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Purification of human galectin-1 produced in high-cell density cultures of recombinant *Escherichia coli*: a comparison with classic shake flask cultivation

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Abstract

The aim of the present work was to develop a highly productive and simplified process for active human galectin-1 (Gal1) production. Gal1 is a β-galactoside binding lectin that differentially affects biological and cellular functions such as immune surveillance and apoptosis. These effects have attracted the attention of researchers in cell biology, biochemistry and immunology. However, the production of sufficient amounts of recombinant human Gal1 (rhGal1) is needed to study of the effects of Gal1 during cell treatments. To this end, an high-yield expression of rhGal1 was achieved by high-cell density fed-batch cultivation using an exponential glycerol feeding strategy and rhGal1 was purified by a one-step purification scheme using affinity chromatography.

Keywords: *Escherichia coli*; Galectin-1

1. Introduction

The subject of high-cell density cultivation of microorganisms was reviewed recently [1,2]. The aim of this approach is to increase the production of the compound of interest per unit volume. Here, we report on the expression of recombinant human galectin-1 (rhGal1) by high-cell density fed-batch cultivation of *Escherichia coli*, and on the purification and characterization of rhGal1.

Galectin-1 (Gal1) is a member of a highly conserved family of β-galactoside-binding animal lectins defined on the basis of structural analysis and binding specificity studies. Membership in this family requires fulfillment of two criteria—binding affinity for β-galactoside and a conserved sequence elements in the carbohydrate-binding site [3]. To date, 14 mammalian galectins have been identify.

Gal1 is a homodimer with a subunit molecular weight (Mr) 14,500 and an isoelectric point (p*I*) of 5.1 [4,5]. Gal1 has no transmembrane domain and is devoided of recognizable signal sequence [6]. However, it can be externalized from cells by a mechanism independent of the normal secretory process (for review see [7]).

Gal1 regulates some aspects of cell cycle progression [8] and it has become clear that it can differentially affect cellular function and activation. Its role as a putative modulator of immune surveillance, apoptosis, cell adhesion and chemotaxis has been demonstrated in several cell models. These biological effects attracted attention of researchers in cell biology, biochemistry, glycobiology and immunology. Due to this growing interest and to the fact that the knowledge of the mechanisms governing these effects involves studies using cells treated with exogenous Gal1, production of large quantity of rhGal1 is needed.

2. Experimental

2.1. Materials and reagents

Except when overwise indicated, chemicals and other reagents were obtained from Sigma–Aldrich, (Saint-Quentin Fallaviers, France). PD-10 columns containing Sephadex

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G-25 M were from Amersham-Bioscience (Uppsala, Sweden). Vertical slab Mini Protein II cell used for electrophoresis and densitometer GS-700 were from Biorad (Richmond, CA, USA).

2.2. Strain and growth media

The host organism, *E. coli* M15(pREP4) and the pQE-60 procaryotic expression vector were obtained from Qiagen (Courtaboeuf, France). The host cell contains multiple copies of plamid pREP4, which carries the lacIq gene encoding the lac repressor for tight regulation of protein expression.

The human Gal1 cDNA was prepared as described earlier [9]. A template total RNA was extracted from human substantia nigra and kindly provides by Dr. H. Chneiweiss (INSERM, U144). A reverse transcription of 25 mg of total RNA were primed with oligodT12-18 and an aliquot of the reaction was amplified by polymerase chain reaction (PCR) (30 cycles; 94 °C, 1.5 min; 56 °C, 1.5 min; 72 °C, 2 min) with primers corresponding to positions 27–46 and 501–482 of the published human cDNA sequence (GenBank: HUMLEC). The 475 bp long fragment was purified by gel electrophoresis and an aliquot was subjected to another round of amplification using the same down stream primer (501–482) and a mutator upstream primer (GACTCAACCATGGCTTGTGG) creating a *Nco*I restriction site at the level of initiator ATG. After restriction with *Nco*I (a natural *Nco*I site is located after the stop codon, at position 469 of the published sequence), the 421 bp long fragments was gel-purified and inserted into a *Nco*I-cleaved dephosphorylated pQE-60 vector. Twelve M15[pREP4] transformants were grown in liquid medium and those expressing human Gal1 were identified after isopropyl-D-thiogalactoside (IPTG) induction and Western blotting using a polyclonal anti-human Gal1 rabbit antiserum. A positive plasmid was selected and its cDNA insert was sequenced in order to check the absence of mutations modifying the primary structure of the recombinant protein.

2.3. Shake flask cultivation procedure

Unless otherwise stated all cultivation media contained 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin at 37 °C. The ratio between medium and shake flask volumes was 1/10. Fresh colonies of recombinant *E. coli* were obtained after growth on Luria Bertani (LB) agar plates. A single colony of *E. coli* (pREP4) cells carrying the construct was inoculated in 10 ml of LB medium (50 ml shake flask) and incubated for 8 h at 250 rpm and 37 °C. From these cultures, 100 ml of LB were inoculated to an OD_{600} of 0.1. After overnight incubation, the culture was used to inoculate 1.5 l of medium at an OD_{600} of 0.1. At an OD_{600} of 0.6, rhGal1 production was induced by 2 mM IPTG. The cells were collected by centrifugation (4 °C, 14,000 × *g*, 20 min) for protein extraction after 2 h of induction at a final OD_{600} of 2.

2.4. High-cell density cultivation procedure

The high-density cultivation was accomplished, with minor modifications, as already described [10]. Cell growth was followed by measurement of the optical density at 600 nm, using a Hitachi U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan). One unit was found to correspond to 0.5 g l^{-1} of cells dry weight (obtained after lyophilisation). An Applikon fermentor system (Applikon France, Les Mureaux, France) in fed-batch cultures with 1500 ml of the defined medium was inoculated at an OD_{600} of 0.1. The cultivation was performed at 37 °C and at pH 7, with dissolved oxygen concentration (pO_2) over than 25%. Culture samples were taken for determining rhGal1 expression and cell dry weight. In addition, pO_2, off-gas concentrations of O_2, base consumption were measured on-line and recorded by an external data acquisition and control system.

Culture of 1500 ml was carried out in a synthetic medium containing 0.108 M glycerol, 0.2 M KH_2PO_4, 0.14 M KOH, 30 mM $(NH_4)_2SO_4$, 1.62 mM $MgSO_4$, 0.68 mM $CaCl_2$, 3.7 μM $FeSO_4$, 1.4 μM $ZnSO_4$, 2.3 μM $MnSO_4$, 0.16 μM $CuSO_4$, 1 μM $NaMoO_4$, 6.4 μM H_3BO_3, 0.6 μM KI and 11.85 μM thiamine.

After 15 h at 37 °C, pH 7, 800 revolution per minute (rev min^{-1}), 1 volume of air per volume of culture per minute (v.v.m.), 50 times concentrated medium was added to obtain a specific growth rate of 0.25 h^{-1} and a theoretical biomass yield of 0.5 g per gram of glycerol. The stirring rate was then increased to 1200 rev min^{-1} to maintain sufficient aeration.

After the culture reached an OD_{600} of 30, the IPTG induction was carried out. The production of rhGal1 was induced by 2 mM IPTG during the exponential phase of the bacterial growth and the bacteria were pelleted (8000 g, 10 min, 4 °C) 2 h later at a final OD_{600} of 40.

2.5. Extraction

Cells collected by centrifugation (4 °C, 8000 × *g*, 10 min), were suspended with ice-cold extraction buffer (50 mM Tris–HCl, pH 7.4, 10 mM EDTA, 4 mM β-mercaptoethanol, 0.25 mM phenylmethyl sulfonylfluoride (PMSF), one minitablet of antiprotease (Roche, Basel, Switzerland)/10 ml buffer, and then sonicated. After sedimentation of cell debris at 14,000 × *g* for 30 min at 4 °C, the clear supernatant was collected. The extraction procedure was repeated. The protein content of soluble extracts was determined by the method of Bradford [11]. rhGal1 was purified on a column of lactosyl-agarose [12].

2.6. Purification of Gal1 by lactose immobilized affinity chromatography

Following extraction and removal of the particulate material by centrifugation, 50 ml of bacteria supernatant were applied at room temperature to a column containing 5 ml

of lactosyl-agarose equilibrated with 50 mM Tris-HCl, pH 7.4 containing 4 mM β-mercaptoethanol, 0.25 mM PMSF, a cocktail of antiprotease and 0.02% sodium azide (TBS). The column was washed extensively with TBS in order to remove weakly bound proteins, and rhGal1 was eluted at 4 °C with TBS that contained 100 mM lactose. Fractions containing rhGal1 were dialysed against TBS and stored a −20 °C. Multiple chromatographies were performed to purify whole of the extraction products. The purified protein was analyzed by SDS–PAGE under reducing conditions in a vertical slab Mini Protein II (Biorad, Richmond, CA, USA) cell. Proteins were visualized by Coomassie staining according to standard procedures. Gel imaging was achieved using a GS-700 densitometer piloted by Quantity One software (Biorad, Richmond, CA, USA).

2.7. Biological activity tests

Biological activity tests were performed as previously described [9]. Briefly, before performing the assay, gel permeation chromatography of rhGal1 was carried out on (Amersham-Biosciences, Uppsala, Sweden) PD-10 column equilibrated with sterile/apyrogenic 0.15 M NaCl solution. The sample (total volume of 1 ml) was eluted in the same solution, and used immediately. Jurkat cells (1×10 cells ml^{-1} in RPMI 1640 medium containing 10% FCS) were seeded into flat-bottomed 96-well plates (40,000 cells wells^{-1}) and cultured for 24 h at 37 °C. RhGal1 (2 mM) was added and apoptosis was analyzed by labeling with the annexin-V FITC apoptosis detection kit (BD Biosciences-Pharmingen, San Diego, USA), which recognizes phosphatidylserine exposure on outer leaflet of the plasma membrane. Apoptosis was also evaluated by propidium iodide staining.

3. Results and discussion

Recombinant protein production at a research scale is generally performed using complex medium such as the LB. However, for producing protein in high-cell density cultures a defined medium is required. Therefore, production of rhGal1 in high-cell density cultures was compared with shake flask cultures using LB medium.

In flask cultures, rhGal1 was accumulated during the first 2 h after induction to 15% of the total cell protein (Fig. 1). For maximizing the volumetric productivity of rhGal1, a high-cell density cultivation procedure with a pre-determined exponential feeding strategy was applied. This strategy has been used successfully for the production of various heterologous proteins [13–15].

The exponential feeding strategy allows cells to grow at a constant specific growth rate by using glycerol as a growth-limiting nutriment and thus, causes minimum perturbation on cellular carbon metabolism. As shown in Fig. 2, during the exponential growth period, the specific growth rate could be successfully controlled at a constant value of

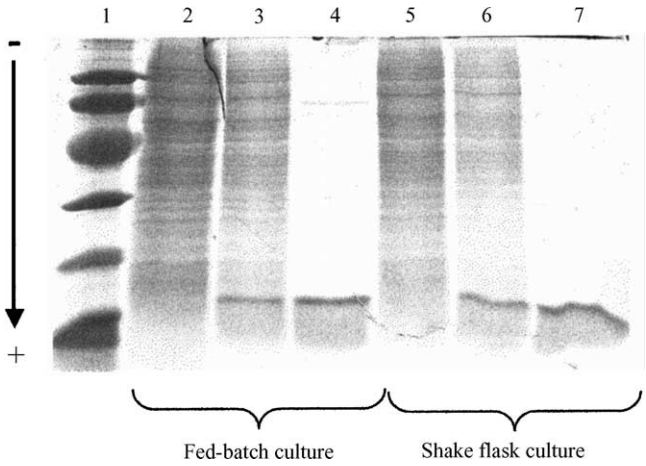

Fig. 1. SDS–PAGE analysis of rhGal1 overexpressed in *E. coli* and isolated on a lactosyl-agarose column. Samples were electrophoresed on a 15% gel, stained and scanned using a densitometer. RhGal1 expression analysis from *E. coli* induced or not with IPTG (2 mM) during fed batch culture and shake flask cultivation are shown lanes 2–4 and lanes 5–7, respectively. Lane 1, molecular weight markers; lane 2, 0 h induced; lane 3, 2 h induced; lane 4, purified rhGal1; lane 5, 0 h induced; lane 6, 2 h induced; lane 7, purified rhGal1.

0.25 h^{-1}. This growth profile is what one typically observes during fed-batch culture of *E. coli*, not only at laboratory scale but also in industrial-scale fed-batch fermentation with exponential feeding [16–18].

Cultivation of recombinant *E. coli* REP4 started as a batch process at 37 °C with a middle specific growth rate and a biomass to glycerol yield of about 0.3 h^{-1} and 0.5 g g^{-1}, re-

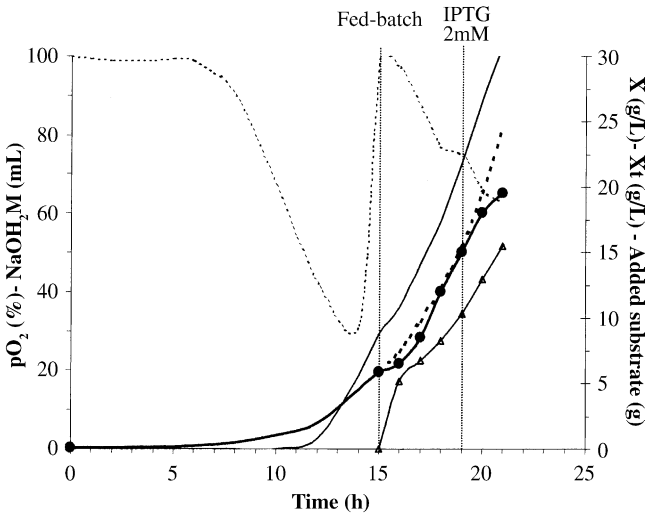

Fig. 2. Kinetic study of growth (●) of *E. coli* M15[pREP4] in fed-batch cultivation according to the exponential feeding profile imposed (g h^{-1}, △). Analysis of the O_2 (%,· · ·) and base consumption (ml, —). The dashed curve (– – – –) represents the theoretical kinetic of growth (*Xt*), calculated according to: a theoretical growth yield $Y_{X/S} = 0.5$ g g^{-1}; a specific growth rate μ of 0.25 h^{-1}—a feeding solution of glycerol of 500 g l^{-1}; a volume of 1.5 l. Vertical lines indicate, respectively, the beginning of the fed-batch process and the moment of induction. Others cultivation conditions: 37 °C, pH 7, in presence of the M63mGly500 medium.

Table 1
Comparison of rhGall quantity and volumetric yield obtained with fed-batch and shake flask cultivation

	Fed-batch cultivation	Shake flask cultivation
Volume of culture (l)	1.5	1.5
Biomass (g)	42	2.2
Crude extracts	4.5 g	245 mg
rhGal1 purified (mg)	675	39.2
Volumetric yield (mg l^{-1})	450	26.1

spectively (Fig. 2). At a biomass concentration of 10 g l^{-1}, the end of the batch phase was noticed by a steep increase in pO_2 and the absence of glycerol in the medium. At this moment, the exponential addition of concentrated feed solution was started aiming at a theoretical specific growth rate of $\mu_{setpoint} = 0.25\,h^{-1}$ (the middle practical value is about $\mu_{real} = 0.255\,h^{-1}$). After the culture reached an OD_{600} of 30, the production was induced by 2 mM IPTG. The protein expression was concomitant with a reduction of the specific growth rate during the last hour of induction ($\mu_{real} = 0.08\,h^{-1}$).

After extraction, rhGal1 was purified from clear supernatant by affinity chromatography on a column of lactosyl-agarose in one-step and with a high-purification fold.

The volumetric yield obtained corresponds to 450 mg rhGal1 per liter for fed-batch cultivation procedure (i.e. a production yield $Y_{rhGal1/X}$ of about 22.5 mg of protein per gram of biomass) and to 26 mg rhGal1 per liter (i.e. a production yield $Y_{rhGal1/X}$ of about 26 mg of protein per gram of biomass) for classical shake flask procedure (Table 1). Production yield was not so different for the two different processes of cultivation whereas the batch cultivation medium (LB medium) is enriched in amino acids in comparison with the composition of the synthetic fed batch medium.

It has been shown that Gal1 is involved in the induction of apoptosis in Jurkat T cells [19,20]. To determine whether affinity-purified rhGal1 retained or not its biological activity, Jurkat T cells were treated with 2 mM of rhGal1. RhGal1 was used as a sterile solution of 0.15 M NaCl and controls were performed using sterile NaCl solution instead rhGal1. As shown in Fig. 3, fed-batch obtained rhGal1 induced death by apoptosis in Jurkat T cells.

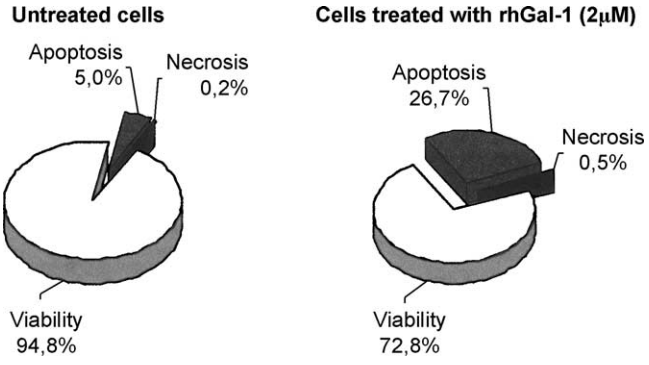

Fig. 3. Affinity-purified rhGal1 induced an increase of cell death in Jurkat cells. The cells were treated without or with rhGal1, stained with annexin-V FITC, and analyzed by flow cytometry.

4. Conclusion

Summarizing our results on this work, a simple method has been proposed permitting to obtain a powerful tool for studying of Gal1 induced cellular events. *E. coli* is the most widely used organism for recombinant protein production because of its rapid growth to high-cell densities on inexpensive substrates, of its well-characterized genetics and proteomics [21] and of the availability of a large number of cloning vectors. For cost-effective reasons, it is important to maximize protein production by development of efficient microbial process coupled to appropriate chromatography methods. This can be achieved by increasing the amount of protein per cell per time and/or by increasing cell concentration per time. The high-cell density fermentation by fed batch strategy employed in this work is probably one of the most cost-effective means to enhance cell mass and proteins production. RhGal1 was produced in form of soluble protein representing 15% of the total cell protein. Taking advantage of high-volumetric yields obtainable by high-cell density cultivation, 450 mg of rhGal1 per liter of culture broth was produced. To our knowledge, the procedure described give the highest overall yield reported so far for rhGal1 production.

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