Synthesis of functionally active proteins containing disulfide bonds using the new PURE system (PUREfrex™)

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PUREfrex™を用いた活性型ジスルフィド結合含有タンパク質の合成
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Abstract
The PURE system is a recombinant cell-free protein synthesis system, which contains only purified factors necessary for transcription, translation, and energy regeneration. The PURE system has the unique feature that it contains less contaminants such as nucleases and proteases and that composition of the reagents can be easily adjusted in accordance to the purposes. Therefore, the PURE system is now used not only preparation of the larger protein but also in vitro display technology such as ribosome display (RDb). But, we found that the original PURE system contains a large quantity of lipopolysaccharide (LPS) from incompletely purified components. We modified the preparation methods of all components that were purified from E. coli and developed the new PURE system (PUREfrex™). In PUREfrex™, the amount of contaminants such as LPS and RhesA is reduced and thus both protein synthesis activity and secretion efficiency in BD were improved.

2. Development of PUREfrex

Table 2-1. Comparison of the preparation methods of components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Original</th>
<th>PUREfrex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tag</td>
<td></td>
<td>Disulfide bond-containing proteins</td>
</tr>
<tr>
<td>Number of column</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Wash with detergent</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ribosome</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wash with detergent</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>tRNA</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

LPS: Lipopolysaccharide, RhesA: RhesA (a ribosome effector), pGAP: pGAP (a cell-free protein synthesis system).

RhesA is a ribosome effector that modulates the activity of ribosomes.

Table 2-2. Comparison of the results of ribosome display among cell-free protein synthesis systems.

<table>
<thead>
<tr>
<th>System</th>
<th>Temperature</th>
<th>Reaction rate</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUREfrex</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
</tr>
<tr>
<td>PUREfrex</td>
<td>3°C</td>
<td>3°C</td>
<td>3°C</td>
</tr>
<tr>
<td>PUREfrex</td>
<td>2°C</td>
<td>2°C</td>
<td>2°C</td>
</tr>
</tbody>
</table>

2.5-3.5°C is the optimal temperature for PUREfrex.

3. Model proteins

- vTPA (truncated tissue plasminogen activator) 9 SS bonds (8 non-consecutive)
- AppA (E. coli acid phosphatase) 5 SS bonds (1 non-consecutive)
- AP (E. coli alkaline phosphatase) 2 SS bonds
- Fab (Heavy chain + Light chain) 2 SS bonds in each chain

4. Supplemented factors

- GSSG (oxidized glutathione)
- DsbC (disulfide bond isomerase in E. coli)

5. Synthesis of proteins containing disulfide bonds using PUREfrex SS

Active vTPA can be synthesized in the presence of both GSSG and DsbC.

Active vTPA can be synthesized without DsbC.

Figure 5-1. Synthesis of vTPA in the presence of GSSG and DsbC.

- vTPA was expressed using PUREfrex supplemented with 1 mM GSSG and the indicated concentration of DsbC.
- A. 3 µg/mL of the ribosome mixture was used to observe changes in the activity of vTPA by SDS-PAGE/SDS UV assay. The maximum activity was set at 1.00.
- B. ½ of the ribosome mixture was addition to 100 µM GSSG and the gel was gelled with TCA and stained with Coomassie Brilliant Blue. The band corresponding to vTPA is indicated by a triangle.

Figure 5-2. Effect of GSSG and DsbC on the translation of vTPA.

- vTPA was synthesized for 2 hours in the presence or absence of GSSG and DsbC. After stopping translation reaction by addition of chloramphenicol, tRNA, and tRNA were added to the ribosome mixture and all samples were further incubated for 2 hours.
- Activity of synthesized vTPA was measured as in Figure 5-1.
- Synthesized vTPA was first purified by TCA acetate.

Figure 5-3. Synthesis of acid phosphatase in the presence of GSSG and DsbC.

- Acid phosphatase (AppA) was synthesized using PUREfrex supplemented with 1 mM GSSG and the indicated concentration of DsbC.
- A. 3 µg/mL of the ribosome mixture was used to observe changes in the activity of acid phosphatase by SDS-PAGE/SDS UV assay. The maximum activity was set at 1.00.
- B. ½ of the ribosome mixture was addition to 100 µM GSSG and the gel was gelled with TCA and stained with Coomassie Brilliant Blue. The band corresponding to AppA is indicated by a triangle.

Figure 5-4. Synthesis of alkaline phosphatase.

- Alkaline phosphatase (AP) was synthesized using PUREfrex supplemented with the indicated concentration of GSSG in the presence of 10 µM DbsC or in the absence of DsbC.
- A. 3 µg/mL of the ribosome mixture was used to observe changes in the activity of alkaline phosphatase by SDS-PAGE/SDS UV assay. The maximum activity was set at 1.00.
- B. ½ of the ribosome mixture was addition to 100 µM GSSG and the gel was gelled with TCA and stained with Coomassie Brilliant Blue. The band corresponding to AP is indicated by a triangle.

Figure 5-5. Summary

- We succeeded to synthesize proteins containing disulfide bonds with activities using PUREfrex supplemented with oxidized glutathione and DsbC protein.

Optimum concentration of oxidized glutathione and DsbC was different from each target protein.

“PUREfrex SS”