STUDIES ON YEAST PROTEINASES

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CONTENTS

ABBREVIATIONS

ATEE	N-Acetyl-L-tyrosine ethylester
BAEE	α-N-Benzoyl-L-arginine ethylester
CGT	Carbobenzoxy-L-glutamyl-L-tyrosine
DFP	Diisopropylfluorophosphate
DTNB	5,5'-Dithio-bis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
HCl-Hb	Acid-denatured hemoglobin
PCMB	p-Mercuribenzoate
PITC	Phenylisothiocyanate
TCA	Trichloroacetic acid
CD	Circular dichroism
ORD	Optical rotatory dispersion

CHAPTER I

INTRODUCTION

In general, the yeasts excrete little extracellular proteinases, differently from bacteria.[1] Therefore, for example, when they grow on wort gelatin, the liquefaction of the gelatin is very slow. However, a full complement of proteinases and peptidases is contained in yeast cells and can be liberated after the cells are broken by autolysis.

Yeast peptidases have been studied in some details and characterized by Grassman et al.,[2-4] Johnson[5] and Félix and Labouesse-Mercouroff.[6] On the other hand, the nature of yeast proteinases has not been made clear and inconsistent results have been obtained. Yeast proteinases were described at first by Dernby[7] as "Hefetryptase" and "Hefepepsin", with optimum pH at 7.0 and 4.5, respectively. According to Grassman et al.,[2-4] yeast contained only one proteinase similar to papain, which was most active at pH 5.0 for many protein substrates. Thereafter, Hecht and Civin[8] described a pepsin-like proteinase acting at pH 1.8 in yeast autolysate. More recently, Lenney[9] reported that yeast proteinase consisted of two enzymes, by measuring the activity according to the method of Kunitz.[10] He described that the two proteinases, A and B, were liberated after the autolysis of four different strains of Saccharomyces cerevisiae with chloroform. Proteinase A exhibited an optimal pH at 3.7 for acid-denatured hemoglobin and was extremely labile in urea solution. Proteinase B exhibited an optimal pH at 6.2 for urea-denatured protein. This enzyme was stable in urea solution and inhibited by sulfhydryl reagents.

These conflicting results concerning yeast proteinases are considered

to be mainly based on the experiments without any purification of the enzymes. Under these circumstances, the present author wanted to clarify fully the system of intracellular proteinases in yeast by systematical fractionation with chromatography on ion-exchangers. The present thesis firstly demonstrates the fractionation and characterization of three different proteinases, named as yeast proteinases A, B and C.[11-13]

It is of great interest whether or not the intracellular proteinases play any significant role in living cells. Sylvén et al.[14] have suggested that yeast proteinases and peptidases may be operative in the replenishment of the metabolic amino acid pool necessary for protein synthesis. The present author demonstrated the occurrence of inactive forms of yeast proteinases B and C, named as pro-proteinases B and C.[15-17] The active and inactive forms of yeast proteinases may be involved in cellular control of such a system. To approach for such problem, the present thesis successively describes the isolation procedure of one of the inactive proteinases,[18] pro-proteinase C, together with the investigations on its activation mechanism.

CHAPTER II

FRACTIONATION AND PROPERTIES OF YEAST PROTEINASES

In the preliminary works, at least three different proteinases were found in the autolysate of baker's yeast, and named as proteinases A, B and C.[11] The fractionation method of these enzymes involved alcohol fractionation and ion-exchange chromatography on TEAE-cellulose and DEAE-Sephadex A-50. Thereafter, it became clear that the three proteinases can be effectively separated from each other only by chromatography on DEAE-Sephadex A-50,[13] as shown in Fig. 1.

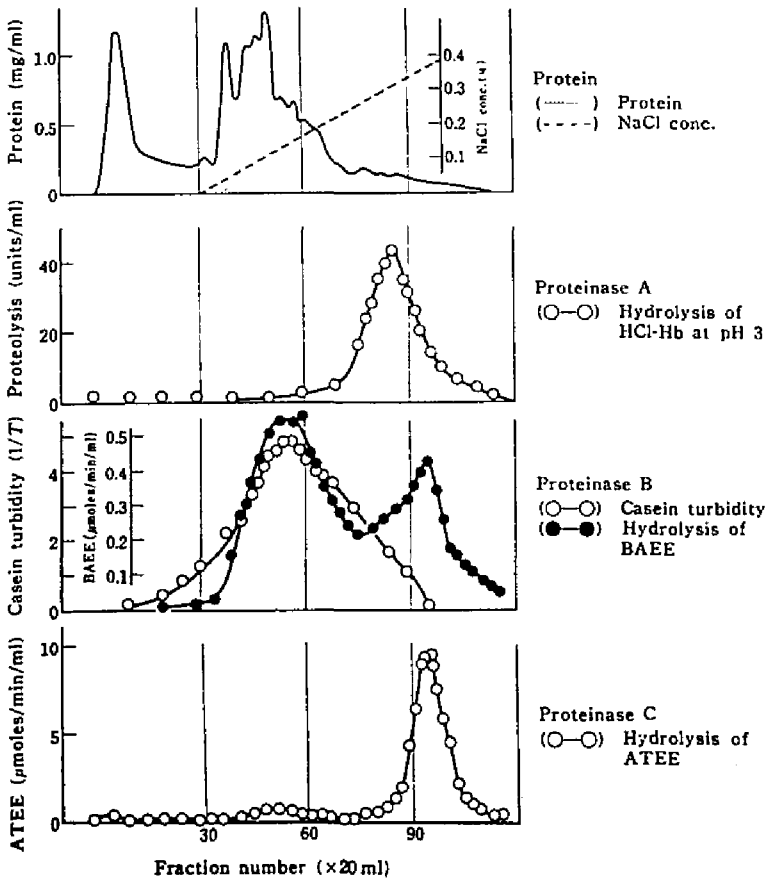

Fig. 1. Demonstration of three proteinases with chromatography on a DEAE-Sephadex A-50 column. The activity of each enzyme was determined by characteristic method described in Section 4.

In this chapter, the systematic method for fractionation of each enzyme is developed on the basis of this finding and characteristics of each enzyme are clarified.

1. Fractionation and Purification of Proteinases[13)]

Autolysis and extraction of yeast proteinases were carried out as described by Lenney[9)] with slight modification. Twenty one kg of compressed baker's yeast (Oriental Yeast Co.) were plasmolyzed with chloroform (9.6 l). After 30 to 60 min at room temperature, water (9.6 l) was added and the pH was adjusted to 7.0. After the autolysis was carried out at about 25°C overnight, cell debris was removed from the suspension and the supernatant solution was obtained. The solution was adjusted to pH 5.0, then allowed to stand under toluene at about 25°C overnight. After centrifugation, the supernatant solution (18 l) was obtained as the crude extract. The fractionation and purification procedures are summarized in Fig. 2.

By the first chromatography on DEAE-Sephadex A-50, proteinases A and C were completely separated from each other, as shown in Fig. 3. Proteinase C was obtained in pure form by the second chromatography. When cold 99 % ethanol was added to the effluent of the second chromatography up to 50 % (v/v) concentration and the solution was maintained with stirring at about -10°C, crystals of proteinase C appeared. Proteinase A still contained some impurities of protein after the second chromatography. So, the third chromatography was conducted.

Purification steps and yields of proteinases A and C are summarized in Table 1.

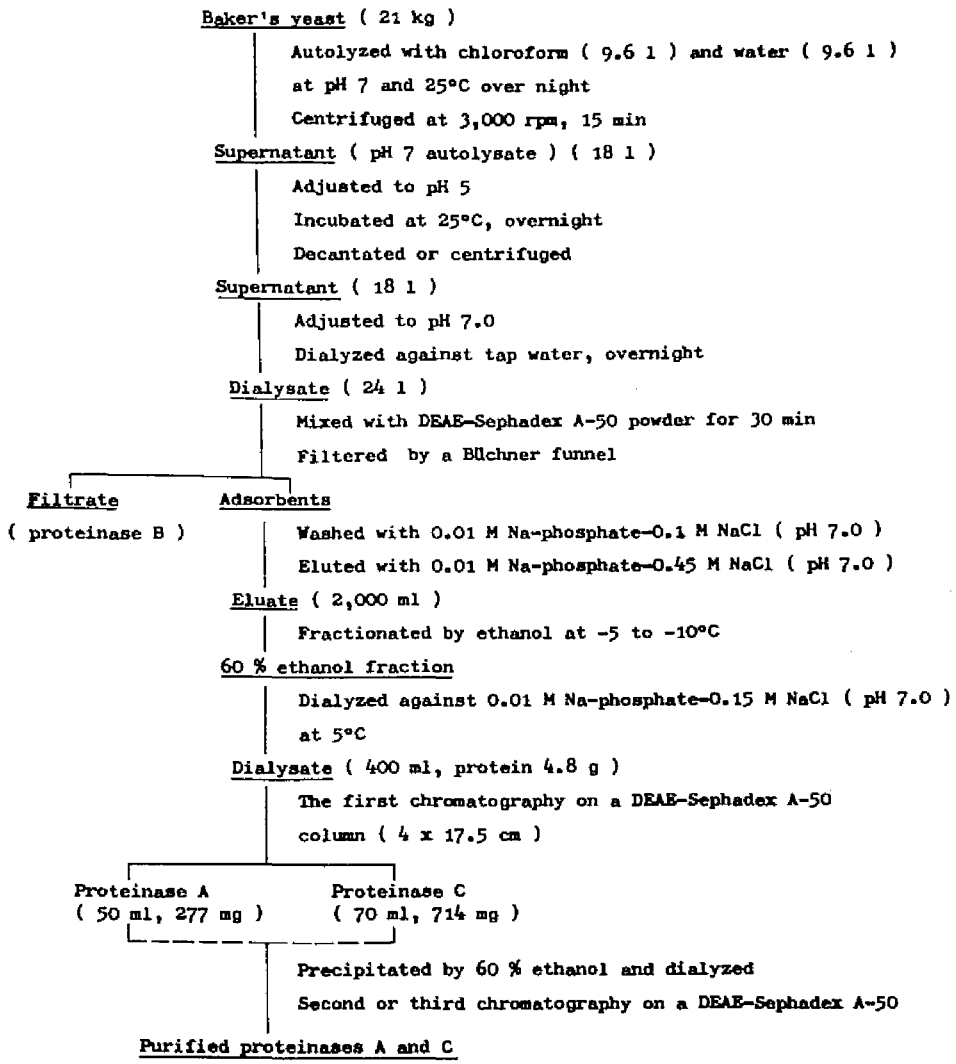

Fig. 2. Fractionation and purification procedures of yeast proteinases

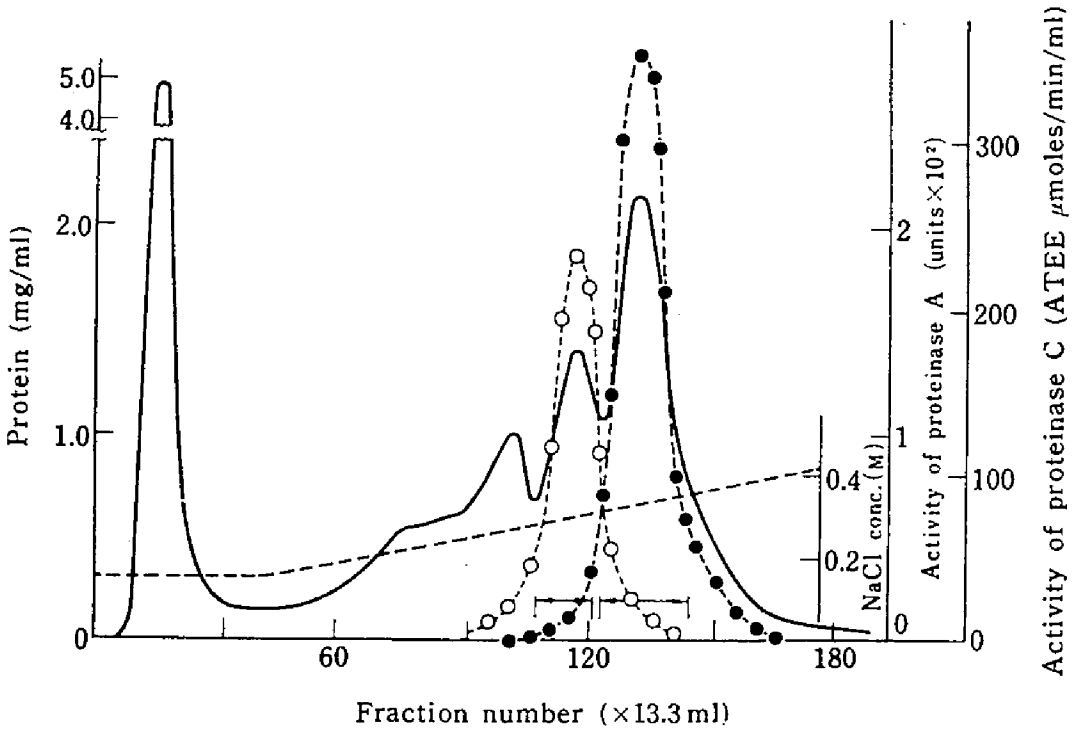

Fig. 3. The first chromatography on a DEAE-Sephadex A-50 column (4 x 17.5 cm).

(———) Protein (○—○) Activity of proteinase A
(- - - - -) NaCl conc. (●—●) Activity of proteinase C

Table 1. Summary of the purification of proteinases A and C

Steps	Proteinase A					Proteinase C				
	Volume	Total Protein*	Total PU**	PU/mg Protein	Yield	Volume	Total Protein*	Total EU***	EU/mg Protein	Yield
	ml	mg	×10³		%	ml	mg	×10³		%
Autolysis at pH 7	18,000	391,000	11.5	0.74	—	18,000	391,000	7.1	0.02	—
Autolysis at pH 5	18,000	250,000	20.2	2.05	100	18,000	250,000	178	0.71	100
Dialysis	24,000	179,000	19.4	2.67	95.3	24,000	179,000	164	0.96	92.1
DEAE-Sephadex treatment	2,000	11,720	17.1	36.1	84.5	2,000	11,720	150	12.7	84.3
Alcohol precipitation	400	4,824	12.8	66.0	64.5	400	4,824	102	20.4	57.3
First chromatography on DEAE-Sephadex	300	336	10.7	762	50.8	450	746	101	135	56.7
Alcohol precipitation	50	277	9.4	833	45.6	70	714	86.8	123	48.3
Second chromatography on DEAE-Sephadex	150	169	8.6	1265	42.7	160	544	68.0	125	37.1
Alcohol precipitation	16	153	8.4	1350	41.3	34	439	55.8	127	31.4

* Protein concentration was determined by the method of Lowry et al.[32] using bovine serum albumin as the standard. ** Proteinase units. *** Esterase units (μmoles ATEE/min.).

2. Enzymatic Properties of Proteinases A and B[11)]

Enzymatic properties of proteinase A were examined with the fraction obtained by the third chromatography. Properties of proteinase B were investigated with the "break-through " fraction of the batchwise chromatography. Because of the unstable nature of proteinase B, the fraction was prepared in the day of the experiment.

a. Optimum pH

Optimum pH of proteinases A was at pH 2 and 3 for hydrolysis of casein and acid-denatured hemoglobin (HCl-Hb), respectively. The enzyme showed stronger activity on HCl-Hb than on casein.

Optimum pH of proteinase B was at about 9 for hydrolysis of casein.

b. pH and temperature-stability

Proteinase A was stable from pH 5 to 6 after 15 hr incubation at 25°C, and about 90 % of the initial activity remained at pH 5 after 30 min incubation at 50°C. The enzyme was stable under freezing at least six months.

Proteinase B was very unstable enzyme. Only 40 % of the initial activity remained at the most after pre-incubation at 37°C for 30 min. Many metals (Ca^{+2}, Mg^{+2}, Ba^{+2}, Mn^{+2}, Fe^{+2} and Co^{+2}), EDTA and cysteine showed no effect on stability of the enzyme.

C. Effect of inhibitors and activators

Proteinase A was not inhibited by DFP, SH-reagents and soybean inhibitor. EDTA and urea (final conc. 6 M) showed little effect on the activity of the enzyme.

Proteinase B was strongly inhibited by DFP and SH-reagents (PCMB

and $HgCl_2$). However, L-cysteine (10^{-3} M) and 2,3-dimercaptoethanol (10^{-3} M) did not activated the inhibited enzyme.

d. Hydrolysis of synthetic substrates

Hydrolyzing activity of proteinases A and B for various synthetic substrates was examined by detecting the products with thin-layer chromatography, ninhydrin method or titration method.

Proteinase A did not hydrolyze any synthetic substrates listed in Table 2.

Proteinase B showed weakly esterolytic activity on ATEE and BAEE at pH 8.0. The Km values for ATEE and BAEE were 30-40 mM and 6.5 mM, respectively. Specific activity for both substrates exhibited almost the same value (0.21-0.23 μmoles/min/ml of the enzyme). These esterolytic activities were completely inhibited by DFP and PCMB.

e. Milk-clotting activity of proteinase B

Milk-clotting activity was the marked characteristic of proteinase B which was not found in the cases of proteinases A and C. Usually, this activity is determined by measuring the time until particles of casein are formed on the wall of the test tube, using skim milk or dry milk as the substrate.[19] However, such activity of proteinase B was so weak and long time was needed to produce particles of casein. Thus, the clotting activity of the enzyme was determined according to the method described in Section 4. This method was based on the fact that the enzyme produces turbidity of casein solution at pH 6.0 in the presence of 10^{-3} M $CaCl_2$. Milk-clotting activity was also inhibited by DFP and PCMB.

3. Enzymatic Properties of Proteinase C[12)]

Enzymatic characteristics of the enzyme were studied with the fraction obtained by the second chromatography.

a. Proteolytic activity

The enzyme could hydrolyze only a few per cent of casein under the experimental conditions standardized by Hagihara et al.[20)] Further increase of hydrolysis was not observed by using more quantity of the enzyme (up to 400 μg per reaction mixture). This suggests that the enzyme has narrow substrate specificity for peptide and only susceptible bonds in casein can be splitted under the experimental conditions. Then, higher concentration of casein as 4 % was used as the substrate solution. In this case, linear dependence on the enzyme concentration and increase in optical density at 280 mμ up to 0.6 was observed. Thus, 4 % casein was used as the standard concentration of substrate for activity assay of the enzyme.

Optimum pH of the casein hydrolysis could not be determined under the experimental conditions, because of isoelectric precipitation of concentrated casein solution. However, optimum pH was estimated at pH 6 or below for casein hydrolysis. In the presence of 8 M urea, the activity was depressed to 1/4 of its initial activity.

The enzyme was relatively stable at pH 6-8, and could be stored in frozen state at least for 6 months at pH 7.0.

b. Peptidase and esterase activities[28)]

Hydrolyzing activities of proteinase C on various synthetic substrates were summarized in Table 2. Proteinase C shows relatively broad specificity for the synthetic substrates. However, acyl-L-amino acid esters

Table 2. Hydrolysis of synthetic substrates by proteinase C

Substrate	Optimum pH	Assay pH	Specific activity (μmoles/min/ml)	Km (mM)	Vmax (calculated)
Ac-L-Tyr-OEt	8.0	8.0	103	2.4	128
Ac-L-Phe-OEt	8.0-8.5	8.0	115	1.0	126
Bz-L-Tyr-OEt	8.3-8.7	6.0	81.5	0.32	84
		8.0	103.5	0.12	105
Bz-L-Arg-OEt	9.0	9.0	5.03	1.6	5.8
L-Tyr-OEt	-	8.0	0.69	-	-
Gly-L-Tyr-OEt	ca. 8.0	8.0	53	-	-
Ac-L-Tyr amide	ca. 8.0	8.0	0.025	-	-
Bz-L-Arg amide	-	5.0	0.004	-	-
		8.0	0.008	-	-
Cbz-L-Glu-L-Tyr	5.0	5.0	17.5	0.14	18.5
		5.5	16.0	0.50	19.5
		6.0	12.0	3.0	23.4
Cbz-Gly-L-Phe	6.0-7.0	5.0	1.66	12	1.9
		8.0	1.68	0.65	3.7
Cbz-Gly-L-Leu	6.5	6.0	2.51	-	-

Ac=acetyl, Bz=Benzoyl, Cbz=carbobenzoxy.

containing aromatic side chain, e.g. N-acetyl-L-tyrosine ethylester (ATEE), were strongly hydrolyzed at pH 8.0. The specific activity for 10 mM ATEE and the calculated Vmax at pH 8.0 and 25°C were 103 and 128 μmoles/min/mg, respectively. From the Vmax and the molecular weight, the apparent first-order rate constant, $k_{2(app.)}$, of this enzyme was calculated to be 160 per second. This value can be compared with that of chymotrypsin, 193 per second,[21] at pH 7.9 and 25°C. Hydrolysis of ATEE was about 1,000 times faster than N-acetyl-L-tyrosine amide.

Peptide substrates were hydrolyzed near neutral or weakly acidic pH. Especially, N-carbobenzoxy-L-glutamyl-L-tyrosine (CGT) was most strongly splitted among the peptide substrates tested. Hydrolyzing rate for CGT was sharply dependent on pH; the hydrolysis occurred rapidly at pH 5 but little

at pH 7. This rapid change from pH 5 to pH 7 was responsible for Km change but not Vmax change of the enzyme. Probably, affinity of the enzyme for the substrate will increase when ω-carboxyl group in CGT is dissociated. It may be considered that dissociated form of glutamyl residue is a susceptible residue for proteinase C. Considering that proteinase C shows limited proteolysis below pH 6, it is suggested that the enzyme splits specifically the glutamyl bond of protein.

Dipeptides (Gly-Gly, L-Leu-L-Leu) and tripeptides (L-Leu-Gly-Gly, L-Ala-Gly-Gly) were not hydrolyzed by proteinase C.

c. Effects of inhibitors and activators[27,40]

The effects of certain inhibitors on the caseinolytic activity of the enzyme are shown in Table 3 (A). Sulfhydryl reagents such as PCMB and $HgCl_2$ were powerful inhibitor for the enzyme. However, alkylating reagents such as iodoacetamide and N-ethylmaleimide caused only weak inhibition. DFP inhibited completely the activity.

Reactivation of the inhibited enzyme were examined as shown in Table 3

Table 3. Effects of inhibitors and activators on proteinase C

(A)

Inhibitor	μ mole	Relative activity %
None	—	100
PCMB	0.4	28
PCMB	0.04	7
PCMB (pH 8)	0.4	2
PCMB (pH 8)	0.04	31
$HgCl_2$	0.4	2
Iodoacetamide	0.4	83
N-Ethylmaleimide	0.4	68
EDTA	0.04	94
DFP	0.4	4
DFP	0.04	0

(B)

Activator was added after addition of inhibitor	μ mole	Relative activity %
PCMB Cysteine	0.04 0.4	35
PCMB Glutathione	0.04 0.4	14
PCMB Cysteine EDTA	0.04 0.4 0.4	37
PCMB Cysteine	0.04 40.0	115
$HgCl_2$ Cysteine	0.04 40.0	62
DFP Cysteine	0.04 40.0	0

(B)。 The inhibitors and the enzyme were incubated for 30 min prior to addition of the activator, and after further incubation for 30 min, the enzyme was assayed。 The enzyme inhibited by PCMB or $HgCl_2$ could be activated by higher concentration of L-cysteine。 However, L-cysteine showed no effect on the enzyme inhibited by DFP。 Sometimes, SH-proteinases are inactivated by impurities contaminated in commercial samples of DFP。[28-30] In such cases, L-cysteine is possible to protect the enzymes from DFP inhibition。 However, pre-incubation of proteinase C with L-cysteine could not hinder the inhibition by DFP。 This fact indicates that proteinase C suffers inhibition by DFP itself but not the contaminated impurities。

The same results as above were also noticed for esterase actvity of proteinase C。

Proteinase having esterolytic activity is roughly divided into " serine enzymes " and " sulfhydryl enzymes "。[23] Since yeast proteinase C is inhibited not only by DFP (specific inhibitor of the formers), but also by PCMB (powerful inhibitor of the latters), this enzyme can not be classified in these categories, in spite of its powerful esterase activity。 So, behaviors of proteinase C toward these inhibitors were further studied。[40]

Stoichiometry of the inhibition of proteinase C by DFP and PCMB is shown in Fig。 4。 It is clear that both esterase and peptidase activities of the enzyme were completely inhibited when one mole of DFP or PCMB per mole of the enzyme was present。 Furthermore, PCMB titration according to Boyer[24] revealed the presence of one mole of sulfhydryl group per mole of both native and denatured (in 8 M urea) enzymes。 As described in Chapter IV, one mole of DFP^{32} was incorporated into one mole of the active enzyme。 Furthermore, the enzyme which was inhibited by PCMB did not react

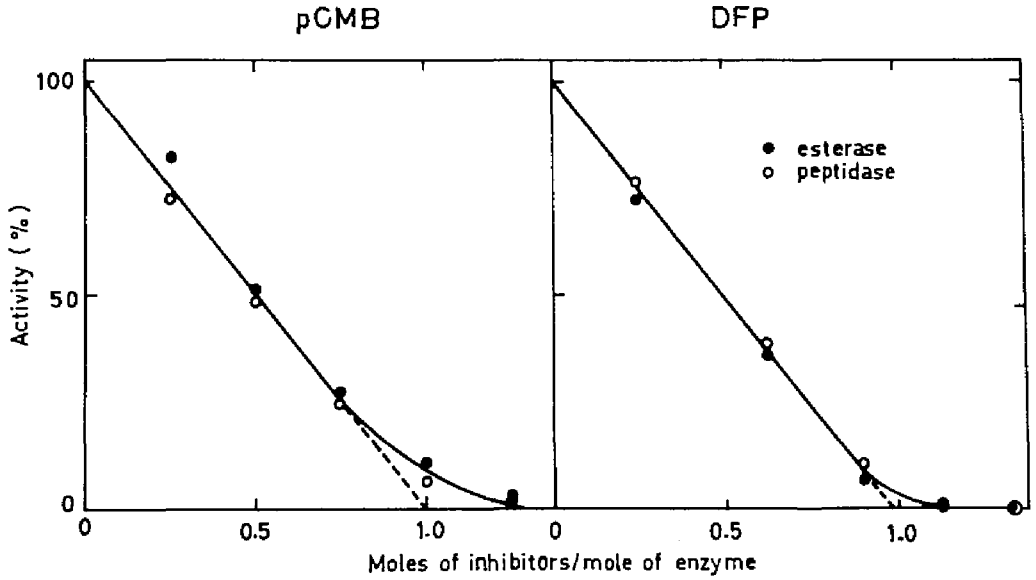

Fig. 4. Inhibition of proteinase C by PCMB and DFP

with DFP. Contrarily, PCMB did not react with the enzyme which was inhibited by DFP, if the inhibited enzyme was denatured with 8 M urea. In this case, diisopropylphosphoryl group was not released with the 8 M urea treatment. Concequently, it was concluded that DFP attacked serine residue but not SH-group in proteinase C.

All the results show that proteinase C possesses one mole of SH-group and serine residue which are essential for the activity of the enzyme. Therefore, yeast proteinase C is a unique enzyme having the property of both "serine enzyme " and " sulfhydryl enzyme ".

4. Characteristic Method for Activity Assay[16)]

Individual activity of proteinases A, B and C could be quantitatively determined, even in mixed states, by applying the characteristics of each enzyme as follows.

Proteinase A was assayed for HCl-Hb[25)] at pH 3.0, according to the

method of Hagihara et al.,[20] in microscale. Prior to the enzymatic assay, the sample was incubated with DFP in order to inactivate proteinases B and C which co-existed in the sample. Activity of proteinase A was expressed as a proteolytic unit with reference to the standard curve obtained from the purified enzyme.

Activity of proteinase B was measured by an increase in turbidity of casein at 610 mμ and expressed by 1/T, where T is the time in minutes requiring 0.5 optical density at 610 mμ. Although increase in absorbancy at 610 mμ was not proportional to the time and the enzyme concentration, 1/T was proportional to the amounts of the enzyme present. This assay method has the advantages of simplicity and high sensitivity, but does not give an accurate value of the activity when strong activities of proteinase A or C co-exist in the assay mixture, as the clotting produced by proteinase B may be digested by proteinase A or C. When necessary, the sample for the assay of proteinase B was passed through a DEAE-cellulose column in order to remove proteinases A and C and the break-through fraction was employed for the assay.

Activity of proteinase C was determined by the rate of hydrolysis of ATEE at pH 8.0 using Radiometer model SBR2/SBU1/TTT1 titration equipment. In certain cases, the activity was determined by the peptidase activity for CGT. The product, tyrosine, was determined by ninhydrin method according to Stein and Moore.[26]

5. Physico-chemical Properties of Proteinases A and C[13]

a. Physical properties

Sedimentation analysis was performed with a Spinco model E ultracentrifuge. Sedimentation patterns of proteinases A and C showed single peaks.

Over different concentration of each enzyme, the sedimentation coefficients of proteinases A and C were 3.3 and 4.3 S, respectively, as shown in Table 4. It can be judged that concentration dependence of $s_{20,w}$ is negligible over the concentration range tested.

Table 4. Sedimentation coefficients of proteinases A and C at different concentrations

Enzymes	Protein conc. (%)	$s_{20,w}$	Temperature (°C)
Proteinase A	0.74	3.32	17.8
	0.35	3.34	19.1
Proteinase C	0.93	4.39	17.8
	0.31	4.36	19.1

Molecular weight of the enzymes was determined by the Archibald's method.[31] The centrifuge was operated at 9,945 rpm. Protein concentration was evaluated from a run in a synthetic boundary cell. Molecular weights of proteinases A and C were calculated as about 60,000 and 61,000, respectively.

In these experiments, partial specific volumes of proteinases A and C were postulated to be 0.75 and 0.70 ml/g, respectively. The latter value was applied taking into consideration of high carbohydrate content (see below).

Free boundary electrophoresis was carried out with a Tiselius' electrophoresis apparatus, Hitachi HT-A, at 0°C. The electrophoretic patterns of the enzymes after 120 min of migration are shown in Fig. 5. The pattern of proteinase C showed a single peak, while that of proteinase A revealed the existence of a small amount of contaminant protein. This minor component was considered not to be proteinase C, because esterolytic activity of this fraction for ATEE was negligible.

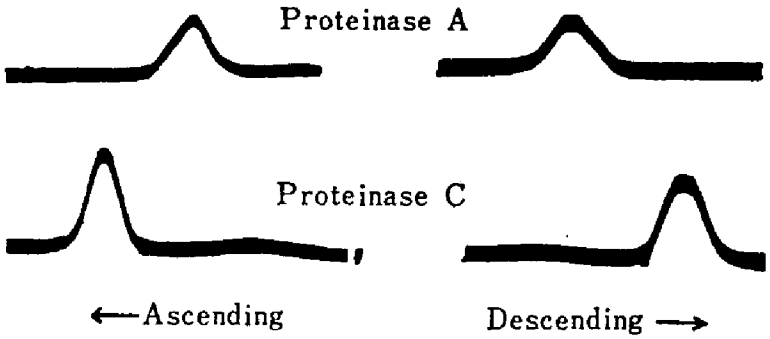

Fig. 5. Electrophoretic patterns of proteinases A and C
Proteinase A: 0.7 % in acetate buffer, pH 5.6, μ=0.1
Proteinase C: 0.9 % in phosphate buffer, pH 6.2, μ=0.1

Isoelectric point of the enzymes was estimated by paper electrophoresis according to the method of Kunkel and Tiselius.[32] The results are shown in Fig. 6. The isoelectric points of proteinases A and C were evaluated at pH 3.8 and 3.4, respectively.

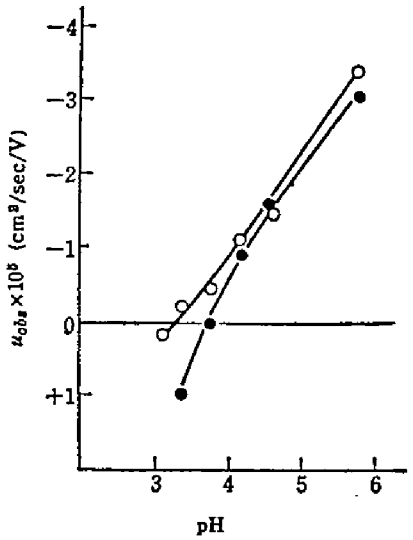

Fig. 6. Mobility-pH curves of proteinases A and C
(—●—) proteinase A
(—○—) proteinase C

b. Chemical properties

Nitrogen contents of proteinases A and C were found to be 14.3 and 12.5 %, respectively. These results suggest that the enzymes were conjugated proteins containing non-nitrogeneous constituents. This was supported by carbohydrate analyses.

The sugars were detected by the combination of Tillman[33,34] and Bial[35] reactions. The absorption spectra of the mixtures of Tillman reactions are shown in Fig. 7. Since Dische's carbazole reaction[36] was negative for both enzymes, it could be concluded that proteinases A and C should contain only hexose, but not pentose and hexuronic acid. The hexose contents of proteinases A and C, as equivalent of mannose, were evaluated as 9.69 % and 20.6 % in dry basis, respectively, from the results of the Tillman reaction. However, sugar content of proteinase C varied between 16 and 21 % with different batches of purified proteinase C. Hexosamine was also detected in acid-hydrolyzate of proteinase C by amino acid analyzer. Amino acid composition of proteinase C is described in Chapter IV, 4.

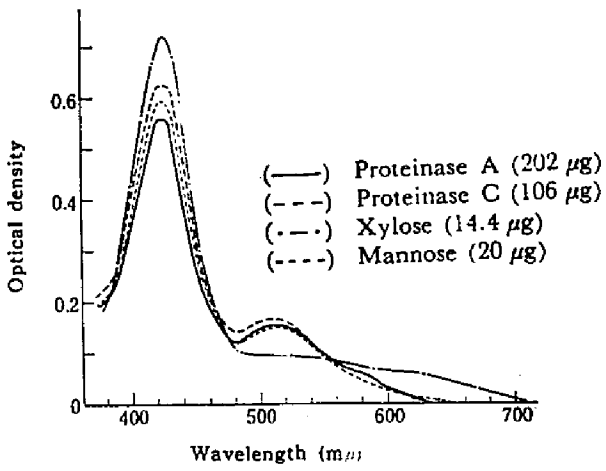

Fig. 7. Absorption spectra of the reaction mixture of Tillman reaction

6. Discussion

The proteinase system in yeast was investigated by a systematic fractionation of yeast autolysate. At least three different proteinases, A, B and C were fractionated and fully characterized. In regard to optimum pH, these enzyme seem to correspond to acid-, neutral- and alkaline proteinases of mold,[37] or to cathepsins A, B and C in animal tissues.[38] However, detailed characteristics are different from those of mold and catheptic enzymes. Only proteinase A shows similarity with acid-proteinase in mold or cathepsin A.

Proteinases A and B seem to be essentially the same enzymes described by Lenney.[9] Since Lenney's experiments were carried out about yeast autolysate itself without any fractionation of each enzyme, properties of Lenney's enzymes will be partly contributed by those of proteinase C. Proteinase C resembles peptidase β purified by Félix and Brouillet[39] from brewer's yeast. Both enzymes split CGT and are inactivated by PCMB or DFP. However, proteinase C belongs to an endopeptidase rather than an exopeptidase, because the enzyme exhibits both caseinolytic and esterolytic activities.

Proteinase C was completely inhibited by the presence of one mole of DFP or PCMB per mole of the enzyme. It is considered that sulfhydryl group and serine residue are involved in catalytic center. In this sence, proteinase C is a unique enzyme having properties of both " SH-enzymes " and " serine enzymes ".[23] The catalytic role of serine residue and SH-group in proteinase C is not known. Hydrolyzing activity of the enzyme for p-nitrophenyl acetate (non-specific substrate for proteinase C) was only partly inhibited by PCMB, while the activity was completely inactivated

in the presence of DFP.[40] Therefore, it is suggested that proteinase C is a serine enzyme in nature and sulfhydryl group may associate with an affinity site rather than a catalytic site of the enzyme.

CHAPTER III

INACTIVE FORMS OF PROTEINASES B AND C

In the course of the purification studies,[11] it was found that activity of proteinase C considerably increased when autolysis of yeast was carried out successively at pH 7 and 5. This increase in the activity may reffered to activation of an inactive precursor. A similar activation of yeast proteinases was also observed by Lenney and Dalbec.[41]

This chapter describes evidence for the presence of inactive forms of proteinases B and C in baker's yeast. The various activation processes are also presented.

1. Activation of Proteinases in Yeast Extracts

a. Activation of proteinases in autolysate

Autolysis of yeast was carried out with chloroform and water, separately adjusted at various pH values, as described in Chapter II, 1. After removing cell debris by centrifugation, each supernatant solution was divided into four portions and re-incubated at various pH values. The resulting clear solutions were obtained as the second autolysate. The first and the second autolysis may be reffered to the extraction and the activation processes of yeast proteinases, respectively. The activity of three proteinases and protein concentration were determined by comparing the first and the second autolysates.

The results are presented in Table 5. A considerable amount of protein disappeared during successive autolysis, probably by digestion with proteinases present in the autolysate. In the first autolysis, the activity of the proteinases emerged only weakly or negligibly at each pH tested, and detectable changes of the activity were not observed when the

Table 5. Extraction and activation of yeast proteinases by autolysis

pH of autolysis 1st	pH of autolysis 2nd	Protein conc. (mg/ml) 1st	Protein conc. (mg/ml) 2nd	Activity of proteinases A (units/ml) 1st	A (units/ml) 2nd	B ($1/T \times 10^2$/ml) 1st	B ($1/T \times 10^2$/ml) 2nd	C (μmoles/min/ml) 1st	C (μmoles/min/ml) 2nd
5	5	1.40	0.6	1.3	2.7	0	0	0.04	0.04
	6		0.9		—		0		0.02
	7		1.0		2.7		0		0.05
	8		1.1		2.7		0		0
6	5	4.23	1.8	5.3	5.3	0.5	2.6	0.05	1.0
	6		2.9		8.0		1.3		0.3
	7		2.9		5.3		1.0		0.3
	8		3.7		5.3		0.5		0
7	5	7.05	2.1	25.3	26.0	1.3	12.5	0.08	5.5
	6		4.4		36.0		2.0		2.1
	7		5.9		21.3		1.0		0.2
	8		5.8		28.0		1.3		0.2
8	5	6.14	1.6	16.7	10.7	0.5	6.0	0.45	1.3
	6		—		18.7		2.6		0.7
	7		4.0		21.3		1.6		0.6
	8		4.4		20.0		1.1		0.5

extract was left at pH 7 or 8. However, the activities of proteinases B and C increased extensively when the second autolysis was carried out pH 5 following the first autolysis at pH 7. On the other hand, the activity of proteinase A showed only small variations during the two step autolysis. From these results, it is obvious that all of proteinases can be maximumly extracted by the first autolysis at pH 7, and activation of proteinases B and C occur extensively in the successive incubation at pH 5. The following experiments were performed using the first autolysate at pH 7, which is the autolysate reffered to in this thesis. The activation profiles of proteinases B and C showed bell type curves as a function of pH; the highest activities of B and C were obtained at pH 4.5 and at pH 5.0, respectively.

Time courses of the activation of proteinases B and C during incubation

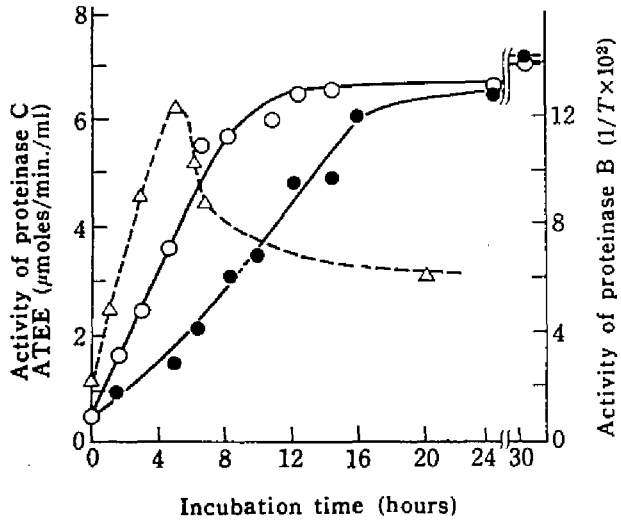

Fig. 8. Time courses of the activation of proteinases B and C

(△---△) proteinase B (25°C), (●—●) proteinase C (25°C), (○—○) proteinase C (37°C).

at pH 5 were shown in Fig. 8. The curves exhibited parabolas but were not sigmoidals. The activation rate of both enzymes was dependent on temperature, being more rapid at 37°C than at 25°C. These results suggest that the activation of proteinases B and C does not autocatalytically but is catalyzed by a specific enzyme which co-exists in the autolysate.

Lenney[9] has already described that proteinases of yeast are rapidly activated by concentrated urea at pH 5. In order to find out whether the present enzymes could also be activated by urea or other organic reagents, the autolysate was incubated with various chemicals, mainly denaturing agents for protein, for appropriate length of time under various conditions. The resulting activity was immediately tested using an aliquot of the pre-incubation mixture. The results are given in Table 6. It will be noted that the activation of proteinase C occurred rapidly with urea and various

Table 6. Activation of proteinases B and C by various reagents

Reagents	Conc.	Incubation conditions	Relative activity
Proteinase B			
None	—	pH 5, 60 min	1
Dioxane	30%	pH 7, 5 min	3.5
Urea	3M	pH 5, 30 min	3.3
Proteinase C			
None	—	pH 5, 60 min	1
Urea	3M	〃	10～14
Ethanol	20%	pH 7, 60 min	3.0
〃	30%	〃	6.0
〃	40%	〃	1
Isopropanol	30%	〃	12.3
n-Butanol (lower layer)	〃	〃	7.5
t-Butanol	〃	〃	12.1
Acetone	〃	〃	12.6
Dioxane	〃	〃	14.7
Sodium lauryl sulfate	0.1%	〃	2.4
Sodium laurylbenzene sulfonate	〃	〃	1.3
Sodium deoxycholate	〃	〃	1.4
Benzylalkonium chloride	〃	〃	0.7
Sodium monooleate	〃	〃	1.0

organic solvents, especially with dioxane. To obtain the highest activation of proteinase C, the required concentration of urea and dioxane were about 3 M and about 30 %, respectively. Activation by urea and dioxane was also observed with proteinase B, although the extent of the activation was less than that for proteinase C. On the other hand, proteinase A was little activated by these reagents under the conditions where proteinases B and C were activated.

b. Activation of proteinases in various extracts

In order to find out whether the activation of proteinases B and C is a specific phenomenon associated with autolysis, the effects of organic reagents and incubation at pH 5 on the activation of yeast proteinases were also tested with extracts from acetone powder and mechanically destructed cells of yeast. The results are shown in Tables 7 and 8. Although the activities of proteinases B and C were very weak or negligible in both extracts, their activities were greatly increased when the extracts were incu-

Table 7. Activation of proteinases B and C in the acetone powder extract

Preincubation conditions	Protein conc. (mg/ml)	Activity		
		A (units /ml)	B ($1/T \times 10^2$ /ml)	C (μmoles /min/ml)
No treatment	13.0	6	Trace	0.16
pH 5, 20 hr	2.4	6	0.5	1.1
3M urea, pH 5, 60 min	—	8	—	0.8
30% dioxane, pH 7, 30 min	—	—	—	1.4

Table 8. Activation of proteinases B and C in the extract from mechanically destructed cells

Preincubation conditions	Protein conc. (mg/ml)	Activity		
		A (units /ml)	B ($1/T \times 10^2$ /ml)	C (μmoles /min/ml)
No treatment	7.8	12.5	Trace	0.13
pH 5, 20 hr	2.0	15.0	0.6	4.34
3 M urea, pH 5, 60 min	—	16.0	—	1.33
30% dioxane, pH 7, 30 min	—	10.0	—	3.13

bated at pH 5 or with urea or dioxane. On the other hand, activity of proteinase A showed no observable variations with these treatments.

From these results, it is concluded that most of proteinases B and C exist in vivo as inactive precursors, which are named as pro-proteinases B and C, respectively, while proteinase A exists in an active form in living cells.

2. Separability of Active and Inactive Proteinases

In order to test whether the active and inactive forms of proteinases B and C be separable or not, the following experiments were performed. The autolysate was concentrated to approximately half its volume with carbowax, and was applied on a DEAE-cellulose column (1.0 x 11 cm). The elution was performed by stepwise increases in concentration of NaCl. As the results, three fractions; I, II and III were obtained. After the activating treatment by incubation at pH 5, activities of the three proteinases in each fraction were compared with those before activation. The results are shown in Fig. 9. Pro-proteinases B and C separately eluted in fractions I and II, respectively. Proteinase C and its precursor were also emerged in different fractions II and III, respectively. However, proteinase A and pro-proteinase C were eluted in the same fraction. Therefore, it can be concluded that proteinase C, pro-proteinases B and C are different in nature.

3. Properties of Partially Purified Pro-proteinase C[17)]

The activation of pro-proteinase C (pro-PC) was further studied by using " Fraction II " prepared in the former section. Pro-PC in this preparation was also activated by incubation with 33 % dioxane or 3 M urea.

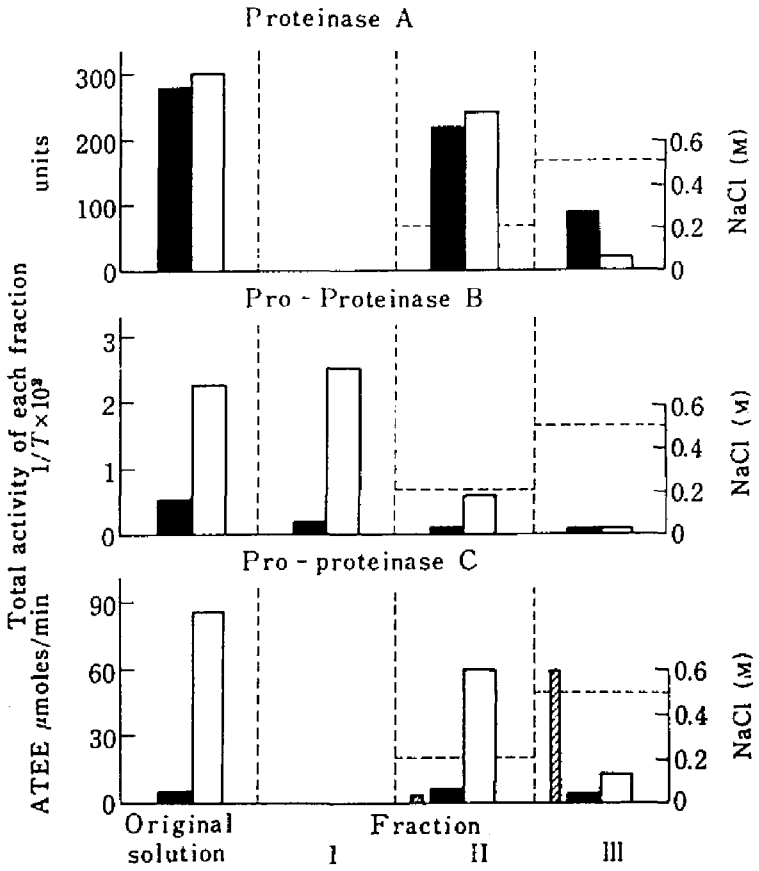

Fig. 9. Separation of active and inactive forms of proteinases by chromatography on a DEAE-cellulose column (1 x 7 cm).

■: Activity before activation
□: Activity after activation
■: Activity of activated proteinase C

a. Activation by acid and alkaline treatments

On incubation at pH 3 or 4, the activity rapidly appeared in initial atage of incubation and was followed by decrease, probably due to irreversible denaturation of the activated enzyme. As described below, the activation which occurred around pH 3 was a different process from that around pH 5, because the activation was not accelerated by addition of purified proteinase A. Probably, the former activation was catalyzed by hydrogen ions and the latter by proteinase A co-existing in the same pre-

paration.

The activation slightly occurred on incubation near pH 9. The activation may be caused by hydroxide ions.

Slight activation occurred spontaneously by heat treatment between 50 and 60°C.

b. Activation by proteinase A

It has been suggested that the activation of pro-PC during incubation at pH 5 is catalyzed by a specific enzyme (Section 1, a). The effect of various proteinases on the activation of pro-PC was tested. Trypsin, chymotrypsin and yeast proteinase C caused little activation of pro-PC. However, the activation rate was increased by addition of increasing amounts of purified proteinase A. The activity obtained was proportional to the amount of pro-PC.

The experiment in Fig. 10 shows that proteinase A causes the activation of pro-PC when yeast extract is incubated at pH 5. Experiment in the figure was performed as follows. The partially purified pro-PC (co-existing proteinase A) 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 addition of purified proteinase A. Thereafter, the activity of proteinase C was compared before and after activation. 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 proteinase A was extensively inactivated as the incubating pH was raised from pH 8 to 9. When activation was carried out without addition of proteinase A, the appearance of activity of proteinase C was suppressed in parallel with the inactivation curve for proteinase A

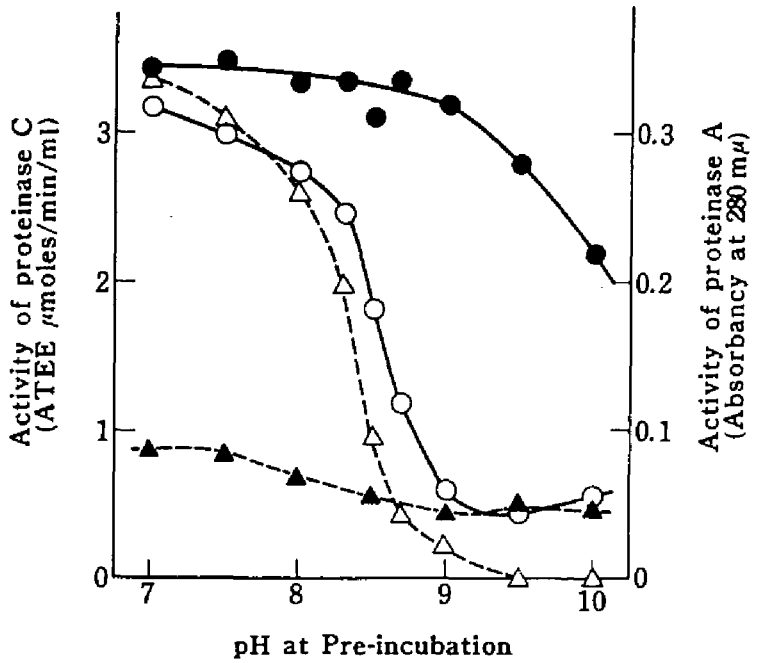

Fig. 10. Effect of preincubation at alkaline pH levels on the activation of pro-proteinase C

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

present in the mixture. However, when activation was performed with the addition of proteinase A, it showed a significant occurrence over a wide pH range. Furthermore, it was shown that 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-PC was prevented unless purified proteinase A was added.

Thus, it may be expected that activation of pro-PC during around 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.

4. Discussion

The activity of proteinases B and C was greatly increased by standing at pH 5. With respect to the increase of activity, there appear to be three possible explanations as follows; (1) destruction or denaturation of some inhibitor present in the homogenate,[42] (2) solubilization of the enzyme from a special cell-granule such as a lysosome[43] and (3) transformation of an inactive precursor to active enzyme. The presence of free inhibitors for proteinase B was described by Lenney and Dalbec.[44] However, the present author could not find free inhibitors for proteinases B and C in yeast extracts. Although no experiments have been made to ascertain the existence of a special particle in which the present proteinases are enclosed by membrane, it is readily conceivable that the second case is not likely, because all the present experiments were carried out under hypotonic conditions where a particle, if any, could be decomposed. Therefore, the last case may be the most reliable.

Such increases in activity were not a specific phenomenon associated with autolysis of yeast cells but were also observed in extracts prepared by different methods. These results may show that most of proteinases B and C exist as inactive forms in intact yeast cells. On the other hand, the activity of proteinase A in the extracts showed little variation before and after various treatments. Thus, it is suggested that proteinase A is present in a fully active form in vivo. Lenney and Dalbec,[41] however, described that proteinase A is also found in the zymogen form. This inconsistency in results may be due to the difference in method of the activity assay.

Active and inactive forms of proteinase C were separately eluted in

column chromatography on DEAE-cellulose. Pro-proteinases B and C were also separated in the same manner. These facts show that the three proteins are different in nature.

Pro-PC can be activatable by treatment with several denaturants such as urea, dioxane and acid. It is also transformed to the active enzyme by action of proteinase A. Similar activation phenomena are observed in the activation of latent phenolase[45-50] and inactive forms of alkaline protease from Aspergillus sojae.[51]

CHAPTER IV

PURIFICATION OF PRO-PROTEINASE C AND ITS ACTIVATION MECHANISM

It became apparent that activation of pro-proteinase C (pro-PC) could be caused by proteinase A and, therfore, spontaneous activation of the crude pro-PC could be effectively prevented by an inactivating treatment of the co-existing proteinase A. Furthermore, a simple estimation of the potential activity of the pro-PC became possible with dioxane treatment.[17] These findings made it possible to isolate and purify the compound.

In the present chapter, a procedure is developed for isolating pro-PC of baker's yeast in homogeneous form. Moreover, its physicochemical properties are investigated and its activation process is studied in chemical and physical respects.

1. Isolation and Purification

In preparing cell-free extracts of yeast, autolysis was carried out with chloroform and water, as described in Chapter III, 1. The supernatant solution obtained after autolysis at pH 7 was subjected to the purification. The procedure of isolation is summarized in Fig. 11.

In order to obtain a good yield of pro-enzyme, it was necessary to repeat the inactivating treatment of proteinase A and, at the same time, to perform the purification procedures as rapidly as possible at low temperature. For inactivation of proteinase A, it was effective to incubate the extract at pH 8.5 and 25°C (see Chapter III, 3). This treatment was repeated in the course of the purification. Finally, pro-PC was purified by chromatography on a DEAE-Sephadex A-50 column. A typical pattern of the second

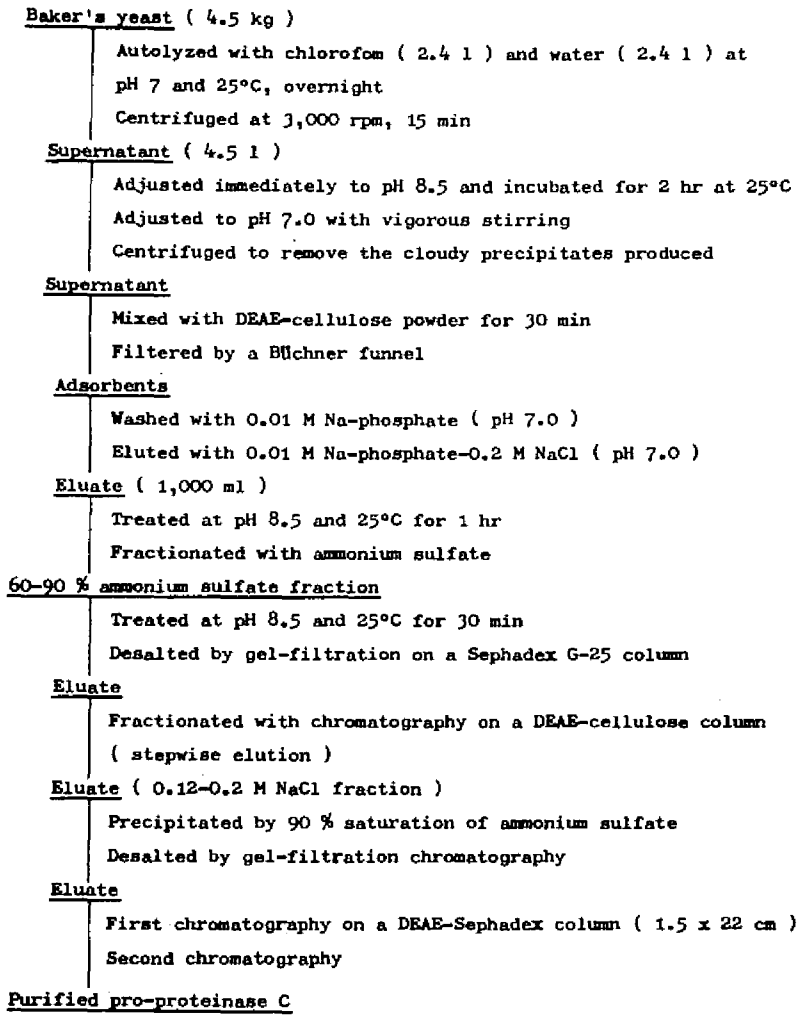

Fig. 11. Isolation and purification procedure of pro-PC

chromatography are shown in Fig. 12. Specific activity before and after the activation treatment and the recoveries at various stages in the purification procedure are summarized in Table 9.

During the course of purification, potentail activity of pro-PC was determined as proteinase C activity immediately after incubating the pro-PC with one third volume of dioxane for 15 min at 20°C. Although 2-4 % of dioxane was present in the assay mixture under these conditions, the activi-

ties of proteinase C and the activated enzyme were not affected by such diluted dioxane.

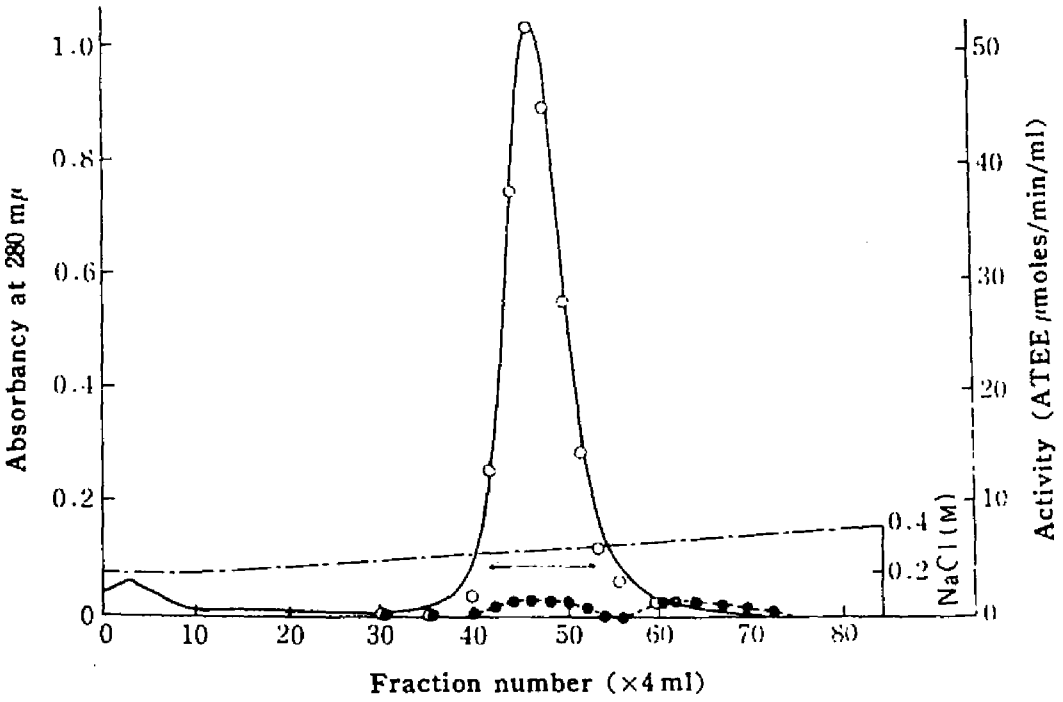

Fig 12. The second chromatography on a DEAE-Sephadex A-50 column (1 x 12 cm).
(———) Protein, (—·—·—) NaCl conc., (—●—) Activity before activation, (—○—) Activity after activation.

Table 9. Summary of the purification of pro-proteinase C

	Purification step	Volume	Total protein*	Potential activity**		Yield
				Total activity	Specific activity	
		ml	mg	μ moles/min	μ moles/min/mg	%
1.	pH 7-autolysate	4150	77,000	10,200	0.15	100
2.	pH 8.5-treatment, dialysate	5700	53,000	9965	0.19	97.7
3.	DEAE-cellulose, batchwise	1000	1040	7850	7.55	77.0
4.	Ammonium sulfate, 60~90%	140	756	7735	10.23	75.8
5.	DEAE-cellulose, 0.12~0.2 M NaCl	295	460	7184	15.62	70.4
6.	DEAE-Sephadex, 1st chromatography	150	113	5630	49.82	55.2
7.	DEAE-Sephadex, 2nd chromatography	47	49	2775	56.63	27.2

* Determined by the method of Lowry *et al.*
** Difference activity for ATEE before and after activation by dioxane.

2. Physico-chemical Properites[18]

a. Stability and potential activity

Pro-PC can be stored frozen at -20°C for at least 6 months with neither activation nor loss of the capacity of the pro-enzyme. However, gradual activation seemed to occur when the solution was frozen and thawed repeatedly. In soluble state, pro-PC was relatively stable when maintained at a neutral pH. Activation occurred extensively at an acidic pH and slightly at an alkaline pH. These activations seemed to be catalyzed by acid and alkali, respectively. Gradual activation occurred at a slightly acidic pH, probably due to a partial contribution of acid-catalyzed activation.

The finally purified preparation possessed a weak activity for ATEE.[52] Time course of this observed activity for ATEE showed a concave curve. The specific acitivity, which was evaluated from the tangent value in the initial reaction process and the used protein concentration, was nearly zero at infinite concentration of the pro-enzyme. This indicate that the pro-enzyme does not possess an inherent activity toward ATEE but a partial activation does occur during the activity assay. This activation seemed not to be due to autocatalytic process, because the pro-enzyme after standing overnight at pH 8 showed the same development of the activity as that of the immediate measurement.

Specific activity of the pro-enzyme after activation by dioxane was about 45 and 9 μmoles/min/mg of protein for ATEE and CGT hydrolysis, respectively. These values are only approximately 60 % of that expected from the specific activity of fully activated pro-PC. However, the activity ratio of ATEE hydrolysis to CGT hydrolysis showed a closer value to that of proteinase C (see Chapter II, 3).

b. Physical properties

Prior to these experiments, the protein solutions were equilibrated with solvent by dialysis overnight at 5°C, and clarified by filtration through Millipore filters (pore size, 0.45 μ).

(i) Sedimentation analysis.

Homogeneity and sedimentation coefficients were investigated by a run at 60,000 rpm in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The plots of $s_{20,w}$ are presented as a function of protein concentrations. From extraporation to infinite dilution, the sedimentation coefficient at zero concentration, $s^{0}_{20,w}$, was evaluated to be 5.27 S.

(ii) Electrophoretic analysis.

For determination of purity, free-boundary and disc-electrophoresis were carried out. The former experiments were performed with 0.905 % solution of the pro-enzyme in phosphate buffer (pH 7.0, μ=0.1) at 0°C. The

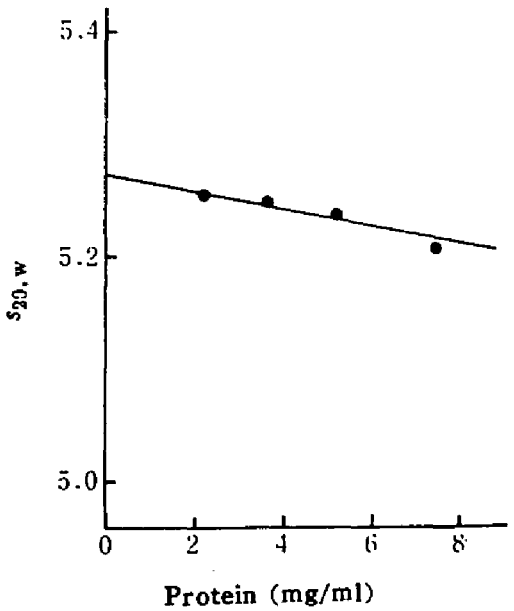

Fig. 13. Concentration dependence of the sedimentation coefficient of pro-proteinase C

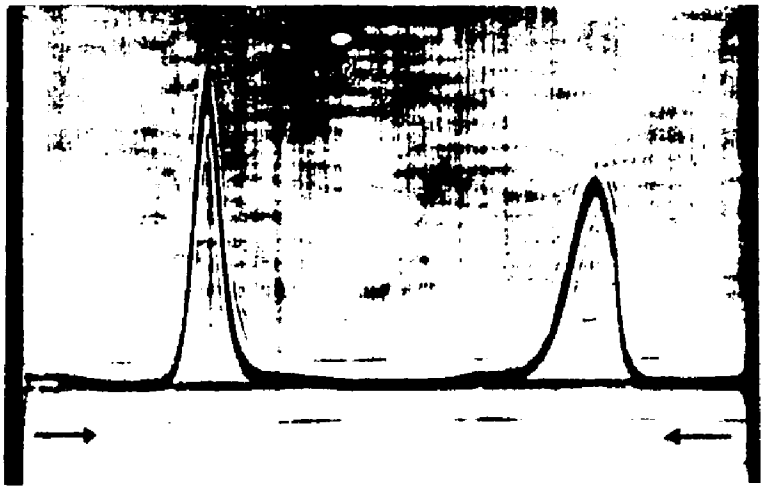

Fig. 14. Electrophoretic patterns of pro-proteinase C

run was made at 0.3°C and 3 mA. Fig. 14 shows electrophoretic patterns obtained after 120 min of migration. The patterns indicate apparent homogeneity of the preparation under the conditions tested. Disc-electrophoresis was also revealed to be homogeneous with the preparation.

(iii) Partial specific volume

The apparent partial specific volume, $\bar{v}$, was calculated for three different concentrations of the pro-enzyme in 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl at 20°C. The results are shown in Table 10. The average value, 0.694, was taken as the partial specific volume.

Table 10. Partial specific volume of pro-proteinase C

Protein concentration (mg/ml)	Density (g/ml)	$\bar{v}$ (ml/g)
0	1.0051	0
4.19	1.0064	0.690
5.47	1.0068	0.689
9.13	1.0078	0.704
	Mean	0.694

(iv) Diffusion study

The experiments were performed with 0.36 and 0.72 % solution of the pro-enzyme in the same buffer as that used in the free-boundary electrophoresis. The diffusion run was made with a Neurath's type cell at 0.3°C for 23 and 30 hr with each respective experiment. Photographs were taken at 2-3 hr intervals after the boundary formed. The diffusion constant was evaluated by three different methods according to Neurath.[53] As listed in Table 11, the values obtained agreed well with each other. This seems to indicate monodispersity of the material. Variations in the diffusion constant were non-observable with respect to protein concentrations. Therefore, at zero concentration, $D^{0}_{20,w}$ of the pro-enzyme was evaluated to be 5.28×10^{-7} $cm^2 sec^{-1}$ by averaging these values.

Table 11. Diffusion constant of pro-proteinase C

Protein concentration (mg/ml)	$D_{20,w} \times 10^{-7}$ cm² sec⁻¹			
	D_1	D_2	D_3	D_{mean}
3.6	5.40	5.27	5.27	5.31
7.2	5.25	5.25	5.21	5.24
			Mean	5.28

D_1: maximum ordinate method
D_2: maximum ordinate-area method
D_3: statistical method

(v) Molecular weight

Molecular weight of the pro-enzyme was calculated from the sedimentation coefficient (s), diffusion constant (D) and partial specific volume ($\bar{v}$) according to the following well-known equation, $M = RTs/D(1 - \bar{v}\rho)$. The molecular weight evaluated was 79,200.

The molecular weight was also deduced by the Archibald method.[31] The centrifuge was operated at 10,490 rpm at 19°C and photographs were taken at

intervals of 10 min. Protein concentration was separately determined from a run in a synthetic boundary cell. The molecular weight was calculated to be 81,000 ± 5,000, which was in fairly good agreement with the value obtained from sedimentation and diffusion measurements.

(vi) Ultraviolet absorption spectrum

The spectrum was measured in 0.01 M sodium phosphate buffer, pH 7.0, with a 0.049 % solution of the pro-PC. A typical absorption spectrum as the protein was obtained and the absorption maximum was at 278 mμ. Absorbancy of the 1 % solution at 280 mμ was 14.1, in dry basis.

c. Chemical properties

Nitrogen content of the pro-enzyme was found to be 13.1 g/100 g of dry weight. This low value suggests that the pro-enzyme contains non-nitrogeneous constituents, probably carbohydrate, as in the case of proteinase C. This was supported by carbohydrate analyses. True sugars were detected by a combination of the Tillman[33,34] and Bial reaction.[35] It was concluded that the pro-enzyme contains only hexose, but neither pentose nor hexuronic acid. The hexose content was calculated as the equivalent of mannose from the results of the Tillman reaction. Eight to twelve per cent carbohydrate was found to be present in different batches of the purified pro-enzyme. Yeast invertase in different preparation has been reported with various amounts of carbohydrate associated with it. Neumann and Lampen[54] suggested that under proper conditions carbohydrate bound to the invertase may be split from the protein moiety by an autolytic enzyme present in yeast cells. Since extraction of pro-PC was made by autolysis of yeast cells, the variation in carbohydrate contents may be attributed to the initial conditions in extracting the pro-enzyme from the cells.

Some physico-chemical properties of pro-PC were summarized and compared with those of proteinase C in Table 12. It is noticeable that molecular weight of pro-PC is larger by about 20,000 than that of proteinase C.

Table 12. Summaries of physico-chemical properties of pro-proteinase C and proteinase C

Properties	Pro-proteinase C	Proteinase C
Sedimentation constant, $s^0_{20,w}$ (S)	5.27	4.3
Diffusion constant, $D^0_{20,w}$ (10^{-7} $cm^2 sec^{-1}$)	5.28	
Partial specific volume, $\bar{V}$ (ml/mg)	0.694	(0.70)
Frictional ratio, f/f_0	1.43	
Isoelectric point	> 3.4	3.4
Absorbancy, $E^{1\%}_{1\,cm}$, at 280 mμ	14.1	16.6
Molecular weight		
Archibald	81,000±5,000	ca. 61,000
Sedimentation & diffusion	79,200	
Nitrogen content (%)	13.1	12.5
Carbohydrate content (%)	8 - 12	16 - 21

3. Activation by Denaturing Agents[56)]

Pro-proteinase C can be converted to an active form by treatment with several denaturing agents or by acid treatment. In order to elucidate the mechanism of the activation, effect of these reagents on the activation of pro-PC was tested.

a. Effect of various denaturants

Two classes of organic substances were added to aqueous solution of

pro-PC: 2-chloroethanol (2-CE), dioxane and dimethylformamide (DFA), all of which are known as helix-forming reagents; guanidine hydrochloride (G·HCl), urea and formamide (FA), all of which have strong tendencies for hydrogen bonding. Pro-PC was preincubated in the presence of various amounts of the reagents for a given time at 25°C and resulting esterolytic activity was immediately determined with using an aliquot of the preincubation mixture. In the mixture of the activity assay, the reagents used for the activation were diluted to 12.5-50 folds, so that little affected on the esterolytic activity. The activating effects of these reagents are illustrated in Fig. 15 (A), (B). These reagents caused qualitatively the same

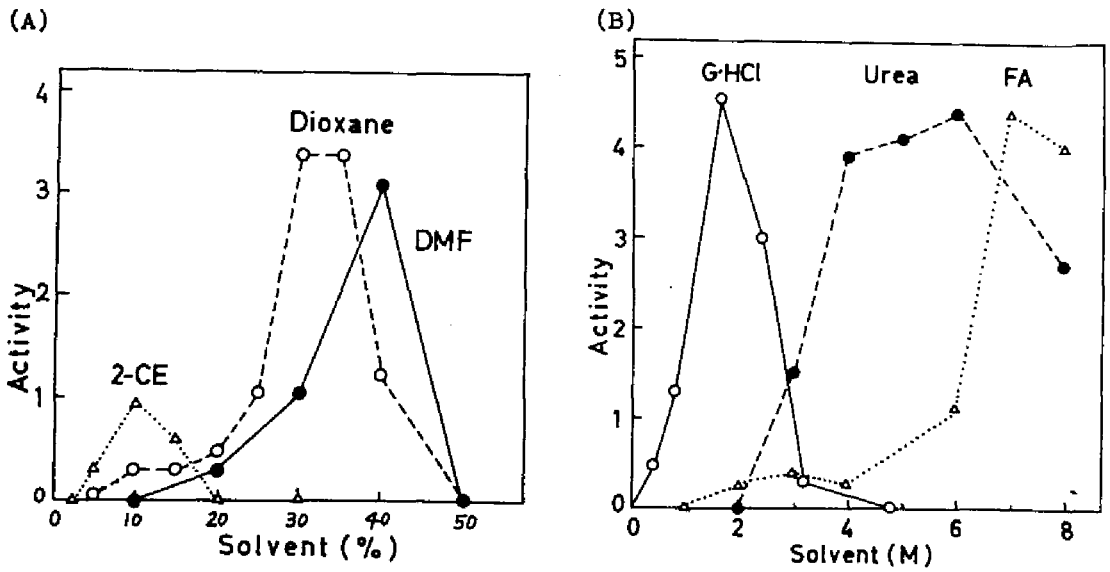

Fig. 15. Effect of some protein denaturants on the activation of pro-proteinase C

effect; activation at an adequate solvent concentration is followed by a decrease at higher concentration. The latter process seems to be decomposition of the over-all structure of pro-PC.

From Fig. 14 (A), it can be seen that concentration necessary to cause

the maximum activation was 10 % for 2-CE, 30 - 35 % for dioxane and 40 % for DMF. The order in effectiveness on the activation of pro-PC is essentially the same as their relative ability to induce conformational changes on synthetic polypeptides and proteins.[55]

Fig. 14 (B) shows that the concentration necessary to cause the maximum activation was 1.6 M for G·HCl, 4-6 M for urea and 7 M for formamide. It is also noted that the activating effectiveness of these reagents are in the same order as that for conformational change of protein.

The effect of aliphatic alcohols on the activation of pro-PC was tested. Although the maximum activity obtained by various alcohols was significantly different from each other, concentration required to cause the maximum activation decreased in order of increase in carbon numbers of alcohols. Maximum activation was obtained at concentration of 20 % for n-propanol and 40 % for ethanol. Methanol little caused the activation even at 70 % concentration. Isopropanol and t-butanol caused the maximum activation at the same concentration as ethanol.

All of these results suggest that splitting of a part of hydrogen bond as well as hydrophobic bonding are involved in these activation process.

Time courses of the activation at acidic pH are shown in Fig. 16. The activation occurred below pH 3.6 and the rate of the activation increased with lowering pH. These activations are caused by hydrogen ions but not by proteinase A, because the rate of the activation at acidic pH was not accelerated by addition of purified proteinase A.

Activity attained by denaturing agents or by acid treatment could not be reversed by removal of the agents or by neutralization, respectively. So, these activation was an irreversible process.

Among various reagents tested, dioxane and urea were the most effective

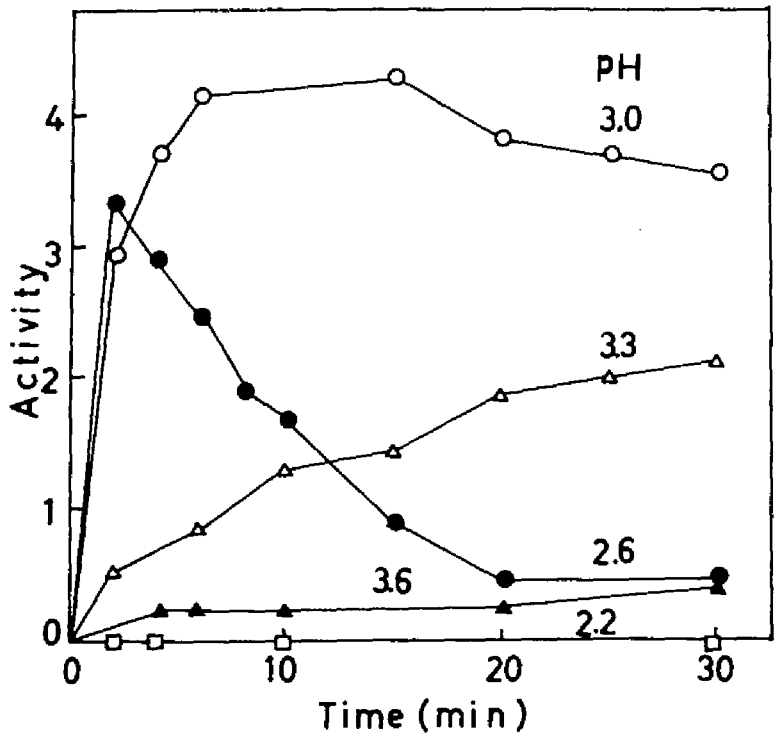

Fig. 16. Activation by acid treatment

for the activation of pro-PC at concentration range of 30-35 % and 4-6 M, respectively. Time courses of the activation by dioxane and urea are shown in Fig. 17 (A) and (B), respectively. At higher concentrations, rapid activation occurred initially, followed by rapid decay of the activity. The maximum activity obtained was at most 60 % of the full activity expectable from the specific activity of proteinase C.

Further investigations were carried out on the activations by 30-33 % dioxane or 4 M urea.

pH-dependency of the activation was tested as follows. Pro-PC solution was adjusted to desired pH values and mixed with dioxane (final conc. 33 %) or with urea (final conc. 4 M). The mixtures were incubated at 25°C for 15 min and 30 min, respectively, and resulting activities were measured.

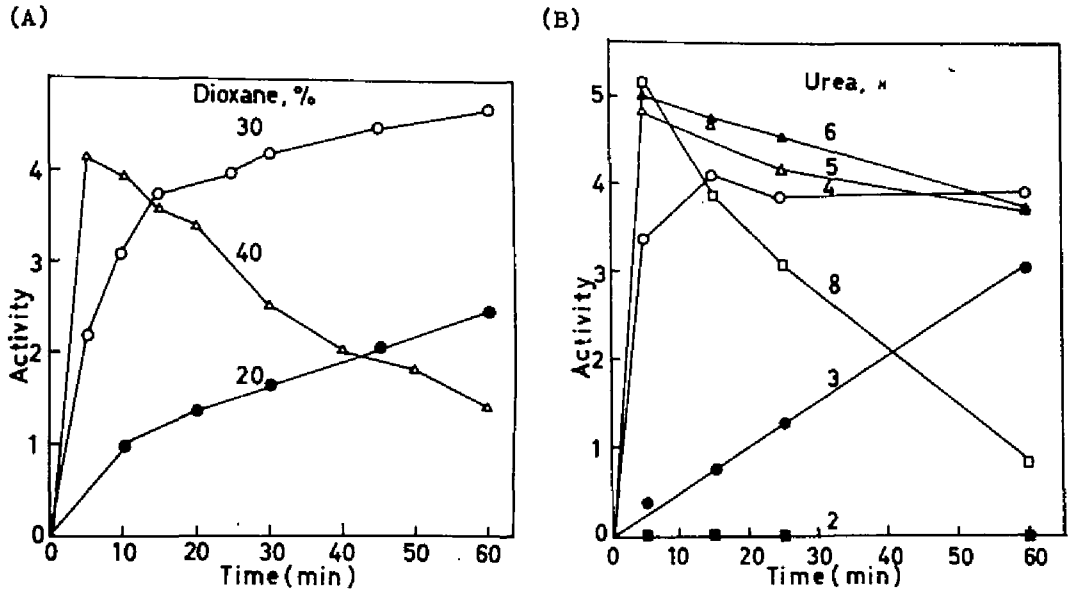

Fig. 17. Activation by dioxane and urea

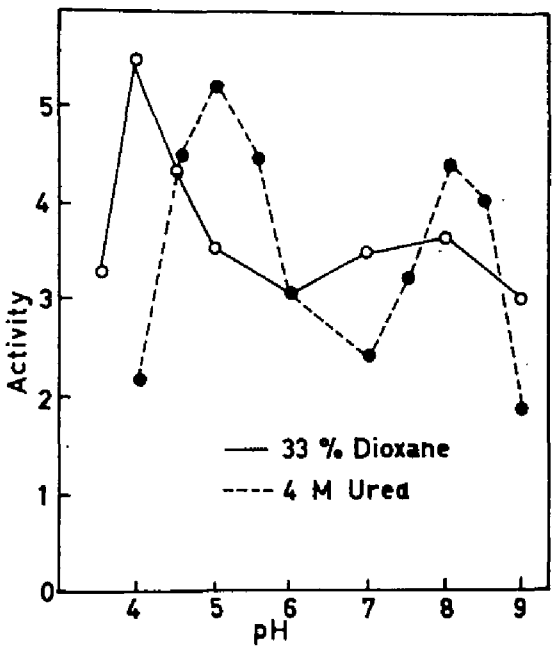

Fig. 18. pH-Dependency of the activation by dioxane and urea

As shown in Fig. 18, the extent of the activation showed two phase profiles toward pH: high at acidic and alkaline pH but low at neutral pH. This

result suggests that splitting in salt linkage between ionized carboxyl group and protonated amino group participates with the activation.

b. Catalytic properties of the activated enzyme

Esterolytic activity of the activated enzyme was compared with that of proteinase C to clarify the nature of the active site of the activated enzyme. Preparation of the activated enzyme was made as follows: pro-PC was incubated with activating reagents for an appropriate time and the mixture was immediately diluted with 0.01 M sodium phosphate buffer, pH 7.0. Since the activity of the preparation gradually decreased on standing, the preparation was used as fast as possible. pH-dependency, Km value and activation energy of the activated enzyme are shown in Fig. 19, in comparison with those of proteinase C. These properties gave good agreement between both

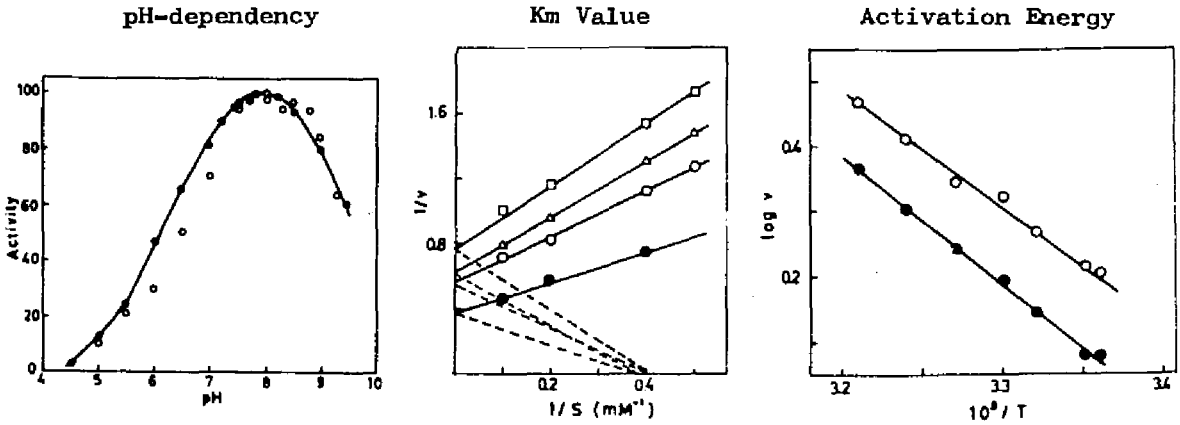

Fig. 19. Comparison of pH-dependency, Km value and activation energy between the activated enzymes and proteinase C

enzymes. Thus, it may be concluded that the activated enzyme hydrolyze ATEE with the same mechanism with proteinase C.

c. Sedimentation studies on the activation process

In order to investigate whether dissociation or association process was

involved or not in the activation process, sedimentation analysis was performed in the presence of dioxane, urea or acid, in the amount at which activation occurred. As shown in Fig. 20, pro-PC in all these conditions sedimented as a single component in the ultracentrifuge. The sedimentation coefficients are summarized in Table 13. These values were experimentally indistinguishable from that of native pro-PC.

These results indicate that neither dissociation nor association of protein is accompanied with the activation process. This conclusion was

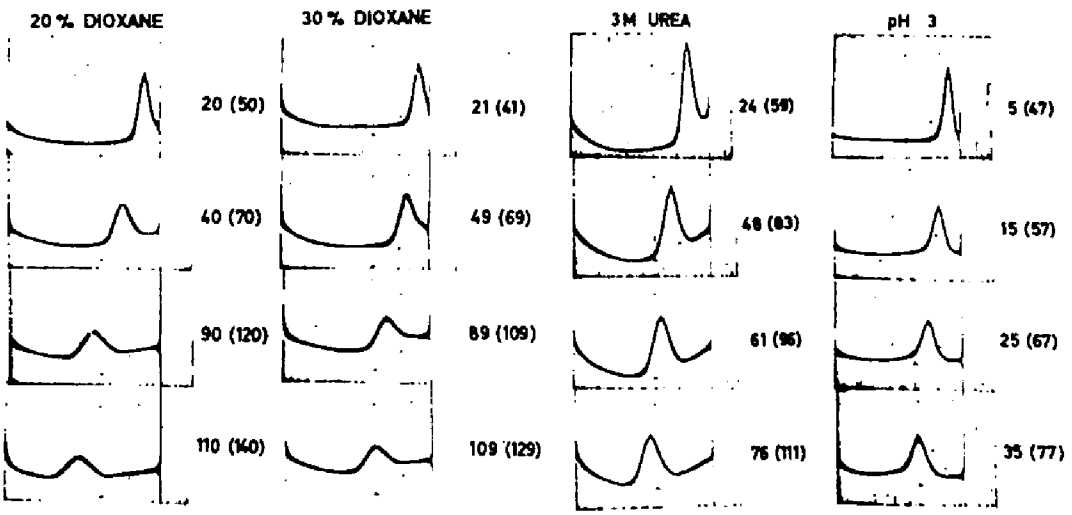

Fig. 20. Sedimentation patterns of pro-PC in various solvents
Figures: time in minutes after reaching at 60,000 rpm

Table 13. Sedimentation constant of pro-PC in varios solvents

Solvents	$s_{20,w}$
0.01 M sodium phosphate-0.1 M NaCl	5.27*
+ 20 % dioxane	5.1
+ 30 % dioxane	5
+ 3 M urea	5.1
+ pH 3	5.4

* $s_{20,w}$ at infinite dilution.

further supported by gel-filtration chromatography of the activation mixtures on Sephadex G-75.

As estimated from difference of molecular weight between pro-PC and proteinase C, possibility of the presence of sub-unit structure in pro-PC still remains. So, electrophoresis of denatured (in 8 M urea) pro-PC and proteinase C was carried out on a cellulose-acetate membrane. The membrane was dipped in 8 M urea solution prior to the electrophoretic run. As seen in Fig. 21, pro-PC was splitted into two subunit proteins; one of these was anionic protein of which mobility resembled that of denatured proteinase C and the other a strong cationic protein.

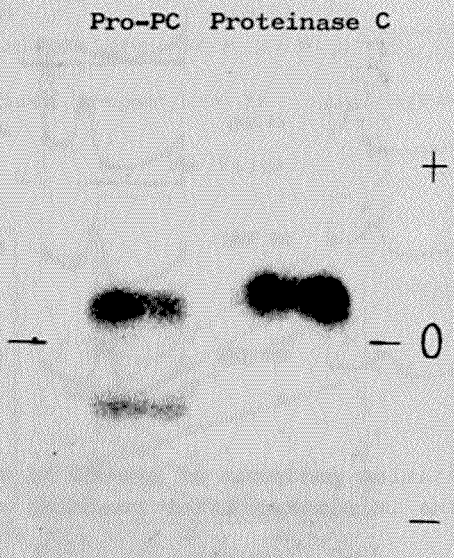

Fig. 21. Electrophoresis in 8 M urea

d. Effects of DFP and SH-reagents on the activated enzyme

When pro-PC was incubated with 10^{-4} M DFP or PCMB for 30 min, the potential activity of the pro-enzyme was fully maintained after removal of the excess reagents. However, dioxane- and urea-activated enzymes were comp-

letely inhibited by both reagents, like proteinase C. This suggests that the activated enzymes also contain both serine residue and SH-group essential for the activity.

Involvement of serine residue was confirmed by incorporation study of DFP-P^{32} into the activated enzymes. The experiment was carried out as follows. The testing proteins (10-50 μg) were incubated with 10^{-2} μmole of DFP-P^{32} at 25°C for about 60 min. The proteins were co-precipitated with 1 % of bovine serum albumin by 10 % TCA. The precipitates were washed several times with 10 % TCA and dissolved in 0.7 ml of 1 N NH_4OH. A portion of the solution was taken into a counting cup, dried under infrared light and counted. The binding quantities of DFP-P^{32} are summarized in Table 14. As expected, DFP-P^{32} was little incorporated into native pro-PC, while it was incorporated into the activated enzymes and proteinase C. The activated enzymes, which were treated under the conditions to cause the maximum activity (33 % dioxane or 4 M urea), reacted with about one mole of DFP-P^{32} per

Table 14. Reaction of pro-PC and the activated enzymes with DFP-P^{32}

Protein, Treatment	DFP^{32} incorporated		Moles of DFP /mole of protein
	cpm	μ moles	
Pro-proteinase C (6.63×10^{-4} μmoles)	381	0.42×10^{-4}	0.06
+ 8 M Urea	91	0.10	0.01
+ 25 % Dioxane, 25 , 15 min.	3646	4.05	0.61
+ 33 % Dioxane, 25 , 15 min.	5793	6.43	0.97
+ 2.7 M Urea, 25 , 30 min.	3416	3.73	0.57
+ 4 M Urea, 25 , 30 min.	5120	5.67	0.85
Proteinase C (5.25×10^{-4} μmoles)	4540	5.05	0.96
+ 8 M Urea	90	0.10	0.01

mole of the protein. One mole of DFP-P^{32} was also incorporated into native proteinase C. On the other hand, denatured (in 8 M urea) pro-PC and proteinase C could not react with DFP-P^{32}. Therefore, it is clear that DFP-P^{32} combines with an active serine residue essential for the activity representation.

Sulfhydryl groups of pro-PC and the activated enzymes were measured by DTNB method.[64] Molar equivalents of SH-group were calculated from the maximum color development and are summarized in Table 15. SH-groups of native pro-PC and proteinase C little react with DTNB. However, one and two moles of SH-group were detected in denatured (in 8 M urea) proteinase C and pro-PC, respectively. These results show that pro-PC contains 2 moles of SH-group per mole of protein which are unreactive for DTNB in the native state. However, one of these became a reactive form in the presence of 30-40 % dioxane or 4 M urea.

Table 15. Reaction of pro-PC and activated enzymes with DTNB

Enzyme, Treatment	DTNB bound moles / mole of protein
Pro-proteinase C	< 0.2
+ 8 M Urea	2.1
+ 4 M Urea	1.1-1.3
+ 30 % Dioxane	1.0
Proteinase C	< 0.2 (1.2)
+ 8 M Urea	1.0 (1.1)

( ) : pCMB titration

As already stated (Chapter II,3), proteinase C contained only one mole of SH-group per mole of the protein which was reactive with PCMB even in the native state. However, this group was unreactive with DTNB in the native state and DTNB little inhibited proteinase C activity. Dioxane- and urea-activated enzymes were also little inactivated by DTNB. Therefore, it is concluded that one of two SH-groups in pro-PC, which becomes highly reactive form by the activation treatments, is independent upon catalytic role of the activated enzymes.

e. Conformational changes in the activation

It has been pointed out the importance of obtaining information concernig the conformational changes which accompany zymogen activation.[57] In regard to this problem as well as in order to obtain the activation mechanism, the conformational changes taking place in the activation of pro-PC were examined by ORD and CD measurements.

In Fig. 22 are shown the Moffitt plots of pro-PC, the activated enzymes (in 30 % dioxane and 4 M urea) and proteinase C. The palameters of a_0 and b_0 were calculated from the Moffitt and Yang equation with assuming λ_0= 212 mμ. For pro-PC 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 latter values, which are experimentally identical, the α-helix contents of both proteins are calculated to be about 14 %. On the other hand, the activation of pro-PC by dioxane or urea was associated with decreases of b_0 values up to -110. It may be assumed from the result that an increase of the helical content is accompanied with the activation of pro-PC. However, this is not necessarily valid when the content of β-structure, if any, changes during the activation, because contribution of the β-structure

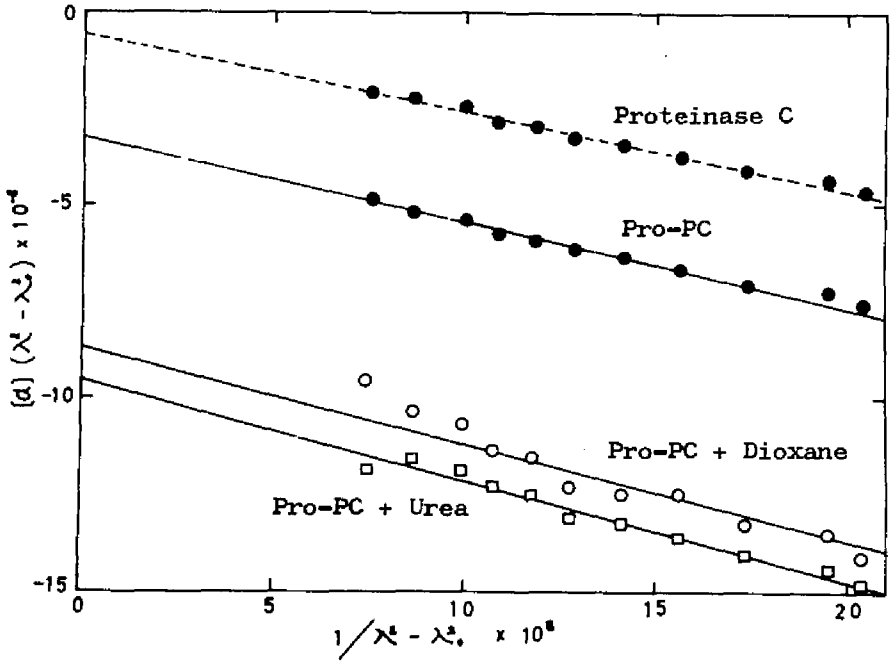

Fig. 22. Moffitt plots of ORD of proteinase C and pro-proteinase C in various solvents

to b_0 value is not fully elucidated.[58] In order to clarify the problem, analyses of ORD and CD in far ultra-violet region were examined.

Fig. 23 shows ORD spectra in 200 to 250 mμ region of native and denatured (in 8 M urea) pro-PC and proteinase C together with dioxane-activated enzyme. The curves for pro-PC and proteinase C in 8 M urea revealed simple dispersion curves, showing denaturation of the proteins. Both native proteins possessed trough at 232-233 mμ and peak near 200 mμ. Shoulders also appeared at 210-215 mμ, suggesting the presence of the α-helical conformation.[59] Dioxane-activated enzyme had a trough at 233 mμ. It was noticeable that a trough near 233 mμ decreased in creation of the active enzymes (proteinase C and dioxane-activated enzyme). However,

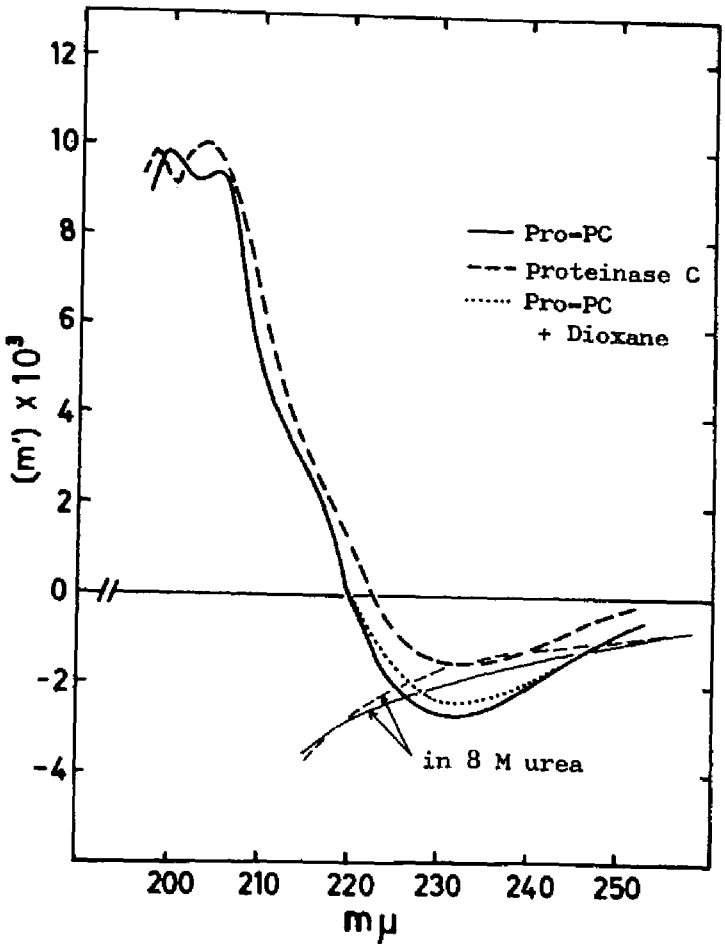

Fig. 23. ORD curves of pro-PC, proteinase C and dioxane-activated enzyme

peaks near 200 mμ remained constant.

Fig. 24 shows CD spectra from 210 to 250 mμ region of native and denatured (in 8 M urea) pro-PC and proteinase C together with dioxane-activated enzyme. The spectra of pro-PC and proteinase C in 8 M urea indicated denaturation of the proteins. The pattern of pro-PC in native state was characterized by an absorbing band typical for β-structure which has a minimum at 217 mμ.[60] In the active enzymes (proteinase C and dioxane-activated enzyme), the negative minimum at 217 mμ disappeared, and new spectra, which have minimum at 200-203 mμ and shoulder near 222 mμ appeared.

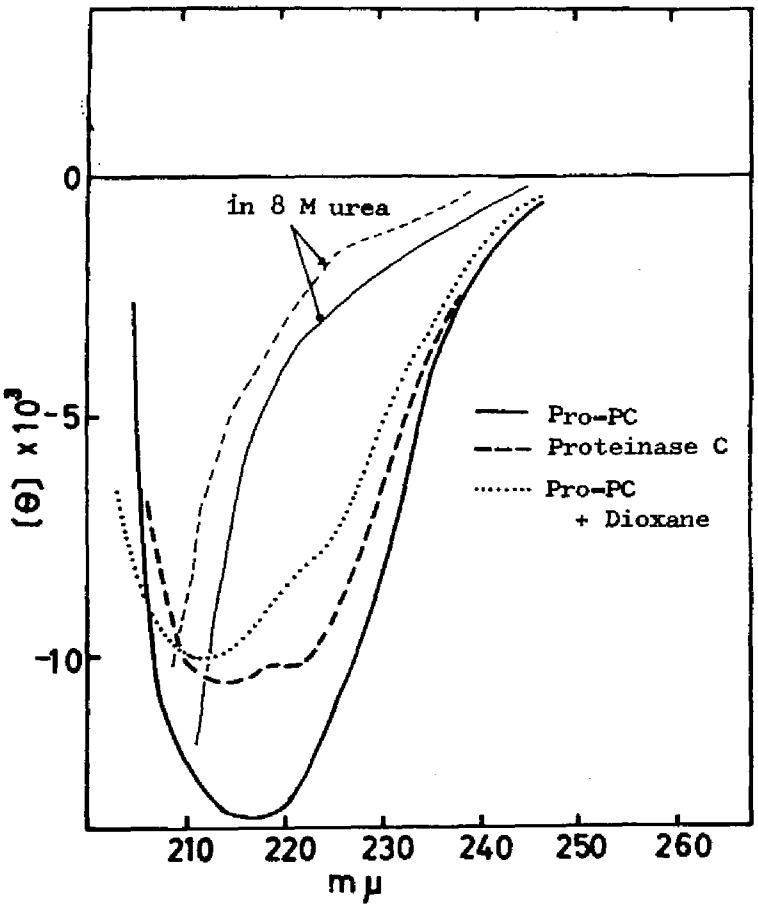

Fig. 24. CD spectra of pro-PC, proteinase C and dioxane-activated enzyme

These spectra suggest the presence of α-helix rather than β-structure. The results lead to the conclusion that β-structure in pro-PC is destroyed during the conversion to the active forms.

Although ORD spectrum of pro-PC indicated the presence of helical conformation, overlapping of the helical and β-structure makes difficult to evaluate the helical content from a trough at 233 mμ. Jirgensons[61] estimated helical content of ribonuclease from the magnitute of the positive extremum near 200 mμ in fair agreement with X-ray data, assuming that contribution of the optical activity of the other conformations is negligi-

ble. Since ORD curves of pro-PC and proteinase C near 200 mμ are experimentally indistinguishable, the α-helix 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 mμ in the activation of pro-PC may be partly contributed by the destruction of β-structure.

By summarizing ORD and CD experiments, it is concluded that the transformation of pro-PC into the active enzymes accompanies the rupture of β-structure in pro-PC without any changes of α-helix content.

4. Activation by Proteinase A

As in other zymogen activation,[57] yeast pro-PC can be transformed to an active enzyme by a proteolytic enzyme, yeast proteinase A. In the activation process, it is anticipated that a limited proteolysis of pro-PC occurred by action of proteinase A, followed by release of peptide(s) which correspond(s) to the difference of molecular weight between pro-PC and proteinase C.

In this section, activation mechanism by proteinase A is investigated by analyzing the chemical compositions of pro-PC and the activated enzyme.

a. Conditions of the activation

Conversion of pro-PC into the active enzyme was catalyzed by proteinase A in incubation at room temperature below pH 5. With lowering pH, the conversion was accerelated as expected from optimum pH of proteinase A. However, since spontaneous activation catalyzed by acid was accompanied below pH 3.5 at room temperature (25°C), it was impossible to distinguish clearly the activation by proteinase A from that by acid catalysis. In order to surpress the activation by acid, experiment was made at 0°C and pH 3.5. As

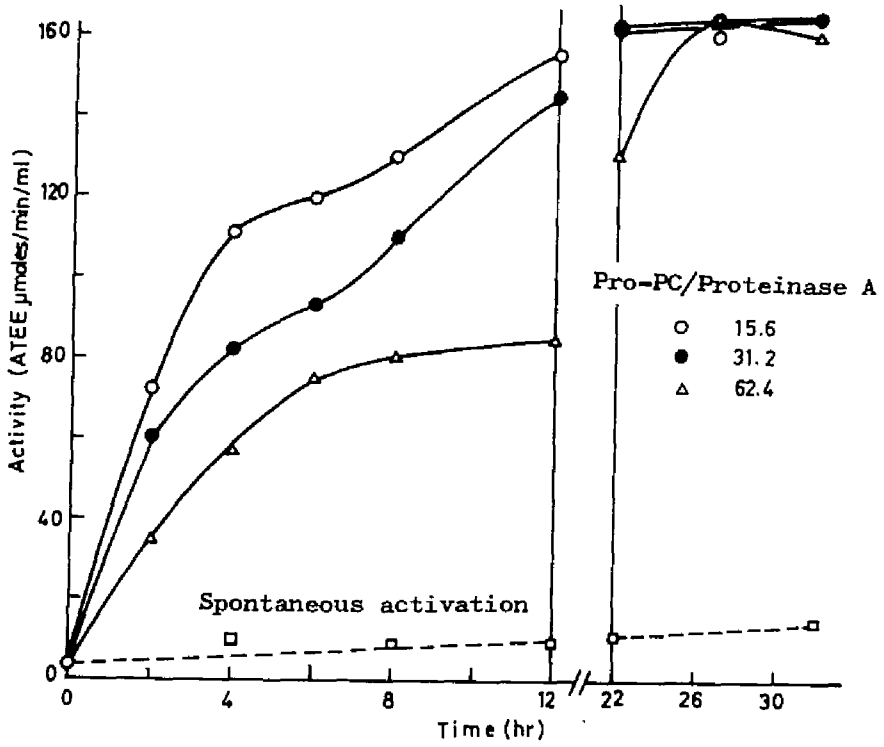

Fig. 25. Activation of pro-proteinase C by proteinase A

seen in Fig. 25, the activation by acid catalysis was substantially avoided and the activation by proteinase A proceeded for about 26 hr.

The mixtures after completion of the activation were analyzed by zone electrophoresis on a strip of cellulose-acetate. As shown in Fig. 26, initial pro-PC was disappeared and two new bands appeared by the activation at pH 3.5 and 0°C. One of these was a strongly basic band and the other was similar to proteinase C. When the activation was performed at 25°C, the rapidly removing band was disappeared, probably digested by proteinase A or the activated enzyme.

b. Analysis of the activation mixture

In order to elucidate the chemical nature of the released protein and the activated enzyme (proteinase C_A), which were derived from pro-PC by the

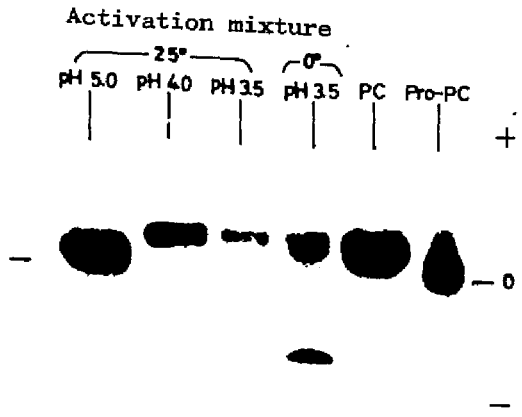

Fig. 26. Electrophoresis of the activation mixture on a cellulose-acetate membrane

PC : Proteinase C

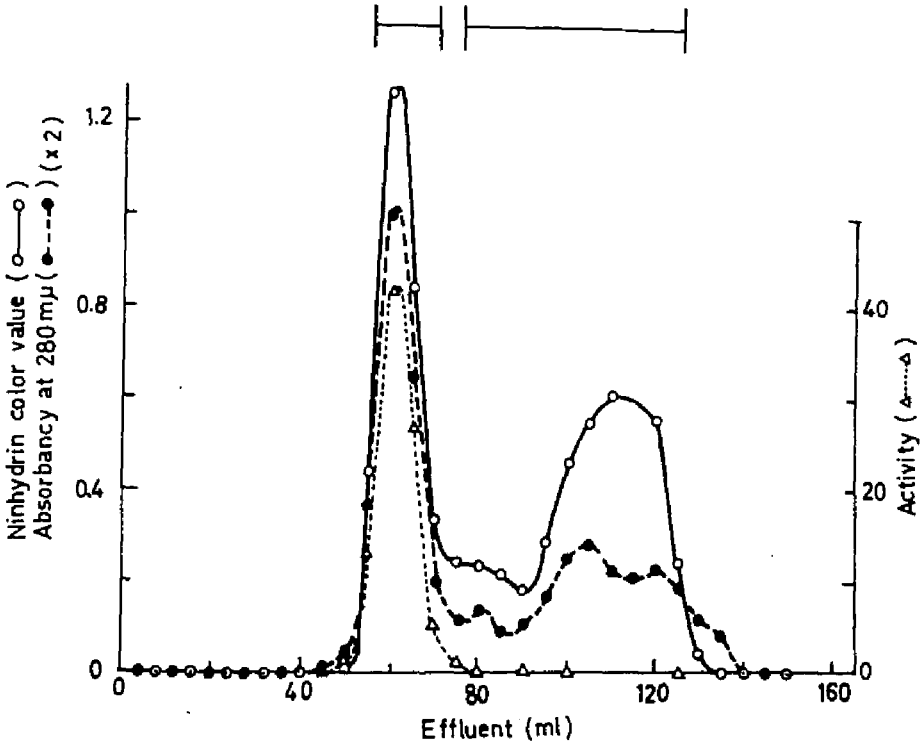

Fig. 27. Gel-filtration of the activation mixture on Sephadex G-100

action of proteinase A, both compounds were separated and purified on a preparative scale as follows: 0.21 mg of proteinase A was added to 16.9 mg of pro-PC (0.025 M acetate buffer, pH 3.5) and the mixture was incubated at 0°C for 26 hr. After completion of the activation, the reaction mixture was transferred to a Sephadex G-75 column (1.5 x 90 cm) and gel-filtration was performed with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.05 M KCl. Fig. 27 shows a gel-filtration pattern of the activation mixture. Fractions having esterolytic activity for ATEE were collected and further purified by chromatography on a DEAE-Sephadex A-50 column. Proteinase C_A was prepared in almost theoretical yield, as judged from the specific activity of proteinase C.

Low molecular weight fractions were collected and used for chemical analysis without further purification. This fraction contained a few contaminants as judged from high-voltage paper electrophoresis.

c. Chemical analysis

Amino acid compositions of pro-PC, proteinase C and C_A were examined. Acid hydrolysis of each sample (ca. 5 mg) was performed pairwise by 3 ml of grass-distilled 6 N HCl for 22 hr at 110°C in a sealed tube under argon gas phase. Amino acid content of the hydrolysates was analyzed by a Hitach KLA-3 amino acid analyzer. The content of half-cysteine was determined as cysteic acid after oxidation with performic acid according to the procedure of Moore.[62] Tryptophan was measured spectrophotometrically by the method of Goodwin and Morton.[63] The results of amino acid analyses are given in Table 16. Difference of amino acid content between pro-PC and proteinase C_A is also shown in the table.

It can be seen that three proteins contain relatively larger quantities

Table 16. Amino acid compositions of pro-PC, proteinase C and C_A

Amino acid	Pro-PC	Proteinase C	Proteinase C_A	Difference between Pro-PC & PC_A
		residues/molecule		
Asp	77.4	56.8	54.3	23
Glu	57.1	34.9	40.4	17
Gly	42.5	29.9	35.3	8
Ala	36.9	19.3	29.3	13
Val	36.1	24.6	23.9	12
Leu	42.5	29	29	14
Ileu	26.2	18.1	17.6	8
Ser	45.4	26.7	33.6	12
Thr	30.5	15.7	17.3	14
Cys	10.1	(7.0)	(7.0)	3
Met	8.7	4.1	3.8	5
Pro	35.7	21.8	22.6	13
Phe	32.5	19.9	20.2	13
Tyr	28.4	21.9	21.2	7
Trp	12.5	10.5	-	-
His	12.7	7.5	8.0	5
Lys	29.4	14.6	16.3	13
Arg	9.9	7.4	7.5	3
Glc.NH_2	4.8	-	-	-
				Total 183

of acidic amino acids in agreement with acidic nature of these proteins. Hexosamine was detected in pro-PC and proteinase C. That content of pro-PC was measured by changing the time of acid hydrolysis.

The content of cysteic acid in pro-PC was 10 moles per mole of protein. Since pro-PC possesses two SH-groups, the residual 8 moles of cysteic acid will constitute 4 sets of disulfide bridge in pro-PC. Cysteic acids were also found in low molecular weight fraction obtained by the gel-filtration. About 3 moles of cysteic acid were recovered in this fraction from one mole of pro-PC. Therefore, this fraction would contain one SH-group and one disulfide bridge. Possibly, this SH-group corresponds to highly reactive SH-group for DTNB which was found in the activation by dioxane or urea.

Amino acid content of proteinase C_A was less by about 183 residues than that of pro-PC. This indicates that the released protein has approximately 20,000 of molecular weight. It is noticeable that this protein contained larger quantities of serine and threonine which have a tendency to form β-structure in synthetic polypeptides.[65] In reference of ORD and CD data, it may be assumed that proteinase A ruptures the conformation, especially β-structure, of this protein.

Amino acid compositions of proteinases C and C_A were very similar to each other, although amino acid in proteinase C, in general, was slightly less than that in C_A. Aspartic acid, glycine, alanine, serine and threonine of proteinase C showed especially smaller value than those of proteinase C_A.

N-terminal amino acids of pro-PC and proteinase C (10-15 mg) were detected by the Edman degradation in an experimental method summarized by Doolittle.[66] In pro-PC, only lysine was detected as N-terminal amino acid in the yield of 0.88 residue per mole of protein. Since pro-PC was composed of two subunits, N-terminal amino acid of one subunit seemed to be blocked not to react with PITC. N-terminal residue of proteinase C revealed lysine and four other amino acids (aspartic acid, glycine, phenylalanine and tyrosine). Heterogeneity of N-terminal amino acid as well as smaller amino acid content in proteinase C suggest that the enzyme is composed of several components as in proteinases[67,68] derived by autolysis, and also suggest that the enzyme may possess different active forms as in chymotrypsin derivatives.[69]

On the other hand, in proteinase C which was prepared by slightly modified method*, only lysine was found as N-terminal amino acid in the yield

of 0.86 residue per mole of protein. Identity in N-terminal amino acids of pro-PC and the activated enzyme would indicate that proteinase A attacks the low molecular weight subunit in the activation but not the precursor subunit of proteinase C.

* prepared from partially purified pro-PC fraction (Fractin II described in Chapter III, 2).

5. Discussion on the Activation Mechanism

Pro-proteinase C consisted of two subunits. One of these was a protein similar to proteinase C so far judged from electrophoretic analysis. This subunit seemed to be a precursor protein of proteinase C. Effects of various reagents on the activation of pro-PC suggest that rupture of several weak bondings other than covalent bond, e.g. hydrophobic, hydrogen and ionic bondings, might be associated with the activation process. However, no dissociation of subunits was demonstrated in these activations. On the other hand, activation by proteinase A accompanied with release of a low molecular weight protein having molecular weight of about 20,000. This protein is considered to be essentially identical with the other small subunit in pro-PC, from the following three facts.

1. Pro-PC contained two SH-groups. These groups seemed to be buried into the protein molecule. One of these will participates in the active site of the activated enzyme. The other SH-group was independent upon exhibition of the activity of the activated enzyme. It became highly reactive in activation treatment by dioxane or urea. The fact indicates that dioxane or urea causes alteration in the over-all structure of pro-PC molecule and the SH-group changes its spatial direction from the inside of the protein

molecule to the outside. A free SH-group, which is attributable to the above SH-group, was also demonstrated in the protein fraction released during the activation by proteinase A.

2. Pro-PC exhibited only one N-terminal amino acid, lysine. N-terminal of proteinase C was also found to be lysine. Several zymogens, e.g. trypsinogen,[70] pepsinogen[71] and procarboxypeptidases,[68] are activated by release of peptide(s) near N-terminal portion. However, activation of pro-PC by proteinase A may retain its N-terminal portion. N-terminal of the small subunit is regarded to be blocked in unknown manner.

3. ORD and CD studies showed that destruction of β-structure was involved in the activation of pro-PC. The destruction may be attributable mainly to the conformational changes of the small subunit, because activations by dioxane and proteinase A caused almost the same structural changes

The enzymes created from pro-PC by several denaturants have the same properties with proteinase C, in respect to catalytic ability as well as active site. Rapid creation of these active enzymes by denaturants suggests that the structural configuration characteristic of the active site pre-exists in pro-PC molecule.

From all of these facts and arguments, following conclusion for the activation mechanism of pro-PC is derived. Several denaturants effective for the activation will destruct several weak bonds making up the pro-PC molecule and lead to a rearrangement of the quaternary structure of pro-PC without splitting of the subunits. At the same time, structural alterations will occur mainly on the small subunit. These changes will result in un-masking of the active site which is buried in the pro-PC molecule. The small subunit will be susceptible to proteinase A. Limited proteolysis by proteinase

A is expected to result in conformational changes of the small protein. As the result, the protein is released and the active site will appear.

Recently, Lenney and Dalbec[44] described that pro-proteinase B is an enzyme-inhibitor complex and its activation is brought about by destruction of the inhibitor. Although the present author could not demonstrate the free inhibitor of proteinase C in yeast extracts, the activation mode of pro-PC is essentially similar to their conclusion for pro-proteinase B. Activation of pepsinogen is also brought about by unmasking of the active site following to release of pepsin inhibitor.[71] Bovine procarboxypeptidase A is composed of two or three subunits.[72] Its activation by trypsin accompanies with the dissociation of these subunits, followed by limited proteolysis of precursor subunit of carboxypeptidase A. In the activation of pro-PC, a problem remains to be clarified whether limited proteolysis of precursor subunit of pro-PC is necessary to its activation or not. A proof on the problem will be made when two subunits of pro-PC are separated in the native state, and furthermore, when reversible dissociation and association of these subunits are demonstrated. Recently, dissociation of procarboxypeptidase A molecule in the native state became possible by succinylation of the parent protein.[73,74]

CHAPTER V

CONCLUSION

Characteristics of intracellular proteinase system in yeast were investigated. The results are summarized as follows.

In Chapter II, three different types of proteinases were fractionated and purified from baker's yeast (Section 1). These enzymes were designated as proteinases A, B and C. Optimum pH for protein hydrolysis was at pH 2-3 for A, about pH 9 for B and below pH 6 for C. Proteinase B was characterized by milk-clotting activity and its very unstable nature (Section 2). Proteinase C had strong esterolytic activity for ATEE and peptidase activity for CGT. This enzyme essentially required both serine and cysteine residues for exhibition of the activity. The activity was completely inhibited by the presence of one mole of DFP or PCMB per mole of protein (Section 3). It became possible to evaluate the activity of each enzyme even in their mixed states by applying characteristics of the individual enzyme (Section 4). Physico-chemical properies of proteinases A and C were also clarified. Both enzymes were glycoproteins. Molecular weights of A and C were estimated to be approximately 60,000 and 61,000, respectively (Section 5).

In Chapter III, evidence for the presence of inactive forms of proteinases B and C was presented. These were named as pro-proteinases B and C, respectively. Proteinase A did not possess the inactive form. These findings will give an important clue for role of intracellular proteinase system in the protein metabolism of yeast (Section 1). Pro-proteinases B and C were chromatographically separable from each other (Section 2). These pro-enzymes were rapidly activated by incubation with various protein

denaturants. The activation of pro-PC was also brought about by acid treatment or by action of proteinase A (Section 3).

In Chapter IV, in the first place, isolation and purification procedures of pro-PC were developed (Section 1). Its physico-chemical properties were elucidated. Pro-PC was a glycoprotein like proteinase C. Molecular weight was evaluated by several methods to be approximately 80,000 (Section 2). Chemical and physical natures associated with the activation by protein denaturants were examined. Neither dissociation nor association of pro-PC molecule was demonstrated during such activation processes. Decrease of β-structure of pro-PC was found to be involved in the activation, as judged from ORD and CD measurements (Section 3). The activation by proteinase A was examined under controlled conditions. The activated enzyme (proteinase C_A) and the released protein were separated. Amino acid compositions as well as N-terminal amino acids were compared between pro-PC and the activated enzyme. Finally, the activation mechanism of pro-PC was discussed by summarizing the experimental results. It was concluded that the activation was caused by unmasking of the active center.

ACKNOWLEDGEMENT

The author would like to express his sincere gratitute to Dr. Tadao Hata, Professor of Kyoto University, for his kind guidance and encouragement during the course of this work. The author is also greatly indepted to Dr. Etsushiro Doi, Assistant Professor of Kyoto University, for his kind guidance and suggestion, and to Mr. Yoshinobu Minami for his cooperation in carrying out a part of this work.

The author is grateful to Dr. Kozo Hamaguchi, Professor of Hokkaido Univesity, and his co-workers for the ORD and CD measurements.

Thanks are also due to the members in the Research Institute for Food Science in Kyoto University for their kind help and discussion.

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