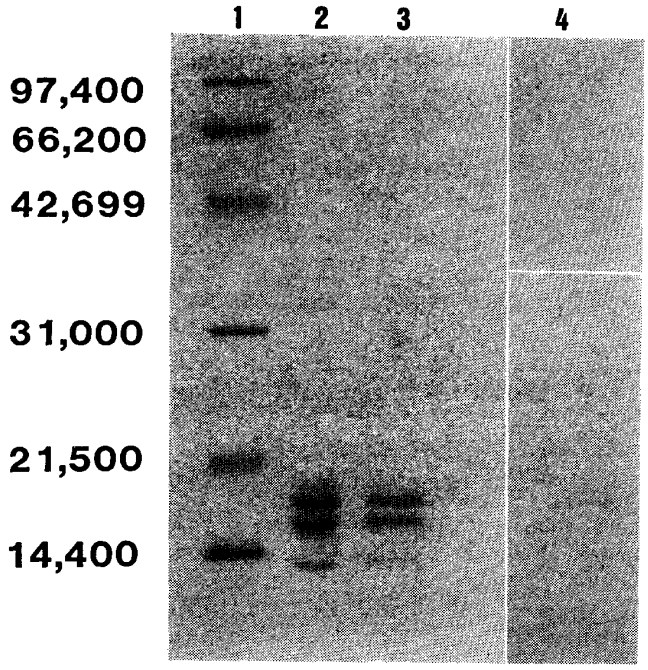


Fig. 3 SDS-PAGE (12.5% slab gel) of the growth inhibitor.

biguous for carbohydrates). The presence of 2-mercaptoethanol did not affect the result, which suggested that the subunits were not linked by disulfide bonds. This agrees with the report by Powers & Whitaker.[8]

The zymographs on SDS-PAGE by the different groups were fairly similar in pattern though the given *Mr* for each band deviated from each other. Table 1 is an attempt to make a correspondence among various bands as judged from the zymographs. Moreno *et al.* did not give the values of *Mr* so that the estimation, in parentheses, was made by us. Lajolo & Filho's bands a′, b′ and c′, Powers & Whitaker's a″, b″ and c″, Moreno *et al.*'s a‴ and/or b‴, c‴ and e‴, seemingly correspond to our bands a, b and c. Lajolo & Filho considered bands a′, b′ and c′ as three different subunits present, giving *Mr* of 47,000 for the undissociated inhibitor. Band d′ (*Mr*=*ca.* 32,000), because of its high *Mr*, was suspected to be an undissociated aggregate of the smaller polypeptides. The corresponding bands were also observed by other workers (d″, e″, p‴, d, Ic and IIc). They concluded the existence of three different subunits. In contrast, Pick & Wober observed only a single band (*Mr* 18,000 or 11,000) on SDS-PAGE and suggested that the inhibitor isolated from white kidney beans (*Mr* 42,600) was composed of three or four identical subunits. Frels & Rupnow studied separately their α-amylase inhibitors I and II for subunits. Although there were some similarities with others, a major band corresponding to b′, b″, c‴ or b (or a′, a″, b‴ or a) was not found. There might be a possibility that the bean which Frels & Rupnow used was a different cultivar (black bean) of *P. vulgaris* having a somewhat different α-amylase inhibitor. Moreno *et al.*[14] made a detailed study of subunits. When they separated the individual polypeptides and subjected them to another round of SDS-PAGE, band p‴ partially dissociated, giving rise to smaller polypeptides, whereas the smaller polypeptides partially associated, giving rise to band p‴. From analysis of the *N*-terminal amino acid sequencing, they thought that the five polypeptides, a‴ to e‴, may be glycoforms of two different polypeptides. Only a‴ was Endo-H sensitive, whereas all others were Endo-H resistant but chemically deglycosylated with TFMS to give the same x‴ (not shown in Table; *Mr*= between d‴ and e‴). c‴ gave a band x‴, indicating the presence of a single complex glycan. b‴ gave also x‴, but the mobility was twice as large, indicating that it had two complex glycans. a‴ gave y‴ (*Mr*: about d‴) with Endo-H and x‴ with TFMS, indicating the presence of a single complex glycan as well as a high-mannose glycan. This interpretation corresponds with our finding in Endo-H cleavage of the growth inhibitor.

7. *Periodate Oxidation*

Periodate oxidation resulted in an initial rapid loss of inhibitory activity for porcine pancreatic α-amylase, followed by a much slower loss (Fig. 4), in agreement with the finding by others,[8,14] suggesting that the carbohydrate moiety of the inhibitor plays an important role for its activity. Although periodate is rather specific to cleave glycols in the carbohydrates, possibility of modification of the protein moiety can not be excluded. Wilcox & Whitaker[12] observed that the partially oxidized inhibitor (on one phenylalanine, one methionine and two tyrosine residues) still retained 2/3 of its original activity. Concerted analysis of carbohydrates, amino acids and α-amylase inhibitory activity will be required to answer the question.

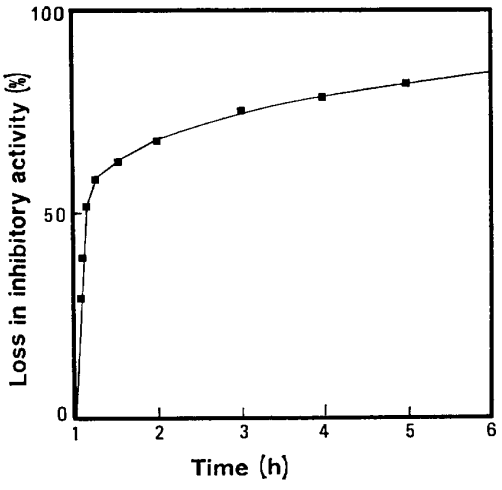


Fig. 4 Effect of periodate oxidation on the inhibitory activity to the porcine α-amylase of the growth inhibitor.

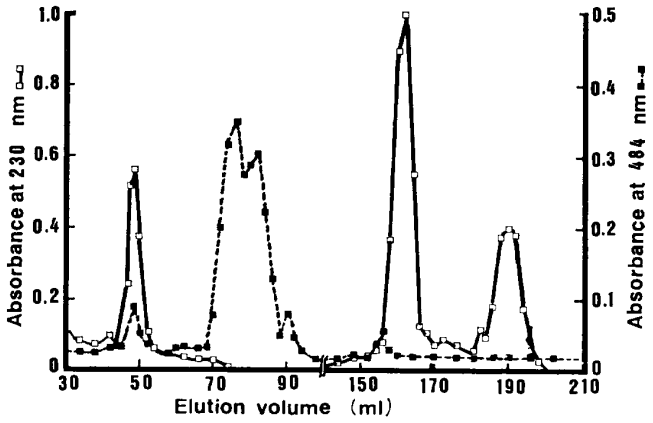


Fig. 5 Chromatography of the growth inhibitor on a Biogel P-4 column after the digestion with pronase E for 48 hr.

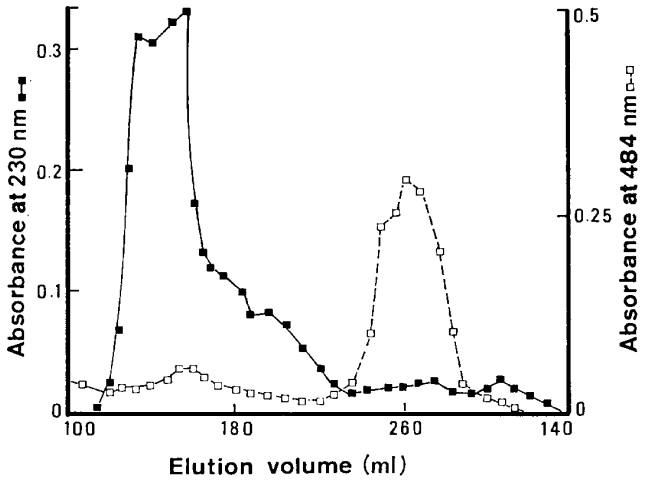


Fig. 6 Chromatography of the growth inhibitor on a Biogel P-4 column after alkaline cleavage (2 M NaOH-1 M $NaBH_4$, 100°C, 6 hr).

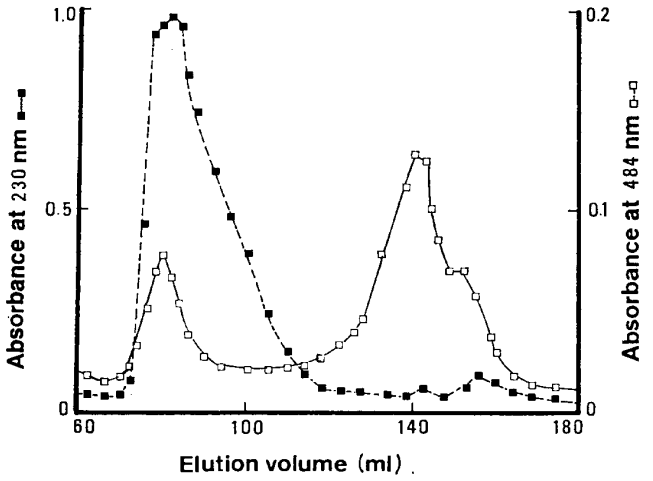


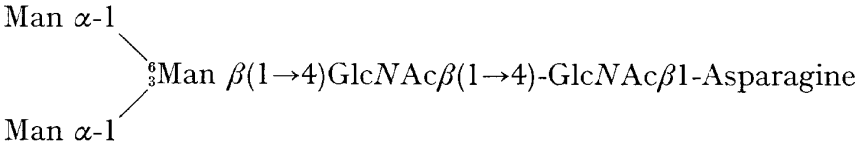
Fig. 7 Chromatography of the endoglycosidase H-treated growth inhibitor on a Biogel P-4 column.

8. Pronase Digestion

It resulted in fragmentation of the polypeptide chain to produce a mixture of glycopeptides (one major peak) and peptides (three peaks) (Fig. 5). The reaction mixture inhibited neither larval growth nor α-amylase. Native protein moiety seems to be required for inhibition.

9. Carbohydrate-Protein Linkage

Powers & Whitaker[8] suspect that the carbohydrates probably is covalently linked *via* an amide linkage to asparagine, but without enough evidence. Lajolo & Filho[13] suspect that the carbohydrate moiety in the α-amylase inhibitor is linked to an asparagine group through linkage with glucosamine, because none of the carbohydrates could be released under conditions for β-elimination in alkaline conditions. In our study, the glyco chain was removed as shown by the appearance of peptide peak (Fig. 6) only under vigorous alkaline conditions, but not under milder conditions. This result indicates that the growth inhibitor is not an *O*-glycosylprotein, but an *N*-glycosylprotein. The only *N*-glycosidic bond presently found in glycoproteins is *N*-acetylglucosaminyl-asparagine. The following inner-core structure is known to be common to all *N*-glycosylprotein.



This was further confirmed by the incubation of the inhibitor with Endo-H in the presence of SDS, which resulted in a maximal 70% loss of the glyco chain as indicated by the appearance of peptide peak (Fig. 7). After hydrolysis with Endo-H as described above, the unliberated carbohydrates consisted of 1 fucose, 2 xylose and 7 mannose residues per mole (*N*-acetylglucosamine was not quantitated). This indicates the removal of most of

the mannose and the retention of all the fucose and xylose, in agreement with the results obtained by Wilcox & Whitaker.[12]

When a mixture of glycopeptides and/or oligosaccharides were eluted by affinity chromatography on a Concanavalin A-Sepharose column with three specified buffers (see materials and methods), they were fractionated into three classes: (i) non-reactive components eluted with the first buffer, (ii) weakly reactive components eluted with the second buffer, and (iii) strongly reactive components eluted with the third buffer. It has been established that the primary structure of glycopeptides and/or oligosaccharides can be predicted by a fraction they appear in. In our study, glycopeptides and the glyco chains of the growth inhibitor obtained by alkaline or Endo-H hydrolysis were eluted in the (iii) fraction, implying the high mannose type nature.

According to Wilcox & Whitaker,[12] the intact inhibitor consists of three major subunits, two of which retain carbohydrates after Endo-H treatment. They suggest the presence of two types of glyco chains insensitive to Endo-H. As discussed in section *6*, Moreno *et al.*[14] indicate that only one subunit is Endo-H sensitive, suggesting the presence of two complex glycans insensitive to cleavage with Endo-H.

The Endo-H insensitive glycoprotein, because of the similarity in unliberated carbohydrate composition (fucose, xylose, mannose and *N*-acetylglucosamine) may have a core structure similar to the one of stem bromelain,[20] which has fucose on asparagine-linked Glc*N*Ac and xylose on Man adjacent to the second GlcNAc.

There are several discrepancies among the data from various groups, which might have resulted from a different variety of *P. vulgaris*, different purification procedures, different analysis conditions and others, but from an overall estimation, we judge that our growth inhibitor is identical with the α-amylase inhibitor in kidney beans.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Foundation for Promotion of Food Science and Technology, Japan and Sumitomo Chemical Co., Ltd. We wish to express our sincere thanks to Prof. Tetsujiro Obara of the Foundation for his constant encouragement.

REFERENCES

1) M. Ishimoto & K. Kitamura: *Jpn. J. Breed* **38**, 367 (1989)
2) M. Ishimoto & K. Kitamura: *Appl. Entomol. Zool.* **24**, 281 (1988)
3) B. Smitanond, K. Tanaka, H. Honda & I. Yamamoto: *J. Pesticide Sci.* **15**, 89 (1990)
4) K. Tanaka, H. Honda & I. Yamamoto: unpublished (1981)
5) D. H. Janzen, H. B. Juster & I. E. Liener: *Science* **192**, 795 (1976)
6) A. M. R. Gatehouse, F. M. Dewey, I. Dove, K. A. Fenton & A. Pusztai: *J. Sci. Food Agric.* **35**, 373 (1984)
7) J. J. Marshall & C. M. Lauda: *J. Biol. Chem.* **250**, 8030 (1975)
8) J. R. Powers & J. R. Whitaker: *J. Food Biochem.* **1**, 217 (1977)
9) J. R. Powers & J. R. Whitaker: *J. Food Biochem.* **1**, 239 (1977)
10) K. H. Pick & G. Wober: *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1371 (1978)
11) J. M. Frels & J. H. Rupnow: *J. Food Biochem.* **8**, 281 (1984)
12) E. R. Wilcox & J. R. Whitaker: *J. Food Biochem.* **8**, 189 (1984)
13) F. M. Lajolo & F. F. Filho: *J. Agric. Food Chem.* **33**, 132 (1985)
14) J. Moreno, T. Altabella & M. J. Chrispeels: *Plant Physiol.* **92**, 703 (1990)
15) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Reber & F. Smith: *Anal. Chem.* **28**, 350 (1956)
16) J. Montreul, S. Bouquelet, H. Debray, B. Fournet, G. Spik & G. Strecker: "Carbohydrate Analysis" ed. by M. F. Chaplin & J. F. Kennedy, IRL Press, Oxford, pp. 143–204, 1986
17) A. L. Tarentino & F. Maley: *J. Biol. Chem.* **249**, 811 (1974)
18) R. B. Trimble & F. Maley: *Biochem. Biophys. Res. Commun.* **78**, 935 (1977)
19) R. B. Trimble & F. Maley: *J. Biol. Chem.* **252**, 4409 (1977)
20) H. Ishihara, N. Takahashi, S. Oguri & S. Tejima: *J. Niol. Chem.* **254**, 10715 (1979)

要　　約

アズキゾウムシ幼虫の生育阻害に係わるインゲン種子中の生態活性物質の性状

Bongotrat Smitanond, Vinai Pitiyont
大沢貫寿，本田　博，山本　出

インゲン種子から単離した幼虫生育阻害物質（収率約3%）は分子量（*Mr.*）約 48,000，等電点 4.46 の *N*-グリコシルタンパクであり，endo-*N*-acetyl-β-D-glucosaminidase H に感受性と非感受性の部分からなっていた．SDS-PAGE で二つの主バンド（*Mr.* 18.000-18,500 と 17,000-17,500）と一つの小さいバンド（*Mr.* 14,400-15,000），その他のうすいバンドを与えた．糖含量はフェノール-硫酸法では 8.5%，構成糖（1モル当たり mannose 20，fucose 1，xylose 2，*N*-Ac-glucosamine 15 分子）からの計算値は 15.2% であった．豚スイ臓，アズキゾウムシ幼虫の α-アミラーゼを阻害するが，植物，微生物起源のそれは阻害しなかった．またトリプシン阻害活性，レクチン活性はなかった．糖部分とタンパク部分の両者が幼虫生育阻害ならびに α-アミラーゼ阻害に必要のようである．結果を総合すると，幼虫生育阻害物質はこれまでインゲン種子から単離されてきた α-アミラーゼインヒビターと同一ないし近似のものといえる．