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Identification of Proteins Binding to Decursinol by Chemical Proteomics

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Decursinol, found in the roots of *Angelica gigas* Nakai, has been traditionally used to treat anemia and other various diseases. Recently, numerous biological activities such as cytotoxic effect on leukemia cells, and antitumor, neuroprotection, and antibacterial activities have been reported for this compound. Although a number of proteins including protein kinase C, androgen receptor, and acetylcholinesterase were proposed as molecular targets responsible for the activities of decursinol, they are not enough to explain such a diverse biological activity mentioned above. In this study, we employed a chemical proteomic approach, leading to identification of seven proteins as potential proteins interacting with decursinol. Most of the proteins contain a defined ATP or nucleic acid binding domain and have been implied to be involved in the pathogenesis and progression of various human diseases including cancer, autoimmune disorders, or neurodegenerative diseases. The present results may provide clues to understand the molecular mechanism of the biological activities shown by decursinol, an anticancer natural product.

Keywords: Anticancer, chemical proteomics, decursinol, enolase, Hsp90

Chemical proteomics, which may represent a focused proteomics, consists of affinity chromatographic purification of potential targets by biologically active molecules immobilized on solid supports and subsequent identification of the purified proteins by mass spectrometry [9, 17, 19]. Together with chemical genetics, therefore, it has been successfully employed to elucidate the molecular mechanism of the biological activity shown by natural products [5, 11] or synthetic compounds [7].

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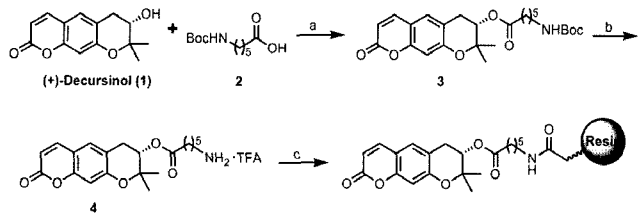
The root of Korean medicinal herb *Angelica gigas* Nakai (also known as Cham Dang Gui) has been used in traditional Oriental herbal medicine for thousands of years [21]. Decursinol and its derivatives, which were isolated from the root of *Angelica gigas* Nakai, have been reported to show various biological activities such as aldolase inhibition [26], neuroprotection [13, 15, 22], antiplatelet [25], antibacterial [3, 24], and anticancer activities [1, 8, 23]. The proposed molecular targets for its biological activities include protein kinase C (PKC) [2, 18, 28], acetylcholinesterase [14], and androgen receptor [10]. Although these proteins account for a part of biological activities shown by decursinol, they are not enough to explain the whole biological activities reported until now. Amongst the biological activities of decursinol derivatives, the anticancer activities and their related molecular targets have been repeatedly reported by many research groups. PKC and androgen receptor have been suggested as potential molecular targets responsible for the anticancer activity. Yim *et al.* [30] have reported that decursinol derivatives induced a strong G1 arrest to human prostate carcinoma cells but not to normal human prostate epithelial cells. In addition, there have also been some contradictory reports for the biological activities of decursinol. Hence, we have tried to mine target proteins of decursinol by employing a chemical proteomic approach in order to dissect the molecular mechanisms and physiological functions of decursinol.

As the first step to prepare the decursinol affinity column, (+)-decursinol (**1**) and 6-*N*-*tert*-butyloxycarbonylaminocaproic acid (**2**) were synthesized following the literature procedure [20]. Compound **3** was prepared by esterification of decursinol with compound **2** using *N*-ethyl-*N'*,*N'*-dimethylaminopropylcarbodiimide (EDC). Treatment of compound **3** with 30% trifluoroacetic acid furnished compound **4** carrying a free amine group. The decursinol affinity column (DAC) was prepared by forming an amide bond between compound **4** and NHS-activated Sepharose resin (Sepharose 4 Fast Flow), as shown in Scheme 1.



Scheme 1. Reagents and conditions: (a) EDC, Et_3N, CH_2Cl_2; (b) 30% Trifluoroacetic acid in CH_2Cl_2; (c) Sepharose 4 Fast flow, Diisopropylethylamine, DMF.

The mouse HT22 hippocampal cell line was maintained in Dulbecco's modified Eagle's medium (GibcoBRL, Gaithersburg, MD, U.S.A.) with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA, U.S.A.) [12, 16]. The cells were gently pipetted and subsequently ultrasonicated in a lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 1 μg/ml leupeptin]. After centrifugation of the cell lysate (16,600 ×*g* for 20 min at 4°C), the supernatant was taken and its protein concentration was determined using a Bradford assay. The 500 μg protein samples were applied onto the affinity column (DAC) at a flow rate of 0.5 ml/min. The column was washed with 10 ml of a binding buffer (50 mM Tris pH 7.5, 200 mM NaCl, 1 mM EDTA, and 1 mM PMSF) and subsequently eluted with 500 mM decursinol in the binding buffer.

Each fraction was loaded and run on a 10% SDS-PAGE gel (18×16 cm) at a constant voltage of 220 V (Fig. 1). The elution fractions (lanes 6 and 7) were compared with total protein fraction (lane 1), flow-through (lane 2), and wash fraction (lanes 3, 4, and 5). The eluted proteins were also compared with the proteins (lane 8) bound to the column that were not eluted by decursinol (Fig. 1). The bands of interest were manually excised. Then, the proteins were destained, and in-gel digested with trypsin. The extracted peptides were put through a desalting/concentration step on μZipTipC18 (Millipore Corp.) before LC-MS/MS (Q-TOF Premier, Micromass) analysis.

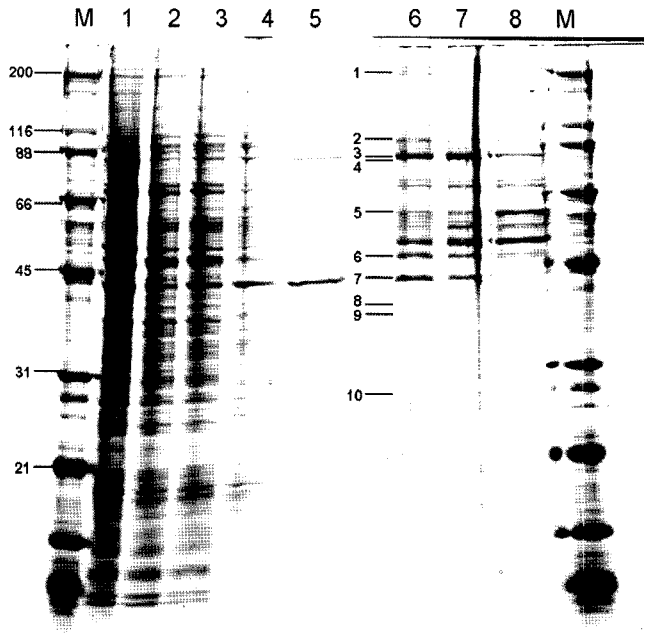


Fig. 1. SDS-PAGE analysis of the enriched proteins by DAC. M, broad range marker; 1, total cell lysate; 2, flow-through; 3, 1st wash; 4, last wash; 5, DMSO wash; 6, 1st elute; 7, 2nd elute; 8, boiled resin.

Protein identification was processed and analyzed by searching the NCBI protein database using the MASCOT search engine of Matrix Science that is integrated in the global protein server (GPS) workstation (http://www.matrixscience.com). The mass tolerance was limited to 0.1 Da. Overall, 14 protein bands that showed specific binding to DAC were excised and subjected to LC-MS/MS analysis as described. Among them, 10 protein bands were successfully identified with a high reliability, corresponding to 7 different proteins (Table 1). The identified proteins have various p*I* values, suggesting that decursinol does not show a notable binding preference toward the total charge of the proteins. Interestingly, all the seven proteins have an ATP-binding domain or bind nucleic acids such as DNA and RNA as well as relevance to cancers.

Table 1. Target identification by LC-MS/MS.

Spot No.	ID	Seq. cov. (%)	Matched peptides	p*I*	MW (kDa)	Expectation value	Mascot score[a]	NCBI
1	Myosin, heavy polypeptide 9, non-muscle	20	35	5.54	227.4	2.0E-07	126	gi|20137006
2	Tumor rejection antigen gp96	36	36	4.74	92.7	1.0E-10	159	gi|14714615
3	Heat-shock protein 1, β	41	40	4.97	83.5	5.0E-20	252	gi|40556608
4	Heat-shock protein 1, β	9	12	4.97	83.5	0.045	65	gi|40556608
5	Heat-shock protein 1	45	23	5.67	61.0	1.6E-06	117	gi|31981679
6	Eno1 protein	33	15	7.67	50.1	0.0087	80	gi|54114937
7	ACTG1 protein	58	25	5.31	42.1	7.9E-24	290	gi|6752954
8	Hnrpa2b1 protein	35	7	8.94	23.0	0.029	74	gi|37747847
9	Tumor rejection antigen gp96	22	21	4.74	92.7	0.029	74	gi|6755863
10	Heat-shock protein 1, β	42	42	4.97	83.5	3.2E-22	274	gi|40556608

[a]Mascot scores greater than 61 are significant ($p<0.05$).

Among the proteins identified as the decursinol-binding partner in this study, enolase 1 is a multifunctional enzyme in the glycolysis pathway as well as various processes including growth control, apoptosis, allergic responses, and hypoxia tolerance [29]. In addition, it is also involved in the intravascular and pericellular fibrinolytic system as a receptor and activator of plasminogen on the cell surface [29]. Furthermore, enolase 1 is often overexpressed in various metastatic cancer cells. Thus, it would be expected that decursinol binds enolase 1 to inhibit the activity, and ultimately exerts its antitumor effect. However, it would be further studied whether decursinol directly binds to enolase 1 or indirectly interacts *via* other decursinol-binding proteins.

The heat-shock protein 1 (also known as Hsp90) is the most abundant molecular chaperone in eukaryotic cells [4]. There are four isomers of Hsp90; Hsp90α (inducible/major form), Hsp90β (constitutive/minor form), 94 kDa glucose-regulated protein (GRP94), and Hsp75/tumor necrosis factor receptor associated protein 1 (TRAP-1). Hsp90 binds many client proteins and functions as a chaperone. These client proteins include protein kinases, nuclear hormone receptors, transcription factors, and a number of essential proteins [4]. Therefore, it is worthy to investigate whether the decursinol affects the chaperone activity of Hsp90β. Hence, a luciferase refolding assay was performed, according to the literature procedure [6]. In this experiment, decursinol inhibited the chaperone activity of Hsp90β in a concentration-dependent manner up to 250 μM, which is the maximum solubility limit in the assay condition. The 250 μM of decursinol showed about 50% inhibition of luciferase refolding activity by Hsp90β. Decursinol has been known to induce the G1 arrest of carcinoma cells [30], which is the typical effect of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), a well-known Hsp90 inhibitor [27]. Together with the previous reports, the present results strongly suggest that decursinol shows the anticancer effect by modulating the interaction of Hsp90 with its client proteins.

In summary, we have enriched and identified decursinol-binding proteins using chemical proteomics that employs a decursinol-linked affinity column and LC-MS/MS mass spectrometry. The identified proteins include chaperones and nucleic acid binding proteins that are highly related to cancers and autoimmune diseases. The results may afford clues to understand the molecular mechanism of the biological activities shown by decursinol.

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