BIOACTIVITIES OF CRUDE PEPTIDE EXTRACTS OF HANWOO *M. LONGISSIMUS*

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***Abstract*—This study was performed to select the most efficient enzyme for the production of bioactive peptides and to search bioactivities of crude peptide extracts produced by the injected enzymes, such as protease type XIII and thermolysin. From the enzymatic proteolysates extracted from prepared Hanwoo beef, large molecules with higher than 3,000 Da were removed by ultrafiltration. The filtrate was lyophilized and concentrated in distilled water to measure ACE inhibitory activity, antioxidative activity, and cancer cell anti-proliferation activity. The crude peptide extract E2 (extract from thermolysin injected Hanwoo beef) showed the highest ACE inhibitory activity with IC_{50} of 2.3 mg/ml and extracts E1 and E3 also showed higher ACE inhibitory activity (IC_{50} 11.9 mg/ml and 21.2 mg/ml, respectively) than that of control (1,370 mg/ml) ($P<0.05$). Though, E2 showed significantly high antioxidative activity (7.63 ± 0.28) than control (6.52 ± 0.26) ($P<0.05$), it didn't show strong antioxidative activities. Moreover, 400 and 800 mg/ml of E1 and E2 showed inhibition effect on the proliferations of HepG2 and HT29 cells by up to 55% ($P<0.05$).**

***Index Terms*—ACE inhibitory activity, antioxidative activity, cancer cell anti-proliferation activity, Hanwoo *M. longissimus*.**

I. INTRODUCTION

Since health-conscious consumers have made functional foods as the leading trends in the food industry, efforts have been taken in many countries to develop new functional foods and to establish regulations for functional foods (Arihara, 2004; Dentali, 2002; Eve, 2000; Hutt, 2000). Biologically active peptides are produced during protein hydrolysis by digestive enzymes such as trypsin, chymotrypsin or pepsin (Liepke, Zucht, Forsmman, and Standker, 2001). In addition to their production by protein hydrolysis in food stuffs during processing and digestion in the digestive tract, bioactive peptides may also be generated by controlled protein hydrolysis and these peptides, containing only a few amino acid residues, are able to cross the digestive epithelial barrier and reach the blood vessels, which allow them to reach peripheral organs and have beneficial effects for the organism (Yust et al., 2003). Numerous food components with such physiological functions have been isolated and characterized (Hasler, 1998). Especially, Jang and Lee (2005) and Jang, Jo, Chang and Lee (2008) had reported the ACE inhibitory activity and cancer cell proliferation inhibition activity of peptides derived from enzymatic hydrolysis of extracted Hanwoo myofibrilar and sarcoplasmic proteins. However, no study was preformed yet about degradation of muscle protein itself. This study is performed to select the enzyme which produce the bioactive peptide most effectively and search bioactivities of crude peptides extracts produced by tested enzymes by measuring ACE inhibitory activity, antioxidative activity, anticancer activity and immunomodulatory effect of selected enzymatic proteolysates of Hanwoo *M. longissimus*.

II. MATERIALS AND METHODS

A. Preparation of crude peptide extracts from Hanwoo *M. longissimus*

M. longissimus of Hanwoo were taken at Gangwon livestock processing center (Gangwon-do, Korea) from 28 to 30 months old castrated Hanwoo bulls raised in Gangwon-do and get grade 1 carcass grading scores by Korean beef grading standards at 24 hrs after slaughter. Based on the report of Jang and Lee (2005), Enzyme protease type XIII (P2143, Sigma, USA) and thermolysin (T7902, Sigma, USA) were injected separately or in combination as shown in Table 1 and store for 3 days in 5℃-chilling room for emzymatic proteolysis of muscle proteins. Beef peptide extracts were separated into a large molecular weight fraction and a low molecular weight fraction by ultrafiltration at 4 ℃ using PM-10 membrane (MWCO, 10,000; Amicon Co., Beverly, MA) and Ultracel 3K membrane (MWCO, 3,000; Amicon Co., Beverly, MA), subsequently lyophilized and measured the weight to calculate the yield. The lyophilized crude peptide extracts were kept in deep-freezer for further use in gel-filtration.

Table 1. Preparation of Hanwoo *M. Longissimus* with enzyme injection

Sample	Treatment
C	No enzyme
E1	100 ppm of protease type XIII
E2	80 ppm of thermolysin
E3	100 ppm of protease type XIII + 80 ppm of thermolysin

B. Cell culture

HeLa human negroid cervix epitheloid carcinoma cells, HepG2 human hepatocarcinoma cells and HT29 human colon adenocarcinoma cells were obtained from Korean Food Research Institute (Sungnam, Korea) and used to measure cancer cell anti-proliferation activity of crude extracts. HT29 cells were grown in Dulbecco's modified Eagle medium (DMEM, high glucose) containing 10% FBS, 100 μg/ml streptomycin, and 100 U of penicillin and HeLa and HepG2 cells were grown in RPMI 1640 with 10% FBS and 100 μg/ml streptomycin, and 100 U of penicillin.

C. Reagents

ABTS as diammonium salt, Angiotensin converting enzyme (ACE, from rabbit lung), hippuryl-L-histidyl-L-leucine (HHL), Culture-grade dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Vitamin C was from Mann Research Laboratories, Inc. (New York, USA). All other chemicals used were analytical grade (Fisher, Springfield, NJ). Media for cells, 100 μg/ml streptomycin, and 100 U/ml of penicillin were from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA).

D. Angiotensin I-converting enzyme (ACE) inhibitory activity

The determination of ACE inhibitory activity was performed by the spectrophotometric method described by Cushman and Cheung (1971). For each assay, 100 μl of hippuryl-L-histidyl-L-leucine (HHL, 12.5 mM in 0.05 M sodium borate buffer) were incubated at 37 ℃ for 5 min. After incubation, 50 μl of bovine peptide extracts and 150 μl of ACE (peptidyldipeptide hydrolase, from rabbit lung acetone extract) was added and the mixture incubated for another hour. The enzymatic reaction was stopped by adding 250 μl of 0.5 N HCl. The hippuric acid forced by the action of the angiotensin-converting enzyme on HHL was extracted from the acidified solution into 1 ml ethle acetate by vortex mixing for 15 s. This was centrifuged at 3290g for 10 min at 4 ℃, and a 0.7 ml aliquot of each ethyl acetate layer was transferred to clean tubes and evaporated by heating at 95 ℃ for 20 min on a water-bath. The hippuric acid was redissolved in 3 ml of 1 M NaCl, and the amount formed was determined by its absorbance at 228 nm. The IC_{50} value, defined as the concentration of a peptide that inhibits 50 % of the ACE activity, was determined by measuring the ACE inhibitory activity and peptide contents of each extracts after regression analysis.

$$\text{ACE inhibitory activity} = \left(1 - \frac{\text{S - S.C.}}{\text{B - B.C.}}\right) \times 100$$

S; O.D. value of sample, S.C.; O.D. value of sample control, B; O.D. value of blank, B.C.; O.D. value of blank control

E. Vitamin C equivalents antioxidant capacity (VCEAC) assay

Vitamin C standard curves that related the concentration of vitamin C and the absorbance of ABTS at 734 nm were obtained using the ABTS assay. The absorbance at 734 nm of four crude peptide extracts was also measured by ABTS assay. The calculation of VCEAC of each crude extract was made using vitamin C standard curve (not shown). The absorbance of four crude peptide extracts was also related to that of vitamin C standard, and the result was calculated as vitamin C equivalents in mg/100ml.

F. Cell viability

Cell viability was assessed using 3-(4-5-dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT) staining as per manufacturer instructions (Roche). Cells (HeLa, HepG2 and HT29) were seeded in 96 well plates at a density of 1×10^4 cells/well and incubated in the CO_2 incubator at 5% CO_2 and 37℃ for 24 h to make the cells adhere to the inner wall of the flask. Subsequently, 150 μl/well of media mixed with sample as concentrations of 50, 200, 200, 400, and 800 μg/ml, respectively. The treated cells were then incubated in the incubator for 24 and 48 h. After incubation, cells were taken out, the culture medium was discarded, and 1 mg/ml MTT was added at 100 μl/well, after which the cells were incubated for 2 h more. After 2 h, the liquid was discarded, DMSO was added at 100 μl/well, after which the samples were mounted on the micromixer for 5 min to make the blue granules in the samples dissolve throughly. The culture plate was then placed on the microplate reader, and OD was measured at 570 nm.

The inhibition rate was calculated according to the following formula: inhibition rate (%) = (1 - average absorbance for treated group / average absorbance for control group) × 100

G. Statistical analysis

Whole experiments were replicated 3 times with 2 observations per each replication. Statistical analysis was performed with the SAS program for Window V9.1 (SAS Institute, Cary, NC, USA). General linear model (GLM) with Duncan's multiple range test was carried out to analyze the significant differences among the treatments ($P<0.05$).

III. RESULTS AND DISCUSSION

A. Yield and angiotensin I-converting enzyme (ACE) inhibitory activity of crude peptide extracts

The yield of crude peptide extracts derived from prepared Hanwoo beef was measured (Table 2). The yield of crude peptide extracts from thermolysin injected Hanwoo (E2, 3.98±0.06%) and both protease type XIII and thermolysin injected Hanwoo (E3, 3.94±0.11%) were significantly higher than that of protease type XIII injected Hanwoo (E1, 2.11±0.22%) and no enzyme injected Hanwoo (C, 1.85±0.08%) ($P<0.05$). This result may caused by the difference of substrate specificity of protease type XIII and thermolysin as Hanwoo myofibrillar and sarcoplasmic proteins (Jang and Lee, 2005). The angiotensin I-converting enzyme (ACE) inhibitory activity (IC_{50}) of crude peptide extracts derived from enzyme injected Hanwoo was measured (Table 2.2). The crude peptide extracts from E2 showed the highest ACE inhibitory activity (the lowest IC_{50} of 2.3 mg/ml). The crude peptide extracts from E1 and E3 showed 11.9 and 21.2 mg/ml, respectively. These results are different to the previous report of Jang and Lee (2005), who showed the proteolysates of enzyme mixture showed higher ACE inhibitory activities than single enzyme proteolysates. This may caused by the difference of storage temperature and time.

Table 2. The yield and angiotensin I-converting enzyme (ACE) inhibitory activity of crude peptide extracts

Extracts*	Yields (%)	IC_{50} (mg/ml)
C	1.85 ± 0.08^{c}	$1{,}370^{c}$
E1	2.11 ± 0.22^{b}	11.9^{b}
E2	3.98 ± 0.06^{a}	2.3^{a}
E3	3.94 ± 0.11^{a}	21.2^{b}

*C; extracts of no enzyme injected Hanwoo beef, E1; extracts of protease type XIII injected Hanwoo beef, E2; extracts of thermolysin injected Hanwoo beef, E3; extracts of protease type XIII and thermolysin injected Hanwoo beef.

[a-c]Means±S.D. in same column with different superscripts are significantly different ($p<0.05$).

B. Vitamin C equivalents antioxidant capacity (VCEAC) of crude peptide extracts

AAPH, a thermolabile water-soluble radical initiator, oxidized $ABTS^{2-}$ to ABTS radical anion (Scott, Chen, Bakac, and Espenson, 1993). The generation of the ABTS radical anion before the addition of antioxidants was reported to eliminated the interference of compounds affecting radical production (van den Berg, Haenen, van den Berg, and Bast, 1995). The reduction of ABTS radical chromogen by an antioxidant decreases absorbance at 734 nm. The calibration curve showed a linear relationship ($R^2=0.9999$) between vitamin C concentration and absorbance reduction at 734 nm (not shown).

The antioxidative activity of crude peptide extracts was measured by vitamin C equivalents antioxidant capacity (VCEAC) assay (Table 3). E2 had significantly higher VCEAC (7.63 ± 0.28, $P<0.05$), however, E1 (5.83 ± 0.16) and E3 (5.33 ± 0.37) had significantly lower VCEACs than that of control (C, 6.52 ± 0.26). Though there were significant differences between treatments, all VCEACs were too low to consider they have antioxidative activity.

Table 3. Vitamin C equivalents antioxidant capacity (VCEAC) of crude peptide extracts

Sample	VCEAC (mg/100ml)
C	6.52 ± 0.26^{b}
E1	5.83 ± 0.16^{c}
E2	7.63 ± 0.28^{a}
E3	5.33 ± 0.37^{c}

*C; extracts of no enzyme injected Hanwoo beef, E1; extracts of protease type XIII injected Hanwoo beef, E2; extracts of thermolysin injected Hanwoo beef, E3; extracts of protease type XIII and thermolysin injected Hanwoo beef.

[a-c]Means±S.D. in same column with different superscripts are significantly different ($p<0.05$).

C. Effect of crude peptide extracts on cell viability of several human cancer cell-lines

Fig. 1. a to c show the viabilities of cancer cell-lines. Cell viabilities of tested three human cancer cell-lines were decreased as time and dose dependant manner. Especially, 48 h after incubation, viabilities of HepG2 human hepatocarcinoma cells and HT29 human colon adenocarcinoma cells incubated with E1 and E2 at the concentration of 400 and 800 μg/ml were significantly decreased by up to 55% than that of not treated cells (N) ($P<0.05$). Moreover, although there was no significance, 400 and 800 μg/ml of E1 and E2 showed decreasing tendency on HeLa cervix epitheloid carcinoma cells. Consequently, E1 and E2 were considered as possessing cancer cell proliferation inhibition activities on HepG2 and HT29 cells.

Jang, Jo, Kang and Lee (2008) reported that the peptide PKb325 derived from enzymatic proteolysates of Hanwoo muscle protein shows linear decrease of cell viability with dose dependant manner on MCF-7 (human breast adenocarcinoma) and AGS (human stomach adenocarcinoma) cells.

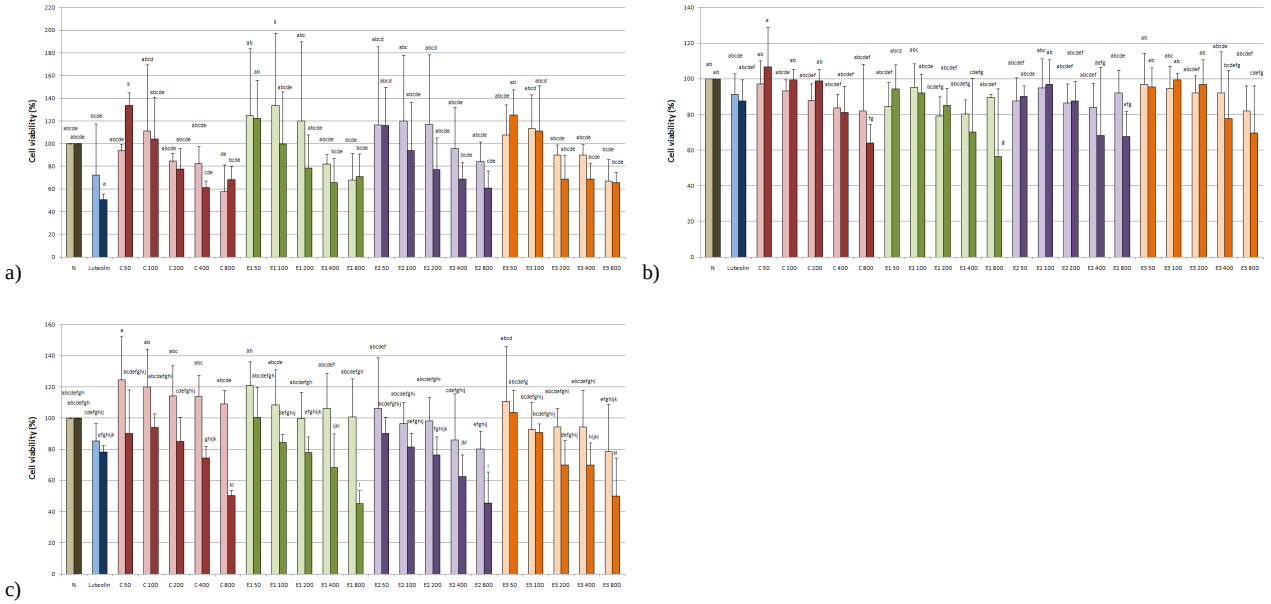

Fig. 1. Viability of human cancer cell-lines after 24 and 48h of incubation with several crude peptide extracts. a; HeLa, b; HepG2, c; HT29, C; extractcs from no enzyme injected Hanwoo beef, E1; extracts from protease type XIII injected Hanwoo beef, E2; extracts from thermolysin injected Hanwoo beef, E3; extracts from protease type XIII and thermolysin injected Hanwoo beef.

IV. CONCLUSION

From those results, thermolysin was considered the most effective enzyme to generate bioactive peptides from Hanwoo muscular proteins and the hydrolysates showed several bioactivities, such as ACE inhibitory and cancer cell anti-proliferation activity. However, there were still remained various kinds of proteases to be tested and also further isolation of crude peptide and purification of active fraction may need to identifty bioactive peptides responsible for those bioactivities.

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