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

Young Nam Jin, Yong-Hoon Choi, and Chul-Hak Yang*

Department of Chemistry, College of Natural Sciences, Seoul National University, San 56-1, Shillim-dong, Kwanak-gu, Seoul 151-742, Korea
Received November 22, 2000

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 Linnê*) 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 (S(+)-allyl-L-cysteine sulfoxide) lysis by alliinase.

Stoll *et al.*[2] were the first to isolate alliinase from garlic clove. Further investigations by others demonstrated that enzymes which catalyzed similar reactions are not only present in *Alliaceae* but also in *Brassicaceae*,[3] *Fabaceae*,[4] broccoli,[5] and even in bacteria.[6] Since the discovery of the alliinases, there have been numerous reports on the identification, purification and characterization of alliinases from several *Alliaceae* species.[7-10] More recently, alliinase cDNA clones from garlic, shallot (*A. ascalonicum L.*), and onion (*A. cepa L.*) were isolated and characterized.[11] The holoenzyme from garlic is a glycoprotein containing 5.5% carbohydrate[9] and consists of two identical subunits,[8] each with a molecular mass of about 55 kDa.[11] There are very few reports citing the importance of specific amino acid residues in the function of this enzyme. Human serum was shown recently to contain antibodies to the two major proteins from cloves of garlic.[12] These antibodies were directed against alliinase and mannose-specific *Allium sativum* agglutinin. Alliinase from garlic has been shown to form a complex with a garlic mannose-specific lectin through a glycosylation site at Asn146 of the enzyme.[13] More recently, pyridoxal 5'-phosphate (PLP) has been shown to modify Lys251 in alliinase from onion,[14] confirming previous report that PLP participates in enzymatic degradation of alliinase.[9] There is neither 3-dimensional structural data nor any information on the identity and roles of residues involved in the catalytic 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 H_2O_2 as

*Corresponding Author. Phone/Fax: +82-2-878-8545; e-mail: chulyang@plaza.snu.ac.kr

described previously.[15] The following chemical modification reagents were purchased from Sigma; dithiothreitol (DTT), succinic anhydride (SA), N-acetylimidazole (NAI), maleic anhydride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), iodoacetic acid, phenylmethylsulfonyl fluoride (PMSF), diethyl pyrocarbonate (DEPC), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (CMC). N-bromosuccinimide (NBS) was obtained from WAKO. Polyvinylpolypyrrolidine, concanavalin A Sepharose 4B, methyl α-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 protein sequencing was obtained from Aldrich. Hydroxyapatite, acetonitrile (HPLC grade), and trichloroacetic acid (TCA) were purchased from Bio-Rad, Fisher, and Lancaster, respectively. Potassium dibasic phosphate, sodium monobasic phosphate, and all other reagents were analytical grade.

Enzyme Purification and Activity Assay. Alliinase was purified from garlic as described previously except for buffer composition.[9,16] The buffer A consisted of 50 mM NaK phosphate (pH 6.5), 5 mM EDTA 1 mM DTT, and 10% glycerol. NaK phosphate buffers were obtained by mixing varying proportions of equimolar solutions of NaH_2PO_4 and K_2HPO_4 to obtain the desired pH. The enzyme preparation was over 95% pure as judged by resolving protein bands by SDS-PAGE (10% gels) and by staining with Coomassie Brilliant Blue.[17] Alliinase activity was measuring using a modification of the method of Nock *et al*..[9] The final reaction volume (0.5 mL) contained 100 mM NaK phosphate buffer (pH 6.5), 0.25 mM PLP, 4 mM SECS, and appropriate aliquots of enzyme. The reaction mixture was incubated for 5 min at room temperature and the reaction was terminated by the addition of 1 mL of 10% (w/v) TCA. Protein precipitates, if present, were removed by centrifugation. The pyruvate produced by the alliinase was measured by the total keto-acid method of Friedemann *et al*..[18] Protein concentrations were determined quantitatively by the method of Bradford[19] using BSA as a standard. For all inactivation and spectroscopic studies, the amount of protein used was expressed as monomer concentration (Mr = 55,000).

General Chemical Modification Procedures. 10 μM of the enzyme protein was incubated with various concentrations of chemical modification reagents in 1 mL of buffers under the following conditions: 10 mM phenylglyoxal, 2 mM SA, 10 mM maleic anhydride, and 10 mM iodoacetate for 15 min and 20 mM NAI for 30 min in 0.1 M borate buffer (pH 7.0); 10 mM EDC and 10 mM CMC in 0.1 M Mes buffer (pH 6.5) for 15 min; 5 mM PMSF in 0.1 M HEPES buffer (pH 6.5) for 15 min; 10 mM DEPC in 50 mM NaK phosphate buffer (pH 6.5) for 30 min; 10 mM DTT and 5 mM NBS (in the dark) in 50 mM NaK phosphate buffer (pH 5.5) for 5 min. The stock solutions of the modifying reagents used above were prepared with distilled water except CMC, DEPC, phenylglyoxal, and NBS that were dissolved in absolute EtOH. The concentrations of the stock solutions of these reagents dissolved in ethanol were adjusted at 2% (v/v), or less, in the final reaction mixture to ensure that the amount of added ethanol does not disturb the enzymatic activity. All these reactions were carried out at room temperature. Each appropriate aliquot out of the respective reaction mixture was subjected to assay for alliinase activity. In the NBS modification reaction, the enzyme solution to react with NBS had to be dialyzed against buffer A without DTT, which will be referred to buffer A' throughout this report, because NBS reacts with DTT faster than tryptophan. Therefore, DTT was used for termination of the modification reaction with NBS. Since the absorbance at 450 nm was slightly increased by the product made by the reaction of NBS and DTT, this product and excess of NBS 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 alliinase was treated with varying concentration of SA, NAI, and NBS with changing reaction time, the residual activity of alliinase was recorded.

Effects of NBS Modification on Alliinase 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 unspecific 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) spectrofluorometer. Fluorescence intensities were determined using an excitation wavelength of 295 nm.[20] 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[20] slightly modified for this experiment. 600 μL of 5 μM protein, 2393 μL of 6 M Gdn-HCl in 0.1% NH_4HCO_3 (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% NH_4HCO_3 (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 NH_4HCO_3 and 2 μL of 0.5 M DTT were added. These samples were incubated in a water bath adjusted to 50 ^oC 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 tdH_2O 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 ration of 1 : 25 (w/w) at 37 ^oC for 24 h. All of the above reagents had to be dissolved in tdH_2O. The digestion was terminated by acidification with 20% TFA to pH 2-2.5.[21] 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 centricon. 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 control, modified, and protected samples was achieved using a Beckman HPLC system (model: system GOLD). Separations were carried out using a Vydac 218TP54 C_{18} reverse phase column (300 Å, 5 μm, 4.6 $\times$ 250 mm). In all cases, solvent A was 0.1% TFA in tdH_2O : acetonitrile (99 : 1) and solvent B was 0.1% TFA in tdH_2O : 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, idoacetic acid) seems not to inhibit the enzymatic activity (not shown here). SA has proved useful in the modification of lysine.[22] Alliinase lost 60% of its activity in 2 mM SA by the method described previously.

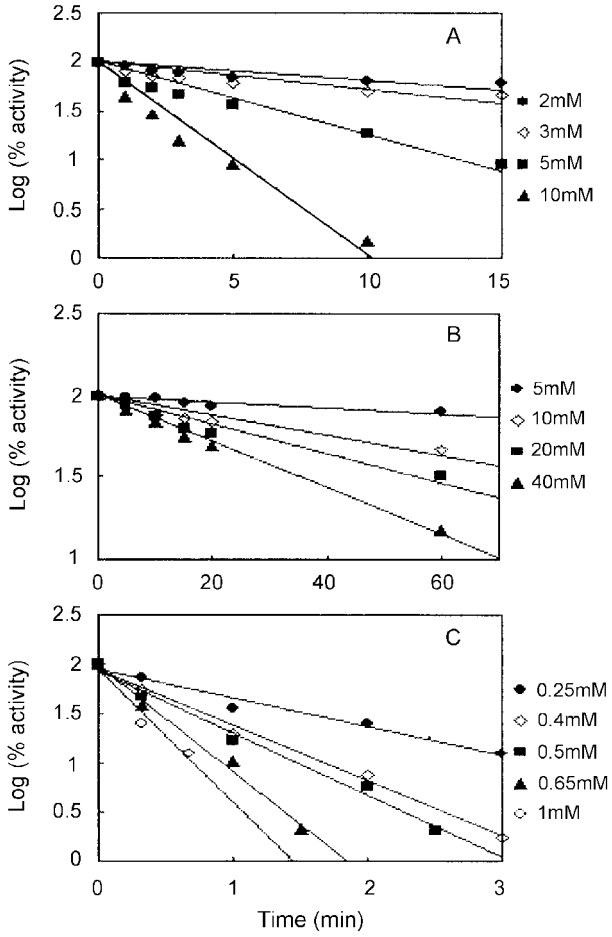

Figure 1. Time course of inactivation of alliinase by chemical reagents; Plots of Log (% activity) against time to determine the pseudo- first order rate constants (k_1). [A] succinic anhydride (SA) and [B] N-acetylimidazole (NAI) in 0.1 M borate buffer (pH 7.0), [C] N-bromosuccinimide (NBS) in buffer A' (50 mM NaK phosphate buffer (pH 6.5), 10% glycerol, and 5 mM EDTA).

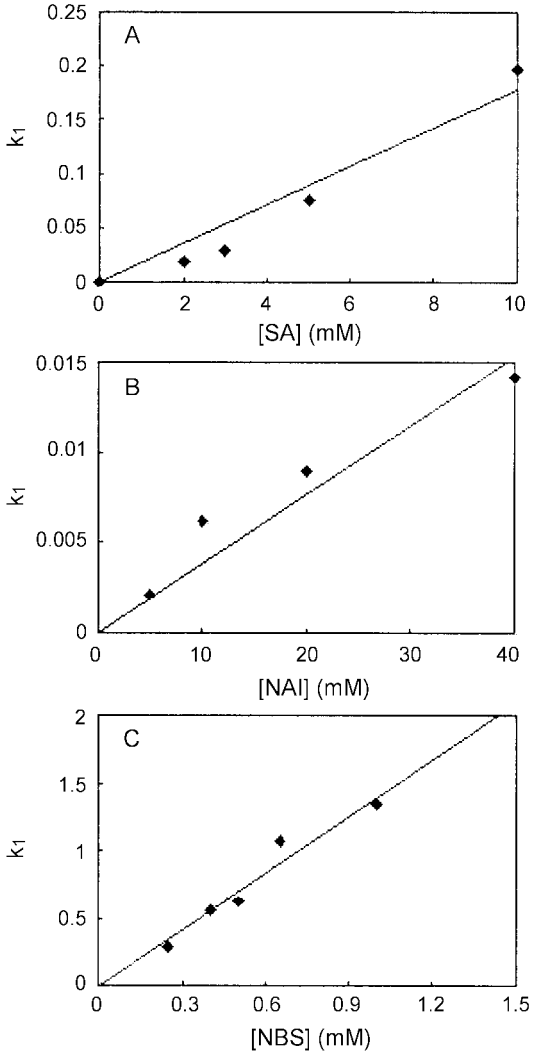

Figure 2. A plot of k_1 against the concentration of inhibitors to determine the second order rate constant k_2 of inactivation of the enzyme by inhibitors. [A] SA, [B] NAI, and [C] NBS.

Lys[251] is already known to participate in binding PLP,[14] an important cofactor and lysine-modifying reagent itself. NAI was used as a reagent for the selective modification of tyrosyl residues.[23] 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.

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

modifying reagents	modified residue (s)	k_2 ($M^{-1}min^{-1}$)	reaction order (n)
Succinic anhydride[b]	Lys, Tyr	17.8	1.51
N-acetylimidazole[b]	Tyr	0.4	0.883
N-bromosuccinimide[c]	Trp	1398	1.14

[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).

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[24]:

$$k_1 = k_2[I]^n \text{ or } \log k_1 = \log k_2 + n\log[I]$$

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.[21,25-27] 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

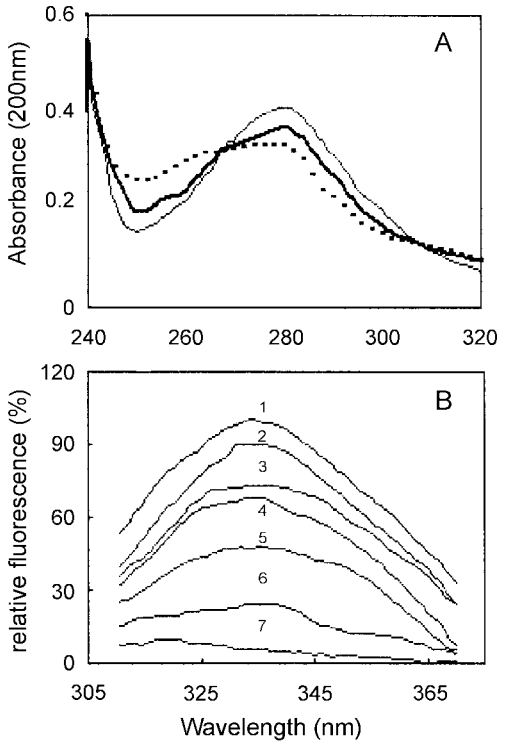

Figure 3. Effects of NBS modification on alliinase spectral properties. [A] UV absorption spectra of native alliinase (thin line) and 100- (thick line) or 200-fold (- - -) molar excess NBS-oxidized alliinase in buffer A' (pH 5.5). The protein concentration in the cuvette was 0.2 mg/mL. [B] Fluorescence 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.

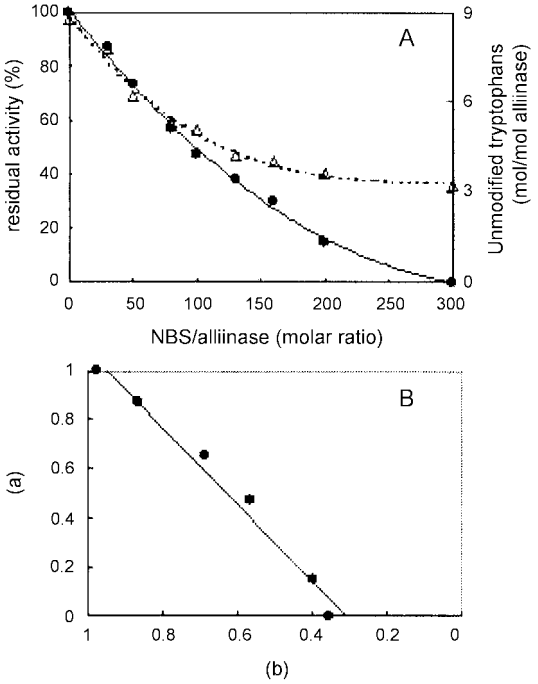

Figure 4. [A] Concentration-dependent effects of NBS modification on the enzymatic activity of alliinase. 5 μM alliinase was treated with variable amounts of NBS for 20 sec in buffer A' (pH 5.0). Measurement of residual activity (●) and titration of unmodified tryptophan residues (△) was performed by experimental procedures described previously. [B] Fraction of alliinase activity remaining (a) plotted against the fraction of residual Trp residues (b).

not shown). 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 6.5) with 1 mM, 200 fold molar excess DTT. But we found that the modified alliinase did not show the decrease of the fluorescence emission, suggesting that tryptophan residues of alliinase were not modified with NBS. This result indicates that SEC and DTT react with NBS faster than tryptophan in pH 6.5. Therefore alliinase in buffer A had to be dialyzed against buffer A' (without DTT) prior to modification with NBS. Since tryptophan residues may be rapidly[28] and specifically modified at mildly acidic pH, which increases both reactivity[29] and selectivity,[30] we had to find the appropriate pH. This is the pH in which alliinase has to be relatively stable and the specific reactivity of NBS for tryptophan must be improved as compared with pH 6.5. The optimal pH was controlled to pH 5.0 in which alliinase shows 75% residual activity, relatively stable.

It is noted that the oxidation of methionine is a possible side reaction of the treatment of proteins with NBS.[31] 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

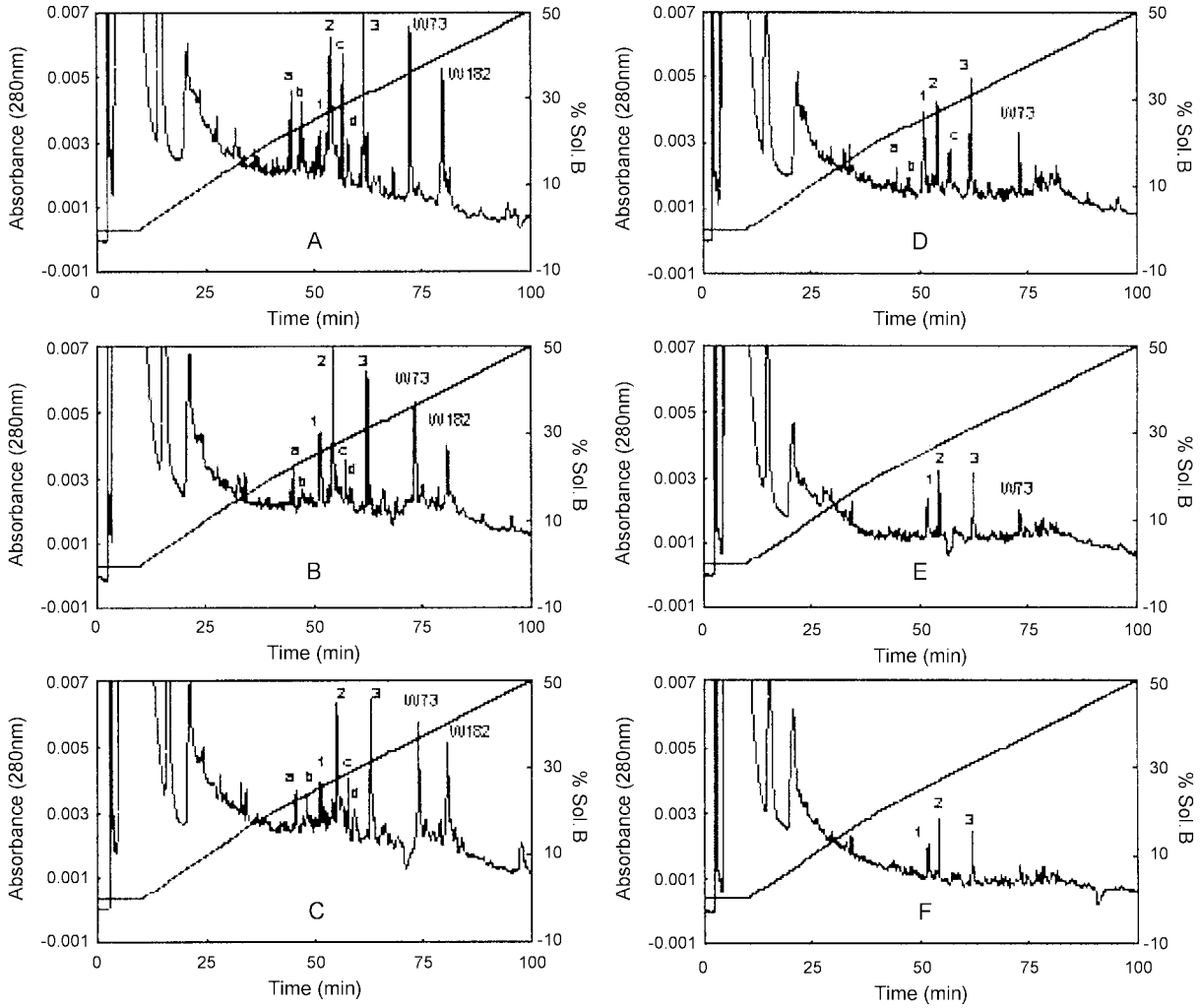

Figure 5. RP-HPLC elution profile of tryptic peptides of Control [A], Protected 1 [B], 2 [C], Modified 1 [D], 2 [E], and 3 [F]; Sol. A was 0.1% TFA in tdH_2O : acetonitrile (99 : 1) and Sol. B was 0.1 % TFA in tdH_2O : acetonitrile (2 : 8). The flow rate was fixed to 1 ml/min.

sec. 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.[32] 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 tryptic 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 nm[a]

Fragment	Sequence
fragment denoted W73	I QGCSADVAS…
predicted fragment 54-74	I QGCSADVASGDGLFLEEYWK
fragment denoted W182	GYV**X**AGNAAN…
predicted fragment 179-213	GYVWAGNAANYVNV…PEGLLR

[a]The 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.

ure 5B, C). As compared with the Control, peaks a, b, c, and d showed about 57%, 86%, 78%, and 70% oxidation, respectively on the average, indicating that these residues were not protected by SEC from modification. In the cases of W73 and W182, these two residues appear to be protected by SEC from modification, suggesting that at least one of the two residues is located in the active center of alliinase. In addition, we prepared samples protected by various procedures. In these samples, the peaks of W73 and W182 were similar in Figure 5B, C (not shown). Therefore these two fragments were collected, lyophilized, and submitted to 10 cycles of protein N-terminal sequencing by automatic Edman degradation. The two obtained N-terminal sequences were identified by comparison with predicted fragments (Table 2).

In order to determine which residue of the two is directly involved in the active site, alliinase was modified with NBS for 3, 7, and 20 sec. The activity of alliinase showed 60% residual activity at 3 sec and was perfectly inactivated at 7 sec (result not shown). Therefore, 8 sec is enough time to inactivate completely the activity of alliinase. In Figure 5D, RP-HPLC profile of sample modified for 8 sec, peaks a, b, c could be observed and especially W73, which could be detected even in Figure 5E, was a little oxidized. This demonstrates that these residues could be excluded from probability that among these residues there might be an essential residue. Consequently, we come to the conclusion that Trp182 is situated in the active, or binding, site of alliinase and plays an important role in the activity, since the modification of W182 alone is sufficient to induce a complete loss of activity.

Figure 6 shows changes of fluorescence intensity (a) and the number of unmodified tryptophan residues (b) in each sample in Figure 5. The total intensity of peaks in RP-HPLC is well in accord with the total fluorescence intensity in Figure 6. In particular, it shows that the decrease of peak intensity in modified samples (Figure 5D, E, F) is modification time-dependent. The number of unmodified tryptophan residues between B and D was approximately two. This demonstrates that the essential Trp residue(s) should be one instead of two residues, for the fluorescence effect of non-essential residues in B that were not modified on account of interference of SEC must be excluded.

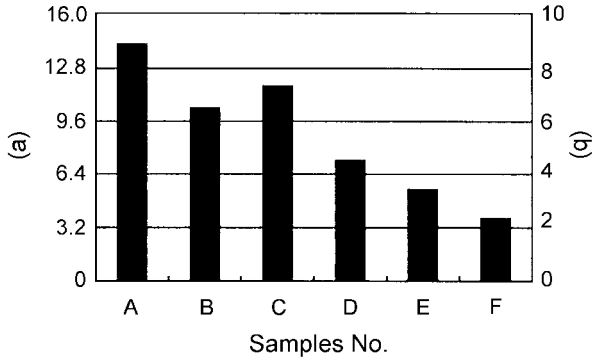

Figure 6. Changes of fluorescence intensity (a) and the number of unmodified tryptophan residues (b) upon each sample in Figure 6.

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.[33] No subunit dissociation is observed in polyacrylamide gel electrophoresis under non-denaturating 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,

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.*)

Species	Alliinase Sequence		
	65	182	191
Garlic	YPVFREQTKYFNKKGYV**W**AGNAANYVN		
Shallot	YPVFREQTKYFDKKGYE**W**KGNAADYVN		
Onion	YPVFREQTKYFDKKGYE**W**KGNAADYVN		

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

only 4 of the 8 tryptophans in xylanase A from *Schizophyllum commune*[37] and 3 of the 11 tryptophans in EGII from *T. reesei*[38] 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 sequestered within the interior of alliinase and may still be accessible 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.[11] Comparison of the aligned sequences of these alliinase (Table 3) indicate that Trp^{182} 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.

Acknowledgment. We are grateful to Hae-Young Kwon and Hoong-Sun Park, Seoul National University, for valuable advice and helpful discussion and Myoung-Hee Nam, KBSI, for performing N-terminal sequencing. This study was supported by a Grant from the Ministry of Education (2000). Financial support in part from the Brain Korea 21 Program is gratefully acknowledged.

References

1. Lancaster, J. E.; Collin, H. A. *Plant Sci. Lett.* **1981**, *22*, 169.
2. Stoll, A.; Seebeck, E. *Helv. Chim. Acta* **1949**, *32*, 197.
3. Mazelis, M. *Phytochemistry* **1963**, *2*, 15.
4. Mazelis, M.; Creveling, R. K. *Biochem. J.* **1975**, *147*, 485.
5. Hamamoto, A.; Mazelis, M. *Plant Physiol*. **1986**, *80*, 702.
6. Nomura, J.; Nishizuka, Y.; Hayaishi, O. *J. Biol. Chem*. **1963**, *238*, 1441.
7. Tobkin, Jr, H. E.; Mazelis, M. *Arch. Biochem. Biophys*. **1979**, *193*, 150.
8. Nock, L. P.; Mazelis, M. *Arch. Biochem. Biophys*. **1986**, *249*, 27.
9. Nock, L. P.; Mazelis, M. *Plant Physiol*. **1987**, *85*, 1079.
10. Fujita, M.; Endo, M.; Sano, M. *Agric. Biol. Chem*. **1990**, *54*, 1077.
11. Van Damme, E. J. M.; Smeets, K.; Torrekens, S.; Van Leuven, F.; Peumans, W. *Eur. J. Biochem.* **1992**, *209*, 751.
12. Tchernychev, B.; Rabinkov, A.; Mirelman, D.; Wilchek, M. *Immunol. Lett.* **1995**, *47*, 53.
13. Rabinkov, A.; Wilchek, M.; Mirelman, D. *Glycoconj. J.* **1995**, *12*, 690.
14. Kitamura, N.; Shimomura, N.; Iseki, N.; Honma, M.; Chiba, S.; Tahara, S.; Mizutani, J. *Biosci. Biotech. Biochem.* **1997**, *61*, 1327.
15. Stoll, A.; Seebeck, E. *Adv. Enzymol.* **1951**, *11*, 377.
16. Won, T.; Mazelis, M. *Physiol. Plantarum* **1989**, *77*, 87.
17. Laemmli, U. K. *Nature* **1970**, *227*, 680.
18. Friedemann, T. E.; Haugen, P. E. *J. Biol. Chem.* **1943**, *147*, 415.
19. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
20. Pajot, P. *Eur. J. Biochem.* **1976**, *63*, 263.
21. Puri, K. D.; Surolia, A. *J. Biol. Chem.* **1994**, *269*, 30917.
22. Klotz, I. M. *Methods Enzymol.* **1967**, *11*, 576.
23. Riordan, J. F.; Wacker, W. E. C.; Vallee, B. L. *Biochemistry* **1965**, *4*, 1758.
24. Hollenberg, P. F.; Flashner, M.; Coon, M. J. J. *Biol. Chem*. **1971**, *246*, 946.
25. Freisheim, J. H.; Huennekens, F. M. *Biochemistry* **1969**, *8*, 2271.
26. Divita, G.; Jault, J. M.; Gautheron, D. C.; Di Pietro, A. *Biochemistry* **1993**, *32*, 1017.
27. Zheng, W. Z.; Chen, Q. X.; Zhao, H.; Zhang, Z.; Zhang, W.; Zhou, H. M. *Biochem. Mol. Biol. Int.* **1997**, *41*, 951.
28. Ohnishi, M.; Kawagishi, T.; Hiromo, K. *Arch. Biochem. Biophys.* **1989**, *272*, 46.
29. Spande, T. F.; Witkop, B. *Methods Enzymol.* **1967**, *11*, 528.
30. Lundblad, R. L.; Noyes, C. M. In *Chemical Reagents for Protein Modification*; Lundblad, R. L., Noyes, C. M., Eds.; CRC Press: Boca Raton, FL, U. S. A., 1984; p 47.
31. Spande, T. F.; Witkop, B. *Methods Enzymol.* **1967**, *11*, 498.
32. Tsou, C. L. *Sci. Sinica.* **1962**, *11*, 1535.
33. Imoto, T.; Forster, L. S.; Rupley, J. A.; Tanaka, F. *Proc. Natl. Acad. Sci.* U.S.A. **1972**, *69*, 1151.
34. Ramachandran, L. K.; Witkop, B. *Methods Enzymol.* **1967**, *11*, 283.
35. Shechter, Y.; Patchornik, A.; Burstein, Y. *Biochemistry*

1976, *15*, 5071.
36. O'Gorman, R. B.; Matthews, K. S. *J. Biol. Chem.* **1977**, *252*, 3565.
37. Bray, M. R.; Clarke, A. J. *Biochemistry* **1995**, *34*, 2006.
38. Macarron, R.; Henrissat, B.; Claeyssens, M. *Biochim. Biophys. Acta* **1995**, *1245*, 187.
39. Bray, M. R.; Johnson, P. E.; Gilkes, N. R.; Mcintosh, L. P.; Kilburn, D. G.; Warren, R. A. J. *Protein Sci.* **1996**, *5*, 2311.
40. Clottes, E.; Vial, C. *Arch. Biochem. Biophys.* **1996**, *329*, 97.