活性型タンパク質合成のための、再構成型無細胞タンパク質合成系(PURE*frex*®)を用いたアプローチ

Investigation on how to synthesize active proteins by using a reconstituted cell-free protein synthesis system (PURE*frex*®)

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<Abstract>

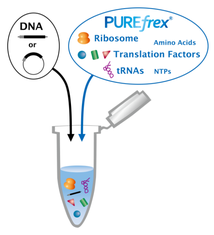
PURE*frex*® is a reconstituted cell-free protein synthesis kit developed based on the PURE system. Now the productivity of PURE*frex*® is up to 1 mg/mL. However, depending on the protein, it may be difficult to synthesize as functional and soluble form by using only PURE*frex*®.

In such cases, we can apply chaperones (DnaK and GroE) for correct folding and isomerases (DsbC and PDI) for disulfide bond formation as additives for PURE*frex*®. Here, we introduce examples of protein synthesis that could not be solved by these additives alone, but could be solved by adding other additives or synthesizing at lower temperature.

We mainly show examples of human acetylcholinesterase (hAChE) synthesis. hAChE has three intramolecular and one intermolecular disulfide bonds. When hAChE was synthesized by using PURE*frex*® with PDI, active hAChE was obtained but there was a problem in solubility. The synthesis under the presence of surfactant Brij 58 increased the solubility of hAChE but decreased its activity. Next, we examined whether the difference in the solubility of hAChE is depending on the synthesis temperature. The solubility of the product was less than 10% at 30ºC although the amount of the product was high. In contrast, the product synthesized at 25ºC was almost soluble although the amount was low. It was also found that DnaK/DnaJ/GrpE are essential for the synthesis of soluble hAChE even at lower temperature.

This result indicates that it is possible to synthesize difficult-to-express proteins by using PURE*frex*® with optimization of conditions. In this presentation, we also discuss the results from other types of proteins and additives.

1. PURE*frex*®; Reconstituted cell-free protein synthesis system based on the PURE system technology



Advantage

- Low level of contamination
- Easy adjustment of the reagent composition
- PCR products usable as a template DNA

Application

- High throughput preparation of proteins (including Fab, scFv, protein toxin etc.)
- Protein science research
- in vitro display (ribosome display, mRNA display etc.)

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

For the synthesis of difficult-to-express protein…

PURE*frex*® + **Additives:**
- Surfactants
- Molecular Chaperones

and / or

Reaction conditions:
- Low temperature (20ºC-25ºC)

→ Functional protein

2. Result 1: Influence of surfactant on protein synthesis by PURE*frex*®

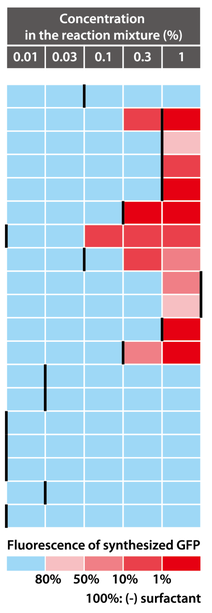
Experimental conditions for protein synthesis

Reaction mixture	Incubation	Template DNA
PURE*frex*®2.1 (4 mM GSH) + Sufractants	37ºC 4 h	sfGFP PCR product (1 ng/μL)

→ Measurement of GFP fluorescence

- Most surfactants did not inhibit the protein synthesis reaction by PURE*frex*® below the CMC.
- Some surfactants such as Triton X-100 and Tween 20 could be used even above the CMC.

Surfactant Name	CMC (%)
Digitonin	0.061
Sodium cholate	0.628
CHAPS	0.492
CHAPSO	0.505
n-Octyl-β-D-glucoside	0.731
n-Octyl-β-D-thioglucoside	0.278
n-Dodecyl-β-D-maltoside	0.009
n-Decyl-β-D-maltoside	0.087
n-Octyl-β-D-maltoside	1.064
Mega-8	1.864
Mega-9	0.839
Mega-10	0.245
Triton X-100	0.016
Triton X-114	0.011
NP-40	0.009
Tween 20	0.007
Tween 80	0.002
Brij 35	0.011
Brij 58	0.009



3. Result 2: Effect of molecular chaperones on the solubilization of the synthesized product by PURE*frex*®

3-1. Increasing the amount of DnaK Mix (DnaK/DnaJ/GrpE) facilitates the solubilization of the products during protein synthesis.

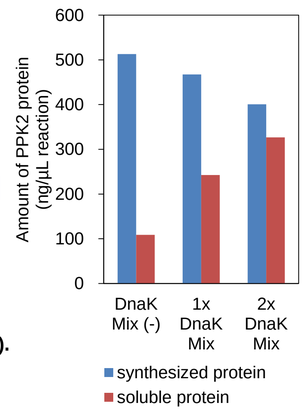
Polyphosphate:AMP phosphotransferase (PPK2)

Organism	*Cytophaga hutchinsonii*
Synthesized region	1Met – 284Ser (+6xHis)
Length	292 a.a.
Molecular weight	33,921 Da

Experimental conditions for protein synthesis

Reaction mixture	Factor	Incubation	Template DNA
PURE*frex*®2.1 (4 mM GSH)	Concentration of DnaK Mix 1x; 5 μM DnaK, 1 μM DnaJ, 1 μM GrpE	30ºC 4 h	PCR product (1 ng/μL)

- Measurement of solubilized products
→ All samples were centrifuged at 20,000 xg for 30 min.
→ Synthesized protein and supernatant were applied to SDS-PAGE gel (12.5%).
→ Gel staining: Oriole Fluorescent Gel Stain (Bio-Rad)
→ Quantitation: LAS-4000 system (GE Healthcare)



The solubility of the synthesized PPK2 increased by adding twice the amount of DnaK Mix to the reaction mixture.

3-2. DnaK Mix and ClpB can solubilize the aggregated products after protein synthesis.

Luciferase

Organism	*Photinus pyralis*
Synthesized region	1Met – 550Leu
Length	550 a.a.
Molecular weight	60,745 Da

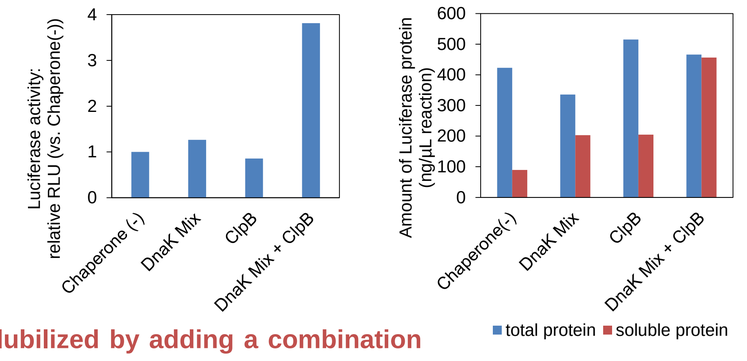
PURE*frex*® reaction mixture containing synthesized luciferase

↓ + Chaperones:
DnaK Mix (5 μM DnaK/ 1 μM DnaJ/ 1 μM GrpE)
1 μM ClpB (*E. coli*)

- Luciferase activity assay
- Measurement of solubilized protein

Experimental conditions for protein synthesis

Reaction mixture	Incubation	Template DNA
PURE*frex*®2.1 (4 mM GSH) without molecular chaperones	37ºC, 4 h	PCR product (2.5 ng/μL)



Aggregated synthesized luciferase could be solubilized by adding a combination of DnaK Mix and ClpB to the reaction mixture after protein synthesis.

4. Result 3: Optimization of reaction conditions for functional protein synthesis by PURE*frex*®

Human Acetylcholinesterase (hAChE)

Organism	*Homo sapiens*
Synthesized region	32Glu – 614Leu (+FLAG – 6xHis)
Length	602 a.a.
Molecular weight	66,812 Da
No. of disulfide bonds	4 (intermolecular: 1)

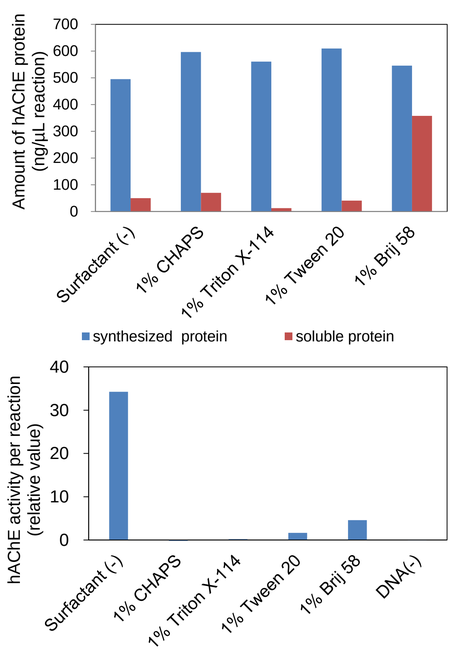
Basic experimental condition for protein synthesis

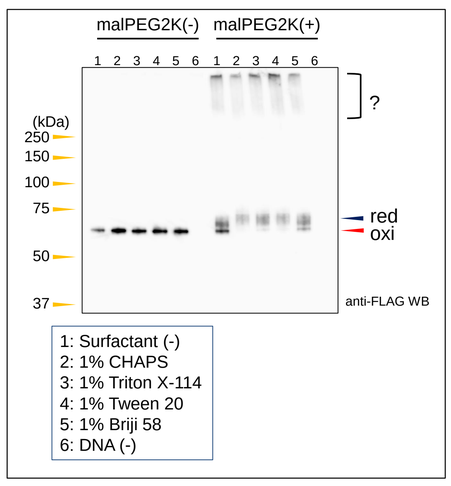
Reaction mixture	Incubation time	Template DNA
PURE*frex*®2.1 (4 mM GSH) 10 μM hPDI/ 0.5 μM hEro1α	24 h	PCR product (1 ng/μL)

- Measurement of solubilized protein
- AChE activity assay: Amplite™ Colorimetric Acetylcholinesterase Assay Kit (AAT Bioquest).

4-1. Influence of surfactants for hAChE synthesis

Factor	Surfactant	DnaK Mix	Temperature
Surfactant	CHAPS/ Triton X-114/ Tween 20/ Brij 58	(+)	30ºC





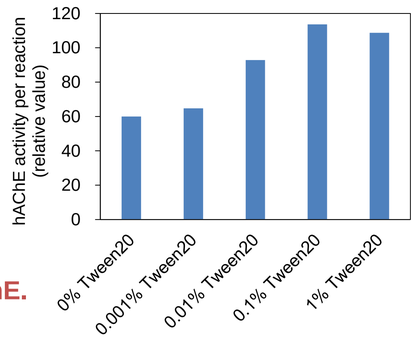
hAChE was synthesized with soluble form in the presence of Brij 58. However, the activity of the synthesized hAChE was reduced.

4-2. The timing of adding surfactant is important for the activity of the product.

PURE*frex*® reaction mixture containing the synthesized hAChE

↓ + Tween 20
(final conc. : 0%/ 0.001%/ 0.01%/ 0.1%/ 1%)

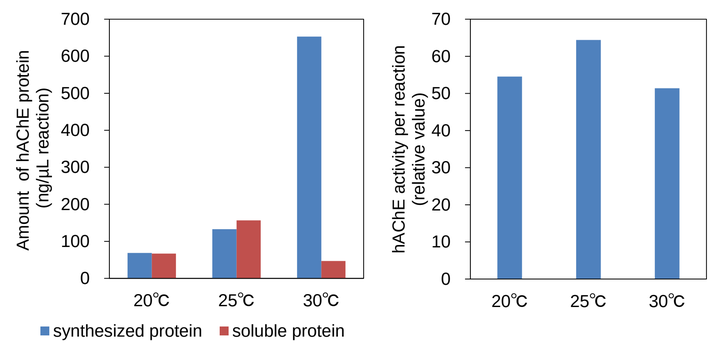
hAChE activity assay



Addition of surfactant after synthesis increased the activity of synthesized hAChE.

4-3. It is possible to synthesize soluble and functional hAChE by synthesis at low temperature.

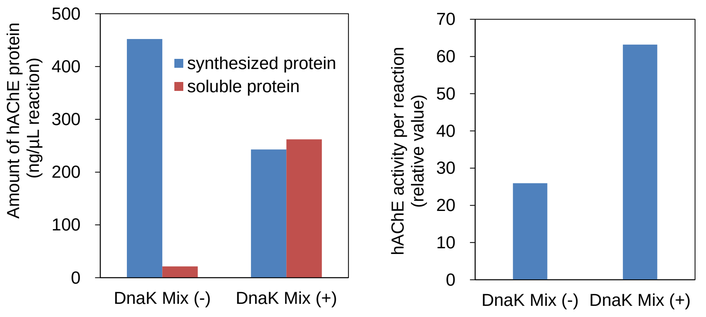
Factor	DnaK Mix	Temperature
Temperature	(+)	20ºC/ 25ºC/ 30ºC



Most of hAChE synthesized at below 25ºC was soluble and had its activity.

4-4. DnaK Mix is necessary for hAChE synthesis even under low temperature condition (25ºC).

Factor	DnaK Mix	Temperature
DnaK Mix	(-)/ (+)	25ºC



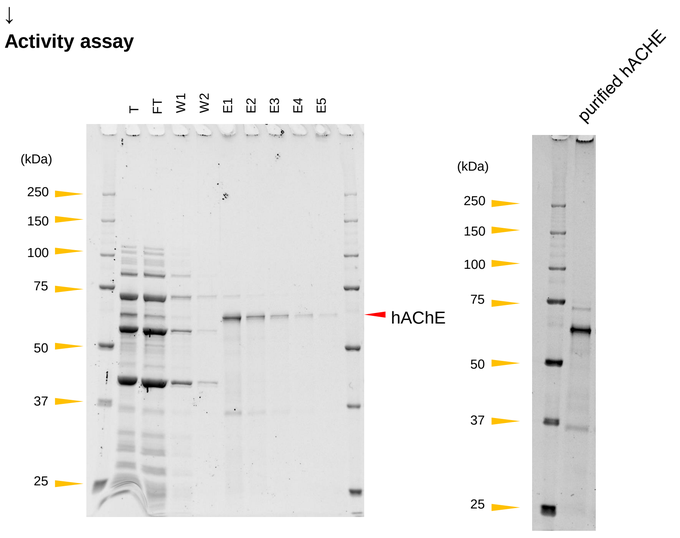
Low temperature (25ºC) + DnaK Mix → Soluble hAChE

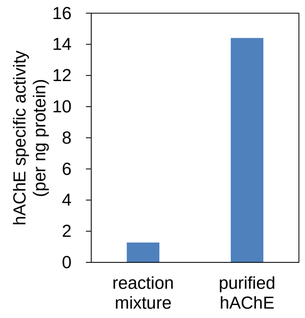
4-5. Purification of synthesized hAChE

Protein synthesis

Reaction mixture	DnaK Mix	Temperature	Incubation time	Template DNA
PURE*frex*®2.1 (4 mM GSH) 10 μM hPDI/ 0.5 μM hEro1α	(+)	25ºC	24 h	PCR product (1 ng/μL)

↓
Purification by Co^{2+}-resin (TALON Metal Affinity Resin (Clontech))
(Binding buffer: 50 mM Tris-HCl (pH 8.0)/ 500 mM NaCl/ 5 mM Imidazole/ 0.1% Tween 20)
↓
Activity assay





Highly active hAChE could be purified from the reaction mixture of PURE*frex*®.

5. Summary

- Surfactants can be used for protein synthesis by PURE*frex*®. However, some proteins require investigation in the timing of the use of surfactants.
- Increasing the amount of DnaK Mix improve the solubility of synthesized PPK2.
- Soluble hAChE is obtained by protein synthesis at lower temperature. DnaK Mix is essential for the soluble protein synthesis even at low temperature.

Optimal conditions for synthesizing proteins with PURE*frex*® differ for each protein.
The great advantage of PURE*frex*® is that it is possible to try various variations of additives and synthesis conditions.

For more information, please contact us.

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