Journal of Protein Chemistry, Vol. 12, No. 1, 1993

Basic Trypsin-Subtilisin Inhibitor from Marine Turtle Egg White: Hydrodynamic and Inhibitory Properties

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Received October 22, 1991; revised March 23, 1992

A basic trypsin-subtilisin inhibitor has been isolated from the egg white of marine turtle (*Caretta caretta* Linn.) and purified to homogeneity by gel filtration followed by ion-exchange chromatography. It has a single polypeptide chain of 117 amino acid residues, having a molecular weight of 13,600. It lacks methionine and tryptophan. Its isoelectric point is at *p*H 10.0 and the sedimentation coefficient ($s_{20,w}$) value of 1.62 S is independent of protein concentration. It has a Stokes radius of 18.8 Å, an intrinsic viscosity of 0.048 dl g^{-1} and a diffusion coefficient of 10.17×10^{-7} cm^2 sec^{-1}. Its fluorescence emission spectrum is similar to that of free tyrosine and the bimolecular quencing rate constant of its tyrosine residues with acrylamide is 3.15×10^9 M^{-1} sec^{-1}. The inhibitor strongly inhibits both trypsin and subtilisin by forming enzyme-inhibitor complexes at a molar ratio of unity. The nature of inhibition toward both enzymes is not temporary. It has independent binding sites for inhibition of trypsin and subtilisin. Chemical modification with tetranitromethane suggests the presence of three tyrosine residues on the surface of the inhibitor molecule.

KEY WORDS: Proteinase inhibitor; trypsin; subtilisin; chemical modification.

1. INTRODUCTION

Protein proteinase inhibitors from avian egg whites have been extensively studied (Laskowski and Sealock, 1971; Lin and Feeney, 1972; Kato *et al.*, 1987; Laskowski *et al.*, 1987, 1990), and the X-ray crystallographic structures of the third domains of ovomucoids have been established (Weber *et al.*, 1981; Papamokos *et al.*, 1982; Bode *et al.*, 1985; Musil *et al.*, 1991). By contrast, information on proteinase inhibitors from reptilian egg whites is limited. The molecular weights of serine proteinase inhibitors from reptilian egg whites have been found to be about one half of those of avian ovomucoids and one fourth of that of ovoinhibitor. During the course of our studies on proteinase inhibitors in the egg whites of tortoise and turtle, we have found that an acidic trypsin-subtilisin inhibitor is present in the egg white of *Geomyda trijuga trijuga* Schariggar (Ray *et al.*, 1982), whereas the egg white of *Caretta caretta* Linn. contains a basic trypsin-subtilisin inhibitor and chymotrypsin inhibitors (Guha and Sinha, 1984). Although an abstract on the amino acid sequence of basic trypsin-subtilisin inhibitor from Red Sea turtle has appeared (Kato and Tominaga, 1979), its physicochemical and inhibitory properties have not yet been reported. Red Sea turtle inhibitor, having a single polypeptide chain of 110 amino acid residues, consists of two domains. Its first domain has a sequence homology with Kunitz pancreatic trypsin inhibitor (Laskowski *et al.*, 1974), and the second domain has two homologous domains of secretary leukocyte proteinase inhibitor (SLPI) (Heinzel *et al.*, 1987; Grutler *et al.*, 1988) in which methionine residues seem to play an important role in its interaction with proteinases (Kramp *et al.*, 1990). Red Sea turtle inhibitor contains a single methionine residue which is absent in the inhibitor studied here. In the present paper, purification and

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0277-8033/93/0200-0071$07.00/0

characterization of a basic trypsin-subtilisin inhibitor from the egg white of marine turtle (*Caretta caretta* Linn.) have been described.

2. MATERIALS AND METHODS

2.1. Materials

Eggs of freshly killed marine turtle (*Caretta caretta* Linn.) were collected from local markets. Bovine trypsin (twice crystallized) was purchased from Worthington Biochemical Corporation. Subtilisin BPN′ (protease type VII), carboxypeptidase B, p-tosyl-L-arginine methyl ester (TAME), N-α-benzoyl-DL-arginine-p-nitroanilide (BAPA), N-acetyl-L-tyrosine ethyl ester (ATEE), 2,4,6-trinitrobenzene sulfonate (TNBS), and acrylamide were purchased from Sigma Chemical Company. Subtilisin BPN′ was purified according to Glazer (1966). Sephadex G-50 (fine), Sephadex G-100 (fine), and CM-Sephadex C-50 were Pharmacia products. All other chemicals used were analytical reagent grade.

2.2. Isolation and Purification of Trypsin-Subtilisin Inhibitor

Trypsin-subtilisin inhibitor was isolated and purified from marine turtle egg white according to the procedure described by Guha and Sinha (1984) for the purification of chymotrypsin inhibitors. Trypsin inhibitor fraction obtained from CM-Sephadex column chromatography was rechromatographed on the same column (2.5 × 23 cm) with a linear gradient from 0 to 0.3 M NaCl in 0.1 M sodium acetate buffer, *p*H 5.0. Active fractions were pooled, dialyzed exhaustively against water, and freeze-dried.

2.3. Assay of Enzymic and Inhibitory Activities

Trypsin and subtilisin activities were determined at *p*H 8.5 and 30°C by the *p*H-stat method (Walsh and Wilcox, 1970) using Radiometer model SBR2/SBUI/TTllb titration equipment. The assay was performed with TAME and ATEE as substrates for trypsin and subtilisin, respectively. In a few experiments, trypsin activity was determined spectrophotometrically with BAPA at *p*H 8.1 and 30°C (Kassell, 1970). The inhibitory activity was calculated as the difference between enzyme activity in the absence and presence of inhibitor. The incubation time of enzyme and inhibitor was 2 min at *p*H 8.5 before the addition of substrate. One unit of inhibitory activity is expressed as milligrams of enzyme inhibited. Specific activity is expressed as units of inhibitory activity per milligram of inhibitor.

2.4. Protein Concentration

Protein concentrations in egg whites and pooled fractions were estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The concentration of purified trypsin-subtilisin inhibitor was determined by measuring absorbance at 280 nm using a value of $E_{1\,cm}^{1\%} = 5.8$ determined experimentally. Bovine trypsin concentration was measured by active site titration with p-nitrophenyl-p′-guanidinobenzoate according to the method of Chase and Shaw (1967). The concentration of subtilisin-BPN′ was calculated from the absorbance at 280 nm using $E_{1\,cm}^{1\%} = 11.7$ (Matsubara *et al.*, 1965).

2.5. Polyacrylamide Gel Electrophoresis

Disc polyacrylamide gel electrophoresis at the *p*H values of 4.5 and 8.3 were carried out according to the methods of Reisfeld *et al.* (1962) and Davis (1964), respectively. Sodium dodecyl sulphate-urea polyacrylamide gel electrophoresis was done according to Swank and Munkres (1971).

2.6. Gel Filtration

The method of gel filtration through Sephadex G-100 was employed for the determination of the molecular weight and Stokes radius of the basic trypsin-subtilisin inhibitor as described by Whitaker (1963) and Seigel *et al.* (1966), respectively. The Stokes radius of the inhibitor was found to be 18.8 Å.

2.7. Isoelectric Focusing

Isoelectric focusing was performed according to Vesterberg and Svenson (1966) in an LKB 8101 electrofocusing column using 1% ampholine in the *p*H range 5–11. The sample was allowed to focus at 600 V at 4°C for 90 hr. After focusing, fractions of 1.25 ml were collected and analyzed for *p*H and absorbance at 280 nm.

2.8. Viscosity

Viscosity measurements were made at 29.6 ± 0.05°C in a Cannon–Manning semimicroviscometer with a flow time of 250.9 sec for solvent.

Intrinsic viscosity was determined by plotting the viscosity number against concentration and extrapolating to zero concentration (Kragh, 1961).

2.9. Analytical Ultracentrifugation

Sedimentation velocity experiments were carried out with a Spinco Model E analytical ultracentrifuge at 60,000 rpm using a synthetic boundary cell. Boundary displacements, heights, and areas of patterns were measured with a Zeiss toolmaker's microscope by the procedure of Baldwin (1957). Diffusion coefficients were measured from boundary spreading using a synthetic boundary cell at 8000 rpm (Schachman, 1957).

Partial specific volume was determined from the amino acid composition according to Cohn and Edsall (1943), and the value was found to be 0.719 ml/g.

2.10. Fluorescence Measurements

Fluorescence measurements were performed with a Perkin-Elmer model MPF-44B spectrofluometer at 25°C, using 6-nm bandwidths. Fluorescence quenching stdies were described with the Stern-Volmer equation (Birks, 1970):

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, K_q is the bimolecular quenching rate constants, τ_0 is the lifetime of the fluorophore in the absence of the quencher, and $[Q]$ is the concentration of quencher. In the case of two classes of fluorophores, one of which was inaccessible to quencher, the modified Stern–Volmer equation (Birks, 1970) was employed:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K [Q]} + \frac{1}{f_a}$$

where K is the Stern-Volmer quenching constant and f_a is the fraction of initial fluorescence which is accessible to quencher. Since acrylamide had strong absorption at the excitation wavelength, the appropriate correction factor was made for the "inner-filter effect" (Lakowicz, 1983).

2.11. Amino Acid and Amino-Terminal Residue Analyses

Amino acid analyses were performed on a Beckman Multichrom amino acid analyzer according to Spackman *et al.* (1958). Samples were hydrolyzed with 6 N HCl at 110°C for 24, 48, and 72 hr in evacuated, sealed tubes. Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1967). The existence of sulfhydryl groups was checked by the method of Ellman (1959). The presence of tryptophan was checked by hydrolysis with p-toluene-sulfonic acid (Liu and Chang, 1971). Serine and threonine contents were calculated by extrapolation to zero time.

Attempts to determine the amino-terminal residue of the S-carboxymethylated derivative of the inhibitor were made by the dansyl chloride method of Hartley (1970) and the phenylisothiocyanate method of Edman (1950).

2.12. Chemical Modification

Modification of free amino groups in the inhibitor was performed with TNBS in 0..1 M sodium borate buffer, *p*H 9.5, at room temperature according to the method of Habeeb (1966). The number of amino groups modified was calculated using a molar extinction coefficient of $\varepsilon_{367\,nm} = 1.1 \times 10^4\ M^{-1}\ cm^{-1}$ for nitrophenyl amino groups.

Modification of arginine residues of the inhibitor was done with 1,2-cyclohexanedione (Liu *et al.*, 1968) in 0.2 M sodium borate buffer, *p*H 9.0 at 37°C.

The tyrosine residues of the inhibitor were nitrated with tetranitromethane in 0.1 M sodium phosphate buffer, *p*H 7.5–8.5, at room temperature according to Riorden and Vallee (1972). The degree

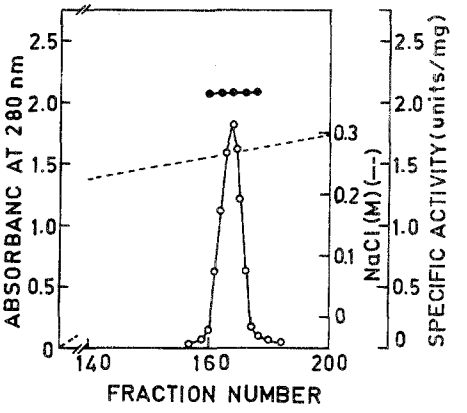

Fig. 1. Rechromatography of trypsin-subtilisin inhibitor from marine turtle egg white on CM-Sephadex C-50. The column (2.5 × 23 cm) was equilibrated with 0.1 M sodium acetate buffer, *p*H 5.0. Elution was performed using a linear gradient of 0–0.3 M NaCl in the same buffer. Fractions of 5 ml were collected at a flow rate 30 ml/hr. ○, absorbance at 280 nm; ●, specific activity toward trypsin.

of nitration was estimated using a molar extinction coefficient of $\varepsilon_{428\,nm} = 4200\ M^{-1}\ cm^{-1}$ at *p*H 9 for nitrophenoxide ion.

3. RESULTS AND DISCUSSIONS

3.1. Purification of Turtle Egg White Trypsin-Subtilisin Inhibitor

Chromatography of active fraction from the gel filtration of egg white on CM-Sephadex C-50 revealed six peaks as described previously (Guha and Sinha, 1984). The last peak contained trypsin inhibitor and its rechromatography gave a single peak (Fig. 1) with constant inhibitory activity across the peak, indicating the homogeneous preparation. The purification procedure is summarized in Table I.

3.2. Homogeneity

The inhibitor gave a single band on polyacrylamide gel electrophoresis at *p*H values of 4.5 and 8.3, and on SDS-urea gel electrophoresis in the presence of 2-mercaptoethanol, as shown in Fig. 2. Isoelectric focusing yielded a single peak with isoelectric point at *p*H 10.0.

The inhibitor gave single symmetrical peaks on ultracentrifugal analysis at different protein concentrations (0.75–1.55%) in 0.004 M NaH_2PO_4–0.032 M Na_2HPO_4–0.1 M NaCl buffer, *p*H 7.48. Its sedimentation coefficient was practically independent of protein concentration and the value of $s_{20,w}$ was determined to be 1.62 S. Results of gel electrophoresis, isoelectric focusing, and ultracentrifugal analysis suggested the homogeneity of the purified inhibitor.

3.3. Diffusion Coefficient

The diffusion coefficient of the inhibitor was calculated from the boundary spreading in the ultracentrifuge at the protein concentration of 1.0% at *p*H 7.48. Its $D_{20,w}$ value was found to be $10.17 \times 10^{-7}\ cm^2\ sec^{-1}$.

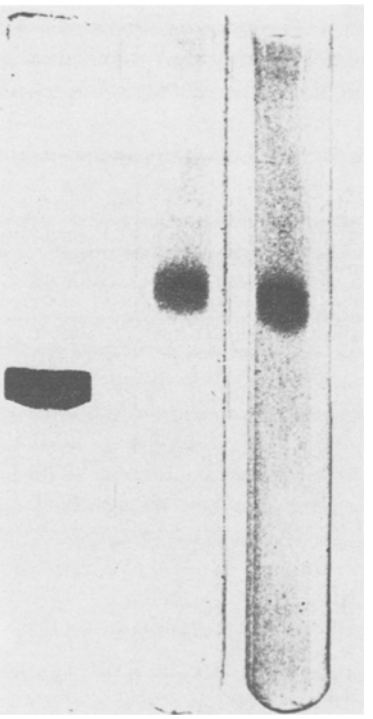

Fig. 2. Polyacrylamide gel electrophoretic patterns of trypsin- subtilisin inhibitor on (left) 10% gel at *p*H 4.5, (middle) 10% gel at *p*H 8.3, and (right) 15% gel containing SDS-urea at *p*H 6.8.

3.4. Viscosity

Viscosity measurements of the inhibitor in 0.1 M NaCl were made at 29.6°C at five different protein concentrations (0.4–2.9%). The viscosity number of the inhibitor was practically independent of protein concentration. A value for the intrinsic viscosity of 0.048 dl g^{-1} was obtained for the inhibitor.

Table I. Summary of Purification of Basic Trypsin-Subtilisin Inhibitor from Marine Turtle Egg White

Step	Protein (mg)	Trypsin inhibitory activity (U)[a]	Specific activity (U/mg)	Recovery of activity (%)	Purification (fold)
Egg White	8100	260	0.03	100	1
$(NH_4)_2SO_4$ precipitation	6787	248	0.04	95	1
Gel filtration through Sephadex G-50	810	242	0.30	93	10
Chromatography on CM-Sephadex C-50	92	172	1.87	66	62
Rechromatography on CM-Sephadex C-50	73	161	2.21	62	73

[a] One unit is expressed as a milligram of trypsin inhibited.

3.5. Molecular Weight

The molecular weights of the inhibitor determined by SDS-urea gel electrophoresis and gel filtration through Sephadex G-100 were 13,600 and 14,000, respectively. These agreed well with the molecular weight of 13,750 calculated from its sedimentation-diffusion data. A somewhat lower value of molecular weight of 12,910 was obtained from its amino acid composition. The average molecular weight of the inhibitor calculated from the above results was found to be 13,600.

3.6. Fluorescence

Turtle egg white inhibitor, which was devoid of tryptophan, showed a peak with an emission maximum at 305±2 nm after excitation at 278 nm (Fig. 3). This was similar to that of free tyrosine, suggesting that the influence of the micro-environment around the excited fluorophore did not alter the normal emission characteristics.

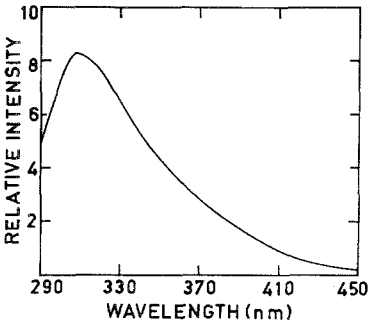

Fig. 3. Fluorescence emission spectrum of trypsin-subtilisin inhibitor in water. Protein concentration was 0.09 mg/ml and excitation was at 278 nm.

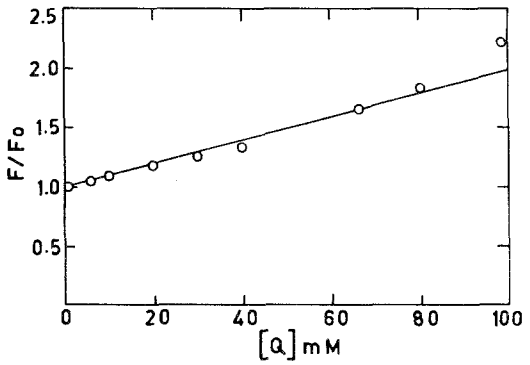

Fig. 4. Stern-Volmer plot of acrylamide quenching of trypsin-subtilisin inhibitor.

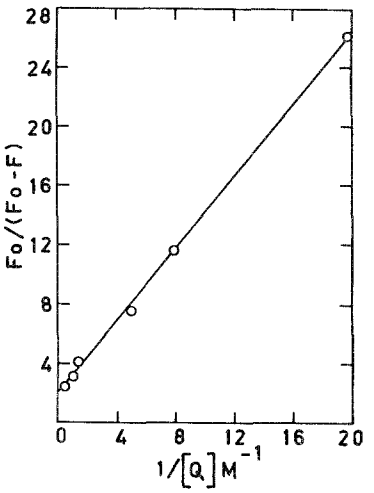

Fig. 5. Modified Stern-Volmer plot of phosphate quenching of trypsin-subtilisin inhibitor.

Fluorescence quenching of the inhibitor was studied by using non-ionic quencher, acrylamide and ionic quenchers, KI, and K_2HPO_4. The linear Stern-Volmer plot as shown in Fig. 4 indicated that all the typrosine residues of the inhibitor were accessible to acrylamide quenching. The biomolecular quenching rate constant calculated from this plot was found to be $3.15 \times 10^9\ M^{-1}\ sec^{-1}$. Potassium iodide did not quench tyrosine fluorescence of the inhibitor, whereas K_2HPO_4, having higher charge density of HPO_4^{2-} than iodide ions, caused quenching. In the case of K_2HPO_4 quenching, the Stern-Volmer plot produced a downward curvature, indicating that all the tyrosine residues of the inhibitor wre not accessible for quenching. The modified Stern-Volmer plot, as shown in Fig. 5, gave an f_a value of 0.5, which suggested that 50% of the total tyrosine residues present in the inhibitor was accessible to K_2HPO_4. Fluorescence emission spectra of the inhibitor in the presence of 8 M urea produced no remarkable change from that in simple solution, suggesting a very compact structure of the protein.

3.7. Amino Acid Composition

The amino acid composition of the inhibitor is given in Table II. It contained 117 amino acid residues, among which 12 are half-cystine residues. No free sulfhydryl group was detected with Ellman's reagent (Ellman, 1959) even in the presence of 8 M urea, suggesting that all the half-cystine residues were present as disulpide bonds. The amino-terminal residue of the inhibitor was found to be blocked.

Table II. Amino Acid Compositions of Basic Trypsin-Subtilisin Inhibitors of Marine Turtle (*Caretta caretta*) and Red Sea Turtle

	Marine turtle (*Caretta caretta*) inhibitor		Red Sea turtle inhibitor[a]
Amino acid	Residues/mol	Nearest integer	Residues/mol
Lys	9.0	9	9
His	1.3	1	1
Arg	8.2	8	8
Asp	9.9	10	10
Thr	3.1	3	3
Ser	6.7	7	7
Glu	10.8	11	8
Pro	13.1	13	12
Gly	13.1	13	14
Ala	5.0	5	4
Half-Cys	12.0	12	14
Val	4.0	4	3
Met	0	0	1
Ile	5.9	6	5
Leu	4.8	5	3
Tyr	5.0	5	4
Phe	5.3	5	4
Trp	0	0	0
Total		117	110

[a] Data from Kato and Tominaga (1979).

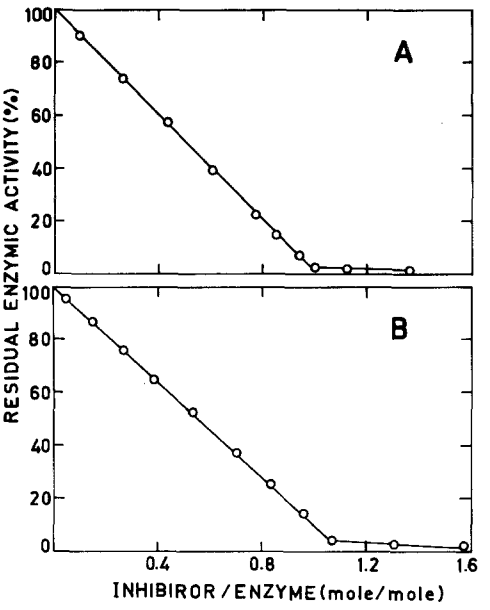

Fig. 6. Inhibition of trypsin (A) and subtilisin (B) by trypsin-subtilisin inhibitor. Assays were performed at *p*H values of 8.1 and 8.5 at 30°C after preincubation of enzyme and inhibitor for 2 min as described in the text. Inhibition was expressed as percentage of residual enzymic activity.

A comparison of these data with those of Red Sea turtle inhibitor (Kato and Tominaga, 1979) shows that two inhibitors had strikingly similar amino acid compositions, though they differed in the contents of several amino acids. The marked feature of the inhibitor studied here is that unlike Red Sea turtle inhibitor, it did not contain methionine. The absence of methionine has been supported by the observation that amino acid analysis of performic acid oxidized sample did not yield methionine sulfone. It appears that this inhibitor is not identical to Red Sea turtle inhibitor, but is another variant of the trypsin-subilisin inhibitor in the chelonianin family.

3.8. Inhibitor Properties

The inhibitor was found to be stable over the *p*H range of 2–10. No loss of inhibitory activity was observed when the inhibitor was heated at neutral *p*H at 100°C for 4 hr. The effect of inhibitor concentration on the amidase activity of bovine trypsin against BAPA at *p* 8.1 and that on the esterolytic activity of subtilisin BPN′ against ATEE at *p*H 8.5 was studied separately at 30°C. The inhibitor was found to inhibit strongly both enzymes to form inactive complexes which contained molar ratios of inhibitor/enzyme close to unity (Fig. 6). The nature of inhibition toward trypsin and subtilisin is not temporary, since the inhibitor retained its inhibitory activity after being incubated with trypsin or subtilisin at *p*H 8 for 48 hr at room temperature.

The inhibitor did not inhibit chymotrypsin, elastase, or plasmin.

3.9. Inhibition Sites

The time course for the modification of amino groups of turtle egg white inhibitor with TNBS and the change in its inhibitory activities against trypsin and subtilisin are shown in Fig. 7. It is evident that its inhibitory activities against these two enzymes were decreased by modification of the free amino groups; however, its subtilisin inhibitory activity was reduced faster than its trypsin inhibitory activity up to 50 min. Since the amino terminal amino acid of the inhibitor was blocked, lysine residues might be involved in the active sites of the inhibitor. This has been confirmed by limited proteolysis of the inhibitor with trypsin at *p*H 3.8 (Ozawa and Laskowski, 1966), followed by digestion of modified inhibitor with carboxypeptidase *B* at *p*H 8.5. A single Lys-X bond was splitted. Identification of its subtilisin reacive site by limited proteolysis with subtilisin at low *p*H was unsuccessful.

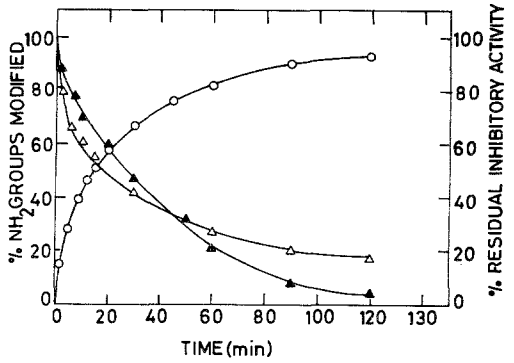

Fig. 7. Time course of modification of amino groups with TNBS and loss of inhibitory activity in trypsin-subtilisin inhibitor. The number of amino groups modified (○) was determined spectrophotometrically and assays were performed at *p*H 8.0 and 30°C for residual trypsin (△) and subtilisin (▲) inhibitory activities.

3.10. Modification of tyrosine residues

Nitration of the inhibitor at *p*H 8.5 with fivefold molar excess of tetranitromethane per mole of protein for about 120 min resulted in the modification of three tyrosine residues with no loss of inhibitory activity (Fig. 8). Increase in reaction time did not modify additional tyrosine residue and the reaction rate of nitration was relatively slow at lower *p*H values. Results indicated that out of five tyrosine residues present in the inhibitor, only three tyrosine residues were on the surface of the molecule. This was consistent with the results of a fluorescence quenching study with K_2HPO_4.

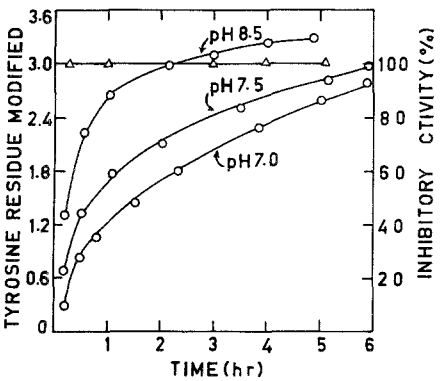

Fig. 8. Chemical modification of tyrosine residues in trypsin-subtilisin inhibitor with tetranitromethane. The number of tyrosine residues modified (○) was determined spectrophotometrically and assays were performed at *p*H 8.0 and 30°C for inhibitory activity (△).

ACKNOWLEDGMENTS

The financial support from the CSIR, New Delhi is gratefully acknowledged. We thank Mr. S. Som for assistance in amino acid analyses.

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