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Expression, high-pressure refolding and purification of human leukocyte cell-derived chemotaxin 2 (LECT2)

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ABSTRACT

Human leukocyte cell-derived chemotaxin 2 (LECT2) is a chemotactic factor for neutrophils and a 16-kDa secreted protein consisting of 133 amino acids with three intramolecular disulfide bonds. Here, we propose an efficient method for the preparation of human LECT2 using a high hydrostatic pressure (HHP, 200 MPa) refolding technique. When LECT2 was over-expressed in *Escherichia coli* cells, most of LECT2 molecules became insoluble inclusion bodies (IBs). HHP was applied to the refolding of LECT2 from insoluble IBs, which dramatically improved the yield of the active LECT2. CD and NMR measurements demonstrated that the refolded LECT2 had a tertiary structure indistinguishable from the solubly expressed LECT2. In addition, both the refolded and solubly expressed LECT2 showed the same level of chemotactic activity.

Introduction

LECT2[1] was originally purified from culture supernatants of phytohaemagglutinin-activated (PHA) human T-cell leukemia SKW-3 cells as a chemotactic factor for human neutrophils [1]. Human LECT2 is preferentially expressed in the adult and fetal livers and human hepatoma cell lines [1,2]. Although LECT2 exhibits chemotactic activity *in vitro*, accumulating evidence suggests that LECT2 plays multifunctional roles in cell growth, differentiation, damage/repair processes, carcinogenesis and autoimmune diseases [3–7]. In addition, LECT2 is also associated with human systemic amyloidosis [8,9]. Proteins homologous to LECT2 have been isolated in many other vertebrates [10,11], such as bovine (*Bos taurus*) and chicken (*Gallus gallus*). Bovine LECT2 was found to be identical to bovine chondromodulin-II, which stimulates the proliferation of chondrocytes and osteoblasts [12–14].

The diverse biological functions of LECT2 should be relevant to its biochemical properties. Human LECT2 is a 16-kDa secreted protein consisting of 133 amino acids and three intramolecular disulfide bonds [15]. Database searches using BLAST suggest that human LECT2 belongs to the zinc metallopeptidase M23 family. Members of this family have a preference for peptides containing polyglycine residues, especially Gly–Gly–Xaa, where Xaa is any aliphatic hydrophobic residue [16]. However, the actual biochemical properties and structural basis of LECT2 are still unknown. A large amount of active LECT2 is required for its structural and functional studies, but the available amount is limited for both the native protein and the recombinant one produced in CHO cells [17]. *Escherichia coli* is the most widely used as a host for producing large quantities of recombinant proteins. However, during over-expression in *E. coli*, target proteins frequently form insoluble and misfolded aggregates, known as inclusion bodies (IBs), in the cytoplasmic or periplasmic space of bacteria [18,19]. This phenomenon also happens in the expression of LECT2. In a report by Ito et al., most of the LECT2 produced in *E. coli* formed insoluble aggregates, even at a low temperature (10 °C) and/or a low IPTG concentration (0.1 mM), which resulted in a small amount of proteins from the soluble fractions. Ito et al. applied the traditional chemical-based refolding methods to LECT2 IBs expressed in *E. coli* cells, and successfully refolded LECT2 [17]. However, this method is complicated and includes two stages: the urea-assisted denaturation and the reconstruction of intramolecular disulfide bonds.

Recently, high hydrostatic pressure (HHP) has been used as a powerful tool for protein dissociation and refolding from IBs [20–22]. Pressure between 100 and 300 MPa has been shown to dissociate multimeric proteins [23,24], whereas pressure between 400 and 800 MPa is usually required to unfold monomeric proteins [25–29]. Aggregates behave like multimers and are readily dissociated with moderate pressure (100–300 MPa). Unlike traditional refolding methods with denaturants, HHP refolding techniques do not require protein denaturation prior to refolding. Thus, the disaggregation and refolding of protein occur simultaneously under HHP.

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[1] *Abbreviations used*: LECT2, leukocyte cell-derived chemotaxin 2; HPP, high hydrostatic pressure; IBs, inclusion bodies; BCA, bicinchoninic acid; CD, circular dichroism; HBSS, Hanks' Balanced Salt Solution.

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Here, we describe an effective method for the preparation of human LECT2 using an HHP refolding technique. Using this method, we successfully refolded LECT2 from IBs expressed in *E. coli*, and showed that the refolded LECT2 had the same conformation and chemotactic activity as the expressed protein in a soluble fraction (solubly expressed LECT2).

Materials and methods

Materials

A cDNA clone of the human LECT2 gene (GenBank Accession No. BC093670) was purchased from Open Biosystems. The kits for PCR purification and gel extraction were purchased from Qiagen. The plasmid extraction kit was purchased from Promega. All other chemicals were of biochemical research grade and were purchased from Wako Pure Chemical Industries, Ltd., Sigma–Aldrich Industries, Ltd., Dojindo Molecular Technologies, Inc. and Calbiochem.

Construction of LECT2 expression plasmid

Consistent with the extracellular location of the protein, the gene for LECT2 encodes a secretory signal at the N terminus. The SignalP 3.0 server [30] estimated that the signal sequence was comprised of the first 18 amino acid residues. In addition, the LECT2 sequence from Gly19 to Thr35 is almost completely consistent with the reported N-terminal sequence of bovine chondromodulin-II [12]. Since the signal peptide is usually cleaved during the post-translational process, the signal sequence is not included in the mature LECT2. Therefore, we selected the LECT2 sequence from Gly19 to Leu133 for this study. The cDNA sequence encoding the mature LECT2 (19–133 residues) was amplified by PCR with KOD Plus DNA polymerase (Toyobo) and the primers 5′-gggccatgggctaatatatgtgctgg-3′ and 5′-cgggatccttacaggtatgcagtaggg-3′. The PCR products were digested with the restriction enzyme BamHI and were then ligated into pET-48b(+) vector (Novagen) between the SmaI and BamHI sites with T4 DNA ligase (Toyobo). The cloning sequence was verified by a DNA sequencing service (FASMAC Co., Ltd.).

Protein expression

LECT2 is a secreted protein containing three disulfide bonds [15], and the cytoplasm of *E. coli* is an unfavorable environment for disulfide-bond formation. In addition, the LECT2 gene sequence includes several rare codons for *E. coil*, such as AGG and AGA. Therefore, we selected pET-48b(+) bearing Trx as an expression vector and Rosetta-gami 2(DE3) with glutathione reductase (gor) and thioredoxin reductase (trxB) mutations ($trxB^-$ gor^-) as an expression host, since this has been shown to be an optimal combination for enhancing the solubility of target proteins and the formation of disulfide bonds [31–33]. *E. coli* strain Rosetta-gami 2(DE3) (Novagen) was transformed with the LECT2 expression plasmid. A freshly transformed colony was grown in 10 mL of Lysogeny–Broth (LB) medium containing 20 mg/L kanamycin, 34 mg/L chloramphenicol and 6 mg/L tetracycline overnight at 37 °C. The cell culture was transferred into 2 L of LB medium supplemented with 20 mg/L kanamycin, 17 mg/L chloramphenicol, 6 mg/L tetracycline and 0.01% (v/v) antifoaming agent (Antifoam 204, Sigma). The cells were grown with a bubbling cultivation method [34] at 37 °C until the OD_{600} reached 0.7. Protein expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM at 25 °C. After further incubation overnight, the cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C. The cell pellets were rapidly frozen in liquid nitrogen and stored at −80 °C.

For expression of ^{15}N-labeled LECT2, the expression method was slightly modified. 10 mL of cell culture was transferred into 2 L of LB medium and incubated at 37 °C until the OD_{600} reached 0.5. The cells were harvested by centrifugation at 4000*g* for 10 min at 25 °C. The harvested cells were then dissolved in 2 L of ^{15}N-labeled C.H.L. medium (SI Science Co., Ltd.) supplemented with 20 mg/L kanamycin, 17 mg/L chloramphenicol, 4 mg/L tetracycline and 0.01% (v/v) antifoaming agent and were incubated at 37 °C until the OD_{600} reached 0.7. A final concentration of 0.5 mM IPTG was added to the cell culture, and further incubation was carried out at 25 °C overnight.

Preparation of the soluble fraction and IBs of LECT2

E. coli cell pellets (5.7 g wet weight) from 2 L culture were resuspended in 180 mL of lysis buffer (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole) including 0.1% (v/v) protease inhibitor cocktail and 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (ABESF) and were sonicated using a Branson 250-D at 4 °C. The lysis suspension was centrifuged at 40,000*g* for 30 min at 4 °C to separate the soluble fraction and IBs. After centrifugation, the supernatants were directly used for protein purification. The IBs were washed once with 10 mL of wash buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 0.125 M NDSB-201, and 5% glycerol) per gram of wet cell pellet and twice with 10 mL of the wash buffer without NDSB-201. The suspensions of IBs were centrifuged at 8000*g* for 15 min at 10 °C following each washing and the supernatants were discarded. The purified IBs were resuspended in 10 mL of Milli-Q water per gram of wet cell pellet and stored frozen at −80 °C.

HHP refolding

Before the refolding experiments, the protein concentration of IBs suspension was determined by using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc.) and then diluted to 5 mg/mL with Milli-Q water. 50 μL of IBs suspension was diluted in 450 μL of refolding buffer containing 50 mM Tris–HCl (pH 8.0) and some additives, such as redox reagents, L-arginine and urea. The solution was mixed well and then loaded into a high pressure 'caisson' (ProVENT^{TM}). Refolding was performed in a PreEMT M150 pressure chamber (BaroFold Inc.). Pressure was increased to 200 MPa and was then kept for 16 h at room temperature. After depressurization at a rate of 25 MPa/5 min, the samples were transferred to microfuge tubes and centrifuged for 30 min at 20,000*g* at 4 °C. The refolding efficiency was examined by SDS–PAGE. The gel was stained with Coomassie Brilliant Blue (CBB) R-250. Large-scale refolding of LECT2 (60 mL) was performed under the optimal conditions established in the small-scale screening experiments.

Purification of solubly expressed and refolded LECT2

After HHP treatment the sample was dialyzed against 20 mM Tris–HCl (pH 8.0), 300 mM NaCl at 4 °C to remove L-arginine and then centrifuged for 30 min at 40,000*g* at 4 °C. The other purification procedures were the same for both the solubly expressed and refolded LECT2. The LECT2 solution was loaded onto Ni–NTA Superflow resin (Qiagen) equilibrated with the lysis buffer as described above. The resin was washed with the lysis buffer to remove nonspecifically bound proteins. In order to obtain the recombinant LECT2 with only two additional residues Gly and Pro at the N-terminus, the N-terminal thioredoxin (Trx)−hexahistidine (His_6) tag was cleaved off with HRV3C protease in an on-resin, overnight treatment at 4 °C, which was expressed and purified by the method described previously with some modifications [35-