A dimeric inhibitor or insect α-amylase from barley

Cloning of the cDNA and identification of the protein

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A cDNA clone, designated pUP-44, whose longest open reading frame codes for a protein that is homologous to the wheat α-amylase inhibitors, has been isolated from a library obtained from developing barley endosperm. The deduced sequence for the mature protein, which is 122 residues long, is preceded by a sequence of 30 residues which has the typical features of a signal peptide. A closely corresponding protein, designated BDAI-1, has been isolated from mature endosperm. BDAI-1 behaves as a dimer and inhibits the α-amylase from the insect *Tenebrio molitor* at concentrations that have no effect on salivary or pancreatic α-amylases.

Proteinaceous inhibitors of proteinases and α-amylases are widespread in the plant kingdom, where quite a number of different types have been identified (see [1]). Although their possible physiological functions in the plant remain obscure, several lines of evidence indicate that possibly a majority of them, which are active against heterologous enzymes, play a protective role against the attacks of animal predators, insects, fungi, bacteria and viruses. This circumstance justifies the current increased interest in these molecules as potential protective agents which are susceptible to genetic manipulation by recombinant-DNA techniques.

A protein family that includes inhibitors of heterologous α-amylases and of trypsin is among those that have been more actively studied [1 – 3]. Inhibitors of heterologous α-amylases were first described in wheat endosperm by Kneen and Sandstedt [4]. Fractionation of crude inhibitor preparations from wheat allowed the identification of monomeric, dimeric and tetrameric types, whose subunits are encoded by a single multi-gene family which is dispersed over several chromosomes [5 – 10]. Evidence for the three size-classes of α-amylase inhibitors in barley has been presented recently and only the tetrameric inhibitor has been studied in some detail [9]. Two trypsin inhibitors from barley have been also shown to be members of the same protein family [11 – 13]. We report here the characterization of a cDNA clone from barley and of a closely corresponding protein which has the expected properties of a dimeric inhibitor and is active against insect α-amylases.

MATERIALS AND METHODS

Plant material

Hordeum vulgare cv. Bomi was the gift of H. Doll (Riso National Laboratory, Denmark). Developing endosperms were collected at approximately 20 days post-anthesis by mechanical extrusion into liquid nitrogen [14].

Reagents

Oligo(dT)-cellulose was obtained from Collaborative Research. AMV reverse transcriptase was from Life Sciences Inc. Terminal transferase and S_1 nuclease were obtained from P-L Biochemicals. Restriction endonucleases and other enzymes were supplied by Boehringer or Amersham. [^{35}S]Cysteine, [α-^{32}P]dATP and [γ-^{32}P]ATP were from Amersham.

Construction and screening of a cDNA library from developing barley endosperm

A cDNA library was constructed from total RNA extracted from barley endosperm at about 20 days after anthesis, according to published procedures [15, 16]. A collection of about 10^4 independent colonies of *Escherichia coli* Mc1061, harbouring pBR322 plasmids with cDNA inserts at the *Pst* site, were identified by screening for ampicillin sensitivity and tetracycline resistance (Amp^s Tet^r). These colonies were further screened by hybridization with a radioactive single-stranded cDNA (ss-cDNA) probe synthesized with AMV reverse transcriptase, using as template a fraction of poly(A)-rich RNA enriched for mRNAs encoding this protein family, which was obtained by density-gradient ultracentrifugation [15 – 17]. Clones resulting from this preliminary screening were subjected to two rounds of hybrid-release translation: first in pools of five clones and then as individual clones [16]. The method of Maxam and Gilbert [18] was used for DNA sequencing of appropriate subclones in plasmids pUC-12 or pUC-13 [19].

Protein purification and characterization

A crude inhibitor preparation was obtained as previously described [9]: ground endosperm from *Hordeum vulgare* cv. Bomi was extracted with 150 mM NaCl, and the extract was precipitated with 50% saturated $(NH_4)_2SO_4$, suspended in

water, dialyzed and lyophilized. Preparative gel filtration under non-dissociating conditions was carried out on a Sephadex G-100 (85 × 2.5-cm column) using 100 mM ammonium acetate (pH 6.8) as elution buffer. Appropriate aliquots of the eluted fractions were heated for 40 min at 60 °C to inactivate endogenous amylases and assayed for their inhibitory activities against α-amylases from the larvae of the insect *Tenebrio molitor*.

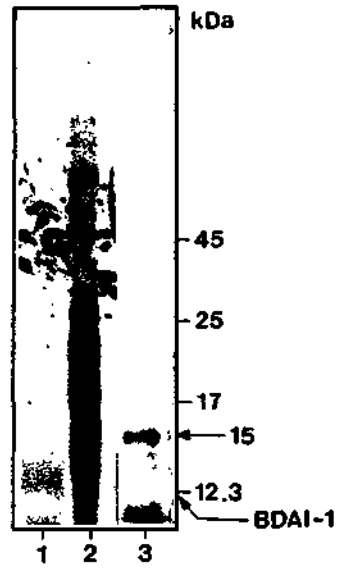

Fig. 1. *Translation of mRNA selected by DNA of clone pUP-44.* SDS-PAGE and fluorography of translation products obtained in the wheat-germ system as in [17] (1), no RNA; (2) poly(A)-rich RNA; (3) mRNA selected by DNA of clone pUP-44. [^{35}S]Cysteine was used as labelled amino acid. Reference proteins were ovalbumin (45 kDa), chymotrypsinogen (25 kDa), myoglobin (17 kDa), and cytochrome *c* (12.3 kDa)

Isolation of the barley dimeric α-amylase inhibitor (BDAI-1) was performed by preparative HPLC of the Sephadex G-100 dimeric inhibitors fraction (Nucleosil 300-5 C4, particle size 5 µm, 250 × 8-mm column; elution with a linear gradient 10–85% acetonitrile in 0.1% trifluoroacetic acid, at a flow of 0.5 ml/min). Association of BDAI-1 protein was analyzed by HPLC gel filtration on Spherogel TSK-G 300 SWG (50 µg purified protein inserted; elution with 0.1 M sodium phosphate buffer, pH 7.0; flow of 0.5 ml/min, 300 × 21.5-mm column), using as standards a monomeric and a dimeric inhibitor from wheat endosperm, isolated as in [8].

SDS-PAGE gel electrophoresis was performed according to Laemmli [20], and two-dimensional electrophoresis and isoelectrofocusing (pH 4–9) starch gel electrophoresis (pH 3.2) were carried out as in [9].

Inhibition of α-amylases was tested as in a previous report [13]. Hydrolysis and performic acid oxidation of purified BDA-1 protein were carried out as described [21, 22]. Amino acid analyses were carried out on a HPLC Beckman system.

RESULTS

Characterization of a cDNA clone encoding a putative α-amylase inhibitor

Following a previously described strategy [16] a cDNA clone (pUP-44) corresponding to a member of the family of trypsin/α-amylase inhibitors from barley endosperm was identified by hybrid-selected translation as shown in Fig. 1. DNA from this clone selected a mRNA that encoded a protein

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                                                                    32
                                        TA ATC AAA AAT AAC CGA TAA TAT TTC TTA AGC

                                                                    92
CGA ATA TCC AGC TTT GAA GAT AAG CAT ATA AGC AAC TGA TTA AGA TAG TAG TAG ATG GGA

                                                                   152
ATG GGA GCA ATG TGG ATG AAG AGC ATG TTG TTG GTG CTC CTG CTA TGC ATG CTC ATG GTG
Met Gly Ala Met Trp Met Lys Ser Met Leu Leu Val Leu Leu Leu Cys Met Leu Met Val
1  LEADER
                                                                   212
ACT CCA ATG ACG GGC GCA CGG AGT GAT AAC AGT GGC CCT TGG ATG TGG TGT GAT CCT GAG
Thr Pro Met Thr Gly Ala Arg Ser Asp Asn Ser Gly Pro Trp Met Trp Cys Asp Pro Glu
21                                     MATURE PROTEIN
                                                                   272
ATG GGC CAC AAG GTG AGC CCG CTC ACG CGC TGC CGA GCA TTG GTG AAG CTC GAG TGT GTG
Met Gly His Lys Val Ser Pro Leu Thr Arg Cys Arg Ala Leu Val Lys Leu Glu Cys Val
41
                                                                   332
GGC AAC CGG GTG CCC GAG GAT GTC CTA AGA GAC TGC TGC CAG GAG GTG GCC AAC ATC AGC
Gly Asn Arg Val Pro Glu Asp Val Leu Arg Asp Cys Cys Gln Glu Val Ala Asn Ile Ser
61
                                                                   392
AAC GAG TGG TGC AGG TGC GGC GAC CTC GGC AGC ATG CTG CGC AGC GTG TAT GCG GCG CTC
Asn Glu Trp Cys Arg Cys Gly Asp Leu Gly Ser Met Leu Arg Ser Val Tyr Ala Ala Leu
81
                                                                   452
GGC GTG GGG GGC GGA CCG GAG GAG GTG TTC CCG GGT TGC CAG AAG GAT GTG ATG AAG CTC
Gly Val Gly Gly Gly Pro Glu Glu Val Phe Pro Gly Cys Gln Lys Asp Val Met Lys Leu
101
                                                                   512
TTG GTT GCA GGC GTG CCT GCG CTC TGC AAT GTC CCC ATT CCC AAC GAG GCG GCA GGC ACC
Leu Val Ala Gly Val Pro Ala Leu Cys Asn Val Pro Ile Pro Asn Glu Ala Ala Gly Thr
121
                                                                   572
AGA GGG GTC TGC TAC TGG TCA GCG TCT ACG GAC ACC TAG TCA AGC GAT TAT TAT GCT TTT
Arg Gly Val Cys Tyr Trp Ser Ala Ser Thr Asp Thr ter
141
                                                                   632
TTA ATA ATT TGT GTG GTG TGT GTG TGC GCG CAC AAG ATG AAT AAA ATG AAA TAA TGG TGT
                                                    Poly A
                                        661
ATC TGC CTA TGC GAC GCA TGC GTC ATG TA
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Fig. 2. *Nucleotide sequence of the cDNA insert in clone pUP-44 and deduced amino acid sequence of the longest open reading frame.* N terminals of the leader sequence and of the mature protein, as well as the polyadenylation signal (Poly A) are indicated

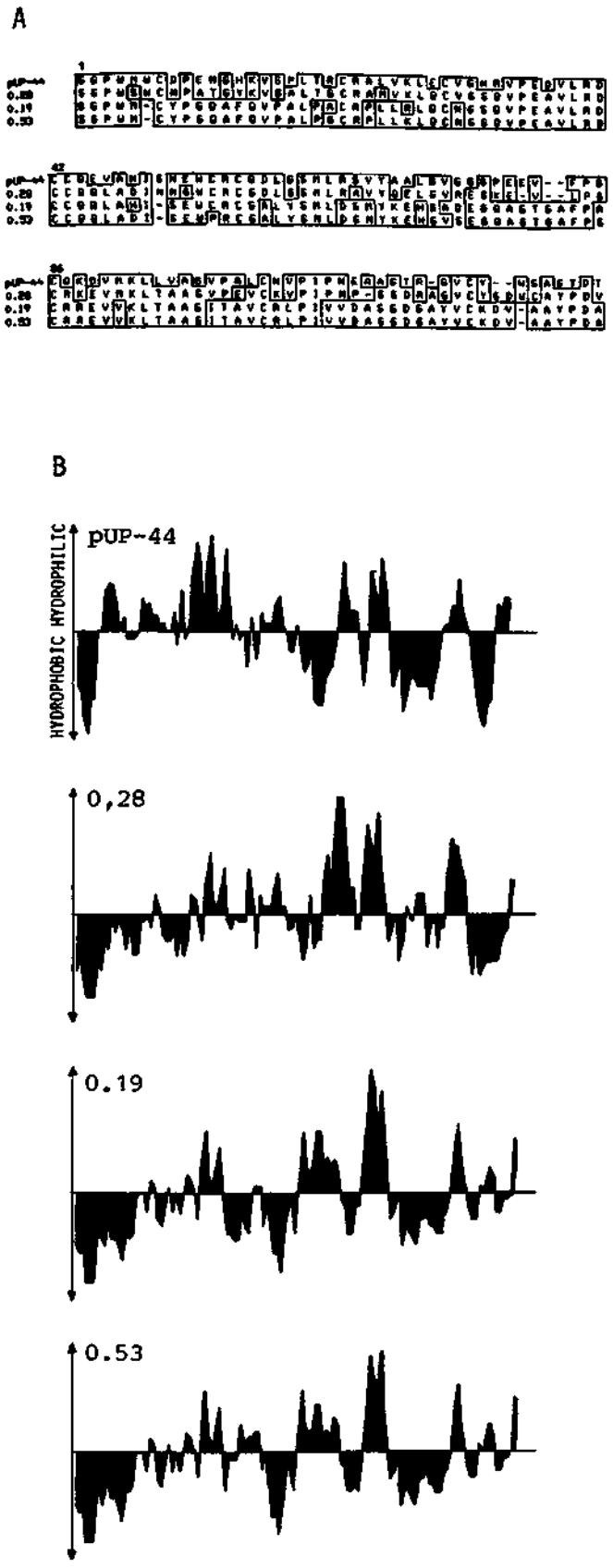

Fig. 3. *(A) Alignment of the sequence deduced from clone pUP-44 with those of the 0.28 monomeric inhibitor [6] and the 0.19 and 0.53 dimeric inhibitors [7] from wheat. (B) Comparison of the calculated hydropathy profiles of the four sequences (Microgenie program, Beckman)*

Table 1. *Comparison of the amino acid analysis of protein BDAI-1 with the amino acid composition predicted for the mature protein encoded by the insert in clone pUP-44*
The index of relative compositional differences (RCD) for the binary comparison is that of Cornish-Bowden [25] as modified in [26]. Trp was not analyzed and not included in the RCD calculation

Amino acid	BDAI-1		pUP-44
	experimental	nearest integer	
Lys	5.6	6	4
His	1.3	1	1
Arg	6.5	7	7
Asx	10.6	11	11
Thr	4.5	5	4
Ser	6.1	6	7
Glx	12.0	12	10
Pro	9.2	9	9
Gly	12.4	12	13
Ala	10.6	11	9
Val	11.0	11	14
$^1/_2$ Cys	10.8	11	10
Met	1.9	2	4
Ile	2.4	2	2
Leu	9.3	9	10
Tyr	2.4	2	2
Phe	1.4	1	1
Trp			4
RCD		0.30	

Table 2. *Inhibitory activity of BDAI-1 against α-amylases from different sources*
A crude mixture of barley dimeric inhibitors, obtained by gel filtration as indicated in Fig. 4, and the wheat dimeric 0.19 inhibitor were used for comparison

Inhibitor	Amount	Inhibition of α-amylase		
		insect	salivary	pancreatic
	µg	%		
BDAI-1	5	21	0	0
	10	35	0	0
Barley mix	25	93	80	–
Wheat 0.19	5	76	95	–

which was efficiently labeled with [^{35}S]cysteine, had an apparent M_r within the range observed for the precursors of these inhibitors [17], and gave a weak antigenic reaction with antibodies raised against a mixture of several components of this protein family (not shown). The longest open reading frame in the nucleotide sequence of the insert in clone pUP-44 coded for a protein whose N-terminal sequence had the typical features of a signal peptide and was followed by the sequence of a putative mature protein which was clearly homologous to previously described members of the family of trypsin/α-amylase inhibitors from cereal endosperm (Fig. 2). There were several Met codons upstream from the sequence encoding the hydrophobic core of the leader peptide, the second of these codons was compatible with the rules deduced by Kozak [23] and was therefore the most likely initiator. Four potential cleavage sites were identified for the leader sequence following

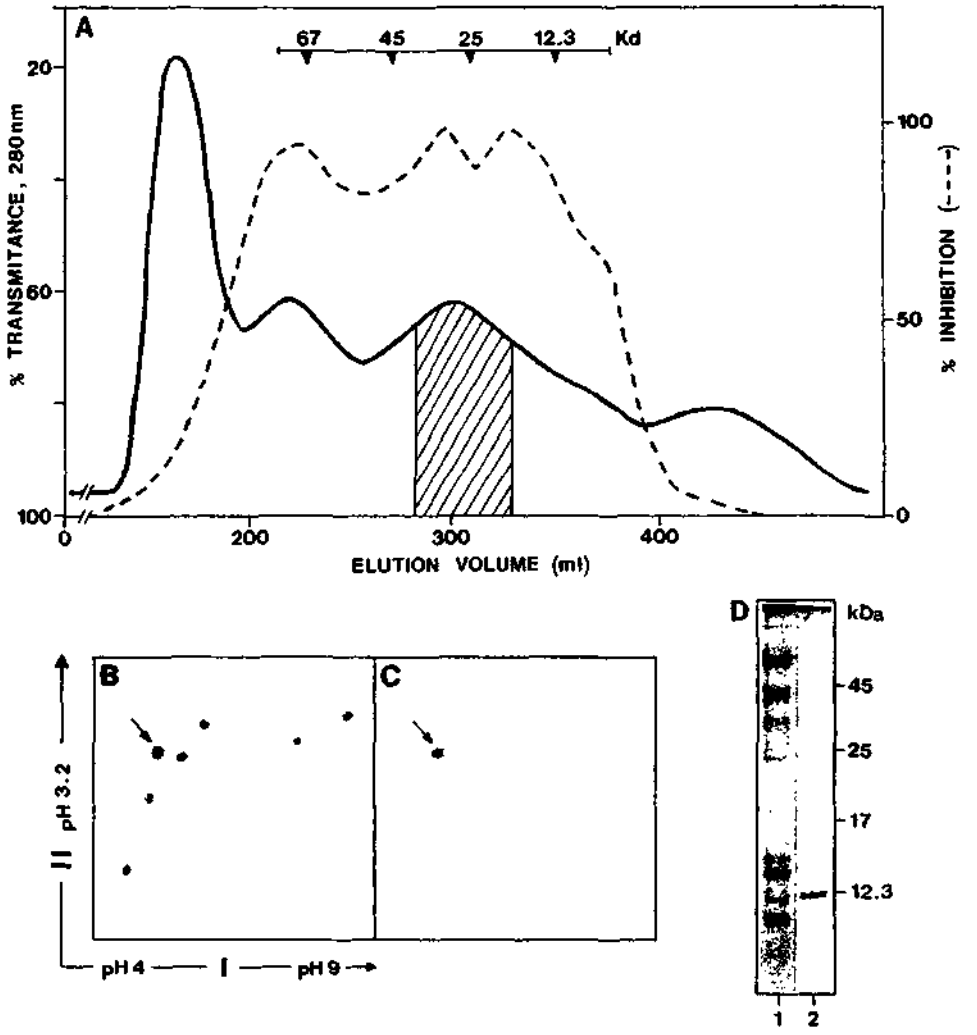

Fig. 4. *Purification of protein BDAI-1.* (A) Gel filtration on Sephadex G-100 of a crude inhibitor preparation obtained by precipitation with 50% $(NH_4)_2SO_4$ of a 150 mM NaCl extract from mature endosperm of barley cv. Bomi. The crude inhibitor (450 mg) was applied to a 85 × 2.5-cm column and eluted with 100 mM ammonium acetate, pH 6.8, at 25 ml/h. Bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), and cytochrome *c* (12.3 kDa) were used to calibrate the column. The fraction indicated by shaded area was used for subsequent purification steps. (B, C) Two-dimensional electrophoresis of crude inhibitor preparation and of purified BDAI-1. (D) SDS-PAGE of (1) crude inhibitor preparation and (2) purified BDAI-1. Reference proteins as in Fig. 1

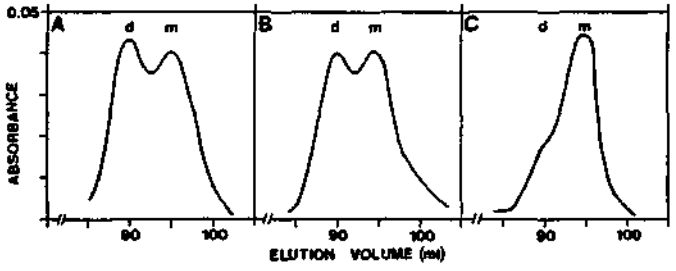

Fig. 5. *Self-association of protein BDAI-1.* HPLC molecular filtration on a Spherogel TSK-G 300 SWG column of the following samples (50 µg each): (A) Protein BDAI-1; (B) dimeric wheat inhibitor [9]; (C) monomeric wheat inhibitor [9]. Peaks correspond to (d) dimeric and (m) monomeric forms

the criteria of von Heijne [24]. One of them, albeit that with the lowest probability, would generate a mature protein with an N-terminal amino acid sequence which was identical to that of the monomeric and dimeric α-amylase inhibitors from wheat. The deduced amino acid sequence has been aligned with those of the two types of α-amylase inhibitors, and their hydropathy profiles have been compared in Fig. 3.

Isolation and characterization of a dimeric α-amylase inhibitor

In the course of a systematic charcterization of monomeric and dimeric α-amylase inhibitors from barley, a protein was isolated which closely corresponded to that encoded by clone pUP-44. This protein, which before full identification was labeled CMb′ [1], is now designated BDAI-1. A crude inhibitor preparation, obtained by salt extraction and $(NH_4)_2SO_4$ precipitation, was subjected to gel filtration as indicated in Fig. 4A. The gel-filtration, which approximately corresponded to the dimeric inhibitors, was subjected to HPLC on a reverse-phase column, yielding 12 major and several minor peaks (not shown). Amino acid analysis of the purified components of this fraction was carried out, and the composition of component BDAI-1, eluting at 32% acetonitrile, did not differ significantly from that deduced from clone pUP-44, as their compositional divergence index was not higher

Table 3. *Binary comparisons (homology) of the protein corresponding to clone pUP-44 with members of the cereal α-amylase/trypsin inhibitor family*

Species	Proteins	Ref.	pUP-44	0.53	0.19	0.28	CMe	RBI	MTI	pUP-13
				%						
Barley	pUP-44			48	48	65	23	26	26	22
Wheat	0.53	[7]			94	58	28	29	30	22
	0.19	[7]				56	30	29	30	23
	0.28	[6]					25	30	29	23
Barley	CMe	[27]						56	51	36
Ragi	RBI	[28]							64	34
Maize	MTI	[29]								38
Barley	pUP-13	[16]								

than that expected for two purification batches of the same protein (Table 1). Protein BDAI-1 was found specifically to inhibit the α-amylase from the insect *Tenebrio molitor* (Table 2). A protein with identical characteristics to BDAI-1 has been isolated by preparative HPLC of the total salt extract from barley endosperm and found to have an N-terminal sequence which is identical to that deduced for BDAI-1 from the cDNA clone in at least 21 out of 23 positions sequenced (D. Barber and E. Mendez, unpublished results). The ability of BDAI-1 to form dimers was demonstrated by subjecting the purified protein to gel filtration under non-dissociating conditions on a HPLC column, using wheat monomeric and dimeric inhibitors as controls (Fig. 5). In the case of proteins BDAI-1 and the wheat dimeric inhibitor, aliquots of the two peaks (m and d in Fig. 5) were analyzed by SDS-PAGE and the same protein band was observed in each case (not shown). On the other hand, when the crude inhibitor preparation was fractionated using non-dissociating conditions in the electrofocusing step of the two-dimensional electrophoretic procedure (i.e. 6 M urea omitted), no other component of the extract comigrated with BDAI-1 in the first dimension (not shown).

DISCUSSION

The product of *in vitro* synthesis encoded by the mRNA selected by the DNA of clone pUP-44 has an apparent molecular mass that is about 3 kDa greater than that of the putative mature protein (Figs 1 and 4), which is in agreement with previous evidence that members of the family of α-amylase/trypsin inhibitors are synthesized as precursors by membrane-bound polysomes [16, 17] and is consistent with the amino acid sequence deduced from the nucleotide sequence of the insert in clone pUP-44 (Fig. 2), where the mature protein is preceded by a typical 'leader' sequence of 30 amino acid residues.

The sequence deduced from clone pUP-44 encodes a protein that is more closely related to the wheat monomeric and dimeric α-amylase inhibitors than to the trypsin inhibitors from barley and maize or to the bifunctional inhibitor from ragi (Table 3).

Protein BDAI-1 is identical or very closely related to that encoded by clone pUP-44 on the basis of its amino acid composition and N-terminal sequence. The protein is recovered as a dimer after gel filtration on Sephadex G-100 of a crude inhibitor extract, where it does not seem to be associated with other components, as judged from our failure to detect such an association by a two-dimensional electrophoretic procedure that does detect the interaction between subunits of the tetrameric inhibitors [9]. The fact that BDAI-1 is able to self-associate, to an extent similar to the wheat 0.19 dimeric inhibitor, lends further support to the idea that it is a homodimer in its native state. Although the amino acid sequence of BDAI-1 is closer to that of the monomeric wheat inhibitor than to those of the dimeric ones (Table 3), secondary-structure predictions (not shown) and, especially, their hydropathy profiles (Fig. 3B) are quite divergent, and BDAI-1 has a C-terminal half which is quite hydrophobic and probably determines its self-association.

The insect α-amylase is inhibited by BDAI-1 at concentrations that have no effect on salivary or pancreatic α-amylases, whereas the gel-filtration fraction that corresponds to the dimeric inhibitors (Fig. 4) is active against both the insect and the human enzymes, which means that other dimeric inhibitors with specificities different from that of BDAI-1 must be present in this fraction. Isolation and characterization of these inhibitors is in progress.

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