Structure-Function Relationships of Proteinase Inhibitors from Soybean (Bowman-Birk) and Lima Bean

MODIFICATION BY N-ACETYLMIDAZOLE*

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Contributions of tyrosyl residues to trypsin- and chymotrypsin-inhibitory activities in two homologous proteinase inhibitors were investigated by modifying them with N-acetylimidazole under various conditions. In Bowman-Birk soybean proteinase inhibitor, Tyr 55, immediately following the antichymotryptic site, Leu 53-Ser 54, is relatively inaccessible to N-acetylimidazole and can only be acetylated in the presence of 6 M guanidine hydrochloride but not in 8 M urea. The acetylation of Tyr 55 is accompanied by 60% loss in antichymotryptic activity. Deacetylation with hydroxyamine restores the activity to the original level. Tyr 69, located in the antitryptsin portion of the inhibitor, is exposed relatively to N-acetylimidazole and can be acetylated without denaturing agent. The acetylation of Tyr 69 parallels decrease in antitryptic activity. The inhibitor acetylated at Tyr 69 is fully active toward chymotrypsin and has 30 to 40% antitryptic activity of the native. The original level of antitryptic activity is restored upon deacetylation.

Tyr 69 of lima bean proteinase inhibitor is relatively inaccessible to N-acetylimidazole: 75% acetylation in the presence of 6 M guanidine hydrochloride and 17% without the denaturing agent. The acetylated inhibitor is fully active toward chymotrypsin but retains only 29% (acetylated without guanidine hydrochloride) and 17% (acetylated with guanidine hydrochloride) of the original antitryptic activity. Deacetylation partially restores the lost antitryptic activity in the inhibitor acetylated without the denaturing agent.

The total and irreversible loss of antitryptic activity in samples acetylated in the presence of 8 M urea or 6 M guanidine hydrochloride is attributed to the acetylation at the ε-amino group of Lys 26 at the trypsin-inhibitory site.

Bowman-Birk soybean proteinase inhibitor and lima bean proteinase inhibitor are "double-headed" inhibitors which inhibit trypsin and chymotrypsin at independent reactive sites (1-6). A close homology between the inhibitors can be recognized by a near-identity in their amino acid sequences; only six positions are substituted from residues 13 to 73 (5, 7, 8).

BBI can be cleaved into three fragments by cyanogen bromide treatment followed by pepsin digestion. One fragment, residues 11 to 37 and 67 to 77 held together by four disulfide bonds, contains the trypsin-inhibiting site Lys 26-Ser 27. It has 84% antitryptic activity of the native and no antichymotryptic activity. The second consists of residues 38 to 66, which include the chymotrypsin-inhibitory site Leu 53-Ser 54 and three disulfide bonds. The antichymotryptic fragment possesses no antitryptic activity and 16% antichymotryptic activity of the intact inhibitor. The third fragment is a tetrapeptide from the COOH terminus of the native inhibitor (9). Spectrophotometric study of BBI showed that one of two tyrosyl phenolic groups is relatively exposed and can be O-acetylated by 100- to 1500-fold molar excess of N-acetylimidazole. The other is inaccessible to the reagent even in the presence of 8 M urea, but can be acetylated in the presence of 6 M guanidine hydrochloride. Which of the 2 tyrosyl residues is exposed could not be determined (10). LBI contains only one tyrosine, Tyr 69, which is relatively inaccessible to N-acetylimidazole. A 100-fold excess of the reagent in the presence of 6 M guanidine hydrochloride elicited about 75% O-acetylation (11).

Radiation studies of BBI and LBI suggest that damage to BBI Tyr 55, adjacent to the antichymotryptic site, leads to loss of chymotrypsin-inhibitory activity. Radiation damage to Tyr 69, located in the antitryptic fragment, has no effect on either of the antiproteinase activities in both inhibitors (11, 12).

In the present study, antiproteinase activities of BBI and LBI derivatives acetylated under various conditions have been determined and the roles of tyrosyl residues in the activities have been evaluated.

EXPERIMENTAL PROCEDURES

BBI and LBI were purified as described (10, 11). Acetylation and deacetylation of the inhibitors were performed according to reported methods (13, 14). Unreacted N-acetylimidazole was removed by dialysis against water at 4°C. Inhibitory activities of the inhibitors were measured spectroscopically, as described (12).

RESULTS

Fig. 1 presents relationship between the O-acetylation of tyrosyl side chains and the change in inhibitory activities in BBI. The treatment of the inhibitor with increasing amounts of N-acetylimidazole resulted in a progressive acetylation of tyrosyl side chains. However, only 1 of 2 tyrosyl residues in BBI could be modified by 100- to 1500-fold molar excess of the reagent. Urea (8 M) did not affect the tyrosine acetylation, as one tyrosine remained unmodified by its presence (Table I). When BBI was acetylated in the presence of 6 M guanidine...
hydrochloride, however, both tyrosines were almost completely acetylated (Table I).

Antichymotrypsin activity of BBI was not affected by the acetylation of the “accessible” tyrosine, as inhibitor modified by N-acetylimidazole alone was fully active toward chymotrypsin, even after treatment with 1500-fold molar excess of the reagent (Fig. 1). Antiprotease activity, however, paralleled the acetylation of the accessible tyrosine and decreased as the tyrosyl side chain was acetylated. At 100-fold molar excess of the reagent, antiprotease activity was about 40% of the original; further increase did not reduce the trypsin-inhibitory activity much. At 1500-fold excess, modified BBI retained about 25% antiprotease activity of the native. The lost antiprotease activity of the modified inhibitor acetylated without denaturing agent could be recovered fully by deacetylating the O-acetylated tyrosyl side chain with hydroxylamine (Table I).

Although essentially no enhancement in tyrosine acetylation was observed in samples modified in the presence of 8 M urea, antiprotease activity was completely lost by this treatment, while antichymotrypsin activity remained relatively unaffected (Table I). Desacetylation of the sample acetylated in 8 M urea restored the original antichymotrypsin activity, but not the antiprotease activity.

A sample acetylated in the presence of 6 M guanidine hydrochloride and with both tyrosyl side chains O-acetylated lost all of its antiprotic activity and more than half of antichymotrypsin activity (Table I). Antiprotease activity could not be regained by deacetylation of O-acetyltirosines but antichymotrypsin activity was fully restored by this process.

The acetylation of LBI with a 100-fold molar excess of N-acetylimidazole induced 17% modification of tyrosine (Table I). The modified LBI lost more than two-thirds of the original antiprotic activity but retained full activity toward chymotrypsin. The deacetylation of O-acetyltirosine by hydroxylamine realized only a partial recovery of the lost antiprotic activity.

The presence of 6 M guanidine hydrochloride during acetylation enhanced the extent of tyrosine modification to 75% from 17% (Table I). An increased loss in the antiprotic activity was also noted, but the antichymotrypsin activity was unaffected by the presence of the denaturing agent. The lost antiprotic activity could not be regained by the restoration of tyrosyl side chains by hydroxylamine.

The treatment of the inhibitors with only 8 M urea, 6 M guanidine hydrochloride, or hydroxylamine without N-acetylimidazole did not have any effect on either of the antiprotease activities.

**Table I**

<table>
<thead>
<tr>
<th>Molar excess N-acetylimidazole</th>
<th>Acetylated</th>
<th>Deacetylated</th>
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</thead>
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<tr>
<td>Per cent tyrosine acetylated</td>
<td>Antiprotic</td>
<td>Antichymotrypsin</td>
</tr>
<tr>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4.1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>17</td>
<td>100</td>
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</table>

**Discussion**

Of 2 tyrosyl residues in BBI, Tyr 55 is located next to antichymotrypsin site Leu 53-Ser 54. The other, Tyr 69, resides in the antiprotease fragment but the proximity of this tyrosine to the trypsin-inhibitory site is unknown (9). In I.BI, Tyr 69 is the sole tyrosine, as position 55 has isoleucine in the inhibitor (15).

From results of the present study, the tyrosyl residue exposed to N-acetylimidazole in BBI may be assigned to Tyr 69 in the antiprotease fragment because: (a) the loss of antiprotease activity follows closely the acetylation of the accessible tyrosine, (b) antiprotease activity can be restored to the original level by deacetylating the BBI derivative which was acetylated without denaturing agent; and (c) the acetylation of the accessible tyrosine does not have any effect on antichymotrypsin activity. The other tyrosyl residue that is relatively inaccessible to N-acetylimidazole must then be Tyr 55, adjacent to the antichymotrypsin active site. Additional evidence is that the acetylation of the “inaccessible” tyrosine results in 60% reduction of antichymotrypsin activity which can be restored upon deacetylation.

The acetylation of Tyr 69 in BBI without denaturing agent induces 60 to 70% loss in antiprotease activity which is fully recoverable by deacetylation. The presence of 8 M urea during the acetylation does not influence the degree of tyrosine modification, but it produces a complete and irreversible loss in antiprotease activity. The presence of 8 M urea must have promoted the acetylation of other amino acid residues(s) essential to trypsin inhibitory activity. Although N-acetylimidazole is used primarily for acetylation of tyrosyl side chains, acetylation of amino groups has been demonstrated as well (13). e-Amino groups of lysines can be modified by such a treatment (16), but only O-acetytyrosine is deacetylated by the procedure used (13). The substitution of Lys 26 at the trypsin inhibitory site may then be suggested for the irreversible loss in antiprotease activity. Similarly, a total and irreversible loss of antiprotease activity in samples acetylated in the presence of 6 M guanidine hydrochloride may also be attributed to the irreversible acetylation of the lysyl residue.

The results of I.BI acetylation support the assignment of tyrosyl residues in BBI. The acetylation of Tyr 69 in LBI, with or without 6 M guanidine hydrochloride, has no effect on antichymotrypsin activity at all while antiprotease activity
reduced. A part of the lost antitryptic activity in the sample modified without the denaturing agent can be recovered upon deacetylation. The irreversible acetylation of Lys 26 might also be assumed for unrecoverable loss of antitryptic activity in LBI.

A conclusion that modification of Tyr 55 results in the loss of antichymotryptic activity is in accord with a previous study using free radicals as modifying agents. In that study radiation damage to Tyr 55 was suggested to be accompanied by decline in antichymotryptic activity without affecting antitryptic activity (12). However, the modification of Tyr 69 by free radicals had no effect on either of the antiproteinase activities in both BBI and LBI (11, 12), a conclusion which seems contradictory to results of the acetylation study.

Since radiation and acetylation produce different products, the modification of the same residue in a protein by these two techniques may be expected to induce different effects on the functions. Derivatives of Br₂⁻ and (CNS)₂⁻ attack on tyrosine have not been well characterized. By analogy with hydroxyl radicals, however, a major product of tyrosine degradation by these radical anions may be assumed to be 3,4-dihydroxyphenylalanine (17-19), a minor structural modification. On the other hand, acetylation introduces a relatively bulky acetyl group at the hydroxyl group. Such a bulky substitution may sterically hinder the formation of a proper enzyme-inhibitor complex.

Although the modification of Tyr 55 in BBI by either radiation or acetylation results in the loss of antichymotryptic activity, it is not an "essential" amino acid and probably does not participate directly in chymotrypsin binding. Thus it is replaced by other amino acids in inhibitors homologous to BBI without losing antichymotryptic activity, isoleucine in LBI (15) and methionine in garden bean proteinase inhibitor II (20). However, the integrity of Tyr 55 is essential to chymotrypsin-inhibitory activity in BBI.

REFERENCES