Expression, isolation and purification of the antiviral peptide Alloferon-1

Volker Klix[1], Marian Beshara[1,2] and Monier Tadros[1*]

1 - International Laboratory for Biotechnology and Consulting (ILBC GmbH), Hermannswerder 14, 14473 Potsdam
2 - The German University in Cairo, El Tagamoa El Khames, New Cairo City, Egypt
* Corresponding author: ILBC GmbH, phone: +49 331 2300 412, email: monier.tadros@gmx.de

Summary

The development of new antiviral products is very much needed because viral diseases present a worldwide problem. Many natural products contain biologically active substances which can inhibit viruses. Alloferon-1 is derived from the blow fly *Calliphora vicina* and has an inhibitory effect on the reproduction of *Human Herpes Virus-1* (HHV-1) and *Coxsackie B in vivo* (1). No negative effects on human cell lines were observed.

In this report, we present two different genetic systems for the production of native, natural Alloferon-1. For the successful overexpression we used a fusion construct of Alloferon-1 with the signal peptide ompA, directing the antiviral peptide to the outer cellular space or using the ubiquitin-like SUMO-protein respectively. In both cases Alloferon-1 was purified using reverse phase C_{18} HPLC. It was necessary to rechromatograph the Alloferon-1 peak using the same column to obtain highly pure Alloferon. We were also able to purify Alloferon-1 using cation exchange chromatography (FPLC), followed by removing residual salts by dialysis or acetone precipitation, followed by rechromatography on reverse phase C_{18} HPLC. Both methods worked in a satisfactory and reliable manner. The yield was about 4 mg/L culture to 3 mg/L culture respectively.

Introduction

Alloferon-1 is a tridecapeptide (H-His-Gly-Val-Ser-Gly-His-Gly-Gln-His-Gly-Val-His-Gly-OH) isolated from the bacteria-challenged larvae of the blow fly *Calliphora vicina* (2). *In vitro* experiments revealed that Alloferon-1 has a stimulatory activity toward natural killer lymphocytes and induces the IFN synthesis in mice *in vivo* (1). Further studies showed that Alloferon-1 is able to inhibit the replication of DNA and RNA viruses *in vivo* as demonstrated for *Human Herpes Virus* Type-1 and *Coxsackie B* (1, 3). In contrast, no toxicity effects were found on different human cell lines (3). These findings lead to the conclusion that Alloferon-1 may be used as a potential antiviral agent in human healthcare.

In former studies it has been shown that biologically active Alloferon-1 can be synthesized *in vitro* (2,3). Here we present the possibility to overexpress Alloferon-1 in the heterologous system of *E. coli* without any modifications compared to the amino acid sequence of the native peptide. The signal peptide of the outer membrane protein ompA from *E. coli* was used for secretion of the peptide into the extracellular space. The SUMO protein tag system (Invitrogen, Germany) was used as a control system for the overexpression of Alloferon-1. For purification purposes of both systems reverse phase C_{18} (RP18) HPLC or cation exchange chromatography (CIEX) were used. With the presented system it is possible to reliably produce Alloferon-1 in great amounts for a comparably low price.

Materials & Methods

All plasmids used in this study were obtained from the manufacturer MWG Eurofins GmbH or cloned according to manufacturers instructions (pET-SUMO; Invitrogen).

E. coli BL21 strain was used for the overexpression of the fusion construct. Cells were grown in LB-medium to an optical density of 0.6 at 600 nm and induced with 1 mM IPTG. After 5 h of growth the culture was harvested by centrifugation at 6000 rpm for 20 min, 4 °C. For the ompA construct the supernatant was lyophilized and resuspended in water 1/10 of starting volume. The SUMO construct was extracted by sonication of the cell pellet followed by batch immobilized metal ion affinity chromatography (NiNTA) purification. The NiNTA purified material was cleaved using SUMO protease (Invitrogen, GmbH) to separate the SUMO fusion from the target peptide.

Separation of Alloferon-1 from lyophilised medium or from SUMO and SUMO protease, respectively, was done by two-step RP18 HPLC (AppliChrom® OTU LipoMare C_{18}, 105 Å, 5 µm, 250 x 4.6 mm) on two different gradients 0.05 % TFA/Acetonitrile or cation exchange chromatography (CIEX) (HiTrap CM FF 5ml, GE Healthcare Life Sciences) after dialysis against water followed by RP18 HPLC.

Purified peptide was analyzed on Tricine-SDS-PAGE and via mass-spectrometry and amino acid sequence analysis. Synthetic Alloferon-1 was synthetised following the peptide sequence of natural Alloferon-1 by the manufacturer Peptides & Elephants GmbH.

Results and Discussion

Secretion of recombinant Alloferon using ompA signal peptide

In order to overproduce Alloferon-1, the gene sequence encoding Alloferon-1 was cloned in-frame with the *E. coli ompA* (outer membrane protein A) signal sequence. Under aerobic growth conditions Alloferon-1 was secreted into the medium, with the ompA signal peptide being correctly processed as revealed by N-terminal sequence analysis. Alloferon-1 was observed in induced cultures compared to non-induced ones (Fig. 1 A, B). The maximum expression was observed after 6 hours.

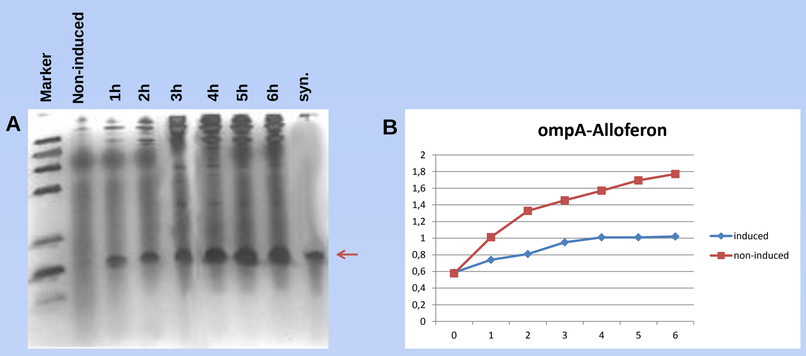

Figure 1: A) Visualisation of Alloferon-1 in precipitated culture supernatant of induced and non-induced ompA-Alloferon in *E. coli* BL21 for 6 h after induction. Synthetic Alloferon-1 (syn.) was used a positive control. Separated on Tricine-SDS-PAGE, visualized by silver staining. B) Growth curve of induced cultures compared to non-induced ones at 600 nm wavelength for 6 h.

Method 1: Purification of Alloferon-1 using RP18 (HPLC)

Culture supernatant of the induced culture was collected after 6 hours, cells were removed by centrifugation and lyophilised. The lyophilised material was dissolved in H_2O + 0.05 % TFA (1/10 of the original volume). Alloferon was purified using RP18 (HPLC). Peaks containing Alloferon-1 (as confirmed by mass spectrometry) were collected and re-chromatographed using the same column under the same conditions (Fig. 2A). The identity and purity of the peak containing Alloferon-1 was confirmed by SDS-PAGE (data not shown), mass spectrometry and N-terminal sequence analysis (Fig. 2B).

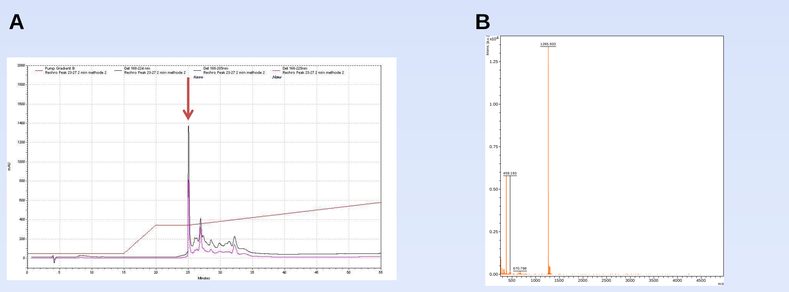

Figure 2: A) Elution pattern of isolated Alloferon-1 fraction separated by RP18 HPLC. Elution was performed with H_2O containing 0.05 % TFA as solvent A and acetonitrile as solvent B. The gradient is indicated in red (gradient 1). After the major Alloferon-1 peak (red arrow) small minor peaks were detected. The flow-rate was 1 ml/min at room temperature. B) Mass spectrometry data of the Alloferon-1 fraction obtained from RP18 column chromatography.

Method 2: Purification of Alloferon-1 using Cation Exchange Chromatography (CIEX)

Lyophilized culture supernatant was dissolved in H_2O and dialysed against water to get rid of residual salts present in the sample and applied to CIEX column. Fractions 14-17 (Peak 2) were collected during the elution gradient (Fig. 3A). The obtained material was lyophilized and resuspended in about 0.5 ml H_2O. The resolved material was applied on RP18 HPLC (see Fig. 3B).

Three main peaks were resolved and their polypeptide content was analyzed by SDS-PAGE (data not sown). We concluded that peak 2 contains Alloferon-1. Mass spectrometry and N-terminal sequence analysis confirmed this result.

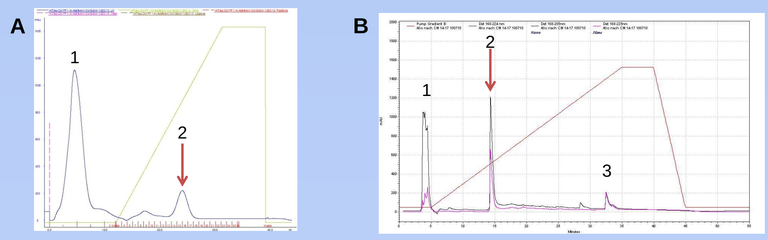

Figure 3: A) Chromatogram of dialysed medium supernatant on CIEX at 215 nm. B) Chromatogram of CIEX peak 2 on RP18 HPLC with gradient 2. The position of Alloferon-1 is marked by red arrows.

Over expression of Alloferon-1 using SUMO

The commercially available SUMO expression system was used to verify Alloferon-1 data. After expression and harvesting of the cells, the SUMO-Alloferon was extracted and separated from cell debris by centrifugation and NiNTA chromatography.

After NiNTA purification of SUMO-Alloferon-1, the fusion construct was precipitated with acetone and afterwards cleaved by addition of SUMO protease (Invitrogen, Germany). The cleaved material was applied on RP18 HPLC. For the cleaved material a signal was detected at the same time point as for synthetic Alloferon in the given gradient (Fig. 4).

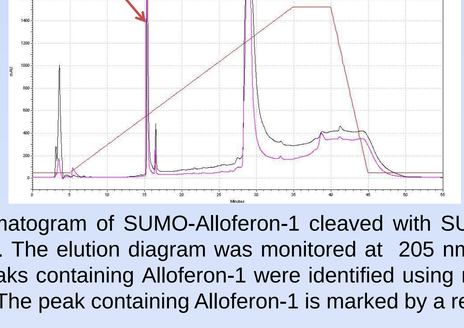

Figure 4: Chromatogram of SUMO-Alloferon-1 cleaved with SUMO protease on RP18 HPLC. The elution diagram was monitored at 205 nm and 225 nm wavelength. Peaks containing Alloferon-1 were identified using mass spectrometry analysis. The peak containing Alloferon-1 is marked by a red arrow.

Conclusion

We were able to overexpress Alloferon-1 in *E. coli* fused to a signal sequence leading the mature peptide to the outer cellular space. From the culture supernatant we purified Alloferon-1 to homogeneity (4 mg/l culture) using either a two-step RP18 HPLC system or cation exchange column chromatography (CIEX) followed by RP18 HPLC. Because of the necessity to remove residual salt from the samples prior to CIEX column chromatography we favour the two-step RP18 as the most effective system of purification for Alloferon-1 from the medium supernatant. The present work opens the way for the production of antiinfective and antitumoral biopharmaceuticals and for new possibilities in the development of much needed antiviral substances.

Literature

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