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α-Amylase Inhibitors from Wheat: Amino Acid Sequences and Patterns of Inhibition of Insect and Human α-Amylases

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Four α-amylase inhibitors, WRP24, WRP25, WRP26, and WRP27, were purified from wheat flour by preparative, reversed-phase high performance liquid chromatography. All have polypeptide molecular masses of about 14 kDa and are members of the cereal superfamily of protease and α-amylase inhibitors. Sedimentation velocity analysis indicated that WRP25 and WRP27 are monomeric proteins, whereas WRP24 is a dimer. WRP24 is identical in N-terminal amino acid sequence to the well characterized 0.19 dimeric inhibitor from wheat kernels. WRP25 and WRP26 differ in sequence from each other at only three positions and represent previously unseparated forms of the 0.28 wheat inhibitor. WRP27 is a previously uncharacterized inhibitor and is more similar in sequence to the 0.28 inhibitor than to the 0.19 inhibitor. WRP25 and WRP26 inhibited α-amylases from the rice weevil, red flour beetle, and the yellow meal worm, but did not inhibit human salivary α-amylase. WRP24 inhibited the human as well as the insect α-amylases, but inhibited one of the two rice weevil α-amylases much more strongly than the other. WRP27 was notable in that, of the enzymes tested, it strongly inhibited only the rice weevil α-amylases. We observed that the growth rate of red flour beetle larvae was slowed when purified WRP24 was included in the diet at a level of 10%. Addition of WRP24 to corn starch resulted in greater weight loss of red flour beetle adults than occurred on control diets. Our results support the hypothesis that these α-amylase inhibitors provide wheat seeds with a selective evolutionary advantage since the inhibitors can slow the growth of insect pests that attack cereal grains. Copyright © 1996 Published by Elsevier Science Ltd.

α-Amylase inhibitors Amino acid sequence Stored product insect pests Plant protection Wheat proteins

INTRODUCTION

Proteinaceous enzyme inhibitors from plants have been extensively studied, in part because they may play a role in host-plant resistance to insect and microbial pests (Yetter *et al.*, 1979; Ryan, 1984, 1990; Gatehouse *et al.*, 1986; Silano, 1987; Mills, 1988; Richardson, 1991). Because α-amylases are essential enzymes for insect growth and development, inhibitors of α-amylase may have detrimental effects on the insect's life cycle when present in the diet. Wheat kernels are particularly rich in inhibitors of α-amylases of animals such as insects and mammals (Kneen and Sandsted, 1943, 1946; Buonocore and Silano, 1986; Baker, 1988a; Baker and Lum, 1989; Gomez *et al.*, 1989; Feng *et al.*, 1991a). Some of these are selective* for insect enzymes, in that they inhibit α-amylases from insects strongly but inhibit mammalian salivary or pancreatic α-amylases only weakly or not at all. Insect-selective α-amylase inhibitors in wheat are primarily monomeric proteins (Silano *et al.*, 1975), and, based on an electrophoretic mobility of 0.28 of the first of these proteins to be sequenced, this set of proteins has been called the 0.28 group of inhibitors (Silano, 1987). The insect-selectivity, monomeric architecture, and small

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* By "selectivity" we mean an inhibitor's ability to inhibit enzymes (α-amylases in our case) from some sources much more strongly than the corresponding enzymes from other sources.

size (approx. 14 kDa) of these inhibitors make their genes attractive candidates for introduction into plants to increase resistance to attack by insect and microbial pests.

Not only are we interested in developing α-amylase inhibitors as insect control proteins, we also want to elucidate structure–function relationships in these proteins. Thus, we would ultimately like to understand the physico–chemical basis for the selectivity exhibited by individual α-amylase inhibitors. That is, how do the structures of these proteins determine their patterns of inhibition against different α-amylases? The selectivity exhibited by a particular inhibitor can be much more subtle than simply distinguishing between insect and mammalian α-amylases. α-Amylases from different insect species can differ substantially in their sensitivity to particular inhibitor fractions (Feng *et al.*, 1991a,b). For example, the two α-amylases from the rice weevil exhibited marked differences in their sensitivities to inhibitor fractions or purified inhibitors (Baker, 1987, 1988b, 1989; Feng *et al.*, 1991a; Chen *et al.*, 1992).

Achieving the goal of relating structure to function will require assembling a group of inhibitors whose structures are known and whose patterns of inhibition against a repertoire of insect and mammalian enzymes have been determined. Previously we demonstrated the effectiveness of reversed-phase HPLC in fractionating α-amylase inhibitors from wheat and rice (Feng, 1990; Feng *et al.*, 1991a,b). Several chromatographic fractions from both sources exhibited selectivity towards insect α-amylases. Because of the much greater abundance of the inhibitors in wheat than in rice (Baker, 1988a; Feng *et al.*, 1991a,b), we have chosen to purify and characterize more fully the structures and activities of several wheat inhibitors.

MATERIALS AND METHODS

Purification of α-amylase inhibitors from wheat

Newton wheat kernels were kindly provided by Dr C. R. Hoseney, Department of Grain Science and Industry, Kansas State University. Kernels were ground into a flour and the flour extracted with 0.15 M NaCl. The extract was heat-treated and subjected to ammonium sulfate precipitation as described by Feng *et al.* (1991a). From ammonium sulfate fraction AS 1.0–1.5, we isolated several inhibitors—WRP24, WRP25, WRP26 and WRP27 in HPLC peaks 24, 25, 26 and 27, respectively. The numbering scheme for HPLC peaks is that of Feng *et al.* (1991a). HPLC was carried out on a Vydac 218 TP 1022 C-18 column (2.2 × 25 cm, 10 μm particle size, 300 Å pore size) at a flow rate of 8 ml/min. Individual peaks were collected, lyophilized, and stored at −20°C.

Peak 27 exhibited two bands after electrophoresis in the presence of sodium dodecyl sulfate, and the inhibitor in this peak was further fractionated by Mono Q ion-exchange chromatography (Feng, 1990). The chromatography was conducted at room temperature in 50 mM ethanolamine buffer, pH 9.5, using an NaCl gradient to 0.5 M. The inhibitor peak from the Mono Q column was analyzed by denaturing and non-denaturing polyacrylamide gel electrophoresis and found to be essentially homogenous and to have an apparent molecular mass of approx. 14 kDa (Feng, 1990). Minor contaminants were removed from the inhibitors in HPLC peaks 25 and 26 by gel permeation chromatography (Feng, 1990).

Assay of α-amylase inhibitor activity

Assays of inhibitor activity were performed as described by Feng *et al.* (1991a) against six test enzymes: human salivary α-amylase (HSA) and five insect α-amylases—rice weevil (*Sitophilus oryzae*) α-amylases 1 and 2 (SOA1 and SOA2), red flour beetle (*Tribolium castaneum*) α-isoamylases 1 and 2 (TCA1 and TCA2) and yellow mealworm (*Tenebrio molitor*) α-amylase (TMA). The purification and properties of the insect α-amylases have been described elsewhere (Chen *et al.*, 1992).

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel (15%) electrophoresis (SDS–PAGE) was conducted as described by Fling and Gregerson (1986). Rainbow protein molecular weight markers were from Amersham Life Science Products (Arlington Heights, IL). Non-denaturating gel electrophoresis was carried out as described by Davis (1964) using a pH 8.8 separating gel. Bromophenol blue was used as the tracking dye.

Sedimentation velocity analysis

Ultracentrifugation was carried out on a Beckman Model E analytical ultracentrifuge equipped with ultraviolet optics. Samples ($A_{280} = 0.5$) in 0.020 M Tris–Cl, pH 8.0, 0.15 M NaCl were centrifuged at 52,000 rpm at 20°C and the movement of the protein boundary over time used to calculate sedimentation coefficients (Chervenka, 1970).

Amino acid sequence determination and analysis

Proteins were reduced and *S*-carboxymethylated by the method of Crestfield *et al.* (1963). N-terminal sequences (from 35 to 52 residues) of the *S*-alkylated proteins and the sequences of some larger fragments derived from chemical cleavages were determined by using a standard Edman degradation sequenator program with 2–5 nmol amounts in a Model 477A automatic pulsed liquid phase protein sequencer/Model 120A PTH HPLC analyzer (Applied Biosystems Ltd).

Cleavage at methionine residues was achieved by treatment of a sample (2 mg) with cyanogen bromide (100-fold molar excess) in 2 ml of 70% formic acid under nitrogen for 48 h at 20°C. After lyophilization, the residue was dissolved in 1 ml of 0.1 M ammonium bicarbonate containing 6 M guanidine HCl and fractionated on a column (1 × 200 cm) of Biogel P-6 in 0.1 M ammonium bicarbonate. Each fraction was further purified by

reversed-phase HPLC on a column (4.6 mm × 25 cm) of Vydac C18 using a linear gradient of 0–70% acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid. Smaller peptides were obtained from the proteins by enzymatic digestion with: trypsin (2% w/w enzyme/substrate in 0.2 M *N*-ethyl morpholine–HCl, pH 8.1, for 3 h at 37°C); the Lys-specific endoproteinase from *Lysobacter enzymogenes* [2% (w/w) in 0.2 M phosphate, pH 7.0, for 4 h at 37°C]; chymotrypsin [2% (w/w) in 0.1 M ammonium bicarbonate, pH 8.0, for 2 h at 37°C]; pepsin [1% (w/w) in 5% formic acid for 2 h at 37°C]; and endoproteinase Glu-C from *Staphylococcus aureus* V8 [2% (w/w) in 0.2 M phosphate, pH 8.0, for 24 h at 37°C]. Peptides obtained from those digests were purified by reversed-phase HPLC on a column (4.6 mm × 25 cm) of Vydac C18 using gradients (5–50%) of acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid. The amino acid sequences of the smaller peptides were determined by the manual dimethylaminoazobenzene isothiocyanate/phenylisothiocyanate double coupling method of Cheng *et al.* (1978).

To determine amino acid compositions, samples of proteins and peptides were hydrolyzed with 5.6 μHCl containing 0.02% (v/v) *o*-cresol at 108°C for 24 h. Amino acids in the hydrolysates were derivatized with phenylisothiocyanate and analyzed by HPLC using the Waters Pico-Tag method.

Bioassay of inhibitor WRP24

WRP24 was bioassayed with a laboratory colony of *T. castaneum* by using both larvae and adults. For larvae, the diet consisted of a mixture of corn starch, wheat gluten (7% wt/wt), cellulose, Torula yeast and a salt mixture (Applebaum and Konijn, 1965; Medici and Taylor, 1966). WRP24 (10% wt/wt) was added to the diet and ground in a mortar with a pestle with water (20% v/wt). Twelve neonate larvae were placed in a disposable beaker (about 12 mm dia. and 21 mm deep) with 300 mg of diet at 27°C and 75% relative humidity. A similar group of newly hatched larvae was weighed on a Cahn C-31 Microbalance to provide the mean initial weight of the larvae. After 9 days on the diet, 10 larvae were selected randomly for the bioassay. The weight of each larva was recorded, after which the larvae were placed in individual 1.5 ml disposable centrifuge tubes containing approx. 30 mg amounts of the diet. Larval weight was recorded at several timed intervals.

For the feeding test with adults, newly eclosed *T. castaneum* adults were weighed, placed in 20 mg corn starch with or without the test material (10% wt/wt), and weighed again 29 days later. Test materials were a wheat albumin/globulin fraction and purified WRP24. The wheat albumin/globulin fraction (AS 0–1.8) was prepared by extracting wheat flour with 0.15 M NaCl, heating, and precipitating with 1.8 M ammonium sulfate according to Feng *et al.* (1991a). The bioassay data were analyzed using the independent *t*-test, Duncan's multiple range test, and linear regression analysis (Wilkinson, 1989; SAS, 1982).

RESULTS

Purification of wheat α-amylase inhibitors

We previously reported that reversed-phase HPLC resolved ammonium sulfate fractions of extracts of wheat flour into numerous peaks of absorbance at 280 nm (Feng *et al.*, 1991a). The fractions AS 1.0–1.5 and AS 1.5–2.5 were found to be particularly rich in HPLC fractions 24, 25, 26 and 27. Of these, fraction 24 inhibited both insect and human salivary α-amylases, and fractions 25, 26 and 27 were selective for insect α-amylases.

Using a preparative reversed-phase HPLC column, we have now fractionated larger samples (about 200 mg of protein) of AS 1.0–1.5 and obtained chromatographic resolution (Fig. 1) comparable to that achieved on an analytical column (Feng *et al.*, 1991a). Peaks 24, 25 and 26 from the preparative column were each found to contain one major band when analyzed by SDS–PAGE (not shown). For amino acid sequence determination, the inhibitors in peaks 25, 26 and 27 (each of which contained a minor band) were further purified as described in Materials and Methods. WRP24, WRP25, WRP26 and WRP27 were essentially homogeneous with relative mobilities of 0.17, 0.24, 0.24 and 0.06, respectively, in non-denaturing gel electrophoresis.

Sedimentation coefficients of WRP24, WRP25 and WRP27

Whereas the polypeptide masses of these preparations, as estimated by SDS–PAGE, were all about 14 kDa, the sedimentation coefficients were 2.5 S, 1.7 S and 1.7 S for WRP24, WRP25 and WRP27, respectively, at a concentration of approx. 0.5 mg/ml in 0.020 M Tris–Cl, pH 8.0,

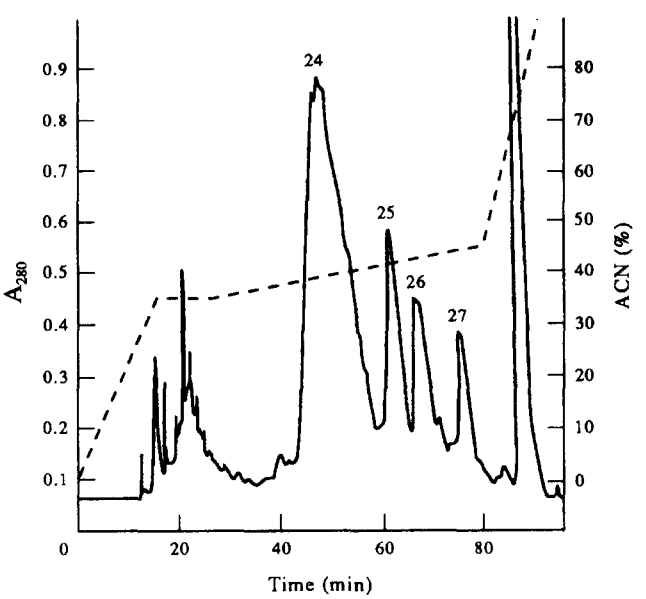

FIGURE 1. Preparative reversed-phase HPLC of wheat albumin/globulin fraction AS 1.0–1.5. The separation was carried out in a Vydac 218 TP10 (2.2 × 25 cm) preparative column with flow rate 8 ml/min. HFBA (0.1%) was used as ion-pairing agent and the dashed line indicates the acetonitrile gradient. 40 ml of wheat albumin fraction AS 1.0–1.5 (A_{280} = 3.9) was loaded.

0.15 M NaCl. Thus, WRP24's sedimentation behavior was consistent with its being larger under nondenaturing conditions than either WRP25 or WRP27.

Amino acid sequences of WRP24, WRP25, WRP26 and WRP27

We determined the complete amino acid sequences of WRP25, WRP26 and WRP27, as well as the sequence of the N-terminal 55 residues of WRP24. In Fig. 2 we compare these sequences with the previously determined sequences of the 0.28, 0.19 and 0.53 α-amylase inhibitors obtained from wheat kernels (Kashlan and Richardson, 1981; Maeda *et al.*, 1983, 1985). The 0.19 and 0.53 inhibitors are closely related, dimeric proteins, whereas the 0.28 inhibitor is monomeric. The N-terminal sequence of WRP24 exactly matched that of the wheat 0.19 inhibitor (Fig. 2). The sequences of WRP25 and WRP26 differed from each other at only three positions and were very similar to the sequence of the wheat 0.28 inhibitor. WRP27 was clearly distinct from previously reported sequences. Of the inhibitors of known sequence, WPR27 most closely resembled the 0.28 inhibitor.

TABLE 1. Sequence similarities (measured as % positional identity) of α-amylase inhibitors from wheat

Inhibitors	0.28	WRP25	WRP26	WRP27	0.53	0.19
0.28		97	98	74	64	62
WRP25			98	75	65	63
WRP26				75	64	62
WRP27					56	56
0.53						94

Pairwise similarities, as reflected by per cent identities, of several sequences of α-amylase inhibitors from wheat are summarized in Table 1.

Selectivities of the purified inhibitors

The inhibitory properties of WRP24, WRP25, WRP26 and WRP27 towards human salivary α-amylase and several insect α-amylases were assessed *in vitro* as shown in Fig. 3. In these assays, WRP25 and WRP26 had virtually the same selectivity; each of these proteins inhibited each of the insect α-amylases, but not human

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                                    20                                  40
 0.28   S G P W S W C N P A T G Y K V S A L T G C R A M V K L Q C V G S Q V P E A V L R
WRP25   . . . . . . . D . . . . . . . . . . . . . . . . . . . . . K . . . . . . . . .
WRP26   . . . . M . . D . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
WRP27   . . . . M . . D . . M . H R . R P . M . . . . . . . . . . . N . . . . . I Q .
 0.53   . . . . M - . Y . G Q A F Q . P . . P . . . P L L . . . . N . . . . . . . . .
 0.19   . . . . M - . Y . G Q A F Q . P . . P A . . P L L R . . . N . . . . . . . . .
WRP24   . . . . M - . Y . G Q A F Q . P . . P A . . P L L R . . . N . . . . . . . . .

                                    60                                  80
 0.28   D C C Q Q L A D I N N E W C R C G D L S S M L R A V Y Q E L G V R E G - - - K E
WRP25   . . . . . . . . . . . . . . . . . . . . . . . . S . . . . . . . . . . - - - . .
WRP26   . . . . . . . . . . . . . . . . . . . . . S . . S . . . . . . . . . . - - - . .
WRP27   . . . . E . . N . T . N . . . . H . . G . . . N S . . . . . . A . . . - - - T .
 0.53   . . . . . . . . . S - . . P . . . A . Y . . . D S M . K . H . . S . . Q A G T G
 0.19   . . . . . . . H . S - . . . . . . A . Y . . . D S M . K . H . A Q . . Q A G T G
WRP24   . . . . ? . . H . S - . . . . . .

                                   100                                 120
 0.28   V L P G C R K E V M K L T A A S V P E V C K V P I - P N P S G D R A G V C Y G D
WRP25   . . . . . . . . . . . . . . . . . . . . . . . . . . . - . . . . . . . . . . .
WRP26   . . . . . . . . . . . . . . . . . . . . . . . . . . . - . . . . . . . . . . - -
WRP27   . F . . . . . . . . . . . V . . . . A . . . . . . . - . . L Q R G G E . . . . >
 0.53   A F . S . . R . . V . . . . . . I T A . . R L . . V V D A . . . G . Y . . - K .
 0.19   A F . R . . R . . V . . . . . . I T A . . R L . . V V D A . . . G . Y . . - K .

                    127
 0.28   W A A Y P D V >
WRP25   . . . . . . A >
WRP26   . . . . . . >
 0.53   V . . . . . A >
 0.19   V . . . . . A >
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FIGURE 2. Amino acid sequence comparison of WRP24, WRP25, WRP26 and WRP27 with the sequences of wheat 0.28 inhibitor (Kashlan and Richardson, 1981) and the wheat 0.19 and 0.53 inhibitors (Maeda *et al.*, 1983, 1985). (---) indicates a gap; (.) indicates an amino acid residue that is identical to that in the 0.28 inhibitor.

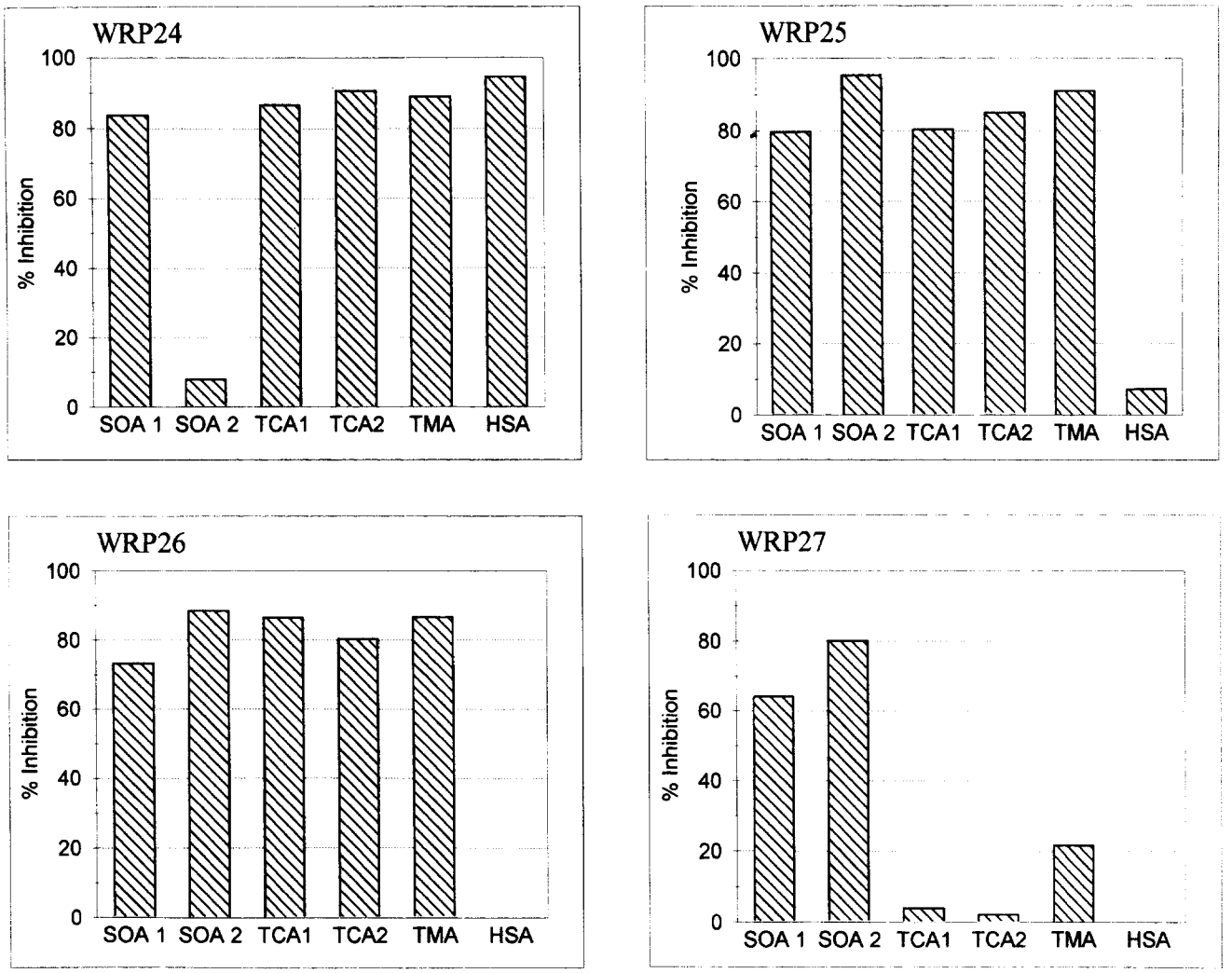

FIGURE 3. Activities of several reversed-phase HPLC fractions towards insect α-amylases and human salivary α-amylase. SOA1 and SOA2 are α-amylases 1 and 2 from rice weevil; TCA1 and TCA2 are α-amylases 1 and 2 from *Tribolium castaneum*; TMA is *Tenebrio molitor* α-amylase; and HSA is human salivary α-amylase.

salivary α-amylase. WRP24 and WRP27 were very different in selectivity from those inhibitors and from each other as well. WRP24 inhibited all of the test enzymes except rice weevil α-amylase 2. It thus discriminated between the two rice weevil amylases. WRP27 was notable in that it strongly inhibited only the two rice weevil α-isoamylases. Shainkin and Birk (1970) previously reported on two wheat inhibitors that differ markedly in selectivity towards human and *Tenebrio* α-amylases.

Inhibition of the growth of the red flour beetle by WRP24

The effect of WRP24 mixed with a diet of starch, wheat gluten, cellulose, yeast and salts on the growth of *T. castaneum* larvae was determined. Mean weights of larvae reared on the diet supplemented with the inhibitor were substantially lower than those reared on the control diet (Fig. 4). For example, 10% WRP24 suppressed larval growth by more than four-fold after 24 days, the time when the control larvae had finished feeding and were beginning to pupate.

In a separate experiment we tested the effect of a 10% supplemental level of wheat gluten (thus, at a level of 17% since the control diet contained 7% wheat gluten). We observed no difference in larval growth rate compared to that in the control diet (results not shown). This result indicated that the growth inhibition observed with diets supplemented with WRP24 was caused by WRP24 and not by a difference in total dietary nitrogen between the control diet and the WRP24-containing diet.

We also tested WRP24 for an effect on the weight of *T. castaneum* adults by mixing it with a diet of corn starch. When maintained on corn starch alone for one month, red flour beetles lost about 11% of their body weight (Table 2). When a wheat albumin/globulin fraction (AS 0–1.8) was added to the diet (at 10% wt/wt), a 7% weight loss occurred. However, when purified WRP24 (10% wt/wt) was mixed with corn starch, the beetles lost 1.6 times more weight than was observed with beetles fed corn starch alone and 2.8 times more weight than beetles fed the corn starch supplemented with the wheat albumin/globulin fraction. In this experiment, the diet supplemented with the wheat albumin/

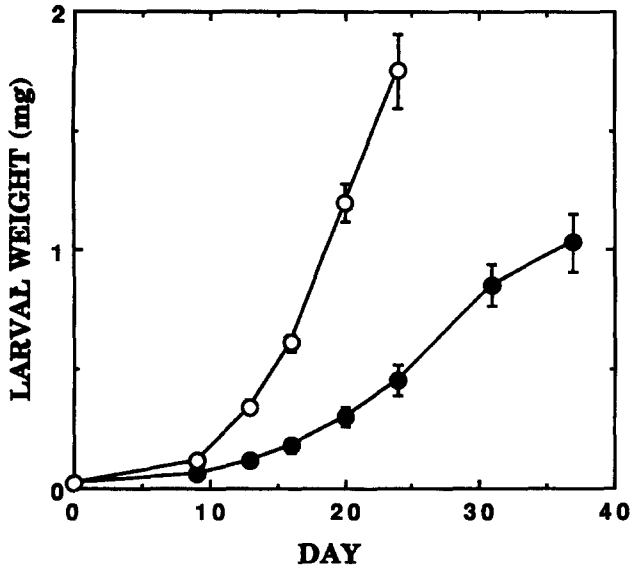

FIGURE 4. Growth curve of *T. castaneum* fed diets containing WRP24 (○) or a control diet (●). Means ± SEM ($n = 9$–10). The growth rate of larvae fed the WRP24 diet was significantly different ($\alpha < 0.01$) from that of larvae fed the control diet as determined by the independent *t*-test on individual regression coefficients of larval weights between days 9 and 24.

TABLE 2. Effect of a wheat albumin/globulin fraction and α-amylase inhibitor WRP24 on weight loss of adults of the red flour beetle, *T. castaneum*[*]

Treatment	Weight loss (%)[†]
Control	11.1 ± 2.8[a]
Wheat albumin fraction	6.5 ± 1.1[a]
WRP24	17.7 ± 2.8[b]

[*]Newly eclosed adults were weighed, placed in 20 mg corn starch with or without test material (10% wt/wt), and weighed again after 29 days. The wheat albumin fraction was prepared by extraction with 0.15 M NaCl and precipitation with 1.8 M ammoniun sulfate. WRP24 is a wheat α-amylase inhibitor purified by reversed-phase HPLC as described in the text.

[†]Means ± SEM with the same letter were not significantly different ($\alpha \leq 0.05$; $n = 8$–10; ANOVA: Duncan's multiple range test).

globulin fraction served as an isonitrogenous control for the diet supplemented with purified WRP24.

DISCUSSION

Wheat flour contains a complex mixture of polypeptides with molecular masses of 13–14 kDa, many of which individually inhibit α-amylases or are components of oligomeric proteins that inhibit α-amylases (Silano *et al.*, 1975; Silano, 1987). We have previously reported that reversed-phase HPLC is very useful for fractionating these polypeptides (Feng *et al.*, 1991a) and the corresponding set of polypeptides in rice flour as well (Feng *et al.*, 1991b). In this paper, we used preparative reversed-phase HPLC as the main step in purifying relatively large quantities of several inhibitors from wheat flour for detailed structural studies, particularly the determination of their amino acid sequences, and also for insect feeding studies.

Genes that encode α-amylase inhibitors may prove useful as part of a multi-gene strategy to genetically engineer increased resistance of plants to attack by insects (Silano, 1987; Garcia-Olmedo *et al.*, 1987; Richardson, 1991). The recent report of Shade *et al.* (1994) indicates that a gene that encodes an α-amylase inhibitor from the common bean is capable of enhancing the resistance of transgenic pea seeds to bruchid beetles. Anticipating that insects will in turn develop resistance to increased levels or different types of inhibitors in transgenic plants, it is important that we learn to correlate the structures of α-amylase inhibitors with their selectivities as a basis for altering inhibitors' activities in response to adaptations by insects. The work reported here is a step in that direction and provides data that should eventually contribute to understanding the relationship between structure and inhibitor activity, at least for inhibitors in the cereal superfamily.

We have determined the amino acid sequences and the inhibitory properties of four proteins, WRP24, WRP25, WRP26 and WRP27, from wheat flour. WRP24 is the most abundant inhibitor obtained by reversed-phase HPLC of extracts from wheat flour, and it appears to be identical to the protein previously characterized as the 0.19 inhibitor (Silano, 1987). The 0.19 inhibitor occurs as a dimer, and our sedimentation coefficient of 2.5 S for WRP24 is consistent with a dimeric architecture.

The sequences of WRP25 and WRP26 differ from each other at only 2 positions and are very similar to the previously reported sequence of the 0.28 inhibitor (Kashlan and Richardson, 1981). They can, then, be considered variants of the 0.28 inhibitor, which is a monomeric protein. The sedimentation coefficients of WRP25 (1.7 S) is consistent with its being monomeric. It may be that reversed-phase HPLC provided resolution of previously unrecognized microheterogeneity within the 0.28 inhibitor preparations.

WRP27 does not appear to correspond directly to any previously reported protein. Among other known inhibitors from wheat, WRP27 is most similar in sequence to the 0.28 inhibitor (74% positional identity). At least provisionally, we consider it to be a member of the 0.28-inhibitor group. Its sedimentation coefficient (1.7 S) indicates that it is a monomeric protein.

Our feeding studies with a diet supplemented with purified WRP24 are some of the few such studies conducted with purified inhibitors of α-amylases (for another, see Gatehouse *et al.*, 1986). WRP24 slowed the growth of *T. castaneum* larvae and caused a substantial weight loss in adults. Since WRP24 inhibits both major α-amylases of *T. castaneum in vitro*, it is possible that its effect *in vivo* is achieved by causing incomplete digestion of dietary starch. Howewever, there is no evidence for this mode of action other than Baker's finding that weevils feeding on cereals with high levels of α-amylase inhibitors have reduced levels of α-amylase compared to those

feeding on cereals with low levels of inhibitors (Baker, 1988b).

It is interesting to note that our wheat albumin/globulin fraction (AS 0–1.8), which would contain WRP24 and other inhibitors, did not cause significant weight loss on adults of the red flour beetle, whereas WRP24 alone did. The wheat albumin/globulin fraction is a complex mixture of proteins (Feng, 1990; Feng *et al.*, 1991a), about two-thirds of which have been estimated to be inhibitors of α-amylases (Petrucci *et al.*, 1974). We interpret the results of the feeding studies to indicate that A0–1.8 supplied not only inhibitors to the flour beetle adults, but also nutritional, dietary protein. In contrast, the purified inhibitor WRP24 may act principally through an inhibitory effect on the digestion of starch, even though WRP24 may also be a nutritional protein. The growth-inhibitory activity of WRP24 supports the hypothesis that this protein could provide a selective advantage to the wheat plant by slowing the attack of stored product insect pests on wheat seeds.

The interactions of α-amylase inhibitors with α-amylases are not well understood at this point. We have very little insight, for instance, into what portions of the amino acid sequences of an inhibitor establish contact with α-amylase. The goal of studying chemical structure and biological reactivity among related inhibitors, such as members of the cereal superfamily, would be to obtain correlations between variations in the inhibitors' structures and their selectivities towards various α-amylases. The current study contributes to what might become a database of such structure–activity information. From our data alone, however, major insights into the correlation between sequence and selectivity cannot be made. We can conclude, however, that the very few differences in the sequences of WRP25 and WRP26 do not appear to influence selectivity, at least against the α-amylases used in our work and as judged by our assays of the proteins' inhibitory properties. The selectivities of WRP24 and WRP27 clearly differ substantially, both from each other and from those of WRP25 and WRP26, but the differences in sequence among these proteins are too great to allow us to attribute the observed differences in selectivity to any particular differences in amino acid sequence. Our studies support the hypothesis that the proteins studied here, which have all evolved from a common ancestral protein, have acquired protective functions against insect predators and are components of a plant's defense against insect attack. As more information is added to the incipient database, hypotheses will no doubt emerge with regard to the relationship of structure to selectivity in the α-amylase inhibitors that belong to the cereal superfamily of proteins.

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