KINETICS OF THE INHIBITION OF CALMODULIN-DEPENDENT PROTEIN KINASE II BY PEA PROTEIN-DERIVED PEPTIDES

BY

HUAN LI

A Thesis submitted to
The Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Calmodulin (CaM) is a ubiquitous protein present in all living cells and is involved in calcium-mediated activation of various physiologically important enzymes. Agents that bind to CaM can prevent activity of CaM-dependent protein kinase II (CaMKII), an enzyme that has been implicated in the initiation and propagation of chronic diseases. Agents that decrease or block the activity of CaMKII can reduce or eliminate excessive protein phosphorylation and, therefore, minimize or prevent associated pathological conditions. Inhibitors of CaM usually have either a net positive charge or a net hydrophobic character. Therefore, pea protein isolate with high levels of positively charged amino acids was enzymatically hydrolyzed to generate peptides with net positive charges. The peptides were separated on a cation-exchange column to obtain two peptide fractions that differed in level of positive charges. CaM-binding determination showed that fraction #2 had higher contents of basic amino acid residues and almost three times affinity for CaM when compared with fraction #1. Enzyme inhibition kinetics was studied for peptides to see if they could inhibit CaMKII activity. Inhibition by the two peptide fractions followed a competitive manner and fraction #2 had a higher inhibition constant than fraction #1. Fluorescence spectrophotometry and circular dichroism (CD) were used to determine the structural changes of CaM in the presence of enzyme and inhibitory peptides. The results showed that interactions between CaM and inhibitory peptides led to the

unfolding of CaM and exposure of previously buried hydrophobic groups. Peptide #2 was more effective than peptide #1 in causing unfolding of CaM. CD studies showed that the inhibitory peptides reduced the amount of α-helix structure, a conformation that seems to be required for optimum interaction of CaM with target enzymes. Excessive unfolding of CaM by the inhibitory peptides led to increased interaction with CaMKII followed by unfolding of enzyme structure and reduction in enzyme activity. It was concluded that pea protein-derived low molecular weight peptides with net positive charges interacted strongly and caused excessive unfolding of CaM, which reduced the ability of CaM to activate CaMKII; however, this inhibition can be removed by increasing the concentrations of CaM.

ACKNOWLEGMENT

First and foremost, I would sincerely like to thank my advisor, Dr. Rotimi Aluko, who was so kind to take me as his graduate student and provide all the necessary facilities that enable me to finish this research. I particularly appreciate his nice, patience, professional knowledge and friendly atmosphere that gave me lots of help during my work. I also thank my advisory committee members, Dr. R. Przybylski and Dr. T. Beta, for their contribution to the completion and success of my work.

Thank you Dr. J.O.J. O'Neil, Dr. I.J. Oresnik, Dr. P. Dibrov and Dr. Ricky Yada (University of Guelph) for providing some necessary equipments and technical expertise that enable me finish my laboratory work.

I would also like to thank my parents for all their love and support over the two years.

Finally, I want to thank Natural Sciences and Engineering Research Council of Canada for giving the financial assistance.

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

Nutraceuticals are naturally derived, bioactive compounds that have health promoting, disease preventing, or medicinal properties and have an impact on human genes that control cellular metabolisms (Rafi, 2004). Vegetables, fruits, herbs all contain certain nutraceuticals that have these properties and impact. Epidemiologic studies have shown that environmental factors, especially food components, have a major impact on cancer prevention and, a low intake of vegetables is associated with high mortality in cardiovascular diseases (Rissanen et al, 2003, Temple et al, 2003). Thus interests in nutraceuticals from vegetables has arisen

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 (Cho et al, 1998). Thus agents that can inhibit the CaM activity can also inhibit these reactions. CaM is a negatively charged protein and exposes a hydrophobic surface in its activated state, which suggests that important structural features for CaM inhibitors are: a number of basic amino acids which give a net positive charge, and hydrophobic surface. Inhibitors bind to CaM directly to form a CaM-inhibitor complex that is unable to bind and activate corresponding enzymes (Comte et al, 1983), therefore, the CaM-dependent

processes are blocked.

CaM-dependent protein kinase II (CaMKII) is an enzyme that is dependent on CaM for activity. It can be activated by binding to calcium and CaM, and promote autophosphorylation of its two subunits (Schoworer et al, 1986). This autophosphorylation is a key point in cellular proliferation which can be caused by excessive or abnormal activity of CaMKII (Li et al, 1992). So agents that can decrease or block the activity of CaMKII can decrease or eliminate the autophosphorylation process and therefore, minimize or prevent cellular proliferation diseases such as cancer.

Pea (Pisum sativum *L.*) has a high content of protein, which can be hydrolyzed to yield peptides containing large high level of basic amino acids with net positive charges such as lysine and arginine, as well as amino acids with hydrophobic surface such as phenylalanine and tyrosine (Hudson, 1991). This makes it possible to use pea protein-derived peptides to inhibit CaM activity and, therefore, inhibit CaM-dependent processes such as cell proliferation.

1.1 OBJECTIVES

1. To optimize hydrolysis of pea protein by alcalase to produce potent CaM-binding peptides.
2. To determine the kinetics of CaMKII inhibition by CaM-binding peptides.
3. To determine the quantitative structure-activity relationships of CaM-binding peptides with respect to inhibition of CaMKII.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 PEA PROTEINS

The development of protein products with high nutritional value has become a major focus of research worldwide. Seeds from the pea crops, with relatively high contents of total protein (Fan et al., 1994), have drawn increasing attention in recent years. However, peas also contain a variety of antinutritional and antiphysiological factors which may negatively influence its protein quality (Fredrikson et al., 2001). Therefore, it is important to produce high-quality pea protein isolate with improved nutritional properties. The technology for production of pea protein concentrates and isolates has been well studied and developed (Adsule et al., 1989). The protein content of pea seeds is between 18 to 30% (Schroefer, 1982; Gueguen & Barbot, 1988). The pea proteins are composed mainly of albumins and globulins as the major storage proteins. The pea proteins are deficient in tryptophan as well as sulfur-containing amino acids such as cysteine and methionine. In contrast, the levels of lysine and arginine, which have positive charges, are quite high. The amounts of aspartic acid + asparagine and glutamic acid + glutamine are also high as most of the other seed storage proteins. The nutritional value of pea protein has been the subject of less study compared with other food proteins. Mariotti and his research team have demonstrated that albumin and globulin fractions of pea protein were of good nutritional value for

humans (Mariotti et al., 2001), and some nutritional values have also been previously reviewed (Savage and Deo, 1989).

Table 2.1 Amino acid composition (grams of amino acid per 16 g of nitrogen) of pea proteins *

	Protein Fraction	
	Albumin	Globulin
ASX	11.90	12.99
THR	5.66	3.34
SER	5.03	5.30
GLX	14.95	18.66
PRO	4.46	4.36
GLY	5.97	3.89
ALA	5.85	3.97
CYS	3.15	0.80
VAL	4.41	4.73
MET	1.34	0.70
ILE	3.86	4.59
LEU	4.87	8.23
TYR	4.71	3.37
PHE	4.52	5.40
TRP	1.47	0.67
LYS	9.34	6.41
HIS	2.63	2.55
ARG	5.67	8.00

* Gueguen (1991)

ASX = aspartic acid + asparagine

GLX = glutamic acid + glutamine

2.2 PROTEIN HYDROLYSIS AND PROTEIN HYDROLYSATES

Natural food proteins can be modified by enzyme hydrolysis to change their structures. The purpose is to create new products with better functional and physiological properties (Hamada, 1992). Protein hydrolysates are mixtures of oligopeptides, polypeptides and free amino acids. They are easily available protein sources used in dietary or medicinal therapies (Schmidl et al., 1994). They are also made into 'formula' diets for children and elders, or athletes. Because of their solubility, they are also used in beverages and fruit juices (Frokjaer, 1994).

Enzymes are biocatalysts that accelerate or induce the rate of chemical reactions; they act on a limited number of substrates and will catalyze only one specific type of reaction (Howell, 1996). Enzymes catalyze the hydrolysis of peptide bonds in protein to give rise to products with lower molecular weight than the initial substrate. Compared with chemical modification, enzyme hydrolysis can be used at a moderate temperature and have less side effects. Thus, enzyme hydrolysis is preferred by most of researchers.

Alcalase belongs to the serine proteases family. The serine proteases have their maximum activity at alkaline pH (Ward, 1983), and the catalytic activity is pretty well understood in its basic features (Polgar and Halasz, 1982). Alcalase is very soluble at most concentrations of use and the typical pH range for catalysis is 6 to 10. The enzyme cuts peptide bonds where the carboxyl group is donated by a hydrophobic amino acid, and has been observed to have high specificity for

aromatic (Phe, Tyr, Trp) and basic (Lys) residues (Adamson, 1996; Doucet, 2003).

Protein hydrolysis can only be carried out when the four hydrolysis parameters have been specified, which are the substrate concentration, the enzyme-substrate ratio, temperature and pH. These four parameters are the major determinants of how fast the hydrolysis reaction proceeds, as well as other aspects of the reaction such as yield of and amino acid composition of the peptides.

The substrate concentration usually refers to the concentration of the original protein, because in the whole process both original protein and soluble peptides formed during the initial stages may serve as the substrates in the further hydrolysis reaction to produce smaller peptides. The 'true' value of the substrate concentration during the reaction can usually not be assessed (Nissen, 1985). The enzyme-substrate ratio, which is the concentration of enzyme relative to the concentration of substrate, is more important than the enzyme concentration alone in the reaction. Effect of enzyme-substrate ratio on hydrolysis of pea protein isolate has been studied (Karamac et al., 2002). It has been observed that pea protein isolates, *Pisane* and *Propulse*, when hydrolyzed by trypsin, achieved highest degree of hydrolysis (DH) at enzyme/substrate ratios of 35 mAU/g and 15 mAU/g, and temperature of 45 and 50°C, respectively. In most protein hydrolysis process the reaction is stopped by inactivating the enzyme through a heat treatment, a change of pH or combination of both (Nissen, 1985). The soluble and insoluble parts of the hydrolysates can be separated by centrifuge, or the

hydrolysates can be separated according to their molecular weights using a membrane. The separated fractions usually have different functions from the mixture.

Bioactive peptides derived from food proteins have received lots of attention because of their potent physiological effects. A hexapeptide, ovokinin (RADHPF), derived from ovalbumin, was found to contain anti-hypertensive properties through inducing nitric oxide mediated vasorelaxation (Matoba et al., 2001). Oral administration at 10 mg/kg lowered the systolic blood pressure in rats. Some other peptides obtained after digestion of ovalbumin by trypsin and chymotrypsin displayed anti-microbial activity (Pellegrini et al., 2004). These bioactive peptides included SALAM (residues 36-40), SALAMVY (residues 36-42) YPILPEYLQ (residues 111-119), ELINSW (residues 143-148), NVLQPSS (residues 159-165), AEERYPILPEYL (residues 127-138), GIIRN (residues 155-159) and TSSNVMEER (residues 268-276). Peptides derived from milk proteins, such as α_{s1}-casokinin-10, immunopeptides β –CN(f63-68) and β –CN(f191-193), have immunomodulatory effects. Casein-derived peptides, such as β–casokinin-7 and α_{s1}-casokinin-5, are potent angiotensin converting enzyme (ACE) inhibitors, thus can regulate high blood pressure. Several peptides derived from milk, such as caseinphosphopeptides β–CN(f1-25)4P and α_{s1}–CN(f59-79)5P, can also act as mineral-binding carriers that help mineral transportation, especially calcium (Meisel, 1997). Nutraceutical and functional food both have health-promoting,

disease-preventing, or medicinal properties, while the former one is isolated from foods and the later one is similar to a conventional food. Health Canada has proposed the following definitions: (i) Functional foods are products that may look like or be a conventional food and be consumed as part of a usual diet, but have physiological benefits or can reduce the risk of chronic disease beyond basic nutritional functions; (ii) Nutraceuticals are products isolated or purified from food, generally sold in a medicinal form not usually associated with food and has physiological benefit or provides protection against chronic disease. Therefore, bioactive peptides can be isolated from foods to make nutraceuticals, or incorporated with foods to produce functional foods.

2.3 CALMODULIN: STRUCTURES AND PROPERTIES

Calmodulin (CaM) is a small Ca^{2+}-binding protein that acts to transduce second messenger signals into a wide array of cellular responses (Zielinski, 1998). It acts by binding to short peptide sequences within target proteins, leading to structural changes which alter the activity of the proteins.

Vertebrate CaM has 148 amino acids with a molecular weight of about 16.7 kDa. The typical amino acid sequence of CaM is shown in Figure 2.1. The sequence shown in Figure 2.1 suggests the hydrophobic and charge-charge interactions between CaM and peptides. CaM comprises lots of hydrophobic residues, Phe (8), Leu (8), Ile(7) and Val(7), as well as acidic residues such as Asp (17) and Glu (21).

CaM is an acidic protein and has net negative charges at physiological pH (7.0-7.3). It has four isoforms, with isoform 1 being the major isoform and the only isoform found in animals. Isoforms 2, 4, 6 are found in plants. It has four functional EF-hands motifs (helix-loop-helix) that bind Ca^{2+} and are named I, II, III and IV (underlined sequences in Fig. 2.1). Sites I and II combine to form the globular domain of N-terminal, and III and IV sites form globular domain of C-terminal. The two globular domains are separated by a short flexible linker (Chin & Means, 2000). Sites III and IV have a 10-fold higher affinity for Ca^{2+} than sites I and II. Figure 2.2 shows the Ca^{2+}-regulated conformational changes of CaM.

X-ray crystallography revealed the CaM structure as a dumbbell-like shape with an extended, solvent-exposed α–helical region joining the two globular domains (Babu, 1988; Kretsinger, 1986), with the EF-hand domains embedded within two separate globular regions. NMR and biochemical studies revealed that CaM is a more globular shape (Persechini, 1988; Barbato, 1992). The current view is that Ca^{2+}-loaded and apo-CaM can adopt a variety of conformations that are determined by the shape of the central α-helix, which acts as a region of variable expansion and contraction to allow CaM to bind different protein targets (Kuboniwa, 1995; Zhang, 1995; Persechini, 1988).

In the absence of Ca^{2+}, the N-terminal domain adopts a 'closed' conformation while the C-terminal domain adopts a 'semi-open' conformation in which a partially exposed hydrophobic pocket is accessible to solvent (Chin & Means, 2000).

Figure 2.1 Amino acids sequence of CaM.

ADQLTEEQIAEFKEAFSLF

DKDGDGTITTKELGTVMR

SLGQNPTEAELQDMINEV

DADGNGTIDFPEFLTMMARK

MKDTDSEEEIREAFRVF

DKDGNGYISAAELRHVMTN

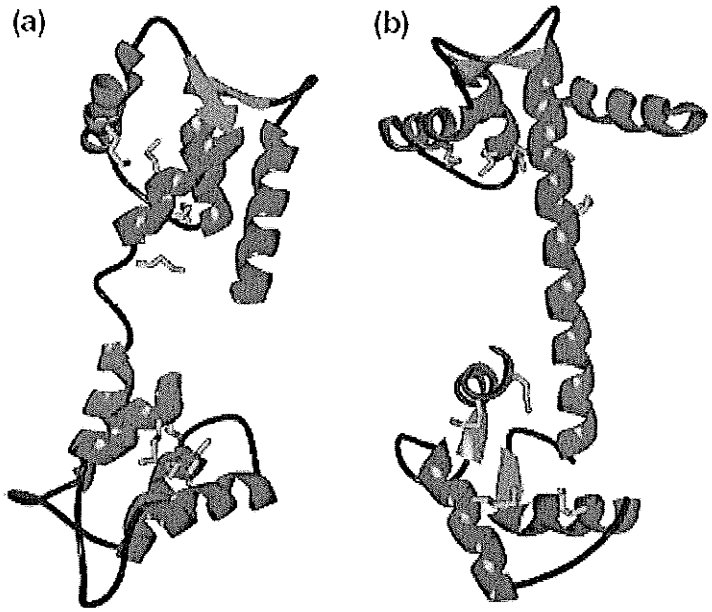
LGEKLTDEEVDEMIREA

DIDGDGQVNYEEFVQMMTAK

Figure 2.2 The Ca^{2+}-regulated conformational changes in CaM*, with N-terminal on top.

(a) structure of Ca^{2+}-free (apo) CaM

(b) structure of Ca^{2+}-CaM



*: Chin & Means (2000).

This enables CaM to interact with some protein targets at resting levels of intracellular free Ca^{2+}. When binding the Ca^{2+}, the EF-hands pull away from one another and expose a hydrophobic pocket within the domains, which enables CaM to recognize the binding domains of target proteins (Chin & Means, 2000; Hook et al., 2001). Ca^{2+} binding to CaM not only induces local conformational changes within the EF-hands, but also causes a global structural change where the α–helical content of the protein is increased (Dedman et al., 1977; Hennesey et al., 1987). The CaM-binding domains on the target proteins are usually 9-26 residues in length.

CaM is expressed in all eukaryotic cells where it participates in signaling pathways that regulate many processes such as proliferation, growth and movement (Chin & Means, 2000). It constitutes at least 0.1% of the total protein present in cells (10^{-6} M - 10^{-5} M), and even higher in rapidly growing cells, especially those undergoing cell division and differentiation (Chin & Means, 2000).

CaM can bind to various targets, which potentiates various biological reactions that influence activities of the cells, tissues and organs. Inhibition of CaM activity can therefore, inhibit many important cellular reactions. Basic polypeptides and proteins, such as polylysine (D and L), histone H2B, myelin basic protein, melittin, apamin and mastoparan have been found to inhibit phosphodiesterase through inhibition of CaM activity (Itano et al., 1980; Barnette et al., 1983). The wide variety of basic polypeptides and proteins that inhibit CaM activity indicates that

these interactions are not specific. Peptides from casein hydrolysates have also been found to inhibit CaM-induced phosphodiesterase activity by binding to CaM (Kizawa et al., 1995; Kizawa, 1997). The common features for these peptides were the presence of basic and hydrophobic groups in the primary structure. Some neuropeptides such as β–endorphin, adrenocorticotrophic hormone and dynorphin also have inhibitory effects on CaM. Drugs that are potent CaM inhibitors have been studied to alter CaM activity for potential use in clinical applications to treat human diseases (Weiss et al., 1982). The most potent of these CaM inhibitors include certain antipsychotic drugs, smooth muscle relaxants, α-adrenergic blocking agents and neuropeptides (Wiess et al., 1982). These compounds all carry positive charges at physiological pH and have hydrophobic groups. For example, the diphenylbutylpiperidiners (e.g. pimozide) and phenothiazines (e.g. chlorpromazine) both contain two aromatic rings which are hydrophobic regions. Butyrophenones (e.g. haloperidol) contains one aromatic ring and thus less potent. These drugs influence central nervous system, neuroendocrine system, gastrointestinal system and cardiovascular system. Some of these actions can be explained by the inhibition of CaM (Weiss et al, 1980). Most of the potent inhibitors were those that were positively charged at neutral pH and contained large hydrophobic regions. Some cancer chemotherapeutic agents also showed CaM inhibition activity (Watanabe et al., 1979; Katoh et al., 1981). Table 2.2. shows some of the CaM inhibitors and the

enzyme they inhibit. Some of them have been shown to have physiological impact on humans.

The role CaM plays in biology suggests that inhibition of this protein may provide a wide field for clinical and nutritional applications.

Table 2.2. CaM inhibitors and inhibited CaM-dependent enzymes.

Name	Enzyme Inhibited	Physiological Impact	References
α_{S2}-casein(164-179) α_{S2}-casein(183-206) α_{S2}-casein(183-207)	CaM-induced phosphodiesterase		Kizawa et al., 1995
polylysine (D & L) polyarginine (L)	CaM-induced phosphodiesterase		Itano et al., 1980
histone H1 histone H2A histone H2B histone H3 histone H4	CaM-induced phosphodiesterase		Itano et al., 1980
protamine myelin basic protein	CaM-induced phosphodiesterase		Itano et al., 1980
melittin, apamin mastoparan	CaM-induced phosphodiesterase		Barnette et al., 1983
Vinblastine	CaM-induced phosphodiesterase	cancer preventive	Watanabe et al., 1979
Adriamycin	CaM/calcium protein kinase adenylate cyclase	cancer preventive	Katoh et al., 1981 Brostrom et al., 1977
Chlorpromazine	myosin light chain kinase	anti-psychotic	Hidaka et al., 1979
Trifluoperazine	phospholipase A2 phosphorylase b kinase	anti-psycotic	Wong et al, 1979 Walsh et al, 1980
Prenylamine	Calcium-stimulated protein kinase	Smooth muscle relaxed	Hidaka et al., 1980

2.4 CALMODULIN/CALCIUM-DEPENDENT PROTEIN KAINSE II: STRUCTURES, ACTIVITIES AND ROLES IN CLINICAL DISEASES

Among the CaM/Ca^{2+}-dependent enzymes, protein kinases are a prominent class of proteins that alter the function of key cellular proteins throughout the cell by phosphorylation. CaM/Ca^{2+}-dependen protein kinase II (CaMKII) is one of the most studied proteins among these kinases.

CaMKII comprises a family of isoforms derived from four closely related genes, $\alpha,\beta,\gamma,\delta$. It is highly expressed in brain and neurons; in cells, it is localized in cytoplasm. CaMKII requires CaM/Ca^{2+} for activity and after binding to CaM/Ca^{2+}, the enzyme undergoes an important autophosphorylation, a process that regulates its dependence on and affinity for the activator as well as its intracellular targets (Hudmon & Schulman, 2002). CaMKII is a Serine/Threonine protein kinase like other CaM/Ca^{2+}–dependent kinases, which means it phosphorylates serine and threonine positions on the substrates. Substrates phosphorylated by CaMKII are involved in many cellular functions, such as cell proliferation and apoptosis, membrane excitability, neurotransmitter synthesis and release, intracellular calcium homeostasis, etc (Hudmon & Schulman, 2002).

Basal activity of CaMKII is 10-100 folds below its maximal activity. CaMKII is activated by CaM/Ca^{2+} through displacement of an autoinhibitory domain from the active sites (LeVine et al., 1986; Kwiatkowski et al., 1989; Yamagata et al., 1991). Activation is achieved by relieving an inhibitory constraint rather than by stabilizing

a conformation of the kinase that has higher activity (Hudmon & Schulman, 2002). ATP-binding pocket and the protein substrate-binding site are blocked by the autoregulatory domain at the inactivated state.

CaM/Ca^{2+} binding disrupt the interaction of autoinhibitory domain and the ATP and protein substrate-binding domains. After binding to calcium, CaM undergoes conformational changes which increase its affinity for CaMKII (O'Neil et al., 1990; Hook et al., 2001). By reacting and releasing some of the residues from their autoinhibitory domains, CaM/Ca^{2+} activate the active site of the CaMKII. Half maximal activation of CaMKII occurs at 0.5-1.0 μM free calcium (Rostas et al., 1992). At saturated calcium level, half maximal activation of CaMKII occurs at 25-100 nM CaM concentration (Brickey et al., 1990; Kotah et al., 1991). However, once activated CaMKII-based catalysis is independent of CaM/Ca^{2+} concentrations.

Following CaM/Ca^{2+}-binding, CaMKII requires ATP for autophosphorylation; affinity of ATP for CaMKII is directly influenced by the CaM/Ca^{2+} complex. The binding affinity of ATP can be determined by measuring the rate of autophosphorylation. Previous works have demonstrated that CaM/Ca^{2+}-binding to CaMKII enhanced ATP binding (Hanson et al., 1992; Colbran, 1993). It is considered that conformational changes in the ATP-binding domain as well as the CaM/Ca^{2+}-binding domain within the CaMKII occur at the molecular level, possibly via elimination of the autoinhibitory domain, which enhances reciprocal binding of

the molecules (Hudmon & Schulman, 2002).

After binding to CaM/Ca^{2+} and ATP, CaMKII undergoes autophosphorylation and becomes an active enzyme that does not require CaM/Ca^{2+} for converting the substrate to the product. CaMKII undergoes autophosphorylation at several different sites, among which phosphorylation of threonine 286 (Thr286) is the best explored and understood so far. Phosphorylation of Thr286 enables CaMKII to become autonomous of calcium, as calcium declines to baseline level and CaM/Ca^{2+} complex dissociates from CaMKII. Secondly, phosphorylation of Thr286 increases the affinity of CaMKII for CaM/Ca^{2+} by 1000 times, which is called CaM trapping. Finally, this autophosphorylation exposes another site on CaMKII that allows its binding to other proteins, especially substrates (Hudmon & Schulman, 2002).

After autophosphorylation, CaMKII becomes CaM/Ca^{2+}–independent and thus CaM/Ca^{2+} complex dissociates from CaMKII; this dissociation initiates phosphorylation of Thr305 and Thr306 in the CaM binding site, which blocks rebinding of CaM/Ca^{2+} (Hudmon & Schulman, 2002).

Protein phosphorylation is related to many proliferation diseases such as cancer, inflammatory diseases, metabolic diseases and neurological diseases (Lu, 2004, Rickle et al., 2004, Kammer et al., 2004). CaMKII autophosphorylation leads to phosphorylation of membrane proteins and is a key point in undesirable cellular proliferation, which can be caused by excessive or abnormal activity of CaMKII (Li

et al., 1992). Although the studies are limited, previous researches have shown that excessive phosphorylation of cellular proteins is related to development of cancer, cardiovascular diseases, obesity and Parkinson's disease. In breast cancer cells, CaMKII was found to be associated with ß1 integrin, leading to dissociation of actin from integrin, which caused poor adhesion of human cancer cells (Kazuhide, 2001); poor adhesion is directly related to migration, i.e., metastasis of the cancer cells to other organs. CaMKII was found to regulate proliferation of small cell lung carcinoma (SCLC) cells and was sensitive to the anti-proliferative effects of KN-62 (1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenyl piperazine), which was an antagonist of the kinase (Williams et al., 1996). Incubation of SCLC cells with KN-62 potently inhibited DNA synthesis, and slowed progression through S phase. Such effects were also observed in SK-N-SH human neuroblastoma cells and K562 human chronic myelogenous leukemia cells, which both expressed CaMKII (Williams et al., 1996). Apart from cancers, CaMKII was also found related to the pathogenesis of some cardiovascular diseases. It was observed to directly phosphorylate cytosolic phospholipase A2, which resulted in vascular smooth muscle cells (VSMC) proliferation. Inhibition of CaMKII activity by CaM inhibitor KN-93 led to decreased blood pressure and ameliorated kidney vascular pathology (Mubarack, 2002). Other studies also support the fact that CaMKII plays a role in the cardiovascular diseases (Hagemann et al., 2001; Yousif et al.,

2003; Kirchhof et al., 2004). Studies also indicated that abnormal CaMKII autophosphorylation plays a causal role in the alterations of striatal plasticity and motor behavior that follow dopamine denervation. Normalization of CaMKII activity may be an important underlying mechanism of the therapeutic action of L-DOPA in Parkinson's disease (Picconi et al., 2004).

Therefore, agents that can decrease or block the activity of CaMKII can decrease or eliminate the autophosphorylation process and thus, minimize or prevent cellular proliferation diseases such as cancer. By far, only one paper using peptides derived from food protein to inhibit a CaM-dependent enzyme has been published. This study was done by Kizawa and his colleagues (Kizawa et al., 1995). They used peptides derived from pepsin digest of α–casein and the CaM-dependent enzyme was cyclic nucleotide phosphodiesterase. Their results showed that these peptides from food could inhibit CaM-dependent process of cellular regulation. So it seems that such peptides could be taken daily as therapeutic agents against diseases that are due to excessive activity of CaMKII. However, in order for the peptides to have therapeutic effects they must escape degradation by proteases in the gastrointestinal tract and be absorbed intact into the blood circulatory system.

CHAPTER THREE

3.0 ENZYME HYDROLYSIS AND PEPTIDES FRACTIONATION

3.1.0 ENZYMATIC HYDROLYSIS OF PEA PROTEIN ISOLATE

3.1.1 INTRODUCTION

Protein hydrolysis can be accomplished with enzymes, acid or alkali, but enzymatic hydrolysis is preferred over strictly chemical methods for producing hydrolysates in nutritional applications (Lahl and Braun, 1994). Enzymatic hydrolysis is preferred because it produces hydrolysates with well-defined peptide profiles. On the other hand acid and alkali hydrolysis can destroy L-form amino acids, produce D-form amino acids and can form toxic substances like lysino-alanine (Finot et al., 1978). Peptides produced by enzymatic hydrolysis of food proteins have also been shown to have physiological activities such as inhibition of angiotensin converting enzyme, a principal causative agent of hypertension (Yamamoto, 1997; Kim et al., 2001). Other types of physiologically active food-derived peptides include those that have immunomodulating, antithrombic, ion binding, and opioid properties (Meisel, 1997). A novel class of peptides can be produced to inhibit activity of calmodulin (CaM), a ubiquitous protein that is involved in the regulation of most cellular processes.

The rate of protein hydrolysis and peptide composition of the hydrolysate is dependent parameters such as the substrate concentration, enzyme-substrate ratio, pH and temperature. Thus, different combinations of the four parameters

can be used to optimize protein hydrolysis and produce tailor-made peptides. The enzyme-substrate ratio is the concentration of enzyme relative to the concentration of substrate and is usually more important than the enzyme concentration. Enzyme-substrate concentration can be simply expressed as weight percentage. During the hydrolysis reaction, the substrate concentration, the enzyme-substrate ratio and pH may change from their initial values, so the specific values of the substrate concentration and the enzyme-substrate ratio usually refer to the initial values, and the pH can be kept at the fixed value during the whole reaction. Alcalase is a proteolytic enzyme that has broad substrate specificity and cut peptide bonds where the –COOH group is donated by a hydrophobic amino acid. It is a water-soluble alkaline protease with typical application within the pH range of 6 to 10. Soluble peptides formed at the initial stage of hydrolysis can be further degraded into smaller peptides as the reaction progresses. The alcalase-catalyzed reaction can be stopped by a change of pH to acid conditions (less than 5.0) or by heat treatment, or a suitable combination of the two. The inactivated reaction solution can be further processed by centrifugation to separate the soluble part and insoluble part, or by filtration to separate peptides according to molecular size using a specific molecular weight cut-off (MWCO) membrane. The objectives of this project were to:

1. Optimize the enzymatic hydrolysis of pea proteins with alcalase to produce low molecular weight cationic peptides that will pass through a 1,000 MWCO

membrane.

2. To obtain calmodulin-binding peptides by fractionating the low molecular weight (LMW) cationic peptides according to charge density on an ion-exchange column.

3.1.2 MATERIALS

Pea protein isolate (85% protein content) was a gift from Parrheim Foods, Portage la Prairie, Manitoba. Alcalase was purchased from Sigma Chemicals (St. Louis, MO, USA), while ultrafiltration membranes (1,000 MWCO) and other analytical reagents were obtained from Fisher Scientific (Oakville, ON).

3.1.3 METHODS

3.1.3.1 Enzymatic hydrolysis of pea protein isolate: This process involved the use of alcalase at different enzyme/substrate ratios and different hydrolysis time. The substrate concentration, the enzyme/substrate ratio and the hydrolysis time were based on previous works by various researchers. There were 7 different combinations of substrate concentration, enzyme/substrate ratios and hydrolysis time. 1) 5% (w/v) substrate, 4% (w/w) enzyme, 6h; 2) 5% (w/v) substrate, 4% (w/w) enzyme, 2h; 3) 5% (w/v) substrate, 4% (w/w) enzyme, 4h; 4) 5% (w/v) substrate, 2% (w/w) enzyme, 6h; 5) 5% (w/v) substrate, 6% (w/w) enzyme, 6h; 6) 3% (w/v) substrate, 4% (w/w) enzyme, 6h; 7) 8% (w/v) substrate, 4% (w/w) enzyme, 6h. A slurry of the pea protein isolate was prepared in distilled water and adjusted to pH

9.0 with 2.0 M NaOH solution. Then the slurry was heated to 50°C and a fixed concentration of alcalase added. The sample was hydrolyzed at 50°C for a fixed time while maintaining constant pH at 9.0 by addition of NaOH if necessary. After digestion, the pH was adjusted to 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 10,000g for 15 minutes and the supernatant saved. The supernatant was passed through Amicon stirred cell ultrafiltration set-up using 1,000 molecular weight cut-off membrane. The permeate obtained was freeze-dried and stored at -20°C until used.

3.1.3.2 Protein content determination: Modified Lowry method was used to determine protein content (Markwell et al., 1978). Three reagents were used. Reagent A included 2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate, 1% sodium dodecyl sulfate. Reagent B was 4% $CuSO_4.5H_2O$. Reagent C included 100 part of reagent A with 1 part of reagent B. 2.0N Folin-Ciocalteu phenol reagent was diluted to half concentration using distilled water. Sample of 1 ml was prepared containing 10-100 µg of protein concentration. Reagent C of 3 ml was added to the sample and incubated at room temperature for 1h. Diluted Folin-Ciocalteu phenol reagent of 0.3 ml was added to the mixture and mixed vigorously immediately using a vortex. The mixture was kept at room temperature for 45 minutes and the absorbance at 660 nm measured. Absorbance was measured at room temperature using 1 cm pathlength cuvettes on a spectrophotometer

(MILTON ROY, SPECTRONIC 3000 ARRAY). Standards containing 10-100 µg/ml bovine serum albumin were prepared and measured following the same steps. Triplicate determinations were used to calculate protein concentrations of samples.

3.1.4 RESULTS AND DISCUSSION

The 1,000 MWCO membrane was used to isolate the low molecular weight (LMW) peptides from the protein hydrolysate; the LMW peptides can be absorbed from the small intestine at a higher rate when compared to the high molecular weight peptides (Grimble et al., 1987, Roberts et al., 1994).

Real sample concentrations were obtained from standard curves using the measured absorbance. Protein content was calculated using equation,

Protein Content = (real sample concentration / weighed sample concentration) x 100%.

Real sample concentration refers to the average value of the concentrations of the sample obtained from the standard lines (shown in appendix). Weight sample concentration refers to the sample concentration that was calculated from weighed amount of sample. Average crude freeze-dried protein concentration was 71.64%.

In conclusion, pea protein isolate could be hydrolyzed to yield high concentration of peptides with molecular weights that are less than 1,000 Da.

Table 3.1.1. Mean absorbances of bolvine serum albumin at different concentrations.

Bovine serum albumin concentration (μg/ml)	Absorbance (660 nm)
10	0.042
20	0.086
30	0.131
40	0.176
50	0.221
60	0.257
70	0.305
80	0.348
90	0.386
100	0.434

Table 3.1.2. Mean absorbances of samples at different concentrations.

Weighed sample concentration (µg/ml)	Absorbance (660 nm)
10	0.025
20	0.058
30	0.095
40	0.136
50	0.168
60	0.199
70	0.235
80	0.249
90	0.277
100	0.304

3.2.0 CALMODULIN BINDING ACTIVITY AND STABILITY OF HYDROLYSATE TO GASTRIC ENZYMES

3.2.1 INTRODUCTION

CaM is negatively charged in physiological conditions and has many hydrophobic amino acids, such as leucine (L), isoleucine (I), phenylalanine (F), valine (V), so the contacts between CaM and its target peptides are charge-charge interactions and hydrophobic interactions. CaM-peptide complex is less soluble and thus more turbid, leading to increased absorbance within visible light wavelength range. The tighter the peptides bind to CaM, the higher the absorbance. Therefore, the affinity of peptides towards CaM can be determined using spectrophotometry.

It is possible that these peptides are damaged by gastrointestinal tract (GI tract) enzymes after consumption, so it is of great importance to test their stability. Pepsin is the major enzyme in stomach that breaks the protein into smaller pieces. Pancreatin contains many enzymes such as amylase, trypsin, chymotrypsin, lipase, protease and ribonuclease. In this method, pepsin and pancreatin were used to mimic the enzyme digestion in stomach and intestine, respectively.

3.2.2 METHODS

3.2.2.1 Determination of calmodulin-binding activity: Equal volumes of 200 µg/ml CaM and 1mg/ml permeate from ultrafiltration were mixed together at room temperature. Absorbance was recorded at room temperature using 1cm pathlength cuvette on a spectrophotometer (MILTON ROY, SPECTRONIC 3000 ARRAY). The absorbance (Ab1) at 500 nm was measured using 30 mM PIPES as blank (pH 6.5). Equal volumes of 200 µg/ml CaM and 30 mM PIPES (pH 6.5) were mixed together. The absorbance (Ab2) at 500 nm was measured using 30 mM PIPES as blank (pH 6.5). Determination of CaM-binding activity was measured both before and after incubation with gastric enzymes. Duplicate determinations were used.

3.2.2.2 Pre-incubation with gastric enzymes: 32 mg permeate were incubated in 20 ml of pepsin solution (0.2 mg/ml in 0.1 M KCl-HCl buffer, pH 2.0) for 4 hours at 37°C with shaking water bath. This reaction was stopped by neutralizing with 10 ml of 2.0 M NaOH. 10 ml pancreatin solution (4.47 mg/ml in 0.2 M MOPS, pH 8.0) was added to the reaction system mixture and incubated for 20h at 37°C. The reaction was stopped by boiling for 15 minutes and the mixture filtered through 1,000 MWCO membrane. The permeate was freeze-dried and tested for CaM-binding activity using the turbidity method.

3.2.3 RESULTS AND DISCUSSION

PIPES buffer was used as blank when determining the absorbance of CaM-peptide complex. CaM-binding ability of the permeate could be determined using (Ab1-Ab2). Duplicate determinations were used to take the average value. Table 3.2.1. shows the CaM-binding ability of hydrolysates before and after gastric enzyme treatment. It showed that hydrolysate number 1 (5% substrate, 4% enzyme, 6h) had the highest binding affinity to CaM both before and after gastric enzymes digestion, suggesting that it is not damaged by the GI tract enzymes digestion, which ensures its safe arrival to the small intestine and absorption by the small intestine. Hydrolysate number 5 which used 5% substrate, 6% enzyme and 6h showed almost the same binding affinity to CaM as hydrolysate number 1. Although the enzyme/substrate ratio is higher in hydrolysate number 5 than number 1, number 5 had a very close and even a little bit lower affinity than number1. It is not necessary to use more enzymes to hydrolyze the protein, which implies a waste of enzyme. All the other hydrolysate were less closely bind or not bind to CaM, which may be due to either the less hydrolysis time or lower enzyme/substrate ratio.

Generally it takes about 2 hours and 4 hours for the foods to pass through the stomach and small intestine. In our method we used 4 hours and 20 hours each to give the enzymes fully enough time to digest the protein. For the hydrolysate that was not damaged, they wouldn't be damaged by the enzyme through the GI tract

after consumption, either.

In conclusion, pea protein isolate could be successfully hydrolyzed to produce short-chain peptides that have high affinity for CaM. Some of these peptides were stable enough to resist the damage by GI tract enzymes, which ensures their activities after consumption.

Table 3.2.1. Calmodulin-binding ability of hydrolysates before and after gastric enzyme treatment.

Hydrolysate No.	Absorbance (at 500 nm) Before treatment with gastric enzymes	Absorbance (at 500 nm) After treatment with gastric enzymes
1	0.0045	0.0045
2	0	0
3	0	0
4	0.0005	0
5	0.0035	0.0045
6	0.0005	0.0005
7	0.0005	0.0005

3.3.0 SEPARATION OF CALMODULIN-BINDING PEPTIDE FRACTIONS AND CALMODULIN-BINDING ACTIVITY OF PEPTIDE FRACTIONS

3.3.1 INTRODUCTION

Protein hydrolysate from previous studies contained many different peptide fractions with different CaM-binding abilities (Kizawa, et al., 1996, Kizawa, 1997). In order to obtain peptides with high affinity towards CaM, it is necessary to separate the protein hydrolysate obtained from enzymatic hydrolysis of pea proteins into fractions that have different molecular properties such as net charge or hydrophobicity. The protein hydrolysate that had the highest CaM-binding affinity was used in this study. Since positively charged peptides have high affinity for CaM, the protein hydrolysate was fractionated using a cation-exchange column on a Fast Protein Liquid Chromatography (FPLC) system. In this method, the protein hydrolysate was passed through a column that is packed with negatively charged polymers. During sample loading all positively charged peptides bind onto the column; unbound peptides are removed by washing the column with appropriate buffer. To release the bound peptides, a linear gradient of increasing pH concentration is applied to neutralize the positively charged peptides and cause them to elute from the column. Weakly-bound peptides are eluted first followed with low gradient while the strongly-bound peptides will elute later at high gradient. Therefore, the objective was to separate the pea protein hydrolysate into peptide fractions that differed in net positive charge.

3.3.2 MATERIALS

The FPLC used was the Bio-Rad Biologic LP Chromatography System (Bio-Rad, Hercules, CA). SP Sepharose column (HiPrep 16/10 SP FF), a strong cation exchanger was purchased from Amersham Biosciences (Montreal, PQ).

3.3.3 METHODS

3.3.3.1 Separation of CaM-binding peptide fractions: The pea protein hydrolysate with highest CaM-binding activity was used. Hydrolysate was made into 140 mg/ml solution using 0.1 M ammonium acetate (pH 7.5) buffer and filtered through 0.2 µm membrane. An aliquot (1 ml) of the sample solution was injected onto the column. Prior to sample loading, the column was equilibrated with 60 ml of 0.1 M ammonium acetate buffer (pH 7.5). The column was then washed with 60 ml of ammonium acetate buffer to remove unbounded peptides. Adsorbed peptides were then eluted using a linear gradient between 0 and 0.5 M ammonium carbonate (pH 8.8) in 0.1 M 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.

3.3.3.2 CaM-binding activity of peptide fractions: Equal volumes of 200 µg/ml calmodulin and 1 mg/ml peptide fraction were mixed together. The absorbance (Ab1) at 500 nm was measured using 30 mM PIPES buffer as blank (pH 6.5).

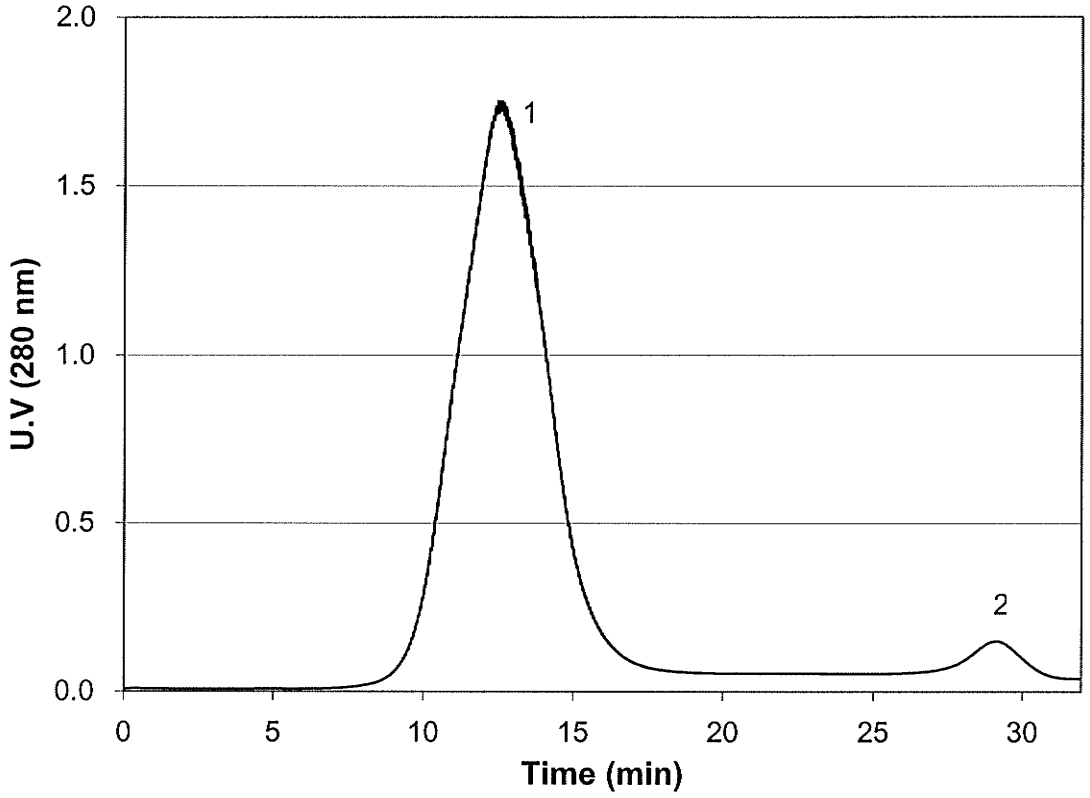
Equal volumes of 200 µg/ml calmodulin and 30 mM PIPES (pH 6.5) were also mixed together. The absorbance (Ab2) at 500 nm was measured using 30mM PIPES as blank (pH 6.5). Absorbances were all measured using 1cm pathlength cuvette on a spectrophotometer (MILTON ROY, SPECTRONIC 3000 ARRAY). Duplicate determinations were used.

3.3.3.3 Amino acids composition analysis: Amino acid analysis was performed at the Department of Animal Science, University of Manitoba according to the Association of Official Analytical Chemists (AOAC) Official Method 994.12.

3.3.4 RESULTS AND DISCUSSION

Two peptide fractions were obtained. The first fraction eluted at about 10 minutes while the second fraction eluted at about 27 minutes after start of the gradient, which means that peptide fraction #2 was adsorbed to the cation-exchanger much stronger than peptide fraction #1; thus fraction #2 has higher net positive charge than fraction #1. However, fraction #1 was about 10 times more abundant than fraction #2, which could be seen from the UV absorption values. Figure 3.3.1. shows the two peptide fractions obtained using FPLC. The proteins that are adsorbed to the ion-exchanger can usually be eluted from the column by a change in pH. In our method where cation exchanger was used, an increase in pH made the peptides less positively charged, leading to their elution from the column. It is often advantageous to use volatile buffers such

Figure 3.3.1. Separation of peptide fractions using FPLC.



1: peptide fraction #1

2: peptide fraction #2

Flow rate: 2 ml/min

Gradient: 0 – 0.5M ammonium carbonate (pH 8.8) from 0 – 40 min

Sample concentration: 140 mg/ml

Volume of peptide fraction #1: 20 ml

Volume of peptide fraction #2: 8 ml

Table 3.3.1. Duplicate measurements of absorbance of the complex of CaM-peptide fractions at 500 nm.

	Peptide fraction 1		Peptide fraction 2	
	1	2	1	2
Sample(Ab1)	0.003	0.003	0.008	0.007
Blank(Ab2)	0.001	0.000	0.001	0.000
Ab1-Ab2 *	0.002	0.003	0.007	0.007

*: Average absorbance for peptide fraction #1 was (0.002+0.003)/2=0.0025.

Average absorbance for peptide fraction #2 was (0.007+0.007)/2=0.007.

as ammonium acetate and ammonium carbonate that allow direct lyophilization of the pooled fractions after chromatography. Usually the concentration of buffer used during protein adsorption is low. The concentration used in our method was according to that used in a previous study (Kizawa, et al., 1995), and seemed to work very well under the separation conditions. Table 3.3.1 shows the abilities of the two peptides fractions to bind to CaM. The absorbance of CaM-peptide complex was determined by subtracting absorbance of the blank from that of the sample. The average value from two measurements was taken. Average absorbance of peptide fraction #1 was 0.0025 and was 0.007 for peptide fraction #2. It can be deduced that peptide fraction #2 has much higher CaM-binding affinity than peptide fraction #1.

Amino acids composition is shown in Table 3.3.2. It is clear to see that both peptides have amino acids with positive charges, 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 #2 peptides were, respectively, 5 and 6 times higher than the levels found in peptides present in fraction #1. The results are consistent with reported studies that strong CaM-binding peptides have high content of positively-charged groups and hydrophobic groups (Itano et al., 1980; Kizawa et al., 1995; Kizawa et al., 1996; Kizawa, 1997). Fraction #1 contained higher level of ASx and GLx than fraction #2. Aspartic acid and glutamic acid are acidic amino acids that contribute to the

amount of ASx and GLx. Therefore, peptide fraction #1 had more negatively charged amino acid residues as a result of the higher content of glutamic and aspartic acid residues, which contributed to the decreased affinity for the negatively charged CaM, when compared to fraction #2.

In conclusion, two positively-charged peptide fractions were obtained from separation of pea protein hydrolysates on a cation-exchange column. The peptides in fraction #1 were more in quantity but peptides in fraction #2 had higher CaM-binding affinity. Both peptide fractions show amino acid compositions that are consistent with high contents of positively-charged groups and hydrophobic groups that facilitate binding to CaM. However, fraction #2 which showed a higher affinity for CaM had higher contents of basic amino acid residues and lower contents of acidic amino acids when compared to fraction #1.

Table 3.3.2. Amino acids composition for peptide fractions #1 and #2*.

Amino acids	Peptide fraction #1 (%)	Peptide fraction #2 (%)
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	4.29	4.47
ALA	3.85	3.99
CYS	1.09	0.32
VAL	4.83	2.88
MET	0	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

*: ASx = asparagine + aspartic acid

G Lx = glutamine + glutamic acid

CHAPTER FOUR

4.0 ENZYME INHIBITION KINETICS

4.1 INTRODUCTION

$$\text{ATP + casein} \rightarrow \text{phosphopeptide + ADP} \text{ ---------- (1)}$$

$$\text{ADP + phosphoenolpyruvate} \rightarrow \text{ATP + pyruvate} \text{ ----------(2)}$$

$$\text{pyruvate + NADH} \rightarrow \text{NAD}^{+} \text{ + lactate} \text{ ---------- (3)}$$

Assay of activity of CaM-dependent protein kinase II (CaMKII) was carried out according to the method described by Roskoski (1985). Equation (1) is catalyzed by CaMKII, (2) is catalyzed by phosphoenolpyruvate kinase while (3) is catalyzed by pyruvate dehydrogenase, all in a sequential fashion. Therefore, the rate of the reaction is directly proportional to the disappearance of NADH, which can be monitored by measuring the decrease in absorbance at 340 nm. Since equation (1) is the rate-limiting step, inhibition of CaMKII activity is directly proportional to the rate of decrease in absorbance at 340 nm. Because CaMKII requires CaM for activation, it follows that the binding affinity of peptides can be determined by calculation of the rate of decrease in enzyme activity. The higher the binding affinity of the peptide, the greater the decrease in enzyme activity; if sufficient amount of inhibitory peptide is present, the enzyme could be totally inhibited and there will be no change in absorbance at 340 nm.

4.2 MATERIALS

Phosphodiesterase 3':5'-cyclic nucleotide activator (CaM), β -nicotinamide adenine di-nucleotide (β -NADH, reduced form), α -lactic dehydrogenase, pyruvate kinase, casein (from bovine milk, 5% solution, dephosphorylated and hydrolyzed), phospho(enol)pyruvate were purchased from Sigma Chemical Co. CaMKII was purchased from BioLabs. Adenosine-5'-triphosphate (ATP) was purchased from Roche Applied Science.

4.3. METHODS

4.3.1. Reagents: Both peptide fractions obtained from cation-exchange chromatography were used. The following stock solutions were prepared: 100 mM MOPS (pH 7.0), 5 mM $CaCl_2$, 50 mM $MgCl_2$, 1 mM dithiothreitol, 3000 units/ml of lactic dehydrogenase, 1400 units/ml of pyruvate kinase, 100 mM NADH, 10 mM phosphoenolpyruvate, 10 mM ATP, 5% casein solution (dephosphorylated and hydrolyzed), 50 units/ml CaMKII, and 300 µg/ml CaM. The reaction mixture (2 ml) contained 1 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM dithiothreitol, 12 units of lactate dehydrogenase, 8 units of pyruvate kinase, 200 µ M NADH, 1 mM phosphoenolpyruvate, 2 mM ATP, 1 mM casein, 10-70 µg CaM and 1 unit CaMKII all in MOPS buffer, pH 7.0.

4.3.2. Preparation and assay of inactivated enzyme (blank): CaMKII was first put in boiling water for 15 minutes to inactivate the enzyme protein. After cooling

the inactivated enzyme was incubated with CaM at room temperature for 15 minutes. This inactivated CaM/CaMKII mixture was then mixed with other components of the reaction mixture and absorbance at 340 nm was measured using 1 cm pathlength cuvette on a spectrophotometer (MILTON ROY, SPECTRONIC 3000 ARRAY) until there was no more decrease in absorbance.

4.3.3. Preparation and assay of enzyme activity in the absence of peptides (control): CaMKII was first mixed with CaM and incubated at room temperature for 15 minutes and then mixed with other components of the reaction mixture. Absorbance at 340 nm was measured as described above.

4.3.4. Preparation and assay of enzyme activity in the presence of peptides (samples): A stock solution of fraction #1 was diluted to obtain concentrations of 1.5 mg/ml, 2.5 mg/ml and 3.5 mg/ml using MOPS buffer, pH 7.0. Similarly, a stock solution of fraction #2 was prepared to obtain concentrations of 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml using the same MOPS buffer, pH 7.0. These concentrations were used because they enabled determination of rate of decrease in absorbance at 340 nm in a reasonable time frame. CaM was first incubated with an inhibitory peptide fraction at different levels for 15 minutes and then mixed with CaMKII for another 15 minutes before addition of 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.

4.4 RESULTS AND DISCUSSION

Following a reaction depends on using a property of one of the reactants, which is changed measurably by the reaction (Engel, P.C, 1996). Spectrophotometry is one of the most commonly used methods to monitor enzyme reactions. The decrease in absorbance at 340 nm is positively related to the reaction speed.

Figures 4.4.1 and 4.4.2 show Lineweaver-Burk plot of 1/velocity versus 1/CaM concentration at different concentrations of inhibitory peptide fractions; CaM concentration was 15 μg/ml. The plots suggest that the inhibitory peptide fractions were bound to CaM in a competitive manner because all the lines passed through the Y-axis at the same point; and the higher the inhibitor concentration, the higher the slope which suggests reduction in ability of CaM to activate CaMKII. It is possible that the inhibitory peptides were bound to the same sites on CaM that would normally interact with CaMKII, giving rise to mutual exclusive binding. It is also possible that binding of the inhibitory peptides caused changes in CaM structure that reduced its ability to interact with and activate CaMKII. These considerations were discussed by Engel in details (Engel,P.C., 1996). Previous study (Barnette, et al, 1982) 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.

Figure 4.4.1. Lineweaver-Burk plot of 1/velocity versus 1/CaM concentration at different concentrations of inhibitory peptide fraction #1.

CaM concentration is 15µg/ml.

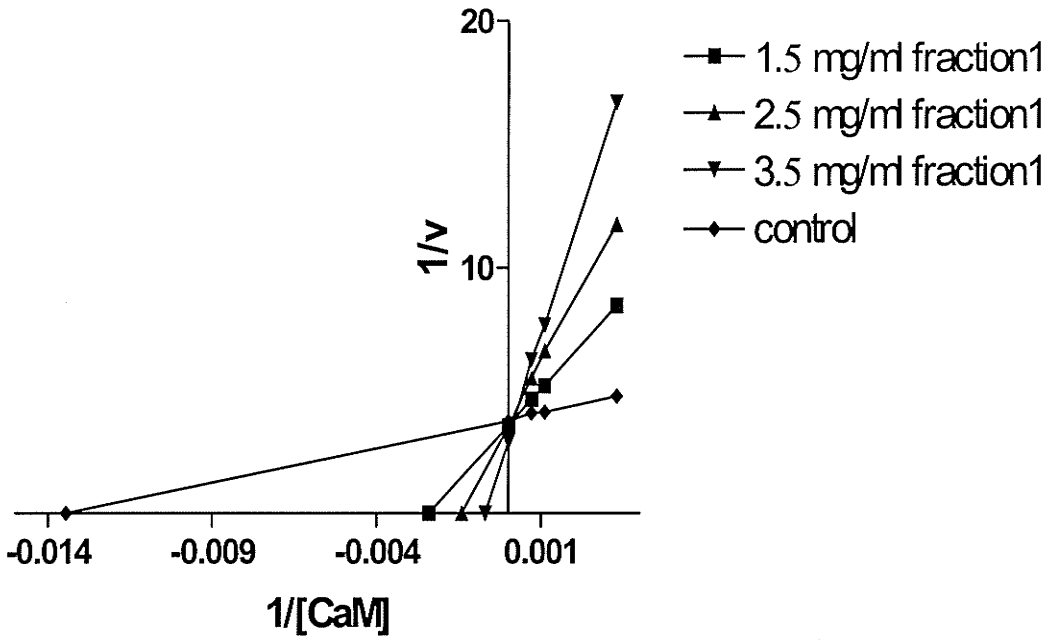
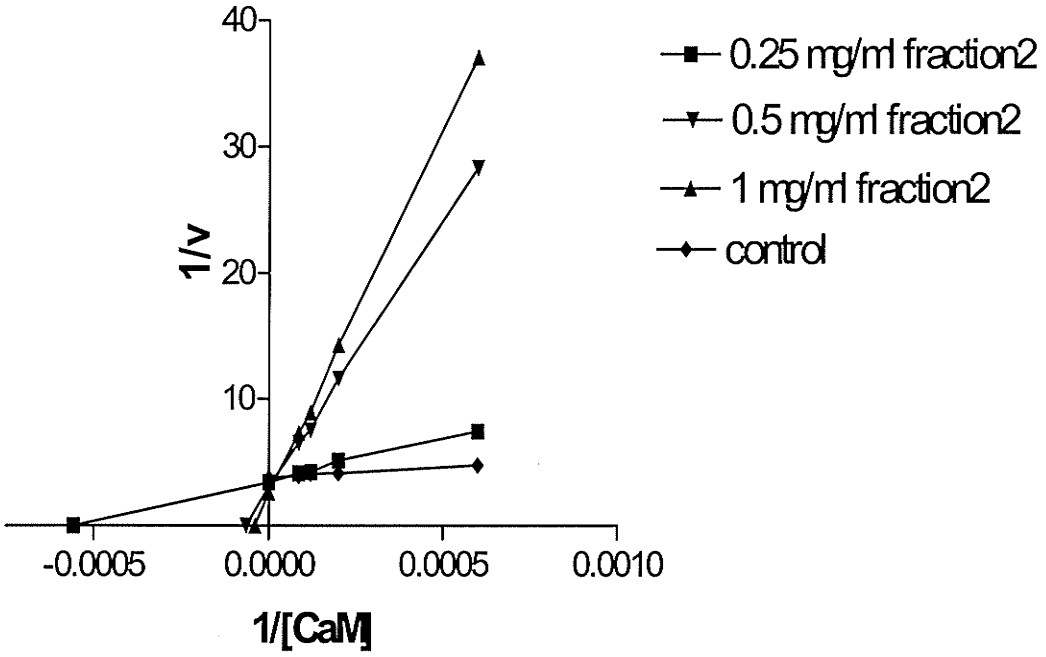


Figure 4.4.2. Lineweaver-Burk plot of 1/velocity versus 1/CaM concentration at different concentrations of inhibitory peptide fraction #2.

CaM concentration is 15µg/ml.

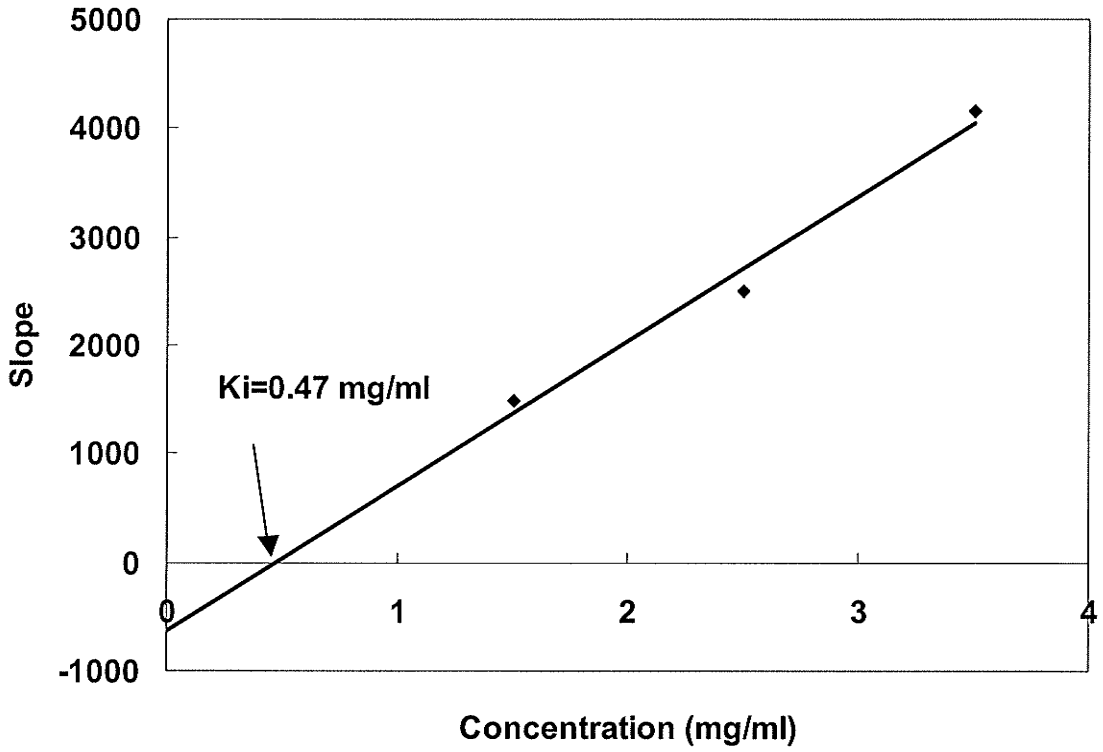


Another study found that melittin inhibited myosin-light chain kinase, another calcium/CaM-dependent kinase, competitively with respect to CaM (Ki=0.08mM), suggesting that melittin inhibited myosin light-chain kinase by interacting with a site on the enzyme the same as, or proximal to, the calmodulin-binding site (Katoh, et al, 1982).

Figures 4.4.3 and 4.4.4 show the secondary plots from Lineweaver-Burk results using slope of the lines on the Y-axis and peptide concentrations on the X-axis. Inhibition constant (Ki) is the intercept of the line on the X-axis. The smaller the Ki value, the stronger the affinity of the peptide for CaM and the greater the reduction in CaMKII activity. The results showed that peptide fraction #2 had a higher binding affinity for CaM then fraction #1. These Ki values are similar as those obtained by Katoh and his teammates, using melittin, an amphipathic polypeptide, to inhibit phospholipid-sensitive Ca^{2+}-dependent protein kinase and myosin light-chain kinase (Katoh, et al, 1982), but lower than the value obtained from another study using melittin to inhibit CaM-stimulated phosphodiesterase activity (Barnette, et al, 1983).

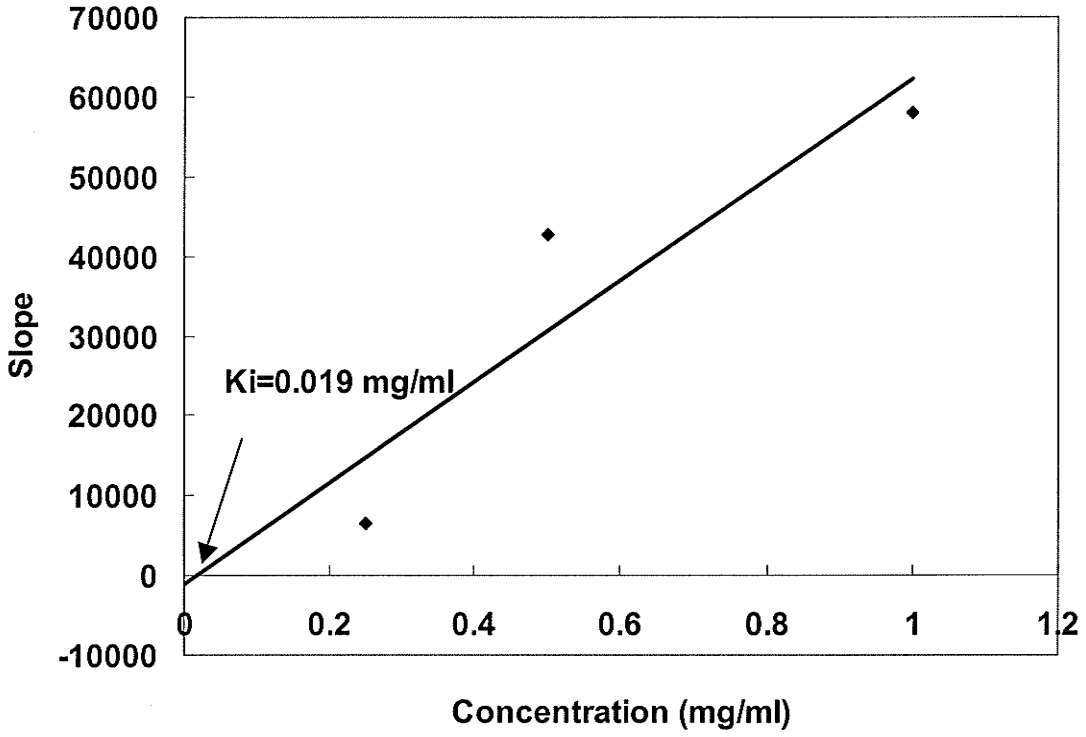
Figures 4.4.5 and 4.4.6 show the residual percentage activity of CaMKII at different concentrations of peptide fractions #1 and #2 using different CaM levels. At fixed CaM concentration, CaMKII activity decreased with increase in peptide concentration and up to 90% reduction in enzyme activity was obtained. At fixed peptide concentrations, CaMKII activity increased with increased CaM

Figure 4.4.3. Determination of inhibition constant (Ki) for peptide #1*.



*: Slope was calculated from Figure 4.4.1.

Figure 4.4.4. Determination of inhibition constant (Ki) for peptide #2*.



*: Slope was calculated from Figure 4.4.2.

Figure 4.4.5. CaMKII activity (%) of different concentrations of peptide fraction #1 at different CaM levels.

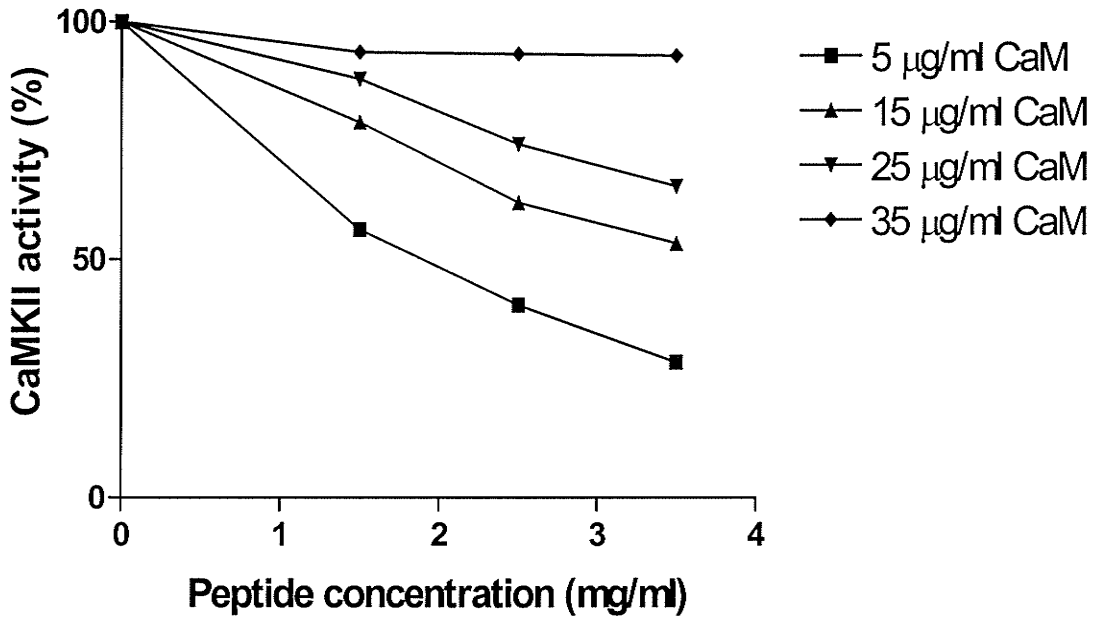
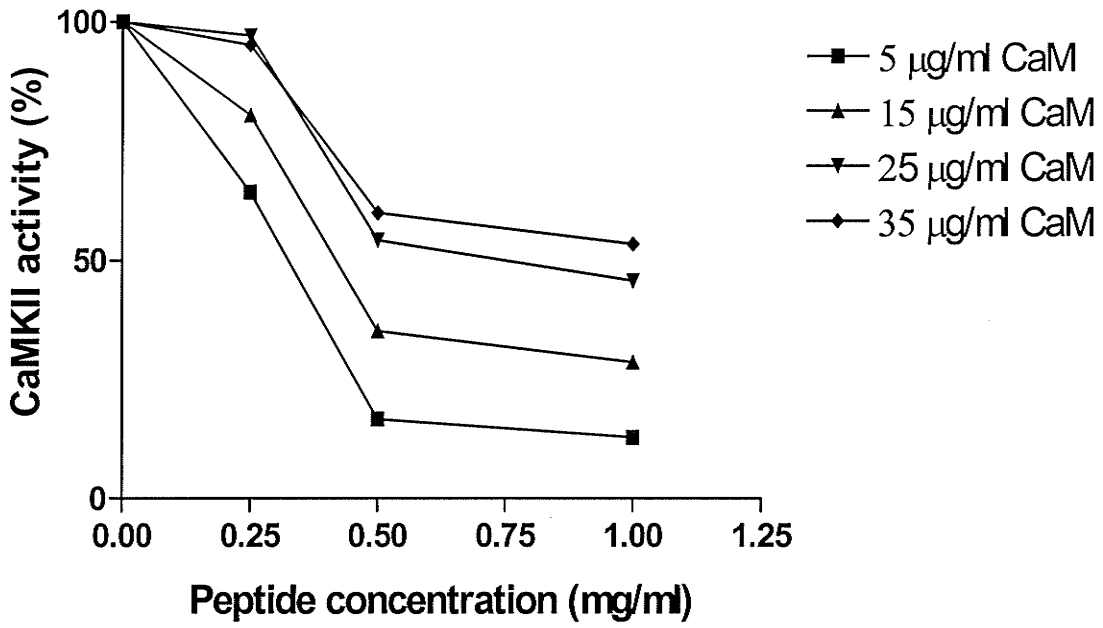


Figure 4.4.6. CaMKII activity (%) of different concentrations of peptide fraction #2 at different CaM levels.



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.

Figures 4.4.7 and 4.4.8 show the maximum reaction velocity (Vmax) at different peptide concentrations and CaM concentrations. It was obvious that at fixed CaM concentration, higher peptide concentration led to less Vmax, while at fixed peptide concentration, higher CaM concentration led to higher Vmax. Vmax could even be kept unchanged when there was enough CaM.

Table 4.4.1 shows the concentration of peptides that reduced enzyme activity by 50% (IC_{50}) for both peptide fractions at different CaM concentrations. Peptide fraction #1 has an IC_{50} that is almost 10 times than that of peptide fraction #2 at each CaM concentration, which means that fraction #2 has a much stronger inhibitory power properties than fraction #1. Our values are essentially consistent with previously reported values for the peptides isolated from αs2-casein (Kizawa et al, 1996), α-casein pepton (Kizawa, 1995) and insect venom (Katoh, et al, 1982). Barnette and his colleagues using peptides isolated from insect venom obtained much lower IC_{50} values to inhibit CaM or CaM-stimulated phosphodiesterase activity. Table 4.4.2. shows the Michaelis-Menten constant (Km) and Vmax values of both peptides. Km is the substrate concentration when velocity is half of the maximal velocity. It is an inverse measure of affinity or

strength of enzyme binding to the substrate. The lower the Km value, the greater the affinity or the strength. Usually, when substrate concentration is fixed, the Km value increases with increased inhibitor concentration, which means higher substrate concentration is needed to reach the velocity half of the maximum. Km value can be determined from X-intercept on the X-axis from LineWeaver-Burk plot. Km= -(1/ X-intercept). In our research, peptide fraction #2 had greater Km values compared with peptide fraction #1, suggesting that higher CaM concentration was required to overcome the inhibition of CaMKII induced by peptide fraction #2 compared with peptide fraction #1. Therefore, peptide fraction #2 showed a greater inhibitory effect on CaMKII than peptide fraction #1. Maximal velocity (Vmax) is the initial rate achieved as $[s] \rightarrow \infty$. Thus, ideal Vmax should be the same for control group and both peptide fractions. In Tale 4.4.2, velocities at 35μg/ml CaM was used as Vmax values for control group and both peptide fractions. Therefore, Vmax reduced as peptide fraction concentrations increased, with peptide fraction #2 reduced more than peptide fraction #1, which showed higher inhibitory properties.

In conclusion, the two peptide fractions obtained from cation-exchange chromatography were able to inhibit CaMKII activity. Fraction #2 exhibited higher inhibitory property towards CaMKII than fraction #1. The inhibition by both fractions was competitive in nature, which means that high concentrations of CaM can nullify the inhibitory property of the peptides.

Figure 4.4.7. The maximal reaction rate at different peptide fraction #1 concentrations at different CaM levels.

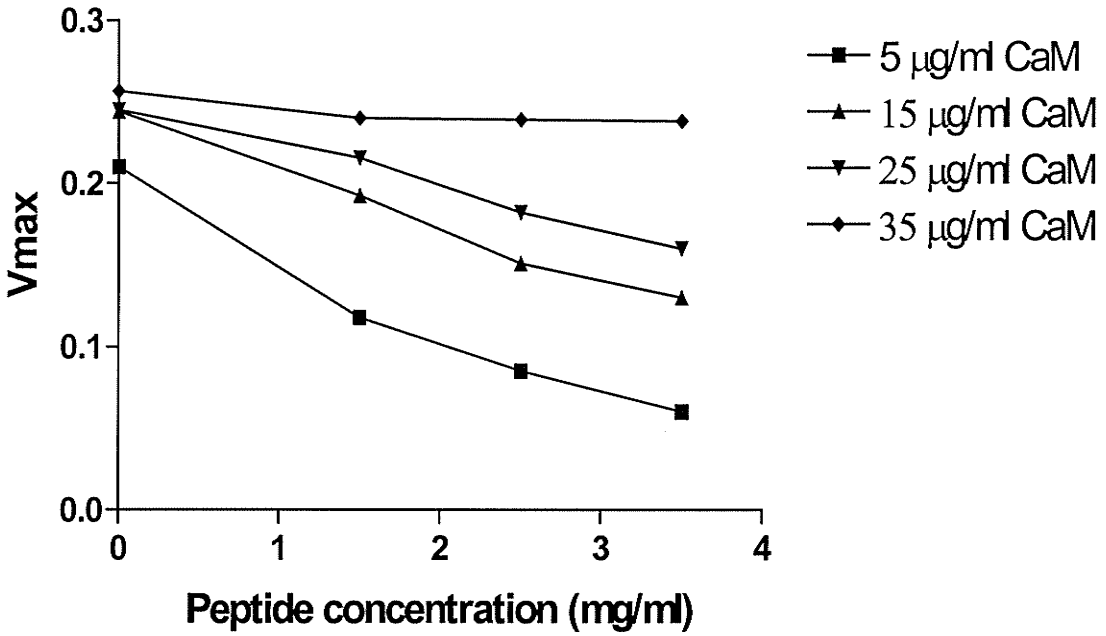
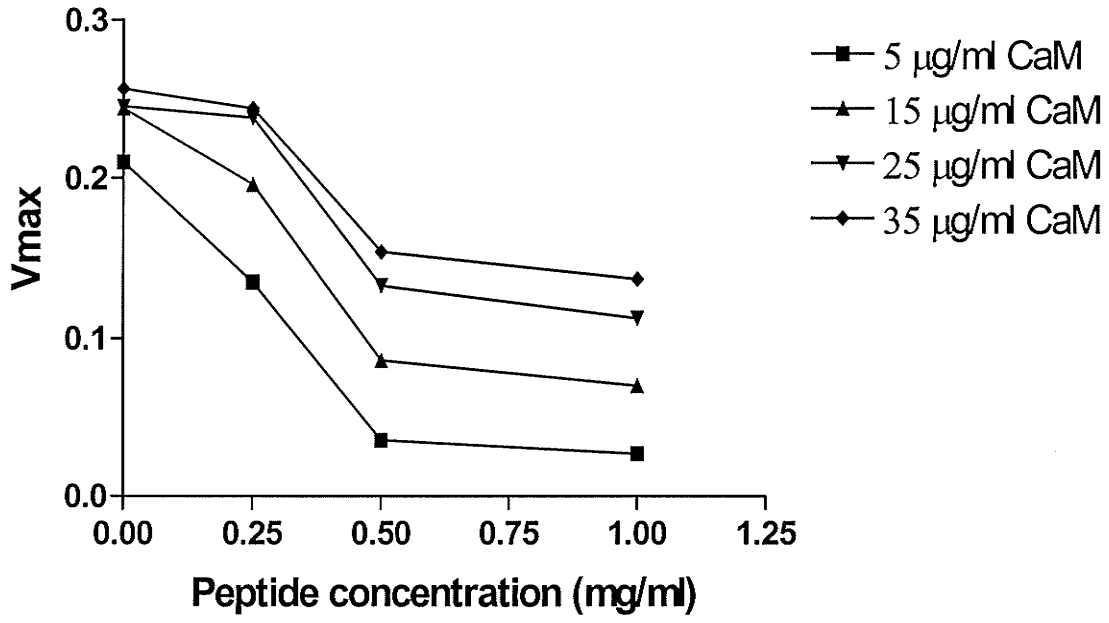


Figure 4.4.8. The maximal reaction rate at different peptide fraction #2 concentrations at different CaM levels.



Talbe 4.4.1. IC_{50} (mg/ml) of peptide fraction #1 and peptide fraction #2 at different CaM amount.

CaM concentration (μg/ml)	Peptide fraction #1	Peptide fraction #2
5	2.78	0.24
15	4.0	0.3
25	5.2	0.37
35	22.98	0.4

IC_{50} is the peptide concentration that reduces 50% of CaMKII activities.

Table 4.4.2. Km and Vmax values of both peptides.

	control	Peptide fraction #1			Peptide fraction #2		
Concentration (mg/ml)		1.5	2.5	3.5	0.25	0.5	1.0
Km (mM)	0.414	0.415	0.708	1.430	1.796	15.489	25.259
Vmax (uM/min)	0.262	0.240	0.239	0.238	0.244	0.154	0.137

Km is the substrate concentration when v = Vmax / 2.

CHAPTER FIVE

5.0 DETERMINATION OF CaM STRUCTURE BY SPECTROFLUORIMETRY AND CIRCULAR DICHROISM

5.1 INTRODUCTION

Determination of CaM structure in the presence and absence of inhibitory peptides could provide information on the molecular conformation that is required for CaM-induced activation of metabolic enzymes. CaM lacks tryptophan (W) residue but has 8 phenylalanine (F) residues and 2 tyrosine (Y) residues. Phenylalanine and tyrosine have maximum ultraviolet (UV) light absorption when excited at 257 and 275 nm, respectively. Thus changes in the UV-absorption properties of phenylalanine and tyrosine can be used to determine the changes in the structure of CaM in the presence and absence of inhibitory peptides.

Fluorescence is used based on the principle that, a molecule absorbs a high-energy photon, jumps to a higher energy level and soon re-emits it as a lower-energy (longer-wavelength) photon (Gore, 2000). The energy difference between the absorbed and emitted photons ends up as molecular vibrations (heat). Usually the absorbed photon is in the ultraviolet, and the emitted light (luminescence) is in the visible range (Gore, 2000). These lights are collected by wavelength and amplified to be converted to the electric signals. Fluorescence investigation can be used in the protein structure-function studies because three aromatic amino acids (phenylalanine, tyrosine and tryptophan) absorb light in the

ultraviolet spectra range (Kilhoffer et al., 1981; Kim et al., 1993).

8-Anilinonaphthalene-1-sulfonic acid (ANS) fluoresces weakly in aqueous solution, but brightly in hydrophobic environments (Brand and Gohlke, 1972). It has been widely used as 'hydrophobic probe' for protein structure (Tanaka et al., 1986; Moorthy et al., 1999; Andressa et al., 2003). It binds to hydrophobic groups of the protein, leading to increase in fluorescence intensity. Therefore, it can be used to monitor the exposure of the hydrophobic domains or unfolding of CaM polypeptide structure.

Circular dichroism (CD) refers to the differential absorption of the left and right circularly polarized components of plane-polarized radiation. This effect will occur when a chromophore is chiral either a) intrinsically by reason of its structure, or b) by being covalently linked to a chiral center, or c) by being placed in an asymmetric environment (Kelly et al., 1997). Proteins form regular secondary structure because the peptide O=C-N- link between amino acids is planar and rigid, but it has a large degree of rotational freedom about its bonds to the rest of the protein chain (Gore, 2000). This allows a variety of intramolecular hydrogen bond arrangements, which results in the chiral secondary structure that can be defined by CD signals. CD spectropolarimetry can be used to qualify secondary structures in protein, detect the presence of tertiary structure, and follow structural changes during protein folding and unfolding. UV spectra can be divided into the near-UV and far-UV regions. The near-UV refers to the 250-300 nm and far-UV

means less than 250 nm. Near-UV is used to estimate changes in the tertiary structure of proteins. It is also described as the aromatic region because transitions of aromatic amino acid groups (phenylalanine, tyrosine and tryptophan) contribute to this region. Far-UV is dominated by transitions of peptide backbone, such as α–helix and β–sheet and is used to estimate changes in secondary structure of proteins. Near-UV CD spectra of ram testis CaM in the presence and in the absence of calcium was shown by Kilhoffer and his colleagues (Kilhoffer et al., 1981). Two negative signals were shown, corresponding to the CD spectra of phenylalanine and tyrosine. Calcium binding to CaM was accompanied by an increase of the CD of the aromatic amino acids, which indicated a change in the tertiary structure. Many other researches also used CD to carry out structure-function studies (Bouvier et al., 2003; Hu et al., 2004; Yuan et al., 2004). This method was used in our research to study the changes in CaM structure arising from binding of inhibitory peptides obtained from pea protein hydrolysate.

5.2 MATERIALS AND METHODS

Peptide fractions #1 and #2 obtained from FPLC using cation-exchange chromatography as previously described were used. Fluorescence spectra were recorded on a spectrofluorophotometer (RF-1501 spectrofluorophotometer, SHIMADZU, Japan). Circular dichroic spectra were measured using a JASCO J-600 Spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo, Japan) and a

MILTON ROY SPECTRONIC 3000 ARRAY Spectropolarimeter (MILTON ROY, USA).

5.2.1 Assay of CaM structure by fluorescence: The reagents included 100 mM MOPS (pH 7.0), 400 µg/ml CaM, 392 mg/ml $CaCl_2$, 20 mg/ml peptide fraction #1 and 20 mg/ml peptide fraction #2. Each measurement was made using a total volume of 2 ml. The final concentrations of CaM and $CaCl_2$ were 20 µg/ml and 0.196 mg/ml, respectively. The final concentrations of peptide fraction #1 were 0.0005%, 0.001%, 0.002% and 0.003%, and peptide fraction #2, 0.00025%, 0.0005%, 0.001% and 0.002%. The signal for phenylalanine fluorescence was too weak to be detected; therefore, tyrosine fluorescence was used to measure changes in CaM structure. Excitation wavelength was fixed at 275 nm and emission wavelength range was from 290-400 nm using a 1 cm pathlength quartz cuvette. Fluorescence intensity (FI) is expressed in arbitrary units. Fmax is the maximum FI obtained during the wavelength scan, while λmax is the wavelength at Fmax. The following spectra analyses were performed in order to determine the effect of calcium and peptides on CaM structure.

i) Structure of CaM = spectrum of CaM in buffer – buffer spectrum

ii) Structure of calcium = spectrum of calcium in buffer - buffer spectrum

iii) Structure of CaM in the presence of calcium = (calcium/CaM spectrum) – calcium spectrum

iv) Structure of peptides = spectrum of peptides in buffer – buffer spectrum

v) Structure of calcium/CaM complex in the presence of peptide = (spectrum of calcium/CaM/peptides) – peptide spectrum

5.2.2 Assay of enzyme structure by fluorescence: A total of 1.3 units of CaMKII were used for all fluorescence assays, which were performed with the reagents described in section 5.2.1 except that the wavelengths used for measurements were different as follows. Excitation wavelength was fixed at 295 nm (tryptophan absorption) and emission wavelength range was from 310-450 nm. Since CaM contains no tryptophan residues the results are indicative of the changes in the structure of CaMKII only. Therefore, this method allowed probing of the structural modification of CaMKII by CaM in the absence and presence of inhibitory peptides. The following spectra analyses were performed in order to determine the effect of calcium/CaM complex and inhibitory peptides on the structure of CaMKII.

i) Structure of calcium/CaM complex = spectrum of calcium/CaM in buffer – buffer spectrum

ii) Structure of CaMKII alone = spectrum of CaMKII in buffer – buffer spectrum

iii) Structure of CaMKII in the presence of calcium/CaM complex = Spectrum of calcium/CaM/CaMKII in buffer - calcium/CaM spectrum

iv) Peptide structure = spectrum of peptides in buffer – buffer spectrum

v) Structure of calcium/CaM/CaMKII complex in the presence of inhibitory peptides = spectrum of calcium/CaM/CaMKII/inhibitory peptides complex in buffer – peptide spectrum

5.2.3 Assay of CaM structure using ANS by fluorescence: A stock solution that contained 169.4 μM of ANS was prepared and stored at 4°C in a container wrapped in aluminum foil to prevent damage by light. All other reagents were the same as described in section 5.2.1. The final concentration of ANS that was used for fluorescence measurement in a total volume of 2 ml was 60 μ M. Fluorescence spectra were recorded between 400 and 650 nm with excitation at 385 nm. Pathlength of the quartz cell was 1 cm. The following spectra analyses were performed in order to determine the effect of calcium and peptides on CaM structure.

i) Structure of ANS = spectrum of ANS in buffer – buffer spectrum

ii) Structure of calcium in the presence of ANS = spectrum of calcium/ANS complex – spectrum of ANS in buffer

iii) Structure of CaM in the presence of ANS = spectrum of CaM/ANS complex – spectrum of ANS in buffer

iv) Structure of calcium/CaM complex in the presence of ANS = spectrum of calcium/CaM/ANS complex – spectrum of calcium in the presence of ANS

v) Structure of peptides in the presence of ANS = spectrum of peptides/ANS complex – spectrum of ANS in buffer

vi) Structure of calcium/CaM/peptide in the presence of ANS = spectrum of calcium/CaM/peptide/ANS complex – spectrum of peptides in the presence of ANS

5.2.4 Determination of secondary structures by CD: The following stock solutions were made in 10 mM MOPS buffer, pH 7.0; CaM (1.0 mg/ml); $CaCl_2$ (50.0 mM); peptide fractions #1 and 2 (20 mg/ml). CD measurements were performed using 0.25 mg/ml, 10 mM and 1 mg/ml final concentrations of CaM, $CaCl_2$ and peptides, respectively. MOPS buffer, CaM alone, CaM and $CaCl_2$, CaM and $CaCl_2$ and peptide 1, and CaM and $CaCl_2$ and peptide 2 were all measured at far-UV respectively. Far-UV spectra were recorded from 200 to 240 nm. Pathlength of the quartz cell for far-UV was 0.05 cm. The spectra in terms of millidegrees were calculated using the following equation;

millidegrees=(mv1-mv2) x 41.38 x sensitivity,

where mv1 is the protein CD spectra, mv2 is the buffer spectra and sensitivity is 2 m^{o}/cm.

5.2.5 Determination of tertiary structures by CD: The following stock solutions were made in 10 mM MOPS buffer, pH 7.0; CaM (0.2 mM); $CaCl_2$ (20.0 mM); peptide fractions #1 and 2 (20 mg/ml). CD measurements were performed using 0.1 mM, 1.0 mM and 1 mg/ml final concentrations of CaM, $CaCl_2$ and peptides, respectively. MOPS buffer, CaM alone, CaM and $CaCl_2$, CaM and $CaCl_2$ and peptide 1, and CaM and $CaCl_2$ and peptide 2 were all measured at near UV respectively. Near-UV spectra were recorded from 250 to 320 nm. Pathlength of the quartz cell for near-UV was 1 cm. The spectra in terms of mean residue weight

(MRW), ellipticity, $[\theta]_{MRW,}$ were calculated using the following equation;

$$[\theta]_{MRW} = (\theta_{obs} \times MRW) / (10 \times c \times l),$$

where θ_{obs} is the measured ellipticity in degree, MRW is the mean residue weight of CaM, c is the protein concentration in moles per liter (mol/L) and l is the pathlength in centimeters. The units of $[\theta]_{MRW}$ is degrees x cm^2 x $dmol^{-1}$.

5.3 RESULTS AND DISCUSSION

5.3.1 Fluorescence properties of CaM in the presence and absence of peptides: Figures 5.3.1 and 5.3.2 show the effects of different concentrations of peptide fractions #1 and #2, respectively on CaM structure. Higher peptide concentration led to higher fluorescence intensity (FI), which suggested more exposure of hydrophobic groups, especially tyrosine. Interactions between peptides and CaM might have resulted in unfolding of CaM, thus leading to the increased exposure of hydrophobic groups. The ratios of FI of CaM in the presence or absence of calcium and peptide inhibitors (Fmax) to FI of CaM (Fo) as well as λmax values are shown in Tables 5.3.1. and 5.3.2., respectively for fraction #1 and 2. Fmax/Fo was higher after addition of inhibitors and increased when peptide concentration increased. The higher the value of Fmax/Fo ratio, the greater the exposure of hydrophobic groups in CaM structure. The results suggest that the presence of inhibitory peptides may have caused unfolding of the CaM structure, which confirms the results shown in Figures 5.3.1. and 5.3.2.

Figure 5.3.1. Effect of peptide fraction #1 (different concentrations) on CaM structure changes.

Excitation wavelength was 275 nm.

CaM concentration was 20 μg/ml and calcium concentration was 0.196 mg/ml.

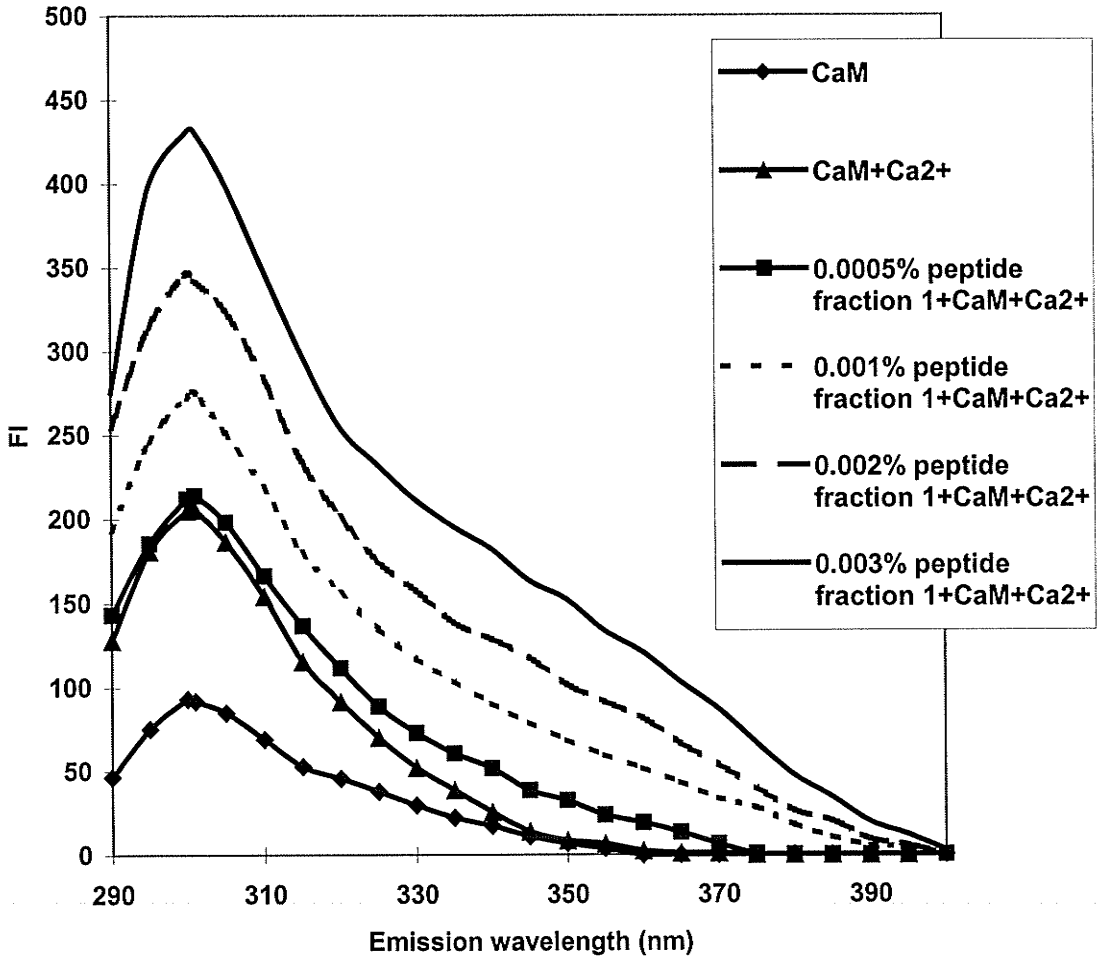


Figure 5.3.2. Effect of peptide fraction #2 (different concentrations) on CaM structure changes.

Excitation wavelength was 275 nm.

CaM concentration was 20 µg/ml and calcium concentration was 0.196 mg/ml.

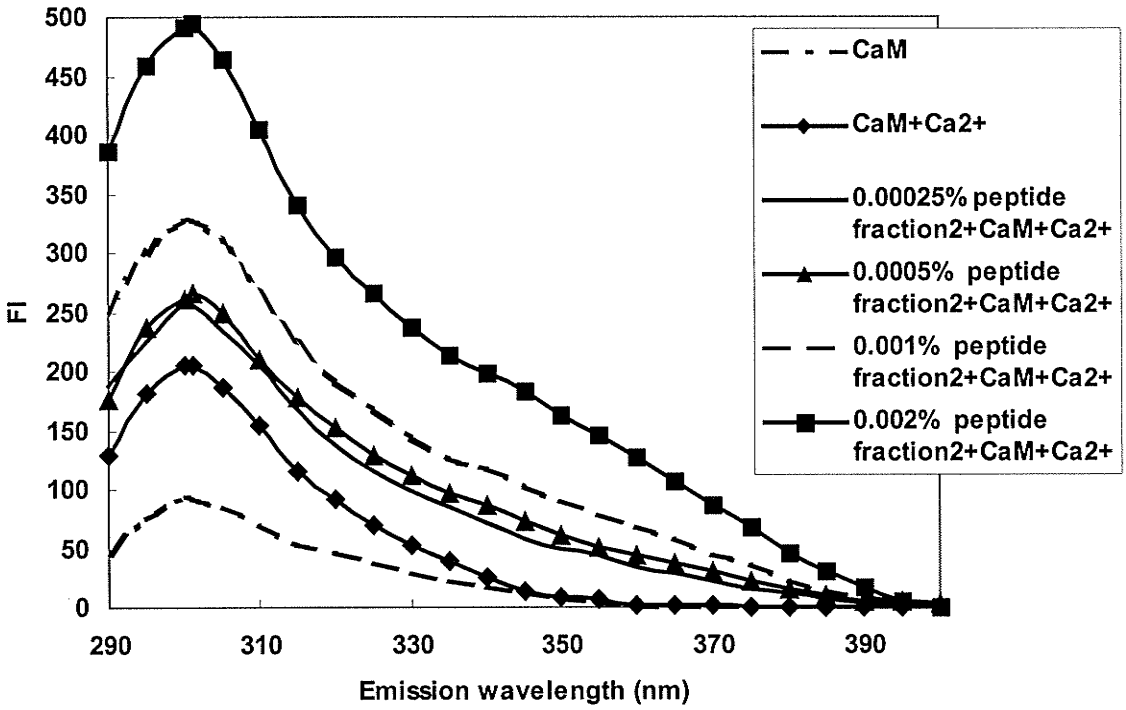


Table 5.3.1. Fmax/Fo of CaM+ Ca^{2+}, or CaM+ Ca^{2+}+ peptide fraction #1 at different concentrations.

Sample	Fmax/Fo	λmax (nm)
CaM	2.23	301
CaM + 10 μg peptide	2.33	301
CaM + 20 μg peptide	3.00	301
CaM + 40 μg peptide	3.72	300
CaM + 80 μg peptide	4.71	301

F_{max}: Fluorescence Intensity (FI) of CaM+ Ca^{2+}, or CaM+ Ca^{2+}+ peptide 1

F_o: FI of CaM

λ_{max}: wavelength of maximum FI

Table 5.3.2. Fmax/Fo of CaM+ Ca^{2+}, or CaM+ Ca^{2+}+ peptide fraction #2 at different concentrations.

Sample	Fmax/Fo	λmax (nm)
CaM	2.23	301
CaM + 5 μg peptide	2.76	300
CaM + 10 μg peptide	2.90	301
CaM + 20 μg peptide	3.56	301
CaM + 40 μg peptide	5.38	301

F_{max}: Fluorescence Intensity (FI) of CaM+ Ca^{2+}, or CaM+ Ca^{2+}+ peptide 2

F_o: FI of CaM

λ_{max}: wavelength of maximum FI

Peptide fraction #2, which was used at much lower concentrations, had higher Fmax/Fo ratios than fraction #1. All these data indicate that peptide fraction #2 had a much greater effect on CaM structure than fraction #1. There were no changes in the λmax values at different peptide concentrations, which indicate that the exposed hydrophobic groups interacted with the environment to the same degree.

Previous research using fluorescence showed that binding of calcium to CaM led to solvent exposure of hydrophobic patches, which thus became ready to interact with the targets; these hydrophobic patches were actually involved in peptide binding in the experimental structure of the calmodulin-peptide complex (Yang et al., 2004). CaM-binding peptides have been shown by previous researches using fluorescence that they bound to CaM by hydrophobic interactions which were reflected from the increased fluorescence intensity, and the higher the concentrations, the higher the fluorescence intensity (Bouvier et al., 2003). This increased fluorescence intensity was accompanied by a blue shift of the maximum emission wavelength, which demonstrated the hydrophobic environment and the formation of the calcium/CaM-peptide complex. Our results are consistent with these previous works.

5.3.2 Fluorescence properties of CaMKII in the presence and absence of peptides: The effect of calcium/CaM on CaMKII structure is shown in Figure 5.3.3. CaM at higher concentrations led to increased FI. The results indicate that when

CaM is bound to CaMKII, it promoted unfolding of the enzyme protein structure, leading to increased exposure of hydrophobic groups, especially tryptophan. Since interaction with CaM is required for enzyme activity, it would seem that unfolding of the enzyme protein structure promotes the autocatalytic (autophosphorylation) properties of CaMKII. Figures 5.3.4 and 5.3.5 show the effects of peptide fraction #1 and #2 (at different concentrations) on structure of CaMKII. In the plots, FI increased with increased peptide concentration, suggesting that the CaM-peptide complex increases the unfolding of CaMKII beyond the level possible with CaM alone, which led to greater exposure of hydrophobic domains. This influence on enzyme structure was stronger when peptides from fraction #2 were used than when fraction #1 was used. The results are consistent with enzyme kinetics studies that showed greater inhibitory properties of fraction #2 when compared to fraction #1. Tables 5.3.3 and 5.3.4 showed that the value of Fmax/Fo ratio was increased as the peptide concentration increased, which is consistent with results of the FI plots. Therefore, the results suggest that CaM binding is required to unfold CaMKII structure prior to autophosphorylation. Beyond this initial unfolding, binding of inhibitory peptides causes excessive changes in enzyme structure that are detrimental to catalysis. Peptide fraction #2 with a higher binding affinity towards CaM was more efficient in promoting excessive unfolding (and possible denaturation) of CaMKII structure when compared to fraction #1.

Figure 5.3.3. Effect of calcium/CaM (different concentrations) on structural changes of CaMKII.

Excitation wavelength was 295 nm.

CaM concentration was 20 µg/ml and calcium concentration was 0.196 mg/ml.

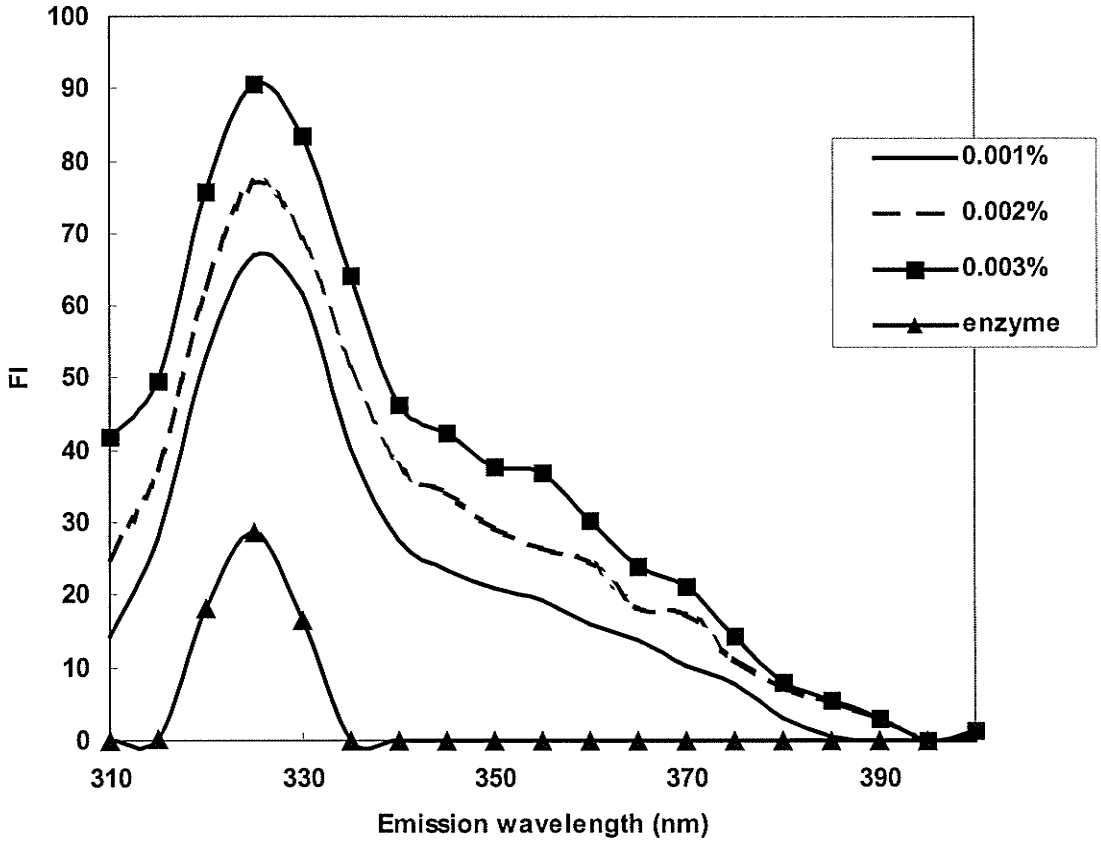


Figure 5.3.4. E ffect of peptide fraction #1 (different concentrations) on structural changes of CaMKII.

Excitation wavelength was 295 nm.

CaM concentration was 20 µg/ml and calcium concentration was 0.196 mg/ml.

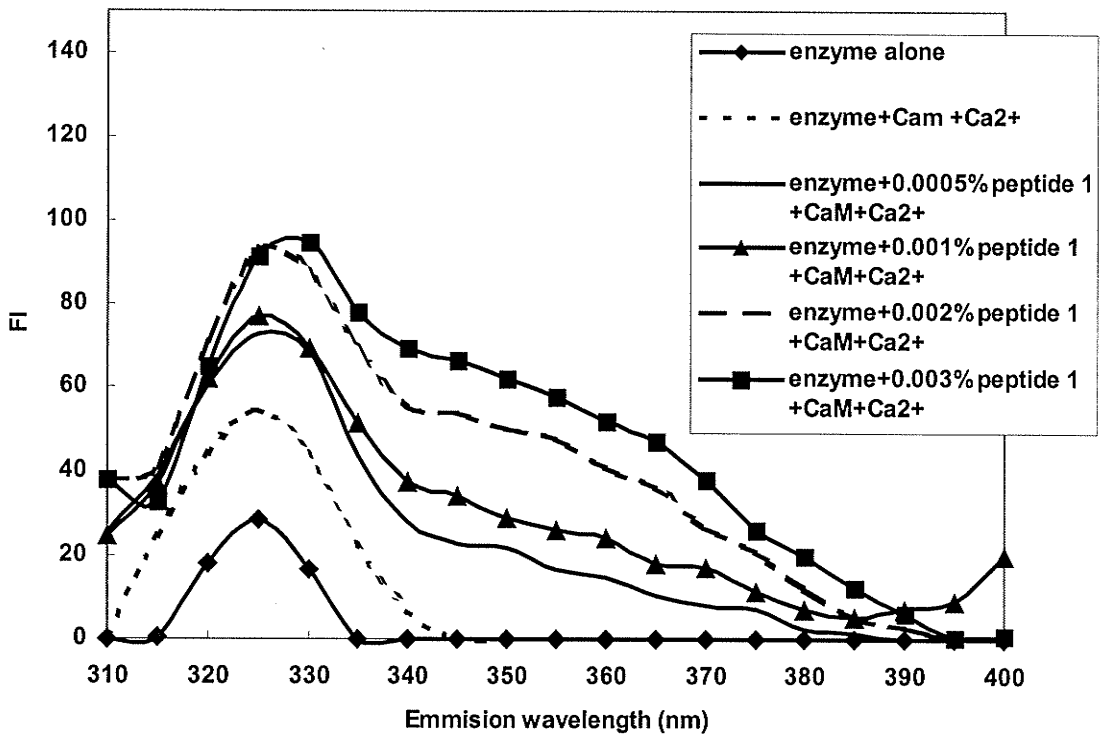


Figure 5.3.5. E ffect of peptide fraction #2 (different concentrations) on structural changes of CaMKII.

Excitation wavelength was 295 nm.

CaM concentration was 20 µg/ml and calcium concentration was 0.196 mg/ml.

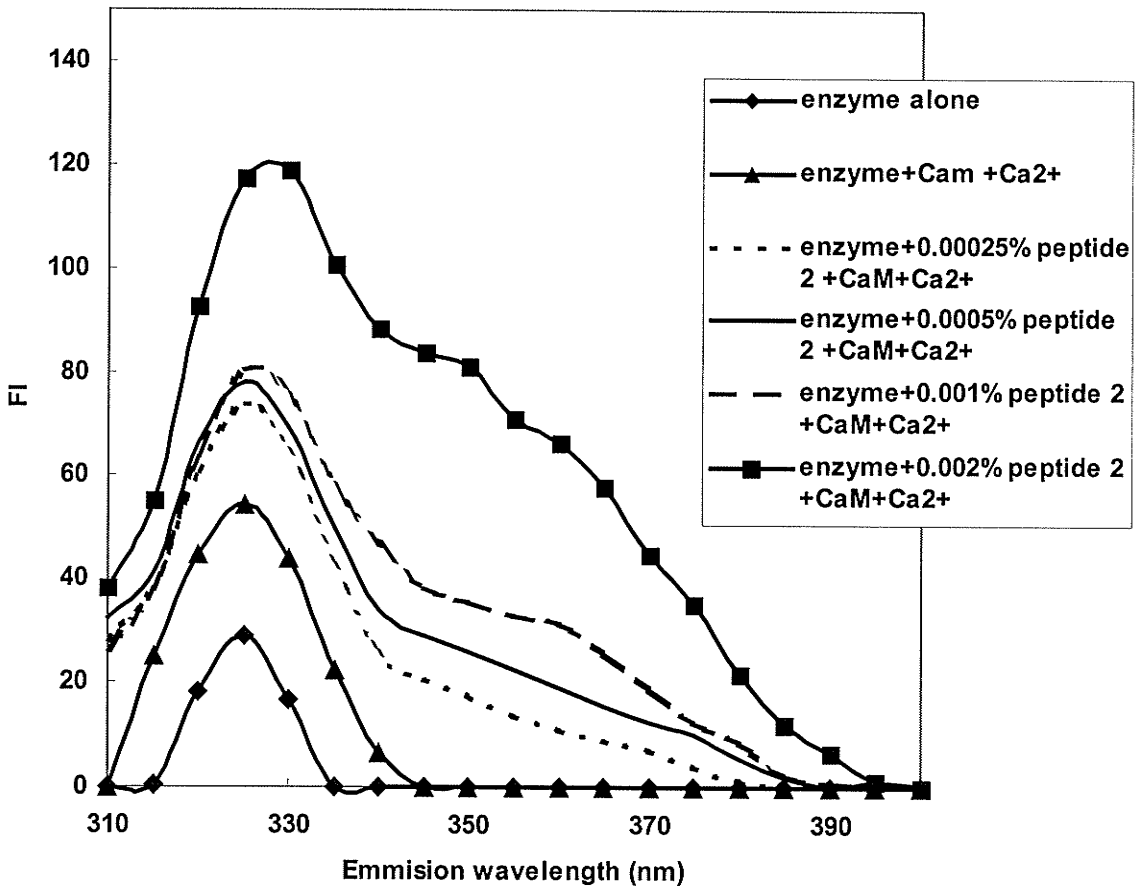


Table 5.3.3. Fmax/Fo of CaM+ CaMKII, or CaM+ CaMKII+ peptide fraction #1 at different concentrations.

Sample	Fmax/Fo	λmax (nm)
CaM/kinase	1.89	325
CaM + 10 μg peptide	2.52	325
CaM + 20 μg peptide	2.68	325
CaM + 40 μg peptide	3.22	325
CaM + 80 μg peptide	5.71	330

F_{max}: Fluorescence Intensity (FI) of CaM+ CaMKII, or CaM+ CaMKII+ peptide 1

F_o: FI of CaMKII

λ_{max}: wavelength of maximum FI

Table 5.3.4. Fmax/Fo of CaM+ CaMKII, or CaM+ CaMKII+ peptide fraction #2 at different concentrations.

Sample	Fmax/Fo	λmax (nm)
CaM/kinase	1.89	325
CaM + 5 μg peptide	2.56	325
CaM + 10 μg peptide	2.71	325
CaM + 20 μg peptide	2.79	325
CaM + 40 μg peptide	7.14	330

F_{max}: Fluorescence Intensity (FI) of CaM+ CaMKII, or CaM+ CaMKII+ peptide 1

F_o: FI of CaMKII

λ_{max}: wavelength of maximum FI

5.3.3 Determination of CaM structural changes using ANS fluorescence: Figure 5.3.6 shows the CaM structural changes in the absence and presence of calcium as determined by ANS fluorescence values. In the presence of calcium there was a substantial increase in the ANS FI for CaM, which indicates that binding of calcium causes conformation changes that made more hydrophobic groups available for binding to ANS. The result is consistent with FI data shown above that indicates exposure of more hydrophobic groups in calcium/CaM complex when compared to CaM alone. Figures 5.3.7 and 5.3.8 show the effects of peptide fractions #1 and #2 on the binding of ANS to CaM. It was obvious that interactions between peptides and CaM made more hydrophobic groups to be exposed, which led to increased binding by ANS and greater FI values. The use of ANS in our research confirms the intrinsic fluorescence results that these inhibitory peptides really change CaM structure through protein unfolding and increased exposure of hydrophobic groups. Table 5.3.5 shows the FI peak wavelengths (λmax) for different samples. The λmax value of ANS was shifted slightly towards the blue spectrum of light (lower wavelengths) upon binding to the samples. The blue shift in ANS fluorescence is because the interaction with hydrophobic groups in the target proteins shields the ANS from an aqueous environment, which reduces wavelength of emission. Generally, increased interaction with aqueous environment will increase the wavelength of light emission. However, this blue shift was more pronounced when the inhibitory

Figure 5.3.6. Structure changes of CaM using ANS as a hydrophobic probe.

Excitation wavelength was 385 nm.

CaM concentration was 20 μg/ml and ANS concentration was 60 μM.

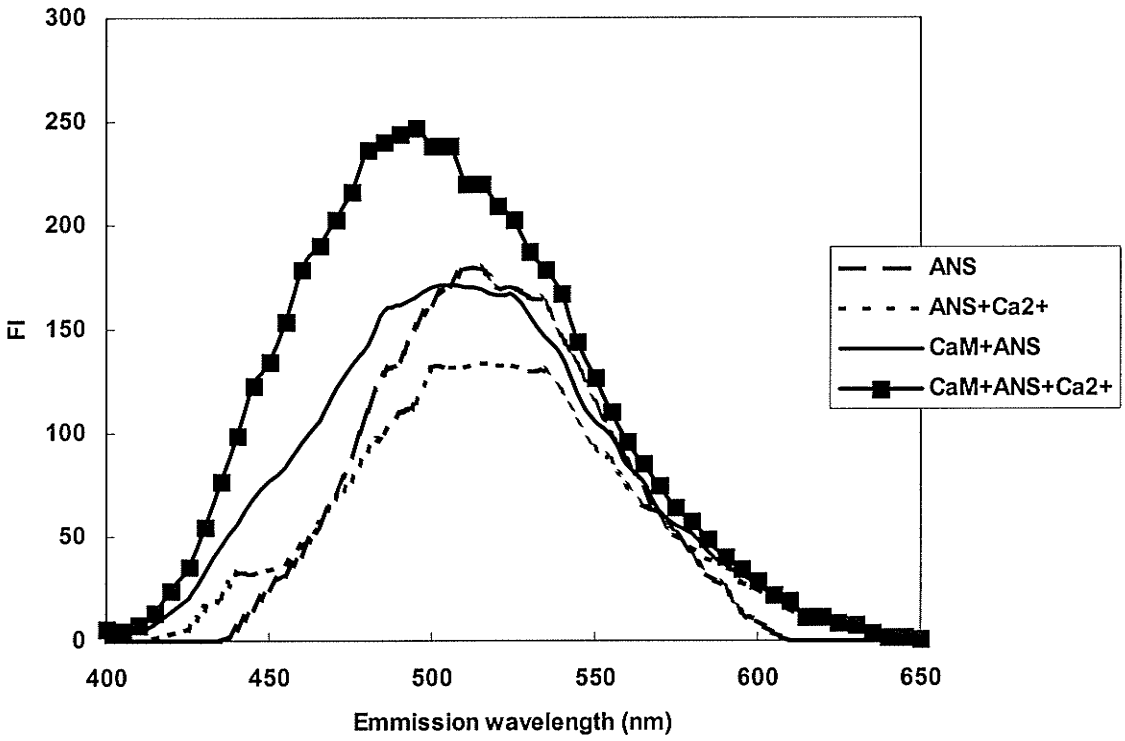


Figure 5.3.7. Structure change of CaM at fixed peptide fraction #1 concentration using ANS as a protein probe.

Excitation wavelength was 385 nm.

CaM concentration was 20 µg/ml and ANS concentration was 60 µM.

Peptide concentration was 0.0005%.

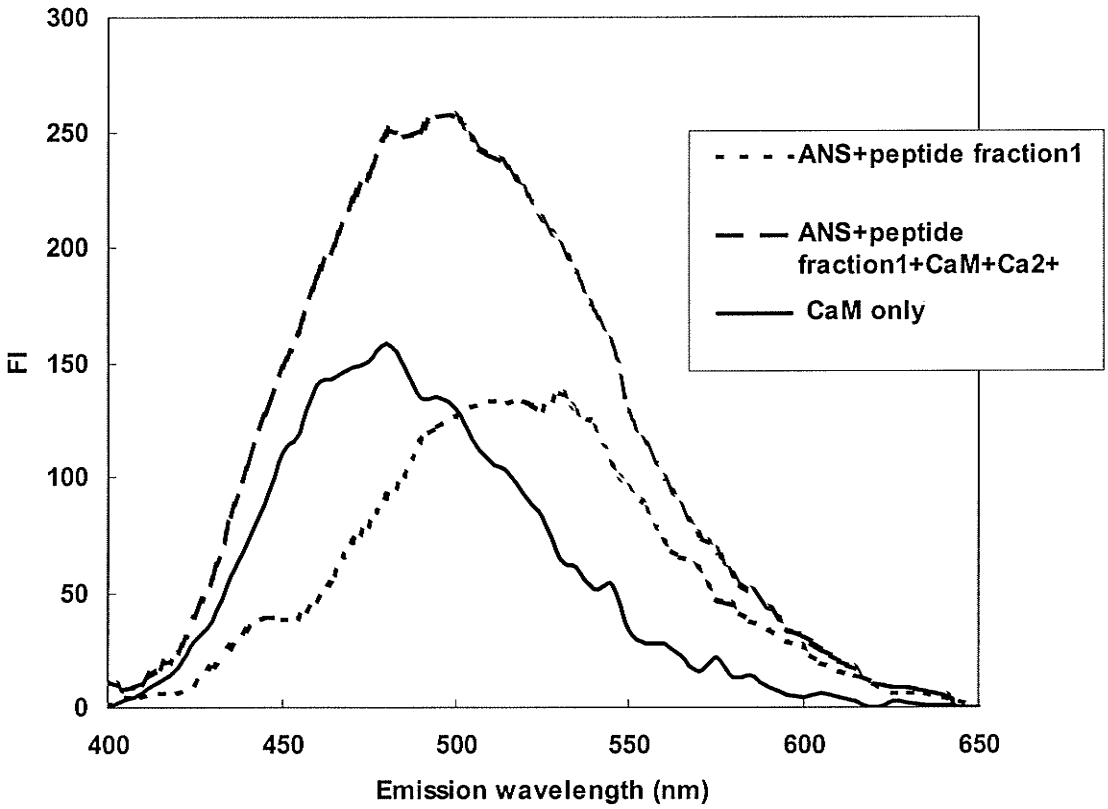


Figure 5.3.8. Structure change of CaM at fixed peptide fraction #2 concentration using ANS as a protein probe.

Excitation wavelength was 385 nm.

CaM concentration was 20 µg/ml and ANS concentration was 60 µM.

Peptide concentration was 0.00025%.

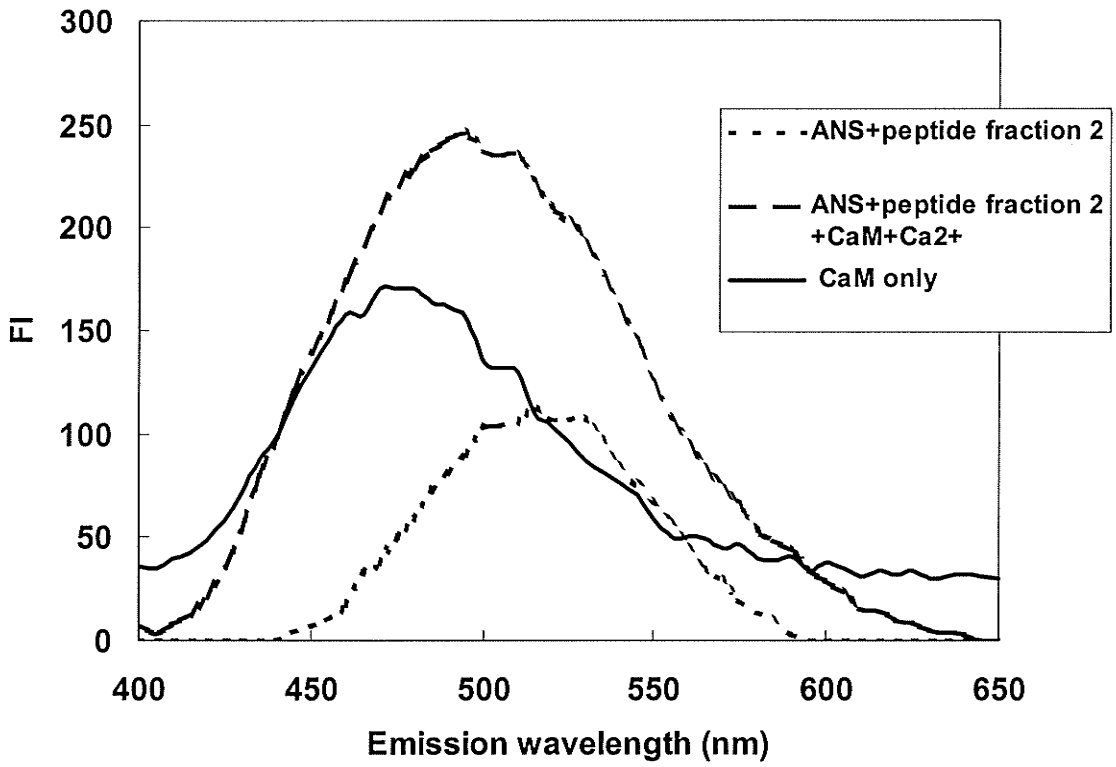


Table 5.3.5. Wavelength shifts obtained during CaM structure changes using ANS as a protein probe.

λmax: wavelength of maximum Fluorescence Intensity

Sample	λmax (nm)
ANS	515
ANS+ Ca^{2+}	515
ANS+CaM	502
ANS+CaM+ Ca^{2+}	498
peptide 1+ANS	515
peptide 1+ANS+CaM+ Ca^{2+}	487
peptide 2+ANS	515
peptide 2+ANS+CaM+ Ca^{2+}	494

peptides are present, which suggests the presence of greater number of hydrophobic groups that shields the ANS probe from the aqueous environment.

5.3.4 Determination of CaM structural changes using CD: The far UV CD spectra of the samples at pH 7.0 are shown in Figures 5.3.9. The negative CD intensities at 208 and 222 nm are used as a measure of the amount of α-helix in a protein. Beta-sheet also contributes to this negative band at about 216 nm with less magnitude than α–helix (Gore, 2000). In the presence of calcium, the negative band was slightly of higher intensity than that of CaM only. This is consistent with previous studies that have shown increased α-helix structure of CaM as a result of binding with calcium (Zielinski, 1998). Addition of peptides to the calcium/CaM complex decreased the negative intensities at 208 and 222 nm, which indicates a reduction in the amount of α-helix in structure of CaM. The proportions of secondary structures are shown in Table 5.3.6. The results show that an increase in α-helix structure of CaM is promoted in the presence of calcium, which indicates that this structural conformation is required for activation of CaMKII. Addition of inhibitory peptides reduced the α-helix structure of CaM, which further confirms the role of this structural conformation in CaMKII activity. Apart from the decrease in percentage of α-helix structure, peptides in fraction #1 modified CaM structure to contain substantially higher level of randomness when compared to calcium/CaM complex or CaM alone. It is possible that such an increase in random structure resulted from unfolding of CaM structure upon

binding of the peptides. The result is consistent with the observed increases in fluorescence properties of CaM in the presence of fraction #1 peptides. Conversely, peptides in fraction #2 modified CaM structure to contain a very high proportion of beta-sheet conformation. Beta-sheet represents a highly stretched structure when compared to α–helix (Fennema, 1985). The level of β-sheet conformation of CaM upon binding of peptides in fraction #1 was slightly reduced when compared to CaM alone or the calcium/CaM complex. This may explain previous results that showed increased exposure of aromatic groups and thus greater inhibitory property of peptides in fraction #2 when compared to fraction #1. The results suggest that conformation changes that produce more β-sheet are more effective than random structure in exposing the hydrophobic groups of CaM.

The near UV CD spectra of CaM in the absence and presence of calcium and inhibitory peptides at pH 7.0 are shown in Figure 5.3.10. Near UV region, which is from 250 to 320 nm is described as the aromatic region because aromatic amino acids (phenylalanine, tyrosine, and tryptophan) have absorbance in this region (Gore, 2000) and the spectra obtained are used to describe the tertiary structure of proteins. CaM does not contain tryptophan residue, therefore tertiary structure is estimated from the responses obtained at 250-260 nm for phenylalanine and 265-275 nm for tyrosine. Positive CD signal is as a result of increased interaction between aromatic groups and indicates a compact protein structure. In contrast a negative CD signal means less interaction between the aromatic groups and

indicates protein unfolding. The results showed that CaM did not give any significant CD signal whereas addition of calcium provided two distinct negative peaks for phenylalanine at 261 and 268 nm which meant that the phenylalanine residues were located in two different environments. The one absorbing at 261 nm was in a more hydrophobic environment while the one absorbing at 268 nm was in a more hydrophilic environment. The intensity of these two peaks increased with addition of peptides; fraction #2 induced more negative intensity than fraction #1. These results indicate gradual loss of compact structure of CaM as calcium and peptides were added; the change produced by calcium induced optimum unfolding that is required for CaM to activate CaMKII activity. However, as the CaM progressively unfolds with addition of peptides, CaM interacts more strongly with CaMKII, which results in enzyme denaturation and loss of activity. The results are consistent with the fluorescence that showed peptides in fraction #2 produced greater structural changes in CaM when compared to fraction #1. Previous near-UV studies of CaM showed two negative signals corresponding to tyrosine and phenylalanine respectively, and calcium binding to CaM was accompanied by an increase of the CD spectra (Kilhoffer et al., 1981; Harmat et al., 2000; Sun et al., 2001). In our study, we didn't see negative peaks contributed by tyrosine. This might be because that tyrosine absorption was cancelled out, i.e., the amount of left rotation was equal to the right rotation.

In conclusion, these peptides obtained from pea protein can interact with CaM,

and thus lead to the structural changes of CaM. These structural changes are mainly expressed as exposure of hydrophobic residues and changes of secondary fractions proportions.

Figure 5.3.9. Far-UV circular dichroism spectra of CaM, CaM/calcium and CaM/calcium/peptide comlex at pH 7.0.

CaM concentration was 0.25 mg/ml.

Calcium concentration was 10 mM.

Peptides concentrations were 1 mg/ml.

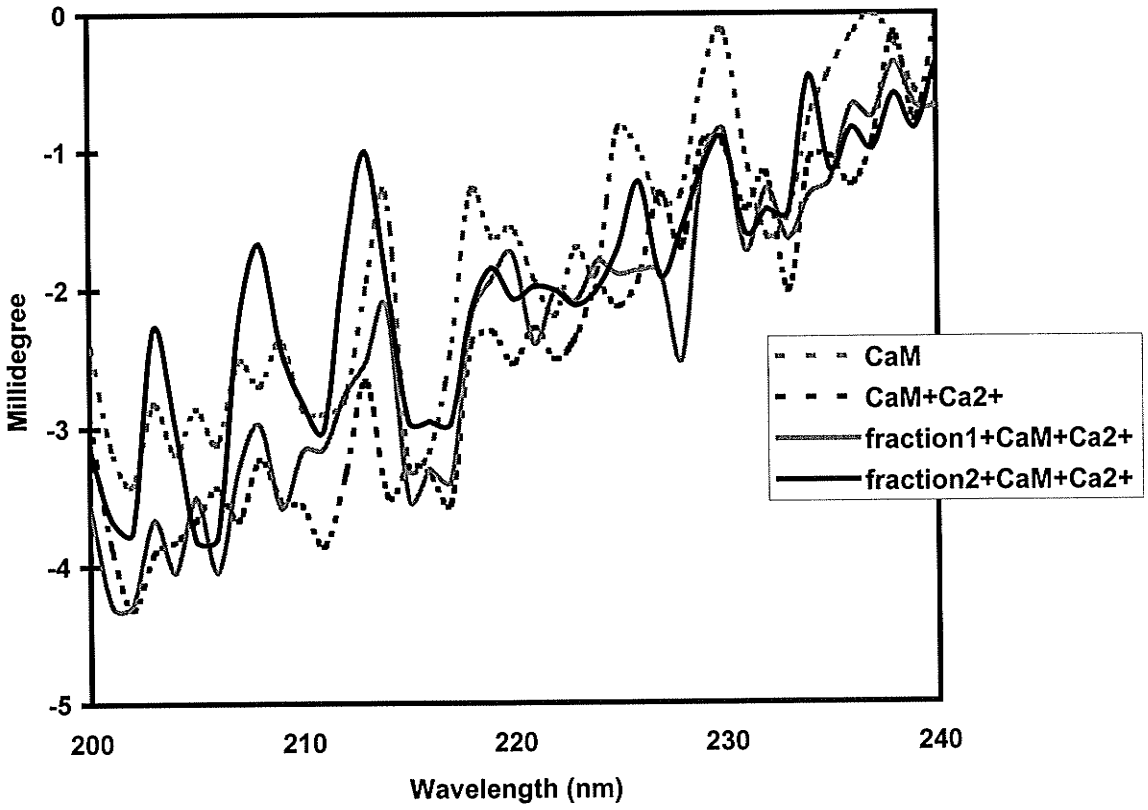


Figure 5.3.10. Near UV circular dichroism spectra of CaM, CaM/Ca^{2+} mixture and CaM/Ca^{2+}/peptides mixtures at pH 7.0.

CaM concentration was 0.1 mM.

Calcium concentration was 1.0 mM.

Peptide concentration was 1 mg/ml.

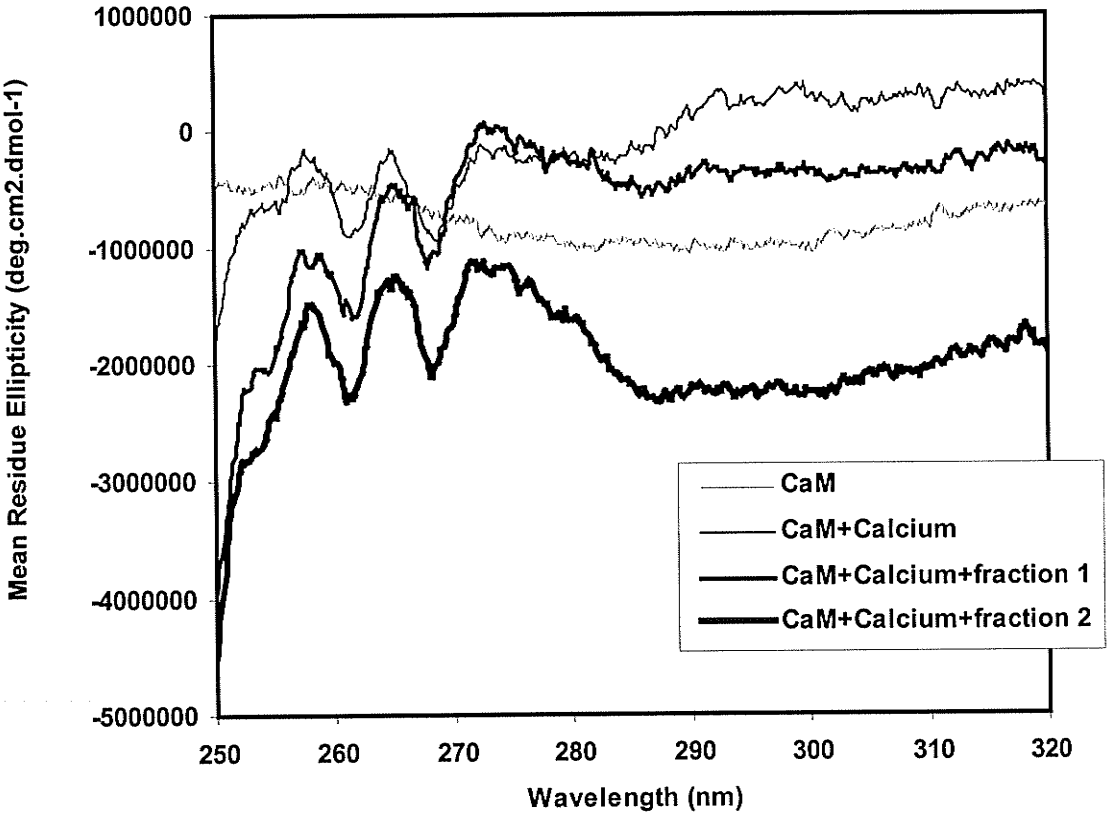


Table 5.3.6. Proportions of secondary structure fractions of different solutions *.

	α-helix (%)	β-sheet (%)	β-turn (%)	random (%)
CaM	2.3	60.9	13.6	23.2
CaM+ Ca^{2+}	7.8	50.8	16.5	24.9
peptide 1+ CaM+ Ca^{2+}	2.1	45.2	12.5	40.2
peptide 2+ CaM+ Ca^{2+}	3.9	73.9	2.7	19.5

* Mean of values from 4 scans per replicate.

The proportions of the secondary structures (α-helix, β-sheet, β-turn, random) were determined using the JASCO protein secondary structure estimation program (Japan Spectropolarimetry Co.) based on the method of Chang et al. (Chang, et al, 1978).

CHAPTER SIX

6.0 SUMMARY AND CONCLUSIONS

The present work was carried out to produce CaM-binding peptides through enzymatic hydrolysis of pea proteins, study kinetics of CaMKII inhibition by these peptides and changes in the secondary and tertiary structures of CaM in the presence of these inhibitory peptides.

Enzymatic hydrolysis with alcalase was optimized using different enzyme/substrate/time ratios to produce CaM-binding peptides from pea protein isolate. After filtration through a 1,000 MWCO membrane, these peptides were incubated with gastric enzymes to test their potential stability against digestive enzymes of the gastrointestinal tract. The results showed that a combination of 5% (w/v) substrate, 4% (w/w) enzyme, and 6 hr of hydrolysis produced hydrolysate that had highest binding affinity to CaM before and after gastric enzyme digestion. The low molecular weight and the stability against gastric enzymes will ensure that the peptides escape digestion in the small intestine and pass into the blood circulatory system.

Two peptide fractions were obtained after separation of the hydrolysate on a cation-exchange column using an FPLC, suggesting that these two peptides contained net positive charges. Peptide fraction #1 was much more abundant than peptide #2, but peptide #2 was more positively charged than peptide #1. CaM-binding determination showed that peptide #2 had almost three times affinity

for CaM than peptide #1, indicating a stronger interaction between CaM and peptide #2.

Enzyme inhibition kinetics was studied for both peptides #1 and #2. Both fractions inhibited autophosphorylation of CaMKII and subsequent phosphorylation of a polypeptide substrate. The inhibitions were competitive, which suggested that this inhibition could be overcome with high concentrations of CaM. Data showed that the IC_{50} values for both peptides increased with increased level of CaM. Peptide #2 had a higher inhibitory property toward CaMKII than peptide #1 and this could be seen from the inhibition constant (Ki) values.

Fluorescence spectrophotometry showed that interactions between CaM and inhibitory peptides led to the unfolding of CaM and thus more exposed hydrophobic groups. Peptide #2 had greater effects on CaM structure than peptide #1, and higher concentrations had greater effects than lower concentrations. Fluorescence property of CaMKII was examined in the presence and absence of inhibitory peptides. At fixed peptide concentration, CaM with higher concentration led to higher fluorescence intensity, indicating that CaM promoted unfolding of the CaMKII structure and exposed more hydrophobic residues. Since CaMKII needs CaM for activity, it could be considered that unfolding of the enzyme structure is required for CaMKII autophosphorylation. Fluorescence intensity increased at higher peptide concentrations, which means increased unfolding of CaMKII structure that may be excessive and detrimental to

normal enzyme activity. Peptide #2 had stronger influence on unfolding of CaMKII structure than peptide #1, which was consistent with kinetics studies. Studies using ANS as a protein probe showed that binding of calcium and peptides changed CaM structure, leading to unfolding of structure and more exposed hydrophobic groups which were accompanied by a blue shift of maximal wavelength. The results from the ANS probe were consistent with excessive unfolding of enzyme structure in the presence of inhibitory peptides, which agrees with data from the intrinsic fluorescence studies.

Secondary structures of CaM in the presence and absence of peptides were determined by far-UV CD. The presence of calcium led to the increase of α–helix while addition of inhibitory peptides led to reduced α–helix, which confirms the importance of this conformation to normal enzyme activity. Peptide #1 increased randomness structure of CaM and peptide #2 increased β-sheet structure of CaM. Random structure may come from the unfolding of CaM when binding to peptides. Beta-sheet is more stretched than α–helix and can explain the increased exposure of hydrophobic groups caused by peptide binding to CaM. Since peptide #2 has higher affinity for CaM than peptide #1, it would seem that β-sheet is more effective than random structure in exposing hydrophobic groups of CaM. Tertiary structures of CaM in the presence and absence of peptides were measured by near-UV CD. Results showed that addition of calcium gave two negative peaks for phenylalanine, and the intensities of these two peaks increased in the presence of

inhibitory peptides, with peptide #2 producing greater intensity than peptide #1 at the same concentration. This suggests that interaction with calcium makes CaM lose its compact structure and induce optimum unfolding that is needed to activate CaMKII activity; interactions with peptides leads to excessive unfolding and stronger interaction with CaMKII which might denature the enzyme and thus loss of activity.

In conclusion, the present work has shown that it is possible to produce specific peptides with desirable CaMKII inhibitory properties by using an alcalase-catalyzed hydrolysis of pea proteins. The mechanism of inhibition by the peptides is through strong interactions with CaM to induce protein unfolding. The resulting CaM-peptide complex then interacts strongly with CaMKII, which causes excessive unfolding of enzyme structure and subsequent loss of catalytic activity.

CHAPTER SEVEN

7.0 SUGGESTIONS FOR FURTHER RESEARCH

Future work is required to further purify peptide fractions #1 and #2 to get homogenous peptide preparations which might be more potent. Amino acids sequence analysis of the peptides may also need to be done. It is necessary to do quantitative structure-function analysis using amino acids composition to better understand the inhibitory properties of the peptides. Except in vitro studies, in vivo studies, such as absorption, transportation and metabolism of these peptides are also important to do, which can provide information on potency of the peptides in the body. Biological testing using specific animal disease models such as cancer, kidney malfunction and cardiovascular impairment are also desirable to determine the potential physiological impact. Finally, the amounts that are suitable for human consumption without serious negative impact on the consumer need to be determined in order to achieve the optimum therapeutic effect against disease conditions.

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APPENDIX

Figure 1. Standard curve I of bolvin albumin in protein content determination.

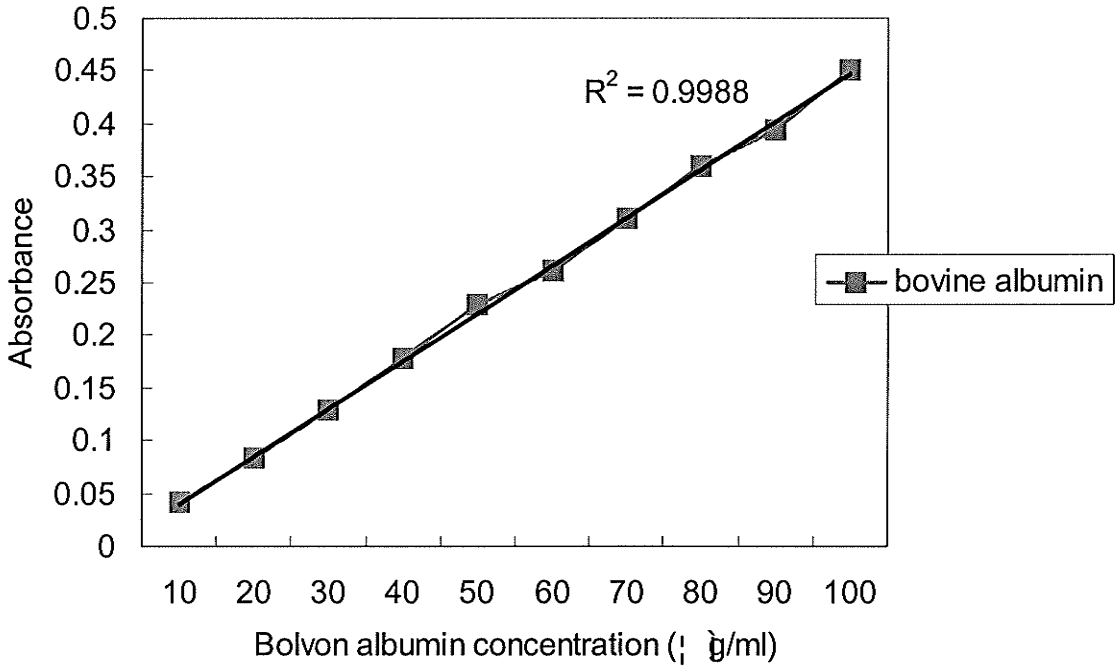

Figure 2. Standard curve II of bolvin albumin in protein content determination.

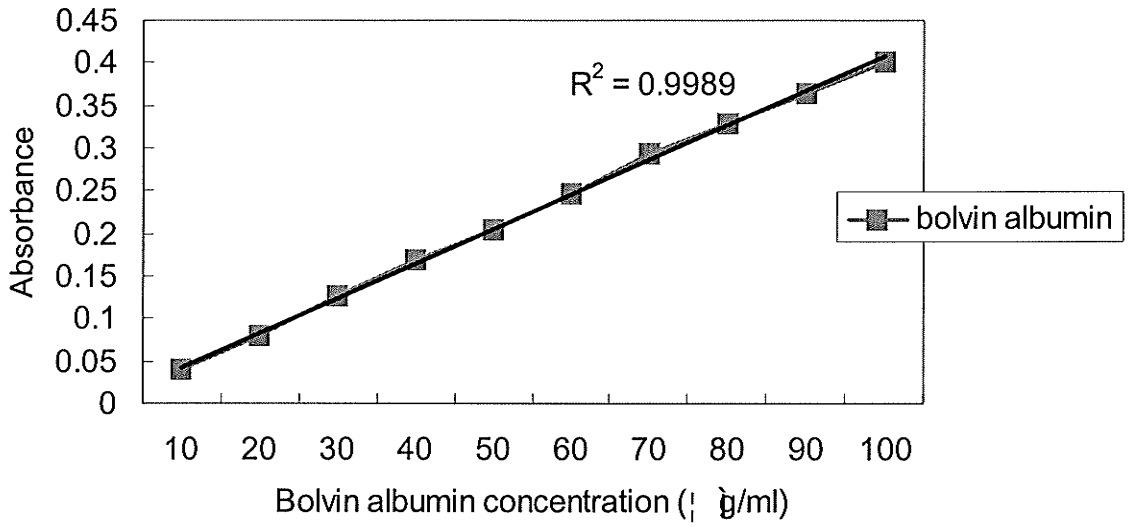

Figure 3. Standard curve III of bolvin albumin in protein content determination.