再構成型無細胞タンパク質合成系 (PURE*frex*®) および糖転移酵素を用いた糖タンパク質合成の試み

Synthesis of glycosylated proteins using PURE*frex*® with glycosyltransferase

○松本 令奈[1], 丹羽 達也[2], 田口 英樹[2], 金森 崇[1] ([1]ジーンフロンティア株式会社、[2]東工大・研究院・細胞センター)

○ Rena Matsumoto, Tatsuya Niwa, Hideki Taguchi and Takashi Kanamori ([1]GeneFrontier Corp., [2]Cell Biology Center, IIR, Tokyo Tech)

<Abstract>

PURE*frex*®は、PURE systemを基にした大腸菌で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。そのため、反応条件や追加因子によって、合成したタンパク質の翻訳後修飾の調節が容易である。これまでに酸化剤とプロテインジスルフィドイソメラーゼ (PDI) を追加して活性を有したジスルフィド結合含有タンパク質の合成などが可能であることを示してきた。本発表では、糖転移酵素および基質となる糖供与体を用いてN型糖鎖修飾タンパク質をPURE*frex*®で合成することが可能かを検討した。

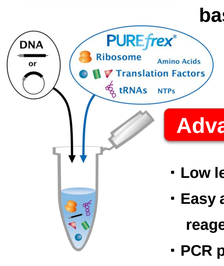
糖鎖修飾反応はJewettらの方法を参考にした (Kinghtlinger et al. (2019) *Nat. Commun*., **10**, 5404)。大腸菌Colicin-E7 immunity proteinにN型糖鎖付加配列を挿入したモデルタンパク質 (Im7-6)、ならびに数種類の糖転移酵素をPURE*frex*®でそれぞれ合成し、これらと糖供与体を混合してIm7-6の糖鎖修飾反応 (in vitro glycosylation (IVG)) を行った。IVG後のSDS-PAGEおよび質量分析の結果、Im7-6の糖鎖付加配列中のアルギニン残基へのグルコース付加を起点とし、酵素依存的にガラクトース、GlcNAcあるいはシアル酸の付加を確認した。さらに、Im7-6と糖鎖転移酵素の合成および糖鎖修飾反応を同時に行うone-pot合成・糖鎖修飾反応 (cell-free protein synthesis (CFPS)-IVG one-pot)を行ったところ、この方法でも糖鎖修飾されたIm7-6を確認できた。

また、ジスルフィド結合タンパク質や膜タンパク質についても、IVGの方法を変えることによって、糖鎖付加が可能であることが分かった。

以上の結果より、 PURE*frex*®でも糖タンパク質を合成できることが示された。

in vitro Glycosylation (IVG) of target proteins combined with Cell-Free Protein synthesis (CFPS) by PURE*frex*®

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.)

Cell-free Protein Synthesis (CFPS) PURE*frex*®

↓

in vitro Glycosylation (IVG)

The glycosylation reaction was based on the method of Jewett et al. (Kinghtlinger et al. (2019) *Nat. Commun*., 10, 5404).

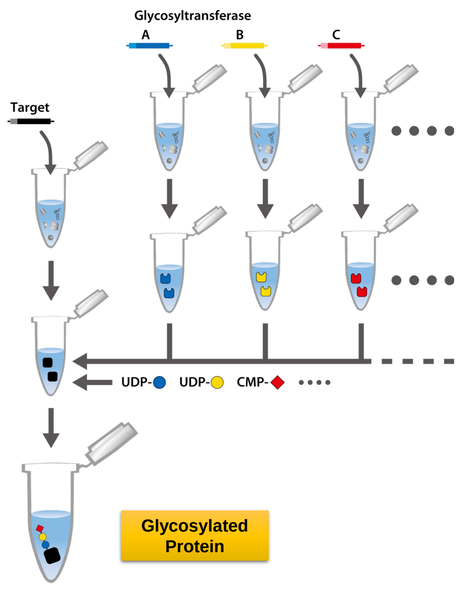
↓

Glycosylated Protein

1. in vitro Glycosylation of model protein

1-1. IVG

Cell-free Protein synthesis (CFPS) of target protein and Glycosyltransferases (GTs)



→ All IVG samples were purified using Ni-Sepharose 6 FF.

→ Eluates were applied to SDS-PAGE(10-20%).

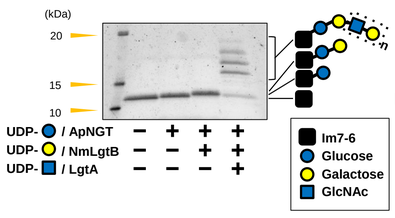
Gel staining: Oriole Fluorescent Gel Stain (Bio-Rad)

Quantitation: LAS-4000 system (GE Healthcare)

→ The rest of the samples were subjected to mass spectrometry. (Q-Exactive & Proteome Discoverer 2.4)

Colicin-E7 immunity protein mutant (Im7-6) (*Escherichia coli*)

Length 101 a.a.
Molecular weight 11,366 Da



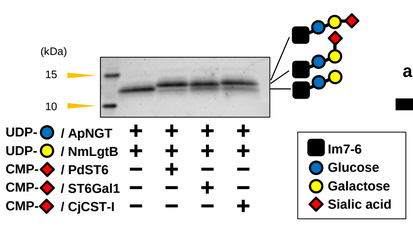
MS analysis

Im7-6 [21-49] EIEKEATTGGNWTTAGGDVLDVLLEHFVK

Modification	Theo. MH+ [Da]	Number of PSMs* lane 1	lane 2	lane 3	lane 4
-	3129.5739	50	1	8	2
Glc	3291.6267	0	33	7	9
Glc-Gal	3453.6795			106	48
Glc-Gal-(GlcNAc-Gal)$_1$	3818.8117			0	29
Glc-Gal-(GlcNAc-Gal)$_2$	4183.9439			0	49
Glc-Gal-(GlcNAc-Gal)$_3$	4549.0761			0	30

*Number of PSMs (Peptide Spectrum Matches) was calculated as the average value of three measurements.

(The same applies to the following experiments.)



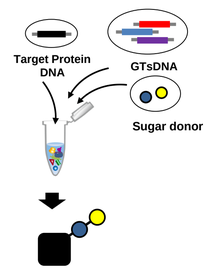
MS analysis

Im7-6 [21-49] EIEKEATTGGNWTTAGGDVLDVLLEHFVK

Modification	Theo. MH+ [Da]	Number of PSMs (control)	lane 2	lane 3	lane 4
-	3129.5739	92	7	5	4
Glc	3291.6267	0	30	25	30
Glc-Gal	3453.6795	0	36	74	69
Glc-Gal-Sia	3744.7750	0	77	74	72

IVG with PURE*frex*® could provide the N-glycosylation of Im7-6.

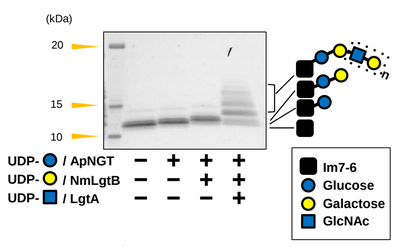
1-2. One-pot IVG



CFPS PURE*frex*® + IVG

One-pot CFPS-IVG condition

Template DNA	Reaction mixture	Sugar donor
Im7-6 (3.7 nM) ApNGT (0.2 nM) NmLgtB (0.1 nM) LgtA (0.5 nM)	PURE*frex*® 2.1 (4 mM GSH) + DnaK Mix	UDP-glucose (2.5 mM) UDP-galactose (2.5 mM) UDP-GlcNAc (2.5 mM)



One-pot IVG by PURE*frex*® could provide the N-glycosylation of Im7-6.

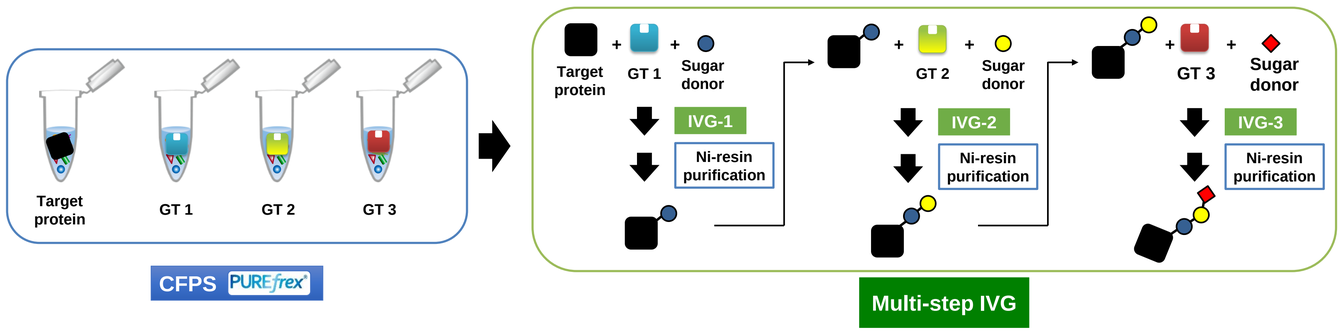
MS analysis

Im7-6 [21-49] EIEKEATTGGNWTTAGGDVLDVLLEHFVK

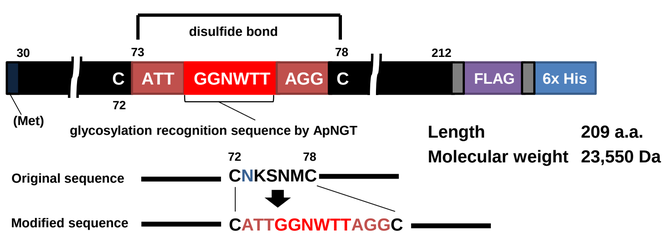
Modification	Theo. MH+ [Da]	Number of PSMs lane 1	lane 2	lane 3	lane 4
-	3129.5739	90	7	50	70
Glc	3291.6267	1*	114	31	21
Glc-Gal	3453.6795	1*	0	102	51
Glc-Gal-(GlcNAc-Gal)$_1$	3818.8117	0	0	0	71
Glc-Gal-(GlcNAc-Gal)$_2$	4183.9439	0	0	0	34
Glc-Gal-(GlcNAc-Gal)$_3$	4549.0761	0	0	0	8

*It is most likely detecting a carryover from the previous measurement.

2. in vitro Glycosylation of disulfide bonded protein



Human Interleukin-6 (hIL6)

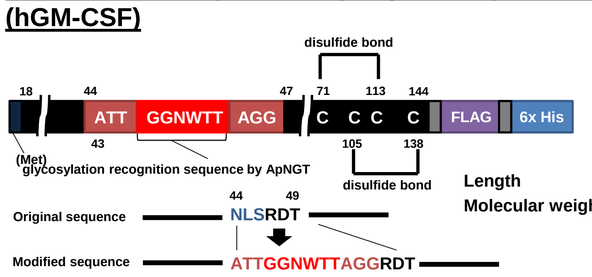


Length 209 a.a.
Molecular weight 23,550 Da

Original sequence CNKSNMC

Modified sequence CATTGGNWTTAGGC

Human Granulocyte-macrophage colony-stimulating factor (hGM-CSF)

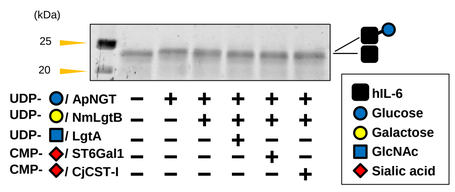


Length 155 a.a.
Molecular weight 17,475 Da

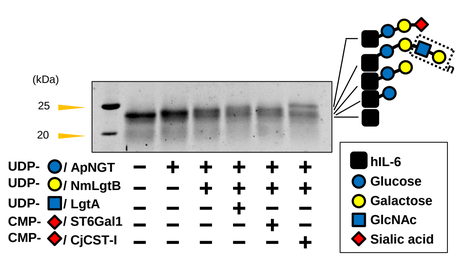
Original sequence NLSRDT

Modified sequence ATTGGNWTTAGGRDT

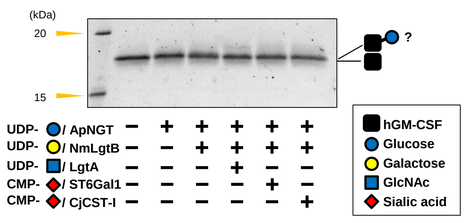
IVG



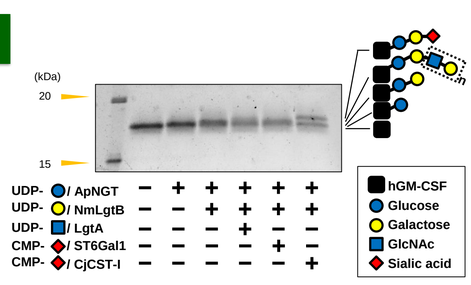
Multi-step IVG



IVG

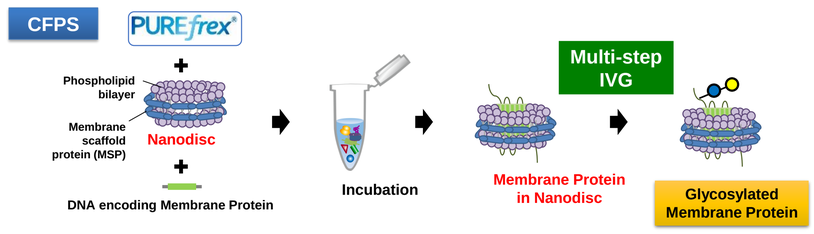


Multi-step IVG

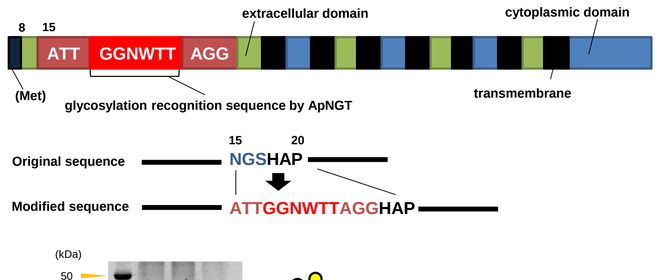


Multi-step IVG with PURE*frex*® was effective for disulfide bonded proteins.

3. in vitro Glycosylation of membrane protein



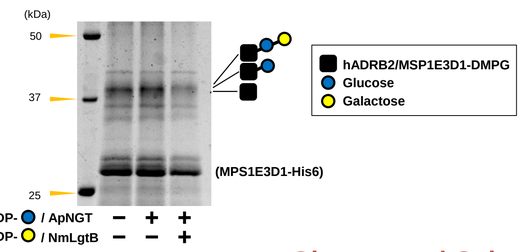
Human Beta-2 adrenergic receptor (hADRB2)



Length 416 a.a.
Molecular weight 46,764 Da

Original sequence NGSHAP

Modified sequence ATTGGNWTTAGGHAP



Glucose and Galactose were added to asparagine in hADRB2 by multi-step IVG with PURE*frex*®.

<Summary>

- **N-glycosylation of Im7-6 were succeeded IVG and One-pot IVG with PURE*frex*®.**
- **Depending on the target protein, glycosylation could be achieved by arranging the method as Multi-step IVG.**

↓

Glycoproteins can be synthesized by IVG using PURE*frex*®.

Future Study

- **Application of current glycosylation methods to other proteins.**
- **Examination of oligosaccharide glycosylation by IVG with PURE*frex*® .**

For more information, please contact us
URL: www.genefrontier.com
E-mail: purefrex@genefrontier.com

GeneFrontier