ARTICLES

Purification, Characterization, and Utilization of Pig Plasma Factor XIIIa†

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To investigate the properties of pig plasma factor XIII (a zymogen of factor XIIIa, EC 2.3.2.13, TGase) and consequently to utilize the pig blood, factor XIII was purified to electrophoretical homogeneity after DEAE-Sephacel chromatography. The molecular weights were 320 000 estimated by Sepharose CL-6B and 75 000 by SDS–PAGE, suggesting this zymogen contains four subunits with identical molecular weights of 75 000. This proenzyme was activated by thrombin and Ca^{2+}. Accordingly, the purified proenzyme was identified as factor XIII. The optimal temperature for incorporating monodansylcadaverine to β-substrate was 55 °C. Factor XIIIa (activated factor XIII) was inhibited by *N*-ethylmaleimide, *p*-(chloromercuri)benzoate, and iodoacetic acid in the presence of Ca^{2+}. The rate constants for thermal denaturation (K_d) at 55 °C of factor XIII and XIIIa were 5.0×10^{-5} and 0.6×10^{-5} s^{-1}, respectively. Factor XIIIa catalyzed the covalent cross-linking of myosin heavy chain. The addition of crude plasma factor XIII (including thrombin) substantially increased the gel strength of minced mackerel.

INTRODUCTION

Transglutaminase (TGase), which catalyzes the intermolecular ϵ-(γ-glutamyl)lysine cross-linkage, is widely distributed in various tissues and body fluids (Folk and Finlayson, 1977). TGase has been purified from liver (Connellan et al., 1971) and hair follicle (Chung and Folk, 1972) of guinea pig, rabbit liver (Abe et al., 1977), and epidermis (Hanigan and Goldsmith, 1978) and erythrocyte (Brenner and Wold, 1978) of human and also isolated from muscle and surimi of Alaska pollack (Seki et al., 1990). The properties of the enzyme have been extensively examined (Folk and Cole, 1966; Connellan et al., 1971; Folk and Chung, 1973; Abe et al., 1977; Folk and Finlayson, 1977; Folk, 1980; Lorand and Conrad, 1984). The enzymatic cross-linking of proteins has usually been employed to develop the texturized food products (Whitaker, 1977). Some studies indicated that TGase could catalyze the cross-linking among food proteins, such as casein, β-lactoglobulin, and soybean proteins, which consequently improved their functionalities (Ikura et al., 1980a,b; Motoki and Nio, 1983; Nio et al., 1985; Tanimoto and Kinsella, 1988). According to Ikura et al. (1981, 1985), TGase could incorporate essential amino acids into food proteins and consequently improve their amino acid composition. It was also found to catalyze the binding of myosin with nonmeat proteins and produce new food proteins (Kurth and Rogers, 1984). From the study by Yoshikawa et al. (1982), a coenzyme, NAD^+ analogue, could be immobilized to casein by the action of TGase. However, the tissues and/or organs used in these studies are still not good sources for the isolation of TGase, because of the difficulty in the collection of these sources. Factor XIII (protransglutaminase) was purified from human plasma (Loewy, 1961a) and platelet (Schwartz et al., 1973). The catalytic properties of factor XIII were found to be different from those of transglutaminase obtained from tissues (Chung, 1972) and microorganisms (Ando et al., 1989).

In Taiwan, the total slaughtered pork was about 1 million metric tons in 1990 (*Taiwan Agricultural Yearbook*, 1991). Although the quantity of blood is around 2.5–3.5% of the body weight, about 30 thousand metric tons of blood was produced each year. So far, there is still no appropriate utilization for it. This, accordingly, caused heavy pollution. This study aimed to purify and characterize the factor XIII from pig blood and to investigate the effects on gelation of fish muscle. Utilization of this enzyme on the processing of minced fish meat was also evaluated, consequently, to consume the pig blood.

Table I. Factors Used in the Gelation Study of Minced Mackerel Meat

independent variables	code	level −1	level 0	level 1
pH value	X_1	7.0	7.5	8.0
setting time at 37 °C, min	X_2	0	40	80
crude enzyme added, %	X_3	0	0.2	0.4

MATERIALS AND METHODS

Materials. DEAE-Sephacel, Sepharose CL-6B, and electrophoresis and gel filtration calibration kits were purchased from Pharmacia (Uppsala, Sweden). Acrylamide, bis(acrylamide), Coomassie blue G-250, dithiothreitol (DTT), and thrombin (from bovine plasma, 50 NIH units/mg) were obtained from Merck (Darmstadt, Germany). Dephosphorylated β-casein, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), iodoacetic acid (IAA), monodansylcadaverine, *N*-ethylmaleimide (NEM), and *p*-(chloromercuri)benzoate (PCMB) were products of Sigma (St. Louis, MO). All other chemicals were of reagent grade.

Purification of Pig Plasma Factor XIII. Fresh blood was obtained from a slaughterhouse in northern Taiwan. During collection, 15% of an anticoagulant (mixture of 2.2% trisodium citrate, 0.8% citric acid, and 2.5% dextrose) was added to the

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Table II. Summary of the Purification of Pig Plasma Factor XIII

procedure	total protein, mg	total activity, units	specific activity, units/mg	yield, %	purification factor, x-fold
plasma	128 000	87 040	0.68	100	1.0
precipitate 20% satn AS	30 030	38 485	1.27	44.2	1.9
precipitate 16% satn AS[b]	7 410	34 605	4.67	39.8	6.9
heating at 56 °C for 3 min	2 300	31 970	13.9	36.7	20.4
precipitate 36% satn AS[c]	1 920	27 264	14.2	31.3	20.9
DEAE-Sephacel chromatography	270	22 275	82.5	25.6	121.3

[a] 20% satn AS, 20% saturation ammonium sulfate at pH 7.0. [b] 16% satn AS, 16% saturation ammonium sulfate at pH 5.4. [c] 36% satn AS, 36% saturation ammonium sulfate at pH 7.5.

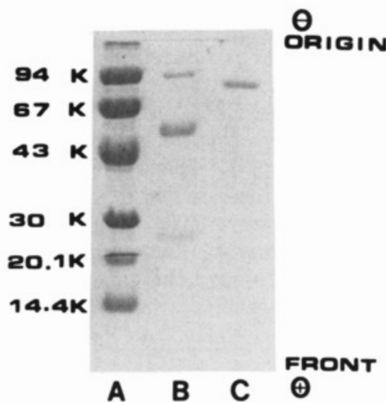

Figure 1. Electrophoretic pattern of purified pig plasma factor XIII using sodium dodecyl sulfate–polyacrylamide gel electrophoresis [12.5% acrylamide; standard (A); washed fraction on DEAE-Sephacel chromatography (B); eluted fraction with monodansylcadaverine activity on DEAE-Sephacel chromatography (C)].

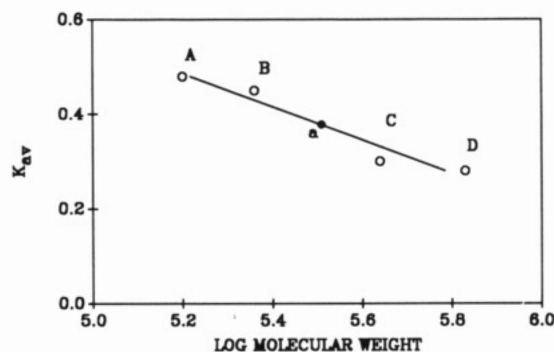

Figure 2. Calibration curve for determination of the molecular weight of pig plasma factor XIII by Sepharose CL-6B chromatography [aldolase, 158 000 (A); catalase, 232 000 (B); ferritin, 440 000 (C); thyroglobulin, 669 000 (D); S: pig plasma factor XIII, 320 000 (S)].

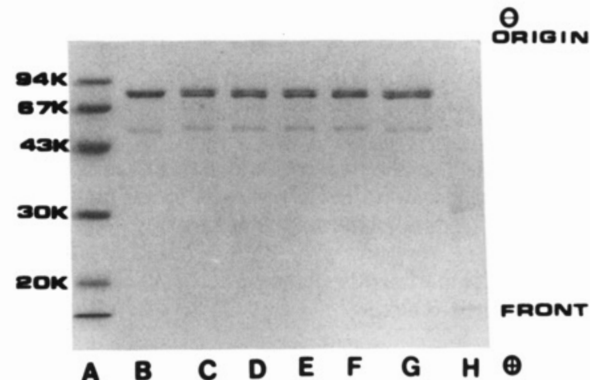

Figure 3. Effect of thrombin activation on the pattern of disc sodium dodecyl sulfate–polyacrylamide gel electrophoresis of pig plasma factor XIII [standard proteins (A); factor XIII (B); factor XIII incubated with thrombin–calcium at 37 °C for 20 min (C) and 1 (D), 2 (E), and 3 h (F); factor XIII incubated with thrombin–EGTA at 37 °C for 3 h (G); thrombin (H)].

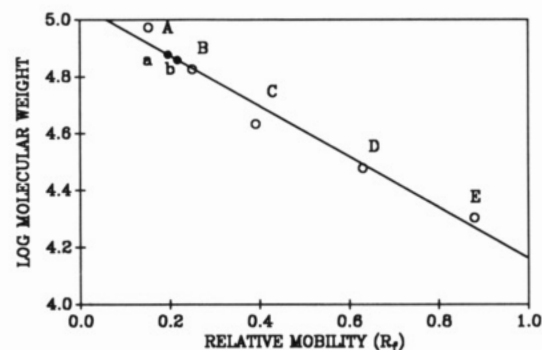

Figure 4. Calibration curve for determination of the molecular weight of subunits of pig plasma factor XIII [using SDS–PAGE on 10% polyacrylamide gel; phosphorylase *b*, 94 000 (A); bovine serum albumin, 67 000 (B); ovalbumin, 43 000 (C); carbonic anhydrase, 30 000 (D); soybean inhibitor, 20 100 (E); α-lactalbumin, 14 400 (F); 75 000 (a); 72 000 (b)].

fresh blood. After 10 min of centrifuging at 1000*g* twice, the supernatant was subjected to ammonium sulfate fractionation. The precipitate on 20% saturation of ammonium sulfate was collected and then dissolved in 10 volumes of 0.15 M KCl. After the pH was adjusted to 5.4 using 1.0 N acetic acid, the factor XIII and fibrinogen were precipitated on 16% saturation of ammonium sulfate. The precipitate was then dissolved in 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl and then heated at 56 °C for 3 min to remove the fibrinogen. After cooling to 10 °C using ice–water, the rubbery ball of denatured fibrinogen was squeezed to remove the included liquid. The factor XIII was again precipitated with 36% saturation of ammonium sulfate and dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 1.0 mM EDTA (buffer I) again. After dialyzing against the same buffer at 4 °C for 48 h, the sample was chromatographied onto the DEAE-Sephacel column (2.6 × 85 cm) which was equilibrated by buffer I. After the column was washed with about 5 bed volumes of buffer I, the factor XIII was eluted with a linear gradient of 0.0–0.3 N NaCl in buffer I. Fractions of 10 mL were collected with a flow rate of 36 mL/h. Fractions with monodansylcadaverine incorporating activity were collected and subjected to the following experiments.

Determination of Enzyme Activity. The dephosphorylated β-casein was acetylated according to the method of Cooke and Holbrook (1974a). About 15 mL of dephosphorylated β-casein in 50 mM Tris-HCl buffer (pH 7.5, 30 mg/mL) was mixed with 35 mL of 8.5 M urea, 5 mL of pyridine, and 7 mL of acetic anhydride. After dialyzing against distilled water at 4 °C for 36 h, the acetylated β-casein was freeze-dried and is referred to as β-substrate. β-Substrate was dissolved in 0.1 M Tris-HCl (pH 7.5, 30 mg/mL) before use. The enzyme activity was measured according to the method of Takagi et al. (1986). When samples were subjected to measurement of enzyme activity before heat treatment during purification, the crude enzymes were desensitized by heating with ethylene glycol monomethyl ether at 56 °C for 3 min and fibrinogen was excluded by centrifuging at 8000*g* for 10 min. To 0.1 mL enzyme sample were added the

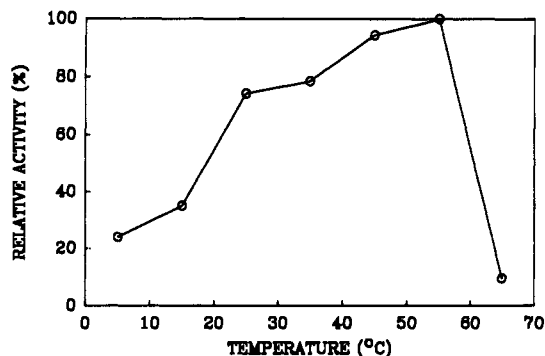

Figure 5. Effect of temperature on plasma factor XIIIa activity.

Table III. Effect of Metal Ions on Plasma Factor XIII[a]

metal ions[b]	5 mM		10 mM	
	A[c]	B[c]	A	B
K^+	94.0[d]	2.0	102.1	1.2
Na^+	93.7	2.0	106.7	0.7
Mg^{2+}	105.9	2.9	117.9	2.4
Sr^{2+}	87.8	13.6	91.0	20.0
Zn^{2+}	12.2	13.2	0	0
Ca^{2+}	100.0	100.0	100.0	100.0
Cd^{2+}	0	0	0	0
Ba^{2+}	62.6	3.1	67.4	1.9
Mn^{2+}	61.3	3.1	84.8	0
Co^{2+}	0	0	0	0
Ni^{2+}	0	0	0	0
Cu^{2+}	0	0	0	0
Hg^{2+}	0	0	0	0
Fe^{2+}	0	0	0	0
Fe^{3+}	0	0	0	0

[a] Factor XIII was incubated with thrombin at 37 °C for 20 min. [b] The counterion of all metals was chloride. [c] A, with 10 mM Ca^{2+} added; B, with 1 mM EGTA added. [d] Values in this table are expressed as percentage ratio relative to that measured at 10 mM $CaCl_2$.

following solutions in turn: 50 μL of DTT (0.2 M), 100 μL of thrombin (125 NIH units/mL), 1.2 mL of 0.1 M Tris-HCl buffer (pH 7.5), 0.2 mL of 0.1 M calcium chloride, and 50 μL of 0.25 mM monodansylcadaverine. The enzyme mixture was incubated at 37 °C for 30 min after the addition of 0.2 mL of β-substrate. After the reaction was terminated with 100 μL of 1.0 M ammonium sulfate, the fluorescence intensity, which caused incorporation of monodansylcadaverine into β-substrate, was measured by a fluorescence spectrophotometer (Model 650-10S, Hitachi). The excitation and emission wavelengths were 350 and 480 nm, respectively. The blank was performed under the same conditions, except the enzyme solution was replaced with 0.1 M Tris-HCl buffer (pH 7.5). The specific activity was defined as the amount of enzyme that caused 1.0% increase in fluorescence intensity compared with the blank.

Determination of Protein Concentration. Protein concentration was determined according to the protein-dye binding method (Bradford, 1976) using bovine serum albumin as a standard.

Sepharose CL-6B Chromatography. The molecular weight (MW) of the purified enzyme was determined by Sepharose CL-6B chromatography on a 2.6 × 85 cm column which was equilibrated with about 5 bed volumes of 0.1 M NaCl–50 mM Tris-HCl buffer (pH 7.5). One milliliter of sample (0.8 mg/mL) was loaded onto the column and eluted with the same buffer at a flow rate of 12 mL/h. Fractions of 3 mL were collected. Thyroglobulin (669 000), ferritin (440 000), catalase (232 000), and aldase (158 000) were used as standards.

Proteolysis of the Purified Enzyme by Thrombin. After DEAE-Sephacel chromatography, the purified enzyme (0.1 mL) suspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ or 1 mM EGTA was incubated with 10 NIH units/mL thrombin at 37 °C for 20 min and 1, 2, and 3 h. About 20 μg of the resulted samples was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The purified enzyme dissolved in sample buffer [62.5 mM Tris-HCl buffer containing 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, and 0.002% bromophenol blue] was incubated at 100 °C for 5 min. The dissociated samples were subjected to disc SDS–PAGE analysis according to the method of Laemmli (1970). Gel staining was carried out according to the procedure of Neuhoff et al. (1988). Phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), and α-lactalbumin (14 400) were used as standards.

Table IV. Effect of Inhibitors on Plasma Factors XIII and XIIIa

inhibitors	factor XIIIa		factor XIII	
	EGTA[b]	Ca^{2+}	EGTA	Ca^{2+}
control[a]	100.0	100.0	100.0	100.0
NEM[b]	59.7[c]	0	51.0	77.9
PCMB[b]	32.2	0	58.5	60.8
IAA[b]	35.2	0	39.4	58.7

[a] Control: without inhibitors. [b] EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; NEM, *N*-ethylmaleimide; PCMB, *p*-(chloromercuri)benzoate; IAA, iodoacetic acid. [c] Values in this table are expressed as percentage ratio relative to control.

Table V. Thermodynamic Parameters[a] for Thermal Inactivation of Plasma Factors XIII and XIIIa

enzyme	temp, °C	$K_d \times 10^5$, 1/s	ΔG^*, kcal/mol	E_a, kcal/mol	ΔS^*, cal/(K mol)	ΔH^*, kcal/mol
XIII	55	5.0	25.7	94.9	209.1	94.3
XIII	70	2576.0	22.7	94.9	208.5	94.2
XIIIa	55	0.6	24.1	60.9	110.2	60.3
XIIIa	70	4552.0	22.3	60.9	110.5	60.2

[a] K_d, thermal denaturation rate; ΔG^*, change in free energy; E_a, activation energy; ΔS^*, change in entropy; ΔH^*, change in enthalpy.

Optimal Temperature. After being activated by thrombin at 37 °C for 20 min, 0.25 mL of enzyme solution was added to the reaction mixture [1.2 mL of 0.1 M Tris-HCl buffer (pH 7.5), 0.2 mL of 0.1 M calcium chloride, 0.05 mL of 0.25 mM monodancylcadaverine, and 0.2 mL of β-substrate] and then incubated at 5, 15, 25, 35, 45, 55, and 65 °C for 30 min. The reaction was terminated by 0.1 mL of 1.0 M ammonium sulfate solution. The enzyme activity was measured according to the method of Takagi et al. (1986).

Effect of Metal Ions. The purified factor XIII was first modified by thrombin, and then various metals (5 or 10 mM) suspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ or 1 mM EGTA were added. The resulted samples were incubated at 37 °C for 5 min, and then the activity was measured according to the method of Takagi et al. (1986).

Effect of Inhibitors. Factor XIII and factor XIIIa suspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ or 1 mM EGTA were incubated with 0.1 mM blocking reagents *N*-ethylmaleimide (NEM), *p*-(chloromercuri)benzoate (PCMB), or iodoacetic acid (IAA) at 37 °C for 10 min. The residues of blocking reagents were neutralized by 5 mM dithiothreitol (DTT). The remaining activities were then measured according to the method of Takagi et al. (1986).

Thermal Stability. Factors XIII and XIIIa suspended in 0.1 M Tris-HCl buffer (pH 7.5) were incubated at various temperatures (50–70 °C) for 80 s–4 h. At definite time intervals, the enzyme solutions were cooled immediately in iced water for 10 min. The remaining activities were measured according to the method of Takagi et al. (1986). The rate constants for thermal inactivation (K_d) of factors XIII and XIIIa at various temperatures were calculated as follows: $K_d = (\ln A_0 - \ln A_t)1/t$ (A_0 and A_t, activities before and after *t*-s incubation). The energy of activation (E_a) was obtained from the slopes [slope $= -E_a/R$] of the inactivation curves in Arrhenius plot. The thermodynamic parameters were calculated according to Eyring transition-state theory. ΔH^*, ΔG^* and ΔS^* were calculated as

$$\Delta H^* = E_a - RT \quad (1)$$

$$\Delta G^* = RT(\ln (K_b/h) + \ln T - K_d) \quad (2)$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (3)$$

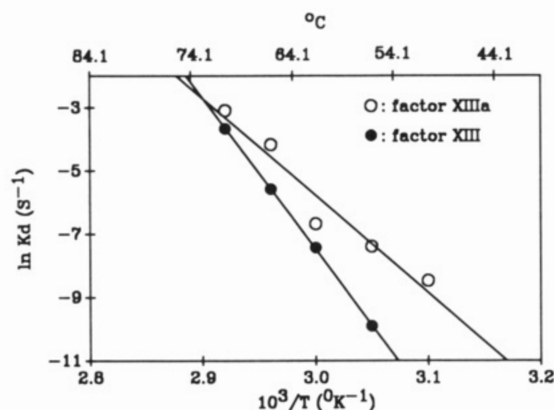

Figure 6. Arrhenius plots for thermal inactivation of plasma factors XIII and XIIIa.

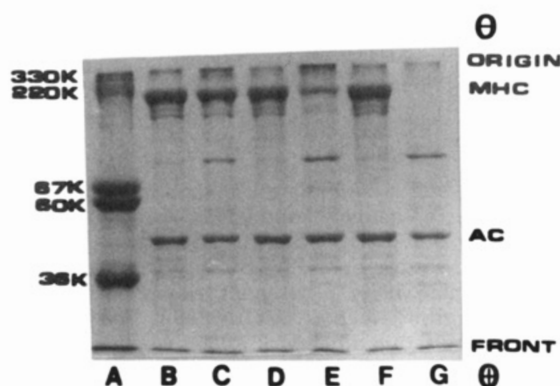

Figure 7. Electrophoretic pattern of polymerization of actomyosin by pig plasma factor XIIIa [standard proteins (A); actomyosin incubated at 5 °C for 3 days (B), at 30 °C for 15 min (D), and at 37 °C for 15 min (F); actomyosin with factor XIIIa incubated at 5 °C for 3 days (C), at 30 °C for 15 min (E), and at 37 °C for 15 min (G); MHC, myosin heavy chain; AC, actin].

where ΔH^*, ΔG^*, and ΔS^* are the enthalpy, free energy, and entropy changes, respectively; R, K_b and h are the gas constant (1.98 cal/deg), Boltzmann constant (1.38×10^{-16} erg/deg), and Planck constant (6.62×10^{-27} erg s), respectively; and T is the absolute temperature (K).

Cross-Linking of Actomyosin. Actomyosin (AM) was extracted from dorsal muscle of mackerel according to the method of Noguchi (1974). To the AM dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 0.6 M NaCl and 10 mM $CaCl_2$ was added factor XIIIa. The reaction mixture was incubated at 5 °C for 3 days and at 30 and 37 °C for 15 min. The resulted samples were boiled with an equal volume of sample buffer [2% SDS, 5% mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8)] for 3 min. About 25 μg of protein was loaded on the gels of SDS–PAGE. The electrophoretic pattern was used to evaluate the degree of cross-linking caused by factor XIIIa.

Preparation of Minced Surimi. Frozen mackerel, which was stored at −20 °C for 2 months on board, was purchased from a fish market in northern Taiwan. After thawing to a body temperature of 0 °C using running tap water (about 25 °C), samples were headed, eviscerated, skinned, and deboned. The resulted muscle was washed with 4 volumes of chilled 0.4% $NaHCO_3$ solution for 5 min and then drained by centrifugation. Samples were again washed with 4 volumes of chilled water and finally with 2 volumes of 0.3% NaCl solution. The washed meat was drained to a moisture content of 78% by centrifugation and mixed with 3% sucrose, 3% sorbitol, and 0.2% polyphosphate (50% sodium polyphosphate and 50% potassium pyrophosphate) using a mechanical pestle–mortar. The minced mackerel was packaged in polyethylene bags (5 kg each) and stored at −40 °C until use.

Preparation of Crude Plasma Factor XIII. Fresh pig blood was obtained immediately after slaughter from a local slaughterhouse. The anticoagulant EDTA was added to a concentration of 1000 ppm. After 10 min of centrifuging at 1000*g* twice, the supernatant was used as crude factor XIII. After lyophilizing at a plate temperature of 30 °C for 48 h, the crude enzyme powder was stored at −40 °C until use.

Effects of Crude Factor XIII on the Gelation of Minced Mackerel. The effect of the levels of crude enzyme added, pH, and setting time at 37 °C on the gelation of minced mackerel was investigated using 3^3 factorial experimental design and analyzed using response surface analysis (RSA): $y = f(x_1,x_2,x_3)$, where y is a dependent variable (gel strength) and x_1, x_2, and x_3 are independent variables (x_1, pH of minced meat; x_2, setting time; x_3, crude enzyme added) (Table I). Regression coefficients were calculated by fixing one value and solving the equation for a combination of the other two factors using the SAS program. The three-dimensional surface response curves were plotted using the Statgraph program (Statgraph Software). Frozen surimi was thawed to a temperature of −3 °C and sliced. The pH of sliced surimi was adjusted to 7.0, 7.5, and 8.0, and the surimi was ground with 0, 0.2, and 0.4% crude plasma factor XIII powder for 10 min and 2.5% NaCl was added. Grinding was resumed for another 30 min. Finally, 0.2% $CaCl_2$ was added, and samples were stuffed into a poly(vinyl chloride) tubes (diameter, 1.5 cm). Samples were then heated at 90 °C for 30 min and cooled at 5 °C for 12 h. The gel strength of the samples was measured using a rheometer (Model CR-200D, Sun Scientific Co., Ltd.; plunger, 5 mm; speed, 60 mm/min).

RESULTS AND DISCUSSION

Purification and Properties. Since calcium could induce the formation of blood clots and consequently causes the loss of plasma factor XIII activity, the EDTA was employed as a stabilizing agent during purification in this study. In the preliminary study, although the total dansylcadaverine incorporating activity of the precipitate on 30% saturation of ammonium sulfate (AS) was slightly higher than that on 20% saturation of AS, the specific activity of that on 30% saturation of AS was significantly lower than that on 20% saturation (data not shown). Accordingly, 20% saturation of AS was selected to precipitate the crude plasma factor XIII in this study. As summarized in Table II, the purification of plasma factor XIII from pig blood included three main procedures: ammonium sulfate fractionation, heat treatment, and DEAE-Sephacel chromatography.

During the ammonium sulfate fractionation, although the total activity and yield of enzyme decreased, most of the other proteins such as albumin were removed. According to Loewy et al. (1961b), the native factor XIII usually forms a complex with fibrinogen and the isoelectric point (p*I*) of fibrinogen is around 5.4. Thus, the second AS fractionation was performed at pH 5.4 to precipitate the factor XIII–fibrinogen complex. After the removal of fibrinogen by heating at 56 °C for 3 min, the specific activity of factor XIII increased significantly. The enzyme was finally purified by DEAE-Sephacel chromatography eluting with a linear gradient of 0.0–0.3 M NaCl in 50 mM Tris-HCl buffer (pH 8.0). A 121-fold purification was achieved at this step (Table II).

After the DEAE-Sephacel chromatography, the purified enzyme revealed a single band on SDS–PAGE (Figure 1). The molecular weight (MW) of the purified enzyme was 320 000, estimated by Sepharose CL-6B chromatography (Figure 2). However, when the enzyme was subjected to SDS–PAGE, only a 75 000 single band (Figure 1) was observed. The monodansylcadaverine incorporating activity of this purified enzyme could not be detected without thrombin–calcium activation (data not shown). After limited proteolysis at 37 °C for 20 min by thrombin, the MW of one subunit of this enzyme decreased from 75 000 to 72 000 (Figure 4b). Furthermore, the densities of these two subunits were identical even after 3 h of proteolysis by thrombin (Figure 3C–F). These phenomena suggested that the purified enzyme was factor XIII and had two different subunits: one (a chain) could and another (b

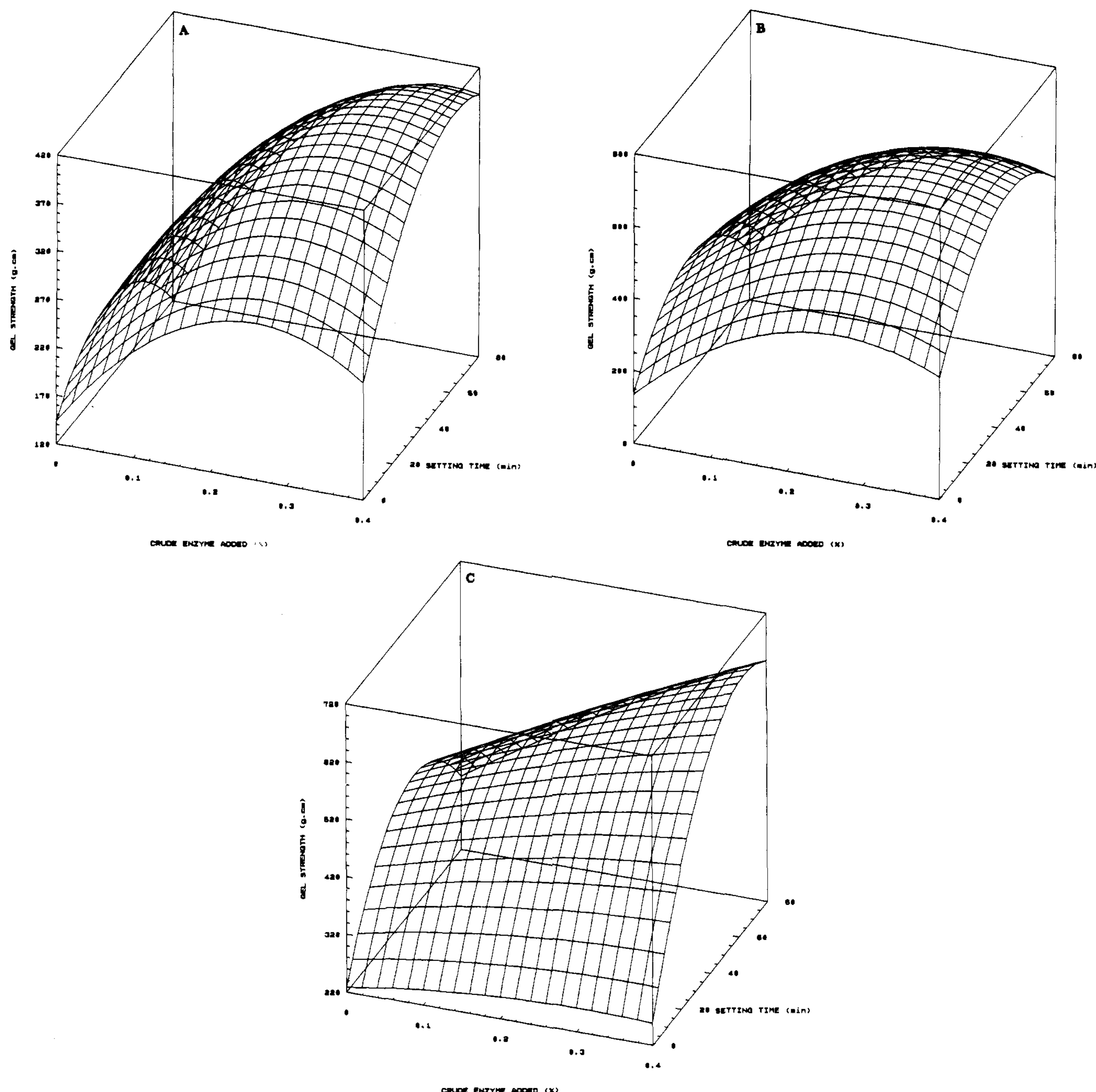

Figure 8. Response surface plots of gel strength of minced mackerel as a function of crude enzyme added and setting time at pH 7.0 (A), pH 7.5 (B), and pH 8.0 (C).

chain) could not be proteolyzed by thrombin. From the MWs of 320 000 and 75 000 estimated by Sepharose CL-6B chromatography and SDS-PAGE, respectively, the purified factor XIII was considered to have a structure with a_2b_2 subunit. Both a and b chains had identical MWs of 75 000.

Human plasma factor XIII was purified to electrophoretical homogeneity after DEAE-cellulose (Loewy et al., 1961a) and Sepharose 6-B chromatographies (Cooke and Holbrook, 1974b). The MW of this zymogen had been determined to be 310 000 (Schwartz et al., 1971), 320 000 (Schwartz et al., 1973), 340 000 (Loewy et al., 1961a), and 350 000 (Cooke and Holbrook, 1974b). Although there were different molecular weights of human plasma factor XIII reported by researchers, this zymogen contained four subunits (a_2b_2) with identical MWs of 81 000 ± 4000.

Optimal Temperature. The optimal temperature for incorporating the monodansylcadaverine to β-casein of the purified factor XIIIa (activated by thrombin–calcium at 37 °C for 20 min) was 55 °C, which was higher than that of microbial transglutaminase (50 °C using hydroxylamine and CBZ-glutaminylglycine as substrates) (Ando et al., 1989). However, the activity of this enzyme around 25–55 °C was over 70% relative to that at 55 °C (Figure 5). This phenomenon suggested that the physiological coagulation of blood can occur in rather broad environmental temperatures.

Effect of Various Metal Ions. The purified factor XIIIa was inhibited by Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, and Fe^{3+} but not by K^+, Na^+, Mg^{2+}, and Sr^{2+}, when it coexisted with 10 mM calcium (Table III). According to Curtis and Lorand (1976), the calcium induced the dissociation of thrombin-modified factor XIII and consequently exposed the cysteine in the active center of the a chain to substrates. The metals (Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+}, or Fe^{3+}) might react with the functional residues on active center and therefore inhibit the activity. Although some studies observed that Mn^{2+}, Sr^{2+}, Ba^{2+}, and Mg^{2+} could replace Ca^{2+} to initiate the formation of insoluble fibrin or the exposure of reactive sulfhydryl of

factor XIII treated by thrombin (Robbin, 1944; Gottlieb et al., 1961; Curtis et al., 1974), the thrombin-treated factor XIII could only be activated by calcium in this study.

Effect of Inhibitors. The factor XIIIa was completely inhibited by NEM, PCMB, and IAA in the presence of 10 mM Ca^{2+} and partially inhibited by these reagents in the presence of EGTA. However, the factor XIII was only partially inhibited by these thiol-blocking reagents (Table IV). According to these data, the active center of this enzyme might contain cysteine and exist inside the molecule. The inhibition of factor XIIIa by thiol reagents in the presence of Ca^{2+} suggested that the conformation of factor XIIIa changed after the addition of Ca^{2+} and subsequently caused the exposure of the masked SH to thiol reagents. From the study of Brenner and Wold (1978), the erythrocyte TGase was also completely inhibited by thiol-blocking reagents.

Thermal Stability of Factors XIII and XIIIa. The inactivation curves of factors XIII and XIIIa in Arrhenius plot were crossected at 71.8 °C (Figure 6). From these two inactivation curves, the stability of factor XIII was higher than that of factor XIIIa. The thermodynamic parameters (ΔG^*, ΔS^*, ΔH^*, and E_a) of factor XIII at 55 and 70 °C were significantly higher than those of factor XIIIa (Table V). The higher ΔH^* of factor XIII indicated that during denaturation the factor XIII absorbed more heat and caused higher randomness. Assuming the average ΔH^* per bond to be 5 kcal/mol (Richardson and Hyslop, 1985), the number of noncovalent bonds broken during denaturation would be 19 for factor XIII and 12 for factor XIIIa. The difference in free energy (ΔG^* between factors XIII and XIIIa decreased with the increase of temperature. Both free energies (ΔG^*) would be equal when the temperature reached 71.8 °C. Accordingly, the thermal stability of factor XIII was higher than that of factor XIIIa at temperatures below 71.8 °C. This might be because factor XIII underwent limited proteolysis by thrombin and dissociated into four subunits (two a, two b) by calcium, which consequently lost the protective subunits (released peptides or b chain).

Cross-Linking of Actomyosin. As indicated in Figure 7, the myosin heavy chain in mackerel AM decreased after 15 min of incubation with factor XIIIa at 30 and 37 °C, while the corresponding polymers were observed on the top of gels. No distinct change in the density of actin was obtained. These phenomena suggested that the purified factor XIIIa could catalyze the cross-linking of myosin heavy chains. The actin might not be involved in this cross-linking reaction. According to Cohen et al. (1979), the maximum cross-links per mole of human platelet myosin caused by human plasma factor XIIIa was 19. The Ca-ATPase was almost not altered, even though 50–60% of myosin was cross-linked (Cohen et al., 1979). However, according to Mui and Ganguly (1977) and Kahn and Cohen (1981), human plasma factor XIIIa catalyzed the cross-linking of actin with fibrin. It is well-known that myosin contains both acyl acceptor (γ-glutamine) and acyl donor (ϵ-lysine side chain). It was considered to be a good substrate for TGase. Since the glutaminyl–acyl cross-linking reaction would be influenced by amino acid residues around the reactive glutamines (Gorman and Folk, 1980), the primary structures of proteins were more important than their absolute lysine and glutamine contents in evaluating their reaction ability (Kurth and Rogers, 1984).

Generally, the setting temperature of minced fish products is about 40–50 °C. The quality was highly correlated with the degree of cross-linking of myosin heavy chain (Numakura et al., 1985; Nishimoto et al., 1987). Accordingly, the purified factor XIIIa would be useful for improving the gel-forming ability of minced fish products. Although plasma factor XIII existed as zymogen and required thrombin–calcium to activate, calpain (an indigenous protease to fish muscle) and other proteases could also activate this zymogen (data not shown). This phenomenon suggested the possibility of utilization of this zymogen for minced fish processing.

Effect of Crude Factor XIII on the Gelation of Minced Mackerel. Catalysis of myosin heavy chain cross-linking suggested that plasma factor XIII could improve the quality of minced fish meat. At pH 7.0, the gel strength of samples without and with plasma crude TGase increased with the duration of setting time and reached a maximum after 60 min of setting at 37 °C. However, during longer setting, deterioration was observed on the samples without plasma crude TGase and almost no deterioration occurred on samples with 0.2 and 0.4% crude enzyme. According to the gel strength, the optimal crude TGase for minced mackerel at pH 7.0 was 0.2% (Figure 8A). Similar results were obtained on samples at pH 7.5 and 8.0; however, the gel strength of these samples was higher than that at pH 7.0 (Figure 8B,C). This might be because the alkaline pH was good for the TGase transfer reaction.

No deterioration occurred in mackerel sol with crude plasma factor XIII during setting process. The deterioration that occurred in heat-induced fish gel was considered to result from the action of indigenous alkaline proteases (Makinodan and Ikeda, 1971; Ikeda et al., 1974; Iwata et al., 1979; Deng, 1981; Lainer et al., 1981; Makinodan et al., 1985; Boye and Lainer, 1988). Accordingly, the cross-linkage of myosin heavy chain formed by plasma factor XIIIa might have a steric hindrance to prevent proteolysis. In addition, the isopeptide bond formed by TGase had resistance against hydrolysis by proteases such as papain, chymotrypsin, trypsin, leucine aminopeptidase, and pronase (Folk and Finlayson, 1977).

In summary, factor XIIIa had cross-linking ability (Figure 7), high thermal stability (Figure 6), and broad optimal temperature for activity (Figure 5). The addition of crude plasma factor XIII significantly increased the gel strength of minced mackerel. These data suggested that this enzyme could be applied to minced fish processing.

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