Purification and characterization of recombinant murine immune interferon

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The recombinant murine immune interferon (rMu-IFN-γ) was purified to homogeneity from *Escherichia coli* harboring the expression vector of murine IFN-γ. The purified rMu-IFN-γ showed an M_r of 15000 in SDS-polyacrylamide gel electrophoresis. Results of amino acid analysis, amino- and carboxyl-terminal analyses and peptide mapping of rMu-IFN-γ suggest that it has the complete protein sequence predicted on the basis of cDNA except for lack of four amino acid residues from the mature carboxyl-terminus.

Recombinant murine immune interferon *Purification* *Terminal analysis* *Peptide mapping*

1. INTRODUCTION

As the immune interferon (IFN-γ) has a strict species specificity, murine IFN-γ (Mu-IFN-γ) should be used in murine model systems which have been used for the study on the action of IFN-γ. Mu-IFN-γ has been isolated from spleen cultures [1] and T-cell lines [2]. The cDNA of Mu-IFN-γ was cloned and expressed by Gray and Goeddel [3] in order to clarify the possibility of contamination of natural IFN-γ with other lymphokines and also to provide sufficient material. However, detailed molecular characterization of both natural and recombinant Mu-IFN-γs has not yet been performed. Here, we describe the purification and characterization of recombinant murine IFN-γ (rMu-IFN-γ) produced in *Escherichia coli*.

Abbreviations: rMu-IFN-γ, recombinant murine immune interferon; HPLC, high-performance liquid chromatography; SDS-PAGE, polyacrylamide gel electrophoresis

2. EXPERIMENTAL

2.1. *Isolation of Mu-IFN-γ cDNA and construction of expression plasmid*

Mu-IFN-γ cDNA was isolated from the cDNA library constructed from concanavalin A-induced B5 cells [4] by the method of Okayama and Berg [5], which will be described in detail elsewhere. The nucleotide sequence encoding mature Mu-IFN-γ was placed under the control of the *trp* promoter essentially as described by Gray and Goeddel [3].

2.2. *Expression of rMu-IFN-γ in* E. coli *and purification*

LB and M9 media were prepared as in [8]. *E. coli* K-12 strain C600 was used for the expression of rMu-IFN-γ. The cultures (100 ml) were grown overnight in LB medium supplemented with ampicillin (40 mg/l), and then diluted into 10 l of M9 medium containing 0.5% glucose, 0.5% casamino acid, 40 mg/l ampicillin. After growth at 37°C under vigorous agitation and aeration, *E. coli* cells were harvested by centrifugation. The rMu-IFN-γ was purified by a combination of anion-exchange,

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hydrophobic and size-exclusion chromatographies after cell disruption and ammonium sulfate fractionation.

2.3. *Amino acid analysis and determination of terminal sequences*

Samples were hydrolyzed according to Simpson et al. [7] and amino acids were analyzed with a Hitachi model 835 amino acid analyzer. Edman degradation was performed as described by Iwanaga et al. [8] with slight modifications. The carboxyl-terminal sequence was characterized by digestion with carboxypeptidase P (Protein Research Foundation).

2.4. *Peptide mapping of rMu-IFN-γ*

Reduced and *S*-carboxymethylated rMu-IFN-γ (RCm-Mu-IFN-γ) prepared according to Crestfield et al. [9] was dissolved in 6 M guanidine hydrochloride and chemically modified with succinic anhydride. The modified protein was incubated for 4 h at 37°C with *Staphylococcus aureus* protease [Miles Laboratories, 2% (w/w) of rMu-IFN-γ], and then directly subjected to reversed-phase HPLC for peptide mapping.

3. RESULTS

3.1. *Purification of rMu-IFN-γ from* E. coli

About 80 mg pure rMu-IFN-γ protein was obtained from 100 g wet cells with an overall recovery of 12%. The specific activities of some final preparation batches were $2–6 \times 10^6$ units/mg protein (vesicular stomatitis virus and mouse LO cells with mouse α/β international reference standard) and in close agreement with that reported by Burton et al. [10].

3.2. *Purity of the final rMu-IFN-γ preparation*

One major protein band at 15 kDa and another faint band at 30 kDa were observed by SDS-PAGE (fig.1). The minor band at 30 kDa increased when samples were treated without 2-mercaptoethanol (2-ME) prior to electrophoresis and seemed to be an undissociated dimeric form as observed in the case of natural human [11] and murine [12] IFN-γs. This undissociated dimeric form was less than 3% in the presence of 2-ME, and any other impurity bands were not observed in the dye-stained gel. The single symmetrical peak appeared in an analytical HPLC pattern (not shown) suggesting the purity of almost 100%.

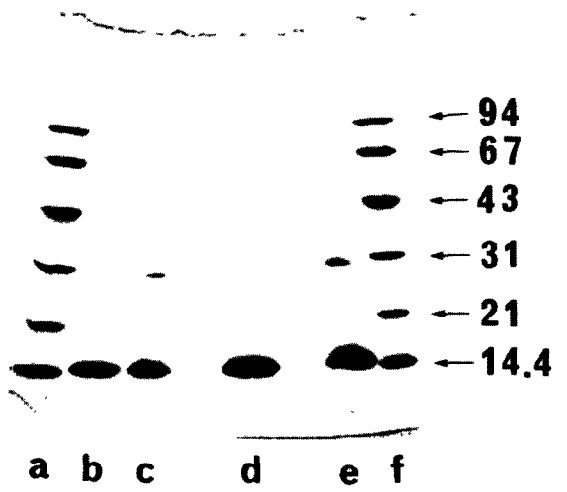

Fig.1. SDS-PAGE pattern of rMu-IFN-γ. Purified rMu-IFN-γ (b and c, 10 μg; d and e, 20 μg) was treated with (b,d) or without 2-ME (c,e) and subjected to SDS-PAGE. The gel was stained with Coomassie brilliant blue R-250. Molecular masses (in kDa) of marker proteins (a,f) are indicated by arrows.

3.3. *Protein chemical characterization of purified rMu-IFN-γ*

Table 1 shows the amino acid composition of the final preparation. Most values were in good agreement with the theoretical ones predicted from cDNA, while Ser, cysteic acid and Arg were one or two residues less than the theoretical values. Edman degradation of RCm-Mu-INF-γ suggested the single amino-terminal sequence of NH_2-carboxymethylcysteine (CmCys)-Tyr-CmCys-His-Gly- consistent with that predicted from cDNA. Fig.2 shows the liberation of amino acids from RCm-Mu-IFN-γ by carboxypeptidase P, suggesting a sequence of -Ser-Leu-(Arg 2, Lys 2)-COOH as the carboxyl-terminus. Considering the theoretical protein sequence and the results of amino acid and carboxyl-terminal analyses, the actual carboxyl-terminus of purified rMu-IFN-γ should be Lys 132 and four residues from the mature carboxyl-terminus (Arg 133-Ser-Arg-Cys 136) should be completely truncated. Fig.3 shows the peptide mapping pattern of RCm-Mu-IFN-γ by HPLC. All peaks were collected, rechromatographed and

Table 1

Amino acid composition of rMu-IFN-γ

	Observed	Theory
Asp	17.0	17
Thr	2.9[a]	3
Ser	14.3[a]	16
Glu	17.1	17
Pro	2.2	2
Gly	3.3	3
Ala	5.4	5
Cys/2	1.7[b]	3
Val	8.0[c]	8
Met	2.0	2
Ile	11.5[c]	12
Leu	13.0	13
Tyr	2.7	3
Phe	8.9	9
Lys	10.0	10
His	2.8	3
Trp	1.9	2
Arg	6.0	8
Total		136

[a] Values corrected to 0 h hydrolysis
[b] Value of cysteic acid after performic acid oxidation
[c] Values of 72 h hydrolysis

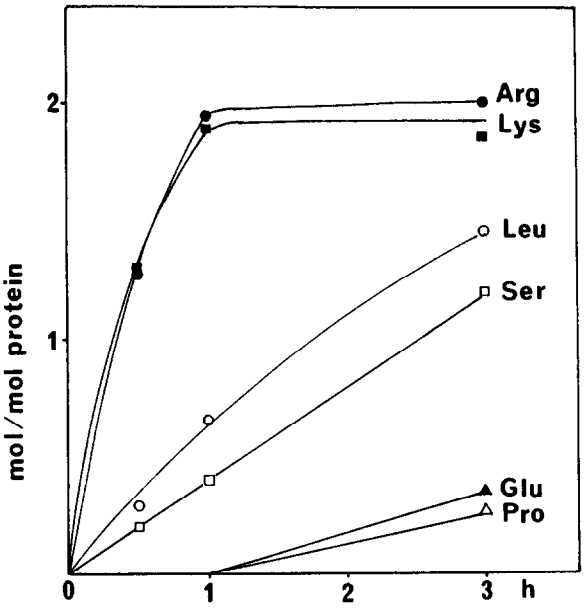

Fig.2. Liberation of amino acids from RCm-Mu-IFN-γ by carboxypeptidase P. RCm-Mu-IFN-γ was incubated with carboxypeptidase P [0.1% (w/w) rMu-IFN-γ] in 50 mM sodium acetate buffer (pH 3.7) containing 0.1% Triton X-100. At appropriate times aliquots were withdrawn and subjected to amino acid analysis.

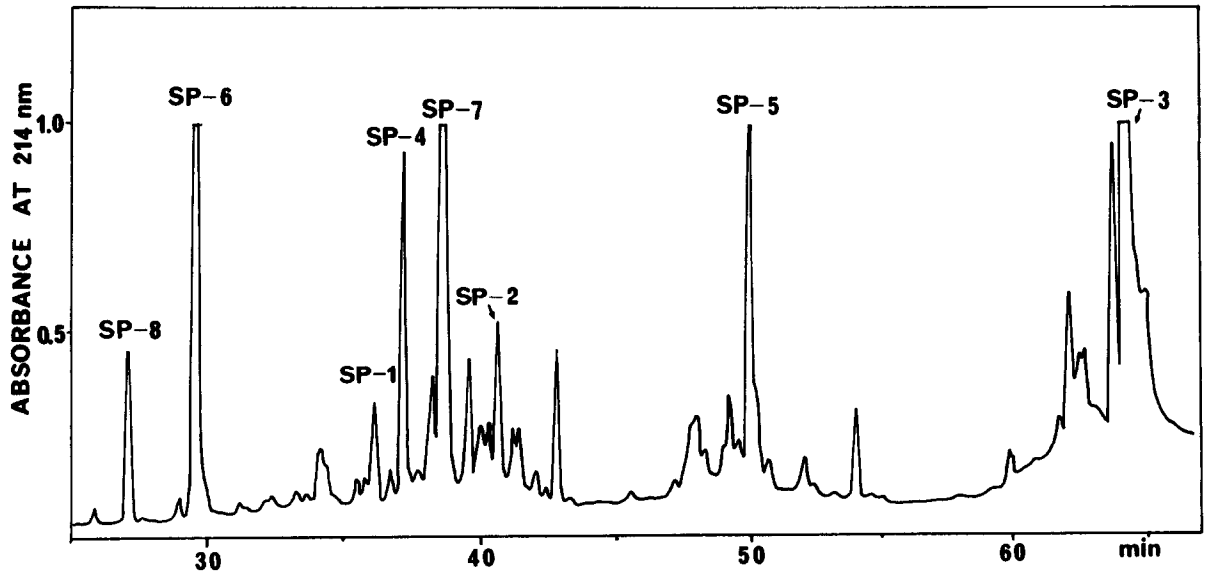

Fig.3. Reversed-phase HPLC pattern of succinyl RCm-Mu-IFN-γ after digestion with *S. aureus* protease.

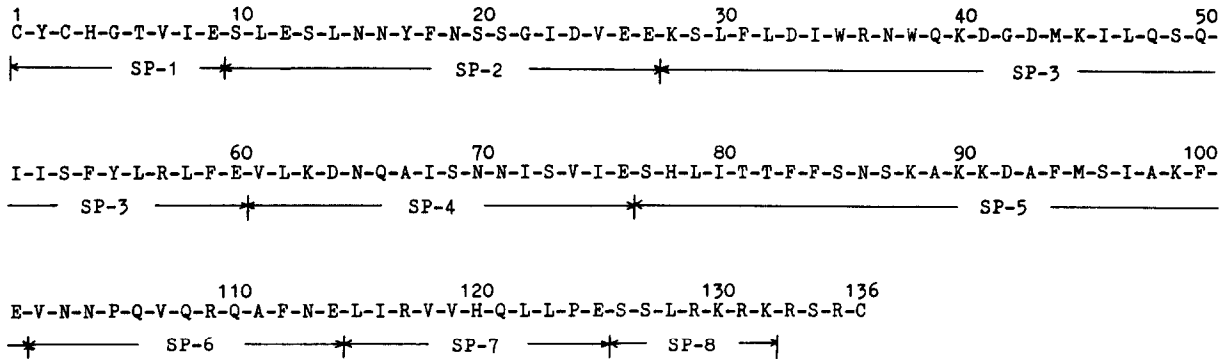

Fig.4. Assignment of peptide fragments obtained by the peptide mapping shown in fig.3 according to their amino acid compositions.

subjected to amino acid analysis for assignment. Only eight peaks (SP-1 to SP-8) were assignable because other small peaks were non-proteineous or not enough to be analyzed. Results of amino acid analyses (not shown) suggested that those eight peptides explained the complete protein sequence except for four residues truncated from the mature carboxyl-terminus (fig.4). There was no detectable peptide containing the Arg 133-Cys 136 sequence.

4. DISCUSSION

The purified rMu-IFN-γ consisted of the protein sequence expected from cDNA with the exception of the lack of four amino acid residues from the mature carboxyl-terminus. It is not clear that the processing of carboxyl-terminus like this occurred during fermentation or further purification. We added some protease inhibitors such as benzamidine and phenylmethanesulfonyl fluoride to buffers for purification to prevent the proteolytic processing during the purification procedure. We also tried to isolate rMu-IFN-γ from the fresh *E. coli* cells harvested at 15 h instead of the usual 24 h expecting that a small amount of rMu-IFN-γ of full size might be obtainable, but the carboxyl-terminus of the obtained rMu-IFN-γ was also completely truncated. Therefore, the processing seemed to occur at the earlier stage of expression in cells. The possibility that some amount of full-size protein existed but was removed during the purification procedure is not deniable. Rinderknecht et al. [13] reported that both 20 kDa and 25 kDa species of natural human IFN-γ derived from peripheral blood lymphocytes were heterogeneous at their carboxyl-termini, having forms which terminated at all residues from Gly 130 to Met 137 of the mature protein sequence predicted from cDNA. It is not clear whether all forms are active nor which carboxyl-termini are predominant in vivo. The physiological meaning of the carboxyl-terminal processing is also still unknown. Gribaudo et al. [12] also suggested the post-translational proteolytic processing of natural murine IFN-γ. Rinderknecht and Burton [14] showed that about 12 carboxyl-terminal amino acid residues were not so important for antiviral activity by limited proteolysis of recombinant human IFN-γ. This is also supported by the fact that protein homology between human and murine IFN-γs is around 40% and a characteristic difference is the deletion of nine carboxyl-terminal amino acid residues [3]. Moreover, the carboxyl-terminal regions of IFN-γs are supposed to be subject to attack by some proteolytic enzymes.

Availability of rMu-IFN-γ will be of great value in understanding the biological and physiological roles of IFN-γ. Some studies with natural IFN-γ have been performed, but the possibility that other contaminated lymphokines might have affected the observations does exist. The rMu-IFN-γ will clarify those observations and also give sufficient opportunities to perform studies in vitro as well as in vivo.

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