Inhibitory serpins from rye grain with glutamine as P_1 and P_2 residues in the reactive center

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Abstract Six of seven serpins detected in grains of rye (*Secale cereale*) were purified and characterized. The amino acid sequence close to the blocked N-terminus, the reactive center loop sequence and the second order association rate constant (k_a') for irreversible complex formation with chymotrypsin were determined for each serpin. Three of four serpins containing the unusual reactive center P_2–P_1' QQ↓S and one with P_2–P_1' PQ↓M were equally efficient inhibitors of chymotrypsin ($k_a' \sim 10^5$ M^{-1} s^{-1}). One serpin with P_2–P_1' PY↓M was a faster inhibitor ($k_a' \sim 10^6$ M^{-1} s^{-1}). Similar but differently organized glutamine-rich reactive centers were recently found in grain serpins cloned from wheat [Østergaard et al. (2000) J. Biol. Chem. 275, 33272] but not from barley. The prolamin storage proteins of cereal grains contain similar sequences in their glutamine-rich repeats. A possible adaption of hypervariable serpin reactive centers late in *Triticeae* cereal evolution as defence against insects feeding on cereal grains is discussed.

Key words: Chymotrypsin inhibitor; Prolamin; Serpin; *Secale cereale*

1. Introduction

The serpins constitute a superfamily of proteins found in animals, plants and some viruses, but not in fungi or bacteria [1]. Most serpins are irreversible *ser*ine *p*roteinase *in*hibitors with regulatory functions based on diverse reactive center loop (RCL) sequences allowing for specific association with the substrate binding subsites of the cognate proteinases. A few serpins are inhibitors of cysteine proteinases [2]. In mammals, serpins are involved in regulation of numerous intra- and extracellular events, including blood coagulation and other complex proteolytic cascades [1]. Although putative serpin genes and mRNAs have been identified in many plants, only serpins of the *Triticeae* cereals barley and wheat have been cloned or isolated from seeds and characterized in some detail [3–5]. The cereal serpins have inhibitory properties similar to those of mammalian serpins, but physiologically relevant target proteinases have not been identified.

In a recent study, the inhibitory specificities of serpins representing the complement of major serpins in wheat grain were characterized [5]. Unexpectedly, five of the six serpins with distinct RCLs contained Gln as either one or both of the P_1–P_1' residues in the reactive center. A single Gln residue is found in the reactive center of only a few of more than 200 previously characterized non-plant serpins [6]. The reactive centers of the wheat serpins resemble sequences in the Gln- and Pro-rich repeats of the major prolamin storage proteins of the endosperm, indicating evolutionary adaption of the serpins to inhibition of proteinases specifically involved in prolamin degradation. To support this hypothesis we have now characterized six major serpins expressed in the grain of rye, a related *Triticeae* cereal containing similar classes of prolamins as the major storage proteins. As in wheat [5], five of the six serpins studied were found to contain one or two Gln residues at the reactive center bond. However, the Gln residues were differently positioned in the rye and wheat RCL sequences, suggesting inhibitory specificities distinct from those of the wheat serpins. Therefore a kinetic comparison was made using chymotrypsin as a common target enzyme.

In the wheat serpin study [5], complete separation of the six major serpin forms of grain extracts was not obtained, and kinetic characterization was based on cloning and heterologous expression of $(His)_6$ affinity tagged proteins in *Escherichia coli*, which lacks endogenous serpins. In the present study, purification from a grain extract using an improved strategy based on numerous pilot experiments resulted in efficient separation of the seven molecular forms detected by native polyacrylamide gel electrophoresis (PAGE) after a few chromatographic steps.

2. Materials and methods

2.1. Materials

Mature grains of rye (*Secale cereale* cv. Dominator) were obtained from a commercial mill. Serine proteinases were gifts from NOVO Nordic. Porcine chymotrypsin and human cathepsin G were measured with Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (Bachem), porcine trypsin with Sar-Pro-Arg-*p*-nitroanilide (Bachem) and human plasmin with D-Val-Leu-Lys-*p*-nitroanilide (Chromogenix) as substrate. Secondary antibodies and streptavidin labelled with alkaline phosphatase were from DAKO.

2.2. Serpin purification

Finely ground rye flour (40 g) was extracted at 4°C with 400 ml 0.1 M Tris–HCl, pH 8.0 containing one complete proteinase inhibitor tablet (Roche). After vigorous stirring for 30 min, a viscous, greenish

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Abbreviations: BSZ, RSZ and WSZ, barley, rye and wheat serpins (protein Z family); DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; RCL, reactive center loop; SDS, sodium dodecyl sulfate

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supernatant solution was obtained by centrifugation for 20 min at 9000×*g*. After addition of solid $(NH_4)_2SO_4$ to 35% saturation and centrifugation, the serpins were precipitated with $(NH_4)_2SO_4$ at 70% saturation. The pellet was redissolved in 40 ml 25 mM Tris–HCl, pH 8.0 containing 1 M Na_2SO_4 and subjected to thiophilic adsorption chromatography on Fractogel EMD TA (Merck) as detailed in Fig. 1. Selected fractions were pooled and treated with 20 mM dithiothreitol (DTT) for 30 min at 22°C to dissolve possible serpin–serpin and serpin–β-amylase aggregates [7]. The major serpin forms were separated by anion exchange chromatography at pH 8.0 on a MonoQ column (Pharmacia) and rechromatographed under the same experimental conditions, as described in Fig. 2. Purification was monitored by native PAGE in 8% Tris–glycine gels using the Xcell II Mini Cell system (Novex). Prior to application, samples were treated with 20 mM DTT for 15 min at 50°C. Silver staining and immunoblotting were performed as described previously [3]. For detection of complex formation with serine proteinases on blots, the nitrocellulose membrane was incubated for 1 h at 22°C with biotinylated proteinase. Subsequent treatment with alkaline phosphatase-labelled streptavidin and staining was made under the same conditions as immunoblotting.

2.3. Inhibition assays and kinetic analysis

Screening for inhibition of serine proteinases during and after purification was made at pH 8.0 in microtiter plates after preincubation of the serpin with proteinase for 30 min at 22°C [3,4]. The association rate constant (k_a) and stoichiometry of inhibition (SI) were determined under second order conditions according to the simplified branched pathway of suicide substrate inhibition [1] as described in detail previously [3,5]. In brief, serpin was incubated with proteinase at 22°C. The reaction was stopped after various times by 10- or 20-fold dilution of aliquot samples with 0.5–1.0 mM *p*-nitroanilide substrate, and residual activity was determined using a Shimadzu 2101PC spectrophotometer. All reactions were performed in 50 mM Tris–HCl, pH 8.0 containing 0.1 M NaCl and 0.1% Tween 20. Concentration of active enzyme was determined by active site titration or titration with a tight-binding protein inhibitor as described previously [4]. Concentration of serpin was estimated by amino acid analysis [8].

2.4. Complex formation and sequence determination

For visualization of complex formation, serpin was incubated with proteinase at a molar ratio of ~2:1 for 5–30 min at 22°C, proteins were separated by tricine sodium dodecyl sulfate (SDS)–PAGE in 10–20% precast gels (Novex), transferred to polyvinylidene difluoride (PVDF) membranes by semidry electroblotting and stained with Coomassie Blue [3]. Reactive centers were identified by N-terminal sequencing of the excised ~4 kDa peptide bands released from the irreversible complex by boiling with SDS [3]. Similar methods were used to obtain overlapping RCL sequences after incubation of a 20- to 50-fold molar excess of serpin with trypsin for 3 h at 37°C. N-terminal serpin sequences were obtained from excised ~43 or ~40 kDa protein bands, either directly or after chymotrypsin treatment as in the complex formation experiments.

3. Results

3.1. Purification

After salt extraction and $(NH_4)_2SO_4$ precipitation, the serpins were separated from other major albumins, the predominant being β-amylase, by thiophilic adsorption chromatography (Fig. 1, top). Native PAGE, using a procedure developed to separate barley and wheat serpins [5], showed that the rye serpins were eluted in the first part of the gradient at relatively high Na_2SO_4 concentrations, whereas β-amylase was the main protein of the major peak appearing at lower salt concentrations in the gradient (Fig. 1, bottom). Immunoblotting using a monospecific antibody raised against recombinant WSZ1a confirmed the presence of at least six immunochemically related serpins in the rye grain extract. Treatment of a similar protein blot with biotinylated chymotrypsin showed that all six molecular forms made stable complexes with this enzyme,

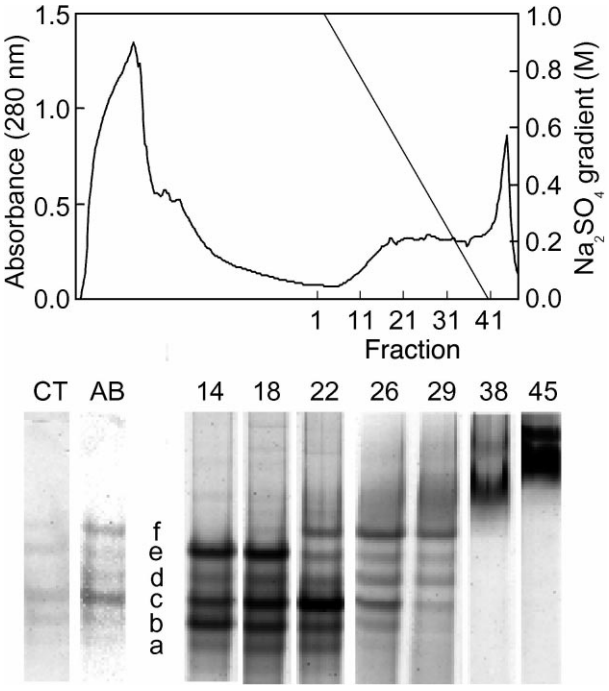

Fig. 1. Partial separation of rye serpins by thiophilic adsorption chromatography. The Fractogel EMD TA column (13×142 mm) was equilibrated at 25°C with 25 mM Tris–HCl, pH 8.0 containing 1 M Na_2SO_4, and the protein concentrate from the $(NH_4)_2SO_4$ precipitation was applied. After washing with equilibration buffer, the adsorbed protein was eluted by a 1–0 M linear gradient of Na_2SO_4 in the Tris buffer at 1 ml/min. Fractions of 2.5 ml were collected (top panel). Aliquots of selected fractions (14–45) were subjected to native PAGE followed by silver staining (bottom panel). Proteins of the native pattern of fraction 26 were electrotransferred to a nitrocellulose membrane and stained for binding of biotinylated chymotrypsin (CT) and a monospecific WSZ1a antibody (AB). The RSZ bands identified are marked a to f. Protein bands with lower electrophoretic mobility (fractions 38 and 45) were identified as β-amylases by immunoblotting.

whereas no binding of biotinylated trypsin was observed. These preliminary tests suggested that the rye serpins, like the cloned and characterized wheat serpins [5], were inhibitors of serine proteinases with chymotrypsin-like specificity. The six serpin forms detected were provisionally termed RSZa–f (Fig. 1). For further purification and separation of the serpins, fractions 10–20 (enriched in RSZb and RSZe) and 21–29 (enriched in RSZc and RSZf) were pooled and subjected to anion exchange chromatography in separate experiments (not shown). Although the serpins were dominant in the native PAGE patterns (Fig. 1), other proteins were present in the serpin pools. The major protein peaks with chymotrypsin inhibitor activity were confirmed to contain serpins by SDS–PAGE immunoblotting. Native PAGE showed that fractions containing RSZb, RSZd, RSZe and RSZf as the main serpin had been separated. Two distinct peaks contained serpins migrating as RSZc in native PAGE (Fig. 1), and they were termed RSZc1 and RSZc2. Only minute amounts of RSZa were detected and this molecular form was not further studied. Pooled peak fractions representing the six separated molecular forms were further purified by anion exchange rechromatography. The serpins corresponding to distinct bands of the native pattern were eluted in almost symmetrical peaks at distinct salt concentrations in the gradient (Fig. 2). Only

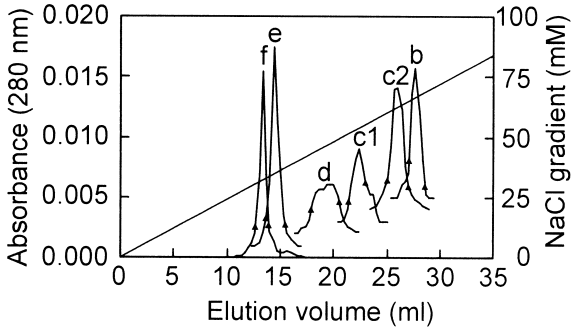

Fig. 2. Anion exchange rechromatography of major RSZ forms. DTT-treated serpin fractions from the first anion exchange chromatography (not shown) were applied in 25 mM Tris–HCl, pH 8.0 to a MonoQ column (5×50 mm) and eluted at 25°C with a 0–0.1 M gradient of NaCl in the buffer. The figure displays elution of RSZb through RSZf as the major protein peak in each of six chromatograms. Arrows (▲ to ▲) mark the central part of each peak collected for further serpin characterization.

RSZd eluted as a broader peak; this serpin also gave a diffuse peak in native PAGE, indicating microheterogeneity. SDS–PAGE confirmed that the serpins were essentially pure. In addition to the main band at ~43 kDa, many preparations contained protein (5–15%) in a band at ~40 kDa representing the RCL-cleaved form of the serpin. Further attempts to remove this inactive form of the serpin resulted in substantial loss of inhibitory activity. Small amounts of a ~20 kDa protein present in some preparations, especially of RSZb and RSZc1, could be separated by gel filtration chromatography. The N-terminal sequence DPPPV–, as well as tests with specific barley antibodies, identified this protein as a rye homologue of barley BASI and wheat WASI, which are bifunctional inhibitors of endogenous α-amylases and microbial subtilisins [9]. This type of serine proteinase inhibitor is specific for subtilisin-like serine proteinases and is therefore not a potential source of error in kinetic experiments with chymotrypsin-like serine proteinases.

For further characterization, 100–200 μl aliquots of the serpin fractions were immediately vacuum-dried at room temperature and stored at −20°C. Previously, this procedure was found to give the best conservation of inhibitory activity of the cereal serpins [3].

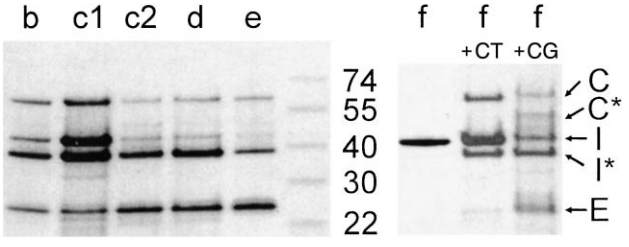

Fig. 3. Complex formation between rye serpins and serine proteinases. Serpin was incubated with proteinase at a molar ratio of about 2:1 for 15 min at 22°C. The reaction was stopped with PMSF prior to addition of sample buffer and boiling for 5 min. After SDS–PAGE in 10–20% tricine gels, the proteins were transferred to a PVDF membrane and stained with Coomassie Blue. Left panel, incubation of the indicated RSZ forms with chymotrypsin. Molecular mass markers of the See Blue standard are indicated (kDa). Right panel, purified RSZf (control) and incubation of RSZf with chymotrypsin (CT) and cathepsin G (CG). C, intact serpin proteinase complex; C*, complex with partially degraded enzyme [3]; I, intact inhibitor; I*, cleaved inhibitor; E, residual enzyme.

3.2. Complex formation and reactive center sequences

Among the many families of serine proteinase inhibitors, only serpins form stable covalent complexes with their target proteinases. All the rye serpins were found to form SDS stable complexes with about equimolar amounts of chymotrypsin in accordance with their inhibitory activity (Fig. 3). The rye serpins were also inhibitors of cathepsin G, another animal serine proteinase with chymotrypsin-like specificity, and complex formation with this enzyme could also be demonstrated, as shown for RSZf (Fig. 3). In addition to the intact serpin proteinase complex (C), which lacks the C-terminal ~4 kDa peptide of the serpin after RCL cleavage and boiling with SDS, complexes of intermediate size (C*) were observed (Fig. 3). In a previous study of barley and wheat serpins [3], these bands were found to represent serpin in complex with proteolytically modified proteinase, presumably cleaved by free proteinase after complex formation.

Sequencing the ~4 kDa peptide released from the serpins during complex formation with chymotrypsin provided a 10-residue amino acid sequence (P_1'–P_{10}') of the C-terminal part of the RCL (Fig. 4). In accordance with the conserved P_5 Lys in the cloned wheat serpins, cleavage with catalytic amounts of trypsin could be used to obtain the P_4–P_1 sequence of the reactive centers (Fig. 4). In these experiments, sequencing performed on the ~4 kDa band excised from the PVDF membrane gave two overlapping sequences, one starting with P_4 and the other starting with P_4'. The latter was due to cleavage at P_3' Arg or Lys, in accordance with the sequences obtained after reactive center cleavage (Fig. 4).

The RCL sequences of the rye serpins were similar to those of the wheat serpins, but characteristically different from

	P_8	P_{10}'	k_a'
Conserved	T KM ↓ ARPPS MDF		($mM^{-1}s^{-1}$)
RSZb	:ISQQ S.....D...		81
RSZc2	:ISQQ S.....D...		120
RSZd	:.SQQ S.....D...		nd
RSZe	:.SQQ S.....D...		47
RSZc1	:.VPQ M.K...VI..		83
RSZf	:.VPY M.KL..VI..		1100
WSZ1a	.AI..VLQ Q.....V...		120
WSZ1b	.AI..VPQ Q.....V...		31
WSZ1c	.AI..ALL Q.....V...		2400
WSZ2a	.IA.AVLL S.S...D...		370
WSZ2b	.IA.VVL RQ.P...V...		8
	↑		

Fig. 4. RCL sequences and kinetic constants for chymotrypsin inhibition. RCL sequences of the rye grain serpins and the related wheat serpins [5] are compared between P_8 and P_{10}'. The top line indicates highly conserved residues and a dot (.) indicates identity with these residues. A colon (:) indicates that Lys was inferred in this position from cleavage with trypsin after P_5 and conservation of Lys in the wheat serpins. An arrow (↓) indicates the reactive center bond, as identified in complex formation experiments (Fig. 3 and [5]), except in WSZ2b where chymotrypsin interacts at the overlapping inhibitory site at P_2 (↑). The overall second order association rate constant (k_a) and stoichiometry of inhibition (SI) were determined at 22°C by fitting the experimental data to the expression for residual proteinase activity [3]. From these results the apparent association rate constant (k_a') for formation of stable complex [1] was calculated as discussed in the text. nd, not determined (see text).

those of other serpins. For example, the rye and wheat serpins were found to have a conserved basic residue P_5 Lys not found in the barley serpins and rarely in animal serpins. The length of the loop between the conserved residue P_8, which is important for loop insertion during complex formation [10], and the highly conserved P_9'–P_{10}' Asp–Phe is 15 residues, whereas the corresponding loop of the barley serpins is 16 residues (BSZ4 and BSZx) or 13 residues (BSZ7). Five of the six rye serpins share the very unusual property of containing Gln residues in the reactive center with four of the previously cloned and characterized wheat serpins [5]. However, an important difference is that the wheat serpins contain the reactive center bonds Q↓Q or X↓Q, while the corresponding bonds of the rye serpins are QQ↓S or XQ↓S, with Ser in the P_1' position as found in most animal serpins [6].

3.3. Kinetics of chymotrypsin inhibition

In accordance with a previous characterization of wheat serpin specificity using a large number of animal, plant and microbial serine proteinases [5], the rye serpins were found to inhibit proteinases with chymotrypsin-like specificity, including cathepsin G, but not pancreas elastase or enzymes with trypsin-like specificity, as tested with trypsin and plasmin.

The second order association rate constant (k_a) and the stoichiometry of inhibition were determined for inhibition of porcine chymotrypsin with each of the six rye serpins according to the simplified branched pathway of suicide inhibition [1,3]. When inhibition of the purified recombinant cereal serpins was studied [3,5], an amino acid analysis could be used to determine the concentration of active serpin. However, the presence of small amounts of cleaved serpin and other protein impurities, which do not directly affect the kinetics of inhibition, made the determination of the concentration of active serpin unreliable, such that SI values varying between 1.5 and 3.5 were obtained for different preparations of the same serpin. In addition, our previous studies showed that during incubation with proteinase, significant cleavage may occur in RCL positions other than the inhibitory site, adding pathways parallel to those of the branching mechanism [3]. Not directly solving these problems, we found that kinetic experiments with different preparations of the same rye serpin gave reproducible results when the apparent association rate constant $k_a' = k_a \times SI$ [1], which is limited to represent the rate of association leading to stable complex formation only, was calculated (Fig. 4). Values for this constant, based on the determination of active chymotrypsin by active site titration and the assumption that formation of the chymotrypsin–serpin complex was rate limiting, were compared with the corresponding wheat k_a' values (recalculated from [5]). In assays with RSZd, the curves obtained indicated slow release of enzyme from an initial reversible complex, even after incubation for 23 h, and a reliable overall rate constant could not be determined.

As expected, the serpins containing hydrophobic residues in the P_1 position, WSZ1c and WSZ2a with P_1 Leu and RSZf with P_1 Tyr, showed the highest rates of association ($k_a' \sim 10^6$ M^{-1} s^{-1}), whereas WSZ2b interacting with chymotrypsin at the overlapping reactive center P_2 Leu was the slowest inhibitor ($k_a' \sim 10^4$ M^{-1} s^{-1}). The four rye and two wheat serpins with the hydrophilic P_1 Gln were all efficient inhibitors of chymotrypsin with $k_a' = 0.3$–1.2×10^5 M^{-1} s^{-1} (Fig. 4). Apparently, identity of the P_2 residue (Gln, Leu or Pro) or of the P_1' residue (Gln, Ser or Met) in these serpins did not significantly affect the rate of association with chymotrypsin.

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                    ↓
Consensus    <ATTLATDVRLSIAHQTRF
RSZb              .......
RSZc2             ........
RSZd              .......M......
RSZe              .......M...
RSZc1             ........
RSZf              ..........
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Fig. 5. N-terminal sequences of rye grain serpins. An arrow (↓) indicates cleavage of the native, N-terminally blocked rye serpins with chymotrypsin to obtain a partial N-terminal sequence. Consensus denotes the sequence conserved in the five cloned wheat serpins [5] and barley BSZ4 [3]. A dot (.) indicates identity with the consensus sequence. < indicates that the N-terminal Ala is blocked, presumably by acetylation as demonstrated for BSZ4 and BSZ7 [11].

3.4. N-terminal sequences

The serpins previously purified from barley and wheat grain were blocked in their N-terminus, although partial deblocking was sometimes observed, depending on the extraction and purification procedure used [3,5]. The rye serpins were generally found to be blocked. To obtain a partial N-terminal sequence, the native serpins could be deblocked by cleavage with chymotrypsin. Sequencing of the reactive center-cleaved rye serpins obtained in the complex formation experiments (Fig. 3, band I*) provided sequences from cleavage at a hydrophobic residue, presumably Leu located four residues from the blocked N-terminus (Fig. 5). As expected, the rye serpins had N-terminal sequences very similar to that conserved in all the cloned wheat serpins. Only RSZd and RSZe contained a substitution of residue 12 (Ile→Met), indicating a closer similarity of these two serpins with identical reactive center sequences (Fig. 4). For barley BSZ4 and BSZ7 [11] it was shown by mass spectrometry that the N-terminal Met residue was removed after translation and the following Ala residue acetylated. The highly conserved N-terminal sequences (Fig. 5) suggest that all the blocked cereal serpins contain an N-terminal acetyl-Ala residue.

4. Discussion

The sequence surrounding the scissile bond of the RCL is the main determinant of the inhibitory specificity of serpins [1]. Several studies have demonstrated hypervariability of the amino acids in the RCL of animal serpins, indicating that positive selection is leading to increased functional diversity in this superfamily of proteinase inhibitors [12,13].

Prolamins are the main storage proteins in seeds of wheat, rye and barley, the cultivated cereals of the grass tribe *Triticeae* [14]. The sulfur-poor prolamins (rye ω-secalins) consist predominantly of repeated sequences based on the octapeptide consensus motif PQQPFPQQ. Similar octa- or heptapeptide repeats, all containing the consensus sequence PQQP, are present in the sulfur-rich prolamins (rye γ-secalins). In the prolamin repeats Pro, but not Gln, is often substituted by Ser, giving the tripeptide sequences SQQ or QQS as found in the reactive centers of four of the rye serpins. In the central dipeptide Pro–Phe of the octapeptide motif, the Pro is highly conserved, while Phe is often substituted by another hydro-

phobic residue. Frequently, this dipeptide is identical with P_2–P_1 Pro–Tyr in the reactive center of RSZf. Other repeats with Val as the hydrophobic residue contain the sequence VPQ(Q) found in the reactive centers of RSZc1 and WSZ1b.

These similarities suggest that during evolution the serpins of the closely related cereals rye and wheat have evolved reactive centers resembling repetitive sequences of their major prolamin storage proteins. Most likely, the Gln-rich RCLs represent attractive baits for proteinases specifically adapted to prolamin degradation. Reactive center Gln residues are not present in the major barley grain serpins BSZ4 [3] and BSZ7 [11] and are extremely rare in ~25 putative plant serpins identified in the EMBL/GenBank databases, indicating that the nucleotide substitutions leading to Gln-rich RCLs have been fixed late in the evolution of the *Triticeae* tribe of grasses. If the highly unusual Gln-rich sequences are the result of rapid positive selection, it seems unlikely that accelerated co-evolution of one or more endogenous target proteinases has been the driving selective force. Most serpins are inhibitors of chymotrypsin-like proteinases but only (genes for) subtilisin-like serine proteinases have been identified in plants. Very few serpins are efficient inhibitors of subtilisins [15] and no inhibition of two plant subtilisins, cucumisin D from honeydew melon and hordolisin from germinated barley grain, could be detected with any of the wheat serpins [5]. These results suggest that the serpins participate in plant seed defence against invading pests, in cooperation with a number of 'standard mechanism' inhibitors of serine proteinases also abundantly present in cereal seeds [14,16]. In contrast to several low molecular weight protein inhibitors of wheat and barley grain, the serpins were found not to inhibit microbial serine proteinases, either of the chymotrypsin family or the subtilisin family [3–5]. Most likely, the complement of serpins in rye or wheat grain could impair digestion based on serine proteinases in insects specifically adapted to the use of prolamins as their main amino acid source. Unfortunately, isolated digestive proteinases of cereal insect pests were not available for inhibition studies. In addition, no specificity studies of insect proteinases have, to our knowledge, elucidated a possible selection for or adaption to specific degradation of the major storage proteins of a preferred feed plant species.

The different positions of Gln residues relative to the scissile bond in rye and wheat serpins may be accidental. Kinetic studies with a non-physiological target proteinase, porcine chymotrypsin, did not reveal major differences in inhibitory properties. However, considerable variation is found in protein digestive systems of insects [16] and it can not be excluded that the observed differences in the repertoire of serpin RCLs present in the two cereals may reflect evolutionary adaption to defence against distinct populations of insect pests.

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