Yeast Pro-proteinase C

Part IV Activation by Yeast Proteinase A and the Activation Mechanism

By Rikimaru HAYASHI and Tadao HATA

The Research Institute for Food Science, Kyoto University, Uji City, Kyoto

Received October 1, 1971

Activation of pro-proteinase C by yeast proteinase A was examined under controlled conditions. Maximum activation occurred at pH 3.5 and 0°C, releasing a cationic protein. The active enzyme and the protein was separated and chemically analyzed.

The same N-terminal amino acid, lysine, was found in both the active enzyme and proenzyme. Amino acid analysis revealed that the released protein is a protein of small molecular weight about 19,000 containing one SH-group and one disulfide bond. These results strongly suggested that the protein would correspond to the cationic subunit of the proenzyme.

In both activation processes by denaturant and proteinase A, a decrease of β -structure was found as determined from ORD and CD measurements.

All of these results supported the idea that activation of the proenzyme occurred by denaturant- or enzyme-modification of the inhibitor protein, followed by demasking of the active site.

The preceding paper¹⁾ described yeast pro-proteinase C was composed of two subunit proteins and was activated by exposure to various denaturants without their dissociation. This observation suggested that the activation may occur by non-covalent change in the proenzyme molecule, followed by demasking of the active site. On the other hand, it has been demonstrated in the crude preparation that the proenzyme was activated by action of yeast proteinase A. This activation, in contrast to denaturant-activation, shows that splitting of the covalent-bond would be involved in the activation process. In order to correlate these different phenomena and to elucidate the activation mechanisms, more precise experiments are necessary.

In the present paper, the purified proenzyme is activated by proteinase A in controlled con-

ditions and the active enzyme (proteinase C_A) is isolated to compare physically and chemically with denaturant-activated enzymes as well as pro-proteinase C. Here, proteinase C_A is terminologically distinguished from proteinase C, which is obtained from yeast autolysate,^{2,3)} though the latter is produced from the proenzyme by action of proteinase A in the autolytic process of the cell extract. In physical investigation, however, proteinase C will be used sometimes as the example of the active enzyme, instead of C_A which is difficult to prepare.

MATERIALS AND METHODS

Materials. Pro-proteinase C,⁴⁾ proteinase C and proteinase A,⁵⁾ prepared from commercial baker's yeast as described previously and stored in a refrigerator, were used after gel-filtration chromatography on a Sephadex G-100 column. The ORD and CD were measured using lyophilized enzymes. In special cases described, proteinase C was prepared from partially

Abbreviation used: ATEE, N-Acetyl-L-tyrosine ethylester; PTH: 3-Phenyl-2-thiohydantoin; CD, Circular dichroism; ORD, Optical rotatory dispersion.

purified pro-proteinase C.³⁾

Reagents used for Edman degradation were purified according to procedures described by Edman and Begg.⁶⁾

Determination of enzyme activity. Activities of proteinases A and C were determined by proteolytic activity for acid-denatured hemoglobin and esterase activity for ATEE, respectively.⁵⁾ Potential activity of pro-proteinase C was determined as in the preceding paper.¹⁾

Chemical analysis. NH_2 -terminal of proteins (10~15 mg) was estimated by the method of Edman, whereas the PTH-amino acid was identified by thin layer chromatography.⁷⁾

Amino acid compositions of protein (about 5 mg) were analyzed and compared pairwise with 22 hr-acid hydrolysate using a Hitach KLA-3 amino acid analyzer. Acid hydrolysis was performed at 110°C for 20 hr with 6N HCl (3 ml) in a sealed tube under an argon gas phase. Cysteine and half-cystine were determined as cysteic acid after oxidation with performic acid according to the procedure described by Moore.⁸⁾ Tryptophan and tyrosine was determined spectrophotometrically by the method of Goodwin and Morton.⁹⁾

Measurements of ORD and CD. To measure ORD and CD in the ultraviolet region, the reagent-activated enzyme was prepared as follows. The lyophilized proenzyme was dissolved in 0.01 M sodium phosphate, pH 7.0, and mixed with dioxane or with 8 M urea dissolved in 0.1 M sodium phosphate, pH 5.0. Final concentration of dioxane and urea was 30% (v/v) and 4 M, respectively. The ORD and CD were immediately measured with these aforementioned solutions. ORD in the visible region was measured with the activation mixture without dilution.

ORD and CD measurements were made using a Jasco spectropolarimeter model QRD/UV-5 equipped with CD attachment. The ORD data in ultraviolet region were measured with 0.4% of protein concentrations using cells of 0.1 mm path length and expressed in terms of $[m']$, the reduced mean residue rotation, defined as

$$[m'] = \frac{3}{(n^2 + 2)} \frac{M_0}{100} [\alpha]_\lambda$$

where $[\alpha]_\lambda$ is the specific rotation at wavelength λ , M_0 is the average residue weight (assumed to be 115),

and n is the refractive index of the solvent at wave-length of λ . The refractive indices as function to the solvent were obtained from those tabulated by Fasman.¹⁰⁾ ORD measurements in visible region were made in the 300 to 600 m μ wave-length, using a cell of 0.5 cm path length. The protein concentration used was 0.78% for pro-proteinase C and proteinase C and 0.4% for the dioxane- and urea-activated enzymes. The ORD properties was interpreted by means of the Moffitt-Yang equation.¹¹⁾

$$[m'] = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

The a_0 and b_0 values were calculated from the intercepts and slopes of $[\alpha]$ ($\lambda^2 - \lambda_0^2$) versus $(\lambda^2 - \lambda_0^2)^{-1}$ plots with λ_0 taken as 212 m μ .

CD measurements were carried out with the same spectropolarimeter with a CD attachment, using cells of 0.2 cm path length at 200~250 m μ and 1 cm at 250~300 m μ . The protein concentrations used were 1.6%. Molecular ellipticity, $[\theta]$, was obtained by the equation,

$$[\theta] = 3300 (\epsilon_L - \epsilon_R)$$

where $(\epsilon_L - \epsilon_R)$ is the difference between the molar extinction coefficients for left and right circularly polarized light. In the calculation of $[\theta]$, average residue weight was used instead of molecular weight. The units of $[\theta]$ are degree decimole⁻¹ cm².

Electrophoresis on cellulose acetate membrane. Using Oxoid cellulose acetate membrane (Oxo Ltd.), electrophoresis was carried out under conditions previously described.¹⁾

RESULTS

Activation conditions by proteinase A

Conversion of the proenzyme into the active enzyme was catalyzed by proteinase A in incubation at 25°C below pH 5. Lowering the pH of the incubation mixture, conversion was accelerated as expected from the optimum pH of proteinase A.⁵⁾ However, as spontaneous activation catalyzed by acid was accompanied below pH 3.5 at 25°C, it was impossible to distinguish clearly activation by proteinase A from that of acid catalysis. In order to suppress the latter activation, experiment was

FIG. 1. Activation of the Proenzyme by Proteinase A.

The proenzyme (252 μ g) was mixed with 150 μ l of 0.05 M sodium acetate, pH 3.5 and various amounts of proteinase A (4.1~16.4 μ g) in total volume of 200 μ l and incubated at 0°C. At appropriate time intervals, 10 μ l aliquot of the mixture was withdrawn and immediately assayed for esterase activity. The ratio of the proenzyme to proteinase A was 15.6 (O—O), 31.2 (●—●) or 62.4 (Δ — Δ).

FIG. 2. Electrophoresis of the Activation Mixture on a Cellulose Acetate Membrane.¹⁾

The activation was performed on similar conditions as in Fig. 1 at various pH's and temperatures for 24 hr. The ratio of the proenzyme to proteinase A was 31. The incubation conditions; A, pH 5.0 and 25°C; B, pH 4.0 and 25°C; C, pH 3.5 and 25°C; D, pH 3.5 and 0°C. E and D show proteinase C and the proenzyme, respectively.

made at 0°C with a pH of 3.5. As seen in Fig. 1, the activation by acid catalysis was substantially avoided activation by proteinase A allowed to continue for about 26 hr.

The mixtures after completion of the activation were analyzed by zone electrophoresis on a strip of cellulose-acetate membrane. As shown in Fig. 2, the initial proenzyme disappeared and two new bands appeared by the activation treatment at pH 3.5 and 0°C. One of them was a strongly basic band, the mobility of which suggested that this band would correspond to the basic subunit protein which appeared in similar electrophoresis in 8 M urea-denaturation of proenzyme. Conceivably, the other band in the electrophoresis would correspond to the active enzyme produced from the proenzyme. When the activation was performed at 25°C, the basic band disappeared, no doubt digested by proteinase A and/or the

FIG. 3. Gel-filtration of the Activation Mixture on Sephadex G-100.

Experimental conditions are described in the text. Effluent was analyzed with the ninhydrin reaction¹³⁾ following the alkaline hydrolysis¹²⁾ (O—O), absorbancy at 280 $m\mu$ (●—●) and esterase activity (Δ ... Δ).

activated enzyme.

Purification of the activated enzyme

Separation and purification of the activated enzyme were attempted. To prepare the activated enzyme, 0.21 mg of proteinase A was added to 16.9 mg of the proenzyme (0.025 M acetate buffer, pH 3.5) and the mixture was incubated at 0°C for 26 hr. Under these conditions, proteinase C_A was prepared in an almost theoretical yield, as judged from the specific activity. Immediately after activation, the reaction mixture was transferred to a Sephadex G-75 column (1.5 × 90 cm) and gel-filtration was performed with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.05 M KCl. Figure 3 shows a gel-filtration pattern of the activation mixture. Fractions having esterolytic activity for ATEE (Fraction A) were collected and further purified by chromatography on a DEAE-Sephadex A-50 column. As seen from Fig. 4,

FIG. 4. Chromatography of the Activated Enzyme in a DEAE-Sephadex A-50 Column (0.9 × 46 cm).

Fraction A in Fig. 3 was applied on the column previously equilibrated with 0.01 M sodium phosphate, pH 7.0 and eluted stepwise with the buffer containing 0.2 M and 0.4 M NaCl. The main peak shows the active fractions.

a chromatographically pure fraction of proteinase C_A was obtained. The specific ac-

tivity of the purified proteinase C_A was 130 μ moles ATEE hydrolyzed/min/mg: little higher value than that of proteinase C.⁵¹ Low molecular weight fractions (Fraction B) in chromatography on Sephadex G-75 were also collected and used for chemical analysis without further purification. This fraction contained several minor peptides and/or amino acids as judged from high-voltage paper electrophoresis at pH 3.6.

Chemical analysis of the proenzyme and the activated enzyme

The results of amino acid analyses and difference of amino acid content between the proenzyme and proteinase C_A are given in Table I. It can be seen that the proenzyme, proteinase C and C_A contain relatively more quantities of acidic amino acids in agreement with acidic nature of these proteins. Amino acid compositions of proteinases C and C_A were temporarily similar to each other except aspartic acid, glutamic acid, glycine, alanine and serine, although each amino acid content in proteinase C showed slightly less than that in C_A . Glutamic acid, glycine, alanine and serine in proteinase C showed particularly less value than those of proteinase C_A . Hexosamine was also commonly detected in these proteins.

The content of cysteic acid in pro-proteinase C was estimated to be 10 moles per mole of protein. As the proenzyme possesses two SH-groups as described in the preceding paper,¹¹ the residual 8 moles of cysteic acid would constitute 4 sets of disulfide bridge in the proenzyme. On the other hand, proteinases C and C_A contained 7 moles of half cystine per mole of each protein. As proteinase C contained only one mole of cysteine residue per molecule¹¹ proteinase C would contain 3 sets of disulfide bridges. This evaluation would be valid in proteinase C_A . Cysteic acid was also detected in low molecular weight fraction obtained by the gel-filtration: approximately 3 moles of cysteic acid were recovered in this

TABLE I. AMINO ACID ANALYSES OF 22-HR HYDROLYSATE OF PROTEINS

Amino acid	Pro-enzyme	Proteinase C_A	Proteinase C_A ^{a1}	Difference between proenzyme and C_A
residues/molecule				
Asp	77.4	58.8	56.2	21.2
Glu	57.1	36.1	41.8	15.3
Gly	42.5	30.9	36.5	6.0
Ala	36.9	20.0	30.3	6.6
Val	36.1	25.4	24.7	11.4
Leu	42.5	30.0	30.0	12.5
Ileu	26.2	18.7	18.2	8.0
Ser ^{b1}	45.4	27.6	34.8	10.6
Thr ^{b1}	30.5	16.2	17.9	12.6
Cys ^{c1}	10.1	7.0	7.0	3.1 ^{d1}
Met	8.7	4.2	3.9	4.8
Pro	35.7	22.6	23.4	12.3
Phe	32.5	20.6	20.9	11.6
Tyr ^{b1}	28.4	22.7	21.9	6.5
Trp	12.5	10.9	—	—
His	12.7	7.8	8.3	4.4
Lys	29.4	15.1	16.9	12.5
Arg	9.9	7.7	7.8	2.1
Glc. NH ₂	4.8	Yes ^{e1}	Yes ^{e1}	—
Total ca. 163				

^{a1} Calculated as leucine equals 30.

^{b1} Corrected for decompositions during 22-hr acid hydrolysis. The factors applied were: serine, 0.90; threonine, 0.95; tyrosine, 0.95. (cf. A. M. Crestfield, S. Moore and W. H. Stein, *J. Biol. Chem.*, **238**, 622 (1963).)

^{c1} Measured as cysteic acid after performic acid oxidation.⁸¹

^{d1} Approximately 3 moles per mole of the proenzyme were recovered in Fraction B of Fig. 3.

^{e1} not determined.

fraction from one mole of pro-proteinase C. This indicates that the small protein which contains one sulfhydryl group and one disulfide bridge would be released in the activation. It is also expected that this sulfhydryl group corresponds to highly reactive sulfhydryl group for DTNB which was found in the activation by dioxane or urea.¹¹

Amino acid content of proteinase C_A was less than that of pro-proteinase C by about 163 residues. This indicates that the protein fraction released from the proenzyme in the activation by proteinase A has approx. 19,000 of molecular weight, which corresponds to the difference between the proenzyme and

proteinase C.

Edman degradation of pro-proteinase C revealed only lysine as NH_2 -terminal amino acid in the yield of 0.88 residue per mole of protein. Since the proenzyme was composed of two subunits, NH_2 -terminal amino acid of one subunit seemed blocked so as not to react with phenyl-isothiocyanate. On the other hand, proteinase C which was prepared from partially purified proenzyme (see Materials) also revealed only lysine as NH_2 -terminal amino acid in the yield of 0.86 mole per mole of protein. (There was too little proteinase C_A to detect NH_2 -terminal amino acids.)

Conformational changes in the activation

Conformational changes in the activation of the proenzyme were examined by ORD and CD measurements.

Figure 5 shows the Moffitt plots of the proenzyme, proteinase C and the dioxane- and urea-activated enzymes. The parameters of a_0 and b_0 were calculated from the Moffitt and Yang equation with assuming $\lambda_0 = 212 \text{ m}\mu$. For the proenzyme and proteinase C, a_0 values

were -69.0 and -12.0 , respectively, and b_0 values were -95.1 and -90.5 , respectively. From the b_0 values which are experimentally identical, the α -helix contents of both proteins are calculated to be about 14%. On the other hand, activation by dioxane or urea resulted in decreases of b_0 values up to -110 . From these results, an increase of the helical content appeared to accompany the activation of the proenzyme. This is not necessarily valid when the content of β -structure changes during the activation.¹⁵⁾ In order to clarify the problem, analyses of ORD and CD in far ultra-violet region were examined.

Figure 6 shows ORD spectra in 200 to 250 $\text{m}\mu$ region of proenzyme and proteinase C together with dioxane-activated enzyme in their native and denatured (in 8 M urea) states. The curves of both protein in 8 M urea showed denaturation of the proteins. Both native proteins had trough at $232\sim 233 \text{ m}\mu$ and peak near $200 \text{ m}\mu$. Shoulders also appeared at $210\sim 215 \text{ m}\mu$ suggesting the presence of the α -helix. It was noticeable that troughs near $233 \text{ m}\mu$ decreased in both proteinase C and dioxane-

FIG. 5. Moffitt Plots of ORD of Proteinase C and the Proenzyme in Various Solvents.

Experimental details are described in the text. ○—○ proteinase C; ●—● proenzyme △—△ proenzyme + 30% dioxane; ▲—▲ proenzyme + 4 M urea.

FIG. 6. ORD Curves of the Various Forms of the Enzyme.

Experimental conditions are described in the text. —, the proenzyme; ---, proteinase C, ..., dioxane-activated enzyme.

activated enzyme, but peaks near 200 $m\mu$ remained constant.

Figure 7 shows CD spectra from 210 to 250 $m\mu$ region of native and denatured (in 8 M urea) proenzyme and proteinase C together with dioxane-activated enzyme. The spectra of the proenzyme and proteinase C in 8 M urea indicated denaturation of the proteins. The pattern of the proenzyme in native state was characterized by an absorbing band typical for β -structure which has a minimum at 217 $m\mu$.¹⁶⁾ In active enzymes (proteinase C and dioxane-activated enzyme), the negative minimum at 217 $m\mu$ disappeared and new spectra, which have minimum at 200–203 $m\mu$ and a shoulder near 222 $m\mu$ appeared. These spectra suggest the presence of α -helix rather than β -structure. The results lead to the conclusion that β -structure in the proenzyme is destroyed during the conversion to the ac-

FIG. 7. CD Spectra of the Various Forms of the Enzyme.

Symbols and the experimental condition are the same as in Fig. 6.

tive forms.

In summarizing ORD and CD experiments, it is concluded that the transformation of the proenzyme into the active enzymes accompanied the change in β -structure in the proenzyme without any changes of α -helical content.

DISCUSSION

Pro-proteinase C consists of two subunits: anionic and cationic ones.¹¹⁾ Their properties and the activation features are summarized from the present work as follows.

Anionic subunit would be in itself an enzyme protein of molecular weight approx. 60,000. As a result of activation treatment, it can be activated without any covalent changes. This concept is supported from the facts that NH_2 -terminal amino acid, lysine, was the same between active and inactive forms of the enzyme and that the activation was brought about by denaturants which no doubt have

no effect on the covalent bonding of protein. Activation of bovine procarboxy-peptidase A by trypsin accompanies dissociation of the subunits, followed by limited proteolysis near NH_2 -terminal portion of the precursor subunit.¹⁸⁾ Usually, proteinase zymogens are activated by release of a peptide near its NH_2 -terminal.¹⁹⁾ At present, however, it may be concluded that such limited proteolysis is not a case of activation of the present proenzyme. Proof of this is the concept made when reversible dissociation and association of the proenzyme subunits correlates with the activity and further two subunits are separated into their native states as to be subjected to chemical and physical analyses.

Cationic subunit should be an inhibitor protein which tightly binds to the anionic subunit and covers the active site. This protein has a molecular weight of approx. 19,000 including one SH-group and one disulfide bridge. It is noticeable that this small protein fraction contained larger quantities of serine and threonine which have a tendency to form β -structure in synthetic polypeptides¹⁴⁾ (see below). Its NH_2 -terminal should be blocked so as not to react with phenylisothiocyanate in usual conditions for Edman degradation. This subunit protein appeared to have a distinct amount of β -structure and to be sensitive to many protein denaturants. Its structural alteration was reflected on destruction of the β -structure observed in the denaturant-activation. Change of the structure would result in change of the arrangement of the small subunit, followed by unmasking of the active site which is located in the large subunit. In addition, small subunit seemed also to be susceptible to proteinase A. Since the other proteinases, *e.g.* trypsin and chymotrypsin, did not cause activation of pro-proteinase C, action of proteinase A would be limited. Proteinase A would cleave the critical bond of the small subunit and release it from the large one, thereby leading to the unmasking of the active site. Release of minor peptides and/or amino

acids were detected in the proteinase A-activation mixture by high voltage paper electrophoresis. Their identification and characterization in further work will clarify the crucial bond cleaved by proteinase A.

Although ORD spectrum of the proenzyme indicated the presence of helical structure, overlapping of the helix and β -structure makes difficult to evaluate the helical content from a trough at 233 $\text{m}\mu$. Jirgensons¹⁷⁾ estimated helical content of ribonuclease from the magnitude of the positive extremum near 200 $\text{m}\mu$ in fair agreement with X-ray data, assuming that contribution of the optical activity of the other conformation is negligible. Since ORD curves of the proenzyme and proteinase C near 200 $\text{m}\mu$ are experimentally indistinguishable, the helical content of both proteins can be estimated, on the Jirgensons proposition, to be about $9,500/75,000=0.12$ or 12%. This evaluation is in fair agreement with the estimation from b_0 values. Therefore, the change of trough near 233 $\text{m}\mu$ in the activation of the proenzyme may be contributed partly by the destruction of β -structure but not helical structure.

Amino acid content was different among the active enzymes. Glutamic acid, glycine, alanine and serine in proteinase C, which was obtained from yeast autolysate, revealed a smaller value than that in proteinase C_A , whereas serine and glycine in proteinase C, which was obtained from partially purified pro-proteinase C, showed intermediate values of the aforementioned enzymes. NH_2 -terminal analysis of proteinase C in the autolysate revealed lysine and minor amounts of four amino acids (aspartic acid, glycine, phenylalanine and tyrosine), whereas the other proteinase C revealed only one amino acid, lysine, as NH_2 -terminal amino acid. Different amino acid contents as well as heterogeneity of NH_2 -terminal suggest that the enzyme may suffer a modification in the course of its preparation resulting in different active forms as in chymotrypsin derivatives. Further, the fact that

the enzyme with a higher specific activity was obtained from the purified proenzyme-proteinase A system shows that the original enzyme was obtained from this system.

Acknowledgements. The authors express their sincere gratitude to Professor K. Hamaguchi, and his co-workers of the Faculty of Science, Osaka University, for the ORD and CD measurements, thanks is also due to Mr. T. Higasa and Miss H. Takebayashi for the technical assistance as well as to Miss M. Ohara for assistance with the manuscript.

REFERENCES

- 1) R. Hayashi, Y. Minami and T. Hata, *Agr. Biol. Chem.*, **36**, 621 (1972).
 - 2) *ibem, ibid.*, **32**, 367 (1968).
 - 3) S. Aibara, R. Hayashi and T. Hata, *ibid.*, **35**, 658 (1971).
 - 4) R. Hayashi, Y. Oka and T. Hata, *ibid.*, **33**, 196 (1969).
 - 5) T. Hata, R. Hayashi and E. Doi, *ibid.*, **31**, 357 (1967).
 - 6) P. Edman and G. Begg, *European J. Biochem.*, **1**, 80 (1967).
 - 7) P. Edman, "Protein Sequence Determination," ed. by S. B. Needleman Springer-Verlag, Berlin, 1970, p. 211.
 - 8) S. Moore, *J. Biol. Chem.*, **238**, 235 (1963).
 - 9) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).
 - 10) G. D. Fasman, "Methods in Enzymology," vol 6, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, 1963, p. 928.
 - 11) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, **42**, 596 (1956).
 - 12) R. G. Fruchter and A. M. Crestfield, *J. Biol. Chem.*, **240**, 3868 (1965).
 - 13) W. H. Stein and S. Moore, *ibid.*, **176**, 367 (1948).
 - 14) K. Imahori and I. Yahara, *Biopolymers Symposia*, No. **1**, 421 (1964).
 - 15) E. Iizuka and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, **55**, 1175 (1966).
 - 16) R. Townend, T. F. Kumosinski, S. N. Timasheff, G. D. Fassman and B. Davidson, *Biochem. Biophys. Res. Comm.*, **23**, 163 (1966).
 - 17) B. Jirgensons, *J. Am. Chem. Soc.*, **89**, 5980 (1967).
 - 18) J. H. Freisheim, K. A. Walsh and H. Neurath, *Biochem.*, **6**, 3010 (1967).
 - 19) H. Neurath, *Federation Proc.*, **23**, 1 (1964).
-