Heterologous Expression of Three Plant Serpins with Distinct Inhibitory Specificities*

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For the first time, inhibitory plant serpins, including WSZ1 from wheat, BSZ4, and the previously unknown protein BSZx from barley, have been expressed in Escherichia coli, and a procedure for fast purification of native plant serpins has been developed. BSZx, BSZ4, and WSZ1 were assayed for inhibitory activity against trypsin, chymotrypsin, and cathepsin G, and cleavage sites in the reactive center loop were identified by sequencing. BSZx proved to be a potent inhibitor with overlapping reactive centers either at P1 Arg for trypsin or at P2 Leu for chymotrypsin. At 22 °C, the apparent rate constant for chymotrypsin inhibition at P2 (k a = 9.4 × 10^5 m⁻¹ s⁻¹) was only four times lower than for trypsin at P1 (k a = 3.9 × 10^6 m⁻¹ s⁻¹), and the apparent inhibition stoichiometries were close to 1. Furthermore, our data suggest that cathepsin G was inhibited more favorably at P2 Leu. BSZ4 inhibited cathepsin G (k a = 3.9 × 10^6 m⁻¹ s⁻¹) at both P1 Arg and P2 Leu. These results indicate a unique adaptability of the reactive center loop of BSZx. WSZ1 inhibited chymotrypsin (k a = 1.1 × 10^5 m⁻¹ s⁻¹) and cathepsin G (k a = 7.6 × 10^6 m⁻¹ s⁻¹) at P1 Gln and not, as for BSZx, at the more favorable P2 Leu. BSZ4 inhibited cathepsin G (k a = 2.7 × 10^6 m⁻¹ s⁻¹) at P1 Met but was hydrolyzed by trypsin and chymotrypsin. The three plant serpins formed stable SDS-resistant complexes with the proteases in accordance with the kinetic data.

The serpins constitute a superfamily of serine protease inhibitors with regulatory properties. Most serpins may interact both as substrates and as suicide inhibitors forming inhibitor-proteinase complexes, which are unusually stable toward SDS, urea, and other denaturants. In mammals, serpins are the main proteinase inhibitors controlling complex activation, blood coagulation, fibrinolysis, and many other physiological processes. Insect and viral serpins with defined inhibitory roles have also been described (1, 2).

Since the first evidence for presence of serpins in plants appeared a decade ago (3), several immunochemically related serpins have been purified from cereal grain and characterized with respect to molecular properties (4–6), gene structure, and expression (7, 8). Molecular stability of the reactive site cleaved form of the major barley serpin BSZ4 toward extreme pH, high temperatures, and proteinases (4) as well as a weak, time-dependent inhibitory action of the native serpin BSZ7 toward chymotrypsin (5) indicated that plant serpins might undergo conformational transitions characteristic of inhibitory serpins. Only recently, an inhibitory wheat serpin WSZ1, which forms SDS-stable complexes with chymotrypsin at the unusual P1 residue Gln, was identified (6), and the gene was cloned (9). Serpins have not been described from other plants or from other cereal tissues, but a barley gene for a putative serpin BSZx, which could not be detected in the grain, has been identified and sequenced (8).

Isolation of intact, homogeneous plant serpins from barley and wheat grain has suffered from difficulties, mainly due to: (a) proteolytic activity in the grain extracts, (b) the presence of several isoforms, (c) formation of aggregates, and (d) instability at pH < 6 (4–6). In order to obtain native, homogeneous plant serpins for systematic studies of interactions with serine proteinases, BSZ4, BSZx, and WSZ1 were selected for heterologous production on basis of their different putative P1 residues (Arg, Met, and Gln, respectively). Because BSZ4 and WSZ1 contain N-glycosylation sites but are not glycosylated in the plant, an Escherichia coli expression system was selected. After isolation, novel inhibitory properties could be assigned to all three serpins. Interestingly, BSZx, which has not been detected in the plant, was found to be a potent inhibitor with overlapping reactive sites at either P1 Arg (trypsin) or P2 Leu (chymotrypsin).

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, Klonef fragment, calf intestinal phosphatase, and T4 DNA ligase were obtained from Amersham Corp., Promega, and Pharmacia Biotech Inc. AmpliTag was from Perkin-Elmer, and oligonucleotides were from DNA Technology (Aarhus, Denmark). Ampicillin, chloramphenicol, trans-epoxysuccinyl-l-leucylamidomethyl-4-guanidino)butane, EDTA, isopropyl-b-D-thiogalactoside, phenylmethanesulfonyl fluoride, and Tween 20 were obtained from Sigma, and polyvinylidene difluoride membrane from Millipore. Complete protease inhibitor tablets were from Boehringer Mannheim, Ni-NTA-agarose was from Qiagen, and gel filtration media and Resource Q column were from Pharmacia. Porcine trypsin, human leukocyte cathepsin G, and leech eglin C were gifts from Novo Nordisk (Bagsvaerd, Denmark), and bovine a-chymotrypsin was obtained from Sigma. The substrates N-p-tosyl-Gly-Pro-Arg-p-nitroanilide and succinyl-Ala-Ala-Pro-Phe-p-nitroanilide were obtained from Sigma and Bachem, respectively. Other chemicals used were of analytical grade.

BSZ4 cDNA Isolation and Characterization—A cDNA library of mRNA from immature barley (Hordeum vulgare L., cv Bomi) endosperm (10) was screened using a partial BSZ4 cDNA. A full-length BSZ4 clone pc05B01 was sequenced in its entirety on an Applied Bio- systems 373 DNA sequencer. Sequence reactions were performed using

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X97636.

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1 The abbreviations used are: Ni-NTA, nickel nitrolotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.
the Dy terminator cycle sequencing FS kit (Perkin-Elmer) according to the manufacturer's protocol. Both strands were sequenced using internal primers. The sequence data were analyzed using the GeneWorks and Wisconsin Genetics Computer Group software packages. The protein sequence predicted from the cDNA represents a variant of that predicted from the genomic BSZ4 clone (7). The cDNA encoded BSZ4 differs in three positions: Arg^24→Ala, I^175→Val, and Ala^57→Val (EMBL accession number Z49890) and a genomic clone from a cross between barley cv. Hiproly and cv. Villa encoding BSZ4 (EMBL accession number Z15116) have been presented previously (8, 9).

Expression and Purification—Heterologous WSZ1, BSZ4, and BSZx were produced using the pET3d/E. coli BL21(DE3) pluS expression system (Novex). The transformed cells were grown in conical flasks with two baffles containing superbroth (12) with 100 μg/ml ampicillin and 20 μg/ml chloramphenicol. 1 or 2 liters of medium was inoculated with 2% (v/v) of overnight culture at 37°C. At an optical density of 2.5 at 600 nm, the broth was cooled to 15°C in ice water, and expression was induced with 0.5 mM isopropyl thiogalactoside for 18 h. The cell was harvested by centrifugation at 5,000 g for 10 min and resuspended in 10 ml of 50 mM Tris-HCl, pH 8.0, 0.1% Tween 20, and 5 mM EDTA. Half of a Complete inhibitor tablet was added prior to lysis by French pressure, and the cell supernatant was isolated by centrifugation at 40,000 g for 15 min. Subsequently, EDTA and low molecular weight thiols compounds were removed by gel filtration on a 2.5 x 40-cm G-25 column equilibrated in 50 mM Tris-HCl, pH 8.0, and 0.5 mM NaCl at a flow rate of 1 ml/min. The product was collected in the first 30 ml after void volume. The eluate was batch incubated at 5°C for 1 h with 2 ml of Ni-NTA-agarose previously charged with 100 μM NiCl2 and equilibrated in the G-25 buffer. The resin was loaded into a 1.0 cm inner diameter column and washed with approximately 20 column volumes of the equilibration buffer at a flow rate of 0.5 ml/min before retained protein was released with equilibration buffer containing 10 mM EDTA. The Ni-NTA eluate was loaded on a G-100 column after addition of half of a Complete inhibitor tablet and 10 mM diethiothreitol. The serpins eluted at ~1.9–2.4 x void volume, and the exact location of the inhibitor serpin was determined by microriter plate inhibition assay or, before the inhibitory activities were discovered, by Western blotting using polyclonal antibodies (4, 6). BSZx cross-reacted with antibodies raised against BSZ4. Fractions containing active serpin were pooled, reduced with diethiothreitol, and subjected to anion exchange on a Resource Q column mounted on a fast protein liquid chromatography apparatus (Pharmacia) (see legends to Figs. 3 and 4 for details).

Inhibition Assays—For screening of fractions during purification, assays for trypsin and chymotrypsin in inhibitory activity were performed in microtiter plates as described previously (13). Determinations of association rate constants were performed by preincubating approximately equimolar amounts of enzyme and inhibitor for 0.25–16 min at either 0 or 22°C in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20, and 10 mM CaCl2, before mixing aliquots of 100 or 200 μl with 800–900 μM of 0.55 mM Np-tosyl-Gly-Pro-Arg-p-nitroanilide (trypsin) or 0.55 mM succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (chymotrypsin and cathespin G) in a 1 ml cuvette. The dilution of reactants and competition with substrate effectively stopped further complex formation, and the linear change in absorbance at 405 nm was recorded for 1–2 min. The concentrations of active trypsin and chymotrypsin were determined by active site titration (14, 15), and active cathespin G was determined relative to chymotrypsin by titrations with eglin C. The concentration of purified serpin was determined by amino acid analyses using cation exchange chromatography and postcolumn derivatization with o-phthaldialdehyde (16).

Complex Formation and Determination of Cleavage Sites—Enzyme-serpin complexes were formed by incubation of serpin with proteinase in 20 mM Tris-HCl, pH 6.6, 100 mM NaCl for 5 min (trypsin and chymotrypsin) or 15 min (cathespin G) at 22°C. The preparations were subsequently boiled in reducing SDS-PAGE sample buffer and subjected to Tricine SDS-PAGE (17) using 10–20% precast gradient gels from Novex. The C-terminal fragments (~4 kDa) were separated from the complexes and the cleaved inhibitors during electrophoresis, and after blotting onto a polyvinylidene difluoride membrane (18), the active center loop sequences were determined by N-terminal amino acid sequencing on a Procise 494 from Applied Biosystems.

Kinetic Analysis of Inhibition Data—The kinetic assays were performed under second order conditions. Serpins interact with proteases as suicide inhibitors (2, 19), and a kinetic expression for inactivation of proteases must correct for the proportional (but not equimolar) decrease in the concentrations of free protease and free, uncleaved inhibitor during complex formation. Ignoring intermediate states, the suicide inhibition reaction scheme (Scheme I) may be simplified in which the enzyme E may interact with the inhibitor I either by cleaving it, which forms E*, or by participating in a complex EI that subsequently breaks down into native enzyme and cleaved inhibitor. In the cleavage reaction, complex formation and complex dissociation proceed with the apparent rate constants k_1 and k_2, respectively. Assuming that k_1 > k_2, the following stoichiometric links apply:

E + I ⇌ EI → E* + I

SCHEME 1

In which X_{in} (0 < X_{in} < 1) is the final fraction of protease in complex with the inhibitor; E, I, and C are the concentrations of free enzyme, free uncleaved serpin, and enzyme-serpin complex, respectively; and E^* and P are the corresponding concentrations at time 0. Accordingly, the rate of complex formation is given by:

\[
\frac{dC}{dt} = k_1 \cdot (E^* - C) \cdot P \cdot \left(1 - \frac{C}{E^* \cdot X_{in}}\right)
\]

(Eq. 3)

In which the time t and C are the only variables. Integration of Equation 3 results in Equation 4, which gives the relation between incubation time and complex concentration:

\[
C = E^* \cdot X_{in} \cdot \exp\left(1 - \frac{X_{in} \cdot P \cdot k_1 \cdot t}{X_{in} \cdot P \cdot k_1 \cdot t - X_{in}}\right) - 1
\]

(Eq. 4)

The right side of the equation contains two unknown parameters, k_1 and X_{in}. The fraction of inhibited protease, X_{in}, was determined either from the steady-state level of residual proteinase activity after complete turnover of the serpin or, as k_1, by nonlinear regression to the experimentally determined values of C. Regression was performed by automatic minimization of the root mean square deviation between the observed and calculated values for C. Finally, X_{in} was used to calculate the fraction of inhibitor that participate in complex formation, X_{in} (0 < X_{in} < 1):

\[
X_{in} = X_{in} \cdot \frac{E^*}{P}
\]

(Eq. 5)

RESULTS

Subcloning and Engineering—The region encoding WSZ1 was ligated into pET3d (11) as a Neo-BamHI fragment as previously outlined (9). Subsequently, a synthetic fragment coding for a new start codon and six histidine residues was generated by annealing the complementary oligonucleotides 5'-CATGCACCATCATCACATCA-3' and 5'-CATGTTGATGATGATGATG-5', and the fragment was inserted into the NcoI site overlapping the ATG start codon. A clone, pEH1, carrying a single insert of the six histidines in the correct orientation was identified by sequencing. The procedure for subcloning BSZ4 was more elaborate, because this clone did not contain a BamHI site downstream from the stop codon. First, the BSZ4 cDNA clone and the pET3d vector were cut with EcoRI or BamHI, respectively, and the newly generated termini were made blunt-ended with Klenow fragment. The BSZ4 cDNA and pET3d vector were then purified and cut with NcoI at the start codons, and the vector was dephosphorylated with alkaline phosphatase. Both preparations were purified on low melting agarose before ligation with T4 DNA ligase over night at 5°C. Positive clones were identified by restriction digest analyses (results not shown). Insertion of the synthetic
used to amplify the exon regions neighboring the intron. The 5′ overlap extension PCR (38). Primers a (5′CGTGGACTTCCAAACTAAGGCTCCTGAAGTTGCTGGTCA-3′) was digested with restriction enzyme SalI, extended, and finally amplified using primer band c. This PCR product was digested with SalI and HindIII and cloned into pBluescript KS for sequencing using T3 and T7 primers before the 194-base pair HinI and SalI sites, respectively (panel II). The fragment did not contain the downstream NcoI site, and the synthetic fragment encoding a histidine tag (indicated by 6xHis) could be inserted into the unique NcoI site at the start codon generating pET/His-5′. The downstream exon was subcloned as a SalI-NcoI fragment replacing the T7 RNA polymerase promoter region (indicated by T7) (step b). In the resulting construct, the intron was confined between unique SalI and BstXI sites, and this region was subsequently replaced by a PCR-generated SalI-BstXI fragment in which the intron had been deleted (see panel II). Finally, pET/His-5′ and pET/PCR-3′ were digested with AspI, and the expression vector pEHBx was generated by combining the fragments from pET/His-5′ and pET/PCR-3′ containing the intact upstream and downstream exons, respectively (step c). Panel II, removal of the intron by splicing overlap extension PCR (38). Primers a (5′GGTGAAGGATCTTGCCGTCG-3′) and b (5′GCGACCCTCCTCGGCGT-3′) and primers c (5′GTTGAAGATCTTGCCGTCG-3′) and d (5′CGAGACCCAGTCCGTTACCCAAAATTTGCGTTAGTTGCTGA-3′) were used to amplify the exon regions neighboring the intron. The 5′ end of primer d was complementary to the last 31 bases of the template strand of the upstream exon. Following digestion of the upstream and downstream PCR products with Ddel and AspI, respectively, and purification by agarose gel electrophoresis, the coding strand of the upstream and the template strand of the downstream PCR product were annealed, extended, and finally amplified using primer b and c. This PCR product was digested with SalI and HindIII and cloned into pBluescript KS for sequencing using T3 and T7 primers before the 194-base pair SalI-BstXI fragment was used to generate pET/PCR-3′.

Inhibitory Protein Z-type Plant Serpins—The serpin-enzyme association rates were measured at low inhibitor and proteinase concentrations and under second order conditions to slow down the association and facilitate characterization of the process. Trypsin was a likely target for BSZx due to an Arg residue in the putative P1 position, and inhibition of trypsin (Fig. 5) proceeded with a rate constant of 3.9 × 10^6 M^-1 s^-1 at 22°C (Table I). Interestingly, BSZx was also an inhibitor of chymotrypsin (k_a = 9.4 × 10^6 M^-1 s^-1) and of cathepsin G (k_a = 2.4 × 10^6 M^-1 s^-1) (Figs. 5 and 6). The rate constants for inhibition of trypsin and chymotrypsin were also measured at 0 °C, and whereas the rate for inhibition of chymotrypsin was largely unaffected by temperature, that for trypsin was decreased by a factor of 4 at 0°C (Fig. 5 and Table I). However, the fraction X_inh of complex forming BSZx was similar for trypsin and chymotrypsin at 22 °C (98%) and 0 °C (75-72%) (Table I). WSZ1 exhibited much faster inhibition of chymotrypsin (k_a = 1.1 × 10^6 M^-1 s^-1) than of cathepsin G (k_a = 7.6 × 10^6 M^-1 s^-1), and the apparent stoichiometry of inhibi-
tion was $1.2$ for chymotrypsin (i.e., $1.2$ mol of WSZ1 were turned over per mole of complex formed) and $10$ for cathepsin G. BSZ4 has a Met in the putative P$_1$ position but did not exhibit inhibitory activity against chymotrypsin or trypsin. However, cathepsin G was inhibited with a $k_a$ of $2.7 \times 10^4$ M$^{-1}$ s$^{-1}$ (Fig. 6 and Table I). Complex Formation and Loop Cleavage—The kinetic data were supported by formation of SDS-stable complexes (Fig. 7) and by partial characterization of the products by N-terminal sequencing of peptides after SDS-PAGE and transfer to polyvinylidene difluoride membranes. BSZx formed well defined complexes with trypsin, chymotrypsin, and cathepsin G (Fig. 7, lanes 2–4). In addition, partially degraded complexes and the products of reactive center cleavage, the large N-terminal fragments I*, and the C-terminal peptides P were observed. Sequencing of the complex C between BSZx and trypsin (Fig. 7, lane 2) resulted in the two expected N-terminal sequences in about equal amounts, and no other peptides were detected. The intermediate band C' contained the same two sequences but in amounts corresponding to a BSZx:trypsin molar ratio of $7:1$ and, in addition, several other unidentified minor sequences. Apparently, the relatively distinct C' band represents complex in which especially trypsin is partially degraded. In accordance with the kinetic data (Table I), BSZ4 only formed complex with cathepsin G, and as for BSZx, I*, C, and faint C' bands were observed (Fig. 7, lane 6). Complex formation between WSZ1 and chymotrypsin has been demonstrated previously (6, 9). C-terminal peptides ($4$ kDa) released by loop cleavage and SDS-PAGE were partially sequenced after similar experiments with all relevant serpin/proteinase combinations (Fig. 8).
The interaction of WSZ1 with chymotrypsin suggests that inhibition of cathepsin G takes place at both the over substrate by cathepsin G (Table I), the cleavage results of experiments with cathepsin G were more complex as in different mammalian serpin families (28–35%). The known plant serpin gene structures, i.e. the structures of the BSZ4 (7) and WSZ4 (8) genes, are identical with respect to the position of their single intron but differ from the gene structure of other serpins (22). Furthermore, none of the known plant serpins possess cleavable signal peptides in contrast to most mammalian serpins (7, 8). BSZ4, previously termed protein Z4, is the major endosperm serpin in barley and was the first plant serpin to be characterized (3, 4). For these reasons, we consider BSZ4 to be the archetype of a family of protein Z-type plant serpins that to date only contains members from barley (BSZ) and wheat (WSZ). However, inhibitory serpins have been detected in the related cereals rye and oats, and a database search clearly identified DNA fragments of plant origin representing (pseudo)genes for BSZ4-related serpins. The available cDNA, genomic DNA, and amino acid sequences indicate that the members of the protein Z-type plant serpin family have evolved from a common ancestor.

**DISCUSSION**

Expression of the plant serpins in E. coli has solved the problems with multiple isoforms and reactive center loop cleavage that were associated with purification from mature seed (4–6). In addition, we have obtained a new inhibitory barley serpin detected neither in the grain nor in vegetative tissues. The presented purification protocol results in rapid isolation of homogeneous, uncleaved plant serpins from E. coli and is a major improvement over a preliminary protocol published recently (9). Gel filtration of the preparation before anion exchange chromatography had a great impact on the purity and homogeneity of the product. A major fraction of the contaminating E. coli proteins formed colloids at the low ionic strength (20 mM Tris-HCl) and eluted near the column void volume, well separated from the serpins (Fig. 3). In addition, small amounts (−10%) of polymerized serpin, previously eluted over a wide sodium chloride gradient interval on the anion exchanger, were removed. The final ion exchange step served to concentrate the preparation and to remove trace contaminants (Fig. 4). WSZ1, BSZ4, and BSZ5 behaved similarly during purification, and no adjustment of the purification protocol was required. Due to the relatively low expression levels, high quality recombinant serpin preparations were only obtained in moderate yields. From one liter of fermentation broth, 0.5–1 mg of pure serpin was recovered.

Inhibitory activities of BSZx, BSZ4, and WSZ1 were tested against the selected putative target enzymes trypsin, chymotrypsin, and cathepsin G, and new activities were assigned to chymotrypsin as a substrate and equal amounts of peptides released by cleavage after P1 Met and P4 Leu were obtained. Reactive center loop cleavage of BSZ4 by cathepsin G occurred at P1 Met (−60%) and P4 Lys (−40%). Undoubtedly, P1 is the inhibitory site in BSZ4 and P4 is a substrate site. In contrast to results with chymotrypsin, no cleavage of BSZ4 was observed after P4 Leu (Fig. 8).

The Family of Protein Z-type Plant Serpins—Alignments of serpin sequences using ClustalW (21) show that BSZx, BSZ4, and WSZ1 are −70% identical with −60% of the residues being conserved in all three proteins. The sequence similarity between the plant serpins is at level with identities observed between antitrypsins as well as between antichymotrypsins (65–70%) but lower than for the more specific antithrombin III, plasminogen activator inhibitor 1, and leukocyte elastase inhibitor families (80–90%). The similarity between plant and animal serpins is comparable with the similarity between different mammalian serpin families (28–35%). The known plant serpin gene structures, i.e. the structures of the BSZ4 (7) and WSZ4 (8) genes, are identical with respect to the position of their single intron but differ from the gene structure of other serpins (22). Furthermore, none of the known plant serpins possess cleavable signal peptides in contrast to most mammalian serpins (7, 8). BSZ4, previously termed protein Z4, is the major endosperm serpin in barley and was the first plant serpin to be characterized (3, 4). For these reasons, we consider BSZ4 to be the archetype of a family of protein Z-type plant serpins that to date only contains members from barley (BSZ) and wheat (WSZ). However, inhibitory serpins have been detected in the related cereals rye and oats, and a database search clearly identified DNA fragments of plant origin representing (pseudo)genes for BSZ4-related serpins. The available cDNA, genomic DNA, and amino acid sequences indicate that the members of the protein Z-type plant serpin family have evolved from a common ancestor.

**FIG. 7.** Formation of SDS-stable serpin-proteinase complexes. Barley serpin was incubated with proteinase at 22 °C for 5 min (trypsin and chymotrypsin) or 15 min (cathepsin G) before the addition of reducing tricine SDS-PAGE sample buffer and boiling for 5 min. Lanes 1–4, 0.9 μg of BSZx incubated with enzyme. Lane 1, control; lane 2, 0.6 μg of trypsin; lane 3, 0.6 μg of chymotrypsin; lane 4, 0.6 μg of cathepsin G. Lanes 5 and 6, 0.8 μg of BSZ4 incubated with enzyme. Lane 5, control; lane 6, 0.6 μg of cathepsin G. The gel was stained with Coomassie Blue R-250. The positions of intact complexes (C), degraded complexes (C'), intact inhibitors (I), cleaved inhibitors (I'), and released C-terminal peptides (P) are indicated to the right.

| P1 | P10 | P2 | P3 | P4 | P5 | P9 |
|----|-----|----|----|----|----|----|
|    |     |    |    |    |    |    |
| Cons. | GTEAZAAATA*... -S...PE...... |     |     |     |     |     |
| BSZx | GTEAZAAAKARVTGAR-SLTVMPVVKVD |     |     |     |     |     |
| BSZ4 | GTEAZAXGTVMSGVM-V-SMPLKVLVLDV |     |     |     |     |     |
| WSZ1 | GTEAZAAATKRLIMALQ-QARPSPVMD |     |     |     |     |     |

**FIG. 8.** Reactive center loop sequences and cleavage sites. The interactions between the serpins BSZx, BSZ4, and WSZ1 and proteinases were investigated by N-terminal sequencing of C-terminal peptides released after incubation (P in Fig. 7). Cleavage sites identified for trypsin (T), chymotrypsin (C), and cathepsin G (G) are indicated by arrows. The consensus sequence based on all inhibitory serpins (Cons.) is shown at the top. An asterisk indicates conservation of hydrophobic residues, and a dash indicates the P3-P1 bond.

*J. Hejgaard, unpublished results.*
each of the serpins. BSZx is by far the most potent inhibitor of the enzymes investigated (Table 1), and the ease with which BSZx may interact with proteinases of different specificity at either its P1 Arg or P2 Leu is unique among serpins (Fig. 8). α2-Antiplasmin and C1 inhibitor have previously been shown to inhibit chymotrypsin by interaction at secondary, overlapping sites, but the rates of inhibition and the low ability of the inhibitors to form SDS-resistant complexes with chymotrypsin revealed a clear preference for their natural Arg-specific target proteinases (23–27). The second order association rate constant at 22 °C for inhibition of chymotrypsin with BSZx was only four times lower than the rate measured for trypsin, and at 0 °C the rates were very similar (Table 1). At 22 °C, the apparent stochiometries of inhibition of trypsin and chymotrypsin by BSZx were close to 1, and chymotrypsin readily formed a SDS-resistant complex with BSZx and did not interact at secondary substrate sites in the reactive center loop as reported for α2-antiplasmin (24). In addition, the present results with cathepsin G suggest that both P1 Arg and P2 Leu of BSZx may fit in an inhibitory mode in the less specific substrate pocket of this serine proteinase (20). Apparently, BSZx has a unique adaptability to serine proteinases of different specificity.

Loebermann et al. (28) were the first to show that the even-numbered reactive center loop residues from the cleavage site to P14 are accommodated within the serpin structure when the loop is embedded in the A-sheet. Complete loop insertion has only been observed for inhibitory serpins (29, 30) either in the cleaved form or in the latent state (31). It is currently believed that partial insertion of the hinge region (P14-P9) is required for inhibitory activity (32–36). The reactive center loop of BSZx has an Arg residue in the P6 position (Fig. 8), which is generally occupied by a hydrophobic residue in inhibitory serpins. Inspection of structures of cleaved serpins and identification by alignment of residues in BSZx that would get close to the P6 residue upon loop insertion have not uncovered compensating mutations that would permit or facilitate accommodation of the Arg in the core. This observation is in accordance with the assumption that only partial insertion is required for inhibition, but we have not investigated the impact of the P6 Arg residue on the stability of the complex or on the structural stabilization of BSZx upon cleavage. Previously, BSZ4 was not found to activate trypsin or chymotrypsin, (3) but the cleaved form was unaffected by incubation at pH 1 to 13 or at temperatures up to 100 °C (4), indicating that BSZ4 was stabilized by loop insertion and hence was a potential proteinase inhibitor (36). Our studies are in accord with these results. BSZ4 is a moderately good inhibitor of cathepsin G with a $k_{in} = 2.7 \times 10^4$ s$^{-1}$ and with an apparent stoichiometry of inhibition of 2.4. WSZ1 was the first plant serpin shown to form SDS-stable complexes with a serine proteinase, chymotrypsin (6, 9). We have now demonstrated that WSZ1 also possess inhibitory activity against human cathepsin G. Interestingly, WSZ1 formed complex with both chymotrypsin and cathepsin G at its P1 Gln and not, as observed for BSZx, at Leu in P2 (Fig. 8). Thus, the two closely related serpins BSZx and WSZ1 are very different in their proteinase interaction where conformational adaption between the proteinase reactive site and the bait loop is essential for formation of a stable complex (35, 36).

The plant serpins characterized in this study belong to a family of protein Z-type serpins that differ from other known serpins (37). It is concluded that serpins from cereals are to be regarded as potent inhibitors of serine proteinases of the trypsin family. In many aspects, BSZx is the most interesting of the known plant serpins, but preliminary immunochemical inves-

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3. It is hypothesized that BSZx promoters in transgenic tobacco using the uidA gene as reporter show expression in tobacco seed. If expressed, BSZx may have other functions than those proposed for the abundant grain serpins BSZ4, BSZ7, and WSZ1, i.e. either as storage proteins or as inhibitors directed against digestive proteinases of insect pests, and work is in progress to elucidate if BSZx has a regulatory function, e.g. associated with plant development or pollination.

3. S. K. Rasmussen, unpublished results.