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Variation of seed α-amylase inhibitors in the common bean

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Abstract Variation of seed α-amylase inhibitors was investigated in 1 154 cultivated and 726 non-cultivated (wild and weedy) accessions of the common bean, *Phaseolus vulgaris* L. Four α-amylase inhibitor types were recognized based on the inhibtion by seed extracts of the activities of porcine pancreatic α-amylase and larval α-amylase and larval α-amylase of the Mexican bean weevil, *Zabrotes subfasciatus* Boheman. Of the 1 880 accessions examined most (1 734) were able to inhibit porcine pancreatic α-amylase activity, but were inactive against the *Z. subfasciatus* larval α-amylase; 41 inhibited only the larval α-amylase activity, 52 inhibited the activities of the two α-amylases, and 53 did not inhibit the activity of either of the α-amylases. The four different inhibitor types were designated as αAI-1, αAI-2, αAI-3, and αAI-0, respectively. These four inhibitor types were identified by the banding patterns of seed glycoproteins in the range of 14–20 kDa by using SDS-polyacrylamide gel electrophoresis. Additionally, four different banding patterns were recognized in accessions with αAI-1, and were designated as αAI-1a, 1b, 1c, and 1d. Two different patterns of the accessions lacking an α-amylase inhibitory activity were identified and designated as αAI-0a and αAI-0b. The largest diversity for seed α-amylase inhibitors was observed in non-cultivated accessions collected from Mexico where all eight inhibitor types were detected. The possible relationships between the variation of seed α-amylase inhibitors and bruchid resistance are discussed.

Key words Common bean · *Phaseolus vulgaris* α-Amylase inhibitor · Bruchid resistance · Genetic diversity

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Introduction

Seeds of plants possess a protective mechanism against vertebrates, insects, and microorganisms, by virtue of the presence of secondary compounds. Some of the compounds in the common bean (*Phaseolus vulgaris* L.) have been implicated in conferring protective properties against insect pests (Applebaum et al. 1969, 1970; Jaffe et al. 1973; Janzen et al. 1976). Ishimoto and Kitamura (1989) demonstrated that a seed α-amylase inhibitor protein in several cultivars of the common bean plays a protective role against bruchid pests. The α-amylase inhibitor strongly inhibited the larval midgut α-amylase activities of both the adzuki bean weevil (*Callosobruchus chinensis* L.) and the cowpea weevil (*C. maculatus* F.), non-pest species of the common bean. Larvae of the two bruchids died before the second instar when fed artificial beams containing the inhibitor at the same levels as that found in common bean seeds, 0.2–0.5%. The bean weevil (*Acanthoscelides obtectus* Say) and the Mexican bean weevil (*Zabrotes subfasciatus* Boheman) are tolerant to the inhibitor (Ishimoto and Kitamura 1992), and cause serious post-harvest loss in the common bean. Recently, a novel α-amylase inhibitor, which inhibits only the larval α-amylase activity of *Z. subfasciatus*, has been identified in seeds of several wild common bean accessions resistant to *Z. subfasciatus* (Gatehouse et al. 1987; Ishimoto and Kitamura 1993). Since only some of the

resistant accessions contain the inhibitor, it is considered that the inhibitor is not the only factor responsible for the resistance (Minney et al. 1990). The resistance has been shown to be mainly associated with the presence of arcelin variants, and the concurrent reduction of phaseolin, a major storage protein in the common bean (Minney et al. 1990). However, these findings suggested that variation of seed α-amylase inhibitors, in terms of specific inhibitory activity against the α-amylases of some insect pests, occurs in the common bean and may be involved in the bruchid resistance. This study describes the specificity and electrophoretic variations in the types of seed α-amylase inhibitors in the common bean.

Materials and methods

Seeds

A total of 1 154 cultivated and 726 non-cultivated, wild and weedy (Toro et al. 1990) common bean accessions were used in this study. Twenty local cultivars in Japan were obtained from the Hokkaido Prefectural Tokachi Agricultural Experimental Station (Memuro, Japan). Other accessions were obtained from the *Phaseolus* world collection at CIAT.

Preparation of α-amylases

Midguts dissected from the last instar larvae of *Z. subfasciatus* were homogenized in 20 mM of sodium phosphate buffer, pH 6.7, containing 20 mM $NaCl_2$ and 0.1 mM $CaCl_2$ (50 µl/midgut), and centrifuged at 10 000 g for 20 min. The supernatant was filtered through a 0.2-µm filter to remove small suspended particles and bacteria, and diluted with the extract buffer to liberate 100 µg maltose/min in 100 µl. Porcine pancreatic α-amylase was obtained from Sigma Chemical Co. Ltd. The α-amylase was diluted with the buffer solution to obtain the same activity as that of the *Z. subfasciatus* larval α-amylase. The diluted α-amylases were used immediately.

Analysis of α-amylase inhibitory activity

At least two seeds of each accession were used individually for the analysis of the α-amylase inhibitory activity. The distal portion (10 mg) of individual seeds was extracted with 500 µl of 20 mM phosphaste buffer (pH 6.7) for 60 min at room temperature. After centrifugation, each 100 µl of the supernatant was used for the analysis of the α-amylase inhibitory activity against porcine pancreatic α-amylase and the *Z. subfasciatus* larval α-amylase by a modification of the Bernfeld method (Bernfeld 1955) as previously described (Ishimoto and Kitamura 1989). The α-amylase preparation in 100 µl was pre-incubated with a seed extract at 30° C for 15 min before the addition of 250 µl of substrate solution (1% potato starch solution in 0.1 M sodium phosphate buffer, pH 5.8). After 5 min the reaction was termminated by the addition of 500 µl of 3,5-dinitrosalicylic acid reagent, followed by boiling for 10 min in a water bath. After 5 ml of water had been added, the solution was mixed and allowed to stand at room temperature for 15 min. The absorbance of the solution was measured at 546 nm, and the α-amylase activity was expressed in µg of maltose liberated/min. The α-amylase inhibitory activity was expressed as a relative α-amylase activity without pre-incubation with the seed extract.

Detection of glycoproteins

The above seed extract was mixed with an equal volume of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.2% (w/v) sodium dodecyl sulphate (SDS), 5 M urea and 2% (v/v) 2-mercaptoethanol, and subjected to polyacrylamide-gel electrophoresis (SDS-PAGE), using a 13.5% acrylamide gel, by the method of Laemmli (1970). After separation by SDS-PAGE, the proteins in the gel were transferred onto a polyvinylidene difuoride (PVDF) membrane (Imobilon, Millipore, USA), and reacted with peroxidase-coupled concanavalin A (Honen, Japan) according to the procedure described by Kijimoto-Ochiai et al. (1985).

Results

Variation in specificity

The present method does not enable us to analyze the α-amylase inhibitory activity quantitatively. All the seed extracts showed either an almost complete inhibition (more than 80%) or no inhibition (less than 10%). No accession showed an intermediate inhibitory activity.

Variation in seed α-amylase inhibitory activities was recognized for the specific inhibition of the activities of porcine pancreatic α-amylase and the *Z. subfasciatus* larval α-amylase. Of the 1 880 accessions examined, most of the seed extracts were able to inhibit the procine pancreatic α-amylase activity, but were inactive against the *Z. subfasciatus* α-amylase. The type of inhibitory activity was the same as that of a cultivated common bean, Taishoukintoki described previously as αAI-1 (Ishimoto and Kitamura 1993). Forty-one accessions inhibited the activity of the *Z. subfasciatus* larval α-amylase only, 52 inhibited the two α-amylase activities, and 53 did not inhibit the activity of either of the α-amylases (Table 1). These three different types of in-

Table 1 Variation in specificity of seed α-amylase inhibtor (αAI) in the common bean, *Phaseolus vulgaris* L

Type of seed α-amylase inhibitor	α-Amylase inhibitory activity[a]		No. of accessions examined[b]
	Porcine pancreas	*Zabrotes subfasciatus*	
αAI-1	+	−	1734
αAI-2	−	+	41
αAI-3	+	+	52
αAI-0	−	−	53

[a] Inhibitory activity is expressed as +, more than 80% inhibition, or −, less than 10% inhibition
[b] These numbers include both non-cultivated, wild and weedy, and cultivated common beam accessions

hibitory activity were designated as αAI-2, αAI-3, and αAI-0, respectively (Table 1).

Electrophoretic variation

As previously reported (Ishimoto and Kitamura 1991), the seed α-amylase inhibitor of "Taishou-kintoki" yielded a pattern of three glycopolypeptide bands in the range of 14 to 20 kDa by the peroxidase-coupled concanavalin-A staining test after electroblotting on SDS-PAGE gels. In our study four different α-amylase inhibitor types were identified by the banding patterns observed in the same size range using this test (Fig. 1). Some of these patterns (Fig. 1, lanes 1, 6, and 10) were reported previously (Ishimoto and Kitamura 1989, 1991; Suzuki et al. 1993). Based on differences in banding patterns, four distinct αAI-1 types (Fig. 1, lanes 2, 3, 4, and 5) were found, namely, αAI-1a, 1b, 1c, and 1d. These four types showed a major polypeptide band and a few faintly stained bands in common, although the electrophoretic mobility and the number of polypeptide bands were different from each other. The banding pattern of lane 6 in Fig. 1, which was observed in accessions with the αAI-2 inhibitory activity type, was characterized by two major bands separated from each other. The accessions with the αAI-3 type showed a characteristic banding pattern consisting of one major polypeptide band and three minor bands. Two cultivars, Ofuku-5 (Fig. 1, lane 10) and Ofuku-8, were reported previously to lack the α-amylase inhibitor protein (Ishimoto and Kitamura 1991). Of the 53 accessions lacking inhibitory activity against the two α-amylases, 15 did not contain any glycopolypeptides in the same region of the inhibitors, whereas 38 contained some polypeptides in the region. The two different banding patterns of the accessions lacking α-amylase inhibitory activity were designated as αAI-0a (containing bands in the region of the seed α-amylase inhibitor) and αAI-0b (lacking bands).

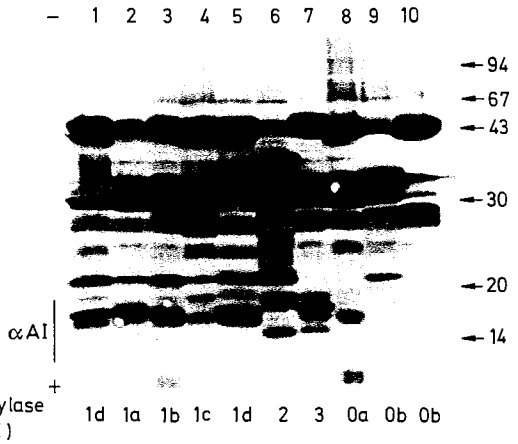

Fig. 1 Electrophoretic banding patterns of seed glycoproteins in the common bean. *Lane 1*, Taishou-kintoki; *2*, G11025A; *3*, G10009; *4*, G12915; *5*, G23456A; *6*, G12952; *7*, G13026; *8*, G12898; *9*, G12882B; *10*, Ofuku-5. *Numbers* in the right margin indicate Mr × 10^{-3}

Geographical distribution

The non-cultivated common bean accessions used in this study were classified as wild or weedy according to Toro et al. (1990). The largest diversity for the types of seed α-amylase inhibitors occurred in wild common bean accessions (Table 2). Thus, all eight inhibitor types were observed in the accessions classified as wild. Accessions containing either αAI-1b or αAI-0b were not observed in the weedy accessions. Non-cultivated, wild and weedy accessions were collected from nine countries in Central and South America (Table 3). Accessions containing either αAI-1a or αAI-1d were widely distributed

Table 2 Frequency of common bean accessions showing different types of seed α-amylase inhibitor

Biological status	Type of αAI								Total
	1a	1b	1c	1d	2	3	0a	0b	
Wild	282	3	4	72	19	16	20	11	427
Weedy	148		2	92	22	17	18		299
Cultivated	685			446		19	1	3	1154

Table 3 Geographical distribution of seed α-amylase inhibitor types in non-cultivated common bean accessions examined

Country	Type of αAI								Total
	1a	1b	1c	1d	2	3	0a	0b	
Mexico	284	2	6	5	41	13	37	10	398
Guatemala	14	1		12					27
El Salvador	1			1					2
Costa Rica	6								6
Colombia	7			5					12
Ecuador				7					7
Peru	59			86					145
Bolivia	2			6					8
Argentine	10			23		1			34
Unknown	47			19		19	1	1	87
Total	430	3	6	164	41	33	38	11	726

Table 4 Geographical distribution of seed α-amylase inhibitor types in cultivated common bean accessions examined

Region or country	Type of αAI					
	1a	1d	3	0a	0b	Total
Central America						
Mexico	326	60	13		1	400
Belize	4					4
Guatemala	52	9		1		62
El Salvador	21	2				23
Honduras	25	2				27
Nicaragua	16	1				17
Costa Rica	19	8				27
South America						
Colombia	26	37	1			64
Ecuador	23	44				67
Peru	25	77				102
Bolivia	5	18				23
Argentine	3	25				28
Brazil	41	33	1			75
Chile	1					1
North America and Caribbean						
countries	13	27			1	41
Europe	3	10				13
Africa	1	6				7
Asia and Oceania	22	31	1		1	55
Unknown	59	56	3			118
Total	685	446	19	1	3	1 154

in Central and South America. The banding patterns of αAI-1b, 1c, 2, 3, 0a, and 0b, in contrast, were detected only in the accessions from Mexico, except for one αAI-1b accession from Guatemala and one αAI-3 accession from Argentina.

In cultivated common beans, only five types of inhibitors were detected and the frequency of αAI-1a and 1d accounted for 98% of all the accessions examined (Table 2). The cultivated common bean accessions examined were collected from 25 countries around the world (Table 4). Cultivars containing either αAI-1a or αAI-1d were widely distributed. The distribution of the αAI-3 cultivars was restricted to Mexico, Colombia, Brazil, and Australia. Cultivars with αAI-0b originated from Mexico, Canada and Japan, while the one cultivar with αAI-0a came from Guatemala.

Discussion

Four different types of seed α-amylase inhibitors were observed in common bean accessions based on the inhibition of the activities of porcine pancreatic α-amylase and the *Z. subfasciatus* larval α-amylase (Table 1). The four inhibitor types could be readily distinguished from each other by comparing the banding patterns of glycopolypeptides in the range of 14 to 20 kDa (Fig. 1). In addition, αAI-1 was subdivided into four types based on difference in banding pattern, and αAI-0 was subdivided into two types. The α-amylase inhibitors in several cultivated varieties have been studied comprehensively and shown to be dimeric or tetrameric structures with total molecular weights of 28 or 49 kDa (Marshall and Lauda 1975; Powers and Whitaker 1977; Moreno et al. 1990; Yamaguchi 1991), although it remains to be determined which of these types would have corresponded to the various inhibitors. Furthermore, another inhibitor protein, αAI-2, has recently been isolated and characterized (Suzuki et al. 1993). These two inhibitor proteins were both found to display insecticidal effects (Ishimoto and Kitamura 1989; Suzuki et al. 1993).

The two inhibitor types, αAI-2 and αAI-3, exhibited activity against the *Z. subfasciatus* larval α-amylase. Gatehouse et al. (1987) and Minney et al. (1990) showed that seed extracts of some accessions classified as resistant to *Z. subfasciatus* (Schoonhoven et al. 1983) inhibited larval α-amylase activity. In the present study, we confirmed that these accessions contained αAI-2. The αAI-2 protein has been purified from seeds of a line resistant to *Z. subfasciatus*, and the growth-inhibitory effects of the protein on the bruchid pests *Z. subfasciatus* and *C. chinensis* have been confirmed by using artifical beans containing the protein (Suzuki et al. 1993). The αAI-3 inhibitor type was characterized by a novel inhibitory activity against the two α-amylases, the combined activities of αAI-1 and αAI-2. Therefore, this inhibitor, which exhibits such wide effects, is considered to be a useful source for the production of insect-resistant crops. However, the banding pattern of αAI-3 seems to result from the co-existence of only one of the αAI-1 inhibitor types and αAI-2. The two types of αAI-0 were characterized by the lack of inhibitory activity against both porcine pancreatic α-amylase and the larval α-amylase. Ishimoto and Kitamura (1991) reported that several cultivars do not contain any seed α-amylase inhibitor proteins. These cultivars were classified as αAI-0b in the present study. Accessions classified as αAI-0b presumably lack the inhibitor proteins. In contrast, accessions classified as αAI-0a showed some polypeptide bands in the region of inhibitors on a blotting membrane. The protein consisting of these polypeptides may display a inhibitory activity against other α-amylases.

It is clearly necessary to study the mode of inheritance of the inhibitors and to isolate the inhibitor proteins for an analysis of their properties.

Non-cultivated common bean accessions exhibited a larger diversity of seed α-amylase inhibitors. In contrast, only four α-amylase inhibitor types were detected in cultivated common bean accessions. The domenstication and subsequent evolution under cultivation may have affected the diversity of some genetic traits and caused the reduction in variability. Phaseolin, a major storage protein in common bean, also exhibits higher levels of variability among wild common beans compared with cultivars (Gepts and Bliss 1986; Gepts et al. 1986). The level of diversity of α-amylase inhibitors in non-cultivated accessions was also higher than that of cultivars.

Wild and weedy common beans occur widely from northern Mexico to northwestern Argentina (Toro et al. 1990). It has been suggested that common bean cultivars result from domestication in two primary centers, Central America and the southern Andes, based on the parallel geographic distribution for phaseolin types and seed sizes between wild and cultivated common beans (Gepts et al. 1986; Singh et al. 1991). The parallel geographic distribution between non-cultivated common beans and cultivars was also observed in the α-amylase inhibitor types (Tables 3 and 4). Non-cultivated and cultivated common bean accessions containing αAI-3 were mainly distributed in Mexico. Cultivars and non-cultivated common beans in South America exhibited predominantly an αAI-1d type. The center of diversity of the seed α-amylase inhibitors appeared to be located in Central America rather than South America. All the eight α-amylase inhibitor types were detected from non-cultivated accessions in Mexico, and αAI-1c and 2 were only found among non-cultivated accessions from Mexico. The greater diversity of seed α-amylase inhibitor types in germplasm from Mexico may be attributed to the larger number of samples tested from this country in our study. However resistance to *Z. subfasciatus* has been confirmed in several accessions of wild common beans collected from the central part of Mexico (Schoonhoven et al. 1983) and was dependent on the presence of two seed proteins, arcelin variants and αAI-2 (Osborn et al. 1988; Minney et al. 1990; Suzuki et al. 1993). It appears that the large diversity of seed α-amylase inhibitor types, and the presence of arcelin variants, both reflect the co-evolutionary adaptation of the common bean to brunchid attacks in this region. By the application of conventional plant breeding methods or genetic manipulation, some types of inhibitor proteins could be used for enhancing the levels of resistance against the Mexican bean weevil, *Z. subfasciatus*, and other bruchid species, not only among common beans but also among other crops.

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