BBA 66417

SOME PROPERTIES OF PROTEINASE *b* IN THE VENOM OF *AGKISTRODON HALYS BLOMHOFFII*

GENICHIRO OSHIMA*, SADAAKI IWANAGA AND TOMOJI SUZUKI**
Institute for Protein Research, Osaka University, Osaka (Japan)

(Received June 2nd, 1971)

SUMMARY

The properties of proteinase *b* (endopeptidase) isolated from the venom of snake, *Agkistrodon halys blomhoffii*, were studied Proteinase *b* contained approx 2 g-atoms of calcium per molecule as estimated by atomic absorption spectrophotometry and spectrophotometric titration with EDTA After the removal of the calcium by electrodialysis or by treatment with EDTA (10^{-2} M), the resulting enzyme molecule underwent some conformational change, followed by loss of the proteolytic activity This conformational change and the concomitant loss of enzyme activity were found to be irreversible

The activity of venom proteinase *b* was slightly inhibited by –SH reagents, including *p*-chloromercuribenzoate (PCMB), monoiodoacetic acid, *N*-ethylmaleimide and 5,5'-dithio-bis-(2-nitrobenzoic acid) On prolonged incubation with the enzyme, PCMB caused significant inhibition In the presence of 8 M urea and 10^{-2} M EDTA, one mole of –SH group was found per enzyme molecule, while in the absence of urea and EDTA, only 20% of the –SH group was measurable

Thus, it may be suggested that the metal is probably involved in the stabilization of the enzyme molecule, and that the –SH groups are closely related to the enzyme activity of proteinase *b*

INTRODUCTION

The snake venom of *Agkistrodon halys blomhoffii* (Japanese trivial name, "Mamushi") contains three proteinases differing in molecular weight, electrophoretic mobility and chromatographic behaviour[1–3] One of these, proteinase *b*, has been highly purified by relatively simple procedures[2], and the purified enzyme was found to be a glycoprotein with a molecular weight of 95 000 (ref 2) The characteristic of this enzyme is its strong hemorrhagic action resulting in petechiae after intra-

Abbreviation PCMB, *p*-chloromercuribenzoate

* Present address School of Pharmaceutical Sciences Kitasato University, Minato-ku, Tokyo
** To whom correspondence may be addressed

cutaneous injection It is thought that this enzyme is one of the lethal principles of this venom[4,5]

Preliminary studies showed that extraneous addition of divalent cations usually inhibited the activities of the venom proteinases, and the only exception was the addition of Ca^{2+} and Mg^{2+}, which were slightly stimulatory[1] The proteinase and hemorrhagic activities of venom proteinase *b* were inhibited by EDTA and cysteine[4] Unlike the well characterized mammalian serine proteinases[7], the enzyme is insensitive to DFP[1] Moreover, the hydrolytic sites of venom proteinase *b* toward the oxidized insulin B-chain, glucagon, bradykinin or synthetic peptides are quite different from those of trypsin (EC 3 4 4 4), α-chymotrypsin (EC 3 4 4 5) and pancreatopeptidase (EC 3 4 4 7), and no exopeptidase action, like that of leucine aminopeptidase (EC 3 4 1 1) and carboxypeptidase A (EC 3 4 2 1) and B (EC 3 4 2 2), was found[6] These findings prompted us to further studies on the properties of venom proteinase *b*, and this paper reports the effects of EDTA, cysteine and sulfhydryl reagents on the biological activities of proteinase *b*

MATERIALS AND METHODS

Proteinase b

Proteinase *b* was highly purified from the lyophilized venom of *A halys blomhoffii* using gel filtration on a Sephadex G-100 column and chromatography on a DEAE-Sephadex column as described previously[2] The material obtained was homogeneous in ultracentrifugation, free-boundary electrophoresis and chromatography on a DEAE-cellulose column Specific activity, expressed as proteinase units (P U)[1], of purified proteinase *b* was 52 5

Chemicals

Casein (Hammarsten) was a product of E Merck Sephadex G-25 (Fine type) was purchased from Pharmacia, Uppsala, Sweden Urea of analytical grade was recrystallized from 70% aq ethanol Commercial *p*-chloromercuribenzoate (PCMB) was purified by the method of BOYER[7] Monoiodo[^{14}C]acetic acid was a product of Radiochemical Center, Amersham, England The other chemicals used were commercial preparations of analytical grade Water was deionized by passage through a column of Amberlite ion exchange resin (a mono-bed mixture of IRA-400 and IR-120) and distilled in a quartz distiller The deionized water was stored in polyethylene bottles

Determination of protein concentration

Protein concentration was determined at 280 nm with a Hitachi Model 124 spectrophotometer The protein was diluted to an absorbance of less than 1 0 with 0 05 M Tris–HCl buffer (pH 7 5) and the extinction coefficient[2], $E^{1\%}_{1cm} = 7\ 4$, was used throughout these studies The molar concentration of venom proteinase *b* was calculated taking the molecular weight as 95 000 (ref 2)

Assay of proteinase and hemorrhagic activity

Caseinolytic activity was estimated as described previously[1] and one unit of proteinase, expressed as P U , was defined as the amount of enzyme yielding an

increase in color equivalent to 1.0 μg of tyrosine per min with Folin's reagent. Specific activity was expressed as units per mg protein. Hemorrhagic activity was assayed essentially according to the method of KONDO *et al*.[8]. The skin of the back of albino rabbits, weighing 2–2.5 kg, was depilated after treatment with a paste of 20% barium sulfide and this area was thoroughly washed with warm water. 24 h after depilation, 0.1 ml of a sample, diluted serially 3-fold with physiological saline, was injected intracutaneously into the depilated skin. 24 h later, the rabbits were killed by ether inhalation and the skin was removed immediately and fixed on a glass plate. The cross diameter of each hemorrhagic spot was measured and the hemorrhagic activity was expressed as μg of protein per MHD (Minimum Hemorrhagic Dose) as previously described[4].

Assay of calcium

Spectrophotometric titration with EDTA in the presence of murexide as indicator, was carried out according to the method of ZAK *et al*.[9], with the modification that all reagents except KOH solution were diluted 10-fold, and the full range of absorbance was scaled up to from 0 to 0.2 with a Cary spectrophotometer Model 14. Calcium was also estimated by atomic absorption spectrophotometry, with a Hitachi Model 139 apparatus. A linear standard curve was obtained from 10 to 60 μg/ml and about 100 mg of protein sample were used in one analysis. For qualitative analysis of metals, lyophilized protein was converted to ash in a platinum crucible, and after adding 0.1 M HCl, it was redried, dissolved in deionized water and examined in a Shimazu flame spectrophotometer, Model 63-071.

Determination of free sulfhydryl groups

The amount of free –SH groups was measured by incorporation of monoiodo-[^{14}C]acetic acid and by spectrophotometric titration with *N*-ethylmaleimide. For incorporation of monoiodo[^{14}C]acetic acid, 6.78 mg of enzyme were dissolved in 0.5 ml of solution containing 8 $\cdot 10^{-2}$ M EDTA and 8 M urea and the mixture was stood for 60 min at 37°. Then, 0.8 ml of 0.4 M Tris–HCl buffer (pH 8.5) and 0.7 ml of monoiodo[^{14}C]acetic acid (7.90 $\cdot 10^5$ counts/min per 0.4 μmole per ml) were added and the mixture was incubated at 37° for 60 min. Excess reagent was removed by gel filtration with a Sephadex G-25 column (1.0 cm × 86 cm) washed with distilled water. Then the column was eluted with water at a flow rate of 11 ml/h and 1.6-ml fractions were collected. An aliquot of the fractions eluted in the void volume was transferred to a planchet and analyzed in a gas-flow counter, Model EA-102, from Kobe Kogyo Corp., Japan. In the spectrophotometric method[10], 10.2 mg of enzyme were dissolved in 0.5 ml of solution containing 8 M urea and 8 $\cdot 10^{-2}$ M EDTA, and the mixture was stood for 60 min at 37°. Then, 0.5 ml of 1.0 M sodium acetate buffer (pH 7.0) and 0.5 ml of *N*-ethylmaleimide (4.5 $\cdot 10^{-4}$ M) were added. After standing the mixture at room temperature for 15 min, the absorbance at 300 nm was compared with that of the reference sample, containing all the reagents except *N*-ethylmaleimide.

Electrodialysis

Electrodialysis was carried out by a modification of the method of IMANISHI[11] essentially according to the technique of STEIN *et al*.[12]. The enzyme solution was subjected to electrodialysis with constant stirring in a cold room against running

deionized water for a definite time The initial concentration of enzyme was about 0 5% and the pH was adjusted to 7 5 with 0 1 M NaOH After electrodialysis, the protein concentration, calcium content and caseinolytic activity of an aliquot of the enzyme solution were measured

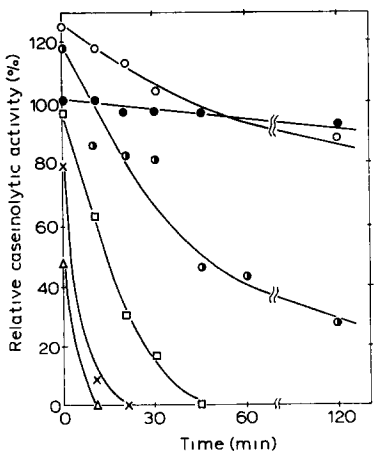
Measurement of difference spectra and sedimentation

Difference spectra were recorded with a Cary automatic spectrophotometer, Model 14 Paired cells with two compartments were used to compensate for the absorption of the solvent Sedimentation measurements were performed in a Hitachi, Model UCA-1, ultracentrifuge at 52 000 rev /min at 15 8°

RESULTS

Effect of urea

Venom proteinase *b* was treated with various concentrations of urea at 37° (Fig 1) When treated with 1 M urea, no significant loss of activity was observed With 2 M and 4 M urea, the effect seemed to have two phases an increase in activity immediately after the addition of urea and then a gradual decrease With concentrations of more than 5 4 M urea, the proteinase activity disappeared within 1 h Table I shows the caseinolytic and hemorrhagic activities of proteinase *b* after exposure to various concentrations of urea for 4 h The two activities seemed to decrease in parallel



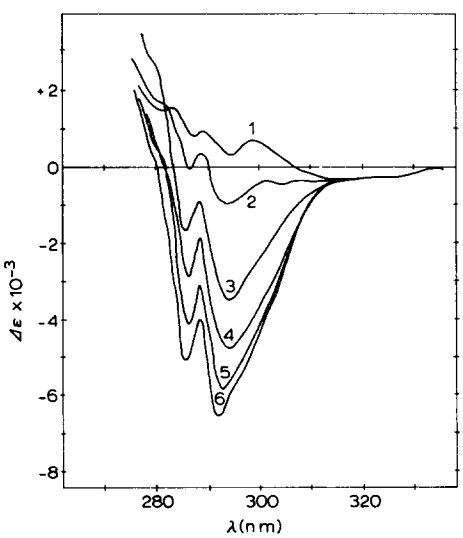


Fig 1 Effect of urea on the caseinolytic activity of venom proteinase *b* Enzyme (0 32 mg) was incubated at 37° in 4 0 ml of 0 05 M sodium acetate (pH 7 0) containing various concentrations of urea Activity was assayed using 0 5 ml of incubation mixture Urea (M) ●—●, 1 ○—○, 2 ◑—◑, 4, □—□, 5 4, ×—×, 6 75, △—△, 8 1

Fig 2 Ultraviolet absorption difference spectra of venom proteinase *b* treated with urea The reference cell contained native enzyme (3 24 mg/ml) dissolved in 0 1 M sodium acetate (pH 7 0) The sample cell contained the same concentration of enzyme in 0 1 M sodium acetate (pH 7 0) containing 8 M urea 1, immediately after dissolving the enzyme, 2, after 5 min, 3, after 30 min 4, after 1 h, 5, after 24 h, 6, after 48 h

TABLE I

EFFECTS OF UREA ON HEMORRHAGIC AND CASEINOLYTIC ACTIVITIES OF VENOM PROTEINASE *b*

Proteinase *b* (0 3 mg) was treated with 1 0 ml of 0 1 M sodium acetate (pH 7 0) containing urea incubated at 37° After 4 h, the samples were diluted 2 2-fold with 0 9% NaCl and the activities were estimated

Urea concentration (M)	*Relative activity*	
	Caseinolytic	*Hemorrhagic*
None	100 0	100 0
1	103 0	98 0
2	71 0	71 5
3	25 5	22 6
4	7 6	8 4
5	2 2	Undetectable

with increase in the concentration of urea The ultraviolet difference spectra of proteinase *b* in the presence of 8 M urea were measured at room temperature and the result is given in Fig 2 A blue shift was found 5 min after the addition of urea, which reached to about 70% of the maximum after 1 h The blue shift was not reversed and the enzyme activity was not restored on removal of urea by dialysis overnight against 0 1 M sodium acetate buffer (pH 7 0) containing 10^{-4} M calcium This suggests that urea denatures the enzyme irreversibly

Content and removal of calcium

A significant amount of calcium was detected by flame spectrophotometry in the purified proteinase *b* Emission lines for magnesium, manganese, zinc and other metals, which were found significantly in the crude venom of *A halys blomhoffii*, were also seen, but these seemed to be present in only trace quantities In quantitative assay of calcium, about 2 moles of calcium were found per enzyme molecule, although the content in different preparations varied somewhat as shown in Table II

On electrodialysis, calcium content decreased progressively but complete removal of it was not achieved even after dialysis for 24 h (Table III) The caseinolytic activity of proteinase *b*, decreased gradually during dialysis, and was not recovered by extraneous addition of various amounts of calcium, even at a final concentration of 10^{-3} M When the enzyme was treated with EDTA (8 10^{-3} M) or EDTA (8 10^{-2} M) containing 8 M urea and then subjected to gel filtration through a Sephadex G-25 column, calcium was no longer detectable (Table IV) On pretreatment of the enzyme with 8 M urea containing no EDTA only about half the total amount of calcium was removed

TABLE II

METAL CONTENT OF VENOM PROTEINASE *b*

Proteinase b	*Calcium (μg equivalent per mg protein)*
Lot 1	0 91
Lot 2	0 84
Lot 3	0 78

TABLE III

REMOVAL OF CALCIUM FROM VENOM PROTEINASE *b* BY ELECTRODIALYSIS

About 5 mg of proteinase *b* in dialysis tubing was subjected to electrodialysis as described in MATERIALS AND METHODS After dialysis for the indicated time, the protein concentration, calcium content and caseinolytic activity were measured The last column shows the relative activity after addition of $CaCl_2$ at a final concentration of 10^{-3} M

Electrodialysis time (h)	*Calcium content (%)*	*Relative caseinolytic activity*	
		—	+ $CaCl_2$
0	100 0	100 0	110 0
3	84 0	78 0	89 0
7	73 5	66 0	71 5
12	56 0	48 5	55 5
24	35 2	20 0	23 6

TABLE IV

REMOVAL OF CALCIUM FROM VENOM PROTEINASE BY VARIOUS TREATMENTS

Proteinase *b* (14 9 mg) was treated with the indicated amounts of urea or EDTA in 1 5 ml of 0 4 M Tris–HCl buffer (pH 8 5) at 37° for 1 h After desalting by passage through a Sephadex G-25 column (1 2 cm × 90 cm), the calcium content was measured by spectrophotometric titration

Treatment	*Calcium content (moles/mole protein)*
None	2 04
8 10^{-2} M EDTA	Nil
8 M urea	0 95
8 10^{-2} M EDTA containing 8 M urea	Nil

TABLE V

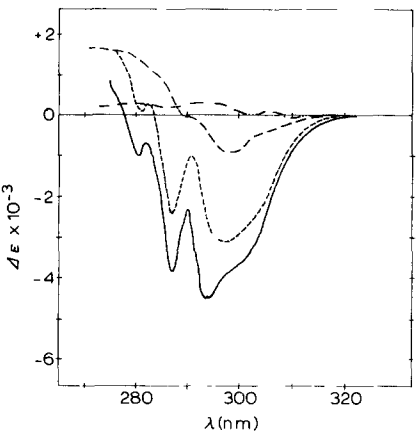
EFFECTS OF EDTA ON THE HEMORRHAGIC AND CASEINOLYTIC ACTIVITIES OF VENOM PROTEINASE *b*

Proteinase *b* (2 10^{-6} M) was treated with EDTA in 0 5 ml of 0 1 M sodium acetate (pH 7 0) at 37° After standing the mixture for 4 h, the caseinolytic and hemorrhagic activities were estimated on samples appropriately diluted with 0 9% NaCl

EDTA concentration (equimolar)	*Relative activity*	
	Caseinolytic	*Hemorrhagic*
None	100 0	100 0
0 5	—	100 0
1 0	80 2	83 0
1 5	—	67 5
2 0	43 5	49 0
2 5	—	35 5
3 0	19 5	18 0
4 0	8 0	Trace
5 0	4 5	Trace
6 0	1 7	Trace
7 0	1 4	Undetectable
8 0	1	Undetectable

Effect of EDTA

Table V shows the effect of EDTA on venom proteinase *b* Inactivation of the enzyme depended on the EDTA concentration and, with four equimolar concentrations of EDTA under the conditions described in Table V, the caseinolytic activity was almost completely, and hemorrhagic activity was completely lost On treatment with EDTA, the hemorrhagic activity and the caseinolytic activity decreased nearly in parallel Moreover, in parallel with the disappearance of caseinolytic and hemorrhagic activities, the ultraviolet difference spectra of protein treated with EDTA showed a distinct blue shift, and the value of $\Delta\varepsilon$ reached to maximal over three molar equivalents of EDTA (Fig 3) Although the maximum value of $\Delta\varepsilon$ was smaller than



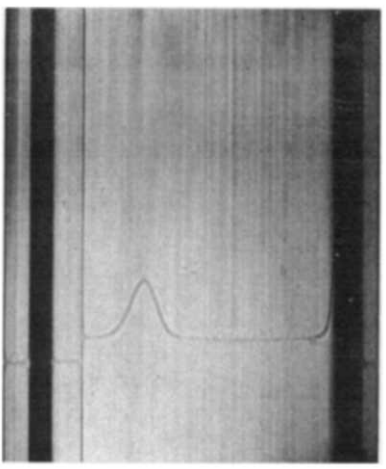


Fig 3 Ultraviolet absorption difference spectra of venom proteinase *b* treated with EDTA The enzyme (2 48 mg) was treated with 0 5–4 molar equivalents of EDTA at 37° for 4 h in 2 0 ml of 0 1 M sodium acetate (pH 7 0) The difference spectrum of EDTA-treated enzyme *versus* native enzyme was taken — —, 0 5 — — 1, — — —, 2 ——, 3 and 4 molar equivalents

Fig 4 Ultracentrifugal pattern of venom proteinase *b* inactivated with EDTA Enzyme inactivated with EDTA (see text) was dialysed overnight against 3 l of phosphate buffer (pH 7 0) *I* 0 1 in a cold room Centrifugation was carried out at 52 000 rev /min at 15 8° for 90 min at 0 48% protein concentration The photograph was taken after 45 min centrifugation

that obtained with 8 M urea (see Fig 2), the effect caused by the addition of EDTA was considered to be essentially similar to that of urea The blue shift was not reversible even on addition of calcium at a final concentration of 10^{-3} M, suggesting that denaturation of the enzyme by EDTA is irreversible like that by urea

Venom proteinase *b* denatured by EDTA was applied to ultracentrifugal analysis To avoid autodigestion the intact enzyme (14 6 mg) was treated at 4° with 0 2 mmole of EDTA in 2 3 ml of 0 1 M sodium acetate buffer (pH 6 8) for 4 days until the caseinolytic activity disappeared The inactive protein was then dialysed overnight against 3 l of phosphate buffer (pH 7 0), *I* 0 1, in a cold room Fig 4 shows the ultracentrifugal pattern of this inactive enzyme It gave a single symmetrical peak with a sedimentation constant of 4 49 S, a value identical with that for intact enzyme reported in the previous paper[2] Thus it is unlikely that removal of calcium causes dissociation of the enzyme and change in its subunits

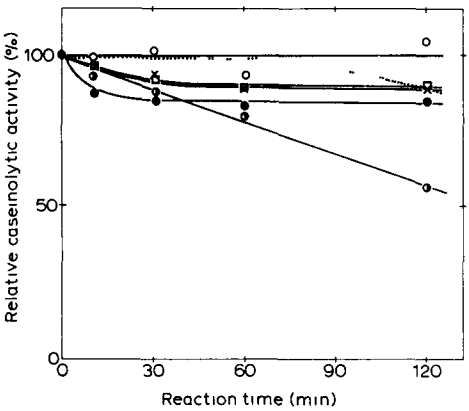


Fig 5 Effects of –SH reagents on caseinolytic activity of venom proteinase *b* Enzyme (0 32 mg) was treated with one of the reagents (10^{-3} M) in 0 8 ml of 0 04 M Tris–HCl buffer (pH 8 5) at 37° After standing the mixture for the indicated time the caseinolytic activity was measured control, ●—●, 5,5′-dithio-bis-(2-nitrobenzoic acid), ◑—◑, PCMB, ○—○, monoiodoacetic acid, ×—×, *N*-ethylmaleimide, □—□, monoiodoacetamide

Existence of a free sulfhydryl group and its reactivity

Fig 5 shows the effects of –SH reagents on the caseinolytic activity of venom proteinase *b* The enzyme was inactivated by treatment with 10^{-3} M PCMB, but other reagents, such as 5,5′-dithio-bis-(2-nitrobenzoic acid), monoiodoacetic acid and *N*-ethylmaleimide at concentrations of 10^{-3} M had no significant effect To examine the possibility of the existence of a free –SH group, the ultraviolet difference spectrum of a mixture of enzyme and PCMB *versus* enzyme alone in the presence of 8 M urea was measured and a typical spectrum of a PCMB–mercaptide was obtained The number of free –SH groups in the enzyme was determined by titration with *N*-ethylmaleimide and incorporation of monoiodo[^{14}C]acetic acid after denaturation of the enzyme with urea or EDTA As shown in Table VI, one mole of –SH group was found per enzyme molecule and the values obtained by the two methods were in good accordance However, this –SH group is not titrated completely even in the presence of 8 M urea or 8 10^{-2} M EDTA (Table VI) The maximum value of titratable –SH groups was obtained only in the presence of 8 M urea containing 8 10^{-2} M EDTA When venom

TABLE VI

REACTIVITY OF THE FREE SULFHYDRYL GROUP IN VENOM PROTEINASE *b*
The procedures used are described in MATERIALS AND METHODS

Treatment	*Sulfhydryl reagent*	
	N-Ethylmaleimide	*Monoiodo[^{14}C]acetic acid*
	(moles of –SH group per mole protein)	
None	0 18	0 21
8 10^{-2} M EDTA	0 39	0 28
8 M urea	0 45	0 36
8 M urea containing 8 10^{-2} M EDTA	1 08	1 11

proteinase *b* was exposed to various sulfhydryl compounds at final concentrations of 10^{-3} M, the caseinolytic activity was strongly inactivated by β-mercaptoethylamine and cysteine, and slightly inactivated by glutathione, β-mercaptoethanol and thioglycolate (Fig 6) The activity disappeared after the treatment with 10^{-2} M of β-mercaptoethylamine or cysteine for 10 min Also, the enzyme treated with cysteine showed a distinct blue shift, suggesting that the cysteine treatment resulted in the conformational change of the enzyme molecule (Fig 7) The blue shift at 286 nm

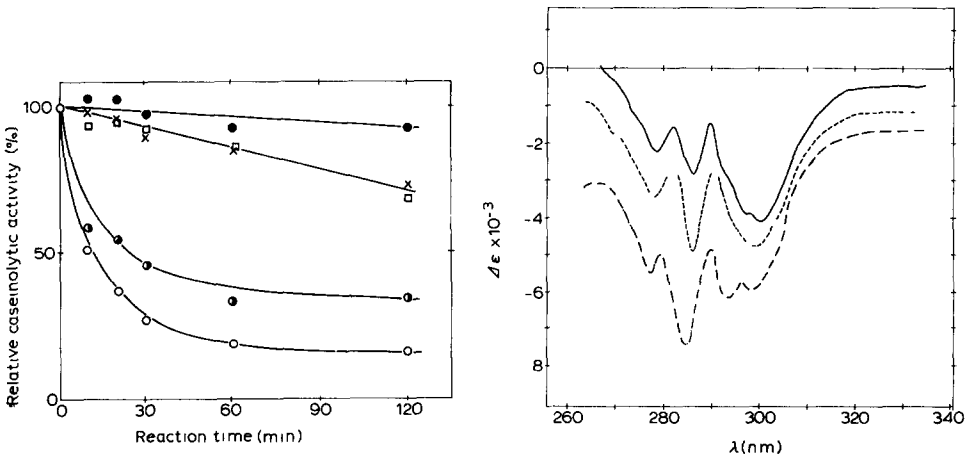


Fig 6 Effects of sulfhydryl compounds on venom proteinase *b* Enzyme (0 34 mg) was treated with 10^{-3} M of one of the sulfhydryl compounds for the indicated time in 4 0 ml of 0 1 M Tris–HCl buffer (pH 8 5) at 37° and the caseinolytic activity was then measured ●—●, thioglycolate, □—□, β-mercaptoethanol, ×—×, glutathione, ◑—◑, cysteine, ○—○, β-mercaptoethylamine

Fig 7 Ultraviolet absorption difference spectra of venom proteinase *b* inactivated with cysteine The enzyme (10 mg) was inactivated with 0 1 M cysteine for 1 h in 1 0 ml of 0 4 M Tris–HCl buffer (pH 8 5) at 37° and the inactive enzyme was subjected to gel filtration through a Sephadex G-25 column (1 2 cm × 90 cm) Using the fraction of the eluate containing protein, the ultraviolet difference spectra of the inactive enzyme *versus* native enzyme were measured — —, immediately after desalting, — — —, after standing at room temperature in 10^{-4} M $CaCl_2$ at pH 8 8 for 16 h and ———, for 48 h

did not disappear by the addition of calcium at a final concentration of 10^{-4} M, and by this treatment enzyme activity was not restored

Exchange reaction between cysteine and the disulfide groups in venom proteinase b

Enzyme (21 9 mg) dissolved in 6 0 ml of 0 1 M Tris–HCl buffer (pH 8 5), containing 5 10^{-3} M cysteine, was incubated at 37° After the indicated times an aliquot (0 03 ml) of the incubation mixture was taken and its caseinolytic activity was estimated Another 0 5-ml aliquot of the mixture was mixed with 0 5 ml of 1 M HCl to stop the exchange reaction The mixture was then dialyzed for 3 days against 3 l of 0 01 M HCl, with six changes of the HCl Then the dialyzed solution was lyophilized and the residue was dissolved in 1 0 ml of 0 4 M Tris–HCl buffer (pH 8 5) containing 8 M urea and 10^{-2} M EDTA, and 0 2 ml of monoiodo[^{14}C]acetic acid (1 33 10^5 counts/min per μmole per ml) was added After the incubation at 37° for 2 h, the mixture was dialyzed for 6 days against 10 l of distilled water, changing the water four times

TABLE VII

EXCHANGE REACTION BETWEEN CYSTEINE AND THE DISULFIDE GROUPS IN VENOM PROTEINASE *b*
The procedures used are described in the text

Incubation time with cysteine (min)	*Relative caseinolytic activity*	*–SH groups appearing (moles per mole protein)*
0	100 0	1 10
5	69 3	1 62
10	59 0	1 69
20	51 5	2 09
60	39 5	2 58
180	28 5	2 10
600	18 0	2 32

Then, the radioactivity incorporated was measured with 0 2–0 4 ml of the dialyzed solution, and the moles of –SH groups newly appeared were calculated

Table VII shows the results of exchange reaction between cysteine and the disulfide groups in the enzyme protein The numbers of free –SH groups in the enzyme molecule increased inversely with decrease in the caseinolytic activity, reaching about twice the initial number after 10 h This may suggest that there are disulfide bonds which are easily exchangeable with cysteine and that the exchange reaction accompanies the irreversible denaturation of the enzyme molecule

DISCUSSION

Recently several workers have independently isolated highly purified proteolytic enzymes (endopeptidases) from venoms of snakes of the genera Crotalus[13], Agkistrodon[2,3,14] and Trimeresurus[15,16] These proteinases were shown to be homogeneous by currently available criteria Their isoelectric points differ, but their molecular weights all range between 22 000 and 25 000, except for those of the proteinases isolated from *A halys blomhoffii*[2] A common feature of proteinases from the Crotalidae venoms is that they all hydrolyze peptide bonds with amino groups contributed by leucine and phenylalanine residues, and require more than a pentapeptide sequence for hydrolysis of this bond[6,13,15–17] Another common characteristic of the venom proteinases is that they are easily inactivated by EDTA and cysteine[1,14,15,18] In these properties they differ from well known mammalian endopeptidases

Proteinase *b* from *A halys blomhoffii* venom contains approx 2 gatoms of calcium per enzyme molecule The removal of the metal results the conformational change of the enzyme protein, as judged from the blue shift in its ultraviolet difference spectra This conformational change of the protein with concomitant loss of enzymatic and hemorrhagic activities is irreversible, and addition of Ca^{2+} does not result in reactivation of the biological activities or reversal of the denaturation Protein freed from metal does not differ in its $s_{20,w}$ value from native enzyme, suggesting that loss of calcium does not lead to dissociation or association of proteinase molecules Thus, the calcium atom in venom proteinase seems very important in retaining the native state of the enzyme molecule It is also possible to consider that the enzyme undergoes denaturation in the presence of EDTA, loses its activity and as a consequence of the denaturation, it loses also its calcium Thus, it is supposed that Ca^{2+} has some role

to maintain the tertiary structure related to caseinolytic activity of proteinase *b* The decrease of caseinolytic activity accompanies the decrease of hemorrhagic activity of proteinase *b*

When the enzyme is exposed to monoiodoacetic acid, *N*-ethylmaleimide or 5,5′-dithio-bis-(2-nitrobenzoic acid) (each of 10^{-3} M) for a relatively long period, enzymatic activity is slightly inhibited PCMB is especially inhibitory and this led to the finding of a free –SH group, one mole of –SH group per enzyme molecule However, in the native state of the enzyme, the –SH group only partially reacts with –SH reagents (*cf* Table VI), suggesting that some conformational change of enzyme may be necessary before the –SH group becomes reactive It seems, therefore, that there must be steric hindrance of the –SH group by a rigid conformational structure

As previously reported[1,4], venom proteinase is markedly inactivated by various kinds of sulfhydryl compounds, especially by cysteine This inactivation, like that by EDTA, seems to induce a concomitant conformational change of the enzyme molecule (*cf* Fig 7) At present, there is some evidence that cysteine can be exchanged with an internal disulfide bond in the molecule, because the number of –SH groups increases after treatment of the enzyme with cysteine reaching about twice the initial number This suggests that venom proteinase contains disulfide bridges which are easily exchangeable with cysteine, and that this exchange reaction participates in the irreversible denaturation of the enzyme molecule

From the above results, it seems that the –SH group and calcium atom in venom proteinase *b* are closely related to enzyme activity and to retaining the conformation of the enzyme molecule The state of the –SH group in the molecule is uncertain, but this group may be deeply buried and chelated with calcium Further investigations are required on the role of the –SH group and metal in the presence of the substrate

ACKNOWLEDGEMENTS

The authors are greatly indebted to Professor Kozo Hamaguchi, for his valuable suggestions and helpful discussion This work was supported by a grant from the Ministry of Education, Japan

REFERENCES

1 M SATAKE, Y MURATA AND T SUZUKI, *J Biochem Tokyo*, 53 (1963) 438
2 G OSHIMA, S IWANAGA AND T SUZUKI, *J Biochem Tokyo*, 64 (1968) 215
3 G OSHIMA, Y MATSUO S IWANAGA AND T SUZUKI, *J Biochem Tokyo*, 64 (1968) 227
4 S IWANAGA, T OMORI G OSHIMA AND T SUZUKI, *J Biochem Tokyo*, 57 (1965) 392
5 T OMORI, S IWANAGA AND T SUZUKI, *Toxicon*, 2 (1964) 1
6 M SATAKE, T OMORI S IWANAGA AND T SUZUKI, *J Biochem Tokyo*, 54 (1963) 8
7 P D BOYER, *J Am Chem Soc*, 76 (1954) 4331
8 H KONDO, S KONDO, H IKEZAWA R MURATA AND A OHSAKA, *Jap J Med Sci Biol*, 13 (1960) 43
9 B ZAK, W H HINDMAN AND M FISHER *Am J Clin Pathol*, 26 (1956) 1081
10 N M ALEXANDER, *Anal Chem*, 30 (1958) 1292
11 A IMANISHI, *J Biochem*, 60 (1966) 381
12 F A STEIN J HSIU AND E H FISHER, *Biochemistry*, 3 (1964) 56
13 V R ZWILLING AND G PFLEIDERER, *Z Physiol Chem*, 348 (1967) 519
14 F W WAGNER, A M SPIFKERMAN AND J M PRESCOTT, *J Biol Chem* 243 (1968) 4486

15 T TAKAHASHI AND A OHSAKA, *Biochim Biophys Acta*, 207 (1970) 65
16 T TAKAHASHI AND A OHSAKA, *Biochim Biophys Acta*, 198 (1970) 293
17 T SUZUKI, S IWANAGA, S NAGASAWA AND T SATO, *Hypotensive Peptides*, Springer Verlag New York, 1966, p 149
18 G PFLEIDERER AND G SUMYK, *Biochim Biophys Acta*, 51 (1961) 482.