Synthesis of proteins containing disulfide bonds using a reconstituted cell-free protein synthesis system (PURE*frex*®)

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Abstract

PURE*frex*® is a cell-free protein synthesis system based on the PURE system, which is reconstituted only from factors involved in protein synthesis in *E. coli*. It has an advantage that the reaction can be controlled by adjusting the components of the reaction mixture freely. We have optimized the synthesis condition for disulfide bond-containing proteins taking advantage of this point. For example, we have succeeded in maximizing the amount of IgG with antigen-binding activity by optimizing the reaction conditions; the type of reducing agent, the ratio (concentration) of reducing agent and oxidizing agent, the concentration of protein disulfide isomerase (*E. coli* DsbC) and molecular chaperones, reaction temperature and reaction time (1).

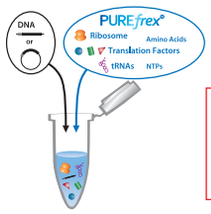
In this presentation, we report the results of investigating whether human PDI and its related proteins can be used in place of DsbC. First, a truncated version of tissue plasminogen activator (vtPA), which has nine complex disulfide bonds, was synthesized as a model protein. When vtPA was synthesized using PURE*frex* containing reduced glutathione (GSH), oxidized glutathione (GSSG) and PDI, the activity of the synthesized vtPA was almost the same as using DsbC with GSH and GSSG. In the absence of GSSG, the synthesized vtPA showed high activity as well by adding PDI oxidase with PDI. This indicates that either the addition of GSSG or PDI oxidase is necessary for PDI to function. Furthermore, it was confirmed that other disulfide bond-containing proteins could be also synthesized as active forms using PDI.

These results showed that even in a heterogeneous system in which PDI was added to an *E. coli*-derived translation system, disulfide-bonded proteins could be efficiently synthesized by adjusting its conditions via controlling redox environment or adding PDI oxidase.

(1) Murakami *et al.* (2019) *Sci. Rep.* vol.9, p.671.

PURE*frex*®; based on the PURE system technology

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
- PCR product usable as a template DNA

(Ref; Shimizu Y. *et al.* (2001) *Nat. Biotechnol.*, vol. 19, p. 751)

PURE*frex*®2.0
Regular kit for the protein synthesis containing DTT as a reducing agent

PURE*frex*®2.1
Regular kit for the protein synthesis capable of selecting a reducing agent

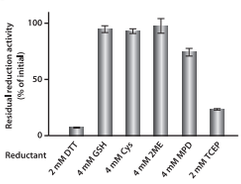
DsbC Set / PDI Set
Supplement for the synthesis of proteins containing disulfide bonds

DnaK Mix / GroE Mix
Supplement for the synthesis of aggregate-prone proteins

1. Effect of reducing reagents on the redox state

< Comparison of residual reduction activity after long incubation >

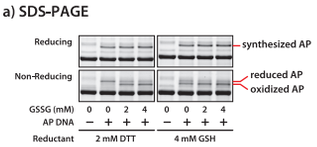
Buffer solution of PURE*frex*
↓ + indicated reducing agent
↓ incubation at 37°C for 24 h
↓ Ellman' s assay



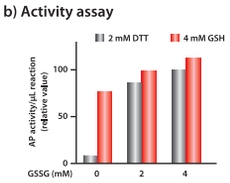
< Effect of reducing agents on the synthsesized AP >

PURE*frex*®2.1 containing DTT or reduced glutathione (GSH)
↓ + oxidized glutathione (GSSG) with indicated concentration
↓ +/- Alkaline phosphatase (AP) DNA
↓ incubation at 37°C for 4 h
↓ a) SDS-PAGE under reducing and non-reducing condition
b) AP enzymatic activity assay

a) SDS-PAGE



b) Activity assay

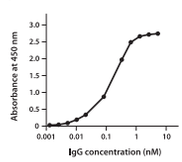


Reducing agents influence on the formation of disulfide bonds.

2. Synthesis of functional IgG (Trastuzumab)

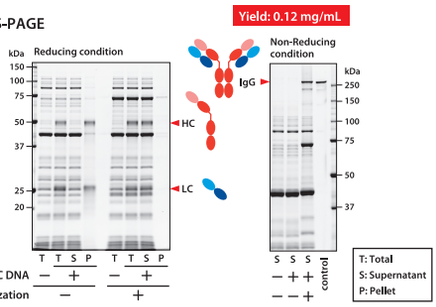
PURE*frex*®2.1 containing GSH
↓ + DsbC Set + DnaK Mix
↓ + HC/LC DNA (PCR product)
↓ incubation at 37°C for 28 h

b) ELISA



a) SDS-PAGE

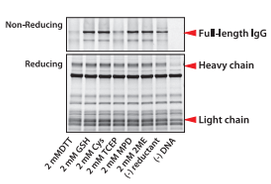
Yield: 0.12 mg/mL



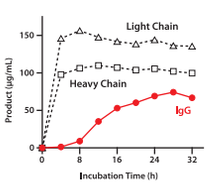
Key Factors for IgG synthesis

- HC/LC DNA ratio (4:1-1:2)
- Redox state (+GSH/GSSG)
- Molecular chaperones (+DnaK, +DsbC)
- Incubation temperature (30-37°C)
- Incubation time (>24 hours)

< Reducing agents >



< Incubation time >

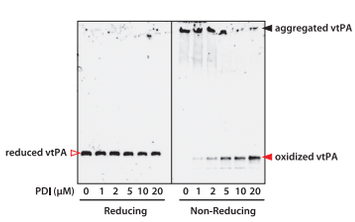


Functional aglycosylated IgGs can be synthesized using PURE*frex* under the optimized condition.

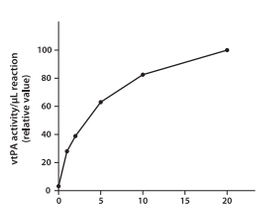
3. Effect of PDI concentration

PURE*frex*®2.1 containing 4 mM GSH / 2 mM GSSG
↓ + DnaK/DnaJ/GrpE
↓ + indicated concentration of PDI
↓ + Plasminogen activator (truncated) (vtPA-FLAG) DNA
↓ incubation at 30°C for 24 h
↓ a) SDS-PAGE under reducing and non-reducing condition and western blotting with anti-FLAG Ab
b) vtPA enzymatic activity assay

a) SDS-PAGE (Western blotting)



b) Activity assay



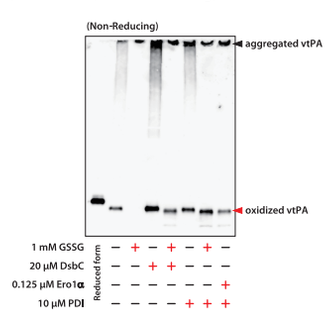
More than 10 μM PDI is required for the synthesis of functional vtPA.

4. Synthesis of several proteins

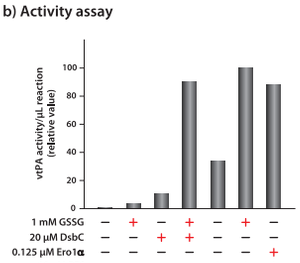
Plasminogen activator (truncated) (*Homo sapiens*)
9 disulfide bonds

PURE*frex*®2.1 (4 mM GSH) + DnaK Mix

a) SDS-PAGE (Western blotting)



b) Activity assay

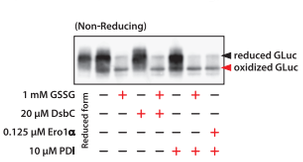


Functional proteins containing disulfide bonds can be synthesized using PURE*frex* supplemented with PDI.

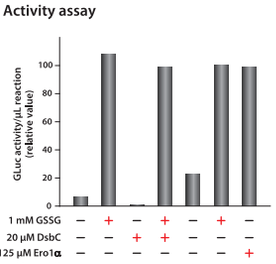
Luciferase (*Gaussia princeps*)
5 disulfide bonds

PURE*frex*®2.1 (4 mM GSH)

a) SDS-PAGE (Western blotting)



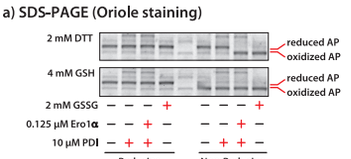
b) Activity assay



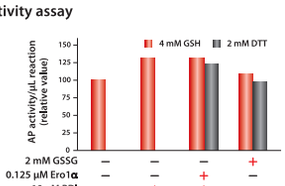
Alkaline phosphatase (*E. coli*)
2 disulfide bonds

PURE*frex*®2.1

a) SDS-PAGE (Oriole staining)



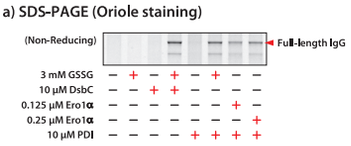
b) Activity assay



Trastuzumab

PURE*frex*®2.1 (4 mM GSH) + DnaK Mix

a) SDS-PAGE (Oriole staining)



Conclusion

- The efficiency of disulfide bonds formation is affected by a reducing agent in the reaction mixture.
- Reduced glutathione is more suitable than DTT when synthesizing proteins containing disulfide bonds.
- Functional aglycosylated IgG can be synthesized using PURE*frex* under the optimized condition.
- Human PDI can be used as an isomerase with PURE*frex*.
- The optimal condition for synthesizing a protein containing dilsufide bonds depends on the protein.