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ORIGINAL PAPER

Expression and aggregation of recombinant human consensus interferon-α mutant by *Pichia pastoris*

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Abstract A recombinant human consensus interferon-α mutant (cIFN) was expressed in *Pichia pastoris*. The maximum dry cell weight, cIFN concentration and antiviral activity were 160 g l^{-1}, 1.24 g l^{-1} and 4.1×10^7 IU ml^{-1}, respec tively. The cIFN secreted into the medium was in the form of aggregates dominantly by non-covalent interaction and partially by disulphide bond. When the fermentation supernatant was disaggregated with 6 M guanidine hydrochloride, the antiviral activity of cIFN achieved 2.2×10^8 IU ml^{-1}.

Keywords Aggregates · Consensus interferon · *Pichia pastoris*

Introduction

Recombinant human consensus interferon-α (IFN-Con1) is a synthetic type of interferon-α (IFN) developed by scanning fourteen interferon-α subtypes and assigning the most frequently observed amino acids in each position (Blatt et al. 1996). The specific antiviral activity of IFN-Con1 is 5- to 20-fold higher than that of other types of IFN-α and has been expressed in *Escherichia coli* with a high protein level (Alton et al. 1983, Fieschko and Ritch 1986). To improve the stability of IFN-Con1, two IFN-Con1 mutants, cIFN (R164S) and cIFN (R22S, R164S), were constructed by comparing about thirty types of known interferon-α (Liu et al. 2002). The mutants were resistant to proteases degradation and expressed by *Pichia pastoris*.

To obtain high cell density and high protein productivity, a fermentation technique of expressing cIFN (R164S) by *Pichia pastoris* was developed in our laboratory. The production level of cIFN reached grams per liter. However, no cIFN monomer could be isolated from the supernatant. This work has investigated the form of cIFN in the fermentation medium: cIFN was as non-covalent aggregates and disulphide bond aggregates. In addition, the level of non-covalent aggregated cIFN was higher than other recombinant proteins expressed by engineered cell lines. To the best of our knowledge, the non-covalent aggregation of interferon-α has not been reported previously.

Materials and methods

Cell line and media

The recombinant strain *Pichia pastoris* GS115/pPIC9-cIFN used in this study was constructed by

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Dr. Liu (Institute of bioengineering, Academy of Military Medical Sciences, China). The cIFN gene was integrated into the chromosome of *P. pastoris* GS115. The expression of the cIFN gene was under the control of alcohol oxidase 1 gene (AOX1) promoter and induced by methanol.

The seed and fermentation media, as well as the fermentation procedures, were as described by Stratton et al. (1998). Briefly, the medium was a basal salt medium (BSM) and glycerol was used as a carbon source in the medium. The fermentation was carried out in a 5 l fermentor starting with 2 l BSM. The inoculum was 2.5% of the fermentor's starting volume. The fermentation started with a batch phase followed by a fed-batch phase. The end of the batch phase was indicated by a sharp increase of the dissolved O_2 (DO) level caused by the exhaustion of glycerol. Typically, the batch phase and the fed-batch phase lasted for about 20 h and 5 h, respectively. During the fed-batch phase, the glycerol-feeding rate was adjusted to keep the DO between 30 and 40% of air saturation. During methanol induction period, the methanol feeding rates were adjusted to control the methanol at less than 5 g l^{-1} and the DO between 20 and 40% of air saturation. The pH was maintained at 5.0 by adding 28% NH_4OH.

Purification of extracellular cIFN

The fermentation supernatant was harvested by centrifuging at 5000 × *g* for 20 min. The cIFN was purified from the supernatant by a hydrophobic coulmn, an affinity column and a gel filtration column as described in the literature (Liu 2002).

Electrophoresis and Western blotting

SDS-PAGE and native-PAGE were performed according to the standard procedures (Wang and Fan 2000). Western blot analysis was performed with a nitrocellulose membrane. Rabbit anti-human interferon-α polyclonal antibody was used as the primary antibody. It was diluted at a 1:2000 ratio in PBS supplemented with 0.05% (v/v) Tween20 and 5% (w/v) fat-free milk. The secondary antibody was the horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin (Sino-American Biotechnology Co.), which was diluted at a 1:500 ratio before use.

Analytical methods

Methanol concentration in the medium was measured by a methanol sensor (FC-2002, East China University of Science and Technology, China). Based on the measured concentration, the feeding rate of methanol was adjusted to ensure no over-feeding occurred during the induction.

Cell concentration was monitored as OD_{600} and converted to the dry cell weight (DCW) via an appropriate calibration curve.

Total amount of protein in the medium was assayed by the Bradford method.

Quantity of cIFN was determined by scanning the area of each band on SDS-PAGE gel, and then calculating with ImageMaster TotalLab software (Amersham Biosciences) using consensus interferon (Infergen, Amgen) as a standard.

Determination of the biological activity of cIFN

Briefly, the activity of cIFN was determined by an antiviral activity assay the Quality Inspection Laboratory of Wanxing Bio-Pharmaceutical Co. Ltd. (Shanghai, PR China). The assay was performed with a WISH cell/VSV system in which the WISH cells were challenged by vesicular stomatitis virus (Hou 1985). The activity units of cIFN were calculated to be the reciprocal of the maximum dilution of cIFN when 50% of the cells in the well were protected by cIFN.

Results and discussion

Expression of cIFN by Pichia pastoris

High cell density fermentation is one of the most important strategies for improving the expression level of recombinant proteins (Guo et al. 2002). Generally, a 100–200 g DCW l^{-1} is regarded as a high-density fermentation. In this study, the biomass reached 160 g DCW l^{-1} (OD_{600} = 270) at

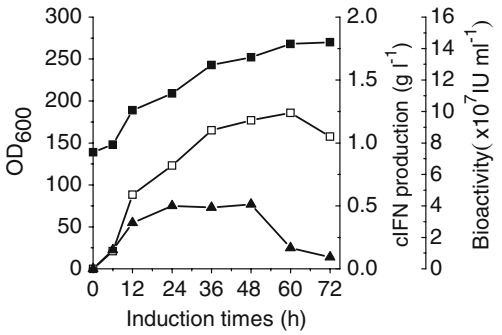

Fig. 1 Time course of various state parameters during fed-batch culture of Pichia pastoris for production of consensus interferon (cIFN). (■) OD_{600}; (□) cIFN production (g l^{-1}); (▲): cIFN bioactivity × 10^7 IU ml^{-1}.NB: OD_{600} of 270 = 160 g DCW l^{-1}

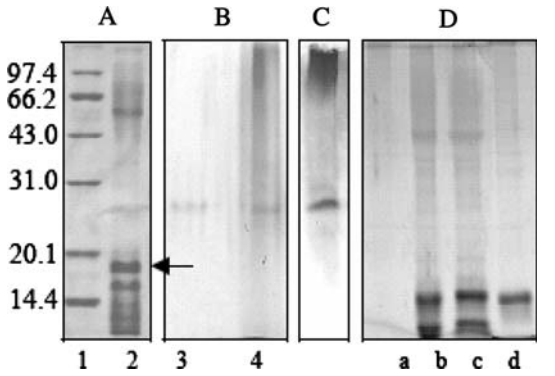

Fig. 2 Analysis of cIFN aggregate in fermentation broth by Pichia pastoris. (**A**) Reduced SDS-PAGE, resolving gel concentration was 15%, Coomassie Brilliant Blue staining. Lane 1: protein molecular size marker; lane 2: supernatant at 60 h after induction. Arrowhead indicated cIFN band, those bands below the arrowhead were the degraded cIFN fragments (data not shown). (**B**) Native–PAGE, resolving gel concentration was 6%. Lane 3: purified cIFN, 5 μl/well; Lane 4: supernatant at 60 h after induction. (**C**) Western blotting of the lane 4 in (**B**). (**D**) Supernatant was filtered with 50 kDa of gel filtration membrane and followed by reduced SDS-PAGE. Lane a: filtrate, Lane b: retentate, Lane c: supernatant not filtered, Lane d: standard cIFN. The lower band is the degradation fragment of cIFN

the end of the methanol induction in a 5 l fermentor (Fig. 1).

The cIFN started to be secreted into the medium after 6 h of induction, and gradually increased to 1.24 g l^{-1} at 60 h and then decreased (Fig. 1). The activity of cIFN was maximal at 4.1×10^7 IU ml^{-1} between 24 h and 48 h but the reason why activity stopped to increase after 24 h while its concentration increased until 60 h is unknown.

Although the expression level of cIFN exceeded 1 g l^{-1}, no cIFN monomer was obtained when the supernatant directly went through a series of the chromatography columns as previously described in Materials and methods and in the literature (Liu 2002). All protein fractions collected from the gel filtration column were over 80 kDa (data not shown). This observation was not consistent with the results of SDS-PAGE gel, which showed that most of the proteins had molecular weight less than 20 kDa. To explain the conflicting phenomena, it is highly desirable to understand the natural form of cIFN existing in the fermentation broth.

Native-PAGE analysis of supernatant

To determine out the natural form of cIFN in fermentation broth, a native PAGE was performed and compared with a reduced SDS-PAGE. On the native-PAGE gel (Fig. 2B), the protein bands appeared as a smear instead of individual bands shown on the reduced SDS-PAGE gel (Fig. 2A). More interestingly, the size of the smear was much bigger than that of cIFN monomer. Furthermore, Western blotting revealed that the proteins with large molecular weight on the upper part of the smear possessed the immuno-activity of cIFN (Fig. 2C). These results suggested that the proteins with large molecular weight were possibly the aggregates of the extracellular cIFN. Since all proteins should retain their natural forms on the native PAGE gel, it strongly suggests that the aggregates were likely to be the natural form of cIFN in fermentation broth. Due to the large size, the aggregates were hard to run into the stacking gel or resolving gel (Schokker et al. 1999) and even hampered the normal movement of other small proteins on the gel and resulted in a smear.

Ultrafiltration analysis of supernatant

The supernatant was filtered with 20, 30, 40 and 50kDa cut-off membranes, and followed by a

SDS-PAGE assay. The cIFN could be only detected in the retentate (Fig. 2D, lane b), not in the filtrate (Fig. 2D, lane a). Since the molecular weight of cIFN is 19.6 kDa, this result indicated that the cIFN in the medium did not exist as a monomer.

Identification of non-covalent aggregation

The aggregation of protein could be formed by non-covalent interaction and/or covalent bond. The denaturants, such as guanidine hydrochloride (GdnHCl) and urea, could be used to determine whether the aggregation is formed by covalent bond or not. If the protein aggregates could be dissolved in the denaturant, the aggregates were probably formed by non-covalent interaction. Otherwise, the covalent aggregation would be suggested (Wang 1999). To identify how the cIFN aggregates were formed, the supernatant was treated with either GdnHCl or urea followed by native-PAGE analysis. Figure 3 showed that the smear in the previous native-PAGE gel almost disappeared and a clear protein band showed up at the position of the cIFN monomer. The cIFN aggregates were mainly formed by non-covalent interaction. In addition, by comparing the strength of the cIFN bands (Fig. 3), the disaggregation effect of GdnHCl was stronger than that of urea and the effect of 6 M GdnHCl was better than 3 M GdnHCl (Table 1). When the sample was treated by 6 M GdnHCl, cIFN activity reached 2.2×10^8 IU ml^{-1} (Table 1), which was five times that without treatment.

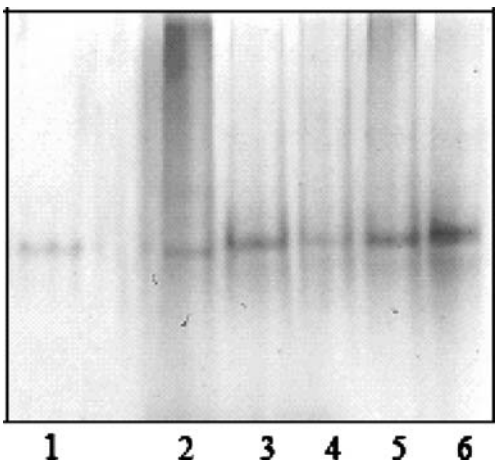

Fig. 3 Analysis of non-covalent aggregates. Supernatant at 60 h after methanol induction was treated with GdnCHl or urea, followed by native-PAGE and stained by Coomassie Brilliant Blue. Lane 1: purified cIFN, Lane 2: fermentation supernatant, Lane 3: treated with 6 M urea, Lane 4: treated with 3 M urea, Lane 5: treated with 3 M GdnCHl, Lane 6: treated with 6 M GdnCHl

Analyses of non-reduced SDS-PAGE and Western blotting

To determine if there were the cIFN aggregates formed by disulfide bonding, a reduced SDS-PAGE and a non-reduced SDS-PAGE were compared and their western blot analyses were performed. Although the disulfide bond formed aggregates could hardly be observed on the non-reduced SDS-PAGE gel stained by Coomassie Brilliant Blue (Fig. 4 lane 10), they could be detected by western blotting when the sample was not treated with 2-mercaptoethanol (Fig. 4, lane 8 and 9). The disulfide bond formed aggregates included dimer, trimer and oligomer. This result was consistent with the observation in Fig. 3 where there was only a clear monomer band, and no obvious dimer or trimer band possibly because the amount of aggregates formed by disulphide bonding was very small, and SDS-PAGE has lower sensitivity than western blotting. Similar oligomerization formed by disulphide bond was also observed in other native type?interferon-α (IFN) (Wetzel et al. 1983, Morehead et al. 1984) and IFN-αA expressed by *S. cerevisiae* (Wang et al. 1995).

Table 1 Activity analysis of supernatant treated with or without GdnHCl

Sample	Untreated[a]		Treated[b]	
Induction time[c] (h)	24	48	48	48
GdnHCl[d] (M^{-1})			3	6
Activity ($\times 10^7$ IU ml^{-1})	4	4	17	22

[a]Supernatant untreated

[b]Supernatant treated with GdnHCl

[c]Cultivation time after adding methanol

[d]Concentration of GdnHCl used

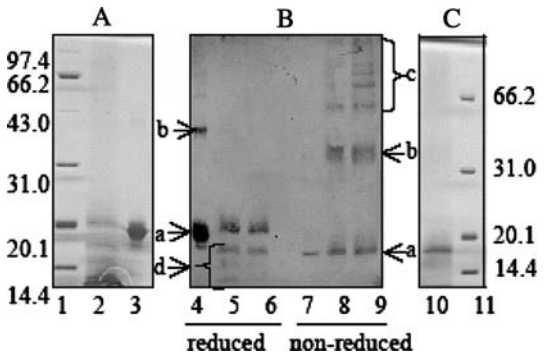

Fig. 4 Analysis of disulphide bond aggregates. Supernatant at 60 h of induction was treated with or without 2-mercaptoethanol, followed by SDS-PAGE analysis and western blot analysis. **A** and **C**: SDS-PAGE profile, **B**: Western blotting profile. Lane 1–6: reduced sample, Lane 7–11: non-reduced sample; Lane 1 and 11 are molecular size markers; Lane 2: supernatant after 59 h of induction; Lane 3: purified cIFN; Lane 4: corresponding to lane 3; Lane 5: corresponding to lane 2; Lane 6: supernatant after 48 h of induction; Lane 7: purified cIFN; Lane 8: supernatant after 48 h of induction; Lane 9; supernatant after 59 h of induction; Lane 10: corresponding to lane 9; a: cIFN monomer; b: cIFN dimmer; c: cIFN trimer and other higher molecular weight oligomers; d: degradated fragments of cIFN

Conclusion

The experimental results showed that the cIFN secreted into the medium formed the aggregates. The aggregation of cIFN occurred mostly by non-covalent interaction and partially by disulphide bond. This is the first report of a recombinant protein that has intensive non-covalent aggregation in *Pichia pastoris* expression system. To optimize and scale up the production of the cIFN, the studies for determining the factors that affect the conformation of cIFN in the fermentation medium are in progress.

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