Cell-free production and stable-isotope labeling of milligram quantities of proteins

Takanori Kigawa[1,a], Takashi Yabuki[a,b], Yasuhiko Yoshida[a,c], Michio Tsutsui[c], Yutaka Ito[d], Takehiko Shibata[d], Shigeyuki Yokoyama[a,b,*]

[a] *Cellular Signaling Laboratory, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*
[b] *Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[c] *Central Laboratory, Amersham Japan, 2802-1 Hiratsuka, Shiroi, Inba, Chiba 270-1402, Japan*
[d] *Cellular and Molecular Biology Laboratory, The Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan*

Received 22 October 1998; received in revised form 30 November 1998

Abstract We have improved the productivity of an *Escherichia coli* cell-free protein synthesis system. First, creatine phosphate and creatine kinase were used as the energy source regeneration system, and the other components of the reaction mixture were optimized. Second, the *E. coli* S30 cell extract was condensed by dialysis against a polyethylene glycol solution to increase the rate of synthesis. Third, during the protein synthesis, the reaction mixture was dialyzed against a low-molecular-weight substrate solution to prolong the reaction. Thus, the yield of chloramphenicol acetyltransferase was raised to 6 mg/ml of reaction mixture. Stable-isotope labeling of a protein with $^{13}C/^{15}N$-labeled amino acids for NMR spectroscopy was achieved by this method.

Key words: Cell-free protein synthesis; In vitro translation; Chloramphenicol acetyltransferase; Ras; Dialysis; Nuclear magnetic resonance

1. Introduction

Biochemical and biophysical studies of proteins usually require large-scale preparations of the proteins of interest. A number of unnatural amino acids have been incorporated site-specifically into proteins by the chemical acylation method [1]. Moreover, specifically stable-isotope-labeled proteins for Fourier transform infrared [2] and NMR [3,4] analyses can be produced. It is also expected that challenging proteins, such those prone to aggregation or with toxic properties, can be expressed by a cell-free system. Despite these merits, the low productivity of cell-free protein synthesis systems has limited their application. Many attempts to improve the productivity have been made, mainly for the *Escherichia coli* and wheat germ systems. First, the duration of the reaction has been increased by substrate supplementation during protein synthesis, by methods such as the continuous-flow method [5]. Recently, a semi-continuous flow system using a dialysis chamber [6] and a conventional dialysis system using a disposable dialyzer [7] have been reported. Second, the rate of protein synthesis has been increased by the use of condensed cell extract. The condensation was achieved by ultrafiltration [8,9] or polyethylene glycol (PEG) precipitation [10]. In addition to these improvements, optimization of the reaction conditions was reported [9,11,12]. Thus, it has been established that about 1 mg of protein can be produced per ml of *E. coli* cell-free reaction mixture [6].

In this study, we have improved the productivity of the *E. coli* coupled transcription-translation cell-free protein synthesis system. First, the energy source regeneration system was changed, and the other components were optimized. Second, the *E. coli* cell extract was condensed by dialysis against a PEG-containing solution. Third, this improved cell-free system was applied to protein synthesis with a disposable dialyzer [7]. As a result, about 6 mg of chloramphenicol acetyltransferase (CAT) protein was synthesized per ml of the reaction mixture in 21 h. Moreover, we applied the improved cell-free system with dialysis for the production of a $^{13}C/^{15}N$-labeled Ras protein for NMR spectroscopy, by using a labeled algal amino acid mixture, and successfully measured the HSQC spectrum.

2. Materials and methods

2.1. Template DNA for cell-free protein synthesis

Plasmids pK7-CAT [9] and pK7-Ras [3], which have the T7 promoter and the gene for the CAT protein and the human c-Ha-Ras protein, respectively, were used as the DNA templates. The Ras protein used in this study consisted of 171 amino acid residues, and lacked the C-terminal 18 amino acid residues, which is a better NMR sample than the full-length form [13–16]. The truncated Ras protein has been shown to have the same guanine-nucleotide binding and GTPase activities, and the same NMR chemical shifts and nuclear Overhauser effects for the corresponding residues, as the full-length Ras protein [13,14].

2.2. Reaction conditions for the batch system

The *E. coli* S30 cell extract used for the cell-free protein synthesis was prepared according to Pratt [17] from *E. coli* strain A19 (*metB*, *rna*). The T7 RNA polymerase was prepared according to Zawadzki and Gross [18]. Acetyl phosphate (AP) was purchased from Kohjin, and acetyl kinase (AK) was from Boehringer-Mannheim. The system used as the starting point of our study (the 'initial' system [3]) consisted of (per 15 μl) 55 mM HEPES-KOH (pH 7.5), 1.7 mM DTT, 1.2 mM ATP, 0.8 mM each of CTP, GTP, and UTP, 27 mM phospho*enol*pyruvate (PEP) (Boehringer-Mannheim), 2.0% polyethylene glycol (PEG) 8000 (Sigma), 0.64 mM 3′,5′-cyclic AMP, 68 μM L(−)-5-formyl-5,6,7,8-tetrahydrofolic acid, 175 μg/ml *E. coli* total tRNA (Boehringer-Mannheim), 210 mM potassium glutamate, 27.5 mM ammonium acetate, 13.3 mM magnesium acetate, 0.46 mM L-[^{14}C]leucine (267 MBq/mmol, Amersham), 0.5 mM of each of the

*Corresponding author. Fax: (81) (48) 462-4675.
E-mail: yokoyama@y-sun.biochem.s.u-tokyo.ac.jp

[1] The first two authors contributed equally to this work.

Abbreviations: AK, acetyl kinase; AP, acetyl phosphate; CAT, chloramphenicol acetyltransferase; CK, creatine kinase; CP, creatine phosphate; DTT, dithiothreitol; MWCO, molecular weight cut off; PEG, polyethylene glycol; PEP, phospho*enol*pyruvate; PK, pyruvate kinase

0014-5793/99/$19.00
PII: S0014-5793(98)01620-2

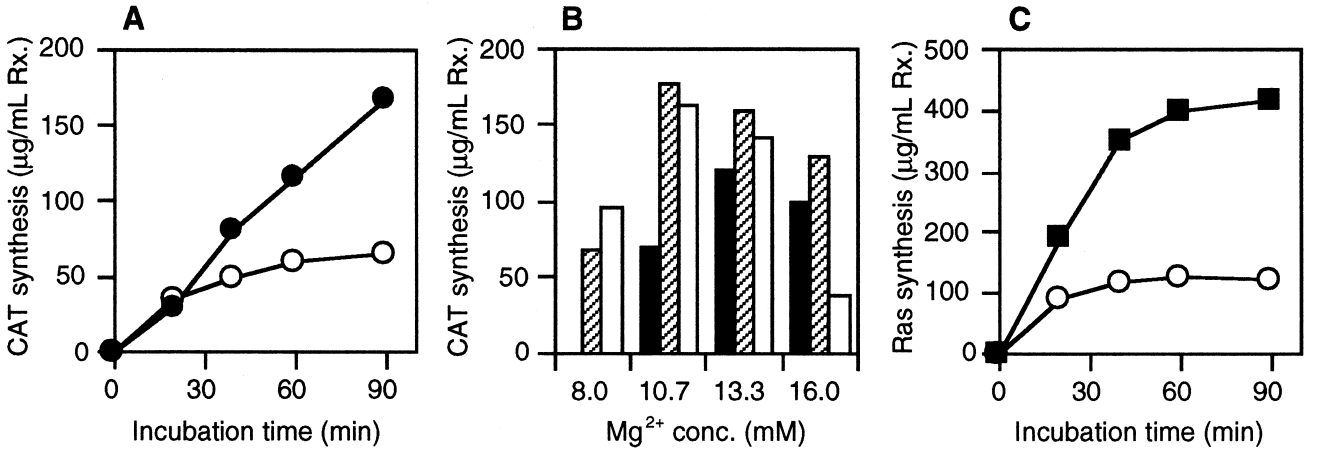

Fig. 1. Improvements of the *E. coli* cell-free system. A: Time courses of CAT synthesis using the system with PEP-PK (○) and the system with CP-CK (●). B: Dependence of CAT synthesis on PEG 8000 concentration (filled bars, 2.0%; hatched bars, 4.0%; open bars, 6.0%) at different magnesium ion concentrations. The incubation time was 1 h. C: Time courses of Ras synthesis by the 'initial' (○) and the 'improved' (■) cell-free systems.

other 19 amino acids, 6.7 μg/ml of either the pK7-Ras plasmid for Ras expression or the pK7-CAT plasmid for CAT expression, 93 μg/ml T7 RNA polymerase, and 3.6 μl S30 extract [3]. On the other hand, the 'improved' system consisted of (per 15 μl) 55 mM HEPES-KOH (pH 7.5), 1.7 mM DTT, 1.2 mM ATP, 0.8 mM each of CTP, GTP, and UTP, 80 mM creatine phosphate (CP) (Boehringer-Mannheim), 250 μg/ml creatine kinase (CK) (Boehringer-Mannheim), 4.0% PEG 8000, 0.64 mM 3′,5′-cyclic AMP, 68 μM L(−)-5-formyl-5,6,7,8-tetrahydrofolic acid, 175 μg/ml *E. coli* total tRNA, 210 mM potassium glutamate, 27.5 mM ammonium acetate, 10.7 mM magnesium acetate, 0.64 mM L-[^{14}C]leucine (193 MBq/mmol, Amersham), 1.0 mM of each of the other 19 amino acids, 6.7 μg/ml of either the pK7-Ras plasmid for Ras expression or the pK7-CAT plasmid for CAT expression, 93 μg/ml T7 RNA polymerase, and 4.5 μl S30 extract. The reaction mixture was incubated at 37°C for 1 h.

2.3. Condensation of S30 extract

The S30 cell extract was placed in a dialysis tube (Spectra/Por, molecular weight cut off (MWCO) 12 000–14 000), and was dialyzed against 10 volumes of an equal-weight mixture of PEG 8000 (Sigma) and the S30 dialysis buffer (10 mM Tris-acetate (pH 8.2), 14 mM magnesium acetate, 60 mM potassium acetate, and 1 mM DTT) in a Heat Seal Bag (Yamamoto) for 45 min at 4°C on a rotator. Then, the S30 extract was dialyzed against 100 volumes of the S30 dialysis buffer for 15 min at 4°C. Typically, the protein was condensed 2–2.5-fold.

2.4. Reaction conditions for the dialysis system

The reaction unit was constructed according to Davis et al. [7] using a DispoDialyzerCE (1 ml, MWCO 10 000 or 50 000, Spectra/Por). The internal solution (300 μl) consisted of the same components used for the improved batch system, 0.05% sodium azide, and 0.5 U/μl RNase Inhibitor (Toyobo). The external solution (3 ml) contained the components of the internal solution except for the creatine kinase, the plasmid vector, the T7 RNA polymerase, the S30 extract, and the RNase inhibitor, and also contained an additional 4.2 mM magnesium acetate corresponding to the magnesium carry over from the S30 extract. The reaction unit was incubated at 37°C with shaking at 160 rpm.

2.5. Assay for reaction products

The incorporation of L-[^{14}C]leucine into the Ras protein was determined by liquid scintillation counting of the trichloroacetic acid-insoluble material. The amount of CAT protein was determined by a colorimetric assay as described [19]. The reaction products were also analyzed by a modified SDS-PAGE [20,21].

2.6. Synthesis of $^{13}C/^{15}N$-labeled Ras protein and NMR analysis

The reaction was carried out using three units of the dialysis system (each unit contained 500 μl of the internal solution and 5 ml of the external solution) with the condensed S30 extract. The amino acids in the internal and external solutions were replaced by 3 mg/ml of the

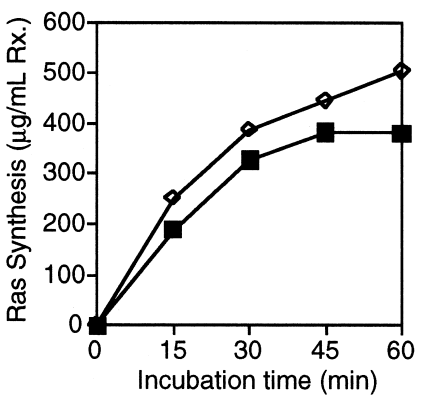

Fig. 2. Time courses of Ras synthesis by the 'improved' cell-free systems with original S30 cell extract (■) and the condensed S30 cell extract (◇).

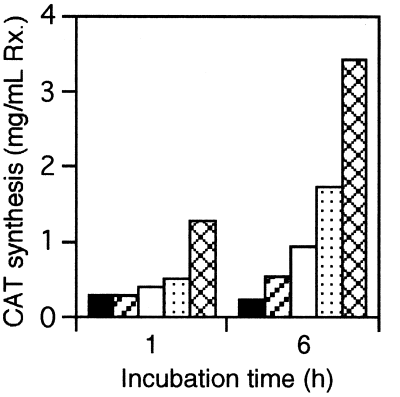

Fig. 3. Dependence of CAT synthesis on the concentration of the S30 extract and the MWCO of the dialysis membrane (filled bars, batch system with original S30; hatched bars, MWCO 10 kDa with original S30; open bars, MWCO 10 kDa with condensed S30; dotted bars, MWCO 50 kDa with original S30; checked bars, MWCO 50 kDa with condensed S30).

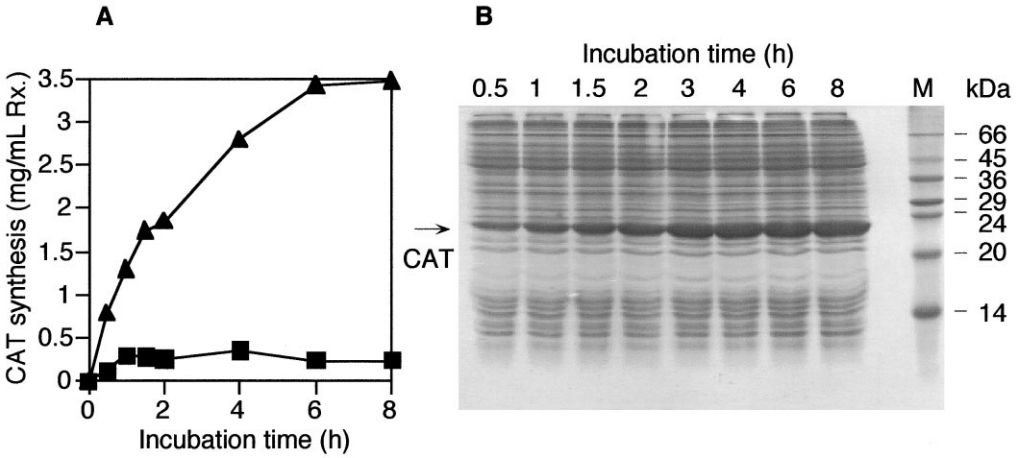

Fig. 4. A: Time courses of CAT synthesis by the 'improved' cell-free batch system (■) and the dialysis system with the condensed S30 cell extract (▲). B: SDS-PAGE analysis of the reaction products of the dialysis system with the condensed S30 cell extract (modified CBB staining [23]).

$^{13}C/^{15}N$-labeled algal amino acid mixture (Chlorella Industries Inc.) supplemented with 1 mM each of L-[^{13}C,^{15}N]phenylalanine and L-[^{13}C,^{15}N]arginine, and with 1 mM each of L-cysteine, L-glutamine, and L-asparagine. The reaction tubes were incubated at 37°C for 4 h. For comparison, the Ras protein was also synthesized in vivo in *E. coli* cells as described [16]. The two samples of the Ras protein were purified and sampled for NMR spectroscopy, and the HSQC spectra were recorded as described [3] except for the pH value (=6.5 in the present study). As the recombinant Ras protein synthesized in vivo retains the N-terminal Met residue, the present Ras protein synthesized in the cell-free system was shown to have the terminal Met residue by Edman sequencing (data not shown).

3. Results and discussion

3.1. Improvement of the productivity of the E. coli cell-free batch system

During the experiments to optimize the 'initial' system, we found that PEP, which was used to regenerate the energy sources, such as ATP and GTP, in the *E. coli* cell-free system, and/or a derivative of PEP, had an inhibitory effect on the system (data not shown). Therefore, instead of using the PEP-PK energy source regenerating system, we examined the AP-AK energy source regenerating system, which enhanced the cell-free protein synthesis over that with PEP-PK [11]. The system with AP-AK (40 mM and 870 μg/ml, respectively, were optimal) was twice as productive as the system with PEP-PK for CAT expression (data not shown). The CP-CK system, which is usually used in eukaryotic cell-free systems [22], was also examined. The cell-free system with CP-CK (80 mM and 250 μg/ml, respectively) gave maximum productivity, and was about 2.5-fold more productive than the system with PEP-PK for CAT expression (Fig. 1A). As the cell-free system with CP-CK was more productive than the system with AP-AK, CP-CK was used in the 'improved' system.

As previously demonstrated by our group [9], the optimal concentrations of PEG and magnesium ion were interdependent. Thus, these concentrations were co-optimized (4.0% PEG and 10.7 mM magnesium acetate were optimal), which increased the CAT expression by about 1.5-fold (Fig. 1B). Furthermore, the volume of the extract used for the cell-free reaction was increased from 3.6 μl/15 μl reaction to 4.5 μl/15 μl reaction, and the concentration of the amino acids was increased from 0.5 mM to 1 mM.

Finally, we compared the system with the newly developed conditions (the 'improved' system, see Section 2) to the system with the previously developed conditions (the 'initial' system) [3] for Ras expression. The 'improved' system was approximately 4-fold more productive than the 'initial' system, and could synthesize more than 0.4 mg of the Ras protein per ml reaction mixture (Fig. 1C).

3.2. Condensation of S30 extract and dialysis system

In our system, condensation with PEG precipitation [8] did not work well, because of the difficulty in dissolving the precipitate, while the reproducibility of condensation with ultrafiltration was not so amenable to being scaled up because of membrane clogging. Therefore, we adopted condensation by dialysis against a PEG-containing solution. This method is simple and can easily be scaled up. The condensed S30 extract appreciably increased both the initial rate of protein synthesis and the total amount of synthesized Ras protein (Fig. 2).

3.3. Dialysis system

By the use of the dialysis system, the amount of synthesized CAT protein was dramatically increased, and the productivity of the dialysis system was dependent on the concentration of the S30 extract and the MWCO of the dialysis membrane

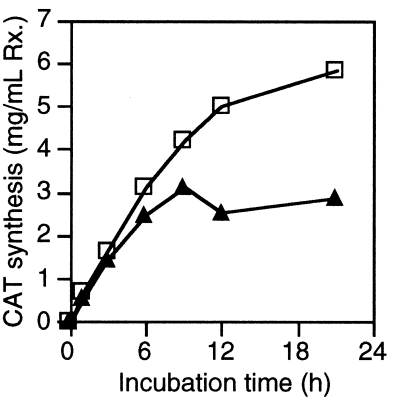

Fig. 5. Time courses of CAT synthesis by the dialysis system using the condensed S30 extract without an exchange of the external solution (▲) and with an exchange at 6 h (□).

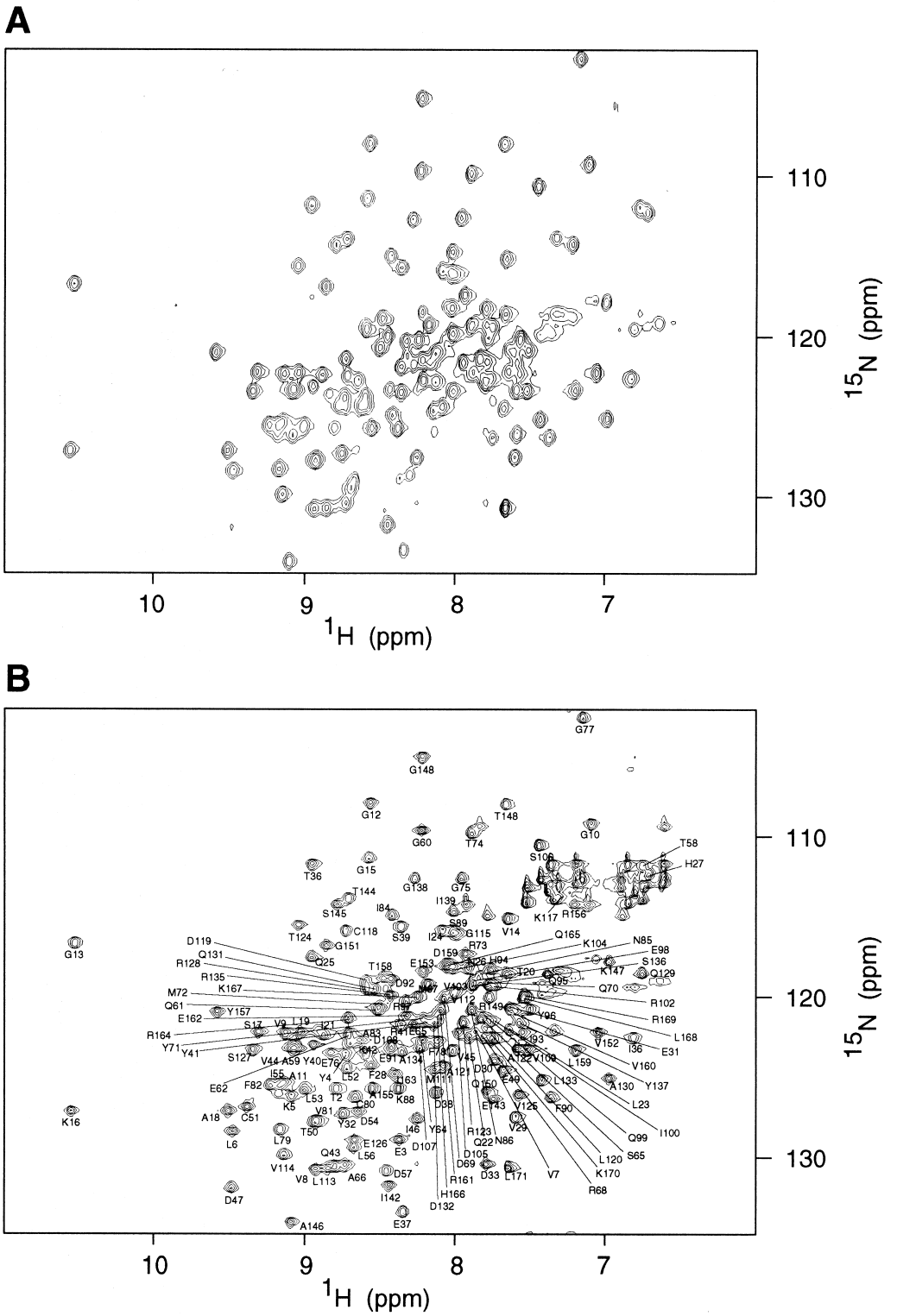

Fig. 6. The ^{15}N-HSQC spectra of the labeled Ras proteins. The backbone amide resonances and the folded sidechain amino resonances are observed. A: The Ras protein synthesized by the cell-free dialysis system with the [^{13}C,^{15}N]algal amino acid mixture. B: The Ras protein synthesized by conventional overexpression in vivo with ^{13}C-glucose and ^{15}N-ammonium chloride.

(Fig. 3). The condensed S30 extract increased the productivity of the CAT protein about 2-fold with 10 kDa and 50 kDa MWCO membrane after 6 h of incubation. The MWCO of the dialysis membrane strongly affected the production of the CAT protein. The amount of CAT protein synthesized with the 50 kDa MWCO membrane was about 3.5 times greater than that with the 10 kDa MWCO membrane, in both the original and condensed S30 extracts. With the 10 kDa MWCO membrane, the exchange of substrate seemed to be insufficient, due to membrane clogging. With the combination of the condensed S30 extract and the 50 kDa MWCO membrane, the concentration of the synthesized CAT protein

reached over 3 mg/ml in the reaction mixture. The translation kinetics of the dialysis system with the condensed S30 extract and 50 kDa MWCO membrane indicates that a high production rate was sustained for 6 h (Fig. 4A). The synthesized CAT protein was observed as a thick, major band in the SDS-PAGE analysis (Fig. 4B). The concentration of CAT protein in the external solution after 6 h of incubation was 16 μg/ml, and most of the CAT protein (MW 25 634) was retained in the internal solution, in spite of the 50 kDa MWCO. Finally, about 6 mg of the CAT protein was synthesized per ml of the reaction mixture in 21 h, by an exchange of the external solution (and supplementation of the plasmid vector and the T7 RNA polymerase) at 6 h (Fig. 5).

The amount of the CAT protein synthesized by the dialysis system with the condensed S30 extract and the 50 kDa MWCO membrane (6 mg/ml) was about 10 times larger than that with the batch system and with the 1 h incubation. The ratio of the product to the substrate in the dialysis system was comparable to that in the batch system. On the other hand, 1 μl of the original S30 extract (before condensation) produces about 1 μg of CAT protein in the batch system and about 10 μg in the dialysis system. The higher purity of the product in the reaction mixture with the dialysis system is advantageous for purification of the product protein.

The high productivity of our cell-free protein synthesis system is comparable to the productivity of the in vivo expression methods. Thus, cell-free protein synthesis will become a powerful protein production method for biochemistry and biophysics.

3.4. $^{13}C/^{15}N$-labeled Ras protein

In order to produce a $^{13}C/^{15}N$-labeled Ras protein by the cell-free system, we used $^{13}C/^{15}N$-labeled algal amino acid mixture instead of unlabeled amino acids in the reaction mixture. The algal amino acid mixture contained no cysteine, glutamine, asparagine, and tryptophan, and only small amounts of tyrosine, phenylalanine, and arginine. Therefore, these amino acids, except tryptophan, which is not contained in the Ras protein, were supplemented. Second, the potassium glutamate contained in the original reaction mixture was eliminated, because it interferes with the labeling of the glutamate residues of the Ras protein, and the other salt concentrations were optimized to eliminate the potassium glutamate. These modifications did not decrease the production of the Ras protein (data not shown).

The ^{15}N-HSQC spectrum of the labeled Ras protein synthesized in vitro was consistent with that of the uniformly labeled protein synthesized in vivo (Fig. 6). The ^{13}C-HSQC spectra were also measured successfully (data not shown). All of the observed backbone amide ^{15}N resonances for the amino acid residues, which had originally been labeled amino acids in the reaction mixture, were successfully assigned according to the resonance assignment for the uniformly labeled recombinant Ras (Fig. 6), indicating that the in vitro and in vivo preparations of the Ras protein have the same tertiary structure. As unlabeled L-glutamine, L-asparagine, and L-cysteine were used in the cell-free synthesis, the backbone amide resonances of the Asn and Cys residues and the side chain resonances of the Gln and Asn residues were missing, while the backbone amide resonances of the Gln residues were very weakly observed probably because of metabolic conversion of L-glutamate to L-glutamine (Fig. 6). In addition, the amide resonances of the Glu and Asp residues were somewhat weakened probably by metabolic dilution of stable isotopes. If all of the amino acids, including L-glutamine and L-asparagine, in the cell-free protein synthesis are labeled with stable isotopes, the Ras protein may be labeled uniformly. Therefore, cell-free protein synthesis will become a powerful protein production method for NMR spectroscopy.

Acknowledgements: We thank Drs. K. Takio and N. Dohmae (Division of Biomolecular Characterization, RIKEN, Japan) for N-terminal sequencing of Ras proteins. This work was supported in part by a grant for the Biodesign Research Program and a Special Grant for Promotion of Research, from the Institute of Physical and Chemical Research (RIKEN), Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (08260222, 08558076, 08780570), a Grant-in-Aid (Bio Media Program) from the Ministry of Agriculture, Forestry and Fisheries, Japan (BMP 97-V-4-2), and a Grant-in-Aid ('Research for the Future' Program) from the Japan Society for the Promotion of Science, Japan (JSPS-RFTF 96100305).

References

[1] Ellman, J., Mendel, D., Anthony-Cahill, S., Noren, C.J. and Schultz, P.G. (1991) Methods Enzymol. 202, 301–336.
[2] Sonar, S., Lee, C.P., Coleman, M., Patel, N., Liu, X., Marti, T., Khorana, H.G., RajBhandary, U.L. and Rothschild, K.J. (1994) Nature Struct. Biol. 1, 512–517.
[3] Kigawa, T., Muto, Y. and Yokoyama, S. (1995) J. Biomol. NMR 6, 129–134.
[4] Yabuki, T., Kigawa, T., Dohmae, N., Takio, K., Terada, T., Ito, Y., Laue, E.D., Cooper, J.A., Kainosho, M. and Yokoyama, S. (1998) J. Biomol. NMR 11, 295–306.
[5] Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S.Y. and Alakhov, Y.B. (1988) Science 242, 1162–1164.
[6] Kim, D.M. and Choi, C.Y. (1996) Biotechnol. Prog. 12, 645–649.
[7] Davis, J., Thompson, D. and Beckler, G.S. (1996) Promega Notes Mag. 56, 14–18.
[8] Nakano, H., Tanaka, T., Kawarasaki, Y. and Yamane, T. (1994) Biosci. Biotechnol. Biochem. 58, 631–634.
[9] Kim, D.M., Kigawa, T., Choi, C.Y. and Yokoyama, S. (1996) Eur. J. Biochem. 239, 881–886.
[10] Nakano, H., Tanaka, T., Kawarasaki, Y. and Yamane, T. (1996) J. Biotechnol. 46, 275–282.
[11] Ryabova, L.A., Vinokurov, L.M., Shekhovtsova, E.A., Alakhov, Y.B. and Spirin, A.S. (1995) Anal. Biochem. 226, 184–186.
[12] Kawarasaki, Y., Kawai, T., Nakano, H. and Yamane, T. (1995) Anal. Biochem. 226, 320–324.
[13] Ha, J.M., Ito, Y., Kawai, G., Miyazawa, T., Miura, K., Ohtsuka, E., Noguchi, S., Nishimura, S. and Yokoyama, S. (1989) Biochemistry 28, 8411–8416.
[14] Fujita-Yoshigaki, J., Ito, Y., Yamasaki, K., Muto, Y., Miyazawa, T., Nishimura, S. and Yokoyama, S. (1992) J. Protein Chem. 11, 731–739.
[15] Muto, Y., Yamasaki, K., Ito, Y., Yajima, S., Masaki, H., Uozumi, T., Walchli, M., Nishimura, S., Miyazawa, T. and Yokoyama, S. (1993) J. Biomol. NMR 3, 165–184.
[16] Ito, Y., Yamasaki, K., Iwahara, J., Terada, T., Kamiya, A., Shirouzu, M., Muto, Y., Kawai, G., Yokoyama, S., Laue, E.D., Walchli, M., Shibata, T., Nishimura, S. and Miyazawa, T. (1997) Biochemistry 36, 9109–9119.
[17] Pratt, J.M. (1984) in: (Hemes, B.D. and Higgins, S.J., Eds.), pp. 179–209, IRL Press, Oxford.
[18] Zawadzki, V. and Gross, H.J. (1991) Nucleic Acids Res. 19, 1948.
[19] Shaw, W.V. (1975) Methods Enzymol. 43, 737–755.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Odom, O.W., Kudlicki, W., Kramer, G. and Hardesty, B. (1997) Anal. Biochem. 245, 249–252.
[22] Pelham, H.R. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247–256.
[23] Choi, J., Yoon, S., Hong, H., Choi, D. and Yoo, G. (1996) Anal. Biochem. 236, 82–84.