Biosynthesis of Mutant β-D-Galactosidases Containing Nonnatural Aromatic Amino Acids by *Escherichia coli*

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Mutant β-D-galactosidases containing nonnatural aromatic amino acids were produced by misincorporation during protein overproduction by *Escherichia coli*. The incorporation of 3-(1-pyrenyl)-L-alanine (pyrAla) was confirmed by fluorescence measurement of the produced enzyme, although the replacement frequency was low. Some of mutant enzymes showed improved thermostability.

Chemical treatment and protein engineering have produced many modified or mutant proteins, which are more effective in enzymatic activity than the original ones. In the meantime, the introduction of functional residues by means of chemical modification is limited only to functional groups on the protein surface, where modifying reagents are sterically reachable. On the other hand, site-directed mutagenesis provides a means of substitution at precise position of the sequence with natural amino acids. If protein engineering also enables utilization of nonnatural amino acids, we can design novelly functionalized enzymes easier.

Proofreading system prevents protein biosynthesis from mistranslation in every step of biosynthetic process.[1] However, the system could not function properly against nonnatural amino acids because it works most strictly against naturally occurring amino acids. So far, many kinds of nonnatural amino acids, whose structure is very similar to a natural one, have been introduced into proteins by using *Escherichia coli*. The substitution rate of a target amino acid with a nonnatural amino acid was found to be about 10% to 90% in the cases of alkaline phosphatase,[2-8] aspartate transcarbamylase,[9] β-D-galactosidase[10] human epidermal growth factor (hEGF).[11] Schlesinger and co-workers have succeeded in incorporation of various amino acid analogs into bacterial alkaline phosphatase.[2-7] Alkaline phosphatase gene is expressed under the control of *phoA* promoter that is induced by lowering the phosphate concentration of the culture medium. The biosynthesis of the other probably toxic abnormal proteins is not active under these conditions except the production of abnormal alkaline phosphatase. In this method, Miyazawa and co-workers succeeded in preparing hEGF substituted with one norleucine for methionine by biosynthesis of alkaline phosphatase signal peptide-hEGF fusion protein.[11] On the other hand, incorporation of nonnatural amino acids, whose structure is very different from a natural one, is a difficult task. One of approaches to realize this purpose is that the protein-biosynthesis system is accelerated to obtain the mutant enzymes containing nonnatural amino acids as much as possible.

In this paper, incorporation of fluorescent nonnatural amino acids into β-D-galactosidases was studied by using *E.coli* in the overproduced state on the protein synthesis. 3-(1-Pyrenyl)-L-alanine (pyrAla) was incorporated into β-D-galactosidases and relatively high thermostability was found in some of the mutant enzyme.

Experimental

o-Nitrophenyl β-D-galactopyranoside (ONPG), bovine serum albumin (BSA), and *E.coli* β-D-galactosidase (M.W. 130 kDa) were purchased from Sigma, USA. Bacterial endonucleases BamHI and EcoRV, bacterial alkaline phosphatase (BAP), and T4 DNA ligase were purchased from Toyobo Co., Ltd., Japan. Bacto-Trypton and Bacto-Yeast-Extract were purchased from DIFCO Laboratory, USA. *E.coli* B357 (wild type) and *E.coli* DH5α (F^-, *endA*1, *hsdR*17 (rk^-, mk^+), *supE*44, *thi*$^{-1}$, *recA*1, *gyrA*96, *relA*1, Δ (*argF*-laczya) U169, φ 80d*lacZ* λ M15, λ^-) were used for expression of β-D-galactosidase.

Ultraviolet absorption spectra were recorded by a Hitachi Model 200-10 spectrometer. Fluorescence emission and excitation spectra were recorded by a Hitachi MPF-4 autocorrected fluorescence spectrometer. Fluorescence excitation spectra were monitored at wavelength 397 nm for pyrAla and at wavelength 415 nm for 3-(9-anthryl)-L-alanine (antAla).

The recombinant plasmid pUC19Z was synthesized from the plasmid pUC19 and pMC1871 (Pharmacia, Sweden). A 3.0 kbp fragment containing the lacZ gene was excised from the pMC1871 plasmid with BamHI, and ligated into the pUC19, digested with BamHI and dephosphorylated with BAP. The resultant plasmid pUC19Z was transformed into DH5α strain and the transformants exhibiting β-D-galactosidase activity were picked on 2% agar plates of LB-medium (1% (w/w) bacto-trypton/0.5% (w/w) bacto-yeast-extract) in the presence of ampicillin, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and isopropyl β-D-galactopyranoside. The plasmid DNA was isolated with the method of alkaline treatment.[15]

The *E.coli* strain DH5α harboring plasmid pUC19Z was precultured in a 500 ml flask containing 100 ml of LB-medium at 37 °C, with shaking for 16 h. The cells were collected by centrifugation at 3,000 rpm for 5 min and suspended in 30 ml of deionized water. An aliquot (1 ml) of the precultured cell

suspension was inoculated into 100 ml of LB-medium in a 500 ml flask and cultivated to the exponential growth phase. Then 10 mg of chloramphenicol was added and the flask was shaken for further 1 h. The cells were harvested by centrifugation and suspended in 30 ml of deionized water again. A portion of cell suspension (20 ml) was inoculated into 2.0 liter of LB-medium in the presence of 50 μg ml^{-1} of nonnatural fluorescent amino acid and 0.1% lactose instead of glucose and cultured to the exponential growth phase at 37 °C. After cultivation, the cells washed with deionized water were suspended in 400 ml of 0.3 M (1 M=1 mol dm^{-3}) potassium phosphate buffer (pH 7.2) in the presence of 3 mM $MgCl_2$. Cell growth was monitored at absorbance of 600 nm. 1 OD corresponds to 10^8 cells ml^{-1}.

Cell-free extracts were prepared by disintegrating the cells with a Braun cell homogenizer (Type 853023, B. Brown, Germany). The cell homogenate was centrifuged at 12,000 rpm for 5 min, the supernatant obtained being further centrifuged at 45,000 rpm for 60 min at 4 °C. The cell-free extract obtained finally was concentrated through a DIAFLOW membrane filter (Ultrafiltration Cell Model 12, AMICON Corp., USA). The total amount of protein was estimated by the Lowry's method.[16] The β-D-galactosidase protein was analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The proteins were recovered from the cell-free extracts with saturated ammonium sulfate. It was solubilized again with 100 mM potassium phosphate buffer (pH 7.2) and dialyzed for 24 h at 4 °C against 500-fold volume of the potassium phosphate buffer to remove ammonium sulfate. Thereafter, the fluorescence excitation spectra of the protein solution and the dialyzing buffer were measured at every 2.5 h of dialysis against 500-fold volume of the buffer at 4 °C. After the dialysis for ten times (25 h), the protein solution was added to the same volume of dimethyl sulfoxide (DMSO) and stirred overnight at 4 °C to wash out the nonnatural amino acid followed by further 20 times of the dialysis. The content of the nonnatural amino acid was monitored by following the fluorescence spectrum during the dialysis treatment.

β-D-Galactosidase was purified with a gel-filtration chromatography followed by nondenaturing polyacrylamide gel electrophoresis. The proteins were applied to a Sephacryl S-300 column (2.5×85 cm) equilibrated with 100 mM potassium phosphate buffer (pH 7.2) containing 10 mM $MgCl_2$. A single peak of the β-D-galactosidase activity was found and the fractions were collected and concentrated. The concentrated solution was applied to a nondenaturing polyacrylamide gel electrophoresis (7%) and the gel band corresponding to β-D-galactosidase was excised and eluted out with an Extraphore electrophoretic concentrator (Pharmacia). The preparation finally obtained was used as a purified β-D-galactosidase.

The nonnatural amino acid contents in the β-D-galactosidases were estimated by the fluorescence measurement. The fluorescence from the enzyme is attributed to both the original tryptophan residues and the incorporated nonnatural amino acid residues. Assuming that the fluorescence intensity of the tryptophan residues ($F_{trp}(\lambda)$) gives a maximum at wavelength λ_{trp} and the intensity of the nonnatural amino acid residues ($F_{aa}(\lambda)$) gives a maximum at wavelength λ_{aa}, we have a ratio (R) of the fluorescence intensity from the nonnatural amino acid residues to that from the tryptophan residues as follows.

$$F_{obs}(\lambda) = XF_{trp}(\lambda) + YF_{aa}(\lambda)$$

$$R_{obs} = F_{obs}(\lambda_{aa}) \,/\, F_{obs}(\lambda_{trp})$$

$$R_{trp} = F_{trp}(\lambda_{aa}) \,/\, F_{trp}(\lambda_{trp})$$

$$R_{aa} = F_{aa}(\lambda_{aa}) \,/\, F_{aa}(\lambda_{trp})$$

$$R = YF_{aa}(\lambda_{aa}) \,/\, XF_{trp}(\lambda_{trp})$$
$$= R_{obs}(1 - R_{trp}/R_{obs}) \,/\, (1 - R_{obs}/R_{aa})$$

where $F_{obs}(\lambda)$ represents the observed fluorescence intensity at wavelength λ and X and Y represent the contributions of each fluorescent residue. The nonnatural amino acid content C (mol/mol tryptophan residue) was estimated from a calibration curve for 1 mg ml^{-1} bovine serum albumin solution.

The β-D-galactosidase activity was measured at 30 °C by following the increase in absorbance of *o*-nitrophenol produced through the hydrolysis of ONPG at 405 nm.

Results

The construction of plasmid pUC19Z to express β-D-galactosidase is illustrated in Fig. 1. *E.coli* DH5α was transformed with the pUC19Z. Several transformants exhibiting β-D-galactosidase activity (blue-colored col-

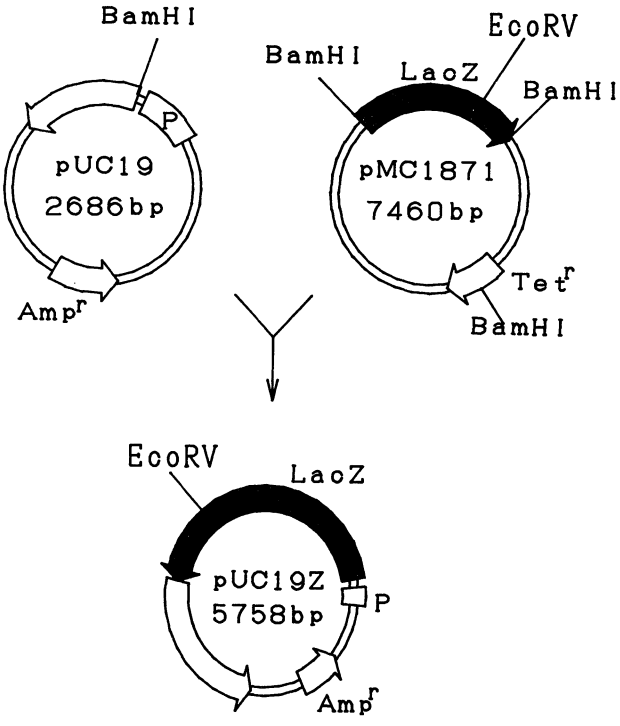

Fig. 1. Construction of β-D-galactosidase expressing plasmid pUC19Z. BamHI and EcoRV represent the restriction sites on the plasmid. P is the promoter, and lacZ is the structural gene of β-D-galactosidase. Some of the colonies were screened for the presence of lacZ gene by agalose electrophoresis. A positive colony had a band corresponding to the open-circular plasmid DNA band digested at the site of EcoRV in lacZ gene.

onies) and having 5.7 kbp fragment of plasmid pUC19Z were obtained. The enzymatic activities of β-D-galactosidase in various *E.coli* strains, grown in the absence of nonnatural amino acids, were measured and are summarized in Table 1. The activity of the wild strain B357 was 73-fold higher in lactose-grown cells than in glucose-grown cells. The protein produced in the presence of lactose from the DH5α strain, transformed with pUC19Z and treated with chloramphenicol, showed two thousands-fold activity of that in the presence of glucose. The content of β-D-galactosidase in the total protein was estimated over 65% judging from the calculation of specific activity (126 μmol min^{-1} mg^{-1} in the cell free extract; 171 μmol min^{-1} mg^{-1} in the purified preparation).

The reduction in growth of strain B357 was within 20% when the concentration of each nonnatural amino acid added in medium was lower than 50 μg ml^{-1}. The amount of proteins produced and β-D-galactosidase activity are shown in Table 2. In the presence of 3-(1-naphthyl)-L-alanine (napAla), both the protein production and the activity per cell increased slightly. In this case, the effect of incorporation of napAla was not observed. In comparison, in the presence of antAla, the protein production per cell increased, while the activity per cell did not increase significantly, and in the presence of pyrAla, the protein production per cell did not decrease, while the activity per cell was reduced considerably. These results might be due to the incorporation of the nonnatural amino acid into β-D-galactosidase.

The amount of a nonnatural amino acid in a protein was estimated by the fluorescence intensity of the nonnatural amino acid taking that of tryptophan in the protein as standard. For the calibration of fluorescence intensity, fluorescence of nonnatural amino acids was measured in the presence of BSA.

The fluorescence intensity ratio R and the residual content C were plotted on log–log graph paper and a calibration curve was obtained by the least-squares method. The curve was expressed by $C=9.28R^{0.615}$ (mmol/mol tryptophan residue) for antAla in the range of $R<15$ and by $C=0.795R^{1.55}$ (mmol/mol tryptophan residue) for pyrAla in the range of $R<10$.

The protein solution from the wild strain B357 grown in the presence of the nonnatural amino acid was added to the same volume of DMSO and the nonnatural amino acid that nonspecifically bound to the protein was removed by dialysis. The fluorescence intensity ratio during the dialysis was followed and is shown in Fig. 2. The ratio was decreased exponentially, but a certain intensity was remained after the sufficient dialysis for 75 h. The minimum R was 0.30 for antAla and

Table 1. β-D-Galactosidase Activity of Various *E. coli* Strains

Strain	Plasmid[b]	Induction[c]	CP[a,d]	Specific activity[e]
B357	None	None	None	0.0645
B357	None	+	None	4.73
DH5α	pUC19Z	+	+	126
DH5α	None	+	+	0.00

a) CP represents chloramphenicol treatment b) Transformation with the plasmid. c) Induction with lactose. d) CP treatment for 1 h. e) Specific activity was expressed in units per milligram of the β-D-galactosidase (μmol min^{-1} mg^{-1}).

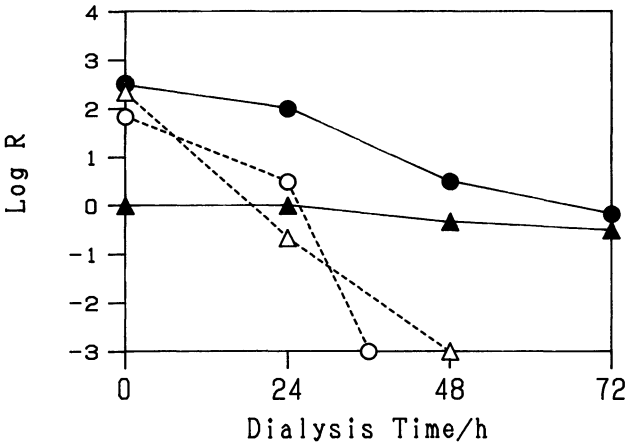

Fig. 2. Change in fluorescence intensity ratio during dialysis treatment. The fluorescence intensity ratio of the protein solutions obtained from the cells of strain B357 were followed during the dialysis. ●, Sample from the cells grown in the presence of pyrAla; ○, mixture of sample from the cells grown without nonnatural amino acid and 40 μg ml^{-1} of pyrAla; ▲, sample from the cells grown in the presence of antAla; △, mixture of sample from the cells grown without nonnatural amino acid and 40 μg ml^{-1} of antAla.

Table 2. The Relative Amount of Proteins Obtained from the Cells of Strain B357 and the Relative β-D-Galactosidase Activity

Nonnatural amino acid[a]	Growth[b]	Amount of protein[c]	Specific activity[d]	Change[e]
None	1	1	1	—
Nap	1.04	1.06	1.09	+9%
Ant	0.91	1.38	0.95	−5%
Pyr	0.80	1.35	0.75	−25%

a) Nap, Ant, and Pyr represent 3-(1-naphthyl)-L-alanine, 3-(9-anthryl)-L-alanine, 3-(1-pyrenyl)-L-alanine, respectively. b) The relative growth of the cells. c) The relative amount of proteins per cell. d) The relative specific activity of β-D-galactosidases. e) The change in the specific activity.

was 0.65 for pyrAla. The residual content C was obtained using the calibration curve as 4.5 mmol mol^{-1} for antAla and 0.42 mmol mol^{-1} for pyrAla. In comparison, the protein solution of the B357 grown without nonnatural amino acids was mixed with a solution of 40 μg ml^{-1} nonnatural amino acids and stirred at 4 °C for 24 h. The fluorescence intensity ratio during the dialysis was followed after washing with DMSO. As shown in Fig. 2, the fluorescence of the nonnatural amino acids disappeared in 48 h, indicating that the nonspecifically bound nonnatural amino acids were completely removed by the dialysis.

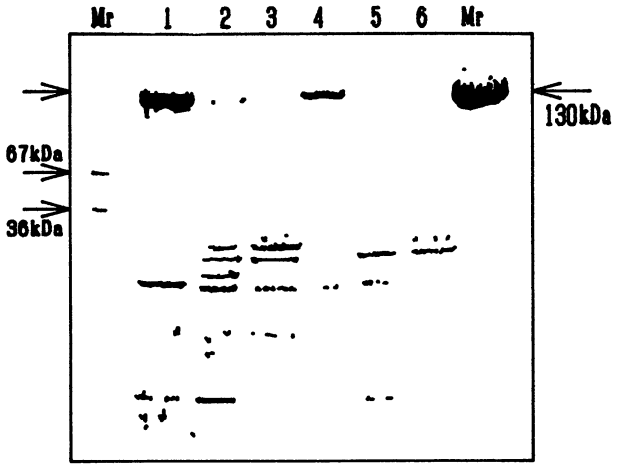

Fig. 3. The SDS-PAGE profile of produced β-D-galactosidases. β-D-Galactosidase was obtained from various strains without nonnatural amino acid. Mr, Marker; lanes 1 and 4, DH5α strain harboring pUC19Z; lanes 2 and 5, DH5α strain harboring pUC19; lanes 3 and 6, DH5α without transformation. Lanes 1, 2, and 3, 20 μg protein each; lanes 4, 5, and 6, 10 μg protein each.

β-D-Galactosidase was purified from the protein solution obtained from the DH5α cells harboring pUC19Z without nonnatural amino acid (Fig. 3). Similarly, β-D-galactosidase was purified from the protein solution obtained from the DH5α cells harboring pUC19Z grown in the presence of 50 μg ml^{-1} nonnatural amino acids. Each 200 μg of purified β-D-galactosidase was obtained from 1 mg of the crude preparation through the nondenaturing polyacrylamide gel electrophoresis.

A significant fluorescence of pyrenyl moiety was observed in the purified β-D-galactosidase obtained from the cells grown in the presence of pyrAla (Fig. 4). It was found that the fluorescence intensity ratio R_{obs} was 0.796 from the fluorescence excitation spectrum of the purified β-D-galactosidase monitored at wavelength 397 nm. The residual content C was determined as 0.556 mmol mol^{-1} from the calibration curve.

β-D-Galactosidases were incubated in a water bath at 60 °C. Aliquots were removed at various times over 180 s period, placed on ice and then measured for the residual activity (Fig. 5). The activity of the purified β-D-galactosidases obtained from the cells grown in the presence of pyrAla or antAla remained after the incubation of 120 s at 60 °C, while that obtained from the cells grown without nonnatural amino acids was lost completely in 120 s. This indicates that the thermostability of β-D-galactosidase was improved by the incorporation of nonnatural amino acids.

It can be derived from the Michaelis-Menten's reaction kinetics that a specific activity of an enzyme is proportional to the rate constant k_{cat} in the case of sufficiently high substrate concentration. The activation energy of the catalytic reaction was calculated from the

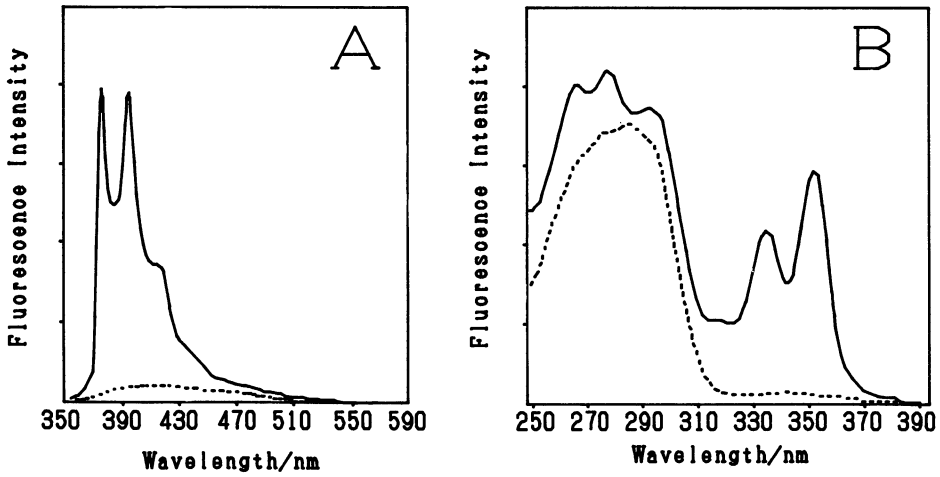

Fig. 4. Fluorescence emission and excitation spectra of the purified β-D-galactosidase obtained from the cells of strain DH5α grown with or without pyrAla. A, fluorescence emission spectra excited at 345 nm; B, fluorescence excitation spectra monitored at 395 nm. —, spectra of the purified β-D-galactosidase obtained from the cells grown in the presence of pyrAla; ···, spectra of that obtained from the cells without nonnatural amino acid.

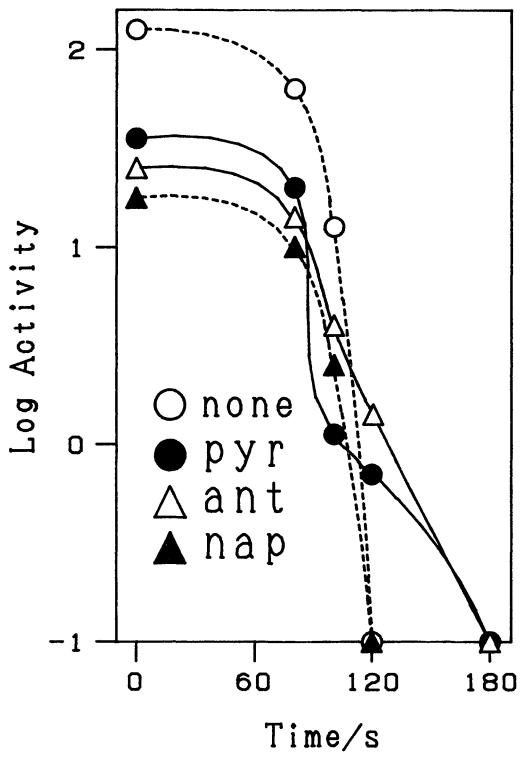

Fig. 5. Thermostability of purified β-D-galactosidases. Log specific activity is plotted against incubation times of 80 s, 100 s, 120 s, and 180 s. ○, Sample obtained from the cells of strain DH5α grown without nonnatural amino acid; ●, △, and ▲, samples obtained from the cells of strain DH5α grown in the presence of pyrAla, antAla, and napAla, respectively.

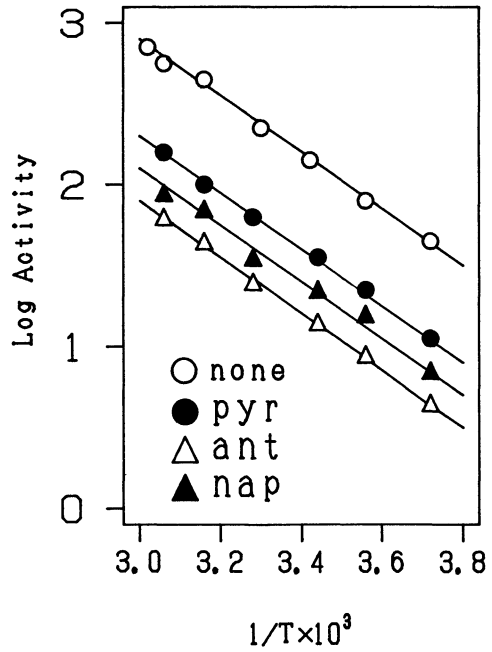

Fig. 6. The log specific activity vs. $1/T$ plot of purified β-D-galactosidases. ○, Sample obtained from the cells of strain DH5α grown without nonnatural amino acid; ●, △, and ▲, samples obtained from the cells of strain DH5α grown in the presence of pyrAla, antAla, and napAla, respectively.

plot of log specific activity against $1/T$ as shown in Fig. 6. The activation energy of β-D-galactosidase obtained from the cells grown in the presence of various nonnatural amino acids was identical with that for β-D-galactosidase obtained from the cells grown without the nonnatural amino acids.

Discussion

In general, a tRNA is not mischarged with nonnatural amino acids, because aminoacyl-tRNA synthetase precisely discriminates the corresponding amino acid from the others. Nonnatural amino acids found in natural polypeptides is usually formed in the process of the posttranslational modification. In some cases, the nonnatural amino acid-containing proteins show resistance to the digestion by proteases. The natural amino acid analogs, such as canavanine, 2-azetidinecarboxylic acid, and 4-fluorophenylalanine, were shown to be incorporated during the accelerated biosynthesis of protein after viral infection of bacteria.[12] The mutant proteins inhibited the self-assembly of the viral proteins. On the other hand, it has been reported that cleavage of the signal sequence of pre-prolactin synthesized in the presence of hydroxynorvaline is inhibited by incorporation of the analog.[12] In most cases, incorporation of analogs resulted in reduction of protein functions. Abnormal (mutant) proteins accumulated in a cell are known to be digested by intracellular proteases. The accumulation of abnormal proteins leads to an expression of protease La, which digests the abnormal proteins.[13] In addition, cell growth will be inhibited because high frequency of production of abnormal proteins may cause reduction of normal protein production. For these reasons, it was difficult to prepare abnormal proteins incorporated with nonnatural amino acids.

Parker reported that the amino acid substitutions did not lead to proteins recognized as grossly abnormal by the proteolysis systems in cells. Abnormal proteins in which substitution frequency was under 10% were not eliminated in a cell, and abnormal subunits of ribosome were found in cells grown normally.[14] Taking these results into consideration abnormal proteins may be prepared by lowering substitution frequency. Abnormal β-D-galactosidase was prepared in the presence of various fluorescent nonnatural amino acids with low frequency of incorporation. In order for the protein not to be recognized as a foreign abnormal protein, β-D-galactosidase was synthesized in *E.coli*. The nonnatural amino acids used in this study were not analogous to any natural amino acids, so that the nonatural amino acids were mischarged to tRNAs to be incorporated into proteins in sufficiently low frequency. Fluorescent amino acids can be detected in abnormal proteins even in low frequency of incorporation.

We have succeeded in overproduction of β-D-galactosidase using the DH5α strain with plasmid

pUC19Z in the level of two thousands times higher than the wild strain (Table 1). AntAla and pyrAla were found in the proteins of the wild strain synthesized in the presence of these nonnatural amino acids (Fig. 2), indicating that *E.coli* utilized these nonnatural amino acids to some extent in protein biosynthesis. Fluorescence emission of pyrenyl moiety was observed in the purified β-D-galactosidase synthesized in the presence of pyrAla, and the residual content was estimated to be 0.556 mmol mol^{-1} tryptophan residue. Thus, one pyrAla residue per 46 molecules of β-D-galactosidase was found because there are 39 tryptophan residues in a β-D-galactosidase molecule. However, pyrAla may be attached to the hydrophobic surface of BSA used in the calibration experiment, so that this value is the minimum in the case of the absence of pyrenyl moiety quenching by surrounding charges or dipoles.

The specific activity of the proteins obtained in the presence of antAla or pyrAla was reduced (Table 2), while the amount of proteins per cell was not affected. This indicates that protein biosynthesis was not lowered by the addition of the nonnatural amino acids. The β-D-galactosidase content of the total proteins was found to increase continuously throughout the cultivation, indicating that the reduction of the specific activity was not the result of proteolytic digestion of abnormal β-D-galactosidase but the result of the production of β-D-galactosidase with low activity. On the other hand, the activation energy of the purified β-D-galactosidase did not decrease (Fig. 6). Thus, it is considered that the nonnatural amino acids were incorporated into the active site of β-D-galactosidase and the activity of some β-D-galactosidase was lost. In this case, incorporated amino acids are thought to be on the protein surface and easily quenched. In addition, incorporation of a highly hydrophobic amino acid into the hydrophilic surface may cause instabilization of β-D-galactosidase.

At least 2.2% of β-D-galactosidase was estimated to have one pyrAla residue. As shown in Fig. 5, several per cent of the mutant β-D-galactosidases resulted in the improved thermostability of the enzyme, indicating that the nonnatural amino acids were incorporated into the hydrophobic region of β-D-galactosidase and a higher-order structure of the enzyme was stabilized. In this case, the incorporated nonnatural amino acids should be located apart from the protein surface and are hardly quenched.

In conclusion, we have succeeded in preparing mutant β-D-galactosidases substituted by the nonnatural amino acids. By lowering substitution frequency, cell growth and protein biosynthesis rate were not reduced.

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