Chemical Synthesis of the Gene for Microbial Transglutaminase from *Streptovorticillum* and Its Expression in *Escherichia coli*

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The gene coding for microbial transglutaminase (TGase) from *Streptovorticillum*, which consists of 331 amino acids, was chemically synthesized. The codons have been substituted for those mainly favored in yeast. Our strategy involved the construction of the TGase gene in five sections (54 oligomers) that contained unique restriction enzyme sites at both ends, which could readily be ligated to form the full-length product. The chemically synthesized gene was inserted downstream from the ompA signal peptide of the *E. coli* expression vector, pN-III-ompA, which carries *lpp* and *lac* promoters. The resultant plasmid directed the expression of TGase, with the activity being secreted mainly into the periplasmic space of *E. coli*. The induced gene product was identical with native TGase in size and in immunological properties, though the enzyme activity was low.

Transglutaminases (glutamyl-peptide : amino γ-glutamyltransferase, EC 2.3.2.13) are acyl-transfer enzymes that catalyze the cross-linking of proteins by promoting the formation of isopeptide bonds (ε-(γ-Glu)Lys bonds) between protein-bound glutamine and lysine residues. These enzymes are widely distributed in various cells and tissues of mammals, and their enzymatic and physiological properties have been studied. The best characterized transglutaminase (TGase) is plasma TGase (Factor XIII), which has been purified, cloned, and sequenced. Ikura et al. have cloned the cDNA for TGase from guinea pig liver (tissue TGase), and examined its expression in *E. coli*. A TGase derived from a microorganism has been identified, and its enzymatic properties have been characterized. The purified TGase from a culture filtrate of *Streptovorticillum* S-8112 has been analyzed by automated Edman degradation and its complete amino acid sequence has been analyzed by Kanaji et al. Consequently, we made it possible to synthesize the microbial TGase gene using synthetic oligonucleotides.

In this study, we synthesized the microbial TGase gene encompassing the entire coding region for the protein. The synthesized gene was expressed in *E. coli* using the pN-III-ompA vector and the gene product had the same properties as native TGase.

**Materials and Methods**

*Bacterial strains and plasmids*. Recombinant plasmids were constructed in *E. coli* JM109 from pUC18. E. coli JA221 (hisD + lacY recA1 lacTV pro + ) was used as a host for expression of the synthetic TGase gene. The pN-III-ompA vector was used as an expression vector.

*Reagents. All enzymes for DNA manipulation were from Takara Shuzo, used under the conditions recommended by the supplier. The Vectastain ABC kit was obtained from Vector Laboratories Inc. An anti-TGase polyclonal antibody raised in a rabbit was obtained from Nippon Bio-Test Laboratories Inc. CBZ-g-glutaminylglycine was obtained from Kokusan Chemical Works, Inc.*

Oligonucleotide synthesis. The synthetic TGase gene was divided into 54 oligonucleotides (A1–E10 in Fig. 1). Among them, some oligomers (A1, A2, A11, A12, B1, B2, B9, B10, C1, C2, C11, C12, D1, D2, D9, D10, E1, E2, E9, and E10) have additional base(s) at one end for the formation of EcoRI or HindIII cleavage sites (Fig. 2). The 54 oligomers, ranging in size from 18 to 47 nucleotides were synthesized using phosphoramidite chemistry on an Applied Biosystems models 380A DNA synthesizer. Crude oligomers were purified in two steps. First, the tritylated oligomers were passed through the Applied Biosystems OPCs, then purified by reversed phase HPLC on an Inertsil ODS-2 column (4.6 mm x 150 mm), and then assembled into five fragments to form the synthetic TGase gene.

**Assembly of the component fragments.** Standard DNA techniques were used. The synthetic TGase gene was divided into five separate fragments (A, B, C, D, and E), which were assembled as follows. All oligomers comprising each fragment, except for the two final 5′ termini were phosphorylated using T4 polynucleotide kinase, then complementary pairs of individual oligomers were constructed by annealing. Fragment A comprised oligomers A1–A12, all of which, except A1 and A12 were phosphorylated, and the annealed oligomer pairs, A1/A2 and A3/A4, A5/A6 and A7/A8, A9/A10 and A11/A12, were ligated, respectively. The reaction mixtures were examined by 10% polyacrylamide gel electrophoresis (PAGE), and the predicted components were isolated. The three components obtained (A1/A2/A3/A4, A5/A6/A7/A8, and A9/A10/A11/A12) were further ligated in a single reaction, to yield an entire fragment, and purified by 10% PAGE (Fig. 3). The other fragments also were assembled in the same two-step manner. The final fragments were ligated to EcoRI + HindIII-digested pUC18 and those sequences were confirmed by dideoxy chain-termination sequencing. Almost all of the clones sequenced were correctly assembled.

Expression of the TGase in *E. coli*. The synthetic TGase gene was expressed in *E. coli* JA221, which was cultivated at 37°C in M9 medium supplemented with ampicillin (50 μg/ml) and casamino acids (2%). When the cell growth (A600) reached 0.4, the culture temperature was shifted to 23°C and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium to a final concentration of 1 mM to induce gene expression. After culture for 3 h at 23°C, the cells were harvested by centrifugation. The periplasmic fraction was prepared by the method of Koshland and Bovee.

Transglutaminase assays. TGase activity was measured by a colorimetric
hydroxamate procedure using N-carbobenzyoxy-L-glutaminyll-y-glycine. Hydroxylamine and CBZ-glutaminyll-y-glycine were used as substrates, and the reaction proceeded for 10 min at 37°C. One unit caused the formation of one micromole of hydroxamic acid per min. A calibration curve was prepared using l-glutamic acid-y-monohydroxamic acid. Protein concentrations were measured using the Bio-Rad Protein Assay kit.

Immunoblotting. The culture medium and periplasmic fractions except that of cytosol were concentrated by trichloroacetic acid (10%) precipitation, and all fractions (0.5 µg protein) were separated using a 10–20% gradient gel (Daiichi Pure Chemicals Co., Tokyo, Japan) by SDS-polyacrylamide gel electrophoresis. The gel was stained with silver. The recombinant TGase was detected by western blotting using an anti-TGase polyclonal antibody.

Results

Design of the TGase gene

A major advantage of synthetic genes is the ability to alter the nucleotide sequence encoding the protein of interest. We have designed a synthetic TGase gene based on the known amino acid sequence of TGase derived from the Actinomyces strain, Streptococcus S-8112, which consists of 331 amino acid residues. The amino acid sequence of TGase and the synthetic gene fragments are shown in Fig. 1. The amino acid codons used in this synthesis were selected mainly from those favored in Saccharomyces cerevisiae, to increase the translational efficiency in yeast. As indicated in Fig. 1, sequences for the restriction enzymes HpaI, XbaI, BglII, and NcoI were designed for unique sites.

Fig. 1. Nucleotides and Amino Acid Sequence of the Synthetic TGase Gene.

Synthesized oligomers are numbered A1 to E10. Fragment designations and lengths are specified (refer to Fig. 2 for more detail about fragment designations). The restriction enzyme sites used for construction of the gene are indicated.
Fig. 2. The Five Component Fragments of the Synthetic TGase Gene. Fragments were cloned into the EcoRI and HindIII sites of pUC18, and other restriction sites were used to construct the gene. Each fragment comprised 10-12 oligomers.

without disrupting the amino acid sequence of native TGase. The termination codons were also added at the C-terminus twice.

Because of the length of the TGase DNA (993 bp), it was divided into five separate fragments to assemble and subclone. The subassemblies, designated A, B, C, D, and E were comprised of 12, 10, 12, 10, and 10 oligomers, respectively, arranged in complementary pairs. The overlaps joining the pairs consisted of six to eight bases to facilitate annealing, and were designed to be unique. Each fragment was about 180–220 bp long and contained unique EcoRI and HindIII sites at the 5′ and 3′ ends, respectively, for subcloning into pUC18 and plN-III-ompA (Fig. 2). These EcoRI and HindIII restriction sites were added as extensions, flanking the natural sequence. Restriction sites internal to these (HpaI, XbaI, BglII, and NcoI) were used for assembly of the intact gene.

Construction of the TGase gene and the expression plasmid

Assembly of the five fragments to form a total synthetic TGase gene is shown in Fig. 3. These fragments were made of two or three components containing 2–3 annealed oligo pairs, respectively, and each was assembled in two steps as described in Materials and Methods. The five assembled component fragments were each cloned into pUC18 which had been digested with EcoRI + HindIII. The final assembly was confirmed by nucleotide sequencing.

The synthetic TGase gene was constructed as follows (Fig. 3). Restriction enzyme sites were used to join the five fragments. The Scal–XbaI fragments containing Frags B or C were ligated, and the Scal–NcoI fragments containing Frags D or E were also ligated, to yield about 400-bp synthetic gene segments (B–C or D–E), respectively. Next, the Scal–BglII fragments containing Frags B–C or D–E were ligated to yield about an 800-bp segment (B–C–D–E).

Finally, a 0.2-kb EcoRI–HpaI fragment containing Frag A and a 0.8-kb HpaI–HindIII fragment containing Frag B–C–D–E were ligated into the expression vector plN-III-ompA which had been digested with EcoRI + HindIII. The resulting plasmid pOMP-TG carries the entire 993-bp synthetic TGase gene. The TGase gene was inserted downstream from the ompA signal peptide sequence under the control of the lpp and lac promoters. The correctly assembled TGase expression plasmid, pOMP-TG, was identified and characterized by restriction enzyme mapping, and the sequence was confirmed by deoxy sequencing. No insertions or deletions were detected following ligation of the fragments to assemble the TGase gene.

Expression of the TGase gene in E. coli

E. coli JA221 was transformed with the expression vector pOMP-TG, and the expression of the synthetic TGase gene was examined in the transformant by induction with IPTG. At 23°C, cell growth was inhibited by the addition of IPTG within 1 h, and apparently cells lysed 3 h after induction (data not shown). This is considered to be the reason for TGase activity in the cells. The culture broth was separated into culture medium, periplasmic space, and cytosol. Each fraction (0.5 μg protein) was analyzed by western blotting (see Fig. 4 and Materials and Methods). The induction conditions were examined at several IPTG concentrations and temperatures, however there was no significant difference in production (data not shown). When TGase gene expression was induced with 1 mM IPTG at 23°C, a protein (about 37 kDa), which was immunoreactive with anti-TGase antisera, was detected in every fraction of the transformant harboring pOMP-TG (Fig. 4b, lanes 3, 6, and 9). Furthermore, every fraction contained an immunoreactive band and a little larger band (about 40 kDa), which was the precursor (ompA signal peptide fused TGase) resulting from not undergoing proteolytic processing, was mainly observed in the cytosol fraction (Fig. 4b, lane 9). From the relative intensity of the immunoreactive bands (Fig. 4b, lanes 3, 6, and 9), its concentration was estimated to be about 5 mg/liter of culture. When induced with 1 mM IPTG at 37°C, the immunoreactive band was only slight (Fig. 4, lanes 7 and 10).

The 37 kDa protein was further substantiated by the fact that TGase activity was measured in all fractions using the TGase assay (see Table and Materials and Methods). Enzymes in all fractions were assayed without further purification. When induced with IPTG, TGase activity was also detected in all fractions. The TGase activity of the periplasmic fraction were the highest (0.22 U/mg protein). On the other hand, a significant decrease in the specific activity occurred in the cytosol and the culture medium. This is probably because that the precursor form in the cytosol is almost inactive and the cells lyse markedly 3 h after induction. However, compare with the specific activity of the purified TGase from Streptococcus (22.6 U/mg

<table>
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<tr>
<th>Plasmids</th>
<th>Fraction</th>
<th>Induction (IPTG)</th>
<th>TGase activity (U/mg)</th>
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<tbody>
<tr>
<td>plN-III-ompA</td>
<td>Control</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>pOMP-TG</td>
<td>Culture medium</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>Periplasmic space</td>
<td>+</td>
<td>0.025</td>
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<tr>
<td>Cytosol</td>
<td>+</td>
<td>0.22</td>
<td>0.033</td>
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Table Specific Activity of TGase in Each Fraction from E. coli JA221 Carrying Plasmids

Assays were done as described in Materials and Methods.
protein, the activity level even in the periplasmic space was only 1% of the purified enzyme.

**Discussion**

This study showed that a totally synthesized *Streptovercillium* TGase gene was expressed in *E. coli* under the control of the lpp and lac promoters. The product of the synthetic TGase gene retained all the properties of the natural TGase despite having 3 extra amino acid residues upstream of the N-terminal end of mature TGase, which resulted from the construction of the TGase expression vector. However, the level of TGase gene expression in this experiment was quite low, in the range of about 5 mg/liter of culture in *E. coli* harboring pOMPA-TG. At present, it should be noted that TGase produced by *E. coli* has not been purified to homogeneity, and therefore, we cannot compare the enzymatic properties of native TGase from *Streptovercillium* with those of recombinant enzyme from *E. coli* in detail. The plN-III-ompA vector used in this study is expressed by induction with IPTG. In our previous work,
we found that there are two essential conditions for a higher yield of active subtilisin of Bacillus subtilis, in the periplasmic space of E. coli: lower inducer concentrations and lower culture temperature,\(^{16}\) so we examined several induction conditions, such as the concentration of IPTG and the temperature in different host strains. However, we failed to cause overproduction. We considered that direct expression of TGase gene in E. coli cells in an active form did not lead to production at high levels because the typical TGase activity of the product, which introduces covalent crosslinks between proteins, might have a harmful influence on the cells, leading to death. Therefore, we selected the method of production in which the product is secreted into the periplasmic space of E. coli using the ompA signal peptide.

Generally, there are several problems associated with gene expression, such as the efficiency of transcription and translation, or the stability of the plasmid, mRNA, and product. We studied how the designed DNA structures influence transcription and the mRNA stability. The results showed that they did not affect gene expression (data not shown). To express the TGase gene at high levels, it might be effective to use different promoters and produce an inactive form such as a fused protein. Yeast, which has an efficient secretory system, might be useful as the host in which to express and secrete products into the culture medium, instead of E. coli.

This TGase is of interest not only in regard to its structure-functional relationship, but also for its medicinal and food-industry applications. The study of TGase using recombinant DNA technology depends on the ability of the expression system to produce engineered proteins. Much more research in yeast as well as E. coli is required to construct an effective expression system and is currently in progress.

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References