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PURIFICATION AND CHARACTERIZATION OF RECOMBINANT HUMAN INTERLEUKIN-1β PRODUCED IN ESCHERICHIA COLI

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SUMMARY: Recombinant human interleukin-1β(rIL-1β) produced in Escherichia coli was purified to homogeneity by a combination of mass ion exchange column chromatography, ion exchange and gel filtration high performance liquid chromatography. The purified rIL-1β had a molecular weight of 18 kD on SDS-polyacrylamide gel electrophoresis and an isoelectric point of 6.9 on analytical isoelectric focusing. These values were almost same as those of natural interleukin-1β. The amino acid composition and amino acid sequence of the amino terminal region were consistent with those deduced from the cDNA sequence. In addition, the primary structure was confirmed by peptide mapping with lysyl-endopeptidase on reverse phase HPLC. Besides rIL-1β with amino terminal Ala, two molecular species, [Met^{0}]rIL-1β and [$desAla^{1}$]rIL-1β, were also obtained. Biological and physicochemical properties of the three species of rIL-1β were compared. © 1987 Academic Press, Inc.

Interleukin-1(IL-1) is a polypeptide hormone produced by activated macrophage and several other cell types. It mediates a wide range of biological activities involved in immune and inflammatory responses (1). Recently, two kinds of IL-1, α and β, have been cloned by several groups (2,3,4,5). We also have succeeded in purification of IL-1β as a potential tumor growth inhibitory factor (GIF) and cloning the cDNA from activated human monocyte and have succeeded in cloning the cDNAs of IL-1α and β from mRNA of a human histiocytic lymphoma cell line, U937 (6), using the Okayama-Berg expression vector by transfection method (7). In this paper, we describe the purification of rIL-1β produced in high yields in an Escherichia coli expression system. The comparison of biological activities and physicochemical properties among the three molecular species obtained here, rIL-1β, [Met^{0}]rIL-1β and [$desAla^{1}$]rIL-1β, are also discussed.

Abbreviations: rIL-1β, recombinant human interleukin-1β; GIF, growth inhibitory factor; cDNA, complementary DNA.

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MATERIALS AND METHODS

Cloning of the IL-1β gene and construction of the expression plasmid: IL-1βcDNA (pIL-1β) was cloned from the cDNA library constructed from mRNA of activated human monocyte, which will be described in detail elsewhere. After cleaving pIL-1β with AccI and ClaI, the formed 1.2kb fragment containing IL-1β gene was isolated, treated with DNA polymeraseI (Klenow Fragment), joined with BamHI linker (pCGGATCCG) and cleaved with MspI and BamHI. The obtained fragment which specifies amino acids 127-269 of IL-1β precursor was ligated together with synthetic oligonucleotides, pCGATAATGG CTCCTGTACGTTCTCTGAACTGCACTCTC and pCGGAGAGTGCAGTTCAGAGAACGTACAGGAGCCATTAT. The fragment above obtained was cleaved with ClaI and BamHI and inserted into the ClaI and BamHI site of pTM1 which was constructed using E.coli tryptophan promoter and pBR322 (8). The expression plasmid was designated to be ptrpIL-1β.

Expression of rIL-1β in E.coli: E.coli K-12 strain HB101 harboring plasmid ptrpIL-1β was grown in a 10 l jar fermenter in M9 medium containing 0.5% glucose and 0.5% casamino acids. After growth for 10 hrs at 37°C under vigourous agitation and aeration, E.coli cells were harvested by centrifugation.

Purification of rIL-1β: After disruption of E.coli cells (330 g wet weight) in 5 mM Tris-HCl (pH 8.0), the pH of the solution was adjusted to 4.5 with acetic acid and the precipitate was removed by centrifugation for 30 min at 16,000 g. The supernatant fluid (2100 ml) was applied at a flow rate of 10 ml/min to a ZetaPrep SP-100 (LKB) previously equilibrated with 50 mM sodium acetate (pH 5.3). The proteins were eluted with 50 mM sodium acetate (pH 5.5) containing 0.1M NaCl and 50 mM sodium acetate (pH 5.5) containing 1M NaCl. Fractions containing the IL-1β activity were pooled and purified with cation exchange high performance liquid chromatography. GILSON HPLC System equipped with TSK gel SP-5PW column, 200X55mm I.D. (Toyo Soda manufacturing Co.,Ltd.) was used. Elution was performed with a linear gradient of NaCl concentration in 50 mM sodium acetate (pH 5.5). At this purification step, three molecular species with biological activity, [Met^{0}]rIL-1β, rIL-1β and [$desAla^{1}$]rIL-1β, could be separated. Fractions of [Met^{0}]rIL-1β, rIL-1β, and [$desAla^{1}$]rIL-1β were subjected to gel filtration high performance liquid chromatography, respectively. LKB chromatography system equipped with TSK gel G2000SW, 600X21.5mm I.D.(Toyo Soda manufacturing Co.,Ltd.) was used and phosphate buffered saline was used as eluent. Fractions containing the rIL-1β were pooled to give 670 mg of purified product.

Biological activity: IL-1 activity was determined its ability to inhibit the growth of A375 human melanoma cells (9). This property of IL-1β is refered to as its growth inhibitory factor (GIF) activity. Briefly, A375 cells were added into 96 wells microtiter trays ($2x10^{3}$ cells/well) in Eagle's MEM supplemented with 10% fetal calf serum containing the test sample. After the incubation at 37°C for 4 days, viable cells were measured by neutral-red dye-uptake method.

One GIF unit per ml represents the reciprocal of the dilution of GIF causing 50% cytostasis or cytolysis for 4 days cultures.

SDS-polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis on 15% gel was performed by the methods of Laemmli (10). Molecular weight standards used to calibrate the gels were as follows; Phosphorylase b, 94 kD; Bovine Serum Albumin, 67 kD; Ovalbumin, 43 kD; Carbonic anhydrase, 30 kD; Soybean Trypsin Inhibitor, 20.1 kD; α-Lactalbumin, 14.4 kD. The gels were fixed and stained with silver (Bio-Rad) or coomassie brilliant blue.

Isoelectric focusing: Isoelectric focusing was performed on an Ampholine PAG plate (LKB) with a pH range of 3.5-9.5 according to the manufacture's instructions. The pH gradient was determined by slicing a lane into 1 cm sections, eluting the sections in water at 4°C and measuring the pH of these solutions.

Amino acid analysis: Protein samples were hydrolyzed in 4M methane sulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 130°C for 4 hrs. Hydrolyzates were analyzed on a Hitachi amino acid analyzer using o-phthalaldehyde as a coloring reagent.

Amino acid sequence analysis: Edman degradation was performed with a gas phase sequencer (Applied Biosystems Inc., Forster City). Phenylthiohydantoin derivatives of amino acids from each cycles were analyzed by reverse phase HPLC.

Peptide mapping: A solution of rIL-1β (100 μg) in 10mM Tris-HCl pH 8.7 (110 μl) were treated with a lysyl-endopeptidase (Wako Pure Chemical Industries, Ltd.) at 37°C for 4 hrs. Whole mixture was applied to the reverse phase HPLC System. The resulting peptides were subjected to amino acid analysis and amino acid sequence analysis.

RESULTS AND DISCUSSION

The expression level of rIL-1β reached was approximately 20% of the total cellular proteins as shown in Fig.1. After disruption of the E.coli cells, the purification of rIL-1β was carried out by a combination of mass

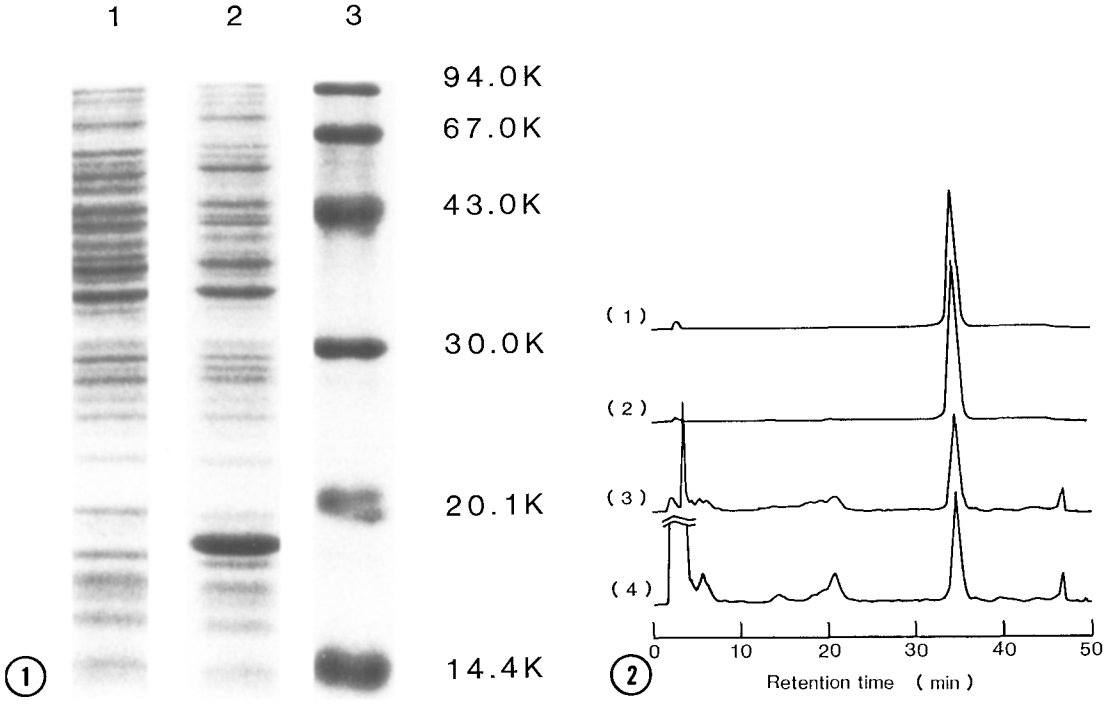

Fig.1. The expression of rIL-1β on SDS-polyacrylamide gel electrophoresis. Lane 1, total proteins from E.coli HB101/pTM1 (without IL-1β gene; lane 2, total proteins from E.coli HB101/ptrpIL-1β (with IL-1β gene); lane 3, marker proteins.

Fig.2. The profiles of SP-HPLC of rIL-1β at various stages of purification. (1), GF-HPLC pool; (2), SP-HPLC pool; (3), Mass ion exchange chromatography pool; (4), Cell extract. Each sample was applied to a TSK gel SP-5PW column. Elution was performed with 50mM sodium acetate buffer (pH 5.5) containing sodium chloride, which was increased gradiently from 0 to 0.5 M. Absorbance at 280nm was used as the monitoring.

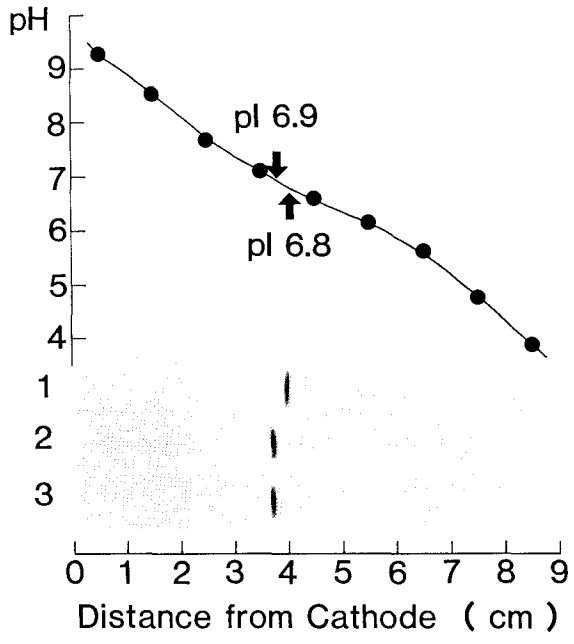

Fig.3. Isoelectric focusing of purified rIL-1β and its derivatives. Lane 1, [Met^{0}]rIL-1β; lane 2, rIL-1β and lane 3, [desAla^{1}]rIL-1β.

ion exchange column chromatography, cation exchange HPLC with TSK gel SP-5PW (SP-HPLC) and gel filtration HPLC with TSK gel G2000SW (GF-HPLC). The profiles of analytical SP-HPLC of preparations at various stages in purification were shown in Fig.2.

The purified rIL-1β showed a single peak on analytical SP-HPLC (Fig.2), a single silver stained band at a molecular weight of 18 kD on SDS-PAGE, a single silver stained band at isoelectric point of 6.9 on analytical isoelectric focusing (Fig.3) and a single amino terminal amino acid sequence on a gas phase sequencer. As the amino terminal amino acid, only Ala was detected. The amino acid composition (Table 1) and amino terminal amino acid sequence were consistent with those deduced from the cDNA sequence. To confirm the entire amino acid sequence of rIL-1β, peptide mapping of rIL-1β was performed using the enzyme lysyl-endopeptidase and peptides separated from one another using RP-HPLC (Fig.4). The resultant fragments were aligned by their amino acid compositions and sequences (Fig.5). These fragments (K1-K14) corresponded to a complete set of the peptides containing the entire amino acid sequence from the amino terminus to carboxyl terminus of the IL-1β.

The SP-HPLC preparation containing the rIL-1β had two additional molecular species with biological activities. These two molecular species were also purified to homogeneity. The molecular weights of these proteins were estimated to be 18 kD on SDS-PAGE. The amino terminal amino

Table 1. Amino acid compositions of rIL-1β, [Met^0]rIL-1β and [desAla^1]rIL-1β

	rIL-1β	[Met^0]rIL-1β	[desAla^1]rIL-1β
Asp	17.9(17)	17.5(17)	17.9(17)
Thr	5.8 (6)	5.8 (6)	5.8 (6)
Ser	12.3(14)	12.8(14)	12.9(14)
Glu	24.4(23)	24.0(23)	23.4(23)
Pro	ND[a)] (8)	ND[a)] (8)	ND[a)] (8)
Gly	8.3 (8)	8.4 (8)	8.1 (8)
Ala	5.0 (5)	5.3 (5)	4.0 (4)
Cys	ND[a)] (2)	ND[a)] (2)	ND[a)] (2)
Val	10.5(11)	9.7(11)	9.6(11)
Met	5.8 (6)	6.7 (7)	5.7 (6)
Ile	4.8 (5)	4.8 (5)	4.7 (5)
Leu	15.3(15)	15.5(15)	15.7(15)
Tyr	3.9 (4)	4.0 (4)	4.0 (4)
Phe	9.0 (9)	9.0 (9)	9.0 (9)
Lys	14.7(15)	15.2(15)	14.9(15)
His	0.9 (1)	1.0 (1)	1.0 (1)
Trp	ND[a)] (1)	0.6 (1)	0.7 (1)
Arg	3.1 (3)	3.0 (3)	3.0 (3)
Total	(153)	(154)	(152)

The calculated values were shown in parentheses.
a)ND means not to be determined.

acid sequences of these proteins as determined by a gas phase sequencer were Met-Ala-Pro-Val-Arg- and Pro-Val-Arg-Ser-Leu-. The peptide mappings of these two proteins were identical to that of rIL-1β, except for their amino terminal fragments. These results revealed that one is [Met^0]rIL-1β with an additional Met attached to amino terminus, the other one is [desAla^1]rIL-1β lacking the amino terminal Ala of IL-1β. The amino acid compositions of [Met^0]rIL-1β and [desAla^1]rIL-1β were consistent with the calculated values (Table 1).

On gel isoelectric focusing, [Met^0]rIL-1β, rIL-1β and [desAla^1]rIL-1β gave a single silver stained band with isoelectric points of 6.8, 6.9 and 6.9, respectively (Fig.3). The pI of rIL-1β is in good accordance with natural IL-1β (Table 2).

As shown in Table 2, the in vitro biological activity of rIL-1β, as measured by the ability of growth inhibition for human melanoma A375 cells was 4.4×10^7 u/mg of protein, which was comparable to that of natural human IL-1β purified from the culture supernatant of the LPS stimulated human

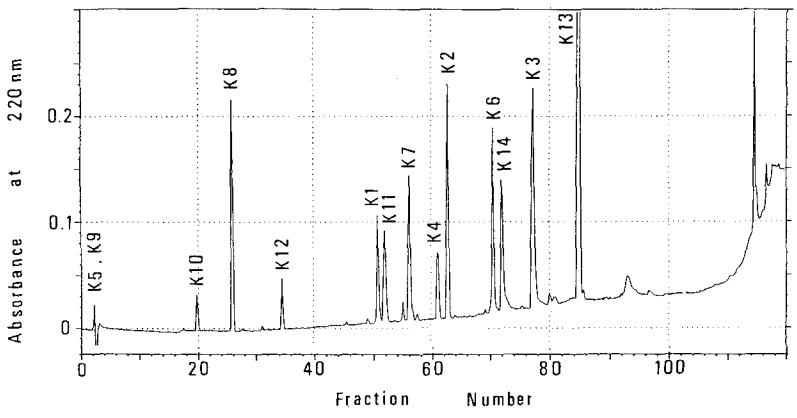

Fig.4. Peptide mapping of rIL-1β with lysyl-endopeptidase on reverse phase HPLC. After digestion of rIL-1β with lysyl-endopeptidase (See Materials and Methods), the hydrolyzate was applied on Cosmosil 5C18 column. The elution was performed with TFA/acetonitrile buffer system.

monocytes (Table 2). But surprisingly, the biological activity of [Met^{0}]rIL-1β is only one-tenth of rIL-1β; this result is different from that reported by Wingfield et al.(11). They reported that the biological activity of [Met^{0}]rIL-1β was similar to that of rIL-1β. This discrepancy may be caused by a difference in the purity of the proteins or because of a differential response of the assay systems.

1 5 10 15 20
Ala-Pro-Val-Arg-Ser-Leu-Asn-CYS-Thr-Leu-Arg-Asp-Ser-Gln-Gln-Lys-Ser-Leu-Val-Met
K1

21 25 30 35 40
Ser-Gly-Pro-Tyr-Glu-Leu-Lys-Ala-Leu-His-Leu-Gln-Gly-Gln-Asp-Met-Glu-Gln-Gln-Val
K2

41 45 50 55 60
Val-Phe-Ser-Met-Ser-Phe-Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys-Ile-Pro-Val-Ala-Leu
K3 K4

61 65 70 75 80
Gly-Leu-Lys-Glu-Lys-Asn-Leu-Tyr-Leu-Ser-CYS-Val-Leu-Lys-Asp-Asp-Lys-Pro-Thr-Leu
K5 K6

81 85 90 95 100
Gln-Leu-Glu-Ser-Val-Asp-Pro-Lys-Asn-Tyr-Pro-Lys-Lys-Lys-Met-Glu-Lys-Arg-Phe-Val
K7 K8 K9 K10

101 105 110 115 120
Phe-Asn-Lys-Ile-Glu-Ile-Asn-Asn-Lys-Leu-Glu-Phe-Glu-Ser-Ala-Gln-Phe-Pro-Asn-Trp
K11 K12

121 125 130 135 140
Tyr-Ile-Ser-Thr-Ser-Gln-Ala-Glu-Asn-Met-Pro-Val-Phe-Leu-Gly-Gly-Thr-Lys-Gly-Gly
K13

141 145 150
Gln-Asp-Ile-Thr-Asp-Phe-Thr-Met-Gln-Phe-Val-Ser-Ser
K14

Fig.5. Assignment of peptide fragments obtained by the peptide mapping shown in Fig.4 according to their amino acid compositions and amino acid sequences.

Table 2. The comparison of biological activities and physicochemical properties of natural human IL-1β, rIL-1β, [Met^0]rIL-1β and [desAla^1]rILβ

	natural IL-1β	[Met^0]rIL-1β	rIL-1β	[desAla^1]rILβ
Molecular weight	18 kD	18 kD	18 kD	18 kD
Isoelectric point	6.9	6.8	6.9	6.9
Specific activity (units/mg)	2.1×10^7	2.7×10^6	4.4×10^7	2.8×10^7

NaturalIL-1β was purified from the culture supernatant of the activated human monocytes. Molecular weights were estimated by SDS-PAGE. Isoelectric points were determined by isoelectric focusing. Protein contents were measured by amino acid analysis. Biological activity was determined with the ability of growth inhibition for human melanoma A375 cells.

As described above, large quantities of rIL-1β with biological and physicochemical properties similar to those of natural IL-1β were obtained. Now it is possible to use this IL-1β for elucidating various biological properties of IL-1. On the other hand, in the course of purification of rIL-1β, we found that minor variation of amino terminal portion affects the biological activity. We are going to study the relationship between structure and function in detail.

REFERENCES

1. Durum, S.K., Schmidt, J.A. and Oppenheim, J.J. (1985) Annu. Rev. Immunol. 3, 263-287.
2. Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M. and Dinarello, C.A. (1984) Proc. Natl. Acad. Sci. USA 81, 7907-7911.
3. March, C.J., Mosley, B., Larsen, A., Cerretti, D.P., Braedt, G., Price, V., Gillis, S., Henney, C.S., Kronheim, S.R., Grabstein, K., Conlon, P.J., Hopp, T.P. and Cosman, D. (1985) Nature 315, 641-647.
4. Furutani, Y., Notake, M., Yamayoshi, M., Yamagishi, J., Nomura, H., Ohue, M., Furuta, R., Fukui, T., Yamada, M. and Nakamura, S. (1985) Nucleic Acid Res. 13, 5869-5882.
5. Gubler, U., Chua, A.O., Stern, A.S., Hellmann, C.P., Vitek, M.P., Dechiara, T.M., Benjamin, W.R., Collier, K.J., Dukovich, M., Familletti, P.C., Fiedler-Nagy, C., Jenson, J., Kaffka, K., Kilian, P.L., Stremlo, D., Wittreich, B.H., Woehle, D., Mizel, S.B. and Lomedico, P.T. (1986) J. Immunol. 136, 2492-2497.
6. Nishida, T., Nishino, N., Takano, M., Kawai, K., Bando, K., Masui, Y., Nakai, S. and Hirai, Y. (1987) Biochem. Biophys. Res. Commun. 143, 345-352.
7. Okayama, H. and Berg, P. (1983) Mol. Cell. Biol. 3, 280-289.
8. Maekawa, T. and Imamoto, F. unpublished data.
9. Nakai, S. and Hirai, Y. manuscript in preparation.
10. Laemmli, U.K. (1970) Nature 227, 680-685.
11. Wingfield, P., Payton, M., Tavernier, J., Barnes, M., Shaw, A., Rose, K., Simona, M.G., Demczuk, S., Williamson, K. and Dayer, J-M. (1986) Eur. J. Biochem. 160, 491-497.