Growth Inhibitory Effect of an $\alpha$-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 $\alpha$-amylase inhibitor, designated as $\alpha$AI-2, in seeds. An $\alpha$-amylase inhibitor, designated as $\alpha$AI-1, from the cultivated common bean inhibited the activity of the larval midgut $\alpha$-amylase of Callosobruchus chinensis but not that of Z. subfasciatus, whereas $\alpha$AI-2 strongly inhibited the activity of Z. subfasciatus.

The addition of the purified $\alpha$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 $\alpha$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 $\alpha$-amylase was not appreciably inhibited by $\alpha$AI-2 in vitro. It appears that $\alpha$AI-2 exhibits a broader spectrum of the inhibitory effect on the growth of bruchid pests than $\alpha$AI-1.

KEY WORDS: Phaseolus vulgaris, Zabrotes subfasciatus, insect resistance, $\alpha$-amylase inhibitor.

Introduction

Most cultivars of common bean (Phaseolus vulgaris L.) contain a proteinous $\alpha$-amylase inhibitor, which inhibits the activity of some mammal and insect $\alpha$-amylases, but not that of endogenous plant enzymes (Jaffe 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 $\alpha$-amylase inhibitor ($\alpha$AI-1) from common bean inhibited the activity of the larval midgut $\alpha$-amylases of the azuki 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, $\alpha$AI-1 did not inhibit the larval growth and the activity of the larval $\alpha$-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 $\alpha$-amylase inhibitor, designated as $\alpha$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 αAI-2 in the seeds has not been well-documented so far. In the present study, we characterized αAI-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 αAI-1. For the purification of αAI-2, the F₁ seeds were harvested from an F₂ line with αAI-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 αAI-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-1A) 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 αAI-1 and αAI-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 αAI-1 or αAI-2 were pre-incubated with the above three different α-amylase preparations, respectively.

**Purification of α-amylase inhibitors**

αAI-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 αAI-2 was almost the same as that of αAI-1 with some modifications as follows. Thirty grams of the F₁ 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 αAI-1 and αAI-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 F₄ 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 F₄, 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 αAI-1 and αAI-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 αAI-1 and αAI-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 F₄ seeds derived from the crosses between Ofuku-5 and G12953, artificial beans were prepared from the seed flour of the F₄, Taishou-kintoki and PI165486, respectively. The percentage of survival on the artificial beans from the F₄ 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 F₄ seeds within 30 days (Table 1). The artificial beans from the seeds of Taishou-kintoki or PI165486, which did not contain αAI-2, were severely damaged by *Z. subfasciatus*. The results suggested that the F₄ seeds were strongly resistant to *Z. subfasciatus*.

*Purification of αAI-2*

A fraction adsorbed on Con A-Sepharose 4B, which inhibited the α-amylase activity of
Z. subfasciatus, was applied to the DEAE-Sepharcel 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, αAI-2 was purified from the second peak fraction by gel filtration on the Sephacryl-S200 column. It was estimated that the content of αAI-2 in the F_4 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. αAI-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 αAI-2, in comparison with that of αAI-1, each protein was applied to the column of Superose 12 by using the FPLC system (Pharmacia). αAI-2 was eluted at the position cor-

<table>
<thead>
<tr>
<th>Seed flour</th>
<th>Survival (%)</th>
<th>Adult emergence (%)</th>
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<tr>
<td>F_4 seeds^1)</td>
<td>58.45 ± 2.65^2)</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Taishou-kintoki</td>
<td>81.25 ± 3.87</td>
<td>60.70 ± 3.44</td>
</tr>
<tr>
<td>PI65486</td>
<td>81.69 ± 5.01</td>
<td>75.88 ± 5.12</td>
</tr>
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^1) The seeds derived from crosses between Ofuku-5 and G12953 contained αAI-2.

^2) The standard errors are indicated.

![Fig. 1. Separation of Con A Sepharose-adsorbed fraction containing αAI-2 by ion-exchange chromatography on a column of DEAE-Sepharcel. 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.](image)

![Fig. 2. SDS-PAGE analysis of αAI-1 and αAI-2. Lane 1, αAI-1; Lane 2, αAI-2. Thirty µg of each protein was applied to a 13.5% polyacrylamide gel. Numbers indicate the approximate molecular weight in kDa.](image)
responding to a Mr of 49,000, while αAI-1 was eluted at approximately half of the Mr value (Mr, 24,400). These results suggest that αAI-2 must have a hetero- or homo-tetrameric structure, whereas it was reported that αAI-1 had a heterodimeric structure (YAMAGUCHI 1991).

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

αAI-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, αAI-2 strongly inhibited only the α-amylase activity of Z. subfasciatus. The α-amylase activity of C. chinensis was not appreciably inhibited by αAI-2. The amount of αAI-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 αAI-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 αAI-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 αAI-1 as anticipated from the lack of inhibitory effect of αAI-1 on the larval α-amylase activity in vitro. The larval growth of C. chinensis was strongly suppressed by both αAI-1 and αAI-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 αAI-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 αAI-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 αAI-1 or αAI-2. Preparations of each α-amylase in sufficient amount to liberate 80 μg 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 αAI-1; B, inhibition by αAI-2.
Fig. 4. Effect of αAI-1 or αAI-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 αAI-1 on the larval growth of *Z. subfasciatus*; B, effect of αAI-2 on the larval growth of *Z. subfasciatus*, C, effect of αAI-1 on the larval growth of *C. chinensis*; D, effect of αAI-2 on the larval growth of *C. chinensis*.

However, αAI-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 αAI-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 αAI-1 and αAI-2 were not digested by the midgut extract of *C. chinensis* even after incubation for 12h (Fig. 5). αAI-1 was restrictively digested to polypeptide fragments by the extract of *Z. subfasciatus* after incubation for 1h, whereas αAI-2 was not digested even after incubation for 12h. αAI-2 was highly resistant to the digestive
enzymes in the larval midgut extract of *Z. subfasciatus*.

**Discussion**

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

The purified αAI-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 αAI-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 αAI-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 αAI-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 αAI-2.

Ishimoto and Kitamura (1989) confirmed that the inhibitory effect of αAI-1 on the activity of the larval α-amylases in vitro was correlated with the effect of αAI-1 on the larval growth of the bruchids in vivo. Although the results obtained in the present study regarding αAI-1 agreed with the previous findings, the effect of αAI-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 αAI-2. Nevertheless, the larval growth of the bruchid was strongly suppressed by the addition of αAI-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 αAI-1 at a concentration of 0.3%, whereas the larval growth of *Z. subfasciatus* was not appreciably suppressed by the addition of αAI-2 at such a concentration. Second: αAI-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 αAI-2, it is necessary to investigate the inhibitory effect of αAI-2 on the activity of the larval α-amylase in the first or second instar. Third: αAI-2 may inhibit the growth of *C. chinensis* through an unknown mechanism. Further studies should be carried out to analyze the effect of αAI-2 on the growth inhibition of the two bruchid species. It appears that αAI-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 αAI-1 due to the ability of the larvae to digest the inhibitor (Ishimoto and Kitamura 1992). On the
other hand, αAI-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 αAI-2 was distinctly different from that of αAI-1. The inhibitory effect of αAI-2 may be attributed to the structure of the protein.

Literature Cited


ブラジルマメザムツ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

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インゲンマメ種子に含まれるα-アミラーゼ活性を阻害することにより、アズキソ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ソムツムシは、αAI-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、ブラジルマメソムツムシ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ーターパク質（αAI-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ソムツム抵抗性とαAI-2との関係を明らかにするため、αAI-2を精製し、ブラジルマメソムツムの生育に及ぼす影響を調査した。

ブラジルマメソムツム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αAI-2を含むF2種子から、αAI-2を精製した。精製に供試した種子は、ブラジルマメソムツムに強い抵抗性を有していた（Table 1）。αAI-2は、Con A-Sepharoseに吸着し、DEAE-Sephacel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αAI-2は、SDS-ゲル電気泳動でαAI-1と同じ分子量（Mr 14,400-20,100）にαAI-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るαAI-2の分子量は、αAI-1（Mr 24,400）の約2倍量の49,000であった。

精製されたαAI-2は、ブラジルマメソムツム幼虫のα-アミラーゼ活性を強く阻害し、アズキソウムシ幼虫やアスタリスク幼虫のα-アミラーゼ活性をほとんど阻害しなかった（Fig.3）。αAI-1はブラジルマメソムツム幼虫の消化管消化液により短時間で分解されたが、αAI-2は分解されなかった（Fig.5）。一方、アズキソウムシ幼虫の消化管消化液には、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、αAI-2の有するα-アミラーゼ阻害特性は、αAI-2がブラジルマメソムツム幼虫の消化作用に耐性を備えていることに加え、ブラジルマメソムツム幼虫のα-アミラーゼの陰性化に対する特異性を有していることによると考えられた。

αAI-2およびαAI-1がマメソムツムの生育に及ぼす影響を調べるため、人工土を用いた生物検定を行った。αAI-2を1％（w/w）混入した人工土では、ブラジルマメソムツムの幼生が完全に阻害されなかった（Fig.4）。一方、αAI-1は、これまでの報告のように、アズキソウムシの生育を強く阻害し、ブラジルマメソムツムの生育は阻害しなかった。

αAI-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。αAI-2をこの濃度で混入させた人工土においては、ブラジルマメソムツムの生育は、強くは阻害されなかった。このため、αAI-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、αAI-2はブラジルマメソムツムだけでなく、アズキソ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、耐性タンパク質としての有用性が高いと考えられた。