Mechanism of the Inhibition of Calmodulin-Dependent Neuronal Nitric Oxide Synthase by Flaxseed Protein Hydrolysates

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ABSTRACT: This work was aimed at producing potential nutraceutical peptides from flaxseed protein hydrolysate that can bind to calmodulin (CaM) and inhibit the activity of CaM-dependent neuronal nitric oxide synthase (nNOS), an enzyme that has been implicated in some forms of human diseases. Flaxseed protein isolate was hydrolyzed with alcalase, and the resultant protein hydrolysate was passed through a 1000-Da M.W. cut-off membrane to isolate low-M.W. peptides. The permeate from the membrane was loaded onto a cation-exchange column, and adsorbed peptides were separated into fractions I and II that had a content of 42 and 51% basic amino acids, respectively. Kinetic analyses showed that both fractions were capable of binding to CaM, which led to reductions in the activity of nNOS; the inhibition constant (K_i) was 5.97 and 2.55 mg/mL for fractions I and II, respectively. Double reciprocal plots showed that the mode of enzyme inhibition was mostly noncompetitive. Estimation of nNOS structure by fluorescence spectroscopy indicated that binding of the peptides to CaM led to a gradual unfolding of enzyme structure as levels of the fractions were increased. We concluded that the flaxseed protein-derived peptides may be used as ingredients for the formulation of therapeutic foods.

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KEY WORDS: Calmodulin, enzyme inhibition kinetics, flaxseed, fluorescence spectroscopy, nitric oxide synthase, nutraceuticals, protein hydrolysate.

Calmodulin (CaM)-dependent neuronal nitric oxide synthase (nNOS) catalyzes the production of nitric oxide (NO) by converting L-arginine to L-citrulline (1). The nNOS reaction involves NADPH, FMN (flavin mononucleotide), FAD, heme, tetrahydrobiopterin (BH_4), and CaM as important cofactors (2,3), each having its own binding site on the NOS enzyme. Binding of CaM to the target enzyme requires the presence of calcium ions. NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes including neurotransmission, vasodilation, immune response, and smooth muscle contraction-relaxation (4,5). Although NO has several important physiological functions, excessive levels are detrimental and have been implicated as a crucial factor in the pathogenesis of several diseases (6). Excessive activity of nNOS has been reported in a number of diseases including acute and chronic neurodegenerative disorders such as stroke, Alzheimer's, and Parkinson's as well as migraine headaches, convulsion, and pain (1,6–8). Therefore, inhibition of the activities of nNOS may be used to prevent and/or treat pathological conditions that are due to uncontrolled production of NO. To this end, pharmacological inhibition of nNOS activity has emerged as a new therapeutic strategy for the treatment of various clinical conditions associated with NO overproduction (3). A large number of NOS inhibitors have been developed, e.g., L-arginine analogs, such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-L-nitro-L-arginine methyl ester (L-NAME) (9). Synthetic CaM-binding peptides that interfere with the interaction of CaM with nNOS also are potential therapeutic tools (10).

Apart from drugs, current research efforts have focused on producing compounds such as nutraceuticals that can be used against diseases without the need for a physician's prescription. Nutraceuticals are products that are isolated or purified from food, that are generally sold in a medicinal form not usually associated with food, and that have physiological benefit or provide protection against chronic disease. Protein hydrolysates, especially those that contain short-chain peptides, are potential candidates for the formulation of functional foods and nutraceutical products because they can cross the digestive epithelial barrier and reach the blood vessels. From the blood vessels the peptides can be transported to peripheral organs and elicit beneficial effects on the organism (11). Interest in nutraceuticals from vegetables has arisen in recent years because of the abundance of plant food proteins.

CaM has a net negative charge at physiological pH; therefore, the strategy is to produce compounds with positively charged amino acids as inhibitors. Evidence is available that some food-derived peptides with positively charged amino acids have CaM-binding activity and could be useful as inhibitors of certain CaM-dependent enzymes (12–14). Three peptides that inhibit CaM-dependent phosphodiesterase (CaM-PDE) have been isolated from a pepsin digest of a-casein (14). Similarly, Li and Aluko (15) showed that two positively charged protein hydrolysate fractions with CaM-binding activities can be isolated from pea protein enzyme digest by cation exchange chromatography; both fractions inhibited the activity of CaM-dependent protein kinase II (CaMKII). However, there is lack of information on the inhibition of nNOS by food protein-derived peptides.

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The present study determined the inhibition of CaM-dependent nNOS by cationic protein hydrolysate fractions obtained from enzymatic hydrolysis of flaxseed proteins. Fluorescence spectroscopy was used to study changes in nNOS structure as a result of interactions with CaM and the protein hydrolysate fractions.

EXPERIMENTAL PROCEDURES

Materials. Defatted flaxseed meal was a gift from Bioriginal Foods (Saskatoon, Canada). Alcalase, CaM, FAD, FMN, and BH_4 were purchased from Sigma Chemicals (St. Louis, MO). ATP, NADPH, and catalase were from Roche Applied Science (Mississauga, Ontario, Canada), and nNOS was purchased from Cayman Chemical (Ann Arbor, MI). Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, Ontario, Canada).

Enzymatic hydrolysis of flaxseed protein isolate. Protein isolate was prepared from defatted flaxseed meal by alkali extraction that was followed by acid-induced protein precipitation (16). Flaxseed protein isolate was hydrolyzed for 6 h with alcalase according to the modified method of Aluko and Monu (17) at an enzyme/substrate ratio of 1.5:25. A slurry of the protein isolate (5% wt/vol, protein basis) was prepared in distilled water and the pH adjusted to 9.0 with 2 M NaOH solution. The slurry was heated to 50°C and the enzyme added while gently stirring. The sample was digested at 50°C while the pH was kept constant at 9.0 by addition of 2 M NaOH when necessary. After digestion, the pH of the protein hydrolysate was adjusted to 4.0 with 2 M HCl to stop the enzyme reaction and precipitate undigested materials; digested sample was cooled to room temperature and centrifuged at 10,000 × *g* for 15 min. The resulting supernatant, which contained target peptides, was passed through an Amicon stirred ultrafiltration cell setup using a 1000-Da M.W. cut-off membrane (Fisher Scientific). The permeate, which contains low-M.W. peptides (<1000 Da) was freeze-dried as the protein hydrolysate and the protein content determined using the modified Lowry method (18).

Production of protein hydrolysate fractions. The protein hydrolysate was fractionated according to the previously described method of Kizawa *et al*. (14), with slight modifications. The freeze-dried permeate was dissolved in 0.1 M ammonium acetate and the solution (100 mg protein/ml of buffer) adjusted to pH 7.0 using 2 M ammonium hydroxide. The sample solution was loaded onto a HiLoad 26/10 SP-Sepharose High Performance cation exchange chromatography column (58 mL bed volume) connected to an AKTA Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences, Montréal, Canada). The column was washed with 3 vol (180 mL) of 0.1 M ammonium acetate buffer (pH 7.0) to remove unbound peptides; 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; protein content of each fraction was determined using the modified Lowry method (18).

Determination of amino acid composition. The freeze-dried pooled fractions were hydrolyzed under vacuum at 110°C for 24 h using 6 M HCl containing 1% phenol. Amino acid composition of the hydrolysate was determined by HPLC according to the method of Bidlingmeyer *et al.* (19). The cysteine and methionine content was determined using performic acid oxidation (20), and tryptophan content was determined by alkaline hydrolysis (21).

Enzyme inhibition kinetics. nNOS activity was determined by measuring the rate of decrease in NADPH absorption at 340 nm according to the modified method of Cho *et al.* (4) using an Ultrospec 4000 spectrophotometer (Amersham Biosciences, Montréal, Canada) that was fitted with a water circulation bath. The assay was carried out at 37°C with different CaM concentrations in the absence (uninhibited) or presence (inhibited) of protein hydrolysate fractions in a total volume of 2 mL. The final reaction mixture contained 50 mM HEPES buffer (pH 7.0), 1 mM DTT, 4 μM BH_4, 4 μM FAD, 4 μM FMN, 1 mM $CaCl_2$, 10 units catalase, 1 mM L-arginine, 0.1 mM NADPH, CaM (6.25, 25, and 50 nM), and three units of nNOS. The blank contained all the above except NADPH. The rate of decrease in absorption at 340 nm was linear during the first 30 min of enzyme assay; absorbance of inhibited reaction was subtracted from absorbance of control to obtain the degree of inhibition. There was no change in absorbance when CaM was omitted from the reaction mixture; therefore, nNOS activity was dependent on the presence of CaM. Enzyme activity was expressed as the net decrease in absorbance at 340 nm after 30 min reaction time. The type of inhibition was determined from Lineweaver-Burk (L-B) plots using GraphPad version 3.0. (GraphPad Software Inc., San Diego, CA). K_m and V_{max} were determined by nonlinear regression of the data, and the ratio was used to estimate the catalytic efficiency of the enzyme in the absence and presence of peptide fractions. The inhibition constant (K_i) was determined as the X-axis intercept of a plot of the slope of the L-B lines against peptide concentration (15).

Intrinsic fluorescence of nNOS. Fluorescence spectroscopy was used to determine changes in the structure of nNOS as a result of interaction with CaM and various concentrations of protein hydrolysate fractions. The excitation wavelength was fixed at 295 nm, and the emission wavelength range was from 310 to 450 nm. An excitation wavelength of 295 nm was used because this is the wavelength of tryptophan absorption; since CaM has no tryptophan residues, all spectra measurements indicate the changes in the structures of nNOS and the protein hydrolysate fractions. All measurements were taken using a total volume of 2 mL with final concentrations of $CaCl_2$, CaM, and nNOS as 1 mM, 50 nM, and 0.5 units, respectively. All measurements were performed in a 1-cm path length quartz sample cuvette using a Shimadzu RF-1501 recording spectrofluorimeter (Shimadzu Corp., Kyoto, Japan) at 24°C. Spectra of the peptides alone were subtracted from the spectra of samples that contained both nNOS and peptides.

RESULTS AND DISCUSSION

FPLC fractionation of protein hydrolysate. Fractionation of the protein hydrolysate on a cation exchange column yielded two fractions (I and II) as shown in Figure 1. The fact that fraction II eluted later from the column means it was bound more tightly to the column and has a higher content of positively charged amino acids than fraction I; this was confirmed by amino acid analysis. The amino acid composition of the fractions is shown in Table 1. Fraction I had a higher percentage of arginine compared with fraction II; however, fraction II had higher levels of histidine and lysine when compared with fraction I. Overall, fraction II had 51% total content of basic amino acids when compared with 42% for fraction I. The results in this study are similar to those reported by Li and Aluko (15) for pea protein cationic hydrolysates, in which the fraction that eluted later from the cation-exchange column had higher levels of positively charged amino acids when compared with the early eluting fraction. Therefore, fraction II would be expected to have a higher affinity for CaM and produce greater inhibition of nNOS than fraction I.

Kinetics of nNOS inhibition. The initial rate of the enzyme reaction (control and inhibited) was linear over a 30 min time period, as illustrated in Figure 2. Figures 3A and B show the percentage residual activity of nNOS at different levels of inhibition with fractions I and II, respectively, at different CaM concentrations. The results show that fraction II was a more effective inhibitor at lower concentrations (0.25–1.0 mg/mL) when compared with fraction I (1.5–3.5 mg/mL). At fixed CaM concentration, nNOS activity decreased as peptide concentration increased. As CaM concentration increased at fixed peptide concentration, nNOS activity increased, suggesting that inhibition could be overcome by increasing the concentration of CaM; therefore, the peptides most likely interacted with CaM and not the enzyme. These findings are consistent with previous studies on peptide-induced inhibition of CaM-dependent enzymes. Kizawa *et al*. (14) showed a reduction in CaMPDE with increasing concentrations of casein-derived peptides, and and Li and Aluko (15) showed a similar inhibition pattern for CaMKII using pea protein-derived peptides. Also similar to our findings, Doyle *et al*. (10) showed that the rate of inhibition of nNOS by amphibian peptides was reduced in the presence of increasing CaM concentrations.

Figures 4A and B show the double reciprocal plots of the enzyme reaction velocity for nNOS at different peptide concentrations. The results showed that both fractions inhibited nNOS in a mostly noncompetitive manner, with K_i values of 5.97 and

TABLE 1
Amino Acid Composition of Peptide Fractions

Amino acid	Fraction I (%)	Fraction II (%)
Asx (Asp + Asn)	3.61	3.01
Thr	2.59	0.89
Ser	4.40	2.88
Gx (Glu + Gln)	10.67	11.30
Pro	2.36	2.95
Gly	4.75	4.55
Ala	3.24	1.20
Cys	0.34	1.24
Val	5.08	2.60
Met	1.56	1.68
Ile	2.78	2.73
Leu	6.21	4.36
Tyr	1.59	2.86
Phe	4.66	0.91
His	2.03	10.78
Lys	4.70	11.44
Arg	35.65	28.79
Trp	3.76	5.85

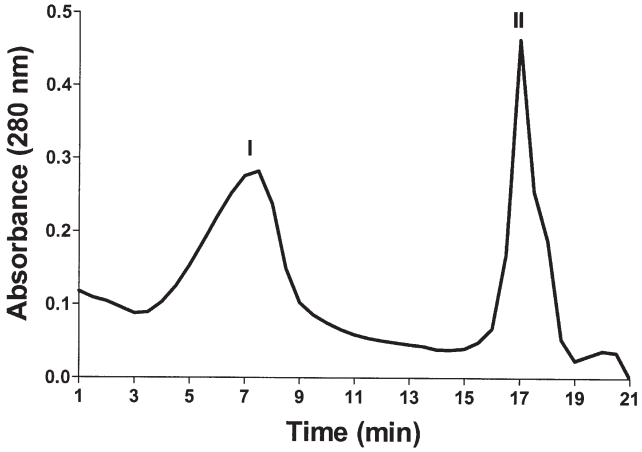

FIG. 1. Fast protein liquid chromatogram showing separation of the flaxseed protein hydrolysate on an SP-Sepharose cation-exchange column using buffers A (0.1 M ammonium acetate, pH 7.0) and B (0.5 M ammonium carbonate). After sample was loaded, the column (58-mL capacity) was washed with 180 mL of 0.1 M ammonium acetate buffer (pH 7.0) to remove unbound peptides. Adsorbed peptides were eluted with a gradient of 0–60% of buffer B at a flow rate of 5 mL/min; 2-mL fractions were collected, and pooled peak fractions were freeze-dried.

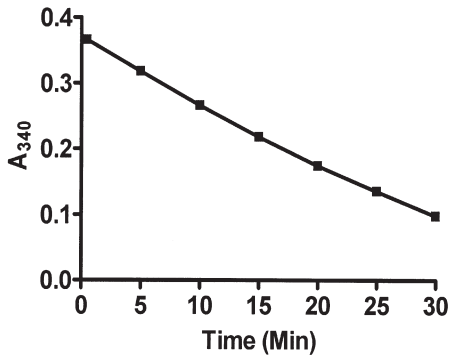

FIG. 2. Change in absorbance of neuronal nitric oxide synthase (nNOS)-catalyzed reaction in the absence of inhibitory peptide fractions as a function of time. Similar linear decreases in absorbance were also obtained in the presence of peptide fractions. Enzyme reaction was carried out at 37°C in a total volume of 2 mL that contained required cofactors in addition to 1 mM L-arginine, 0.1 mM NADPH, calmodulin (CaM), and three units of nNOS, all dissolved in 50 mM HEPES buffer (pH 7.0); progress of enzyme reaction was monitored as the decrease in absorbance at 340 nm for 30 min.

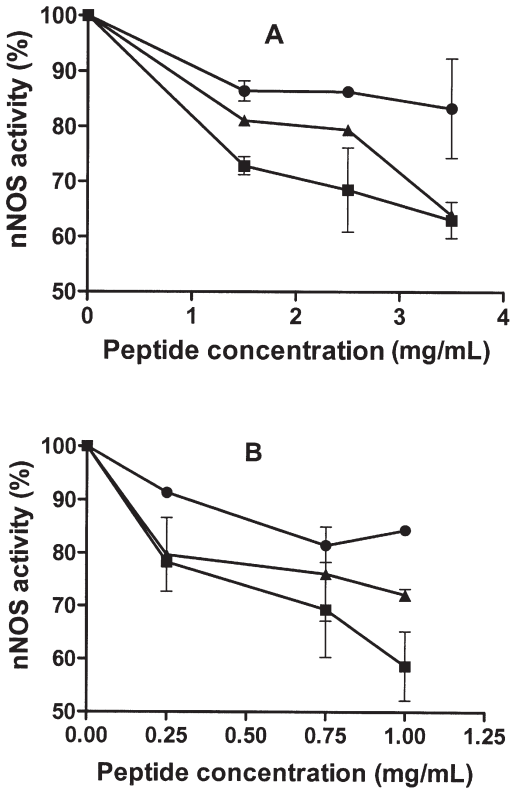

FIG. 3. Effect of peptide concentration on activity of nNOS at different concentrations of calmodulin. See Figure 2 for details of the enzyme reaction. (A) Fraction I (B) fraction II. CaM concentrations (nM): 6.25, ■; 25, ▲; 50, ●. Error bars represent SD. For abbreviations see Figure 2.

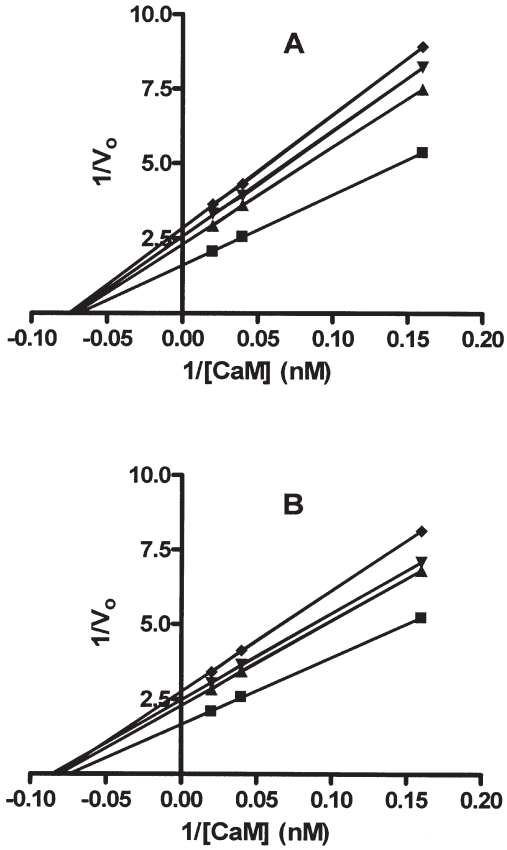

FIG. 4. Lineweaver-Burk plots for the inhibition of nNOS at varying concentrations of CaM and fixed concentrations of inhibitory peptides; V_o is the initial velocity, which is the net decrease in absorbance at 340 nm after 30 min reaction time. (A) Fraction I peptide concentrations (mg/mL): 0.0, □; 1.5, ■; 2.5, ○; 3.5, ●. (B) Fraction II peptide concentrations (mg/mL): 0.0, □; 0.25, ■; 0.75, ○; 1.0, ●. For abbreviations see Figure 2.

2.55 mg/mL for fractions I and II, respectively. The lower K_i for fraction II indicates it is a more potent inhibitor when compared with fraction I, which has a K_i value that is more than twice that of fraction II. Catalytic efficiency (V_{max}/K_m) of the enzyme reaction was greater in the absence of peptides (control = 0.009) when compared with the presence of inhibitory peptides (0.006); this confirms that the enzyme was more active in the absence of the peptides. The higher the inhibitor concentration, the higher the slope, and this suggests a reduction in the ability of CaM to activate nNOS. Noncompetitive inhibition means

TABLE 2
Changes in Intrinsic Fluorescence Properties of Neuronal Nitric Oxide Synthase (nNOS) in the Presence and Absence of Protein Hydrolysate Fractions

Sample	λ_{max} (nm)[a]	F_{max}[a]	F_{max}/F_o
nNOS alone	331 ± 1.4[c]	256.43 ± 2.3[d]	—
nNOS + calmodulin (CaM)	330 ± 0.0[c]	274.31 ± 1.3[d]	1.07
nNOS + CaM + 6.25 µg/mL fraction I	332 ± 0.0[b,c]	381.65 ± 4.5[c]	1.49
nNOS + CaM + 12.5 µg/mL fraction I	334 ± 1.4[b]	414.66 ± 11.6[b]	1.62
nNOS + CaM + 25 µg/mL fraction I	337 ± 0.7[a]	503.21 ± 14.5[a]	1.96
nNOS + CaM + 1.25 µg/mL fraction II	333 ± 0.7[a,b]	312.99 ± 3.9[b]	1.22
nNOS + CaM + 2.5 µg/mL fraction II	334 ± 0.0[a]	532.95 ± 7.5[a]	2.08
nNOS + CaM + 5 µg/mL fraction II	329 ± 0.0[d]	188.42 ± 11.3[e]	0.73

[a]Values are mean ± SD. Excitation wavelength = 295 nm; F_{max} = maximum fluorescence intensity (FI); F_o = FI of nNOS only; λ_{max} = wavelength of maximum FI. Samples that contained CaM also contained 1 mM $CaCl_2$. Each analysis was done in duplicate, and ANOVA and Duncan's multiple-range test were carried out using the Statistical Analysis Systems software, Version 9.1 (Statistical Analysis System, Cary, NC). For each column of F_{max} and λ_{max}, values with different letters are significantly different ($P < 0.05$).

that the inhibitory peptides and nNOS bind to different sites on CaM. It is also possible that peptide binding caused changes in CaM structure such that its ability to properly activate nNOS was reduced. Our results are similar to that of Barnette *et al*. (22), which showed that melittin inhibited the binding of chlorpromazine to CaM in a noncompetitive manner. However, our results differ from that of a previous study by Katoh *et al*. (23) in which melittin inhibited CaM-dependent myosin light chain kinase in a competitive manner with respect to CaM. Li and Aluko (15) also reported competitive inhibition of CaMKII by pea protein-derived peptides, which is different from the noncompetitive inhibition of nNOS observed in this work.

Intrinsic fluorescence of nNOS. Fluorescence studies on the structural characteristics of nNOS in the absence or presence of CaM and peptides were carried out to understand the structural basis for the interaction between enzyme and CaM/peptides complex. Since the excitation wavelength used was specific for only tryptophan, the changes observed are those of the enzyme and peptides because CaM does not have any tryptophan residue. Varying concentrations of both peptides were added to nNOS in the presence of CaM. Since kinetic experiments (Fig. 3) showed that the peptides interacted mostly with CaM, the fluorescence intensity of the CaM/peptide complex (the peptides contain some tryptophan) at 295 nm was subtracted from that of the CaM/peptide/nNOS complex to obtain data that reflect mostly the conformation of the enzyme.

Addition of CaM to nNOS resulted in an increase in fluorescence intensity (FI) as represented by F_{max} (maximum FI) (Table 2). The increase in FI indicates greater exposure of the tryptophan molecules in the nNOS protein complex with CaM when compared with the native enzyme structure. This suggests that the enzyme adopts a globular compact structure in its native state, and its activation by CaM results in a partial unfolding of the enzyme structure to expose previously buried hydrophobic residues. However, there was no change in the wavelength of maximum intensity (λ_{max}), which indicates that binding of CaM did not change the local environment of the tryptophan residues. This finding differs from those of Sheta *et al*. (2) in which there was a reduction in nNOS tryptophan fluorescence on addition of CaM. The observed quenching of tryptophan fluorescence was interpreted as a change in the conformational structure of nNOS and may reflect a folding in the enzyme that switches some tryptophan residues to the internal surface (2).

F_{max} of nNOS increased with addition of fraction I in the presence of CaM. As fraction I concentration increased, F_{max} of nNOS also increased, indicating exposure of more hydrophobic groups in nNOS, especially tryptophan. The ratios of FI of nNOS in the presence or absence of CaM and peptide fractions (F_{max}) to FI of nNOS (F_o) as well as maximum emission wavelength (λ_{max}) values are also shown in Table 2. Addition of fraction I at a concentration of 25 μg/mL produced approximately twofold increases in FI of nNOS (F_{max}/F_o of 1.96) and a shift in maximum emission wavelength from 331 to 337 nm (Table 2). The increase in λ_{max} values at 25 μg/mL of fraction I indicate that apart from unfolding of the native nNOS structure, the tryptophan residues were in a more polar environment when compared with the native state. Interactions of fraction II with nNOS resulted in a different fluorescence pattern when compared with fraction I. In the presence of 1.25 and 2.5 μg/mL of fraction II, the F_{max} and λ_{max} of nNOS also increased, suggesting an unfolding of the enzyme structure and increased polar nature of the tryptophan environment. A fraction II concentration of 2.5 μg/mL increased FI of nNOS by twofold (Table 2), similar to the effect produced by 25 μg/mL of fraction I; therefore, fraction II has an almost tenfold higher unfolding effect than fraction I. The red shift in tryptophan fluorescence at 1.25 and 2.5 μg/mL levels of fraction II is similar to the effect produced by fraction I and indicates increased polarity of the tryptophan environment as the enzyme was unfolded. These results show that binding of CaM led to a rearrangement of nNOS structure such that previously buried tryptophan residues became exposed, demonstrating the optimal level of enzyme unfolding required for activity. The increased FI of the enzyme on addition of the protein hydrolysate fractions indicates excessive unfolding of the enzyme structure beyond the level required for catalysis and may explain the basis for the peptide-induced inhibition of the enzyme. These results are in agreement with a previous finding that showed an increase in the FI of CaMKII as it interacted with CaM in the presence of inhibitory pea protein-derived peptides (24).

However, unlike fraction I, the F_{max} of nNOS was reduced at a higher concentration (5 μg/mL) of fraction II and was accompanied by a blue shift in λ_{max} (Table 2). The results indicate that the tryptophan residues are in more hydrophobic environment in the presence of 5 μg/mL of fraction II when compared with the effects of fraction I and lower concentrations of fraction II. The observed decrease in nNOS FI on addition of 5 μg/mL fraction II indicates a folding of the enzyme such that some tryptophan residues became buried in its globular structure. A plausible reason for this conformational change in nNOS structure at this peptide concentration could be that the water molecules interacted preferentially with the positively charged amino acids in fraction II, thereby reducing the degree of polarity of the enzyme microenvironment. Such an event would increase folding of the enzyme into a more globular structure with reduced FI and a shift of λ_{max} to lower wavelengths.

In conclusion, flaxseed protein-derived cationic protein hydrolysate fractions were able to interact with CaM, which resulted in modulation of activity of nNOS in a noncompetitive way. The effects of the fractions were dependent on the level of positively charged amino acid groups, with high content being favorable to higher inhibition of enzyme activity. The results confirm previous findings that positively charged molecules can bind to CaM and may be used as potential modulators of CaM-dependent enzyme activities. Our results show promise for the use of flaxseed protein as a source for the production of peptides that may be used as ingredients for the formulation of therapeutic foods, especially to manage diseases associated with overproduction of NO.

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