Growth Inhibitory Effect of an α -Amylase Inhibitor from the Wild Common Bean Resistant to the Mexican Bean Weevil (*Zabrotes subfasciatus*)

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Some accessions of the wild common bean resistant to *Zabrotes subfasciatus* which is an important pest of common bean in storage contained a novel α -amylase inhibitor, designated as α AI-2, in seeds. An α -amylase inhibitor, designated as α AI-1, from the cultivated common bean inhibited the activity of the larval midgut α -amylase of *Callosobruchus chinensis* but not that of *Z. subfasciatus*, whereas α AI-2 strongly inhibited the activity of *Z. subfasciatus*.

The addition of the purified α AI-2 protein into artificial beans at concentrations higher than 1% resulted in the strong growth inhibition of the larvae of *Z. subfasciatus*. It was suggested that α AI-2 was responsible for the bruchid resistance to some extent. On the other hand, the larvae of *C. chinensis* could not grow on the artificial beans containing the purified protein at a concentration of 0.3% and died at 1%, although the activity of the larval midgut α -amylase was not appreciably inhibited by α AI-2 in vitro. It appears that α AI-2 exhibits a broader spectrum of the inhibitory effect on the growth of bruchid pests than α AI-1.

KEY WORDS: *Phaseolus vulgaris*, *Zabrotes subfasciatus*, insect resistance, α -amylase inhibitor.

Introduction

Most cultivars of common bean (*Phaseolus vulgaris* L.) contain a proteinous α -amylase inhibitor, which inhibits the activity of some mammal and insect α -amylases, but not that of endogenous plant enzymes (JAFKE *et al.* 1973, MARSHALL and LAUDA 1975). Therefore, the inhibitor has been considered to play an important role in plant defense against insects and herbivores.

ISHIMOTO and KITAMURA (1989) confirmed that a proteinous α -amylase inhibitor (α AI-1) from common bean inhibited the activity of the larval midgut α -amylases of the adzuki bean weevil (*Callosobruchus chinensis* L.) and the cowpea weevil (*C. maculatus* F.), and suppressed strongly the larval growth of the two bruchids at concentrations higher than 0.2% (w/w) on artificial beans. However, α AI-1 did not inhibit the larval growth and the activity of the larval α -amylase of the Mexican bean weevil (*Zabrotes subfasciatus* BOHEMAN), which causes serious post-harvest losses in common bean (ISHIMOTO and KITAMURA 1989, 1992).

Some accessions of the wild common bean highly resistant to *Z. subfasciatus* were identified by SCHOONHOVEN *et al.* (1983). Attempts have been made to detect and characterize the compounds responsible for the resistance (OSBORN *et al.* 1988, GATEHOUSE *et al.* 1987, MINNEY *et al.* 1990). A novel α -amylase inhibitor, designated as α AI-2, which inhibited the activity

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of the larval midgut α -amylase of *Z. subfasciatus*, was detected in some of the resistant accessions (GATEHOUSE *et al.* 1987, MINNEY *et al.* 1990). Nevertheless, the relationship between the resistance and the presence of α AI-2 in the seeds has not been well-documented so far. In the present study, we characterized α AI-2 derived from one of the resistant wild accessions, G12953, in relation to the growth inhibitory effect *in vivo* as well as the inhibition of the α -amylase activity *in vitro* on the two bruchid species, *Z. subfasciatus* and *C. chinensis*.

Materials and Methods

Seeds and insects

Seeds of cultivated common bean (cv. Taishou-kintoki) were used for the purification of α AI-1. For the purification of α AI-2, the F₄ seeds were harvested from an F₃ line with α AI-2 derived from the crosses between cv. Ofuku-5, lacking seed α -amylase inhibitors (ISHIMOTO and KITAMURA 1991), and G12953, a resistant wild accession containing α AI-2.

Z. subfasciatus and *C. chinensis* were reared on cowpea (cv. PI165486) seeds and adzuki bean (cv. Erimo-shozu) seeds respectively, at 30°C and 70% RH.

Assay for inhibition of α -amylase activity

Porcine pancreatic α -amylase (Type-IA) was purchased from Sigma Chemical Co. Ltd. The larval midgut α -amylases of *Z. subfasciatus* and *C. chinensis* were prepared through the extraction from dissected midguts of the fourth instar larvae (ISHIMOTO and KITAMURA 1989). The inhibitory effect of α AI-1 and α AI-2 on the α -amylase activity was determined using a modification of the Bernfeld method (BERNFELD 1955) as described by ISHIMOTO and KITAMURA (1989). Different amounts of α AI-1 or α AI-2 were pre-incubated with the above three different α -amylase preparations, respectively.

Purification of α -amylase inhibitors

α AI-1 was purified from the seeds of cv. Taishou-kintoki according to the procedure previously reported (ISHIMOTO and KITAMURA 1989), except for the absence of heat treatment. The procedure of purification of α AI-2 was almost the same as that of α AI-1 with some modifications as follows. Thirty grams of the F₄ seed flour were extracted with a 800 ml of 0.02 M sodium phosphate buffer solution (PBS), pH 6.7 at 4°C for 4 h. After centrifugation at 11,000 g for 20 min, the supernatant was subjected to ammonium sulfate fractionation. The precipitate obtained between 20 and 80% saturation was dissolved with 200 ml of 0.02 M PBS and dialyzed against the buffer solution. After dialysis for 24 h, the resulting precipitate was removed by centrifugation and the supernatant was applied to a column of DEAE-Sephacel (Pharmacia) equilibrated with 0.02 M PBS. The column was washed with the buffer solution. Then, the adsorbed proteins were eluted with 0.25 M PBS, pH 6.7 and applied to a column of Con A-Sepharose 4B (Pharmacia) equilibrated with 0.02 M PBS, pH 6.7 containing 0.5 M sodium chloride. Proteins adsorbed on Con A-Sepharose 4B were eluted with the buffer solution containing 0.1 M methyl-D-mannopyranoside, and dialyzed against 0.02 M PBS, pH 6.7 for 24 h and applied to the DEAE-Sephacel column again. A linear gradient elution (0.02~0.25 M PBS, pH 6.7) was carried out and the eluates were monitored by measuring the absorbance at 280 nm and assaying the inhibitory effect on the activity of the larval

α -amylase of *Z. subfasciatus*. The fraction exhibiting the α -amylase inhibition was further purified by a gel filtration step on a Sephacryl-S200 (Pharmacia) column equilibrated with 0.05 M PBS, pH 6.7 containing 0.15 M NaCl.

The purified inhibitors were analyzed using a native polyacrylamide gel electrophoresis (PAGE) gel and a sodium dodecyl sulfate (SDS)-PAGE gel (13.5% acrylamide), and were dialyzed against distilled water and lyophilized.

Estimation of molecular weight of native α -amylase inhibitors

The molecular weight of the native α AI-1 and α AI-2 proteins was estimated by gel filtration on a column of Superose 12 (Pharmacia) equilibrated with 0.05 M PBS, pH 6.7 containing 0.15 M NaCl. The following proteins were used as standard samples, bovine albumin, egg albumin, ovalbumin, chymotrypsinogen A, myoglobin equine, ribonuclease A, cytochrome C.

Feeding tests

The resistance to *Z. subfasciatus* of the F_4 seeds derived from the crosses between Ofuku-5 and G12953 was confirmed by a feeding test using artificial beans. The artificial beans were prepared from the seed flour of the F_4 , Taishou-kintoki and cowpea (cv. PI165486), respectively as described previously (ISHIMOTO and KITAMURA 1989). The lyophilized artificial beans were weighed and coated with a 9% gelatin solution. Each treatment was conducted with six replications using an artificial bean weighing approximately 0.5 g. After 30 days of first oviposition by *Z. subfasciatus*, the numbers of adults, living larvae and pupae were recorded, and the results were expressed in terms of survival (%) and adult emergence (%).

The effect of α AI-1 and α AI-2 on the bruchid growth was also investigated by the feeding test using the artificial beans prepared from the seed flour of PI165486 containing the purified proteins at various concentrations ranging from 0.0 to 2.0% (w/w). Each treatment was also conducted with six replications in the feeding tests with *Z. subfasciatus* or *C. chinensis*.

In vitro proteolysis of α -amylase inhibitors

The digestive activities of the larval midgut extracts of *Z. subfasciatus* and *C. chinensis* against the α AI-1 and α AI-2 proteins were examined in vitro according to the method previously described (ISHIMOTO and KITAMURA 1992)

Results

In order to confirm the resistance to *Z. subfasciatus* of the F_4 seeds derived from the crosses between Ofuku-5 and G12953, artificial beans were prepared from the seed flour of the F_4 , Taishou-kintoki and PI165486, respectively. The percentage of survival on the artificial beans from the F_4 seeds was lower than that on the artificial beans from the seeds of the other cultivars and no adults emerged from the artificial beans from the F_4 seeds within 30 days (Table 1). The artificial beans from the seeds of Taishou-kintoki or PI165486, which did not contain α AI-2, were severely damaged by *Z. subfasciatus*. The results suggested that the F_4 seeds were strongly resistant to *Z. subfasciatus*.

Purification of α AI-2

A fraction adsorbed on Con A-Sepharose 4B, which inhibited the α -amylase activity of

Z. subfasciatus, was applied to the DEAE-Sephacel column and separated into three peaks by gradient elution (Fig. 1). The first peak exhibited an hemagglutination activity, and the second peak showed an inhibitory effect on the α -amylase activity of *Z. subfasciatus*, while the third peak did not display either the hemagglutination activity or the α -amylase inhibitory effect. Finally, α AI-2 was purified from the second peak fraction by gel filtration on the Sephacryl-S200 column. It was estimated that the content of α AI-2 in the F₄ seeds ranged from 0.4 to 0.5% (w/w) (data not shown).

The two purified inhibitors were examined by native PAGE and SDS-PAGE. α AI-2 gave a single protein band on the native gel and three polypeptide bands with molecular weights ranging from 14,400~20,100 on the SDS gel (Fig. 2). To estimate the molecular weight (Mr) of α AI-2, in comparison with that of α AI-1, each protein was applied to the column of Superose 12 by using the FPLC system (Pharmacia). α AI-2 was eluted at the position cor-

Table 1. Survival of *Z. subfasciatus* reared on artificial beans prepared from seed flour

Seed flour	Survival (%)	Adult emergence (%)
F ₄ seeds ¹⁾	58.45 ± 2.65 ²⁾	0.00 ± 0.00
Taishou-kintoki	81.25 ± 3.87	60.70 ± 3.44
PI165486	81.69 ± 5.01	75.88 ± 5.12

¹⁾ The seeds derived from crosses between Ofuku-5 and G12953 contained α AI-2.

²⁾ The standard errors are indicated.

Fig. 1. Separation of Con A Sepharose-adsorbed fraction containing α AI-2 by ion-exchange chromatography on a column of DEAE-Sephacel. The arrow indicates the fraction exhibiting the inhibitory effect on the larval midgut α -amylase of *Z. subfasciatus*. Broken line indicates the molar concentrations of sodium phosphate (pH 6.8). Volume of fraction is 12.5 ml.

Fig. 2. SDS-PAGE analysis of α AI-1 and α AI-2. Lane 1, α AI-1; Lane 2, α AI-2. Thirty μ g of each protein was applied to a 13.5% polyacrylamide gel. Numbers indicate the approximate molecular weight in kDa.

responding to a Mr of 49,000, while α AI-1 was eluted at approximately half of the Mr value (Mr, 24,400). These results suggest that α AI-2 must have a hetero- or homo-tetrameric structure, whereas it was reported that α AI-1 had a heterodimeric structure (YAMAGUCHI 1991).

Effect of α -amylase inhibitors on the activity of the larval α -amylases

α AI-1 strongly inhibited both the activity of the larval α -amylase of *C. chinensis* and porcine pancreatic α -amylase, but not that of *Z. subfasciatus* (Fig. 3). On the other hand, α AI-2 strongly inhibited only the α -amylase activity of *Z. subfasciatus*. The α -amylase activity of *C. chinensis* was not appreciably inhibited by α AI-2. The amount of α AI-2 required for reaching a 40% inhibition of the α -amylase activity of *C. chinensis* was approximately 100 times that for *Z. subfasciatus*. In practice, however, the extent of the inhibitory effect of α AI-2 on the α -amylase activity of the *Z. subfasciatus* larvae was about 38 times larger than that of the *C. chinensis* larvae, when considering the difference in the degree of activity of the larval α -amylases of the two bruchids, i.e., the activity of *Z. subfasciatus* was about 2.6 times higher than that of *C. chinensis* (data not shown).

Effect of α -amylase inhibitors on the growth of larvae

The growth of the larvae of *Z. subfasciatus* was strongly suppressed by the addition of the purified α AI-2 protein to the artificial beans used for larval rearing. No adults were observed in the beans containing the purified protein at a concentration of 1.0% (Fig. 4). In contrast, the larval growth was hardly suppressed by α AI-1 as anticipated from the lack of inhibitory effect of α AI-1 on the larval α -amylase activity in vitro. The larval growth of *C. chinensis* was strongly suppressed by both α AI-1 and α AI-2 (Fig. 4). The addition of the inhibitors into artificial beans at concentrations of more than 0.3% resulted in the absence of adult emergence or the complete mortality of the bruchid, although α AI-2 hardly inhibited the activity of the larval α -amylase of *C. chinensis*.

Proteolytic activity of the larval midgut extracts.

The larval midgut extracts contain not only α -amylases but also other digestive enzymes such as proteases. It was shown that the larval midgut extract from *Z. subfasciatus* digested immediately α AI-1 to small molecules and suppressed the inhibitory effect (ISHIMOTO and KITAMURA 1992).

Fig. 3. Inhibition of the activity of the larval midgut α -amylases of *Z. subfasciatus* and *C. chinensis*, and porcine pancreatic α -amylase by α AI-1 or α AI-2. Preparations of each α -amylase in sufficient amount to liberate 80 μ g maltose/min at 30°C were used. The inhibition (%) shows the relative value against the activity of each α -amylase without pre-incubation with the inhibitor. A, inhibition by α AI-1; B, inhibition by α AI-2.

Fig. 4. Effect of α AI-1 or α AI-2 on the larval growth of *Z. subfasciatus* and *C. chinensis*. The effect is expressed as the survival rate of the bruchids on artificial beans at 30 days after the first oviposition. A, effect of α AI-1 on the larval growth of *Z. subfasciatus*; B, effect of α AI-2 on the larval growth of *Z. subfasciatus*; C, effect of α AI-1 on the larval growth of *C. chinensis*; D, effect of α AI-2 on the larval growth of *C. chinensis*.

Fig. 5. SDS-PAGE analysis of α AI-1 and α AI-2 after incubation with the larval midgut extracts of *Z. subfasciatus* or *C. chinensis*. Lane 1, α AI-1; lanes 2 and 3, α AI-1 incubated with the extract of *Z. subfasciatus* for 5 min and 12 h, respectively; lane 4, α AI-1 incubated with the extract of *C. chinensis* for 12 h; lane 5, α AI-2; lanes 6 and 7, α AI-2 incubated with the extract of *Z. subfasciatus* for 5 min and 12 h, respectively; lane 8, α AI-2 incubated with the extract of *C. chinensis* for 12 h.

However, α AI-2 specifically inhibited the α -amylase activity of *Z. subfasciatus* (ISHIMOTO *et al.* 1993). Therefore, in order to determine whether the midgut extracts of *Z. subfasciatus* and *C. chinensis* digested α AI-2 for suppressing the inhibitory effect, the two inhibitors were incubated with the larval extracts of the two bruchids.

After incubation, the inhibitors were examined by SDS-PAGE. Both α AI-1 and α AI-2 were not digested by the midgut extract of *C. chinensis* even after incubation for 12h (Fig. 5). α AI-1 was restrictively digested to polypeptide fragments by the extract of *Z. subfasciatus* after incubation for 1h, whereas α AI-2 was not digested even after incubation for 12h. α AI-2 was highly resistant to the digestive

enzymes in the larval midgut extract of *Z. subfasciatus*.

Discussion

The wild common bean accession, G12953, was found to be highly resistant to *Z. subfasciatus* (SCHOONHOVEN *et al.* 1983). In the present study, a novel α -amylase inhibitor, α AI-2, was purified from the bruchid-resistant F₄ seeds derived from the crosses between Ofuku-5 and G12953.

The purified α AI-2 strongly inhibited the activity of the larval midgut α -amylase of *Z. subfasciatus* and the larval growth of the bruchid. No adults emerged from the artificial beans containing the purified protein at a concentration of 1% within 30 days, although the addition of α AI-2 into artificial beans at the levels present in the seeds, 0.4~0.5%, did not result in an appreciable inhibition of the larval growth. Therefore, it is likely that α AI-2 is responsible, at least in part, for the bruchid resistance. The seeds of G12953 and the F₄ seeds also contain arcelin-4 which at a high level has been considered to confer the resistance (MINNEY *et al.* 1990). The gene controlling the presence of arcelin-4 was closely linked to that controlling the expression of α AI-2 (ISHIMOTO *et al.* 1993). It thus seems reasonable to assume that the resistance to *Z. subfasciatus* in G12953 is associated with the presence of not only arcelin-4 but also α AI-2.

ISHIMOTO and KITAMURA (1989) confirmed that the inhibitory effect of α AI-1 on the activity of the larval α -amylases in vitro was correlated with the effect of α AI-1 on the larval growth of the bruchids in vivo. Although the results obtained in the present study regarding α AI-1 agreed with the previous findings, the effect of α AI-2 in vitro was not correlated with the effect in vivo. The activity of the larval α -amylase of *C. chinensis* was hardly affected by the incubation with α AI-2. Nevertheless, the larval growth of the bruchid was strongly suppressed by the addition of α AI-2 into artificial beans at levels above 0.3%. The apparent discrepancies in the results could be explained as follows. First: the larval growth of *C. chinensis* may be very sensitive to the inhibition of the α -amylase activity by the inhibitors, compared with *Z. subfasciatus*. The larvae of *C. chinensis* could not develop and died by the addition of α AI-1 at a concentration of 0.3%, whereas the larval growth of *Z. subfasciatus* was not appreciably suppressed by the addition of α AI-2 at such a concentration. Second: α AI-2 may be able to inhibit the α -amylase activity of the *C. chinensis* larvae in the early instar. Since the larval midgut α -amylase prepared from only the last instar larvae was used for examining the inhibitory effect of α AI-2, it is necessary to investigate the inhibitory effect of α AI-2 on the activity of the larval α -amylase in the first or second instar. Third: α AI-2 may inhibit the growth of *C. chinensis* through an unknown mechanism. Further studies should be carried out to analyze the effect of α AI-2 on the growth inhibition of the two bruchid species. It appears that α AI-2 could be used for the control of insect pests through both conventional breeding methods and genetic engineering techniques, because it exhibits a broad spectrum of growth inhibitory effects on bruchid pests.

It has been suggested that the larval growth of *Z. subfasciatus* was not affected by α AI-1 due to the ability of the larvae to digest the inhibitor (ISHIMOTO and KITAMURA 1992). On the

other hand, α AI-2 from the resistant accession tolerated the digestion by the larval midgut extract of *Z. subfasciatus*, and inhibited the larval growth of the bruchid. Both the polypeptide composition and molecular weight of the native proteins in the two inhibitors suggested that the structure of α AI-2 was distinctly different from that of α AI-1. The inhibitory effect of α AI-2 may be attributed to the structure of the protein.

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ブラジルマメゾウムシに抵抗性の野性インゲンマメに見い出された α -アミラーゼインヒビターの生育阻害特性鈴木一典¹⁾・石本政男²⁾・菊池文雄³⁾・喜多村啓介^{1),2)}¹⁾筑波大学連携大学院 (農学), つくば市, 〒 305²⁾農業研究センター, つくば市, 〒 305³⁾筑波大学農林学系, つくば市, 〒 305)

インゲンマメ種子に含まれる α -アミラーゼインヒビタータンパク質 (α AI-1) は, α -アミラーゼ活性を阻害することにより, アズキゾウムシなどの生育を阻害する。しかし, インゲンマメを食害するブラジルマメゾウムシは, α AI-1 による α -アミラーゼ活性阻害に耐性を有している。最近, ブラジルマメゾウムシに抵抗性を示すインゲンマメ野性系統が見い出され, それらのいくつかは本幼虫の α -アミラーゼ活性を強く阻害するインヒビタータンパク質(α AI-2) を含むと報告された。

本報では, 野性インゲンマメのブラジルマメゾウムシ抵抗性と α AI-2 との関係を明らかにするため, α AI-2 を精製し, ブラジルマメゾウムシの生育に及ぼす影響を調査した。

ブラジルマメゾウムシ抵抗性の野性インゲンマメ系統と栽培インゲンマメ品種の交配から得た α AI-2 を含む F_4 種子から, α AI-2 を精製した。精製に供試した種子は, ブラジルマメゾウムシに強い抵抗性を有していた (Table 1)。 α AI-2 は, Con A-Sepharose に吸着し, DEAE-Sepharose によるイオン交換クロマトグラフィーでレクチンの後に溶出した (Fig. 1)。Sephacryl-S 200 のゲルろ過により最終的に精製された α AI-2 は, SDS-ゲル電気泳動で α AI-1 と同じ分子量域 (Mr 14,400-20,100) に α AI-1 とは異なるバンドパターンを示した (Fig. 2)。また, ゲルろ過により推定された α AI-2 の分子量は, α AI-1 (Mr 24,400) のほぼ2倍量の49,000であった。

精製された α AI-2 は, ブラジルマメゾウムシ幼虫の α -アミラーゼ活性を強く阻害し, アズキゾウムシ幼虫やブタすい臓の α -アミラーゼ活性をほとんど阻害しなかった (Fig. 3)。 α AI-1 はブラジルマメゾウムシ幼虫の消化管磨砕液により短時間で分解されたが, α AI-2 は分解されなかった (Fig. 5)。一方, アズキゾウムシ幼虫の消化管磨砕液には, これらインヒビターに対する分解作用は認められなかった。これらの結果から, α AI-2 の有する α -アミラーゼ阻害特性は, α AI-2 がブラジルマメゾウムシ幼虫の分解作用に耐性を備えていることに加え, ブラジルマメゾウムシ幼虫の α -アミラーゼ分子種に対し特異性を有しているためによると考えられた。

α AI-2 および α AI-1 がマメゾウムシの生育に及ぼす影響を調べるため, 人工豆を用いた生物検定を行った。 α AI-2 を1% (w/w) 混入した人工豆では, ブラジルマメゾウムシの羽化, そしてアズキゾウムシの生存が全く認められなかった (Fig. 4)。一方, α AI-1 は, これまでの報告のように, アズキゾウムシの生育を強く阻害し, ブラジルマメゾウムシの生育は阻害しなかった。

α AI-2 は, 本抵抗性系統の種子に重量当たり0.4-0.5% (w/w) 含まれている。 α AI-2 をこの濃度で混入させた人工豆においては, ブラジルマメゾウムシの生育は, 強くは阻害されなかった。このため, α AI-2 が単独で本抵抗性の主因を果たしているとは考え難かった。しかしながら, α AI-2 はブラジルマメゾウムシだけではなく, アズキゾウムシに対しても強い生育阻害物質として作用したため, 耐虫性タンパク質としての有用性が高いと考えられた。