RBI, a one-domain α-amylase/trypsin inhibitor with completely independent binding sites

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Abstract The bifunctional inhibitor from *Ragi* (*Eleusine coracana* Gaertneri) (RBI) is the only member of the α-amylase/trypsin inhibitor family that inhibits both trypsin and α-amylase. Here, we show that both enzymes simultaneously and independently bind to RBI. The recently solved three-dimensional NMR structure of RBI has revealed that the inhibitor possesses a hitherto unknown fold for serine proteinase and α-amylase inhibitors. Despite its different fold, RBI obeys the standard mechanism observed for most protein inhibitors of serine proteinases and is a strong, competitive inhibitor of bovine trypsin ($K_i = 1.2 \pm 0.2$ nM). RBI is also a competitive inhibitor of porcine α-amylase ($K_i = 11 \pm 2$ nM) when a disaccharide is used as a substrate of α-amylase. However, the inhibition mode becomes complex when larger (≥7 saccharide units) α-amylase substrates are used. A second saccharide binding site on porcine α-amylase may enable larger oligosaccharides to displace RBI from its binding site in an intramolecular reaction.

Key words: *Ragi* bifunctional inhibitor; Trypsin; Serine proteinases; α-Amylase; *Eleusine coracana* Gaertneri

1. Introduction

A large number of serine protease inhibitors have been isolated from bacterial and eukaryotic sources during the last decades [1–4]. There is a relatively new class of inhibitors found in cereal seeds, designated the α-amylase/trypsin inhibitor family or cereal inhibitor family [2,3,5]. These proteins have a size of about 120 amino acids and contain a large number of conserved cysteines forming four or five intramolecular disulfide bridges. All functional members of the family are either inhibitors of α-amylase or inhibitors of trypsin. The only member possessing both functions simultaneously is the bifunctional α-amylase/trypsin inhibitor (RBI) from *Ragi* (*Eleusine coracana* Gaertneri, Indian finger millet) [6,7]. The monomeric, basic protein (pI > 10) consists of 122 residues and contains five disulfide bridges [5,8]. RBI shares 25–66% sequence identity with the other functional members of the inhibitor family [5]. We have recently solved the three-dimensional structure of RBI in solution by NMR. The inhibitor possesses a hitherto unknown fold, which essentially consists of four central α-helices with up-and-down topology and a short antiparallel β-sheet [5]. Detailed studies of the inhibition mechanism of trypsin and investigations of the inhibition of mammalian α-amylases have not been reported so far for members of the cereal inhibitor superfamily. In this paper, we describe the interaction of recombinant RBI with bovine trypsin and porcine α-amylase and show that both binding sites of this one-domain inhibitor are completely independent.

2. Materials and methods

2.1. Materials

Seeds of *Eleusine coracana* Gaertneri were kindly provided by the ICRISAT Institute, Patancheru, Andhra Pradesh, India. Restriction enzymes, DNA modifying enzymes, α-amylase from hog pancreas, BSA, the α-amylase PNP assay kit (including *p*-nitrophenyl-α-D-maltoheptaoside (NPMH) and α-glucosidase) and *N*-α-benzoyl-L-arginine-4-nitroanilide (L-BAPA) were purchased from Boehringer. *p*-Nitrophenyl-α-D-maltoside (NPM) was from Sigma. Zulkowsky starch and bovine trypsin were obtained from Merck.

2.2. Expression and purification of recombinant RBI

We used a synthetic RBI gene for expression of RBI in *E. coli*, which codes for one of the natural RBI isoforms (STRT at residues 25–28 and S at residue 70 [8]) and was fused to the bacterial OmpA signal sequence (accession number A22416; [11]). The NMR structure of RBI [5] has also been determined for this RBI isoform. For functional expression of RBI in *E. coli* JM83 [11] the plasmids pRBI [10] or pRBI-PDI-T7 [5] were used. RBI was purified from periplasmic extracts either by anhydrotrypsin affinity chromatography [5] or by conventional chromatography, which was performed as follows. The periplasmic extract of a 10 liter culture of *E. coli* JM83/pRBI-PDI was dialyzed against 10 mM Tris-HCl pH 8.0 and applied to a DE52 anion exchange column (45 ml, Whatman). The flow through was applied to a CM52 cation exchange column (15 ml, Whatman) equilibrated with 10 mM Tris-HCl pH 8.0. Proteins were eluted with a linear gradient from 0–0.4 M NaCl in 10 mM Tris-HCl pH 8.0. Purified inhibitor was obtained after hydrophobic chromatography on phenyl Superose as described [5].

2.3. Purification of RBI from seeds of Indian finger millet

10 g of *Ragi* seeds were powdered and mixed with 20 ml 150 mM NaCl. After centrifugation (12 000 × *g*, 10 min, 4°C), the pellet was re-extracted with 20 ml 150 mM NaCl. The supernatants were pooled, mixed with 1/50 volume 1 M sodium phosphate pH 7.0 and heated to 60°C within 20 min. After cooling on ice and centrifugation (17 000 × *g*, 30 min, 4°C), the supernatant was filtered (0.2 μm pore size) and ammonium sulfate was added to 60% saturation. After centrifugation (17 000 × *g*, 30 min, 4°C), the inhibitor was purified from the solubilized pellet as described above.

2.4. Protein concentration

The concentration of native RBI was determined by its absorbance at 280 nm ($A_{280nm,1mg/ml,1cm} = 0.680$; [5]). The N-termini of natural, recombinant and cleaved RBI were verified by Edman degradation.

2.5. Analytical gel filtration

Analytical gel filtration studies were performed at room temperature on a Superose 12 column (30 × 1 cm, Pharmacia) equilibrated with 20 mM MOPS/NaOH pH 6.9, 100 mM NaCl, 1 mM $CaCl_2$ (flow rate: 0.2 ml/min; sample volume 50 μl). RBI was incubated

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Abbreviations: BSA, bovine serum albumin; L-BAPA, *N*-α-benzoyl-l-arginine-*p*-nitroanilide; K_i, inhibition constant; K_M, Michaelis constant; NPM, *p*-nitrophenyl-α-d-maltoside; NPMH, *p*-nitrophenyl-α-d-maltoheptaoside; PVDF, polyvinylidenedifluoride; *Ragi*, *Eleusine coracana* Gaertneri (Indian finger millet); RBI, *Ragi* bifunctional inhibitor

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with the target enzymes for 1 h in the same buffer before application to the column. Identical concentrations of RBI, α-amylase and trypsin (8.9 μM) were used.

2.6. Trypsin inhibition assay

Trypsin inhibition assays were performed using L-BAPA as substrate. The assay was performed in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 20 mM $CaCl_2$, 0.005% (v/v) Triton X-100 with trypsin at a concentration of 16 or 60 nM and varying RBI concentrations. The mixture was incubated for 24 h. After addition of L-BAPA (10 mM, dissolved in 1 mM HCl) to a final concentration of 0.2 mM, the increase in absorption at 405 nm was recorded ($\varepsilon_{405nm,pH8} = 10\,300$ M^{-1} cm^{-1}). The concentration of active trypsin was determined prior to the assay by active site titration with *p*-nitrophenyl-*p'*-guanidinobenzoate as described ($\varepsilon_{410nm,pH8.3} = 16\,600$ M^{-1} cm^{-1}; [12]).

2.7. α-Amylase inhibition assays

All assays were performed in α-amylase test buffer (20 mM MOPS/NaOH pH 6.9; 100 mM NaCl; 1 mM $CaCl_2$) at 25°C. Isoforms I and II of porcine α-amylase were separated by ion exchange chromatography as described by Wiegand et al. [13]. Assays with the disaccharide substrate NPM ($K_M = 6$ mM) were performed at an α-amylase concentration of 0.385 μM, NPM concentrations between 1 and 6 mM and varying RBI concentrations (0–1 μM). The assay was started by addition of 25 μl α-amylase stock solution to a pre-equilibrated mixture of RBI and substrate in 1 ml. Substrate hydrolysis was followed by the increase in absorbance at 405 nm ($\varepsilon_{405nm,pH6.9} = 8200$ M^{-1} cm^{-1}; [14]). It was verified that identical results were obtained when α-amylase and RBI were mixed first and the reaction was initiated by addition of NPM.

A linked enzyme assay with α-glucosidase as second enzyme was applied when the heptasaccharide substrate NPMH ($K_M = 61$ μM) was used, also allowing on-line detection of α-amylase activity at 405 nm ($\varepsilon_{405nm,pH6.9} = 8200$ M^{-1} cm^{-1}). Typically, α-amylase (0.18 nM) was incubated with varying concentrations of RBI (0–0.11 μM) in 1 ml buffer containing 100 μg/ml BSA for 16–24 h. The reaction was started by the addition of 40 μl of a solution containing NPMH (0.6–10 mM) and α-glucosidase (125 U/ml) in test buffer.

Assays with Zulkowsky starch (~27 saccharide units; $K_M = 0.17$ mM) were performed in test buffer containing BSA (100 μg/ml) at final concentrations of 1.8 nM α-amylase, 0–0.2 μM RBI and 0.1–0.8 mM substrate. RBI and α-amylase were incubated for 16–24 h in 4 ml buffer and the reaction was started by addition of 1 ml substrate solution (0.5–4 mM in test buffer). Samples of 0.5 ml were removed after different incubation times, mixed with 0.5 ml 1% (w/v) dinitrosalicylic acid in 0.4 M NaOH, incubated at 100°C for 5 min and then cooled on ice. Product formation was detected by the absorbance at 546 nm ($\varepsilon_{546nm} = 1230$ M^{-1} cm^{-1}). The results were independent of the order of addition of RBI and Zulkowsky starch to the enzyme.

3. Results

3.1. Purification of recombinant RBI

Recombinant RBI was expressed in the periplasm of *E. coli* JM83 [11] harboring the expression plasmids pRBI [10] or pRBI-PDI-T7 [5]. After application of the periplasmic extracts to an anhydrotrypsin affinity column, acid elution of bound RBI yielded a mixture of native RBI and RBI cleaved at the reactive peptide bond between Arg^{34} and Leu^{35} at a ratio between 3:1 to 10:1, presumably due to residual trypsin activity on the affinity resin. The native and the cleaved inhibitor were separated by subsequent chromatography on a phenyl Superose column. To avoid partial cleavage of RBI by trypsin, the native inhibitor was alternatively purified from periplasmic extracts by conventional chromatography.

3.2. Formation of complexes with the target enzymes

The interaction of RBI with its target enzymes was first analyzed by analytical gel filtration on a Superose 12 column (Fig. 1). When RBI was incubated with one molar equivalent of trypsin or α-amylase before application to the column,

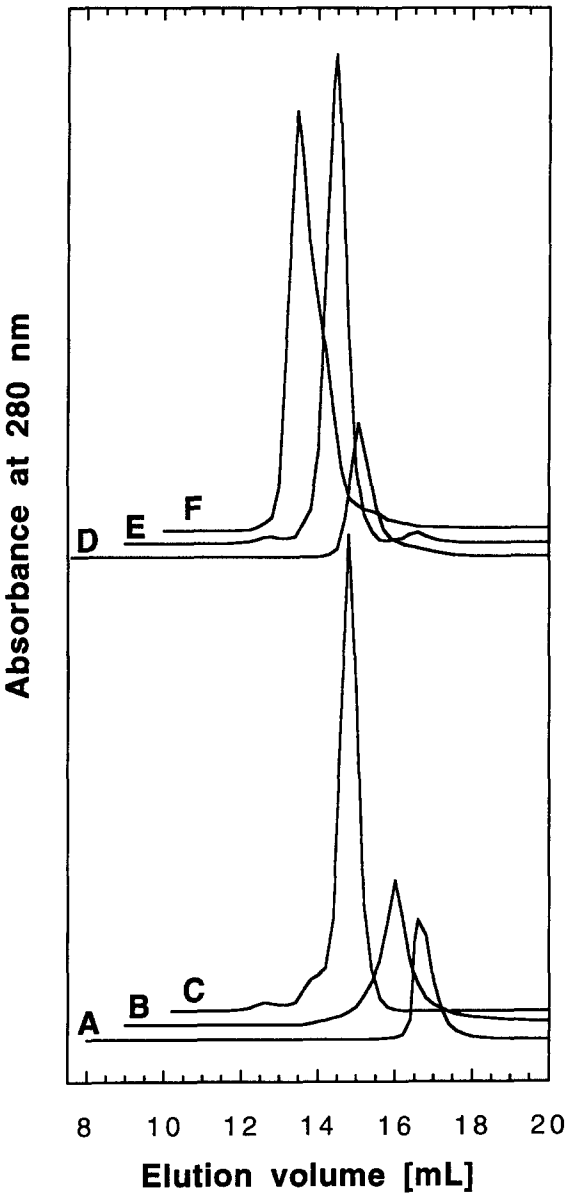


Fig. 1. Analytical gel filtration of complexes between recombinant RBI and its target enzymes on Superose 12 at pH 6.9 and 25°C. Proteins were applied to the column at a concentration of 8.9 μM. A: Recombinant RBI (13.1 kDa); B: bovine trypsin (23.5 kDa); C: porcine α-amylase (56 kDa); D: 1:1 complex between RBI and trypsin; E: 1:1 complex between RBI and α-amylase; F: ternary complex between RBI, α-amylase and trypsin.

complete complex formation with each of the target enzymes could be demonstrated. Two peaks corresponding to the enzyme/RBI complex and the free inhibitor were obtained, when the inhibitor was used at a 2-fold molar excess over the enzymes, supporting a 1:1 stoichiometry of both the trypsin/RBI and α-amylase/RBI complex (data not shown). Moreover, complete formation of the ternary complex between RBI, trypsin, and α-amylase was achieved when the three proteins were incubated at a 1:1:1 stoichiometry before application to the gel filtration column (Fig. 1F). Interaction between α-amylase and trypsin was not detectable under the same conditions (data not shown). Therefore, RBI binds to its target enzymes at different sites of the molecule, and the binding sites are not mutually exclusive.

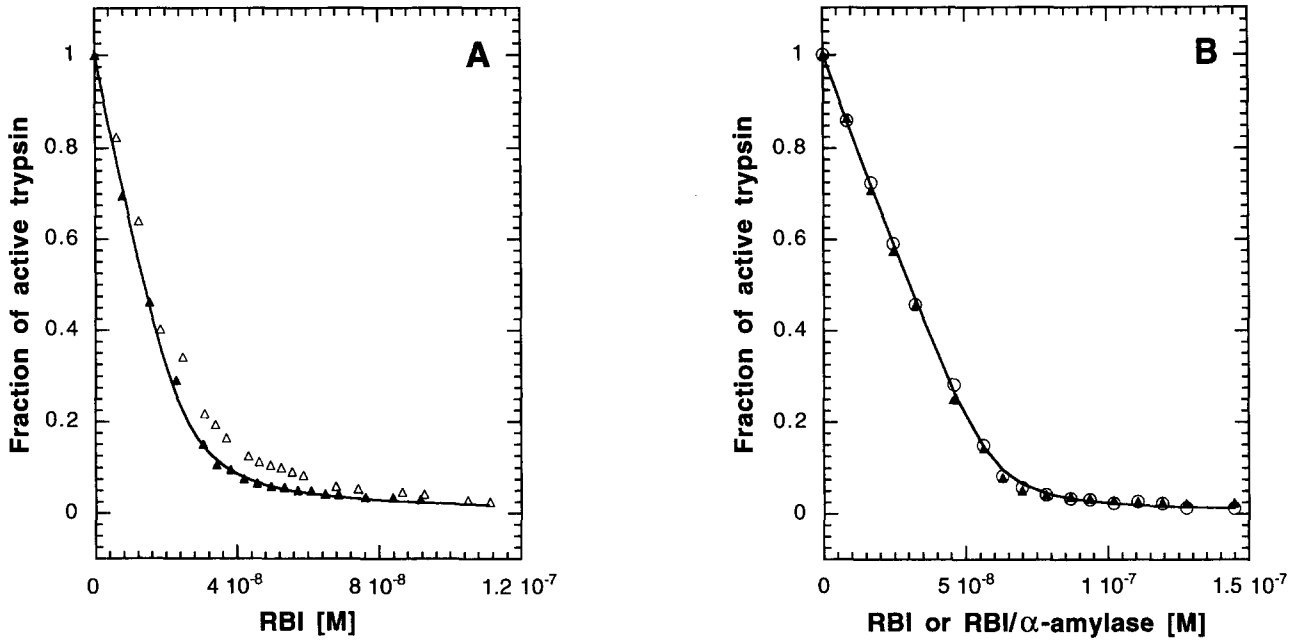


Fig. 2. Inhibition of bovine trypsin by RBI and by the α-amylase/RBI complex at pH 8 and 25°C. A: Comparison of trypsin inhibition by natural RBI (△) and recombinant RBI (▲). The trypsin concentration was 16 nM. The solid line results from a nonlinear regression according to [15] yielding an inhibition constant of 1.2±0.2 nM. B: Inhibition of trypsin by the α-amylase/RBI complex: RBI (final concentrations: 0–145 nM) was incubated for 1 h in trypsin test buffer with a high molar excess of α-amylase (isoform II, final concentration: 430 nM in all experiments) to guarantee formation of the α-amylase/RBI complex to ≥90%. After addition of trypsin (final concentration: 60 nM), the samples were incubated for another 24 h. An identical set of samples was prepared without α-amylase. ○, α-Amylase/RBI complex; ▲, RBI.

3.3. Interaction of RBI with trypsin

The inhibition of bovine pancreatic trypsin was determined by measuring the residual trypsin activity after incubation of trypsin with different amounts of RBI, using the chromogenic substrate L-BAPA [17]. A K_i of 1.2±0.2 nM was measured for RBI by titration of trypsin with increasing amounts of the inhibitor (Fig. 2A). The K_i value was independent of the substrate concentration (data not shown). Therefore, the trypsin/RBI complex dissociates slowly and recombinant RBI was not displaced by the substrate during the time of the assay. Comparison of recombinant RBI with RBI isolated from seeds of *Ragi* revealed a slightly weaker affinity of the natural inhibitor for trypsin (Fig. 2A).

To probe the influence of bound α-amylase on the inhibitory properties of RBI towards trypsin, titration experiments with free RBI and the α-amylase/RBI complex were performed. At saturating concentrations of α-amylase (see below), identical inhibition profiles were obtained, demonstrating that trypsin inhibition by RBI is fully independent of the occupation of its α-amylase binding site (Fig. 2B).

After incubation of native RBI (2 μM) for 3 days in the presence of catalytic amounts (1/100 molar equivalent) of trypsin, a pH-dependent cleavage of the inhibitor within its trypsin binding loop was found. The fraction of cleaved RBI was about 10% between pH 6.5 and 8, about 50% at pH 5.5 and approximately 90% between pH 3 and 4.5 (data not shown). To probe the reversibility of the cleavage of the scissile peptide bond between Arg^{34} and Leu^{35} in RBI, the cleaved inhibitor was purified [5] and incubated with one molar equivalent of trypsin at pH 8 for 3 h. The trypsin/RBI complex was dissociated by addition of different amounts of HCl (final pH values: 3.5–0.8) and analyzed for the content of native and cleaved inhibitor by reducing SDS-PAGE [9] and Western blotting (Fig. 3). Below pH 2.5, native RBI was quantitatively recovered from cleaved RBI, showing the complete reversibility of the cleavage of the Arg^{34}/Leu^{35} peptide bond.

3.4. Interaction of RBI with α-amylase

To investigate the inhibition of porcine α-amylase by RBI, different oligosaccharide substrates with varying lengths were used. In a first set of experiments, the disaccharide NPM was used as substrate. Since porcine α-amylase occurs as a mixture of two isoforms [18], the ability of RBI to inhibit both isoforms was investigated. Isoforms I and II could be completely

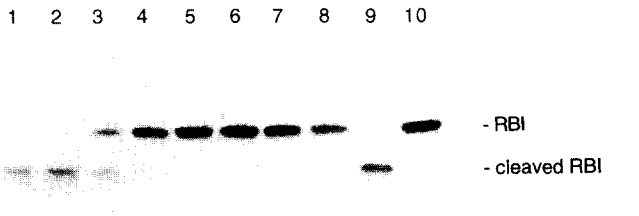
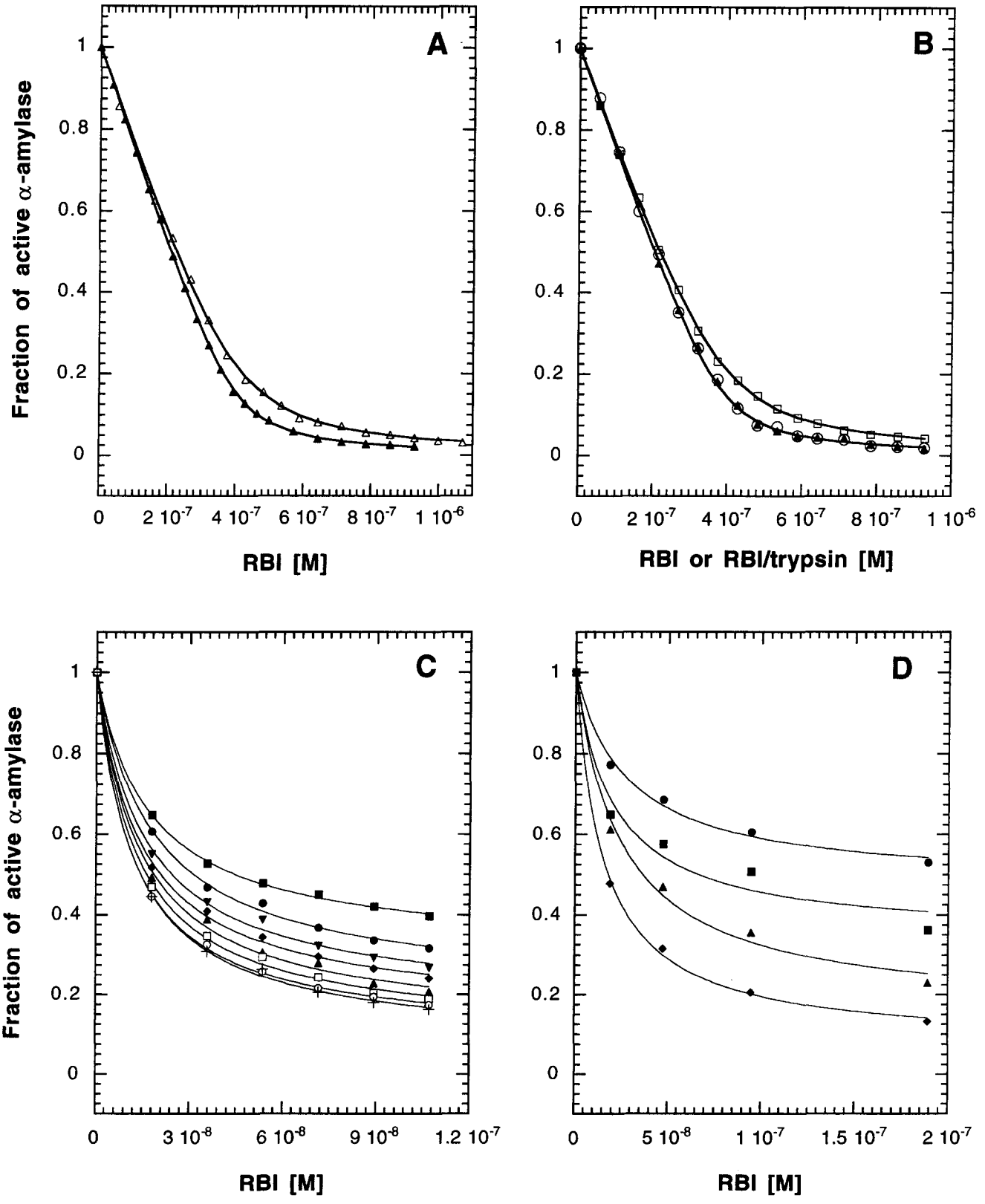


Fig. 3. Western blot analysis of the reversible cleavage of the reactive peptide bond Arg^{34}/Leu^{35} of RBI by trypsin. Cleaved RBI (45 μM) was incubated at 25°C and pH 8.0 for 3 h with one molar equivalent of active bovine trypsin. Samples were mixed with 1/4 volume HCl solution (0.1–1 M). After addition of 1/2 volume 10% (w/v) SDS and 2/3 volume sample buffer, samples were boiled and applied to a reducing SDS-PAGE (15% (w/v) acrylamide). Native RBI yields a single band corresponding to 13.1 kDa, and cleaved RBI yields two fragments corresponding to residues 1–34 and 35–122, respectively. Only the larger fragment (9.5 kDa) can be detected under the conditions applied. Proteins were transferred to a PVDF membrane and RBI bands were stained immunospecifically as described [10]. RBI after dissociation of the RBI/trypsin complex at pH 8.0 (lane 1), pH 3.5 (lane 2), pH 3 (lane 3), pH 2.5 (lane 4), pH 2 (lane 5), pH 1.5 (lane 6), pH 1 (lane 7), and pH 0.8 (lane 8), cleaved RBI (lane 9) and native RBI (lane 10).

inhibited by RBI. The affinity of the inhibitor for isoform II was found to be slightly higher than for isoform I (Fig. 4A). The inhibition profiles could be well described by a competitive inhibition mechanism. In contrast to the inhibition of trypsin, the measured inhibition constants were dependent on the concentration of the substrate NPM (Fig. 4B). Using the measured K_M value of 6.0 mM for NPM, the data were evaluated assuming fast binding equilibria for RBI and NPM to α-amylase ([15]; Fig. 4) and yielded consistent K_i values of 21 ± 4 nM for isoform I and 11 ± 2 nM for isoform II of porcine α-amylase. In accordance with the unchanged binding of trypsin in the ternary complex, inhibition of α-amylase by



free RBI and the trypsin/RBI complex was indistinguishable (Fig. 4B). Therefore, the inhibitor's binding sites for the target enzymes are fully independent.

The apparent inhibition mode of RBI for α-amylase became complex when the larger oligosaccharide substrates NPMH (7 saccharide units) and Zulkowsky starch (about 27 saccharide units) were used. With none of these substrates could complete inhibition of α-amylase be achieved, even at a high molar excess of RBI over the enzyme (Fig. 4C,D). The inhibition patterns were very similar with both substrates. Inhibition was strongly dependent on the substrate concentrations and no longer consistent with a pure competitive inhibition mechanism. We evaluated the data according to the known types of inhibition [16]. At a given substrate concentration, the data were in agreement with a partial competitive as well as with a partial mixed type inhibition mode with both substrates. However, the fitted K_i values decreased systematically with increasing substrate concentrations in both inhibition models. The inhibition patterns observed with these oligosaccharide substrates thus did not yield consistent data and could not be explained by known mechanisms of inhibition.

4. Discussion

RBI is the only bifunctional member of the cereal inhibitor superfamily [5]. Gel filtration studies demonstrated that recombinant RBI binds to both target enzymes simultaneously with a 1:1:1 stoichiometry. The stoichiometry was confirmed by inhibition studies, which also revealed the independence of both binding sites.

The competitive inhibition of trypsin and the substrate character of the inhibitor are in full agreement with the so-called standard mechanism of serine proteinase/protein inhibitor interactions (reviewed in [1]), which can be described by the scheme $E+I \rightleftarrows C \rightleftarrows E+I^*$ where E is the protease, I is the native inhibitor, I^* is the inhibitor cleaved at its reactive peptide bond, and C is a stable, Michaelis-type inhibitor/proteinase complex with the inhibitor's reactive peptide bond intact [1,4]. In the presence of catalytic amounts of the protease, the $[I^*]/[I]$ ratio (K_{hyd}) is strongly pH-dependent and in many cases close to 1 at neutral pH. Several lines of evidence indicate that the standard inhibition mechanism also applies to the trypsin/RBI interaction: (i) K_{hyd} varies between 0.1 and 10 in the presence of catalytic amounts of trypsin between pH 3 and pH 8, and is approximately 1 at pH 5.5. (ii) Cleavage of the Arg^{34}/Leu^{35} peptide bond is fully reversible. Native RBI is obtained quantitatively after incubation of the cleaved inhibitor with one molar equivalent of trypsin at pH 8 and rapid dissociation of the complex at pH ≤2, where trypsin is simultaneously inactivated. (iii) The trypsin binding loop in RBI exhibits the canonical conformation observed in all three-dimensional structures of serine proteinase inhibitors obeying the standard mechanism [4,5].

The measured K_i value (1.2 nM) of recombinant RBI is in the range of known values of protein inhibitors of serine proteinases (10^{-7}–10^{-12} M [19]). Since K_i values of other trypsin inhibitors of the cereal inhibitor family have so far not been measured, a direct comparison of RBI with the other trypsin inhibitors of the family is presently not possible. The fact that recombinant RBI displays a higher affinity for trypsin than the inhibitor isolated from *Ragi* may be explained by the presence of about equimolar amounts of the other RBI isoform in the preparation of natural RBI [8], which may bind trypsin with lower affinity.

The competitive inhibition of porcine α-amylase detected with the disaccharide substrate NPM ($K_i = 11$ nM) proves specific binding of RBI next to the active site of α-amylase. In contrast to binding of trypsin, the equilibrium for α-amylase binding is rapid, as demonstrated by the dependence of the apparent inhibition constant on the substrate concentration (Fig. 4B). Surprisingly, the apparent inhibition mode changed to complex inhibition patterns when larger oligosaccharide substrates were used. At all concentrations of the substrates NPMH and Zulkowsky starch tested, the shapes of the inhibition profiles were apparently in agreement with both a partial competitive and a partial mixed type inhibition mechanism. However, both inhibition models include the existence of a ternary complex between inhibitor, enzyme and substrate, which would mean that RBI can occupy different binding sites on α-amylase, depending on the present substrate. We believe that this explanation is very unlikely. The fact that no consistent values for K_i and the parameters α and β could be obtained when the data for the larger substrates were analyzed quantitatively [16] is a strong hint that these inhibition modes indeed do not exist. The most plausible explanation for the observed inhibition patterns of RBI comes from the fact that porcine α-amylase possesses at least two different saccharide binding sites [13,20–22]. One of the binding sites is a long cleft for 5 saccharide units and contains the active site of the enzyme. Hydrolysis of the substrate occurs between the third and the fourth saccharide unit [23]. The other, independent saccharide binding sites are located about 15 Å and 20 Å remote from the active site [21,22]. Therefore, ternary complexes can exist where RBI is bound near the catalytic center of the enzyme within the pentasaccharide binding cleft, and a substrate is bound at a second binding site. The size of the disaccharide NPM is not sufficient to span the distance between both saccharide binding sites. However, larger oligosaccharide substrates bound at the second site can principally displace bound RBI in an intramolecular reaction due to their high effective concentrations. This mechanism is not compatible with any of the commonly used models for

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Fig. 4. Inhibition of porcine α-amylase (isoforms I and II) by RBI and by the trypsin/RBI complex at pH 6.9 and 25°C, assayed with the disaccharide substrate NPM (A, B) and the larger oligosaccharide substrates NPMH ($n = 7$) (C) and Zulkowsky starch ($n \approx 27$) (D). A: Comparison of inhibition of the α-amylase isoforms I (△) and II (▲) by recombinant RBI. The final concentrations were 1 mM for NPM and 0.385 μM for α-amylase. B: Inhibition of α-amylase (isoform II, 0.385 μM) by RBI (▲, □) and the trypsin/RBI complex (○) at 1 mM NPM. Titration experiments in the presence of 1 mM (▲) and 6 mM (□) NPM yielded identical K_i values of 11 ± 2 nM ($K_{app} = K_i\ (1+S/K_M)$) [15], where K_{app} is the apparent inhibition constant and K_M is the Michaelis constant for the substrate NPM (6.0 mM). C: Inhibition of α-amylase (isoform II) (0.18 nM) with RBI at different NPMH concentrations ($K_M = 61$ μM). NPMH was added to final NPMH concentrations of 24 μM (+), 36 μM (○), 48 μM (□), 66.7 μM (▲), 100 μM (♦), 133 μM (▼), 200 μM (●), and 400 μM (■). (D) Inhibition of α-amylase isoform II (1.8 nM) by RBI measured at different concentrations of Zulkowsky starch ($K_M = 0.17$ mM), which was added to final concentrations of 0.8 mM (●), 0.4 mM (■), 0.2 mM (▲) and 0.1 mM (♦). Solid lines correspond to nonlinear fits according to [16] assuming a partial competitive inhibition mode.

enzyme inhibition [16,24]. It can, however, explain the change in the apparent inhibition mode of RBI when larger oligosaccharide substrates are used. The fast equilibrium between RBI and α-amylase suggests that RBI can be readily displaced from its binding site.

Complex inhibition mechanisms have also been reported for many other protein inhibitors of α-amylases [25]. In the case of red kidney bean inhibitor, a noncompetitive mechanism was postulated, where the inhibitor may inactivate the enzyme by inducing a conformational change and binding may proceed through two steps [26]. A two-step binding reaction was also observed for the complex between barley α-amylase/subtilisin inhibitor (BASI) and barley α-amylase [27]. In contrast to the α-amylase/RBI complex, saturation with red kidney bean inhibitor does not lead to complete inhibition of the enzyme in the presence of the disaccharide substrate NPM [26]. Indeed, the full inhibition of α-amylase by RBI in the presence of NPM and the rapid displacement of RBI by NPM (Fig. 4B) provide the strongest evidence that RBI is a competitive inhibitor of α-amylase. The α-amylase inhibition constants of a few homologous members of the α-amylase/trypsin inhibitor family have been measured. The values are in the range of 0.1–10 nM [25,28,29] and thus comparable with the K_i of RBI for porcine α-amylase (11 nM).

In conclusion, a possible intramolecular displacement of a competitive inhibitor by substrates implies that inhibition data obtained for other inhibitors of porcine α-amylase by using large oligosaccharide substrates should be re-examined with smaller substrates such as NPM.

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