DOI 10.1002/mnfr.200600041 Mol. Nutr. Food Res. 2006, *50*, 958 – 966

Effect of cationic flaxseed protein hydrolysate fractions on the *in vitro* structure and activity of calmodulin-dependent endothelial nitric oxide synthase

Adetayo O. Omoni and Rotimi E. Aluko

Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

The objective of this study was to determine the *in vitro* effects of cationic flaxseed protein hydrolysate fractions on calmodulin (CaM) structure as well as the activity of CaM-dependent endothelial nitric oxide synthase (eNOS). Flaxseed protein isolate was hydrolyzed with alcalase, and two peptide fractions were isolated by cation-exchange chromatography. Fraction I, eluted first from the column, and fraction II contained 42% and 51% contents of basic amino acids, respectively. Fractions I and II reduced the activity of CaM-dependent eNOS through a mostly mixed-type inhibition mode. Fraction II was at least ten times more effective as an eNOS inhibitor when compared to fraction I, as evident from the IC_{50} (concentration of protein hydrolysate that reduced eNOS activity by 50%) values. Fluorescence spectroscopy showed that the tyrosine residues in CaM were increasingly exposed upon addition of fraction I, while the opposite was the case when fraction II was added. Circular dichroism studies showed that fractions I and II reduced the α-helix content but increased the rigidity of the active calcium/CaM complex. We concluded that ability of the protein hydrolysate fractions to change the secondary and tertiary structures of CaM may explain their ability to reduce activity of CaM-dependent eNOS.

Keywords: Calmodulin / Circular dichroism / Flaxseed protein hydrolysate / Fluorescence / Nitric oxide synthase

Received: March 27, 2006; revised: May 16, 2006; accepted: May 18, 2006

1 Introduction

Calmodulin (CaM) is a relatively small, acidic, negatively charged (at physiological pH) Ca^{2+}-binding protein made up of 148 amino acids [1, 2], with a molecular weight of approximately 17 000 [3]. CaM functions as an intracellular mediator of the effects of Ca^{2+}, specifically binds four Ca^{2+}, and is structurally stable and yet flexible enough to undergo Ca^{2+}-induced conformational changes, thereby increasing its helical content and exposing hydrophobic regions for binding target molecules [3–5]. Calcium binding to CaM allows it to bind and activate several enzymes, among which are the nitric oxide synthases (NOS) [3, 6].

NO is synthesized from L-arginine and molecular oxygen by a family of three Ca^{2+}/calmodulin-dependent enzymes called the NOS [7], and is important for neurotransmission, immune response and regulation of vascular tone [8]. Although NO is an important molecule in the body's physiological processes, its production is not always beneficial; over- or underproduction of NO can be detrimental. NO synthesized by the endothelial isoform of NOS (eNOS) is responsible for vascular smooth muscle cell relaxation and vasodilation, regulation of blood pressure and inhibition of platelet aggregation [9–11], thus underproduction of endothelial NO, or an impairment of eNOS activity is implicated in the pathophysiology of vascular diseases [8]. On the other hand, recent evidence has implicated eNOS as a determinant factor in the production of tumor necrosis factor-α (TNFα) by human monocytes/macrophages; therefore, eNOS may be regarded as a pro-inflammatory parameter [12]. Inhibition of eNOS activity in such disease conditions could provide beneficial therapeutic effects.

Correspondence: Dr. R. E. Aluko, Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada, R3T 2N2
E-mail: alukor@cc.umanitoba.ca
Fax: +1-204-474-7593

Abbreviations: **BH_4**, tetrahydrobiopterin; **CaM**, calmodulin; **CD**, Circular dichroism ; **eNOS**, endothelial nitric oxide synthase; **FAD**, flavin adenine dinucleotide; **FMN**, flavin mononucleotide; **NADPH**, reduced nicotinamide adenine dinucleotide phosphate; **NO**, nitric oxide; **TNFα**, tumor necrosis factor-α

CaM-induced enzyme activation can be blocked by a number of pharmacological agents [2, 3], natural products and endogenous substances [13]. The binding of CaM to several synthetic and natural peptides has been studied [13 – 16]. It has been shown that CaM binds with high affinity and broad specificity to basic amphiphilic α-helical peptides [4].

Some food-derived peptides have been shown to have CaM-binding activity. Three CaM-binding peptides have been isolated from a pepsin digest of α-casein [13]. These peptides share the same feature common to other CaM-binding sequences such as abundance of basic and aromatic amino acid residues, with an almost complete lack of acidic residues. Similarly, two positively charged peptide fractions isolated from pea protein hydrolysate by cation exchange chromatography have been shown to have CaM-binding activity [17].

Flaxseed (*Linum usitatissimum*) has turned out to be a functional food ingredient with increasing importance in the world market because of its potential to reduce the risk of cardiovascular diseases, cancers, and gastrointestinal disorders [18 – 20]. The protein content in flaxseed is influenced by genetic and environmental factors, and ranges from 20.9 – 48.1%, with a mean of 34.5% [21]. Flaxseed proteins contain high levels of hydrophobic and positively charged amino acids [22, 23], which increases the possibility of generating hydrophobic and positively charged peptides by enzymatic hydrolysis. However, the current market for flaxseed as a functional food is limited to the whole intact meal (rich in lignans) and its oil, rich in alpha-linolenic acid (ALA) [18], as evidenced by the results of a meta-analysis by [23], which showed a dearth of studies on the use of flaxseed proteins as a functional food. The presence of beneficial bioactive phytochemicals in flaxseed has increased interest in the meal, which traditionally has been used as animal feed [22]. However, while there is a plethora of studies attributing the beneficial effects of flaxseed in cardiovascular disease and certain forms of cancer to its oil, rich in ALA and to its lignan content, the potential effects of flaxseed proteins is generally an under-researched area.

There is available evidence that some food-derived peptides have CaM-binding activity and have been used to inhibit some CaM-dependent enzymes. When bound to these peptides, CaM fails to regulate its target enzyme; hence, these peptides can be referred to as CaM inhibitors. In a previous study, we showed that cationic flaxseed protein hydrolysate fractions inhibited CaM-dependent activation of neuronal NOS (nNOS) [24]. The objectives of the present study were to: (i) study the effects of the cationic flaxseed protein hydrolysate fractions on activity of CaM-dependent eNOS; and (ii) determine the structural conformations of CaM in the presence of the protein hydrolysate fractions to gain a better understanding of the structural basis for inhibition.

2 Materials and methods

2.1 Materials

Defatted flaxseed meal was a gift from Bioriginal Foods (Saskatoon, SK, Canada). Alcalase, CaM (from bovine brain), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH_4) were purchased from Sigma Chemicals (St. Louis, MO, USA). Adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and catalase were from Roche Applied Science (Mississauga, ON, Canada), while recombinant bovine eNOS was purchased from Cayman Chemical (Ann Arbor, MI, USA). Other analytical grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

2.2 Production of flaxseed protein hydrolysate fractions

Low molecular mass (< 1000 Da) protein hydrolysate fractions I and II were obtained from enzymatic hydrolysis of flaxseed protein isolate as previously described [24]. In summary, an aqueous slurry that contained 5% flaxseed protein isolate was digested for 6 h at 50°C and pH 9.0 with alcalase using enzyme:substrate ratio of 1.5 : 25. After digestion, the slurry was adjusted to pH 4.0 with 2.0 M HCl to stop the enzyme reaction and the sample was cooled to room temperature. The sample was then centrifuged at $10\,000 \times g$ for 15 min and the supernatant saved. The supernatant was passed through Amicon stirred cell ultrafiltration set-up using a 1000 molecular weight cut-off membrane. The permeate obtained was freeze-dried and stored at −20°C until used. Separation of the ultrafiltration permeate was carried out on an SP-Sepharose High Performance cation-exchange chromatography column connected to an AKTA Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Montreal, PQ, Canada). Unbound peptides were removed using three column volumes of 0.1 M ammonium acetate buffer (pH 7.0); a linear gradient of 0 – 60% 0.5 M ammonium carbonate in 0.1 M ammonium acetate buffer (pH 7.0) was then applied to elute the adsorbed peptides at a flow rate of 5 mL/min. Two eluted peaks were obtained and the fractions within each peak were pooled together and freeze dried. As previously reported, fractions I and II contained approximately 42% and 51% levels of basic amino acids, respectively [24].

2.3 Enzyme inhibition kinetics

In vitro eNOS activity was determined by measuring the rate of decrease in NADPH absorption at 340 nm according to the modified method of [25] using an Ultrospec 4000

spectrophotometer that was connected to a circulatory water bath (Amersham Biosciences). The assay was carried out at 37°C with different CaM concentrations in the absence of peptides (uninhibited) and at varying peptide concentrations (inhibited) in a total volume of 2 mL; cofactors, enzyme, and protein hydrolysate fractions were all dissolved in 50 mM HEPES buffer (pH 7.0). The final control reaction mixture contained the HEPES buffer, 1 mM DTT, 4 μM BH_4, 4 μM FAD, 4 μM FMN, 1 mM $CaCl_2$, 10 U catalase, 1 mM L-arginine, 0.1 mM NADPH, CaM (6.25, 25 and 50 nM), and 0.5 U eNOS. Catalase was added to prevent accumulation of hydrogen peroxide, a by-product of eNOS activity; the peroxide could introduce error into the assay because of its ability to induce oxidation of NADPH. The blank solution contained all the above reagents except NADPH, while the sample solutions contained protein hydrolysate fractions (as part of the buffer volume) in addition to the reagents present in the control reaction mixture. All the reagents (except eNOS) were incubated for 5 min at 37°C in the spectrophotometer; reaction was initiated by addition of eNOS followed by mixing and automated absorbance readings at 30-s intervals for 30 min. Enzyme reaction was linear in the first 30 min and change in absorbance of inhibited reaction was subtracted from that of uninhibited to obtain degree of inhibition. IC_{50} was defined as the concentration of protein hydrolysate that reduced eNOS activity by 50%. Type of inhibition was determined from Lineweaver-Burk (L-B) plots using GraphPad version 3.0. (GraphPad Software Inc., San Diego, CA, USA); inhibition constant (Ki) was determined as X-axis intercept of a plot of the slope of the L-B lines against peptide concentration.

2.4 Intrinsic fluorescence

The fluorescence measurements were recorded using a RF-1501 spectrofluorimeter (Shimadzu Corp., Japan) at 24°C. The intrinsic fluorescence of proteins originates from the absorption properties of the three aromatic amino acids; phenylalanine, tyrosine and tryptophan. There was no emission signal when CaM was excited using the phenylalanine wavelength of maximum absorption. Therefore, tyrosine fluorescence was used to determine the *in vitro* structural changes of CaM since this protein does not have any tryptophan residue. The excitation wavelength was fixed at 275 nm and the fluorescence emission spectra were recorded between 290 and 400 nm. All measurements were taken using a total volume of 2 mL that contained 50 mM HEPES buffer (pH 7.0), 1 mM $CaCl_2$ and 50 nM calmodulin. The concentrations for fraction I were 6.25, 12.5 and 25 μg/mL, while those of fraction II were 1.25, 2.5 and 5 μg/mL. All measurements were taken using a 1-cm path length quartz cuvette. Spectra of the peptides alone were subtracted from the spectra of samples that contained both CaM and peptides.

Fluorescence spectroscopy was also used to determine changes in the *in vitro* structure of eNOS as a result of interaction with CaM and various concentrations of peptide fractions. Excitation wavelength was fixed at 295 nm and emission wavelength range was 310–450 nm. An excitation wavelength of 295 nm was used because this is the wavelength of tryptophan absorption and since CaM has no tryptophan residues, the spectra measurements indicate the changes in structures of eNOS or signal from the peptides. All measurements were taken using a total volume of 2 mL with concentrations of 1 mM $CaCl_2$, 50 nM CaM, and 0.05 U eNOS. Spectra of the peptides alone were subtracted from the spectra of samples that contained both eNOS and peptides.

2.5 Circular Dichroism

Far- and near-UV circular dichroism (CD) spectra were measured using a JASCO model J-810 spectropolarimeter (JASCO Corporation, Tokyo, Japan). Far-UV spectrum was measured at 190–240 nm. Concentrations of $CaCl_2$ and CaM in a total volume of 200 μL were 5 mM and 8 μM, respectively, in 50 mM HEPES buffer (pH 7.0). Concentration of both peptide fractions was 0.5 mg/mL. All spectra measurements were taken using a quartz cell with path length of 0.5 mm. Deconvolution of far-UV spectra to calculate secondary structure fractions was performed using the CDSSTR secondary structure determination algorithm [26, 27] accessed via the DICHROWEB website (http://www.cryst.bbk.ac.uk/cdweb/html/home.html). Near-UV spectrum was measured at 250–320 nm. Concentrations of $CaCl_2$ and CaM in a total volume of 1 mL were 10 mM and 80 μM, respectively, in 50 mM HEPES buffer (pH 7.0) while the peptide fractions were added at the level of 1 mg/mL. All measurements were taken using a quartz cell with path length of 10 mm.

2.6 Statistical analysis

Each analysis was done in duplicate and analysis of variance and Duncan's multiple-range test carried out using the Statistical Analysis Systems software, Version 9.1 (Statistical Analysis System, Cary, NC, USA).

3 Results

3.1 Enzyme inhibition kinetics

As we have shown in a previous report, separation of the flaxseed protein hydrolysate on a cation-exchange column yielded two fractions (I and II), with 42% and 51% contents

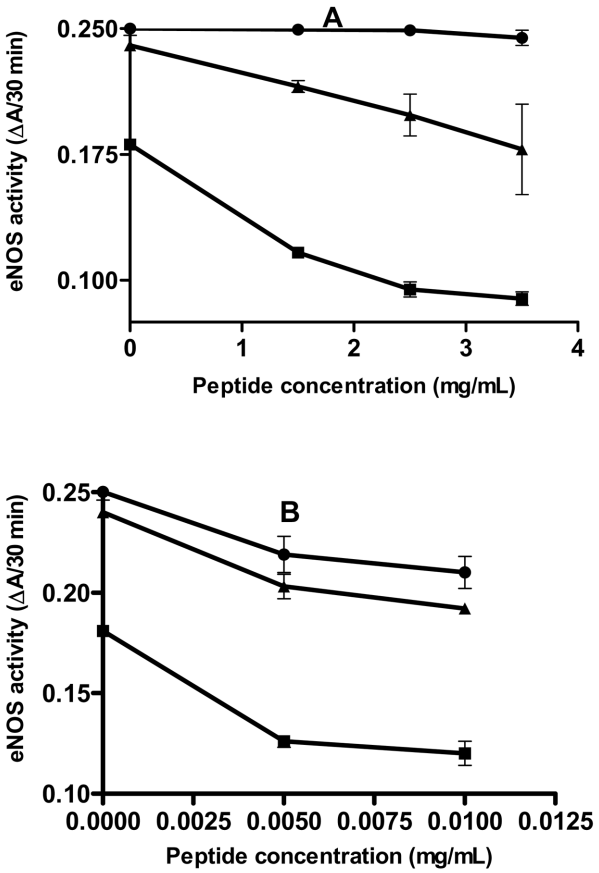

Figure 1. Effect of inhibitor concentration on activity of eNOS at different concentrations of calmodulin (CaM). (A) Fraction I. (B) Fraction II. CaM concentrations (nM): 6.25, ■; 25, ▲; 50, ●.

of basic amino acids, respectively [24]. Figure 1A and B shows the residual eNOS activity at different concentrations of fractions I and II, respectively. At fixed CaM concentration, *in vitro* eNOS activity decreased as protein concentration increased. As CaM concentration increased at fixed protein concentration, eNOS activity increased, and for fraction I (Fig. 1A) the inhibition was almost completely overcome at CaM concentration of 50 nM. The results indicate that the inhibitory fractions interacted mostly with CaM and not the enzyme. Lower concentrations of fraction II were required for inhibition, which was not overcome by 50 nM CaM concentration as was the case with the inhibition pattern for fraction I. Table 1 shows that the IC_{50} values were higher as the CaM concentration was increased; fraction II had values that were less than those of fraction I by a factor of at least 10.

Figure 2A and B shows the double reciprocal (L-B) plots of eNOS reaction velocity at different peptide I and II concentrations. Inhibition pattern for both fractions was mostly of the mixed type (with respect to CaM), which means that the peptides are capable of binding to CaM alone or the

Table 1. IC_{50} values for the inhibition of eNOS by protein hydrolysate fractions at different CaM concentrations

[CaM] (nM)	Fraction I (mg/mL)	Fraction II (mg/mL)
6.25	3.2	0.32
25	14.51	1.06
50	–	5.84

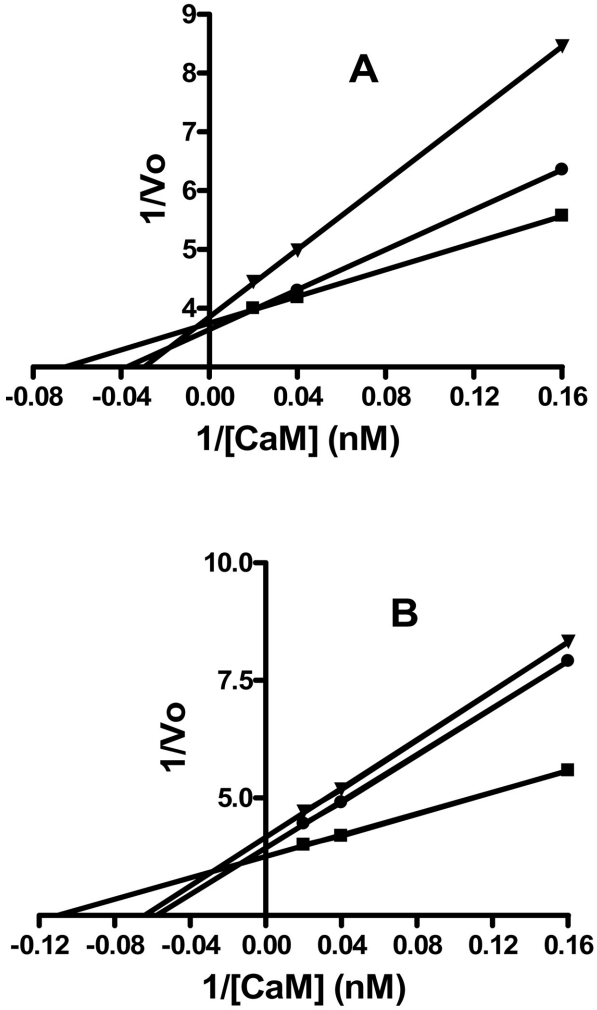

Figure 2. L-B plots for the inhibition of eNOS at varying concentrations of calmodulin and fixed concentrations of inhibitory peptides. (A) Fraction I concentration (mg/mL): 0.0, ■; 1.5, ●; 3.5, ▲. (B) Fraction II concentration (mg/mL): 0.0, ■; 0.005, ●; 0.01, ▲. Vo is the initial velocity of the enzyme reaction.

CaM/eNOS complex. It also means that the peptides bind to CaM at a site that is different from the binding site of eNOS. Fraction I had a Ki value of 2.13 mg/mL in comparison to the value of 0.01 for fraction II.

3.2 Intrinsic fluorescence of CaM

In vitro fluorescence studies in the absence and presence of calcium and protein hydrolysate fractions were conducted to gain an understanding of the structural consequences to CaM structure after interaction with these molecules. Changes in protein conformation such as unfolding often influence the fluorescence emission intensity. The high sensitivity of fluorescence spectroscopy enables detection of very small conformational changes in protein structure [28]. The effects of calcium and different concentrations of protein hydrolysate fractions I and II on CaM structure are shown in Table 2. Addition of calcium to CaM resulted in an increase in maximum fluorescence intensity (Fmax) of CaM, which was a 1.6-fold increase (Fmax/Fo ratio) over that of CaM alone. Our result is comparable with previous findings [29–31], which indicates a partial unfolding of CaM and increased exposure of hydrophobic groups as a result of interaction with calcium. The present results are consistent with the proven fact the calcium-induced unfolding of CaM structure is a prerequisite for activation of CaM-dependent enzymes [2].

Additions of protein hydrolysate fraction I to CaM in the presence of calcium produced increases in Fmax of CaM that were greater than the value obtained with calcium alone. A significant ($p < 0.05$) increase in the wavelength at Fmax (λmax) was obtained at 12.5 μg/mL concentration of fraction I, which is an indication of the residence of the tyrosine residues in a more polar environment than in the native or calcium/CaM complex. However, further increase of fraction I concentration to 25 μg/mL led to a significant ($p < 0.05$) reduction in λmax to a level similar to the inactive CaM. The blue shift at high protein hydrolysate concentration means that the tyrosine residues are in a less polar environment when compare to the effect observed at 12.5 μg/mL. The results provide strong indication that the inhibitory peptides have substantial effect on CaM structure.

Upon addition of protein hydrolysate fraction II to CaM in the presence of calcium, the Fmax of CaM also increased to a greater extent than in the presence of calcium alone, indicating a higher unfolding effect of the peptides on CaM structure. However, in contrast to fraction I, the Fmax of CaM decreased as fraction II concentration was increased, which indicates peptide-induced formation of compact protein structure. For example, at fraction II concentration of 1.25 μg/mL, the Fmax of CaM increased by ~10-fold over that of CaM alone, with a large shift in the λmax from 302 to 349 nm (Table 2). The red shift in λmax indicates transfer of the tyrosine residues to a more polar environment when compared to their environment in the absence of peptides. However, at fraction II concentrations of 2.5 and 5 μg/mL, Fmax of CaM was decreased and accompanied by a blue shift in λmax when compared to the effect of 1.25 μg/mL level (Table 2). The blue shift at higher fraction II concentrations indicates transfer of the tyrosine residues to a more hydrophobic environment possibly because the positively charged peptides attract some water molecules away from the CaM molecule.

Table 2. Changes in intrinsic fluorescence intensity of CaM in the presence of calcium and different inhibitor concentrations[a)]

Sample	λmax (nm)[b)]	Fmax[b)]	Fmax/Fo
CaM alone	302 ± 0.7[d]	59.9 ± 3.8[e]	–
CaM+Ca^{2+}	315 ± 5.7[c]	96.1 ± 0.4[d]	1.60
CaM+Ca^{2+}+12.5 μg/mL fraction I	342 ± 0.7[b]	104.6 ± 9.9[d]	1.75
CaM+Ca^{2+}+25 μg/mL fraction I	305 ± 0.7[d]	164.8 ± 6.0[c]	2.75
CaM+Ca^{2+}+1.25 μg/mL fraction II	349 ± 2.1[a]	582.8 ± 0.8[a]	9.73
CaM+Ca^{2+}+2.5 μg/mL fraction II	343 ± 2.8[ab]	276.3 ± 16.2[b]	4.61
CaM+Ca^{2+}+5 μg/mL fraction II	338 ± 0.0[b]	111.3 ± 0.0[d]	1.86

a) Excitation wavelength, 275 nm; Fmax, maximum FI; Fo, Fmax of CaM; λmax, wavelength at Fmax. Concentration of reagents: 50 mM HEPES buffer (pH 7.0), 1 mM $CaCl_2$, 50 nM CaM. Each analysis was done in duplicate and analysis of variance and Duncan's multiple-range test carried out using the Statistical Analysis Systems software, Version 9.1 (Statistical Analysis System). For each column of Fmax and λmax, values with different letters are significantly different ($p < 0.05$).

b) Values are mean ± SD.

3.3 Intrinsic fluorescence of eNOS

In vitro fluorescence studies on the structural characteristics of eNOS in the absence and presence of CaM and protein hydrolysate fractions was carried out to investigate the structural basis for the enzyme's interaction with CaM and peptides. There was no significant change ($p > 0.05$) in Fmax of eNOS upon addition of CaM/Ca^{2+} though a red shift in maximum emission wavelength from 331 to 334 nm occurred (Table 3). This is an indication of a slight change in the structure of eNOS such that the tryptophan residues became more exposed to the hydrophilic environment when compared to the apo-enzyme. Addition of fraction I led to a significant reduction in λmax such that the tryptophan environment in the presence of 25 μg/mL was similar to that of the apo-enzyme. There was very little change in the Fmax of eNOS as a result of addition of CaM or 12.5 μg/mL concentration of fraction I. However, at 25 μg/mL level of fraction I, there was a substantial increase in Fmax, which is an indication of increased exposure of the tryptophan residues. Similar to fraction I, addition of fraction II also led to significant reduction ($p < 0.05$) in λmax (Table 3); however, the Fmax reduced with increased concentration of fraction II. The decrease in Fmax may be due to increased solvation of the positively charged residues in fraction II, which would reduce the amount of water available to hydrate eNOS with consequent

Table 3. Changes in intrinsic fluorescence properties of eNOS in the presence of calcium and different peptide concentrations[a)]

Sample	λ max (nm)[b)]	Fmax[b)]	Fmax/Fo
eNOS alone	331 ± 0.0[c]	433.8 ± 4.1[b]	–
eNOS+CaM[+]	334 ± 0.0[a]	431.7 ± 0.4[b]	0.99
eNOS+CaM+12.5 μg/mL fraction I	333 ± 0.0[b]	419.3 ± 5.3[c]	0.97
eNOS+CaM+25 μg/mL fraction I	331 ± 0.0[c]	531.3 ± 6.2[a]	1.22
eNOS+CaM+1.25 μg/mL fraction II	333 ± 0.0[b]	400.7 ± 0.0[d]	0.92
eNOS+CaM+2.5 μg/mL fraction II	330 ± 0.0[d]	406.3 ± 2.7[d]	0.94
eNOS+CaM+5 μg/mL fraction II	330 ± 0.0[d]	368.7 ± 3.3[e]	0.85

a) Excitation wavelength, 295 nm; Fmax, maximum FI; Fo, FI of eNOS only; λ max, wavelength of maximum FI. Samples that contain CaM also contained 1 mM $CaCl_2$. Each analysis was done in duplicate and analysis of variance and Duncan's multiple-range test carried out using the Statistical Analysis Systems software, Version 9. 1. For each column of Fmax and λ max, values with different letters are significantly different ($p < 0.05$).

b) Values are mean ± SD.

increased folding of the enzyme structure. The present results differ slightly from those we reported for nNOS using the same peptide fraction concentrations [24]; interaction of the peptide fractions and CaM/Ca^{2+} with nNOS resulted in mostly increases in λ max and Fmax.

3.4 CD spectra of CaM

The far-UV spectrum of CaM (Fig. 3) is characteristic of a typical protein with high content of α-helix structure: two negative peaks at 222 and 208 nm, a positive peak at 196 nm, and a crossover near 200 nm [28]. Addition of Ca^{2+} to CaM resulted in an increase in the negative intensities of the CD spectra at 222 and 208 nm, indicating the induction of a higher amount of α-helical structure. These results are consistent with previous reports showing an increase in α-helical content of CaM upon Ca^{2+} binding [29, 32, 33]. Addition of protein hydrolysate fractions I and II to CaM in the presence of calcium led to decreases in the negative intensities of the CD spectra around 222 and 208 nm. This indicates that binding of the protein hydrolysate fractions to CaM reduced the α-helical content of CaM. Table 4 shows the proportion of secondary structure of CaM in the absence and presence of calcium and peptides as calculated from the spectra in Fig. 3. Addition of calcium to CaM increased α-helix fraction of CaM from 49% to 62%; the level of increase was less in the presence of the protein hydrolysate fractions. The calcium/CaM complex had reduced proportion of random structure when compared to the apo-CaM, while addition of the peptides led to increased random structure of the calcium/CaM complex.

Table 4. Effect of calcium and inhibitors on the proportions of CaM secondary structure[a)]

Sample	α-helix (%)	β-sheet (%)	β-turn (%)	Random (%)
CaM	49	13	15	24
CaM + calcium	62	11	13	16
CaM + calcium + fraction I	53	28	4	19
CaM + calcium + fraction II	54	21	6	19

a) Concentration of reagents: 50 mM HEPES pH 7.0; 5 mM $CaCl_2$; 8 μM CaM; 0.5 mg/mL protein hydrolysate fractions I and II.

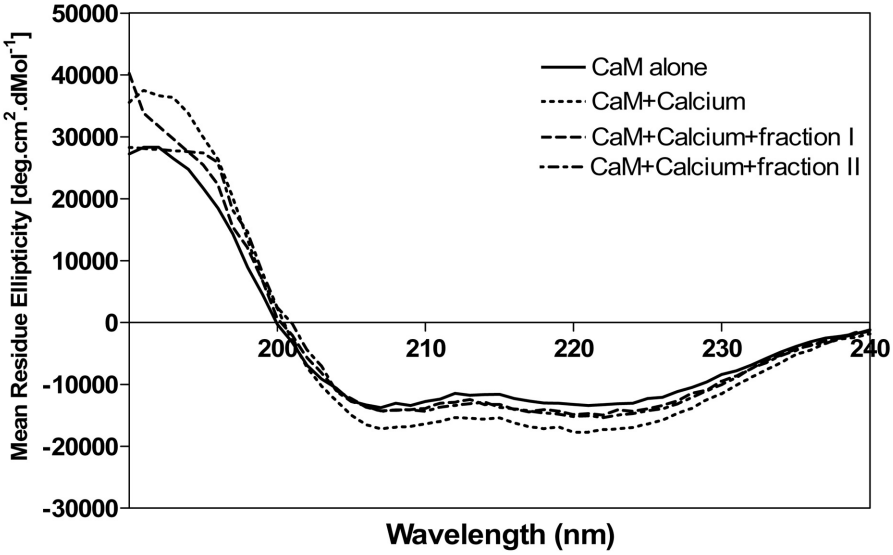

Figure 3. Far-UV CD spectra of calmodulin (CaM), CaM/Ca^{2+} complex, CaM/Ca^{2+}/fraction I complex, and CaM/Ca^{2+}/fraction II complex. Experiments were performed with 8 μM CaM, 5 mM $CaCl_2$, and 0.5 mg/mL protein hydrolysate fractions in 50 mM HEPES buffer (pH 7.0).

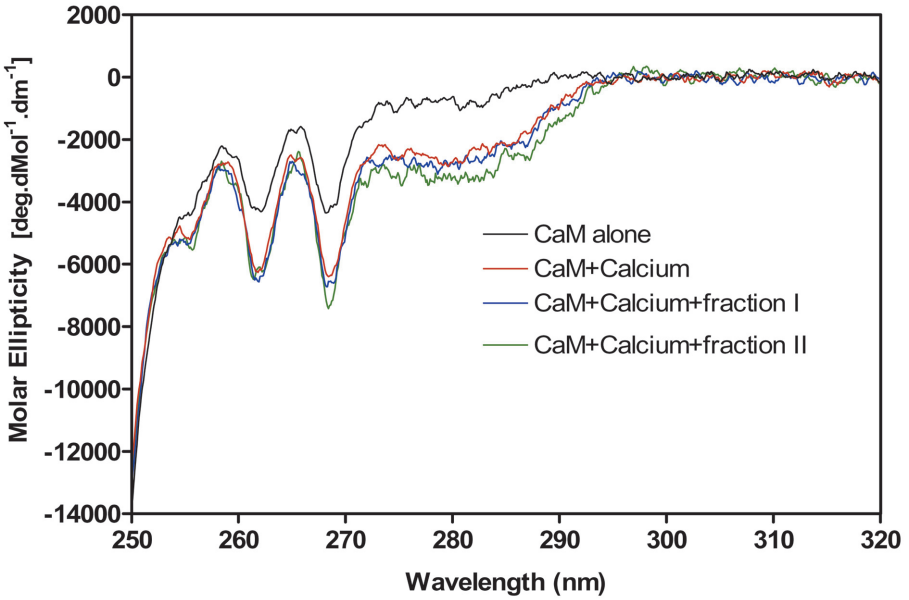

Figure 4. Near-UV CD spectra of calmodulin (CaM), CaM/Ca^{2+} complex, CaM/Ca^{2+}/fraction I complex, and CaM/Ca^{2+}/fraction II complex. Experiments were performed with 80 μM CaM, 10 mM $CaCl_2$ and 1 mg/mL protein hydrolysate fractions in 50 mM HEPES buffer (pH 7.0).

The tertiary structure of CaM can be estimated from the near UV spectra as shown in Fig. 4. Since CaM does not contain any tryptophan residues, tertiary structure is estimated from the responses obtained at 255–270 nm for phenylalanine and 275–290 nm for tyrosine [34]. Figure 4 shows that in the absence of calcium, only the phenylalanine signal was evident and that the residues were located in two different environments as evidenced by the two negative peaks obtained at 262 and 268 nm. The phenylalanine residues that show intensity at 262 nm are in a more hydrophobic environment when compared to those with intensity at 268 nm. Addition of calcium resulted in an increase in the intensity of the negative peaks (but no change in wavelength) (Fig. 4), which indicates increased rigidity of the environments of the phenylalanine residues when compared to that of CaM alone. Similar results that showed increases in the intensity of the negative phenylalanine signal of CaM at similar wavelengths in the presence of calcium have also been reported [32, 35, 36]. Addition of protein hydrolysate fractions to the CaM/Ca^{2+} complex resulted in a slight increase in the intensity of the negative signal of phenylalanine residues, but the wavelength remained unchanged (Fig. 4). The presence of the protein hydrolysate fractions also led to increased intensity of the tyrosine signal at about 280–285 nm, which shows that the tertiary structure of CaM was modified. Fraction II induced more negative intensity at 268 nm than peptide fraction I, which is consistent with findings from the fluorescence studies (Table 2) where fraction II produced greater structural changes in CaM when compared to fraction I. This is in agreement with findings of Li and Aluko [32] that showed the negative intensity of CaM at 262 and 268 nm was increased upon addition of cationic pea protein hydrolysate fractions.

4 Discussion

Interaction of ligands with CaM represents an important mechanism by which inhibitors can modulate activity of CaM-dependent enzymes. We have previously shown that the cationic flaxseed protein hydrolysates that were used in this work exhibited strong inhibitory properties towards nNOS [24]. The present work confirms that the protein hydrolysates also have the capacity to inhibit eNOS, although the mode of inhibition is different. The mixed-type inhibition of eNOS is different from the non-competitive inhibition of nNOS that we previously reported, which indicates that the CaM-ligand interactions differ depending on the nature of enzyme. Inhibition constants (Ki) for the interaction between CaM-dependent eNOS and protein hydrolysates was 2.13 and 0.01 mg/mL for fractions I and II, respectively, which indicates greater affinity of fraction II for CaM. The Ki values obtained in this work for eNOS are lower than the 5.97 and 2.55 mg/mL (fraction I and II, respectively) that we reported for nNOS [24]. Therefore, we can conclude that the protein hydrolysate fractions are more effective inhibitors of eNOS than nNOS. Differences in the mode and level of inhibition for eNOS and nNOS by the protein hydrolysate fractions may be a reflection of the fact

that each NOS isoform interacts with different binding sites on CaM as was previously suggested by Censarek *et al*. [37]. The results suggest that fraction II, which has a higher content of positively charged amino acid residues [24] binds more effectively to CaM when compared to fraction I and is similar to previous reports that have shown greater inhibition of CaM-dependent enzymatic reactions by protein hydrolysate fractions with higher contents of basic amino acids [17, 24].

Our previous work with two pea protein-derived cationic hydrolysate fractions showed that there was no difference in their histidine contents (about 2%) [17], unlike the results obtained in the present work. Fraction II from the flaxseed protein hydrolysates had about 11% histidine content when compared to 2% for fraction I. Additionally, the differences in lysine and arginine contents between the two pea protein hydrolysate fractions [17] was much larger than the differences between the two flaxseed hydrolysate fractions that were used in this work [24]. Overall, fraction I from the pea protein hydrolysate contained about 10% by weight of basic amino acids [17], when compared to 42% for the fraction I from flaxseed protein hydrolysate [24]. The basic amino acid content of pea fraction II was about 42% [17], which is lower than the 51% obtained for fraction II from flaxseed protein hydrolysate [24]. Therefore, it is very likely that the pea protein hydrolysate cationic fractions will inhibit CaM-dependent enzymes at levels that are different from the inhibition caused by flaxseed protein hydrolysate fractions because of the differences in the content of basic amino acids.

Fluorescence data suggest that fraction II has a stronger effect (higher Fmax at lower concentrations) on CaM structure than fraction I. The increase in FI of CaM upon addition of the peptide fractions is similar to that reported by Li and Aluko [32] using pea protein-derived protein hydrolysate fractions. Previous studies on the binding of hormones and neuropeptides to CaM using fluorescence spectroscopy showed higher FI of CaM upon binding to these peptides [29, 38]. Intrinsic fluorescence results have shown that apo-CaM exist in a fairly folded compact structure, which undergoes some unfolding that exposes tyrosine residues when it interacts with calcium ions. The higher FI (exposure of Tyr residues) of the Ca^{2+}/CaM complex when compared to apo-CaM (Table 2) shows that a change in structure is necessary for CaM to interact with and activate various target enzymes. However, the results suggests that excessive unfolding as was induced by the protein hydrolysate fractions led to reduction in the optimum configuration of CaM. Since CaM has a net negative charge and the protein hydrolysate fractions have net positive charges at the pH 7.0 used in this work, it is reasonable to suggest that an interaction between the two reagents was responsible for the structural changes observed for CaM. This is consistent with previous reports that have shown that positively charged peptides and casein hydrolysates can interact with and affect the functional performance of CaM [13 – 15, 39, 40]. The stronger affinity of fraction II is indicated by the significantly ($p < 0.05$) larger Fmax values of CaM when compared to fraction I. However, increases in the concentration of fraction II led to lower Fmax values, which suggest folding of CaM structure into a more globular structure. The change into a more compact structure could have been as a result of dehydration of the CaM protein by the highly charged fraction II peptides.

The changes in secondary structure of CaM as promoted in the presence of Ca^{2+} are consistent with previous reports that holo-CaM is stabilized by increase in the α-helix content [30]. Addition of inhibitory peptides reduced the α-helix structure of CaM (Table 4), which further confirmed the role of this structural conformation in activation of eNOS. The results suggest that the inhibitory mechanism of the protein hydrolysate fractions may be through reduction in the α-helix fraction of CaM and possible increased level of random structure, which could reduce the affinity of CaM for target enzymes such as eNOS.

The near-UV region is described as the aromatic region because aromatic amino acids (phenylalanine, tyrosine, and tryptophan) have absorbance in this region and the spectra obtained are used to describe the tertiary structure (folding) of proteins [34]. The appearance of two distinct negative peaks for Phe at 261 and 268 nm in the presence of Ca^{2+} indicates a structural rearrangement of CaM such that the residues became located in more asymmetric environments, *i. e*., increased rigidity of the tyrosyl side chains. The results confirm the FI data that showed a structure modifying effect of Ca^{2+} on CaM. The intensity of these two peaks increased with addition of peptides; fraction II induced more negative intensity than fraction I. These results indicate stronger modification of CaM structure by the protein hydrolysate fractions when compared to the effect of Ca^{2+}. The change produced by Ca^{2+} may induce optimum unfolding that is required for CaM to bind to and activate target enzymes. However, as the CaM increasingly unfolds with addition of protein hydrolysate fractions, it may result in protein denaturation and loss of ability to activate enzyme reactions. The results are consistent with the fluorescence data that showed fraction II produced greater structural changes in CaM when compared to fraction I. Our results are consistent with those of previous near-UV studies of CaM that showed two negative signals corresponding to Phe and Tyr; calcium binding to CaM was also accompanied by an increase of the CD spectra [35, 41]. These results indicate that the fractions I and II interacted with CaM in the presence of calcium and the resultant structural conformations may not be the optimum required for CaM-induced activation of eNOS.

Financial support for this work was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery grant to Dr. R. E. Aluko.

5 References

[1] Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., *et al.*, *Science* 1992, *256*, 632–638.

[2] Vogel, H. J., *Biochem. Cell Biol.* 1994, *72*, 357–376.

[3] Weiss, B., Prozialeck, W. C., Wallace, T. L., *Biochem. Pharmacol.* 1982, *31*, 2217–2226.

[4] O'Neil, K. T., DeGrado, W. F., *Trends Biochem. Sci.* 1990, *15*, 59–64.

[5] Reddy, A. S. N., Day, I. S., Narasimhulu, S. B., Safadi, F., *et al.*, *J. Biol. Chem.* 2002, *277*, 4206–4214.

[6] Sugimura, M., Sato, T., Nakayama, W., Morishima, Y., *et al.*, *Eur. J. Pharmacol.* 1997, *336*, 99–106.

[7] Sheta, E. A., McMillan, K., Masters, B. S. S., *J. Biol. Chem.* 1994, *269*, 15147–15153.

[8] Albrecht, E. W., Stegeman, C. A., Heeringa, P., Henning, R. H., van Goor, H., *J. Pathol.* 2003, *199*, 8–17.

[9] Szabo, C., Southan, G. J., Thiemermann, C., *Proc. Natl. Acad. Sci. USA* 1994, *91*, 12472–12476.

[10] Salerno, L., Sorrenti, V., Guerrera, F., Sarva, M. C., *et al.*, *Pharm. Pharmacol. Commun.* 1999, *5*, 491–494.

[11] Bryk, R., Wolff, D. J., *Pharmacol. Ther.* 1999, *84*, 157–178.

[12] Muhl, H., Pfeilschifter, J., *Biochem. Biophys. Res. Commun.* 2003, *310*, 677–680.

[13] Kizawa, K., Naganuma, K., Murakami, U., *J. Dairy Res.* 1995, *62*, 587–592.

[14] Kizawa, K., Naganuma, K., Murakami, U., *J. Dairy Sci.* 1996, *79*, 1728–1733.

[15] Kizawa, K., *J. Agric. Food Chem.* 1997, *45*, 1579–1581.

[16] Cox, J. A., Comte, M., Fitton, J. E., DeGrado, W. F., *J. Biol. Chem.* 1985, *260*, 2527–2534.

[17] Li, H., Aluko, R. E., *J. Nutr. Biochem.* 2005, *16*, 656–662.

[18] Li-Chan, E. C. Y., Ma, C. Y., *Food Chem.* 2002, *77*, 495–502.

[19] Bathena, S. J., Ali, A. I., Mohammed, A. I., Hansen, C. T., Velasquez, M. T., *J. Nutr. Biochem.* 2002, *13*, 684–689.

[20] Lei, B., Li-Chan, E. C. Y., Oomah, B. D., Mazza, G., *J. Agric. Food Chem.* 2003, *51*, 814–821.

[21] Oomah, B. D., Mazza, G., *Inform* 1995, *6*, 1246–1252.

[22] Oomah, B. D., Mazza, G., *Food Chem.* 1993, *48*, 109–114.

[23] Oomah, B. D., *J. Sci. Food Agric.* 2001, *81*, 889–894.

[24] Omoni, A., Aluko, R. E., *J. Am. Oil Chem. Soc.* 2006, *83*, 335–340.

[25] Cho, M. J., Vaghy, P. L., Kondo, R., Lee, S. H., *et al.*, *Biochemistry* 1998, *37*, 15593–15597.

[26] Lobley, A., Whitmore, L., Wallace, B. A., *Bioinformatics* 2002, *18*, 211–212.

[27] Whitmore, L., Wallace, B. A., *Nucleic Acids Res.* 2004, *32*, W668–673.

[28] Schmid, F. X., in: Creighton, T. E. (Ed.), *Protein Structure: a practical approach*, Oxford University Press, New York 1990, pp. 251–285.

[29] Malenick, D. A., Anderson, S. R., *Biochemistry* 1982, *21*, 3480–3486.

[30] Burger, D., Cox, J. A., Comte, M., Stein, E. A., *Biochemistry* 1984, *23*, 1966–1971.

[31] VanScyoc, W. S., Sorensen, B. R., Rusinove, E., Laws, W. R., *et al.*, *Biophys. J.* 2002, *83*, 2767–2780.

[32] Li, H., Aluko, R. E., *Int. J. Food Sci. Nutr.* 2006, in press.

[33] LaPorte, D. C., Wierman, B. M., Storm, D. R., *Biochemistry* 1980, *19*, 3814–3819.

[34] Strickland, E. H., *CRC Crit. Rev. Biochem.* 1974, *2*, 113–175.

[35] Kilhoffer, M., Demaille, J. G., Gerard, D., *Biochemistry* 1981, *20*, 4407–4414.

[36] Sun, H., Yin, D., Coffeen, L. A., Shea, M. A., Squier, T. C., *Biochemistry* 2001, *40*, 9605–9617.

[37] Censarek, P., Beyermann, M., Koch, K.-W., *Biochemistry* 2002, *41*, 8598–8604.

[38] Clapperton, J. A., Martin, S. R., Smerdon, S. J., Gamblin, S. J., Bayley, P. M., *Biochemistry* 2002, *41*, 14669–14679.

[39] Barnette, M. S., Daly, R., Weiss, B., *Biochem. Pharmacol.* 1983, *32*, 2929–2933.

[40] Itano, T., Itano, R., Penniston, J. T., *Biochem. J.* 1980, *189*, 455–459.

[41] Harmat, V., Bocskei, Z., Naray-Szabo, G., Bata, I., *et al.*, *J. Mol. Biol.* 2000, *297*, 747–755.

