23. Lorenzo O, Chico JM, Sánchez-Serrano JJ, Solano R: ***JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsi.** *Plant Cell* 2004, **16**:1938-1950.
24. Gallagher E, Gao M, Liu YC, Karin M: **Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change.** *Proc Natl Acad Sci USA* 2006, **103**:1717-1722.
25. Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M: **Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression.** *Plant Cell* 2000, **12**:393-404.
26. Solano R, Stepanova A, Chao Q, Ecker JR: **Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1.** *Genes Dev* 1998, **12**:3703-3714.
27. Dombrecht B, Xue GP, Sprague SJ, Kirkegaard JA, Ross JJ, Reid JB, Fitt GP, Sewelam N, Schenk PM, Manners JM, Kazan K: **MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*.** *Plant Cell* 2007, **19**:2225-2245.
28. Swindell WR, Huebner M, Weber AP: **Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways.** *BMC Genomics* 2007, **8**:125.
29. Guo J, Wu J, Ji Q, Wang C, Luo L, Yuan Y, Wang Y, Wang J: **Genome-wide analysis of heat shock transcription factor families in rice and *Arabidopsis*.** *J Genet Genomics* 2008, **35**:105-118.
30. Kumar M, Busch W, Birke H, Kemmerling B, Nürnberger T, Schöffl F: **Heat shock factors HsfB1 and HsfB2b are involved in the regulation of *Pdf1.2* expression and pathogen resistance in *Arabidopsis*.** *Mol Plant* 2009, **2**:152-165.
31. von Koskull-Döring P, Scharf KD, Nover L: **The diversity of plant heat stress transcription factors.** *Trends Plant Sci* 2007, **12**:452-457.
32. Czarnecka-Verner E, Yuan CX, Scharf KD, Englich G, Gurley WB: **Plants contain a novel multi-member class of heat shock factors without transcriptional activator potential.** *Plant Mol Biol* 2000, **43**:459-471.
33. Czarnecka-Verner E, Pan S, Salem T, Gurley WB: **Plant class B HSFs inhibit transcription and exhibit affinity for TFIIB and TBP.** *Plant Mol Biol* 2004, **56**:57-75.
34. Bergomaz R, Boppré M: **A simple instant diet for rearing Arctiidae and other moths.** *J Lepidopt Soc* 1986, **40**:131-137.
35. **Preparation of genomic DNA.** In *Short protocols in molecular biology* Edited by: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. New York: Wiley; 1997:2.10-12.11.
36. Arimura G, Köpke S, Kunert M, Volpe V, David A, Brand P, Dabrowska P, Maffei ME, Boland W: **Effects of feeding *Spodoptera littoralis* on Lima bean leaves: IV. Diurnal and nocturnal damage differentially initiate plant volatile emission.** *Plant Physiol* 2008, **146**:965-973.
37. Wang L, Halitschke R, Kang JH, Berg A, Harnisch F, Baldwin IT: **Independently silencing two JAR family members impairs levels of trypsin proteinase inhibitors but not nicotine.** *Planta* 2007, **226**:159-167.
38. von Dahl CC, Winz RA, Halitschke R, Kühnemann F, Gase K, Baldwin IT: **Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*.** *Plant* 2007, **51**:293-307.
39. Maffei M, Bossi S, Spiteller D, Mithöfer A, Boland W: **Effects of feeding *Spodoptera littoralis* on lima bean leaves. I. Membrane potentials, intracellular calcium variations, oral secretions, and regurgitate components.** *Plant Physiol* 2004, **134**:1752-1762.
40. Arimura G, Garms S, Maffei M, Bossi S, Schulze B, Leitner M, Mithöfer A, Boland W: **Herbivore-induced terpenoid emission in *Medicago truncatula*: concerted action of jasmonate, ethylene and calcium signaling.** *Planta* 2008, **227**:453-464.
41. Curtis MD, Grossniklaus U: **A gateway cloning vector set for high-throughput functional analysis of genes in planta.** *Plant Physiol* 2003, **133**:462-469.
42. Sawasaki T, Ogasawara T, Morishita R, Endo Y: **A cell-free protein synthesis system for high-throughput proteomics.** *Proc Natl Acad Sci USA* 2002, **99**:14652-14657.
43. Sawasaki T, Kamura N, Matsunaga S, Saeki M, Tsuchimochi M, Morishita R, Endo Y: **Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate.** *FEBS Lett* 2008, **582**:221-228.
44. Masaoka T, Nishi M, Ryo A, Endo Y, Sawasaki T: **The wheat germ cell-free based screening of protein substrates of calcium/calmodulin-dependent protein kinase II delta.** *FEBS Lett* 2008, **582**:1795-1801.
45. Zuo J, Niu QW, Chua NH: **Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants.** *Plant J* 2000, **24**:265-273.
46. Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM: **pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation.** *Plant Mol Biol* 2000, **42**:819-832.
47. Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H: **Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase.** *Plant Cell* 2007, **19**:1065-1080.
48. Jefferson RA, Kavanagh TA, Bevan MW: **GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants.** *Embo* 1987, **6**:3901-3907.

doi: 10.1186/1471-2229-10-97
Cite this article as: Kanchiswamy *et al.*, Regulation of Arabidopsis defense responses against Spodoptera littoralis by CPK-mediated calcium signaling *BMC Plant Biology* 2010, **10**:97

The Wheat-Germ Cell-Free Expression System

Kazuyuki Takai, Tatsuya Sawasaki, and Yaeta Endo*

Cell-free Science and Technology Research Center and Venture Business Laboratory, Ehime University, 3, Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan

Abstract: We have made a dramatic improvement of the wheat cell-free protein synthesis system. The first key improvement is the method for preparation of the cell-free extract that is free of inhibitory factors of translation reaction. Additional improvements include a method for preparation of transcription-ready templates by PCR, an expression vector for the cell-free system, and the "bilayer" mode reaction method that is much more efficient than the batch mode method and at the same time easy to be performed by human hands and by liquid handling machines. We review here the history of the development and describe the protocols for the most handy "bilayer" method and a more efficient but complicated methods. Information on many examples and variations of the wheat cell-free protein synthesis methods already published elsewhere is then provided so that the readers can understand the power and potential applications of the methods.

Keywords: Cell-free protein synthesis, wheat, split-primer PCR, automation, pEU.

INTRODUCTION

Cell-free protein synthesis was first established with mammalian cell extracts in 1950's [1]. It was soon found that extracts from *E. coli* catalyze efficient translation, which led to the discovery of poly(Phe) synthesis directed by poly(U) in 1961 [2]. The first cell-free translation system from wheat embryo, which could catalyze poly(Phe) synthesis, was described in 1964 [3]. It was later shown that an extract from commercial wheat germ can efficiently translate tobacco mosaic virus (TMV) RNA and rabbit globin mRNA [4,5], demonstrating that the extract contains all the ingredients required for protein synthesis. The amounts of proteins synthesized by these cell-free methods were generally small, however. In an attempt to increase the protein production, Spirin's group showed that cell-free protein synthesis with wheat germ extracts can last for very long time when performed in an ultrafiltration chamber with continuous supplementation of the substrates and removal of the products, which was termed the continuous-flow cell-free (CFCF) method [6]. This seemed to mean that the translation apparatus is stable under a steady state-like condition that mimics intracellular environments. As the CFCF method is complicated, it was substituted by the less-complicated continuous-exchange cell-free (CECF) method, in which the reaction proceeds within a semi-permiable membrane bag being dialyzed against the substrate solution (dialysis method). We made a dramatic improvement in yield and achieved automation of the wheat cell-free protein production technology, as summarized in the following sections.

TECHNOLOGY DEVELOPMENTS

1. Extract Preparation

We speculated that the conventional wheat cell-free translation system contains factor(s) that catalytically inactivate(s) the ribosomes during the translation reaction. One such factor is tritin, present in commercial wheat germ, which attacks and modifies the sarcin-ricin loop of the ribosomes [7-9]. We have tested many different methods for inhibiting or removing the intrinsic ribosome inhibitors and found that extensive water wash of embryo particles largely removes white matter originating from endosperm and thereby dramatically improves the efficiency of translation catalyzed by the extract [10]. Such water wash has additional effects: most of other potential catalysts, such as proteases, DNases, and RNases, are also removed, and translation may be activated during the wash, for a substantial amount of water is absorbed into the embryo particles.

The method for preparation of the wheat embryo particles, which will be subsequently subjected to water wash, includes crushing wheat seeds, mesh sieving, flotation, and eye selection. This procedure was problematic because the flotation step needed use of carbon tetrachloride which is tightly regulated in Japan, and because eye selection was too laborious. These problems have been minimized in CellFree Sciences, Co., Ltd. (CFS), Matsuyama, Japan. The conventional method of crushing the washed embryo particles was to grind the liquid nitrogen-frozen particles with mortar and pestle in a cold room, after which oil was removed and the supernatant of the 30,000×g centrifugation (S30) was recovered. We could prepare several milliliter of the S30 extract by this conventional method at a time. This process is now replaced by a quality-controlled method utilizing a blender with rotating blades, and the extract can be obtained from CFS. We have recently described a laboratory-level version of this blender method elsewhere [11].

2. mRNA Preparation

As the cap analog and uncompleted transcripts inhibit cell-free translation, it would be necessary, if a capped mRNA is used for translation, to purify the complete transcript for reproducible results. Instead, an Ω sequence from TMV can be used for activation of downstream translation when placed in the 5'-untranslated region (5'-UTR) of un-

*Address correspondence to this author at the Cell-free Science and Technology Research Center, Ehime University, 3, Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan; Tel: +81-89-927-9936; Fax: +81-89-927-9941; E-mail: yendo@eng.ehime-u.ac.jp

1389-2010/10 $55.00+.00

capped mRNAs [12]. With the Ω sequence, the transcript can be directly introduced into the cell-free translation reaction without purification. The pEU vector that contains an SP6 promoter, the Ω sequence, and a multiple cloning site in this order was then constructed [12]. The wheat cell-free system thus developed produced up to 10 mg of proteins per 1-ml reaction mixture by the dialysis CECF method [12]. We have also developed several artificial sequences that are different from the Ω sequence but have almost the same functionality as Ω. The E01 sequence is one of such artificial sequences, and is regularly used in our group [13]. Furthermore, a method was developed for preparation of transcription-ready templates by PCR reactions without cloning of the target ORF into pEU as below. This facilitated parallel production of many different proteins encoded in a cDNA library [12].

3. Reaction Format

The dialysis method is not simple enough for translation of many different samples in parallel. Thus, a more handy method for CECF translation was developed [14]. The extract is slightly denser than the substrate solution. Thus, the reaction mixture can be put under the substrate solution to form a bilayer without the need for the separating membrane (Fig. (**1**)). Both solutions (*i.e.,* extract and substrate solution) mix together gradually with time, accompanied by supply of fresh substrates to and dilution of the byproducts from the extract layer. While the productivity of this "bilayer" method is lower than that of the dialysis method, it is much higher than that of the simple batch mode method. We have learned at the same time that the dilution of the byproducts, such as pyrophosphate and inorganic phosphate, has a larger effect on the productivity of translation than the supplementation of the substrates. The most important feature of the bilayer method was that it fits automation due to the absence of the semi-permeable membrane.

4. Automation

The bilayer method made automation possible. A robotic machine named "GenDecoder" performs a parallel small-scale bilayer protein synthesis, and a robot named "Protemist" performs a large-scale synthesis of small number of proteins (some Protemist machines also perform tag purification after the synthesis). One of the latest machines can perform gram-scale synthesis based on the "intermittent exchange" or "discontinuous batch" method [15,16]. This can synthesize nearly 1 gram of a model protein by an overnight operation.

In the following section, we describe basic protocols for synthesizing proteins encoded in a cDNA subcloned into a pEU plasmid. The protocols of the bilayer methods are essentially the same as those provided by CFS: basic protocols and typical results are available from the CFS website (www.cfsciences.com). As a lot of variations of these protocols are possible and have already performed by many people, we review such variations in the last section, which we expect will be useful for the readers to determine whether they will try the wheat cell-free system for their purposes.

PROTOCOLS AND EXAMPLES

Transcription of pEU Plasmids

The wheat embryo protein synthesis system is primarily a translation system, although both transcription and translation can be done in one tube. mRNA solutions are prepared by *in vitro* transcription with a DNA template, ribonucleoside 5'-triphosphates (NTPs), and SP6 RNA polymerase. The DNA template to be transcribed can be either a PCR product or a plasmid DNA, if it contains an SP6 promoter with a GAA trinucleotide at the transcription start site, an Ω sequence or an E01 or E02 enhancer sequence, the ORF sequence to be translated, and a long 3'-UTR of >500 nucleotides of any sequence. Such template DNA can be prepared by the split-primer PCR method as below or by subcloning the ORF sequence into the pEU vector. The protocol presented here is for an ORF subcloned in the pEU vector. We have observed that a G between the 5' enhancer sequence and the initiation codon sometimes causes alternative translation initiation that may result in no band or a doublet band in the SDS gel of the final products. Closed circular DNA is directly used for transcription.

For transcription in the small-scale bilayer method (see next section), mix Milli-Q water, 10 μl of 5× TB (400 mM HEPES-KOH, pH 7.8, 80 mM $Mg(OAc)_2$, 10 mM spermidine, and 50 mM DTT), 5 μl of 25 mM NTP, 50 units of RNase Inhibitor from human placenta (Promega), 50 units of SP6 RNA polymerase (Promega), and 5 μg of the plasmid DNA in a 50-μl solution. Incubate the solution at 37°C for 6 h. White pellet will appear during the incubation. For the large-scale method, perform the same reaction in a 250-μl solution. The transcription solution is directly used for translation (without purification).

For checking the transcription product, incubate 1-3 μl of the sample in a standard formamide dye at 65°C for 3 min and load the sample on a standard 1% agarose gel run in 1× TAE (40 mM Tris-acetate, pH 7.5-7.8, 1 mM EDTA). The ethidium bromide stained gel will show a few bands with smear signals in the upper (slowly migrating) region. The major band may be from the RNA molecule stopped near the plasmid replication origin: there is a transcription terminator around there. If there are several bands or a smear signal below the major band, it is possible that the transcription mixture was contaminated with some nuclease that cannot be inhibited by the RNase inhibitor. In such cases, we recommend to further purify the plasmid and to prepare all the solutions freshly.

Bilayer Method

In the bilayer method, the starting translation reaction mixture containing all the components required for translation (*i.e.*, creatine kinase, the wheat extract components, and mRNA) is first layered below a substrate solution. The substrate solution contains the standard 20 amino acids, nucleotides required for translation, an energy source substrate (creatine phosphate), a reducing agent dithiothreitol (DTT), salts, and a buffer. The substrate solution had been called Dialysis Buffer (DB) because it is used for the external solution for the dialysis method below. In this paper, however, we call it Buffered Substrate Solution (BSS). The wheat extract is prepared in BSS.

The efficiency of the bilayer translation may depend on the shape of the container in which the reaction is performed. The ratio of the volume to the contacting surface area can affect the rate of the exchange of the substrates and byprod-

ucts. While the following protocol using a flat-bottomed 96-well microtiter plate will give reproducible results, standard 0.2 ml PCR tubes can also give good results.

For protein synthesis, mix 10 μl of the mRNA solution (the transcription product above) with 0.8 μl of 1 mg/ml creatine kinase and 10 μl of 240 OD/ml wheat embryo extract. Then, pour 206 μl of BSS (30 mM HEPES-KOH, pH 7.6, 100 mM KOAc, 2.7 mM $Mg(OAc)_2$, 0.4 mM spermidine, 2.5 mM DTT, 0.3 mM each amino acid, 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate) into a well of a flat-bottomed 96-well microtiter plate. Put the reaction mixture containing mRNA, creatine kinase, and the extract under the BSS at the bottom of the microtiter well carefully NOT to mix these two solutions together (Fig. **1**). Cover the well using a standard seal in order to minimize evaporation. Incubate the plate at 15°C for 20 h.

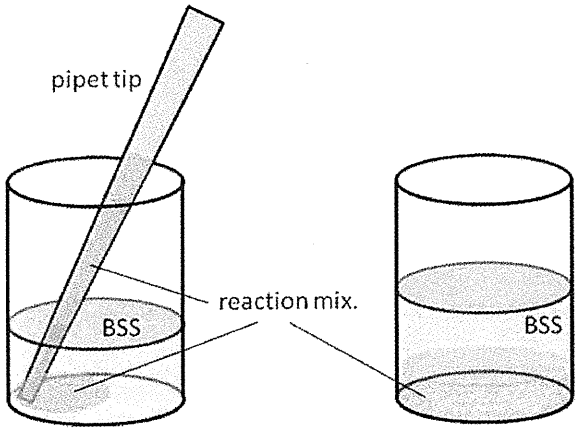

Fig. (1). The bilayer method.
Schematic representations showing how the reaction mixture should be layered under the substrate solution without perturbation (left) and the bilayer to be formed by this operation (right).

After the incubation, mix the sample and analyze 3 μl of it by a standard SDS-PAGE stained with Coomassie Brilliant Blue. The band for the product protein may be visible among many bands of the wheat proteins. If the band overlaps with that of a wheat protein, or the band is too faint to be detected, it is recommended to label the protein with [^{14}C]leucine during the translation reaction and to detect the band by autoradiography. A larger protein tends to give a fainter band.

The above system can be scaled-up in the large-scale bilayer mode useful for sub-milligram synthesis of selected proteins. The reaction is performed in a 6-well plate with 500 μl of the reaction mixture in each well. For translation, first mix 250 μl of the transcription product with 1 μl of 20 mg/ml creatine kinase and 250 μl of the 240 OD/ml extract. Then, pour 5.5 ml of BSS into a well of a 6-well plate (Cat. No. 92006, Techno Plastic Products). Layer the reaction mixture under the solution at the bottom of the well, and seal the well. Incubate the plate at 15°C for 20 h. Mix the solution after the incubation, and analyze the products on an SDS-polyacrylamide gel.

Dialysis Method

The dialysis method is useful for synthesizing a large amount of protein, though the method is rather complicated. The reaction mixture, which is essentially the same as that in the bilayer method, is placed in a dialysis membrane or a dialysis cartridge, and the substrate solution is placed in the outer container. The dialysis membrane containing the reaction mixture is dipped into the outer solution, and the container is sealed and incubated. When utilizing this method, we usually add the mRNA solution every 12 h to the inner solution and exchange the outer solution every 24 h to maximize the productivity per unit amount of the extract. The reaction could be prolonged for more than 2 weeks if maintained carefully, while 60 h may be the most convenient duration time: a longer incubation may cause growth of fungi. For a 50-μl reaction we usually use a dialysis cup (Biotech International, Japan) for the reaction mixture with a 9-ml polypropylene tube (Cat. No. 0204-03, Maruemu Corp., Japan) as the reservoir of BSS. For a 2-ml reaction, a 5-ml Spectra/Por Float-A-Lyzer™ G2 device (MWCO of 8-10 K, Cat. No. G235055, Spectrum Laboratories, Inc.) can be used with about 20 ml of BSS.

ADVANTAGES AND DISADVANTAGES

PCR-Based Template Preparation

The wheat embryo cell-free translation system can utilize not only the pEU-subcloned templates but also PCR-produced templates amplified from cDNA library clones of any format. The PCR-based method is useful for high-throughput parallel production of natural and tag-fused proteins [17-19]. It is required to introduce an SP6 promoter and an enhancer sequence upstream of the ORF and a suitable 3'-UTR sequence by the PCR. For this purpose the "split-primer" PCR method mentioned above is useful [12]. This method utilizes two primers, each of which has an incomplete SP6 promoter sequence, and combination of the two generates a complete promoter. This method seldom generates byproducts containing a complete promoter that could produce an unintended RNA molecule during the following transcription reaction. The sequence in the 3'-UTR can be the sequence in the vector in which the cDNA sequence is inserted. There is no need for the poly(A) sequence characteristic of the eukaryotic mRNA. In the case of the cDNA clones subcloned in pEU or other pUC-related plasmid vectors, reverse primers that define the 3' terminus of the transcript near the plasmid replication origin are useful for the split-primer PCR method.

Modification of the Extract

The extract is highly resistant to treatments with chromatography resins and ultrafiltration membranes. The buffer can be exchanged by passing the sample through a Sephadex G-25 column. The extract may be diluted after passage through a column, while it can be concentrated by the use of an ultrafiltration cartridge, such as Amicon Ultra-15. Taking advantage of this robust nature of the extract, a highly productive gram order synthesis method was developed and automated as mentioned above. In this method, the buffer and substrates surrounding the protein synthesis apparatus are intermittently exchanged by ultrafiltration during the

reaction. The extract can be freeze-dried without severe loss of activity and can be stored at –20°C for years.

Spectrum of Suitable Proteins

We have recently shown that 12,996 (97.2%) of randomly chosen 13,364 human cDNA clones produced detectable amounts of proteins using the wheat embryo cell-free translation system [20]. The results clearly show that in most cases the amino acid sequence of the cDNA-encoded polypeptide could successfully be synthesized in the wheat cell-free system. Of those 12,996 polypeptides, 12,682 were detected in the soluble fraction. It was apparent in Ref. [20] and as shown in Fig. (**2**) that the cell-free system is essential for highly parallel production of many different proteins, and the wheat cell-free system exhibits a very high success rate.

Number	Resource of ORF (FLJ ID)	Mw (kDa)	TM	Wheat germ (*in vitro*)		*E. coli* (*in vitro*)		*E. coli* (*in vivo*)	Cultured cell (DM2)	Cultured cell (CHO)
				5'GST	3'His	5'GST	3'His	3'His	3'His	3'TAP
1	FLJ20659	10.4	0	○	○	○		○	○	○
2	FLJ21903	14.7	0	○	○				○	
3	FLJ20616	15.2	0	○	○		○	○	○	○
4	FLJ20852	17.1	0	○	○		N.D.	○		
5	FLJ22873	17.8	0	○	○			○	○	○
6	FLJ21700	18.9	0	○	○			○	○	○
7	FLJ22881	20.0	0	○	○	○		○	○	○
8	FLJ20819	23.5	0	○	○			△	○	○
9	FLJ22273	25.3	0	○	○	○		○	○	○
10	FLJ22538	29.6	0	○	○	○				
11	FLJ22880	33.3	0	○	○	○		○	○	○
12	FLJ20621	33.3	0	○	○	○		△	○	○
13	FLJ20855	33.5	0	○	○	○		○	○	○
14	FLJ21182	33.7	0	○	○	○		○	○	○
15	FLJ21486	34.9	0	○	○	○		○	○	○
16	FLJ22325	35.7	0	○	○	○		○	○	○
17	FLJ20586	37.0	0	○	○	○	○		○	○
18	FLJ22320	38.2	0	○	○	○			○	○
19	FLJ22491	39.0	0	○	○	○		○	○	N.D.
20	FLJ21861	50.1	0	○	○	○	○	△	○	
21	FLJ21235	50.1	0	○	○	○	○	△		○
22	FLJ22470	52.7	0	○	○	○		△	△	N.D.
23	FLJ22779	57.3	0	○	○	○	○		○	N.D.
24	FLJ20768	66.9	0	○	○	○		△	○	○
25	FLJ22818	91.3	0	○	○		○	○		
26	FLJ21907	101.3	0	○	○	△		△		
27	FLJ21076	7.0	1	○	△			△	○	N.D.
28	FLJ21791	10.1	1	○	○	○		○	○	
29	FLJ21558	15.3	2	○	○			○	○	
30	FLJ20556	17.4	1	○				○		
31	FLJ20489	25.7	1	○	△					○
32	FLJ20507	25.8	4	○	○	○	N.D.	N.D.	○	○
33	FLJ12627	26.0	1	○		○		△		
34	FLJ10199	27.8	2	○		○		○	△	△
35	FLJ10191	28.6	4	○	○					N.D.
36	FLJ12791	32.3	4	○	○	○				○
37	FLJ10099	35.1	1	○	○	○		△	△	N.D.
38	FLJ12133	36.2	3	○	○				○	○
39	FLJ21879	37.7	7	△	△	○				○
40	FLJ11856	45.8	10	○	△	○				N.D.
41	FLJ10583	46.3	3	○	○	○				
42	FLJ12397	48.5	1	○	○				△	△
43	FLJ12769	51.2	1	○	○				○	
44	FLJ10494	53.9	9	○	○	△		△		N.D.
45	FLJ12646	63.1	1	○	○				△	N.D.
46	FLJ22958	64.7	8	○	○	○				N.D.
47	FLJ10375	68.3	9	○	○			△		N.D.
48	FLJ12756	76.1	14	○	○					
49	FLJ10407	76.3	7	△	△					
50	FLJ10572	80.1	1	○				△	○	○

Fig. (2). A comparison of different expression systems in Goshima *et al.* (2008).
Expression of fifty different ORFs was compared for the wheat cell-free, *E. coli* cell-free, *E. coli in vivo*, DM2 cultured cell, and CHO cultured cell systems. The numbers in the "TM" column show the numbers of predicted transmembrane domains. Tags shown below the names of the expression systems were fused to the ORFs by using the Gateway system. The expression levels were determined by visual inspection of the SDS gels stained with CBB. Circles and triangles, high and low expression, respectively; crosses, no expression; N. D., no data. Reproduced from Ref. [20].

While many expression systems suffer from low productivity due to biased codon preferences within the ORF for the target protein, the effects appear to be small for the wheat cell-free system. For example, while malaria proteins are generally difficult to produce partly because the codon preferences are biased extremely, many of them have been successfully produced in the wheat cell-free system [21]. A template with a very high A/T content may rather be troublesome at the level of DNA/RNA technologies. It has also been revealed that the wheat cell-free system is excellent in producing DNases, such as restriction enzymes [22]. Other examples of produced proteins are summarized elsewhere [23-30].

Modifications on Synthesized Proteins

The N-termini of the cell-free synthesized proteins are processed according to the normal eukaryotic "N-end rule", and are acetylated if the cell-free reactions are supplied with acetyl-coenzyme A [31]. N-teminal lipoylation, such as palmitoylation, may also occur if the substrate is supplied [32]. Glycosylation does not occur during wheat cell-free reactions. We have observed that some expressed proteins give SDS-PAGE bands of very high molecular weights, which may correspond to ubiquitination.

Quality of Synthesized Proteins — Protein Degradation and Co-Translational Folding

The proteins produced in the wheat cell-free protein synthesis system are only minimally susceptible to degradation and thus can be used without purification. In fact, the wheat extract does not contain detectable activity of 26S proteasome [33]. This may be why some proteins give bands for ubiquitinated forms that have not been degraded.

Another prominent feature in the wheat cell-free system is that more proteins are produced in soluble forms than in the bacterial expression system [34,35]. It is suggested that the wheat cell-free system has a mechanism to minimize inter-domain interactions, presumably due to both a lower elongation rate than in bacterial systems and the presence of eukaryote-specific chaperones that bind to growing polypeptides [35,36]. In fact, we have successfully produced an active multi-domain RNA ligase that had been difficult to produce in an active form in a bacterial system [37]. Protein kinases are also difficult to produce in bacterial systems, in general, but not in the wheat cell-free system [38]. Successful production of native homopolymer of polyhedrin [39] and heterodimer protein [40,41] was also possible in the wheat cell-free system. Both of the apo- and holo-forms of an enzyme were also successfully produced [42].

It is possible, in the wheat cell-free system, to perform the protein synthesis reaction at lower temperatures than that presented in the above protocol. We have observed that translation proceeds even at 4°C, which facilitates production of proteases (unpublished results). Therefore, it may also be possible to promote correct folding and subunit assembly by performing the translation reaction at a low temperature.

Applications to Structural Biology

The low protease activity within the extract makes the method useful for protein structural analyses. HSQC spectra can be obtained using the product of protein synthesis with ^{15}N amino acids just after a simple buffer exchange without purification [43]. The cell-free labeling is useful also because only smaller amounts of labeled amino acids are required, and because the labeled amino acids can be in part recovered from the reaction mixture, as seen in the structural genomics project at the Center for Eukaryotic Structural Genomics (CESG) in Wisconsin University [44,45].

The wheat cell-free method has been found to be quite useful for amino acid-specific stable-isotope labeling that should accelerate 3D-structure determination. The amino acid-specific labeling technology in general may suffer from "scrambling" of the isotope due to metabolic reactions that simultaneously occur with the protein synthesis reaction. In the case of the wheat cell-free translation system, only a few inhibitors were found to be sufficient for eliminating the effects of such metabolic reactions [46-48]. Therefore, the wheat cell-free method is expected to be also useful for labeling with "SAIL" amino acids that should increase the size limit of NMR protein structure determination from around 20 kDa to 40 kDa [45,49].

The wheat cell-free method is also useful for protein structure determination through X-ray crystallography [22]. Selenomethionine labeling for this purpose is quite easy in the cell-free method: it is achieved just by substituting Met with selenomethionine. The buffer in the extract can be exchanged by simply passing through a Sephadex G-25 column.

Production of membrane proteins may be one of the major technologies in structural biology, protein engineering and drug discovery. Conventionally canine microsomal fraction has been used for *in vitro* production of membrane proteins and glycosylation. However, the microsomal fraction contains many degrading enzymes, and can be used for only limited purposes. It has been demonstrated that by adding a detergent and liposomes prepared from soybean asolectin to the wheat cell-free translation system, a membrane transporter can be produced in an active form on the liposome membrane [50].

Disadvantages

Cell-free methods are not very good at producing proteins with disulfide bonds. This is because the reaction mixture should contain a sufficient amount of reducing agent, such as DTT. We have shown that a single-chain variable fragment with two disulfide bonds can be made using a minimal amount of DTT in the reaction mixture, though the productivity is not very high [51]. This technology needs much more improvement, which may include regulation of reducing potential and introduction of membrane technologies.

The major disadvantage of the wheat cell-free protein synthesis system is the cost of the extract [45]. This is because the extract is prepared from wheat embryo particles selected by human eyes. This process cannot be compromised, as it ensures the quality of the extract. Another possible point is due to the presence of unknown components and mRNA degrading enzymes in the extracts. Such problems are minimal in the PURE system from *E. coli* (see the next

paper in this volume; sold by Wako Pure Chemical Industries, Ltd., Osaka, and New England Biolabs).

ACKNOWLEDGEMENTS

This work is supported in part by the Special Coordination Funds for Promoting Science and Technology (Y. E.) and in part by a Grant-in-Aid for Scientific Research on the Priority Areas (No. 20034040 to K. T.) by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

[1] Zamecnik, P. C.; Keller, E. B. Relation between phosphate energy donors and incorporation of labeled amino acids into proteins. *J. Biol. Chem.*, **1954**, *209*, 337-354.

[2] Nirenberg, M. W.; Matthaei, J. H. Characteristics and stabilization of DNAase-sensitive protein synthesis in *E. coli* extracts. *Proc. Natl. Acad. Sci. USA*, **1961**, *47*, 1588-1602.

[3] Marcus, A.; Feeley, J. Activation of protein synthesis in the imbibitions phase of seed germination. *Proc. Natl. Acad. Sci. USA*, **1964**, *51*, 1075-1079.

[4] Roberts, B. E.; Paterson, B. M. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc. Natl. Acad. Sci. USA*, **1973**, *70*, 2330-2334.

[5] Erickson, A. H.; Blobel, G. Cell-free translation of messenger RNA in a wheat germ system. *Methods Enzymol.*, **1983**, *96*, 38-50.

[6] Spirin, A. S.; Baranov, V. I.; Ryabova, L. A.; Ovodov, S. Y.; Alakhov, Y. B. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science*, **1988**, *242*, 1162-1164.

[7] Endo, Y.; Wool, I. G. The site of action of alpha-sarcin on eukaryotic ribosomes. The sequence at the alpha-sarcin cleavage site in 28 *S* ribosomal ribonucleic acid. *J. Biol. Chem.*, **1982**, *257*, 9054-9060.

[8] Endo, Y.; Mitsui, K.; Motizuki, M.; Tsurugi, K. The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification in 28 *S* ribosomal RNA caused by the toxins. *J. Biol. Chem.*, **1987**, *262*, 5908-5912.

[9] Endo, Y.; Tsurugi, K. RNA *N*-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. *J. Biol. Chem.*, **1987**, *262*, 8128-8130.

[10] Madin, K.; Sawasaki, T.; Ogasawara, T.; Endo, Y. A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 559-564.

[11] Takai, K.; Sawasaki, T.; Endo, Y. Practical cell-free protein synthesis system using purified wheat embryos. *Nat. Protoc.*, **2010**, *5*, 228-238.

[12] Sawasaki, T.; Ogasawara, T.; Morishita, R.; Endo, Y. A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA*, **2002**, *99*, 14652-14657.

[13] Kamura, N.; Sawasaki, T.; Kasahara, Y.; Takai, K.; Endo, Y. Selection of 5'-untranslated sequences that enhance initiation of translation in a cell-free protein synthesis system from wheat embryos. *Bioorg. Med. Chem. Lett.*, **2005**, *15*, 5402-5406.

[14] Sawasaki, T.; Hasegawa, Y.; Tsuchimochi, M.; Kamura, N.; Ogasawara, T.; Kuroita, T.; Endo, Y. A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett.*, **2002**, *514*, 102-105.

[15] Sawasaki, T.; Endo, Y. Protein expression in the wheat-germ cell-free system. In: *Expression Systems*; Dyson, M. R., Durocher, Y., Eds.; Scion Publishing Ltd.: Oxfordshire, Oxford, UK, **2007**, pp. 87-108.

[16] Sawasaki, T.; Endo, Y. The wheat germ cell-free protein synthesis system. In: *Cell-free Protein Synthesis*; Spirin, A., Swartz, J., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA; Weinhein, Germany, **2008**; pp. 111-139.

[17] Kanno, T.; Komatsu, A.; Kasai, K.; Dubouzet, J. G.; Sakurai, M.; Ikejiri-Kanno, Y.; Wakasa, K.; Tozawa, Y. Structure-based *in vitro* engineering of the anthranilate synthase, a metabolic key enzyme in the plant tryptophan pathway. *Plant Physiol.*, **2005**, *138*, 2260-2268.

[18] Kobayashi, T.; Kodani, Y.; Nozawa, A.; Endo, Y.; Sawasaki, T. DNA-binding profiling of human hormone nuclear receptors via fluorescence correlation spectroscopy in a cell-free system. *FEBS Lett.*, **2008**, *582*, 2737-2744.

[19] Sawasaki, T.; Kamura, N.; Matsunaga, S.; Saeki, M.; Tsuchimochi, M.; Morishita, R.; Endo, Y. Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate. *FEBS Lett.*, **2008**, *582*, 221-228.

[20] Goshima, N.; Kawamura, Y.; Fukumoto, A.; Miura, A.; Honma, R.; Satoh, R.; Wakamatsu, A.; Yamamoto, J.; Kimura, K.; Nishikawa, T.; Andoh, T.; Iida, Y.; Ishikawa, K.; Ito, E.; Kagawa, N.; Kaminaga, C.; Kanehori, K.; Kawakami, B.; Kenmochi, K.; Kimura, R.; Kobayashi, M.; Kuroita, T.; Kuwayama, H.; Maruyama, Y.; Matsuo, K.; Minami, K.; Mitsubori, M.; Mori, M.; Morishita, R.; Murase, A.; Nishikawa, A.; Nishikawa, S.; Okamoto, T.; Sakagami, N.; Sakamoto, Y.; Sasaki, Y.; Seki, T.; Sono, S.; Sugiyama, A.; Sumiya, T.; Takayama, T.; Takayama, Y.; Takeda, H.; Togashi, T.; Yahata, K.; Yamada, H.; Yanagisawa, Y.; Endo, Y.; Imamoto, F.; Kisu, Y.; Tanaka, S.; Isogai, T.; Imai, J.; Watanabe, S.; Nomura, N. Human protein factory for converting the transcriptome into an *in vitro*-expressed proteome. *Nat. Methods*, **2008**, *5*, 1011-1017.

[21] Tsuboi, T.; Takeo, S.; Iriko, H.; Jin, L.; Tsuchimochi, M.; Matsuda, S.; Han, E.; Otsuki, H.; Kaneko, O.; Sattabongkot, J.; Udomsangpetch, R.; Sawasaki, T.; Torii, M.; Endo, Y. Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. *Infect. Immun.*, **2008**, *76*, 1702-1708.

[22] Miyazono, K.; Watanabe, M.; Kosinski, J.; Ishikawa, K.; Kamo, M.; Sawasaki, T.; Nagata, K.; Bujnicki, J. M.; Endo, Y.; Tanokura, M.; Kobayashi, I. Novel protein fold discovered in the PabI family of restriction enzymes. *Nucleic Acids Res.*, **2007**, *35*, 1908-1918.

[23] Endo, Y.; Sawasaki, T. High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *Biotechnol. Adv.*, **2003**, *21*, 695-713.

[24] Endo, Y.; Sawasaki, T. High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *J. Struct. Funct. Genomics*, **2004**, *5*, 45-57.

[25] Sawasaki, T.; Hasegawa, Y.; Morishita, R.; Seki, M.; Shinozaki, K.; Endo, Y. Genome-scale, biochemical annotation method based on the wheat germ cell-free protein synthesis system. *Phytochemistry*, **2004**, *65*, 1549-1555.

[26] Sawasaki, T.; Gouda, M. D.; Kawasaki, T.; Tsuboi, T.; Tozawa, Y.; Takai, K.; Endo, Y. The wheat germ cell-free expression system: methods for high-throughput materialization of genetic information. *Methods Mol. Biol.*, **2005**, *310*, 131-144.

[27] Endo, Y.; Sawasaki, T. Advances in genome-wide protein expression using the wheat germ cell-free system. *Methods Mol. Biol.*, **2005**, *310*, 145-167.

[28] Endo, Y.; Sawasaki, T. Cell-free expression systems for eukaryotic protein production. *Curr. Opin. Biotechnol.*, **2006**, *17*, 373-380.

[29] Sawasaki, T.; Morishita, R.; Gouda, M. D.; Endo, Y. Methods for high-throughput materialization of genetic information based on wheat germ cell-free expression system. *Methods Mol. Biol.*, **2007**, *375*, 95-106.

[30] Takai, K.; Sawasaki, T.; Endo, Y. Development of key technologies for high-throughput cell-free protein production with the extract from wheat embryos. *Adv. Protein Chem. Struct. Biol.*, **2008**, *75*, 53-84.

[31] Kanno, T.; Kitano, M.; Kato, R.; Omori, A.; Endo, Y.; Tozawa, Y. Sequence specificity and efficiency of protein N-terminal methionine elimination in wheat-embryo cell-free system. *Protein Expr. Purif.*, **2007**, *52*, 59-65.

[32] Lingappa, J. R.; Hill, R. L.; Wong, M. L.; Hegde, R. S. A multistep, ATP-dependent pathway for assembly of human immunodeficiency virus capsids in a cell-free system. *J. Cell. Biol.*, **1997**, *136*, 567-581.

[33] Takahashi, H.; Nozawa, A.; Seki, M.; Shinozaki, K.; Endo, Y.; Sawasaki, T. A simple and high-sensitivity method for analysis of ubiquitination and polyubiquitination based on wheat cell-free protein synthesis. *BMC Plant Biol.*, **2009**, *9*, 39.

[34] Netzer, W. J.; Hartl, F. U. Recombination of protein domains facilitated by co-translational folding in eukaryotes. *Nature*, **1997**, *388*, 343-349.

[35] Hirano, N.; Sawasaki, T.; Tozawa, Y.; Endo, Y.; Takai, K. Tolerance for random recombination of domains in prokaryotic and eukaryotic translation systems: Limited interdomain misfolding in a eukaryotic translation system. *Proteins*, **2006**, *64*, 343-354.

[36] Kramer, G.; Boehringer, D.; Ban, N.; Bukau, B. The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nat. Struct. Mol. Biol.*, **2009**, *16*, 589-597.

[37] Makino, S.; Sawasaki, T.; Tozawa, Y.; Endo, Y.; Takai, K. Covalent circularization of exogenous RNA during incubation with a wheat embryo cell extract. *Biochem. Biophys. Res. Commun.*, **2006**, *347*, 1080-1087.

[38] Masaoka, T.; Nishi, M.; Ryo, A.; Endo, Y.; Sawasaki, T. The wheat germ cell-free based screening of protein substrates of calcium/calmodulin-dependent protein kinase II delta. *FEBS Lett.*, **2008**, *582*, 1795-1801.

[39] Ikeda, K.; Nakazawa, H.; Shimo-Oka, A.; Ishio, K.; Miyata, S.; Hosokawa, Y.; Matsumura, S.; Masuhara, H.; Belloncik, S.; Alain, R.; Goshima, N.; Nomura, N.; Morigaki, K.; Kawai, A.; Kuroita, T.; Kawakami, B.; Endo, Y.; Mori, H. Immobilization of diverse foreign proteins in viral polyhedra and potential application for protein microarrays. *Proteomics*, **2006**, *6*, 54-66.

[40] Matsumoto, K.; Tomikawa, C.; Toyooka, T.; Ochi, A.; Takano, Y.; Takayanagi, N.; Abe, M.; Endo, Y.; Hori, H. Production of yeast tRNA (m^7G46) methyltransferase (Trm8-Trm82 complex) in a wheat germ cell-free translation system *J. Biotechnol.*, **2008**, *133*, 453-460.

[41] Kanno, T.; Kasai, K.; Ikejiri-Kanno, Y.; Wakasa, K.; Tozawa, Y. *In vitro* reconstitution of rice anthranilate synthase: distinct functional properties of the alpha subunits OASA1 and OASA2. *Plant Mol. Biol.*, **2004**, *54*, 11-22.

[42] Abe, M.; Ohno, S.; Yokogawa, T.; Nakanishi, T.; Arisaka, F.; Hosoya, T.; Hiramatsu, T.; Suzuki, M.; Ogasawara, T.; Sawasaki, T.; Nishikawa, K.; Kitamura, M.; Hori, H.; Endo, Y. Detection of structural changes in a cofactor binding protein by using a wheat germ cell-free protein synthesis system coupled with unnatural amino acid probing. *Proteins*, **2007**, *67*, 643-652.

[43] Morita, E. H.; Sawasaki, T.; Tanaka, R.; Endo, Y.; Kohno, T. A wheat germ cell-free system is a novel way to screen protein folding and function. *Protein Sci.*, **2003**, *12*, 1216-1221.

[44] Vinarov, D. A.; Lytle, B. L.; Peterson, F. C.; Tyler, E. M.; Volkman, B. F.; Markley, J. L. Cell-free protein production and labeling protocol for NMR-based structural proteomics. *Nat. Methods*, **2004**, *1*, 149-153.

[45] Vinarov, D. A.; Loushin Newman, C. L.; Markley, J. L. Wheat germ cell-free platform for eukaryotic protein production. *FEBS J.*, **2006**, *273*, 4160-4169.

[46] Morita, E. H.; Shimizu, M.; Ogasawara, T.; Endo, Y.; Tanaka, R.; Kohno, T. A novel way of amino acid-specific assignment in ^{1}H-^{15}N HSQC spectra with a wheat germ cell-free protein synthesis system. *J. Biomol. NMR*, **2004**, *30*, 37-45.

[47] Kohno, T. Production of proteins for NMR studies using the wheat germ cell-free system. *Methods Mol. Biol.*, **2005**, *310*, 169-185.

[48] Kohno, T.; Endo, Y. Production of protein for nuclear magnetic resonance study using the wheat germ cell-free system. *Methods Mol. Biol.*, **2007**, *375*, 257-272.

[49] Kainosho, M.; Torizawa, T.; Iwashita, Y.; Terauchi, T.; Ono, M. A.; Güntert, P. Optimal isotope labelling for NMR protein structure determinations. *Nature*, **2006**, *440*, 52-57.

[50] Nozawa, A.; Nanamiya, H.; Miyata, T.; Linka, N.; Endo, Y.; Weber, A.P.M.; Tozawa, Y. A cell-free translation and proteoliposome reconstitution system for functional analysis of plant solute transporters. *Plant Cell Physiol.*, **2007**, *48*, 1815-1820.

[51] Kawasaki, T.; Gouda, M. D.; Sawasaki, T.; Takai, K.; Endo, Y. Efficient synthesis of a disulfide-containing protein through a batch cell-free system from wheat germ. *Eur. J. Biochem.*, **2003**, *270*, 4780-4786.

Received: June 30, 2009 **Revised: August 15, 2009** **Accepted: September 05, 2009**

Tohoku J. Exp. Med., 2010, **220**, 199-206

Evaluating the Role of Rheumatoid Factors for the Development of Rheumatoid Arthritis in a Mouse Model with a Newly Established ELISA System

Yuki Tanaka,[1,2] Hiroaki Komori,[1*] Shiro Mori,[3] Yoshiko Soga,[1] Takahito Tsubaki,[1] Miho Terada,[1] Tatsuhiko Miyazaki,[1] Takahiro Fujino,[2] Satoshi Nakamura,[4] Hiroyuki Kanno,[5] Tatsuya Sawasaki,[6] Yaeta Endo[6] and Masato Nose[1,7]

[1]Department of Pathogenomics, Ehime University Graduate School of Medicine, Ehime, Japan
[2]Integrated Center for Sciences, Ehime University, Ehime, Japan
[3]Department of Oral and Maxillofacial Surgery, Tohoku University Graduate School of Dentistry, Sendai, Japan
[4]Department of Bioengineering, Tokyo Institute of Technology, Tokyo, Japan
[5]Department of Pathology, Iwate University School of Medicine, Morioka, Japan
[6]Cell-Free Science and Technology Research Center, Ehime University, Ehime, Japan
[7]Proteo-Medicine Research Center, Ehime University, Ehime, Japan

Enzyme-linked immunosorbent assays (ELISA) have been widely used to determine quantitatively autoantibodies. However, the processes for the purification and immobilization of antigens in conventional ELISA methods include multiple steps, which have hampered the application for screening of autoantibodies. Here, we have developed a novel ELISA system using the plates pre-coated with glutathione casein to capture recombinant proteins fused to N-terminal glutathione S-transferase (GST). The GST-fused proteins were synthesized with the wheat germ cell-free protein production system. Thus, the present system combined the GST-capture ELISA with the cell-free protein production system, which allowed immobilization of the recombinant proteins with one-step purification. Using this ELISA method, we determined whether rheumatoid factors (RF), which have been considered as one of the representative disease – specific autoantibodies for rheumatoid arthritis (RA), were genetically associated with severity of arthritis in a mouse model for RA, MRL/Mp-*lpr/lpr* (MRL/lpr). GST-fused human IgG1-Fc (GST-Fc), synthesized with the robotic protein synthesizer, were used as reactants for RF. Serum samples for RF were prepared from 11 lines of a recombinant inbred mouse strain, MXH/lpr, which was established from intercrosses between MRL/lpr and non-arthritic C3H/HeJ-*lpr/lpr* (C3H/lpr) strains, composed of a different genomic recombination derived from the parental strains in each line. A correlation of RF titers with the severity of the arthritis in these lines was not significant, indicating genetic dissociation of RF from arthritis and that RF is not necessarily required for the development of RA. The present method may provide high-throughput screening for determining the disease-specific autoantibodies in autoimmune diseases.

Keywords: MRL/lpr; recombinant inbred strain; MXH/lpr; GST; IgG-Fc
Tohoku J. Exp. Med., 2010, **220** (3), 199-206.

Enzyme-linked immunosorbent assays (ELISA) have been widely used to determine quantitatively the reactant proteins for autoantibodies in various diseases involving autoimmunity and infection (Gawryl et al. 1986; Moore et al. 1986; Tan 1989; Valdés Veliz et al. 2003). However, conventional ELISA methods are not suitable for the wide screening of autoantibodies because various processes and conditions are necessary for the purification and immobilization of each protein.

The wheat germ cell-free protein production system was established in our laboratory, in which any proteins could be synthesized in the presence of the cDNA template by using wheat germ ribosome more effectively than in *E. coli*-based system (Madin et al. 2000; Sawasaki et al. 2002a), and made it possible to synthesize proteins in a high-throughput manner (Sawasaki et al. 2002b). In fact, almost 380 kinds of proteins could be automatically synthesized at once overnight based on cDNA templates by using the robotic protein synthesizer GenDecorder 1000A® (CellFree Sciences, Matsuyama, Japan). Among cell-free

Received October 14, 2009; revision accepted for publication January 20, 2010. doi:10.1620/tjem.220.199
*Deceased March 7, 2009
Correspondence: Masato Nose, M.D., Ph.D., Department of Pathogenomics, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan.
e-mail; masanose@m.ehime-u.ac.jp

systems for protein synthesis, this wheat germ-based system is of special interest for its eukaryotic nature; it has the significant advantage of producing a large amount of eukaryotic multi-domain proteins in folded state (Endo and Sawasaki 2006; Goshima et al. 2008; Takai et al. 2010). Thus, this system may be suitable for high-throughput synthesis of any reactant proteins for autoantibodies. Moreover, when recombinant proteins fused to N-terminal glutathione S-transferase (GST) are prepared in this system and captured on glutathione-coated ELISA plates (Sehr et al. 2001), the multi-step processes for the purification and immobilization of each protein required in conventional ELISA methods may not be necessary.

This report describes the development of a capture ELISA system using synthesized proteins fused to GST in the wheat germ cell-free protein production system. This method allowed the simple immobilization and purification of the proteins in one-step on ELISA plates pre-coated with glutathione casein. Then, this system was used to measure the rheumatoid factor (RF) in a murine model for rheumatoid arthritis (RA), MRL/Mp-*lpr/lpr* (MRL/lpr) (Murphy and Roths 1978; Nose et al. 1989). This mouse strain spontaneously develops arthritis resembling RA associated with high titers of IgG-RF, supporting the concept that IgG RF is one of disease-specific autoantibodies in RA (Theofilopoulos and Dixon 1985). Our results may reveal that the RF activity does not genetically correlate with the severity of arthritis in this model.

Materials and Methods

IgG-Fc with GSTtag in cell free system

The Fc portion of human IgG1 (IgG-Fc) was prepared as RF reactant according to a cell-free protein synthesis system using wheat germ ribosomal RNA (Madin et al. 2000; Sawasaki et al. 2002a; Soga et al. 2009). In brief, the Fc portion of human IgG1 cDNA (Kitai et al. 1988) was inserted into a pEUE01-GST-N2 expression vector (CellFree Sciences, Matsuyama, Japan) containing a GST region and SP6 promotor. The GST-IgG-Fc proteins were automatically synthesized by the Robotic Protein Synthesizer Protemist® DT (CellFree Sciences) as previously described (Soga et al. 2009). Namely, 250 μl of transcription mixture containing 25 μg of the plasmid DNA, 80 mM HEPES-potassium hydroxide, pH 7.8, 16 mM magnesium acetate, 2 mM spermidine, 10 mM dithiothreitol, 2.5 mM each of nucleoside triphosphates, 250 U of SP6 RNA polymerase (Promega, Madison, WI, USA) and 250 U of RNasin (Promega) was incubated for 6 hr at 37°C. After the incubation, the transcription solution containing transcribed mRNA was mixed with 250 μl of wheat germ extract WEPRO1240 (CellFree Sciences) supplemented with 2 ml of 20 mg/ml creatine kinase in a single well of a six-well plate. The 5.5 ml substrate mix (30 mM HEPES-potassium hydroxide, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM dithiothreitol, 0.3 mM amino acid mix, 1.2 mM ATP, 0.25 mM GTP and 16 mM creatine phosphate; CellFree Sciences) was then added on top of the translation mix and then was incubated at 17°C for 20 hr. The reaction mixture was used without any purification procedures as IgG-Fc-GST to be captured on the ELISA plates. In some experiments, the IgG-Fc-GST in the mixture was purified by glutathione Sepharose 4B (GE Healthcare, Uppsala, Sweden) according to the instructions for use.

GST capture ELISA

GST capture ELISA was performed basically according to the previously described method (Sehr et al. 2001). In brief, to conjugate glutathione to casein, casein (Wako, Osaka, Japan) at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) was incubated at room temperature (RT) with 0.4 mM N-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO, USA) and then the single cysteine residue was blocked in casein. Next, sulfosuccinimidyl 4-[p-maleimidophenyl] butyrate (SSMBP; Pierce, Rockford, IL) was added at 4 mM as a cross-linker. Free SSMBP and NEM were separated from casein on PD10 columns (GE Healthcare). The protein fraction was then supplemented with 10 mM glutathione (Wako) and the coupling reaction was executed for 1 h at RT. The glutathione casein was separated from unbound glutathione by gel filtration with PD10, using PBS as buffer and stored at –20°C until use. Next, plastic plates, consisting of 96 wells (Thermo Labsystems, Franklin, MA), were coated overnight at 4°C with 100 μl/well of glutathione casein, 2 μg/ml in 50 mM carbonate buffer, pH 9.6. The optimal concentration of glutathione casein was determined in a titration study by using plates pre-coated with it in a various concentration (0.5 to 8 μg/ml). Thereafter, the wells were incubated for 1 h at 37°C with blocking buffer (0.2% casein in PBS, 0.05% Tween 20), followed by incubation for 1 h at 4°C with the reaction mixture (100 μl/well) of IgG-Fc-GST proteins synthesized in cell free system diluted in blocking buffer.

The coated ELISA plates were then incubated for 1 h with 100 μl/well of goat anti-human IgG-Fc antibodies conjugated with alkaline phosphatase (AP; Sigma-Aldrich) or diluted sample solution of mouse monoclonal antibodies and mouse sera. Bound antibodies of the later were detected by polyclonal goat anti-mouse immunoglobulin IgG (Sigma) or IgM (Zymed Laboratories Inc., San Francisco, CA), conjugated to AP, diluted 1/2000 in blocking buffer. In some experiments, the conventional ELISA using *E. coli.*-generated IgG-Fc with the same construct (Nose et al. 1990) which were purified by protein A column (GE Healthcare) was performed in the same procedure. Commercialized glutathione-coated ELISA plates for GST-proteins, which are pre-coupled with glutathione via polyethylene glycol as a spacer, were reacted overnight at 4°C with GST or IgG-Fc-GST proteins synthesized in cell free system according to the manufacturer instructions.

Mouse sera

Serum samples for RF were prepared from MRL/MpJ-*lpr/lpr* (MRL/lpr) and C3H/HeJ-*lpr/lpr* (C3H/lpr) mice and 11 lines of a recombinant inbred (RI) strain of mice, MXH/lpr, established by intercrosses between an MRL/lpr and a C3H/lpr strain of mice. These lines had a different genome recombination each other derived from the parental strains in homozygote. Sera from C3H/HeJ mice without the *lpr* gene were used as normal control for RF. The parental strains of mice were originally obtained from Jackson Laboratory (Bar Harbor, ME). All of the mice used in this study were bred and housed in an animal facility in a pathogen-free and climate-controlled environment with 12 hr light/dark cycles. All experiments were done according to the Guidelines for the Care and Use of Laboratory Animals at Ehime University.

RF monoclonal antibodies

Hybridomas producing IgM-RF monoclonal antibodies (clones; Zao2 and Zao7) were prepared by the cell fusion of NS-1 myeloma cells with unmanipulated spleen cells from a 20 wk-old male C57BL/6-*lpr/lpr* mouse, which were originally obtained from the Jackson Laboratory (Bar Harbor), according to a method described elsewhere (Takahashi et al. 1993). The selection of IgM-RF-producing hybridoma cells was performed in conventional ELISA using *E. coli.*-generated IgG-Fc (Nose et al. 1996). Ant-TNP monoclonal IgM antibodies (a mouse hybridoma clone; Sp6) (Nose and Wigzell 1983) were used as a murine IgM control.

Histopathology of arthritis

The mice were killed under ether anesthesia, and their hind limbs were processed for histopathology. Whole ankle joints were fixed in 10% formalin in 0.001 M phosphate buffer, pH 7.2, decalcified in 10% formic acid, and embedded in paraffin. Serial sections 2-3-μm thick were taken sagittally through the talus and stained with hematoxylin and eosin for examinations by light microscopy. The lesions, including the calcaneus bone and anterior and posterior synovial tissue at the ankle joints, were evaluated histopathologically. To evaluate the severity of synovitis, the following grading was used: normal, grade 0; thickening and proliferation of synovial lining, grade 1; and grade 1 with granulomatous and/or fibrous lesions in synovial sublining tissue, grade 2 (Kamogawa et al. 2002). Pannus formation was categorized as follows: normal, grade 0, extending to bone cortex, grade 1; and further to bone marrow, grade 2. Each score of synovitis and pannus formation in the individual mice was represented as the maximum score in the bilateral ankles.

Statistical analysis

Student's *t*-test was applied to analyze the statistical significance of the results. A p value of < 0.05 was considered to be significant. Correlation between any two parameters of the severity of arthritis and RF activity; synoviti vs. pannus, IgG-RF vs. IgM-RF, IgG-RF vs. synovitis/pannus, and IgM-RF vs. synovitis/pannus, was estimated by the Spearman rank-correlation coefficient as indicated r.

Results

One-step capture of IgG-Fc-GST fusion proteins on the ELISA plates

Synthetic IgG-Fc-GST in the reaction mixture was successfully captured on ELISA plates coated with glutathione-conjugated casein. The reaction mixtures of GST or IgG-Fc-GST prepared in cell free system themselves showed multiple bands on PAGE as shown in Fig. 1A. However, each fraction obtained after reacting on and then eluting from the ELISA plates coated with glutathione-conjugated casein, but not with casein only, clearly showed a single band with the expected molecular sizes corresponding GST or GST-IgG-Fc, except a few extra bands with 22-25kD in molecular sizes derived from wheat germ extract (Fig. 1B).

The dose dependency of IgG-Fc capture on the glutathione casein-coated plates was measured by using anti-human IgG-Fc antibodies (Fig. 2). Synthetic IgG-Fc-GST in the reaction mixture was reacted with the glutathione casein-coated plates at a series of concentrations generated by serial dilution. The results showed the capture of IgG-Fc-GST to be saturated at concentrations of less than 1.0 μg/ml under this condition. The background coating only with glutathione casein was quite low and GST itself did not react at any concentrations.

Reactivity of monoclonal RF on the capture ELISA

Next, to confirm the specificity of RF reactivity of the

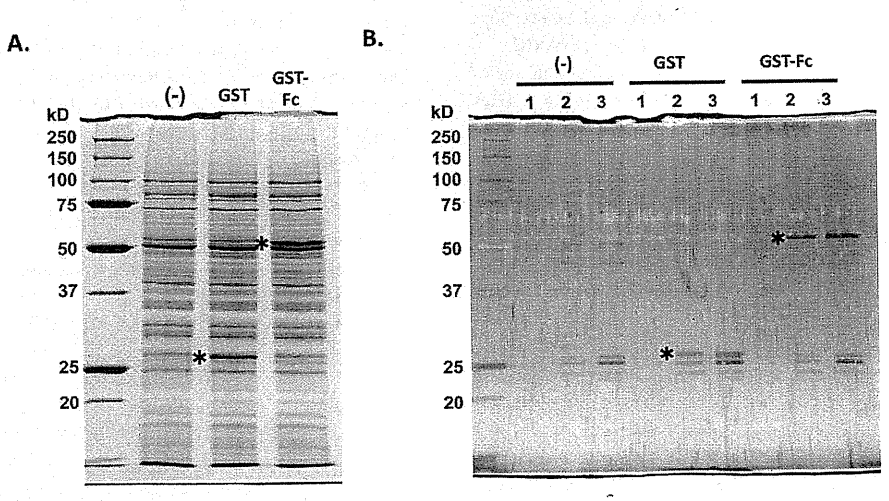

Fig. 1. Capture of the synthetic IgG-Fc-GST on glutathione casein-coated ELISA plates. A. The reaction mixture (0.5 μl) of vector free (–), GST or IgG-Fc-GST was loaded on SDS-PAGE. B. The same mixture of samples (100 μl) was loaded after reacting on and then eluting by using 10 μl of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH8.0) from the ELISA plates coated with; casein only (lane 1) or glutathione-conjugated casein (10 mg/ml; lane 2, 1 mg/ml; lane 3). Each eluate (0.5 μl) was loaded on SDS-PAGE. GST and IgG-Fc-GST seemed to be specifically bound to the glutathione casein-coated plates. Extra bands around 25 kDa seemed to be from wheat germ extract. Asterisk indicates the expected band on each lane.

capture ELISA plates, the reactivity of mouse monoclonal IgM RF, clones Zao2, Zao7 and Sp6 (see Materials and Methods) was compared with that of the conventional ELISA plates coated with *E. coli.*-generated IgG-Fc (Fig. 3). Both monoclonal IgM RF showed almost the same reactivity between the capture ELISA and the conventional ELISA systems. And, mouse IgM control showed no reactivity in both ELISA systems.

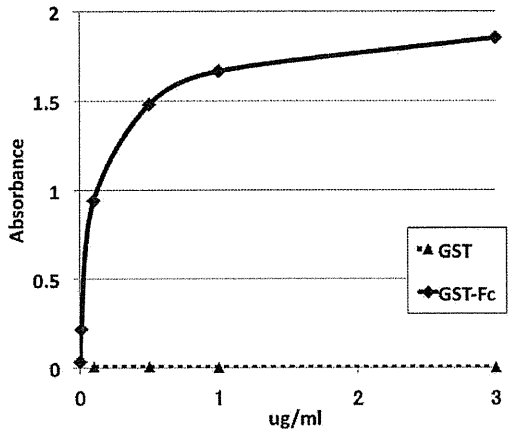

Fig. 2. Dose dependency of IgG-Fc to be captured on the glutathione casein-coated plates. IgG-Fc-GST in the reaction mixture was loaded on the glutathione casein coated plates. The binding of IgG-Fc-GST was measured by using AP-conjugated anti-human IgG-Fc antibodies. The capture of IgG-Fc-GST was in dose-dependent manner, and saturated at concentrations of less than 1.0 μg/ml. The coating background with glutathione casein was quite low and GST itself did not react at any concentration. Absorbance indicates the mean value of OD_{405}-OD_{510} in triplicate samples.

Measurement of RF activity of mouse sera

Finally, the IgG RF activity in mouse sera was compared between the arthritis-prone strain of mice MRL/lpr and the non-arthritic strain of mice C3H/lpr, both of which possess the Fas deletion mutant lpr (Nagata, 1994) (Fig. 4A). MRL/lpr mice showed a higher reactivity than C3H/lpr mice as previously reported (Theophilopoulus and Dixon 1985). And, sera from C3H/HeJ mice without the *lpr* gene showed no reactivity for IgG-Fc. At the same time, the results were compared with those obtained using commercialized GST-ELISA plates (Fig. 4B). Unexpectedly, GST-ELISA did not show any IgG-Fc specificity in both mouse sera.

Genetic dissociation of RF activity from the severity of arthritis in the MXH/lpr mice

The capture ELISA system was used to determine the correlations between the RF activity and the severity of arthritis in the recombinant inbred strains of mice, MXH/lpr. MXH/lpr mice were established by intercrosses between MRL/lpr and C3H/lpr strains of mice, among which genetic dissociation of arthritis might be generated due to genome recombination between the parental stains, based on a previous study of chromosomal mapping of the susceptibility loci to arthritis (Nose et al. 1989; Kamogawa et al. 2002). Expectedly, genetic dissociation of arthritis was observed among the MXH/lpr mice (Fig. 5); that is, several lines among the MXH/lpr mice showed a significantly higher score in the severity of synovitis (lines; 07, 10, 25 and 36) ($p < 0.05$ vs. C3H/lpr) and pannus formation (lines; 10, 25 and 36) ($p < 0.05$ vs. C3H/lpr) while others such as lines 51

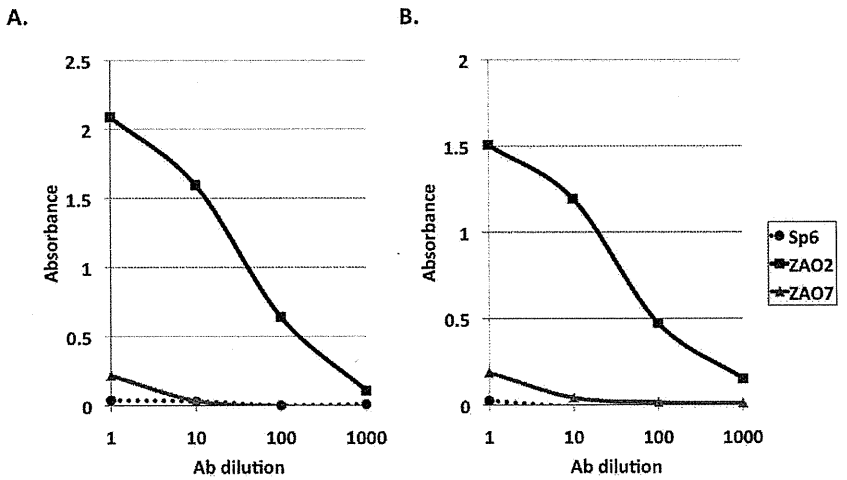

Fig. 3. Reactivity of monoclonal RF on the capture ELISA and the conventional ELISA systems. The culture supernatant of the hybridomas producing IgM monoclonal RF (clones; Zao2 and Zao7) and IgM monoclonal anti-TNP antibodies (clone; Sp6) was reacted on the capture ELISA (A) and the conventional ELISA (B) systems in a serial dilution. These antibodies showed almost the same reactivity between both ELISA systems. Absorbance indicates the mean value of OD_{405}-OD_{510} in triplicate samples.

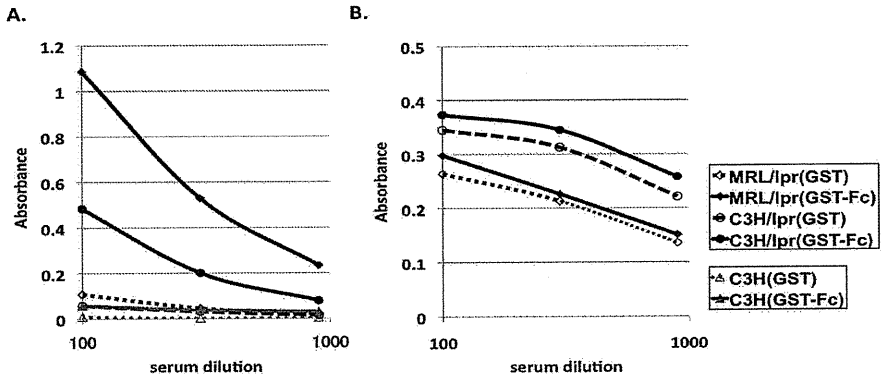

Fig. 4. RF activity of the mouse sera. The mouse sera from male mice of an arthritis-prone stain of mice, MRL/lpr and non-arthritic strain of mice, C3H/lpr (pooled sera at 16-20 weeks old, $n = 5$ each) were compared in a serial dilution. A. The capture ELISA plates, showed the sera from MRL/lpr mice was greater than those from C3H/lpr mice. The normal control sera from C3H/HeJ mice (pooled sera at 8 weeks old, $n = 12$) showed no reactivity. B. The same samples were measured on commercialized GST-ELISA plates. The results showed higher background, thus indicating the reaction to be non-specific for IgG-Fc. Absorbance indicates the mean value of OD_{405}-OD_{510} in triplicate samples.

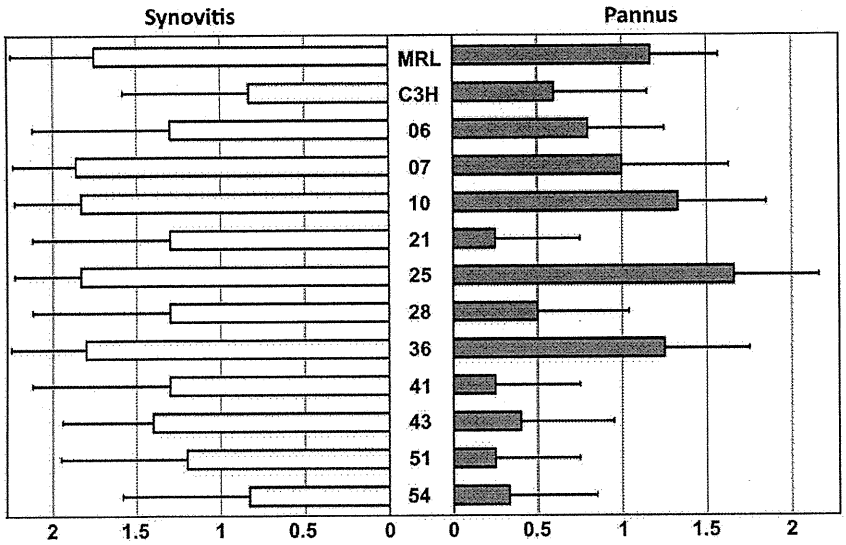

Fig. 5. Genetic dissociation of arthritis among the recombinant inbred strains MXH/lpr. The severity of arthritis of individual mice was graded at 16-20 weeks old ($n = 4$-6 each) and the results were represented as the average total score +/– S.D. of each strain (see Materials and Methods).

and 54 showed a lower score in the both severity. In addition, the severity between synovitis and pannus formation was highly correlated ($r = 0.725$, Fig. 7A), indicating that both lesions are on the same pathological sequence leading arthritis.

Then, the IgM- and IgG-RF activity of each stain of MXH/lpr mice was measured. As shown in Fig. 6, it was clearly demonstrated that IgM- and IgG-RF activity was also genetically dissociated among the MXH/lpr mice. IgG-RF activity of the line 41 was extremely higher than even the arthritis-prone parental strain of mice MRL/lpr ($p < 0.01$) and IgM-RF activity of the lines 06 and 36 was also higher than that of both parental strains ($p < 0.01$). There was only a slight negative correlation between both activities ($r = -0.286$, Fig. 7B).

Finally, correlation between the severity of arthritis and RF activity was examined. There was only a slight positive correlation between any two parameters; IgM-RF or IgG-RF vs. synovitis or pannus formation ($r < 0.265$ in any combinations, Fig. 7C and D).

Discussion

This study presented a novel method combining the GST-capture ELISA and the wheat germ cell-free protein

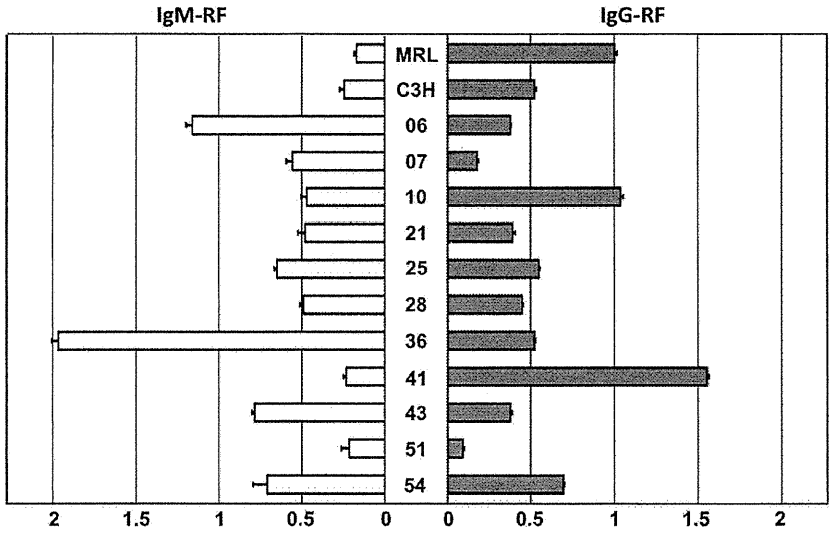

Fig. 6. Genetic dissociation of the RF activity among the recombinant inbred strains MXH/lpr. Mouse sera from each strain of male MXH/lpr mice were pooled at 16-20 weeks old, and they were reacted on the capture ELISA plates following a 100 × dilution. The results were represented as the average total score +/– S.D. in triplicate samples. Each of the studies was repeated three times and the similar results were obtained in all experiments.

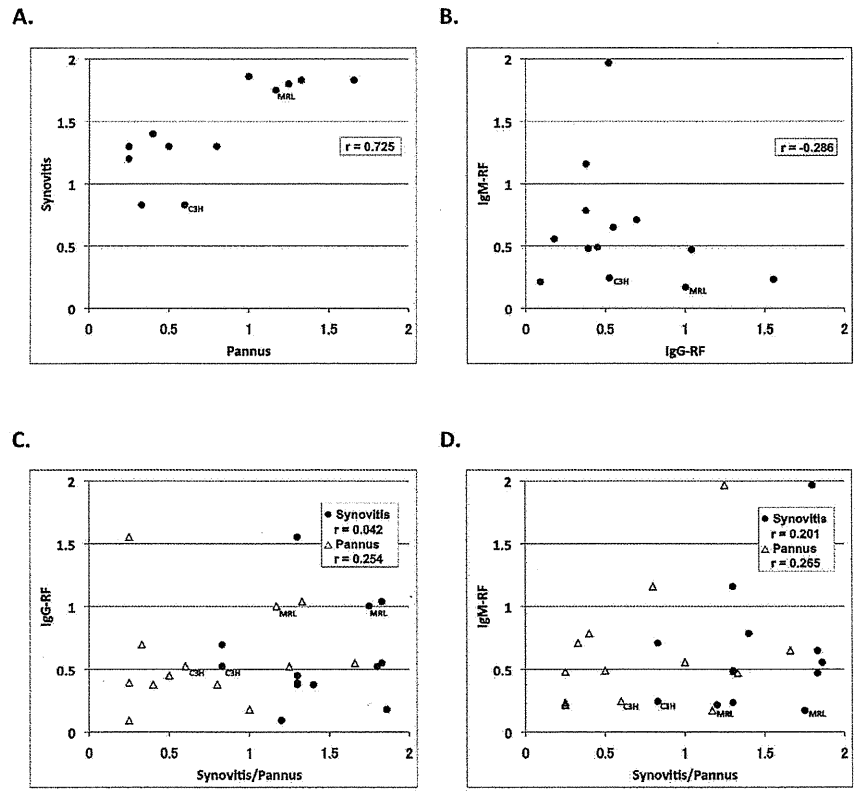

Fig. 7. Correlations of the severity of arthritis and RF activity. A correlation between any two parameters of the severity of arthritis and RF activity was estimated with the Spearman rank-correlation coefficient as indicated by *r*. A; synoviti vs. pannus, B; IgG-RF vs. IgM-RF, C; IgG-RF vs. synovitis/pannus and D; IgM-RF vs. synovitis/pannus.

production system, which thus provides high-throughput screening to clarify disease-specific autoantibodies in autoimmune diseases. The key points of this system are, first, to be able to use any kind of recombinant proteins fused to GST synthesized in the cell-free system without any purification processes, and second, to immobilize them on ELISA plates pre-coated with glutathione casein in one-step and at once.

This system was used to actually measure RF reacting with recombinant IgG-Fc with GST synthesized in the cell-free system. First, the RF specificity of this system was compared with a conventional ELISA containing *E. coli*-generated IgG-Fc using two types of monoclonal IgM-RF with a higher affinity and lower affinity. The results were similar between the two systems (Fig. 3). Next, the IgG-RF activity in mouse sera derived from MRL/lpr and C3H/lpr mice, which has higher RF activity in the former and lower in the later (Theofilopoulos and Dixon 1985), was measured, and compared their specificity with commercialized glutathione-coated ELISA plates. The results showed that the commercialized ELISA showed not only increased background but also absence of specific reactivity (Fig. 4B).

Then, this method was used to clearly demonstrate the genetic dissociation of RF from arthritis in a mouse model for RA. RI strains of mice MXH/lpr mice were prepared using two different parental inbred strains as progenitors, MRL/lpr and C3H/lpr mice, followed by an F1 intercross and more than 20 generations of strict brother-sister mating. This breeding protocol allows the production of a family of new inbred strains with special properties relative to each other since the genome of each RI strain consists of a random combination of genomes. MXH/lpr mice are the first RI strains in autoimmune disease model mice. In this study, the severity of arthritis was different among the strains, thus indicating that genome recombination between the parental strains regulates the severity of arthritis (Fig. 5). A previous study (Kamogawa et al. 2002) suggested that arthritis in MRL/lpr mice is under the control of multiple gene loci with an allelic combination derived from the original inbred strains by chromosomal mapping of the susceptibility loci to arthritis by using the backcross progeny of MRL/lpr × (MRL/lpr × C3H/lpr) F1 mice. The present results verified this proposed concept more clearly by using RI strains on the ground of the reason as described below.

In general, the genetic analysis of disease phenotypes by crossing disease-prone and non-disease prone strains allows for the examination of only the phenotype of one individual in an association with genotypes. This may make it difficult to analyze complex disease phenotypes in a reproducible fashion (Theofilopoulos et al. 1989). In this respect, a set of inbred strains having a genomic mosaic of progenitor strains such as MXH/lpr is considered to be a highly appropriate tool for analyzing the disease phenotypes in a reproducible fashion. In fact, the current study demonstrated genetic dissociation of the severity of arthritis and the RF activity (Figs. 6 and 7). RF has been considered as the argument that RA is an autoimmune disease, and also as the disease-specific autoantibodies in RA. However, the specificity and sensitivity of RF to RA are still controversial and a pathological basis of RA negative for RF, so called sero-negative RA, remains unclear. Our results may indicate that RF activity is not associated with the development of arthritis at least in this mouse model, and other autoantibodies, if present, could be considered as pathogenic autoantibodies.

Not only arthritis, but also other pathological phenotypes might be genetically segregated from autoantibodies, which have been reported as disease-specific antibodies. Therefore, the system using the capture ELISA with many synthetic proteins in cell free system may thus make it possible to perform a screening study of the protein reactants of autoantibodies and verify their significance for disease phenotypes in mouse models and also humans.

Acknowledgments

This work was supported by Research Funds from the Ministry of Health and Welfare of Japan and the Grant-in Aid for Scientific Research (B) of the Ministry of Education, Science and Culture of Japan (#18390123).

References

Endo, Y. & Sawasaki, T. (2006) Cell-free expression systems for eukaryotic protein production. *Curr. Opin. Biotech.*, **17**, 373-380.

Gawryl, M.S., Simon, M.T., Eatman, J.L. & Lint, T.F. (1986) An enzyme-linked immunoabsorbent assay for the quantitation of the terminal complement complex from cell membranes or in activated human sera. *J. Immunol. Methods*, **95**, 217-225.

Goshima, N., Kawamura, Y., Fukumoto, A., Miura, A., Honma, R., Satoh, R., Wakamatsu, A., Yamamoto, J., Kimura, K., Nishikawa, T., Andoh, T., Iida, Y., Ishikawa, K., Ito, E., Kagawa, N., Kaminaga, C., Kanehori, K., Kawakami, B., Kenmochi, K., Kimura, R., Kobayashi, M., Kuroita, T., Kuwayama, H., Maruyama, Y., Matsuo, K., Minami, K., Mitsubori, M., Mori, M., Morishita, R., Murase, A., Nishikawa, A., Nishikawa, S., Okamoto, T., Sakagami, N., Sakamoto, Y., Sasaki, Y., Seki, T., Sono, S., Sugiyama, A., Sumiya, T., Takayama, T., Takayama, Y., Takeda, H., Togashi, T., Yahata, K., Yamada, H., Yanagisawa, Y., Endo, Y., Imamoto, F., Kisu, Y., Tanaka, S., Isogai, T., Imai, J., Watanabe, S. & Nomura, N. (2008) Human protein factory for converting the transcriptome into an in vitro–expressed proteome. *Nat. Methods*, **5**, 1011-1017.

Kamogawa, J., Terada, M., Mizuki, S., Nishihara, M., Yamamoto, H., Mori, S., Abe, Y., Morimoto, K., Nakatsuru, S., Nakamura, Y. & Nose, M. (2002) Arthritis in MRL/lpr mice is under the control of multiple gene loci with an allelic combination derived from the original inbred strains. *Arthritis Rheum.*, **46**, 1067-1074.

Kitai, K., Kudo, T., Nakamura, S., Masegi, T., Ichikawa, Y. & Horikoshi, K. (1988) Extracellular production of human immunoglobulin G Fc region (hIgG-Fc) by *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, **28**, 52-59.

Madin, K., Sawasaki, T., Ogasawara, T. & Endo, Y. (2000) A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc. Natl. Acad. Sci. USA*, **97**, 559-564.

Moore, T.L., Dorner, R.W. & Zuckner, J.J. (1986) 19S IgM rheu-

matoid factor-7S IgG rheumatoid factor immune complexes isolated in patients with rheumatoid arthritis. *J. Lab. Clin. Med.*, **107**, 465-470.

Murphy, E.D. & Roths, J.B. (1978) Autoimmunity and lymphoproliferation: induction by mutant gene lpr, and acceleration by a male-associated factor in strain BXSB mice. In Rose, N.R., Bigazzai, P.E. & Warner, N.L., editors. Genetic control of autoimmune disease. *New York: Elsevier North Holland*, 207-220.

Nagata, S. (1994) Fas and Fas ligand: a death factor and its receptor. *Adv. Immunol.*, **57**, 129-144.

Nose, M., Nishimura, M., Ito, M.R., Itoh, J., Shibata, T. & Sugisaki, T. (1996) Arteritis in a novel congenic strain of mice derived from MRL/lpr lupus mice: Genetic dissociation from glomerulonephritis and limited autoantibody production. *Am. J. Pathol.*, **149**, 1763-1769.

Nose, M., Nishimura, M. & Kyogoku, M. (1989) Analysis of granulomatous arteritis in MRL/Mp autoimmune disease mice bearing lymphoproliferative genes. The use of mouse genetics to dissociate the development of arteritis and glomerulonephritis. *Am. J. Pathol.*, **135**, 271-280.

Nose, M., Takano, R., Nakamura, S., Arata, Y. & Kyogoku, M. (1990) Recombinant Fc of human IgG1 prepared in an Escherichia coli system escapes recognition by macrophages. *Int. Immunol.*, **2**, 1109-1112.

Nose, M. & Wigzell, H. (1983) Biological significance of carbohydrate chains on monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, **80**, 6632-6636.

Sawasaki, T., Hasegawa, Y., Tsuchimochi, M., Kamura, N., Ogasawara, T., Kuroita, T. & Endo, Y. (2002a) A bilayer cell-free protein synthesis system for high-throughput screening of gene products. *FEBS Lett.*, **514**, 102-105.

Sawasaki, T., Ogasawara, T., Morishita, R. & Endo, Y. (2002b) A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA*, **99**, 14652-14657.

Sehr, P., Zumbach, K. & Pawlita, M. (2001) A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: Validation for HPV serology. *J. immunol. Methods*, **253**, 153-162.

Soga, Y., Komori, H., Miyazaki, T., Arita, N., Terada, M., Kamada, K., Tanaka, Y., Fujino, T., Hiasa, Y., Matsuura, B., Onji, M. & Nose, M. (2009) Toll-like receptor 3 signaling induces chronic pancreatitis through the Fas/Fas ligand-mediated cytotoxicity. *Tohoku J. Exp. Med.*, **217**, 175-184.

Takahashi, S., Itoh, J., Nose, M., Ono, M., Yamamoto, T. & Kyogoku, M. (1993) Cloning and cDNA sequence analysis of nephritogenic monoclonal antibodies derived from an MRL/lpr lupus mouse. *Mol. Immunol.*, **30**, 177-182.

Takai, K., Sawasaki, T. & Endo, Y. (2010) Practical cell-free protein synthesis system using purified wheat embryos. *Nat. Protoc.*, **5**, 227-238.

Tan, E.M. (1989) Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv Immunol.*, **44**, 93-151.

Theofilopoulos, A.N. & Dixon, F.J. (1985) Murine models of systemic lupus erythematosus. *Adv. Immunol.*, **37**, 269-390.

Theofilopoulos, A.N., Kofler, R., Singer, P.A. & Dixon, F.J. (1989) Molecular genetics of murine lupus models. *Adv. Immunol.*, **46**, 61-109.

Valdés Veliz, R., García, J., Reyes, B., Muñoz, L., Alvarez, T., Padilla, S., Abrahantes Mdel, C., Zubiaurrez, J., Agraz, A. & Marrero, A. (2003) A very sensitive enzyme-linked immunoabsorbent assay to Staphylococcal protein A in the presence of immunoglobulins. *Biochem. Biophys. Res. Commun.*, **303**, 863-867.

Chapter 13

Cell-Free Protein Synthesis for Structure Determination by X-ray Crystallography

Miki Watanabe, Ken-ichi Miyazono, Masaru Tanokura, Tatsuya Sawasaki, Yaeta Endo, and Ichizo Kobayashi

Abstract

Structure determination has been difficult for those proteins that are toxic to the cells and cannot be prepared in a large amount in vivo. These proteins, even when biologically very interesting, tend to be left uncharacterized in the structural genomics projects. Their cell-free synthesis can bypass the toxicity problem. Among the various cell-free systems, the wheat-germ-based system is of special interest due to the following points: (1) Because the gene is placed under a plant translational signal, its toxic expression in a bacterial host is reduced. (2) It has only little codon preference and, especially, little discrimination between methionine and selenomethionine (SeMet), which allows easy preparation of selenomethionylated proteins for crystal structure determination by SAD and MAD methods. (3) Translation is uncoupled from transcription, so that the toxicity of the translation product on DNA and its transcription, if any, can be bypassed. We have shown that the wheat-germ-based cell-free protein synthesis is useful for X-ray crystallography of one of the 4-bp cutter restriction enzymes, which are expected to be very toxic to all forms of cells retaining the genome. Our report on its structure represents the first report of structure determination by X-ray crystallography using protein overexpressed with the wheat-germ-based cell-free protein expression system. This will be a method of choice for cytotoxic proteins when its cost is not a problem. Its use will become popular when the crystal structure determination technology has evolved to require only a tiny amount of protein.

Key words: Structural genomics, Toxic protein, Wheat germ, Restriction enzyme, Selenomethionine, SAD

1. Introduction

In general, it is difficult to determine crystal structure of proteins toxic to cells because the toxicity does not allow their preparation in a large amount for crystallization. Many of those cytotoxic proteins may play important biological roles, but their structural diversity may be left unexplored, especially in the context of the

Y. Endo et al. (eds.), *Cell-Free Protein Production: Methods and Protocols,* Methods in Molecular Biology, vol. 607
DOI 10.1007/978-1-60327-331-2_13,

structural genomics. In addition to novel structural folds that expand the universe of protein structure, elucidation of their structure may reveal novel modes of protein–DNA interaction and protein–protein interaction among others (1, 2).

Restriction endonucleases, essential tools for molecular biology, form a good example of such groups of cytotoxic proteins with important biological roles and structural diversity (3–5). A restriction endonuclease recognizes a specific DNA sequence and introduces a double-stranded break, while a paired modification enzyme can methylate a specific base of the same sequence. Their genes are usually tightly linked and form a restriction–modification gene complex. Various lines of evidence indicate that they behave as selfish mobile genetic elements just as viral genomes and transposons. Comparison of two closely-related genome sequences, such as those of two species within the same hyperthermophilic archaeon genus, *Pyrococcus abyssi* and *Pyrococcus horikoshii*, has indicated their mobility and linkage with various types of genome rearrangements (6). This property makes it possible to search restriction enzymes of a novel fold by bioinformatics methods. While the restriction endonucleases show little sequence conservation, the methyltransferases belong to a conserved protein family. Therefore, the bioinformatics strategy is to first identify methyltransferase genes and then search for their cognate restriction endonuclease genes in the neighboring open reading frames (ORFs) (7). If genome comparison reveals that the candidate gene has been moving between genomes together with the methyltransferase homolog, it is likely a restriction gene. This strategy allowed identification of PabI from *P. abyssi* as a unique 4-bp cutter restriction enzyme with novel properties, generation of TA3′ restriction terminus, and Mg^{2+} ion independence (7, 8).

PabI was predicted to adopt an entirely novel structure (7). However, PabI, like most of the restriction enzymes, is cytotoxic when expressed in vivo in the absence of appropriate and sufficient methylation on the genome. The strategies to bypass this toxicity include use of tightly repressible expression systems and expression of the cognate methyltransferase, but they did not work well. In contrast, in vitro translation systems can synthesize almost any protein, often with high accuracy and at a speed approaching in vivo rates. Among the cell-free systems, the wheat-germ-based system is of special interest: the translation machine has little codon preference and, especially, has little discrimination between methionine (Met) and selenomethionine (SeMet), a characteristic that is useful in crystal structure determination by SAD and MAD methods; translation is uncoupled from transcription, so that digestion of the template DNA by the produced restriction enzyme does not take place (8). The only problem with the system is the cost, especially in a large scale.

In this chapter, we describe successful preparation of PabI protein in this wheat-germ-based cell-free expression system in the native and SeMet-labeled forms (Fig. 13.1), their crystallization (Fig. 13.2), and structure determination. The crystal structure, mutant analysis and *in silico* modeling revealed that the protein adopts a novel fold and participates in a novel mode of protein–DNA interaction (8). The new nomenclature and abbreviations for restriction enzymes and their genes were used (9).

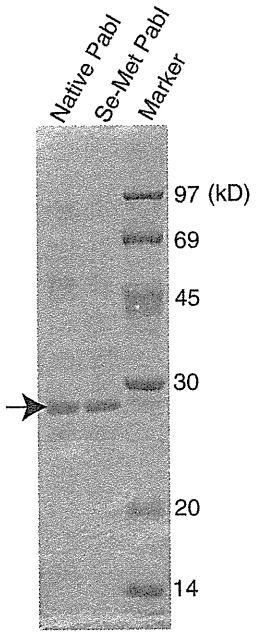

Fig 13.1 PabI restriction enzyme and its selenomethionine-substituted version expressed in a wheat-germ-based cell free expression system. SDS-PAGE.

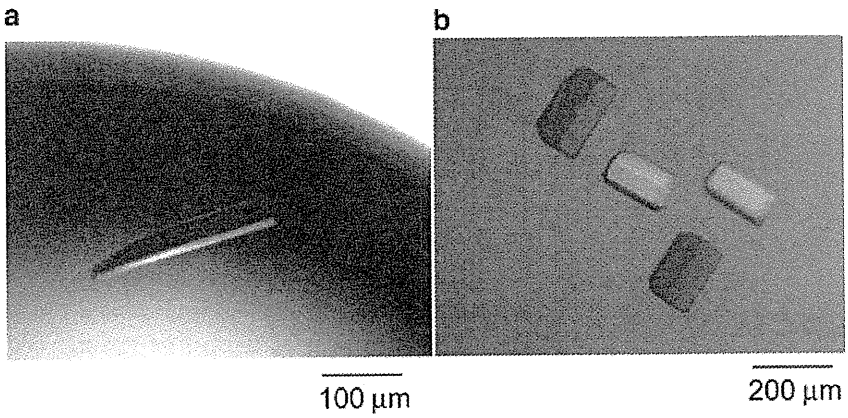

Fig 13.2. Crystals of PabI (**a**) and its seleomethionine-substituted version (**b**) prepared in a wheat-germ-based cell free expression system.

2. Materials

2.1. Production and Purification of the Native and Selenomethionine-Substituted Forms of the Protein

1. High-quality circular preparation of plasmid pEU-*pabIR*: the PabI gene (*pabIR*) is inserted into the multiple cloning site of pEU3b specialized for cell-free expression (10) prepared from *Escherichia coli* strain: JM109 {*recA1 endA1 gyrA96 thi hsdR17* (rK- mK+) e14 (*mcrA*) *supE44 relA1* Δ(*lac-proAB*)/[F' *traD36 proAB+ lacIq lacZ* ΔM15]} (11) (see Note 1).
2. Milli-Q water, freshly prepared.
3. 5× Transcription buffer (TB): 400 mM HEPES-KOH, pH 7.8, 80 mM magnesium acetate, 10 mM spermidine, and 50 mM DTT.
4. Nucleotide tri-phosphates (NTPs) mix: a solution containing 25 mM each of ATP, GTP, CTP, and UTP.
5. SP6 RNA polymerase (80 units/μL, Promega).
6. RNasin (80 units/μL, Promega).
7. Translational substrate buffer (TSB): 30 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM DTT, 0.3 mM amino acid mix (20 standard amino acids), 1.2 mM ATP, 0.25 mM GTP, and 16 mM creatine phosphate.
8. SeMet buffer: 30 mM HEPES-KOH, pH 7.8, 100 mM potassium acetate, 2.7 mM magnesium acetate, 0.4 mM spermidine, 2.5 mM DTT, 0.3 mM each of 20 standard amino acids except for methionine, 1.2 mM ATP, 0.25 mM GTP, 16 mM creatine phosphate, and 250 μM selenomethionine.
9. 6-Well plate (3.2 cm in diameter, Whatman Inc., Clifton, NJ).
10. Wheat embryo extract (240 OD/mL) or WEPRO®1240 (CellFree Sciences Co., Ltd.).
11. 20 mg/mL creatine kinase (Roche Diagnostics K. K.).
12. Sephadex G-25 (fine) column (Amersham Biosciences).
13. Heparin–sepharose purification buffer: 10 mM Tris–HCl pH7.5, 1 mM DTT, 0–2 M NaCl gradient.
14. ÄKTA purifier chromatography system (GE healthcare).
15. Protein storage buffer: 10 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 1 mM DTT.

2.2. Crystallization and Structure Determination

1. INTELLI-PLATEs and CrysChem plates for crystallization were purchased from Art Robbins Instruments and Hampton research, respectively.