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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1. General Introduction (written in Chapter 1)

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. The different layers of the gut wall work synergistically and in coordination to preserve GI homeostasis. Since intestine epithelial is supported by the lumen structure, the coordination of biological response to inflammation is important. 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 dietary active components may effectively promote intestinal health. Extensive studies have shown the homeostatic regulation of intestinal protection by biologically active peptides. Peptides exert merit in alleviating inflammation by inhibiting the production of pro-inflammatory cytokines and improving the intestinal tight junctions.

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 that bring 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.

It has been established that the etiology of inflammatory bowel disease (IBD) is related to intestinal inflammation, including Ulcerative colitis (UC) and Crohn's disease (CD). UC showed characteristics

distinctively associated with the inflammation starting from the rectum and extending to the proximal colon. UC will crucially damage the intestinal tight junction, leading to disruption of the intestinal barrier. This will enable the passage of bacteria and its harmful metabolite to the bloodstream, which results in the secretion of multiple inflammatory cytokines (IL-1β, IL-6, and TNF-α) to promote inflammation. Extensive studies have reported that plant-derived dietary peptides play an important role in the treatment of colitis. Therefore, the anti-inflammatory activity from functional peptide-derived jack bean protein hydrolysates (JBPH) was evaluated to mitigate the progression of IBD in colitis mice induced by dextran sodium sulfate (DSS).

The present study aimed to produce anti-inflammatory peptides from JB protein by enzymatic digestion using alcalase and a combination of pepsin and pancreatin. 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 is performed using an *in silico* approach.

2. Identification and molecular mechanism of anti-inflammatory peptides isolated from jack bean protein hydrolysates using pepsin-pancreatin enzyme: *in vitro* studies with human intestinal Caco-2BBe cells.

   In Chapter 3, we investigated the molecular mechanism of anti-inflammatory activity from JBPH derived from pepsin-pancreatin enzyme (JBPH-PP) in Caco-2BBe induced TNF-a and identified the potential anti-inflammatory peptide using an *in silico* approach. This study found that JBPH-PP reduced the 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. Separation of JBPH-PP with three-step acetonitrile gradient elution indicated anti-inflammatory activity at a fraction of 30% acetonitrile. Further separation with ultrafiltration revealed that small peptides (<3kDa) from JBPH-PP 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 potential anti-inflammatory peptides.

3. Identification and molecular mechanism of anti-inflammatory peptides isolated from jack bean protein hydrolysates using alcalase enzyme: *in vitro* studies with human intestinal Caco-2BBe cells.

   In Chapter 4, we sought the molecular mechanism from JBPH derived from alcalase enzyme (JBPH-ALC) exerting anti-inflammatory effect in Caco-2BBe induced TNF-a and identify the potential anti-inflammatory peptides. This study showed that JBPH-ALC inhibits the production of IL-8 protein expression in Caco-2BBe cells stimulated with TNF-α. Immunoblot indicated that JBPH-ALC

attenuated inflammation by suppressing TNF-α-induced phosphorylation of NF-κB and p38, but not JNK proteins. Separation of JBPH-ALC with three-step acetonitrile gradient elution indicated anti-inflammatory activity at a fraction of 65% acetonitrile. Further separation with ultrafiltration suggested that peptides with molecular weight <3kDa from JBPH-ALC had a potent inhibitory effect on IL-8 production. Purification of the peptides by RP-HPLC indicated two fractions (F1 and F5) showing potent suppression of IL-8 secretion. Using the *in silico* approach with PeptideRanker, BIOPEP, and PreTP-EL, six peptides were identified as potential anti-inflammatory peptides in JBPH-ALC.

4. Anti-inflammatory effect of jack bean protein hydrolysates using pepsin-pancreatin enzyme: *in vitro* studies with mouse macrophage RAW 264.7 cells.

   In Chapter 5, we investigated the anti-inflammatory effect of JBPH-PP using mouse macrophage RAW 264.7 cells stimulated with lipopolysaccharide (LPS). 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

5. Identification and molecular mechanism of anti-inflammatory peptides isolated from jack bean protein hydrolysates using alcalase enzyme: *in vitro* studies with mouse macrophage RAW 264.7 cells.

   In Chapter 6, we elucidated the molecular mechanism of anti-inflammatory activity from JBPH-ALC using mouse macrophage RAW 264.7 cells stimulated with LPS and identified the potential anti-inflammatory peptide. This study revealed that 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.

6. Anti-inflammatory effect of jack bean protein hydrolysates using pepsin-pancreatin enzyme in DSS-induced colitis mice.

   In Chapter 7, we elucidate JBPH-PP's protective effects in alleviating the pathological symptoms of DSS-induced colitis in mice, particularly intestinal inflammation and barrier function. 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.

7. Anti-inflammatory effect of jack bean protein hydrolysates using alcalase enzyme in DSS-induced colitis mice.

   In Chapter 8, we verified the anti-inflammatory effect of JBPH-ALC in DSS-induced colitis mice. This study demonstrated that although administration of JBPH-ALC at a concentration of 200 mg/kg BW/day for two weeks tends to improve the body weight change, we did not find substantial evidence of the JBPH-ALC-mediated protective effect on colonic inflammation, even our previous studies found the anti-inflammatory activity *in vitro* study. Further investigation is needed to evaluate the beneficial effect of the JBPH-ALC on intestinal health.