Absolute Side-chain Structure at Position 13 Is Required for the Inhibitory Activity of Bromein*

Received for publication, September 2, 2008, and in revised form, October 10, 2008. Published, JBC Papers in Press, October 23, 2008, DOI 10.1074/jbc.M806748200

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Bromelain isoinhibitor (bromein), a cysteine proteinase inhibitor from pineapple stem, has a unique double-chain structure. The bromein precursor protein includes three homologous inhibitor domains, each containing an interchain peptide between the light and heavy chains. The interchain peptide in the single-chain precursor is immediately processed by bromelain, a target proteinase. In the present study, to clarify the essential inhibitory site of bromein, we constructed 44 kinds of site-directed and deletion mutants and investigated the inhibitory activity of each toward bromelain. As a result, the complete chemical structure of Leu13 in the light chain was revealed to be essential for inhibition. Pro12 prior to the leucine residue was also involved in the inhibitory activity and would control the location of the leucine side chain by the fixed $\phi$ dihedral angle of proline. Furthermore, the five-residue length of the interchain peptide was strictly required for the inhibitory activity. On the other hand, no inhibitory activity against bromelain was observed by the substitution of proline for the N terminus residue Thr15 of the interchain peptide. In summary, these mutational analyses of bromein demonstrated that the appropriate position and conformation of Leu13 are absolutely crucial for bromelain inhibition.

Cysteine proteinases are involved in specific processing or more general degradation of proteins in a wide variety of organisms, including viruses, fungi, plants, and animals (1). Their activity is regulated by limited proteolysis of inactive precursors, by the pH of the surroundings (2), and by tight binding with proteinaceous inhibitors (3). With regard to proteinaceous inhibitors, six structurally different families of cysteine proteinase inhibitors have been reported so far: bromelain isoinhibitors (bromein) (4), cystatins (5), soybean trypsin inhibitor-like inhibitors (6), thyropins (7), inhibitors homologous to the propeptide regions of cysteine proteinases (8), and clitocypins (9).

Bromelain is known as cysteine proteinases in the stem and fruit of *Ananas comosus*, while the inhibitors exist only in the stem and have been classified into eight isoforms based on their amino acid sequences (10). The major component of bromelains, stem bromelain, has been sequenced (11) and shown to be a member of the papain superfamily (12). On the other hand, the presence of inhibitory fractions has also been confirmed in pineapple stem (13), and the amino acid sequence of the seventh bromein (bromein-7) was the first sequence determined among the inhibitory fractions (4). Hatano et al. (14) revealed the complete primary structures of all eight bromein isoforms: each isoform is composed of a light chain (10–11 residues) and a heavy chain (40–41 residues), which are cross-linked by five disulfide bridges.

The three-dimensional solution structure of the sixth bromein with the two chains (bromein-6N) is characterized by inhibitory and stabilizing domains, each of which is formed by a three-stranded antiparallel $\beta$-sheet (15, 16). As shown in Fig. 1, A and B, the inhibitory domain consists of the light chain and two parts of the heavy chain (Glu20-Cys26 and Asp51-Lys60). This domain is thought to be the major bromein inhibitory site, and it has a relatively flexible structure that appears to allow itself to fit well into the active site cleft of the target proteinase (17). On the other hand, the structure of the stabilizing domain (Thr29-Ile48) is thought to contribute mainly to the conformational stability of the inhibitory one, because the NMR structures calculated were well converged (17). Surprisingly, bromein-6N shares the same fold and disulfide bridge connectivity as the Bowman-Birk serine proteinase inhibitor (BBI) (17). For instance, BBI from soybeans is a 71-residue inhibitor that has two independent inhibitory sites for the serine proteinases trypsin and chymotrypsin (18). It is noteworthy that bromein-6N exhibits relatively weak inhibitory activity against these serine proteinases (19).

The genomic DNA of a bromein precursor protein (27.5 kDa) was found to encode three homologous isoinhibitor domains, each of which contains an interchain peptide (five residues) between the light chain and heavy chain, two interdomain peptides (19 residues each), and a C-terminal pro-peptide (18 residues) (20). The precursor protein would be converted into the interchain peptide; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

*This work was supported in part by Grants-in-Aid for Scientific Research and by the 21st Century Center of Excellence Program from the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: bromein, bromelain isoinhibitor; BBI, Bowman-Birk serine proteinase inhibitor; bromein-6N, native bromein-6 with light and heavy chains; bromein-6, recombinant single-chain bromein-6 with an interchain peptide; bromein-6H, native bromein-6 processed incompletely by bromelain; bromein-6R, recombinant single-chain bromein-6 without inhibitor–like inhibitors (6), thyropins (7), inhibitors homologous to the propeptide regions of cysteine proteinases (8), and clitocypins (9).
into mature iso-inhibitors (6 kDa) by proteolytic processing. Moreover, we constructed a recombinant single-chain sixth bromelain with the interchain peptide (bromein-6R, Fig. 1B) and revealed that it shows almost the same inhibitory activity and secondary structure as bromein-6N (20). Interestingly, bromelain-digested bromein-6R (bromein-6R) exhibited much more bromelain inhibitory activity than bromein-6R (19). However, the essential inhibitory site has not been precisely specified so far. In the present report, to identify the inhibitory site of bromein, we prepared 44 kinds of site-directed and deletion mutants and investigated the inhibitory activity of each toward bromelain. For the mutants with low inhibitory activity, the secondary structures were examined using their circular dichroism (CD) spectra.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Bromein-6R and Its Mutants—His-tagged bromein-6R was expressed in Escherichia coli and was purified by Ni-NTA agarose and MonoQ chromatography as described previously (20). The expression vectors for the mutants were constructed from a pET32′-bromein-6R plasmid by using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). The mutant proteins were expressed in E. coli and were purified according to the same method used for bromein-6R. The identification and purity of the samples were confirmed by both matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and Tricine SDS-PAGE (16.5% gels) without dithiothreitol steps of chromatography to give a single band on Tricine SDS-PAGE (data not shown). Furthermore, the purity of the mutants was confirmed to be more than 95% by MALDI-TOF MS analysis (data not shown). As shown in Fig. 2, almost complete loss of the inhibitory activity was observed in the Pro12- and Leu13-substituted mutants as well as in the mutants without the interchain peptide. It is remarkable that the mutant T15P showed no inhibitory activity against bromelain. In the following sections, we will discuss the inhibitory activity of these mutants in greater detail.

Here, we examined the secondary structures of the mutants with low inhibitory activity by using their CD spectra. As a result, the spectra of the mutants V10A, P12A, L13A, L13I, R14D, and R14I were roughly identical to the spectrum of bromelain-6R (Fig. 3). This indicates that the secondary structures of these mutants and bromelain-6R are similar to the secondary structure of bromelain-6N, because bromelain-6R has almost the same secondary structure as bromelain-6N (20). Interestingly, the CD spectrum of the non-inhibitory mutant T15P was almost identical to that of bromelain-6R (Fig. 3). On the other hand, the spectrum of the non-inhibitory mutant L13V was different from that of bromelain-6R (Fig. 3), indicating that the L13V mutant did not retain the same secondary structure as bromelain-6R.

Site-directed Mutagenesis of the Light Chain—In a previous study (14), we constructed a binding model between bromelain and papain by computer modeling. According to this model, the C-terminal region of the light chain, especially Arg34, protrudes into the solvent from one edge of the inhibitor and fits well into the active site cleft region of the target protease.
Therefore, we focused the mutational analyses on the C-terminal region of the light chain (Fig. 2). As a result, the inhibitory activities of the mutants S7A, E8A, and V10A were only slightly decreased as compared with that of bromelin-6R. On the other hand, the activity of the P12A and P12R mutants fell to less than 20 and 3%, respectively. This indicates that the Pro12 residue, the angle of which is fixed, plays an important role in restricting the backbone movement in the C-terminal region of the light chain and the conformation of Leu13.

With the exception of the L13Y mutant, the inhibitory activity for each of the Leu13-substituted mutants fell to less than about 5% (Fig. 2). Interestingly, even the substitution of leucine to isoleucine or valine, whose chemical structure is similar to that of leucine, resulted in about 95% inhibitory activity loss. The secondary structure of these mutants, with the exception of L13V, was almost identical to that of bromelin-6R as judged by CD analysis (Fig. 3). It seems likely that the secondary structure of the L13V mutant is disrupted (Fig. 3), and thus may be misfolded. However, the NMR analysis indicates that the whole structures of the mutants L13F and L13V were almost the same as the structure of bromelin-6R (Fig. 4). Taken together, these findings demonstrated that bromelain strictly recognizes the chemical structure of Leu13 in bromelain.

Furthermore, the fixed dihedral angle of Pro12 was also found to be important for keeping the proper side-chain conformation of Leu13 for bromelain inhibition.

Previously, we proposed that the Arg14 residue is a putative reactive site for bromelain (14, 24), since leupeptin, another cysteine protease inhibitor, has the chemical structure of N-acetyl-Leu-Leu-argininal and exhibits trypsin inhibitory activity (23). However, the present study revealed that the Arg14-substituted mutants, especially the mutant R14E, did not lose a substantial amount of inhibitory activity (Fig. 2). This indicates that the positive charge at this position is not important for bromelain.
inhibition. On the other hand, the bromelain inhibitory activity of the R14D and R14I mutants were 40 and 28%, respectively. The results suggested that the different sizes of the side chain of aspartic acid or isoleucine might influence the side-chain conformation of Leu^{13}, thereby affecting the inhibitory activity.

Site-directed Mutagenesis of the Interchain Peptide—We previously revealed that stem bromelain performs stepwise processing of the interchain peptide, and that the 50% inhibitory (IC_{50}) value of bromein-6^{R} was ~10-fold higher than that of bromein-6^{RP} (19). Accordingly, the full inhibitory activity might require cleavage between the light chain and interchain peptide or the removal of some residue(s) in the interchain peptide. In this study, we prepared several recombinants, the mutants T15A, T15D, T15P, S16A, S17A, S18A, D19A, and D19S, which are mutated on the region of the interchain peptide. These mutants except for the T15P mutant did not show any activity loss (Fig. 2), indicating that the removal of some residue(s) in the interchain peptide is not important for bromelain inhibition.

Here, a question arises as to whether or not the cleavage at Arg^{14}-Thr^{15} is essential for inhibitory activation. We examined the bromelain cleavage site of the non-inhibitory mutants L13I and T15P by SDS-PAGE and N-terminal sequencing analyses. The results showed that bromelain hydrolyzed easily at Arg^{14}-Thr^{15} of L13I within 2 h of incubation, while Arg^{14}-Pro^{15} of T15P was not cleaved by bromelain even after 20 h of incubation (data not shown). We propose that the fixed φ dihedral angle of Pro^{15} likely restricts the conformation of the putative cleavage site of T15P. Considering that bromein-6^{R} was processed at Arg^{14}-Thr^{15} within 4 h (19), we concluded that this cleavage is not essential for the activation of inhibition and the free C-terminal motility in the light chain region would be required for full inhibitory activity.

Deletion Analysis of the Interchain Peptide—In the previous section, we confirmed that the amino acid sequence of the interchain peptide is not particularly important for the inhibitory activity. Here, we investigated whether or not the length of the interchain peptide can affect the activity. We constructed deletion mutants with various lengths of the interchain peptide and examined the activity of each mutant. The deletion mutant lacking one residue in the interchain peptide, the dD19 mutant, showed ~30% loss of activity, and the mutants lacking more than two residues, the dSD19 and dSSSD19 mutants and bromein-6^{R}, lost almost all inhibitory activity (Fig. 2). This indicates that at least four residues of the interchain peptide were necessary for the inhibitory activity. The five-residue length in the interchain peptide thus would maintain the proper conformation of the Leu^{13} side chain for a good fit into the catalytic site of the target proteinase. Considering that the double-chain inhibitor bromein-6^{N} showed higher inhibitory activity than the single-chain inhibitor bromein-6^{R}, the free movement of the Leu^{13} side chain appears to be very important for inhibition.

Site-directed Mutagenesis of the Heavy Chain—We next performed site-directed mutagenesis for the charged residues and terminal regions in the heavy chain. Only the mutant D28A showed a more than 30% loss of inhibitory activity by this analysis (Fig. 2), indicating that the other residues in the heavy chain are not directly involved with proteinase inhibition. In previous studies (3, 25), the carboxyl groups of the side chains of Asp^{58} and Asp^{51} were revealed to form hydrogen bonds with the...
amide protons of Ser7 and Lys38 above pH 4, respectively. In the molecule, two linking regions (Thr27-Asp28 and Cys49-Leu50) connect the inhibitory domain to the stabilizing one (17). Accordingly, these hydrogen bonds might act as hooks between the linking regions and the domains, resulting in rigid binding between the inhibitor and the enzyme. It is probable that these mutations do not lead to conformational change of the Leu13 side chain directly, consistent with the small inhibition loss of the mutants D28A and D28A/D51A.

Deletion Analysis of the Disulfide Bridges in the Inhibitory Domain—Thus far, we have demonstrated that almost all the residues essential to bromelin inhibition exist on the inhibitory domain, especially near Leu13 (Fig. 1A). We previously proposed that the conformational stability of the inhibitory domain is important for the inhibitory activity (17). Therefore, we here made two double mutants, C6A/C26A and C11A/C24A, lacking the disulfide bridges near Leu13, Cys6–Cys26 and Cys11–Cys24, respectively (Fig. 2). The secondary structures of these mutants appeared to be a little different from the secondary structure of bromelin-6R as judged by CD analysis (Fig. 3), indicating that a part of the antiparallel β-sheet on this domain may be broken down.

The NMR spectra of these mutants were a little different from the spectrum of wild-type bromelin-6R (Fig. 4). For example, almost all NH resonances of Ser7, Cys26, Lys38, and Cys49 in the mutant C6A/C26A were not observed in the lower magnetic field region (Fig. 4). This suggests that the hydrogen bonds of Ser7(NH)→Asp26(β-CO2H), Cys26(NH)→Asp31(C=O), Lys38(NH)→Asp51(β-CO2H), and Cys49(NH)→Asp26(C=O) disappeared or weakened on the molecule (3, 25). In particular, the hydrogen bond Cys26(NH)→Asp51(C=O) is a component of the β-sheet on the inhibitory domain (15), indicating that the formation of this β-sheet is involved in bromelin inhibition. It is noteworthy that the deletion of the disulfide bridge Cys6–Cys26 affected the formation of the hydrogen bond Cys49(NH)→Asp28(C=O) on the stabilizing domain. As concerns the mutant C11A/C24A, two of these NH resonances were observed in the downfield region (Fig. 4). Perhaps, the hydrogen bonds of Ser7(NH)→Asp26(β-CO2H) and Cys26(NH)→Asp51(C=O) might have disappeared or weakened on the molecule, because these hydrogen bonds are located closely to the disulfide bridge Cys11–Cys24.

These disulfide bridges thus would play an adjunctive role in the inhibitory mechanism, since these mutants showed more than 50% inhibition loss (Fig. 2). Particularly, the disulfide bridge Cys11–Cys24 seems to be important for bromelin inhibition, because the inhibitory activity of the mutant C11A/C24A was lower than that of the C6A/C26A mutant. In addition to the hydrogen bonds mentioned above, the β-sheet structure would also be important for keeping the proper geometry of the Leu13 side chain.

Substitution Analysis of the Loop Peptide Corresponding to the Inhibitory Loop of BBI—As described earlier, bromelin-6R shares similar folding and disulfide bridge connectivity to BBI and possesses slight inhibitory activities toward trypsin and chymotrypsin (17, 19). In this study, to examine whether the trypsin-inhibitory activity exists on the inhibitory or stabilizing domain, we prepared the mutants bromelin-6BBI-1 and bromelin-6BBI-2, in each of which the putative inhibitory loop in the stabilizing domain is substituted by the trypsin-inhibitory loop of BBI from soybeans (18). As shown in Fig. 1B, the amino acid sequence in bromelin-6BBI-1 corresponding to the stabilizing domain is 4TKSNPPO49, while the bromelin-6BBI-2 sequence is 4ACTSNPQPCR51. The residues Lys13 and Leu44 in soybean BBI were the reactive residues against trypsin and chymotrypsin, respectively (18). The structural difference between bromelin-6BBI-1 and bromelin-6BBI-2 is that in the latter an additional inner disulfide bridge is integrated into the stabilizing domain. As a result, the 50% inhibitory (IC50) values of bromelin-6N and bromelin-6BBI-2 were 24.2 ± 0.3 and 4.69 ± 0.13 μM against trypsin, respectively. In a control experiment, the IC50 value of soybean BBI was 1.69 ± 0.02 μM under the same condition. On the other hand, the trypsin-inhibitory activities of bromelin-6R, bromelin-6RP, and bromelin-6BBI-1 were not detectable.

These results thus revealed that the trypsin-inhibitory site in bromelin-6N exists not on the stabilizing domain but on the inhibitory one. Because bromelin-6R and bromelin-6RP did not exhibit trypsin inhibitory activity, the flexible C-terminal region of the light chain would be necessary for trypsin inhibition. However, the terminal-ragged interchain peptide digested by bromelain might interfere with the inhibitory activity. Moreover, the inner disulfide bridge (Cys42–Cys50) in bromelin-6BBI-2 was revealed to be important for trypsin inhibition, indicating that the inhibition requires the conformational stability of the inhibitory loop (Cys40–Cys49). This is applicable to the case of bromelain inhibition, where the C11A/C24A mutant lost more than 60% inhibitory activity as compared with bromelin-6R (Fig. 2).

BBI interacts with serine proteinases in a substrate-like manner, and the proteinase-binding loop is kept in a well-ordered conformation. There is a consensus sequence, CT-P4-SXP-PPC, among most of the reactive site loops of BBI. The consensus sequence corresponds to P5-P4-P3-P2-P1-P0-(P5-P4-P3-P2-P1-P0)-based on the nomenclature of Schechter and Berger (26). P1 indicates a residue that determines the specificity of inhibition; for instance, the optimal residues for trypsin and chymotrypsin are Lys/Arg and Phe/Trp/Leu, respectively (27, 28). Bromelain-6R also has two highly conserved cysteines and leucine in the reactive loop of 12CPLREYKC19; however, this protein did not exhibit any chymotrypsin inhibitory activity (data not shown). In BBI, a cis-formed proline at the P3 position is reported to be essential for biological activity and to constitute the center of a type-VI β-turn (29). On the other hand, bromelin-6R does not have such a proline at the P3 position; therefore this protein could not inhibit chymotrypsin.

Inhibitory Mechanism of Bromelin—Bromelin belongs to the papain superfamily of cysteine proteinases (12); papain (30) and bromelain (11) are each composed of a 212-residue polypeptide chain cross-linked by three disulfide bridges at essentially the same locations, and they are about 40% identical in sequence. Functionally important residues in papain, such as Cys22, His159, and several other residues at the active site cleft, including Val133 and Val157, are largely conserved in bromelain as Cys26, His198, Val136, and Leu148, respectively. Accordingly, the three-dimensional structure of bromelain is assumed to be very
similar to that of papain, although the structure of bromelain so far has not been solved. On the other hand, the crystal structure of a papain-leupeptin complex has been determined by x-ray crystallography (31); therefore, we can discuss the inhibitory mechanism of bromelain-6N using papain instead of bromelain, since papain is known to be inhibited by bromelain-6N (3).

In the three-dimensional structure of the papain-leupeptin complex, the Leu$^1$ side chain in leupeptin is involved in hydrophobic interactions with the side chains of Tyr$^{61}$ and Tyr$^{67}$ in papain (31). The Leu$^2$ side chain in leupeptin firmly binds the hydrophobic pocket enclosed by the side chains of Tyr$^{67}$, Pro$^{68}$, Val$^{133}$, and Val$^{157}$ in papain. On the other hand, the guanidino group of leupeptin does not interact with any residue in the complex. Now, we can discuss a putative papain-bromein complex based on these interactions in the papain-leupeptin complex. In this model, the Leu$^{13}$ side chain in bromelain-6N would be placed in the hydrophobic pocket of the proteinase and Pro$^{12}$ was noted with the inhibition of bromelain. In consideration of the leupeptin-papain complex, the hydrophobic interaction between the hydrophobic pocket of the proteinase and Pro$^{12}$ and Leu$^{13}$ of bromelain appears to be the most important factor in bromelain inhibition.

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