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Cloning and expression of an interleukin-1β precursor and its conversion to interleukin-1β

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A gene coding for a N-terminal precursor of interleukin-1β (IL-1β) was cloned and expressed in *E. coli*. The isolated Met-Glu-Ala-Glu-IL-1β precursor was enzymatically converted to IL-1β by means of dipeptidylaminopeptidase (DAP I). This method ensured a correct N-terminal residue and the often observed expression of Met-IL-1β was thus avoided. The pure and physically homogeneous product exhibited the characteristic properties of natural IL-1β. The in vitro biological activity was measured in the lymphocyte-activating factor assay and was compared to that of natural IL-1β isolated from stimulated monocyte culture using exactly the same purification procedure. The specific biological activity of both products was 2×10^{-8} U/mg indicating that the recombinant product exhibits full biological activity.

Interleukin-1β; Gene expression; Enzymatic conversion; Dipeptidylaminopeptidase

1. INTRODUCTION

Interleukin-1 (IL-1) is a common designation for the α and β type of IL-1 molecules with a broad range of biological activities related to the acute-phase response to microbial infection or injury. Recently in vitro studies have demonstrated that IL-1β selectively affects the insulin producing β-cells of the pancreas, resulting in decreased synthesis of insulin and finally destruction of the β-cells [1]. Access to large amounts of well characterized recombinant IL-1β is required for further elucidation of the above-mentioned cytotoxicity. Here we report a simple and efficient large scale procedure for the production of IL-1β based on a method in which an *E. coli* produced precursor to IL-1β is converted to a natural IL-1β in vitro using the enzyme dipeptidylaminopeptidase I (DAP-I, EC 3.4.14.1). See fig.1 for detailed explanation of the strategy. To enable a specific determination of the biological activity of the recombinant IL-1β, interleukin-1β was purified from activated monocytes using the same purification procedure as described for the bacterial protein, thus eliminating the uncertainty present when different purification procedures are used.

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Abbreviations: r-IL-1β, recombinant interleukin-1β; m-IL-1β, monocyte interleukin-1β; DAP-I, dipeptidylaminopeptidase I; PDMS, plasma desorption mass spectrometry

2. EXPERIMENTAL

2.1. *Construction of expression vector and conditions for expression*

The hGH expression plasmid pHD86SP13 (SP9b) [2] was cut with *Bam*HI/*Sal*I, treated with Klenow DNA polymerase I + dNTP and religated, resulting in the plasmid pHD166SP9b. This plasmid was cut with *Pvu*II/*Eco*RI to remove the hGH gene. Instead the *Eco*RI/*Hin*dIII poly linker fragment from pUC18 filled in at the *Hin*dIII site was introduced, resulting in the expression vector pHD162SP9b. The synthetic IL-1β gene was inserted between the *Cla*I and *Xma*I sites to obtain the expression plasmids pHD228 or pHD230. *E. coli* MC1061 [3] harbouring the plasmid pHD228 or pHD230 was propagated in TB medium containing 50 mg ampicillin/l at 37°C to an A_{600} between 15 and 20. The cells were harvested by centrifugation.

2.2. *Isolation of MEAE-IL-1β and its conversion to IL-1β*

MEAE-IL-1β was extracted from bacterial suspension as described by Kronheim [4] except that the buffer used was 0.2 M Na-citrate, pH 3.5. The extract was filtered on 0.8 μm Millex filter, dialyzed against 20 mM Na-citrate, 0.5 mM

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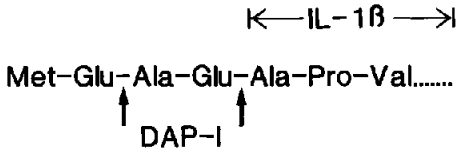

Fig.1. Conversion of MEAE-IL-1β to IL-1β. The Ala-Pro sequence will not be cleaved as DAP-I does not recognize Pro as the second amino acid.

EDTA, pH 4.0, and applied to a FF-S-Sepharose HR 16/10 column.

The pooled MEAE-IL-1β fraction was converted to IL-1β by adding 0.38 U of DAP-I (Cathepsin C, Boehringer Mannheim GmbH) per mg protein directly to the FF-S pooled fractions. After 45 min at 37°C, the reaction mixture was dialyzed overnight against 10 mM Na-citrate, 1 mM EDTA, pH 4.0, at 4°C. The dialysate was adjusted to pH 9.5 and applied to a FF-Q-Sepharose HR 10/10 column. The recombinant IL-1β fraction was concentrated by ultrafiltration using a 10 ml Nova cell (8 kDa cut off) to a volume of 2.0 ml and applied to a G-75 Sephadex K16 (1.6 × 90 cm) column. The product was stored in 50 mM Tris-Cl, pH 8.0, at −20°C. Monocyte IL-1β from activated human monocytes [5] was purified using exactly the same methods as for recombinant IL-1β, except that the DAP-I conversion was omitted.

2.3. *Analytical methods*

The IL-1β was subjected to amino acid analysis (LKB 451 Alpha Plus) after 24 h hydrolysis at 110°C in constant boiling HCl. Free cysteine was determined as the pyridyl-ethyl cysteine derivative. The protein was sequenced by automated Edman degradation (Applied Biosystems 477A). The molecular mass was determined by the plasma desorption/time of flight technique [6]. Electrophoresis was carried out using 8–25 gradient Phastgels and IEF 3–9 Phastgels (Pharmacia-LKB Biotechnology). Reverse-phase chromatography was performed on a Brownlee column (Applied Biosystem 130A) (RP300, C8 (2.1 × 30 mm)). $E^{277}_{0.1\%} = 0.59$ was used for protein determination. The amount of monocyte-derived IL-1β was determined by means of an internal ELISA assay calibrated against the WHO international reference IL-1β standard. The biological activity and test for endotoxins were performed as described in [5] and [7].

3. RESULTS AND DISCUSSION

3.1. *Design and construction of the IL-1β gene*

The synthetic gene was designed on the basis of the published amino acid sequence of the protein [8] using the codons most frequently found in highly expressed *E. coli* genes [9]. To allow for further manipulation, convenient cloning of the coding region and insertion of the gene in a suitable expression vector, unique restriction cleavage sites were introduced into the coding region as well as at the 5′- and 3′-ends. The gene was N-terminal extended with a sequence encoding the amino acids Met-Glu-Ala-Glu (MEAE) and followed by two tandemly arranged stop codons (TAG, TAA) at the C-terminus.

The synthetic promoter and Shine and Dalgarno sequence [10] was chosen as it has been shown to direct highly efficient synthesis of B-hGH in *E. coli* in excess of 10 μg/ml ($A_{600} = 1$) [2]. However, the total extract from cells harbouring the initial expression plasmid pHD228 was found in a IL-1β-specific ELISA only to contain immunoreactive material corresponding to approx. 250 ng IL-1β/ml ($A_{600} = 1$).

Recent studies have highlighted the key role of preventing secondary structure formation on the mRNA, particularly in the region comprising the Shine and Dalgarno sequence and the translation initiation codon [11,12]. During the DNA sequencing of the synthetic IL-1β gene strong secondary structure was found to be formed at the beginning of the gene (amino acid 4–7, GAG GCG CCG GTT). If this secondary structure is formed in vivo it may inhibit the translation efficiency. Alteration of the sequence by in vitro mutagenesis to be similar to the corresponding sequence from the published IL-1β cDNA [8] (GAA GCA CCT GTA)

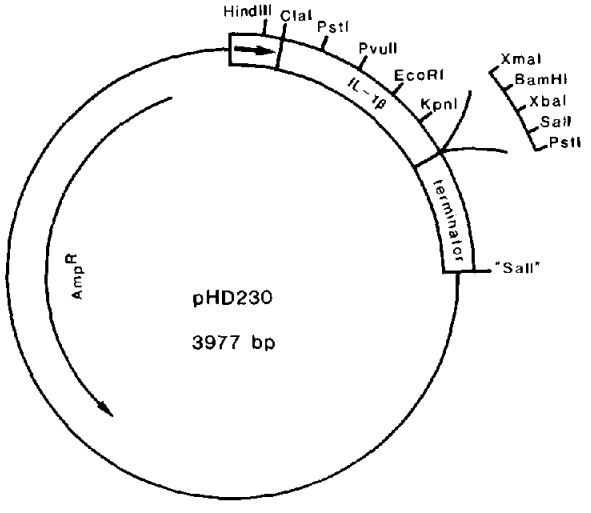

Fig.2. Schematic drawing of the expression plasmid. The plasmid consists of a synthetic promoter Shine-Dalgarno sequence [10], the synthetic MEAE IL-1β gene, part of the poly linker from pUC19, the fd transcription terminator, and the vector pAT153 (Δ*Sal*I/Δ*Eco*RI).

resulted in a plasmid (pHD230) which very efficiently expressed IL-1β to a level of approx. 10 μg/ml ($A_{600} = 1$) (fig.2).

3.2. *Extraction and purification*

The purification profiles of the three chromatographic separations are shown in fig.3. The frac-

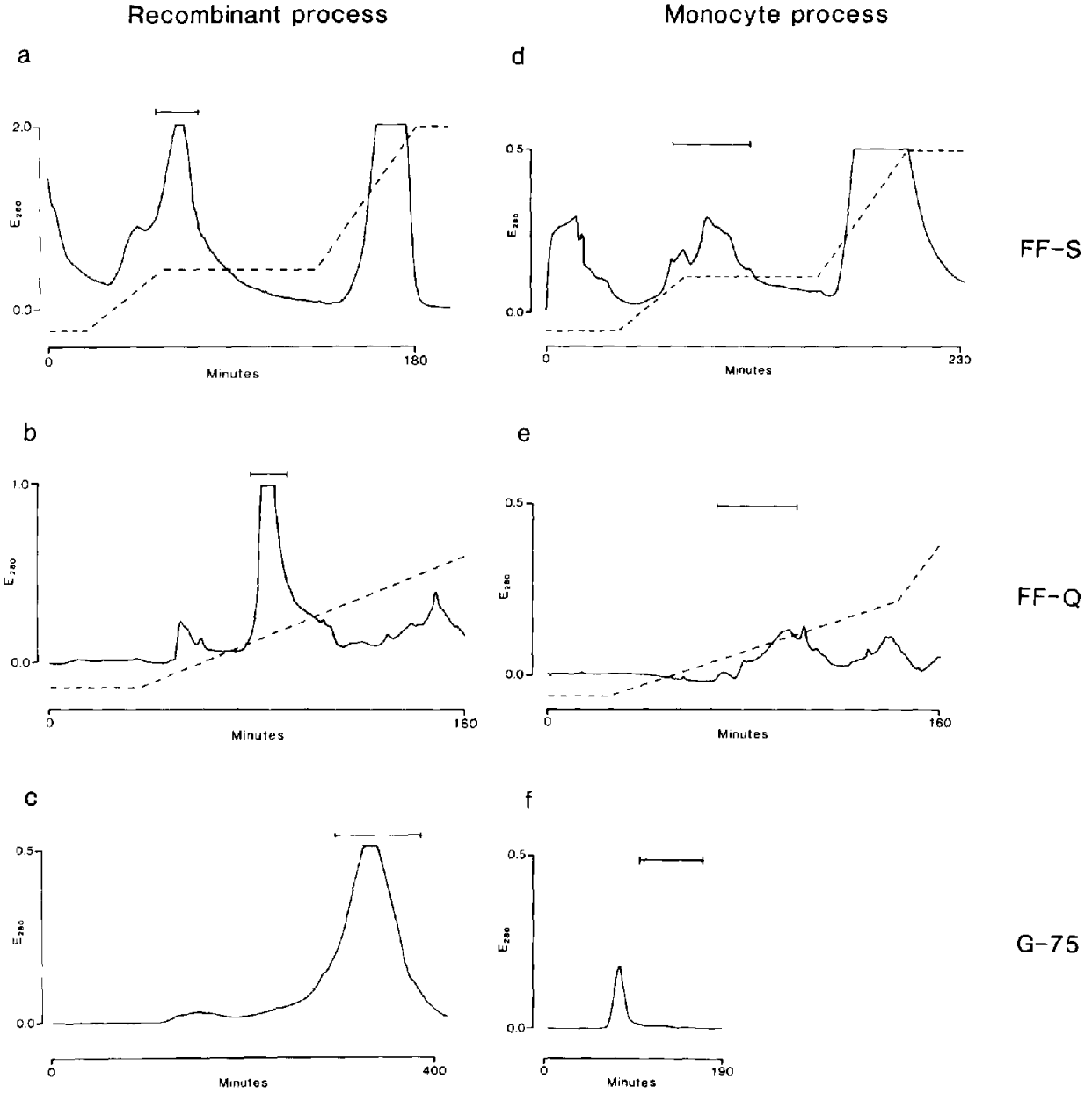

Fig.3. Purification of r-IL-1β and m-IL-1β. (Upper panel) FF-S-Sepharose chromatography in 30 mM Na-citrate, pH 4.0, 0–1 M NaCl gradient at 1.3 ml/cm^2 per min, 4°C of r-IL-1β (a) and m-IL-1β (d). (Middle panel) FF-Q-Sepharose chromatography in 50 mM Tris-Cl, pH 9.5, 0–0.4 M NaCl gradient at 1.3 ml/cm^2 per min, 4°C of r-IL-1β (b) and m-IL-1β (e). (Lower panel) G-75 gel filtration in 0.5 M Na-acetate, pH 3.5, of r-IL-1β on a 1.6 × 90 cm column at 0.1 ml/min, 4°C (c). m-IL-1β was purified on a 0.9 × 60 cm column at 0.1 ml/min, 4°C (f). The peak observed at 280 nm comprises high-molecular-mass proteins. No absorbance is observed in the immunoreactive fractions due to the very low protein concentration (ng/ml). (|——|) Fractions pooled; (– – –) gradient profile.

tions pooled in each of the six panels are indicated. Two different columns were used for gel filtration, thus retention times cannot be compared. The peak observed in the purification of m-IL-1β is a high-molecular-mass fraction which eluted before IL-1β. The recombinant process was followed by SDS electrophoresis (fig.4). Due to the acidic extraction procedure used only a few other proteins are co-extracted. The 14 kDa band present is lysozyme added during extraction (lanes 2 and 3). A slightly higher mobility of the product is observed in lanes 4 and 5 due to the decrease of molecular mass after DAP-I conversion. The purified IL-1β is shown in lane 6.

3.3. *Characterization*

Total amino acid analysis (table 1) and positive identification of the first 42 amino acids by sequence analysis confirmed the primary sequence of the IL-1β. Sequence analysis of 2600 pmol IL-1β (from absorption measurement) indicated Ala as the only N-terminal amino acid (with an initial yield of 80%) in accordance with the primary structure and gene sequence proving that the enzymatic cleavage with DAP-I resulted in removal of the MEAE sequence. The PTH detection limit is 5 pmol for the stable derivatives and 10 pmol for the rest. A result of 1.6 mol sulphydryl per mol

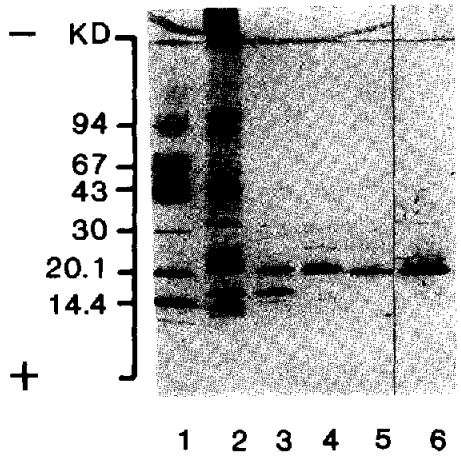

Fig.4. SDS electrophoresis. Samples for SDS electrophoresis were diluted in 0.1 M Tris-Cl, 1 mM EDTA, 2.5% SDS, 0.1 M DTT, 0.01% bromophenol blue, pH 8.0. 1 μl was applied. Lanes: 1, low molecular mass markers; 2, total cell extract from *E. coli* MC1061/pHD230; 3, extract; 4, FF-S pool; 5, FF-Q pool; 6, G-75 pool.

Table 1

Amino acid composition

Amino acid	Found (nmol)	Composition (mol/mol)	Theoretical (mol/mol)
Asx	3.97	17	17
Thr	1.32	5.6	6
Ser	2.71	11.6	14
Glx	5.59	23.9	23
Pro	2.34	10.0	8
Gly	1.98	8.5	8
Ala	1.21	5.2	5
PE-Cys		1.6	2
Val	2.59	11.1	11
Met	1.40	6.0	6
Ile	1.17	5.0	5
Leu	3.55	15.2	15
Tyr	0.92	3.9	4
Phe	2.07	8.9	9
His	0.25	1.1	1
Lys	3.54	15.2	15
Arg	0.80	3.4	3
Trp	n.d.		1
Total			153

Samples were subjected to amino acid analysis after 24 h hydrolysis at 110°C in constant boiling HCl. The mol/mol composition was calculated relative to Asx

IL-1β indicates that the two cysteine groups do not form a disulphide bond. The theoretical molecular mass of IL-1β from the amino acid sequence considering the content of 13-C and 15-N was calculated to be 17 359 a.m.u. The molecular mass determined experimentally by PDMS was 17 360.1 a.m.u. The composition and purity of the recombinant material was evaluated by means of reverse-phase chromatography on a RP300 C8 column and electrophoresis (fig.5). The mean specific biological activity was calculated to 3.24×10^8 U/mg with a standard deviation of 0.6 using the WHO reference standard as reference. Results were based on IL-1β from three different batches. This is in agreement with the specific biological activity calculated for the purified natural monocyte-derived IL-1β of 2.0×10^8 U/mg and with the reported specific biological activities of recombinant IL-1β, 4.6×10^8 U/mg [13], 1.95×10^8 U/mg [4] and of natural IL-1β, 3.2×10^8 [14]. The level of endotoxins was found to be 13 pg/μg IL-1β, an amount which does not interfere with the biological assay.

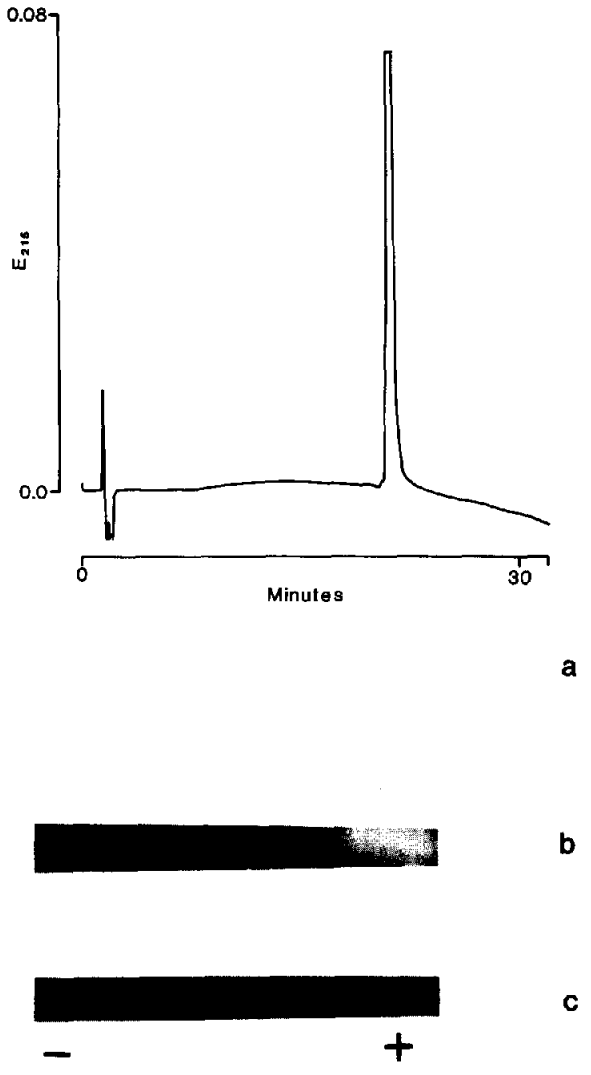

Fig.5. Characterization of recombinant IL-1β. (a) RP-HPLC on RP300, C8 column. A buffer, 0.1% trifluoroacetic acid; B buffer, 0.085% trifluoroacetic acid, 80% CH_3CN. Flow, 0.1 ml/min; temp., 26°C. (b) Native PAGE. Samples were diluted in 0.1 M Tris-Cl, 1 mM EDTA, 0.01% bromophenol blue, pH 8.0. 1 μl was applied. (c) Isoelectric focusing. Samples were diluted in 7 M urea. 1 μl was applied.

In conclusion the procedure described results in a highly purified IL-1β bulk material possessing the correct N-terminal amino acid, alanine. The process is feasible for large scale production of IL-1β thus avoiding the tedious separation of Met-IL-1β, IL-1β and desAla-IL-1β described earlier [13,15].

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