Inhibitory Serpins from Wheat Grain with Reactive Centers

Resembling Glutamine-Rich Repeats of Prolamine Storage Proteins

CLONING AND CHARACTERIZATION OF FIVE MAJOR MOLECULAR FORMS*

Henrik Østergaard‡§¶, Søren K. Rasmussen§, Thomas H. Roberts‡, and Jørn Hejgaard‡|

From the ‡Department of Biochemistry and Nutrition, Building 224, Technical University of Denmark, DK-2800 Lyngby, Denmark and §Plant Biology and Biogeochemistry Department, PBK-301, Risø National Laboratory, DK-4000 Roskilde, Denmark

Running title: Inhibitory Serpins from Wheat Grain.

Copyright 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Summary

Genes encoding proteins of the serpin superfamily are widespread in the plant kingdom, but the properties of very few plant serpins have been studied, and physiological functions have not been elucidated. Six distinct serpins have been identified in grains of hexaploid bread wheat (*Triticum aestivum* L.) by partial purification and amino acid sequencing. The reactive centers of all but one of the serpins resemble the glutamine-rich repetitive sequences in prolamine storage proteins of wheat grain. Five of the serpins, classified into two protein Z subfamilies, WSZ1 and WSZ2, have been cloned, expressed in *E. coli* and purified. Inhibitory specificity towards 17 proteinases of mammalian, plant and microbial origin was studied. All five serpins were suicide substrate inhibitors of chymotrypsin and cathepsin G. WSZ1a and WSZ1b inhibited at the unusual reactive center P$_1$-P$_1′$ Gln-Gln, and WSZ2b at P$_2$-P$_1$ Leu-Arg—one of two overlapping reactive centers. WSZ1c with P$_1$-P$_1′$ Leu-Gln was the fastest inhibitor of chymotrypsin ($k_a = 1.3 \times 10^6$). WSZ1a was as efficient an inhibitor of chymotrypsin as WSZ2a ($k_a \sim 10^5 \text{M}^{-1}\text{s}^{-1}$), which has P$_1$-P$_1′$ Leu-Ser—a reactive center common in animal serpins. WSZ2b inhibited plasmin at P$_1$-P$_1′$ Arg-Gln ($k_a \sim 10^3 \text{M}^{-1}\text{s}^{-1}$). None of the five serpins inhibited *Bacillus subtilis*in A, *Fusarium* trypsin, or two subtilisin-like plant serine proteinases, hordolisin from barley green malt and cucumisin D from honeydew melon. Possible functions involving interactions with endogenous or exogenous proteinases adapted to prolamine degradation are discussed.
Introduction

The serpins constitute a superfamily of versatile proteins participating in the regulation of complex proteolytic systems (1, 2). Most serpins are serine proteinase inhibitors of chymotrypsin-like enzymes, but a few have been found to inhibit cysteine proteinases (3), and some are non-inhibitory. Many functionally distinct serpins have been identified in higher eukaryotes, some in viruses, but none in yeast or bacteria (4, 5). Mammalian serpins have been shown to participate in a growing number of extra- and intracellular physiological processes including blood coagulation, complement activation, remodelling of extracellular matrix and hormone transport.

The only plant serpin characterized in detail with respect to inhibitory specificity is recombinant barley (Hordeum vulgare) serpin rBSZx (6-8), but BSZx has not been detected in the plant. In addition, two serpins from barley grain, BSZ4 (7, 9-11) and BSZ7 (12, 13), and one from wheat (Triticum aestivum) grain (7, 14, 15) have been studied. Despite the high concentration of these inhibitors in the grains of monocot cereals (up to 4% total protein), the physiological functions of plant serpins remain unknown. Increasing numbers of putative serpin genes and serpin mRNAs have been identified from other monocot plants, including rice and wild oats, from eudicot plants, including tomato, cotton and the model plant Arabidopsis thaliana, and from the non-vascular plant Physcomitrella patens (EMBL/GenBank), suggesting that serpins are widespread in the plant kingdom.

Inhibitory serpins are metastable proteins (>40 kD) employing a unique “suicide substrate” mechanism of irreversible inhibition very different from the reversible “standard mechanism” used by other proteinase inhibitors (<25 kD) (1). The serpin in its native, active conformation has a flexible reactive center loop (RCL) located near the C-terminus and protruding from the main body of the protein as a bait. The proteinase recognizes a specific peptide bond (P1-P1') in the RCL, and a Michaelis-type encounter complex is formed between enzyme and inhibitor. The subsequent
formation of an acyl-ester bond between the proteinase and serpin (16) is associated with insertion of the RCL (as strand 4A) into β-sheet A of the serpin. There is evidence that loop insertion results in the translocation of the proteinase—linked to the P₁ residue of the serpin—from its docking position to the opposite end of the serpin molecule (17), but a serpin-proteinase crystal structure has not yet been obtained. This dramatic, irreversible conformational change in the serpin separates the P₁ and P₁′ residues by ~70 Å and generates a C-terminal serpin fragment which remains in the protein structure. The complex is only kinetically stable, dissociating after hours or days into active enzyme and cleaved, inactive inhibitor. The identity of the P₁ residue is the major determinant of serpin inhibitory specificity, but other RCL residues, as well as serpin body–proteinase body interactions, are also involved (18, 19). Thus empirical testing and kinetic studies are required to identify target proteinases.

Serpins are major albumins of wheat endosperm, with ~3-4 mg serpin/g grain found in some bread wheat varieties (14). About 40% total serpin can be extracted from flour with buffer or salt solutions. Subsequent extraction with a thiol reagent, e.g. 20 mM DTT, added to the buffer releases most of the remaining “bound” serpin together with “bound” β-amylase. One serpin with chymotrypsin inhibitor activity has been isolated from the thiol extract of bread wheat grain and partially sequenced (14). A cDNA encoding a serpin (WSZ1, renamed WSZ1a in the present study) with an identical RCL sequence (P₁′-P₁₉′) was subsequently cloned and characterized kinetically as an inhibitor of enzymes with chymotrypsin-like specificity (7, 15).

We have now adapted a native PAGE system to characterize the serpin complement of bread wheat grain. Six serpins with distinct reactive center sequences were identified in the thiol extract. Four serpin cDNAs, in addition to that encoding WSZ1a, were cloned and expressed in E. coli. As a step towards identification of their natural target enzymes, the inhibitory specificities of the five recombinant wheat serpins towards a panel of proteinases were determined. Interestingly, the
reactive center of two of the five cloned serpins was found to be P$_1$-P$_1'$ Gln-Gln, whereas two of the other serpins contain a Gln residue at P$_1'$. Reactive center Gln residues are extremely rare among serpins, and to our knowledge, no other naturally occurring serpins for which inhibitory properties have been demonstrated contain P$_1$ Gln.
EXPERIMENTAL PROCEDURES

Screening of a Wheat Grain cDNA Library and Serpin cDNA Cloning—A λZAPII cDNA library (15), derived from mRNA isolated from immature grains (15-20 d after flowering) of wheat cv. Chinese Spring, was screened at high plaque density by standard methods (20). Hybridization was performed at low stringency (5 × SSC, 60 °C overnight) using a [32P]-labelled C-terminal 1032-bp HindIII/BamHI fragment of the gene encoding BSZx (6), as done previously (15). Hybridizing plaques were identified and retained as partially pure phage stocks. By repeated rounds of plaque purification, two novel cDNAs encoding serpins (WSZ1b and WSZ2b) were isolated. The remaining phage stocks were screened by PCR using forward (5’-GGGAGCTCAGTTCAAACACC-3’) and reverse (5’-GATGAAATCCATGACCGAGG-3’) primers (from T-A-G-Copenhagen) complementary to conserved regions flanking the RCL of plant serpins. These regions were identified by alignment of the two cDNAs with the previously identified WSZ1a sequence (15). In each case a PCR product corresponding to the expected distance (0.5 kbp) between the two primers was obtained. Sequencing of the PCR products revealed the presence of two additional novel serpin cDNAs (encoding WSZ1c and WSZ2a). These were isolated by plaque purification, as described above, using the corresponding [32P]-labelled PCR product as the hybridization probe.

The four novel cDNAs were fully sequenced on both strands by the dideoxynucleotide chain termination method using a Perkin Elmer ABI Prism 377 DNA sequencer. The coding region of each novel cDNA was amplified by PCR (10 cycles: 30 s at 94 °C, 30 s at 48 °C, 2 min at 72 °C). In this process, each serpin was equipped with a N-terminal His-tag by use of an upstream primer (5’-GCCATGGGCCACCATCATCACCATCACATGGCAACCACCC-3’) with a 5’ extension (underlined) encoding a new initiating Met and the sequence Gly-(His)6- (overlapping an NcoI restriction site shown in bold). The 3’ primer (5’-TTAACCCTCACTAAAGGGA-3’) was designed...
to anneal at a position immediately downstream from a unique *Bam*HI site in the vector. The WSZ1b cDNA had the opposite orientation with respect to the *Bam*HI site, and was thus amplified using a 3′ primer (5′-GCAGGATCCATTTATTGGAATTGTATGG-3′) with a *Bam*HI extension (in bold). The four PCR products were digested with *Nco*I and *Bam*HI and inserted into the multiple cloning site of a pre-cut pET-15b expression vector (Novagen). The final constructs were sequenced, and were subsequently transformed into the *E. coli* strain BL21(DE3)pLysS (Novagen).

*Purification from E. coli and from Mature Grain*—Heterologous expression and purification of soluble recombinant His-tagged protein were performed as previously described (7). In brief, a one-litre culture of *E. coli* was grown in Super Broth to an optical density of 2.5 at 600 nm. Expression of recombinant protein was induced by addition of 0.5 mM isopropyl-β-thiogalactoside (IPTG). After incubation for 18 h at 15 °C, cells were harvested by centrifugation and lysed by sonication. The crude lysate was subjected to gel filtration on a Sephadex G-25 (Pharmacia Biotech) column, and the eluate containing soluble serpin was loaded onto a Ni-NTA (Qiagen) column. Recombinant protein was released from the column by 10 mM EDTA. For purification of WSZ2b, the desalting step was omitted and the protein was eluted from the Ni-NTA column with a 0-0.5 M imidazole gradient in the buffer. Gel filtration on a Sephadex G-100 (Pharmacia Biotech) column enabled the removal of small amounts of polymerized serpin along with a major fraction of the contaminating *E. coli* proteins (forming colloids) eluting near the void volume. Finally, anion exchange chromatography on a ResourceQ column (Pharmacia Biotech) yielded essentially pure recombinant serpin in the native form. Aliquots of 50-100 µl were vacuum-dried at room temperature and stored at −20 °C. Prior to use, the samples were reconstituted with deionized water and kept on ice.

The “bound” serpins of 50 g flour from wheat cv. Hereward (obtained from Havnemøllerne, Denmark) were extracted with 20 mM DTT, and separated from the co-extracted β-amylase by
thiophilic adsorption chromatography using a previously described purification protocol (14). The broad peak of partially separated serpins (see Fig. 2 in (14)) was collected in six fractions, each of which was desalted and subjected to anion exchange chromatography on a MonoQ column (Pharmacia Biotech) equilibrated with 25 mM Tris-HCl, pH 8.0, and eluted with a 0.0-0.3 M NaCl gradient. Purification was monitored by native-PAGE in precast Tris-glycine gels (8% acrylamide) using the Xcell II Mini Cell system (Novex). Samples were treated with 20 mM DTT for 15 min at 50 °C prior to application. Silver staining and immunoblotting were performed as described previously (7).

**Inhibition Assays and Kinetic Analysis**—Initial screening for inhibition of serine proteinases with the purified recombinant serpins was made at pH 8.0 in microtiter plates after preincubation at 22 °C for 30 min with a molar ratio of serpin to enzyme >10 (7). Concentration of active enzyme was determined using either active site titrants or tight-binding inhibitors as described previously (8). Concentrations of purified serpins were determined by amino acid analysis (21). The same screening procedure was used for detection of inhibitory activity in serpin fractions isolated from wheat grain and for titration of enzyme with serpin (initial determination of stoichiometry of inhibition, SI). The association rate constant ($k_a$) and SI were determined under second order conditions according to the simplified branched pathway of suicide substrate inhibition (1) depicted in Scheme I.

**Insert Scheme I**

Here $EI^*$ represents the long-term stable covalent complex between enzyme and serpin, $I^*$ is the cleaved inactive serpin, and $(k_a+k_s)/k_s$ corresponds to SI. In brief, serpin and proteinase were allowed to react for various times, the reaction was stopped by a 10- to 25-fold dilution with 0.5-1.0
mm p-nitroanilide substrate, and residual activity was determined using a Shimadzu UV-2101PC spectrophotometer. All reactions were performed at 22 °C in 50 mM Tris-HCl, pH 7.6 or 8.6, containing 0.1 M NaCl and 0.1% Tween 20. CaCl₂ (5 mM) was added in assays with coagulation factor VIIa/sTF. The serine proteinases and their specific substrates were as described previously (8), except that in the present study trypsin activity was measured with Ser-Pro-Arg- p-nitroanilide (Bachem) as substrate. Curve fitting and data treatment have been described in detail previously (7).

The recombinant wheat serpins were also tested for their ability to inhibit the cysteine proteinase EP-B (endoproteinase B) (22) and the subtilisin-like serine proteinase hordolisin (23), both from barley malt, as well as the subtilisin-like serine proteinase cucumisin D from honeydew melon (24), which was purified from the tissue surrounding the seeds of the ripe fruit. In these assays the serpin was incubated with enzyme for 20 min at 22 °C and residual activity measured using a Kontron SFM25 spectrofluorometer. EP-B activity was measured in 50 mM sodium acetate buffer, pH 4.5 or pH 5.0, containing 5 mM cysteine and 0.1% Tween 20, with the internally quenched substrate Abz-Gly-Pro-Phe-Arg-Gln-Gln-Ala-Glu-Tyr(NO₂)-Asp (22). For hordolisin and cucumisin D, the buffers used were 100 mM MES, pH 6.0, or 50 mM HEPES, pH 7.4, each containing 100 mM KCl, 0.05% Tween 20, 2 mM CaCl₂, and the substrate was Abz-Phe-Ala-Pro-Phe-Gly-Gly-Gly-Tyr(NO₂). The purified EP-B and hordolisin and the substrates were obtained from Dr. D. J. Simpson, Carlsberg Research Laboratory, Copenhagen, Denmark.

Complex Formation and Reactive Center Cleavage—Serpin was incubated with proteinase at a molar ratio of approximately 2:1 for 5-30 min at 0 °C or 22 °C. Tricine SDS-PAGE, semidry electroblotting to PVDF membranes, Coomassie Blue staining and N-terminal sequencing of peptides in excised bands have been described in detail (7). Similar methods were used to identify RCL sequences of the purified grain serpins after incubation for ~3 h at 37 °C with non-target proteinases using a 50-fold molar excess of serpin.
RESULTS

Expression Profile of Serpins in Wheat Grain—The thiol extract obtained after exhaustive buffer extraction of finely milled flour is a convenient source for characterization of the grain serpins. Serpins and β-amylases are the only major protein components present, and more than 50% of the total serpin is recovered in this extract (14). Native PAGE of the thiol extract in 8% gels allowed for effective separation of the bands of the microheterogenous β-amylases from those of the serpins. The seven bands observed in the protein-stained electrophoresis pattern (Fig. 1) all represent serpins, as documented by immunostaining with polyclonal antibodies raised against purified recombinant barley serpin rBSZx (Fig. 1) (7). The thiol extract of the varieties Chinese Spring, a hard red spring wheat used for cloning, and Hereward, a soft white winter wheat used for purification, gave identical patterns apart from minor quantitative differences (Fig. 1), as did those of 10 other randomly selected hexaploid bread wheats, of which only Kadet, previously used for purification (14), is included in Fig. 1. Immunostaining also confirmed that the native PAGE serpin pattern of the more complex salt extract (not shown) was similar to that of the thiol extract.

Insert Fig. 1

The serpins of the Hereward grain thiol extract were partially separated by thiophilic adsorption chromatography followed by anion exchange chromatography, according to a previously described protocol (14). Poorly separated major peaks were rechromatographed using adapted narrow salt gradients. All major peak fractions were subjected to native PAGE, and six fractions representing single major bands of the native pattern (Fig. 1) were characterized by partial sequencing. The wheat serpins were blocked in the N-terminus, presumably by acetylation, as observed for barley serpins (12). However, by taking advantage of the potential tryptic cleavage site corresponding to P
Lys in the RCL of the previously cloned WSZ1a (15), five distinct reactive center sequences could be associated with major bands in the native pattern (Fig. 1). These five serpin fractions, WSZ1a-c and WSZ2a-b (see the following section for the basis of the nomenclature), were all inhibitors of chymotrypsin. WSZ2b appeared to be cleaved with trypsin in the reactive center and not at P5 Lys, a result supported by a weak trypsin inhibitor activity associated with the WSZ2b fraction. A sixth serpin fraction, WSZ3, was resistant to trypsin cleavage but was not an inhibitor of trypsin. WSZ3 also lacked the ability to inhibit chymotrypsin, but this proteinase cleaved the serpin in the RCL to give a sequence entirely different from those of the other serpins (Fig. 1).

The staining intensities of the individual serpin bands observed with thiol extracts of 12 bread wheat cultivars indicated that the level of grain expression of the six major wheat serpins can generally be ranked as follows: 1b > 2b ≈ 1a > 3 > 2a ≈ 1c. This ranking was supported by comparisons of peak areas from the chromatograms obtained during purification of the Hereward grain serpins (not shown). The partial sequencing showed that serpins migrating as a single band in native- and SDS-PAGE often contained 5-15% of one or two of the other serpins. This previously observed difficulty in isolating homogeneous serpin isoforms from grain extracts (7) prompted us to clone the wheat grain serpins for complete sequencing and expression in *E. coli* for kinetic studies.

Insert Fig. 2

**Wheat Grain Serpin Sequences**—Screening of the λZAPII cDNA library resulted in the isolation of 38 partially pure phage clones. Each of the clones contained either the previously cloned WSZ1a cDNA (15) or one of four novel serpin-encoding cDNAs, all of which were sequenced (Fig. 2). The deduced reactive center sequences of the cloned Chinese Spring serpins were identical to those of five of the six serpins identified in the grain thiol extract (Fig. 1). Identity
with the Hereward grain serpins was further supported by partial sequencing of selected peptides isolated by reverse phase HPLC after CNBr-cleavage of the grain serpins (Fig. 2). Methionine residues are present only in the C-terminal part of the serpins, and in total, the sequenced peptides correspond to 11% of the C-terminal half of the five serpin sequences. Only one amino acid polymorphism was detected: Lys-233 of the cloned WSZ1b was found to be Ala in WSZ1b isolated from grain. The results of a phylogenetic analysis (Fig. 3a) and the percentage amino acid identity among the serpins (Fig. 3b) suggested a classification of the five wheat serpins into two subfamilies, WSZ1 and WSZ2. Accordingly, the proteins encoded by the four novel cDNAs were named WSZ1b, WSZ1c, WSZ2a, and WSZ2b. This subdivision was further substantiated by immunostaining, as polyclonal antibodies raised against rWSZ1a reacted only with the three WSZ1 serpins (Fig. 1). A monoclonal antibody raised against BSZ7 purified from barley grain reacted only with WSZ1b and WSZ1c (Fig. 1). Sequences encoding the sixth serpin, provisionally named WSZ3 (Fig. 1), were not identified among the cDNAs, and this serpin was not further characterized. However further sequencing of the C-terminal fragment of WSZ3 purified from grain (Fig. 1) showed the presence of a Gln-Gln-Gln reactive center sequence, followed by a sequence similar to that of BSZ7 (↓ indicates the putative reactive center):

WSZ3  SVAIEQQ↓QMPIVMDF
BSZ7  ↓SLPIRMDF.

The barley and wheat grain serpins belong to the family of protein Z-type cereal serpins (7) and can be divided into five subfamilies sharing ~75% amino acid sequence identity (Fig. 3b). Within a subfamily, members share at least 89% identity. The highest degree of identity of a serpin from barley or wheat to any of the 12 annotated putative serpin sequences from Arabidopsis thaliana is 50%. Among animal serpins, the highest degree of identity to a cereal serpin is 34% (EMBL/GenBank). All of the highly conserved regions in serpin sequences are present in the cereal
serpins (Fig. 2) (7), which are thus very likely to have overall conformations similar to those of serpins whose structures have been determined (25). For comparisons, the sequence and standard template numbering of \( \alpha_1 \)-proteinase inhibitor (\( \alpha_1 \)-PI) are included in Fig. 2.

**Insert Fig. 3**

All five wheat serpins contain a potential N-glycosylation sequence at Asn-168, and the two WSZ2 serpins have an additional site at Asn-340 (Fig. 2, wheat serpin numbering). However, BSZ4, BSZ7 and WSZ1a purified from grain were found not to be glycosylated in earlier studies (9, 14). An *E. coli*-based expression system was thus appropriate for production of the recombinant wheat serpins (7).

*Recombinant Wheat Grain Serpins*—The four new recombinant wheat grain serpins were purified from the cell lysate by successive use of Ni-NTA affinity chromatography, gel filtration and anion exchange chromatography. Amino acid analysis and SDS-PAGE confirmed isolation of >95% pure native (uncleaved) serpin, but recoveries were moderate (0.2-1.5 mg/l culture broth). In all productions most of the serpin was entrapped in inclusion bodies, but attempts to obtain active serpin from this fraction according to procedures established for some mammalian serpins (26) were unsuccessful.

The five wheat serpins contain Cys-123, as do barley serpins BSZ4 and BSZ7 (10, 12). Cys-123 is surface-exposed with reference to the \( \alpha_1 \)-PI structure (27). The WSZ1 serpins contain another surface-exposed Cys-301/302, and an additional Cys-99 is present in WSZ1a. As observed previously during purification of rBSZ4 and rWSZ1a (7), sample treatment with DTT (10 mM) prior to gel filtration and anion exchange chromatography was essential to prevent disulfide-based polymerisation. Storage of the purified serpins at –20 °C or –80 °C in Tris buffer, pH 8.0, with or
without 50% glycerol, resulted in 15-20% reduction in activity after each thawing (or prolonged storage) and all activity was lost after lyophilization. When aliquots (50-100 µl) of the serpin peak fractions from the final anion exchange step were vacuum-dried at room temperature immediately after collection and stored at –20 °C, more than 90% of the activity was retained, allowing reproducible kinetic measurements to be made.

**Inhibitory Specificity**—The P$_2$-P$_1$ residues of rWSZ2b (Fig. 4) are the same as those in rBSZx, which was found to inhibit enzymes with trypsin-like specificity at P$_1$ Arg, as well as chymotrypsin-like enzymes at the overlapping site P$_2$ Leu (7, 8). Therefore rWSZ2b was screened for inhibitory activity towards a panel of serine proteinases from human blood, human leukocytes and porcine or bovine pancreas. Assuming irreversible inhibition in these assays, performed with ~100 nM rWSZ2b and 30 min incubation at 22 °C, an intermediate percentage inhibition may indicate slow binding and/or a relatively large SI. In assays with plasma kallikrein (1 nM), ~65% inhibition was observed. With plasmin (15 nM), bovine chymotrypsin (5 nM) and cathepsin G (60 nM), ~40% inhibition was obtained. Porcine trypsin and coagulation factor VIIa (10 nM)/sTF (25 nM) were ~10% inhibited by rWSZ2b, whereas no inhibition of the following proteinases was detected: thrombin (0.1 NIHU/ml), coagulation factors Xa (1 nM) and XIIa (10 nM), uPA (5 nM), leukocyte elastase (10 nM) and porcine pancreas elastase (10 nM).

Previously, rWSZ1a (P$_1$ Gln) was characterized as an inhibitor of chymotrypsin and cathepsin G (7). The remaining serpins, rWSZ1b (P$_1$ Gln), rWSZ1c (P$_1$ Leu) and rWSZ2a (P$_1$ Leu), were also found to inhibit these two proteinases. rWSZ1a-c interacted very weakly with pancreas elastase and leukocyte elastase, whereas rWSZ2a could inactivate the pancreas enzyme. As a representative of
these four serpins with inhibitory specificity for chymotrypsin-like enzymes, rWSZ1a was tested for its ability to inhibit enzymes with trypsin-like specificity, as described above for rWSZ2b. No interactions were found except for a weak inhibition of coagulation factor Xa. These preliminary observations were used to select enzymes for kinetic characterization of the recombinant wheat serpins.

Hordolisin from barley green malt (23) and cucumisin D from honeydew melon (24) were the only plant serine proteinases available. Incubation of each of these subtilisin-like enzymes with a large excess of the wheat serpins for 15 min at 22 °C, both at pH 6.0 (optimum for hordolisin) and pH 7.4 (optimum for cucumisin), resulted in no detectable inhibition. When barley malt endoproteinase B (EP-B), a cysteine proteinase involved in endosperm storage protein degradation during seed germination (22), was incubated with excess of wheat serpin at pH 5.0 (slightly above the pH optimum for EP-B), no inhibition was observed. Incubation of rWSZ2a for up to 3 h at 37 °C with catalytic amounts of EP-B resulted in slow cleavage in the RCL solely at P′ Ser (Fig. 4). Neither a trypsin-like proteinase from *Fusarium oxysporum* nor subtilisin A from *Bacillus licheniformis* was inhibited by any of the recombinant wheat serpins.

**Insert Fig. 5**

*Kinetics of Inhibition*—As all of the five recombinant serpins were inhibitors of chymotrypsin, each was titrated with this proteinase to obtain preliminary SI values. Preincubations for up to 2 h at 22 °C were used to approach complete inhibition. Micromolar amounts of chymotrypsin were then incubated with stoichiometric amounts of each of the inhibitors for 30 min at 0 °C. After 1000- to 2000-fold dilution into substrate solution, spectrophotometric measurements at 22 °C showed no release of active enzyme during 90-120 min incubation. The five serpins formed SDS-stable
complexes with chymotrypsin, as previously shown for WSZ1a (14) and here demonstrated for rWSZ2a (Fig. 5). The same experiments were used to identify the inhibitory site, as well as possible substrate cleavage sites in the RCL, by N-terminal sequencing of the ~4 kD C-terminal peptides released by boiling with SDS after complex formation. Cleavage with chymotrypsin was detected only in the putative inhibitory site (Gln or Leu), which for rWSZ2b was at the overlapping site P2 Leu (Fig. 4). From these experiments we conclude that inhibition of chymotrypsin by each of the recombinant wheat serpins was irreversible under the experimental conditions used. Second order association rate constants \( k_a \) and SI values were determined (Fig. 5 and Table I) according to the kinetic model used previously (7). The fastest inhibitor of chymotrypsin was rWSZ1c \( (k_a \sim 10^6 \text{M}^{-1}\text{s}^{-1}) \). rWSZ1a (P2-P1′ Leu-Gln-Gln) and rWSZ2a (P2-P1′ Leu-Leu-Ser) were equally efficient inhibitors of chymotrypsin \( (k_a \sim 10^5 \text{M}^{-1}\text{s}^{-1}) \), whereas slow inhibition \( (k_a \sim 10^3 \text{M}^{-1}\text{s}^{-1}) \) was observed for rWSZ2b at P2 Leu.

**Insert Table I**

Similar kinetic experiments were made with cathepsin G, the other proteinase inhibited by each of the recombinant serpins (Table I). rWSZ2a inhibited cathepsin G and chymotrypsin with equal efficiency \( (k_a \sim 10^5 \text{M}^{-1}\text{s}^{-1}) \). SDS-stable complexes between intact, as well as partially auto-degraded, cathepsin G were detected (Fig. 5) as previously shown for rBSZx (7), and cleavage was observed only at P1 Leu. Cathepsin G can cleave substrates with both chymotrypsin-like and trypsin-like specificity (28). Inhibition of cathepsin G by rWSZ2b was slow \( (k_a \sim 10^3 \text{M}^{-1}\text{s}^{-1}) \), as found for inhibition of chymotrypsin, and only small amounts of SDS-stable complex were observed. In this experiment, cleavage was detected at P1 Arg (~50%), P2 Leu (~45%) and P5 Lys (~5%), and the observed SI ~1.6 may indicate that inhibition occurred at both of the overlapping sites P1 and P2.
The three WSZ1 serpins were relatively slow inhibitors of cathepsin G \( (k_a \sim 10^3 \text{ M}^{-1}\text{s}^{-1}) \). SDS-stable complexes were observed only after incubation with a five- to 10-fold molar excess of inhibitor.

The main cleavage sites observed in these experiments were at P₃ Lys and P₄ Met, whereas only 10-40% was cleaved at P₁ Gln/Leu (Fig. 4).

Results of the kinetic experiments (Table I) include estimates of SI varying between 1.0 and 10, indicating substrate cleavage in most cases according to Scheme I. However, the SI values above unity may in part be due to experimental problems. The serpin concentration determined by amino acid analysis may include inactive or latent molecular forms and errors may be introduced by long incubation times (up to 24 h) necessary when \( k_a \sim 10^3 \text{ M}^{-1}\text{s}^{-1} \). Only in the experiments with cathepsin G and rWSZ1a was extensive substrate cleavage observed (SI \sim 10). In the remaining kinetic experiments with cathepsin G, values for SI were 1-2, similar to those obtained in the parallel experiments with chymotrypsin. Thus the substantial cleavage at several RCL sites observed in the complex formation experiments with cathepsin G (Fig. 4) was not considered to be a determining factor in the kinetic experiments, which were performed with much lower proteinase concentrations.

Additional kinetic experiments were performed with rWSZ2a (vs. pancreas elastase) and rWSZ2b (vs. plasmin and trypsin). Initial titrations of pancreas elastase with rWSZ2a indicated an SI \sim 20, and attempts to demonstrate complex formation were unsuccessful. RCL cleavage was observed at P₄ Ala (\sim 50%), P₁⁺ Ser (\sim 25%), P₃ Val (\sim 15%) and P₂⁺ Ala (<10%), but not at P₁ Leu (Fig. 4). However, despite this substrate cleavage at several sites in the RCL, rWSZ2a was found to be a relatively fast inhibitor of pancreas elastase \( (k_a = 1.2 \times 10^4 \text{ M}^{-1}\text{s}^{-1}) \) in kinetic experiments using...
a 20-fold excess of inhibitor. Inhibition of plasmin with rWSZ2b was found to be irreversible in substrate dilution experiments. While cleavage was observed only at P$_1$ Arg, the size and heterogeneity of plasmin prevented complex formation from being detected. Initial titration experiments suggested an SI >10, and using a 40-fold excess of inhibitor in kinetic experiments, inhibition was found to be very slow ($k_a \sim 8 \times 10^2$ M$^{-1}$s$^{-1}$).

When trypsin (75 nM) was incubated with a 15-fold excess of rWSZ2b for 5 min at 22 °C and diluted 1200-fold with 0.25 mM substrate, complete inhibition was initially observed, but all enzyme was gradually released during 25 min continued incubation. This dilution effect is characteristic of slow-binding reversible inhibition, a finding further supported by progression curve experiments where trypsin was either added to a mixture of rWSZ2b and substrate (Fig. 6a) or incubated with inhibitor for various times before addition of substrate (Fig. 6b). Both experiments resulted in biphasic curves characteristic of slow reversible inhibition with relaxation to a steady-state (linear) release of product after ~10 min. To test whether this behaviour was due to very slow formation of irreversible complex after the initial reversible step, the reaction mixture with rWSZ2b and trypsin (Fig. 6b) was further incubated for 1, 2 and 4 h at 22 °C. In these assays, linear progression curves corresponding to the uninhibited reaction were observed. After incubation of trypsin with a two-fold molar excess of rWSZ2b at 0 °C or 22 °C for 10 min followed by boiling with SDS, no complex formation but substantial cleavage solely at P$_1$ Arg was observed. These findings may support slow complex formation combined with a high SI or rapid breakdown of the covalent complex. However, in the initial cleavage experiment (Fig. 1) with catalytic amounts of trypsin, WSZ1a-c and WSZ2a were cleaved at P$_3$ Lys. This site is also present in WSZ2b, but cleavage was detected only at P$_1$ Arg. A slow cleavage at P$_3$ Lys followed by faster release of the P$_1$-P$_4$ peptide may partly explain the experimental results. Based on the experimental data of Fig. 6
and a calculated $K_m = 0.15 \text{ mM}$ for hydrolysis of methyl-Gly-Pro-Arg-$p$-nitroanilide by trypsin, the equilibrium constant for the initial reversible complex formation was estimated to be $K_s \sim 2 \text{ nM}$. 
DISCUSSION

Six serpins with distinct reactive centers have been separated and identified among the major albumins in mature grain of hexaploid bread wheat (Fig. 1). Five of these serpins have been cloned, expressed in *E. coli*, and purified for characterization of their inhibitory specificity and elucidation of their physiological role(s) in the grain. Based on the classification of barley serpins into three subfamilies, BSZ4, BSZ7 and BSZx, sharing ~75% amino acid identity, the sequences obtained in the present study (Fig. 2) suggested a classification of the five cloned wheat serpins into two new subfamilies, WSZ1 and WSZ2 (Fig. 3).

The inhibitory specificity of a serpin is determined mainly by the sequence surrounding the RCL scissile bond cleaved during inhibition, especially the identity of the P1 residue (1). Major differences between the barley and wheat serpin sequences are found in the RCL. The three barley serpins contain RCL inhibitory-site residues commonly found in animal serpins, i.e. Arg, Leu or Met in P1 and a conserved Ser in P1′ (1). Surprisingly, two of the five cloned wheat serpins contain P1-P1′ Gln-Gln, and two others have P1′ Gln, as confirmed by studies of inhibitory specificity and complex formation (Figs 4 and 5). None of the >200 sequenced serpins of animal or viral origin contains a Gln in the P1 position, and only one inhibitory serpin, from the insect *Manduca sexta* (29), contains a Gln in P1′. Thus it was relevant to determine the inhibitory specificity of the wheat serpins with a selection of serine proteinases with well-characterized specificity. Comparing the $k_a$ values for inhibition of chymotrypsin, especially with WSZ1a, WSZ1b and WSZ2a (Table I), suggests that serpins with Gln in P1 and/or P1′ are inhibitors as efficient as WSZ2a with P1-P1′ Leu-Ser, a reactive center found in many mammalian serpins, including $\alpha_1$-antichymotrypsin (1). Notably, WSZ1a and WSZ2a had very similar association rates ($k_a \sim 10^5 \text{M}^{-1}\text{s}^{-1}$), and WSZ1c was the fastest inhibitor of chymotrypsin ($k_a \sim 10^6 \text{M}^{-1}\text{s}^{-1}$). A correlation between $k_a$ values for inhibition of chymotrypsin and cathepsin G was not obvious (Table I), but the fastest inhibitor of cathepsin G
was WSZ2a with $P_1'$ Ser ($k_a \sim 10^5 \text{ M}^{-1} \text{s}^{-1}$). As found for barley BSZx (7), WSZ2b could inhibit proteinases at overlapping sites in the RCL. Plasmin was irreversibly inhibited at $P_1$ Arg, chymotrypsin at $P_2$ Leu and cathepsin G possibly at both sites. In contrast, inhibition of trypsin at $P_1$ Arg was found to be reversible after incubation for <10 min (Fig. 6). A slow formation of stable complex combined with a high SI or cleavage at $P_3$ Lys may explain the experiment results. Reversible inhibition has been previously associated with $\alpha_2$-antiplasmin, the primary plasmin inhibitor of the blood coagulation system, which inhibits plasmin irreversibly at $P_1$-$P_1'$ Arg-Met but trypsin reversibly at the same site (30). WSZ2a could also inhibit pancreas elastase, but due to substrate cleavage at several sites, the SI value was high (>20) and the inhibitory site, presumably at $P_1$ Leu, could not be confirmed.

Prolamines form the gluten protein fraction of crucial importance for breadmaking. The Pro- and Gln-rich prolamines account for approximately half the total grain nitrogen, and include the $\alpha$-, $\gamma$-, and $\omega$-gliadins and the LMW and HMW subunits of polymeric glutenin (31, 32). The residue Gln, and more specifically, the Gln-Gln sequence, is found in abundance in the repetitive sequences of the prolamine wheat grain storage proteins. The reactive centers of many of the serpins resemble these repetitive sequences. The $P_2$-$P_1'$ sequence of WSZ1b (Fig. 4) appears twice in the repeated octapeptide sequence PQQPFPQQ in the N-terminal domain of $\gamma$-gliadin. Similarly, the $P_2$-$P_1'$ sequence of WSZ1a is identical to the last three residues of the repeat motif GYYPTSLQQ in the HMW subunit of glutenin, which also contains repeats of PGQGQQ and related sequences (31, 32). Most obvious is the similarity between the putative $P_2$-$P_3'$ sequence QQQMPIV of WSZ3 and sequences such as QQQIPIV present in repeats of LMW glutenin. The $P_4'$-$P_5'$ Pro-Pro sequence found in all five wheat serpins is also common in wheat prolamine repeats. During evolution, the wheat serpins may have adapted to inhibit proteinases involved in storage protein breakdown, either those from grain pests or those endogenous to the grain. Another possibility is that the RCL of the
serpins acts as a substrate for a physiologically relevant conversion of the serpins from the native, stressed form to the cleaved, relaxed form, as shown recently for antithrombin (33).

To our knowledge, no chymotrypsin-like serine proteinases, or their genes, have been identified in plants. The plant serine proteinases with the most well-characterized specificity are cucumisin from ripe melon mesocarp (34) and hordolisin from barley green malt (23). These plant subtilisin-like enzymes have a broad substrate specificity similar to that of bacterial subtilisins, but no inhibition was observed after incubation of either of the two enzymes with each of the five serpins. A few serpins display cross-class inhibition of cysteine proteinases (3), the most well-characterized being the inhibition at pH 5.5 of the lysosomal cysteine proteinases cathepsins K, L and S by human squamous cell carcinoma antigen 1 (SCCA1) (3). In wheat and barley, homologous cysteine proteinases with optimal activity slightly below pH 5 play a central role in degradation of the prolamine storage proteins during germination (22). The specificity of barley malt cysteine proteinase EP-B is determined mainly by a large hydrophobic residue in P2 of the substrate, and Gln is among the readily accepted residues at P1. One of the preferred cleavage sites for EP-B in the highly repetitive prolamine sequences is LQ↓Q, and the most abundant site is PQ↓Q (22). Thus, the reactive centers of the wheat serpins should represent a selection of baits for EP-B, but none of the serpins inhibited EP-B at pH 5.0.

The RCL sequences of certain mammalian serpins have been shown to accumulate amino acid substitutions at a higher rate than the rest of the sequence during evolution (35). The presence of multiple α1-proteinase inhibitors with different target enzyme specificities in some animals has been suggested to constitute a defence response to exogenous proteinases from infectious parasites or predators, which developed by accelerated evolution of the RCL sequence (35). Plant seeds, including cereal grains, contain numerous LMW protein inhibitors of proteinases (32, 36). The majority of these inhibitors are abundantly present, and in most cases endogenous target enzymes
have not been identified. The LMW inhibitors of serine proteinases in cereal grains are efficient inhibitors of subtilisin-like proteinases from microbes and/or chymotrypsin-like digestive proteinases of insects, and there is growing evidence for their participation in an integrated broad spectrum defence system against invading fungal or insect pests (32, 37). The repertoire of at least six distinct reactive centers in the abundant serpins of wheat grain may indicate that these inhibitors have similar functions. Kinetic studies showed that while barley BSZx was a fast inhibitor of subtilisins, the inhibition was inefficient, as ≥98% of the serpin was cleaved via the substrate pathway (Scheme I) (7). In the present study no inhibitory activity was found towards a fungal trypsin and a bacterial subtilisin, and it appears unlikely that the serpins are central components in defence against microbial attack of the grain.

Many proteinase inhibitors, as well as inhibitors of other hydrolases, have been demonstrated to reduce the activity of insect digestive enzymes in vitro (37). Notably, grains of cereals, including wheat, contain specific insect α-amylase inhibitors which do not affect plant or mammalian amylases (32, 38). Additional evidence for protection of seeds against insect infestation comes from feeding tests using isolated proteinase inhibitors or seed lines with markedly different levels of inhibitor expression, as well as from studies using transgenic plants (32, 37). Notably, a serpin from the hemolymph of the insect *Manduca sexta*, as well as active-site engineered variants of the same protein, have been heterologously expressed in several plants at levels up to 0.1% of total plant protein and shown to limit plant damage by insect pests in some experiments (39).

The storage tissues of plant seeds are attractive feed sources for many insects. Evolutionary adaption of the proteolytic digestion system of some insects to efficient degradation of the abundant glutamine- and proline-rich repetitive structures of the cereal grain prolamines seems likely to have occurred. Here we have shown that the reactive centers of wheat grain serpins contain unique glutamine-rich sequences resembling repetitive sequences of wheat prolamines. A working
hypothesis for further studies to elucidate the functions of the grain serpins might be that the RCL sequences have evolved into a complement of baits for irreversible inactivation of serine proteinases, and possibly also cysteine proteinases, from the digestive systems of insect pests, resulting in reduction of damage to seeds and thus in their increased survival.

Acknowledgements—We thank Tran Duc Tuan Tung, Charlotte Koutras, Mette Madsen, Susanne Blume and Bente Isbye for excellent technical assistance; Dr Lars I. Hellgren for preliminary serpin cloning; Dr Lars C. Petersen, Novo Nordisk, for initial screening of rWSZ2b with the blood coagulation enzymes; and Dr David J. Simpson, Carlsberg Research Laboratory, for samples of barley proteinases and their fluorometric substrates.
REFERENCES


Footnotes

*This study was supported by Grant 9601066 from the Danish Agricultural and Veterinary Research Council.

The nucleotide/protein sequences reported in this paper have been deposited in the EMBL/GenBank™ database under accession numbers Z49890 (WSZ1a), Y11485 (WSZ1b), AJ245878 (WSZ1c), AJ245879 (WSZ2a), Y11486 (WSZ2b). Other important sequences referred to have accession numbers P01009 (α₁-PI), X97636 (BSZ4), CAA64599 (BSZ7), Z15116 (BSZx).

¶ Current address: Department of Microbiology, Technical University of Denmark, DK-2800 Lyngby, Denmark.

| To whom correspondence should be addressed. Fax: 45-45886307; E-mail: hejg@mimer.be.dtu.dk.

¹ The abbreviations used are: Abz, 2-aminobenzoyl; α₁-PI, α₁-proteinase inhibitor (α₁-antitrypsin); BSZ, barley serpin (Z family); DTT, dithiothreitol; HMW, high-molecular-weight; LMW, low-molecular-weight; Ni-NTA, nickel nitrilotriacetic acid; r (prefix), recombinant; RCL, reactive center loop; SI, stoichiometry of inhibition; sTF, soluble domain of tissue factor; uPA, urokinase type-plasminogen activator; WSZ, wheat serpin (Z family).
Figure legends:

FIG. 1. **Major serpins of wheat grain.** Serpins in thiol extracts of Kadet (lane 4), Chinese Spring (lane 5) and Hereward (lane 6) wheat grain flour were separated by native PAGE and detected by silver staining. A Hereward native PAGE blot was immunostained with polyclonal antibodies raised against rBSZx (lane 1) and rWSZ1a (lane 2), and a monoclonal BSZ7 antibody (lane 3). The major serpins from the thiol extract of Hereward grain were partially purified by thiophilic chromatography followed by anion exchange chromatography. Fractions representing the major bands of the native PAGE pattern were cleaved with trypsin or chymotrypsin in the RCL. After SDS-PAGE, electroblotting to PVDF membrane and staining, the ~4 kDa C-terminal cleavage product bands were excised and subjected to five cycles of N-terminal sequencing. ↓ indicates cleavage by trypsin and ↑ by chymotrypsin. The previously identified reactive center of WSZ1a is marked by /GD8/. The serpin nomenclature is based on comparison of the amino acid sequences obtained after cloning (Figs 2 and 3).

FIG. 2. **Amino acid sequence alignment of the five cloned wheat serpins.** The sequence for WSZ1a is given in full. Dots indicate identity to WSZ1a, and for human α₁-proteinase inhibitor (α₁-PI), to at least one of the wheat serpins. Dashes indicate a gap. The active site residues at the P₁ and P₁’ positions are shown in bold. Numbers on the right correspond to residues for the individual sequences. The sequence for α₁-PI, beginning with residue 13, and the corresponding standard serpin template residue numbering, are shown below for comparison. The alignment was produced using ClustalX (40). Amino acid sequences confirmed by partial sequencing of the serpins purified from wheat grain are shown with a grey background.

FIG. 3. **Putative evolutionary relationship among the cereal serpins.** (a) Unrooted tree showing the putative phylogenetic relationship among barley and wheat serpins. The amino acid sequence
alignment was done using ClustalX (40). The phylogenetic tree was constructed using a bootstrap value of 1000 and visualized using TreeView 1.5.2. (b) Percent amino acid sequence identity between the cereal serpins.

**FIG. 4. Reactive center loop sequences and proteinase cleavage sites.** RCL sequences of the wheat serpins are compared between the conserved residues P₈ and P₉. After incubation of serpin with proteinase to demonstrate formation of SDS-stable complexes (Fig. 5), the released ~4 kD C-terminal fragments were sequenced to determine the cleavage site(s). Reactive site cleavage: \(\text{\textbullet}\), chymotrypsin and cathepsin G;\(\text{\textleftarrow}\), trypsin, plasmin and cathepsin G. Other cleavage sites discussed in the text: \(\text{\textdownarrow}\), cathepsin G; \(\text{\textuparrow}\), pancreas elastase; \(\text{\textrightarrow}\), barley cysteine endopeptidase EP-B. Substrate cleavage with trypsin at P₅ Lys is shown in Fig. 1.

**FIG. 5. Irreversible inhibition of chymotrypsin and cathepsin G by rWSZ2a.** Residual enzyme activity was measured after incubation of proteinase with serpin at pH 7.4 and 22 °C. Chymotrypsin (38 nM) was incubated with rWSZ2a (83 nM) (O). Cathepsin G (44 nM) was incubated with rWSZ2a (44 nM) (●). The second order association rate constant \(k_a\) and the stoichiometry of inhibition (SI) were determined as described previously (7). The curves depicted are best fits and the results are included in Table I. Insert, Formation of complexes between rWSZ2a and the two proteinases. rWSZ2a (25 pmoles) was incubated at a molar ratio of ~2:1 with proteinase for 20 min at 22 °C prior to addition of Tricine SDS-PAGE sample buffer and boiling for 5 min. After SDS-PAGE in 10-20% Tricine gels, the proteins were transferred to PVDF membrane and stained with Coomassie Blue. rWSZ2a + chymotrypsin (lane 1); rWSZ2a alone (lane 2); rWSZ2a + cathepsin G (lane 3); molecular mass standards (lane 4). C, intact serpin-proteinase complex; C\(^{\dagger}\), complex with partially degraded cathepsin G (7); I, intact inhibitor; I\(^*\), cleaved inhibitor; E, residual enzyme.
Fig. 6. **Reversible inhibition of trypsin by rWSZ2b.** (a) Trypsin (0.15 nM) was mixed with rWSZ2b (14 or 42 nM) and 0.24 mM substrate in Tris buffer, pH 8.6, at 22 °C. After 20 s incubation, hydrolysis (monitored at 405 nm) was recorded for 40 min. An enzyme control (0 nM) was included for calculation of $K_i$. (b) Trypsin (5.7 nM) and rWSZ2b (55 nM) were mixed in Tris buffer, pH 8.6. After 30, 60 and 300 s at 22 °C, an aliquot was diluted nine-fold in 0.5 mM substrate and hydrolysis recorded for 20 min. An enzyme control (0 s) was included.
TABLE I.

Inhibition of chymotrypsin and cathepsin G by recombinant wheat serpins.

The second order association rate constant ($k_a$) and stoichiometry of inhibition (SI) for inhibition of bovine chymotrypsin and human cathepsin G at 22 °C were determined by fitting the experimental data to the expression for residual proteinase activity, as described in detail previously (7).

<table>
<thead>
<tr>
<th>Serpin</th>
<th>$P_2P_1P_1'$</th>
<th>Chymotrypsin</th>
<th>Cathepsin G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_a$ (M$^{-1}$s$^{-1}$)</td>
<td>SI</td>
<td>$k_a$ (M$^{-1}$s$^{-1}$)</td>
</tr>
<tr>
<td>rWSZ1a</td>
<td>L Q Q</td>
<td>$1.1 \times 10^5$</td>
<td>1.3</td>
</tr>
<tr>
<td>rWSZ1b</td>
<td>P Q Q</td>
<td>$1.3 \times 10^4$</td>
<td>1.5</td>
</tr>
<tr>
<td>rWSZ1c</td>
<td>L L Q</td>
<td>$1.3 \times 10^6$</td>
<td>1.9</td>
</tr>
<tr>
<td>rWSZ2a</td>
<td>L L S</td>
<td>$1.6 \times 10^8$</td>
<td>2.3</td>
</tr>
<tr>
<td>rWSZ2b</td>
<td>L R Q</td>
<td>$2.5 \times 10^3$</td>
<td>3.2</td>
</tr>
</tbody>
</table>

aData from (7).
Scheme I

\[ E + I \xrightarrow{k_s} E + I^* \]
\[ E + I \xrightarrow{k_a} EI^* \]
\[ EI^* \xrightarrow{} E + I^* \]
Figure a shows the absorption at 405 nm (A₄₀₅) vs. time (s) for different concentrations of a substance: 0 nM, 14 nM, and 42 nM. The slope of the lines indicates the rate of increase in absorbance with time.

Figure b shows the absorption at 405 nm (A₄₀₅) vs. time for different time points: 0 s, 30 s, 60 s, and 300 s. The curves illustrate the decrease in absorbance over time at each time point.
Inhibitory Serpins from Wheat Grain with Reactive Centers Resembling Glutamine-Rich Repeats of Prolamine Storage Proteins Cloning and Characterization of Five Major Molecular Forms
Henrik Ostergaard, Soren K. Rasmussen, Thomas H. Roberts and Jorn Hejgaard

J. Biol. Chem. published online June 28, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M004633200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2000/06/28/jbc.M004633200.citation.full.html#ref-list-1