# BIOLOGICAL ACTIVITIES OF ACE INHIBITORY PEPTIDES DERIVED FROM BEEF HYDROLYSATES

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## Background

Recently, the interest in the composition of food has been increased because potential anti-carcinogens and other therapeutic agents of food have been reported (Messina et al, 1994; Gibbs et al., 2004). Of particular interests in nutrition and food science are bioactive peptides that are present in the amino acid sequence of food proteins. These peptides are inactive within the sequence of the parent proteins but can be released by enzymatic proteolysis, for example, during gastrointestinal digestion or during food processing. Also, they are produced partly by the hydrolytic action of commercial enzymes (Gibbs et al., 2004). Once they are liberated in the body, the peptides may act like regulatory compounds. Thus, these peptides could be the potential health enhancing nutraceuticals for food and pharmaceutical applications. (Meisel, H.1997). From the nutritional point of view, meat contains many valuable components and biologically active substances (Korhonen et al., 1998). Arihara et al.(2001) reported that ACE inhibitory peptides were purified from porcine skeletal muscle proteins. However, very few studies have been done on beef protein (Jang et al., 2000). In this respect, we separated ACE inhibitory peptides from beef protein hydrolysates by enzyme hydrolysis and suspected that these peptides may have other biological activities.

## Objectives

The aim of this study was to separate ACE inhibitory peptides from beef protein hydrolysates using seven commercial enzymes and investigate whether these ACE inhibition active peptides have any other biological activities, such as cytotoxic activity, antimicrobial activity, and machrophage stimulating activity.

## Methods

Protein digestion was carried out with 7 enzymes, thermolysin at pH7.5 and 37°C, proteinase K at pH7.5 and 37°C, pepsin at pH3.0 and 37°C, protease at pH7.5 and 37°C, trypsin at pH 7.6-8.0 and 25°C, tyrosinase at pH6.5 and 25°C, and papain at pH6.2 and 25°C for 8 hours. The enzymatic hydrolysates were purified further with ultrafiltration and gel filtration. Of each hydrolysate, the fraction with the highest inhibitory activity was selected and identified by RP-HPLC. The amino acid sequence of those peptides was revealed by protein sequencer and the biological activities were determined. The ACE inhibitory activity was measured by a method described in our previous report ( Jang et al.,2001). Antimicrobial activity was examined against six pathogens, *Escherichia coli, Staphylococuus aureus, Salmonella thyphimurium, Pseudomonas aeruginosa, Bacilus cereus,* and *Listeria monocytogenes* with tryptic soy agar (Difco. U.S.A). The sensitivity of pathogen to the peptides was determined by the disk diffusion method (Ponce et al., 2003) The diameter of the inhibition halos were as: not detected for diameters less than 8 mm; sensitive (+) for diameters 9-12 mm; very sensitive (++) for diameters 13-18 mm and extremely sensitive (+++) for diameters larger than 19mm. The cytotoxic effect of the peptides was assayed in vitro against MCF-7(breast cancer cell), AGS(stomach cancer cell), and LLC(lung cancer cell) by using the MTT assay (Carystinos, et al.,2001). Percent cell viability of test samples was determined as : % Cell Viability = (average OD for test group/average OD for control group) x 100. To acquire tumor cytotoxicity, machrophages should be activated by stimulants such as lipopolysaccharide. The activated macrophage secreted tumor necrosis factor, interleukin-1 (IL-1), and nitric oxide as soluble effectors. In this respect, the nitrite formation was taken as an index of the macrophage (RAW 264.7) stimulating activity (Miwa et al., 1990). Nitric oxide production was estimated by measuring the accumulation of the stable NO metabolite, nitrite, in the culture supernatants using the Griess assay (Green et al., 1982).

## Results and discussion

Four peptides were separated from the hydrolysates for 8hrs incubation with 7 enzymes. The ACE inhibition activities of the separated peptides 1155, 325, 1152, 714, and 1134 were 14.64%, 34.77%, 25.09%, 31.66%, and 53.36%, respectively (Tab. 1). Their $IC_{50}$ values were 117.27, 64.27, 52.92, 50.35, and 75ug/ml. The peptide 714 showed that the smallest concentration needed to inhibit 50% of ACE. Although the structure-activity relationships of many bioactive peptides have not yet been fully established, peptides with a defined bioactivity are known to have common structural features. Structure activity correlations among different peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide

sequence of the substrate.(Meisel, H.1997). These peptides characterized were studied for biological activity. A wide variety of organisms produce antimicrobial peptides as a primary innate immune strategy. Typically, peptides that are relatively short(less than 100 amino acids), positively charged, and amphiphillic acid are reported to be active against bacteria, fungi, viruses and protozoa(Farnaud, et al., 2004).
Table. 2 shows the effects of peptide 1155 on 6 pathogens. All the treated concentrations of peptide 1155 showed a sensitive inhibition on *S.typh., B.cer., E.coli, and L.mono* but not *S.aureus and P.aeru*. Peptide 325 showed very sensitive inhibition activity, especially on *E.coli* and *P.aeru* with concentration of 200 and 400ug/ml (Tab.3). However, peptide 1152 was very sensitive to *P.aeru*., only with 200ug/ml and 400ug/ml. Peptide 714 showed sensitive activity to *E.coli* at all treated concentrations(Tab.4). From this result, small peptides (3-4 amino acids), 1152 and 325 in particular, resulted in very sensitive activity to *P.aeru.* but the reason was not clear. Bellamy et al.(1993) suggested that the antimicrobial activity of lactoferricin and synthetic analogs seems to be correlated with the net positive charge of the peptides. These cationic peptides kill sensitive microorganisms by increasing cell membrane permeability.
The cytotoxic effect of separated peptides on breast cancer, lung cancer, and stomach cancer was measured by MTT assay. Peptide 325 showed a slight decrease of MCF-7 cell viability dose dependently( Fig. 1). The peptide 325 reduced cell viability by 85% at 400ug/ml of concentration. However, the rest of peptides showed no significant decrease of cell viability. Fig. 2. shows the effect of separated peptides on stomach cancer(AGS) cell. When 400ug/ml of peptide 325 and peptide 1155 were added into the AGS cell, the viability was decreased by 75% and 25%. The peptide 1155 showed significant reduction by up to 20%. However, peptide 714 seemed to act as a nutrient to AGS cell where it increased viability of AGS cell. Also, peptide 325 showed 4% reduction, while the other peptides had no cytotoxic effect on LLC(Fig. 3). Peptide 325 inhibited viability of those 3 tumor cells. Thus, this peptide was selected to investigate NO production by stimulation of machrophage. To investigate machrophage stimulation activity, 100, 300, and 1000ug/ml of peptide 325 were treated at machrophage. However, nitric oxide was not produced in all treatments(data not shown). Miwa et al.(1997) examined if nitric oxide formation by macrophage was stimulated with water extracts from meats and offal. They suggested that a water extract of beef didn't produce nitric oxide. Low concentrations of NO from activated macrophages are beneficial as, along with other reactive nitrogen intermediates, they are responsible for cytostatic and cytotoxic activity against infectious organisms and tumor cells (Komutarin, et al., 2004). However, overproduction of NO has been found to be associated with various disease such as septic shock, autoimmune disease, chronic inflammation by increasing vascular permeability, and the extravasations of fluid and proteins at the inflammatory site (Moncada, et al., 1991; Komutarin, et al., 2004).

## Conclusions

ACE inhibitory peptides Separated from beef hydrolysates by enzymes were found to have cytotoxic activity on tumor cells and antimicrobial activity on 6 pathogens while macrophage stimulating activity was not shown.

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Tab. 1. ACE inhibition activity and $IC_{50}$ value of separated peptides

| Peptide | 1155 | 325 | 1152 | 714 | 1134 |
|---|---|---|---|---|---|
| ACE inhibition activity(%) | 14.64 | 34.77 | 25.09 | 31.66 | 53.36 |
| $IC_{50}$ (ug/ml) | 117.27 | 64.27 | 52.92 | 50.53 | 75.0 |

Tab.2. Antimicrobial activity of peptide 1155 (octomer)

| | *S. typh.* | *B. cer.* | *E. coli* | *L. mon.* | *S. aureus* | *P. aeru.* |
|---|---|---|---|---|---|---|
| 100 | + | + | + | + | ND | ND |
| 200 | + | + | + | + | ND | ND |
| 400 | + | + | + | + | ND | ND |
| DMSO | ND | ND | ND | ND | ND | ND |
| 2% CA* | ++ | +++ | ++ | ++ | ND | +++ |

Tab. 3. Antimicrobial activity of peptide 325 (tetramer)

| | *S. typh.* | *B. cer.* | *E. coli* | *L. mon.* | *S. aureus* | *P. aeru.* |
|---|---|---|---|---|---|---|
| 100 | ND | ND | + | ND | ND | ++ |
| 200 | ND | ND | ++ | ND | ND | ++ |
| 400 | ND | ND | ++ | ND | ND | ++ |
| DMSO | ND | ND | ND | ND | ND | ND |
| 2% CA* | ++ | ++ | + | ND | ND | ++ |

Tab. 4. Antimicrobial activity of peptide 1152 (trimer)

| | *S. typh.* | *B. cer.* | *E. coli* | *L. mon.* | *S. aureus* | *P. aeru.* |
|---|---|---|---|---|---|---|
| 100 | ND | ND | ND | ND | ND | + |
| 200 | ND | ND | ND | ND | ND | ++ |
| 400 | ND | ND | ND | ND | ND | ++ |
| DMSO | ND | ND | ND | ND | ND | ND |
| 2% CA* | ++ | ++ | + | ND | ND | ++ |

Tab. 5. Antimicrobial activity of peptide 714 (hexamer)

| | *S. typh.* | *B. cer.* | *E. coli* | *L. mon.* | *S. aureus* | *P. aeru.* |
|---|---|---|---|---|---|---|
| 100 | ND | ND | + | ND | ND | ND |
| 200 | ND | ND | + | ND | ND | ND |
| 400 | ND | + | + | ND | ND | + |
| DMSO | ND | ND | ND | ND | ND | ND |
| 2% CA* | ++ | ++ | + | ND | ND | ++ |

*2% CA: citric acid, +++: extremely sensitive, ++:very sensitive, +: sensitive, ND: not detected

Fig. 1. Cytotoxicity of peptides on MCF-7 cell

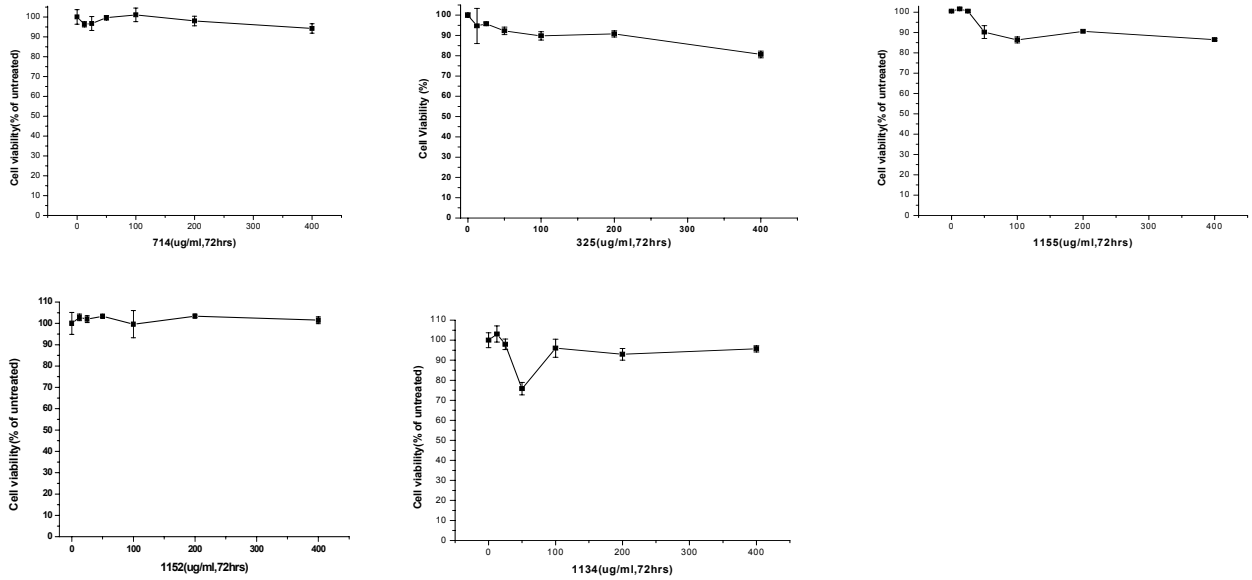


Fig.2. Cytotoxicity of peptides on AGS cell

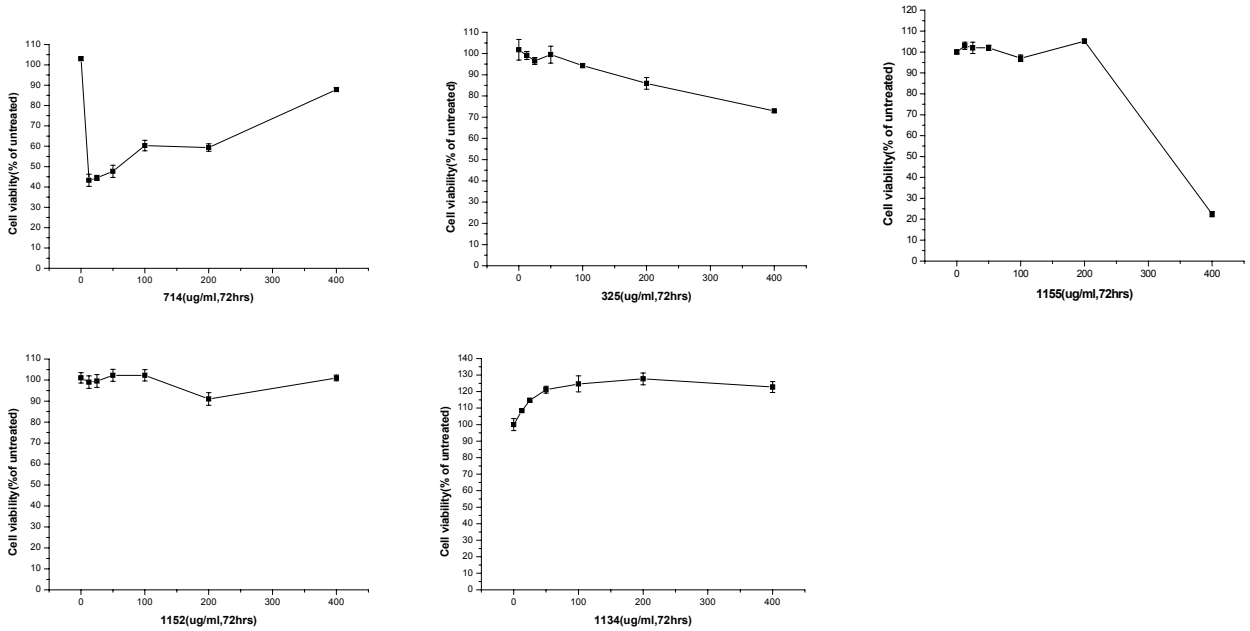
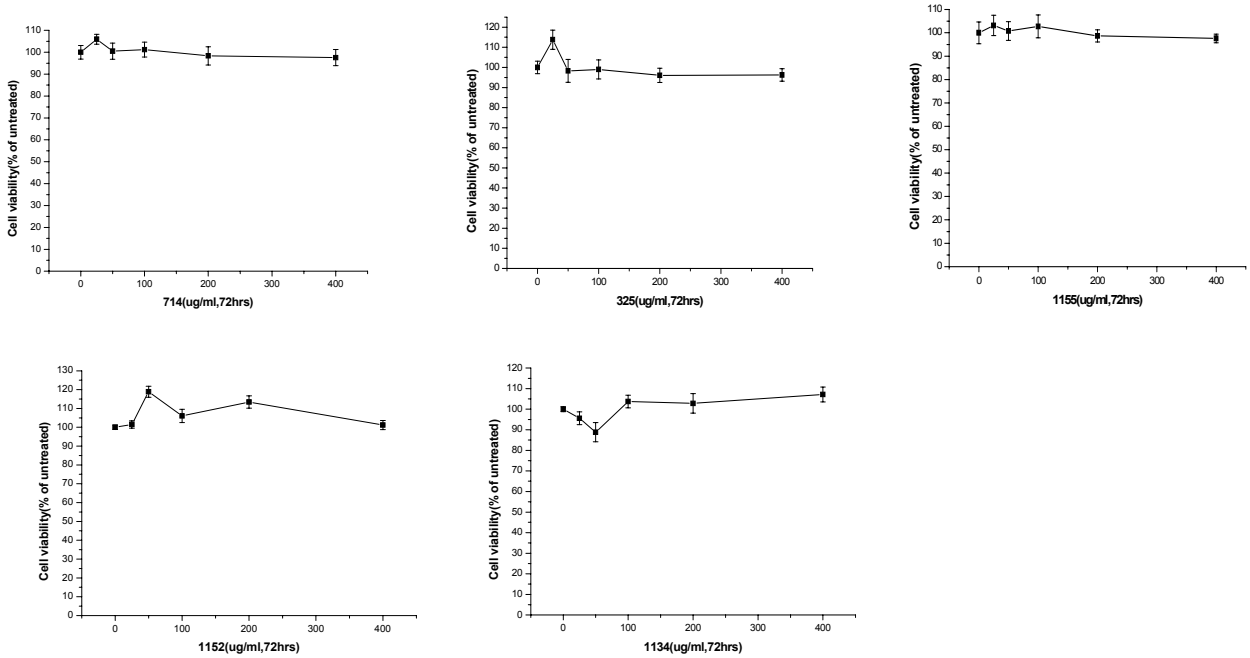



Fig. 3. Cytotoxicity of peptides on LLC cell




* Peptide 1155( octomer), 325(tetramer), 1152(trimer), 714(hexamer), 1134(hexamer)