Inhibitory Effect of Plant Extracts on VHR Dual Specificity Protein Phosphatase

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Received november 16, 2007; Accepted December 5, 2007

Key words: *VHR, Dual specificity protein phosphatase, plant extracts, inhibition*

Vaccinia H1-related phosphase (VHR) is a member of the Dual specificity protein phosphatases (DSPs) that dephosphorylate both phospho-tyrosyl and phospho-seryl/threonyl residues and is involved in the mitogen-activated protein kinase (MAPK) signaling cascade in mammalian cells [Denu *et al.*, 1995; Alonso *et al.*, 2001]. When subjected to the various stimulations such as epidermal growth factor (EGF) and stress, the extracellular-signal-regulated kinase 1 (ERK1) or ERK2 is activated within 5 min, and then inactivated within 20 min by the administration of VHR in COS-1 cells [Todd *et al.*, 1999]. The activated ERKs phosphorylate several important cytoplasmic substrates and also translocate to the nucleus, where they phosphorylate some transcription factors that cause changes in the gene expression [Marshall, 1995]. VHR can suppress T cell antigen receptor signaling and inhibits T cell activation [Alonso *et al.*, 2003]. Loss of the VHR causes the hyperactivation of JNK and ERK, resulting in cell-cycle arrest and senescence [Rahmouni *et al.*, 2006]. Specific inhibitors of VHR are valuable candidates for the treatment of several human diseases and are expected to be useful for revealing the physiological functions of VHR [Usui *et al.*, 2001; Bae *et al.*, 2004; Shi *et al.*, 2007]. In this present paper we report on the investigation of 20 plant extracts collected from Jeju Island for their inhibitory effects on VHR. Freshly picked plants were dried at 55°C using a convection dry oven for 1 day and shredded. The samples were extracted with 80% (V/V) methanol, concentrated under reduced pressure, and freeze-dried.

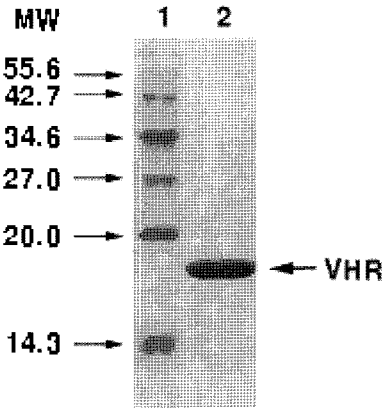

Fig. 1. Expression and purification of VHR. VHR was expressed in BL21 (DE3) *E.coli* and purified by chromatography. The purified protein (2 μg) was analyzed by SDS-PAGE. Positions of the molecular mass markers (kDa) are shown on the left.

The full-length VHR gene was amplified by PCR using the human cDNA (Clontech, Mountain View, CA). The amplified PCR product was subcloned into the *Nde*I - *Bam*HI site of the pET28a(+) vector (Novagen, Darmstad, Germany), resulting in the N-terminal His-tagged protein. BL21(DE3) *E.coli* cells harboring the VHR gene were grown at 18°C, and the protein expression was induced with 0.1 mM IPTG for 16 h. The cell lysate was prepared by sonication with a buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5 mM PMSF, 0.04% (v/v) 2-mercaptoethanol. The His-tagged VHR protein was purified by nickel-affinity chromatography. The His-tag was removed from the protein by thrombin digestion, and the protein was further purified by CM-Sepharose ion exchange chromatography (Fig. 1)

The inhibitory effects of the 20 plant extracts on the VHR activity were measured using the para-nitrophenyl-phosphate (pNPP) as a substrate [Denu *et al.*, 1995b]. The reaction mixture (total volume 100 μL) contained 40 mM Tris-Cl (pH 6), 150 mM NaCl, 2 mM EDTA, and 0.1 mM pNPP with or without the plant extracts. The reactions were performed at 30°C for 10 min and were stopped by

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Abbreviations: PCR, polymerase chain reaction; *E.coli*, *Escherichia coli*; PMSF, phenyl methyl sulfonyl fluoride; IPTG, isopropyl-β-D-thiogalactopyranoside; CM-Sepharose, carboxymethyl-Sepharose

Table 1. Inhibitory effects of Jeju plant extracts on VHR

Voucher Specime No.	Sample name	Part used	Inhibition (%)
K- 1	*Zanthoxylum planispinum* S. et Z.	Leaves	54 ± 5.1
K- 2	*Crinum asiaticum* var. *japonicum* Baker	Leaves	1 ± 4.6
K- 4	*Viscum album* var. *coloratum* (Kom.) Ohwi	Aerial Part	70 ± 9.2
K-16	*Viburnum awabuki* K. KOCH	Stem	41 ± 3.4
K-20	*Leonurus sibiricus* L.	Root	34 ± 7.2
K-24	*Portulaca oleracea* L	Aerial Part	43 ± 5.5
K-25	*Viola Mandshurica* W.Becker	Aerial Part	27 ± 2.9
K-27	*Ligularia fischeri* (Ledeb.) Turcz.	Aerial Part	61 ± 9.8
K-28	*Empetrum nigrum* L. var. *japonicum* K. Koch	Aerial Part	92 ± 7.5
K-30	*Geranium nepalense* subsp.thunbergii	Aerial Part	89 ± 9.1
K-31	*Torilis japonica* (Houttuyn) DC	Root	1 ± 7.7
K-40	*Halorrhagis micrantha* R. Br.	Aerial Part	94 ± 3.6
K-51	*Caesalpinia japonica* Siebold. et Zuccarini.	Stem	48 ± 7.5
K-52	*Calystegia soldanella* Roem. et Schult.	Aerial Part	37 ± 3.2
K-56	*Platycarya strobilacea* Siebold et Zuccarini	Stem	95 ± 4.1
K-58	*Acorus calamus* Linne var. angustataus	Root	35 ± 8.7
K-63	*Oenothera biennis* L.	Aerial Part	95 ± 3.8
K-72	*Zingiber Mioga* (Thunb.) Roscoe	Root	76 ± 8.7
K-74	*Angelica japonica* A. Gray	Aerial Part	46 ± 8.6
K-81	*Glehnia littoralis* Fr. Schm	Root	38 ± 4.5

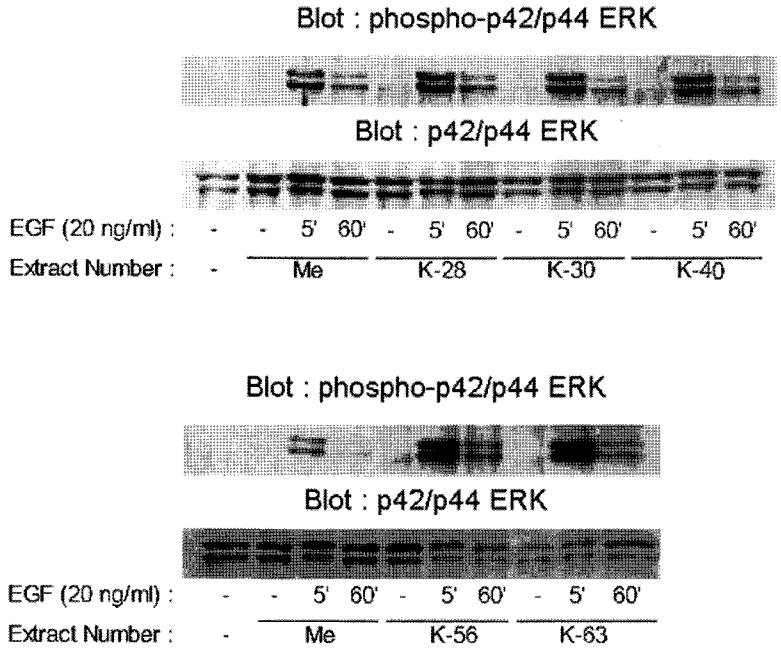

Fig. 2. Effects of plant extracts on ERK. Serum-starved 293T cells were incubated with 0.02 mg/mL plant extracts for 1 h at 37°C and stimulated with EGF for the indicated times (min). Me: Methanol. (a) Cell lysates were analyzed with antibodies against the phospho-p42/p44 ERK and p42/p44 ERK. (b) Level of phospho-p42/p44 ERK remaining at the indicated times was densitometrically quantitated (ImageJ software).

adding 100 μL of 2 N NaOH. The inhibition of VHR activity was calculated as a decrease in the absorbance at 405 nm of the product para-nitrophenolate, compared to that of the control not containing the plant extract. At a concentration of 200 μg/mL, marked inhibitory effects (>70%) were observed from the methanol extracts of the

aerial parts of *Viscum album* var. *coloratum* (Kom.) Ohwi (K-4), *Empetrum nigrum* L. var. *japonicum* K. Koch (K-28), *Geranium nepalense* subsp.thunbergii (K-30), *Halorrhagis micrantha* R. Br. (K-40), *Oenothera biennis* L. (K-63), as well as from the stems of *Platycarya strobilacea* Siebold et Zuccarini (K-56), and the roots of *Zingiber Mioga* (Thunb.) Roscoe (K-72). The extracts of *Crinum asiaticum* var. *japonicum* Baker (K-2) and *Torilis japonica* (Houttuyn) DC (K-31) had no inhibitory effects on the VHR activity, whereas the other remaining plant extracts showed moderate inhibitions on the VHR activity (Table 1).

Because ERK has been identified as a substrate for VHR, we tested whether the plant extracts have inhibitory effects on the VHR activity toward the dephosphorylation of ERK in 293T cells. The serum-starved 293T cells were incubated with the plant extracts (0.02 mg/mL) such as K-28, K-30, K-40, K50, and K60 for 1 h and treated wi the epidermal growth factor (EGF). The cell lyastes were prepared, and the levels of phosphorylated ERKs were determined by Western blotting using antibodies against phospho-p42/p44 ERK and p42/p44 ERK (Fig. 2a). Densitometric analysis was employed to quantitate the level of phospho-p42/p44 ERK (Fig. 2b). EGF induced the phosphorylation of p42/p44 ERK within 5 min in 293T cells (Fig. 2), followed by the dephosphorylation of p42/p44 ERK. After 1 h, only the weak band of phospho-p42/p44 ERK could be detected (Fig. 2a, lanes 1-4). K-40 showed little increase in the phosphorylation of ERK in 293T cells, whereas K-28, K-30, K-56, and K-63 enhanced the phosphorylation of ERKs at 5 min (Fig. 2a and 2b). Among the extracts tested, K-63 showed the strongest accumulation of the phosphorylated ERKs at 5 min, and a strong band of the phospho-p42/p44 ERK was detected after 1 h, compared to those of the control and the other extracts (Fig. 2). Although the extract of *Platycarya strobilacea* Siebold et Zuccarini (K-56) has been used for various medicinal purposes [Choi *et al.*, 2003], the inhibitory effect on phosphatase has not yet been reported. Our results demonstrate that K-56 is a potent candidate for the development of a VHR inhibitor.

Acknowledgment. This work was supported by the Education program for environment favorable agriculture & subtropical bio-industry.

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