Inhibition of coagulation factors by recombinant barley serpin BSZx

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Abstract Barley serpin BSZx is a potent inhibitor of trypsin and chymotrypsin at overlapping reactive sites (Dahl, S.W., Rasmussen, S.K. and Hejgaard, J. (1996) J. Biol. Chem., in press). We have now investigated the interactions of BSZx with a range of serine proteinases from human plasma, pancreas and leukocytes, a fungal trypsin and three subtilisins. Thrombin, plasma kallikrein, factor VIIa/tissue factor and factor Xa were inhibited by BSZx at heparin independent association rates (k_{ass}) of 4.5×10^3–1.3×10^5 M^{-1} s^{-1} at 22°C. Only factor Xa turned a significant fraction of BSZx over as substrate. Complexes of these proteinase with BSZx resisted boiling in SDS, and amino acid sequencing showed that cleavage in the reactive center loop only occurred after P_1 Arg. Activated protein C and leukocyte elastase were slowly inhibited by BSZx ($k_{ass}=1$–2×10^2 M^{-1} s^{-1}) whereas factor XIIa, urokinase and tissue type plasminogen activator, plasmin and pancreas kallikrein and elastase were not or only weakly affected. The inhibition pattern with mammalian proteinases reveal a specificity of BSZx similar to that of antithrombin III. Trypsin from *Fusarium* was not inhibited while interaction with subtilisin Carlsberg and Novo was rapid but most BSZx was cleaved as a substrate. Identification of a monoclonal antibody specific for native BSZx indicate that complex formation and loop cleavage result in similar conformational changes.

Key words: Complex formation; *Hordeum vulgare*; Plant serpin; Serine proteinase; Subtilisin

1. Introduction

Members of the serpin superfamily of serine proteinase inhibitors have been identified in higher animals, insects, viruses and plants [1]. Most mammalian serpins function as regulators of complex physiological processes such as blood coagulation, fibrinolysis and complement activation [2,3] whereas little is known about the biological activity and function of serpins from plants. Recently, three of the four identified plant serpins (BSZx [4] and BSZ4 [5] from barley and WSZ1 [6] from wheat) were expressed in *E. coli* and shown to possess inhibitory activities against trypsin (BSZx), chymotrypsin (BSZx and WSZ1) and cathepsin G (BSZx, BSZ4 and WSZ1) [7]. The atypical specificity of BSZx was due to overlapping reactive centers. Inhibition of trypsin at P_1 Arg proceeded with a second order rate constant of 3.9×10^6 M^{-1} s^{-1} and the rate of chymotrypsin inhibition, exclusively at P_2 Leu, was only four times lower. In both cases ~1:1 molar inhibition stoichiometry was observed. Previously, overlapping centers have only been observed in α_2- antiplasmin [8] and C1 inhibitor [9]. In the present study, interactions of BSZx with mammalian and fungal proteinases of the chymotrypsin family as well as bacterial proteinases of the subtilisin family have been characterized.

2. Materials and methods

2.1. Biological materials

BSZx was expressed from a genomic clone [4]. The genetic engineering, expression in *E. coli* and purification to homogeneity by a combination of metal-chelate chromatography on a Ni^{2+}-NTA column, gel filtration and anion exchange chromatography has been described recently [7]. Human urokinase-type plasminogen activator (u-PA) and porcine pancreas elastase were from Serva and human pancreas kallikrein and leech eglin C from Sigma. Human antithrombin III (AT-III) and plasmin were from Kabi and human plasma kallikrein and human factor Xa were gifts from Dr. I. Schousboe, Copenhagen, Denmark, and Dr. W. Kisiel, Albuquerque, NM, USA, respectively. All other enzymes, recombinant soluble domain of human tissue factor (sTF) and heparin were gifts from Novo Nordisk. Mono- and polyclonal antibodies towards BSZ4 [10] and BSZ7 [11] from barley grain and a polyclonal antibody against a pool of wheat serpins [12] were produced and purified according to standard procedures.

2.2. Chemicals

Substrates (see Table 1) were from Chromogenix except methoxy-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide and succinyl-Ala-Ala-Ala-*p*-nitroanilide which were from Sigma and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide from Bachem. Secondary antibodies were from DAKO. Other chemicals used were of analytical grade.

2.3. Inhibition assays and kinetic analyses

Association rate constants were determined in discontinuous assays under either second or pseudo-first order conditions as previously described [7]. In short, BSZx and the proteinase were allowed to react for the times indicated after which the reaction was stopped by 5–25-fold dilution with 0.55 mM substrate solution. All reactions were performed at 22°C in 50 mM Tris-HCl, pH 8.0 and 100 mM NaCl. Tween 20 (0.1%) or bovine serum albumin (0.05%) was included during assays with trypsins or subtilisins, respectively. 5 mM $CaCl_2$ was included in assays with factor VIIa/sTF. The fraction (X_{inh}) of BSZx forming stable complex with an enzyme according to the suicide inhibition scheme [13,14] was also determined except in cases of very slow inhibition.

Concentrations of active trypsin, chymotrypsin, *Fusarium* trypsin and subtilisins were determined by active site titrations [15,16]. Concentrations of the proteinase inhibitors BSZx, AT-III and eglin C and were determined by titrations against trypsin or chymotrypsin and, subsequently, titrated inhibitors were used to determine thrombin and factor Xa (AT-III) and leukocyte elastase (eglin C). Since no titration method was available for plasma kallikrein, the concentration of active enzyme was estimated by regression analysis of experimental data according to the model [7] assuming 1:1 inhibition stoichiometry.

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Abbreviations: APC, activated protein C; AT-III, antithrombin III; BSZ, protein Z-type barley serpin; DTT, dithiothreitol; NTA, nitrilotriacetic acid; sTF, soluble domain of human tissue factor; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; WSZ, protein Z-type wheat serpin

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Concentrations of the remaining enzymes were estimated by amino acid analyses of highly purified preparations.

2.4. Complex formation and characterization

Formation of enzyme-serpin complexes, tricine SDS-PAGE, immunological detection of BSZx on nitrocellulose membranes and amino acid sequencing of proteins and peptides blotted onto polyvinylidene difluoride membranes have been described previously [7,12]. For sequencing of C-terminal peptides and complexes, samples were prepared by incubating 140 pmol of BSZx (6 μg) with ~120 pmol proteinase for 20–30 min.

3. Results

3.1. Inhibition kinetics

A range of serine proteinases from human blood, leukocytes and pancreas, one fungal trypsin and three *Bacillus* proteinases were analyzed for sensitivity to inhibition by BSZx (Table 1). BSZx was found to be a potent inhibitor of plasma kallikrein, thrombin and coagulation factors VIIa/sTF and Xa and the interactions could be characterized under second order conditions (Fig. 1). Among the proteinases studied, kallikrein was most rapidly inactivated ($k_{ass} = 1.3 \times 10^5$ M^{-1} s^{-1}). The kinetics of BSZx inhibition of thrombin and factor VIIa/sTF were very similar with k_{ass} values of 8.0×10^3 M^{-1} s^{-1} and 4.5×10^3 M^{-1} s^{-1}, respectively, and inhibition stoichiometries close to 1 ($X_{inh} \geq 0.80$). Factor Xa was inhibited at a similar association rate ($k_{ass} = 6.9 \times 10^3$ M^{-1} s^{-1}) but ~80% was turned over as a substrate ($X_{inh} = 0.17$). Addition of 10 μM heparin did not accelerate BSZx inhibition of thrombin or factor Xa in contrast to inhibition with AT-III [17].

Activated protein C and leukocyte elastase were only slowly inhibited by BSZx ($k_{ass} = 1\text{–}2 \times 10^2$ M^{-1} s^{-1}) which required determination of the association rates constants under pseudo first order conditions and prevented estimation of X_{inh}. Coagulation factor XIIa and u-PA were only weakly affected after incubation with BSZx in large excess, and no change in activity was observed for plasmin, t-PA and for the pancreas enzymes elastase and kallikrein.

In contrast to the fast inhibition of both pancreas trypsin and chymotrypsin [7], trypsin from the barley seed rot *Fusarium oxysporum* was not inactivated by BSZx which was cleaved as a substrate. Subtilisin Carlsberg and Novo (BPN′) were rapidly inhibited (Table 1) but the majority of BSZx molecules were turned over as a substrate ($X_{inh} \leq 0.02$). The reactions were completed in less than two minutes and no subsequent change in activity was observed for at least 20 min. The subtilisin from *B. pumilus* showed no inhibition by BSZx.

3.2. Serpin-proteinase complexes and cleavage sites

Complexes of BSZx with thrombin, plasma kallikrein, factor VIIa and factor Xa were resistant to boiling in 1% SDS and subsequent SDS-PAGE (Fig. 2). Formation of stable complexes was further confirmed by Western blotting as in Fig. 3C and by amino acid sequencing of excised upper bands. In five sequencer cycles, only the N-terminal sequence of the proteinase domain (B-chain) of thrombin, kallikrein, factor VIIa and factor Xa, respectively, was detected besides the sequence of the histidine tag of BSZx. Reactive center loop cleavage sites were determined for the four enzymes by sequencing the ~4 kDa C-terminal peptides released by boiling in SDS. With all enzymes, a single sequence: Ser-Leu-Pro-Val-Glu- was observed confirming that the Arg-Ser bond at P_1 was the target site recognized by these proteinases.

To further characterize complex formation with BSZx a

Table 1
Serpin-proteinase association rate constants (k_{ass}) and fractions of BSZx forming complex with proteinases (X_{inh})

Proteinase[a]	Source[b]	C_{BSZx} (nM)	C_{enz} (nM)	*p*-Nitroanilide substrate[c]	k_{ass}[d] (M^{-1} s^{-1})	X_{inh}
fVIIa/sTF	H plasma	200	200	D-Ile-Pro-Arg-	4.5×10^3	0.80
fXa	H plasma	42.5	42.5	Z-D-Arg-Gly-Arg-	6.9×10^3	0.17
fXIIa	H plasma	100	3	D-Pro-Phe-Arg-	wi	nd
Thrombin	H plasma	50	50	D-Phe-Pip-Arg-	8.0×10^3	1.0
Kallikrein	H plasma	10	3.2	D-Pro-Phe-Arg-	1.3×10^5	
APC	H plasma	500	50	pyroGlu-Pro-Arg-	1.7×10^2	nd
u-PA	H plasma	100	3	pyroGlu-Gly-Arg-	wi	nd
t-PA	H plasma	100	5	D-Ile-Pro-Arg-	ni	
Plasmin	H plasma	100	10	D-Val-Leu-Lys-	ni	
Elastase	P pancreas	100	5	suc-Ala-Ala-Ala-	ni	
Kallikrein	H pancreas	100	0.3U/ml	D-Val-Leu-Arg-	ni	
Trypsin[a]	P pancreas	7.5	12.5	*p*-tosyl-Gly-Pro-Arg-	3.9×10^6	0.98
Chymotrypsin[a]	B pancreas	7.5	12.5	suc-Ala-Ala-Pro-Phe-	9.4×10^5	0.98
Elastase	H leukocyte	500	50	msuc-Ala-Ala-Pro-Val-	1.3×10^2	nd
Cathepsin G[a]	H leukocyte	150	220	suc-Ala-Ala-Pro-Phe-	2.4×10^4	1.0
Fungal trypsin	*F. oxysporum*	60	30	Bz-Ile-Glu(γ-OR)-Gly-Arg-	ni	
Sub SP226	*B. pumilus*	100	10	suc-Ala-Ala-Pro-Phe-	ni	
Sub Carlsberg	*B. licheniformis*	110	10	suc-Ala-Ala-Pro-Phe-	nd	0.02
Sub Novo	*B. amyloliquefaciens*	100	10	suc-Ala-Ala-Pro-Phe-	nd	0.006

The data were obtained under either second or pseudo-first order conditions at 22°C. See text for further information on individual enzymes and data analysis.
[a]Results for trypsin, chymotrypsin and cathepsin G have been published previously and are included for comparison [7]. sub: subtilisin.
[b]Source organism: H, human; P, porcine; B, bovine; *F.*, *Fusarium*; *B.*, *Bacillus*.
[c]N-terminal blocking groups: Z, carbobenzoxy; pyroGlu, pyroglutamyl; suc, succinyl; msuc, methoxysuccinyl; Bz, benzoyl.
[d]wi, weak inhibition (~10%) or ni, no inhibition (<5%) after 30 min; nd, not determined.

number of mono- and polyclonal antibodies towards native and cleaved forms of the barley serpins BSZ4 and BSZ7 were screened for interactions with BSZx by Western blotting. One monoclonal mouse antibody (8E8) raised against BSZ4 exhibited high affinity to native BSZx while no recognition of cleaved BSZx or of BSZx in complex with either thrombin, plasma kallikrein, factor VIIa, factor Xa and trypsin (Fig. 3) could be observed. Preincubation of BSZx (50 nM) with 8E8 in ten-fold molar excess for 30 min at 22°C reduced the rate of thrombin inhibition (k_{ass}) by less than 20%.

4. Discussion

The barley serpin BSZx has not been identified in the plant and only obtained in a recombinant form [7]. The observed flexibility of the reactive center loop, which allowed for efficient inhibition of both trypsin and chymotrypsin at overlapping reactive centers, prompted us to investigate the inhibition of a range of serine proteinases. The inhibitory specificity of BSZx at P_1 Arg towards coagulation factors (Table 1) was shown to be similar to that of AT-III. In addition to inhibition of thrombin, plasma kallikrein, factor Xa and trypsin, BSZx and AT-III are, to our knowledge, the only reported serpin inhibitors of factor VIIa/sTF and both with association rates of $\sim 5\times10^3$ M^{-1} s^{-1} [18]. However, BSZx is independent on heparin. The experiments with factor Xa illustrate that a large fraction of the serpin molecules may be cleaved as a substrate at the inhibitory site in accordance with the suicide inhibition scheme and the kinetic model. BSZx contains a potential substrate site at P_6 Arg, a position generally occupied by a hydrophobic residue [1], but no cleavage at this site could be detected with any of the four coagulation factors.

The monoclonal antibody 8E8 was specific for native BSZx and showed no affinity for cleaved BSZx or for any of five proteinase complexes (Fig. 3). BSZx retained nearly full activity after preincubation with 8E8 in excess and, presumably, the epitope recognized by 8E8 is not located in the loop region. More likely, partial or complete loop insertion caused by complex formation or cleavage results in a structural rearrangement disrupting the epitope [19–21]. This suggestion is

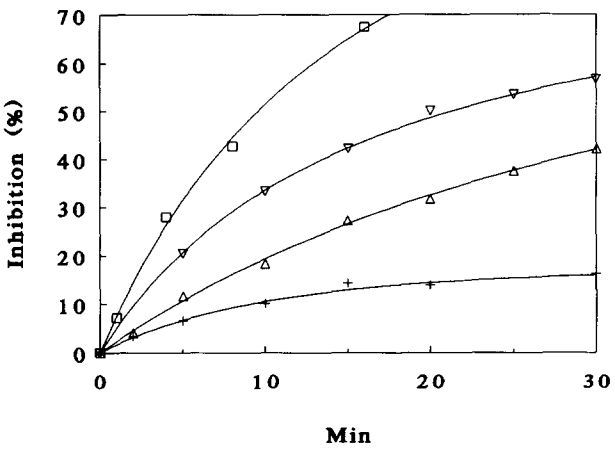

Fig. 1. Inhibition of human coagulation factors by BSZx. □, plasma kallikrein; ▽, factor VIIa/sTF; △, thrombin and +, factor Xa. Determination of k_{ass} and X_{inh} by non-linear regression was done as reported previously [7]. Experimental details and the derived kinetic constants are given in Table 1.

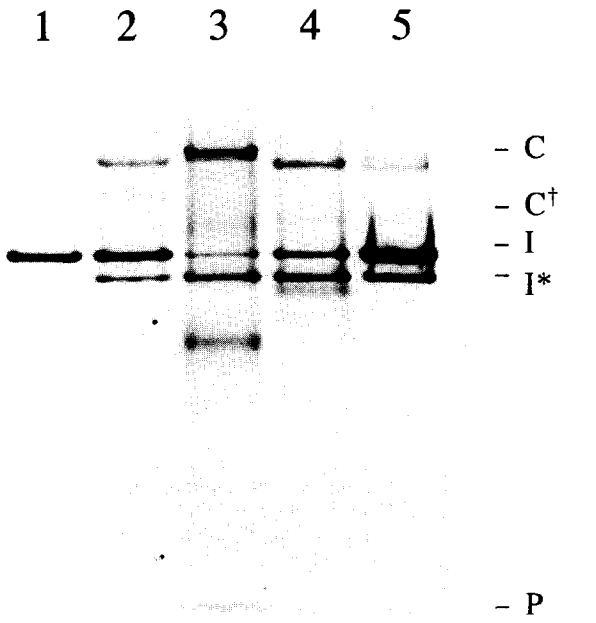

Fig. 2. Formation of SDS-stable complexes with coagulation factors. BSZx was incubated with 15 pmol enzyme at 22°C for 20 min before addition of reducing sample buffer, boiling for 4 min and tricine SDS-PAGE. Lane 1: 6 pmol BSZx; lanes 2–4, 15 pmol BSZx incubated with thrombin, plasma kallikrein and factor VIIa/sTF, respectively; lane 5: 30 pmol BSZx incubated with factor Xa. The bands were visualized by silver staining. The positions of intact complexes (C), partially degraded complexes (C†), intact BSZx (I), cleaved BSZx (I*) and released C-terminal peptides (P) are indicated on the right.

corroborated by reports on monoclonal antibodies recognizing neoantigenic epitopes on C1 inhibitor and PAI-1 generated both by cleavage of the reactive center loop and by complex formation [22,23].

Mammalian and *Fusarium* trypsins are very similar in their secondary structural elements and overall conformation but significant differences are observed in the active site region [24]. Although *Fusarium* trypsin preferentially binds Arg in the specificity pocket and is inhibited by the very different Kunitz inhibitors of pancreas and soy bean [24], no inhibition by BSZx was observed. Recently α_1-proteinase inhibitor and α_1-antichymotrypsin were shown to inhibit and form SDS stable complexes with subtilisins [25]. The interactions of subtilisins both with BSZx and the mammalian serpins were characterized by fast associations but inefficient inhibition as more than 90% of the serpin molecules were turned over as substrate (X_{inh} was 0.02 (BSZx) and 0.08 (α_1-proteinase inhibitor [25]) for interaction with subtilisin Carlsberg at 22°C).

In conclusion, kinetic studies have shown that recombinant BSZx inhibits and forms SDS-stable complexes with several human coagulation factors. The specificity is similar to that of antithrombin III and BSZx interacted with the proteinases and pancreas trypsin exclusively at P_1 Arg. However, BSZx is also an effective inhibitor of chymotrypsin and cathepsin G at P2 Leu [7] and the slow interaction with leukocyte elastase is assumed to occur at this overlapping site.

The other known plant serpins, BSZ7 and BSZ7 from barley and WSZ1 from wheat, are expressed in large amounts in the cereal grain endosperms [10–12]. Target proteinases have not been identified in the resting or germinating grain and a

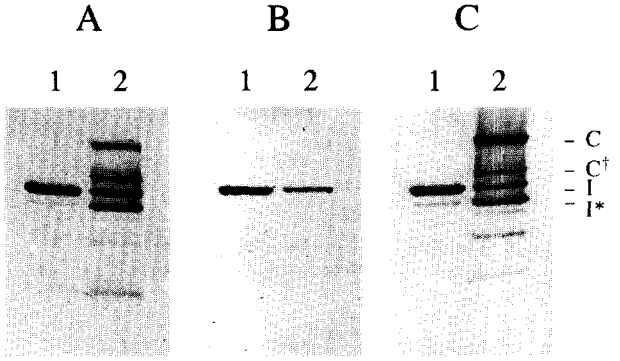

Fig. 3. Immunological recognition of native, cleaved and complex-bound BSZx. Samples of 13 pmol BSZx (lane 1) or 26 pmol BSZx incubated with 23 pmol trypsin (lane 2) were subjected to SDS-PAGE and detected by either silver staining (A) or by Western blotting. The monoclonal antibody 8E8 (B) only reacted with native BSZx whereas the polyclonal antibody 337 (C) recognized native and all proteolytically modified forms of both BSZx and BSZx in complex with trypsin. The different molecular species are indicated as in Fig. 2.

role in plant defence has been suggested. Neither BSZx nor a cDNA encoding this serpin has been detected in grain tissues but the BSZx promoter is functional in transgenic tobacco (S.K. Rasmussen, unpublished). BSZx is a potent inhibitor but was inefficient against the tested microbial enzymes of both the chymotrypsin and the subtilisin family. These observations indicate that BSZx may have a specific regulatory role in the plant and a search for tissue and time specific expression of BSZx as well as endogenous target proteinases has been initiated.

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