Aggregates Formed During Storage of β-Galactosidase in Solution and in the Freeze-Dried State

Sumie Yoshioka,[1,2] Yukio Aso,[1] Ken-ichi Izutsu,[1] and Tadao Terao[1]

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Aggregates formed during storage of freeze-dried β-galactosidase were compared with those formed in solutions. Freeze-dried β-galactosidase aggregated during storage in the presence of moisture, producing a protein precipitate which was soluble in guanidine hydrochloride solution but not in buffer solution. The protein precipitate dissolved in guanidine solution exhibited a large molecular size by high-performance size exclusion chromatography and converted to proteins of original size in the presence of dithiothreitol. It is suggested that the aggregation involves chemical interaction via covalent disulfide bonding. In contrast, β-galactosidase in aqueous solution aggregated without formation of protein precipitates. Soluble aggregates were converted to proteins of original size in guanidine solution without dithiothreitol, suggesting noncovalent bonding. The difference in aggregation behavior may be ascribed to the difference in the water:protein ratio. We propose that inactivation of β-galactosidase is due to formation of thermally denatured (unfolded) protein, which aggregates dependent on the water:protein ratio, either via noncovalent interactions at a high water:protein ratio in solution or via covalent interaction at a low water:protein ratio in the freeze-dried state.

KEY WORDS: β-galactosidase; aggregation; solution; freeze-dried.

INTRODUCTION

The inactivation rate of β-galactosidase lyophilized from phosphate buffer solution was affected by the mobility of water molecules, as determined by the spin-lattice relaxation time, T_1, of water (1). The inactivation rate of β-galactosidase in the aqueous solution was also found to be related to the T_1 of water, but the inactivation mechanisms of β-galactosidase have not been clarified.

Aggregation is an important degradation pathway of pharmaceutical proteins. It has been studied for various lyophilized proteins: bovine serum albumin, ovalbumin, glucose oxidase, lactoglobulin (2), ribonuclease A (3–5), human growth hormone (6), and recombinant tumor necrosis factor (7). Thermally denatured proteins tend to aggregate regardless of their structure.

We studied the inactivation mechanism of freeze-dried β-galactosidase in the presence of moisture compared to that in aqueous solution. The inactivation of β-galactosidase was due to denaturation (unfolding) of the protein, followed by aggregation in the freeze-dried and solution state. The present paper describes the nature of aggregates formed during storage of freeze-dried β-galactosidase in the presence of moisture in comparison with aggregates formed in aqueous solutions.

[1] National Institute of Hygienic Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan.
[2] To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

β-Galactosidase from *Aspergillus oryzae* was purchased from Toyobo Co. (Osaka) and used without further purification. 2-Nitrophenyl-β-galactopyranoside, guanidine hydrochloride, and dithiothreitol were purchased from Wako Chemical Industry Co. (Osaka).

Degradation of β-Galactosidase

Preparation of freeze-dried samples was carried out as described previously (1). β-Galactosidase was lyophilized from, pH 7.4, phosphate buffer solution at a protein concentration of 1 mg/mL. The buffer solution was prepared by adding an adequate volume of 500 m*M* Na_2HPO_4 solution (ionic strength, $\mu = 1.5$) to 500 m*M* KH_2PO_4 solution containing 1 *M* NaCl ($\mu = 1.5$). Sample tubes (14-mm diameter) containing 200 μL of the protein solution were immersed in liquid nitrogen for 1 min, and the frozen samples were dried at 5 Pa for 15 hr in a lyophilizer (Tozai Tsusho Co., Tokyo).

The freeze-dried samples were stored at 60°C and 50% RH (adjusted with NaBr saturated solution). The water content of these samples was determined to be 0.05 mg/mg of the freeze-dried sample by the Karl Fisher method (684 KF Coulometer, Switzerland). The freeze-dried samples were also stored at 50 and 55°C after 50 μL of distilled water was added so that samples contained 3.1 mg of water/mg of the freeze-dried sample. (These samples appeared to be solutions at 50 and 55°C, and the pH was determined to be 7.3.) Samples were removed at appropriate intervals for activity assay, soluble protein assay, and HPSEC measurement.

Phosphate buffer solutions (50 m*M*, pH 7.4) of β-galactosidase containing 0.1 *M* sodium chloride were stored at 50 and 55°C. The protein concentration in the solution was 0.1 mg/mL. Enzyme activity, total amount of soluble proteins, and HPSEC were measured as a function of time.

The activity of β-galactosidase was determined by using 2-nitrophenyl-β-D-galactopyranoside as a substrate, as described previously (1).

High-Performance Size Exclusion Chromatography (HPSEC)

The freeze-dried samples stored under various conditions were dissolved with 4 mL of 200 m*M* phosphate buffer (pH 6.2) to make 50 μg/mL protein solutions, and injected through a 20-μL loop to a column (Tosoh G3000SW, 30 cm × 7.5 mm, Tokyo) maintained at 30°C after filtration (0.45 μm). The mobile phase was 200 m*M* phosphate buffer (pH 6.2), which was delivered at a rate of 1 mL/min. The column eluate was monitored at 230 nm.

A 200 m*M* phosphate buffer solution containing 10 m*M* dithiothreitol and 1 m*M* EDTA, a 200 m*M* phosphate buffer solution containing 6 *M* guanidine hydrochloride, and a 200 m*M* phosphate buffer solution containing 10 m*M* dithiothrei-

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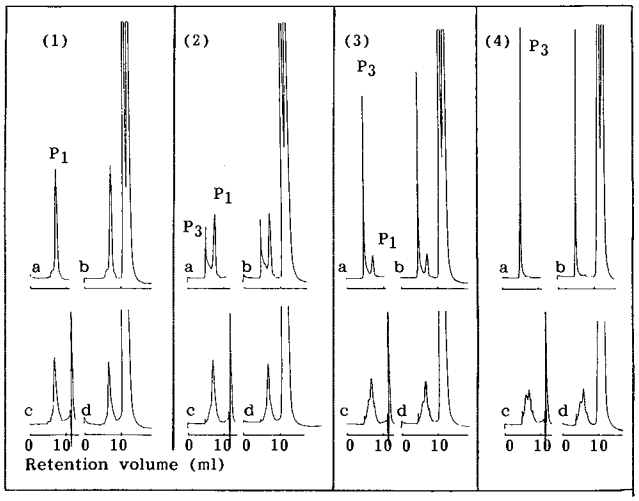

Fig. 1. Size exclusion chromatogram of β-galactosidase in pH 7.4 phosphate buffer solution (50 m*M*) containing 0.1 *M* sodium chloride, stored for 0 (1), 30 min (2), 240 min (3), and 360 min (4) at 50°C. The concentration of β-galactosidase was 0.1 mg/mL. Sample solutions were diluted with mobile phase buffer solution (a), buffer solution of 15 m*M* dithiothreitol and 1.5 m*M* EDTA (b), buffer solution of 6 *M* guanidine hydrochloride (c), and buffer solution of 15 m*M* dithiothreitol, 1.5 m*M* EDTA, and 6 *M* guanidine hydrochloride (d). The diluted sample solutions (b–d) were kept at room temperature for 30 min before injection to HPSEC. The large peaks observed at a retention volume of about 11 mL for the sample diluted with the dithiothreitol solution were due to dithiothreitol and EDTA.

tol, 1 m*M* EDTA, and 6 *M* guanidine hydrochloride were separately used to dissolve the stored freeze-dried samples. The dissolved sample solutions were kept at room temperature for 1 or 18 hr, then injected to the HPSEC.

For HPSEC measurements of β-galactosidase solutions stored under various conditions, 2 mL of 200 mM phosphate buffer solution was added to 1 mL of the sample solution, and injected to the HPSEC. A 200 m*M* phosphate buffer solution containing 15 m*M* dithiothreitol and 1.5 m*M* EDTA, a 200 m*M* phosphate buffer solution containing 6 *M* guanidine hydrochloride, and a 200 m*M* phosphate buffer solution containing 15 m*M* dithiothreitol, 1.5 m*M* EDTA, and 6 *M* guanidine hydrochloride were also used to dilute their respective sample solutions after storage. The diluted sample solutions were kept at room temperature for 30 min, then injected to the HPSEC.

Spectrophotometric Assay for Soluble Proteins

The samples were dissolved or diluted with 2 mL each of the mobile phase buffer solution, the dithiothreitol solution, the guanidine solution, or the dithiothreitol–guanidine solution, as described in the section for HPSEC measurement. The solution was kept at room temperature for 1 hr (for freeze-dried samples) or 30 min (for solution samples) and centrifuged at 20,000*g* for 30 min to remove undissolved protein. After filtration (0.45 μm), absorbance of the supernatant was measured at 280 nm. A Shimadzu UV-260 spectrophotometer and 1-cm-lightpath quartz cuvettes were used.

RESULTS AND DISCUSSION

Degradation of β-Galactosidase in Buffer Solution

Figure 1 shows the HPSEC chromatograms obtained for the β-galactosidase solution stored at 50°C, for various time periods. Before storage, the β-galactosidase sample solution diluted with the mobile phase buffer solution gave a peak representing intact β-galactosidase at a retention volume of 7.2 mL (P_1). The peak was deformed by the addition of guanidine hydrochloride but not by the addition of dithiothreitol. A broader peak at a smaller retention volume (6.8 mL) was observed when the sample solution was diluted with the

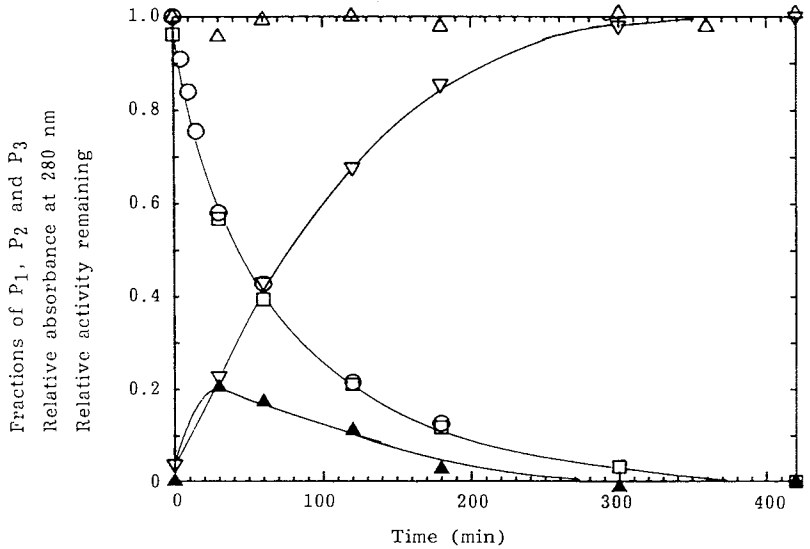

Fig. 2. The time courses of fractions of P_1 (□), P_2 (▲), and P_3 form (▽), relative absorbance at 280 nm (△), and percentage activity remaining (○) for β-galactosidase degradation in pH 7.4 phosphate buffer (50 m*M*) containing 0.1 *M* of NaCl at 50°C. The concentration of β-galactosidase was 0.1 mg/mL.

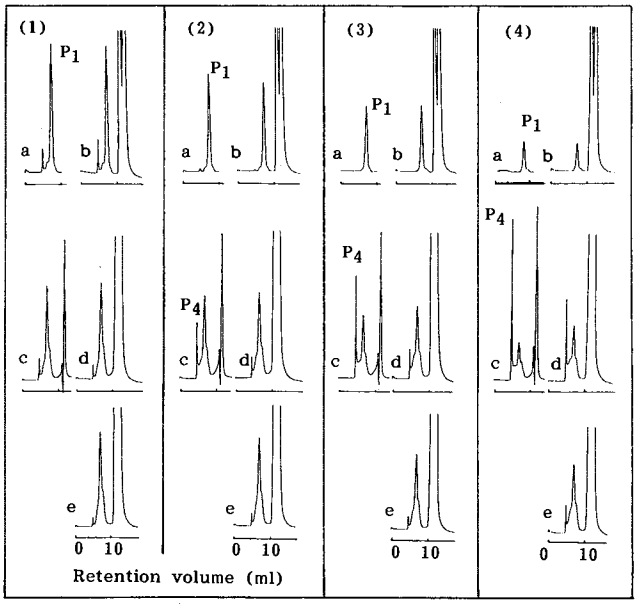

Fig. 3. Size exclusion chromatogram of freeze-dried β-galactosidase of 3.1 mg/mg of water content, stored for 0 (1), 15 min (2), 60 min (3), and 180 min (4) at 55°C. The stored samples were dissolved with mobile phase buffer solution (a), buffer solution of 10 m*M* dithiothreitol and 1 m*M* EDTA (b), buffer solution of 6 *M* guanidine hydrochloride (c), and buffer solution of 10 m*M* dithiothreitol, 1 m*M* EDTA, and 6 M guanidine hydrochloride (d and e). The dissolved samples were kept at room temperature for 1 hr (b–d) or 18 hr (e) before injection to HPSEC.

guanidine solution or the dithiothreitol–guanidine solution. This peak can be ascribed to the denatured form of β-galactosidase.

For the stored sample solution diluted with the buffer solution or the dithiothreitol solution (a and b in Fig. 1), a larger sharp peak (P_3) was observed at a retention volume near the void (4.8 mL), as the main peak representing intact β-galactosidase (P_1) became smaller during storage. The small retention volume suggests that P_3 represents aggregation products of larger size than intact β-galactosidase. It cannot be excluded that P_3 consists of more than one product, since the retention volume was near the void. However, P_3 accounts for the final products of β-galactosidase in solution, since it remained constant after the other peaks disappeared. An unresolved absorbance activity (P_2) appeared between P_1 and P_3 during the initial phase of storage and later disappeared. The P_2 absorbance may be due to intermediates of molecular size larger than intact β-galactosidase and smaller than the final products. Since the retention volume of P_2 is similar to that of the denatured form of β-galactosidase in the presence of guanidine, P_2 may be assigned to the denatured form which undergoes subsequent aggregation.

Peaks P_1 and P_3, which were observed when the stored sample solution was diluted with the mobile phase buffer solution or the dithiothreitol solution, disappeared when these samples were diluted with the guanidine solution or the dithiothreitol–guanidine solution (c and d in Fig. 1). Unresolved broad peaks at retention volumes corresponding to the denatured form were observed instead. This result indicates that the aggregation products (P_3) were converted to the denatured protein in the presence of guanidine, and it suggests that the aggregation resulted from noncovalent interaction, and not from covalent bonding.

HPSEC measurement was also carried out with the β-galactosidase solution stored at 55°C. The chromatograms obtained were similar to those observed at 50°C.

Figure 2 shows the time courses of the fractions of P_1, P_2, and P_3 observed in the chromatograms (Fig. 1) for the stored β-galactosidase solution diluted with the mobile phase buffer solution. Similar time courses were also observed when the sample solution was diluted with the dithiothreitol solution. The fraction of P_1 was calculated from the ratio of

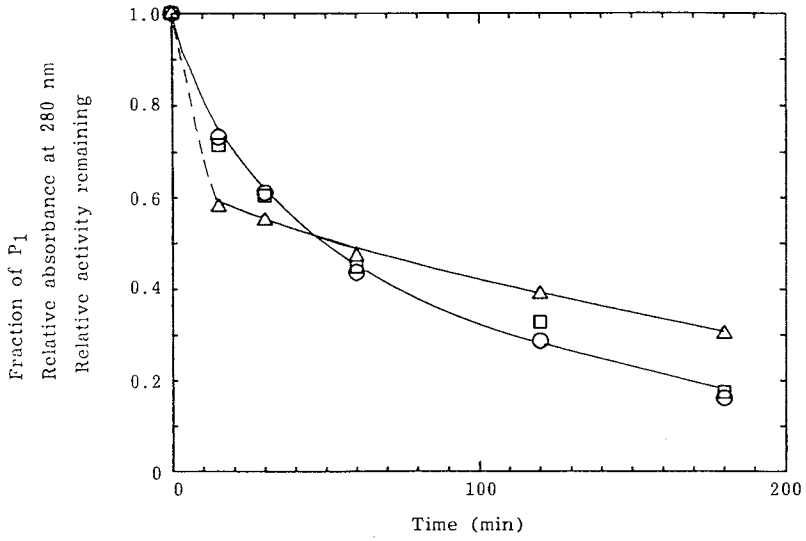

Fig. 4. The time courses of fractions of P_1 (□), relative absorbance at 280 nm (△), and percentage activity remaining (○) for degradation of freeze-dried β-galactosidase with 3.1 mg/mg water content at 55°C. The samples were dissolved with mobile phase buffer solution.

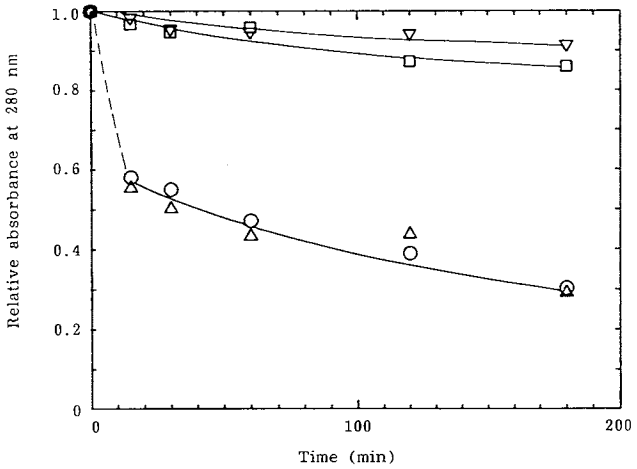

Fig. 5. The time courses of relative absorbance at 280 nm for degradation of freeze-dried β-galactosidase with 3.1 mg/mg of water content, stored at 55°C . The samples were dissolved with mobile phase buffer solution (○), buffer solution of 10 m*M* dithiothreitol and 1 m*M* EDTA (△), buffer solution of 6 *M* guanidine hydrochloride (□), and buffer solution of 10 m*M* dithiothreitol, 1 m*M* EDTA, and guanidine hydrochloride (▽).

the peak height to the initial peak height. The fraction of P_3 was calculated from the peak height by assuming that 100% of the initial P_1 had been converted to P_3, when the peak height of P_3 reached a constant value. Since P_2 was not a single resolved peak, the fraction of P_2 was calculated by subtracting the sum of P_1 and P_3 fractions from unity. Figure 2 also shows the time courses of the enzyme activity remaining. The activity vs time profile coincided with the fraction of P_1 vs time profile, suggesting that both P_2 and P_3 were inactive.

The total amount of soluble proteins determined by spectrophotometry was not changed during storage (Fig. 2), arguing against the presence of insoluble proteins. The total amount of soluble proteins also remained constant during storage when the sample solution was diluted with the dithiothreitol solution, the guanidine solution, or the dithiothreitol–guanidine solution (data not shown).

These results suggest that β-galactosidase in aqueous solution is converted to the denatured (unfolded) form (P_2), from which aggregation products (P_3) are formed via noncovalent interaction.

Degradation of Freeze-Dried β-Galactosidase

Figure 3 shows the HPSEC chromatogram for the freeze-dried β-galactosidase with 3.1 mg/mg of water content, stored at 55°C, as a function of time. The freeze-dried sample exhibited a peak corresponding to P_3 (designated as P_4) as well as P_1 even before storage. This result suggests that β-galactosidase degraded to some extent during the freeze-drying process.

The chromatogram of the freeze-dried sample underwent different changes from those observed for degradation in solutions. When the stored sample was dissolved in the mobile phase buffer solution or the dithiothreitol solution (a and b in Fig. 3), P_4 was not detected. Even the P_4 peak observed before storage disappeared during storage, and P_1 decreased in height with storage time. When the sample was dissolved in the guanidine solution (c in Fig. 3), P_4 appeared and increased with storage time as the peak corresponding to the denatured protein decreased. When the sample was dissolved in the dithiothreitol–guanidine solution (d in Fig. 3), a smaller P_4 peak and higher peak corresponding to the denatured protein were observed, compared to those in the presence of guanidine only. When the sample was stored at room temperature for a longer time period after being dissolved in the dithiothreitol–guanidine solution (e in Fig. 3), P_4 became smaller and the peak corresponding to the denatured protein became larger, while no change was observed in the peaks in the presence of guanidine only.

It is suggested that the storage of freeze-dried β-galac-

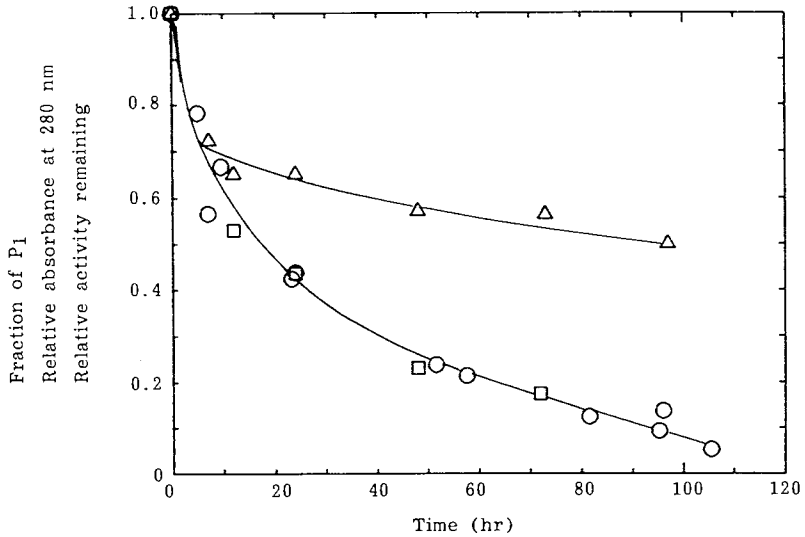

Fig. 6. The time courses of fractions of P_1 (□), relative absorbance at 280 nm (△), and percentage activity remaining (○) for degradation of freeze-dried β-galactosidase exposed to 50% RH at 60°C. The samples were dissolved with mobile phase buffer solution.

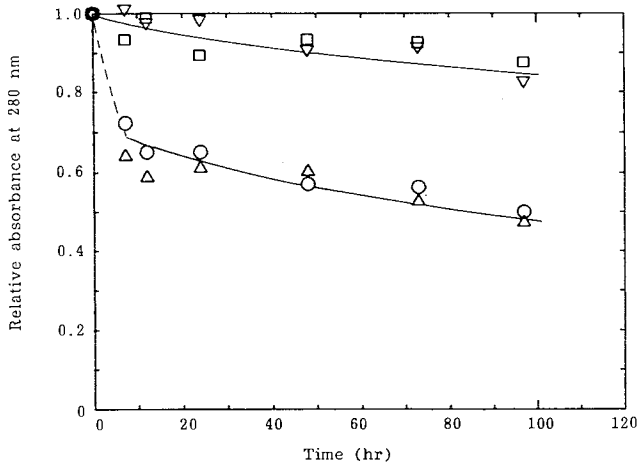

Fig. 7. The time courses of relative absorbance at 280 nm for degradation of freeze-dried β-galactosidase exposed to 50% RH stored at 60°C. The samples were dissolved with mobile phase buffer solution (○), buffer solution of 10 m*M* dithiothreitol and 1 m*M* EDTA (△), buffer solution of 6 *M* guanidine hydrochloride (□), and buffer solution of 10 m*M* dithiothreitol, 1 m*M* EDTA, and guanidine hydrochloride (▽).

tosidase resulted in protein precipitation, since no peaks other than P_1 were observed in the HPSEC chromatograms even when P_1 was significantly decreased. Peak P_4, which appeared in the chromatograms in the presence of guanidine, may represent aggregates solubilized with guanidine. The observation that P_4 was converted to proteins of original size in the presence of both dithiothreitol [which is known to reduce disulfide bonds (2)] and guanidine suggests that covalent disulfide bonding contributes to the formation of the aggregates.

HPSEC measurement was also carried out with the freeze-dried β-galactosidase stored at 50°C. The chromatograms obtained were similar to those observed at 55°C.

Figure 4 shows the time courses of the fraction of P_1 observed in the chromatograms (Fig. 3) obtained when the stored freeze-dried sample was dissolved in the mobile phase buffer solution. The activity remaining agreed well with the fraction of P_1. The total amount of soluble proteins showed a rapid decrease at the initial stage, followed by a slower decrease with storage time, confirming the formation of insoluble proteins in the mobile phase buffer solution.

The decrease in the total amount of soluble proteins was also observed when the sample was dissolved in the dithiothreitol solution. The rapid decrease, however, was not observed when the sample was dissolved in the guanidine solution or the dithiothreitol–guanidine solution, as shown in Fig. 5. Thus the protein precipitates formed during storage were insoluble in the mobile phase buffer solution or the dithiothreitol solution but were soluble in the guanidine solution or the dithiothreitol–guanidine solution.

These results suggest that the freeze-dried β-galactosidase with 3.1 mg/mg of water content forms aggregation products during storage via covalent disulfide bonding. The freeze-dried β-galactosidase exposed to an atmosphere of 50% RH at 60°C (0.05 mg/mg of water content) revealed HPSEC chromatograms similar to those obtained for the freeze-dried sample with 3.1 mg/mg of water content. Figure 6 shows the time courses of the fractions of total protein, P_1, and remaining activity, observed for the freeze-dried sample stored at 50% RH and 60°C, dissolved in the mobile phase buffer solution. The rapid decrease in total amount of soluble proteins did not occur in the presence of guanidine (Fig. 7).

In conclusion, the freeze-dried β-galactosidase was found to aggregate in a different way from β-galactosidase in aqueous solution; the former aggregation involves chemical interaction via covalent bonding, while the latter does not.

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