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Large-Scale Purification of Human Apolipoprotein Kringle Domain V (rHualkV) Expressed in *Pichia pastoris*

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ABSTRACT
Human apolipoprotein kringle domain V (rHualkV) that has an anti-angiogenic activity was expressed and purified in large scale from *Pichia pastoris*. rHualkV was captured by SP-Streamline resin directly from 40 L culture broth through batch type adsorption with about 80% yield (n = 8). Captured rHualkV was further purified by SP-Sepharose column chromatography and concentrated by membrane filtration. On average 5 g of rHualkV was purified per batch with the 56% final yield. The result of analytical High-Performance Liquid Chromatography (HPLC) of the purified rHualkV showed the single peak. ESI mass-spectrometry analysis of the rHualkV has proved that the purified rHualkV has the 9676 kDa that is the correct molecular weight as the one calculated by amino acid sequence. In addition, rHualkV has the same N and C-terminal residue as the expected one from DNA sequence of rHualkV gene in *Pichia pastoris*. Thus, through three steps of process, the anti-angiogenic rHualkV was successfully expressed and purified from *Pichia pastoris*, which will be able to provide a basis for much larger expression and purification for production needed for clinical evaluation later.

Key words: Large scale, purification, apolipoprotein, kringle, *Pichia pastoris*

INTRODUCTION

Angiogenesis, the formation of new blood vessels is required for many normal physiological processes such as embryonic development, wound healing, tissue or organ regeneration (Stack *et al*., 1999; Kamphaus *et al*., 2000; O'Reilly *et al*., 1997; Lucas *et al*., 1998; Cao *et al*., 1996; Folkman and Shing, 1992). However, the abnormal growth of new blood vessel can lead to many diseases such as diabetic retinopathy and tumor growth (O'Reilly *et al*., 1997; Cao *et al*., 1996; Folkman and Shing, 1992). Recently it was reported that the abnormal angiogenesis in tumor could be sufficiently inhibited by some kind of polypeptides. These polypeptides include angiostatin (Stack *et al*., 1999; Lucas *et al*., 1998; Cao *et al*., 1996), endostatin (Trinh *et al*., 2000) and canstatin (Kamphaus *et al*., 2000). Among these, angiostatin is a polypeptide composed of three repeated kringle domains originated from human protein and has been reported to have a high anti-angiogenic activity in various kinds of tumor. Kringle domains exist in many human proteins such as plasminogen, prothrombin, tissue type plasminogen activator (t-PA), urokinase type plasminogen activator (u-PA), factor XII and hepatocyte growth factor (Chenivesse *et al*., 1996; Ryan *et al*., 2003; Shepard *et al*., 2001).

Recently, human apolipoprotein kringle domain V (rHualkV) is reported to inhibit tumor development by blocking angiogenesis in several kinds of cancer *in vitro* and *in vivo*. rHualkV is known to show a similar anti-angiogenic activity as other anti-angiogenic protein drug candidates (Zhu *et al*., 2003). For the purpose of developing new anti-cancer therapeutics, we needed to prepare the purified rHualkV in a large amount and attempted to scale up the production process. Like other protein drugs, rHualkV has been tried to be expressed as a recombinant proteins from culturing microorganism such as bacteria and yeast. In this study, rHualkV was expressed from *Pichia pastoris* which is a kind of methylotrophic yeasts known as a powerful host for recombinant protein industry. Also, other advantages of *Pichia pastoris* are high productivity and secretion ability of the target recombinant proteins (Shepard *et al*., 2000;

Trinh *et al*., 2000). In contrast, the expression system using *Pichia pastoris* often cause its downstream process to be complicated due to its high cell density and viscosity (Shepard *et al*., 2000; Ryan *et al*., 2003; Shepard *et al*., 2001; Trinh *et al*., 2000; Vasquez-Alvarez *et al*., 2001; Ohashi *et al*., 2002). Thus, in this study, we tried to circumvent these possible downstream process problems in our own methods and successfully produced rHualkV from *Pichia pastoris* in high yield.

MATERIALS AND METHODS

Reagents: SP-Streamline and SP-Sepharose fast flow were obtained from Pharmacia. SDS-PAGE gel and IEF-gel were from Invitrogen. Carboxypeptidase P was from Sigma. Membrane filter with molecular weight cutoff of 300 Da was purchased from Sartorius.

Culture condition: Total 5 L of the seed culture was prepared for the 40 L scale m ain culture in a 75-L bioreactor. One milliliter of the frozen stock was inoculated to flasks containing YPD medium (10 g of yeast extract (Difco, Mo., USA), 20 g of bactopeptone (Difco), 20 g of glucose (Sigma, Mo., USA) per liter) and incubated at 30°C with shaking at 200 rpm for 24 h. Then, this seed culture was transferred to the 75-L bioreactor containing medium (40 g glycerol, 27 mL H_3PO_4, 0.9 g $CaSO_4$, 18 g K_2SO_4, 10.24 g $MgSO_4$, 4.13 g KOH, 30 mL NH_4OH, 4.4 mL Yeast Trace Metal (YTM) solution per liter). The YTM was composed with 2.4 g $CuSO_4Æ5H_2O$, 0.09 g KI, 0.6 g $MnSO_4ÆH_2O$, 0.2 g $NaMoÆH_2O$, 0.02 g H_3BO_3, 0.5 g $COCl_2$, 15.0 g $ZnCl_2$, 63.5 g $FeSO_4ÆH_2O$, 0.2 g biotin and 10.0 mL H_2SO_4 per liter.

SP-Streamline capturing: Depending on fermentation conditions, capturing process was performed in two ways (Expanded Bed Adsorption (EBA) or batch type adsorption). At the relatively low cell density of fermentation broth (below 100 g L^{-1}), rHualkV was captured with EBA method but at high cell density (over 100 g L^{-1}), rHualkV was captured with batch type adsorption. Capturing process with EBA adsorption is as follows. SP-Streamline resin was packed in Pharmacia streamline column and equilibrated with sodium phosphate buffer (20 mM, pH 4.8). Prior to loading, fermentation broth was diluted to 2-fold with equilibration buffer and adjusted to pH 4.0 with 1 N HCl. Then the diluted broth was loaded at a flow of 500 mL min^{-1} to the streamline column in bottom to top direction expanding the packed resin. After loading, flow direction was reversed to settle down the expanded resin and washed with sodium phosphate buffer (20 mM, pH 6.8) by 5-bed volumes. Captured rHualkV was eluted with sodium phosphate buffer (20 mM, pH 7.2 containing 150 mM NaCl). Capturing process with batch type adsorption is as follows. Prior to adsorption, fermentation broth was diluted the same way as EBA method and poured to 5 L SP-Streamline resins equilibrated with the sodium phosphate buffer (20 mM, pH 4.8) in 200 L tank with stirring. Then, SP-Streamline resin was packed into Pharmacia streamline column. SP-Streamline resin was washed with sodium phosphate buffer (20 mM, pH6.8) by 5-bed volumes. Captured rHualkV was eluted with sodium phosphate buffer (20 mM, pH 7.2 containing 150 mM NaCl).

SP-Sepharose column chromatography: SP-Sepharose fast flow was packed and equilibrated with sodium phosphate buffer (20 mM, pH 4,8). Prior to loading, the eluted rHualkV from SP-Streamline was diluted to 3-fold with equilibration buffer and loaded to SP-Sepharose column at a flow of 500 mL min^{-1}. The SP-Sepharose resin was washed with sodium phosphate buffer (20 mM, pH 6.8) by 5-bed volume. rHualkV was eluted with sodium phosphate buffer (20 mM, pH 7.2 containing 150 mM NaCl).

Analytical HPLC: Alltech C4 reversed phase column (2.1 mm×150 cm) was equilibrated with 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The 100 uL of purified rHualkV was loaded onto the column and washed with equilibration buffer. rHualkV was eluted with increasing the percentage of acetonitrile from 5-95% (v/v). UV absorbance was recorded at 214 nm.

Mass spectrometry analysis: Purified rHualkV and carboxyl peptidase P treated rHualkV 8 was desalted with reversed phase HPLC and analyzed with Electrospray Ionization Mass Spectrometry (ESI-MS) using Micromass model Platform II instrument.

N-terminal sequencing: N-terminal protein sequencing was performed on an Applied Biosystems model 492 instrument according to the manufacturer's instruction. Purified rHualkV were electro-blotted onto a PVDF membrane and visualized with coomassie brilliant Blue R-250. Bands of interest were excised and washed with methanol to remove dye and detergent and then placed on the sequencer.

C-terminal sequencing: Purified rHualkV was dialyzed against sodium acetate buffer (30 mM, pH 4.0) and digested with carboxypeptidase P at 37°C. At time intervals, aliquot was taken to be mixed with trifluoroacetic acid to final 1% (v/v) and loaded onto Vydac C8 reversed phase column equilibrated with 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Eluted pool from the reversed phase column was concentrated with speed vac and analyzed with ESI-MS. The sequence from C-terminus was determined by the lower molecular weights derivatives emerged with the time order of carboxylpeptidase P enzyme reaction time from intact rHualkV.

RESULTS

Purification of rHualkV: Although *Pichia pastoris* is known as a powerful host for recombinant protein industry, it often makes the downstream process be complicated due to high cell density and viscosity of the culture broth. To overcome these

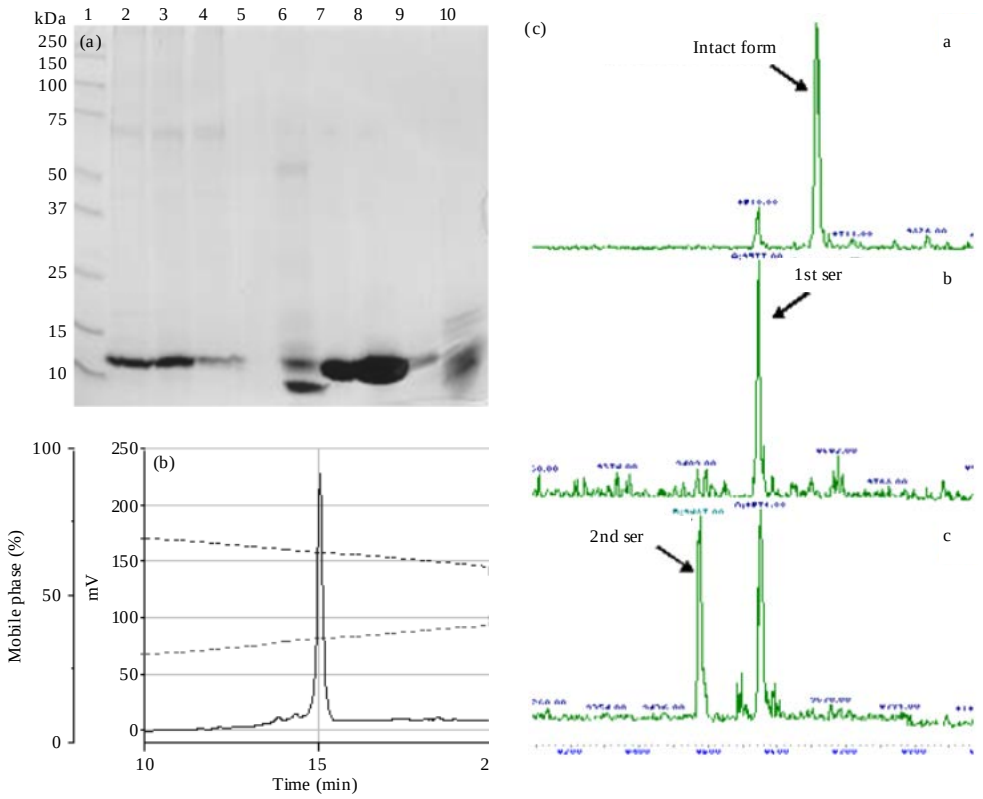

Fig. 1(a-c): Analysis of the purified rHualkV, (a) SDS-PAGE analysis of rHuakV from *Pichia pastoris* during SP-Streamline capture process. Lane 1: Molecular weight markers, Lane 2: Fermentation supernatant (early time), Lane 3: Fermentation supernatant (late time), Lane 4: Diluted fermentation supernatant, Lane 5: 1st washing fraction, Lane 6: 2nd washing fraction, Lane 7: 1st elution fraction, Lane 8: 2nd elution fraction, Lane 9: 3rd elution fraction, lane 10: 10 N NaOH cleaning fraction, (b) Reversed phase analytical HPLC profile of the purified rHualkV and (c) a: Mass spectrum of the purified rHualkV, a, b and c: Carboxylpeptidase treated rHualkV5

Table 1: Summary of the purification process of rHualkV

Purification step	Concentration (mg mL^{-1})	Volume (L)	Amount (g)	Yield (%) step (overall)
Fermentation		40.00	9	
Streamline SP	3.5	2.00	7	78
SP-sepharose	4.0	1.50	6	86 (67)
Filtration	20.0	0.25	5	83 (56)

possible downstream process problems, depending on fermentation conditions, capturing process was performed in two different ways (Expanded Bed Adsorption (EBA) or batch type adsorption). From the 8 times operation, at the relatively low cell density of fermentation broth (below 100 g L^{-1}), rHualkV was captured with EBA method but at high cell density (over 100 g L^{-1}), rHualkV was captured with batch type adsorption. EBA was impossible due to cell clogging in column at high cell density over 100 g L^{-1}. As shown in Table 1, 80% of rHualkV was captured in either method from the culture broth. Then, the rHualkV fraction was further purified by SP-Sepharose and formulated by membrane filtration yielding 5 g/40 L.

Analysis of rHualkV: Most of host proteins were removed by SP-Streamline capturing process (Fig. 1). The purity of finally purified rHualkV eluted from SP-Sepharose column chromatography was above 95% on reversed phase HPLC (Fig. 1b). The molecular weight of rHualkV was found to be 9676 Da from the result of ESI-MS (Fig. 1c (a)). The order of amino acid residue released from N-terminus by Edman degradation was E-Q-D which is identical with the expected one from the cloned DNA sequence (data not shown). When rHualkV was digested with carboxylpeptidase P, 80 and 160 Da reduced derivatives compared to rHualkV were observed with time-dependency (Fig. 1c (b) and (c)). This result confirms that rHualkV has two consecutive serine residues from C-terminus which sequence is -C-S-S. PI value of rHualkV was about 7.8 in IEF and this value is nearly identical with the calculated value from the primary sequence (data not shown).

DISCUSSION

Angiogenesis is the physiological phenomena by that the new blood vessel capillaries are formed from the existing blood vessels and involved in various normal physiological processes including wound healing as well as disease processes such as ischemia and tumor development

(Folkman, 1971). Also, angiogenesis is known as one of the essential steps for providing oxygen and nutrients to tumor cells and well related to the tumor stage and poor prognosis (Oostendorp *et al*., 2008; Carmeliet, 2003; Meitar *et al*., 1996).

Kringle domains have been known to be involved in a various kinds of function such as growth stimulating and proteases activity of the blood clotting (Cao *et al*., 2002). It has been reported that several kinds of kringle domains have the ability to inhibit tumor angiogenesis. For example, Angiostatin, the 38 kDa protein fragment (the first three kringle domains) from human plasminogen, has been identified to block angiogenesis in cancer cells and tumor growth in animal models that has been tried in preclinical stages (O'Reilly *et al*., 1994). However, during clinical stage, the treatment of Angiostatin to cancer patients could not show the significant anti-tumor activity nor improve any clinical results (Kurup *et al*., 2006). Thus, for the purpose of developing the novel kringle domain based protein drugs with the anti-tumor angiogenesis activity, an endogenous human apolipoprotein kringle domain V was selected.

In this study, rHualkV, apolipoprotein kringle domain V as an anti-angiogenic protein, was expressed from *Pichia pastoris* and purified in large-scale. In a pressurized 40 L fermentor, a modified DO-stat fed-batch culture was performed to produce rHualkV using methylotrophic yeast *Pichia pastoris* as a host. rHualkV was captured directly from culture broth by expanded adsorption capturing or batch type capturing method using SP-Streamline. After capturing, rHualkV was further purified with SP-Sepharose, cation exchanger. The overall purification yield of rHualkV was approximately 50%. The finally purified rHualkV was free of endotoxin. Thus, after a simple and efficient purification steps, rHualkV was purified to homogeneity from *Pichia pastoris* which can provide a basis for much larger expression and purification for production needed for clinical evaluation later.

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