Overproduction of DnaJ in *Escherichia coli* Improves in Vivo Solubility of the Recombinant Fish-derived Transglutaminase

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The overexpression of red sea bream (*Pagrus major*) transglutaminase (TGase, E.C. 2.3.2.13) in *Escherichia coli* mostly leads to the accumulation of biologically inactive enzyme. Although the solubility of the gene products could be improved by cultivation at a lower temperature (26–28°C), most of the synthesized TGase was still in the form of insoluble aggregates. The effects of overproduction of molecular chaperones on the intracellular solubility of newly produced recombinant TGase were examined. The overexpression of dnaK or groES/EL did not improve solubility. However, DnaJ greatly increased the solubility of the recombinant TGase, resulting in active enzyme in the presence of calcium ions. Co-expression of dnaK along with dnaJ further increased the content of soluble TGase. Under our experimental conditions, supplementation with both DnaJ and Dnak elevated the TGase activity in the producer cells by roughly 4-fold, compared with the control strain cultured at 30°C. Thus, we found that DnaJ is important in controlling the solubility of protein overproduced in *E. coli*.

Key words: *Escherichia coli*; DnaJ; transglutaminase; co-expression; texture

Transglutaminases (TGase: protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) are a family of enzymes that catalyze acyl transfer between the γ-carboxyamide groups of glutamine residues within peptides and lysyl ε-amino groups, resulting in the formation of ε-(γ-glutamyl)lysine cross-links. Plasma factor XIII a subunit is one of the best characterized TGases. TGase is widely distributed from humans to microorganisms. The participation of TGases in gel formation from mince sols of fish has been studied, and it was reported that endogenous TGases catalyze the cross-linking of myosin heavy chains in fish paste, resulting in viscoelastic properties of the paste. Therefore, TGases including fish-derived enzymes may be applied to food products to improve texture.

We previously reported the purification of tissue-type TGase from red sea bream (*Pagrus major*), and the cloning of its cDNA. We demonstrated that red sea bream TGase was composed of 695 amino acids, and found that soluble and functionally active TGase could be produced in *Escherichia coli* cells when cultured at the reduced temperature of 28°C, while at 37°C, only aggregates of inactive TGase protein were accumulated in the cells. Hence, we investigated the temperature-sensitive intracellular solubility of the TGase synthesized in this system, and undertook the construction of a more effective production system to obtain large amounts of enzymatically active fish-derived TGase.

It has been reported that many proteins require the assistance of molecular chaperones to attain their final structure and that the intracellular solubility of some recombinant proteins can be improved by the co-expression of chaperones. Blum et al. (1992) reported that co-overproduction of Dnak significantly reduced inclusion body formation of human growth hormone protein, and identified Dnak as an important factor controlling heterologous protein aggregation in *E. coli*. Lee and Ollins (1992) described the overexpression of *dnaK* and *groES/EL* on procollagenase production, and found a diverse range of effects of each chaperone on both solubility and accumulation of the protein.

In this study, we examined the abilities of the *E. coli* molecular chaperones GroES/EL, Dnak, and DnaJ to improve the solubility of recombinant red sea bream TGase. The results obtained here indicated that overexpression of *dnaJ* predominantly improved the solubility of the TGase, and therefore the active enzyme could be prepared efficiently.

Materials and Methods

*Bacterial strains and plasmids. * *E. coli* strain HB101 was used as the host for DNA manipulation and for the TGase producer. *E. coli* strains NRK233 (*groES2*), NRK117 (*groEL4*), and KY1453 (*danJ259*), which are derivatives of MC4100 (*groE*′*, araD139*, Δ(argF-lac)U169, rpsL150, *relA1*, *flbB5301*, *deoC1*, *ptsF25*, *rbsR*), were kindly provided by Dr. M. Kanemori, and were used for complementation testing of individual heat shock genes cloned by polymerase chain reaction (PCR) techniques. The expression plasmid pTTG2-22 (Fig. 1) and the vector plasmid pTTNco were used for the production of red sea bream TGase under control of the trp promoter and as a control plasmid, respectively. These plasmids were derived from pBR322 and were ampicillin resistant. Plasmids pGroE-01, pDnak-01, and pDnaJ-01 and pDnaKJ-01 for expression of *groES/EL*, *dnaK*, *dnaJ*, and *dnaKJ*, respectively were constructed as described below.

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Isolation of E. coli heat shock genes. The genes encoding chaperones were prepared from E. coli K-12 chromosomal DNA by PCR. DNA fragments of groES/EL, dnaK and danJ were amplified with the synthetic oligonucleotide primer pairs, GROESL-01 (5'-GACAAGCCTGATAGCGACGCAAT-3'); GROESL-02 (5'-GAGCCACCTCGGCTGCAGT-3'); DNAK-01 (5'-GATGACGGCCATCCGCGCTGCAGT-3'); DNAK-02 (5'-TTCCGCTTCGCGCCGCGCTGCAGT-3'); and DNAJ-01 (5'-CAGCTGGAATTTGACGAATCG-3'), DNAJ-02 (5'-CTGACGAGGAGAATTCCGTCGCAGT-3'), respectively. The amplified DNA fragments were cloned into a chloramphenicol-resistant plasmid pST28 (Takara Shuzo Co., Kyoto, Japan) derived from pACYC184 which is compatible with pBR322. The resulting plasmids were designated pGroE-01, pDnaK-01, and pDnaJ-01, respectively. pDnaKJ-01 was constructed by ligation of a fragment including dnaJ from pDnaJ-01 and a fragment containing dnaK and ori regions from pDnaK-01.

Expression of transglutaminase in E. coli. Transformed E. coli cells were aerobically grown at 32°C for 14 h in 2 x YT medium with ampicillin (200 μg/ml) and/or chloramphenicol (30 μg/ml), and then this seed culture was transferred into the production medium (15.1 g Na₂HPO₄·12H₂O, 3.9 g KH₂PO₄, 8 g casamino acids, 0.2 g yeast extract, 0.2 g L-leucine, 0.2 g L-proline, 2 mg thiamin-HCl, 0.5 g MgSO₄·7H₂O, 14.5 mg CaCl₂·2H₂O, and 5 g glucose per liter) in Erlenmeyer flasks. TGase synthesis was spontaneously induced by depletion of tryptophan in the growth medium. Cultivation was continued for 20 h at the temperatures indicated, and growth of transformants was monitored by optical density measurements at 660 nm. Cells were harvested by centrifugation at 5,000 x g for 10 min and stored at -85°C.

Preparation and fractionation of cell extract. The cell pellets harvested from 50-ml cultures were suspended in 30 ml of buffer R (20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 5 mM EDTA). Then, the cells were disrupted by ultrasonication at 4°C with a Branson model-250 sonifier (100 watts, 15 sec, 6 times). The cell lysates were centrifuged at 20,000 x g for 20 min at 4°C to segregate the soluble and the insoluble fractions. The insoluble protein fraction was resuspended in 30 ml of buffer R, and after sonication of the suspension, portions were solubilized with SDS-containing loading buffer. Soluble and insoluble protein fractions containing equal volumes of original culture were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Assay of transglutaminase activity. TGase activity measurement was done as described previously. Briefly, the activity was measured by the incorporation of monodansylcadaverine (MDC) (Sigma Chem. Co., St. Louis, U.S.A.) into N,N'-dimethylcasein (Sigma Chem. Co.), and the fluorescence intensity of protein-bound MDC was measured. One unit of TGase activity was defined as catalyzing the incorporation of 1 nmol of MDC into N,N'-dimethylcasein in 1 min at 37°C. For the activity calculation, a specific TGase of 2000 U/mg was taken as a basis for the purified recombinant red sea bream enzyme (unpublished result).

SDS-PAGE analysis and Western blotting analysis. The E. coli cell extracts were solubilized and electrophoresed on a SDS-polyacrylamide gel as described by Laemmli (1970), with a gradient gel from 4% to 20% acrylamide (TEFCO Co., Tokyo). Then the proteins were transferred onto nylon membranes (Trans-Blot Transfer Medium, BioRad Labs, Richmond, U.S.A.) in an electrophoretic blotting apparatus. The primary antibodies used as probes were anti-GroEL mouse monoclonal antibody (StressGen Biotech. Co., Victoria, Canada), anti-DnaK mouse monoclonal antibody (StressGen Biotech. Co.), and anti-DnaJ polyclonal antiserum (a gift from Dr. C. Wada). The secondary antibodies were biotinylated anti-mouse IgG and anti-rabbit IgG (Vector Labs. Inc., CA), and then avidin and biotinylated alkaline phosphatase were reacted under the conditions recommended by the manufacturer (Vector Labs. Inc.). The reactants were stained using the BCIP/NBT phosphatase substrate system (Funakoshi Co., Tokyo).

Results
Effects of culture temperature on productivity of transglutaminase in E. coli
The productivity of red sea bream TGase in E. coli harboring pTTG2-22 (Fig. 1) was investigated at various culture temperatures to identify the temperature at which the most active enzyme could be recovered from the producer cells. As shown in Figure 2A, the total
production level increased with elevation of culture temperature from 21°C to 32°C, and reached maximum productivity from 32°C to 37°C. Intracellular aggregates were formed in large amounts in the transformants cultured at temperatures above 30°C, and the recombinant TGase content in the whole cellular proteins was approximately 15%–20%. However, the maximum enzymatic activity of TGase was obtained from producers grown at 26°C–28°C, and as the culture temperature rose to above 30°C, TGase activity decreased markedly (Fig. 2C). We examined the content of TGase accumulated in the soluble fraction. Figure 2B shows the temperature-dependent solubility of TGase synthesized. By SDS-PAGE analysis of fractionated cell lysates, we found that most of the TGase protein synthesized maximally at 32–37°C could not be recovered in the soluble fraction, and that the soluble TGase contents from each transformant were considerably correlated with the activities in each extract (Fig. 2C). These results indicate that the lowered expression level is related to the increased recovery of enzymatically active TGase, suggesting that extremely rapid synthesis of polypeptides beyond the capacities of the cellular chaperone machinery inherent in the host organism may induce the formation of inactive aggregates.

Construction of groES/EL, dnaK and dnaJ expression plasmids

To examine whether overproduction of E. coli heat shock chaperones can increase the amount of soluble TGase produced, we initially cloned chaperone genes and constructed a second plasmid expressing each groES/EL (synthesizing GroESL complex), dnaKJ, dnaK and dnaJ. These plasmids were named pGroE-01, pDnaKJ-01, pDnaK-01 and pDnaJ-01, respectively, and each chaperone gene was expressed from its own promoter. We assessed the expression levels of GroEL, DnaK and DnaJ in the TGase producer cells. The increases of GroEL and DnaK production by pGroE-01 and pDnaK-01 were easily detected by SDS-PAGE analysis of whole-cell extracts (Fig. 3A), and confirmed by Western blotting analysis using anti-GroEL and anti-DnaK antibodies (data not shown). The amounts of GroEL and DnaK were increased by more than 20-fold by pGroE-01 and pDnaK-01, respectively. The increase of DnaJ production by pDnaJ-01 was detected by Western blotting analysis of whole-cell extract (Fig. 3B). The amount of DnaJ was increased at least 4-to 5-fold by pDnaJ-01.

Effects of overproduction of chaperones on the intracellular solubility of active transglutaminase

We constructed a series of TGase producer strains containing both pTTG2-22 and expression plasmids for each chaperone described above, and explored the effects of overproduction of chaperones on TGase activity. Co-expression of groES/EL or dnaK did not improve the TGase activity. However, supplementation of DnaJ by pDnaJ-01 increased the enzymatic activity by roughly 2-fold compared with the producer cells containing control plasmid (pSTV28) without chaperone genes cultured at 28°C (Fig. 4). In addition, pDnaKJ-01 showed a greater improvement of TGase activity than pDnaJ-01. The maximum activity of synthesized TGase was reached in producers cultured at 30°C, and the level expressed was 4-fold higher than that of the control strain grown at 30°C (Fig. 4).

Evaluation of inclusion bodies within TG producer
Fig. 3. Overproduction of GroEL, DnaK, and DnaJ along with Co-expression of Transglutaminase (TGase) Gene.

Whole cell extracts were prepared from the TGase producer grown at 35°C. (A) SDS-PAGE analysis for estimation of GroEL and DnaK contents. The arrows indicate the positions of GroEL, DnaK and TGase. Lane 1, pSTV28; lane 2, pDnaK-01; lane 3, pDnaJ-01; lane 4, pDnaKJ-01; lane 5, pGroE-01. The position of protein size markers are indicated on the left of the gel. (B) Western blot analysis of expression levels of DnaJ in the TGase producer. The arrow indicates the position of DnaJ. Lanes 1-5 are the same samples as those in (A).

Fig. 4. Effects of Overproduced Chaperones on the Transglutaminase (TGase) Activity Expressed in E. coli.

The soluble fractions of whole cell extracts were prepared from the TGase producer grown at various temperatures. The names of plasmids co-expressed along with pTTG2-22 are shown under the horizontal axis. Relative specific activity (vertical axis) of TGase expressed in the producer cells grown under the indicated conditions (horizontal axis) is shown. Specific activity of TGase in the producer cells grown at 30°C co-expressed along with pDnaKJ-01 is indicated as 100%.

Cells by phase-contrast microscopy found that the overproduction of DnaK and DnaJ significantly suppressed the formation of aggregates (data not shown). Figure 5 shows the intracellular distributions (soluble or insoluble fraction) of produced TGase molecules, indicating that co-expression of dnaJ or dnaKJ increased the soluble TGase content. In cells cultured at 32°C, the contents of soluble TGase increased from 10% to 50% of total TGase protein produced. These results indicated that the increase of the TGase activity recovered is attributable to the improvement of intracellular solubility of TGase protein.

No significant differences in growth rate were observed between TGase producers co-expressing dnaKJ or harboring the control vector, and the maximum optical densities reached were nearly equivalent. This suggested that the overall slowdown of the protein synthesis rate was not caused by overproduced chaperones, although a decrease in the rate of polypeptide chain elongation could generally induce the formation of soluble proteins. In addition, the stable coexistence of both plasmids expressing TGase and DnaKJ during cultivation was shown by examination of antibiotic resistance of colonies at the end of culture (data not shown).

Discussion

Recently, it has been reported that co-expression of chaperones has a positive effect on the production of active enzymes and soluble proteins, or the efficient secretion of particular proteins. Among the chaperones in E. coli, the importance of DnaK and GroEL in folding of overproduced heterologous products has been demonstrated. Therefore, we first assessed the effects of these chaperones on the productivity of active TGase, but the results were poor. In this study, we found that an elevated DnaJ level increased the enzymatic activity of red sea bream TGase in the producer cells. This result was unexpected, since it was not known that DnaJ has a major role in solubilization of overproduced proteins. DnaJ predominantly took part in solubilization of the TGase protein through the suppression of polypeptide aggregation.

Gragerov et al. (1992) showed that both DnaK and DnaJ were required to prevent the aggregation of newly synthesized proteins in rpoH mutants. Thomas et al. (1996) reported that protein folding of preS2-β-galactosidase fusion protein (preS2 is the hepatitis B surface antigen) was partially restored upon co-overexpression of the dnaJ operon, but not when the groE operon or dnaK alone were overproduced. In these cases, it is considered that the aggregation of the protein could be suppressed by overproduction of DnaJ alone.

Langer et al. (1992) proposed that sequential reaction of DnaK, DnaJ and GroEL is important in chaperone-mediated protein folding, and showed that DnaJ itself retarded aggregation of the unfolded target protein. Also, it was suggested that DnaJ is the first chaperone to bind nascent polypeptide chains during protein synthesis and guides DnaK in the substrate complex.
before, we supposed that the nascent polypeptide of red sea bream TGase synthesized rapidly might not be recognized by the DNAJ chaperone, and hence the incompletely folded polypeptides aggregate. In this context, it is speculated that an abundance of DNAJ from overproduction could compensate for the insufficient interaction between DNAJ and the nascent TGase polypeptide. In addition, the co-expression of dnaK along with dnaJ could further increase the TGase activity. This suggests that the efficient suppression of aggregation of overproduced proteins requires an appreciable molar ratio of DnaK and DNAJ.

As a novel function of DNAJ, Axelle et al. (1995) reported that DNAJ catalyzes protein disulfide formation, reduction and isomerization, similarly to thioredoxin, protein-disulfide isomerase and DsbA. The tertiary structure model of red sea bream TGase (rsbTGase) was analyzed using the sequence-structure compatibility (3D-ID) method program based on the X-ray crystal structure of plasma factor XIII a subunit (FXIII a). The model indicated that the tertiary structure of rsbTGase resembles that of FXIII a, and rsbTGase has a disulfide bond (unpublished data). Therefore, it was speculated that the abundance of DNAJ could promote the native disulfide bond linkage in TGase during the course of tertiary structure formation of rsbTGase from the overproduced polypeptide and hence increase its solubility.

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