Doctoral Thesis

Studies on Intestinal Protective Effects of Jack Bean

(*Canavalia ensiformis* (L.) DC) Protein Hydrolysates

（Summary）

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学 位 論 文 の 要 旨

論文題目　Studies on Intestinal Protective Effects of Jack Bean (*Canavalia ensiformis* (L.) DC) Protein Hydrolysates (タチナタマメ由来タンパク質加水分解物による腸管保護作用に関する研究)

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Regulation of intestinal homeostasis is essential since it protects and maintains the functions of the gastrointestinal tract. The importance of maintaining the protective functions of the intestinal epithelial cells is a crucial approach to reduce the progression of inflammation. Adaptive responses through the presence of antigens that induce inflammation are also mediated by different immune cell populations, such as macrophages, which produce the regulatory and effector molecules. Accordingly, suppressing inflammation-related molecule production by active dietary components may effectively promote intestinal health. Extensive studies have shown the homeostatic regulation of intestinal protection by biologically active peptides. Jack bean (JB, *Canavalia ensiformis* (L.) DC) is a protein-rich legume commonly cultivated in Indonesia. Jack bean consists of a significant amount of hydrophobic amino acids, such as leucine, valine, proline, and alanine. The hydrophobic amino acid was expected to generate bioactive peptides with physiological functions. Indeed, it has been reported that protein hydrolysates derived from JB protein exhibit various biological activities such as radical scavenging activity, ferric reducing power, and angiotensin-converting enzyme inhibitory activity. Thus, the JB protein can be a promising source of bioactive peptides. Enzymatic hydrolysis using *in vitro* models has been widely recognized as a method for the production of bioactive peptides. The present study aimed to produce anti-inflammatory peptides from JB protein by enzymatic digestion using pepsin-pancreatin and alcalase enzymes to produce jack bean protein hydrolysates (JBPH). To investigate the anti-inflammatory effect of peptides from JBPH and their underlying mechanisms, the human intestinal cell Caco-2BBe cells, mouse macrophage RAW 264.7 cells, and DSS-induced colitis mice were used in the study. Additionally, the identification of potential peptides responsible for the anti-inflammatory activity was performed using an *in silico* approach.

The anti-inflammatory activity and molecular mechanism from jack JBPH derived from pepsin-pancreatin enzyme (JBPH-PP) and alcalase (JBPH-ALC) were investigated in Caco-2BBe induced tumor necrosis factor (TNF)-a and identified the potential anti-inflammatory peptide using an *in silico*

approach (study 1 and 2). This study found that JBPH-PP and -ALC reduced the interleukin-8 (IL-8) expression at protein and mRNA levels in Caco-2BBe cells stimulated with TNF-α. Immunoblot analysis showed that the JBPH-PP reduced the TNF-α-induced phosphorylation of c-Jun-NH(2)-terminal kinase, nuclear factor kappa B (NF-κB), and p38 proteins. However, JBPH-ALC only reduces (NF-κB) and p38 proteins. Separation of JBPH-PP and -ALC with three-step acetonitrile gradient elution in a Sep-Pak C18 cartridge indicated anti-inflammatory activity at a fraction of 30% and 65% acetonitrile, respectively. Further separation with ultrafiltration revealed that small peptides (<3kDa) from JBPH-PP and -ALC had a potent inhibitory effect on IL-8 production. Purification of the peptides by reversed-phase (RP) and anion-exchange high-performance liquid chromatography (HPLC) obtained three peptide fractions with anti-inflammatory activities. A combination of mass spectrometry analysis and an *in silico* approach by PeptideRanker, BIOPEP, and PreTP-EL identified 23 and 6 potential anti-inflammatory peptides from JBPH-PP and -ALC, respectively.

The anti-inflammatory activity and molecular mechanism of JBPH-PP and -ALC were also evaluated in RAW 264.7 cells (study 3 and 4). This study revealed that hydrolysis of JB protein by pepsin-pancreatin did not produce the peptides that reduced IL-6 and TNF-a production in LPS-stimulated RAW 264.7 cells. In contrast with JBPH-PP, JBPH-ALC attenuated inflammation in RAW 264.7 cells stimulated with LPS by reducing the expression of TNF-a in protein and mRNA levels. Further investigation on the underlying mechanism by immunoblot analysis showed JBPH-ALC inhibited the LPS-mediated phosphorylation of NF-κB and mitogen-activated protein kinase (MAPK), including extracellular signaling-regulated kinase (ERK) and p38 protein. Separation of peptides with ultrafiltration indicated that small peptides (<3kDa) from JBPH-ALC exerted potent inhibition on TNF-a production. Purification of the peptides by RP-HPLC obtained two peptide fractions (F6 and F8) showing potent anti-inflammatory activity. The *in silico* approach revealed novel anti-inflammatory peptides LFLLP and DFFL in JBPH-ALC.

Finally, the intestinal protective effect of JBPH-PP and -ALC in alleviating the pathological symptoms of DSS-induced colitis in mice, particularly intestinal inflammation and barrier function, was also evaluated (study 5 and 6). This study found that administration of JBPH-PP at a concentration of 500 mg/kg BW/day for two weeks moderately reduces the pathophysiological changes of intestinal inflammation after nine days of DSS-induced colitis, which tended to improve clinical score, colon length, body weight change, spleen index, expression of CXCL2 and tight junction proteins. However, further studies are needed to investigate the effective dose of JBPH-PP. Additionally, JBPH-ALC at a concentration of 200 mg/kg BW/day for two weeks tends to improve the body weight change. However, we did not find substantial evidence of the JBPH-ALC-mediated protective effect on colonic inflammation, even though our previous studies found anti-inflammatory activity *in vitro*. Further

experimental strategies can be developed to improve the significance of the anti-inflammatory effect of JBPH-PP and -ALC, including modification of dose and regiment, treatment with novel peptides LFLLP and DFFL, and administration of a mixture of JBPH-PP and -ALC to DSS-induced colitis mice.

Although the present study demonstrated the anti-inflammatory activity of JBPH-PP and JBPH-ALC in Caco-2BBe and RAW 264.7 cells, some limitations still exist. We did not confirm the anti-inflammatory effect of potential bioactive peptides identified using *in silico* approaches. Further investigations are needed to understand the beneficial effects of JBPH-ALC on intestinal health.