Activation of Intracellular Proteinases of Yeast

Part II. Activation and Some Properties of Pro-proteinase C*

By Rikimaru HAYASHI, Yoshinobu OKA, Etsushiro DOI
and Tadao HATA

Research Institute for Food Science, Kyoto University, Kyoto

Received September 18, 1967

Pro-proteinase C, the latent form of yeast proteinase C, was partially purified from the autolysate of baker's yeast. It was strongly activated by incubation at pH 5 or by treatment with urea or dioxane. The former activation was prevented by treatment to inactivate yeast proteinase A, which co-existed with the pro-enzyme in the present preparation, but was promoted by addition of purified proteinase A. Thus, it was confirmed that A could activate pro-proteinase C. Furthermore, it was found that activation could be caused by extremes in pH or by heating to 55~60°C, accompanied by the simultaneous destruction of the enzyme produced. Pro-proteinase C was stable over a range of pH 5 to 8 after 60 min incubation at 50°C.

In the preceding papers,^{1,2)} it has been suggested that, in intact cells of baker's yeast, most proteinases B and C occur in latent or zymogen forms, which are named as pro-proteinases B and C, respectively. Their activation was brought about *in vitro* by incubation at pH 5 or by treatment with certain denaturing agents, such as urea and several organic solvents. It has also been suggested that the former activation may be caused by the action of a specific enzyme present in the same extract.

In the present paper, the activation processes of pro-proteinase C are further investigated using the partially purified preparation. Some properties of this precursor are also described.

MATERIALS AND METHODS

Materials. CGT was purchased from the Institute for Protein Research, Osaka University, Osaka. Other chemicals used in this study were the same as in a preceding paper.²⁾ Purified yeast proteinase A was prepared by the method described in a previous paper.³⁾ Dioxane was reagent grade and urea was recrystallized from 95% ethanol.

Determination of enzymatic activity. The activity of proteinase C was followed by measuring the esterase activity for ATEE with a Radiometer pH-stat as described in a preceding paper.²⁾ In certain cases, the activity was determined by the peptidase activity for CGT in the following way. An assay mixture, containing 0.05 M sodium acetate buffer, pH 5.0, 3.3 mM CGT and 0.2 ml of the enzyme solution, in total volume 1 ml, was incubated for 20 min at 25°C and immediately mixed with 2 ml of ninhydrin solution prepared according to Stein and Moore.⁴⁾ The mixture was heated in a boiling water bath for 20 min, cooled, and after appropriate dilution, the absorbancy at 570 m μ was determined in a Shimadzu QV-50 spectrophotometer.

* This investigation was supported in part by a grant from the Japan Foundation of Applied Enzymology.

Abbreviations used in this paper: CGT, carbobenzoxy-L-glutamyl-L-tyrosine; ATEE, N-acetyl-L-tyrosine ethylester; HCl-Hb, acid-denatured hemoglobin.

1) R. Hayashi, Y. Oka, E. Doi and T. Hata, *Agr. Biol. Chem.*, **31**, 1102 (1967).

2) R. Hayashi, Y. Oka, E. Doi and T. Hata, *ibid.*, **32**, 354 (1968).

3) T. Hata, R. Hayashi and E. Doi, *Agr. Biol. Chem.*, **31**, 357 (1967).

4) W. H. Stein and S. Moore, *J. Biol. Chem.*, **176**, 367 (1948).

Quantitative assay of pro-proteinase C was routinely carried out by incubating the pro-enzyme in 0.01 M sodium phosphate buffer, pH 7.0, with one third volume of dioxane at 25°C. After incubation for 15 min, an aliquot was withdrawn and immediately assayed for activity of proteinase C.

The activity of proteinase A was determined as described previously.³⁾

Partial purification of pro-proteinase C. The crude extract of pro-proteinase C was prepared by autolysis of baker's yeast as described in the preceding paper²⁾ and partial purification of the pro-enzyme was performed as follows. The crude extract was dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, at 5°C overnight and 30 ml of the dialysate was applied on a DEAE-cellulose column (1 × 7 cm), equilibrated with the buffer as was used in the dialyzing solution. After washing the column with the same buffer, elution was performed by 0.01 M sodium phosphate buffer, pH 7.0, containing 0.2 M sodium chloride. The first 3 ml of the eluate were discarded and the next 10 ml of the eluate were collected. Since the pro-proteinase C in this preparation was gradually activated, even standing at pH 7, the sample was prepared on the day of the experiments.

In the experiments on pH- and heat-stability of pro-proteinase C, the crude extract was previously incubated at pH 8.5 for 2 hr at 25°C in order to inactivate proteinase A present in the extract, followed by dialysis and chromatography on a DEAE-cellulose column. The eluate from the column was fractionated with ammonium sulfate and the precipitate, which produced between 60~80% saturation of ammonium sulfate, was collected and desalted by gel-filtration on a Sephadex G-25 column. The resulting preparation of pro-proteinase C contained no detectable activity of proteinase A and the activation of pro-proteinase C was little or negligible while standing at pH 5 overnight.

Determination of protein concentration. Protein concentrations were determined by the method of Lowry *et al.*⁵⁾

RESULTS

1) Activation by Urea and Dioxane

The Pro-proteinase C used in these experiments was strongly activated by dioxane and urea, a maximum activation being obtained

when the pro-enzyme was incubated with 3 M concentration of urea for 60 min or with 33% (v/v) concentration of dioxane for 15 min. The activation by urea was sharply dependent upon pH with its optimum at pH 5. However, the activation to the same degree by dioxane was observed over a range of pH 5 to 8. These properties were the same as those in the crude extract.

2) Activation by Incubation at Various pH

The activation of pro-proteinase C was dependent on pH and maximum activation was obtained when the crude extract was incubated at pH 5 overnight.²⁾ However, these experiments may not reveal an accurate pH-dependency of the activation, since the proteinase C produced, which is very labile below pH 4,⁶⁾ may be accompanied by simul-

FIG. 1. Effect of pH on the Activation of Pro-proteinase C.

Pro-proteinase C (1.3 mg) was incubated with 4 ml of 0.05 M buffer solution (lactate, pH 2.0~3.0; acetate, pH 3.5~5.5; phosphate, pH 6.0~7.5; borate, pH 8.0~9.0) at 37°C. An aliquot of each mixture was withdrawn at various intervals and immediately assayed for peptidase activity of proteinase C using CGT as the substrate.

Incubation time:

—○—: 0 min; —○●—: 30 min;
—●—: 120 min; —△—: 400 min;
—×—: 24 hr.

5) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

6) E. Doi, R. Hayashi and T. Hata, *Agr. Biol. Chem.*, **31**, 160 (1967).

taneous inactivation during a long incubation at an acidic pH. So, time dependency of the activation was tested as function of pH. The results are presented in Fig. 1. At pH 3 to 4, a rapid activation was observed but some inactivation seemed to occur at the same time. With incubation time, the peak of activity moved to a higher pH and reached pH 5 after 24 hr. Time courses of the activation at various pH levels are presented in Fig. 2.

FIG. 2. Time Courses of the Activation of Pro-proteinase C at Various pH Values.

Pro-proteinase C (500 μ l, 890 μ g) was incubated with 1 ml of 0.05 M buffer at 37°C. The activity of proteinase C was determined with esterase activity as described in the text. The other experimental conditions are the same in Fig. 1.

Incubation pH:

- : pH 3; —○—: pH 4;
—△—: pH 5; —□—: pH 7.

On incubation at pH 3 or 4, the activity rapidly appeared in initial stage of incubation and was followed by decrease or suppression probably due to irreversible inactivation of the activated enzyme. On incubation at pH 5 or 7, the activation proceeded linearly, with a faster rate than that of the crude extract.²¹ As described below, the activation which occurred around pH 3 was a different process from that around pH 5. Probably the former activation was catalyzed by hydrogen ions and the latter by the proteinase A co-existing in the same preparation.

3) Activation by Yeast Proteinase A

It has been suggested^{1,21} that the activation of pro-proteinase C during incubation at pH 5 is catalyzed by a specific enzyme as in the case of the well-known proteinase zymogens. Since the present preparation of pro-proteinase C contains proteinase A, the effect of the latter enzyme on the activation of pro-proteinase C was tested. As shown in Fig. 3, the activation

FIG. 3. Effect of Yeast Proteinase A on the Activation of Pro-proteinase C.

Pro-proteinase C (360 μ g) was incubated with 400 μ l of sodium acetate buffer (0.1 M, pH 5.0) and specified amounts of purified proteinase A in a total volume of 800 μ l at 25°C. An aliquot was withdrawn at various intervals and immediately assayed for the activity of proteinase C.

Amounts of proteinase A added: none, 50.4, 100.8, 151.2 and 201.6 μ g for the curve 1 to 5, respectively.

rate was increased by addition of increasing amounts of purified proteinase A. The transformation to the active state during incubation with A seemed to be complete after 2 hr under the conditions given in Fig. 3, as the activity obtained under these conditions was proportional to the amount of pro-proteinase C, as shown in Fig. 4. On the other hand, activation was not brought about by addition of purified proteinase C, but a slight activation was observed with the addition of crystalline trypsin. The experiments in Figs. 5 and 6 also show that proteinase A causes

FIG. 4. Relationship of Varying Amounts of Pro-proteinase C and Activity after Full Activation by A.

Varying amounts of pro-proteinase C were mixed with 200 μ l sodium acetate buffer (0.1 M, pH 5) and purified proteinase A (200 μ g) in a total volume of 600 μ l and incubated at 37°C for 2 hr. An aliquot was withdrawn and immediately assayed for the activity of proteinase C.

—○—: Activity before the activation
—●—: Activity after the activation.

activation of pro-proteinase C when the yeast extract is incubated at pH 5. The partially purified pro-proteinase C was adjusted to various pH values and incubated at 25°C for 2 hr. Then, an aliquot of each reaction mixture was withdrawn and activated by incubation at pH 5 with and without the addition of purified A. Thereafter, the activity of proteinase C was compared before and after activation. The results are presented in Fig. 5. In the first incubation at various pH values, the activity of proteinase C in the reaction mixture showed little variation over the wide pH range tested, but A was extensively inactivated as the incubating pH was raised from pH 8 to 9. When activation was carried out without addition of A, the appearance of activity of C was suppressed in parallel with

FIG. 5. Effect of Preincubation at Alkaline pH Levels on the Activation of Pro-proteinase C.

Pro-proteinase C (1.78 mg/ml), adjusted to various pH values, was incubated at 25°C for 2 hr. An aliquot was withdrawn, mixed with an equal volume of sodium acetate buffer (0.1 M, pH 5.0) and the activation was carried out with and without addition of purified proteinase A (71.2 μ g) at 37°C for 4 hr.

---△---, ---▲---: Activity of proteinases A and C before the activating treatment, respectively; —●—, —○—: Activity of proteinase C after the activation with and without addition of proteinase A, respectively.

the inactivation curve for A present in the mixture. However, when activation was performed with the addition of A, it showed a significant occurrence over a wide pH range. As shown in Fig. 6, the proteinase A present in the preparation was almost completely inactivated when pre-incubation was carried out at pH 8.5 for 2 hr, and consequently activation of pro-proteinase C was prevented unless purified A was added.

Thus, it may be expected that activation of pro-proteinase C during incubation at pH 5 is brought about by the action of proteinase A present in the preparation and that this activation can be prevented if the co-existing proteinase A is destroyed by previous incubation at pH 8.5 for 2 hr.

FIG. 6. Effect of Preincubation at pH 8.5 on the Activation of Pro-proteinase C.

Pro-proteinase C ($1.78 \mu\text{g/ml}$) was incubated at pH 8.5 and 25°C . An aliquot withdrawn at various intervals was mixed with an equal volume of sodium acetate buffer (0.1 M, pH 5.0) and the activation was carried out with and without addition of proteinase A ($35.6 \mu\text{g}$) at 37°C for 4 hr. Symbols are the same as in Fig. 5.

4) Stability of Pro-proteinase C

The stability curves of pro-proteinase C, as a function of pH and temperature, are shown in Figs. 7 and 8, respectively, where the activity was measured immediately before and after activation by dioxane under conditions described in the figures. When pro-proteinase C was allowed to stand at various pH values for 60 min at 25°C , spontaneous activation was observed to occur extensively at pH 3 and slightly above pH 9, whereas no detectable changes took place between pH 5 and 8. Since activity of proteinase A in the present preparation was negligible, the activation at pH 3 seemed to be catalyzed by hydrogen ions but not by A. This estimation was confirmed by the fact that the activation was not accelerated by the addition of purified proteinase A. The activation at an alkaline pH

might be caused by hydroxide ions. On the other hand, activation by dioxane gave the same degree of activity over the wide range

FIG. 7. pH-Stability of Pro-proteinase C.

Pro-proteinase C ($120 \mu\text{g}$) which was prepared after the previous inactivation of proteinase A, was incubated with $100 \mu\text{l}$ buffer (0.02 M) in a total volume of $200 \mu\text{l}$ at 37°C for 60 min. Buffers used were the same as in Fig. 1. An aliquot was mixed with an equal volume of sodium phosphate buffer (0.1 M, pH 7.0) and activated with dioxane (final conc., 33% (v/v)) for 15 min.

—○—: Activity before the activation
—●—: Activity after the activation.

FIG. 8. Heat-Stability of Pro-proteinase C.

Pro-proteinase C ($143 \mu\text{g}$) was incubated with $400 \mu\text{l}$ sodium phosphate buffer (0.02 M, pH 6.0) in a total volume of $500 \mu\text{l}$ at various temperatures for 60 min and immediately cooled. Other experimental methods and symbols are the same as in Fig. 7.

of pH 3 to 8. Therefore, taking into consideration spontaneous activation at acid and alkaline pH values, pro-proteinase C appears to be stable on the incubation between pH 5 and 8 at 25°C, at least for 2 hr. As shown in Fig. 8, pro-proteinase C was stable in incubation at pH 6 and 50°C for 60 min. However, slight activation seemed to occur spontaneously between 50~60°C.

DISCUSSION

Partially purified pro-proteinase C was greatly activated by allowing it to stand at various pH values, gradually activated at pH 5~7 and rapidly at pH 3~4. Activation at pH 5~7 was prevented by treatments to inactivate the activity of proteinase A which co-existed in the preparation, whereas its activity was promoted by addition of purified yeast proteinase A. These results show that A catalyzes the transformation of pro-proteinase C to proteinase C at pH 5~7. Although the apparent optimum pH of A is between pH 2 and 3; toward casein and HCl-Hb, respectively,⁷⁾ the enzyme may act at a higher pH according to the kind of the substrate proteins, as with pepsin.⁸⁾ More rapid activation at pH 5~7 of the present preparation than for that of the crude extract may be explained by the possibility that the removal of contaminating proteins facilitates the action of A toward pro-proteinase C. Since rapid activation at pH 3 could be brought about after removal of A and was not promoted by its addition, the results confirmed that this activation is caused by acid but not by A.

These activation processes of pro-proteinase C seem to be basically similar to those of prorennin⁹⁾ and pepsinogen.¹⁰⁾ Their trans-

formation to active enzymes is brought about by hydrogen ions or by limited proteolysis which proceeds autocatalytically. Activation by exposure to acid has been often described for enzymes other than the above zymogens. Latent phenolase^{11,12)} from broad-bean leaves can be activated by brief exposure to pH 3~3.5. Vogels¹³⁾ has described that allantoate amido hydrolase is reversibly activated by acid-pretreatment below pH 4.5. In these two cases, it has been suggested that the transformation of latent forms to active states is not associated with the dissociation and association process of a certain masking peptide.

Pro-proteinase C in the present preparation was also activated rapidly by treatment with urea or dioxane and slightly activated by exposure to alkaline pH or by heating between 50~60°C. From the fact that all these activations occurred in the absence of A, the possibility may be ruled out that these treatments make the inhibitor susceptible to A, and hence that the activation is caused by proteolytic digestion of the inhibitor by A. Thus, all these treatments seem to cause either a certain structural alteration of the precursor or the direct destruction of a bound inhibitor, which leads to the active form.

The molecular dimensions of the active and inactive forms of pro-proteinase C were estimated by gel-filtration on a Sephadex G-100 or G-200 column. Both forms were eluted simultaneously and showed no changes in their molecular dimensions. However, the results may not be necessarily valid, as differences in sugar content of the active and inactive forms³⁾ may not reflect accurately the mole-

7) T. Hata, R. Hayashi and E. Doi, *Agr. Biol. Chem.*, **31**, 150 (1967).

8) M. Schlamowitz and L. U. Peterson, *J. Biol. Chem.*, **234**, 3137 (1959).

9) H. F. Bundy, N. J. Westberg, B. M. Dummel and C. A. Becker, *Biochemistry*, **3**, 923 (1964); B. Foltmann, *Compt. Rend. Trav. Lav. Carlsberg*, **35**, 143 (1966).

10) F. A. Bovey and S. S. Yanari, "The Enzymes," Vol. 4, ed. by P. D. Boyer *et al.*, Second ed., Academic press, 1960, p. 63.

11) R. H. Kenten, *Biochem. J.*, **67**, 300 (1957).

12) D. A. Robb, L. W. Mapson and T. Swain, *Nature*, **201**, 503 (1964).

13) G. D. Vogels, *Biochim. Biophys. Acta*, **113**, 277 (1966).

cular dimension of each form on gel-filtration, as indicated by Andrews.¹⁴⁾ In order to eluci-

date the chemical and physical nature involved in the activation process of pro-proteinase C, it is necessary to perform a further purification of the pro-enzyme.

14) P. Andrews, *Biochem J.*, **96**, 595 (1965).