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Cell-free protein synthesis at high temperatures using the lysate of a hyperthermophile

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Abstract

Systems for cell-free protein synthesis available today are usually based on the lysates of either *Escherichia coli*, wheat germ or rabbit reticulocyte, and protein synthesis reactions using these extracts are performed at moderate temperatures (20-40 ˚C). We report here the development of a novel system for cell-free protein synthesis that can be operated at high temperatures using a lysate of the hyperthermophilic archaeon, *Thermococcus kodakaraensis*. With the system, cell-free protein synthesis of ChiAΔ4, a derivative of *T. kodakaraensis* chitinase (ChiA), was observed within a temperature range of 40 ˚C to 80 ˚C, with an optimum at 65 ˚C. Corresponding chitinase activity was also detected in the reaction mixtures after cell-free protein synthesis, indicating that the synthesized ChiAΔ4 folded in a proper tertiary structure. The maximum concentration of active ChiAΔ4 synthesized was determined to be approximately 1.3 μg/mL. A time course experiment indicated that the amount of synthesized ChiAΔ4 saturated within 30 min at 65 ˚C, and energy depletion was suggested to be the main cause of this saturation. We further developed a system for transcription and translation-coupled protein synthesis at high temperatures using a combination of *T. kodakaraensis* lysate and thermostable T7 RNA polymerase.

Introduction

The production of recombinant proteins in appropriate host cells is now a routine alternative for studying the function and biophysical properties of a given protein. The variety of host cells available has expanded greatly in recent years, and ranges from the bacterial and archaeal prokaryotic cells to the higher eukaryotic cells. However, recombinant protein production in living-cells sometimes shares a common drawback when the target protein is toxic and/or incompatible with host cell growth. This often leads to growth retardation of the host strain, low protein yield, or destabilization of the expression vector (Marston 1986; Goff and Goldberg 1987; Chrunyk et al. 1993).

Cell-free protein synthesis is a method to synthesize proteins *in vitro* by using mRNA and the active translation machinery in the cell lysate (Matthaei and Nirenberg 1961; Dvorak et al. 1967). One of the advantages of this system is that one can utilize and develop the system focusing only on protein synthesis *per se*, and therefore, highly toxic proteins can readily be produced with *in vitro* systems (Henrich et al. 1982). Another major advantage is that these systems, with the properly charged tRNAs, allow the synthesis of proteins containing unnatural amino acids (Noren et al. 1989). Other notable features are the relatively short periods of time required for protein synthesis and the rather simple purification procedure following protein synthesis.

At present, there are three major sources of lysates utilized for cell-free protein synthesis: *Escherichia coli* (Spirin et al. 1988), rabbit reticulocyte (Hempel et al. 2001) and wheat germ (Endo and Sawasaki 2003). As these lysates originate from organisms living at moderate temperatures, protein synthesis reactions are performed in a temperature range between 20 ˚C and 40 ˚C. Although these systems can be presumed to be sufficient for producing a majority of mesophilic proteins, there are several reasons for one to explore the possibilities of protein synthesis at higher temperatures. A slight elevation in temperature, to an extent that it does not denature the target protein itself, will lead to more rapid protein synthesis. It has been reported that, by using capped mRNA, the reaction temperature of wheat germ extract could be increased up to 37 ˚C (from 20 ˚C), and an increased amount of protein synthesis was observed as a result of high speed protein synthesis (Tulin et al. 1995). In addition, elevated temperatures can be expected to prevent mRNA secondary structures that otherwise might be inhibitory in the translation reaction (Myers and Gelfand 1991).

In order to develop an *in vitro* translation system that functions and exhibits stability at elevated temperatures, the use of (hyper)thermophiles as a source of cell lysate is a practical choice. The *in vitro* incorporation of [^{35}S] methionine into proteins has previously been reported using the lysate of *Sulfolobus solfataricus* strain MT4 (Ruggero et al. 1993; Condo et al. 1999), suggesting that the lysates from hyperthermophiles have the potential to be utilized for *in vitro* translation systems. Besides the stability at moderately high temperatures (~50 ˚C), development of this type of system using the lysate of a hyperthermophile would greatly expand the temperature range at which cell-free protein synthesis can be performed. This should also make possible the production of highly thermostable proteins that cannot be properly folded at ambient temperature.

We report here the development of a system for cell-free protein synthesis using a lysate of *Thermococcus kodakaraensis*. *T. kodakaraensis* KOD1 is a hyperthermophilic archaeon isolated from a solfatara on Kodakara Island, Kagoshima, Japan (Morikawa et al. 1994; Atomi et al. 2004). The organism can grow between 60 and 100 ˚C with an optimal growth temperature of 85 ˚C. The broad temperature range at which this organism grows can be expected to provide an advantage in developing a cell-free system that can function at various extents of elevated temperature. In this study, we have performed an initial examination of various parameters and components that affect the rate and yield of protein synthesis, and with this system we have been able to observe the *in vitro* production of an active protein at temperatures between 40 and 80 ˚C.

Materials and methods

Chemicals - Sulfur, Tris-acetate, ammonium acetate, polyethyleneglycol 8000 and potassium phosphoenolpyruvate were purchased from Wako Pure Chemical Industries (Osaka, Japan). ATP, GTP, CTP and UTP were from Sigma (St. Louis, USA). RNase inhibitor was from Ambion (RNAsecure^{TM}, Texas, USA). All the other reagents were obtained form Nacalai Tesque (Kyoto, Japan).

Plasmids and mRNA preparation - The template DNA, pTRC1, used for preparing ChiAΔ4 mRNA, was constructed as follows. The XbaI site of pUC118 was removed with the Blunting High kit (Toyobo, Osaka, Japan) beforehand, and a BglII-EcoRI

fragment (150 bp) containing a T7 promoter was excised from pET-21a(+) (Novagen, Darmstadt, Germany) and inserted between BamHI and EcoRI sites of pUC118. The resulting plasmid was named pT1. A 45 bp-DNA fragment containing the ribosome-binding site of the *T. kodakaraensis* glutamate dehydrogenase gene (Rahman et al. 1998) was synthesized by a polymerase reaction using the following two primers: GDH-R (5'-AAAATCTAGACGCAGATTACCGAAATGAGGT-3', underlined sequences correspond to XbaI site) and GDH-F (5'-AAAACATATGTACCACCTCATTTCGGTAATCTGCG-3', underlined sequences correspond to NdeI site). The DNA fragment was treated with XbaI and NdeI and inserted into the respective sites of pT1, resulting in the plasmid pT2. A 1,283 bp-DNA fragment containing ChiAΔ4 gene was amplified with genomic DNA of *T. kodakaraensis* KOD1 by PCR using the following two primers, ChiA-Nd (5'-AAAACATATGCTTCCCGAGCACTTCTTCGCCC-3', underlined sequences correspond to NdeI site) and ChiA-T1 (5'-AAAAGAATTCTCCAATTTCATTATGGAC-3', underlined sequences correspond to EcoRI site). After treatment with NdeI and EcoRI, the amplified fragment was inserted into the respective sites of pT2, to make pTRC1 (Fig. 1). mRNA encoding ChiAΔ4 was prepared with the T7 RiboMAX^{TM} Express RNA system (Promega, Madison, USA) using pTRC1 as a template. The synthesized mRNA was suspended in RNase-free water and stored at -80 ˚C until use.

Construction of *T. kodakaraensis* Δ*chiA* strain - Disruption of *chiA* by double-crossover homologous recombination was performed using the technique developed for *T. kodakaraensis* as described previously (Sato et al. 2003; Sato et al. 2004; Sato et al. 2005). The vector used for disruption of *chiA* was constructed as follows. A DNA fragment containing the *chiA* coding region together with its flanking regions (about 1,000 bp) was amplified with the primer sets PCHI-R (5'-ACGAACCTTATTCCTTCTGCATAC-3') and PCHI-F (5'-GGTCAAACTGGAACTGCAACTGCC-3') using genomic DNA of *T. kodakaraensis* KOD1 as a template, and inserted into the HincII site of pUC118. Using the constructed plasmid DNA as a template, the flanking regions of *chiA* along with the plasmid backbone were amplified using primers PDCHIA-R (5'-ACAACACCCCTTGAGCTTTG-3') and PDHIA-F

(5'-TTCCCGAGCACTTCTTCGCCC-3'), and the amplified fragment was designated as L-ChiA. A PvuII-PvuII restriction fragment (763 bp) containing the *pyrF* marker gene was excised from pUD2 (Sato et al. 2005), and ligation was performed with L-ChiA to construct the plasmid for *chiA* disruption (pUChiA). A *T. kodakaraensis* uracil-auxotroph strain, KU216 (Sato et al. 2005), was used as a host cell for transformation, and $pyrF^{+}$ strain with uracil prototrophy was selected. Whether successful recombination had occurred was checked by PCR, and the constructed strain was named KC1.

Preparation of *T. kodakaraensis* S30 extract - *T. kodakaraensis* KC1 was precultured at 85 ˚C for 12 h in a nutrient-rich medium (MA-YT) (Kanai et al. 2005) containing 0.5% (w/v) elemental sulfur under anaerobic conditions. The preculture was used to inoculate 800 mL culture with MA-YT medium supplemented with 0.5% (w/v) sodium pyruvate. This was cultured under anaerobic conditions at 85 ˚C for about 14 h until A_{660} reached 0.6-0.7. Cells were harvested by centrifugation at 3,000 *g* for 15 min and washed two times with 0.8x Marine Art SF solution (Senju pharmaceuticals, Osaka, Japan) and once with S30 buffer (10 mM Tris-acetate pH 7.4, 1 mM dithiothreitol, 1.4 mM magnesium acetate, and 6.0 mM potassium acetate) supplemented with 5% (v/v) 2-mercaptoethanol.

Preparation of S30 extract was performed by a modification of the Pratt method (Pratt 1984) under RNase-free conditions. Cells were suspended in S30 buffer (1.27 mL per gram of wet cells) and disrupted with French Press (FA-003, Thermo Electron Co., Massachusetts, USA) with a pressure of 10,000 psi. Dithiothreitol was added to the resulting lysate to a final concentration of 1 mM. The lysate was then centrifuged at 30,000 *g* at 4 ˚C. The upper four-fifths of the supernatant was collected, and a second 30,000 *g* centrifugation was repeated, again collecting only the upper four-fifths of the supernatant. For each 1 ml of the supernatant collected, 0.3 mL of pre-incubation mixture (300 mM Tris-acetate pH 7.4, 9.3 mM magnesium acetate, 13 mM ATP pH 7.0, 84 mM potassium phosphoenolpyruvate, 0.4 mM dithiothreitol, 1 mM each of 20 amino acids, and 10 units/mL of pyruvate kinase from rabbit muscle (Sigma)) was added, and the mixture was incubated for 80 min at 37 ˚C. The mixture was then dialyzed three times (45 min each) against 40 times volume of S30 buffer using 5,000 MWCO dialysis tubes. After centrifugation at 4,000 *g* for 10 min, the resulting supernatant was used as

the S30 extract. Protein concentration was determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, USA) with bovine serum albumin as a standard. The S30 extract was stored at -80 ˚C until use.

Cell-free protein synthesis - Cell-free protein synthesis was performed in a 30 μL batch reaction containing ChiAΔ4 mRNA, *T. kodakaraensis* S30 extract (8.0 mg/mL, final concentration) and other ingredients shown in the "initial condition" column of Table 1. The reaction was incubated for 90 min at 48 ˚C and terminated by chilling the reaction on ice. In optimizing the cell-free protein synthesis reaction, the ChiAΔ4 mRNA concentration was first varied (0, 0.2, 0.3 or 0.4 mg/mL). Next, with 0.4 mg/mL mRNA added, the concentration of each component was changed within the ranges shown below: 0, 5, 10, 15, 20 or 25 mM for magnesium acetate; 0, 100, 400 or 700 mM for potassium acetate; 0 or 80mM for ammonium acetate; 0 or 56 mM for Tris-acetate (pH 7.4); 0, 1.2 or 2.4 mM for ATP; 0 or 0.85 mM (each) for GTP, CTP and UTP mixture (GCU mix); 0, 30 or 60 mM for potassium phosphoenolpyruvate; 0, 2.5, 5% (w/v) for polyethyleneglycol 8000 (PEG8000); 0, 2 or 4 mM (each) for mixture containing 20 amino acids (20AA mix). With the optimized reaction composition summarized in Table 1, the reaction temperature was examined between 30 ˚C and 80 ˚C. The addition of 0.165 mg/mL *T. kodakaraensis* tRNA prepared with the Nucleobond AX kit (Genetics, Düren, Germany) was also tested. The degree of cell-free protein synthesis in the reactions at 60 ˚C, 65 ˚C, 70 ˚C and 80 ˚C were examined for various time periods between 0 and 120 min. Also, the effect of adding each of the following reagents to the reaction after 45 min at 65 ˚C was tested: 12 μg mRNA, 36 nmol ATP, and 2.0 μmol phosphoenolpyruvate.

Transcription-translation coupled reactions were performed with a 30 μL reaction volume containing 2.0 μg of pTRC1, 0-1500 units of Thermo T7 RNA polymerase (Toyobo), *T. kodakaraensis* S30 extract (8.0 mg/mL, final concentration) as well as the following ingredients: 56 mM Tris-acetate (pH 7.4), 7.5 mM magnesium acetate, 80 mM ammonium acetate, 100 mM potassium acetate, 1.2 mM ATP, 0.85 mM each of GTP, CTP, and UTP, 30 mM potassium phosphoenolpyruvate, 2.0 mM each of 20 amino acids, 5 % (w/v) polyethyleneglycol 8000. The reaction was performed at 40 ˚C for the first 60 min, and continued at 60 ˚C for another 90 min.

Western blot analysis – After incubation, the reaction mixture was subjected to sodium

dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5 % acrylamide concentration) followed by blotting to a polyvinylidene fluoride membrane (Hybond^{TM}-P, Amersham Biosciences, Buckinghamshire, UK). Rabbit anti-ChiAΔ4 antiserum was used as the first antibody (1:100,000 dilution), and HRP-rec-Protein G (Zymed Laboratories, San Francisco, USA) was used as the second antibody (1:100,000 dilution). For detection, the ECL Advance^{TM} Western Blotting Detection System (Amersham Biosciences), Hyperfilm^{TM} (Amersham Biosciences) and Lumi vision PRO 400EX (AISIN, Aichi, Japan) were used.

Enzyme assay – A chitinase activity assay was performed according to the procedure described previously (Tanaka et al. 1999) using a fluorometric substrate, 4-methylumbelliferyl β–D-*N*,*N*'-diacetyl chitobioside (Sigma). After a 30 min reaction at 90 ˚C, the fluorescence of liberated 4-methylumbelliferone was measured (365 nm excitation, 460 nm emission) with a NanoDrop ND-3300 Fluorospectrometer (NanoDrop Technologies, Wilmington, USA). Amount of active ChiAΔ4 synthesized was calculated using the specific activity of purified ChiAΔ4 (0.135 nmol min^{-1} μg^{-1}).

Results

Selection of the target protein

ChiAΔ4, a truncated form of *T. kodakaraensis* chitinase (Tanaka et al. 1999), was selected as the target protein to be synthesized in the *T. kodakaraensis* cell-free translation system. Chitinase from *T. kodakaraensis* (ChiA) contains two catalytic domains (Tanaka et al. 1999; Tanaka et al. 2001). ChiAΔ4 (33.8 kDa) is a ChiA derivative containing only the C-terminal endochitinase domain. As ChiAΔ4 is a highly thermostable enzyme with a half life of over 3 h at 100 ˚C (Tanaka et al. 1999), the effect of heat on protein denaturation during cell-free protein synthesis is negligible. Moreover, as ChiAΔ4 is a protein originating from *T. kodakaraensis*, there is no need for concern with codon preference during protein synthesis. In order to remove any possible effects of the native chitinase present in the cell-free extract, a *chiA*-disrupted strain of *T. kodakaraensis* was constructed and used for preparation of the cell lysate (S30 extract).

Cell-free protein synthesis at high temperatures

In the preparation of the S30 extract of *T. kodakaraensis*, we employed the protocol of

Pratt for the preparation of *E. coli* lysate (Pratt 1984), with some modifications. A comparison of the amounts of protein synthesized with different concentrations of ChiAΔ4-encoding mRNA is shown in Figure 2. Western blot analysis revealed that ChiAΔ4 was synthesized only in reactions where exogenous mRNA was added, and that there was a clear correlation between the amount of mRNA added and that of ChiAΔ4 synthesized (Fig. 2).

Optimization of the reaction mixture composition

It is well known that protein synthesis in cell-free systems is largely affected by the concentration of reaction constituents (Tarui et al. 2001). We therefore examined the optimum concentration of each reaction component within the ranges shown in Table 1. In addition to mRNA, S30 extract and the 20 amino acids mixture, we found that magnesium ions and phosphoenolpyruvate were necessary for protein synthesis. Furthermore, the presence of either ATP or GCU mix was also found to be necessary for ChiAΔ4 synthesis. The optimized mixture composition was determined to be as follows: 5-10 mM magnesium acetate, 100 mM potassium acetate, 80 mM ammonium acetate, 56 mM Tris-acetate pH 7.4, 1.2 mM ATP, 0.85 mM (each) of GCU mix, 30 mM potassium phosphoenolpyruvate, 5 %(w/v) polyethyleneglycol 8000 and 2.0 mM (each) of the 20 amino acids. The addition of *T. kodakaraensis* tRNA was also tested, but there was no enhancement of ChiAΔ4 protein synthesis (data not shown).

Temperature preference

Next, the effects of temperature on the system were examined. Western blot analysis revealed the synthesis of ChiAΔ4 within a temperature range from 40 ˚C to 75 ˚C, with a maximum at 65 ˚C (Fig. 3A). To examine whether the ChiAΔ4 protein was synthesized in an active form, the chitinase activity in the reaction mixture was measured. Significant levels of activity were detected within a temperature range of 40 ˚C to 80 ˚C, and highest activity was observed at 65 ˚C (Fig. 3B). This activity profile was consistent with the results of Western blot analysis, indicating that the ChiAΔ4 protein was most likely synthesized with the proper tertiary structure. Using the specific activity value of purified ChiAΔ4 expressed in *E. coli*, the maximum yield of active ChiAΔ4 was estimated to be approximately 1.3 μg/mL (at 65 ˚C).

Time course of protein synthesis and determination of rate-limiting factors

A time course experiment to monitor ChiAΔ4 protein levels showed that, at 65 ˚C,

synthesis of ChiAΔ4 saturated at approximately 30 min (Fig. 4). At 70 ˚C, a rapid accumulation of ChiAΔ4 was observed in the first 5 min, and neared saturation at 15 min. A slower accumulation of ChiAΔ4 was observed at 60 ˚C, with protein synthesis continuing for over 60 min. On the other hand, no significant accumulation of ChiAΔ4 could be observed at 80 ˚C.

We examined the cause for the short duration of the reaction by performing semibatch reactions at the optimum temperature. After reactions were carried out for 45 min, mRNA, ATP and phosphoenolpyruvate were added individually. Figure 5 shows a time course of the accumulation of ChiAΔ4 protein in each reaction mixture. The addition of ATP and phosphoenolpyruvate had similar effects; the amount of ChiAΔ4 increased in the first 30 min and then leveled off. The addition of mRNA did not lead to enhanced synthesis. From this result, it appears that energy depletion is the main cause of the saturation in protein synthesis.

Transcription and translation-coupled protein synthesis

Using the *T. kodakaraensis* S30 extract, we also developed a coupled reaction system for cell-free transcription and translation at high temperatures. Instead of mRNA, the reaction mixture contained pTRC1 (Fig. 1) as a template DNA harboring a ChiAΔ4-encoding gene under the control of the T7 promoter, and thermostable T7 RNA polymerase. The reaction mixture was incubated at 40 ˚C for 1 h (for transcription), and then the temperature was shifted to 60 ˚C and incubation was continued for another 90 min (for translation). Using the two-step reaction, synthesis of active ChiAΔ4 could be detected. Increasing the levels of T7 RNA polymerase until 750 units led to higher amounts of synthesized protein, but further addition of the enzyme had a slightly negative effect (Fig. 6).

Discussion

The present study reports the development of a system for cell-free protein synthesis at high temperatures using *T. kodakaraensis* S30 extract. Synthesis of ChiAΔ4 was detected by Western blot analysis in a temperature range between 40 and 75 ˚C (Fig. 3A). ChiAΔ4 could not be detected by Western blot analysis at 80 ˚C, while chitinase activity at 80 ˚C was almost the same as that detected at 40 ˚C (Fig. 3B). The activity observed at 80 ˚C may be due to degradation products of ChiAΔ4 that still maintained

enzymatic activity.

There was a significant difference between the optimal temperature of cell-free protein synthesis (65 ˚C) and the optimal growth temperature of *T. kodakaraensis* (85 ˚C). Further experiments are necessary to clearly explain the difference, but the formation of precipitate after incubation of reaction mixture at 85 ˚C may indicate that proteinous components in the S30 extract are undergoing thermal degradation *in vitro*. In living-cells, induction of the chaperon system protects cellular proteins from thermal denaturation (Ideno et al. 2002). It has been reported that low molecular substances such as trehalose have the ability to stabilize proteins in high temperature environments (Carninci et al. 1998). Therefore, addition of such compounds to the reaction mixture might help to increase the optimum reaction temperature.

In this study, we initially employed the reaction conditions and methods of Ellman et al. (Ellman et al. 1991) for cell-free protein synthesis. As we could not detect protein synthesis, the method of Pratt (Pratt 1984) was applied with some modifications, leading to favorable results. This is most likely due to the lower concentration of amino acids in the former system (0.35 mM for each amino acid) than in the latter system (2.0 mM). Indeed, we observed a drastic decrease in protein production levels in our optimized system when amino acid concentrations were decreased (data not shown). On the other hand, several compounds in the Ellman method that were not present in the Pratt method (folic acid, pyridoxine hydrochloride, $NADP^+$, FAD, *p*-aminobenzonic acid and calcium ion) may have had an inhibitory effect against protein synthesis in our *T. kodakaraensis*-based system.

In the *T. kodakaraensis* system, ChiAΔ4 synthesis nearly saturated within 30 min of incubation at 65 ˚C, and the highest concentration of protein obtained was approximately 1.3 μg/mL. When compared with other systems, 100-fold higher protein concentrations have been obtained in 60 min using the optimized *E. coli* system (Kim and Swartz 1999; Kim and Choi 2000). Therefore, the speed of protein synthesis in the *T. kodakaraensis* system is, at least at present, considerably lower than we had expected. Since the addition of energy-supplying substrates supported further synthesis of ChiAΔ4, energy depletion can be regarded as the main cause of the arrest in protein synthesis. In general, high-energy compounds are unstable at high temperatures. The half-lives of ATP and phosphoenolpyruvate at high temperatures have been reported;

the half-life of phosphoenolpyruvate is 20 min at 70 ˚C (Schramm et al. 2000), while that of ATP is 115 min at 90 ˚C (in buffer containing Mg^{2+}) (Kengen et al. 1996). It can be reasonably presumed that the half-lives of these compounds are even shorter in cell lysate, as reported in the *E. coli* system (Kim and Swartz 1999). A simple increase in the initial concentration of phosphoenolpyruvate (from 33 mM to 66 mM) was not effective, and rather had an inhibitory effect, probably due to the excess accumulation of inorganic phosphate (Kim and Swartz 1999). Developing a means to provide a stable supply of energy will be a key factor in increasing the production rate and overall yield of the system.

The use of hyperthermophiles as a source of cell lysate has various advantages. As we have observed protein synthesis at temperatures as low as 40 ˚C, further optimization of the reaction conditions may allow application of the system for the synthesis of mesophilic proteins. A moderate elevation in temperature should lead to an increase in the production rate and may also provide an advantage in preventing inhibitory mRNA secondary structures. On the other hand, the system can also be utilized at higher temperatures ranging from 50 to 75 ˚C. This will provide a means to produce proteins from (hyper)thermophiles at temperatures near the native environment. When proteins from (hyper)thermophiles are synthesized in mesophiles, they are in many cases produced in a “semi-mature” form, exhibiting lower activity than that of the native protein. This is thought to be due to the entrapment of the protein molecule in an intermediary state of the folding process at low temperatures. This may be one of the main reasons why a number of proteins deriving from hyperthermophiles cannot be expressed in an active form in mesophilic hosts (Abd Rahman et al. 1997). The system developed in this study may provide an alternative in synthesizing these proteins in an active form. Hyperthermophilic proteins synthesized in mesophilic hosts can be brought to their optimal, fully active states by incubating them at high temperature (Abd Rahman et al. 1997). Recombinant ChiAΔ4 synthesized in *E. coli* is one example, and the specific activity of the protein increases after an incubation of 10 min at 90 ˚C (data not shown). In contrast, we observed that the specific activity of the ChiAΔ4 synthesized by the *in vitro* system at 65 ˚C did not change after heat treatment, indicating that the protein was produced in the optimal, thermostable form (data not shown).

We also examined the possibilities of coupling a T7 RNA polymerase-dependent transcription reaction with our translation reaction with the *T. kodakaraensis* S30 extract. Initial attempts with a single reaction temperature did not lead to ChiAΔ4 synthesis at 40 ˚C, 50 ˚C or 60 ˚C (data not shown). This was most likely due to the difference in the optimum temperatures between the transcription and translation reactions: the temperature optimum of the *T. kodakaraensis* cell-free translation reaction is 65 ˚C, whereas the T7 RNA polymerase used has an optimum temperature of 50 ˚C, with only negligible activity above 55 ˚C. By utilizing a two-step system (reaction temperature was set at 40 ˚C and later changed to 60 ˚C), production of ChiAΔ4 was observed, and the optimal amount of T7 RNA polymerase was 750 units (in 30 μL of reaction mixture). The decrease in ChiAΔ4 synthesis with greater amounts of T7 RNA polymerase may be due to excess consumption of ATP in RNA synthesis, resulting in a shortage of ATP to be used for translation. Indeed, when we decreased the amount of template DNA (0.6 μg), the apparent optimal amount of T7 RNA polymerase increased to 1250 units (unpublished data). As DNA is much more stable than RNA and can be readily amplified by PCR, optimization of this coupled system will be an important subject to address in future studies.

Among hyperthermophiles, *T. kodakaraensis* is one of the few microorganisms for which the entire genome sequence (Fukui et al. 2005) and genetic transformation technology (Sato et al. 2003; Sato et al. 2005) are both available. This unique feature of *T. kodakaraensis* will enable us to undertake molecular alteration of the species by removing genes encoding proteins that are disadvantageous for cell-free protein synthesis or by overexpressing genes that are favorable for the reaction.

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Figure legend

Fig. 1 Schematic drawing of pTRC1 for preparation of ChiAΔ4 mRNA and for the transcription and translation-coupled reaction. pTRC1 was used as a template for these reactions after treatment with EcoRI.

Fig. 2 Cell-free protein synthesis using *T. kodakaraensis* S30 extract. Reaction mixtures containing 0.2 mg/mL of ChiAΔ4 mRNA (lane 1); 0.3 mg/mL of ChiAΔ4 mRNA (lane 2); 0.4 mg/mL of ChiAΔ4 mRNA (lane 3); in the absence of mRNA (lane 4, negative control) were used. Compositions of the reaction mixtures are indicated in Table 1. Reaction were performed at 48 ˚C for 90 min and ChiAΔ4 was visualized by rabbit anti-ChiAΔ4 antibodies.

Fig. 3 Effect of temperature on cell-free protein synthesis with *T. kodakaraensis* S30 extract. (A) Each reaction mixture containing 0.4 mg/mL of ChiAΔ4 mRNA was incubated at 30, 40, 50, 60, 65, 70, 75 or 80 ˚C for 90 min (lanes 1-8, respectively). A negative control reaction was performed at 60 ˚C in the absence of mRNA (lane 9). ChiAΔ4 was visualized by Western blot analysis using rabbit anti-ChiAΔ4 antibodies. (B) Chitinase activity at various temperatures. Activity measurements were performed with and without the addition of substrate at each temperature, and the difference in values was calculated. Results are the average of n = 3 reactions and error bars represent standard deviations. (C) Protein samples visualized with Coomassie Brilliant Blue (2 μL of each reaction mixture). Lane numbers are the same as in (A). Lane C represents 1 μg of ChiAΔ4.

Fig. 4 Time course of cell-free protein synthesis with *T. kodakaraensis* S30 extract. Reactions were performed at 60 ˚C (circles), 65 ˚C (squares), 70 ˚C (diamonds) and 80 ˚C (triangles). Aliquots of sample were taken at 5, 15, 30, 60, 90 and 120 min after the initiation of the reaction and enzyme activities were measured. Results are the average

of n = 3 reactions and error bars represent standard deviations.

Fig. 5 Determination of rate-limiting factors. Reactions were performed in a total volume of 30 μl at 65˚C for 45 min, and then one of the following components were added, none (diamonds), phosphoenolpyruvate (2.0 μmol, circles), ATP (36 nmol, squares) or ChiAΔ4 mRNA (12 μg, triangles), and incubation was continued for a further 45 min. Results are the average of n = 3 reactions and error bars represent standard deviations.

Fig. 6 Transcription and translation-coupled reaction. Reaction mixtures containing 2.0 μg of pTRC1 were first incubated at 40 ˚C for 60 min. Temperature was then shifted to 60 ˚C, and the reaction was further continued for 90 min. Activity measurements were performed with and without the addition of T7 RNA polymerase, and the difference in values was calculated. Results are the average of n = 3 reactions and error bars represent standard deviations.

Table 1. Composition of reaction mixture

Component	Unit	Initial condition [*1]	Optimized condition [*2]	Necessity
$Mg(OAc)_2$ [*3]	mM	16	5-10 (0–25)	Yes
K(OAc) [*3]	mM	230	100 (0–700)	No
$NH_4(OAc)$ [*3]	mM	80	80 (0, 80)	No
Tris-acetate (pH 7.4)	mM	56	56 (0, 56)	No
ATP	mM	1.2	1.2 (0–2.4)	No [*8]
GCU mix [*4]	mM (each)	0.85	0.85 (0, 0.85)	No [*8]
PEP [*5]	mM	30	30 (0–60)	Yes
PEG8000 [*6]	% (w/v)	2.0	5.0 (0–5.0)	No
20AA mix [*7]	mM (each)	2.0	2.0 (0-4.0)	Yes
mRNA	mg/ml	0–0.4	0.4 (0–0.4)	Yes
S30 extract	mg/ml	8	8	Yes

[*1] Result is shown in Fig. 2.

[*2] The range over which reactant concentrations were optimized is shown in parenthesis and more precisely in Materials and Methods.

[*3] OAc = acetate

[*4] GTP, CTP and UTP mixture

[*5] Phosphoenolpyruvate

[*6] Polyethyleneglycol 8000

[*7] Mixture containing 20 amino acids

[*8] The presence of either component is necessary

Figure(s)

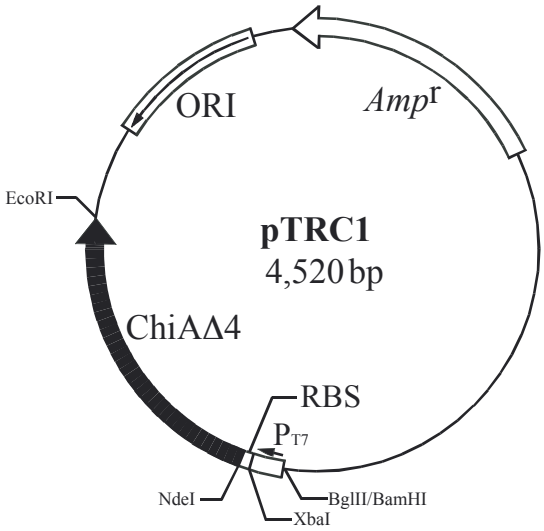


Fig.1 Endoh *et al.*

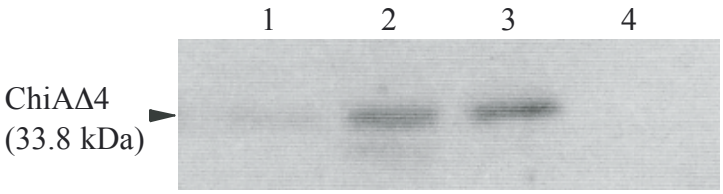


Fig.2 Endoh *et al.*

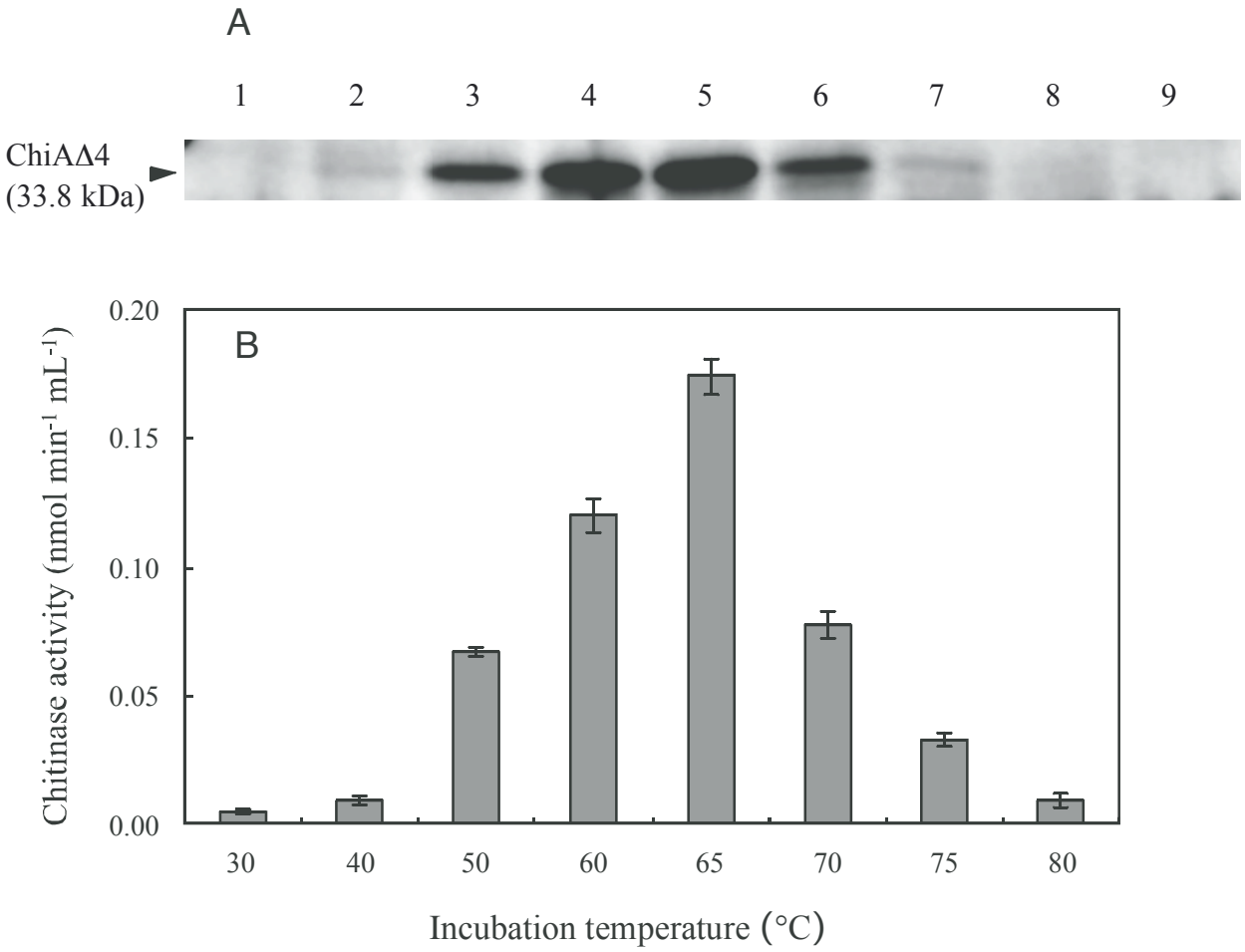


Fig.3 Endoh *et al.*

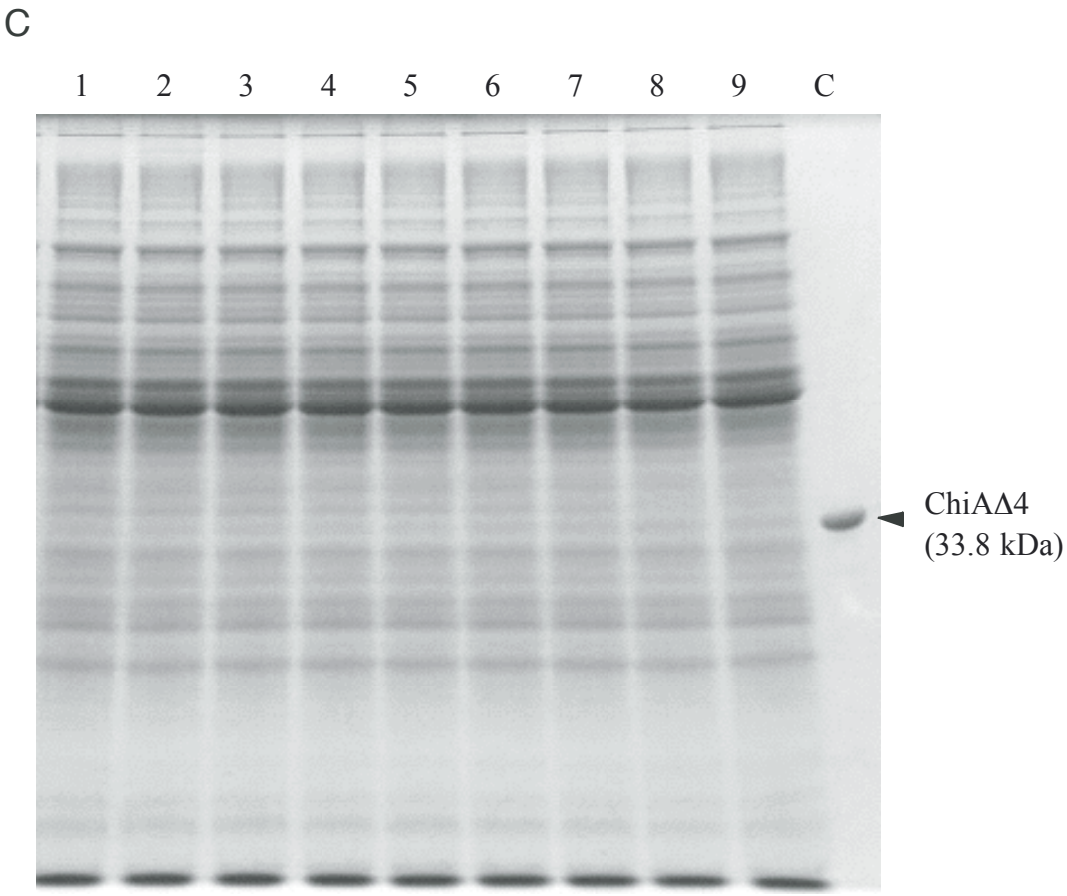
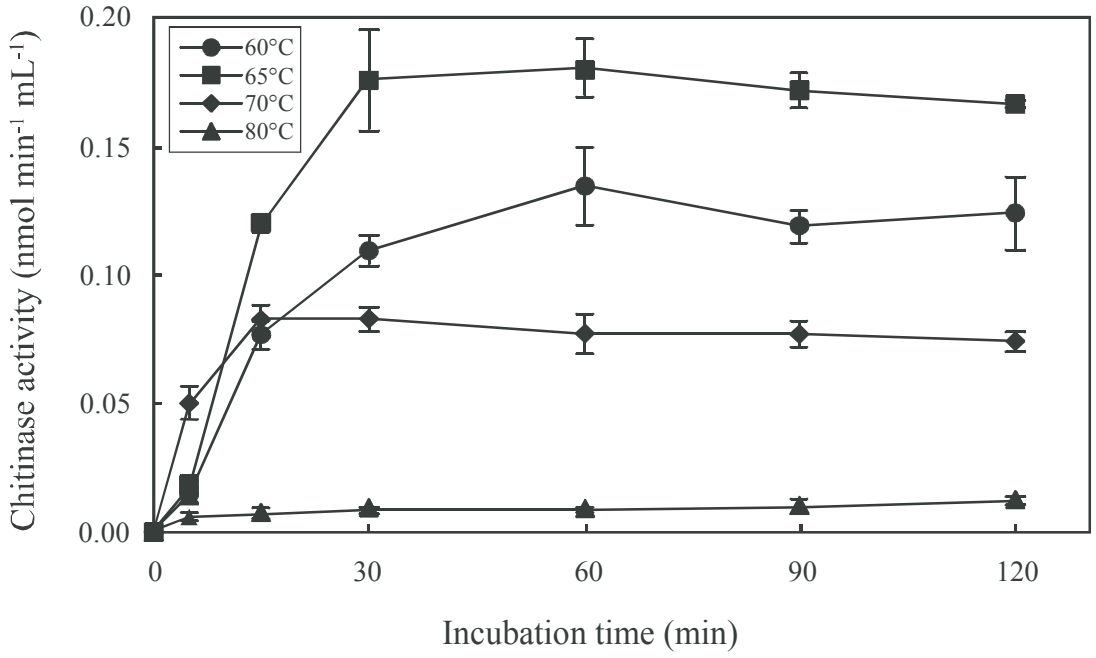


Fig.3 Endoh *et al.*



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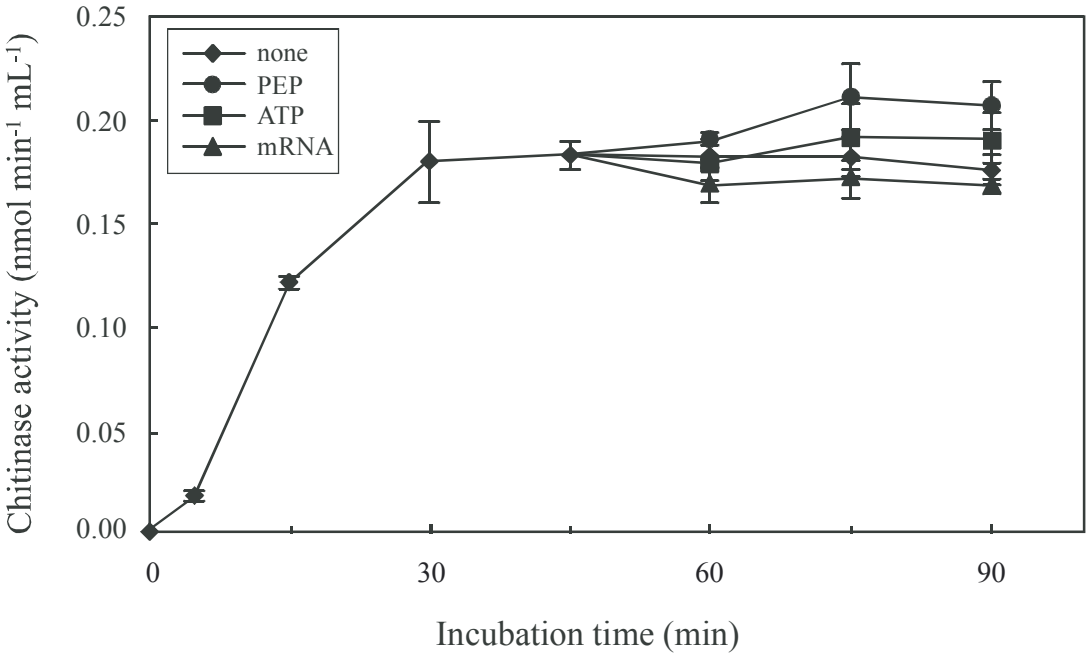

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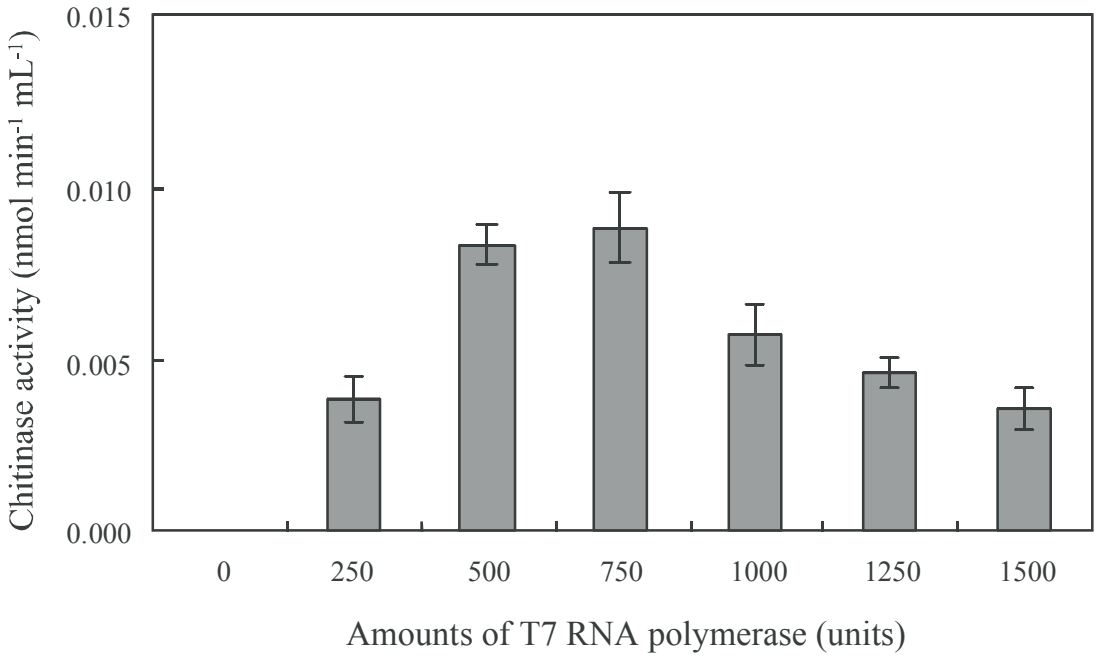

Fig.6 Endoh *et al.*