DOI:10.1021/jf901787e

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Purification, Activity and Sequence of Angiotensin I Converting Enzyme Inhibitory Peptide from Alcalase Hydrolysate of Peanut Flour

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Peanut hydrolysate obtained after 6 h of digestion by Alcalase was used to isolate angiotensin I converting enzyme (ACE) inhibitory peptides. After centrifugation and ultrafiltration through a 0.2 μm nylon filter, the hydrolysate was filtered through the polyethersulfone membrane with a molecular weight cutoff (MWCO) of 10 kDa. The resulting permeate was then separated by primary reverse-phase high performance liquid chromatography (RP-HPLC). Eluate was divided into six major fractions according to eluation time. The fraction with eluting time 50−60 min showed the most potent ACE inhibition and was subjected to further purification by the secondary RP-HPLC. Four peaks were found to have strong ACE inhibitory activities, and their IC_{50} values were determined. Peptide mass for the most potent peak was obtained by matrix-assisted laser desorption and ionization (MALDI), and sequence was determined by MALDI tandem TOF-TOF (time-of-flight) mass spectrometer (MS/MS) to be Lys-Ala-Phe-Arg.

KEYWORDS: Angiotensin I converting enzyme; peanut protein; ACE inhibitory peptides

INTRODUCTION

In living organisms, endogenous peptides usually function through hormone−receptor interactions and signaling cascades. Bioactive peptides from food proteins also exert hormone-like regulatory activities in the human organism (*1*). Among them, those with antihypertensive effect have been extensively studied for different food proteins. It has been shown that short peptide sequences are potentially potent inhibitors of angiotensin I converting enzyme (ACE; EC 3.4.15.1), a dipeptidyl carboxypeptidase with a major role in the regulation of blood pressure (*2*).

ACE inhibitory peptides are usually separated from a hydrolysate mixture by various kinds of membrane-based separations and chromatography techniques. It has been reported that permeates of potato liquid fraction (*3*) and soy protein hydrolysates (*4*, *5*) from a 10 kDa membrane had no significant difference in ACE inhibitory activities when compared to permeates from studied membranes with smaller molecular weight cutoffs (MWCOs), and therefore were selected for further purification. Reverse-phase high performance liquid chromatography (RP-HPLC) separates compounds according to their hydrophobic character and has been the most widely used to isolate ACE inhibitory fractions and peptides. Mass spectrometric techniques can provides molecular mass and amino acid sequence information. They are also effective in confirming the purity of peptides.

Peanut (*Arachis hypogeae*) is an important source of arginine-rich dietary protein as well as vitamins, fiber and other nutrients. Previous research has shown peanut protein to be a potential source of ACE inhibitory peptides (*6*). A recent study showed that ACE inhibition began to plateau after lightly roasted peanut (55.8% protein) was hydrolyzed with Alcalase for 2.5 h with hydrolysis temperature 60 °C, enzyme to substrate ratio 0.3 AU/g flour, pH 7.5 and substrate concentrate 1.25% (w/v), and inhibitory activity remained constant thereafter. It has been proposed that ACE inhibitory peptides from the prolonged hydrolysis by Alcalase, an enzyme with rather broad specificity, might be resistant to digestive enzyme and thus might be allowed for absorption in the GI tract (*7*). In this study a 6 h Alcalase hydrolysate of peanut flour was used as starting material for further purification. The aim of this study was therefore to purify ACE inhibitory peptides from this source by membrane filtration and RP-HPLC and sequence them by mass spectrometry.

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MATERIALS AND METHODS

Materials. ACE reagent *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) was purchased from Trinity Biotech (Wicklow, Ireland). Angiotensin converting enzyme (ACE, EC 3.4.15.1) from rabbit lung was purchased from Sigma-Aldrich (St. Louis, MO).

Lightly roasted peanut (*Arachis hypogaea* L.) flour purchased from Golden Peanut Company (Alpharetta, GA) was defatted by a series of extractions with hexane at room temperature, followed by grinding using a coffee bean grinder (Series CBG5, Black & Decker Co., Towson, MD). Nitrogen content was determined using a Leco FP2000 (model 602-600, LECO Co., Warrendale, PA), and protein was calculated to be 55.8% ($N \times 5.46$).

Microplate Kinetic Assay of ACE Inhibitory Activity. ACE reagent was reconstituted with 5 mL of deionized water. A 125 μL volume

 Published on Web 10/13/2009

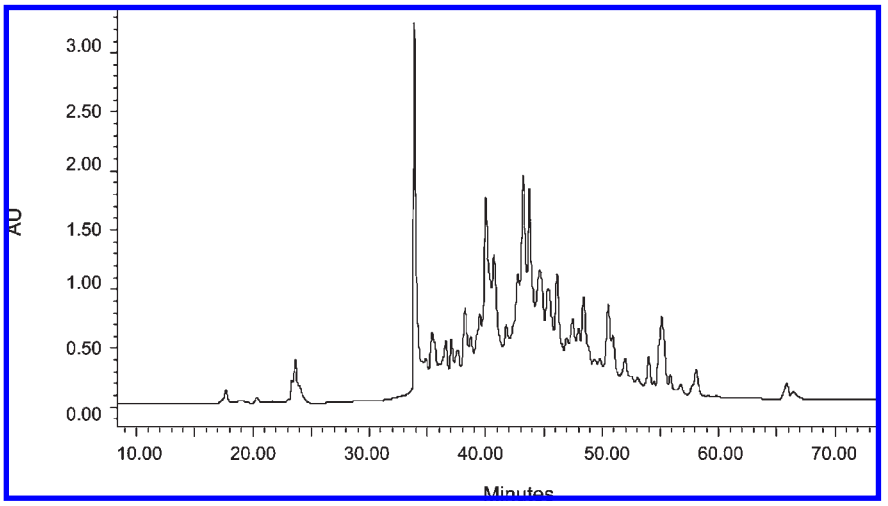

Figure 1. RP-HPLC profile of the 10 kDa membrane permeate of peanut hydrolysate at 215 nm.

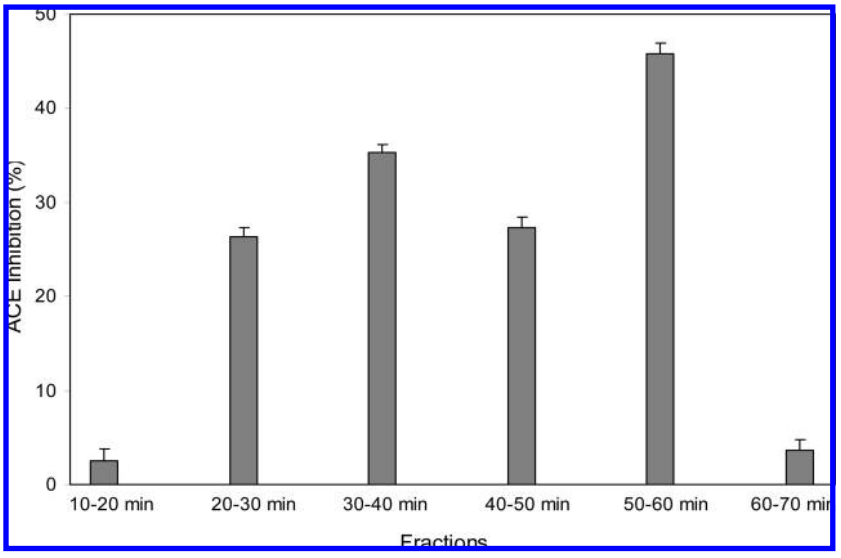

Figure 2. ACE inhibitions of six major fractions after primary RP-HPLC. ACE inhibitions (%) were measured at peptide concentration 45.5 μg/mL. Bars represent the average of three assays.

of the resulting substrate was added to designated microwells of a 96-well Costar microplate (Corning, NY), along with 125 μL of deionized water (control) or inhibitors; the plate was covered by a lid, and preincubated at 37 °C in a FLUOstar Optima microplate reader (BMG LABTECH, Durham, NC) for 10 min. A 250 μL aliquot of deionized water was used as blank. After incubation, the lid was removed and the operation was initiated using OPTIMA 2.10R3 (BMG LABTECH) control software. A 25 μL aliquot of ACE (0.005 units; 18.18 units/L reaction mixture) was injected automatically by the onboard injector with the help of the designated pump to each microwell to start reactions. Absorbance was recorded every minute for 15 min.

The ACE inhibition (%) was calculated as follows:

$$\text{ACE inhibition } (\%) = 100 - 100 \times S_{\text{inhibitor}}/S_{\text{control}}$$

where $S_{\text{inhibitor}}$ was the slope of kinetic curve in the presence of inhibitors, and S_{control} was the slope without inhibitors (*8*).

A plot of log{[100 − ACE inhibition (%)]/[ACE inhibition (%)]} versus log(peptide concentration, μg/mL) was generated using different concentrations of samples. The IC_{50} value was expressed in terms of μg/mL, defined as the concentration of inhibitor which gave 50% ACE inhibition, and calculated using the linear regression equation of the curve. Spectrophotometric assay to determine peptide concentrations was performed on a Thermo Spectronic spectrophotometer (Genesys 6, Thermo Electron Co., Madison, WI) at 215 nm and 225 nm. Peptide concentration (μg/mL) was calculated from UV absorbance of properly diluted solutions using the formula

$$\text{concentration} = (\text{A215} - \text{A225}) \times 144$$

Purification of ACE Inhibitory Peptides. The defatted peanut flour was hydrolyzed at the following parameters: substrate concentration 1.25%; enzyme/substrate ratio 0.3 AU/g peanut flour; pH 7.5; temperature 60 °C; hydrolysis time 6 h. The reacting mixture was preincubated in a water bath for 20 min, and then Alcalase was added. During reaction, pH was monitored and maintained by adding drops of 1 N NaOH. Hydrolysis was terminated by boiling for 15 min. The resulting hydrolysate was centrifuged at 27000*g* for 15 min at 10 °C and then filtered through a 0.2 μm Millipore nylon filter (Billerica, MA). After filtration through the Biomax polyethersulfone membrane (Millipore Co.) with a MWCO of 10 kDa in a Labscale TFF System (Millipore Co.), the resulting permeate (1000 μL) was injected and separated by the primary RP-HPLC system composed of a Jupiter 4u Proteo 90A C12 preparative reversed-phase column (250 × 21.10 mm, Phenomenex, Inc., Torrance, CA), a Waters 2690 Separations Module and a Waters 996 photodiode array detector (Waters Co., Milford, MA) recording absorbance from 210 to 300 nm. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in filtrated deionized water, and solvent B was 0.1% (v/v) TFA in acetonitrile. A linear gradient from 0% to 75% of solvent B within 75 min was applied at a flow rate of 3 mL/min. Eluting peaks were collected as six major fractions using

a Waters Fraction Collector III (Waters Co.), pooled and lyophilized using a Genesis SQ freeze-dryer (VirTis Co., Gardiner, NY) with a dry ice/isopropanol secondary trap to capture any acetonitrile escaping the primary trap in the vacuum line. The fraction with the highest ACE inhibitory activity (100 μL) was injected and further isolated by the secondary RP-HPLC system with a Jupiter 4u Proteo 90A C12 column (250 × 10 mm, Phenomenex, Inc.). Elution was achieved by a linear gradient from 35% to 42.5% solvent B within 30 min at a flow rate of 1 mL/min. Individual peaks were collected, pooled and lyophilized.

Mass Spectrometry and Sequences of ACE Inhibitory Peptides. The molecular mass of the purified ACE inhibitory peptide was determined using an Autoflex matrix-assisted laser desorption and ionization time-of-flight mass spectrometer (MALDI-TOF, Bruker Daltonics Inc., Billerica, MA). The lyophilized sample was first dissolved in 50% acetonitrile containing 0.1% TFA. One microliter of the sample solution was then mixed with 1 μL of matrix solution (α-cyano-4-hydroxycinnamic acid saturated in the mixture of acetonitrile and 0.1% TFA solution in a volume ratio of 1:1) and let dry on a stainless steel MALDI target. The dried sample was introduced into the mass spectrometer where a nitrogen laser (337 nm wavelength) was fired to desorb and ionize the sample. The time-of-flight analyzer separated ions according to their m/z ratios by measuring the time it took for ions to travel through a field free region. The m/z ratio of the mass spectrometer was calibrated with angiotensin II digested by trypsin in external and internal calibration modes. Peptide sequence was determined by a MALDI tandem TOF−TOF mass spectrometer (MS/MS). Sample preparation was the same as that for molecular mass determination using MALDI-TOF. Samples were measured in the reflection and positive ionization mode. The tandem mass spectrum was acquired by an Applied Biosystems 4700 Proteomics Analyzer (Foster City, CA).

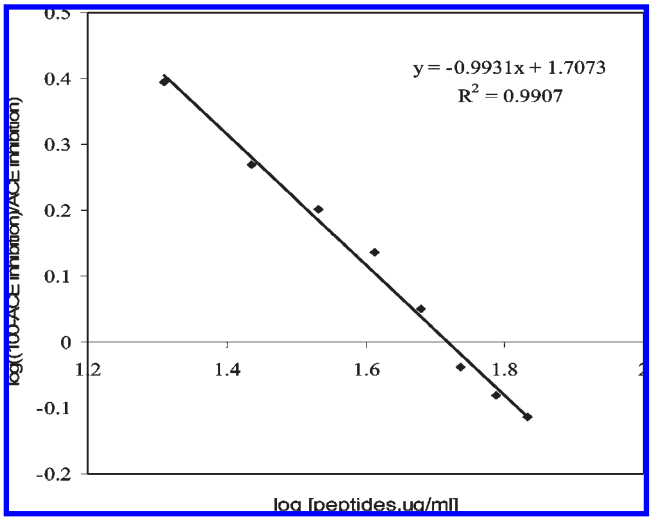

Figure 3. Determination of IC_{50} value for 50−60 min peanut fraction.

RESULTS AND DISCUSSION

Purification and Activity of ACE Inhibitory Peptides from Alcalase Hydolysate of Peanut Flour. As previously reported (*8*), ACE inhibition began to plateau after peanut was hydrolyzed for 2.5 h. The hydrolysate obtained after 6 h of digestion by Alcalase was used to isolate ACE inhibitory peptides. After centrifugation and ultrafiltration through a 0.2 μm Millipore nylon filter, hydrolysate had an IC_{50} value of 134.4 μg/mL, which is lower than that for Alcalase hydrolysates of defatted wheat germ (670 μg/mL) (*9*), isolated soy protein (668 μg/mL) (*5*), mung bean protein isolate (640 μg/mL) (*10*), defatted soy meal (340 μg/mL) (*4*), corn gluten (197 μg/mL) (*11*), and rice protein (140 μg/mL) (*12*), but is higher than that for Alcalase hydrolysates of potato protein (18−86 μg/mL) (*3*). Therefore, peanut protein hydrolysates obtained by Alcalase digestion may be considered as functional foods.

The hydrolysate was filtered through a polyethersulfone membrane with a MWCO of 10 kDa. The resulting permeate had an IC_{50} value of 85.8 μg/mL, which is lower than that for a 10 kDa soy permeate of Alcalase hydrolysate (180 mg/mL) (*4*) but higher than that for another 10 kDa soy permeate of Alcalase hydrolysate (78 mg/mL) (*5*).

The resulting permeate was then separated by primary RP-HPLC (**Figure 1**). The eluate was divided into six major fractions according to eluation time. The eluate with eluting time 50−60 min was found to have the most potent ACE inhibition (**Figure 2**) with the IC_{50} value being 52.4 μg/mL (**Figure 3**) and was used for further purification. It should be pointed out that all other fractions also show ACE inhibitory activities though their capabilities are lower than that of the 50−60 min fraction. After isolation by secondary RP-HPLC, four peaks were found to have the strong ACE inhibition (**Figure 4**), and their IC_{50} values were determined to be 33.1, 27.1, 24.5, and 16.9 μg/mL, respectively.

Mass Spectrometry and Sequence of ACE Inhibitory Peptide. In order to identify peptide sequence, the most potent peak from secondary RP-HPLC was first subjected to MALDI to confirm the purity and molecular mass. The mass spectrum of peanut peak is shown in **Figure 5**. The singly charged ion with m/z 521.4 was found to be the most dominant for peanut peak 4. Two trace

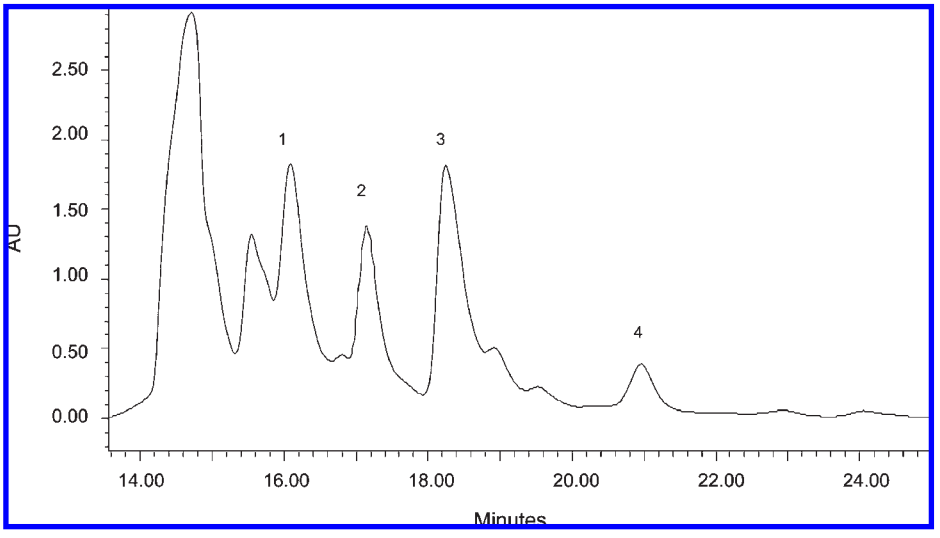

Figure 4. RP-HPLC profile of 50−60 min peanut fraction at 215 nm.

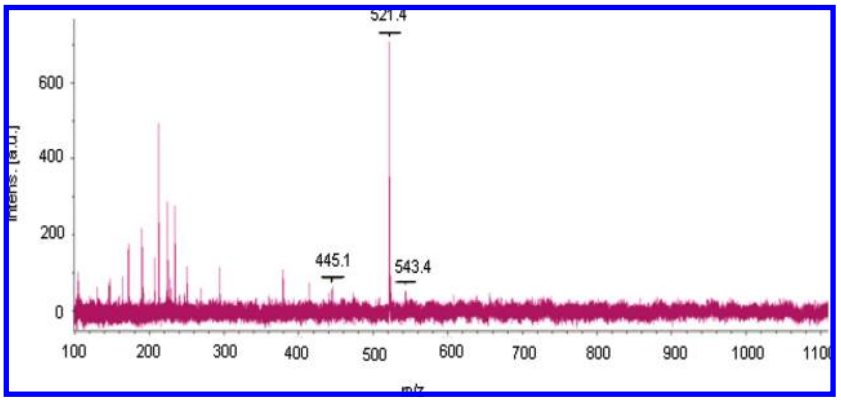

Figure 5. MALDI mass spectrum of peanut peak 4. The *x*-axis shows the mass to charge ratio (*m*/*z*), and the *y*-axis shows the intensity in arbitrary units (a.u.).

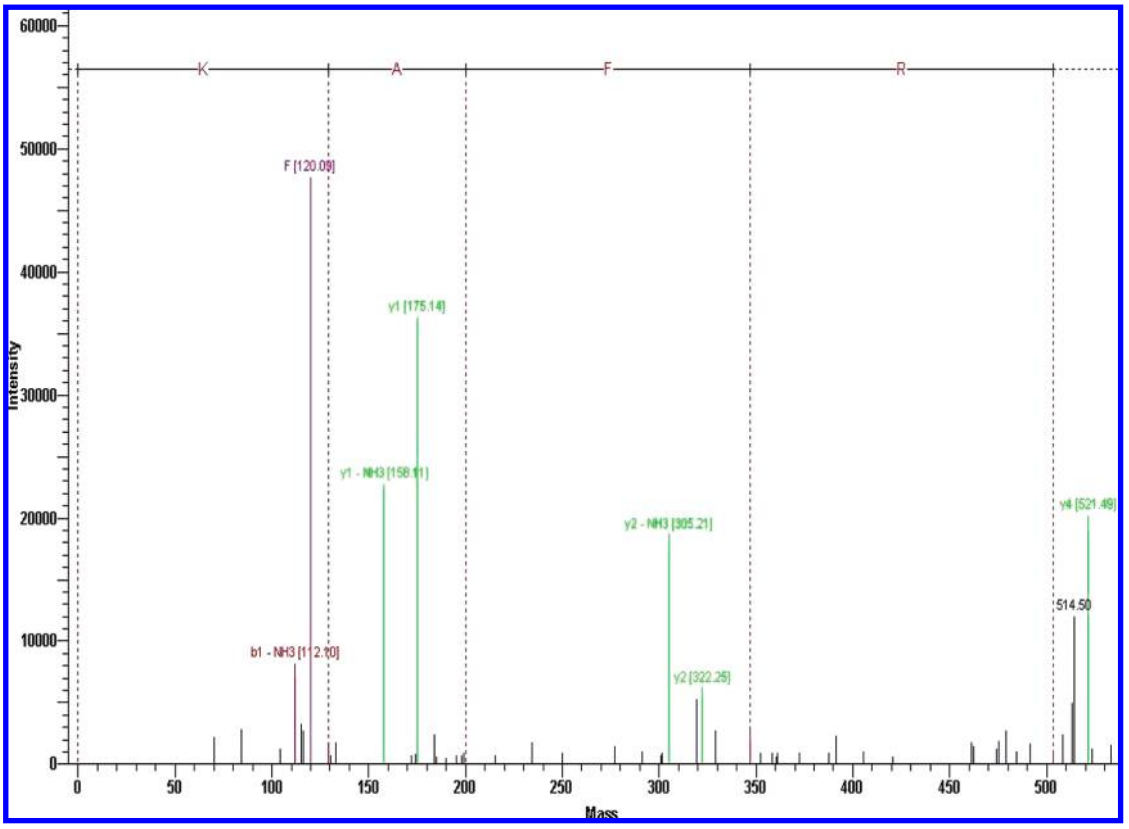

Figure 6. Tandem mass spectrometry (MS/MS) spectrum of ion *m*/*z* 521.4. Dotted lines represent identified b ions of ACE inhibitor.

ions with *m*/*z* 445.1 and 543.4 obtained by MALDI did not appear in the mass spectra obtained by electrospray ionization mass spectrometer and MS/MS, and only the ion with *m*/*z* 521.4 was present in all three mass spectra and was always the most dominant. So only the ion at *m*/*z* 521.4 was subjected to the following fragmentation.

The tandem mass spectrum of the parent ion and its amino acid sequence are shown in **Figure 6**. A major fragment ion was observed at *m*/*z* 120.1, and it is the immonium ion of phenylalanine and was produced as a secondary fragmentation (a combination of a y- and a-type cleavage) of the amide bond during collision induced dissociation and is a strong predictor of corresponding amino acid present in the sequence. The presence of arginine in the C-terminus of peptide KAFR favored the appearance of y-type fragmentation ions (*10*) that are very common and easily identifiable. Dotted lines in the tandem mass spectrum represent b ions, and thus ACE inhibitory peptide is identified as KAFR.

Although ACE appears to have a preference for a substrate or a competitive inhibitor containing hydrophobic (aromatic or branched side-chains) amino acids in the C-terminal tripeptide, structure−activity data suggest that a C-terminal arginine with a positive charge on the ε-amino group seems to contribute substantially to ACE inhibitory potency, which indicates a possible interaction between the inhibitor and an anionic binding site of ACE that is different from the catalytic site. The removal of the arginine residue at the C-terminus may lead to essentially inactive peptide analogues (*13*, *14*). The amino acid arginine also seems to contribute to ACE inhibitory potency when situated at a non-C-terminal position (*15*). For relatively long peptides, the steric structure affects ACE inhibitory activities (*16*). The peptide conformation adopted in a specific environment is also expected to contribute to ACE inhibitory potency (*17*). **Figure 7** shows a compendium of literature data in terms of IC50 values (μM) for tetrapeptides from many sources. Since many sources and

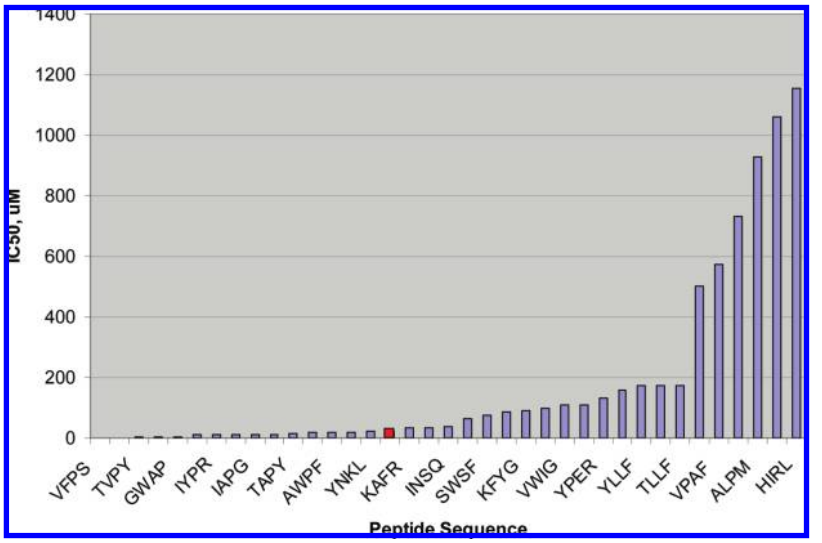

Figure 7. A comparison of ACE inhibitory activity (IC50, μM) for tetrapeptides from various sources. The position and activity of the peanut peptide KAFR is marked in red.

methods were used to determine these values, caution must be exercised in comparing them. However, with this caveat, it is seen that the selected peanut peptide is among the more potent inhibitors in the group. Of course it would be desirable to characterize many more peanut-derived inhibitory peptides, but, although the research continues, that was beyond the scope of this project and this paper.

Conclusion. Peanut has been consumed worldwide due to its nutrition and availability. The present research shows that peanut protein is also a good source of ACE inhibitory peptides. The product from each purification step is comparable in ACE inhibitory activity with products from other sources and may be used as a health enhancing ingredient in functional foods. Purification results in up to an 8-fold increase in ACE inhibitory activity over the crude hydrolysate, which indicates the effectiveness of the isolation procedure. Peptide KAFR is more or less consistent with the structure requirement proposed for ACE inhibitory peptides. Further research is required to confirm its *in vivo* activity.

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Received for review May 26, 2009. Revised manuscript received August 30, 2009. Accepted September 29, 2009.