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Kinetics of the inhibition of calcium/calmodulin-dependent protein kinase II by pea protein-derived peptides

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Abstract

Calcium/calmodulin-dependent protein kinase II (CaMKII) catalyzes the phosphorylation of various cellular proteins and excessive activities have been implicated in the pathogenesis of various chronic diseases. We hypothesized that positively charged peptides can be produced through enzymatic hydrolysis of pea proteins; such peptides could then bind to negatively charged calmodulin (CaM) at a physiological pH level and inhibit CaMKII activity. Pea protein isolate was hydrolyzed with an alkaline protease (alcalase) and filtered through a 1000-mol wt cutoff membrane. The permeate, which contained low-molecular weight peptides, was used to isolate cationic peptides on an SP-Sepharose column by ion exchange chromatography. Separation of the permeate on the SP-Sepharose column yielded two fractions with net positive charges that were subsequently used for enzyme inhibition studies. Fraction I eluted earlier from the column and contained lower contents of lysine and arginine than Fraction II, which eluted later. Results show that both peptide fractions inhibited CaMKII activity mostly in a competitive manner, although kinetic data suggested that inhibition by Fraction II may be of the mixed type. Kinetic analysis (K_m and K_i) showed that affinity of peptides in Fraction II for CaM was more than that in Fraction I, which was directly correlated with the higher inhibitory properties of Fraction II against CaMKII. The results suggest that it may be possible to use pea protein-derived cationic peptides to modulate CaMKII activities.

Keywords: Calcium; Calmodulin; Protein kinase II; Peptides; Pea protein isolate; Enzyme kinetics

1. Introduction

Calmodulin (CaM) is a multifunctional calcium-binding protein that is believed to be a major translator of the intracellular calcium message. It is necessary for calcium-dependent control of many cellular events including cell proliferation, cell division and neurotransmission [1]. Thus, agents that inhibit CaM activity can also inhibit these reactions, therefore reducing disease progression. CaM is a negatively charged protein and exposes a hydrophobic surface in its activated state (i.e., when bound to calcium ions) [2]. Therefore, the important structural features for CaM peptide inhibitors are a number of basic amino acids that give a net positive charge and/or a hydrophobic surface [3–5]. In fact, it has been shown that acetylation of insect venom peptides to reduce the number of positive charges led to a reduction in their inhibitory effect on CaM, which suggests that a net positive charge is an important determinant of anti-CaM activity [4]. Other reports have also demonstrated the CaM-inhibitory activities of various peptides and drugs [3,4,6–8].

CaM-dependent protein kinase II (CaMKII) is an enzyme that requires binding of CaM for activity and is responsible for the phosphorylation of various proteins that are involved in critical metabolic reactions. Interaction of CaM results in autophosphorylation of the 50- and 60-kDa subunits of the protein kinase; once autophosphorylated, the enzyme no longer requires calcium or CaM for activity [9–11]. This autophosphorylation is a key point in cellular proliferation and can lead to excessive or abnormal CaMKII activities [12]. Therefore, agents that bind to and decrease or block the interaction of CaM with protein kinase can reduce or eliminate the autophosphorylation process. Such compounds may be useful as therapeutic agents against diseases that involve excessive activities of calcium/CaM-regulated processes.

Apart from drugs, current research efforts have focused on producing compounds such as nutraceuticals that can be

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used against diseases without the need for a physician's prescription. Nutraceuticals are products isolated or purified from food, are generally sold in a medicinal form not usually associated with food and have physiological benefits or provide protection against chronic diseases. Bioactive peptides, especially those that contain only a few amino acid residues, are potential candidates for the formulation of 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 an organism [13]. Interest in nutraceuticals from vegetables has arisen in recent years because of the abundance of plant food proteins. Pea protein isolate with relatively high contents of positively charged amino acids such as lysine and arginine [14] is potentially a good starting material for the production of cationic peptides that can bind to CaM and inhibit CaM-dependent enzyme reactions. However, casein is the only food protein that has been investigated as a source of peptide sequences that have high affinity for CaM [7].

Therefore, the objective of this work was to generate short-chain cationic peptides through enzymatic hydrolysis of pea proteins; the effectiveness of such peptides as anti-CaM agents was then examined using a series of in vitro enzyme assays.

2. Materials and methods

2.1. Materials

Pea protein isolate (85% protein content) was a gift from Parrheim Foods (Portage la Prairie, Manitoba, Canada). Alcalase, pepsin, pancreatin, CaM, reduced nicotinamide adenine dinucleotide (NADH), lactate dehydrogenase, pyruvate kinase, casein (from bovine milk, 5% solution, dephosphorylated and hydrolyzed) and phosphoenolpyruvate were purchased from Sigma (St. Louis, MO, USA). CaMKII was purchased from New England BioLabs (Beverly, MA, USA). Adenosine-5′-triphosphate (ATP) was purchased from Roche Applied Science (Mississauga, ON, Canada). SP-Sepharose column (HiPrep 16/10 SP FF), a strong cation exchanger, was purchased from Amersham Biosciences (Montreal, PQ, Canada).

2.2. Pea protein isolate hydrolysis

Protein hydrolysis was carried according to a previously described method [15] that was slightly modified as follows. An aqueous slurry that contained 5% (wt/vol, protein basis) of pea protein isolate (substrate) was digested with 4% alcalase (wt/wt, substrate protein basis) for 6 h at 50°C and pH 9.0. During digestion, the pH 9.0 level was maintained constant by the addition of 2.0 M of NaOH solution when necessary. After digestion, the pH level was adjusted to 4.0 with 2.0 M of HCl to stop the enzyme reaction and the sample was cooled to room temperature. The sample was then centrifuged at 10,000*g* for 15 min and the supernatant saved. The supernatant was passed through an Amicon stirred cell ultrafiltration setup using a 1000-mol wt cutoff membrane. The permeate obtained was freeze dried and stored at −20°C until used. Protein content of the dried permeate was determined using a modified Lowry method [16].

2.3. Peptide separation

Separation of the ultrafiltration permeate was carried out on an SP-Sepharose FF column using a Bio-Rad automated Biologic LP chromatography system (Bio-Rad, Hercules, CA, USA) according to the method of Kizawa et al. [7] with slight modifications. Prior to sample loading, the column was equilibrated with 60 ml (three column volumes) of 0.1 M of ammonium acetate buffer (pH 7.5). The freeze-dried permeate from ultrafiltration was made into a 140-mg/ml protein solution using 0.1 M of ammonium acetate (pH 7.5) buffer and filtered through a 0.2-μM membrane; an aliquot (1 ml) of the sample solution was injected onto the column. The column was washed with 60 ml of ammonium acetate buffer to remove unbounded peptides. Adsorbed peptides were then eluted at a flow rate of 2 ml/min using a linear gradient between 0 and 0.5 M of ammonium carbonate (pH 8.8) in 0.1 M of ammonium acetate (pH 7.5) buffer; eluted peptides were monitored through absorbance values at 280 nm. Fractions within each peak were pooled and freeze dried.

2.4. Determination of amino acid composition

The freeze-dried pooled fractions were hydrolyzed under vacuum at 110°C for 24 h using 6 M of HCl containing 1% phenol. Amino acid composition of the hydrolysate was determined by high-pressure liquid chromatography according to the method of Bidlingmeyer et al. [17]. The cysteine and methionine contents were determined using performic acid oxidation [18]; the tryptophan content was determined by alkaline hydrolysis [19].

2.5. Enzyme kinetics

CaMKII activity was determined according to the method of Roskoski [20], which couples formation of ADP in the protein kinase reaction to the pyruvate kinase reaction to produce pyruvate. The pyruvate is in turn coupled to the lactate dehydrogenase reaction with concomitant oxidation of NADH to NAD^+; the decrease in absorbance of NADH at 340 nm was used to determine the rate of reaction [20]. The reaction mixture (2 ml) contained 1 mM of $CaCl_2$, 10 mM of $MgCl_2$, 1 mM of dithiothreitol, 12 U of lactate dehydrogenase, 8 U of pyruvate kinase, 200 μM of NADH, 1 mM of phosphoenolpyruvate, 2 mM of ATP, 1 mM of hydrolyzed and dephosphorylated casein, 10–70 μg of CaM and 1 U of CaMKII, all in a MOPS buffer, pH 7.0. All enzymatic reactions were performed at room temperature (24°C).

Control (uninhibited) reaction was performed by mixing CaMKII with CaM and incubated at room temperature for 15 min to promote interaction between the two molecules. After incubation, the CaM/CaMKII mixture

was then mixed with other components of the reaction mixture as described above to initiate autophosphorylation of CaMKII and subsequent catalysis. The rate of decrease in absorbance at 340 nm was used as a measure of enzyme activity.

Inhibited reactions were carried out by first mixing CaM with the appropriate concentration of peptides, and the mixture was incubated at room temperature for 15 min followed by mixing and incubation with CaMKII for another 15 min before addition to the other reaction components. The initial incubation of CaM with peptides was to facilitate CaM–peptide interactions prior to activation of CaMKII activity. Rate of reaction was monitored by recording the decrease in absorbance at 340 nm. The inhibitor (peptide) concentration (milligrams per milliliter) that is required to reduce CaMKII activity by 50% (IC_{50}) was determined by regression analysis of enzyme inhibition (percentage) versus peptide concentration. Type of inhibition was determined using the Lineweaver–Burk double reciprocal plots method and kinetic constants (K_m and V_{max}) were obtained by nonlinear regression fit of the data using GraphPad version 3.0 (GraphPad Software, San Diego, CA, USA). Inhibition constants (K_i) were calculated as the x-axis intercept from a plot of the slope of the Lineweaver–Burk lines against peptide concentration.

3. Results

3.1. Peptide fractionation

Two peptide fractions (I and II) were obtained from fractionation of the protein hydrolysate on a cation exchange column (Fig. 1). The first fraction eluted at approximately 10 min while the second fraction eluted at approximately 27 min after the start of the gradient, which meant that peptide Fraction II was adsorbed to the cation exchanger much stronger than peptide Fraction I was; thus, Fraction II has a higher net positive charge than Fraction I. However, Fraction I was approximately 10 times more abundant than Fraction II, which could be seen from the UV absorption values.

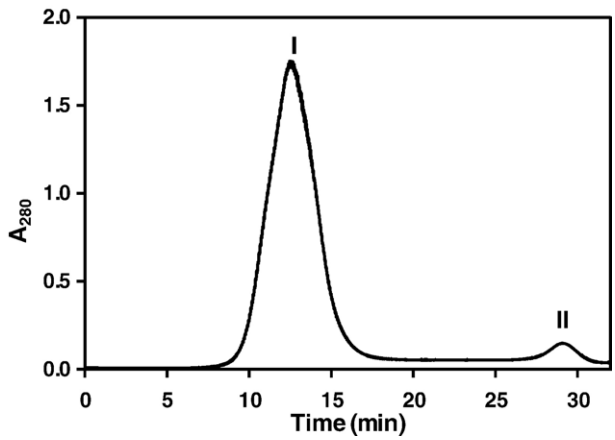

Fig. 1. Separation of cationic peptides on an SP-Sepharose FF column. Details of the protocol are described in Section 2.3.

Table 1
Amino acid composition of peptide fractions

Amino acid	Fraction I (%)	Fraction II (%)
ASx	14.91	6.87
THR	4.46	1.76
SER	5.63	5.20
GLx	24.49	9.33
PRO	5.20	2.64
GLY	4.20	4.47
ALA	3.85	3.99
CYS	1.09	0.32
VAL	4.83	2.88
MET	0.00	0.47
ILE	4.64	2.06
LEU	7.81	6.42
TYR	3.29	3.60
PHE	5.30	6.74
HIS	1.90	1.97
TRP	0.54	0.30
LYS	3.46	15.42
ARG	4.23	25.58

3.2. Amino acid composition

Amino acid compositions of the peptide fractions are shown in Table 1. It was evident that both peptide fractions

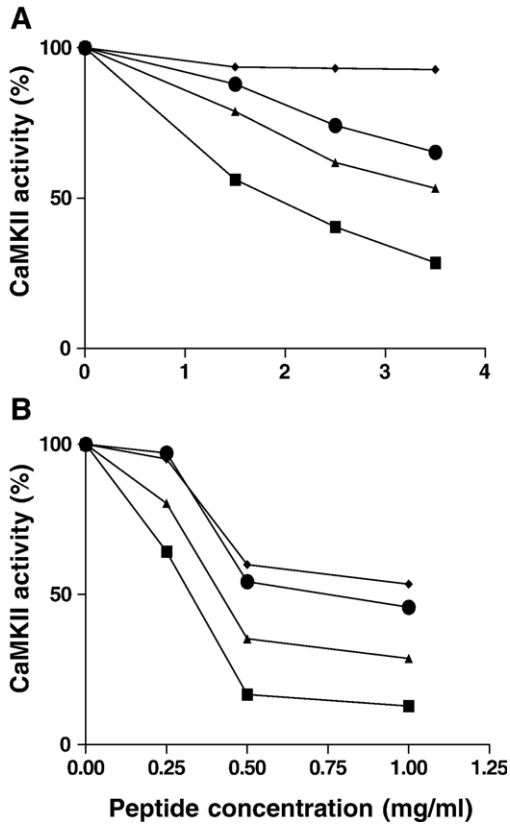

Fig. 2. Effect of peptide concentration on CaMKII activity at different concentrations of CaM. (A) Fraction I. (B) Fraction II. CaM concentrations (μg/ml): 5 (■); 15 (▲); 25 (●); 35 (♦).

have basic amino acids such as lysine and arginine as well as hydrophobic amino acids such as phenylalanine, tyrosine, valine, isoleucine and leucine. However, the levels of lysine and arginine in Fraction II peptides were, respectively, five and six times higher than those found in Fraction I peptides. Fraction I contained higher levels of asparagine+aspartic acid (ASx) and glutamine+glutamic acid (GLx) than Fraction II did; aspartic acid and glutamic acid are acidic amino acids that contribute to the amount of ASx and GLx. Therefore, peptides in Fraction I have more negatively charged amino acid residues as a result of the higher content of glutamic and aspartic acid residues when compared with peptides in Fraction II. Consequently, peptides in Fraction II (higher net positive charges) will be expected to have greater affinity for CaM and inhibit CaMKII to a higher degree than peptides in Fraction I (lower net positive charges).

3.3. CaMKII inhibition kinetics

Fig. 2A and B show the residual percentage activity of CaMKII as a function of peptide concentration for Fractions I and II, respectively, at different levels of CaM. At a fixed CaM concentration, CaMKII activity decreased with an increase in peptide concentration and up to 90% reduction in enzyme activity was obtained using Fraction II. At fixed peptide concentrations, CaMKII activity increased with increased CaM concentration and the inhibition by the peptides could be overcome by high concentrations of CaM. All these data suggest that these peptides compete with CaMKII for the binding sites on CaM; therefore, if the concentration of CaM is high enough, the inhibition of enzyme activity could be abolished.

Table 2 shows the concentration of peptides that reduced enzyme activity by 50% (IC_{50}) for both peptide fractions at different CaM concentrations. Fraction I had an IC_{50} that is greater than that of Fraction II at each CaM concentration, which means that Fraction II has a much stronger inhibitory power than Fraction I. Moreover, the IC_{50} increased (substantially for Fraction I) as the concentration of CaM increased.

Fig. 3A and B show the double reciprocal plots of the velocity of enzyme reaction at different concentrations of inhibitory peptide fractions for Fractions I and II, respectively. The convergence of the lines at approximately the same point on the *y*-axis suggests that the inhibitory activity of the peptide fractions occurred in a mostly competitive manner. The value of the slopes increased at higher concentrations of peptides, which suggested a reduction in the ability of CaM to activate CaMKII. Secondary plots from the double reciprocal results using the slope of the lines versus peptide concentrations were used to estimate the inhibition constant (K_i), which is the intercept of the line on the *x*-axis. Fraction I had a K_i of 0.47 mg/ml while Fraction II had that of 0.019 mg/ml; the smaller the K_i value, the stronger the affinity of the peptide for CaM and the greater the potential reduction in CaMKII activity. The results showed that Fraction II had a higher binding affinity for CaM than Fraction I. The kinetic constants show that Fraction I inhibited the enzyme reaction in a completely competitive manner since the K_m increased with an increase in peptide concentration and the V_{max} remained virtually unchanged (Table 3). The K_m values for inhibition by Fraction II also increased with an increase in peptide concentration; the higher values indicate that Fraction II contained peptides with higher inhibitory potencies than

Table 2
IC_{50} of peptide fractions at different CaM concentrations[a]

CaM (μg/ml)	Fraction I (mg/ml)	Fraction II (mg/ml)
5	2.78	0.24
15	4.00	0.30
25	5.20	0.37
35	22.98	0.40

[a] IC_{50} is the peptide concentration that reduces the activity of CaMKII by 50%.

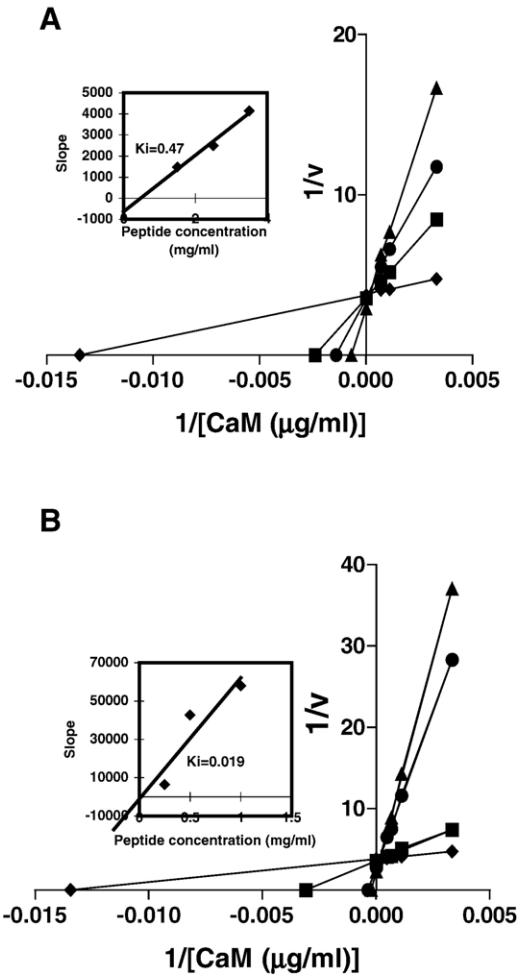

Fig. 3. Lineweaver–Burk plots for the inhibition of CaMKII at varying concentrations of CaM and fixed concentrations of inhibitory peptides. (A) Fraction I peptide concentrations (mg/ml): 0.0 (♦); 1.5 (■); 2.5 (●); 3.5 (▲). (B) Fraction II peptide concentrations (mg/ml): 0.0 (♦); 0.25 (■); 0.5 (●); 1.0 (▲). Insets show the calculations of inhibition constant (K_i).

Table 3
K_m (CaM concentration) and V_{max} values for the inhibition of CaMKII by cationic peptides isolated from pea protein hydrolysate

	Control	Fraction I (mg/ml)			Fraction II (mg/ml)		
		1.5	2.5	3.5	0.25	0.5	1.0
K_m (mM)	0.414	0.415	0.708	1.430	1.796	15.489	25.259
V_{max} (ΔA/min[a])	0.262	0.240	0.239	0.238	0.244	0.154	0.137

[a] Change in absorbance at 340 nm.

Fraction I. However, the V_{max} values for Fraction II were slightly reduced as peptide concentration increased (Table 3), which indicates that the mode of inhibition is not completely competitive and may be slightly different from the mode of inhibition by Fraction I.

4. Discussion

Compared with acid or alkali hydrolysis, enzymatic hydrolysis is the preferred method for producing hydrolysates in nutritional applications [21] (especially as ingredients for the formulation of functional food and nutraceuticals). For example, previous research work have used enzymatic hydrolysis of food proteins to produce bioactive peptides that have physiological activities such as inhibition of angiotensin-converting enzyme [22,23] and immunomodulating, antithrombic, ion binding and opioid properties [24]. In this research, the goal was to produce peptides that possess the ability to inhibit activity of CaMKII, an enzyme that plays an important role in human health and disease. For example, CaMKII has been shown to be involved in one of the critical steps of mitosis, the phosphorylation and activation of tyrosine phosphatase, which triggers cell division [25]. Therefore, high levels of CaMKII activity may result in abnormal cell proliferation, which could lead to the development of tumors. Food protein-derived peptides may serve as potential therapeutic agents to reduce the progression or prevent the development of such abnormal cell divisions. A previous work by Wang et al. [26] also showed that CaMKII is an obligatory requirement for adipogenesis; therefore, inhibition may be a therapeutic tool to reduce excessive accumulation of fatty tissues. To date, only one report has shown the production of CaM-binding peptides from enzymatic hydrolysis of a food protein (casein), which were found to inhibit CaM-dependent phosphodiesterase [7]. Three main casein-derived peptides ranging from 16 to 25 amino acid residues were isolated and evaluated for CaM-binding properties; the large size of these peptides may limit their potential absorption from the gut. In contrast to the work on casein-derived peptides [7], our work was carried out to isolate low-molecular weight (<1000 Da) CaM-binding peptides from an enzymatic digest of pea proteins; the small size of the peptides increases their potential absorption into the blood stream from the intestine. Since a net positive charge has been reported to favor CaM–peptide interactions [3–5], the pea protein hydrolysate was fractionated on a cation exchange column. The profile obtained during separation of the hydrolysate on a cation exchange column indicates that peptides with net positive charges were released during enzyme hydrolysis of pea proteins (Fig. 1). The high contents of basic amino acids such as arginine and lysine, especially in Fraction II (Table 1), are comparable with the results obtained by Kizawa et al. [7], which showed the presence of these amino acids in the CaM-binding peptides isolated from casein digest.

The results showed that the peptide fractions were capable of modulating CaMKII activity to different degrees depending on the amount of CaM that was present in the reaction mixture (Fig. 2). The higher inhibitory activity of Fraction II is consistent with its higher contents of lysine and arginine when compared with Fraction I and confirms previous reports [3–5] that indicated a net positive charge as one of the characteristics of CaM-binding compounds. Fraction II at 1.0 mg/ml was able to reduce the enzyme activity (in the presence of 5 μg/ml of CaM) to less than 10%; at a similar concentration of Fraction I, residual enzyme activity was greater than 50%. The high potency of Fraction II suggests that it has a good potential to exert physiological effects in vivo. At higher CaM concentrations, the level of peptide inhibition was reduced, which is an indication that the peptides compete with the enzyme for similar binding sites on CaM. However, the reduction of potency as CaM concentration increased was more evident for Fraction I where the IC_{50} increased by almost 10-fold when compared with Fraction II where the IC_{50} increased by only less than 2-fold (Table 2).

The ability of peptide fractions to compete with the enzyme for similar binding sites on CaM was further confirmed using double reciprocal plots (Fig. 3). The results are similar to a previous study that found that melittin, a basic peptide found in insect venom, inhibited myosin light chain kinase, another calcium/CaM-dependent kinase, competitively with respect to CaM [27]. It was suggested that melittin inhibited myosin light chain kinase by interacting with a site on the enzyme the same as, or proximal to, the CaM-binding site [27]. Our results are also similar to those of Itano et al. [3], which showed competitive effects of histone during inhibition of CaM-dependent phosphodiesterase. However, our results are different from a previous study [4] that showed that melittin inhibited CaM activity in an uncompetitive way with respect to chlorpromazine, a phenothiazine drug, indicating that melittin and chlorpromazine bind to different sites on CaM. Therefore, it seems possible that the mode of action of inhibitory agents is dependent on the type and/or nature of interactions between

CaM and the target enzyme. An estimate of the binding affinity of the peptides toward CaM was obtained by calculating the inhibition constant (K_i), which also confirms that Fraction II had higher affinity than Fraction I. This is because the lower the K_i value, the higher the ability of the peptides to bind to CaM. The higher binding affinity of Fraction II peptides was also evident in the relatively higher values of K_m when compared with the K_m values obtained for inhibition by Fraction I. Therefore, the amount of CaM that is required to activate the enzyme is consistently higher in the presence of Fraction II than in that of Fraction I.

The peptide products produced in this work have the potential to be used as ingredients in the formulation of functional food and nutraceuticals that can be taken as therapeutic aids by people with chronic diseases arising from excessive activities of CaMKII. This is because food-derived peptides are considered as safer alternatives (fewer side effects) to drugs. Previous work have implicated CaMKII activities in cellular proliferation [12], adipogenesis [26] and, most recently, cardiac hypertrophy [28]. Moreover, CaM antagonists have also been shown to inhibit cell proliferation and are considered as antitumor agents [29–31]. Since the peptides produced in this work showed anti-CaMKII effects, it is reasonable to suggest their potential role as agents in the prevention of metabolic disease symptoms associated with myocardial infarction as well as excessive cellular proliferation and adipogenesis.

In conclusion, the present work has shown that it is possible to produce small-sized peptides with the ability to bind to CaM and to reduce CaMKII activity through alcalase-catalyzed hydrolysis of pea proteins. The results confirmed that peptides with higher contents of positively charged amino acids have greater ability to reduce CaM-dependent enzyme activity when compared with peptides with low contents. The mechanism of inhibition by the peptides is possibly through mutual exclusion of the enzyme from the usual binding site on CaM. However, for Fraction II, it is also possible that binding of the peptides resulted in a change in the conformation of CaM such that subsequent binding to the enzyme was reduced. Spectroscopic analysis of CaM conformations in the presence of inhibitory peptides will be performed to probe the nature of molecular interactions.

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