Expression of proenzyme of microbial transglutaminase and its pressure-dependent unfolding behavior

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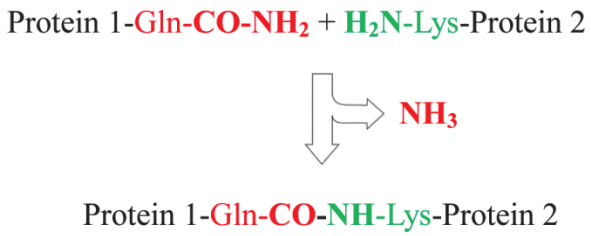
Abstract

Understanding of pressure effect on conformational change of proteins will allow a method by which enzymatic activities are freely manipulated by pressure application. For this goal, we investigated the pressure effect on the proenzyme of microbial transglutaminase (MTG). First, we examined the *E. coli* protein expression system. Then, we found an effective expression conditions. Subsequently, we measured the pressure-induced unfolding of the obtained proMTG. The results indicate that the native conformation of proMTG has a relatively low volume, indicating that structural rigidity is enhanced in the proenzyme state. We suppose that the enhanced rigidity likely contribute to the suppression of its enzymatic activity.

Key words: microbial transglutaminase, proenzyme, pressure-induced unfolding.

1. Introduction

Transglutaminases (TGases, protein-glutamine γ-glutamyltransferases, EC 2.3.2.13) make up a family of enzymes that catalyze the transfer of an acyl group between the γ-carboxyamide group of glutamine residues within peptides and the ε-amino group of lysine residues, resulting in the formation of ε-(γ-glutamyl)-lysine cross-linkages (Scheme 1). [1]



Scheme 1. Protein-protein cross-link reaction by TGase.

TGases are widely distributed in various cells and tissues of mammals, and their physiological properties have been studied. [2] The biochemical function of a microbial transglutaminase (MTG) from *Streptomyces mobaraensis*, which contains 331 amino acid residues and no disulfide bonds and whose activities were independent of calcium, has been studied extensively. [3, 4] The crystal structure of MTG has been determined. [5] MTG is of interest with regard to its applicability to many fields. For example, MTG is used in the meat processing. [6] In addition, applications of its TGase activity to fixation of an enzyme to support

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material [7] and to produce a protein-DNA conjugate, which confer a protein to base sequence-specific binding activity.

MTG is known to be expressed as a preproenzyme (proMTG), which contains a prosequence of 45 amino acid residues at the N-terminus (Fig. 1A). [8, 9] The pro region was thought to be essential for efficient protein folding, secretion, and suppression of its enzymatic activity. The crystal structure of proMTG has also been determined (Fig. 1B). [5, 10] The overall structure of proMTG consists of a single compact domain. A major portion of the prosequence assumes an L-shaped structure, consisting of an extended N-terminal segment with short and long α-helices and blocking the access of the substrates (Fig. 1B). [10] There are a lot of negatively charged residues (Asp1, Asp3, Asp4, Glu249, Asp255, and Glu300) in the substrate-binding cleft. On the other hand, other molecular surface area than the cleft is generally positively charged.

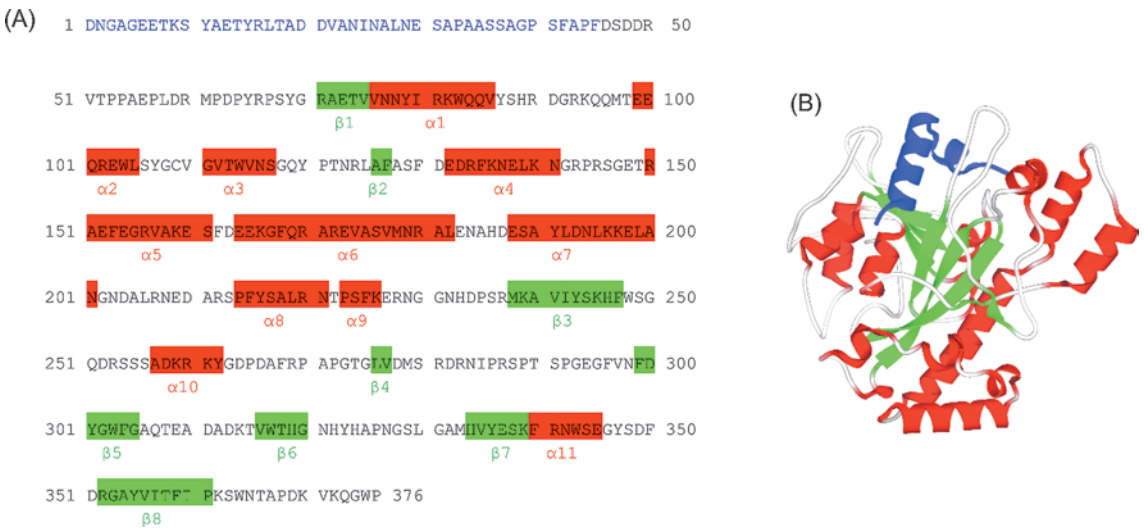

Fig. 1. (A) The position of the secondary structure on the amino acid sequence of proMTG and (B) X-ray crystal structure of proMTG (PDB ID = 3IU0). In both panels, the residues incorporated in the prosequence, α-helix, and β-strand are indicated by blue, red, and green, respectively.

Our goal is to establish methods by which enzymatic activities are freely manipulated by pressure application based on understanding of molecular mechanism of pressure effect on conformational change of proteins. Enzymatic activity is known to be dependent on pressure. For example, a protease activity was found to be raised upon pressure treatment. [11] The enhancement of the activity might be caused by increased molecular fluctuation and partial unfolding of the substrate proteins. Therefore, it is also anticipated that the MTG activity is raised by pressure and, combining the present food processing technique, pressure application might give other effect, such as alternation of texture of foods. For this purpose, molecular mechanism of pressure effect on the MTG structure and function should be studied in detail through spectroscopic and physico-chemical experiments.

For such experiments, an overexpression system of MTG is needed. So far, although proMTG is known to express as the inclusion body in an *E. coli* expression system, the refolding from the inclusion body has

not been successful. [12] However, Suzuki *et al.* [13, 14] recently reported that proMTG can be refolded perfectly from the acid-unfolded state under low-salt conditions.

Here, the purpose of this paper is to establish of proMTG expression system using *E. coli*. proMTG is expected to express as inclusion body. We aimed to obtain correctly folded proMTG by refolding proMTG from the inclusion body by the method of Suzuki *et al.* [13]. After that, the pressure effect on the proMTG structure will be investigated for future applications.

2. Experimental Procedures

2.1 The host-vector system used for the proMTG expression

Fig. 2 illustrates the construction of the vector used for the present experiment, where a cDNA fragment coding the proMTG gene is introduced into the commercial pET-24a plasmid. The vector name is pET24a/proMTG. The competent cell *E. coli* BL21(DE3) was transformed with this plasmid by the standard heat shock method. Subsequent cell suspension was spread on LB agar plate medium containing kanamycin for the selection of the successful transformants.

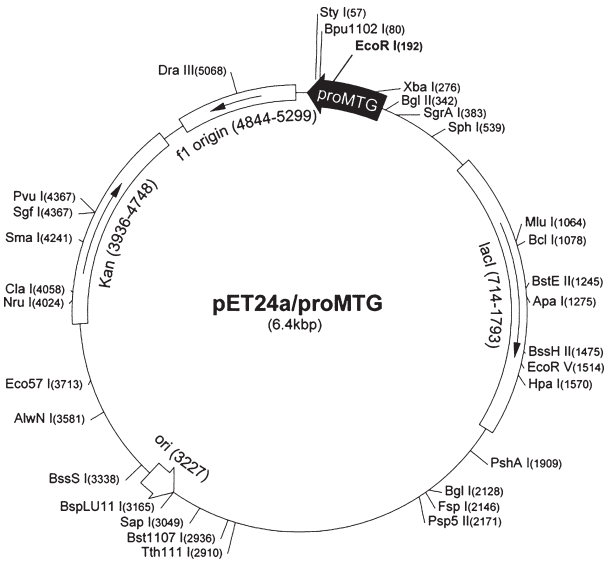

Fig. 2. pET24a/proMTG vector map.

2.2 BugBuster treatments

For the small-scale expression check, the cell collected from 1 mL culture was resuspended by 200 μL BugBuster® (Merck KGaA, Darmstadt, Germany) with 0.2 μL Benzonase solution. After 30 min incubation at room temperature with 1 min vortex in every 10 minutes, the reaction was centrifuged for 5 minutes at 15,000 rpm. After removal of the supernatant, the pellet was dissolved by 200 μL of 8 M urea aqueous solution. Then, the obtained supernatant and pellet solutions were subjected to SDS-PAGE.

2.3 Overexpression of proMTG by *E. coli* system and purification

The colony of the transformant on the kanamycin-LB plate was inoculated into 1 L of M9 medium. The

culture was vigorously shaken at 37℃. When the OD_{600} was reached to 0.6, 1 mL of 0.8 M IPTG was added to the culture. After an overnight incubation at 20℃, the culture was harvested and obtained bacterial cell was frozen. The cell weight was 1.5 g. Refer to Result section about how we determined the induction conditions.

The 1.5 g stored cell was resuspended by 12 mL cell wash buffer (10 mM Tris pH 7.6, 140 mM NaCl) containing 0.2 mM PMSF for inhibition of proteolysis. The resuspension was sonicated for 45 minutes on ice. Subsequent suspension was centrifuged for 30 minutes at 12,000 rpm 4℃ and supernatant was recovered.

The obtained supernatant (12 mL) was diluted with 48 mL of equilibrium buffer (20 mM Tris-HCl pH 7.5, 0.2 mM PMSF) for lowering the salt concentration of the supernatant. It was then flown through a DE52 column equilibrated with 300 mL of equilibrium buffer. After rinsing the column with 100 mL of the equilibrium buffer, the protein was eluted with a 0-0.5 M NaCl gradient.

2.4 Circular Dichroism

The protein concentrations of proMTG were determined spectrophotometrically by using a molar absorption coefficient of 74,205 M^{-1} cm^{-1} at 280 nm. The CD spectra were measured at 25 ℃ and at protein concentrations of 0.08 mg/mL with a 1 mm path length cell from 250 to 190 nm and 0.43 mg/mL with a 10 mm path length cell from 320 to 240 nm with a Model J-820 spectropolarimeter (JASCO Inc., Japan). The results are expressed as mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100\theta_{obs}/lc$, where θ_{obs} is observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters.

2.5 High pressure fluorescence spectrometer

The pressure-dependent conformational transitions were measured by fluorescence spectroscopy at various pHs and 25 ℃. For the measurement, we used a FP-6500 spectrofluorometer (JASCO Inc., Japan) equipped with a high-pressure optical vessel and inner optical cell (Syn-Corporation Co. Ltd., Japan). 0.2 mg/mL proMTG solution containing 10 mM sodium phosphate, 10 mM sodium acetate and 10 mM glycine was first prepared. Then, the solution pH was adjusted to target pH values by adding 1 N HCl or 2 N NaOH. Each measurement was made 3 min after the changing the sample pressure to the target value for temperature equilibrium.

3. Results and Discussion

3.1 Induction conditions

Prior to the over-expression, we performed a small-scale culture to check the proMTG expression. The colony of the transformant on the kanamycin-LB plate was inoculated into 2.5 mL of M9 medium in the test tubes. The culture was vigorously shaken at 37℃. When the OD_{600} was reached to 0.6, 2.5 μL of 0.8 M IPTG was added to the culture. We continued the vigorous shake at 37℃ for 5 hours. Resultant culture was harvested and the obtained cell was subjected to SDS-PAGE after the BugBuster treatments (Fig. 3A). As reported, we observed that proMTG expressed in the precipitated fraction, confirming the formation of inclusion body.

However, we found that the expression behavior of proMTG is dependent on the induction conditions. We subsequently performed a 1-L culture. The culture was vigorously shaken at 37℃ in the growth phase. After the addition of IPTG, we switched the culture temperature to 20℃ and lowered the shaking speed to more gentle speed. After an overnight incubation under these conditions, the culture was harvested and the obtained cell was subjected to SDS-PAGE (Fig. 3B). Then, we found that proMTG expressed in the soluble fraction, indicating that the expressed protein has already attained its native structure. Although the reason of difference in the expression result is unclear, one possibility was that some shaperon molecules were induced by lowering the temperature, which helped the refolding of the expressed proMTG. Seeing the expression result, we skipped the refolding manipulation and proceeded to the purification step.

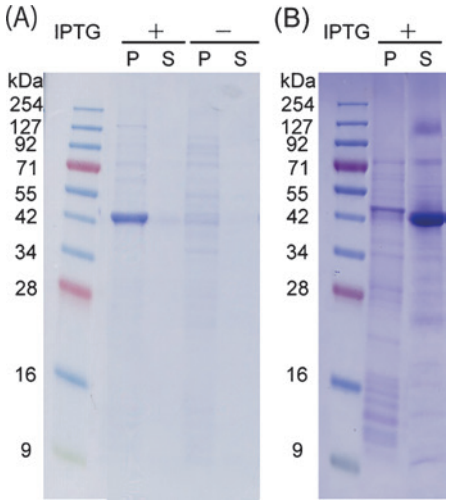

Fig. 3. Expression check of proMTG. The induction conditions were (A) 37℃, 5 hours under vigorous shaking and (B) 20℃, overnight under gentle shaking, respectively. P and S on the top of the gel image stand for the precipitated and soluble fractions, respectively.

After an ultrasonic treatment of the cell suspension, the cell lysate was subjected to anion exchange chromatography. proMTG eluted in early fractions when a 0-0.5 M NaCl gradient was applied (Fig. 4). The absorbance of the proMTG fraction at 260 nm was comparable to that at 280 nm, indicating a contamination of nucleic acids. However, it was found that these species can be removed by ultrafiltration. Based on the elusion pattern, we collected the fractions no.7～11 and concentrated the protein by ultrafiltration. Then, the buffer was replaced by a small gel-filtration column to 10 mM NaAc (pH 5.0) for subsequent spectroscopic measurements.

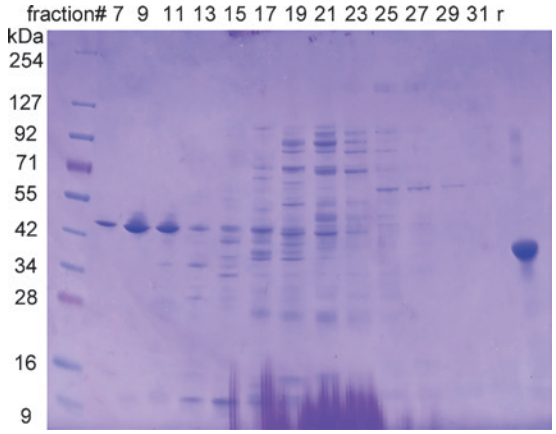

Fig. 4. The elusion result of anion exchange chromatography of expressed proMTG. The rightmost lane "r" is 0.3 mg/mL β-lactoglobulin (36 kDa) for a reference of molecular size and concentration.

Fig. 5 shows the far-UV and near-UV CD spectra of the present sample. These spectra were almost identical to those of the proMTG samples obtained from the corynebacterium expression system CORYNEX® (Ajinomoto Co., Inc.) (Fig. 2A and B in Suzuki *et al.* [13]). Thus, we concluded that the present proMTG sample obtained from *E. coli* has fairly correct secondary and tertiary structures.

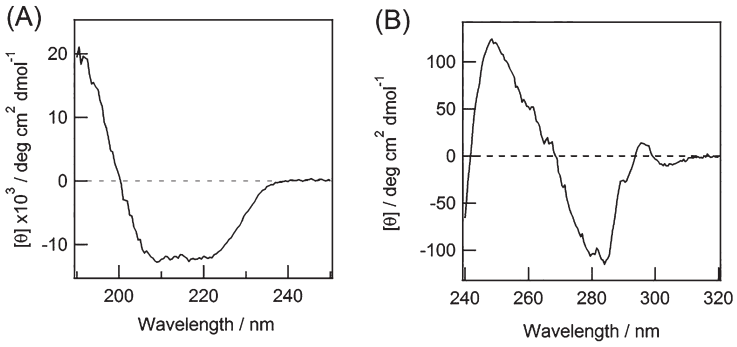

Fig. 5. The far- (A) and near-UV (B) CD spectra of the obtained proMTG.

3.2 Pressure-induced unfolding of proMTG

We measured pressure-induced spectral change of the Trp fluorescence at various pHs (Fig. 6). Although some red shift of the peak wavelength and decrease in intensity were observed at any pHs, degrees of them were not so significant. It indicates that proMTG is relatively insensitive to pressure treatment.

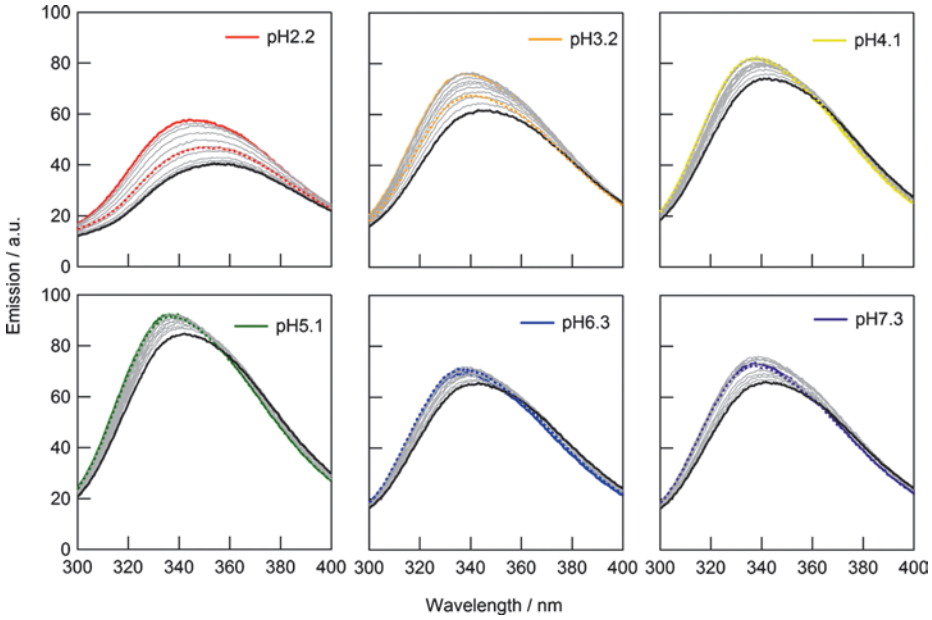

Fig. 6. Pressure-dependent changes of the fluorescence emission spectra of proMTG at pH 2.2～7.3 at 25 ℃ between 5 (colored according to respective pH) and 500 (black) MPa. The colored dotted lines indicate the spectra obtained after pressure release from the last measurement at 500 MPa.

Fig. 7A shows the overlay of the fluorescence spectra obtained at ambient pressure from each pH value. Fig. 7B shows pH dependency of the center of spectral mass (CSM). It can be seen that CSM was constant at pH 4～7 but became higher at pH 2 and 3, indicating proMTG undergoes acid-denaturation under pH 3. This behavior is consistent with the previous report. [13]

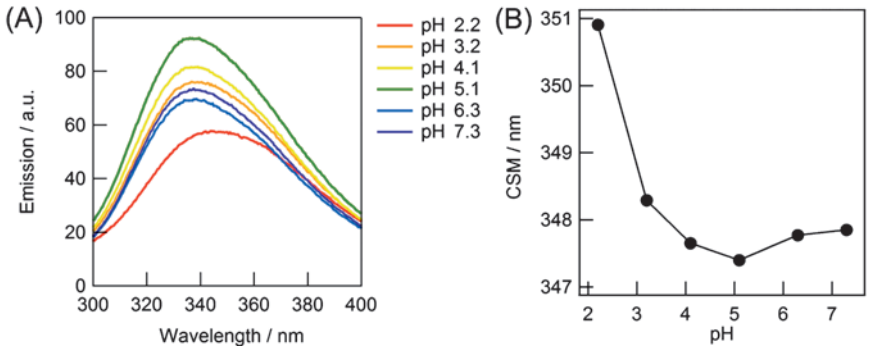

Fig. 7. pH-dependent changes of the fluorescence emission spectra of proMTG at pH 2.2～7.3 at 25 ℃. (A) An overlay of the obtained spectra at respective pHs at ambient pressure. The spectral colors indicate the pH values where the spectra were obtained. (B) The center of spectral mass (CSM) was plotted against measurement pH.

We performed a principal component analysis (PCA) of all of the pH- and pressure-dependent spectral data. The number of the spectra was 6 (pH point) ×12 (pressure point). As a result of the singular value decomposition, we obtained 72 principal components (PCs) and corresponding contribution ratios. Since the accumulative contribution ratio of the first two PCs was 0.87, the two dominant PCs are likely to describe most contributions of the signal changes. Fig. 8A shows a plot of PC1 and PC2. A change in the position of a point on the PC plane corresponds to a fluorescence spectral change. We calculated how the spectral shape changes along with the vertical (PC1) and horizontal (PC2) directions. Fig. 8B shows the calculated spectra corresponding to A～D points on the PC plane indicated in panel (A). The vertical change, corresponding to the change from A to B, includes red shift of the peak wavelength and a strong attenuation of the fluorescence intensity as the pressure increases, whereas the horizontal change, corresponding to the change from C to D, shows a similar red shift but no significant change in intensity. From these spectral changes, it is suggested that an increase in PC1 corresponds to a global unfolding of proMTG because global unfolding induces an exposure of Trp side chain, leading to attenuation of fluorescence and red shift of the peak wavelength, simultaneously. On the other hand, a decrease in PC2 might correspond to water penetration in the proMTG native structure by pressure probably because the interaction between water molecule and Trp side chain in a limited space might lead to red shift by enhanced local polar environment but no significant energy dissipation to the bulk water.

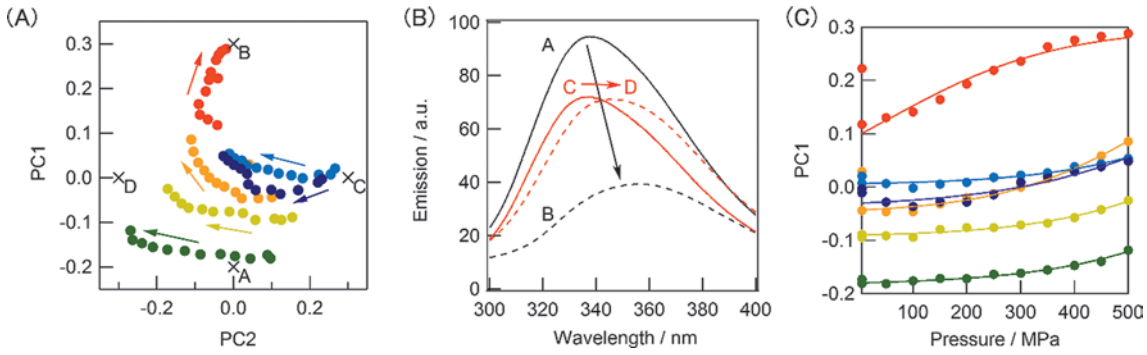

Fig. 8. PCA of the spectral data and subsequent analysis of pressure-induced unfolding reaction. (A) Extracted PCs. A point on the plane corresponds to one spectrum from each measurement. The points in the same color indicate a set of pressure-dependent spectral change in one pH condition. The color indicates the measurement pH values as shown in Fig. 6. The arrows indicate the direction of the spectral change upon pressure increase. (B) The calculated spectra corresponding to the 4 cross points (labelled as A～D) indicated in the panel (A). The spectral change from A to B (indicated by black arrow) and change from C to D (red arrow) correspond to spectral change in PC1 and PC2 directions, respectively. (C) The fitting of the PC1 data for the thermodynamic parameters about pressure-induced unfolding reaction. The solid lines are the fitted theoretical curves based on Eqs. (1) and (2).

Next, we determined the thermodynamic parameters about pressure-induced unfolding reaction. We assumed that the progress of the pressure unfolding is reflected on the PC1 value based on the discussion above and the pressure unfolding of proMTG proceeds in a two-state manner. Then, the theoretical

equation is expressed as follows;

$$f_N = 1/\left(1 + exp\left(-\frac{\Delta G_0 - P\Delta V}{RT}\right)\right) \quad (1)$$

$$PC1_{observed} = f_N \cdot PC1_{Native} + (1 - f_N) \cdot PC1_{Unfolded} \quad (2)$$

where, ΔG_0 ΔV are the Gibbs energy change at ambient pressure and partial molar volume change upon unfolding, respectively. P, R, and T are the measurement pressure, gas constant, and absolute temperature, respectively. f_N is the molar fraction of the native state. Eq. (1) means that, if the ΔV value is larger, the protein is more susceptible to pressure even at lower pressure condition. [15] $PC1_{observed}$ is the PC1 values from the experimental data and $PC1_{Native}$ and $PC1_{Unfolded}$ are PC1 values of the native and unfolded state, respectively. At all pHs, $PC1_{Unfolded}$ was fixed to 0.3, which corresponds to the value of the spectrum obtained at pH 2.2 and 500 MPa, because proMTG is assumed to be completely unfolded under these conditions. On the other hand, at pH 3.2～7.3. $PC1_{Native}$ was set to be variable, whereas, at pH 2.2, it was fixed to -0.0592, which corresponds to the $PC1_{Native}$ value at pH 3.2. The obtained fitting parameters are summarized in Table 1.

Table 1. Results of the thermodynamic analysis of the pressure-induced unfolding reaction.

pH	2.2	3.2	4.1	5.1	6.3	7.3
ΔG_0 / kJ mol^{-1}	-0.632	-8.52	-11.6	-11.7	-11.8	-9.14
ΔV / mL mol^{-1}	-15.3	-15.0	-15.4	-14.0	-16.0	-13.5
$PC1_{Native}$	-0.0592*	-0.0592	-0.0938	-0.184	0.00424	-0.0386
$PC1_{Unfolded}$	0.3*	0.3*	0.3*	0.3*	0.3*	0.3*

*There values were fixed.

It is characteristic that the absolute values of ΔV upon unfolding obtained were smaller than those of other proteins. For example, the ΔV values for chicken ovomucoid and *E. coli* DHFR are -28.9 and -25 mL mol^{-1}. [16, 17] Protein unfolding generally accompanies a reduction in the partial molar volume. The reduction is supposed to be caused by disappearances of void spaces in the native structure upon unfolding. [18] The relatively small ΔV value indicates that proMTG harbors less voids in its native structure. proMTG is a pro-enzyme, where its enzymatic activity is suppressed by the N-terminal prosequence. As mentioned in Introduction, the active site cleft is covered by the prosequence (Fig. 1B). It is suggested that molecular fluctuation is important for protein function. From these points of view, the prosequence might also contribute to raise the rigidity and reduce the fluctuation of the MTG molecule. As a result, the native structure of proMTG contains little void space, leading to relatively small ΔV values. In order to confirm this idea, the ΔV values of mature MTG should be compared with that of proMTG in future. It is expected that the ΔV values of mature MTG is larger than that of proMTG. Furthermore, as all

of proenzymes might have this tendency, the ΔV values of these proteins should be investigated systematically to reveal the general property of proenzymes.

4. Conclusions

We examined the expression of proenzyme of microbial transglutaminase (MTG). We found that, if the protein expression is induced at low temperature, proMTG expresses in the soluble fraction with a correctly folded structure. This finding will enhance the production of proMTG for the laboratory researches. We subsequently measured the pressure-induced unfolding of the obtained proMTG. The experimental results indicate that the native conformation of proMTG has a relatively low volume, indicating little voids in its conformation. This finding suggests that the conferred rigidity might contribute to suppression of the activity of the proenzyme. Our finding is possibly common to other proenzymes.

5. Acknowledgment

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6. References

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和 文 抄 録

大腸菌による proMTG 発現精製と圧力変性

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酵素活性は加圧条件下で変化することが知られている。圧力のこの効果の分子的なメカニズムを理解し加圧による酵素機能の制御が可能になれば、幅広い応用が予想される。そこで、我々は加圧による微生物由来トランスグルタミナーゼ（MTG）の構造への影響を調べることを目指した。タンパク質架橋化酵素、トランスグルタミナーゼ（TG）は幅広く生物界に存在し、基質タンパク質どうしを架橋する機能を持つ酵素である。

はじめに我々は MTG のプロ酵素である proMTG の大腸菌による大量発現の系の確立を目指した。発現条件検討の結果、20℃で長時間の発現誘導を行うと、正しい構造を持った状態で発現することが分かり、効率の良い proMTG 発現条件を決定できた。

得られた proMTG を用いて圧力蛍光測定実験を行ったところ、圧力感受性が非常に低いことが分かった。これは変性に伴う体積変化（ΔV）の絶対値が小さいことを示す。一般的に変性に伴い、蛋白質分子のモル体積は減少し、天然状態の分子構造内にボイドと呼ばれる隙間が多いと ΔV の絶対値は大きくなるが、proMTG ではプロ配列が酵素の活性部位にクレフトにぴったり挟まっており、これが分子構造に隙間のない状態にしているため低い ΔV が得られたと考えられる。構造が密になることで pro 状態の分子の剛直性を上げ、その結果酵素活性が抑制されているものと考えられる。

キーワード：微生物トランスグルタミナーゼ、プロ酵素、蛋白質の圧力変性

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