Characterization of a bifunctional wheat inhibitor of endogenous α-amylase and subtilisin

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A bifunctional α-amylase/serine protease inhibitor which inhibits germination-specific cereal α-amylases of the Graminae subfamily Festucoideae as well as bacterial subtilisins has been isolated from wheat grains. This protein has M_r ~20500 and pI ~7.2. The amino acid composition and N-teminal sequence (45 residues) show that the inhibitor is homologous with cereal and leguminous inhibitors of the soybean trypsin inhibitor (Kunitz) family.

Wheat seed *Bifunctional α-amylase/subtilisin inhibitor* *N-terminal amino acid sequence* *Enzyme inhibitor homology* *Soybean inhibitor (Kunitz)*

1. INTRODUCTION

Preharvest germination is a major problem in the production of wheat, rye and triticale in temperate climates, mainly because the degradation of stored starch by α-amylase produced during germination adversely affects the baking quality of flour milled from the grain. The presence in wheat of inhibitors of endogenous α-amylase, which may be important in regulating this hydrolytic activity, has been suggested [1]. Although the major albumin fractions of wheat grains inhibit α-amylases, numerous studies have only resulted in isolation and characterization of inhibitors active against animal amylases [2–4]. However, an inhibitor from barley (BASI), which is specific for both endogenous α-amylase and for subtilisin, was recently characterized [5–8]. This paper describes how the use of antibodies raised towards this inhibitor facilitated the isolation of a protein with similar antigenic properties from wheat (WASI). Characterization of this protein in terms of amino acid composition, N-terminal sequence, and specificity of inhibition established that wheat grains contain a Kunitz-type protease inhibitor which is also a specific inhibitor of endogenous wheat α-amylase.

Abbreviations: BASI and WASI, barley and wheat α-amylase/subtilisin inhibitor, respectively; RPI, rice protease inhibitor; STI, soybean trypsin inhibitor (Kunitz); SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; 0.19 + 0.28, wheat inhibitors of animal α-amylases (named according to electrophoretic mobility) [4]

2. MATERIALS AND METHODS

2.1. *Materials*

Seeds of wheat (*Triticum aestivum*, cv. Solid) were decorticated in a carborundum dehuller (Schule), ground in a grits mill (Brabender quadrimat junior), sieved through a 600 μm screen, then ground in a laboratory hammer mill (Alpine), and sieved through a 220 μm screen. One kg of the resulting 50% flour was extracted with stirring for 20 h in 10 l of 50 mM Na-phosphate buffer (pH 6.0), 50 mM NaCl. After centrifugation the supernatant was made 60% with

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$(NH_4)_2SO_4$. The resulting precipitate was suspended and dialyzed in several changes of 30 mM phosphate, 15 mM citrate buffer (pH 5.2) and thereafter used as the starting material for purification of the inhibitor. The isolation of BASI and the preparation of cereal α-amylases by glycogen precipitation and heat treatment of extracts of germinated wheat, barley, rye, oats, sorghum, and rice were performed as in [6]. Rabbit antibodies were raised against BASI as in [9]. α-Amylase was purified from the larval midgut of *Tenebrio molitor* (yellow meal worm) as in [10]. Unless otherwise stated, all enzymes and synthetic enzyme substrates were from Sigma.

2.2. *Measurement of α-amylase and serine protease inhibition*

The inhibition of α-amylases by WASI, by BASI and by a commercial preparation of wheat seed α-amylase inhibitor (Sigma) were measured at pH 6.0 using reagents and assay conditions as in [6]. The activities of the different amylase preparations were equalized by dilution and approx. 2.0 μg of the inhibitors were used per assay. Specific amylase inhibition units were calculated as defined by [2].

Inhibition of serine proteases was measured at 25°C after 5 min preincubation of enzyme with inhibitor in 1 ml of 50 mM Tris–HCl (pH 8.0). Inhibition of subtilisins Carlsberg and BPN (Novo Industries), *Aspergillus* protease, and bovine chymotrypsin were measured at 337 nm with 0.75 mM *N*-acetyl-L-tyrosine ethyl ester (ATEE) as substrate; inhibition of bovine trypsin at 353 nm with 0.82 mM *N*-benzoyl-L-arginine ethyl ester (BAEE, Fluka) and of porcine pancreatic elastase (Boehringer Mannheim) at 405 nm with 0.1 mM *N*-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide. The concentration of active enzyme was approx. 20 nM and up to 5-fold equimolar concentrations of the inhibitors were used in the assays.

2.3. *Characterization methods*

The amino acid composition and N-terminal sequence of WASI were determined as described for BASI [6,7]. The procedures for SDS–PAGE, isoelectric focusing and crossed and fused rocket immunoelectrophoresis have been described [6,7,9].

3. RESULTS AND DISCUSSION

The first chromatographic step employed in the purification of the inhibitor was CM Sephadex cation exchange (fig.1A). The major peak (peak CM-1) eluting at 0.3 M NaCl in the gradient was shown by SDS–PAGE and inhibitor activity measurements to contain inhibitors of animal α-amylases of M_r ~13000–15500. When column fractions were analyzed by fused rocket immunoelectrophoresis using antibodies raised towards BASI (fig.2A), a cross-reacting protein was detected at 0.45 M NaCl in the gradient (peak CM-2) immediately after elution of the major basic peroxidases detected by absorbance at 403 nm (fig.1A). Fractions containing this protein were pooled, the buffer changed to 25 mM Tris–HCl (pH 9.3) in an Amicon concentrator fitted with a PM-10 membrane and this solution was then chromatographed on a 1.5 × 30 cm column of QAE–Sephadex equilibrated in the same buffer (not shown). After washing, 600 ml of a 0–0.4 M linear NaCl gradient was applied. A major peak eluting at 0.1 M NaCl was shown by SDS–PAGE to contain two proteins, the cross-reacting protein (M_r ~20500) and an unknown component (M_r ~30000). These two proteins were finally separated by chromatography on hydroxyapatite (fig.1B). Fused rocket immunoelectrophoresis showed only cross-reacting material in the first peak (HA 1) and only a single protein of M_r ~20500 was detected by SDS–PAGE. Isoelectric focusing followed by protein staining or specific staining for subtilisin inhibitory activity [6] showed only one zone at p*I* ~7.2. The single N-terminal amino acid sequence obtained upon analysis by automatic Edman degradation confirmed a high degree of purity.

The amino acid composition of the purified inhibitor (WASI) is compared with that of BASI in table 1. The N-terminal sequence (45 residues) of WASI is compared in fig.3 with those of BASI, RPI [11], and STI [12]. In the first 45 residues, WASI shows 96% homology with BASI, 75% homology with RPI, and 48% homology with STI.

The specificities of inhibition of α-amylases by WASI, BASI, and the commercial preparation of wheat seed α-amylase inhibitor are given in table 2. The latter preparation showed 3 major bands by SDS–PAGE with M_r ~13000, 14000 and 15500.

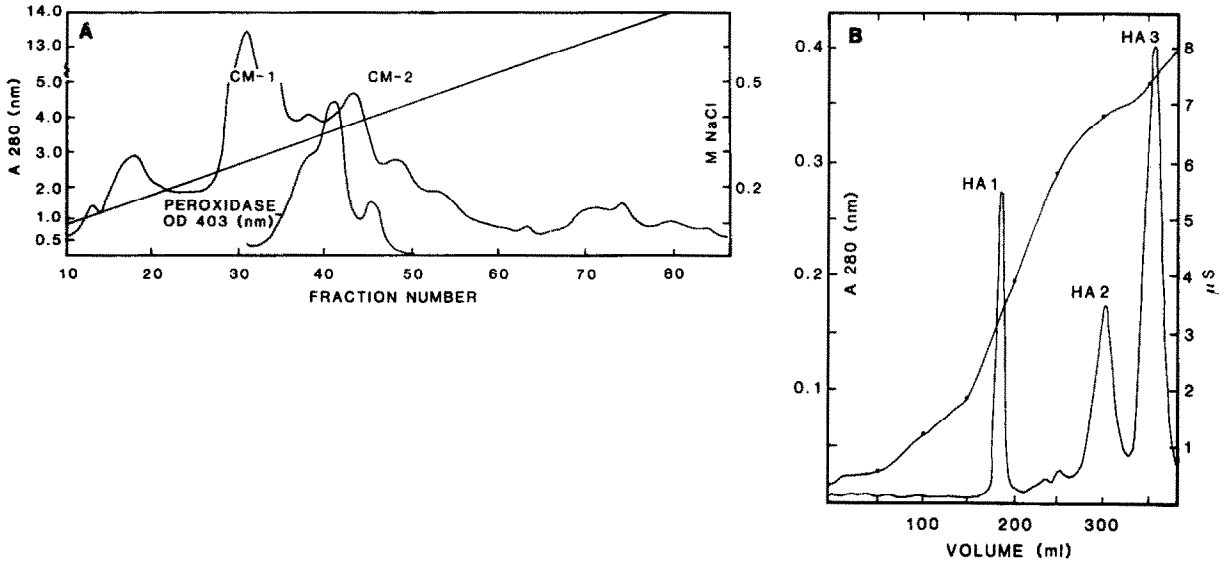

Fig.1. (A) CM-Sephadex C-50 cation exchange chromatography. A wheat protein extract concentrated by $(NH_4)_2SO_4$ precipitation was adsorbed to a 2.5 × 40 cm column of CM-Sephadex equilibrated in 30 mM phosphate, 15 mM citrate buffer (pH 5.2) and eluted with a linear gradient of NaCl (——); 15 ml fractions were collected. After fused rocket immunoelectrophoresis (fig.2A), fractions 43–53 (peak CM-2) were pooled for QAE–Sephadex chromatography. (B) Hydroxyapatite chromatography. Pooled fractions from QAE–Sephadex chromatography which contained WASI were adsorbed in 10 mM Na–phosphate buffer (pH 6.8) to a 1.3 × 25 cm column of Biogel HPT (Biorad) and eluted with a 10–100 mM linear gradient of the same buffer at a flow rate of 25 ml/h. 175–195 ml eluant (peak HA-1) was pooled and used as the WASI preparation. The conductivity of column fractions, expressed in μS, is shown (-----).

As this preparation inhibits both human salivary and *T. molitor* amylases, it presumably contains both the 0.19 and 0.28 inhibitors. WASI and BASI only inhibit α-amylases of the 4 cereals in the Graminae subfamily Festucoideae; they do not inhibit α-amylases of sorghum or rice, both members of the subfamily Panicoideae. Other experiments showed that the inhibition of α-amylase was not affected by the presence of equimolar amounts of subtilisin in the assay mixture. The inhibitor therefore seems to be 'double-headed' containing separate sites directed against α-amylases and subtilisins.

The results (table 2) indicate that WASI is a weaker inhibitor of cereal α-amylases than BASI. In isoelectric focusing experiments with BASI, formation of a stable complex with barley α-amylase

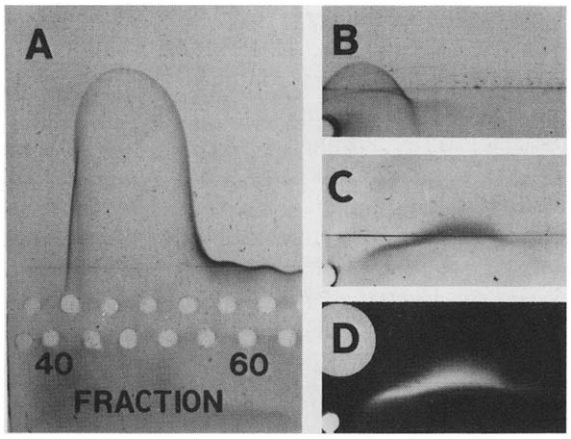

Fig.2. Immunoelectrophoretic characterization of WASI using monospecific antibodies towards BASI [6]. (A) Fused rocket immunoelectrophoresis of fractions from CM-Sephadex ion exchange (fig.1A). Every second fraction was applied (20 μl). (B) Crossed immunoelectrophoresis of wheat extract (20 μl). (C,D) Crossed immunoelectrophoresis of extract of 4-day germinated wheat (20 μl). Note the more extended and diffuse precipitate formed by WASI in the presence of α-amylase. Plates A–C were stained for protein with Coomassie blue. D was stained for amylase activity by a starch-iodine procedure [9].

Table 1

Amino acid composition of WASI and comparison with that of BASI [6]

	WASI	BASI
Asp	19.1	17.3
Thr	7.0	8.7
Ser	9.5	9.9
Glu	15.4	15.7
Pro	15.9	15.3
Met	2.0	2.2
Gly	20.6	20.7
Ala	17.1	16.8
Val	14.7	13.1
Ile	7.1	7.0
Leu	11.9	12.2
Tyr	6.0	6.6
Phe	6.7	7.1
His	7.7	9.4
Lys	6.2	6.8
Arg	13.9	11.9
½Cys	4.1	4.2
Trp	2.9	3.0
Total	187.8	187.9

The compositions are adjusted to M_r 20500 for both proteins

Table 2

Specificity of inhibition of α-amylases of plant, animal and microbial origin by WASI, BASI and a commercial preparation of wheat seed α-amylase inhibitors (0.19 + 0.28)

α-Amylase source	Specific inhibitory activity (IU/mg protein)		
	WASI	BASI	0.19 + 0.28 inhibitors
Wheat	105	480	0
Barley	50	420	0
Rye	70	540	0
Oats	130	720	0
Sorghum	0	0	0
Rice	0	0	0
T. molitor	0	0	760
Hog pancreas	0	0	210
Human saliva	0	0	855
A. oryzae	0	0	0
B. subtilis	0	0	0

0 indicates that inhibition was not measurable

2 could be demonstrated [6]. In similar experiments where WASI was preincubated with barley or wheat α-amylases, the effect of WASI on the isozyme patterns was only to cause a diffuse spreading of the zones, while no effect upon isozymes of the minor α-amylase 1 of wheat and barley (pI ~5.1) could be seen. Complex formation could, however, be demonstrated by amylase staining after crossed immunoelectrophoresis of mixtures of WASI and wheat amylase (wheat malt extract) using antibodies raised against BASI (fig.2B–D).

The specificity of inhibition of serine proteases by WASI was very similar to that of BASI [5,6]. Inhibition of both subtilisin Carlsberg and subtilisin BPN proceeded linearly to above 90% when measured at pH 8.0 with ATEE as substrate.

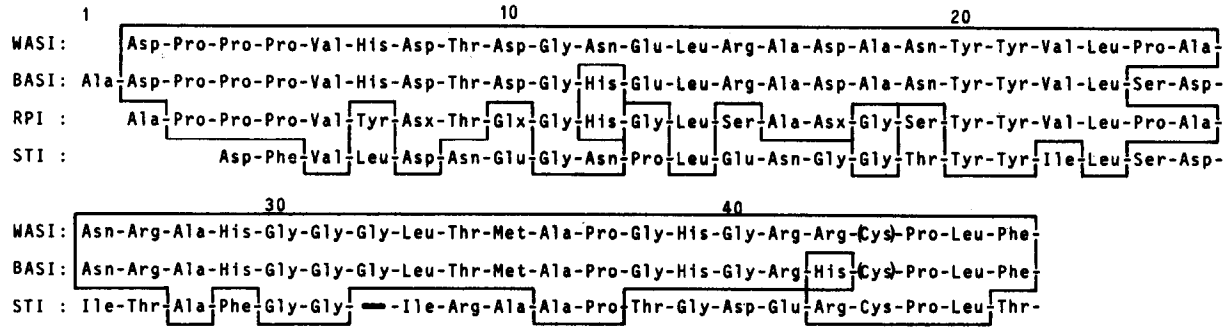

Fig.3. N-terminal amino acid sequence of WASI and comparison with N-terminal sequences of other Kunitz-type plant inhibitors [6,11,12]. (Cys) inferred, (—) deletion.

Under these conditions the inhibition of *Aspergillus oryzae* protease was very weak, and inhibition of porcine pancreatic elastase, bovine chymotrypsin and bovine trypsin was not measurable. Inhibition of subtilisin was not affected by preincubation of the inhibitor with equimolar amounts of barley or wheat malt α-amylase, with 0.1–1% soluble starch, or with mixtures thereof.

In conclusion, our results demonstrate that among the many α-amylase inhibitors of wheat grains [2–4], one protein specifically inhibits α-amylases from wheat and closely related cereals. This protein is also a strong inhibitor of an entirely unrelated enzyme, the bacterial protease subtilisin, and is thus the first bifunctional inhibitor to be isolated from wheat. The other sequenced α-amylase inhibitors of wheat, which are only active towards animal amylases, belong to another family of inhibitors [13]. Bifunctional amylase/protease inhibitors within this family have also been described, but not from wheat [14].

Kunitz-type inhibitors appear to be widespread within the plant kingdom and further studies may elucidate whether their inhibitory activity towards endogenous amylases is a property lost during the divergent evolution of lipid-storing species, such as soybean, or acquired by convergent evolution in some species with starch as the major seed storage material. It also remains to resolve the mechanism of α-amylase inhibition and the physiological importance of this process in cereals.

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