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Identification of anti-inflammatory active peptide from black soybean treated by high hydrostatic pressure after germination

Min Young Kim[a], Gwi Yeong Jang[b], Yoon Jeong Lee[a], Koan Sik Woo[c], Bang Yeon Hwang[d], Junsoo Lee[a], Heon Sang Jeong[a,*]

[a] Depatment of Food Science and Biotechnology, Chungbuk National University, Cheongju 28644, South Korea
[b] Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumseong 27709, South Korea
[c] Department of Central Area Crop Science, National Institute of Crop Science, Rural Development Administration, South Korea
[d] College of Pharmacy, Chungbuk National University, Cheongju 361-763, South Korea

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ABSTRACT

This study was conducted to isolate and identify the anti-inflammatory active peptide in black soybean treated by high hydrostatic pressure after germination. The anti-inflammatory activity was examined using RAW 264.7 macrophages. Black soybean was germinated for 4 days and subjected to 150 MPa for 24 h, followed by preparation of protein extracts. The extracts were sequentially filtered using membranes with a molecular weight cutoff of 30, 10, and 3 kDa. A strong inhibitory effect was observed with the 3–10 kDa fraction. The active peptide from this fraction was purified by gel permeation column chromatography and first and second semi-preparative HPLC. After isolation and purification of the active peptide, secretion of inflammatory markers (nitric oxide, TNF-α, IL-1β, and IL-6) by LPS-stimulated RAW 264.7 macrophages was measured. The purified anti-inflammatory peptide was analyzed by UV, LC–MS, ^{1}H and ^{13}C NMR, COSY, HMBC, and HSQC and was identified as a tripeptide.

1. Introduction

Inflammation is a complex response to local injury or infection. It involves various immune cells and numerous mediators (Kindt et al., 2007). While acute inflammation is essential for combating infection and tissue repair, excessive and uncontrolled inflammation is often associated with chronic diseases, such as metabolic disorders, atherosclerosis, and certain types of cancer (Chakrabarti et al., 2014; Tabas and Glass, 2013). Anti-inflammatory peptides have been well studied in a variety of food sources, including milk, egg, fish, and soy (Chakrabarti et al., 2014). Protein extracts, including bioactive peptide, are used as additional and alternative therapies that inhibit the production of numerous inflammatory factors, such as nitric oxide, prostaglandins, and cytokines, which are involved in the immune response. Proteins and peptides with anti-cancer properties have been found in soybean, including lectins (Abe et al., 1996), Bowman-Birk inhibitor (Kennedy and Wan, 2002), and the most recently discovered molecule, lunasin (Jeong et al., 2007). Biologically active peptides can occur naturally or are derived from soy protein hydrolysates by various methods, such as enzymatic digestion and fermentation. Some bioactive peptides have demonstrated anti-cancer, anti-hypertensive, hypocholesterolemic, anti-obesity, anti-oxidant, immunomodulatory, and anti-inflammatory activities (Wang and de Mejia, 2005).

Sprouting or germination of seeds involves the metabolism of storage proteins to produce simple compounds that support the growing embryo (Mbithi-Mwikya et al., 2001). Furthermore, high hydrostatic pressure (HHP) technology has been used to improve the digestibility and enhance the protein hydrolysate bioactivity of various crops. HHP has been applied to pea (Chao et al., 2013), lentil (Garcia-Mora et al., 2015), and soybean (Penas et al., 2004) to enhance their bioactive peptide production (Garcia-Mora et al., 2015). Modification of protein structure and conformation by HHP causes partial protein unfolding, which facilitates enzyme access to cleavage sites. Enhanced access to cleavage sites increases enzymatic activity and the extent of hydrolysis (Bonomi et al., 2003).

Kim et al. (2017) reported the effects of HHP treatment after germination on soybean protein characteristics, bioactive peptide production, and regulation of inflammatory factors. However, no study has sought to identify active compound from protein extracts of black soybean treated by HHP after germination.

The aims of the present study were to isolate the anti-inflammatory active peptides from protein extracts of black soybean treated by HHP

* Corresponding author.
E-mail address: hsjeong@chungbuk.ac.kr (H.S. Jeong).

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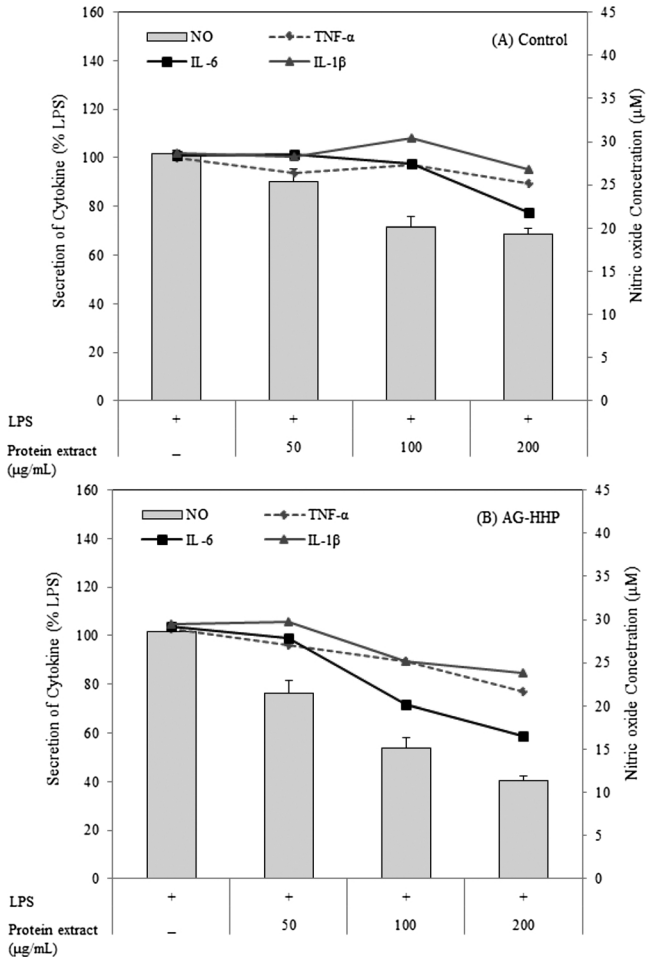

Fig. 1. Effect of soluble protein extracts of black soybean treated by high hydrostatic pressure treatments after germination (AG-HHP) on nitric oxide (NO), TNF-α, IL-6 and IL-1β secretion of RAW 264.7 cell stimulated with LPS (0.5 μg/mL).

after germination through bioassay-guided fractionation techniques, to identify the chemical structures by spectroscopic methods, and to evaluate the regulatory effect of active peptide on inflammatory factors.

2. Results and discussion

2.1. Isolation of anti-inflammatory peptide

Anti-inflammatory activity was preliminarily screened from protein extracts of black soybean with germination periods of 0 to 4 days and applied pressure (0.1–150 MPa). The highest ant-inflammatory activity was found at 150 MPa after germination for 4 days (Kim et al., 2017). Protein extracts of black soybean treated by HHP after germination more effectively inhibited secretion of NO, proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, than raw black soybean (Fig. 1). Therefore, we attempted to isolate the active peptides from protein extracts through bioassay-guided fractionation techniques. Bioactivity of protein hydrolysates was mainly affected by the molecular weight of the peptides. An ultrafiltration membrane system was used to separate the protein extract into defined molecular weight ranges. This approach is effective in the purification of simple peptides from various crude protein hydrolysates (Guo et al., 2009). The protein extract was sequentially passed through 30 kDa, 10 kDa, and 3 kDa MWCO membranes. The protein extract was separated into four fractions and referred to as the < 3 kDa fraction (PM1), 3–10 kDa fraction (PM2), 10–30 kDa fraction (PM3) and > 30 kDa (PM4), respectively. Four fractions (PM1–PM4) were collected and assayed for anti-inflammatory activity (Fig. 2). The NO concentration in the medium markedly increased after treatment with 0.5 μg/mL LPS for 24 h (27.21 μM) compared to the concentration in the medium of the unstimulated control (4.26 μM). The NO concentration in the medium of RAW 264.7 cells was significantly lower in the PM1 (MW < 3 kDa) and PM2 (MW 3–10 kDa) fractions with 9.45 and 7.29 μM, respectively as compared to PM3 (10–30 kDa) and PM4 (MW > 30 kDa) with 15.31 and 19.82 μM at concentration of 200 μg/mL. The strong inhibitory effect was observed in the PM 2 fraction (3–10 kDa). There was a significant increment in the NO inhibition rate of the two fractions (PM1 and PM2) compared to the protein extracts, which was consistent with the results of a previous study in which the isolated peptide fraction showed anti-inflammatory activity compared to the hydrolysate (Yin et al., 2009).

2.2. Purification of anti-inflammatory peptide

Fractions PM1 and PM2 obtained from ultrafiltration with the 10 kDa MWCO with prominent anti-inflammatory activity was further separated into six fractions (PM-S1–PM-S6) using GPC (Sepadex G15;

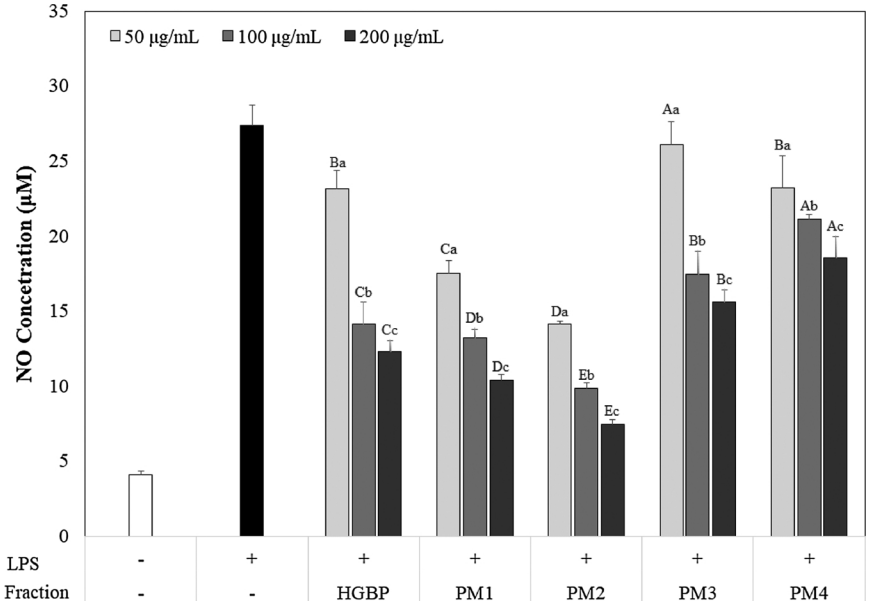

Fig. 2. Effect of molecular weight cutoff fraction (PM1–PM4) isolated from protein extracts treated by high hydrostatic pressure treatments after germination on nitric oxide concentration of RAW 264.7 cell stimulated with LPS (0.5 μg/mL). Different capital and small letters on the error bars indicate a significant difference ($p < 0.05$) among different subfraction of protein extracts and concentrations.