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

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PURE*frex*™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

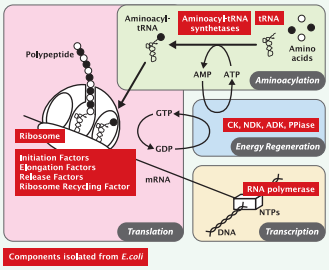
The PURE system is a reconstituted cell-free protein synthesis system, which consists of only purified factors necessary for transcription, translation and energy regeneration. The PURE system has the unique features that it contains less contaminants such as nucleases and proteases and that composition of the reagents can be easily adjusted in accordance to the purpose. Therefore, the PURE system is now used not only in preparation of the target proteins but also in *in vitro* display technology such as ribosome display (RD). But, we found that the original PURE system yet contained 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 (PURE*frex*™). In PURE*frex*, the amount of contaminants such as LPS and RNase is reduced and then both protein synthesis activity and selection efficiency in RD were improved.

However, because PURE*frex* includes only translation factors under reduced condition, it is difficult to synthesize proteins containing disulfide bonds in active form. In this study, we examined the composition of reaction mixture and synthesis condition to synthesize functionally active proteins. We used 4 proteins, which form a different number of disulfide bridges, as model proteins. As a result, we succeeded to synthesize all 4 proteins with activities using PURE*frex* supplemented with oxidized glutathione (GSSG) and DsbC protein (disulfide bond isomerase) and optimum concentration was different from each target protein.

This result shows that disulfide bonds-containing proteins can be also synthesized in active form using PURE*frex* supplemented with optimized concentration of GSSG and DsbC.

1. PURE system

The PURE system is a reconstituted cell-free protein synthesis system, which consists of only purified factors necessary for transcription, translation and energy regeneration.



Advantage

- Low level of contamination
- Easy adjustment of the reagent composition

Application

- High throughput preparation of proteins
- Pretein science research
  - Translation mechanism
  - Folding process
- *in vitro* display
  - Ribosome display
  - mRNA display

The original PURE system yet contains contaminants from incompletely purified components.

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*)

2. Development of PURE*frex*

Contaminants in PURE*frex* are reduced.

	PURE*frex*	Original
Proteins		
Tag	None	His-tag
Number of column	3	1
Wash with detergent	+	−
Ribosome		
Wash with detergent	+	−
tRNA		
Wash with detergent	+	−

(Original; Shimizu *et al.* (2005) *Methods*, vol.36, p.299)

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

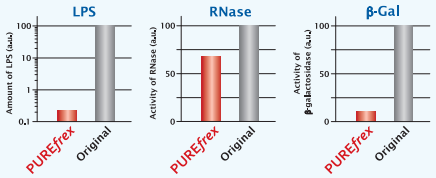
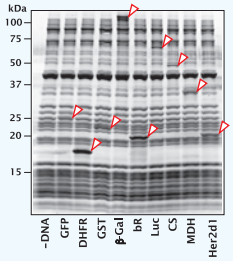


Figure 2-1.
Comparison of the contaminants in the reagents.

The amount of lipopolysaccharide (LPS), RNase and β-galactosidase (β-Gal) in PURE*frex* and the original PURE system was mesured. The amount in the original PURE system was set to 100.

Various proteins can be synthesized using PURE*frex*.



GFP : Green fluorescent protein
DHFR : Dihydrofolate reductase
GST : Glutathione S-transferase
β-Gal : β-Galactosidase
bR : Bacteriorhodopsin
Luc : Luciferase
CS : Citrate synthase
MDH : Malate dehydrogenase
Her2d1 : Domain1 of Her2

Figure 2-2.
Synthesis of various proteins using PURE*frex*.

Various proeins in the figure were synthesized using PURE*frex*. The reaction mixture was subjected to SDS-PAGE and the gel was stained with Oriole (Bio-Rad).

PURE*frex* is the most suitabale system for RD.

	PURE system		*E.coli*
	PURE*frex*	Original	S30 extract
Temperature at selection	4-25ºC	4-25ºC	4ºC
RD complex recovery rate	10-30%	2.5%	0.01-0.2%
Enrichment / round	100,000	1,000-10,000	20-250

Table 2-2.
Comparison of results of ribosome display among 3 cell-free protein synthesis systems.

5. Synthesis of proteins containing disulfide bonds using PURE*frex SS*

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

PURE*frex* +GSSG/DsbC
↓ + vtPA DNA
↓ incubation at 37ºC for 4 h
Assay of chromogenic substrate cleavage activity
SDS-PAGE

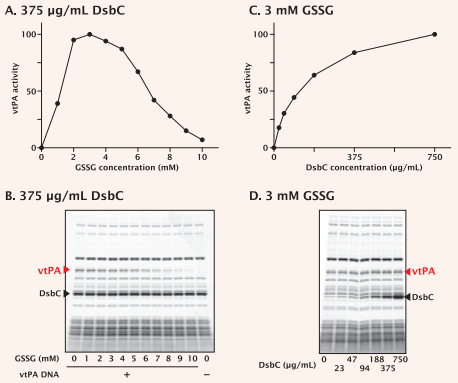


Figure 5-1.
Synthesis of vtPA in the presence of GSSG and DsbC.

vtPA was synthesized using PURE*frex* supplemented with the indicated concentration of GSSG and DsbC.
A, C. 2.5 μL of the reaction mixture was used in a cleavage assay using chromogenic substrate (SPECTROZYME tPA, American diagnostics). The maximum activity was set to 100.
B, D. 1 μL of the reaction mixture was subjected to SDS-PAGE and the gel was stained with SyproOrange (Invitrogen). Synthesized vtPA and supplemented DsbC are indicated by triangles.

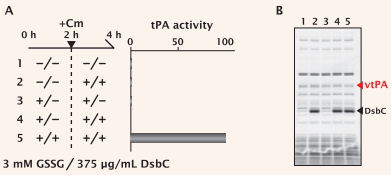


Figure 5-2.
Effect of GSSG and DsbC on the translation of vtPA.

vtPA was synthesized for 2 hours in the presence or absence of GSSG and DsbC. After stopping translation reaction by addition of chloramphenicol (Cm), GSSG and DsbC were added to the indicated samples and all samples were further incubated for 2 hours.
A, Activity of synthesized vtPA was measured as in Figure 5-1.
B, Synthesized vtPA was analyzed by SDS-PAGE as in Figure 5-1.

Active Fab fragment can be synthesized using PURE*frex* with GSSG and DsbC.

PURE*frex* +GSSG/DsbC
↓ + H chain DNA (+FLAG) / L chain DNA
↓ incubation at 37ºC for 4 h
↓ to antigen-coated well
↓ + anti-FLAG/HRP
↓ + TMB

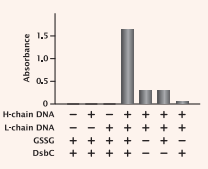


Figure 5-5.
Synthesis of Fab fragment using PURE*frex* with GSSG and DsbC.

Fab fragment was synthesized using PURE*frex* supplemented with 3 mM GSSG and 23 μg/mL DsbC. The diluted reaction mixture was applied to the antigen-coated well and the bound Fab was deteced with anti-FLAG/HRP.

Active phosphatase can be synthesized without DsbC.

PURE*frex* +GSSG/DsbC
↓ + AppA or AP DNA
↓ incubation at 37ºC for 4 h
Assay of PNPP dephosphorylation activity
SDS-PAGE

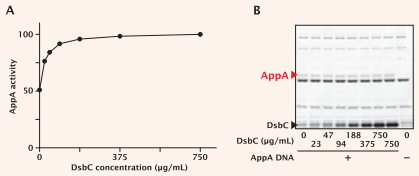


Figure 5-3.
Synthesis of acid phosphatase in the presence of GSSG and DsbC.

Acid phosphatase (AppA) was synthesized using PURE*frex* supplemented with 3 mM GSSG and the indicated concentration of DsbC.
A. 2 μL of the reaction mixture was used in a dephosphorylation assay using *p*-nitrophenol phosphate (PNPP). The maximum activity was set to 100.
B. 1 μL of the reaction mixture was subjected to SDS-PAGE and the gel was stained with SyproOrange. Synthesized AppA and supplemented DsbC are indicated by triangles.

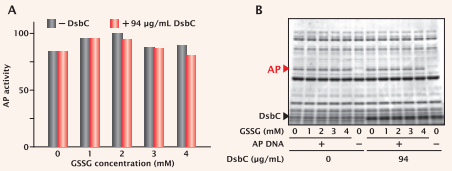


Figure 5-4.
Synthesis of alkaline phosphatase.

Alkaline phophatase (AP) was synthesized using PURE*frex* supplemented with the indicated concentration of GSSG in the presence of 94 μg/mL DsbC or the absence of DsbC.
A. 2 μL of the reaction mixture was used in a dephosphorylation assay using PNPP. The maximum activity was set to 100.
B. 1 μL of the reaction mixture was subjected to SDS-PAGE and the gel was stained with SyproOrange. Synthesized AP and supplemented DsbC are indicated by triangles.

Summary

- We succeeded to synthesize proteins containing disulfide bonds with activities using PURE*frex* supplemented with oxidized glutathione and DsbC protein.
- Optimum concentration of oxidized glutathione and DsbC was different from each target protein.

→ **"PURE*frex SS*"**