Identification of an Essential Tryptophan Residue in Alliinase from Garlic (Allium sativum) by Chemical Modification

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We have employed chemical modification to identify amino acids essential for the catalytic activity of alliinase (EC 4.4.1.4) from garlic (Allium sativum). Alliinase degrades S-alkyl-L cysteine sulfoxides, causing the characteristic odor of garlic. The activity of alliinase was rapidly and completely inactivated by N-bromosuccinimide (NBS) and slightly decreased by succinic anhydride and N-acetylimidazole. These results indicate that tryptophanyl, lysyl, and tyrosyl residues play an important role in enzyme catalysis. The reaction of alliinase with NBA yielded a characteristic decrease in both the absorbance at 280 nm and the intrinsic fluorescence at 332 nm with increasing reagent concentration of NBS, consistent with the oxidation of tryptophan residues. Kinetic analysis, fluorometric titration of tryptophans and correlation to residual alliinase activity showed that modification of only one residue present on alliinase led to complete inhibition of alliinase activity. To identify this essential tryptophan residue, we employed chemical modification by NBS in the presence and absence of the protecting substrate analogue, S-ethyl-L-cysteine (SEC) and N-terminal sequence analysis of peptide fragment isolated by reverse phase-HPLC. A fragment containing residues 179-188 was isolated. We conclude that Trp182 is essential for alliinase activity.

Keywords: Alliinase active site, Tryptophan of alliinase.

Introduction

Garlic (Allium sativum Linné) is a widely distributed plant and is used in all parts of the world not only as a spice and a food, but also as a popular remedy. The odor characteristic of garlic is due to the degradation of S-alkyl-L cysteine sulfoxides by alliinase (EC 4.4.1.4). It is generally believed that the alliinase and the amino acid substrate are present in separated compartments in vivo. Upon rupturing or wounding of the cells, the enzyme located in the vacuole and the alkyl cysteine sulfoxides located in the cytoplasm can react and produce the volatile odorous compounds.1 Allicin (diallyl thiosulfinate or allyl 2-propenethiosulfinate) is the dominant thiosulfinate formed by the rapid condensation of two molecules of 2-propenesulfenic acid, an intermediate of the alliin mechanism of the enzyme from any source.

The goal of this study was to identify amino acids important for the activity of alliinase in garlic. We have chemically modified the enzyme using a variety of reagents to reveal the importance of Trp residues and to a lesser extent Lys and Tyr groups. By modifying the enzyme with NBS in the absence and presence of a substrate analogue, combined with N-terminal sequence analysis of peptides from proteolytic digests, we have identified the essential tryptophan residue as Trp182.

Materials and Methods

Materials. Bulbs of garlic (A. sativum L.) were purchased locally. The standard substrate, S-ethyl-L-cysteine sulfoxide (SECS), was synthesized from S-ethyl-L-cysteine (SEC) purchased from Sigma, by oxidation with acid H2O2 as

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described previously. The following chemical modification reagents were purchased from Sigma; dithiothreitol (DTT), succinic anhydride (SA), N-acetylglutamylamide (NAI), maleic anhydride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), iodoacetic acid, phenylmethylsulfonyl fluoride (PMSF), diethyl pyrocarbonate (DEPC), l-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (CMC), N-bromosuccinimide (NBS) was obtained from Wako. Polyvinylpolypyrrolidone, concanavalin A Sepharose 4B, methyl a-D-mannopyranoside, pyridoxal 5'-phosphate (PLP), EDTA (disodium salt), pyruvic acid (sodium salt), 2,4-dinitrophenylhydrazine, guanidine hydrochloride (Gdn-HCl), and TPCK-treated trypsin were purchased from Sigma. Trifluoroacetic acid (TFA) for dansyl hydrochloride (DANS), maleic anhydride, and DTT had to be removed from the reaction tubes by dialysis, or gel filtration prior to enzyme assay when excess DTT (about more than 300 molar excess) was used. In the experiments for acquiring the kinetic data of inactivation by modifying reagents, after allinase was treated with varying concentration of SA, NAI, and NBS with changing reaction time, the residual activity of allinase was recorded.

**Effects of NBS Modification on Allinase Spectral Properties.**

**UV Absorption Spectra:** The samples for measuring of UV absorption consisted of the following conditions. In all cases of modification reaction with NBS, the final concentration of protein was maintained at 5 μM. A Control not modified with NBS, “Modified 1” oxidized by 0.5 mM NBS, and “Modified 2” oxidized by 1 mM NBS were prepared in buffer A (pH 5.5) at room temperature. The reaction mixture was incubated for 1 min in the dark to minimize unspecified reactions and quenched by addition of a threefold molar excess of DTT. After reaction, these samples were dialyzed against buffer A’ (pH 5.5) and centrifuged to remove, if present, insoluble particles. The sample concentration was adjusted to 0.2 mg/mL and added to a quartz cuvette. Ultraviolet spectra were scanned over the range of 240-320 nm using a Kontron UVikon 930 spectrophotometer at room temperature.

**Fluorescence Emission Spectra:** Fluorescence measurements were carried out with a Kontron Instruments (model SFM 25) spectrophotometer. Fluorescence intensities were determined using an excitation wavelength of 295 nm. The emission spectra were obtained over the range of 310-380 nm at room temperature. The modification procedures were identical with the above cases. It is unnecessary to dialyze reaction mixtures, because only protein could emit fluorescence by excitation at 295 nm. The final protein concentration in the cuvette was adjusted to 1 μM.

**Titration of Unmodified Tryptophan Residues:** The number of tryptophan residues unmodified was determined by the Pajot method slightly modified for this experiment. 600 μL of 5 μM protein, 2393 μL of 6 M Gdn-HCl in 0.1% NH4HCO3 (pH 8.0), and 7 μL of 14 M 2-mercaptoethanol was directly submitted to a fluorescence cuvette. After incubation for 1 h for protein denaturation, its fluorescence was measured at 354 nm as described in the fluorescence emission spectra procedures. The standard curve was obtained.
with known concentrations of free L-tryptophan solution.

**Identification of an Essential Tryptophan Residue.**

**Determination of Modification Condition of Alliinase:** We have employed chemical modification with different concentrations of NBS for 20 sec in buffer A’ (pH 5.0) in order to find the specific condition in which the active site of alliinase could be protected by SEC from modification with NBS. The reaction had to be stopped quickly by addition of a threefold molar excess of DTT after 20 sec. The residual activity of the respective samples was analyzed and the unmodified tryptophan residues were titrated as described previously.

**Trypsin Digestion:** The native alliinase and NBS-modified derivatives were dialyzed extensively overnight against 0.1% NH₄HCO₃ (pH 8.0) and lyophilized. The respective aliquots of 10 nmol were dissolved in 100 μL of 8 M urea, and then 100 μL of 0.4 M NH₄HCO₃ and 2 μL of 0.5 M DTT were added. These samples were incubated in a water bath adjusted to 50 °C for 30 min, cooled to room temperature, and then 20 μL of 0.1 M iodoacetamide was added. After 15 min, sample solutions were diluted by addition of 250 μL of triply distilled water that will be referred to as tdH₂O throughout the rest of this paper. Tryptic cleavage was then carried out with 22 μL of TPCK-treated trypsin (1 mg/mL) at an enzyme/substrate ratio of 1 : 25 (w/w) at 37 °C for 24 h. All of the above reagents had to be dissolved in tdH₂O. The digestion was terminated by acidification with 20% TFA to pH 2-2.5. The insoluble particles were removed by centrifugation prior to separation by reverse phase-HPLC.

**Sampling for Reverse Phase-HPLC:** Samples for sequencing were diversely prepared by the following procedures. All protein samples had to be dialyzed against buffer A’ (pH 5.0) prior to modification with NBS. The concentration of proteins was controlled at 5 μM in all experiments. “Control” was not treated with NBS. “Modified 1, 2, and 3” were modified with 1.5 mM NBS for 8, 20, and 30 sec, respectively, and quenched with a threefold molar excess of DTT. “Protected 1” was incubated with 2.5 mM SEC for 20 min for protection of the active site, dialyzed against buffer A’ (pH 5.0), and concentrated by a centrificon. After the concentration of “Protected 1” was adjusted to 5 μM, it was modified with 5 mM NBS for 20 sec, and then quenched. “Protected 2” was prepared by modification with 10 mM NBS for 20 sec after incubation with 2.5 mM SEC for 20 min. All samples were digested with TPCK-treated trypsin by the method described previously.

**Reverse Phase-HPLC Separation Condition:** Separation of peptides generated by trypsin digestion of alliinase, modified, and protected samples was achieved using a Beckman HPLC system (model: system GOLD). Separations were carried out using a Vydac 218TP54 C18 reverse phase column (300 Å, 5 μm, 4.6×250 mm). In all cases, solvent A was 0.1% TFA in tdH₂O : acetonitrile (99 : 1) and solvent B was 0.1% TFA in tdH₂O : acetonitrile (2 : 8). The flow rate was fixed to 1 mL/min. After sample injection, the solvent mixture was kept constant at 0% sol. B for 10 min and then raised to 20% sol. B over 30 min, 50% sol. B over 60 min, and 80% sol. B over 20 min. Absorbance was monitored at 280 nm to have the advantage of recording absorbance of tryptophanyl residues.

**Sequence Determination:** N-terminal sequence analyses of alliinase were performed by automatic Edman degradation using a model Procise 491 (Applied Biosystems) automatic gas phase sequencer at the Korea Basic Science Institute (KBSI).

**Results**

**Inactivation Kinetics of Alliinase by Selected Chemical Modifying Reagents.** It was revealed that tryptophan, lysine, and tyrosine residues, specifically modified by NBS, SA, and NAI, are critical for the enzymatic activity. Modification by the other reagents (DTT, EDC, PMSF, DEPC, CMC, maleic anhydride, iodoacetic acid) seems not to inhibit the enzymatic activity (not shown here). SA has proved useful in the modification of lysine. Alliinase lost 60% of its activity in 2 mM SA by the method described previously.

![Figure 1](image-url)
Lys is already known to participate in binding PLP, an important cofactor and lysine-modifying reagent itself. NAI was used as a reagent for the selective modification of tyrosyl residues. The treatment of enzyme with 20 mM NAI led to inactivation to about 50%, suggesting that the tyrosyl residues play a role in catalysis by alliinase. Alliinase was completely inactivated by addition of 5 mM NBS, selective for tryptophan, suggesting that the tryptophan residue is the most probable amino acid that participates in the substrate binding or catalysis of alliinase.

To calculate the kinetics of inactivation by modifying reagents, the logarithm of enzyme activity remaining was plotted as a function of time at different concentrations of reagents (Figure 1). These plots give a series of straight lines at different concentrations of reagents. The values of $k_1$, the first order rate constants, can be obtained from the slopes of the straight lines. The results show that with the increase of reagent concentration, the value of $k_1$ increased. The relationship between $k_1$ and the inhibitor concentration $[I]$ can be written:

$$k_1 = k_2[I]^n$$

where $k_1$ and $k_2$ are the first and second order rate constants of inactivation, respectively. $[I]$ is the concentration of the inhibitor. A plot of $k_1$ against $[I]$ gives a straight line which passes through the origin of the coordinate, as shown in Figure 2, where the slopes of the straight lines give the second order rate constants $k_2$ (Table 1). Under these reaction conditions, the rate on inactivation using NBS as a reagent is much faster than observed with SA or NIA. The $n$ is the reaction order, i.e., the number of essential residues modified. Plot of log $k_1$ against log $[I]$ gives a straight line, where the slopes of the straight lines give the values of $n$ (Table 1). The data imply that the loss of alliinase activity is due to the modification of approximately 1 Trp, 1 Lys, and 1 Tyr residue. The stoichiometry of inactivation is slightly higher for SA and consistent with the fact that this lysine specific reagent can also modify Tyr groups, albeit not effectively. Likewise, NBS yielded a reaction order of 1.14, indicating that this reagent can also modify Tyr groups, although at a much slower rate than Trp residues. Out of 9 Trp residues present per monomer in the primary sequence, only the modification of a single Trp results in a loss of enzyme activity.

**Effect of NBS Modification on Alliinase Spectral Properties**

Incubation of alliinase with NBS produced characteristic changes of its spectral properties depending on the NBS/alliinase molar ratio (Figure 3).

**UV Absorption Spectra**: It has been reported that some proteins with tryptophan residues modified by NBS have a decrease of absorbance at 280 nm. Increasing amounts of NBS led to a decrease in the absorbance at 280 nm, but an increase at 250 nm (Figure 3A), showing that tryptophan residues were transformed to their oxindole derivative. SDS-PAGE confirmed that NBS treatment did not result in cleavage of the polypeptide chains (not shown here).

**Fluorescence Emission Spectra**: The intrinsic fluorescence emission spectrum was also strongly affected (Figure 3B). As an excitation wavelength of 295 nm was used, the decrease in fluorescence shows the modification of the tryptophan residues. With the increase of the concentration of NBS, the fluorescence emission intensity at 332 nm decreased rapidly; moreover, when alliinase was treated with 300-fold molar excess of NBS for 5 min, its intrinsic fluorescence emission could be hardly detected at 332 nm (data not shown).

**Table 1. Kinetic data for the reaction of alliinase with succinic anhydride, N-acetylimidazole, and N-bromosuccinimide**

<table>
<thead>
<tr>
<th>modifying reagents</th>
<th>modified residue(s)</th>
<th>$k_2$ (M$^{-1}$ min$^{-1}$)</th>
<th>reaction order $n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinic anhydride$^b$</td>
<td>Lys, Tyr</td>
<td>17.8</td>
<td>1.51</td>
</tr>
<tr>
<td>N-acetylimidazole$^b$</td>
<td>Tyr</td>
<td>0.4</td>
<td>0.883</td>
</tr>
<tr>
<td>N-bromosuccinimide$^c$</td>
<td>Trp</td>
<td>1398</td>
<td>1.14</td>
</tr>
</tbody>
</table>

$^a$For modification conditions, see Experimental Procedures. $^b$These reagents were used in 0.1 M borate buffer (pH 7.0). $^c$The reaction with this reagent was performed in buffer A' (pH 5.5).
This result indicates that all nine tryptophan residues present in alliinase could be oxidized by NBS. For the emission spectra of the modified alliinase with different modified extents, no marked red shift was obtained. This suggests that the tryptophan residues relatively close to the surface of the enzyme have been modified and denaturation, or gross conformational changes, has not occurred in the modified alliinase.

Identification of Essential Tryptophan Residue

Determination of Modification Condition of Alliinase: We tried to protect the active site of alliinase with SEC, a substrate analogue. Alliinase was preincubated with 500 fold molar excess of SEC and then modified with 300 fold molar excess of NBS in buffer A’ (pH 5.5). The protein concentration in the cuvette was 0.2 mg/mL. [B] Fluorescence emission spectra of native alliinase (1) and alliinase oxidized with 10-, 30-, 50-, 100-, 200-, 300-fold molar excesses of NBS (2-7). Excitation wavelength was 295 nm. The protein concentration in the cuvette was 1 µM.

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It is noted that the oxidation of methionine is a possible side reaction of the treatment of proteins with NBS. In order to find out what is produced by the reaction with NBS and SEC, a simple test was performed on the assumption that SEC would be converted to SECS, a substrate of alliinase, by oxidation with NBS, because SEC is similar with methionine in structure. SEC was preincubated with NBS for 5 min, quenched, and added to the alliinase solution. After 5 min, we checked enzyme activity as described in the experimental procedures. The product of the reaction with NBS and SEC showed a similar characteristic with SECS as a substrate (not shown), showing clearly that SEC must be converted to SECS by oxidation with NBS. Since SECS can be located in the active site, both SEC and SECS produced by NBS can sufficiently protect the active site of alliinase. However, if the incubation time of the modification is long enough for the whole SEC to react with NBS, protection cannot be effective, because all the SECS produced was very rapidly degraded by alliinase. Reacting SEC-protected alliinase with NBS for 5 min resulted in complete modification, as evidenced by the absence of absorbing species (280 nm) normally resolved by RP-HPLC (data not shown). Therefore we also had to determine the modification time. Alliinase was modified with different concentrations of NBS for 20
Modification with 300 fold molar excess of NBS led to complete inactivation. On the basis of these data, all the modified samples were prepared under the above conditions, i.e. 300 fold molar excess of NBS for 20 sec in buffer A’ (pH 5.0), except for modification of protected samples. In this condition, the number of unmodified tryptophan residues was 3.2 (Trp mol/alliinase mol), indicating that six of the nine Trp residues are more reactive on NBS than the other three. Figure 5B shows the relationship between the fractional activity remaining (a) and the fractional Trp residue remaining (b) of the enzyme as a Tsou plot. It can be seen that among the six reactive tryptophan residues modified only one is essential for the alliinase activity and three less reactive residues do not have an effect on the enzymatic activity. This fact was evidently proven in the HPLC elution profile (Figure 5).

Tryptic Peptide Analysis for Active Site Tryptophan Identification: On oxidation, the indole chromophore of tryptophan with a strong absorbance at 280 nm is converted to oxindole with a considerably lower absorbance at this wavelength. This decrease in the absorbance at 280 nm was used to distinguish the tryptophan residues in the tryptophan peptides of the control, modified, and SEC-protected peptides (Figure 5). Although tryptophan residues out of the active site in the protected alliinase were not totally modified by NBS (Figure 5B, C), the tryptophan residue in the active site could be efficiently protected from modification by NBS and distinguished from others since it is obvious that tryptophan in the active center is much less reactive for NBS by protection than those out of the active site.

Nine main peaks, denoted 1, 2, 3, a, b, c, d, W73, and W182, were monitored from native alliinase (control) (Figure 5A). Peak 1, 2, and 3 showed a little decrease at 280 nm in the absence of SEC (Figure 5D, E, F), indicating that these residues are buried in the relatively hydrophobic regions of the protein and accordingly slowly modified by NBS in comparison with other residues. Therefore it turns out that these three residues do not have an effect on alliinase activity. This result is in accordance with data above (Figure 4). The absorbance of peak a, b, c, and d was markedly reduced in SEC-protected samples while the peaks of W73 and W182 were slightly decreased in absorbance (Fig-
Table 2. N-Terminal sequence of tryptic alliinase fragments unmodified or less modified from NBS, a little decreased in absorbance at 280 nmε

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>fragment denoted W73</td>
<td>1QGCSADVAS...</td>
</tr>
<tr>
<td>predicted fragment 54-74</td>
<td>1QGCSADVASGDLFLELEYWK</td>
</tr>
<tr>
<td>fragment denoted W182</td>
<td>GYVXAGNAAN...</td>
</tr>
<tr>
<td>predicted fragment 179-213</td>
<td>GYVWAGNAAANYVNV...PEGLLR</td>
</tr>
</tbody>
</table>

εThe peak W73 and W182 obtained in Figure 6 from native alliinase (control) was collected, lyophilized, and submitted to sequencing by automatic Edman degradation. The two obtained N-terminal sequences were identified by comparison with predicted fragments [11]. Even though in the fragment W73 tryptophan was not identified, we could determine that tryptophan is situated at 73 by comparison with the predicted fragment. X was not detected by sequencing, but must be W.

Figure 6 shows changes of fluorescence intensity (a) and the number of unmodified tryptophan residues (b) upon each sample in Figure 6.

Discussion

Chemical Modification of Alliinase with NBS. Oxidation of the tryptophan indole moiety into oxindole by NBS is particularly interesting since the additional steric hindrance of the substituted group is very limited, contrary to other chemical modifiers,30 and since the modified residues become totally nonfluorescent.31 No subunit dissociation is observed in polyacrylamide gel electrophoresis under non-denaturing conditions (not shown), consistent with the lack of red shift of the fluorescence emission spectra. No peptide bond cleavage is apparent under the modifying conditions used, as monitored by subsequent analysis of alliinase on SDS-PAGE (not shown here). This agrees with the observation that proteins are more susceptible than model peptides to NBS-dependent tryptophan modification and cleavage, which additionally require denaturating conditions, strongly acidic pH and a long incubation time.34,35 It is known that NBS can react with not only the indole group of tryptophan but also the SH group of cysteine in a number of proteins.25,36 Our results have shown that the modification of SH groups did not have an effect on the inactivation of catalytic activity of the enzyme by chemical modification with iodoacetate and DEPC, which modify cysteine residues. Therefore, the results presented in the above sections show clearly that the modification of tryptophan residues of alliinase by NBS leads to the complete inactivation of this enzyme. Even though the modification of tyrosine residues, which might be involved in the active site of alliinase, may have occurred, it turns out that the tryptophan residue is much more essential for activity of the enzyme than tyrosine through the chemical modification study. The linearity of the plot and the analysis for inactivation rate and Trp residue modification rate show that among the nine tryptophanyl residues only one is an essential Trp residue.

Properties of Tryptophans in Alliinase. Alliinase modified with NBS for about 5 min becomes nonfluorescent, suggesting that all tryptophan residues become completely oxidized by NBS. In general, NBS oxidizes exposed but not buried indole side chains in folded proteins. For example,
Essential Tryptophan of Alliinase  

Table 3. Comparison of the amino acid sequences surrounding the specific tryptophan residue in alliinase from garlic (A. sativum L.), shallot (A. ascalonicum L.), and onion (A. cepa L.).

<table>
<thead>
<tr>
<th>Species</th>
<th>Alliinase Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garlic</td>
<td>YPVFREQTKYFNKGYWVAGNAANYVN</td>
</tr>
<tr>
<td>Shallot</td>
<td>YPVFREQTKYFDKGYEWKGNADYVN</td>
</tr>
<tr>
<td>Onion</td>
<td>YPVFREQTKYFDKGYEWKGNADYVN</td>
</tr>
</tbody>
</table>

The essential tryptophan residue (W) is indicated in boldface type.

only 4 of the 8 tryptophans in xylanase A from Schizophyllum commune37 and 3 of the 11 tryptophans in EGII from T. reesei38 are oxidized by NBS. Therefore, it is unusual that all nine tryptophans in alliinase are susceptible to oxidation. Three residues (peaks 1, 2, and 3) are located in a relatively hydrophobic region of alliinase and are less reactive for NBS than others. However, they are not completely seques-
tered within the interior of alliinase and may still be accessi-
able to NBS. The others are expected to lie near the surface of the protein.

Sequence Comparison of Alliinase with Others. Amino acid sequences of the alliinase have been deduced from cDNAs isolated from garlic, shallot, and onion.31 Comparison of the aligned sequences of these alliinase (Table 3) indicate that Trp182 in alliinase is perfectly conserved among these species and amino acid sequence surrounding this tryptophan is also highly conserved. Thus, this region, especially W182, may constitute a part of the enzyme active site.

This paper is the first evidence for the localization of an essential tryptophan residue (W182) in alliinase. Another significant value is a new approach of a chemical modification method. By chemical modification, there have been a number of problems in identifying essential residue(s) among the probable residues. In the case of tryptophan modification, there can be three problems. First, it is difficult to obtain the site-specific chemical modification of tryptophan residues in protein without any side reactions of other amino acid residues. Second, since the tryptophan residues in the hydrophobic regions in the enzyme are unreactive, or less reactive, there may be a possibility that essential Trp residues in these regions cannot be identified. Third, in many cases, it is hard to obtain complete protection of the active site of the protein with substrate analogues, or active site binding molecules from the modification with NBS, for NBS is very reactive. For example, Bray et al.39 and Clottes et al.40 could not identify essential tryptophan residue(s), because they could not protect the active, or binding, site effectively. In our study, we overcame the protection problem by regulating the reaction conditions using kinetic information.

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