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Effects of High Concentration of Salts on the Esterase Activity and Structure of a Kiwifruit Peptidase, Actinidain

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Effects of salts on the activity and stability of actinidain were examined. With increasing salt concentration up to 0.5 M, the activity (k_{cat}/K_m) for *N*-α-Cbz-L-lysine *p*-nitrophenyl ester decreased to 40% of that in the absence of salt. The inhibitor constant K_i of LiCl, NaCl, and KCl was 0.16–0.43 M. With 3 M KCl and NaCl, the specificity constant k_{cat}/K_m recovered to 110 and 75%, respectively. No re-activation was observed with LiCl. The inhibition and re-activation were dependent on the changes in both K_m and k_{cat}, whereas no CD change was observed. The tryptophan fluorescence of actinidain was not affected by 0–0.5 M salt, but a considerable decrease in its intensity was observed with increasing salt concentration from 0.5 to 3.0 M. These results suggest that the inhibition observed with the lower salt concentration (<0.5 M) is due to attenuation of the electrostatic interaction between the enzyme and substrate, and the higher concentration (0.5–3.0 M) induces structural change in the states of tryptophan residues, which is associated with the re-activation. Actinidain keeps considerably high activity and stability even in the presence of 3 M salts.

Key words: actinidain, cysteine peptidase, esterase activity, salt effects, tryptophan fluorescence.

Actinidain (previously named actinidin, [EC 3.4.22.14]) is a cysteine peptidase purified from kiwifruit and a member of the papain superfamily, being similar to other plant cysteine peptidases such as papain, bromelain, and ficin in its structure and peptidase activity. Actinidain is composed of 220 amino acid residues with molecular mass of 23.5 kDa (*1–3*). It shows esterase activity as well as peptidase activity, and hydrolyzes most strongly the amide and ester bonds at the carboxyl side of a lysine residue. The enzyme has been used in the food industry for softening meat and digesting meat to produce amino acids (*3*). Recently, we have demonstrated that actinidain can be used for processing the atellocollagen of yellowfin tuna to produce collagen peptides (*4*). This must be related to the high specificity of actinidain for lysyl residues. In the food industry, the enzyme is often used in the presence of high salt concentrations, or at acidic or alkaline pH, high temperature, high viscosity, *etc*. In the purification and characterization of actinidain, we found that its activity is sensitive to the salt concentration added to the reaction medium. This seems to be a suitable clue to investigate the molecular mechanism of the enzymatic activity and structure–function relationship of actinidain, and will also enable us to utilize actinidain effectivelyl in various processes in the food industry. Since actinidain is an enzyme contained in an edible fruit, its safety for food processing is guaranteed. In the present study, we examined the effect of high concentration of salts on the activity and structure of actinidain in order to utilize this enzyme in future for food processing in such unconventional media.

Salt ions have various effects on proteins. They increase the solubility of proteins (salting in) and shield the ionic charges of the protein surface as counter-ions. They also decrease the solubility of proteins as more salt is added (salting out). Hydrophobic interactions between proteins are increased with increasing salt concentrations (*5*, *6*). Generally, we have used low salt concentrations to analyze the interaction of proteins, because the salt ions (Na^+, K^+, Cl^- *etc*.) stabilize the protein structure. However, high salt concentrations would have other effects on the protein-ligand interactions and protein structure. Salt ions added to the enzyme reaction mixture may influence the enzyme activity. It is well known that most salts are an inhibitory factor for enzymes, but some enzyme activities are slightly enhanced (*7*, *8*). Changes in the enzyme structure would be directly responsible for such influences (*9*). On the other hand, the enzymes from extremely halophilic archaea are stable and active in more than 1 M salt concentration (*10*, *11*). It appears that the halophilic enzymes keep the folded structure as a result of reducing electrostatic repulsion and enhancing hydrophobic interaction. We have found that metallopeptidases, thermolysin [EC 3.4.24.27] and matrix metallopeptidase 7 (matrilysin; [EC 3.4.24.23]), are activated in an exponential fashion with increase in salt concentration, and the activity was enhanced 10 times or more at 4 M NaCl in comparison with that in the absence of salt (*5*, *12–16*). The activation effect of these metallopeptidases appear to depend strongly on the Michaelis constant K_m for matrilysin and the catalytic activity k_{cat} for thermolysin (*17*, *18*). However, the

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activation mechanisms of these metallopeptidases remain to be resolved.

Actinidain is enveloped in large negative electric charges at the optimal pH (around 6), and it is considered that the activity and structure might be affected by the presence of high concentration of salt. The X-ray crystallographic analysis was reported at 1.7 Å-resolution (*2*). Amino acid residues Cys25 and His162 constitute the catalytic ion pair (a cysteinyl thiol-histidinyl imidazole dyad) and are located in the L-domain (amino acid sequences 19–115 and 214–220; three α-helices) and the R-domain (amino acid sequences 1–18 and 116–213; a twisted antiparallel β-sheet barrel), respectively (*2*, *3*, *19–22*). The S1 subsite of the active site determines the substrate specificity for a cationic residue. However, the specificity is fairly wide, and bonds involving an amino acid other than proline can be hydrolyzed at the carboxyl side. The S2 subsite prefers a hydrophobic residue (Leu, Val, or Phe, but not Tyr) (*19*). Actinidain contains six tryptophan residues (W7, W26, W122, W178, W184, and W188) (*1*), and these could be a suitable probe for exploring the conformational change of this enzyme.

In the present paper, we describe the effect of neutral salts (KCl, NaCl, and LiCl) on the activity and stability of actinidain. The activity (k_{cat}/K_m) was found to show interesting behavior with the change in the salt concentration: it is minimal at the salt concentration of 0.5 M, but it is re-activated with increasing salt concentration from 0.5 to 3.0 M. This re-activation is suggested to be associated with the conformational changes as examined with fluorescence and circular dichroism (CD) spectra. The results described in this paper may provide useful hints for the application of actinidain in digestion of food proteins, food processing and biochemical processes in the presence of high concentration of salts.

MATERIALS AND METHODS

Materials—Actinidain was purified from kiwifruits by anion-exchange chromatography (TSKgel BioAssist Q, Tosoh, Tokyo) according to the method of Morimoto *et al.* (*4*). The concentration of actinidain was determined by measurements of UV absorbance with the molar absorption coefficient at 280 nm, ε_{280}, of 55,100 $M^{-1}\cdot cm^{-1}$ (*4*). To activate the enzyme, 1 mM actinidain was incubated in 20 mM sodium phosphate buffer (pH 6.5) containing 10 mM dithiothreitol for 1 h at 25°C.

Actinidain-Catalyzed Hydrolysis of N-α-*Cbz-*L*-Lys* p-*Nitrophenyl Ester Substrate*—The esterase activity of actinidain was estimated by measuring the absorbance at 325 nm of *p*-nitrophenol generated by the reaction with *N*-α-Cbz-L-lysine *p*-nitrophenyl ester (Sigma-Aldrich, St. Louis, MO) in 20 mM sodium phosphate buffer (pH 6.5) containing 1 mM EDTA at 25°C. Time courses of the catalytic reaction were monitored with a UV/VIS spectrophotometer UV-2200 (Shimadzu, Kyoto). In this study, we added 0–3.0 M (final concentration) LiCl, NaCl, or KCl into the reaction solution. The final concentration of *N*-α-Cbz-L-lysine *p*-nitrophenyl ester was adjusted to 50, 100, 200, and 300 μM, and 50 nM actinidain was used for the hydrolysis. The initial velocity (v_o) was estimated from the time course of the reaction using the molar adsorption coefficient at 325 nm, ϵ_{325}, of 9,310 $M^{-1}\cdot cm^{-1}$ of *p*-nitrophenol at pH 6.5. The kinetic parameters, K_m and k_{cat}, were determined according to the Michaelis-Menten equation using the Hanes-Woolf plot ($[S]/v_o$ *vs.* $[S]$). The actinidain solution was adjusted to 0.5 mg/ml with 20 mM sodium phosphate buffer (pH 6.5) in the presence of 0–3.0 M LiCl, KCl, or NaCl.

CD and Fluorescence Spectra Measurements—The CD spectra of actinidain (21.3 μM) were measured by scanning in the range of 200–300 nm with a Jasco J-600 CD spectropolarimeter (Tokyo) and the data were analyzed according to the procedures previously described (*23*). The solution was maintained at 25°C by circulating water. The fluorescence changes in actinidain on addition of 0–3.0 M salt were recorded using a Shimadzu RF-5300PC spectrofluorometer (Kyoto). The fluorescence spectra of actinidain (0.426 μM) in 20 mM sodium phosphate buffer (pH 6.5) were measured in the presence of salt at 25°C in a 1-cm path length cell. Actinidain was excited at 295 nm, and the emission spectra were recorded from 310 to 405 nm. Solvent blanks were subtracted from the spectra of actinidain.

RESULTS AND DISCUSSION

Effect of Salts on the Actinidain-Catalyzed Hydrolysis of N-α-*Cbz-*L*-Lys* p-*Nitrophenyl Ester*—Actinidain activity in the hydrolysis of *N*-α-Cbz-L-Lys *p*-nitrophenyl ester was decreased with increasing the concentration of salts examined (LiCl, NaCl, and KCl) (Fig. 1). The minimal activity was found at 1.0–1.5 M LiCl, 0.5–1.2 M NaCl, and 0.5–0.8 M KCl, depending on the substrate concentration used for the activity assay. The minimal activity was 50–70% of that observed in the absence of salt. The activity increased, however, when the salt concentration increased from that (0.5–1.5 M) giving the minimal activity to 3.0 M (Fig. 1). The relative activity to the minimal one is plotted against the salt concentration in order to illustrate clearly the inhibition in the presence of lower salt concentration and the re-activation in the presence of higher salt concentration (Fig. 2).

The inhibition of actinidain by salts in the concentration range of 0–0.5 M was analyzed by Dixon plots ($1/v_o$ *vs.* [inhibitor]) (Fig. 3). The straight lines crossed in the second quadrant but not on the horizontal axis, suggesting that the inhibition type is competitive. The inhibitor constant K_i values of LiCl, NaCl, and KCl were determined to be 0.13 ± 0.02, 0.43 ± 0.03, and 0.35 ± 0.03 M, respectively. The inhibitory activity of the salts is in the order: LiCl > KCl ≈ NaCl.

The re-activation by each salt was more evident at lower substrate concentration, and KCl and NaCl seemed to be more effective in the re-activation than LiCl (Fig. 2). At lower substrate concentration such as 50 μM, the activity increased with increasing salt concentration up to 3.0 M, but at the higher substrate concentration the activity was saturated at the salt concentration of 1.5–2.0 M, and at 3.0 M salt it decreased relative to that at 2.0 M salt. At the concentration of 3.0 M of the three salts examined, the degree of re-activation was higher at the lower substrate concentrations (50, 100, and 200 μM) in the order: KCl > NaCl > LiCl. This is the same order as that reported for the remarkable activation of thermolysin by these salts (*12*, *24*). This order does not follow the orders of chaotropic ions or Hofmeister series, which suggests that the re-activating effect of the cations (Na^+ and K^+) is not

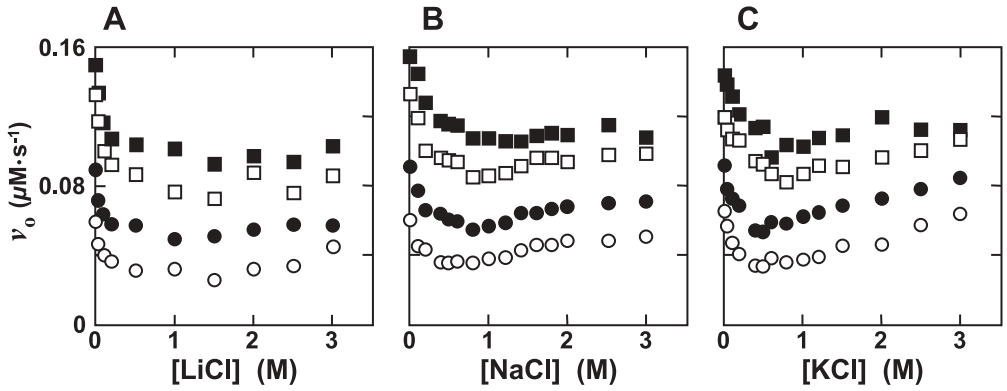

Fig. 1. **Dependence of actinidain activity in the hydrolysis of *N*-α-Cbz-L-lysine *p*-nitrophenyl ester on the salt concentration.** The hydrolysis was performed in 20 mM phosphate buffer (pH 6.5) containing 1 mM EDTA at 25°C at the salt concentration indicated. The reaction was monitored at 325 nm for 100 s, and the initial velocity v_o was estimated from the initial part of measurement. Salts: A, LiCl; B, NaCl; and C, KCl. The concentrations of *N*-α-Cbz-L-lysine *p*-nitrophenyl ester were 50 (open circles), 100 (solid circles), 200 (open squares), and 300 (solid squares) μM.

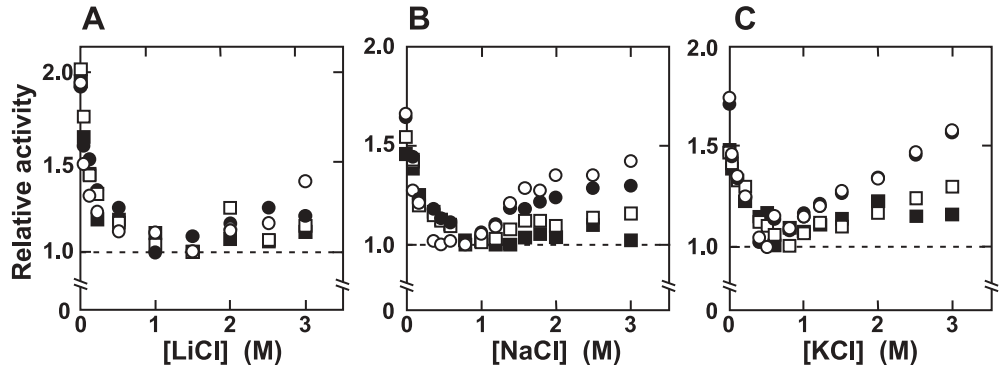

Fig. 2. **Dependence of the relative actinidain activity on the salt concentration.** The data shown in Fig. 1 were re-plotted by setting the minimal activities at the respective substrate concentrations to 1.0. The reaction conditions and symbols for substrate concentrations were the same as shown in Fig. 1.

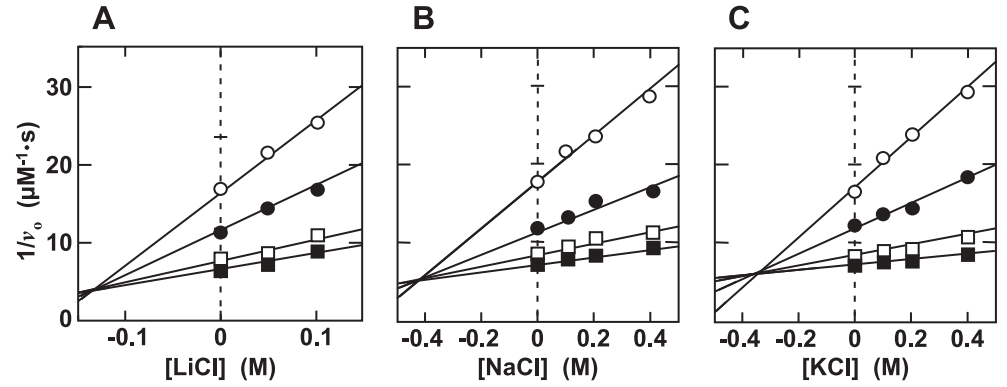

Fig. 3. **Dixon plots for the inhibition of actinidain activities by salts.** The reaction conditions are the same as shown in Fig. 1. Salts: A, LiCl; B, NaCl; and C, KCl. The concentrations of *N*-α-Cbz-L-lysine *p*-nitrophenyl ester were 50 (open circles), 100 (solid circles), 200 (open squares), and 300 (solid squares) μM.

only due to their chaotropic effect on the medium but also related with the specific interaction with actinidain.

Effects of salts on the kinetic parameters (K_m, k_{cat}, and k_{cat}/K_m) were examined (Fig. 4). The effects on K_m were similar with three salts examined. The K_m increased to 140–150% of that in the absence of salt (0.10 mM) with increasing the salt concentration up to 0.5 M, and then decreased to 100% with LiCl and NaCl and to 60% with KCl when the salt concentration increased from 0.5 to 3.0 M (Fig. 4A). The k_{cat} decreased also to 60–80% of that in the absence of salt (2.0 s^{-1}) with increasing the salt concentration from 0 to 0.5 M. With NaCl and KCl, the value increased slightly with increasing salt concentration up to 2.0 M and decreased with its increase from 2.0 to

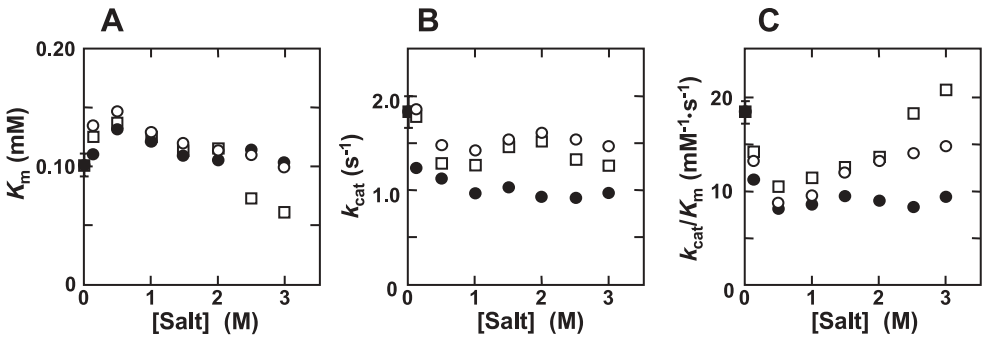

Fig. 4. **The kinetic parameters of actinidain activity in the presence of salts.** Experimental data shown in Fig. 1 were analyzed by Hanes-Woolf plot to determine the kinetic parameters: Panels A, K_m; B, k_{cat}; and C, k_{cat}/K_m. Salts: LiCl (solid circles); NaCl (open circles); and KCl (open squares).

3.0 M; but with LiCl, the value was constant (1.0 s^{-1}) (Fig. 4B). It is obvious that salts used in this study affect both k_{cat} and K_m. Consequently, the k_{cat}/K_m value decreased to 35–50% of that in the absence of salts (18 mM^{-1} s^{-1}) with increasing salt concentration from 0 to 0.5 M, and increased to 110% and 75% with increasing KCl and NaCl concentrations up to 3.0 M. However, almost no change in k_{cat}/K_m was observed with increasing the LiCl concentration from 0.5 to 3.0 M. The re-activation observed with 0.5–3.0 M KCl and NaCl should be due to the decrease in K_m and the increase in k_{cat}. It is considered that K_m is a measure of the stability of enzyme-substrate (ES) complex, while k_{cat} similarly represents the transition state of the enzyme reaction. Accordingly, both the ES complex and the transition state might be destabilized by the addition of 0–0.5 M salt, and both might be stabilized by the addition of 0.5–3.0 M salt. The stabilization of the ES complex is more effective with KCl than NaCl or LiCl, while that of the transition state is effective with salts in the order of NaCl > KCl > LiCl. The order of salts in the re-activation of k_{cat}/K_m is KCl > NaCl > LiCl (Fig. 4C). It is worthwhile noting that the order is different from that in the inhibition observed in the salt concentration of 0–0.5 M (Fig. 3).

As the isoelectric point (p*I*) of actinidain is reported to be 3.1 (*3*), the electrostatic charge of the actinidain surface would be totally negative in the reaction solution of pH 6.5. Indeed, the index of the ratio of the number of negatively charged residues and that of positively charged ones, (Asp+Glu)/(Lys+Arg), of actinidain is calculated to be 2.3 [=(13+12)/(6+5)] (*22*), and therefore, actinidain should be negatively charged under the reaction conditions. Under these conditions, the substrate *N*-α-Cbz-L-lysine *p*-nitrophenyl ester is positively charged. Therefore, the attraction between the enzyme and substrate might be stronger in the absence of salts. However, since counter-ions in the reaction solution shield the charged groups on the enzyme and substrate, and thus reduce electrostatic attraction between them, K_m is expected to increase with increasing the salt concentration. Generally, as the shielding effect is saturated with 0.3–5 M salts (*6*), K_m should be constant in the range of the salt concentration from 0.5 to 3.0 M. The observation that K_m decreases, namely, the ES complex stabilizes, with increasing the salt concentration from 0.5 to 3.0 M (Fig. 4A) suggests that factors other than the charge-shielding effect might be involved in the stabilization of the ES complex. One possible factor is hydrophobic interaction (*6*). A hydrophobic interaction between the P2 residue (the Cbz group in the present case) of substrate and the S2 residue of actinidain is likely to be dependent on the salt concentration. Salt enhances the hydrophobic interaction between enzyme and substrate (*6*, *25*). The contribution of the effect of the electrostatic interaction between the enzyme and substrate to the observed k_{cat}/K_m values was eliminated by extrapolating the plot obtained at 0.5–3.0 M salt to 0 M salt. The k_{cat}/K_m obtained at 0 M salt, being defined as the intrinsic k_{cat}/K_m, is 9 mM^{-1} s^{-1} (Fig. 4C). The increase in k_{cat}/K_m from the intrinsic value at 0 M salt to the values obtained at 3.0 M salt might be caused by a salt effect on the hydrophobic interaction between the active site of actinidain and the substrate. The behavior observed especially with KCl seems to be of an exponential nature, which is similar to the effect of salt observed on the k_{cat}/K_m of thermolysin and K_m of matrilysin (*17*, *18*). This means that the salt activation of the intrinsic k_{cat}/K_m of actinidain is similar to that reported previously, although the thermolysin activation is induced by the increase in k_{cat} and matrilysin activation is induced by the decrease in K_m (*18*).

Effect of Salts on the CD Spectra of Actinidain—When the salt concentration was changed from 0 to 3.0 M, the CD spectrum in the far UV region was not much affected, suggesting that the secondary structure of actinidain was not much changed in the presence of 3.0 M NaCl (Fig. 5). The α-helix content of actinidain was estimated to be 27% from the intensity at 222 nm, and this value is consistent with the value evaluated from the actinidain crystal structure (*2*). The CD spectra of actinidain at LiCl and KCl concentrations up to 3.0 M showed the same features (data not shown). Accordingly, the neutral salts examined have little effect on the conformation of actinidain. These data support the finding that actinidain retains its activity well in the presence of high concentration of salt.

Effect of Salts on the Fluorescence Spectra of Actinidain—Fluorescence spectra of tryptophyl residues of actinidain were measured in the presence of NaCl or KCl concentrations ranging from 0 to 3.0 M (Fig. 6). The maximal intensity was observed at 330 nm. No change in the spectra was observed at the concentration of NaCl or KCl of 0 to 0.5 M, suggesting that 0.5 M NaCl or KCl did not affect the states of the six tryptophyl residues of actinidain. The fluorescence intensity at 330 nm decreased to 80% when the NaCl or KCl concentration increased from 0.5 to 3.0 M. The emission peak at 330 nm was not shifted, but

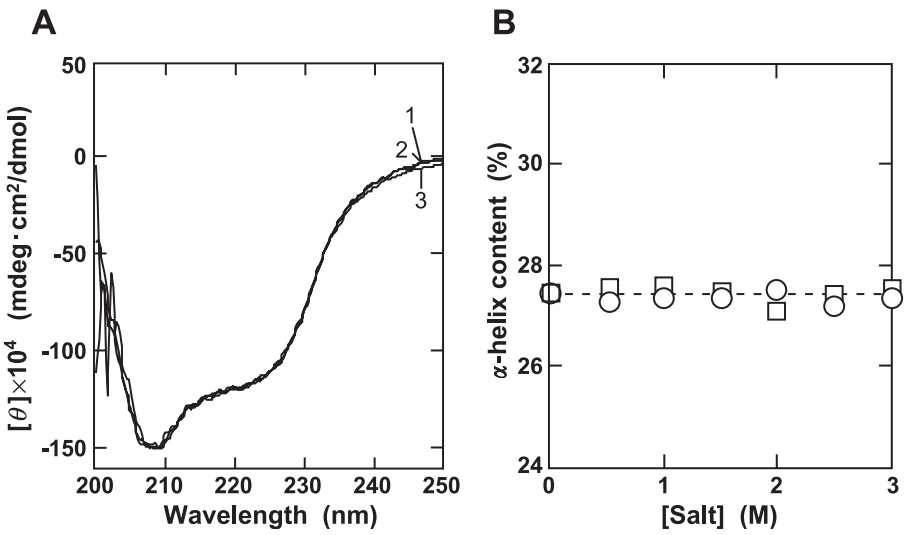

Fig. 5. **Far-UV CD spectra of actinidain and α-helix contents in the presence of salts.** Panel A: CD spectra of actinidain (21.3 μM) were measured at 25°C in 15 mM phosphate buffer (pH 6.5). Spectra 1, 2, and 3 were recorded at the NaCl concentration of 0 M, 1.0 M, and 3.0 M, respectively. Panel B: α-Helix contents in the presence of salts. Salts: NaCl (circles); and KCl (squares).

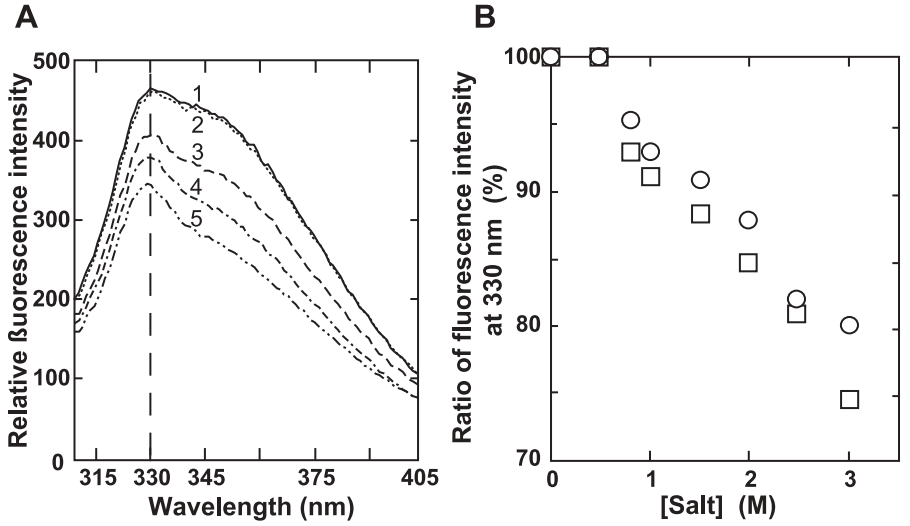

Fig. 6. **Fluorescence spectra of tryptophan residues of actinidain in the presence of salts.** Panel A: Emission spectra of actinidain (0.426 μM) in 0–3.0 M NaCl. The excitation wavelength was 295 nm and the slit widths for excitation and emission were both set to 3 nm. The spectra were recorded from at 25°C. Spectra 1, 2, 3, 4, and 5 were recorded at the NaCl concentration of 0 M, 0.5 M, 1.0 M, 2.0 M, and 3.0 M, respectively. Panel B: Dependence of the relative fluorescence intensity measured at 330 nm on the salt concentration. Salts: NaCl (circles); and KCl (squares).

the shape of the spectra between 310 nm and 360 nm changed with the increase in salt concentration. This indicates that the states of tryptophan residues are changed with an increase of the salts from 0.5 to 3.0 M. The fluorescence intensity of a tryptophan model compound (*N*-acetyl tryptophan amide) is increased with an increase in the salt concentration, and with 3.0 M NaCl it was 20–25% higher than that in the absence of salt (*24*). This increase might be ascribed to an increase in viscosity and in the hydrophobicity of the medium (*6*). It should be noted that, in contrast, the fluorescence intensity of actinidain decreased with the addition of salt. The degree of the decrease was a little larger with KCl than NaCl, and the relative fluorescence intensity was 74% at 3.0 M NaCl and 81% at 3.0 M NaCl. The observation that the intensity decreased without a concomitant change in the maximal fluorescence wavelength suggests that the fluorescence was quenched by the interaction of Na^+ and K^+ ions with the tryptophyls in actinidain. The mechanism of this quenching is unknown, but it is possibile that one or more tryptophyl residues are made more polar by the interaction with Na^+ and K^+ ions. The stronger effect of K^+ than Na^+ on the fluorescence quenching might be correlated with that observed on K_m and k_{cat}/K_m (Fig. 4).

Relationship of the Activity to the CD and Fluorescence Spectra in the Presence of Salts—With an increase in the salt concentration from 0 to 0.5 M, no changes in CD and fluorescence were observed, while the activity (k_{cat}/K_m)

decreased greatly. This suggests that the decrease in the activity is not associated with any conformational change, and might be due to a decrease in the electrostatic attraction between the enzyme and substrate with increasing the ionic strength of the reaction medium. When the salt concentration was increased from 0.5 to 3.0 M, the activity increased and the fluorescence spectrum was decreased, while the CD spectrum was not much changed. The increase in the activity seems to be related to changes in the states of tryptophyl residues, but these are not directly related to a change in the secondary structure of actinidain. Of the six tryptophan residues, five (W7, W122, W178, W184, and W188) are located in R-domain, and one (W26), neighboring the active residue C25, is located in L-domain. The relative solvent accessibility values for the side-chain of these residues were calculated from the X-ray crystallographic data (*2*): 9–16% for W7, 0–4% for W26, 0–4% for W122, 0–4% for W178, 36–49% for W184, and 0–4% for W188. W184 and W188 are in a disordered structure and interact with each other by aromatic stacking. The other four tryptophan residues are in α-helix or β-sheet structures. Because the CD spectra of actinidain were not much affected in the presence of salts up to 3.0 M, the states of these four residues (W7, W26, W122, and W178) are probably not changed; rather, the states of W184 and/or W188 might be changed by salts. On the other hand, it is also possibile that W26 could be the residue responsible for the fluorescent decrease and the activity increase, because of its proximity to the active cysteine. In addition, W184 is located close to H162 in the active site of actinidain, the distance between H162 and W184 being about 3.5 Å (*2*). Thus, change in the micro-environment of the active site might be involved in the increase of the k_{cat}/K_m in 0.5–3.0 M NaCl or KCl. The reason why Na^+ and K^+ are more effective in the activation than Li^+ remains unclear.

In this paper, we have demonstrated that actinidain activity toward *N*-α-Cbz-L-lysine *p*-nitrophenyl ester is inhibited at lower concentrations (0–0.5 M) of NaCl, KCl and LiCl, and recovers considerably at higher concentrations (2–3 M) of NaCl and KCl. Especially at lower concentration (50 μM) of substrate, the activity recovers fully at 3 M KCl. On the other hand, little recovery is seen with LiCl, although LiCl inhibits actinidain most strongly in the concentration range of 0–0.5 M. The inhibition is supposed due to the loss of the electrostatic attraction between the enzyme and substrate, and the re-activation may be due to a change in micro-environment of the active site of the enzyme. The difference in the effect of Na^+, K^+, and Li^+ on the re-activation is currently under investigation. It was found that actinidain retains considerable activity and stability in the presence of 3 M salts, and this evidence could be useful for its application for food processing in the presence of a high concentration of salt. The effect of high concentration of salts on the hydrolysis of collagen or other food proteins (wheat and soy proteins) is also under investigation. Neutral chloride salts examined in this study were selected in view of their applicability to food processing, and thus the selectivity of cations in the inhibition and re-activation of actinidain was demonstrated. The effect of anions (Br^-, I^-, F^-, *etc.*) is worthy of examination. High concentrations of salts are expected to affect the solubility, thermal stability, and autodigestion of actinidain, as has been demonstrated with thermolysin (*5*, *14*, *23*). The evidence described in this paper enables us to investigate the effects of salts on actinidain in various aspects.

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