A Protein Disulfide Isomerase Gene Fusion Expression System That Increases the Extracellular Productivity of Bacillus brevis

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We have developed a versatile Bacillus brevis expression and secretion system based on the use of fungal protein disulfide isomerase (PDI) as a gene fusion partner. Fusion with PDI increased the extracellular production of heterologous proteins (light chain of immunoglobulin G, 8-fold; geranylgeranyl pyrophosphate synthase, 12-fold). Linkage to PDI prevented the aggregation of the secreted proteins, resulting in high-level accumulation of fusion proteins in soluble and biologically active forms. We also show that the disulfide isomerase activity of PDI in a fusion protein is responsible for the suppression of the aggregation of the protein with intradisulfide, whereas aggregation of the protein without intradisulfide was prevented even when the protein was fused to a mutant PDI whose two active sites were disrupted, suggesting that another PDI function, such as chaperone-like activity, synergistically prevented the aggregation of heterologous proteins in the PDI fusion expression system.

A host-vector system for the efficient extracellular production of heterologous proteins, involving Bacillus brevis as the host, has been developed (22). Many proteins of bacterial origin could be produced at high levels without much difficulty. However, certain proteins, especially some mammalian ones, still exhibited low productivity. Sagiya et al. improved the B. brevis protein expression system by means such as modification of the signal sequence (17) and isolation of a protease-deficient mutant (7), which was successfully applied to the secretion and accumulation of not only prokaryotic but also eukaryotic proteins. In fact, a change of the signal peptide sequence in the B. brevis system resulted in the higher secretion of many proteins, for example, growth hormones (7, 17), interleukin 2 (21), and the protease-deficient mutants increased the production of extracellular proteins (6, 7). Still another way of increasing protein productivity is to link the gene of interest to a second gene which is already known to be expressed well in the host to generate a fusion protein (9). In most of the successful fusion protein systems the protein of interest is positioned at the C-terminal end of the highly expressed fusion partner (12, 19) to ensure efficient translation initiation. The thioredoxin gene fusion system also provided the solution for another major problem which has bedeviled heterologous gene expression in E. coli, i.e., the formation of inclusion bodies (11). PDI catalyzes the formation, reduction, and isomerization of disulfide bonds in vitro (5) and facilitates the folding of disulfide-bonded proteins in vivo (18). Even though the physiological roles of these multiple functions of PDI during protein folding in the cell remain obscure, PDI, promoting in vitro folding, can be used to improve an expression system for foreign genes. In fact, in a coexpression system, PDI exhibits chaperone-like activity which suppresses the aggregation and increases the yields of heterologous proteins (3, 4).

In this paper, we describe a novel fusion gene expression system based on the use of thermostable PDI as a fusion partner and also report the effect of the disulfide oxidoreductase activity of the fused PDI as to increases in the production of the light chain of immunoglobulin G (IgG) against 11-deoxycortisol, as a disulfide-bonded protein, and geranylgeranyl pyrophosphate synthase (GGPS) from an extremely thermophilic archaea, Sulfolobus acidocaldarius, as a non-disulfide-bonded protein.

MATERIALS AND METHODS

Strains, plasmids, and media. B. brevis 31-OK was used as the host (7). Plasmid pNU212 is an expression-secretion vector containing a multiple promoter region and the signal peptide-encoding region of the gene that codes for middle wall protein (MWP) (22) of B. brevis 47. pNH326 was constructed from pNH300 (16) by replacing the MWP signal peptide with a modified signal peptide, RL6 (17), and by adding the transcription terminator of pHF926 (2). Plasmids pNU211L4PDI (8) and pMalCGG2 (15), containing the fungal PDI gene and the archaeal GGPS gene, respectively, were described previously. Plasmid pFCA-SCHL (13) contained the gene encoding the LC, a chimeric protein of the Vα-Cα domain of anti-11-deoxycortisol Fab and the two Fe binding domains of protein A from Staphylococcus aureus. For the expression of LC, DNA fragments encoding LC were amplified by PCR, for which pFCA-SCHL was used as a template and synthetic oligonucleotides 5′-CCGCCCATAGGTCACGTGAACAGGTATGAAA-3′ and 5′-GGCCCTGCGAAGGTTTATTTACCATTGCTTACCTTCCGGCGCGTGAAC-3′ (named LCSTOP) were used as primers. The nucleotide sequence of MWPLC comprises the 3′ terminal sequence of the signal-peptide-encoding region of the mwp gene directly connected to the sequence encoding the N terminus of the LC. An NcoI site is present at its 5′ end. The nucleotide sequence of LCSTOP is complementary to that encoding the C terminus of the LC. A stop codon and the HindIII site sequence are attached to its 5′ end. The amplified fragment was digested with NcoI and HindIII and then inserted between the NcoI and HindIII sites of pNH326. The plasmid thus constructed was named pNH326LC (Fig. 1A). The fragment comprising the region from SD to the 3′ end of the LC was amplified by PCR, for which the oligonucleotides 5′-CCGCCCATAGGTCACGTGAACAGGTATGAAA-3′ (named SDMWP) and 5′-GGCCCTGCGAAGGTTTATTTACCATTGCTTACCTTCCGGCGCGTGAAC-3′ (named LCSTOP) were used. The sequence encoding the SDMWP is comprised of that around SD of the mwp gene with a HindIII site attached to its 5′ end. The nucleotide sequence of LCSTOP is complementary to that encoding the C terminus of the LC. A HindIII site sequence was attached to its 5′ end. The amplified fragment was digested with XhoI and HindIII and then inserted between the XhoI site, located immediately downstream of the sequence encoding the LC, and the HindIII site of pNH326LC. The constructed plasmid was named pNH326LC/PDI (Fig. 1A).

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RESULTS

Construction of genes encoding fusion proteins. DNA fragments encoding fusion proteins PDI-LC and PDI-GGPS were constructed by the recombinant PCR method. The DNA fragment encoding PDI was amplified with MWPPDI (5′-CCGCC CCTGTCGCTCGGATTTGTTGTCAGTGTAAGAACGG-3′) and PDIEK (5′-CTTTGCTATCGTATACACCAAGGACAGGAGTTTGGTTTGGTCAAGAACGG-3′) as primers and pNU211L4PDI as a template. The DNA fragment encoding LC was also amplified with a template, pFCA-SCHL, and primers EKLC (5′-GGTTCGTCGTCTGGTGATGACGATGGACATCGACCGCTCCAGCTTCAGGC-3′) and LCSTOP (described under Materials and Methods) as primers and pMalcGG2 as a template.

By essentially the same procedures, a DNA fragment encoding GGPS was amplified from pMalcGG2 as a template with oligonucleotides 5′-GCCGCCCCCATGGGTTTCTGCTGATTAGTACGAAACTA-3′ (named GGPSSTOP) as primers. The DNA fragment was inserted into pNU212, and the resulting plasmid was named pNU212GGPS. The expression vectors for PDI fusion proteins are described under Results. All the plasmids were introduced into B. brevis by electroporation (16).

YCA medium was comprised of 30 g of polypeptone P1 (Nihon Pharmaceutica, Tokyo, Japan), 2 g of yeast extract, 30 g of glucose, 0.1 g of CaCl₂, 2H₂O, 0.1 g of MgSO₄·7H₂O, 10 mg of FeSO₄·7H₂O, 10 mg of MnSO₄·4H₂O, and 1 mg of ZnSO₄·7H₂O per liter. pH 7.2. YCP2 medium was the same as YC medium except that the polypeptone content was 20 g.

Enterokinase cleavage. A supernatant containing the PDI-LC fusion protein was dialyzed against EK buffer (5 mM EDTA, 25 mM HEPES-NaOH [pH 8.0]) prior to enterokinase cleavage. The dialyzed culture supernatant was then mixed with porcine enterokinase (specific activity, 100 U/mg; Sigma) in EK buffer at an enzyme-to-substrate ratio of 1:100 (wt/wt) and then incubated at 37°C for 15 h. The reaction was terminated by the addition of p-aminobenzamidine to 5 mM.

Analysis of the expressed proteins. The amounts of the expressed proteins were routinely determined by Western blot analysis according to the method of Burnette (1), except that an anti-Fab fragment polyclonal antibody, as the primary antibody, and an alkaline phosphatase-conjugated goat anti-rabbit IgG, as the secondary antibody, were used for detection of the LC. Rabbit anti-GPSs fused to maltose binding protein antiserum (15) and an alkaline phosphatase-conjugated goat anti-rabbit IgG monoclonal (Fab′)₂ were used as the primary and secondary antibodies, respectively, for GGPS detection. BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium) was used as the substrate for the color reaction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (10). For nonreducing SDS-PAGE, dithiothreitol was omitted from the sample buffer. The PDI activity of the fused protein was assayed with scissured RNase as a substrate according to the method described previously (20). GGPS activity was assayed by the method described previously (15).
presses the aggregation of secreted LC and GGPS, resulting in increases in the amounts of the extracellular active forms.

The effect of the oxidoreductase activity of PDI on the formation of insoluble aggregates. PDI used as a fusion partner contains two CGHC catalytic sites and can act as an oxidoreductase. When the cysteine residues in each active site of PDI were changed to serine ones (SGHS), the oxidoreductase activity was lost. It has been reported that PDI exhibits chaperone-like activity, e.g., it suppresses rhodanase aggregation in yeast (9), and that the chaperone-like activity of PDI remains unaffected even when both its active sites are changed to SGHS (12). To clarify what function of PDI suppresses aggregation of fusion proteins, LC and GGPS fused with the mutant PDI (mPDI), whose two catalytic site sequences were substituted with SGHS, were expressed in B. brevis. We confirmed that the chimeric proteins fused with mPDI had completely lost their isomerase activity while that of the native PDI fusion remained unaffected. The fusion with mPDI had no effect on the high level of extracellular production of the PDI-GGPS fusion protein (Fig. 3, lanes 3 and 4), indicating that the isomerase activity of PDI is not responsible for the increase in the production of PDI-GGPS, whereas the fusion with mPDI made aggregates appear in the insoluble fraction and decreased the production of soluble PDI-LC in the extracellular fraction (Fig. 3, lanes 1 and 2). These results suggested that the oxidoreductase activity of PDI in the fusion proteins was partly responsible for the suppression of the aggregation of the proteins with disulfides, such as the LC of an IgG, but not those without disulfides, such as GGPS.

Characterization of the fusion proteins. The fusion proteins produced by B. brevis were examined as to their biological functions. The expressed LC could not assemble with the heavy chain (HC) in the fusion form. PDI-LC was incubated with bovine enterokinase. PDI-LC was successfully cleaved specifically at the cleavage site in the linker peptide, resulting in a 40-kDa product corresponding to the native LC, as in the case of that expressed by itself (Fig. 4). The LC cleaved from the fusion protein exhibited significant assembly with HC, forming a 70-kDa product corresponding to the Fab’ form under non-reducing conditions (data not shown), whereas PDI-GGPS produced by B. brevis exhibited the same GGPS activity as the native GGPS throughout the temperature range examined (Fig. 5). PDI-GGPS specifically produced geranylgeranyl diphosphate from an allylic diphosphate (data not shown). These results indicate that the fusion protein showed the same properties in the fusion form as native archaeal GGPS (14).

**DISCUSSION**

We succeeded in the efficient secretion and accumulation of the soluble forms of an LC of IgG and an archaeal GGPS by means of a PDI fusion system in B. brevis. PDI has at least two
characteristics which may make it a particularly suitable choice for the fusion partner. PDI of H. insolens, a thermophilic fungus, can be secreted and accumulated in the culture medium to a concentration of up to 1.1 g/liter by B. brevis, and even at this production level, all of the PDI remains in a soluble form (8). It was thought that the secretion is affected by the sequence of the signal peptide and the following N-terminal sequence of the protein of interest. It is, however, generally undesirable to change the sequence of a protein to increase its secretion since the N-terminal sequence following the signal peptide is often responsible for the function of the protein of interest. When a fusion protein is expressed, more stable secretion can be expected because the signal peptide can be tuned up to result in efficient secretion of the N-terminal fusion partner regardless of the sequence of the partner. Thus, PDI can act as a leader peptide, which allows efficient secretion of heterologous proteins.

There is another advantage that a PDI fusion can provide: it can act as a molecular chaperone which prevents aggregation and facilitates correct folding. The aggregation of secreted proteins is one of the causes of a decrease in extracellular productivity. In some cases of heterologous protein expression by B. brevis, mature-sized heterologous proteins in the insoluble fraction have been obtained. It is possible that by physically linking a heterologous protein to a stable and highly soluble fusion partner such as PDI, these aggregates might be prevented from forming, allowing correct folding to occur eventually. The fact that PDI is secreted first may allow it to fold before its nascent C-terminal fusion partner, and in this way it is able to passively interact with the partner as it emerges from the outer membrane. In contrast to coexpressed PDI, expression as a fusion form would enable close physical contact between PDI and the heterologous protein domain, facilitating any potential interaction. The enhanced extracellular production of the soluble LC by B. brevis required a physical linkage to PDI and could not be accomplished through coexpression of PDI, suggesting that the PDI domain acts as a covalently linked chaperone. The mechanism suppressing the aggregation through fusion with PDI was not elucidated.

We showed that the oxidoreductase activity of PDI was partially responsible for the suppression of the aggregation of the secreted LC, a protein containing intradisulfides, whereas the aggregation of GGPS, a protein without intradisulfides, could be prevented even with the fusion with PDI lacking oxidoreductase activity (SGHS). From these results, we can speculate that PDI prevents the aggregation of a fusion partner through the synergistic effect of its oxidoreductase and its other chaperone-like activity, leading to an increase in the extracellular production of a heterologous protein. Although each domain of PDI and GGPS in the fusion protein exhibited the expected biological function, the LC did not. Partial biological
activity is not unexpected for fusion proteins, since some functional site may be masked by the fusion partner. However, the LC, while inactive in a fusion form, exhibited significant biological activity when cleaved from its partner. This suggests that both PDI and the fused heterologous protein are able to fold correctly when linked together. The PDI used as a fusion partner is a thermostable PDI from a thermophilic fungus and seems to have a very tight tertiary fold, judging from its thermostability. This folding property is probably very resistant to any perturbation that may be caused by the presence of fused heterologous proteins. This may be the underlying reason why thermostable PDI is such a good fusion partner.

REFERENCES