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Serpins from wheat grain

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Abstract

Wheat serpin genes have been identified by Southern blot hybridization with three distinct barley protein Z probes. Immunoblot analysis with a monoclonal antibody towards barley protein Z confirmed expression of related M_r ~40 kDa proteins in wheat grain. The wheat serpins were extracted under reducing conditions and separated from β-amylase and other seed proteins by thiophilic adsorption and anion-exchange chromatography. One molecular form possessing chymotrypsin inhibitory activity was isolated in a reactive site cleaved form on a chymotrypsin affinity column. N-terminal amino acid sequences of a CNBr fragment and of the C-terminal peptide from the cleaved inhibitor (M_r 4574 ± 4 Da) verified homology with barley protein Z and mammalian serpins. The native inhibitory serpin was demonstrated to form an SDS-stable complex with α-chymotrypsin.

Key words: Amino acid sequence; Barley protein Z; Chymotrypsin inhibitor; Complex formation; Serpin; *Triticum aestivum* L

1. Introduction

Serpins are members of a superfamily of proteins involved in regulation of complex physiological processes in mammals, i.e. blood coagulation, fibrinolysis and complement activation [1]. Most serpins are inhibitors of serine proteinases, while others with specific transport functions may interact as substrates. In contrast to the well-characterized low M_r protein inhibitors, the serpins undergo reversible and irreversible conformational changes regulating their activity. Thus, proteolytic cleavage in the reactive site loop results in a protein with new biochemical properties, including increased stability against degradation by proteinases and denaturation at high temperature [1].

Serpins, collectively termed protein Z, are also present in barley grains [2–6] but have not been described from other plants. Neither has a database search identified other peptide or nucleotide sequences of plant origin related to the serpin superfamily. Three barley genes encoding protein Z4 (BSZ4) [2], protein Z7 (BSZ7) (S.K.R., unpublished results) and protein Zx (BSZx) [3], respectively, have been sequenced. Structural and functional properties of two of the gene products, BSZ4 [4] and BSZ7 [5], have been characterized in some detail. Alignments with mammalian members of the serpin family showed overall amino acid sequence identities in the range 25–30%. Amino acid sequences of protein BSZ4, BSZ7, and the putative BSZx are about 70% identical. Except for a weak interaction between BSZ7 and chymotrypsin [5], inhibitory properties have not been associated with the barley serpins and their biological function is unknown. Previous attempts to detect serpins in other cereals by gel precipitation techniques using polyclonal rabbit antibodies against protein Z were not successful [4,6].

In the present work protein Z-like serpins (WSZ) have been isolated from the related cereal wheat. As a first step towards elucidation of putative physiological roles of plant serpins it was demonstrated that one molecular form (WSZCI) reacted with chymotrypsin forming a complex stable towards SDS and boiling, which is a unique property of inhibitory serpins [7].

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Abbreviations: BSZ, barley serpin protein Z; DTT, Dithiothreitol; PVDF, polyvinylidene difluoride; RP-HPLC, reversed-phase high-performance liquid chromatography; SSC, standard saline citrate; T-gel, thiophilic gel; TFA, trifluoroacetic acid; TLCK, tosyl-lysine chloromethyl ketone; WSZ, protein Z-like wheat serpin; WSZCI, protein Z-like wheat serpin with chymotrypsin inhibitor activity.

2. Materials and methods

2.1. Plant material

Mature seeds of wheat (*Triticum aestivum*, cv. Kadett) were used for protein purification. For immunoblot analysis barley plants (*Hordeum vulgare*, cv. Bomi) were grown in the greenhouse with a 16 h light

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(18°C)/8 h dark (12°C) cycle and wheat plants (cv. Pumpe) were grown in the field. Seeds of barley (cv. Bomi) and wheat (cv. Pumpe) were germinated for 7 days in the dark and the primary leaves were used for DNA extractions.

2.2. Chemicals

Hybond-N+ membranes and *Hin*dIII were obtained from Amersham, anhydrochymotrypsin-agarose from Takara Biomedicals and Mini-Leak divinylsulphone activated agarose (High) from Kem-En-Tec. Fractogel TSK DEAE-650(S) was from Merck, bovin α-chymotrypsin, TLCK treated α-chymotrypsin, biotin-labelled α-chymotrypsin and chymostatin from Sigma and PVDF membranes (Immobilon) from Millipore. Alkaline phosphatase conjugated antibodies and streptavidin were obtained from Dako and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide from Bachem.

2.3. Southern blots

DNA was isolated according to [8] and digested with *Hin*dIII. Fragments were separated on a 0.7% agarose gel, transferred to Hybond-N+ membranes and hybridized [9] with BSZ4 [10], BSZx [3] and BSZ7 clones coding for the C-terminal regions. These clones do not cross-hybridize under stringent conditions.

2.4. Protein extraction

To remove the salt soluble proteins flour from seeds of Kadett wheat (150 g) was extracted at 4°C with 1.2 l 100 mM sodium phosphate, pH 8.0, 1 mM EDTA. After centrifugation (20 min at 9,500 × *g*) the extraction was repeated twice. Thereafter the fraction used for purification was obtained by two extractions with 1.2 l of the Tris buffer with 0.2% (w/v) Na_2SO_3 and 0.15 % (w/v) $Na_2S_2O_5$ added. For immunoblot analysis the same extraction sequence was used in small scale on 1 g homogenized tissue samples from developing grains. In these experiments the sulphite/pyrosulphite reductant was replaced by 20 mM DTT (thiol extract). Similar salt and thiol extracts from 0.5 g flour were used for the determination of 'free' and 'bound' WSZ by rocket immunoelectrophoresis as described for the determination of BSZ [4,11]. The pool of WSZ eluted from the T-gel (section 2.5) was used for antibody production as well as standardization, assuming either a composition of molecular forms similar to that of crude extracts or a similar reactivity of the various forms with the antibodies.

2.5. Protein purification

The pooled sulphite extracts (section 2.4) were brought to 70% saturation with solid $(NH_4)_2SO_4$ and the pellet was redissolved and subjected to thiophilic adsorption chromatography as detailed in Fig. 2. The T-gel was prepared by coupling β-mercaptoethanol to Mini-Leak [12]. Fractions containing WSZ were pooled, dialysed and chromatographed on Fractogel TSK DEAE-650(S) as described in Fig. 3. Bovine TLCK-treated α-chymotrypsin (25 mg) was coupled to 2 g Mini-Leak (drained weight) according to the manufacturer's instructions. The column was equilibrated with 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2% (w/v) Na_2SO_3 and 0.15% (w/v) $Na_2S_2O_5$, and fractions from the DEAE-Fractogel containing chymotrypsin inhibitory activity were pooled and applied to the column. Elution was performed with 250 mM sodium phosphate, pH 12, and the fractions were neutralized immediately with acetic acid. Affinity chromatography on a 1.5 ml anhydrochymotrypsin-agarose column was performed as described [13]. Precipitation, dialysis and anion exchange chromatography were performed at 4°C and all other operations at room temperature.

2.6. Protein characterization

SDS-PAGE was performed in 10% acrylamide gels [14] and the proteins were silver stained [15] or electroblotted to nitrocellulose [16]. Monoclonal antibodies towards BSZ7 were produced as described [17]. For immunoblots all incubation and washing steps were carried out in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Tween 20, and in the quenching step 0.5% casein was added. For detection of biotin-labelled chymotrypsin on nitrocellulose, blocking, incubation and washing steps were performed in the above buffer and staining was obtained with alkaline phosphatase conjugated streptavidin. Rocket immunoelectrophoresis was performed as described in [11].

Measurements of chymotrypsin inhibitory activity were made in 96-well microtiter plates with 1.2 nM enzyme and 0.6 mM succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.01% Tween 80. Chymotrypsin was active-site titrated with *N-trans*-cinnamoylimidazole [18]. Enzyme solution (100 μl) was incubated for 30 min at 25°C with inhibitor (100 μl) and substrate (75 μl) was added. Substrate hydrolysis was followed at 405 nm for 12 min using a Bio-Tek Instruments microplate reader. Attempts to detect inhibition of pancreas elastase, trypsin, thrombin and subtilisin were made by essentially the same procedure as described in [19]. WSZ (2.8 mg) was reduced and alkylated with iodoacetamide, cleaved with CNBr [20], and the resulting fragments were separated by RP-HPLC on a Vydac 214TP54 column (250 × 4.6 mm) equilibrated with 0.1% TFA. Elution was performed with a 0–100% linear gradient of acetonitrile (20%) and isopropanol (60%) in 0.1% TFA. Amino acid sequence analysis was carried out on an Applied Biosystems model 470A gas-phase sequencer [21]. The protein eluted from chymotrypsin-agarose at pH 12 was reduced and alkylated and subjected to Tricine-SDS-PAGE [22] followed by electroblotting onto a PVDF membrane [23]. From two individual lanes the stained M_r ~5 kDa bands were excised and one membrane sample was used directly for sequencing on a 477A liquid-phase sequencer as described by the manufacturer (Applied Biosystems). The other membrane sample was extracted with 60% isopropanol, 5% TFA and the supernatant used for mass spectrometry [24]. Amino acid analysis was performed as described in [25].

3. Results

3.1. Identification

Southern blot analysis with the BSZ4 probe at medium stringency (Fig. 1A) showed several strongly hybridizing bands from 2 to 7 kbp in barley and some weaker hybridizing bands from 2 to 8 kbp in wheat. After high stringency washes one strongly hybridizing fragment of 7 kbp was detected in barley and no bands were observed in wheat (Fig. 1A). Similar but distinct wheat restriction fragment patterns were obtained with the BSZ7 and BSZx probes at medium stringency (not shown). A mon-

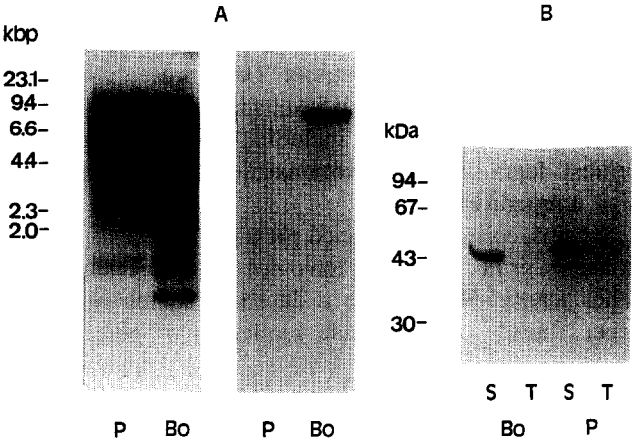

Fig. 1. Southern blot analysis of wheat and barley DNA (A) and immunoblot analysis of wheat and barley protein extracts (B). DNA (10 μg) from the barley cv. Bomi (Bo) and the wheat cv. Pumpe (P) was digested with *Hin*dIII and probed with the ^{32}P-labelled BSZ4 probe. *Hin*dIII-digested lambda DNA was used as size marker. The filter was washed in 1 × SSC at 65°C (left) followed by washes in 0.1 × SSC (right). Extracts of salt soluble proteins (S) and thiol protein extracts (T) from Bomi (Bo) and Pumpe (P) endosperms, respectively, collected 12 days after flowering. Extract (2.5 μl) was applied in each well after treatment with 50 mM DTT in the SDS buffer and monoclonal BSZ antibodies were used for detection after blotting.

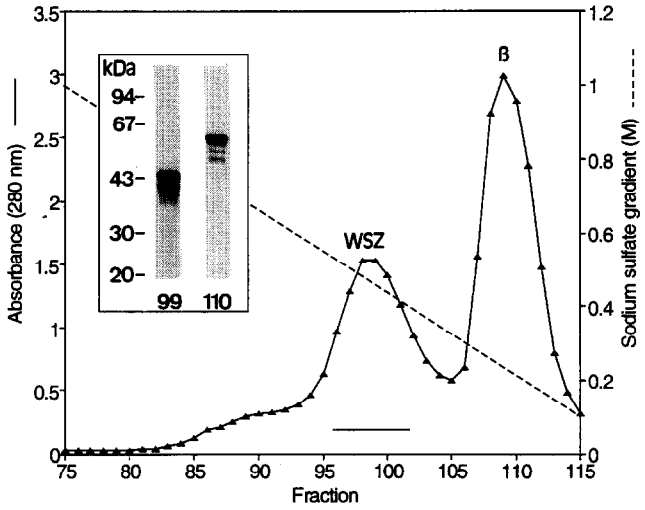

Fig. 2. Separation of WSZ and β-amylase (β) by thiophilic adsorption chromatography. The T-gel column (1.5 × 17 cm) was equilibrated with 25 mM Tris-HCl, pH 8.0 containing 1 M Na_2SO_4, 1 mM EDTA, 0.2% (w/v) Na_2SO_3 and 0.15% (w/v) $Na_2S_2O_5$. The pellet from $(NH_4)_2SO_4$ precipitation was redissolved in 460 ml of the same buffer, centrifuged and applied to the column. Adsorbed material was eluted by a 150 ml 1–0 M gradient of Na_2SO_4 in the buffer at 28 ml/h. Fractions of 4.5 ml were collected and pooled for further purification as indicated by the horizontal bar. The insert shows silver stained SDS-PAGE of the reduced fractions 99 and 110.

oclonal antibody raised against BSZ7 and reacting with native, but not cleaved BSZ4 and BSZ7 was used for immunoblotting experiments (Fig. 1B). Two M_r ~40 kDa bands (BSZ4 and BSZ7) with almost identical mobility in SDS- PAGE were detected in the salt extract of developing barley endosperm. In a similar extract of wheat one band with slightly lower mobility and a weakly stained band with a little higher mobility reacted with the antibodies (WSZ). The faint bands at M_r ~30–35 kDa in both barley and wheat also appeared with control antibodies (not shown). Only low amounts of 'bound' BSZ and WSZ were found in thiol extracts from barley and wheat at this developmental stage. In grain flour of the three wheat varieties Kadett, Cornette and KVL 2420 a total grain WSZ content of about 3–4 mg/g was estimated by rocket immunoelectrophoresis (not shown). About 40% was extractable with buffer or salt solutions, while addition of 20 mM DTT to the buffer also released the 'bound' forms. For purification only the 'bound' forms of WSZ (and β-amylase) were extracted after removal of the salt-soluble fraction.

3.2. Purification

WSZ (M_r 40–45 kDa) and β-amylase (M_r 60 kDa) were separated by thiophilic adsorption chromatography (Fig. 2). The pooled protein immediately inactivated chymotrypsin but not subtilisin, pancreas elastase, thrombin or trypsin. For further separation ~30 mg WSZ from the pool was dialysed and applied to the DEAE-Fractogel and M_r 40–45 kDa proteins were eluted in the 3 main peaks (Fig. 3). In the immunoblotting experiments the monoclonal BSZ antibody did only recognize the M_r 40–45 kDa proteins of the 2 first peaks. However, the polyclonal WSZ antibodies also reacted with the proteins of the third peak, which may represent cleaved forms of WSZ (not shown). Chymotrypsin inhibitory activity was mainly confined to the first peak (pool 1, Fig. 3). Activity measurements indicated that only 4–5% of the protein in pool 1 possessed chymotrypsin inhibitor activity (WSZCI). An attempt to isolate the inhibitor on a column of anhydrochymotrypsin-agarose was not successful. Instead pool 1 was subjected to affinity chromatography on chymotrypsin-agarose. As expected, only a minor fraction of WSZ was retained on the column. Inhibitory activity was neither detected in the run-through nor in the pH 12 eluate from the chymotrypsin column, indicating that all WSZCI was bound and subsequently eluted in a cleaved form which was confirmed by SDS-PAGE (not shown).

3.3. Characterization

When pool 1 (Fig. 3) containing the chymotrypsin inhibitory activitity was incubated with biotin-labelled α-chymotrypsin at 0°C and subjected to SDS-PAGE after boiling, a band corresponding to a complex with chymotrypsin was detected (Fig. 4). Chymotrypsin treated with chymostatin failed to form a complex (not shown), and this inhibitor was used to stop the reaction in the time course study. In agreement with the above results, only a small percentage of WSZ was able to participate in complex formation. Staining for biotin and

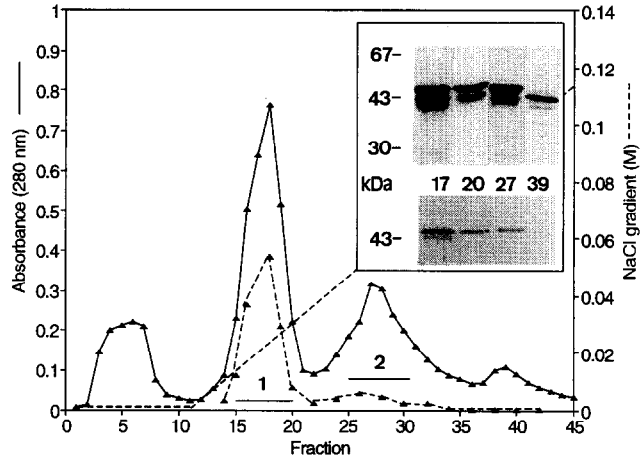

Fig. 3. Anion-exchange chromatography on the DEAE-Fractogel. The WSZ pool from the T-gel (Fig. 2) was applied on the column (1.5 × 6 cm) equilibrated with 20 mM Tris-HCl, pH 8.6 containing 1 mM EDTA, 0.02% (w/v) Na_2SO_3 and 0.015% (w/v) $Na_2S_2O_5$ and eluted at 10 ml/h with a 200 ml 0–0.2 M linear gradient of NaCl in the buffer. Chymotrypsin inhibitory activity was measured (▲---▲, arbitrary units). The insert shows silver-stained SDS-PAGE (top) and immunoblot (bottom) of the reduced fractions 17, 20, 27 and 39. Monoclonal BSZ antibodies were used for detection. Fractions of 2.5 ml were collected and pooled for characterization as indicated by horizontal bars.

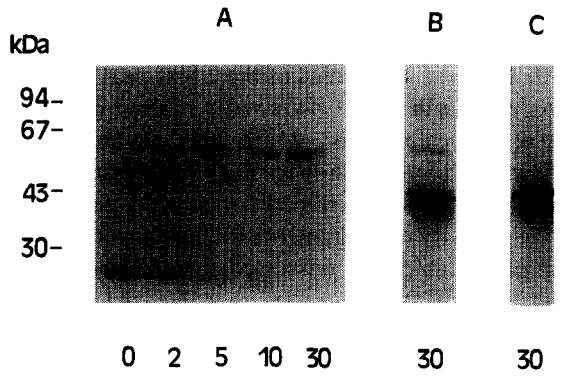

Fig. 4. Complex formation between WSZ and biotin-labelled chymotrypsin. WSZ (16 μg) from pool 1 of the DEAE-Fractogel (Fig. 3) was incubated with 0.25 μg biotinylated chymotrypsin at 0°C for the time indicated (min). The reaction was stopped by addition of chymostatin (10 nmol). After mixing with nonreducing sample buffer the samples were boiled for 3 min and analysed by SDS-PAGE. (A) Detection of biotin (chymotrypsin); (B) Immunoblot with polyclonal antibodies towards WSZ; (C) Immunoblot with monoclonal BSZ antibodies. About 1 μg WSZ was applied in each lane. An arrowhead indicates a faint M_r ~90 kDa band detected by the polyclonal antibodies (presumably dimer WSZ).

immunoblot analysis with the WSZ antibodies showed that both chymotrypsin and WSZ were present in the new band and the size indicated a 1:1 complex (Fig. 4A and B). The complex was not recognized by the monoclonal BSZ antibodies suggesting that the epitope was masked in the complex (Fig. 4C). At 37°C the complex formation was completed within 60 s (not shown) whereafter the complex was converted, presumably by proteolytic action, to intermediate molecular-mass forms as observed with other serpins [26,27].

The amino acid composition of fractions 18, 27 and 39 from the DEAE-Fractogel and of WSZCI eluted from chymotrypsin-agarose were determined (Table 1). Within experimental error the compositions are very similar, but not identical. However, all compositions reveal a close relationship to BSZ4 and BSZ7, e.g. the wheat proteins also contain 2 cysteine residues and are rich in Leu and other hydrophobic residues.

WSZ was expected to be N-terminally blocked like BSZ4 and BSZ7 [5,10]. For amino acid sequence analysis WSZ from pool 2 was cleaved with CNBr and a 8.2 kDa fragment isolated by RP-HPLC.

Comparison of the sequence of 43 residues in the N-terminal of the 8.2 kDa peptide with the BSZ4, BSZ7 and BSZx sequences show 65, 63 and 72% amino acid identity, respectively (Fig. 5A). The C-terminal peptide generated by chromatography on the chymotrypsin-agarose column was isolated by Tricine-SDS-PAGE and blotted onto a PVDF membrane for sequence and mass analysis. An M_r of 4574 ± 4 Da was obtained by plasma desorption mass spectrometry. Sequencing confirmed homology with both barley and mammalian members of the serpin family (Fig. 5B).

4. Discussion

Previously, plant serpins or serpin genes have only

Table 1
Amino acid composition of fractions 18, 27 and 39 from the DEAE-Fractogel (Fig. 3) and of WSZCI

Amino acid	Wheat				Barley		
	18	27	39	WZCI	Z4	Z7	Zx
Asx	34.8	33.6	38.8	35.2	33	35	28
Thr	19.9	20.0	23.8	20.1	21	19	20
Ser	34.8	31.8	30.8	35.8	38	35	42
Glx	39.7	43.0	44.6	38.1	44	41	38
Pro	21.3	20.1	18.4	20.3	15	15	19
Gly	25.8	28.0	27.3	29.9	26	27	27
Ala	44.3	44.7	36.0	44.0	41	37	42
Cys	2.1	2.3	2.6	1.7	2	2	0
Val	32.4	31.7	37.1	32.8	32	31	37
Met	6.6	5.1	4.1	6.3	7	6	8
Ile	13.3	16.6	16.6	13.5	19	16	10
Leu	48.3	48.5	45.8	47.5	45	44	43
Tyr	8.0	8.0	7.5	7.6	6	8	7
Phe	24.2	23.2	23.5	23.9	23	21	24
His	8.6	7.2	8.0	7.9	10	8	9
Lys	18.0	18.5	18.1	17.9	22	19	29
Arg	13.9	13.8	13.0	13.6	12	13	12
Trp	n.d.	n.d.	n.d.	n.d.	3	5	3
Total	396	396	396	396	399	382	398

Values were determined after 20 h hydrolysis and calculated to fit the number of residues in BSZ4. The compositions of BSZ4 [2], BSZ7 [5] and BSZx [3] are shown for comparison. Tryptophan was not determined (n.d.).

A

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            250         260         270         280
             ▾           ▾           ▾           ▾
WSZ     MYILLP EALSGLWSLA EKLSAEPEFL EQHIPRQKVA LRQFKLP
BSZ4    ****** G*QD****** KR**T****I *N***K*T*E VGR*Q**
BSZx    ****** **QD***N** N***T****M *K*M*M***P VG*****
BSZ7    ****** **HD**SR** Q***T**D** *NR**TEE*E VG**M**
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B

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                  P1'   365         375
                   ▾     ▾           ▾
WSZCI               QARPPSVMDFIADHPFLFL
BSZ4               SMPLKVDLV**V*N******
BSZx               SLPVE*VKV**V********
BSZ7               SLPIR---***V*N******
OV                 SVSEE-----*R*******C
PAI-2              TGHGG---PQ*V********
α1-PI              SIPPE-----VKFNK**V**
ATIII              SLNPN--RVT*K*NR***VF
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Fig. 5. Alignment of partial amino acid sequences of WSZ with members of the serpin family. (A) N-terminal sequence of a 8.2 kDa fragment obtained by CNBr cleavage of pool 2 from the DEAE-Fractogel (Fig. 3). (B) N-terminal sequence of the C-terminal peptide from WSZCI. For comparison BSZ4 [2], BSZ7 (S.K.R., unpublished results), BSZx [3], chicken ovalbumin (OV), human plasminogen activator inhibitor (PAI-2), human α_1-proteinase inhibitor (α_1-PI) and human antithrombin III (ATIII) [28] are included. Numbering refers to BSZ4. * indicates identity with the WSZ/WSZCI sequences. In B gaps are introduced according to [28] to maximize similarity. The P_1' position corresponds to residue 359 in α_1-PI.

been detected in barley [2,3,5,10]. We have now identified new members of the serpin family in wheat grain. The BSZ4, BSZ7 and BSZx probes each hybridized to several wheat DNA fragments giving distinct patterns at medium stringency (Fig. 1A). These results indicate that wheat has one or more homologous genes related to each of the three barley serpins. Immunoblotting experiments confirmed the expression of serpins in wheat (Figs. 1B and 3). Preliminary immunoelectrophoretic determinations suggested that field grown wheat varieties may contain about 3–4 mg WSZ/g grain which is similar to or even higher than in common barley varieties (~2,5 mg/g grain) [4,11]. As in barley more than 50% of WSZ could only be extracted in the presence of a reductant together with 'bound' β-amylase [4,11]. Taking advantage of the selective extraction with sulphite/pyrosulphite after removal of the salt soluble proteins, a mixture of WSZ forms could be isolated by thiophilic adsorption chromatography (Fig. 2). Final purification and partial separation was obtained by anion exchange chromatography (Fig. 3). The heterogeneity found with respect to size and charge (Fig. 3) has also been observed for BSZ [2–6] and may be explained by the expression of different genes, posttranslational modifications including reactive loop cleavage, as well as dimer or polymer forms. The characterized forms of WSZ are very similar in size, amino acid composition and sequence to BSZ (Table 1 and Fig. 5). One molecular form, WSZCI, representing <5% of the isolated WSZ, was able to inhibit chymotrypsin and form a SDS-stable complex characteristic for serpins (Fig. 4). Purification of WSZCI on chymotrypsin-agarose generated a M_r 4574 Da fragment of same size as the C-terminal peptides of 42 amino acids obtained after reactive site cleavage of BSZ4 (M_r 4591 Da) and the putative BSZx (M_r 4634 Da). In contrast, the corresponding C-terminal fragment of BSZ7 (M_r 4264 Da) is three residues shorter (Fig. 5B) Thus, WSZ seems more related to BSZ4 (or the putative BSZx) than to the weak chymotrypsin inhibitor BSZ7 [5]. Sequencing of the WSZCI fragment confirmed homology with mammalian serpins (Fig. 5B). Both barley and wheat serpins contain the highly conserved nonpolar strand 4B sequence -Pro-Phe-Leu-Phe-Leu- (residues 373–377 in Fig. 5B) which is buried in the interior B sheet of mammalian serpin structures [28]. The sequence indicates that the P_1' residue in WSZCI is Gln and not Ser or Thr as in BSZ and many other serpins [29], but further proteolytic cleavage may have taken place during dissociation of the WSZCI-chymotrypsin complex.

The functional role of serpins in barley and wheat is not known. Cereals contain numerous low M_r inhibitors of chymotrypsin and other digestive enzymes protecting the grain against invading insects or microbial pests, and more specific regulatory roles of plant serpins would be intriguing.

Work is in progress to identify possible in vivo target proteinases and investigate the presence of serpins in other plants.

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