ヒトプロテインジスルフィドイソメラーゼを添加した
再構成型無細胞タンパク質合成系(PURE*frex*®)によるジスルフィド結合タンパク質合成

Synthesis of proteins containing disulfide bonds using PURE*frex*® with human protein disulfide isomerase

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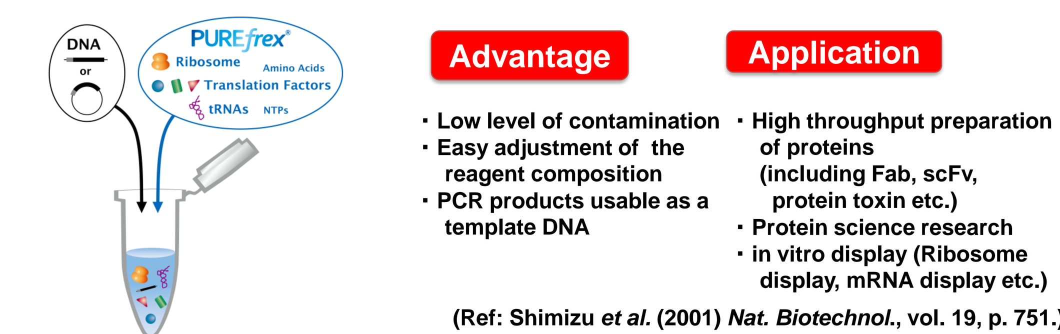
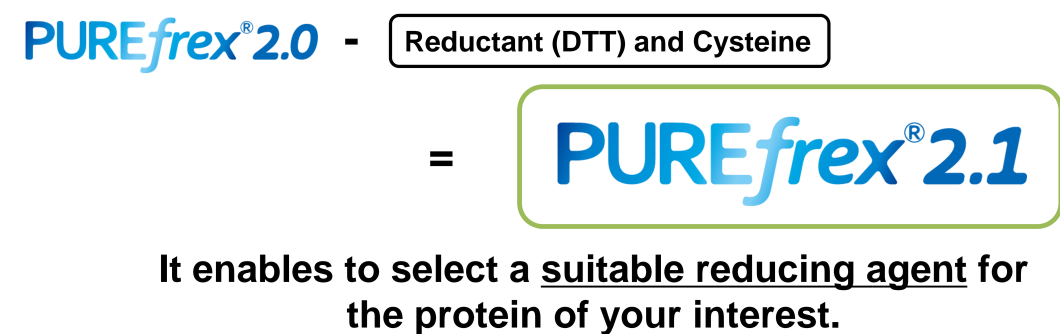
<Abstract>

PURE systemは、大腸菌のタンパク質合成に関与する因子のみから再構成した無細胞タンパク質合成系である。PURE systemにプロテインジスルフィドイソメラーゼ(PDI) として大腸菌DsbCを添加することで、活性を有したジスルフィド結合タンパク質を合成できる。複雑な構造のタンパク質や高等生物のジスルフィド結合タンパク質合成に広く対応するため、本発表ではPURE*frex*® (改良型PURE system) を用い、DsbCに替えてヒトPDI (hPDI) およびその関連タンパク質を利用できるか検討した。モデル系として、9つのジスルフィド結合を持つ組織プラスミノーゲン活性化因子 (tPA) の合成を行った。還元剤に還元型グルタチオン (GSH) を使用したPURE*frex*をベースに、酸化型グルタチオン (GSSG) 存在下でhPDIを添加してtPAを合成すると、合成されたtPAはhPDIの添加濃度依存的に活性が増加した。一方、GSSG非存在下でも、hPDIおよびhEro1α (PDI酸化酵素)を添加しtPAを合成した場合、合成されたtPAは同様に高い活性を示した。すなわち、hPDIを機能させるためにはGSSGあるいはhEro1αの添加が必要であることが分かった。また、GSSG存在下でhPDIとDsbCの添加効果を比較したところ、hPDIはDsbCとほぼ同等の効果を示した。以上より、hPDI添加でも、酸化還元環境の調節やhPDI酸化酵素の添加により効率よくジスルフィド結合タンパク質の合成が可能であることが確認された。さらに他の基質タンパク質の合成についても報告する。

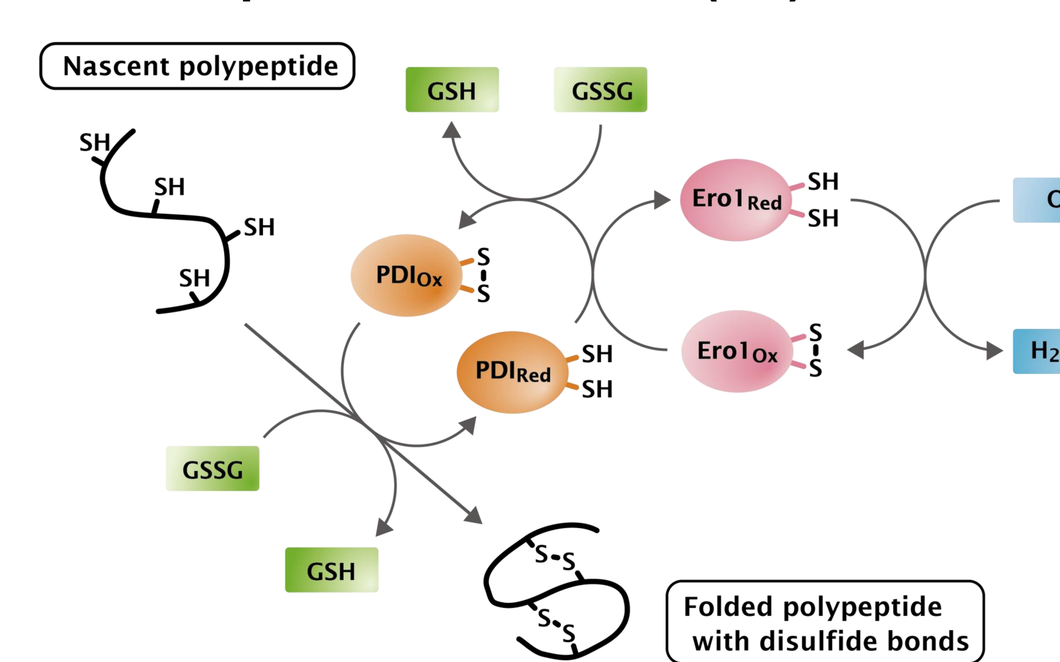
1. PURE*frex*®; based on the PURE system technology





It enables to select a suitable reducing agent for the protein of your interest.

2. Oxidative folding of nascent polypeptide in Endoplasmic Reticulum (ER)



2-1. PDIs and related protein used in this study

hPDI (human protein disulfide isomerase)

UniProt ID	P07237
Organism	*Homo sapiens*
Length	492 a.a.
Molecular weight	55,424 Da

(PDB ID: 4EKZ)

hEro1α (human endoplasmic oxidereductin)

UniProt ID	Q96HE7
Organism	*Homo sapiens*
Length	446 a.a.
Molecular weight	52,122 Da

(PDB ID: 3AHQ)

DsbC (thiol:disulfide interchange protein)

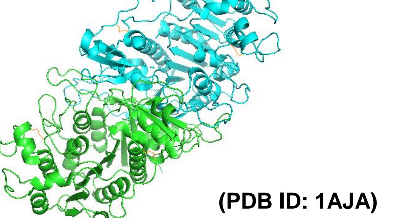
UniProt ID	P0AEG6
Organism	*Escherichia coli*
Length	217 a.a.
Molecular weight	23,591 Da

(PDB ID: 1EEJ)

3. Result 1: Selection of suitable reducing agent

Alkaline phosphatase (AP)

Organism	*Escherichia coli*
Synthesized region	22Arg-471Lys
Length	451 a.a.
Molecular weight	47,330 Da
No. of disulfide bonds	2



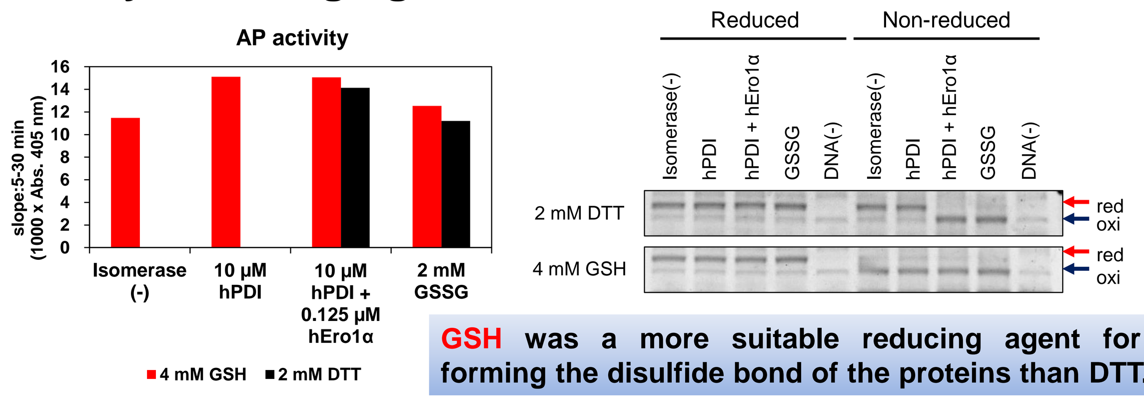
(PDB ID: 1AJA)

Experimental conditions of protein synthesis

No.	Factors	Basic condition	Reductant	hPDI	GSSG	hEro1α	Template DNA	Temp. & Time
1	hPDI	PURE*frex*®2.1 + 0.5 mM Cys	4 mM GSH or 2 mM DTT	10 µM	(-)	(-)	plasmid (10 ng/µL)	37℃, 4 h
2	hPDI + hEro1α			10 µM	(-)	0.2 µM		
3	GSSG			(-)	2 mM	(-)		

・SDS-PAGE (0.5 µL of reaction mix/lane) → Gel staining (Oriole)
・AP activity assay: Assay of PNPP dephosphorylation activity (0.1 µL PURE reaction mix/100 µL assay soln.)

3-1. Difference in synthesized AP activity by reducing agent

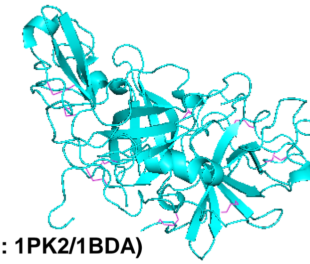


GSH was a more suitable reducing agent for forming the disulfide bond of the proteins than DTT.

4. Result 2: Disulfide bonded protein synthesis using hPDI and hEro1α with PURE*frex*®

truncated version of tissue plasminogen activator (tPA)

Organism	*Homo sapiens*
Synthesized region	36Ser-40Ile/211Gly-562Pro (+FLAG)
Length	368 a.a.
Molecular weight	41,072 Da
No. of disulfide bonds	9



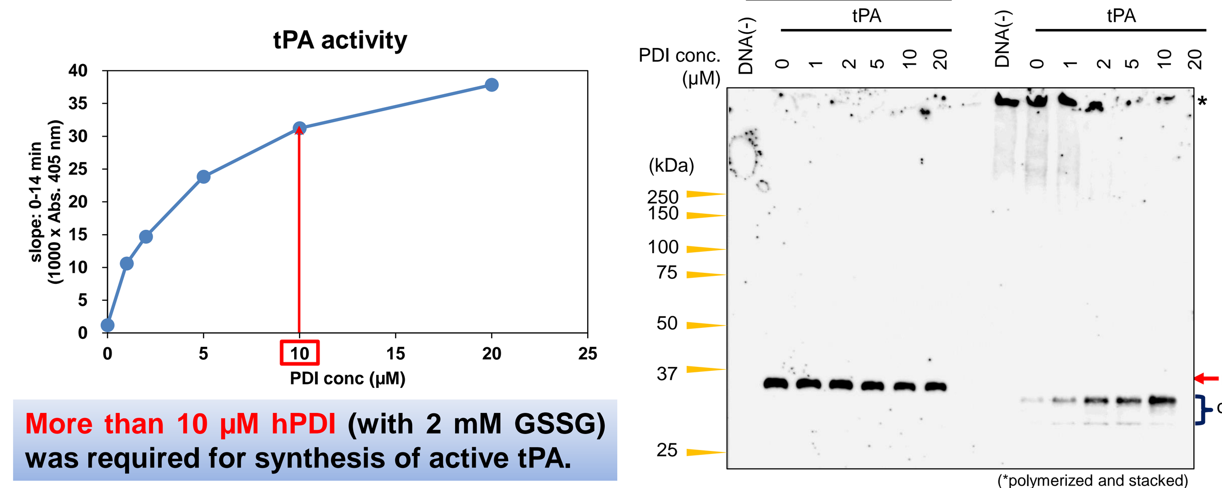
(PDB ID: 1PK2/1BDA)

Experimental conditions of protein synthesis

No.	Factors	Basic condition	hPDI	GSSG	hEro1α	DsbC	Template DNA	Temp. & Time
1	hPDI conc.	PURE*frex*®2.1 + 0.5 mM Cys + 4 mM GSH + DnaK mix (5 µM DnaK, 1 µM DnaJ, 1 µM GrpE)	0-20 µM	2 mM	(-)	(-)	PCR product (1 ng/µL)	30℃, 24 h
2	hPDI + GSSG conc.		10 µM	0-8 mM	(-)	(-)		30℃, 24 h
3	hPDI + hEro1α conc.		10 µM	(-)	0-2 µM	(-)		30℃, 24 h
4	Incubation time		10 µM	2 mM	(-)	(-)		30℃, 0-24 h
				(-)	0.125 µM			
5	DsbC		(-)	0 mM/2 mM	(-)	20 µM		30℃, 24 h

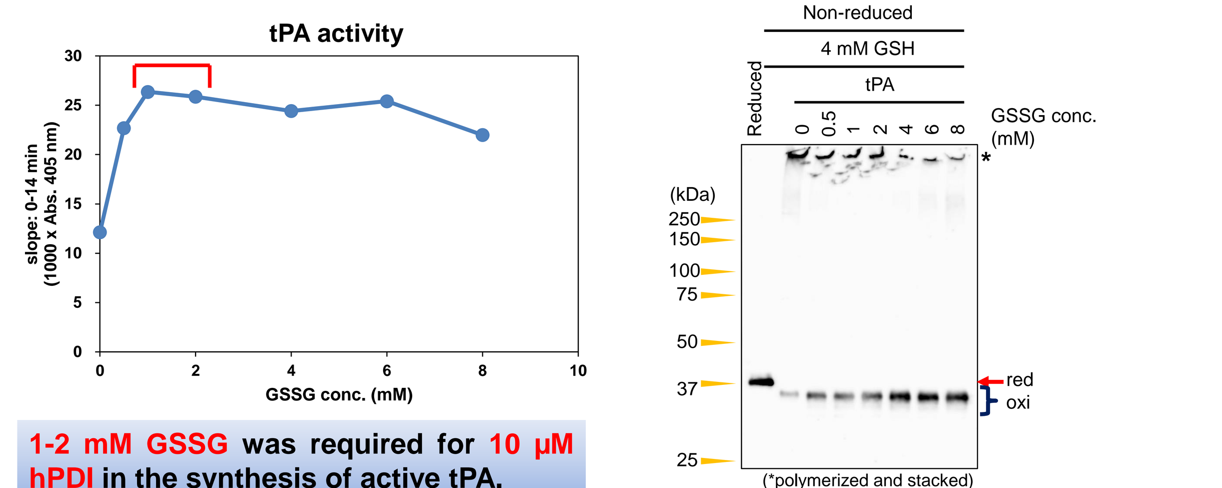
・SDS-PAGE (0.25 – 0.5 µL of reaction mix/lane) → Western Blotting (anti-FLAG) or Gel staining (Oriole)
・tPA activity assay: Assay of chromogenic substrate cleavage activity (2.5 µL PURE reaction mix/ 200 µL assay soln.)

4-1. hPDI concentration



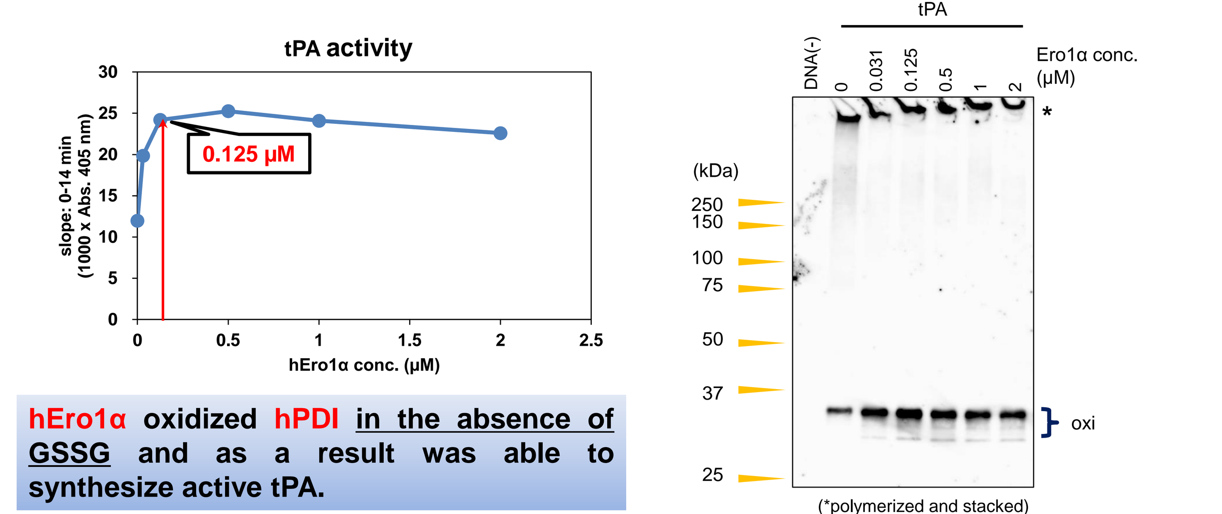
More than 10 µM hPDI (with 2 mM GSSG) was required for synthesis of active tPA.

4-2. hPDI + GSSG concentration



1-2 mM GSSG was required for **10 µM hPDI** in the synthesis of active tPA.

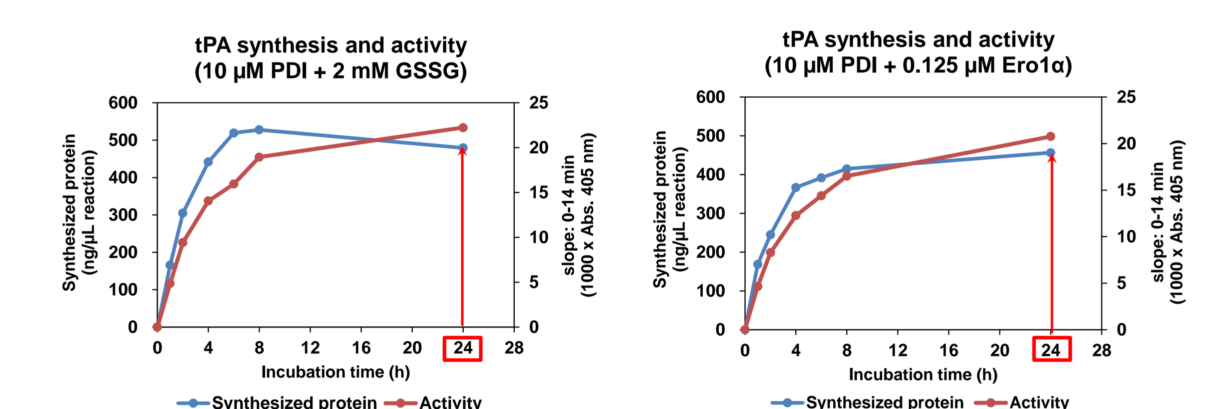
4-3. hPDI + hEro1α concentration

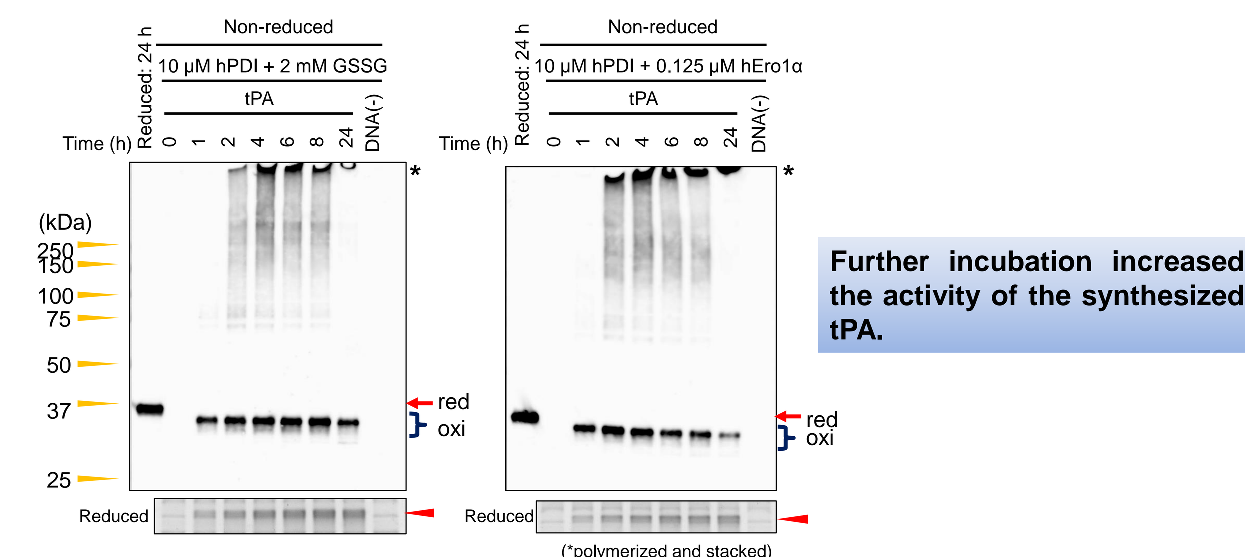


hEro1α oxidized **hPDI** in the absence of GSSG and as a result was able to synthesize active tPA.

hPDI + GSSG = hPDI + hEro1α (- GSSG)

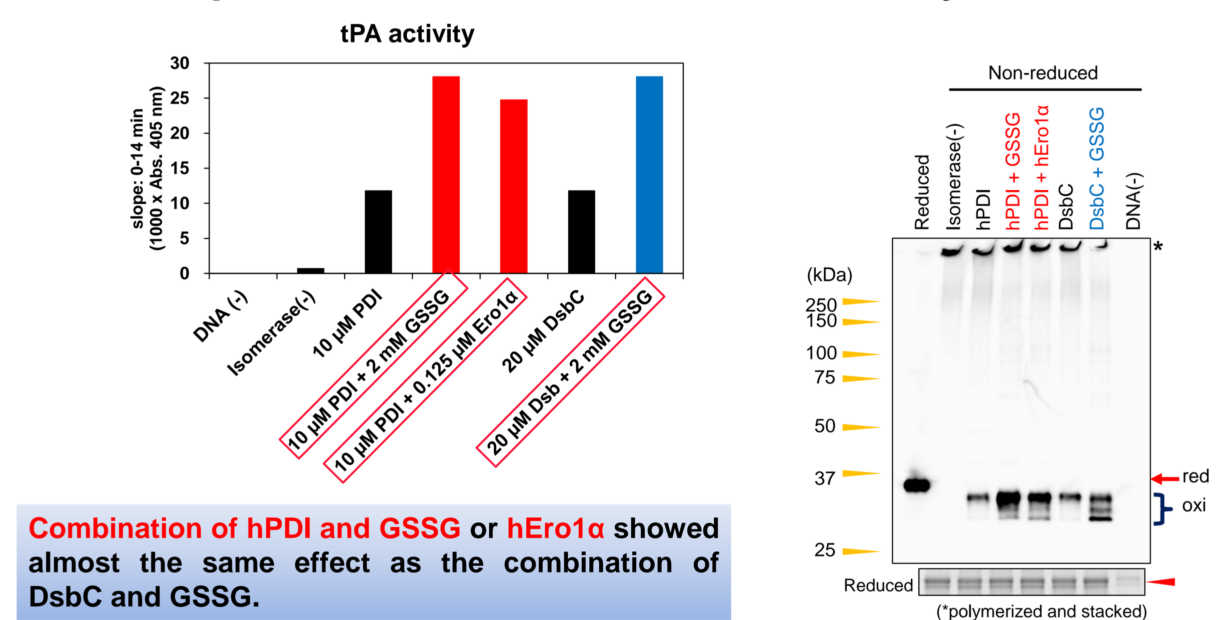
4-4. Incubation time





Further incubation increased the activity of the synthesized tPA.

4-5. Comparison of hPDI with DsbC in tPA synthesis



Combination of hPDI and GSSG or **hEro1α** showed almost the same effect as the combination of DsbC and GSSG.

5. Result 3: Examples of other disulfide bonded protein synthesis

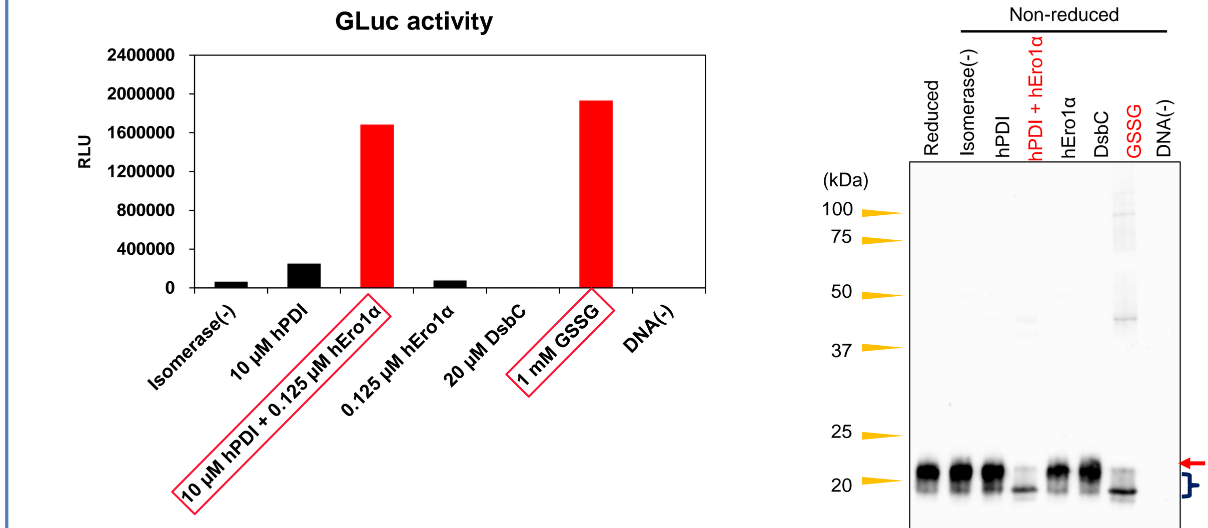
Gaussia Luciferase (GLuc)

Organism	*Gaussia princeps*
Synthesized region	18Lys-185Asp (+FLAG-His6)
Length	187 a.a.
Molecular weight	20,407 Da
No. of disulfide bonds	5

Experimental condition of protein synthesis

No.	Factors	Basic condition	hPDI	GSSG	hEro1α	DsbC	Template DNA	Temp. & Time
1	hPDI	PURE*frex*®2.1 + 0.5 mM Cys + 4 mM GSH	10 µM	(-)	(-)	(-)	PCR product (1 ng/µL)	37℃, 4 h
2	hPDI + hEro1α		10 µM	(-)	0.125 µM	(-)		
3	DsbC		20 µM	(-)	(-)	20 µM		
4	GSSG		(-)	1 mM	(-)	(-)		

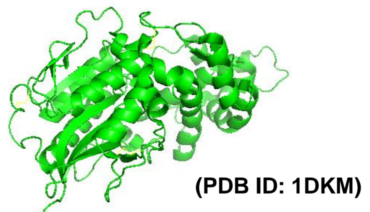
・SDS-PAGE (0.25 – 0.5 µL of reaction mix/lane) → Western Blotting (anti-FLAG) or Gel staining (Oriole)
・GLuc activity assay: BioLux Gaussia Luciferase Assay Kit (NEB#E3300S) (2 µL PURE reaction mix/50 µL assay soln.)



Combination of hPDI and Ero1α was also effective for active GLuc synthesis.
DsbC rather inhibited the activity of synthesized GLuc.

Acid phosphatase (AppA)

Organism	*Escherichia coli*
Synthesized region	23Gln-432Leu (+FLAG)
Length	421 a.a.
Molecular weight	45,959 Da
No. of disulfide bonds	4

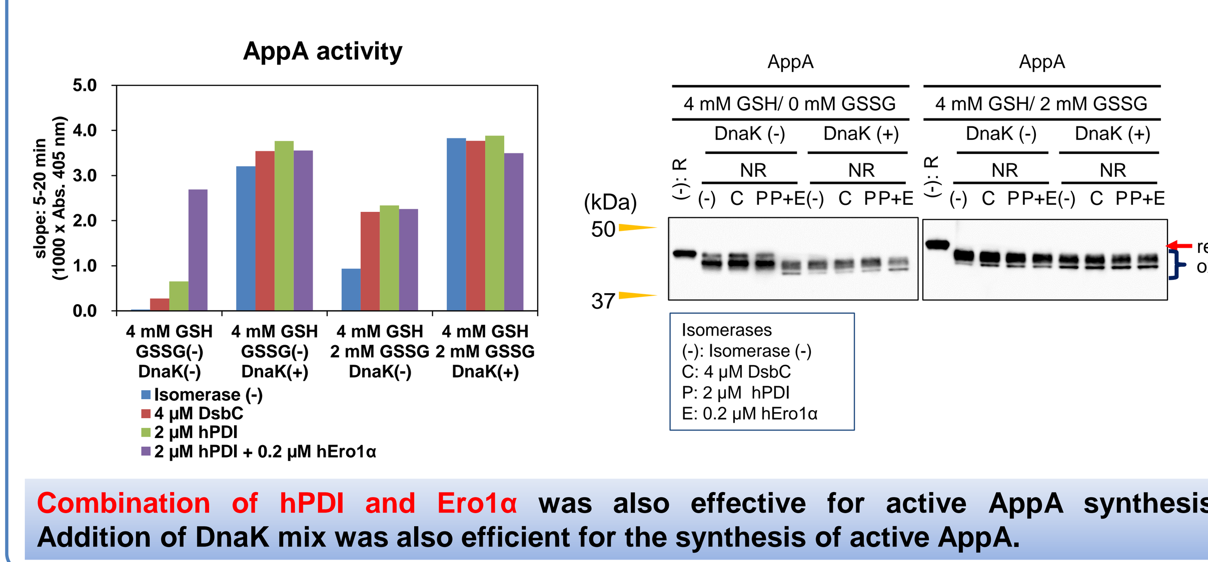


(PDB ID: 1DKM)

Experimental condition of protein synthesis

No.	Factors	Basic condition	hPDI	GSSG	hEro1α	DsbC	Template DNA	Temp. & Time
1	hPDI	PURE*frex*®2.1 + 0.5 mM Cys + 4 mM GSH	2 µM	2 mM	(-)	(-)	PCR product (1 ng/µL)	37℃, 4 h
2	hPDI + GSSG		2 µM	0 / 2 mM	(-)	(-)		
3	hPDI + hEro1α		2 µM	(-)	0.2 µM	(-)		
4	DsbC + GSSG		(-)	0 / 2 mM	(-)	4 µM		
5	DnaK mix (5 µM DnaK, 1 µM DnaJ, 1 µM GrpE)		0 / 2 µM	0 / 2 mM	0 / 0.2 µM	0 /4 µM		

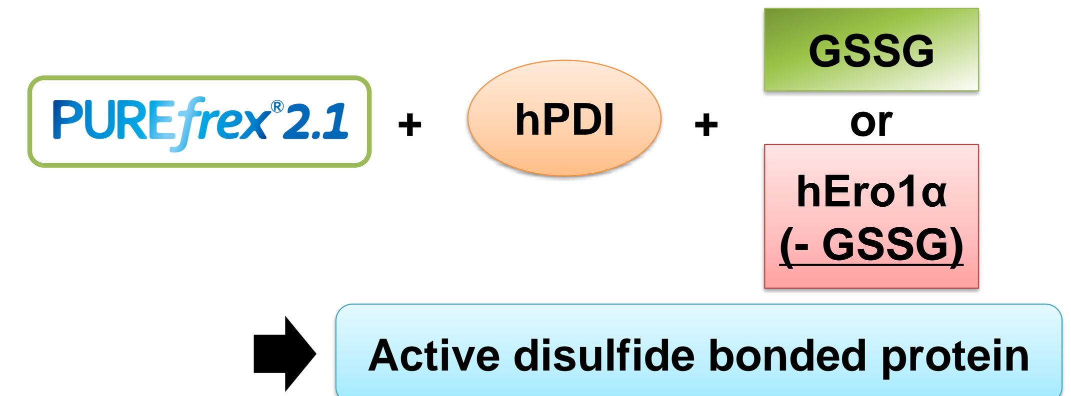
・SDS-PAGE (0.5 µL of reaction mix/lane) → Western Blotting (anti-FLAG)
・AppA activity assay: Assay of PNPP dephosphorylation activity (1 µL PURE reaction mix/100 µL assay soln.)



Combination of hPDI and Ero1α was also effective for active AppA synthesis. Addition of DnaK mix was also efficient for the synthesis of active AppA.

6. Summary

・hPDI can be used as an isomerase with PURE*frex*®*2.1*.



PURE*frex*®*2.1* supplemented with **hPDI** in the presence of **GSSG** or **hPDI oxidase (hEro1α)** can synthesize disulfide bonded proteins with their activities.
GSSG is unnecessary when oxidizing hPDI is applied with hEro1α.

・Necessary conditions for the synthesis of disulfide bonded proteins with activities are different for each proteins.

Protein name	Basic reaction mix	Reductant	Isomerase	Oxidant/Oxidase
Alkaline Phosphatase (AP)	PURE*frex*®2.1 (+ 0.5 mM Cys)	4 mM GSH		
		2 mM DTT		2 mM GSSG
			10 µM hPDI	0.125 µM hEro1α
Trancated version of Tissure Plasminogen Activator (tPA)	PURE*frex*®2.1 (+ 0.5 mM Cys) + DnaK mix	4 mM GSH	10 µM hPDI	2 mM GSSG
			10 µM hPDI	0.125 µM hEro1α
			20 µM DsbC	2 mM GSSG
Gaussia Luciferase (GLuc)	PURE*frex*®2.1 (+ 0.5 mM Cys)	4 mM GSH		1 mM GSSG
			10 µM hPDI	0.125 µM hEro1α
Acid Phosphatase (AppA)	PURE*frex*®2.1 (+ 0.5 mM Cys)	4 mM GSH	2 µM hPDI	2 mM GSSG
			2 µM hPDI	0.2 µM hEro1α
			4 µM DsbC	2 mM GSSG

In PURE*frex*®*2.1*, it is possible to select a suitable isomerase (hPDI etc.) and redox environment depending on the target protein.

For more information, please contact us.
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