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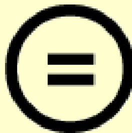

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A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

Fermentation of *Ecklonia cava* and physiological activity change

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GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

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Fermentation of *Ecklonia cava* and physiological activity change

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This thesis has been examined and approved.

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국문초록

최근 우리는 불규칙한 식습관과 과도한 스트레스, 환경오염 등에 노출되어 살아가고 있다. 이로 인한 면역력 저하는 각종 질병을 유발시키고, 고혈압, 동맥경화, 당뇨 등과 같은 성인병, 그리고 암과 같은 큰 질병에 쉽게 노출되는 원인이 되기도 한다. 따라서 이를 해결하기 위한 방법으로 면역력을 증가시키는 기능성 식품이나 천연의약품의 대한 연구가 활발히 이루어 지고 있는 실정이다.

우리나라는 예로부터 젓갈이나 김치, 청국장 및 막걸리 같은 발효공정을 거친 발효식품을 접하고 있다. 이러한 발효공정은 유용성분의 증가뿐만 아니라 소화력과 흡수율이 증가되고, 잔류농약도 미생물의 작용으로 인하여 유기물질로 분해되며, 한편으로 유익한 세균을 증가시킨다고 널리 알려져 있다. 그로 인해 발효는 식품으로뿐만 아니라 면역증강 같은 의약적인 면에서도 더욱 관심을 받고 있다.

한국의 남해안이나 제주 연안에 서식하고 있는 다시마목(*Laminariales*)의 다시마과(*Alariaceae*)에 속한 감태(*Ecklonia cava*)는 이미 이전 연구들을 통해서 혈압 및 혈중 콜레스테롤의 조정, 항염증, 면역활성 자극효능 및 항균이나 항산화, 항바이러스 같은 생리활성이 검증된 해조류이다. 하지만 추출물의 수율이 낮고, 추출하는 과정에서 많은 유효성분이 버려지고 있으며, 그 성분에 있어 대부분을 차지하는 알긴산이나 후코이단 같은 다당류의 특징상 인체흡수가 용이하지 못하다는 단점을 내포하고 있다. 이러한 문제점의 해결 방안으로 이번 연구에서는 해조류 감태의 발효공정을 수행하는 방법을 연구하였다.

총 3 가지의 발효미생물(*Lactobacillus brevis, Saccharomyces cerevisiae* 그리고 *Candida utilis*)를 이용하여 24 시간 동안 발효과정을 수행한 발효감태는 부산에 소제한

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㈜마린바이오프로세스에서 보유한 미생물 발효기를 이용하여 발효하였다. 발효감태의 일반성분을 측정한 결과, 일반 감태보다 오히려 당 함량은 줄어들고, 단백질 함량은 늘어난 것을 확인할 수 있었다. 이것은 발효미생물이 증식함에 따라 단백질 함량이 상대적으로 증가하여 당함량이 줄어든 것으로 보여진다. 이렇게 얻어진 발효감태를 각각 80% 에탄올과 증류수를 이용하여 추출한 결과, 일반감태의 추출물보다 발효감태를 사용한 모든 추출물에서 수율이 1.5 ~ 2 배 이상 증가한 것을 확인 하였고, 당성분 함량 또한 크게 증가한 것을 확인할 수 있었다. 하지만 발효감태 추출물은 일반 감태 추출물보다 폴리페놀의 함량은 감소하였다.

선천면역은 미생물이나 바이러스 같은 외래침입인자가 우리 몸에 들어 오는 것을 방어할 수 있도록 다양한 작용기전을 통해 빠르게 활성화되는 고전적인 숙주방어 형태이다. 그러나 이러한 면역반응이 지속적으로 나타나게 되면, 도리어 조직손상을 촉진하고, 그 결과 일부에서는 패혈증과 만성염증을 유발하게 되기도 한다.

내독소로 우리에게 알려진 lipopolysaccaride(LPS)는 그램 음성 균의 세포외막에 존재하여, RAW 264.7 세포와 같은 대식세포 또는 단핵세포에서 Tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), Interleukin-1β (IL-1β)와 같은 pro-inflammatory cytokin 을 증가시키는 것으로 알려져 있다. 또한 Nucler transcription factor-kappa B (NF-κB)는 사이토카인, 케모카인, 성장인자 그리고 세포부착 등에 관련된 여러 유전자들의 발현에 중요한 역할을 하는 전사인자로, 활성화된 NF-κB 는 iNOS, COX-2, TNF-α그리고 IL-6 등의 여러 염증 매개물질들의 전사를 촉진한다 알려져 있다.

발효감태의 항염증 효과를 알아보기 위해 3 가지 발효미생물을 이용하여 발효된 감태의 물 추출물을 RAW 264.7 에 처리하여 Nitric oxide (NO)를 측정한 결과, LPS 를 자극한 대식세포에서 생성된 NO 가 LPS 무처리 세포군보다 상당히 증가하였으며, 그것을

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기준으로 하여 발효감태들을 처리한 세포군과 비교하였을 때, 약 50%에서 60%까지 NO 생성이 감소된 것을 확인할 수 있었다. 그 중에서 유산균 발효감태 추출물을 가장 우수한 효과를 보였으므로 선택하여 다음 실험에 사용하였다. 동결 건조된 유산균 발효감태로부터 수율을 높이고 유용성분의 량을 증가시키기 위해, 5 종의 당분해효소들과 5 종의 단백질 분해효소들을 사용하여 효소추출을 수행하였고, 물을 이용하여 추출한 경우보다 수율이 약 5%에서 35%까지 증가하였다. 또한, 10 종의 효소추출물들이 모두 LPS 가 유도한 NO 생성을 감소시켰으며, 그 중, Viscozyme 효소추출물이 가장 높은 효과를 보여 다음 실험을 위해 사용하였다.

발효감태 Viscozyme 효소추출물로부터 유효성분을 좀 더 분리하기 위해 먼저 막분리 시스템을 이용하여 분리하였고, 얻어진 30kDa 이상/이하의 분획물을 가지고 LPS 가 증가시킨 NO 의 생성을 억제하는지 확인하였다. 그 결과, 두 분획물 모두, NO 의 생성을 억제하였으나, 30kDa 이상의 분획물이 더 좋은 효과를 보였다. 또한, 30kDa 이상의 분획물로부터 에탄올 분획법을 사용하여 다당류 성분을 침전시켜 분리하였고, 분리된 발효감태 유래 다당류 (Fermented *E. cava* Polysaccharide: FEP)는 농도 의존적으로 NO 생성을 상당히 유의성 있게 감소시켰다.

LPS 의 자극이 유도한 대식세포의 사이토카인의 생성과 PGE_2 의 생성에 대한 FEP 의 억제활성을 확인하기 위해 ELISA 를 각각 수행하였고, 염증매개성 인자들의 단백질 발현 양상을 확인하였다. 또한 신호전달 메카니즘을 확인하고자 웨스턴 블랏을 수행하였다. 그 결과, COX-2 의 발현에 있어 영향을 주는 PGE_2 의 생성에 대한 FEP 의 억제 효과를 확인한 것으로, 농도가 증가할 수록 PGE_2 가 감소되는 것을 알 수 있었으며, LPS 를 대식세포에 처리한 경우, iNOS 와 COX2, TNF-α, IL-6 모두 발현이 상당히 증가하였으나,

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LPS 와 함께 FEP 를 처리한 경우, iNOS 와 TNF-α, IL-6 의 단백질 발현이 모든 농도에서 상당히 감소되었다. 그러나, COX-2 와 IL-1β 발현에서는 별다른 영향을 관찰할 수 없었다.

한편, FEP 가 보인 항염증 반응이 어떤 신호전달에 의해 조절되는 지를 확인하기 위해, NF-κB pathway 와 연관된 단백질들의 발현을 확인하였다. 그 결과, LPS 를 대식세포에 처리하게 되면, 세포질 내에 존재하는 IκBα의 degradation 과 phosphorylation 이 유도되었고, 핵 내로 NF-κB 가 이동하였다. 그러나, FEP 를 처리한 경우, LPS 에 의해 유도된 IκBα의 degradation 과 phosphorylation, NF-κB 의 핵 내 이동이 농도의존적으로 상당히 억제한 것을 확인 할 수 있었다.

이것으로부터 유산균 발효감태의 viscozyme 효소추출물로부터 분리된 당성분이 LPS 가 유도한 대식세포의 활성화에 있어, 활성화된 NF-kB 시그널을 억제함에 따라, iNOS 의 발현 및 TNF-α와 IL-6 의 생성과 발현을 감소시킴으로써 NO 의 생성을 억제하였다는 것을 알 수 있었고, 이것은 FEP 가 항염 활성을 가지고 있음을 확인하였다.

세포에 Gamma ray 를 조사하게 되면 생성되는 전자에 의해 물분자와 반응을 통해 ROS 를 형성하게 되고, apoptosis 관련 단백질들의 발현을 조절함으로써 DNA 의 손상을 유도하여 apoptosis 를 이끌어 세포의 생존과 증식을 감소시키는 것으로 알려져 있다. 따라서, 유산균 발효감태로부터 분리된 당성분이 세포의 생존과 증식에 영향을 미칠 수 있는 지를 확인하기 위해 방사선 조사 실험을 수행하였다.

그 결과로서, 3 종의 유산균 발효추출물을 가지고 세포증식에 대한 효능을 평가한 결과, 유산균 발효감태 추출물이 다른 추출물들보다 더 높은 세포 증식을 유도한 것을 확인하였다.

따라서, 유산균 발효감태로부터 제조된 효소추출물들은 무처리 대조군보다 세포의 생존을 증가시켰으며, 이중 Viscozyme 효소 추출물이 가장 높은 효능을 보였다.

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발효감태의 효소추출물에서 당성분이 세포의 증식과 생존에 영향을 미치는 지를 확인하기 위해, 막분리 시스템을 통해 분자량별 분획물을 제조하였고, 그것과 당, 효소 추출물을 가지고 방사선이 조사된 마우스 면역세포에서 생존율과 세포증식효능을 평가하였다. 그 결과, 방사선 조사에 의해 감소된 세포의 생존율과 증식이 효소 추출물 처리에 의해 증가하였으나, 30kDa 이하 분획물은 거의 활성을 보이지 않았다. 그러나, 30kda 이상과 당 (FEP)은 효소추출물보다 세포의 생존율과 증식을 더 증가시켰으며, 당성분이 다른 성분들보다 효율적으로 상당히 세포의 생존과 증식을 증가시켰다.

그렇다면 방사선 조사된 세포에서 FEP 의 세포 생존과 증식 효능이 초기 발생시키는 ROS 의 억제와 연관되는 지를 확인하기 위해, ROS 의 양을 확인하였다. 그 결과, FEP 을 처리한 경우, 방사선 조사가 유도한 ROS 의 생성이 농도 의존적으로 상당히 감소된 것을 알 수 있었다. 다음으로는 FEP 가 감소시킨 ROS 의 생성에 따른 apoptosis 와의 연관성을 확인하기 위해, PI 염색을 수행하였다. 그 결과, 방사선 조사가 유도한 sub-G1 내 2n 이하의 DNA 가 FEP 처리에 의해 상당히 감소되었으며, 6 시간과 12 시간, 24 시간 모든 시간대에서 효능을 보였다. 이것으로부터 FEP 가 ROS 의 생성을 억제하고, 그것이 apoptosis 의 한 현상인 DNA 손상을 감소시킨 것으로 여겨진다.

다음으로 앞에서 제시된 FEP 의 세포 손상 억제활성과 그 기전을 확인하고자, apoptosis 와 연관된 단백질들의 발현을 확인하였는데, 그 결과, 방사선 조사에 의해 증가된 apoptosis 유도 단백질인 Bax 와 p53 의 발현을 FEP 의 처리로 인해 감소시켰으며, 방사선 조사에 의해 감소된 apoptosis 억제 단백질인 Bcl-2 와 Bcl-xL 의 발현은 FEP 에 의해 증가된 것을 확인하였다. 이것으로부터, FEP 가 apoptosis 단백질의 발현을 조절함으로 방사선 조사가 유도하는 세포의 손상을 억제한 것으로 여겨진다.

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지금까지의 결과를 보면 감태를 유산균으로 발효하게 되면, 수율과 유효성분이 증가하게 되며, 이에 따라, 항염증이나 방사선 보호효과 등과 같은 생리활성 또한 증가한다는 것을 확인하였고, 이것은 발효감태가 연구적인 가치 뿐만이 아니라 산업적으로도 유용될 수있다는 사실을 이번 연구를 통해 확인하였다.

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Figure 2-1. Effects of three kinds of fermented *E. cava*(FE) on the proliferation of primary urine splenocytes. AOE, Aqueous extract of original *Ecklonia cava*; ALFE, Aqueous extract of LFE; ASFE, Aqueous extract of SFE; ACFE, Aqueous extract of ACFE. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05; **, P<0.01; ***, P<0.005.)

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Kojizyme extract prepared from LFE. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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Figure 2-5. Effects of FEP on viability of 2Gy-irradiated splenocytes. The effects of FEP on viability of immune cells were determined by MTT assay. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.)

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Figure 2-6. Effects of FEP on proliferation of 2Gy-irradiated splenocytes. The effects of FEP on proliferation of splenocytes were determined by ^{3}H-thymindine incorporation assay. Experiments were performed in triplicate and the data are expressed as mean ± SE. (**, $P < 0.01$; ***, $P < 0.005$.)

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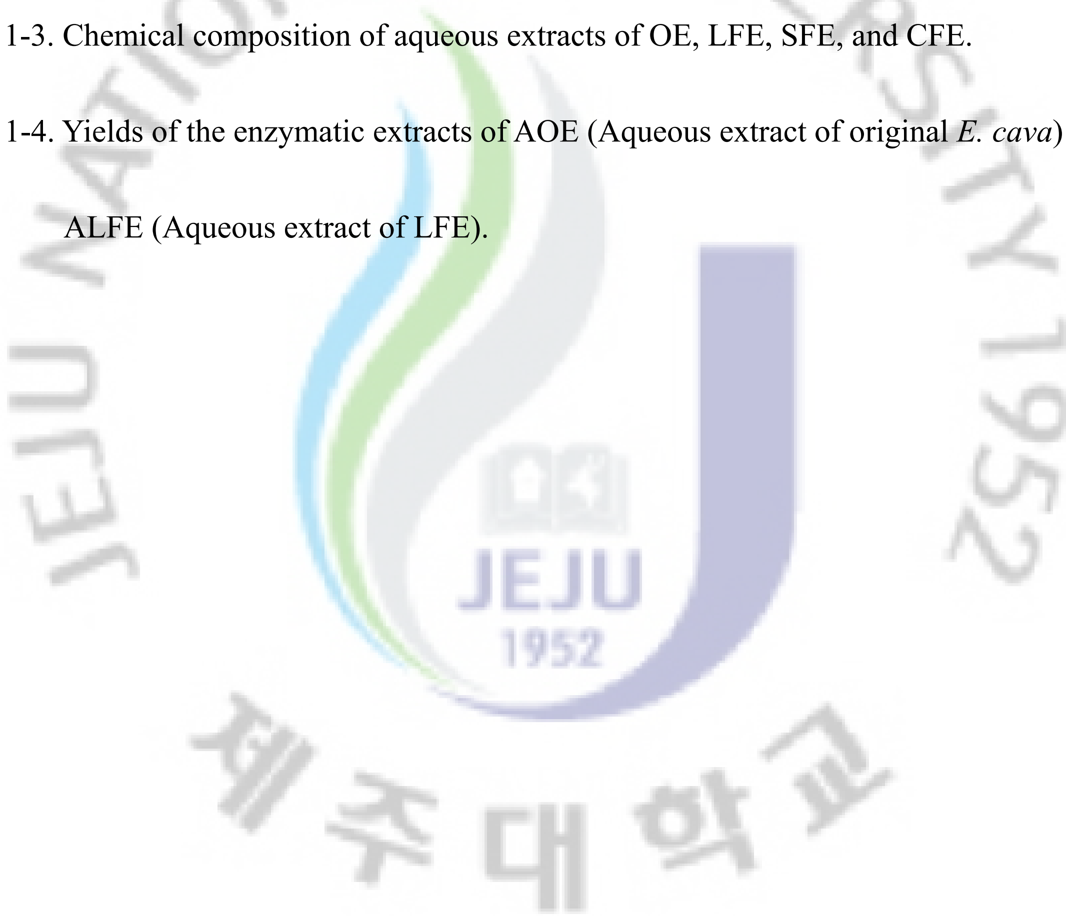

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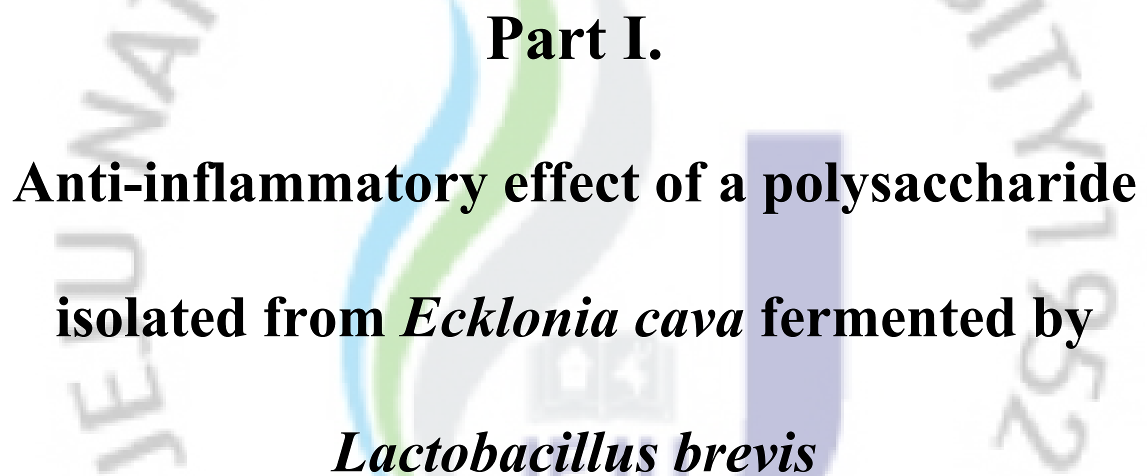

Part I.

Anti-inflammatory effect of a polysaccharide isolated from *Ecklonia cava* fermented by *Lactobacillus brevis*

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ABSTRACT

Fermentation process using several fungi and bacteria plays an important role in increasing the extraction of active compounds such as polysaccharides and peptides, as well as ingestion rate, and body absorption. The antioxidant and anti-inflammatory effects of *Ecklonia cava* (*E. cava*) which contains plentiful polysaccharides and polyphenol has been reported. However, until now, there are no studies about the beneficial capacities of *E. cava* fermented by fungi or bacteria in immune responses. So, here, the potential anti-inflammatory effects of *E. cava* fermented by fungi or bacteria were investigated in LPS-activated RAW 264.7 macrophage cell line.

First, the aqueous extracts of *E. cava* fermented by three kinds of fermentation fungi and bacteria (*Lactobacillus brevis*, *Saccharomyces cerevisiae*, and *Candida utilis*, respectively) (ALFE, ASFE, and ACFE, respectively) showed the increased extraction efficiency and the inhibitory effect on NO production, comparing to the aqueous extract of original *E. cava*. In addition, ALFE led to the higher extraction efficiency and the inhibitory effect on NO production in LPS-stimulated RAW 264.7 cells, compared to the others. And, the viscozyme extract of LFE (LFEV) showed the highest inhibitory effects on NO production, although all enzymatic extracts inhibited NO production after stimulation with LPS in RAW 264.7 cells. Interestingly, LFEV, > 30 kDa fraction of LFEV, and a polysaccharide of LFEV (FEP) significantly decreased NO production, comparing with LPS-stimulated cells. Additionally, FEP down regulated mRNA expression of pro-inflammatory mediator such as inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines such as TNF-α and IL-6 with raising its concentrations, compared to those of only LPS-stimulated cells. In further experiments,

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FEP considerably reduced the phosphorylation and degradation of IκB as well as the translocation of NFκB p65 into nucleus markedly induced by LPS stimulation.

In conclusion, this study suggests that FEP has the anti-inflammatory effects by down-regulating the production and expression of pro-inflammatory cytokines and mediators via the NFκB pathway in LPS-stimulated RAW 264.7 cells.

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1. Introduction

Inflammation, characterized by redness, swelling, pain, and heat, is one of the most important aspects of host defense mechanisms against invading pathogens (Oberyszyn, 2007). Normal inflammatory responses are self-limited by a process that involves the down-regulations of pro-inflammatory proteins and the up-regulations of anti-inflammatory proteins (Lawrence et al., 2002). During the inflammatory response, macrophages play a central role and serve as an essential interface between innate and adaptive immunity (Adams and Hamilton, 1984).

Normally, activation of macrophages by stimuli, such as lipopolysaccharide (LPS) known as the bacterial endotoxin, increases the production and secretion of two pleiotropic inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and a variety of cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α (Larsen and Henson, 1983; Nathan and Root, 1977; Stuehr and Marletta, 1985). Also, nitric oxide (NO), free oxygen radical and prostaglandin E_2 (PGE_2) are over-produced by the iNOS and COX-2 and they play a role as cytotoxic agent in pathological processes, particularly in inflammatory disorders (Vane et al., 1994). In addition, the previous study has reported that NO, PGE_2, COX-2, and iNOS were regulated by a variety of cytokines including TNF-α, IL-1β, IL-6, and IL-10 in LPS-activated RAW 264.7 cells (Chang et al., 2005). Moreover, previous studies have demonstrated that the transcription and secretion of pro-inflammatory mediators and cytokines, for example, iNOS, COX-2, TNF-α and IL-1β, IL-6 and IL-8 were mediated by the activation of IκB/nuclear transcription factor kappa-B (NFκB) signal transduction pathway in LPS-activated RAW 264.7 cells (Surh et al.,

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2001; Lappas et al., 2002). Therefore, searching potential substances which can modulate the transcription and secretion of pro-inflammatory mediators and cytokines via the classical NFκB signal transduction pathway are so important for the development of anti-inflammatory drug.

Normally, fermentation with several fungi and bacteria plays an important role in improvement of nutritional and functional properties of foods as increasing the extraction of active compounds such as polysaccharides and peptides, ingestion rate, and body absorption.

Here, the present study documented whether the polysaccharide isolated from viscozyme extract of *Lactobacillus brevis*-fermented *Ecklonia cava* (*E. cava*), a kind of brown seaweed, has anti-inflammatory effects by down-regulating the production and mRNA expression of pro-inflammatory mediators such as iNOS, COX-2 and PGE_2 and pro-inflammatory cytokines such as TNF-α and IL-6 via the classical NFκB signal transduction pathway in LPS-activated macrophage.

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2. MATERIALS AND METHODS

2.1. Preparation of *E. cava* fermented by fermentation fungi and bacteria

E. cava was collected from the coast of Jeju Island, South Korea, washed with fresh water, freeze-dried and pulverized into powder with a grinder. Then, the freeze-dried *E. cava* was applied to fermentation process using three kinds of fermentation fungi and bacteria (*Lactobacillus brevis* (*L. brevis*), *Saccharomyces cerevisiae* (*S. cerevisiae*), and *Candida utilis* (*C. utilis*), respectively) for 24 h. After freeze-dry, the fermented *E. cava* powders used for next experiments.

2.2. Composition analysis of the *E. cava* fermented by fermentation fungi and bacteria

The chemical compositions of the original *E. cava* and its fermented samples were identified by measuring the contents of moisture, carbohydrates, proteins, ash, and lipids from the weight difference after drying of samples according to the Association of Official Analytical Chemists (AOAC) method.

2.3. Preparation of 80% methanol and aqueous extracts from the original *E. cava* and its fermented samples

Here, 80% methanol and aqueous extracts were prepared from the original *E. cava* and its fermented samples. After freeze-dry, the fermented *E. cava* and its original powders (1 g) were respectively homogenized in 80% ethanol and aqueous (100 ml). After 24 h, the samples were obtained and kept at -20℃ for further experiments.

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2.4. Composition analysis of 80% methanol and aqueous extracts from the original *E. cava* and its fermented samples

To identify the chemical composition of 80% methanol and aqueous extracts from the original *E. cava* and its fermented samples, the contents of carbohydrates, proteins, ash, and lipids from the weight difference after drying of samples were measured according to the Association of Official Analytical Chemists (AOAC) method.

2.5. Preparation of enzymatic extracts from *E. cava* fermented by fermentation fungi and bacteria

The fermented *E. cava* and its original powder were used for the enzymatic extraction technique using several enzymes followed previously reported method (Heo et al., 2005). Among them, *L. brevis*-fermented *E. cava* powders (LFE) (50 g) showed the highest NO inhibitory effect in lipopolysaccharide (LPS)-stimulated macrophage were homogenized in distilled water (2 L) with 500 μl of ten kinds of enzymes. Each reactant was adjusted to the optimum pH and temperature range of the respective enzyme and enzymatic reactions were performed for 24 h. Following digestion, the digest was boiled for 10 min at 100℃ to inactivate the enzymes. After centrifugation (3000 rpm, for 20 min at 4℃), the supernatant were adjusted to pH 7.0 hereafter and designated to enzymatic extract. The sample was kept at -20℃ for further experiments.

2.6. Preparation of molecular weight fractions from viscozyme extract of LFE

To perpetrate different molecular weight fractions, LFE was applied to Lab scale TFF system (PHILOS) using micro-filtration membrane (30 kDa). Then, all the fractions (whole

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extract, > 30 kDa fraction, and < 30 kDa fraction) were separately evaluated the inhibitory effects on NO production in LPS-stimulated RAW 264.7 cells.

2.7. Isolation of a polysaccharide from > 30 kDa fraction of LFE viscozyme extract

A polysaccharide was isolated from > 30 kDa fraction of LFE viscozyme extract by ethanol precipitation according to slightly revised method indicated in previous study (Athukorala et al., 2009). The > 30 kDa fraction of LFE viscozyme extract (1 L) was mixed with 2 L of 99.5% ethanol for 24 h at 4℃. After centrifugation at 10000 rpm for 20 min at 4℃, the crude polysaccharide was collected from its precipitant. Then, the crude polysaccharide isolated from > 30 kDa fraction of LFE viscozyme extract (FEP) was freeze-dried and used for next experiments.

2.8. Analysis of mono-sugar contents

The LFE, > 30 kDa and < 30 kDa fractions of LFE viscozyme extract and FEP were hydrolyzed in a sealed glass tube with 4 M of trifluoroacetic acid for 4 h at 100℃ to analyze neutral sugars. In order to analyze the amino-sugars the samples were digested using 6 N of HCl for 4 h. Then, the samples were separately applied to CarboPac PA1 (4.5 x 250 mm, Dionex, Sunnyvale, CA, USA) with CarboPac PA1 cartrige (4.5 x 50 mm), respectively. The column was eluted using 16 mM of NaOH at 1.0 ml/min flow rate. Each sugar of the samples was detected by using ED50 Dionex electrochemical detector and data were analyzed by Peack Net on-line software.

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2.9. Cell culture and sample treatment

The RAW 264.7 murine macrophage cell line was obtained from the KCLB (Korean Cell Line Bank, Seoul, Korea). These cells were cultured at 37℃ in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere. The cells were treated with LFE, > 30 kDa fraction, < 30 kDa fraction, and FEP at various concentrations from 12.5 to 200 μg/ml and the stimulated with 1 μg/ml of LPS for indicated incubation times.

2.10. Nitrite assay

The cells were plated at a density of 1×10^5 cells/well in 96-well plates for 16 h. The cells were pre-treated with LFE, > 30 kDa fraction, < 30 kDa fraction, and FEP at various concentrations (12.5, 25, 50 and 100 μg/ml or 200 μg/ml) for 2 h and then stimulated by LPS (1 μg/ml) for 24 h. After incubation, the culture media (100 μl/well) was mixed with 100 μl of Griess reagent, an indicator of NO production (1% sulfanilamide in 2.5% phosphoric acid and 0.1% naphthylenediamine dihydrochloride in distilled water) for 10 min and the absorbance of the mixture at 540 nm was measured using an ELISA microplate reader (Amersham Pharmacia Biotech, UK, U.S.A.). The nitrite levels were read off a reference to the standard curve using sodium nitrite. The experimental result represents one of three experiments and is expressed as the mean of triplicates.

2.11. Lactate dehydrogenase (LDH) assay

Among them, FEP, which showed the highest inhibitory effects on NO production caused by LPS stimulation, was used for measuring LDH levels released from RAW 264.7 cells. The cells were plated at a density of 1×10^5 cells/well in 96-well plates for 16 h. The

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cells were treated with various concentrations of FEP (from 12.5 to 100 μg/ml) for 2 h and then stimulated by LPS treatment or not. After an additional 24 h incubation period at 37℃, the LDH level in the culture supernatant was determined by LDH cytotoxicity detection kit according to the manufacturer instructions.

2.12. Determination of PGE_2 production

RAW 264.7 cells (1 x 10^5 cells/well) were pretreated with FEP (from 12.5 to 100 μg/ml) for 2 h and then incubated with LPS (1 μg/ml) for 24 h. PGE_2 levels in macrophage culture media were quantified using the ELISA kits (Biosource International, Camarillo, CA, USA), according to the manufacturer's instructions

2.13. Preparation of cytoplasmic and nuclear proteins

RAW 264.7 cells treated with FEP (from 12.5 to 100 μg/ml) were lysed with buffer A consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1.0% NP-40 and the protease inhibitors (0.5 mM DTT and 0.1 mM PMSF) on ice. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was used as the cytoplasmic protein and then the pellet was followed by lysis with buffer B (20 mM HEPES, 20% glycerol, 0.42 mM NaCl, 1 mM EDTA and the protease inhibitors containing 0.5 mM DTT and 0.1 mM PMSF) for 30 min on ice. Finally, nuclear extracts were obtained by centrifugation at 12,000 rpm for 15 min. And then the contents of the cytoplasmic and nuclear protein were measured using BCA™ protein assay kit. The proteins were used for Western blot analysis.

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2.14. Determination of TNF-α, IL-1β, and IL-6 production

RAW 264.7 cells (1 x 10^5 cells/well) were pretreated with FEP (12.5 and 100 μg/ml) for 2 h and then incubated with LPS (1 μg/ml) for 24 h. After incubation, the supernatants were collected and used for measure the production levels of TNF-α, IL-1β, and IL-6 secreted from macrophage. The production of the cytokines was quantified using the ELISA kits, according to the manufacturer's instructions.

2.15. Western blot analysis

Of cytoplasmic protein, 50 μg from treated and untreated cell extracts were electro-transferred onto a nitrocellulose membrane following separation on a SDS–polyacrylamide gel electrophoresis under denaturing conditions. After blocking with 5% nonfat milk for 1 h, the blots were separately incubated with specific primary rabbit polyclonal anti-rabbit iNOS (1:1000), phospho-IκBα (1:1000) or NFκB p65 (1:1000) or mouse monoclonal anti-mouse COX-2 (1:1000), IκBα (1:1000) or β-actin (1:3000) antibody for 5 min or 24 h and washed twice with Tween 20/Tris-buffered saline (TTBS). Then, the blots were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (1:2000) for 60 min followed by visualization by using ECL reagents.

2.16. RNA preparation and RT-PCR

Total cellular RNA was isolated using Trisol (Molecular Research Center, Inc., Cincinnati, Ohio, U.S.A) and then the cDNA was synthesized with RNA (1 μg) using a Promega A3500 kit, according to manufacturer's instructions, respectively. PCR of this cDNA and the primer displayed in Table 1 was performed for 40 cycles with a 45 sec denaturing step at 94°C, a 45 sec annealing step at 55 to 60°C and a one min extension phase at 72°C using the TaKaRa PCR machine (Takara Bio Inc., Otsu, Japan). PCR products were

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run on a 1.5% EtBr/agarose gel and visualized by UV transillumination.

2.17. Statistical analysis

Data was analyzed using the statistical package for the social science (SPSS) package for Windows (Version 10). Values were expressed as means ± standard error (SE). A p-value of less than 0.05 was considered significant.

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3. RESULTS

3.1. The original *E. cava* and its fermented samples have an abundance of carbohydrate and protein contents

The chemical composition of the original *E. cava* (OE) and its fermented samples using three kinds of fermentation fungi and bacteria (*L. brevis*, *S. cerevisiae*, and *C. utilis*, respectively) (LFE, SFE, and CFE) were determined by measuring the sample's moisture, ash, protein, carbohydrate, and lipid contents according to the AOAC method. As indicated in Table 1-1, the original *E. cava* and its fermented samples (LFE, SFE, and CFE) showed the plentiful carbohydrate and protein contents (50.84, 49.53, and 50.15 % and 29.56 ± 0.34, 31.87 ± 0.45, and 30.75 ± 0.81%, respectively) in comparison with the others (Moisture, Ash, and Lipid). Interestingly, the contents of carbohydrate in fermented samples were slightly decreased, whereas their protein contents were markedly increased, compared to those of original *E. cava* (11.02 ± 1.13%, respectively). These results indicate that the increment of protein and carbohydrate contents in the fermented samples might be induced by increased fermentation fungi or bacteria.

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3.2. The aqueous extracts of LFE increased the extraction yield and carbohydrate contents

Table 1-2 showed that 80% ethanol extracts of LFE, SFE, and CFE (ELFE, ESFE, and ECFE) induced the increased extraction yields (34.33 ± 2, 29.67 ± 1.5, and 31 ± 1%, respectively *vs* 15 ± 1%) and carbohydrate content (8.38 ± 0.08, 5.12 ± 0.17, and 6.17 ± 0.1%, respectively vs 1.95±0.42%), whereas did not increase polyphenol content, as compared to those of OE. Also, aqueous extracts of LFE, SFE, and CFE (ALFE, ASFE, and ACFE) increased extraction yields up to 53.67 ± 2.14, 51.33 ± 1, and 53.33 ± 2%, comparing with that of OE aqueous extract (30.5±0.5%) (Table 1-3). In addition, the carbohydrate contents of ALFE, ASFE, and ACFE (20.82 ± 0.25, 14.29 ± 0.25, and 15.89 ± 0.08%) were increased by fermentation process using fermentation fungi and bacteria as compared with that of AOE (8.63±0.2%). Among them, ALFE showed the highest carbohydrate contents and extraction yields than those of the others.

These results indicated that aqueous extracts of fermented *E. cava* induced the higher extraction yields and carbohydrate contents than those of the 80% ethanol extracts. Also, among aqueous extracts of fermented *E. cava*, the aqueous extract of LFE showed the highest extraction yield and carbohydrate content was used for next experiments.

3.3. ALFE shows the highest inhibitory effects on NO production in LPS-stimulated RAW264.7 cells as well as its higher extraction efficiency

To choose the most potential material showing the highest anti-inflammatory effects, the NO production inhibitory effects of ALFE, ASFE, and ACFE were first checked in LPS-stimulated RAW 264.7 cells.

As shown in Figure 1-1, LPS stimulation markedly increased NO production, in

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comparison with untreated cells as control, whereas the pretreatment of all the extracts showed the strong inhibitory effects on NO production in LPS-stimulated RAW 264.7 cells. Among the tested samples, ALFE (200 μg/ml) markedly reduced the NO production induced by LPS stimulation, compared to control cells (untreated cells). In addition, the extraction yield of ALFE was considerably increased as compared to the aqueous extract of unfermented original *E. cava* up to 53.7% and higher than those of the others. From these results, ALFE shown the highest inhibitory effects on NO production was selected for further experiments.

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Table 1-1. Chemical composition of original *E. cava* (OE), *Lactobacillus brevis*-fermented *E. cava* (LFE), *Saccharomyces cerevisiae* -fermented *E. cava* (SFE), *Candida utilis* -fermented *E. cava* (CFE).

(g/100 g)

Composition	OE	LFE	SFE	CFE
Moisture	9.4±0.1	6.5±0.3	3.5±0.1	4.6±0.2
Carbohydrate	64.35±2.1	50.8±1.8	49.5±1.7	50.2±1.8
Protein	11.0±1.1	29.6±0.3	31.9±0.5	30.8±0.8
Ash	14.0±1.4	10.5±0.7	12.0±0.1	11.5±0.7
Lipid	1.3±0.7	2.6±0.3	3.15±0.5	3.0±0.3

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Table 1-2. Chemical composition of 80% ethanol extracts of OE, LFE, SFE, and CFE.

(g/100 g)

Composition	EOE	ELFE	ESFE	ECFE
Yield	15.0±1.0	34.3±2.0	29.7±1.5	31.0±1.0
Carbohydrate	2.0±0.4	8.4±0.1	5.1±0.2	6.2±0.1
Total phenol	5.8±0.2	3.5±0.2	3.5±0.2	3.6±0.1
etc.	6.0±0.3	20.6±0.1	19.3±0.2	20.2±0.1

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Table 1-3. Chemical composition of aqueous extracts of OE, LFE, SFE, and CFE.

(g/100 g)

Composition	AOE	ALFE	ASFE	ACFE
Yield	30.5±0.5	53.7±2.1	51.3±1.0	53.3±2.0
Carbohydrate	8.6±0.2	20.8±0.3	14.3±0.3	15.9±0.1
Total phenol	2.2±0.5	4.7±0.0	4.5±0.1	4.57±0.1
etc.	17.9±0.1	26.4±0.4	31.2±0.3	30.7±0.2

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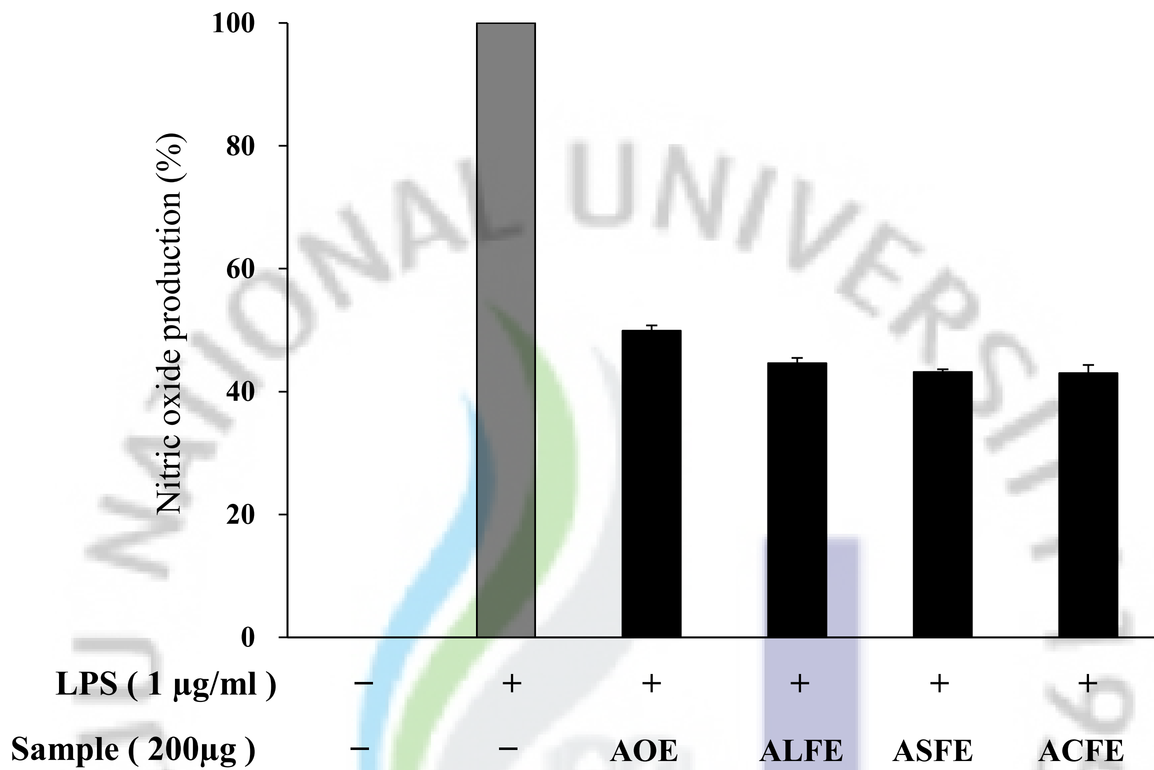

Figure 1-1. Effects of aqueous extracts prepared from fermented *E. cava* on LPS-induced NO production in RAW 264.7 cells. AOE, Aqueous extract of original *Ecklonia cava* (*E. cava*); ALFE, Aqueous extract of *Lactobacillus brevis*-fermented *E. cava* (LFE); ASFE, Aqueous extract of *Saccharomyces cerevisiae* -fermented *E. cava* (SFE); ACFE, Aqueous extract of *Candida utilis* -fermented *E. cava* (CFE). Experiments were performed in triplicate and the data are expressed as mean ± SE.

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3.4. A polysaccharide isolated from Viscozyme extract of ALFE (FEP) significantly inhibits NO production in LPS-stimulated RAW264.7 cells without cytotoxicity

ALFE was enzymatically extracted by several carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) and proteases (Kojizyme, Papain, Pepsin, Protamex and Neutrase) and were used to identify their inhibitory effects on NO production induced by LPS stimulation. As indicated in Table 1-4, all the enzymatic extracts of ALFE showed the higher extraction yields (from 53.7 to 87.5%), compared with that of distilled water extract (30.5%).

Also, all the enzymatic extracts of ALFE strongly inhibited NO production induced by LPS stimulation in RAW 264.7 cells comparing with control cells (Figure 1-2). Especially, among the tested extracts, the Viscozyme and Protamex extracts of ALFE (LFEV and LFEP) showed the higher inhibitory effects on the NO production induced by LPS than those of the others in RAW264.7 cells (Figure 1-2).

Moreover, after LPS stimulation, LFEV and LFEP were markedly decreased NO production with increment of concentrations (from 25 to 200 μg/ml) in comparison with control cells (Figure 1-3).

Particularly, LFEV induced the slightly higher inhibitory effect on NO production induced by LPS than those of Protamex extract from LFE. Therefore, LFEV was selected for further experiments. After its fractionation following Figure 1-4, the > 30 kDa, and < 30 kDa fractions were used to investigate the inhibitory effects on NO production induced by LPS stimulation. As shown in Figure 1-5, both two fractions decreased the NO production increased by LPS stimulation and especially, > 30 kDa fraction led to the markedly high inhibitory activities on LPS-induced NO production, as compared to those of < 30 kDa fractions at all concentrations (from 25 to 200 μg/ml). So, the > 30 kDa fraction was selected for isolation of a crude polysaccharide. Interestingly, the isolated crude polysaccharide (FEP)

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dose-dependently decreased the LPS-induced NO production at all concentrations (Figure 1-6). In addition, FEP did not show cytotoxicity at all concentration. These results indicated that FEP has anti-inflammatory effect via decreasing NO production in LPS-stimulated RAW 264.7 cells.

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Table 1-4. Yields of the enzymatic extracts of AOE (Aqueous extract of original *E. cava*) and ALFE (Aqueous extract of LFE). (%)

	AMG	Ce	Ul	Te	Vi	Ne	Pa	Pe	Pro	Ko	ALFE	AOE
Yield	**56.0**	**62.0**	**58.5**	**62.0**	**57.0**	**63.5**	**62.5**	**87.5**	**66.5**	**68.5**	**53.7**	**30.5**

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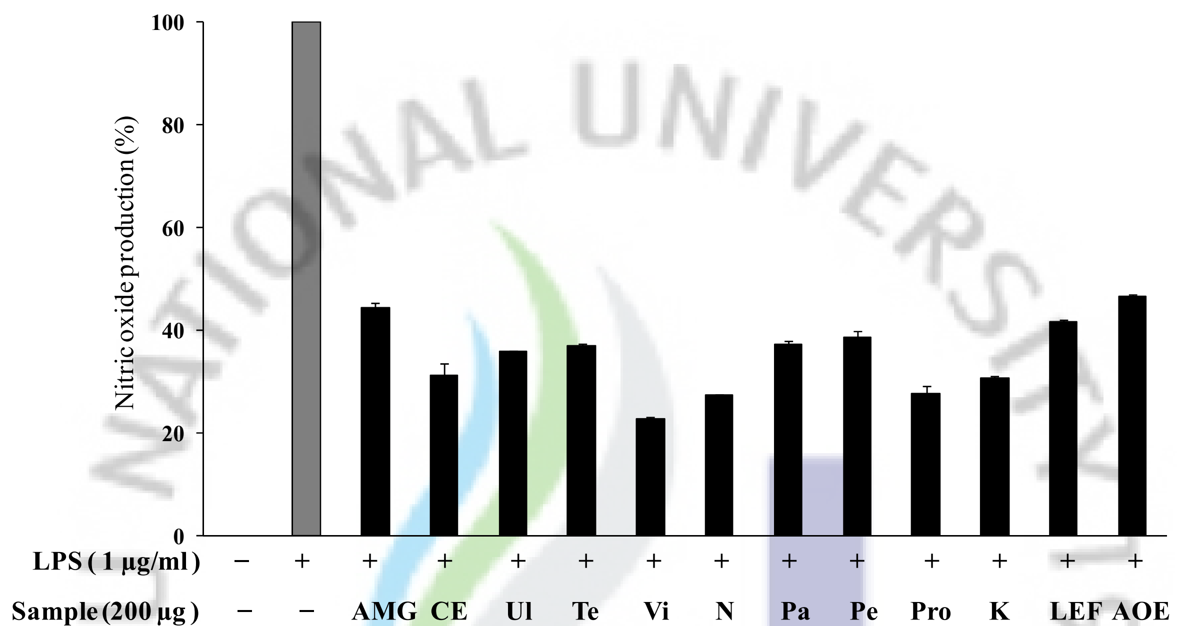

Figure 1-2. Effects of enzymatic extracts prepared from ALFE on LPS-induced NO production in RAW 264.7 cells. AMG; AMG extract prepared from LFE, Ce; Celluclast extract prepared from LFE, Ul; Ultraflo extract prepared from LFE, Te; Termamyl extract prepared from LFE, Vi; Viscozyme extract prepared from LFE,. N; Neutrase extract prepared from LFE. Pa; Papain extract prepared from LFE, Pe; Pepsin prepared from LFE, Pro; Protamex extract prepared from LFE. K; Kojizyme extract prepared from LFE, ALFE; Aqueous extract from LFE, AOE; Aqueous extract from OE. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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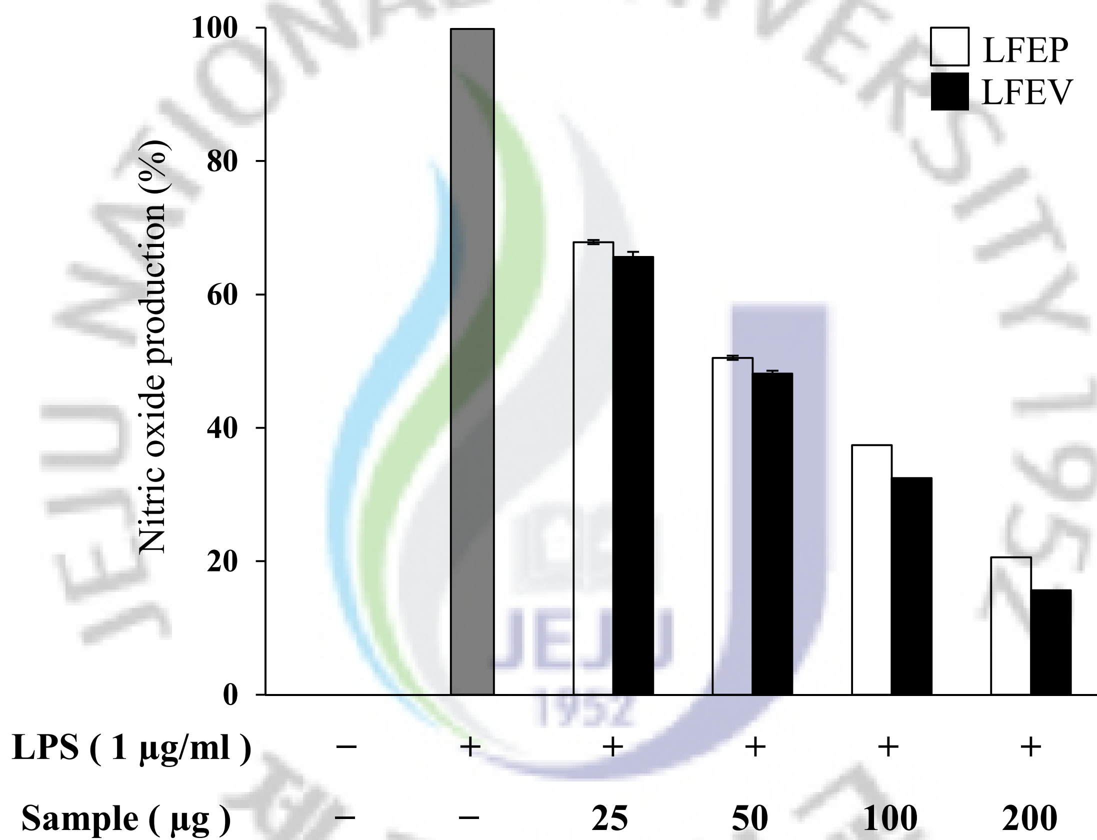

Figure 1-3. Effects of LFEV and LFEP on LPS-induced NO production in RAW 264.7 cells. LFEV; Viscozyme extract prepared from LFE, LFEP; Protamex extract prepared from LFE. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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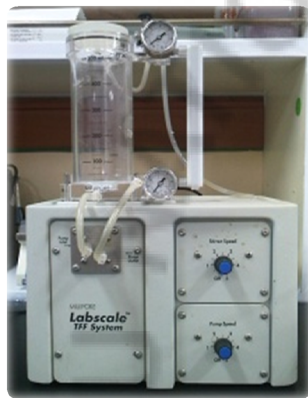

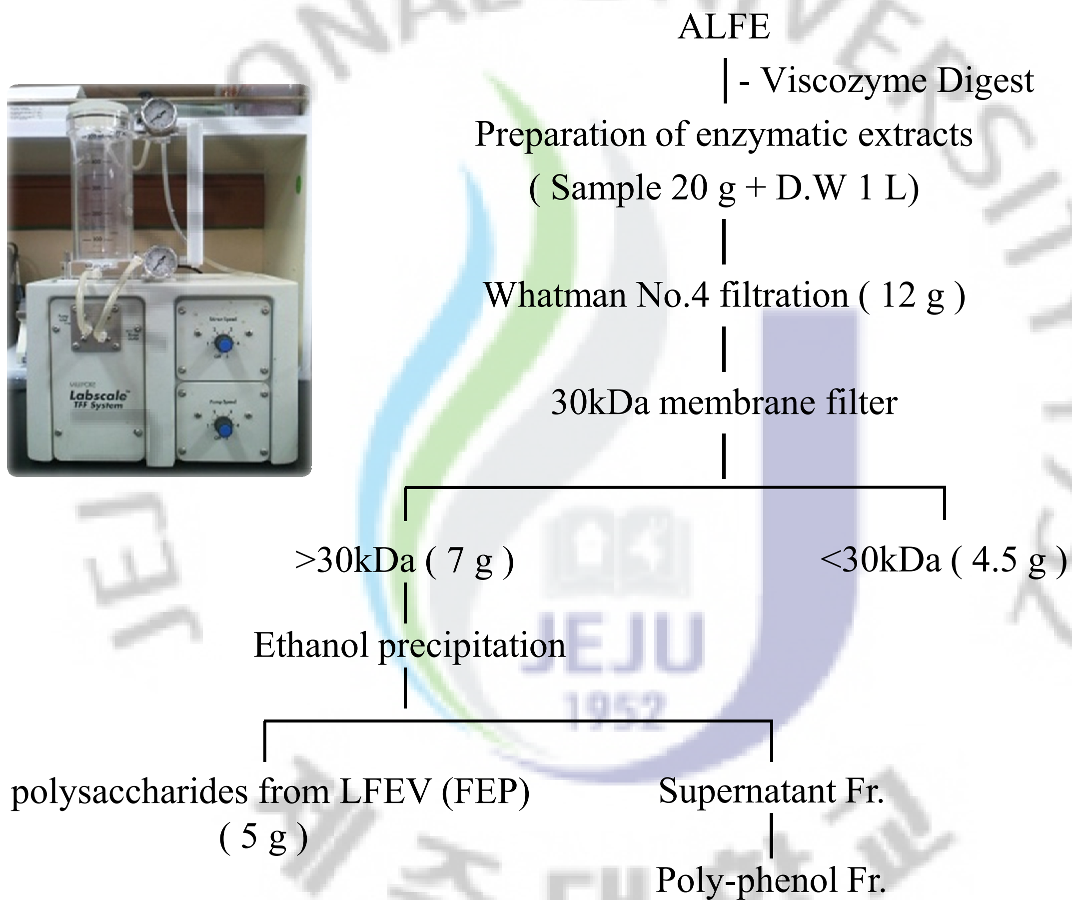

Figure 1-4. Isolation and purification scheme of polysaccharides from LFEV (FEP)

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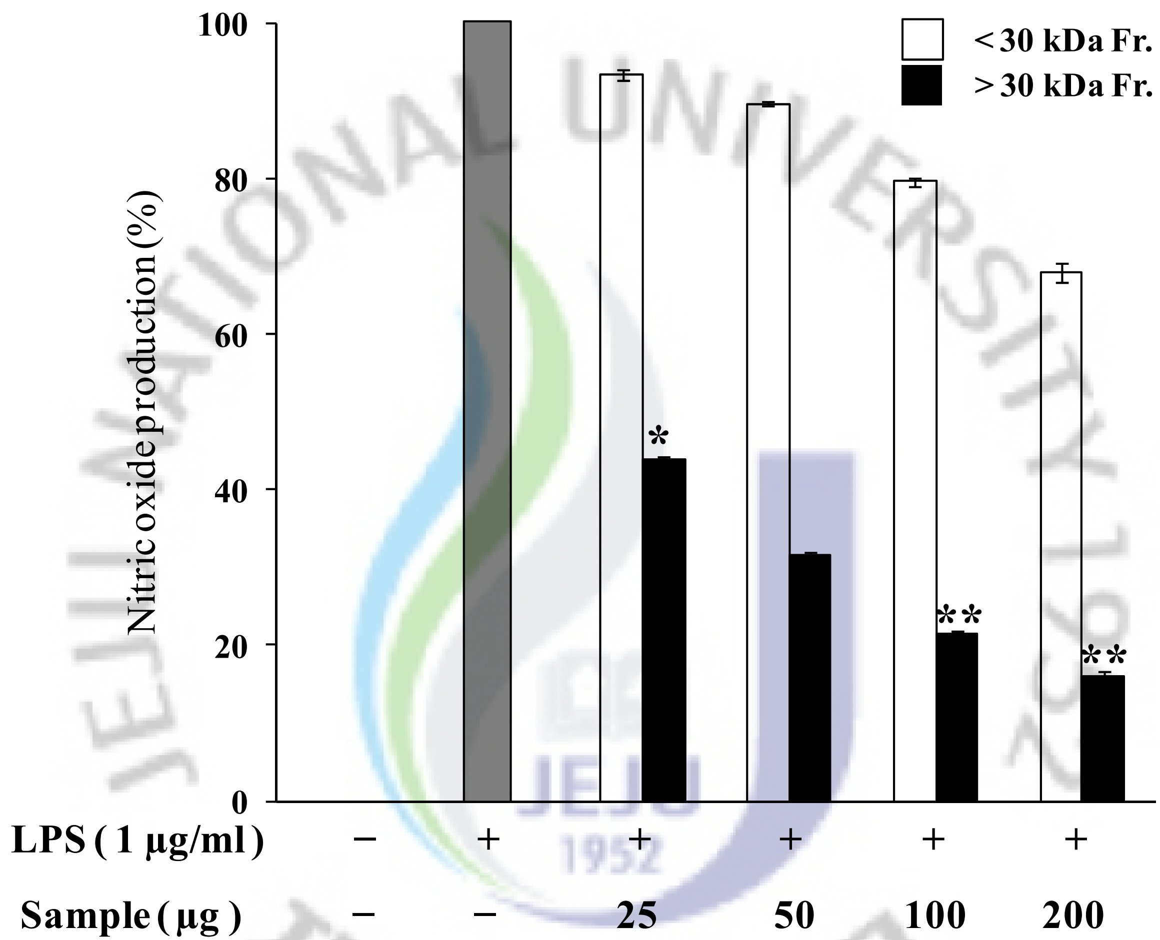

Figure 1-5. Inhibitory effects of <30 kDa and > 30 kDa fractions prepared from LFEV on LPS-induced NO production in RAW264.7 cells. □, <30 kDa fraction; ■, > 30 kDa fraction. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05; **, P<0.01)

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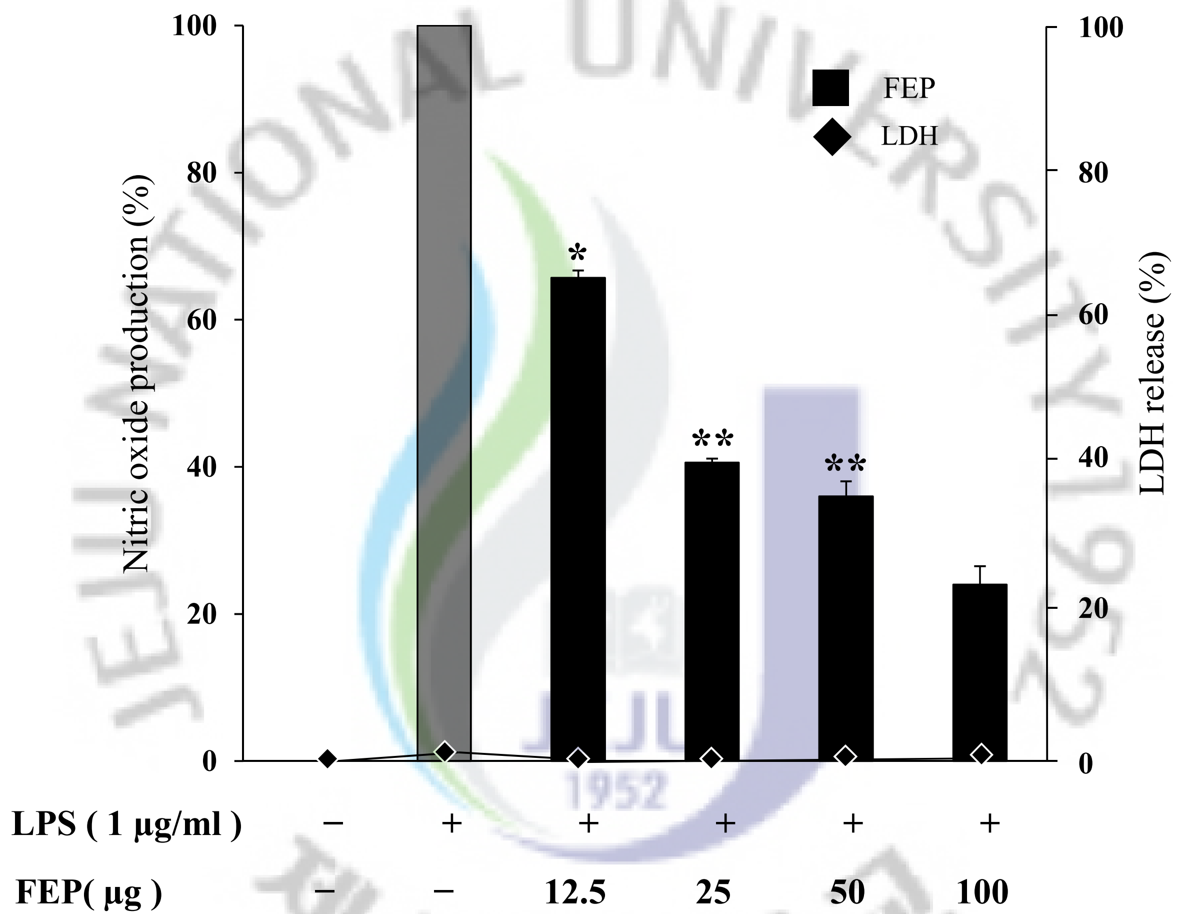

Figure 1-6. Inhibitory effects of FEP on LPS-induced NO production and LDH release in RAW264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, $P<0.05$; **, $P<0.01$)

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3.5. FEP weakly inhibits PGE_2 production in LPS-stimulated RAW264.7 cells

To assess the inhibitory effects of FEP on the production of the inflammatory mediator PGE_2 in LPS-stimulated RAW 264.7 cells, culture media was harvested from the cells treated with/without FEP (12.5, 25, 50, and 100 μg/ml) and LPS (1 μg/ml) and PGE_2 levels were measured. The treatment of LPS markedly increased PGE_2 production compare to control cells, whereas the pretreatment of FEP slightly inhibited LPS-induced PGE_2 production in a dose-dependent manner (Figure 1-7). In addition, the 100 μg/ml of FEP showed the about 20% of weak inhibitory activity on PGE_2 production induced by LPS stimulation. From these results, this study indicates that FEP can decrease NO production through inhibiting PGE_2 production in LPS-stimulated RAW 264.7 cells.

3.6. FEP inhibits LPS-induced iNOS and COX-2 protein and mRNA expression levels

To determine whether the inhibitory effects of FEP on LPS-induced NO and PGE_2 production are related with the protein levels and pretranslational mechanism of iNOS and COX-2, Western blot and RT-PCR were performed. The mRNA expression and protein level of β-actin were used as the internal control. As illustrated in Figures 1-8 and 1-9, the protein and mRNA levels of iNOS and COX-2 were markedly increased in LPS-stimulated cells, as compared to those of control cells. On the other hand, the pretreatment of FEP significantly inhibited iNOS protein expression in a concentration-dependent manner, although did not show significant changes on those of COX-2 (Figure 1-9). Interestingly, the results in RT-PCR analysis correlated with the results shown in Western blot. These results suggest that FEP significantly inhibited the production of NO by reducing the LPS-induced iNOS protein and mRNA expression level in RAW 264.7 cells.

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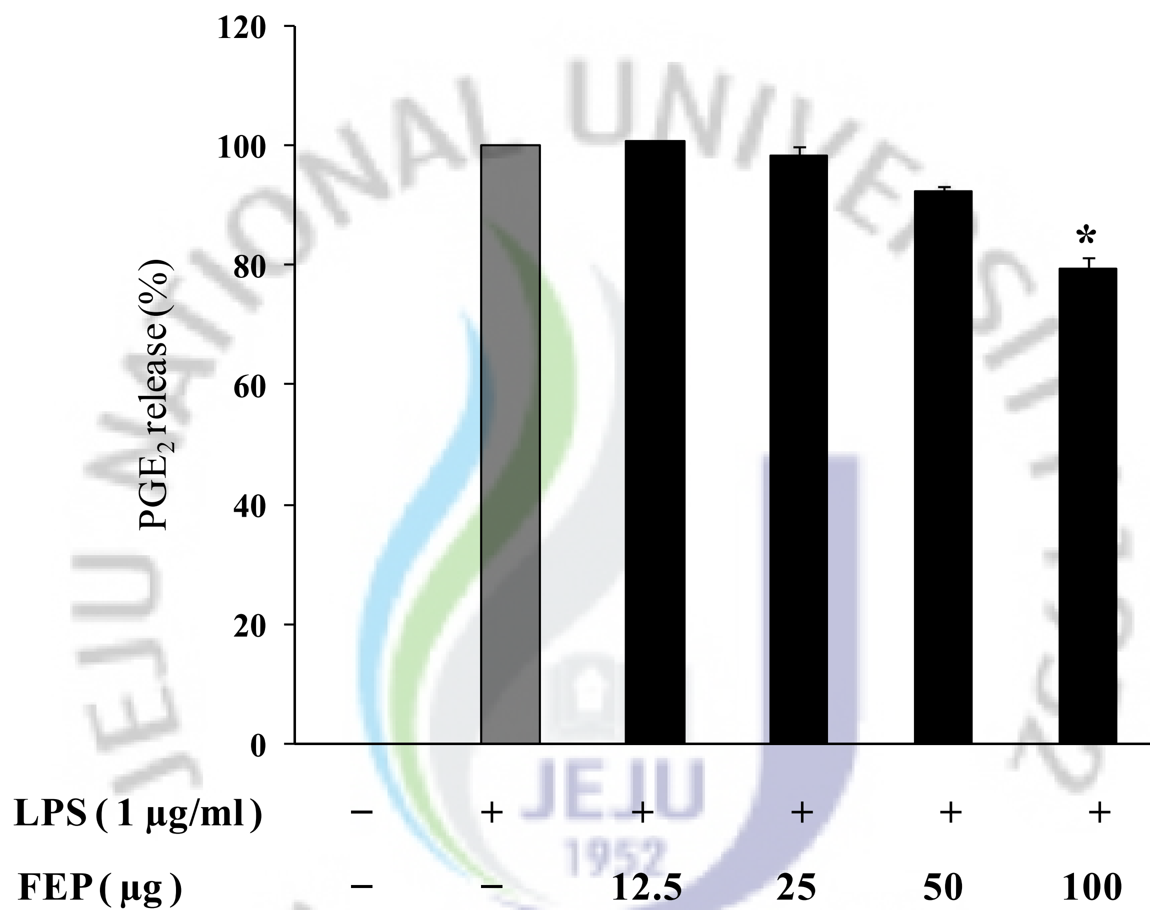

Figure 1-7. Inhibitory effects of FEP on LPS-induced PGE_2 production in RAW264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, $P<0.05$)

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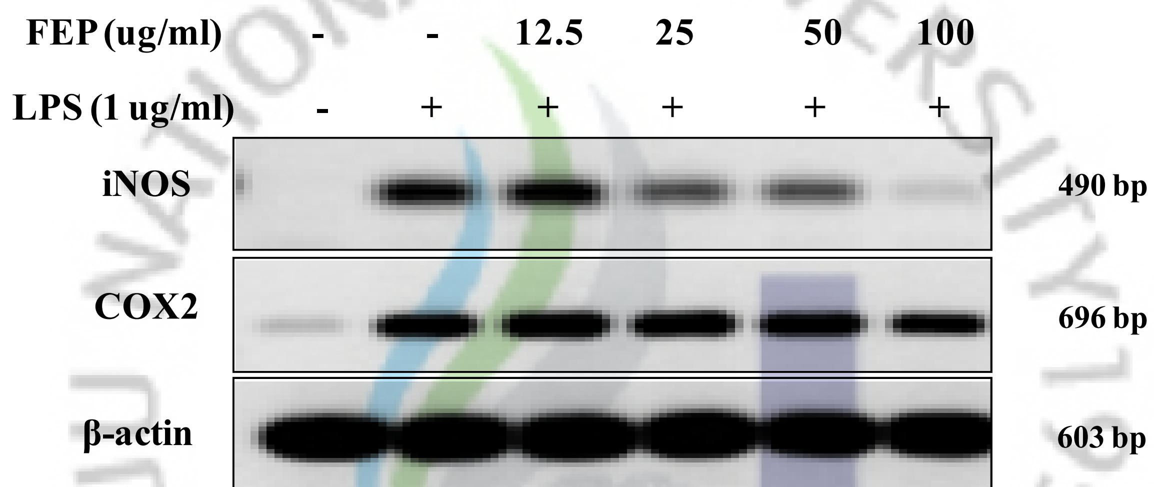

Figure 1-8. Inhibitory effects of FEP on mRNA expression levels of iNOS and COX-2 in LPS- stimulated RAW 264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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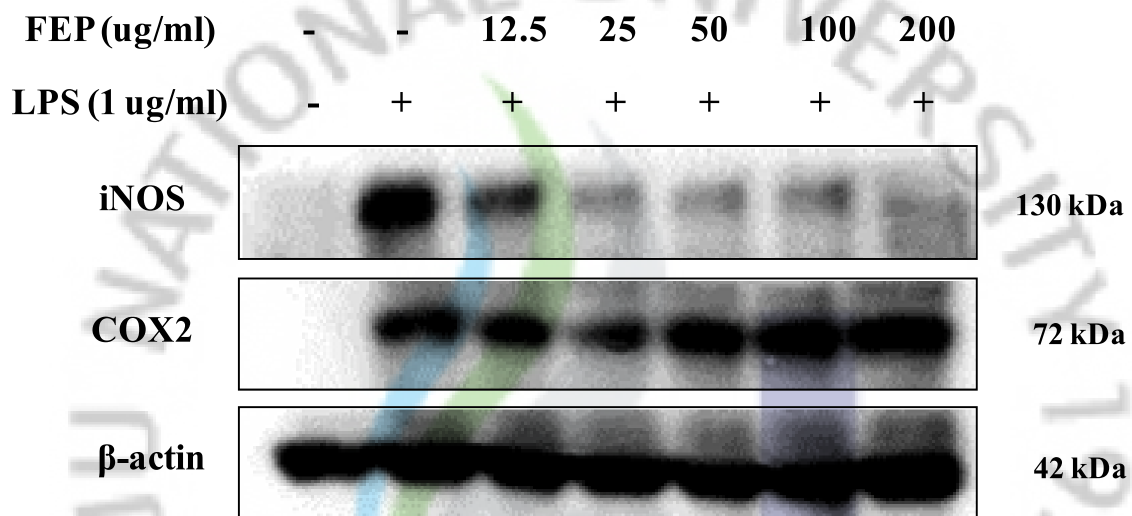

Figure 1-9. Inhibitory effects of FEP on protein expression levels of iNOS and COX-2 in LPS- stimulated RAW264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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3.7. FEP significantly inhibits the protein and/or mRNA expression level of TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells

Since our data showed that FEP inhibited the pro-inflammatory mediators, such as NO, PGE_2, and iNOS, its effects on pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 were further investigated in LPS-activated macrophages. RAW 264.7 cells were incubated in the presence of increasing concentrations of FEP, and quantities of these cytokines secreted into the culture supernatant and the cells were monitored by ELISA. It was found that pretreatment of FEP reduced the production of TNF-α and IL-6, although did not affect to production of IL-1β (Figures 1-10, 1-11, and 1-12). Especially, the protein levels of TNF-α released from LPS-activated RAW 264.7 cells were significantly decreased by FEP with dose-dependent manner at all concentrations from 12.5 to 100 μg/ml (Figure 1-10). Also, FEP showed the inhibitory effects on the mRNA expression of TNF-α at all concentrations, but did not affect to those of IL-1β and IL-6 (Figure 1-13). Taken together, these results demonstrate that FEP shows anti-inflammatory effects as decreasing NO production by down-regulating the protein and mRNA expression level of pro-inflammatory mediator such as iNOS or pro-inflammatory cytokines such as TNF-α and/or IL-6 in LPS-activated macrophages.

3.8. FEP inhibits the degradation and phosphorylation of IκBα as well as translocation of NFκB p65 into nucleus in LPS-stimulated RAW 264.7 cells

Activated nuclear factor-κB (NF-κB), a well-known transcription factor is critically required for the activations of iNOS, COX-2, TNF-α, IL-1β and IL-6 induced by LPS (Surh et al., 2001; Lappas et al., 2002). To identify the effects of FEP on the degradation and phosphorylation of IκBα and translocation of NFκB p65 into nucleus in the classical NFκB

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pathway, the macrophages incubated with FEP and/or LPS were examined by Western blot analysis. β-actin is used as internal control. As Figure 1-14 illustrates, treatment of LPS markedly induced the degradation of IκBα at 15 min after LPS stimulation comparing to control cells, whereas FEP dose-dependently inhibited them. In addition, in parallel, the LPS-stimulated extensive phosphorylation of IκBα was significantly inhibited by the treatment of FEP as well as its degradation. Interestingly, LPS stimulation increased the translocation of NFκB p65 into nucleus, but FEP treatment inhibited the translocation of NFκB p65 increased by LPS in dose-dependent manner (Figure 1-15). Consequently, FEP inhibited the phosphorylation and degradation of IκBα and the translocation of NFκB p65 into nucleus induced by LPS stimulation. These results denote that FEP led to anti-inflammatory effects as inhibiting NO production by down-regulating the expressions of iNOS and pro-inflammatory cytokines such as TNF-α and/or IL-6 via the inhibition of the classical NFκB pathway in activated macrophages.

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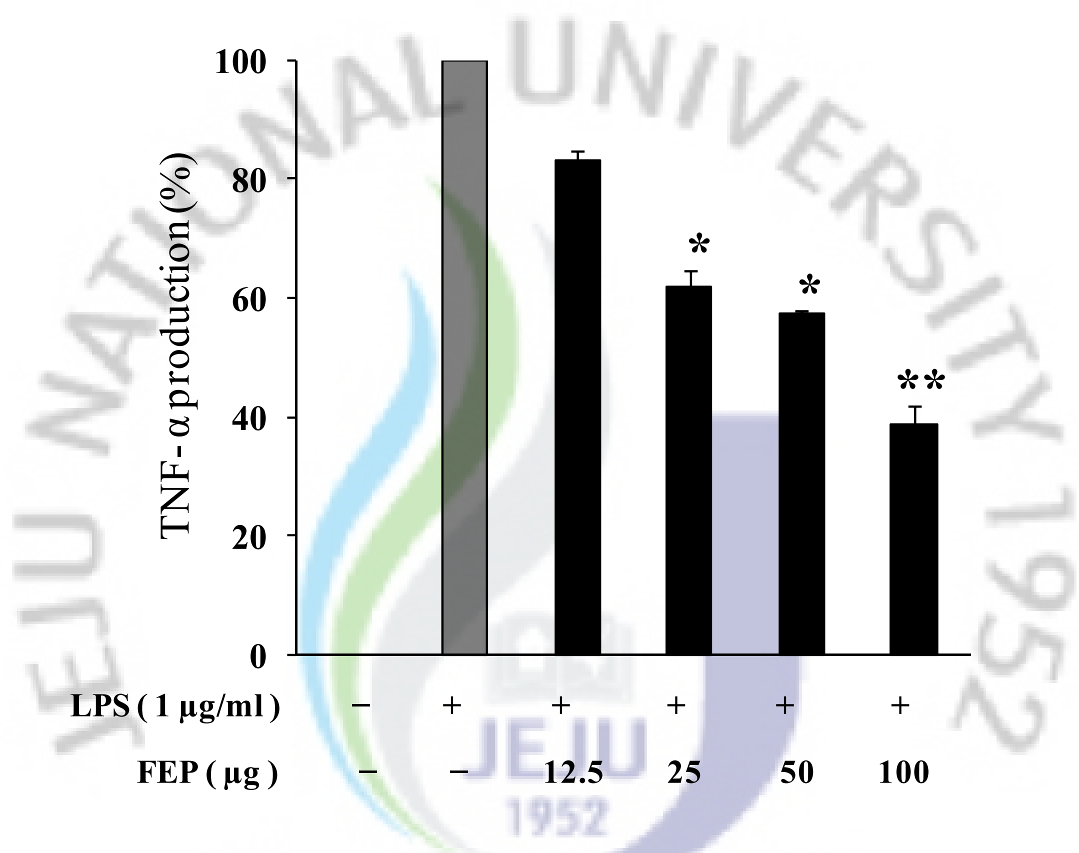

Figure 1-10. Inhibitory effects of FEP on production levels of TNF-α in LPS- stimulated RAW 264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, $P<0.05$; **, $P<0.01$)

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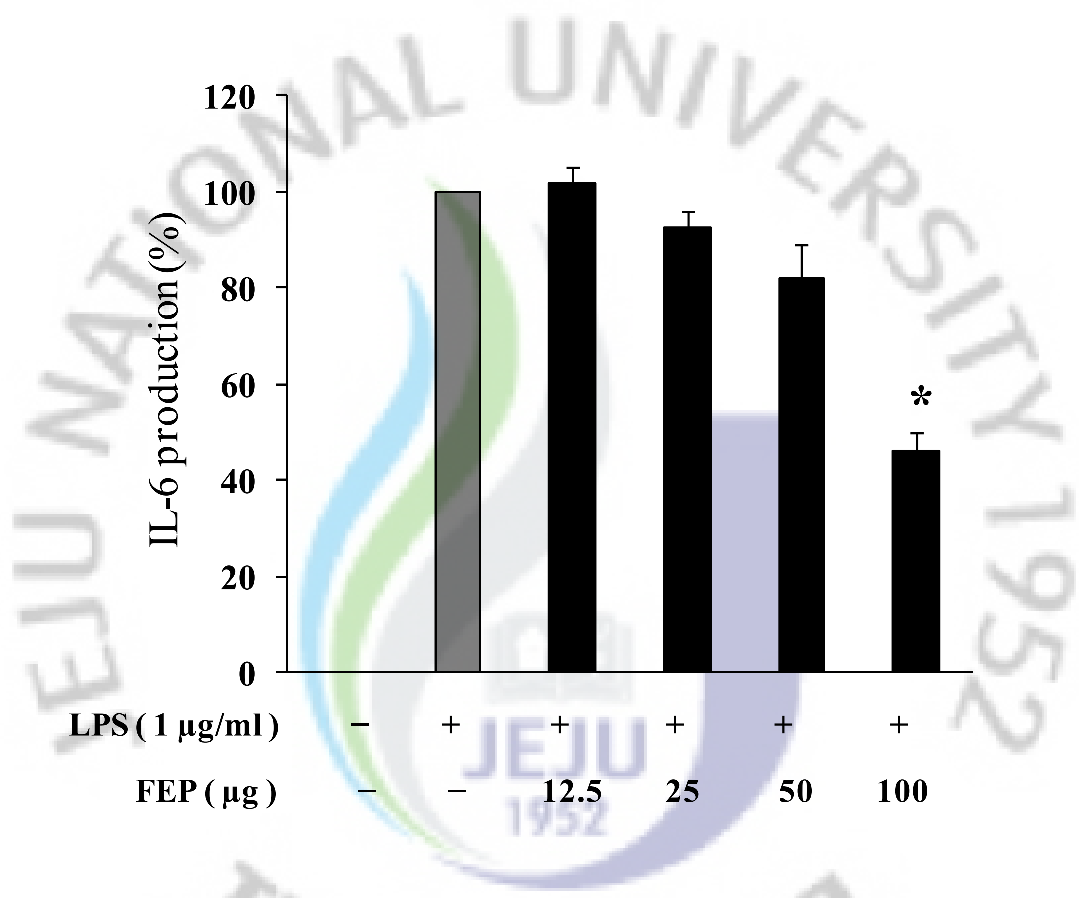

Figure 1-11. Inhibitory effects of FEP on production levels of IL-6 in LPS- stimulated RAW 264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05)

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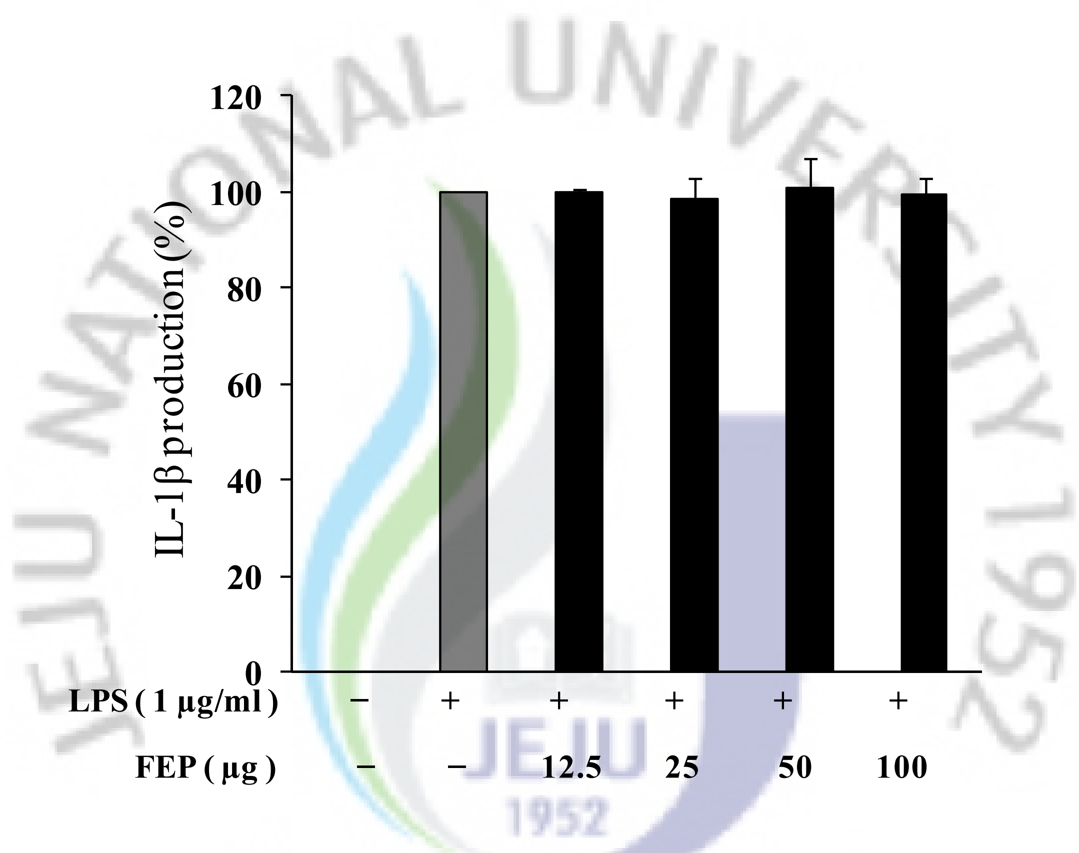

Figure 1-12. Inhibitory effects of FEP on production levels of IL-1β in LPS- stimulated RAW 264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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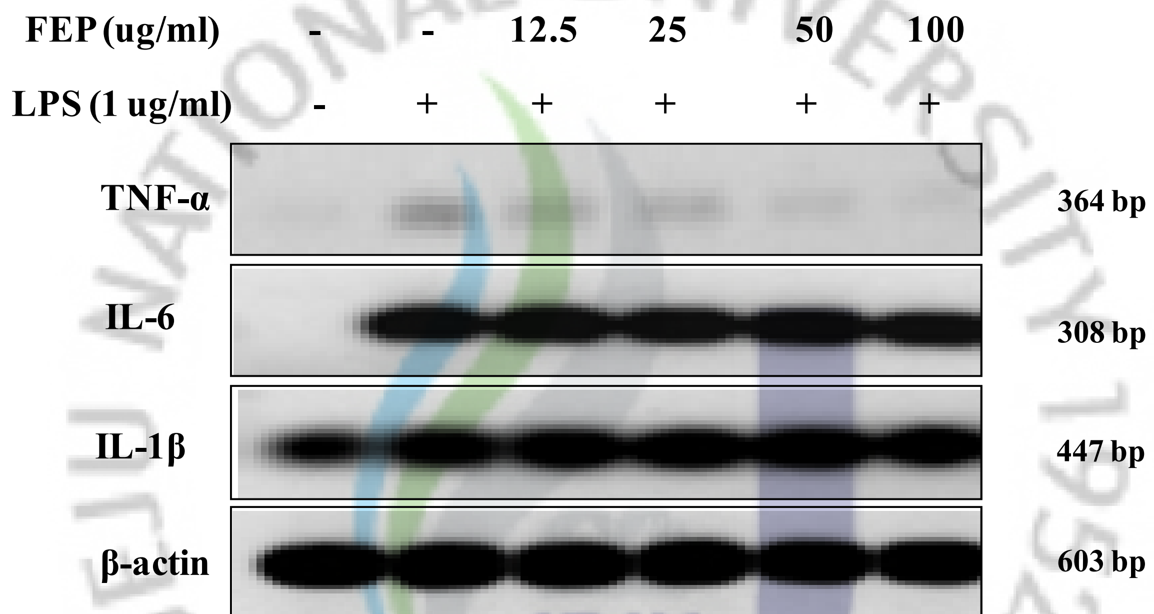

Figure 1-13. Inhibitory effects of FEP on mRNA expression levels of TNF-α, IL-1β and IL-6 in LPS- stimulated RAW 264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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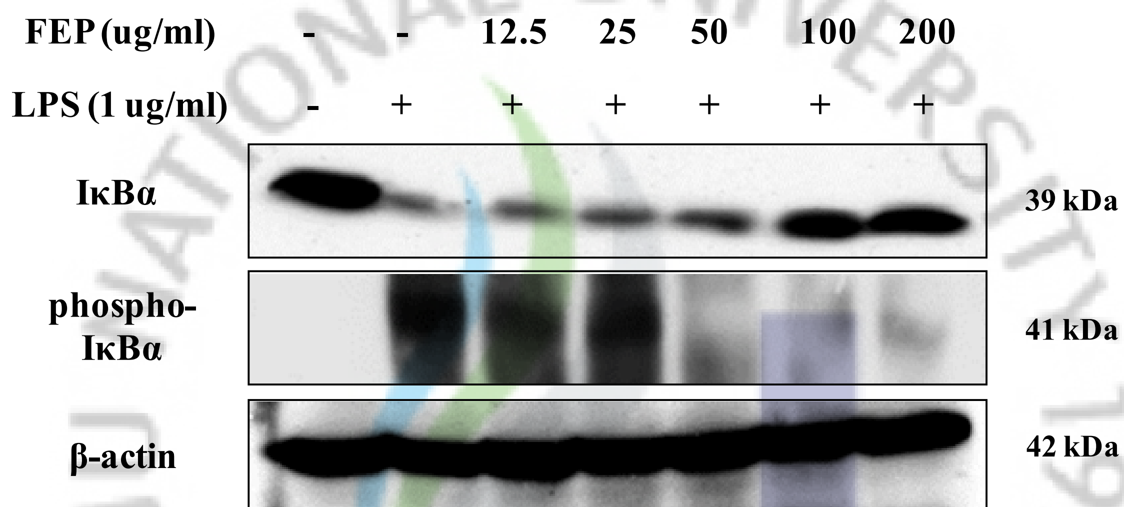

Figure 1-14. Effects of FEP on degradation and phosphorylation of IκB-α in LPS- stimulated RAW 264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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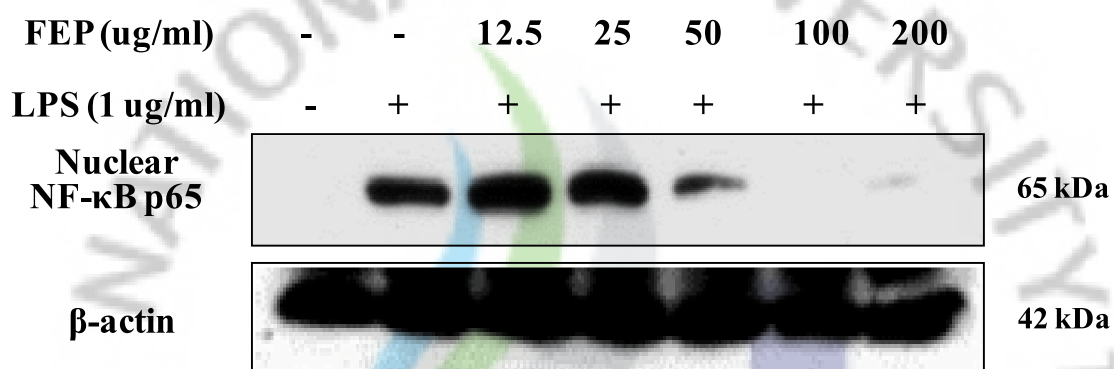

Figure 1-15. Effects of FEP on degradation and phosphorylation of IκB-α in LPS- stimulated RAW 264.7 cells. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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4. Discussion

The present study demonstrates that FEP has anti-inflammatory effects as suppressing the pro-inflammatory cytokines and mediators via inhibiting the activation of the classical NFκB pathway induced by LPS in murine macrophage RAW 264.7 cells.

Although, *E. cava* and its compounds have well-established biological functions such as the anti-inflammatory and anti-oxidant activities for several decades (Ahn et al., 2007 and 2008a), no previous reports have revealed fermented *E. cava*'s participation in the immune response or its underlying mechanism of action.

Normally, fermentation procedure leads to increment of active compounds such as polysaccharide and peptide, digestion rate, and beneficial bacteria in human body. So, the use of fermented materials and evaluation of its biological capacities have been interested by many researchers for few years. Indeed, many researchers have investigated that fermented materials have biological capacities on immune response and oxidative stress in vitro and in vivo (Spanhaak et al., 1998; Hokazono et al., 2010; Joo et al., 2009; Kim et al., 2007; Fan et al., 2009; Kuo et al., 2009). The fermentation process led to the increment of carbohydrate content containing polysaccharide in the extracts of *E. cava*. Interestingly, after the application of enzymatic extraction technique and micro-filtration membrane system, the extracts and molecular fractions prepared from fermented *E. cava* showed the increased yield of extraction and carbohydrate content, as compared to non-fermented *E. cava*. Particularly, its high molecular fraction (> 30 kDa fraction) and polysaccharide showed the higher inhibitory effects on NO production caused by LPS stimulation in Raw 264.7 cells comparing to whole extract and low molecular fraction (< 30 kda fraction). These results demonstrated that the carbohydrate content increased by fermentation process affected to the high

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inhibitory effects on NO production caused by LPS stimulation in Raw 264.7 cells. Furthermore, these resuts indicated that among the carbohydrate contents, polysaccharide was an active compound for the inhibitory effects on NO production caused in LPS stimulated Raw 264.7 cells.

The pharmacological reduction of LPS-induced inflammatory mediators (for example NO, TNF-α, and ILs) is regarded as one of the essential conditions to alleviate a variety of disorders caused by activation of macrophages. Thus, RAW 264.7 macrophages provide us with an excellent model for anti-inflammatory drug screening and for subsequently evaluating the inhibitors of the pathways that lead to the production and induction of pro-inflammatory cytokines and mediators (Yun et al., 2008). Many researchers have reported that LPS stimulates iNOS transcription and transduction, and then the NO production via inducing IκB proteolysis and NFκB nuclear translocation in RAW 264.7 cells (Freeman and Natanson, 2000). Our present study indicated that FEP significantly attenuated the production of NO and the synthesis of its proteins as well as the mRNA levels of iNOS, although has no inhibitory effects on COX-2 in LPS-stimulated RAW 264.7 macrophages.

Normally, TNF-α is a major pro-inflammatory cytokine mainly released by macrophages and plays a considerable role in the pathophysiology of endometriosis and the inflammatory response (Lebovic et al., 2000; Bergqvist et al., 2001; Zhang et al., 2005). IL-6 is also pivotal proinflammatory cytokine, regarded as an endogenous mediator of LPS-induced fever. If FEP inhibits the production of TNF-α and/or IL-6 with the inhibition of NO and iNOS production in LPS-stimulated RAW 264.7 cells, it might be regarded as a potential inhibitor on inflammation response. Indeed, the present study found that FEP significantly inhibits the production of the pro-inflammatory cytokines, TNF-α and IL-6 in LPS-stimulated RAW 264.7 cells. In addition, FEP markedly inhibited the IκB degradation and

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phosphorylation as well as NFκB p65 translocation into nuclei, all of which typify the classical NFκB pathway activated by LPS in RAW 264.7 cells. Interestingly, the previous studies have reported that a large variety of inflammatory conditions, including bacterial and viral infections, rapidly induce the activation of NFκB pathway by activation of IκB kinase (IKK) complex, which phosphorylates IκB, leading to its degradation and translocation of NFκB to the nucleus, where it binds with DNA and activates the transcription of target genes such as pro-inflammatory mediators and cytokines, for example, iNOS, and TNF-α, IL-1β and -6 (Surh et al., 2001; Lappas et al., 2002; Baeuerle et al., 1988; Ghosh and Baltimore, 1990; Verma et al., 1995). Also, it is well known that IκBα phosphorylation at Ser-32 and Ser-36 by IKK complex is the critical step in NFκB activation (Brown et al., 1995; Mercurio et al., 1997). This indicates that the inhibitory effects of FEP on the activation of the NFκB pathway in LPS-stimulated RAW 264.7 cells might be related with the inhibition of the IKK activity and other upstream events required for NFκB activation. Subsequently, further studies regarding with the inhibition of the IKK activity and other upstream events required for NFκB activation is required in future, although this study confirmed that the inflammation responses through the activation of NFκB stimulated by LPS in macrophage were significantly inhibited by FEP.

Taken together, this study suggests that a polysaccharide from the viscozyme extract of *L. brevis*-fermented *E. cava* (FEP) has the anti-inflammatory effects via down-regulating the production and expression of pro-inflammatory cytokines and mediators by inactivating the NFκB pathway in LPS-stimulated RAW 264.7 cells. Also, our conclusion from this evidence is that FEP can be used as a useful anti-inflammatory material to suppress macrophage activation.

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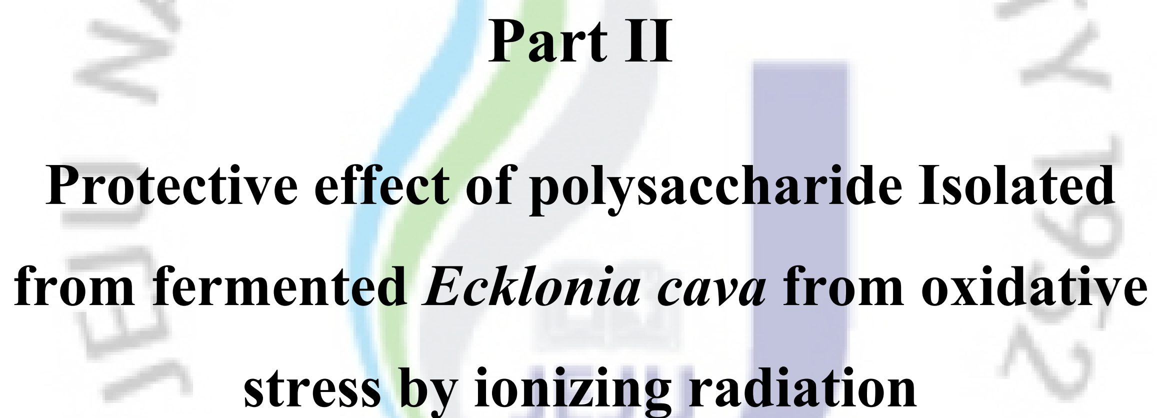

Part II

Protective effect of polysaccharide Isolated from fermented *Ecklonia cava* from oxidative stress by ionizing radiation

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ABSTRACT

Exposure of ionizing radiation induces oxidative stress and inhibits survival and proliferation of cells by causing DNA damage. Fermentation process using several fungi and bacteria increases the extraction of active compounds such as polysaccharides and peptides, ingestion rate, and body absorption and leads to the beneficial effects on immune activation and anti-oxidant. This study investigated whether a polysaccharide from enzymatic extract of fermented *E. cava* can protect cells against damages caused by ionizing radiation and its underlying mechanism in murine splenocytes known as radio-sensitive cells.

Here, the aqueous extracts of *E. cava* fermented by three kinds of fermentation fungi and bacteria (*Lactobacillus brevis*, *Saccharomyces cerevisiae*, and *Candida utilis*, respectively) increased the extraction efficiency and splenocyte proliferation, comparing to the aqueous extract of original *E. cava*. In additon, the aqueous extracts of *E. cava* fermented by *Lactobacillus brevis* (ALFE) showed the highest splenocyte' proliferation and its viscozyme extract (LFEV) also stimulated the proliferation of splenocytesm compared to the others. Interestingly, at 1 or 3 days after exposure of 2 Gy irradiation, LFEV, > 30 kDa fraction of LFEV, and a polysaccharide of LFEV (FEP) significantly enhanced the survival and proliferation of splenocytes. Interestingly, FEP showed the significantly enhanced survival and proliferate effects in 2 Gy-irradiated cells with increasing of concentrations (from 75 to 300 ug/ml), as compared to only-2 Gy-irradiated cells. Also, FEP treatment significantly reduced DNA damage and the formation of apoptotic DNA in sub-G_1 phase as reducing oxidative stress followed by the production of reactive oxygen species (ROS) induced by 2 Gy irradiation. Moreover, FEP decreased the expression level and of Bax, a pro-

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apoptotic protein, whereas increased those of Bcl-2 and Bcl-xL, anti-apoptotic proteins after exposure of 2 Gy irradiation.

Taken together, these results demonstrate that FEP has radio protective effects against damages caused by ionizing radiation by modulating Bcl-2/Bax signal pathway in apoptosis and might be used for a potential materials for radiotherapy of cancer patients.

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1. INTRODUCTION

Normally, exposure of ionizing radiation leads to reactive oxygen species (ROS) production by reacting with H_2O existed in cells and animal. In addition, the reaction of ROS with DNA of cells causes DNA damages undergoing apoptosis and finally leads to the destruction of the lymphoid and hemopoietic tissues, which contain proliferating stem cells such as crypt cells or bone marrow cells and splenocytes known as peripheral immune cells. Especially, when ionizing radiation was irradiated to various cells, radiation induced apoptosis is readily observed in proliferating stem cells and splenocytes including lymphocytes and granulocytes known as the most radio sensitive cell types. Also, for few years, the use of ionizing radiation including gamma ray irradiation has been gradually increasing for radiotherapy of cancer patients. At these points, the investigation of the radio protectors showing beneficial effects on the inhibition of DNA damages induced by ROS production is worthy of attention.

The brown seaweed *Alariaceae Ecklonia cava (E. cava)* grown plentifully in waters surrounding Jeju Island in Korea contains the polysaccharides, fucoidan, alginate, fucan, and laminarin, which are water-soluble dietary fibers, and phycocolloids (Ahn et al., 2010; Guiry and Bulunden, 1991). For few years, many researchers have reported the beneficial effects of *E. cava* such as anti-oxidant *in vitro,* anticancer, anticoagulant, immunomodulation, anti-inflammation, immune activation and matrix metalloproteinase inhibition effects (Ahn et al., 2007, 2008a,b and 2010; Kim et al., 2006a; Athukorala et al., 2006; Kim et al. 2006b). Especially, the recent studies have indicated that *E. cava* is consisted of plentiful polysaccharides and minor phlorotannins (Ahn et al., 2008a). In addition, many researchers

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have been reported that polyphenols and polysaccharides as the radio protectors are related with the beneficial capacities on antioxidant and/or immune activation (Park et al., 2008a,b,c, 2009, 2010; Kang et al., 2005, 2006a,b; Bing et al., 2010). At the moment, searching the potential active compounds showing antioxidant effects, immunomodulatory effects, and immune activation might contribute to development of natural radio protector. Indeed, until now, many researchers have reported the polyphenols of *E. cava* such as eckol, dieckol, phloroglucinol, and Triphlorethol-A have antioxidant and immunological effects and their capacities contribute to radio protective effects in vitro and in vivo (Ahn et al., 2007; Park et al., 2008a,b,c, 2010; Kang et al., 2005, 2006a, Moon et al., 2008). However, there are no studies about the radio protective efficacy of polysaccharides isolated from *E. cava*. From these points, polysaccharides isolated from *E. cava* might be a potential material for development of natural radio protector.

Fermentation process with several fungi and bacteria plays an important role in improvement of nutritional and functional properties of foods as increasing the extraction of active compounds such as polysaccharides and peptides, ingestion rate, and body absorption. Previous reports have demonstrated that after fermentation process, many active compounds led to immune activation and anti-oxidant in vitro and in vivo (Link-Amster et al., 1994; Matsushita et al., 2008; Chen et al., 2009; Fernandez-Orozco et al., 2008).

Therefore, here, this study investigated whether a polysaccharide isolated from viscozyme extract of *Lactobacillus brevis*-fermented *E. cava* have radio protective capacity in gamma ray-irradiated splenocytes.

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2. MATERIALS AND METHODS

2.1. Preparation of enzymatic extracts from fermented *E. cava*

E. cava was collected from the coast of Jeju Island, South Korea, washed with fresh water, freeze-dried and pulverized into powder with a grinder. Then, the freeze-dried *E. cava* was applied to fermentation process using three kinds of fermentation fungi and bacteria (*Lactobacillus brevis*, *Saccharomyces cerevisiae*, and *Candida utilis*, respectively) for 24 h. After freeze-dry, the fermented *E. cava* powders (LFE, SFE, and CFE) were used for the enzymatic extraction technique using several enzymes followed by previously reported method (Heo et al., 2005). Among them, LFE (50 g) shown the highest proliferation of splenocytes was homogenized in distilled water (2 L) with 500 μl of ten kinds of enzymes. Each reactant was adjusted to the optimum pH and temperature range of the respective enzyme and enzymatic reactions were performed for 24 h. Following digestion, the digest was boiled for 10 min at 100℃ to inactivate the enzymes. After centrifugation (3000 rpm, for 20 min at 4℃), the supernatant were adjusted to pH 7.0 hereafter and designated as enzymatic extract. The sample was kept at -20℃ for further experiments.

2.2. Preparation of molecular weight fractions from viscozyme extract of LFE (LFEV)

To perpetrate different molecular weight fractions, Lab scale TFF system (PHILOS) was performed by using micro-filtration membrane (30 kDa). First, LFE viscozyme extract (LFEV) was filtered and applied to the Lab scale TFF system (PHILOS). Then, the filtered LFEV was divided into the two kinds of fractions (> 30 kDa and < 30 kDa fractions). After freeze-dry, the fractions was used for next experiments.

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2.3. Isolation of a polysaccharide from > 30 kDa fraction of LFEV (FEP)

A polysaccharide was isolated from > 30 kDa fraction of LFEV by ethanol precipitation according to slightly revised method indicated in previous study (Athukorala et al., 2009). The > 30 kDa fraction of LFEV (1 L) was mixed with 2 L of 99.5% ethanol for 24 h at 4℃. After centrifugation at 10000 g for 20 min at 4℃, crude polysaccharide was collected from its precipitant. Then, crude polysaccharide isolated from > 30 kDa fraction of LFEV (FEP) was freeze-dried and used for next experiments.

2.4. Analysis of mono-sugar contents

The LFE, > 30 kDa and < 30 kDa fractions of LFEV and FEP were hydrolyzed in a sealed glass tube with 4 M of trifluoroacetic acid for 4 h at 100℃ to analyze neutral sugars. In order to analyze the amino-sugars the samples were digested using 6 N of HCl for 4 h. Then, the samples were separately applied to CarboPac PA1 (4.5 x 250 mm, Dionex, Sunnyvale, CA, USA) with CarboPac PA1 cartrige (4.5 x 50 mm), respectively. The column was eluted using 16 mM of NaOH at 1.0 ml/min flow rate. Each sugar of the samples was detected by using ED50 Dionex electrochemical detector and data were analyzed by Peack Net on-line software.

2.5. Irradiation with ^{60}CO γ-rays

Splenocytes were exposed to γ-irradiation from a ^{60}CO Theratron® (Best Theratronics

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Ltd, Ottawa, Ontaria, Canada) teletherapy unit at Applied radiological Science research Institute, Jeju National University, Korea, at a dose rate of 1.5 Gy/min.

2.6. Preparation of primary splenocytes

Mouse spleens were aseptically removed from 8- to 9-week-old ICR mice purchased from SLC (Yokohama, Japan), and single-cell suspensions were obtained by using a cell strainer. Erythrocytes were depleted in lysis buffer (150 mM NH_4CL, 10 mM $KHCO_3$, and 0.1mM EDTA $Na_2 \cdot H_2O$), which lyses red blood cells, with minimal effect on splenocytes. Ten minutes later, the cells were washed with Dulbecco's phosphate buffered saline (DPBS, Gibco BRL, Raisley, UK) and were suspended in RPMI-1640 medium (Gibco BRL) which contained 10% fetal bovine serum (Gibco BRL) and 100 units/ml penicillin-streptomycin (Gibco BRL). These purified cells were used directly for experiments. Experiments using animals were approved by the Institutional Animal Care and Use Committee of Jeju National University.

2.7. Cell viability

The effects of LFE, LFEV, > 30 kDa and < 30 kDa fractions of LFEV, LEP on the survival of gamma ray (2 Gy) irradiated or nonirradiated splenocytes were assessed by an MTT assay, which is a colorimetric assay that is dependent on the conversion of yellow tetrazolium bromide to its purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mossmann, 1983). The non-irradiated or irradiated

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splenocytes (1×10^5 cells/wells) were incubated with or without LFE, LFEV, > 30 kDa and < 30 kDa fractions of LFEV, LEP at various concentrations (75, 150, and/or 300 μg/ml) for 24 h. Control cells were treated with only RPMI medium. Then, MTT stock solution (10 μl; 5 mg/ml) was applied to each of the wells for 4 h. The absorbance of formazan crystals dissolved in 100 μl of solubilization buffer (pH 4.7) including 50% dimethylformamide (DMSO) and 10% sodium dodecyl sulfate (SDS) was measured at 540 nm using enzyme-linked immunosorbent assay (ELISA) plate reader. The optical density of the formazan generated in control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective control.

2.8. ^{3}H-thymindine incorporation assay

Normally, ^{3}H-thymidine incorporation assay is a standard assay based on the principle that the thymidine base of DNA sequences in these cells is replaced with radioactive ^{3}H-thymidine (Amersham, Arlington Heights, IL, USA). First, the assay was used to identify the effects of *E. cava* fermented by three types of fermentation fungi and bacteria on the proliferation of splenocytes. Next, a polysaccharide (LFE) was isolated from > 30 kDa fraction of LFEV shown the highest proliferation effects in splenocytes and LFEV, > 30 kDa and < 30 kDa fractions of LFEV, and LEP was applied to ^{3}H-thymidine incorporation assay. For this assay, the 4×10^5 cells were cultured with several samples such as fermented *E. cava* extracts (LFE, SFE, and CFE, the fractions and polysaccharide of LFE (LFEV, > 30 kDa and < 30 kDa fractions of LFEV, and LEP) at various concentrations (75, 150, and/or 300 μg/ml) in 96-well round-bottom microtiter plates (Nunc, Copenhagen, Denmark). Concanavalin A (2 μg/ml) was used for positive control cells. After incubation for 72 h at 37°C, 95% humidity

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and 5% CO_2, 1 μCi of ^{3}H-thymidine (specific activity 42 C_i/mmol, Amersham, Arlington Heights IL, U.S.A.) was added to the cells, and the plates were incubated for an additional 18 h. The cells were then harvested onto glass fiber filters by an automatic cell harvester. The amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer (Wallac MicroBeta® TriLux, PerkinElmer, Waltham, MA, U.S.A.).

2.9. DCF-DA assay

To detect intracellular ROS, the DCF-DA assay was used. The irradiated splenocytes were seeded in a 96-well plate at 1×10^5cells/well and were treated with various amounts of FEP (75, 150, and 300 μg/ml). After 2 h incubation, 25 μM of DCF-DA solution was added to the plate for 10 min. The fluorescence of 2', 7'-dichlorofluorescein was detected at 585 nm and 620 nm using a fluorostar (BMG labtech, U.S.A). The intracellular ROS scavenging activity (%) was calculated as 100 × [(optical density of irradiated group) - (optical density of irradiated group with FEP treatment)] / (optical density of irradiated group).

2.10. Propidium iodide (PI) staining assay

To determine whether FEP affected the cell cycle of irradiated splenocytes, phase of cell cycle was assessed by flow cytometry. Splenocytes were collected and washed in ice-cold PBS and pelleted by centrifugation. Splenocytes irradiated with 2.0 Gy were incubated with 75, 150, and 300 μg/ml of FEP for 6, 12, and 24 h. Then, the cells were collected and washed in ice-cold PBS and pelleted by centrifugation. Cells were resuspended in propidium iodide (Sigma) and 100 μg RNase A (Sigma) at a concentration of 1×10^6 cells/100 μl. After 30 min, samples were analyzed by assessing the proportion of sub G_1 cells using a BD

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FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, U.S.A.)

2.11. Western blot analysis

Western blot analysis was performed to assess whether FEP modulates the expressions of apoptosis-related molecules such as p53, Bax, and Bcl-2. Splenocytes irradiated with 2.0 Gy were cultured with FEP at concentrations of 75, 150, or 300 μg/ml. After 12 h, cytoplasmic protein was prepared from the cells by using NE-PER® Nuclear and Cytoplasmic Extraction Reagents according to manufacturer′s instructions. Cytoplasmic (50 μg/well) preparation was loaded into SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and electrophoresed under denaturing conditions. Subsequently, proteins were electro-transferred onto nitrocellulose transfer membrane. After blocking with 5% nonfat milk for 2 h, blots were incubated with primary antibodies such as p53 (1:1000 dilution, Cell Signaling Technology Inc., U.S.A), Bax (1:1000 dilution, Cell Signaling Technology Inc., U.S.A), Bcl-2 (1:1000 dilution, Cell Signaling Technology Inc.) or β-action (1:3000 dilution, Sigma) antibodies for 60 min followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin (Ig)G (Cell Signaling Technology Inc.) for 60 min. Visualization was achieved by using X-ray film and chemiluminescence reagents.

2.12. Statistical analysis

All data were presented as mean ± standard errors (SE) and analyzed by using Student's *t*-test. Differences with a *P* value of less than 0.05 were considered significant.

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3. RESULTS

3.1. LFE showed the highest proliferate effects on splenocytes

The extracts of *E. cava* fermented by several fermentation fungi and bacteria (*Lactobacillus brevis, Saccharomyces cerevisiae* and *Candida utilis*) (LFE, SFE, and CFE) (150 μg/ml) were used to evaluate their proliferative effects on splenocytes. As shown in Figure 2-1, all aqueous extracts of fermented *E. cava* (ALFE, ASFE, and ACFE) increased the proliferation of non-irradiated splenocytes. Especially, of the tested samples, ALFE markedly increased the number of proliferated splenocytes comparing to untreated cells (control cells) (***; $p < 0.001$) and it was higher than others. Therefore, ALFE was selected for further experiments.

3.2. LEP led to the highest effects on the survival and proliferation of splenocytes after exposure of gamma ray irradiation

To obtain the effective compounds on the viabilities of splenocytes, the enzymatic extractions from LFE was prepared by using several carbohydrases (Viscozyme, Celluclast, AMG, Termamyl, and Ultraflo) and proteases (Kojizyme, Papain, Pepsin, Protamex and Neutrase). Then, among them, the Viscozyme extract of LFE (LFEV) (150 μg/ml) showed the markedly increased viabilities in splenocytes, as compared to others (Figure 2-2). Therefore, LFEV was selected for further next experiments.

Next, we fractionated > 30 kDa, and < 30 kDa fractions from LFEV and checked their effects on survival and proliferation of 2 Gy-irradiated splenocytes. As shown in Figure 2-3 and 2-4, 2 Gy irradiation decreased the survival and proliferation of splenocytes, as compared

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with non-irradiated cells (control cells). However, the treatment of LFEV, > 30 kDa fraction, and FEP significantly increased the survival and proliferation of splenocytes decreased by gamma ray irradiation at 150 μg/ml, in comparison with only gamma ray-irradiated cells (*; $p < 0.05$, ***; $p < 0.001$, **; $p < 0.005$ in MTT assay and ***; $p < 0.001$, ***; $p < 0.001$, ***; $p < 0.001$ in ^{3}H-thymidine incorporation assay). However, the treatment of < 30 kDa fraction did not affect the proliferation of 2 Gy-irradiated cells. Interestingly, after exposure of gamma ray irradiation, > 30 kDa fraction and FEP showed the higher proliferation effects in splenocytes than that of LFEV, which is a whole extract. Moreover, FEP showed the highest beneficial capacities on the survival and proliferation of 2 Gy-irradiated splenocytes, compared to the others. In addition, FEP significantly increased the survival and proliferation of splenocytes decreased by gamma ray irradiation with increment of concentrations (from 75 to 300 μg/ml) (Figure 2-5 and 2-6). These results indicate that FEP containing the large amount of carbohydrate can enhanced the survival and proliferation of splenocytes inhibited by gamma ray irradiation.

3.3. FEP decreased the production of intracellular ROS in 2 Gy-irradiated splenocytes

DCF-DA assay was used to evaluate whether FEP reduces the production of intracellular ROS induced by ionizing radiation. As indicated in Figure 2-7, gamma ray-irradiation increased the production of intracellular ROS in splenocytes, compare with non-irradiated cells. However, treatment of FEP dose-dependently reduced the production of intracellular ROS caused by gamma ray irradiation at all concentrations (75, 150 and 300 μg/ml). Especially, 150 and 300 μg/ml of FEP showed the similar scavenging activities on intracellular ROS produced in 2 Gy-irradiated splenocytes, and their scavenging activities were about 46% and 47%, respectively. These results indicate that FEP increased the survival and proliferation of splenocytes via reducing intracellular ROS production caused by ionizing radiation.

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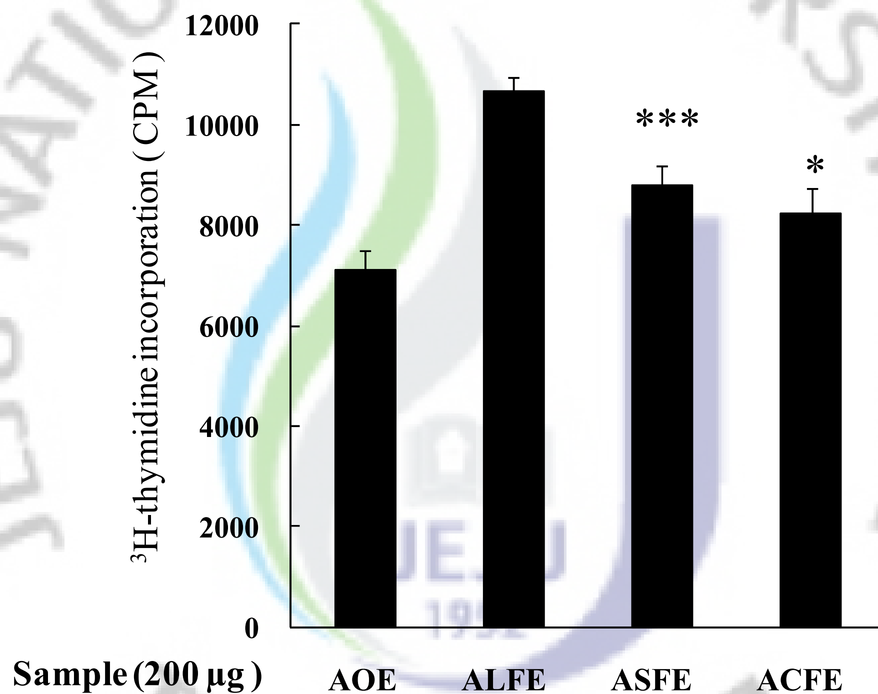

Figure 2-1. Effects of three kinds of fermented *E. cava*(FE) on the proliferation of primary urine splenocytes. AOE, Aqueous extract of original *Ecklonia cava*; ALFE, Aqueous extract of LFE; ASFE, Aqueous extract of SFE; ACFE, Aqueous extract of ACFE. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, P<0.05; **, P<0.01; ***, P<0.005.)

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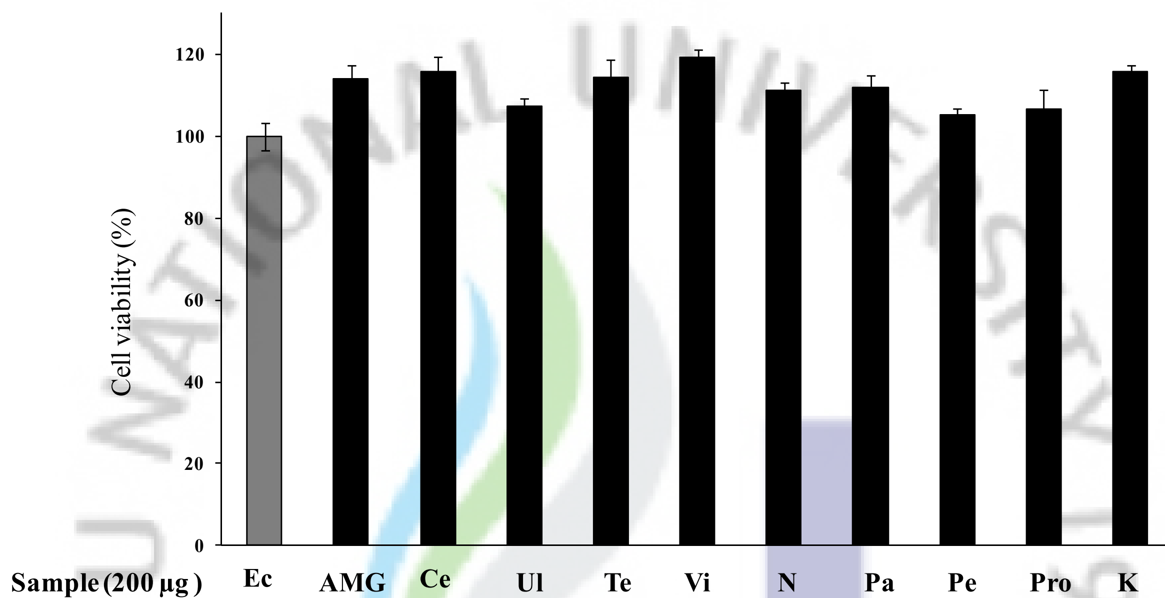

Figure 2-2. Effects of FE enzymatic extracts on the viabilities of nonirradiated immune cells. Ec, *Ecklonia cava*; AMG; AMG extract prepared from LFE, Ce; Celluclast extract prepared from LFE, Ul; Ultraflo extract prepared from LFE, Te; Termamyl extract prepared from LFE, Vi; Viscozyme extract prepared from LFE,. N; Neutrase extract prepared from LFE. Pa; Papain extract prepared from LFE, Pe; Pepsin prepared from LFE, Pro; Protamex extract prepared from LFE. K; Kojizyme extract prepared from LFE. Experiments were performed in triplicate and the data are expressed as mean ± SE.

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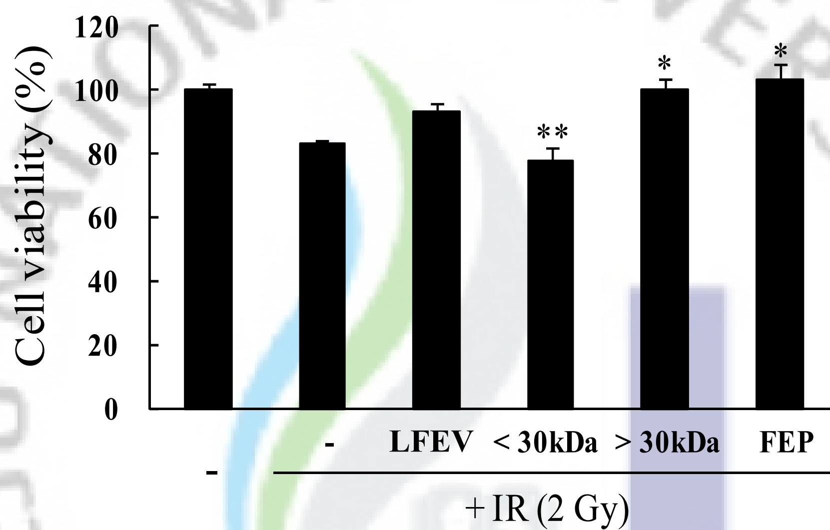

Figure 2-3. Effects of Viscozyme extracts of LFE (LFEV), and it's membrane fractions (<30 kDa and > 30 kDa), and polysaccharide (FEP) prepared from LFEV on the viability of 2Gy-irradiated immune cells. The effects of four samples on viability of immune cells were determined by MTT assay. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.)

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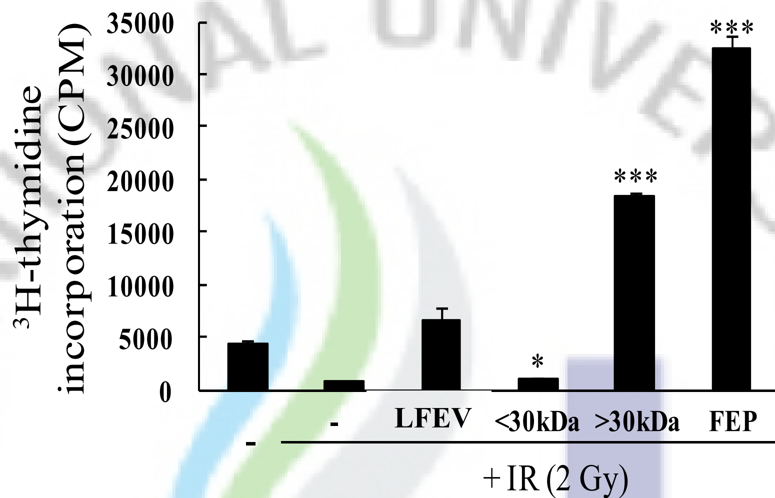

Figure 2-4. Effects of LFEV, it's membrane fractions and FEP on the proliferation of 2Gy-irradiated immune cells. The effects of four samples on viability of immune cells were determined by ^{3}H-thymindine incorporation assay. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.)

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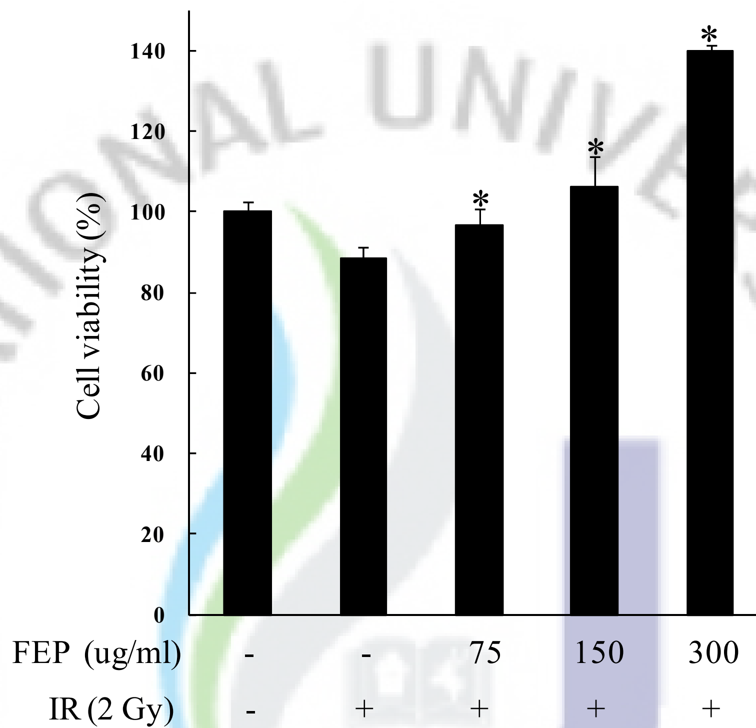

Figure 2-5. Effects of FEP on viability of 2Gy-irradiated splenocytes. The effects of FEP on viability of immune cells were determined by MTT assay. Experiments were performed in triplicate and the data are expressed as mean ± SE. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.)

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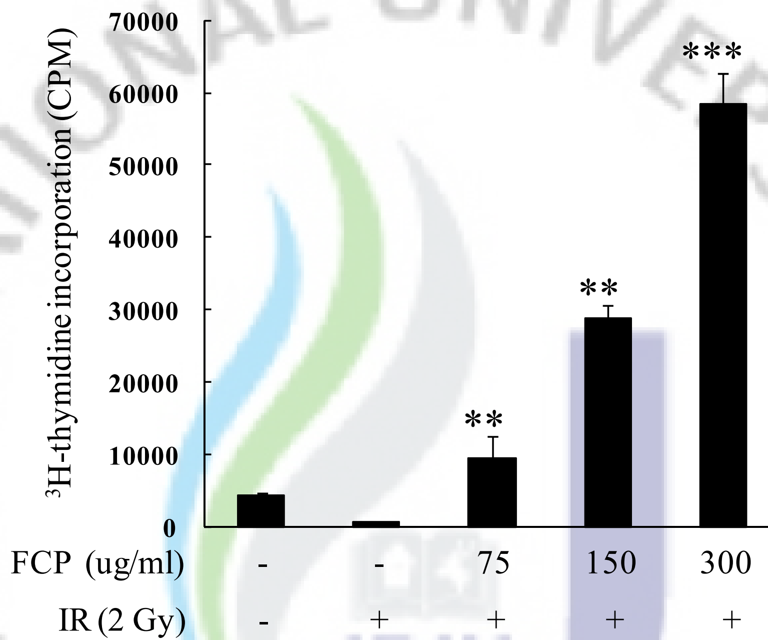

Figure 2-6. Effects of FEP on proliferation of 2Gy-irradiated splenocytes. The effects of FEP on proliferation of splenocytes were determined by ^{3}H-thymindine incorporation assay. Experiments were performed in triplicate and the data are expressed as mean ± SE. (**, $P < 0.01$; ***, $P < 0.005$.)

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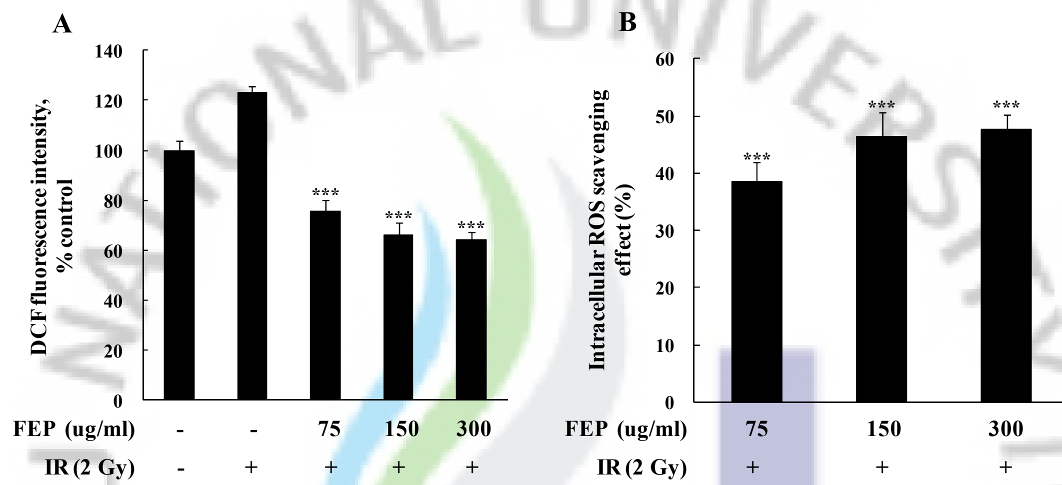

Figure 2-7. The effects of FEP on intracellular ROS produced in gamma ray-irradiated immune cells. The effects of FEP on intracellular ROS produced by gamma ray-irradiation were determined by DCF-DA assay. Experiments were performed in triplicate and the data are expressed as mean ± SE. (***, P<0.005.)

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3.4. FEP decreased the formation of apoptotic DNA of 2 Gy-irradiated splenocytes

The inhibitory effects of FEP on apoptosis induced by gamma ray-irradiation in immune cells was checked by PI staining assay known as a technique measuring the number of cells in the Sub G_1 peak. The results were indicated in Figure 2-8. The accumulation of cells in apoptotic peaks was dramatically increased 6, 12, and 24 h after gamma ray irradiation, compared to non-irradiated cells. However, the cells treated with FEP showed an interestingly lower percentage of cells in apoptotic peak at all concentrations when compared with only-gamma ray-irradiated cells (Figure 2-8). In addition, after 6h of exposure to gamma ray-irradiation, FEP dose-dependently decreased the population of apoptotic DNA increased, although the populations of apoptotic DNA at 12 and 24 h were not dependent on increment of concentrations. These data suggest that FEP showed a cytoprotective effect against gamma ray irradiation-induced cell damage.

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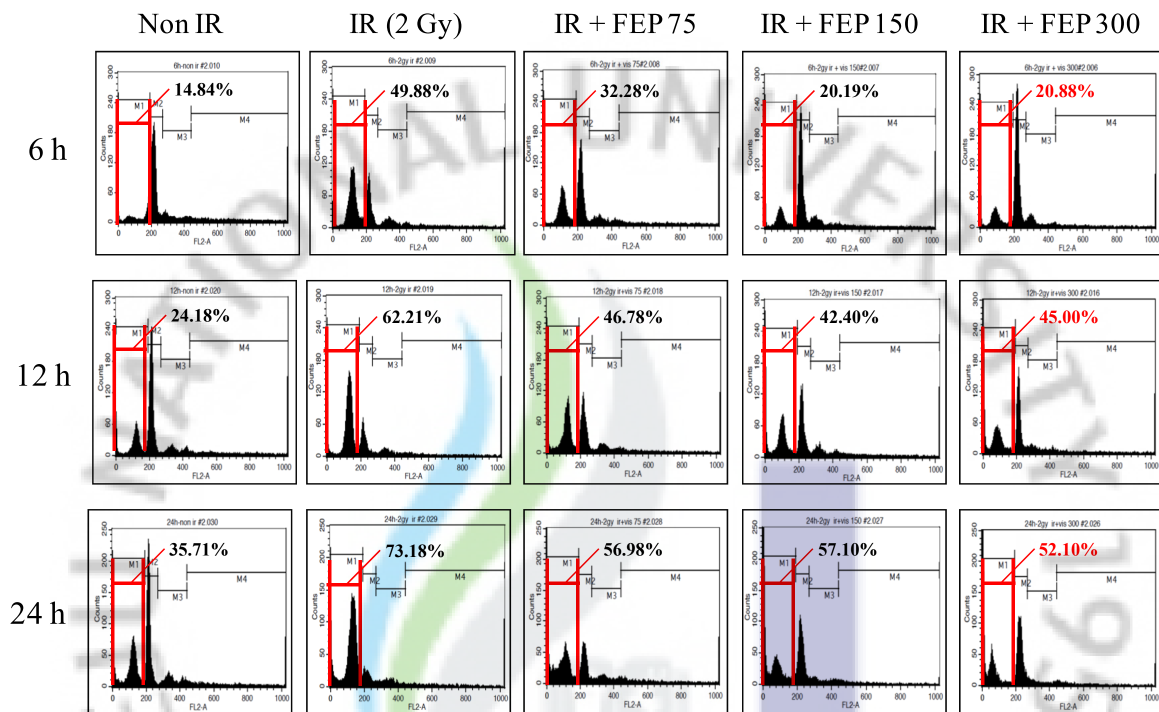

Figure 2-8. The effects of FEP on formation of apoptotic DNA induced in gamma ray-irradiated immune cells. The effects of FEP on the formation of apoptotic DNA (in Sub G_1 peak) induced in gamma ray-irradiated immune cells were determined by PI staining assay. Experiments were performed in triplicate.

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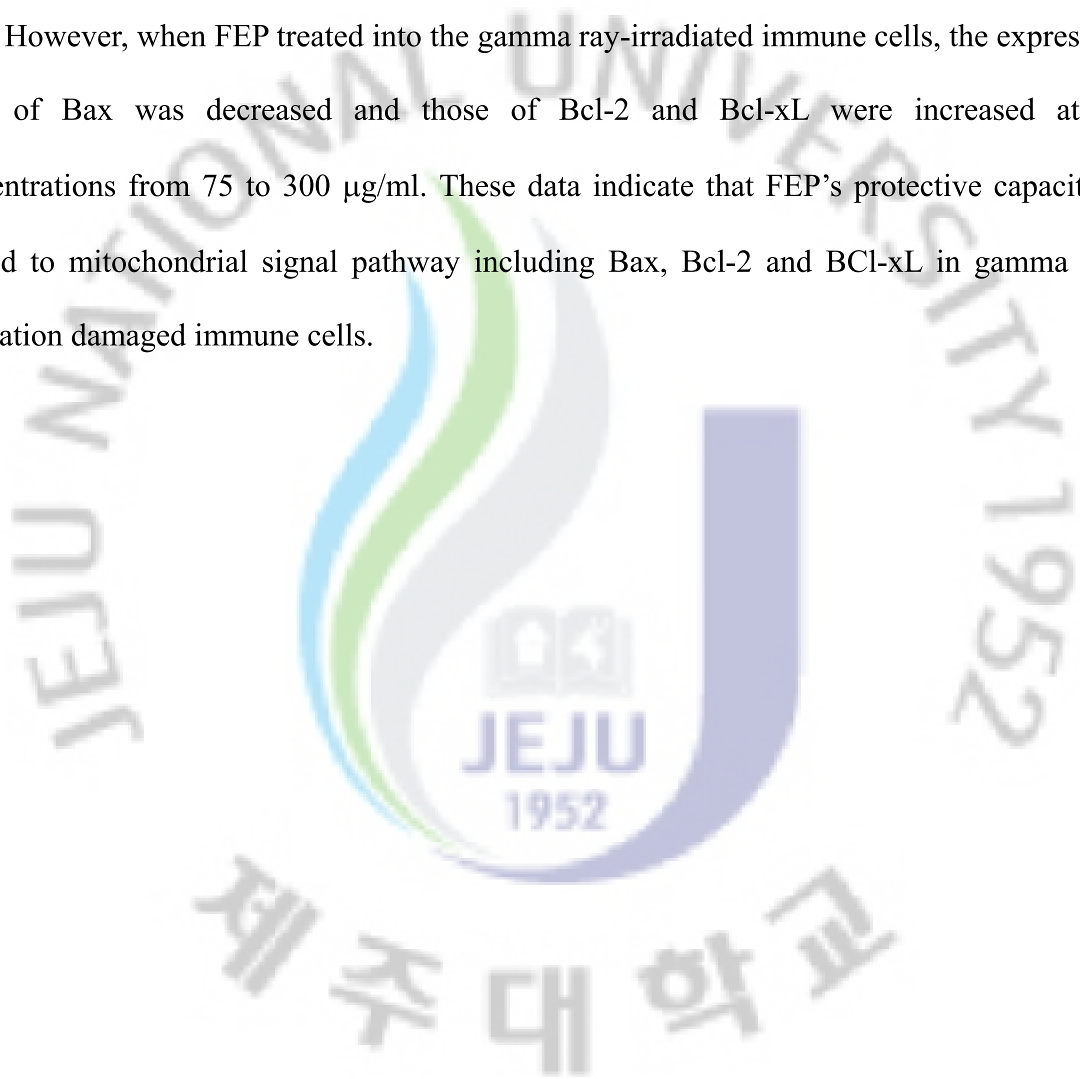
3.5. FEP decreased the formation of apoptotic DNA of 2 Gy-irradiated immune cells

To indentify whether FEP regulates the expression of apoptosis-related proteins in gamma ray-irradiated immune cells, Western blot analysis was carried out. As shown in Figure 2-9, gamma ray irradiation appeared to increase expression of pro-apoptosis molecule, Bax, whereas markedly decreased anti-apoptotic molecules, Bcl-2 and Bcl-xL in immune cells. However, when FEP treated into the gamma ray-irradiated immune cells, the expression level of Bax was decreased and those of Bcl-2 and Bcl-xL were increased at all concentrations from 75 to 300 μg/ml. These data indicate that FEP’s protective capacity is related to mitochondrial signal pathway including Bax, Bcl-2 and BCl-xL in gamma ray-irradiation damaged immune cells.



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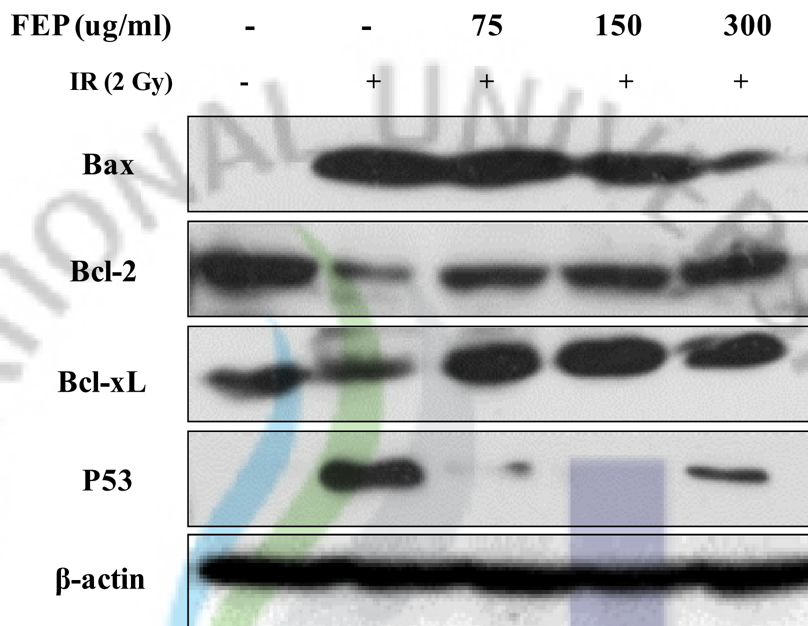

Figure 2-9. The effects of FEP on expression levels of apoptosis related molecules such as Bax, Bcl-2, and Bcl-xL in gamma ray-irradiated immune cells. The effects of FEP on the expression levels of apoptosis related molecules such as Bax, Bcl-2, and Bcl-xL in gamma ray-irradiated immune cells were determined by Western blot. Experiments were performed in triplicate.

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3. DISCUSSION

This study showed that the polysaccharide isolated from viscozyme extract of *Lactobacillus brevis*-fermented *E. cava* (FEP) protects primary murine splenocytes against damage caused by gamma-ray irradiation.

Generally, fermentation procedure leads to increment of active compounds, digestion rate, and beneficial bacteria in human body. At these points, many researchers have interested in use of fermented materials and evaluation of its biological capacities for few decades. Indeed, many researchers have investigated that fermented materials have biological capacities on immune response and oxidative stress (Spanhaak et al., 1998; Hokazono et al., 2010; Joo et al., 2009; Kim et al., 2007; Fan et al., 2009; Kuo et al., 2009). Therefore, in this study, fermentation process was used to isolate active compound from fermented *E. cava* and the extracts of fermented *E. cava* showed the increased carbohydrate content, compared with that of original *E. cava*. In addition, among the fermented extracts, the enzymatic extracts of LFE and its polysaccharide (FEP) markedly enhanced the survival and proliferation of splenocytes decreased by exposure to ionizing radiation, compared to non-fermented *E. cava.* Interestingly, the number of splenocytes decreased by exposure of ionizing radiation was gradually increased with increment of carbohydrate content. Among them, FEP led to the marked increment of cell proliferation in ionizing radiation-exposed splenocytes. Like these results, many previous studies have documented that Tofu fermented with *Pleurotus eryngii* Mycelia and Korean Ginseng fermented with Mushroom Mycelium showed the beneficial effects on immune activation (Lee et al., 2010; Park et al., 2009). Moreover, the previous studies have suggested that polysaccharide content increased by fermentation process induced immune activation *in vitro* and *in vivo* (Kim et al., 2007; Yuan et al., 2006; Makino et al.,

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2006). These results suggest that the fermentation process led to the increased carbohydrate content comparing to non-fermented *E. cava* and its major compound was polysaccharide for the enhancement of survival and proliferation of splenocytes against ionizing radiation.

Natural plant extracts have been gaining importance as radio protective agents due to lesser side effects than other agents. There is a lack of understanding about how FEP protects against DNA damage caused by irradiation. This study determined whether the cytoprotective effects of FEP on splenocytes could be related to DNA repair enhancement property. Normally, exposure to ionizing radiation induces undesirable DNA damage, especially in replicating cells such as immune cells. In this study, FEP led to cellular protective effects by inhibiting apoptosis phenomenon such as the radiation-induced apoptotic cell fraction and DNA damages. Generally, many genes are associated with the regulation of apoptosis under physiological and pathological conditions. Also, previous studies have documented that several natural products protected cells or mice against apoptosis caused by exposure of gamma ray irradiation via modulating apoptosis-related molecules (Park et al., 2008a,b,c, 2010, Kang et al., 2006a, Bing et al., 2010). Generally, p53-dependent pathway participating p53 and Bax, two pro-apoptotic molecules and Bcl-2, an anti-apoptotic molecule plays as important role in apoptosis. The gene of p53 is an upstream regulator of the Bax gene, because it can directly activate Bax and the process causes apoptosis in cells. At these moments, identifying the biological mechanism related with p53, Bax and Bcl-2 in cells undergoing apoptosis is so important. Indeed, it has been shown that synthetic and natural radioprotective agents, such as amifostine, eckol, dieckol and aloe polysaccharides, inhibit radiation-induced apoptosis by modulation of the p53-dependent pathway (Matsuu-Matsuyama et al., 2006; Park et al., 2008a,b, 2009, and 2010; Wang et al., 2005). This present study also revealed that treatment of FEP inhibited DNA damage by apoptosis via increasing

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the expression levels of Bcl-2 and Bcl-xL or decreasing those of Bax and p53 in cells exposed by ionizing radiation. Hence this study speculates that FEP blocks splenocytes, a radio sensitive cell from undergoing apoptosis by modulating p53-dependent pathway after exposure of ionizing radiation.

In conclusion, these results suggest that the polysaccharide isolated from viscozyme extract of *Lactobacillus brevis*-fermented *E. cava* (FEP) has radio protective effects by enhancing the proliferation of cells and inhibiting apoptosis via modulating p53-dependent pathway. Also, for a non-toxic radio protective agent, further studies are required to elucidate the radio protective effect of FEP *in vivo.*

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가장 먼저, 학부를 마치고 방황하던 저를 바로 잡아주시고 큰 꿈을 선물해 주신 전유진 교수님께 진심으로 감사 드리고, 사랑합니다. 또한 바쁘신 중에도 관심과 조언으로 언제나 힘을 주시고, 이번에 부족한 이 논문을 심사해 주신 이기완 교수님, 늘 할아버지 같이 챙겨주시고 아껴주신 김수현 교수님, 항상 밝은 모습으로 열정적으로 지도해주신 허문수 교수님, 어려운 순간마다 격려와 조언을 해주신 송춘복 교수님, 시크하시면서 따듯하신 여인규 교수님, 면역 분야에 눈을 뜨게 해주신 김기영 교수님, 고등학교 대선배님 이시며, 인생의 대선배님이신 최광식 교수님, 큰형처럼 친근하게 감싸주시는 정준범 교수님, 남들 앞에 서면 떨던 저를 바꾸어주신 이경준 교수님, 자신보다 학생들을 먼저 챙겨주시는 이제희 교수님, 언제나 많은 관심과 배움을 주신 이영돈 교수님 정말 감사하고 감사합니다.

연구실 생활에서 항상 힘이 되어주고, 밀어주신 수진이형, 우리 영원한 미소천사 길남이형, 아닌 듯 늘 이것저것 다 챙겨주는 승홍이형, 도박의 신 성명이형, 고생 너무 많이 하는 실험실장 석천이형, 마음 여린 군기반장 선희누나, 친절한 긴내씨 긴내누나, 천사같은 자나카형, 진짜 중국인인지 의심되는 수동이형, 알콜매니아 아름다슬, 파괴신 상원, 실험실 얼짱 민철, 친해지길 바래 주영, 카사노바 혜미, 요요현상 재영, 귀염둥이 막내 나래 모두모두 감사하고, 고맙고, 사랑합니다.

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