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PRODUCTION OF ACTIVE HUMAN INTERFERON-β IN *E. coli*

I. PREFERENTIAL PRODUCTION BY LOWER CULTURE TEMPERATURE

TAMIO MIZUKAMI*, YOSHINORI KOMATSU, NORIKO HOSOI,
SEIGA ITOH, and TETSUO OKA

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
3-6-6 Asahimachi, Machida-shi, Tokyo 194, Japan

SUMMARY

Unusually low culture temperature, such as 20°C, was shown to be preferable for the synthesis of active human interferon-β (IFN-β) in *E. coli* harboring a recombinant plasmid. The *E. coli* cells cultured at 20°C gave 8.6-fold higher IFN-β activity than those cultured at 37°C. However, almost the equal amounts of IFN-β protein were accumulated in both cells cultured at 20°C and at temperature higher than 20°C, suggesting that IFN-β might exist as an active form in the cells cultured at 20°C, while as a rather denatured form in the cells cultured at higher temperature.

INTRODUCTION

The hyperproduction of many useful eukaryotic proteins in *E. coli* has been made possible through the use of recombinant DNA technology. However, it seems to be difficult to produce active molecules of certain eukaryotic proteins, such as immunoglobulins, in *E. coli* because of errors during the process of protein folding (Cabilly et al., 1984). For example, Mark et al. reported that the human IFN-β protein purified from extracts of *E. coli* has lower antiviral specific activity, about 1/10th that of the native glycosylated protein, because the IFN-β protein could not fold correctly into the native conformation in *E. coli* (Mark et al., 1984).

We previously constructed recombinant plasmids which directed the synthesis of a mature human IFN-β under the control of the *E. coli* tryptophan promoter, and achieved the high level expression of human IFN-β in *E. coli* (Itoh et al., 1984). In this report, we describe the effect of culture temperature on the production of active human IFN-β in the *E. coli* cells harboring the plasmid (pMG-1) constructed previously (Itoh et al., 1984). In the cells cultured at lower temperature, such as 20°C, IFN-β seems to exist as an active form, while as a rather denatured form in the cells cultured at higher temperature.

MATERIALS AND METHODS

A bacterial strain. An E. coli K-12 strain HB101 harboring pMG-1 (HB101/pMG-1) (Itoh et al., 1984) was used for the production of human IFN-β.

Culture conditions. The strain HB101/pMG-1 was cultured in a test tube (20 mm x 200 mm) or a 2-liter jar fermenter (Mitsuwa Rikagaku Co., Ltd.) for the production of IFN-β. A culture medium, LGTrpAp (10 g/liter Bactotryptone, 5 g/liter yeast-extract, 5 g/liter Nacl, 1 g/liter glucose, 50 µg/ml L-tryptophan and 50 µg/ml ampicillin) was used for seed culture. A culture medium, MCGAp, which is based on M9 medium and supplemented with 0.5 % casamino acids and 50 µg/ml ampicillin, was used for the production of IFN-β. In a 2-liter jar fermenter, cells were grown in one liter of MCGAp to which glucose was fed continuously to maintain the concentration at approximately 1 %, and the pH was controlled at 6.5 with liquid ammonia. The culture was agitated at 750 rpm and aerated at one liter per minute.

Preparation of bacterial extracts. Bacterial extracts were prepared by freeze-thawing of spheroplasts and subsequent centrifugation as described previously (Nishi et al., 1983).

IFN assays. Antiviral activity of IFN-β was measured using a cytopathic effect inhibition assay (Armstrong, 1971) on FL cells challenged with vesicular stomatitis virus. A unit of IFN-β was calculated by calibration against a National Institute of Health (NIH) reference standard for IFN-β, catalog number G-023-902-527.

Anthranilate synthetase assays. Activity of anthranilate synthetase was measured according to the method of Baker and Crawford (Baker and Crawford, 1966).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Bacterial extracts containing 20 µg of proteins were boiled for 5 min in 2 % SDS, 5 % β-mercaptoethanol and 10 % glycerol, and subjected to SDS-polyacrylamide gel (14 %) electrophoresis (Laemmli, 1970).

RESULTS

The effect of culture temperature on the activity of IFN-β in E. coli cells. The production level of active human IFN-β in the E. coli cells was considerably affected by culture temperature. As shown in TABLE I.A, 8.6-fold higher activity of IFN-β was obtained from the extract of cells cultured at 20°C than that of cells cultured at 37°C. As a control, anthranilate synthetase, a product of trpE gene, whose synthesis is induced by the addition of 3,β-indoleacrylic acid (IAA), was also analyzed. Only 1.5-fold higher activity was obtained from cells cultured at 20°C than those cultured at 37°C, suggesting that the higher activity of IFN-β observed in the cells cultured at lower temperature is specific for IFN-β. The similar result was also obtained in the experiment in which a jar fermenter was used for the culture (TABLE I.B).

TABLE I. Effect of Culture Temperature on the Activity of IFN-β in E. coli Cells.

A, Test tube's scale

culture temperature (°C)	culture time after IAA addition (hr)	OD_{550nm}	activity of IFN-β (units/OD_{550nm})	specific activity of anthranilate synthetase ($x10^{-3}$units/mg)
37	4	3.02	3440 (1.0)	52.3 (1.0)
28	8	3.19	8260 (2.4)	73.2 (1.4)
20	16	3.17	29600 (8.6)	78.3 (1.5)

B, Fermenter's scale

culture temperature (°C)	maximum activity of IFN-β		
	OD_{550nm}	($x10^4$units/ml)	($x10^4$units/OD_{550nm})
30	9.5	35.1	3.69
25	15.8	131	8.29
20	12.0	189	15.8

A; 1.5 ml of an overnight culture of HB101/pMG-1 was diluted into 30 ml of LGTrpAp and incubated at 30°C in a 300-ml Erlenmeyer flask to an OD_{550nm} of 2.0. The culture was washed with MCGAp, and resuspended in MCGAp supplemented with 20 μg/ml of IAA. It was then divided into three equal portions, and 10 ml of each aliquot was cultured at 37°C, 28°C and 20°C in test tubes for the indicated time. Values in parentheses are relative activities. B; 2.5 ml of an overnight culture of HB101/pMG-1 was diluted into 50 ml of LGTrpAp and incubated at 30°C in a one-liter Erlenmeyer flask to an OD_{550nm} of about 5.0. The culture was inoculated into a jar fermenter and cultured as described in MATERIALS AND METHODS. At an OD_{550nm} of about 8.0, IFN-β synthesis was first induced by adding 20 μg/ml of IAA, and subsequently the same amounts of IAA were further added once a day for the following three days.

The change of IFN-β activity in E. coli cells during fermentation. The change of IFN-β activity in the cells cultured at 20°C and 33°C was followed during fermentation (FIGURE 1). After the first addition of IAA, the rapid increase in the activity was observed at both temperatures. When cells were cultured at 20°C, the activity continued to increase gradually and exceeded 10^6 units/ml at 96 hr. On the other hand, the rapid decrease in the activity was observed at 33°C after the maximum activity of 1.3×10^5 units/ml was obtained at 24 hr.

The accumulation of IFN-β protein in E. coli cells during fermentation. To examine the level of IFN-β protein accumulated in the cells, extracts of cells collected at intervals during fermentation were analyzed by SDS-

polyacrylamide gel electrophoresis (FIGURE 2). In both cells cultured at 20°C and 33°C, a protein with a molecular weight of about 20 kilodalton, which was absent in control cells, was detected. The protein was confirmed to be human IFN-β by Western blotting analysis using mouse anti-human IFN-β monoclonal antibody (data not shown). In the cells cultured at 20°C, the amount of IFN-β protein deduced from the analysis by SDS-polyacrylamide gel electrophoresis correlated the IFN-β activity during fermentation. The amount of IFN-β protein accumulated in the cells cultured at 33°C reached the maximum level at 24 hr and did not appear to decline, while the rapid decrease in the activity was observed after 24 hr. The amounts of IFN-β protein accumulated in the both cells seem to be almost equal when they are compared at their maximum levels. These results suggest that the IFN-β protein seems to exist as a rather denatured form with less antiviral activity and denaturation seems to continue in the cells cultured at 33°C.

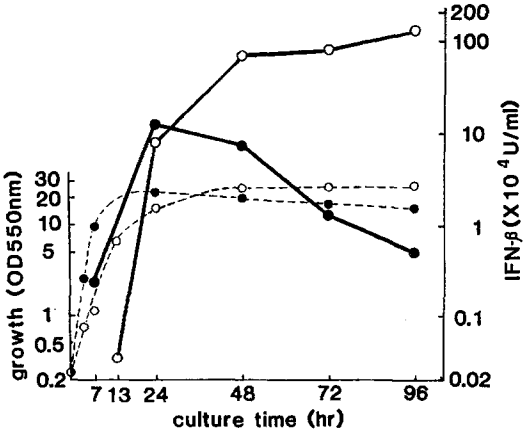

FIGURE 1. The Change of IFN-β Activity in E. coli Cells during Fermentation.
HB101/pMG-1 was grown in a 2-liter jar fermenter at 20°C (O) or 33°C (●). Dotted lines and thick lines represent cell growth and antiviral activity of IFN-β, respectively. IAA (20 μg/ml) was added at 13 hr, 24 hr, 48 hr and 72 hr for the culture at 20°C, and added at 7 hr, 24 hr, 48 hr and 72 hr for the culture at 33°C.

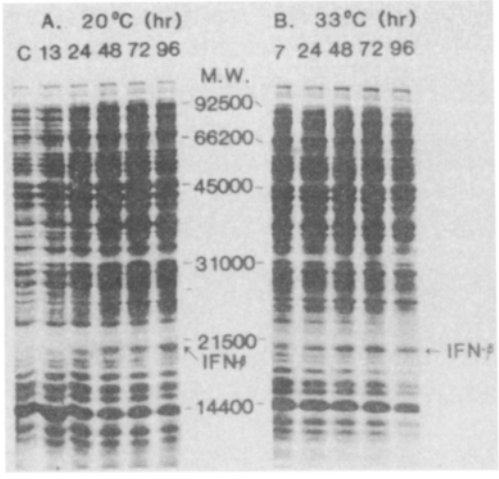

FIGURE 2. The Accumulation of IFN-β Protein in E. coli Cells during Fermentation.
The extracts of cells obtained from the culture of HB101/pMG-1 shown in FIGURE. 1 were subjected to SDS-polyacrylamide gel electrophoresis. Panel A shows the extracts of cells cultured at 20°C, and panel B shows those of cells cultured at 33°C. The numbers on each lane represent the time (hr) after culture. C denotes a control, i.e. the extract of cells of HB101 harboring pBR322.

DISCUSSION

In the present paper, we showed that the highest IFN-β activity was obtained from the cells cultured at low temperature, such as 20°C, while less activity was recovered from the cells cultured at higher temperature (TABLE I.A and I.B). The lower activity in the cells cultured at higher temperatures was not due to lower accumulation of IFN-β protein but probably due to denaturation of the protein during fermentation (FIGURE 1, 2).

Taniguchi et al. reported that the antiviral activity was much lower than that predicted on the basis of the rate at which the IFN-β protein was synthesized in the E. coli (Taniguchi et al., 1980). Mark et al. also reported that the human IFN-β purified from extracts of E. coli had lower specific activity, about 1/10th that of the native glycosylated protein (Mark et al., 1984). However, they created a mutated IFN-β protein in which a cysteine residue (17th residue from the N-terminus) was replaced by a serine residue, and showed that the mutated IFN-β had acquired antiviral activity similar to that of native IFN-β. They suggested from these results that the lower specific activity of intact IFN-β purified from E. coli was due to incorrect inter- and/or intramolecular disulfide bridging. The lower activity which we observed in the cells cultured at higher temperatures might be explained by the above mechanism.

When proteins are overproduced in E. coli using recombinant DNA technique, they are often assembled into insoluble aggregates (Cheng et al., 1981, Schoner et al., 1985). As the IFN-β protein produced in E. coli at 33°C comes to soluble fractions during fractionation procedure, the lower activity can not be ascribed to denaturation by aggregation. A following possibility should not be excluded: several amino acids from the amino and/or carboxyl terminus of IFN-β protein are removed in the cells cultured at higher temperature. On the IFN-β molecule, Nishi et al. previously reported that removal of 5 amino acids from the amino terminus drastically reduced its antiviral activity (Nishi et al., 1983). In any way, the actual mechanism of the lower activity in the cells cultured at higher temperatures remains to be elucidated.

The IFN-β protein purified from the extracts of cells cultured at 20°C has been confirmed to have a high specific activity of 1-2 x 10^8 units/mg which is comparable to that of the native glycosylated protein (Y. Yokoo et al. unpublished results), suggesting that the IFN-β protein exists in an active form in the cells cultured at 20°C. It is of interest to know whether the culture at lower temperature is effective for the production of other unstable proteins besides IFN-β. The results presented in this report demonstrate the significance for investigation of culture conditions for the production of eukaryotic proteins in E. coli without losing their activities.

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