Expression, Purification, and Characterization of Functional Recombinant Human Daintain/AIF-1 in *Escherichia coli*

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Daintain/AIF-1 was identified from injured rat carotid arteries and porcine intestine in the mid 1990s. It is involved in autoimmune disorders, chronic rejection of allografts, gliomas, and breast cancer. Since it is convenient and economical to obtain such a peptide biologically, in this study, we describe the expression, purification, and characterization of recombinant human daintain/AIF-1 (rhdaintain/AIF-1). The backbone of vector pET32a, a high-level expression plasmid, was used to construct the pET32a-daintain/AIF-1 plasmid for daintain/AIF-1 expression in *Escherichia coli*. The recombinant daintain/AIF-1 protein was solubly expressed in the BL21 (DE3) strain and was purified by Ni^{2+} affinity chromatography. After purification, the recombinant protein showed the expected size of 18 kDa on Tricine-SDS-PAGE gels which was further confirmed by Western blotting. A total of 34.0 mg of high purity (over 98%) rhdaintain/AIF-1 was obtained from 1 L culture. The recombinant peptide was able to increase blood glucose elimination rates and enhance the proliferation of human MCF-7 cells. These results suggest that biological activity of the recombinant peptide was preserved after purification.

Key words: Daintain/AIF-1, Blood Glucose Elimination, MCF-7 Proliferation

Introduction

In the mid 1990s, we isolated and characterized a peptide which we initially called daintain due to its activity of a well-sized peptide influencing insulin secretion (Chen *et al.*, 1994, 1997). According to the later investigation of this peptide, we still call it daintain which means "a well-sized inflammatory peptide factor". At the same time, Utans *et al.* (1995, 1996) identified a novel macrophage factor in rat and human hearts that was undergoing chronic rejection and named it allograft inflammatory factor-1 (AIF-1). Daintain and AIF-1 share an almost identical amino acid sequence, so we have termed the peptide daintain/AIF-1.

Daintain/AIF-1 is a 147-amino acid (16.8 kDa) cytoplasmic calcium-binding, EF-hand, interferon-gamma-inducible peptide, which is encoded within the human histocompatibility complex class III genomic region on chromosome 6p21.3 (Utans *et al.*, 1995, 1996; Deininger *et al.*, 2002). Daintain/AIF-1 contains a 44-amino acid segment with the sequence pattern -KR-KK-GKR, which are structural characteristics for peptide hormone precursor proteins (Chen *et al.*, 1997). Daintain/AIF-1 is expressed in macrophage cell lines (Utans *et al.*, 1996), experimental autoimmune encephalomyelitis, neuritis and uveitis models (Schluesener *et al.*, 1998; Pashenkov *et al.*, 2000), human cerebral infarctions (Postler *et al.*, 2000), devascularized skeletal muscles in rat systems (Kuschel *et al.*, 2000), and breast ductal tumour epithelia (Liu *et al.*, 2008). Vascular smooth muscle cells also expressed daintain/AIF-1 when activated by cytokines or injured by balloon angioplasty (Autieri, 1996; Autieri *et al.*, 2000). Microglial response factor-1 and ionized calcium-binding adapter molecule-1 are homologues of daintain/AIF-1 and have been localized to activated infiltrating macrophages in degenerating and inflamed tissues (Tanaka *et al.*, 1998; Imai *et al.*, 1996; Ito *et al.*, 1998). Moreover, in human disease, daintain/AIF-1 is expressed by both the glandular and stromal cells in human eutopic endometrium and endometriosis (Koshiba *et al.*, 2005). Recent studies also indicated that daintain/AIF-1 overexpression enhances endothelial cell

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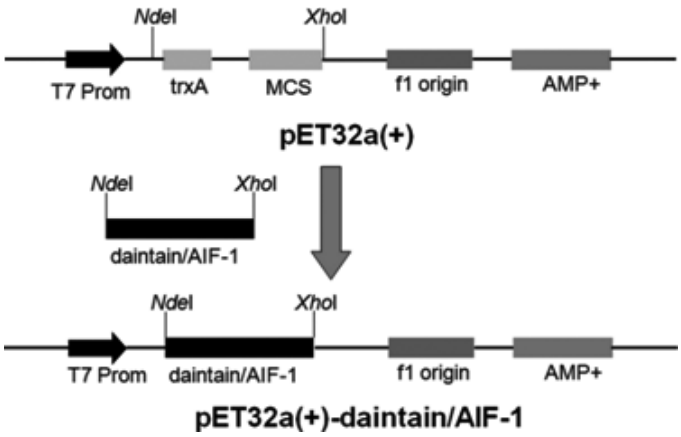

Fig. 1. Construction of recombinant pET32a-daintain/AIF-1 plasmid.

activation, proliferation, and migration (Tian *et al*., 2009; Jia *et al*., 2010).

Therefore, daintain/AIF-1 has been widely investigated in biology and clinical science. Unfortunately, it can currently only be obtained from porcine intestine at very low yields. This greatly limits the application of daintain/AIF-1 in various kinds of researches. To conveniently and economically obtain daintain/AIF-1, we undertook a genetic engineering approach. We optimized the cultivation parameters to express the recombinant protein in soluble form in *Escherichia coli*, obtaining a high yield of recombinant human daintain/AIF-1 (rhdaintain/AIF-1) after purification which maintained its biological activity.

Material and Methods

Materials

The *Escherichia coli* strains DH5*α* and BL21 (DE3) and the breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from China Center for Type Culture Collection (CCTCC) and were used for most routine manipulations. Plasmid pET32a was purchased from Novagen (Madison, WI, USA). T4 DNA ligase and *Taq* DNA polymerase were from TaKaRa Biotechnology (Kyoto, Japan). All restriction enzymes were purchased from Invitrogen (Carlsbad, CA, USA). Ni^{2+}-NTA resin was obtained from GE Healthcare (Uppsala, Sweden).

Construction of pET32a-daintain/AIF-1 expression plasmid

Total RNA was isolated from MCF-7 cells following the standard protocol (Trizol, Invitrogen) and used as a template. First-strand cDNA was synthesized with a first-strand synthesis kit (Invitrogen) and used for reverse transcription-polymerase chain reactions (RT-PCR). The following synthetic oligonucleotide primers: sense, 5'-GCCATATGATGAGCCAAACCAGGGATTT-3', and antisense, 5'-CACTCGAGAATCAGGGCAACTCAGAGAT-3' (underlined sequences indicate restriction endonuclease sites), were synthesized based on the daintain/AIF-1 cDNA sequence (accession No. U19713) to amplify the daintain/AIF-1 coding region by PCR. The resulting fragment consisted of a 5'-*Nde*I site, the daintain/AIF-1 sequence, and a 3'-*Xho*I site. The DNA fragment was then cloned into the pET32a expression vector. The plasmid was used to transform the *E. coli* DH5*α* strain to generate ampicillin-resistant colonies. Clones resistant to ampicillin were selected and plasmid DNA isolated, before examination by restriction endonuclease mapping to confirm the correct orientation of the cloned DNA. The resulting plasmid was named pET32a-daintain/AIF-1 and its structure is shown in Fig. 1.

Rhdaintain/AIF-1 expression and purification

Overnight cultures of *E. coli*, strain BL21 (DE3), transformed with pET32a-daintain/AIF-1 were diluted 1:100 in 1 L LB medium containing ampicillin (50 μg/mL) and incubated at 30 °C with shaking to an OD_{600} of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM. After a further 3 h of growth, cells were pelleted at 6,000 × *g* for 10 min at 4 °C and then resuspended in

30 mL binding buffer [20 mM Na_3PO_4, 500 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, pH 7.4]. Cells were then sonicated and centrifuged at 18,000 × *g* for 20 min at 4 °C and filtered through a 0.45-μm pore-size filter. The cleared lysate was incubated in a batch with 10 mL Ni^{2+}-NTA resin for 2 h at 4 °C. The resin was loaded onto a column and was thoroughly washed with binding buffer, before the bound proteins were eluted by a linear gradient of 40–200 mM imidazole in buffer [20 mM Na_3PO_4, 500 mM NaCl, 10% (v/v) glycerol, pH 7.4]. Fractions containing rhdaintain/AIF-1 were pooled and desalted with Sephadex G25 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Lyophilization was carried out after freezing fractions of Sephadex G25.

SDS-PAGE and Western blotting

Protein samples were dissociated into monomers by the addition of the nonionic detergent Triton X-100 and β-mercaptoethanol, analysed using a 15% (w/v) tricine SDS-PAGE gel, and stained with Coomassie Brilliant Blue R-250 for 4 h at room temperature.

Western blotting was performed as described previously (Fu *et al.*, 2006). Protein samples (0.05 μg) were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and blocked for 1 h with 2% BSA (bovine serum albumin) at room temperature. After repeated washing with PBST (PBS with 0.1% Tween 20), the membranes were incubated with 1:1,000 diluted mouse monoclonal anti-daintain/AIF-1, which was prepared by our laboratory (Fu *et al.*, 2006), overnight at 4 °C. After repeated washing with PBST, the membranes were incubated with 1:2,000 diluted alkaline phosphatase-labeled goat anti-mouse secondary antibody (Boster, Wuhan, China) for 2 h at room temperature. The bands were visualized using the Western Blue Stabilized Substrate (Promega, Madison, WI, USA).

Determination of blood glucose levels

BALB/c mice (18–20 g, from the Standard Animal Center of the China Medical College) were used. D-Glucose was injected intravenously (1 mg/g) into fasted BALB/c mice, alone or combined with rhdaintain/AIF-1 or combined with porcine daintain/AIF-1, at a dose of 5 μg/g (~270 nM/kg) body weight. Blood samples were taken after 1, 5, 15, 30, and 60 min. Individual blood glucose values were measured using an Accu-Chek Advantage blood glucose monitor (Roche Diagnostics, Shanghai, China). This study was approved by the institutional ethics committee of Hubei province, China.

Cell culture and proliferation assay

The human MCF-7 and MDA-MB-231 cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin in a humid atmosphere of 5% CO_2 at 37 °C. For proliferation assays, equal numbers of MCF-7 cells were seeded into 24-well plates at a density of $1 \cdot 10^4$ cells per well. 1 mg of lyophilized rhdaintain/AIF-1 was dissolved in PBS to a final volume of 1 mL and stored at –20 °C after filtration sterilization. Rhdaintain/AIF-1 or porcine daintain at a final concentration of 1 μg/mL was added to the DMEM medium, and the same volume of PBS was added as a control. Cells were cultured for 7 d with media changes on the 3rd and 5th day. After 1, 3, 5 and 7 d, viable cells were counted by trypan blue exclusion using a standard hemocytometer.

Statistical analysis

The statistical significance of differential findings between experimental groups and controls was determined by the Student's *t*-test. For all statistical analyses, significance levels were set at $P < 0.05$.

Results

Design of pET32a-daintain/AIF-1 expression plasmid

pET32a is designed for cloning and high-level expression of peptide sequences (Lavallie *et al.*, 1993). If the daintain/AIF-1 coding sequence has been correctly inserted into the cloning sites of pET32a, the resulting fusion protein will contain cleavable Trx-, His- and S-Tags, and an uncleavable His-Tag. This makes the fusion protein almost twice as large as daintain/AIF-1, by molecular mass, and the protein of interest can be obtained after enterokinase cleavage and another purification process. Furthermore, all the restriction sites in the multiple cloning site of pET32a will introduce some amino acid residues coded by the vector sequence to the *N*-terminal of the

secreted rhdaintain/AIF-1. There is only a single *Nde*I (CATATG) restriction site in the pET32a vector. This ATG is the T7 transcription start, encoding Met, and the *N*-terminal of daintain/AIF-1 is also Met. Therefore, we designed two primers containing an *Nde*I restriction site at the 5'end and a 3'*Xho*I site to amplify the daintain/AIF-1 coding sequence and inserted it into the pET32a vector cleaved by the *Nde*I and *Xho*I restriction enzymes. The resulting recombinant DNA sequence therefore only contained the daintain/AIF-1 coding sequence and the His-Tag coding sequence (Fig. 1). This construction (pET32a-daintain/AIF-1) should produce a recombinant protein of approximately 18 kDa (Fig. 2A) with no redundant amino acid residues attached to its *N*-terminal.

Expression and purification of rhdaintain/AIF-1

After the induction of protein expression with IPTG, the bacteria were pelleted and sonicated, and rhdaintain/AIF-1 was identified by Coomassie Blue staining. We found that the recombinant protein was almost equally distributed in the pellet and supernatant (Fig. 2A). To avoid the tedious denaturing and refolding process, only the supernatant of the induced bacteria cells' lysate was applied to the Ni^{2+}-NTA resin. First, 20 mM imidazole was used to elute components of the supernatant that nonspecifically were bound to the Ni^{2+}-NTA resin column. This was followed by two isocratic steps of 40 mM and 200 mM imidazole, respectively, to elute the bound protein (Fig. 2A). After Ni^{2+} affinity chromatography, the purity of the recombinant proteins (Fig. 2A, lane 4) reached 98%. Then, the 200 mM imidazole eluate was pooled and desalted with Sephadex G25, resulting in 34.0 mg of lyophilized rhdaintain/AIF-1 from 1 L culture.

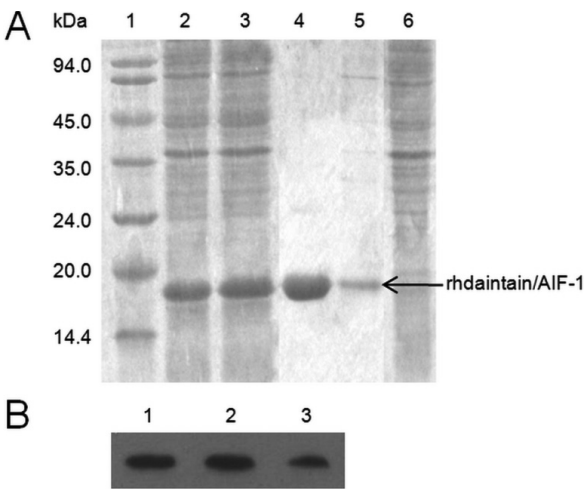

Fig. 2. Analysis of rhdaintain/AIF-1 protein by SDS-PAGE and Western blotting. (A) SDS-PAGE analysis of the expression and purification of soluble fusion protein. Lane 1, protein molecular weight marker; lane 2, pellet of lysate; lane 3, supernatant of lysate; lane 4, eluted with 3× bed volume of 200 mM imidazole; lane 5, washed with 10× bed volume of 40 mM imidazole; lane 6, washed with 10× bed volume of 20 mM imidazole. (B) Anti-daintain/AIF-1 Western analysis of rhdaintain/AIF-1 (0.05 μg loaded in lanes 1 and 2). Lane 1, porcine daintain; lane 2, purified rhdaintain/AIF-1; lane 3, supernatant of lysates from $2 \cdot 10^5$ MDA-MB-231 cells.

The purified protein was assayed by Western blotting to confirm that it was rhdaintain/AIF-1. The native daintain, which was previously purified from porcine intestine (Chen *et al.*, 1997) and the MDA-MB-231 cells, which overexpress the AIF-1 protein (Liu *et al.*, 2008), were used as positive controls (Fig. 2B, lanes 2 and 3). As shown in Fig. 2B, lanes 1–3, the rhdaintain/AIF-1 was recognized by anti-daintain/AIF-1 antibody, just like the native daintain and AIF-1 expressed by MDA-MB-231 cells.

These results showed that *E. coli* BL21 (DE3) harbouring the pET32a-daintain/AIF-1 plasmid expressed the rhdaintain/AIF-1 protein, as expected.

Rhdaintain/AIF-1 increases glucose elimination rates in BALB/c mice

BALB/c mice were used to investigate whether rhdaintain/AIF-1 could influence the blood glucose concentration *in vivo*. The intravenous injection of glucose (1 mg/g) into fasted BALB/c mice rapidly raised the blood glucose levels, with a peak obtained after only 1 min, after which the blood glucose levels returned toward baseline values. The blood glucose levels measured 5 min after injection were significantly lower in mice that received an injection of glucose + rhdaintain/AIF-1 [(14.51 ± 0.19) mM, $n = 8$] compared to an injection of porcine daintain [(12.71 ± 0.18) mM, $n = 8$], and an injection of glucose alone [(19.87 ± 0.16) mM, $n = 8$], with similar results seen after 15 and 30 min (Fig. 3). After 60 min, there was no significant difference in the level of glucose between the treatment groups. This increase in

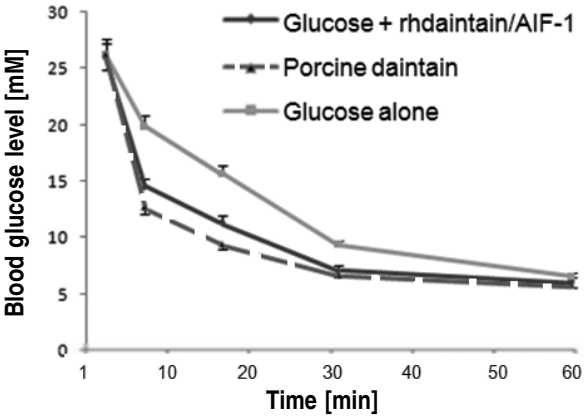

Fig. 3. Rhdaintain/AIF-1 increases blood glucose elimination rates. Blood glucose levels after an intravenous injection of glucose (1 mg/g) with or without the peptides rhdaintain/AIF-1 and porcine daintain (5 μg/g) were measured after 1, 5, 15, 30, and 60 min. BALB/c mice were used in each group (n = 8), and there has been significant difference between injection with the peptides and negative control, $P < 0.05$; error bars show means ± SD.

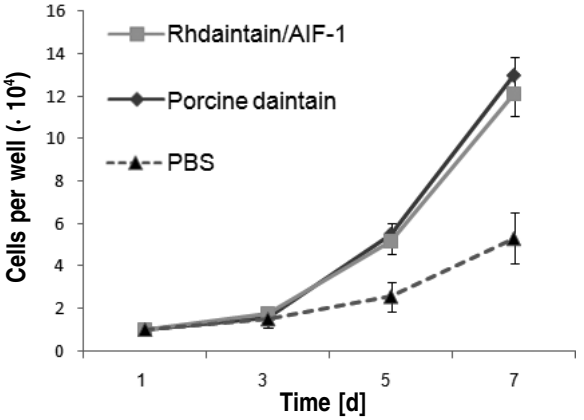

Fig. 4. Rhdaintain/AIF-1 enhances MCF-7 proliferation. Equal numbers of pooled MCF-7 cells were seeded into 24-well plates. The experimental groups received 1 μg/mL rhdaintain/AIF-1 and 1 μg/mL porcine daintain, respectively, another equal volume of PBS was added as a control. After 1, 3, 5, and 7 d, viable cells were counted by trypan blue exclusion in triplicate. Data represent at least three independent experiments (between experimental group and control, $P < 0.05$; error bars show means ± SD).

glucose elimination rates following injection of rhdaintain/AIF-1 demonstrates that rhdaintain/AIF-1 has biological activity *in vivo*.

Rhdaintain/AIF-1 promotes MCF-7 cell proliferation

In order to confirm the bioactivity of rhdaintain/AIF-1 *in vitro*, the effect of rhdaintain/AIF-1 on the proliferation of MCF-7 cells was examined. After 5 d, there was a 2-fold increase in cell number of rhdaintain/AIF-1-treated cells and porcine daintain-treated cells compared to the control PBS [$(5.2 \pm 0.5) \cdot 10^4$, $(5.5 \pm 0.7) \cdot 10^4$, and $(2.6 \pm 0.5) \cdot 10^4$, respectively], which was maintained at day 7 [$(12.1 \pm 1.0) \cdot 10^4$, $(13.0 \pm 1.2) \cdot 10^4$, and $(5.0 \pm 0.8) \cdot 10^4$, respectively; Fig. 4].

Discussion

It is known that daintain/AIF-1 plays an important role in autoimmune diseases (Schluesener *et al*., 1998), cancer proliferation (Liu *et al*., 2008), and vasculopathy (Autieri *et al*., 2000). Both basic research and potential clinical applications may require large quantities of daintain/AIF-1, but the low yields of daintain/AIF-1 obtained from natural material is one of the major restrictions in the study and application of daintain/AIF-1. To provide daintain/AIF-1 for biotechnological application, we developed a daintain/AIF-1 production method using the pET32a expression vector with a T7 promoter in *E. coli.*

In this study, we demonstrated that a higher dose (270 nmol/kg) of rhdaintain/AIF1 can increase blood glucose elimination rates in BALB/c mice as in our previous report (Chen *et al.*, 1997), but lower doses (27 nmol/kg and 2.7 nmol/kg) of rhdaintain/AIF-1 had no effect (data not shown). This might be so because the specific activity of the recombinant protein was lower than that of the native protein, or the dose subdivisions were too extended, or the mice strains differed in their responsiveness. Nevertheless, our results show that rhdaintain/AIF-1 has biological activity *in vivo*. It has been reported that daintain/AIF-1 can promote the proliferation of vascular smooth muscle cells (Autieri *et al*., 2000), T lymphocytes (Kelemen and Autieri, 2005), macrophages (Tian *et al*., 2006), human vascular endothelial cells (Tian *et al*., 2009), and another breast cancer cell line, MDA-MB-231 (Liu *et al*., 2008). These reports demonstrated that overexpression of intracellular daintain/AIF-1 by transfection can promote cell proliferation. However, it has not yet been reported whether extracellular daintain/AIF-1 has effects on cell proliferation. In the

present study, we added rhdaintain/AIF-1 into culture medium and found that it is conducive to MCF-7 cell proliferation. To our knowledge, this is the first report describing the promotion of cell proliferation through the application of high levels of extracellular daintain/AIF-1. This result indicates that the actions of rhdaintain/AIF-1 *in vitro* are similar to the actions observed when daintain/AIF-1 is overexpressed and the activity of rhdaintain/AIF-1 *in vitro* is preserved after purification.

In summary, we have succeeded in expressing and purifying the rhdaintain/AIF-1 protein without redundant amino acid residues attached to its *N*-terminal by biological engineering. The purification of rhdaintain/AIF-1 protein was confirmed by Western blotting. We further demonstrated that rhdaintain/AIF-1 has biological activities *in vivo* and *in vitro*. Thus, the present study provides us with a useful tool to obtain large amounts of functional rhdaintain/AIF-1 protein, which could be utilized as a highly purified biological reagent for structural, biochemical, biological, and clinical investigations.

Acknowledgements

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