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Simple method for analyzing the purity of protease-containing samples by acid-treatment SDS-PAGE

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Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used technique to analyze the purity of a protein. However, it is necessary to denature (via boiling) the samples before subjecting them to electrophoresis. In the case of protease-containing samples, autolysis of the protease can occur, affecting the accuracy of results. In this study, we investigated the methods for analyzing the purity of Dispase I, a thermolysin-like neutral protease. When we analyzed D protease, a neutral metalloprotease component of Dispase I and highly purified Dispase I using the conventional SDS-PAGE method, a large number of bands were detected in both cases. These bands (putative D protease fragments) were assumed to result from autolysis. To inactivate D protease (optimal pH 7–8), 0.05 M sulfuric acid was utilized (pH 0.7–2.5). Using a conventional sample preparation solution, acid-treated Dispase I samples (without boiling) were made, and SDS-PAGE (15% w/v gel) was carried out. Our findings show that autolysis was inhibited under strong acidic conditions, and protein denaturation was achieved by treatment with sulfuric acid and SDS without boiling. Using this modified SDS-PAGE method, the purities of Dispase I and the purified enzyme were determined to be approximately 80% and 98%, respectively. Furthermore, we demonstrated that this method can be applied for the analysis of other samples including non-acidic proteases (e.g., thermolysin, subtilisin, and trypsin) and protease-contaminated samples (a mixed solution of albumin and D protease).

[**Key words:** Electrophoresis; Purity analysis method; Metalloprotease; Neutral protease; Protease inhibitor; Dispase; Autolysis; Subtilisin; Thermolysin; Thermolysin-like protease]

Dispase, a protease preparation reagent, is obtained from *Bacillus polymyxa* (the genus name was changed to *Paenibacillus* in 1994). D protease (32–34 kDa), the main protein component of Dispase, has an optimum pH of 5.0–7.0 and is a neutral metalloprotease that is stabilized by a calcium ion and a coordinating zinc ion at its active center (1,2). Primary sequence analysis has revealed 56% similarity to the sequence of thermolysin. D protease has a synonymous tertiary structure and a similar biochemical properties when compared with those of thermolysin (2).

Currently, Dispase is used in the field of regenerative medicine for various purposes (3,4). For example, it is used for the separation and culture of epithelial cells from the skin tissue grown in the form of sheets (5) or for the preparation of insulin-producing cells from the pancreas (6).

Additionally, investigations are being conducted on the use of Dispase as a pharmaceutical product. For example, Dispase is being used as an auxiliary option for medical purposes, such as vitreous removal during proliferative retinopathy surgery in ophthalmological field (7) or prophylactic therapy for diabetic cataracts.

More recently, non-oral administration of enzymes has become a pharmaceutical treatment option, including supplemental therapy for metabolic disorders or applying a group of enzymes that participate in blood coagulation. These pharmaceutical enzymes are now necessary in high-purity forms owing to their systemic administration. Therefore, allergy-inducing substances or endotoxins must be eliminated. A high purity is also required for enzymes used as bulk drug substances to prevent unexpected adverse side effects. Hence, there is a critical need to develop a high-accuracy method to analyze the purity of thermolysin-like neutral protease Dispase.

Gel electrophoresis has high resolution and is widely used for analyzing the purity of proteins. In the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method, the sample is boiled with SDS to denature the protein, and this prepared sample is electrophoresed in the presence of SDS (8). However, after performing SDS-PAGE with D protease, we detected multiple bands, and our attempt to analyze the purity of D protease in the Dispase preparation was not successful.

In this study, we have developed an SDS-PAGE method for proteases, using Dispase as a representative of neutral metal proteases. We determined that the multiple bands identified by SDS-PAGE were generated by autolysis. The enzymatic chemistry of Dispase and methods to suppress the generation of these degradation bands were investigated. D protease is a neutral

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metalloprotease, and by the addition of 0.05 M sulfuric acid to the sample prior to SDS pretreatment, the autolysis is inhibited, resulting in accurate assessment of the protein on SDS an gel. In addition, this method was applied to samples contaminated with protease and proteases other than Dispase.

MATERIALS AND METHODS

Purity analysis of Dispase I by SDS-PAGE using the conventional pretreatment method Dispase I (1 mg protein/vial; Godo Shusei Co. Ltd., Tokyo, Japan) was dissolved in 50 mM Tris-2 mM calcium acetate (pH 7.5) to attain a final protein concentration of 1 mg/mL. A conventional sample pretreatment solution (100 μL containing 25% w/v glycerin, 2.5% w/v SDS, 0.125 M Tris—HCl pH 6.8, 2.5% w/v 2-mercaptoethanol, and 0.01% bromophenol blue) was added to 100 μL of Dispase I solution, and the samples were boiled for 3 min. The treated samples (10 μL) were then subjected to SDS-PAGE (15% gel).

Measurement of protease activity The protease activity was measured using a general casein degradation method. The protease sample was diluted with 50 mM Tris-2 mM calcium acetate (pH 7.5) to attain a final protein concentration of 5 μg/mL, and then maintained at 30°C for 3 min in a 1-mL test tube. Five milliliters of 0.6% w/v casein solution warmed to 30°C was added to the test tube containing the protease sample, and the mixture was allowed to react for 10 min at 30°C. Subsequently, 5 mL of precipitation reagent (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid; pH 4.0) was added to the reaction mixture and incubated for 30 min at 30°C to terminate the reaction and precipitate intact protein and large polypeptide fragments. The precipitates were filtered using a filter paper (Whatman No. 4A, GE Health Care Co. Ltd., Tokyo, Japan), and the absorbance (At) of the filtrate was measured at 275 nm with a spectrophotometer (Shimadzu U2000; Shimadzu Tokyo, Japan). One milliliter of the diluted protease sample was used as a blank. To inactivate proteases, 5 mL of the precipitation reagent was added, and the reaction was incubated for 10 min at 30°C. For precipitation, 5 mL of 0.6% w/v casein solution warmed to 30°C was added to the test tube, and the mixture was incubated at room temperature for 30 min. Filtration and absorbance (Ab) measurements were carried out as described above. One protease unit (PU) was defined as the amount of enzyme that yields an acid-soluble protein molecule degradation product whose absorbance at 275 nm corresponds to that of 1 μg tyrosine per minute. The tyrosine calibration curve was prepared by measuring the absorbance of samples obtained by adding 6 mL of each precipitation reagent to 6 mL of a solution in which tyrosine was diluted to 400 and 1000 μg/mL using a buffer solution. The absorbance was plotted on the X axis, the amount of tyrosine on the Y axis (μg/mL), and the slope F was calculated.

Purified Dispase I Dispase I was dissolved in 50 mM Tris-2 mM calcium acetate (pH 8.0) to yield a final concentration of 0.4 mg/mL. Using a fast protein liquid chromatography (FPLC) system (ActaPrimePlus, GE Healthcare Japan., Tokyo, Japan), 200 mL of this solution was adsorbed on to an anion exchange resin column (TSK GEL DEAE 650 M; 3 cm × 20 cm; TOSOH Co., Tokyo, Japan) that had been pre-equilibrated with 50 mM Tris-2 mM calcium acetate (pH 8.0). The protein was eluted with a linear gradient of 50 mM Tris-2 mM calcium acetate (pH 8.0) containing 0.1 M sodium chloride. Chromatography was carried out at 4°C. The eluted fractions of D protease were combined and concentrated to 20,000 protease units (PU)/mL using a UF membrane (Microza AIP UF module; Asahi Kasei Corp, Tokyo, Japan). The concentrated D protease was subjected to desalination using 50 mM Tris-2 mM calcium acetate until it ultimately reached no greater than 2 ms/cm electric conductivity (Horiba electric conductive meter D70; Horiba Ltd., Kyoto, Japan). As desalination progressed, the concentrate became opaque and the crystals of D protease precipitated. This crystallized protein was collected by centrifugation and was used as the purified enzyme.

Effect of EDTA, metal ions, and protein denaturant Dispase I was dissolved in 50 mM Tris-2 mM calcium acetate (pH 7.5) to attain a final concentration of 2 mg/mL. Subsequently, 50 μL of 5 mM EDTA, 10 mM EDTA, and metal salts (5 mM $FeCl_2$, 5 mM $NiNO_3$, and 5 mM $CuSO_4$), or a protein denaturant (8 M urea) were added to 50 μL of this Dispase I solution, and 100 μL of conventional sample pretreatment solution was added to the mixture. The samples were prepared with or without boiling. For the former, the resulting mixture was boiled for 3 min. The prepared samples (10 μL) were subjected to SDS-PAGE (15% gel).

Effect of sulfuric acid Dispase I was dissolved in 50 mM Tris-2 mM calcium acetate (pH 7.5) to attain a final concentration of 2 mg/mL. Subsequently, 50 μL of 0.01, 0.05, 0.1, 0.5, or 1.0 M sulfuric acid was added to 50 μL of this Dispase I solution and 100 μL of conventional sample pretreatment solution was added to the mixture. The samples were prepared with or without boiling. The prepared samples (10 μL each) were subjected to SDS-PAGE (15% gel). The above method involving the addition of 0.05 M sulfuric acid is subsequently referred to as acid-treatment SDS-PAGE.

Purity analysis by acid-treatment SDS-PAGE Dispase I and purified Dispase I were dissolved in 50 mM Tris—2 mM calcium acetate (pH 7.5) to concentrations of 0.02—10 mg/mL. Subsequently, 50 μL of 0.05 M sulfuric acid was added to 50 μL of this solution and 100 μL of conventional sample pretreatment solution was added to the mixture. The samples were prepared without boiling. The prepared samples (10 μL each) were subjected to SDS-PAGE (15% gel). Area of each band in the electrophoresis pattern was analyzed by ImageJ (https://imagej.nih.gov/ij/).

Analysis of other proteases by acid treatment SDS-PAGE Chymotrypsin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), trypsin (Wako), subtilisin (Sigma—Aldrich, St. Louis, MO, USA), proteinase K (Wako), papain (Wako), pepsin (Wako), thermolysin (Wako), and Dispase I were dissolved in Milli-Q water (Millipore GmbH, Darmstadt, Germany) to attain a final concentration of 2 mg/mL. Subsequently, 50 μL of 0.05 M sulfuric acid was added to 50 μL of these solutions and 100 μL of the conventional sample pretreatment solution was added to the mixture. The samples were prepared without boiling. The prepared samples (10 μL each) were subjected to SDS-PAGE (15% gel).

Analysis of pseudo protease-contaminated samples by acid treatment SDS-PAGE Bovine albumin (Wako Chemicals) and Dispase I were dissolved in 50 mM Tris—2 mM calcium acetate (pH 7.5) to a concentration of 2 mg/mL individually. Fifty microliters of 0.05 M sulfuric acid were added to 50 μL of this solution and 100 μL of the conventional sample pretreatment solution was added to the mixture. The samples were incubated for 0, 5, 10, 20, and 30 min at 30°C. The prepared samples (10 μL each) were subjected to SDS-PAGE (15% gel).

Capillary electrophoresis The Experion automated electrophoresis system (Experion electrophoresis station, Experion Priming station, Experion software, and Experion PRO 260 Analysis kit, Bio-Rad Laboratories, Hercules, CA, USA) was used for this experiment. Equal amounts of 0.05 M sulfuric acid were added to each sample. To 4 μL of the resulting solution, 2 μL of a dedicated sample treatment solution (from Bio-Rad) was added, and the resulting sample solution was used in the automated electrophoresis system without boiling.

RESULTS AND DISCUSSION

Purity analysis of Dispase I and purified Dispase I by SDS-PAGE using the conventional pretreatment method The two Dispase solutions were subjected to the conventional SDS-PAGE method. As shown in Fig. 1, similar band patterns were obtained in both cases. D protease, which is the main component of Dispase preparations, was detected at 32—34 kDa, but bands of lower molecular weight (less than that of D protease) were also detected. These low-molecular weight bands were considered to result from the degradation of D protease caused by the pretreatment of samples. Thermal decomposition during boiling

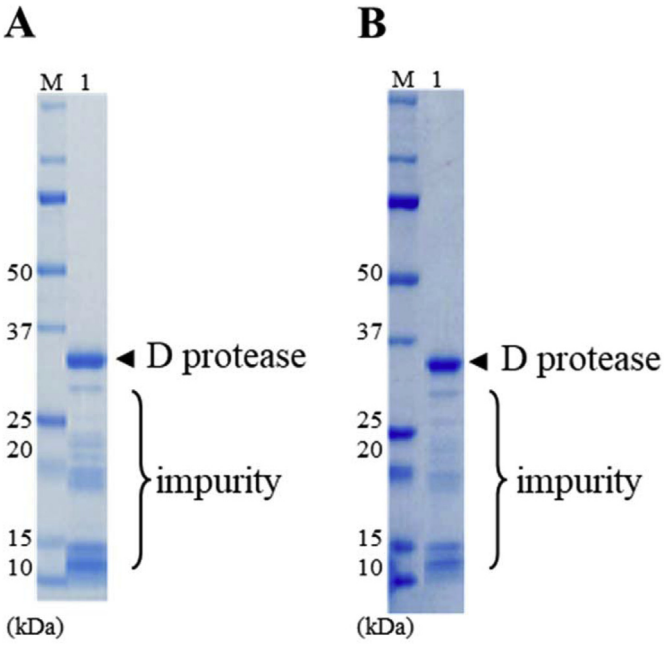

FIG. 1. Purity analysis of Dispase I and purified Dispase I by SDS-PAGE using a conventional pretreatment method. (A) M, molecular weight markers; lane 1, Dispase I. (B) M, molecular weight markers; lane 1, purified Dispase I.

and autolysis during sample pretreatment were hypothesized to cause the degradation. To avoid such degradation of samples, it was necessary to optimize the pretreatment method.

Effect of adding various inhibitors to the Dispase solution before pretreatment and boiling samples on SDS-PAGE band pattern To inhibit the D protease activity, various inhibitors were added to Dispase I solution before pretreatment of the samples, and their effects were assessed by examining differences in the resulting SDS-PAGE band patterns. As D protease is a metalloprotease (Zn), the inhibitors included EDTA and other heavy metals; urea, a common protein denaturant, was also included. After addition of the inhibitors, the samples were prepared with or without boiling, and then electrophoresed.

In the case of pretreatment by boiling, other than the 10 mM EDTA-treated samples, the main band detected in the inhibitor-treated samples was weaker than that detected by conventional electrophoresis (Fig. 2, lanes 1–7). The bands detected with 10 mM EDTA-treated D protease were very strong, suggesting lower degradation. For the samples prepared without boiling, other than the 5 and 10 mM EDTA-treated samples, the main band disappeared. The bands detected with 5 and 10 mM EDTA-treated D protease were very strong, suggesting lower degradation. Additionally, the 10 mM EDTA-treated samples yielded bands stronger than those of the 5 mM EDTA-treated samples (Fig. 2, lanes 9 and 10). Thus, these findings suggest that autolysis is suppressed by the addition of EDTA prior to sample pretreatment. The addition of 10 mM EDTA, with and without boiling, showed similar band patterns; therefore, we decided to conduct the subsequent analyses without boiling.

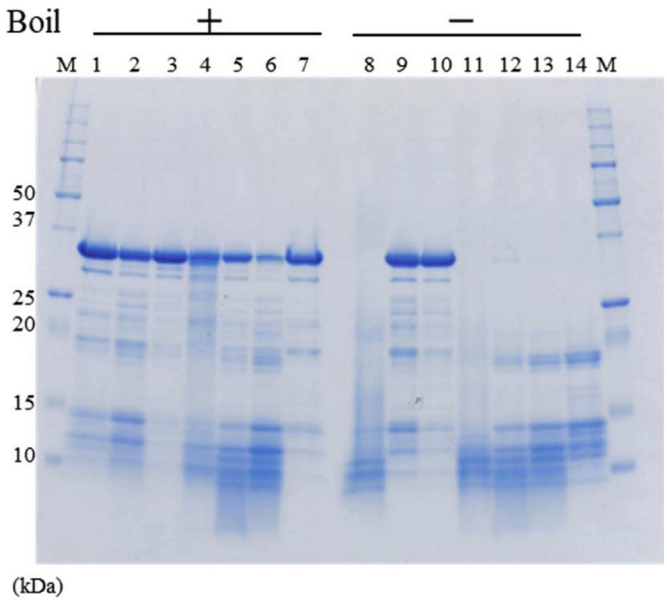

FIG. 2. Comparison of the effects of various inhibitors of D protease with or without boiling on the band pattern in SDS-PAGE. M, molecular weight markers; lanes 1–7, samples prepared with boiling; lanes 8–14, samples prepared without boiling; lanes 1 and 8, buffer; lanes 2 and 9, 5 mM EDTA; lanes 3 and 10, 10 mM EDTA; lanes 4 and 11, 5 mM $FeCl_3$; lanes 5 and 12, 5 mM $NiNO_3$; lanes 6 and 13, 5 mM $CuSO_4$; lanes 7 and 14, 8 M urea.

Effect of sulfuric acid addition to Dispase I before pretreatment As shown in Fig. 2, the addition of EDTA (followed by the addition of pretreatment solution) to Dispase I solution without boiling, resulted in fewer degradation fragments and stronger intact bands of D protease than those obtained with the use of the conventional SDS-PAGE method. These results indicate that EDTA might inhibit protease activity and autolysis. As D protease is a neutral protease, we hypothesized that acidification could inhibit protease activity and autolysis. Therefore, the Dispase solution was pretreated with 0.01–1.0 M sulfuric acid. The conventional sample pretreatment solution was subsequently added, and the resultant mixture was electrophoresed without boiling. Addition of 0.01 M sulfuric acid to Dispase I solution resulted in faint bands for D protease and several bands of low-molecular weight (Fig. 3A, lane 1). However, the addition of 0.05–1.0 M sulfuric acid resulted in stronger bands for D protease

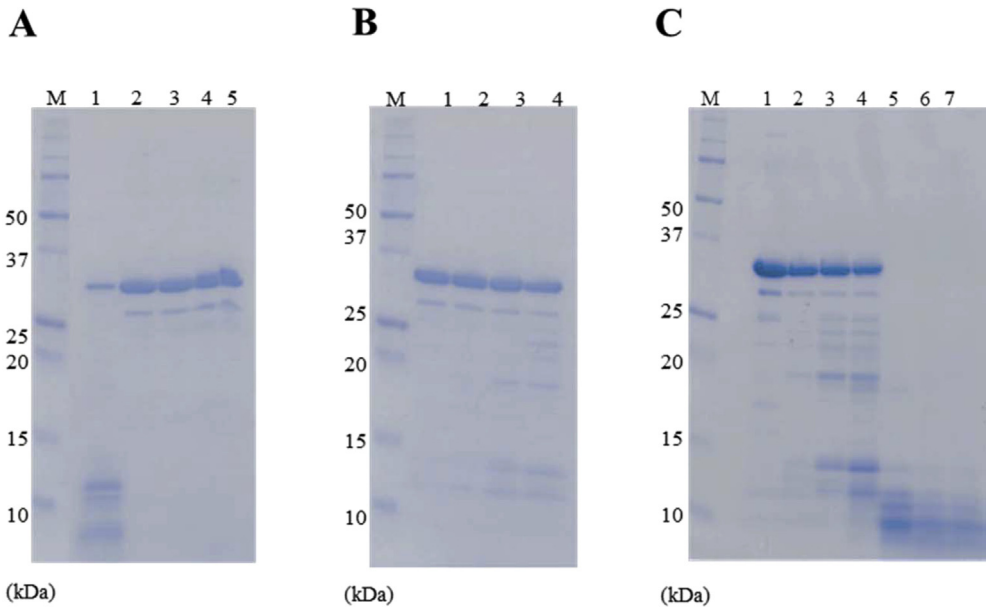

FIG. 3. Effects of the addition of acid or EDTA to Dispase I solution on band pattern in SDS-PAGE. (A) M, molecular weight markers; lane 1, 0.01 M sulfuric acid (after acid addition, sample pH 3.7); lane 2, 0.05 M sulfuric acid (pH 1.9); lane 3, 0.1 M sulfuric acid (pH 1.5); lane 4, 0.5 M sulfuric acid (pH 0.9); lane 5, 1.0 M sulfuric acid (pH 0.7). (B) M, molecular weight markers; lane 1, 0.05 M sulfuric acid (after acid addition, sample pH 2.0); lane 2, 0.05 M hydrochloric acid (pH 2.5); lane 3, 0.05 M phosphoric acid (pH 2.8); lane 4, 0.05 M acetic acid (pH 4.2). (C) M, molecular weight markers; lane 1, 0.05 M sulfuric acid; lane 2, 20 mM EDTA; lane 3, 10 mM EDTA; lane 4, 5 mM EDTA; lane 5, 2 mM EDTA; lane 6, 1 mM EDTA; lane 7, buffer.

and fewer low-molecular weight bands, suggesting that the addition of sulfuric acid to Dispase I solution inhibited the autolysis of D protease (Fig. 3A, lanes 2–5). The addition of 0.01 M sulfuric acid partially inhibited autolysis (Fig. 3A, lane 1) and the addition of 1 M sulfuric acid caused distortion of the D protease band (Fig. 3A, lane 5). Therefore, acidity may negatively influence electrophoresis. The addition of 0.05–0.5 M sulfuric acid produced similar results (Fig. 3A, lanes 2–4), suggesting that 0.05 M sulfuric acid is sufficient to inhibit low-molecular weight band formation or autolysis. This protocol was also used to analyze the purity of D protease using other acids including hydrochloric acid, phosphoric acid, and acetic acid (Fig. 3B). The findings indicate that the formation of contaminating band can be suppressed at a pH of $<$ 2.5 regardless of the acid used. For this reason, the use of hydrochloric acid at the same concentration resulted in similar suppression. However, as hydrochloric acid is highly volatile and prone to decomposition during storage, we selected 0.05 M sulfuric acid, a commonly used acid for neutralization and titration, for the subsequent experiments.

Examination of the effects of adding EDTA and sulfuric acid to Dispase I solution before sample pretreatment As described above, the addition of either EDTA or sulfuric acid to Dispase I solution resulted in fewer bands and suppression of autolysis. Therefore, the effect of EDTA was compared with that of acid addition (0.05 M sulfuric acid). EDTA chelates calcium ions at a molar ratio of 1:1 with D protease. D protease has a molecular weight of ~32 kDa; therefore, the prepared sample (2 mg/mL) had a molar concentration of 2/32 mM. During sample pretreatment, EDTA was added at a molar concentration of 2–20 mM (D protein: EDTA = 1: 32–320).

As shown in Fig. 3C, although the D protease band was detected with the addition of $\geq$5 mM EDTA, and it became stronger at 20 mM (Fig. 3C, lanes 2–6). The strongest band was detected by the addition of 0.05 M sulfuric acid (Fig. 3C, lanes 1). Detailed analysis of the area of each band showed that in the sample to which 0.05 M sulfuric acid was added (Fig. 3C, lanes 1), the D protease band had a value of 9193, the 30 kDa band was 961, and the $\leq$15 kDa band was 1160, whereas in the sample to which 20 mM EDTA was added (Fig. 3C, lanes 2), the D protease band was 6625, the 30 kDa band was 208, and the $\leq$15 kDa band was 2017 (data not shown). The addition of EDTA did not suppress autolysis, and so D protease was broken down to $\leq$15 kDa.

Measurements of the protease activity during sample processing indicated that the addition of EDTA could not completely inactivate D protease immediately and that protease was deactivated completely upon the addition of SDS (Table S1). The addition of SDS deactivated protease, whereas the intensity of the low-molecular weight bands increased after reducing the concentration of EDTA from 20 to 5 mM (Fig. 3C, lanes 2–4). This suggests that the residual activity of D protease, even after inhibitor addition, influenced the generation of degradation bands. Meanwhile, acid treatment induced complete inactivation of D protease (Table S1). Each sample was diluted over 200-fold in Tris buffer and stored at 4°C for 0 and 24 h before measuring the protease activity (0 h: Table S1, 24 h: data not shown). Protease activity was not detected, indicating that the addition of acid irreversibly inactivated D protease. In addition, this showed that autolysis of D protease in the samples and generation of degradation bands were completely inhibited.

Validation of acid treatment The acid treatment (without boiling) successfully inhibited autolysis. The purity analysis of D protease was performed using enzymes with different purities, including Dispase II (partially purified product), Dispase I (purified product), and highly purified Dispase I. The samples were preprocessed by the conventional treatment (with and without boiling) or acid treatment (with and without boiling) (Fig. 4), and then subjected to SDS-PAGE.

The results obtained using conventional sample preprocessing were not consistent with the original purities of the enzymes (Fig. 4, lanes 1–6), indicating that this technique is not suitable for purity analysis of proteases, such as Dispase.

The acid treatment (with boiling) suppressed autolysis to some extent, but the number of degradation bands was increased compared with that obtained by acid treatment (without boiling). As the addition of acid was shown to irreversibly inactivate D protease activity in earlier experiments, we hypothesized that this increase in the number of extra bands likely resulted from heat-induced degradation under acidic conditions (Fig. 4, lanes 7–9).

Meanwhile, acid treatment without boiling resulted in improved purity levels and markedly reduced the low-molecular weight bands observed with the purified enzyme (Fig. 4, lanes 10–12). Furthermore, the samples appeared yellow after treatment with acid and blue after resolving on the gel. As the bromophenol blue indicator dye in the sample pretreatment solution turns yellow at a pH below 3 and blue at a pH above 3 (Fig. S1), the pH of the sample was considered below 3 at the time of acid addition and above 3 post-gel application. After loading on the gel, the pH of the sample was neutralized by the sample buffer (pH 6.8), similar to the conventional sample pretreatment. Thus, acid pretreatment had no effect on electrophoresis, and the post-electrophoresis band pattern was not different from that resulting following the conventional methods.

Moreover, in a pretreatment step of SDS-PAGE, high concentration of TCA is added to the sample; this is followed by washing and collecting the precipitate and dissolving it in neutral sample buffer (9). Although TCA is a powerful protein precipitant, there are differences in the precipitation susceptibilities of proteins to TCA; some proteins are difficult to dissolve after precipitation. Therefore, this method is not suitable for measuring the purity of proteins. In contrast, with the addition of 0.05 M sulfuric acid to the sample, we could more accurately analyze the total protein in the sample. This

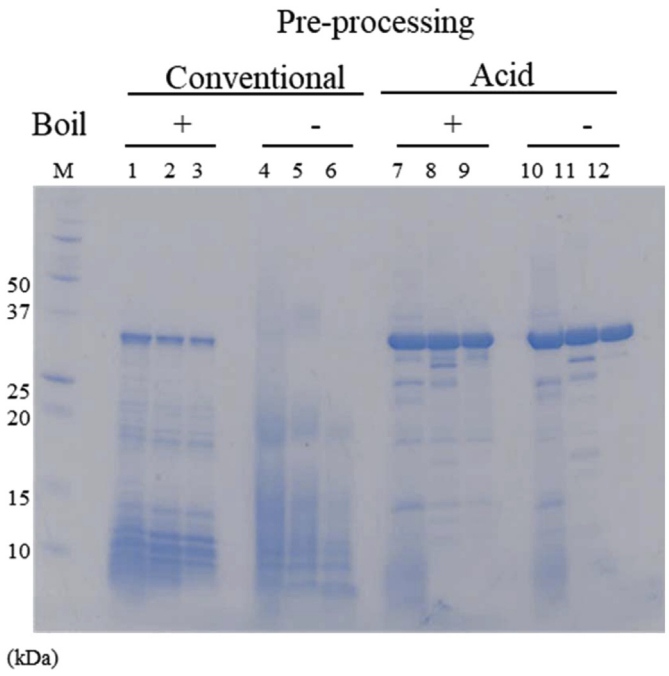

FIG. 4. Comparison of the effects of various types of preprocessing on the band pattern of Dispase with different purities by SDS-PAGE. M, molecular weight markers; lanes 1, 4, 7, and 10, Dispase II (partially purified product); lanes 2, 5, 8, and 11, Dispase I (purified product); lanes 3, 6, 9, and 12, purified Dispase I.

method is suitable for analyzing the purity of a sample by gel electrophoresis.

Purity analysis using acid treatment Acid treatment without boiling has been demonstrated to be suitable for purity analysis, and the content of purified Dispase was determined by a step-wise dilution method using a densitometer. In the purified enzyme, the density of the degradation band of 28–32 kDa was approximately equal to that of the 1:50 diluted D protease band; thus, the 28–32 kDa band constituted ~2% of the purified Dispase (Fig. 5B). Similarly, in Dispase I, the $\leq$ 32 kDa bands constituted 10%–20% of the total bands. Therefore, our purity analysis suggests that Dispase I and the purified enzyme contained 80% and 98% D protease, respectively (Fig. 5A, B). The acid-treatment SDS-PAGE methodology was applied to capillary electrophoresis, where acid was added to the sample, followed by the addition of sample treatment solution (for capillary electrophoresis use), and capillary electrophoresis without boiling. As capillary electrophoresis has a wider measurement range than that of SDS-PAGE, purity can be measured with a densitometer in the same lane without using the step-wise dilution method. As a result, the purity of Dispase I was calculated as 80% and that of purified D protease was calculated as 98%, indicating that the purity is equal to that obtained by the acid-treated SDS-PAGE method.

Using the acid-treatment method, we could measure the purity of D protease in the Dispase preparation for the first time. This preparation, with a purity of 95% or more, is useful for medical applications and has the potential to be used as a treatment option with fewer side effects.

Application to other proteins, proteases, and protease-contaminated samples We analyzed seven proteases other than Dispase with the developed acid-treatment SDS-PAGE methodology and the conventional pretreatment protocol. In comparison with the conventional method, acid-treatment SDS-PAGE resulted in a stronger main band at the target molecular weight (Fig. 6A). The protease activity was likely rapidly inactivated to neutral and alkaline proteases, and suppression of autolysis prevented the degradation of protease. As for papain, the number of bands increased in acid-treatment SDS-PAGE. As the main band was stronger, it was inferred that the band visible with the acid treated samples was digested by the conventional method. In contrast, acidic proteases such as pepsin were predicted to be not inactivated, but the band pattern in both protocols did not differ. The migration distances of trypsin

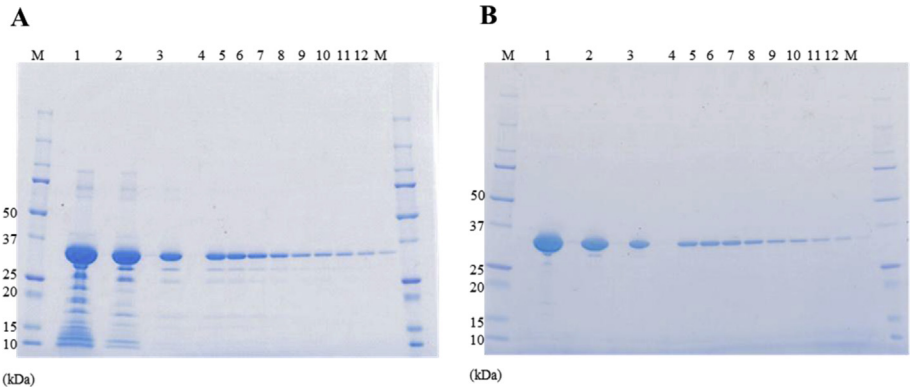

FIG. 5. Purity analysis of samples prepared by acid treatment. (A) Dispase I, (B) purified Dispase. M: molecular weight markers; lane 1, 10 mg/mL; lane 2, 5 mg/mL; lane 3, 1 mg/mL; lane 4, 0.5 mg/mL; lane 5, 0.4 mg/mL; lane 6, 0.3 mg/mL; lane 7, 0.2 mg/mL; lane 8, 0.1 mg/mL; lane 9, 0.08 mg/mL; lane 10, 0.06 mg/mL; lane 11, 0.04 mg/mL; lane 12, 0.02 mg/mL.

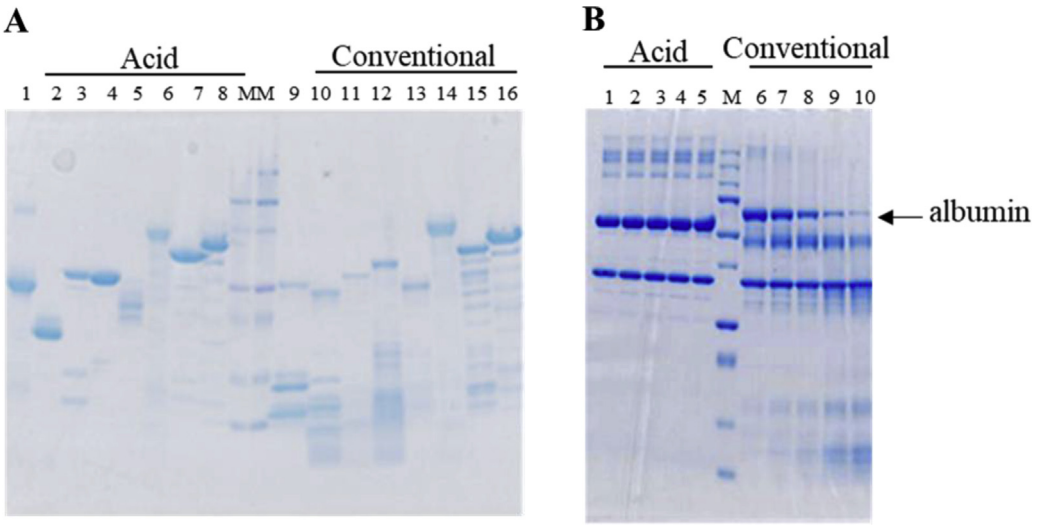

FIG. 6. Application of acid treatment SDS-PAGE method to other samples. (A) Other proteases, (B) albumin and D protease. (A) M, molecular weight markers; lanes 1 and 9, chymotrypsin; lanes 2 and 10, trypsin; lanes 3 and 11, subtilisin; lanes 4 and 12, proteinase K; lanes 5 and 13, papain; lanes 6 and 14, pepsin; lanes 7 and 15, thermolysin; lanes 8 and 16, Dispase I. (B) M, molecular weight markers; lanes 1 and 6, 0 min after addition of sample processing buffer; lanes 2 and 7, 5 min; lanes 3 and 8, 10 min; lanes 4 and 9, 20 min; lanes 5 and 10, 30 min.

(Fig. 6A, lanes 2 and 10), proteinase K (Fig. 6A, lanes 4 and 12), and papain (Fig. 6A, lanes 5 and 13) were considerably different between the acid and conventional treatments, especially the difference with respect to trypsin was significant. As described above, in the acid treatment method, the sample becomes neutral with the addition of buffer during gel loading after sample treatment. Therefore, the pH is almost same as that of the samples in conventional electrophoresis. Furthermore, neutralization increases only salt content, and not all proteins have similar mobility; therefore, we inferred that salt has no effect. We suggest that SDS binding may differ depending on whether the samples are prepared without boiling under acidic conditions (acid method) or with boiling under neutral conditions (conventional method). Although there was some difference in mobility, as long as all proteins could be detected, it would not be an issue in purity analysis.

Further, we prepared a solution containing albumin and D protease mimicking a protease-contaminated protein sample and comparatively analyzed it using both methods (Fig. 6B). Under acid-treatment SDS-PAGE, sample processing resulted in a consistent band pattern even after the samples were left at 30°C for up to 30 min. In contrast, the conventional method resulted in the disappearance of the albumin band over time when sample processing was followed by incubation at 30°C for 0–30 min and boiling prior to electrophoresis, indicating that autolysis still occurred. As yet, for sample processing by the conventional method, the time from the addition of SDS to boiling is not defined, and it was shown that the band pattern changes with the time that the sample is mixed with protease.

In conclusion, the described acid-treatment SDS-PAGE method can be applied to a large number of proteins and proteases. In addition, the protocol can rapidly and simply inactivate protease activity in a variety of samples including those containing microbes and cells and those that are contaminated with proteases. The described methodology is promising as it can be used to analyze proteins in a sample with minimal processing and is likely to have high applicability in a wide range of disciplines.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.04.014.

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TF reports personal fees from GODO SHUSEI Co. Ltd. during the conduct of the study. In addition, TF has an issued patent related to the techniques presented herein (Shiota, K. and Fujita, T., Electrophoretic analysis method, United States Patent No. 8951397 B2, 2015). All other authors declare no conflicts of interest.

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