Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 53 (2007) 331–338
Protein Expression & Purification
www.elsevier.com/locate/yprep

A biologically active angiogenesis inhibitor, human serum albumin–TIMP-2 fusion protein, secreted from *Saccharomyces cerevisiae*

Woo Kyu Kang [a], Eun-Kyu Park [b], Hee Suk Lee [b], Byung-Young Park [b], Jae-Young Chang [a], Min-Young Kim [b], Hyun Ah Kang [c], Jeong-Yoon Kim [a,*]

[a] *School of Bioscience and Biotechnology, Chungnam National University, Daejeon 305-764, Republic of Korea*
[b] *AngioLab Inc., Daejeon 302-735, Republic of Korea*
[c] *Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Republic of Korea*

Received 16 November 2006, and in revised form 31 January 2007
Available online 12 February 2007

Abstract

Tissue inhibitor of metalloproteinase-2 (TIMP-2) is an endogenous and bi-functional inhibitor of angiogenesis. TIMP-2 is expressed in an insoluble form in *Escherichia coli* and secreted at a very low level from yeast. Here, we report on a high level of secretion of TIMP-2 fused with human serum albumin (HSA) from the yeast *Saccharomyces cerevisiae*. The secreted HSA–TIMP-2 fusion protein (87 kDa) was purified to greater than 95% homogeneity. The HSA–TIMP-2 protein inhibited approximately 81% of tube formation of human umbilical vein endothelial cells (HUVECs) when studied at a concentration of 187 μM. The systemic administration of HSA–TIMP-2 at 40 mg/kg to the C57B1/6 mouse inhibited the growth of B16BL6 tumors. Furthermore, a combination treatment of HSA–TIMP-2 with 5-fluorouracil (50 mg/kg) showed significant effects on tumor growth in this model. The high level of secretion of the biologically active angiogenesis inhibitor from *S. cerevisiae* should facilitate fundamental research and application studies of HSA–TIMP-2, as an attractive candidate for therapeutic agents treating angiogenesis-related diseases.

Keywords: HSA–TIMP-2; Angiogenesis inhibitor; Recombinant protein; Secretion; Yeast

Angiogenesis is the process of generating new capillary blood vessels from pre-existing vasculature. The proliferation of endothelial cells in the new capillaries is induced by pro-angiogenic cytokines, inflammation mediators, and activated proteolytic enzymes [1]. Failure to regulate angiogenesis, however, leads to the development of several chronic diseases, including rheumatoid arthritis, retinopathy, psoriasis, and metastatic cancers.

The mammalian tissue inhibitor of metalloproteinase (TIMP)[1] family, consisting of four proteins (TIMP-1, 2, 3, and 4), regulate the activity of matrix metalloproteinases (MMPs) that are capable of degrading the extracellular matrix [2,3]. Of the four TIMPs, TIMP-2 is unique because of its ability to inhibit endothelial cell proliferation through binding to α3β1-integrin receptors independent of the MMP inhibition mediated by the N-terminal domain of TIMP-2 [4,5]. The C-terminal domain of TIMP-2 possessing the MMP-independent anti-angiogenic activity is a potent inhibitor of both embryonic and mitogen-stimulated angiogenesis *in vivo* [6]. This feature is in contrast to the N-terminal domain that does not suppress the neovascularization caused by angiogenic mitogen. The MMP-independent anti-angiogenic activity of TIMP-2 has been mapped to a 24-amino acid peptide corresponding to loop 6 of TIMP-2 (Fig. 1a) [6].

Human serum albumin (HSA) is secreted in large quantities from many yeast strains, and thus the fusion of HSA

* Corresponding author. Fax: +82 42 822 7367.
E-mail address: jykim@cnu.ac.kr (J.-Y. Kim).

[1] *Abbreviations used:* TIMP, tissue inhibitor of metalloproteinase; HSA, human serum albumin; E. coli, Escherichia coli; S. cerevisiae, Saccharomyces cerevisiae; SH2, Src-homology2; PTP, protein tyrosine phosphatase; HUVECs, human umbilical vein endothelial cells; MMPs, metalloproteinases; SC-URA, synthetic complete medium lacking uracil; DO, dissolved oxygen.

1046-5928/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2007.02.001

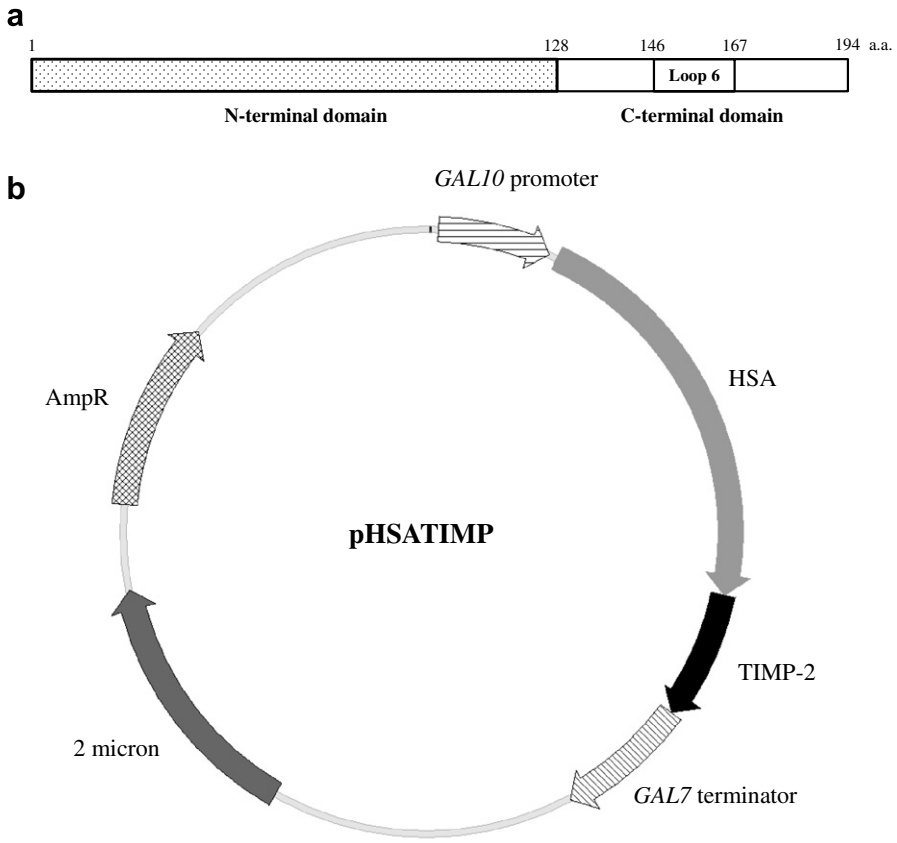

Fig. 1. A protein domain of TIMP-2 (a) and a schematic representation of the pHSATIMP plasmid (b). Loop 6 is known to be responsible for the MMP-independent anti-angiogenic activity of TIMP-2 [6].

to the N terminus of a target protein is a strategy that may lead to the efficient secretion of the protein [7–10]. Furthermore, since the half-life of HSA approximates 19 days, a therapeutic protein fused with HSA can be expected to have an increased circulating half-life [11–14]. Indeed, many therapeutic proteins have been fused to HSA and produced from recombinant cells, and shown to not only retain biological activity but improve *in vivo* stability [12,14,15].

A small amount of active TIMP-2 protein can be prepared by refolding insoluble TIMP-2 expressed in *Escherichia coli* [16,17], but the lack of an efficient expression system has hampered the study of TIMP-2 *in vivo*. Since, the N-terminal domain of TIMP-2 does not have the inhibitory activity against the mitogen-stimulated angiogenesis that closely mimics aberrant angiogenesis *in vivo* [4,6], the fusion of TIMP-2 to the C terminus of HSA (HSA–TIMP-2) was hypothesized not to affect MMP-independent mitogen-stimulated angiogenesis.

In this study, we generated the HSA–TIMP-2 fusion protein for secretion from the yeast *Saccharomyces cerevisiae,* and purified the HSA–TIMP-2 to homogeneity. We found that purified HSA–TIMP-2 could prevent human umbilical vein endothelial cells from forming tubes *in vitro* and effectively suppressed tumor growth in a mouse model. The high level of secretion of a biologically active angiogenesis inhibitor from *S. cerevisiae* can be expected to aid the development of new therapeutic agents for angiogenesis-related diseases.

Materials and methods

Yeast strains and media

The yeast strains used in this study were *S. cerevisiae* 2805 (*MATa pep4::HIS3 prb-Δ1.6R can1 his3-20 ura3-52*), S28G1 (*MATa pep4::HIS3 prb-Δ1.6R can1 his3-20 ura3-52 gal1::tc5*), S28H (*MATa pep4::HIS3 prb-Δ1.6R can1 his3-20 ura3-52 hxk2::tc5*), and S28GH (*MATa pep4::HIS3 prb-Δ1.6R can1 his3-20 ura3-52 gal1::tc5 hxk2::tc5*) [7]. The strains were stored at −80 °C in cryo-vials containing YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) with 30% (v/v) glycerol. The cells were transferred from their glycerol stocks to a synthetic complete medium lacking uracil (SC-URA) and subsequently transferred to YPD medium to generate seed cultures. YPDG medium (1% yeast extract, 2% peptone, 2% dextrose, and 2% galactose) was used to induce the expression of HSA–TIMP-2 from the *GAL* promoter.

Cultivation and analysis

The shake flask studies were carried out at 30 °C using triple-baffled Erlenmeyer flasks. The batch fermentation studies were performed under conditions of controlled pH (5.5) and temperature (30 °C) in a 5 l bioreactor (NBS) containing 3 l of YPDG medium. Fed-batch culture was