

**STUDIES ON INHIBITION OF ANGIOTENSIN-I CONVERTING  
ENZYME (ACE) BY CASEIN NON-PHOSPHORYLATED PEPTIDES  
(Cn-NPP)**

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By

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## DECLARATION

I Seronei Chelulei CHEISON do hereby solemnly declare that the works whose results are reported herein were carried out solely by me under the guidance and supervision of Prof. Zhang WANG of the Food Science Laboratory, School of Food Science and Technology, Southern Yangtze University, Wuxi – China PR and further declare that this work has not been submitted to any other university and/ or research institution for consideration and/or award of a degree and/or academic qualification.

Signed this 27<sup>th</sup> day of June in the year of our Lord 2003.

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## CERTIFICATION

This is to certify that the report presented herein entitled 'Studies on Inhibition of Angiotensin-I Converting Enzyme (ACE) by Casein Non-Phosphorylated Peptides (Cn-NPP)' submitted by one Mr. Seronei Chelulei CHEISON for the requirements of the award of the degree of M.Sc. in Food Science is based on the results of works carried out by him in the Laboratory of Food Science, School of Food Science and Technology, Southern Yangtze University, Wuxi – China PR under my guidance and supervision during the academic season 2001-2003. I certify further that neither the research report nor any part thereof has been submitted previously for consideration and/or award of any other degree.

Signed this 27<sup>th</sup> day of June in the year of our Lord 2003.

Signature.....

Professor Zhang WANG  
(Supervisor)

## DEDICATION

*This Work is dedicated:*

To the loving memory of my departed Grandma, Pot Tera  
Who initiated me into liberal and creative thinking in the hope that I would prosper

To unquenched love for my Baba, Kiptarus  
Who dreamt of this great day but was cruelly denied the joy of beholding it

To my loving Mama, Chemoso  
Whose strength in heart has helped me weather the storms of life  
Whose earnest prayers kept me going

To my loving wife, Ednah  
The love of my youth  
Who remained on her knees to keep me on my feet

To Kiptoo and Cherop  
The two little gifts from God-  
For whom dad was the distant voice on the phone  
My fervent inspirations

To Hon. Kosgey,  
A true father and God's benevolent gift of a reliable friend per excellence

To my brothers and sisters, especially Philister  
Who disobeyed the barrier of age to reclaim her place in the academia

To Cheptabach and Nandi  
Whose relentless urge drove me to achieve even when my knees were yielding

To all and sundry  
Those who will derive something from this work and from me,  
With or without my knowledge

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Who gave me peace within and assurance of health abundant,  
To Him I ascribe Glory, Honour, Power and Majesty

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## ABSTRACT

This research work was concerned with studies on inhibition of angiotensin-I converting enzyme (ACE, peptidyl dipeptide hydrolase, EC 3.4.15.1) by peptides produced from casein (Cn) following hydrolysis by Alcalase 2.4L (EC 3.4.21.62) and selective precipitation of CaCl<sub>2</sub> enriched Cn phosphorylated peptides (CPP) from the mixture by alcohol. The peptides of interest were non-phosphorylated peptides (Cn-NPP) obtained from Cn following enzymatic hydrolysis. The study involved *in vitro* assaying for inhibition of ACE by the peptides with modifications of the traditional spectrophotometric method by use of reversed phase high performance liquid chromatography (RP-HPLC), with a chromogenic substrate, which liberates a detectable product ( $\lambda_{\text{Max}} = 228\text{nm}$ ) following hydrolysis by ACE.

To begin with, Cn was hydrolyzed to various degrees of hydrolysis (DH), basically 10%, 15% and 20% with DH being monitored by addition of NaOH (pH-stat). The hydrolysates were enriched with CaCl<sub>2</sub> and CPP selectively precipitated out to remain with Cn-NPP by use of food grade alcohol to 70% concentration. Assaying to ascertain whether there was an empirical relationship between DH and ACE inhibition by Cn-NPP followed. ACE assay showed that there was a concomitant increase in ACE inhibition by Cn-NPP with increasing DH, being lower at DH 10% ( $\text{IC}_{50} = 1.87\text{mg/mL}$ ) than DH 15% ( $\text{IC}_{50} = 0.9\text{mg/mL}$ ) and being highest at DH 20% ( $\text{IC}_{50} = 0.7\text{mg/mL}$ ).  $\text{IC}_{50}$  was defined as the concentration of peptides to give 50% ACE inhibition. Furthermore, the difference in ACE inhibition between hydrolysates from DH 20% and DH 15% Cn-NPP was not significant and it was found satisfactory to work with peptide mixtures obtained following hydrolysis to DH 15% for further analysis. The ACE inhibition as related to DH implicated generation of short chain, possibly more hydrophobic peptides in Cn-NPP during hydrolysis, which were more potent inhibitors. Quantitative amino acid analysis of Cn-NPP showed a high proportion of highly hydrophobic amino acids (51.63% w/w). Most of the essential amino acids were present representing 49.73% (w/w), which is higher than the recommended level of 33.3% for nutritional significance.

To remove the salt formed during the pH-stat procedure, macroporous adsorption resins (MAR) were employed. Studies on the conditions for adsorption and desorption implicated temperature and flow rate as the main instigators of adsorption and implied that adsorption was typically of hydrophobic interaction in nature. The adsorption gave a Langmuirian –type adsorption isotherm while (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> favoured adsorption which implied a salting out property strengthening the hydrophobic interaction nature of the adsorption. Alcohol could be used to desorb the peptides from the resins and the recovery was high.

When put through size exclusion chromatography (SEC) using Sephadex G-15, the peptides showed a typical nonideal SEC (nSEC) elution which was greatly influenced by buffer pH and concentration. Investigations revealed that 0.02mol/L sodium phosphate buffer pH 7.5 were satisfactory for delineation of peaks while eluting peptides from this column. Furthermore, the nonideal conditions provided a finite range of conditions through which the peptides could be eluted satisfactorily in SEC. Alcohol at 2% did reduce the peptide aggregation and/ or peptide-matrix hydrophobic interaction leading to good peak separation. The

study demonstrated that the nonideal conditions provided a good opportunity which can be exploited for good SEC of peptides in conventional column chromatography.

When the peptide fractions eluting from SEC were characterized for ACE inhibition, the peptides with lower molecular weight, which eluted late in SEC had more potency in ACE inhibition. This implicated shorter peptides in inhibition of ACE with more potency than the higher molecular weight peptides. This result further strengthened the findings reported in the relationship between DH and ACE inhibition.

Further, when peptides were adsorbed on anion exchange resin, the effects of pH and ionic strength of the buffer were found to have significant influence on adsorption. Buffer pH 7.5 and 0.02mol/L sodium phosphate were found to be optimum. The peptides were also adsorbed onto the anion exchange resin with water only; whereas elution was possible with 1.5mol/L NaCl. The adsorbed peptides at pH 7.5 were characterized for ACE inhibition and found to have good inhibition properties. The SEC peak 3 was adsorbed at pH 7.5 and characterized for ACE inhibition and the resulting fraction had improved ACE potency than that for the whole peak. This result confirmed that irrespective of the nature and characteristics of the peptides generated by Cn following hydrolysis by Alcalase 2.4L, there are always some inhibition properties to ACE.

Characterization of ACE inhibition by RP-HPLC method gave good resolution and peak separation between the Hippuryl-L-Histidyl-L-Leucine (substrate) and hippuric acid (product) liberated by action of ACE. The gradient elution was found to be superior to isocratic elution and the study confirmed the superiority of RP-HPLC to spectrophotometric method in assaying for the ACE activity. The RP-HPLC method obviates the extraction procedure for the hippuric acid and eliminated possible entrainment of and contamination by the substrate with the extracted product as has been reported to result in contamination and exaggeration of the quantity of hippuric acid produced, and hence enzyme activity.

As results from our investigations indicate, Cn and peptides deriving from it have diverse ACE inhibition characteristics and potency, and other bioactivities should be investigated for these peptide mixtures to be appreciated fully.

**Key Words:** Alcalase 2.4L, Degree of hydrolysis, pH-stat, macroporous adsorption resin (MAR), gel filtration, anion exchange resin, angiotensin-I converting enzyme (ACE) inhibition, reversed-phase high performance liquid chromatography (RP-HPLC), casein non-phosphorylated peptides (Cn-NPP), nutraceuticals, functional foods, peptide bitterness.

## 摘要

本文主要研究了采用 Alcalase 2.4L (EC 3.21.62)水解酪蛋白并以酒精和氯化钙选择沉淀所得酪蛋白非磷肽(Cn-NPP)对 ACE (血管紧张素-I 转换酶)的抑制作用。通过一种 ACE 可以水解的发色底物对传统分光光度法进行改进, 并采用 RP-HPLC 法评价肽类对 ACE 的抑制作用。

首先将酪蛋白水解, 利用 pH-stat 法检测获得水解度 (DH) 分别为 10%、15%和 20%的三种水解产物 Cn-NPP。然后测定了这三种水解产物 Cn-NPP 对 ACE 的抑制活力, 结果表明随着 DH 的增加, Cn-NPP 的 ACE 抑制活力也相应增加, DH10% ( $IC_{50} = 1.87\text{mg/mL}$ )较之 DH15%( $IC_{50} = 0.9\text{mg/mL}$ )的 Cn-NPP 对 ACE 的抑制活力低, 而 DH20% ( $IC_{50} = 0.7\text{mg/mL}$ )Cn-NPP 的抑制活力则高于 DH15%的 Cn-NPP。而且, DH15%和 DH20%两种 Cn-NPP 产物对 ACE 的抑制活力差别不显著。对 ACE 的抑制活力与 DH 之间的关系显示了短肽的产生, 可能水解所得的疏水肽是主要的抑制因子。通过对 DH15%Cn-NPP 的氨基酸分析表明它含有较高的疏水氨基酸比例 (51.63%), 而且存在诸多的必需氨基酸, 必需氨基酸与总氨基酸的比例为 49.73%, 超过建议的营养需要水平 33.3%。

采用大孔吸附树脂(MAR)来去除由于在控制 DH 时加入了碱而产生的盐类。对吸附和解吸条件的研究表明温度和流速是吸附过程的主要影响因素, 这表明吸附过程的性质属于典型的疏水相互作用。吸附过程表现出有朗缪尔特性的等温吸附性质,  $(\text{NH}_4)_2\text{SO}_4$  对吸附有利显示盐析作用加强了吸附中的疏水相互作用。研究表明采用乙醇从树脂上解吸肽, 肽的回收率较高。

当所得的肽在 Sephadex G-15 上进行排阻洗脱时, 它表现出典型的非理想排阻洗脱特性, 且洗脱受缓冲液的 pH 和浓度影响很大。研究发现在 pH 中性以上, 缓冲液浓度 0.02mol/L 条件下, 洗脱峰的谱图基本令人满意。此外, pH 高于等电点  $pI$  一个单位而离子强度较弱的非理想条件下可以很好地分离肽。另外, 2%的乙醇可以减少肽的聚结而(或者)肽-基质间疏水相互作用可以使峰分离的更好。这表明采用非理想条件也可有效地分离多肽。

测定肽类洗脱组分对 ACE 抑制活力时发现相对分子质量较低的组分, 即出峰时间较晚的组分表现出更强的活力。这表明短肽在抑制 ACE 方面比长肽表现出更强的活力。这个结果进一步验证了在研究 ACE 抑制活力与 DH 之间的关系所得出的结论。

此外, 当采用阴离子交换树脂吸附肽类时, 缓冲液的 pH 和离子强度对吸附效果影响显著。最优的吸附条件是 pH7.5, 缓冲液浓度 0.02mol/L。仅仅采用水时肽类也能吸附, 而洗脱则采用 1.5mol/L NaCl 缓冲液。在 pH7.5 时吸附的肽类表现出良好的 ACE 抑制活力。凝胶排阻色谱分离所得的第三个色谱峰, 即 pH7.5 时吸附的组分, 对 ACE 的抑制作用比整个的肽类强。这个结果证实了由 Alcalase 2.4L 水解酪蛋白后产生的肽都有抑制 ACE 的活力。

HPLC 法测定 ACE 抑制活力具有较好的分辨率，底物马尿酸-组氨酰-亮氨酸和 ACE 作用释放的产物马尿酸两峰分离良好。研究发现梯度洗脱分离效果优于非梯度洗脱，同时确认了 HPLC 法在评价 ACE 抑制活力时优于分光光度法。

研究结果显示了酪蛋白和它所衍生的肽类有不同的 ACE 抑制活力，而其它的生物活性还有待于测定并对它们进行全面评价。

**关键词：**Alcalase 2.4L，水解度（DH），pH-stat，大孔吸附树脂（MAR），凝胶过滤（GF），阴离子交换树脂，血管紧张素-转化酶（ACE）抑制作用，营养因子，功能食品，反相高效液相色谱（RP-HPLC），酪蛋白非磷酸肽（Cn-NPP），肽苦味

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# CHAPTER ONE:

## INTRODUCTION & LITERATURE REVIEW

### 1.0 INTRODUCTION

#### 1.1 Milk

Milk is a perishable food, basically because of its high water activity and near neutral pH. Milk perishability has inspired processing and conversion into various products, unless it is consumed immediately following acquisition from the animal. By its nature, it is a polyphasic secretion of the mammary gland, and remains one of the most elaborately studied of human foods. Its composition within any mammalian species is indicative of the neonatal requirements of its offspring, presenting optimum composition of nutrients required during the neonatal period of that species. Milk and dairy products continue to play an important role in human nutrition in diverse regions of the world. Milk and dairy products have numerous advantages over other competitors when used as ingredients because they are colourless, have a bland taste, are rather stable to processing, are free from toxins as well as having constituents that are easily fractionated, if need be.

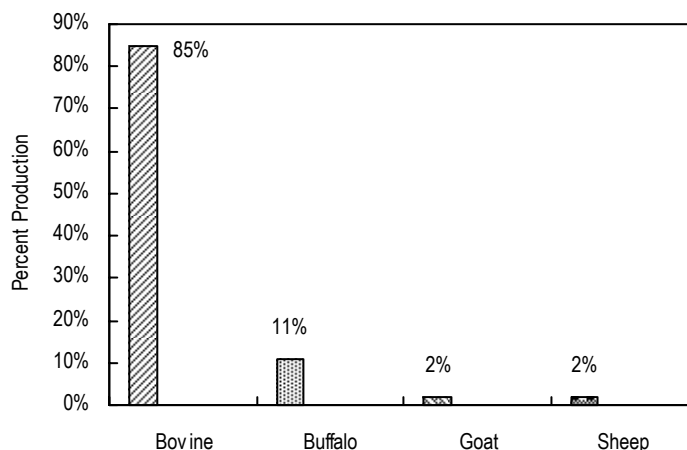


Fig 1. 1 Animal Sources of Milk

The total world milk production is not easy to estimate. However, conservative estimates (Fox, 2001) put the total world production of milk at about 560 million tonnes annually out of which approximately 85% is bovine, 11% buffalo and 2% sheep and 2% goats (Fig 1.1). Other dairy sources include the camel, yak, mare and reindeer, which make insignificant contributions to the total global milk output but are obviously major milk sources in particular geographic zones of the world. In the year 2001, India led in world liquid milk production although buffalo milk is included, such that the USA was the single leading producer when bovine milk is considered per se. The world production of milk in 2001 is presented in

Fig 1.2, whence Russia dominated in the non-EU European countries (IDF, 2003). Meanwhile, China's dairy production rose steadily from under 7 million tonnes in 1997 to just shy of 15 million in 2001 (IDF, 2003). Meanwhile, Kenya is one of the three leading dairying economies in Africa surpassed only by the Republic of South Africa with an annual production in the last five years standing at around 3 million tonnes annually, with promising expansion (Kenya Government, 2003).

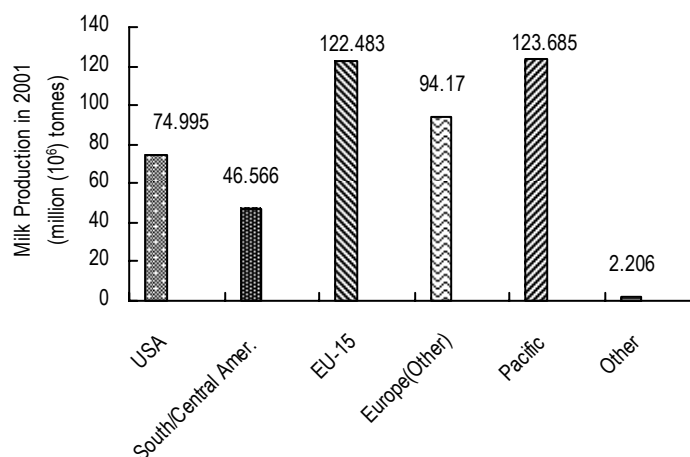


Fig 1. 2 World Milk Production for 2001 (Source IDF, 2003)

Precise data on the uses of various milks are not easily available, although it is probable that some sheep and goats' milk are converted to fermented milk, with the world-famous cheeses such as Roquefort, Romano, Manchego and Feta being produced from sheep milk. More than half the world population of buffaloes are in the Indian sub-continent, which produces about two-thirds of the world buffalo milk. Most of the milk in the traditional dairying civilizations of United States and Europe and Australia/ New Zealand are of bovine in origin.

Globally (Fig 1.3), 39% of bovine milk is used for beverage products, 33% for cheese, and 32% for butter while whole and skim milk powders account for 6% and 9%, respectively (USDA, 2000). Some comparatively small amounts of milk are utilized for concentrated milks (both sweetened condensed and evaporated milk), fermented milks, ice cream products as well as incorporation into infant formula. It is apparent that liquid/beverage milk, cheese and milk powders are the three major dairy products accounting for over 80% of the total milk output (Fox, 2001).

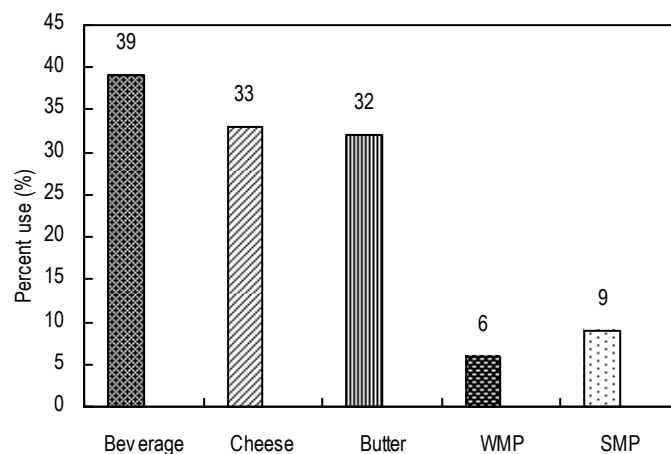


Fig 1. 3 Uses of Milk; WMP = whole milk powder, SMP = skim milk powder

### 1.1.1 Chemical Composition of Milk

By composition, it is an almost complete nutritional package, supplying both to the mammalian neonates some invaluable proteins, ranging from 3.0-3.5% of bovine milk, among other constituents, (Table 1.1). The amount of protein content in milk ranges from 1% in human milk to an abundance in excess of 20% in the small mammals like mice and rats (Hambraeus, 1982). Besides, there is a pronounced variation in the number and types of proteins across species. A correlation has been claimed between the protein content in milk and the growth rate of the neonate of any species (Hambraeus, 1992), the higher the protein content the faster the growth (Table 1.1). Bovine milk is perhaps the most studied of the mammalian milk and as such the issuing review as well as the research carried out in this study will be focussed on the same.

Apart from the main constituents, milk also contains some natural bioactive substances extant in it by virtue of the physiological origin during secretion. These include oligosaccharides, fucosylated oligosaccharides, hormones, growth factors, mucin and gangliosides, and endogenous peptides, which are present in milk at secretion. Nature factored these substances into milk with a scheduled significance; they support two lines of defences (Schanbacher *et al*, 1997; Wong *et al*, 1996). It would seem the first is an elaborate provision for the initial self-defence of milk and perhaps the mammary gland against the degradative army of micro-organisms while the second offers the first line shield for the neonate.

Among the former are lysozyme and lactoperoxidase while the latter comprise a coterie of defensive/protective components, which act as the first line of defence for the newborn, the immunoglobulins. Furthermore, there are also the neuro-active peptides such as bradykinin, which play a fundamental role in the functioning of the nervous system such as the regulation of pain perception and vasodilatation (Lahov and Regelson, 1996).

Table 1.1 Composition of milks obtained from different mammals and growth rate of their offspring

Species	Total Protein <sup>a</sup>	Cn (% Total Protein)	Fat <sup>a</sup>	Lactose <sup>a</sup>	Ash <sup>a</sup>	Days Required to double birth weight
Man	0.9	30	3.8	7.0	0.2	180
Goat	2.9	86	4.5	4.1	0.8	12
Cow	3.4	80	3.7	4.8	0.7	47
Water Buffalo	3.8	84	7.4	4.8	0.8	-
Indian Elephant	4.9	39	11.6	4.7	0.7	-
Sheep	5.5	84	7.4	4.8	1.0	10
Rat	8.4	76	10.3	2.6	1.3	6
Northern Fur Seal	8.9	52	53.3	0.1	0.5	-
Blue Whale	10.9	66	42.3	1.3	1.4	10

Adopted from Hambraeus (1982)

<sup>a</sup> Expressed as g per 100 g whole milk

### 1.1.2 Milk Proteins

Milk proteins are the most important of its constituents from a nutritional and physiological standpoint (Fox, 1992). Their distinctive individual physico-chemical, functional and technological properties are exploited in the food industry. The often-cited feature includes high heat stability, which enables the manufacture of a wide range of heat-sterilized, concentrated and dehydrated products without major changes in the physical and organoleptic properties of milk among others.

The milk proteins have broadly been classified into two (Fig 1.4); Caseins (Cn) are phosphoproteins that are insoluble and precipitate following acidification of raw skim milk to their isoelectric point of pH 4.6 at 20°C, and constitute some 80% of milk proteins (Eigel *et al*, 1984). There have been confusing references in nomenclature of milk proteins (particularly with respect to Cn nomenclature), but these were put to rest following the comprehensive review by the authoritative Eigel *et al* (1984) committee on milk protein nomenclature which identified some fragments in the whey fraction as emanating from proteolytic cleavage of some Cns by milk enzymes.

The second group, the whey proteins constitute a group of milk proteins that remain soluble in milk or skim milk at the precipitation point of Cns, pH 4.6 at 20°C. They have further been shown to comprise of  $\beta$ -lacto-globulin-  $\beta$ -LG- (7-12% of total skim milk protein),  $\alpha$ -lactalbumin-  $\alpha$ -LA- (2-5% of skim milk total protein), serum albumin (SA), immuno-globulins- Ig, lactotransferrin (lactoferrin-Lf) and  $\beta_2$ -microglobulin (Eigel *et al*, 1984; Wong *et al*, 1996).

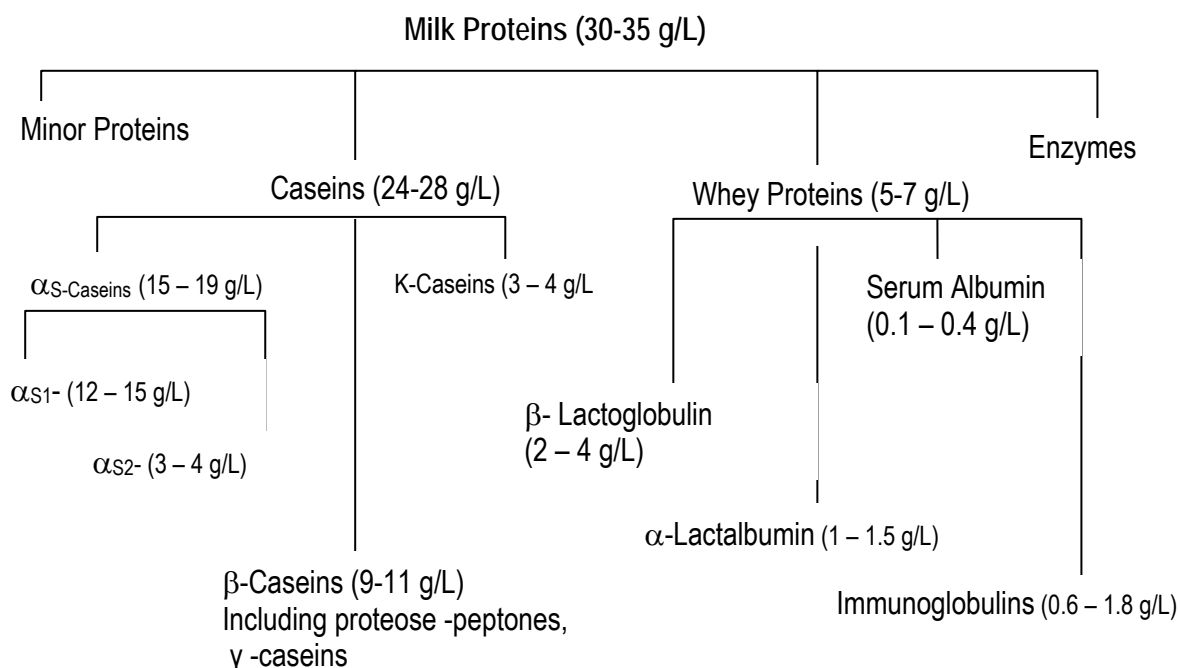


Fig 1. 4 Distribution of fractions and proteins in bovine milk. *Adopted with modifications from Swaisgood (1992)*

### 1.1.3 Caseins and Casein Structure

Casein (Cn) is the dominant milk protein in bovine milk constituting 76-86% of the total milk proteins although it makes up between 20 and 40% in human milk. One of the few known metallo-proteins, Cn is very milk-specific occurring with post-translational modifications exhibited in varying degrees of phosphorylation (Eigel *et al*, 1984; Swaisgood, 1992). Within the Cns exist genetic products which have given rise to further classification according to the homology of their primary structures into:  $\alpha_{s1}$ -Cn,  $\alpha_{s2}$ -Cn,  $\beta$ -Cn and  $\kappa$ -Cn, constituting in proportion 3:0.8:3:1 respectively (Wong *et al*, 1996). Cns differ in the degrees of phosphorylation (Eigel *et al*, 1984) with  $\alpha_{s2}$ -Cn as the most phosphorylated (10-13 residues) and  $\kappa$ -Cn with at least one phosphate (Ser<sub>149</sub>) moiety esterified to the serine amino acid(s), in between lies  $\alpha_{s1}$ -Cn (8-9 residues) and  $\beta$ -Cn (4-5 residues).

Besides limited phosphorylation,  $\kappa$ -Cn is glycosylated and is not precipitated by calcium at concentrations which precipitate the other Cns, rather it stabilizes them. The action of chymosin and several other proteinases specifically hydrolyzes a special peptide bond in  $\kappa$ -Cn (Phe<sub>105</sub>-Met<sub>106</sub> peptide bond) leading to curd formation in the presence of Ca<sup>2+</sup> (Swaisgood, 1992).

The Cns have remarkable differences in their hydrophobicities too, with  $\beta$ -Cn being invariably highly hydrophobic irrespective of its genetic polymorphs available whereas  $\alpha_{s2}$ -Cn is the least hydrophobic besides possessing the highest charge frequency (Swaisgood, 1992). Cn hydrophobicity is thought to play a role in micellar stability of Cn because of hydrophobic association, besides some electrostatic interaction and van der Waals forces (Schmidt, 1982).

Cns may be considered as transport proteins ferrying to the neonate the essential minerals for bone growth as well as preventing precipitation of the same in the mammary gland. Its structure and micellar stability is a subject of passionate academic controversy and is beyond the scope of the present paper to delve into these. Structurally, Cns exist as large colloidal cluster-micelles and although authors are not unanimous on the position of various Cns on the micelle, it is agreed that  $\kappa$ -Cn has a protective effect on the cluster stability with Cn solubility being enabled in concentrations of calcium sufficient to precipitate the other Cns; being probably situated on the periphery covering the micelle. The protective shield is removed resulting in coagulation of the micelles by calcium following cleavage of this protein (Schmidt, 1982; Rollema, 1992; Walstra, 1990).

#### 1.1.4 Milk Proteins & Human Nutrition

Following parturition, the neonate has no other source of nutrients such that milk almost exclusively serves this purpose. The requirements for pronounced initial growth and tissue maturation, which the neonate experiences together with its inability to take up nutrients from other sources demands a self-sufficient supply of the requisite nutrient constituents. Milk represents an almost completely packaged food item for the neonate of a particular species at these formative stages and its use in human diet in the varied forms available takes cognisance of the content of essential nutrients (Hambraeus, 1982 and 1992). The nutritive value of proteins is closely related to its amino acid composition and bioavailability.

Human milk is supplemented or replaced by bovine or ovine and caprine milk, in certain regions and populations of the world from a few months after parturition and this continues till maturity of the human being. Bovine milk constitutes the bulk of the milks consumed in the world and the variety of products available in stock underscores the creativity of its consumers in dealing with the problem of monotony while presenting a cocktail of products that are nutritive, attractive and sensual. That it is one of the most studied foods is no doubt the reason behind the contribution that the study of milk proteins has made to protein and enzyme chemistry because of their great susceptibility to various proteinases.

Milk proteins have a high content of essential amino acids (Table 1.2) and together with egg proteins have been used as a reference for the evaluation of the nutritive value of food proteins (Hambraeus, 1982). There are glaring differences, however, between amino acid constitutions of both egg and milk proteins just as they exist indeed between milks from various species. The latest benchmark for amino acid reference by FAO/WHO/UNU (1985) updates the use of human milk amino acids as a reference pattern from the use of egg proteins which provided a weak benchmark because of species specificity. This means that egg proteins are nature's design for the chicks in the same way as bovine proteins are qualitatively and quantitatively related to the nutritional needs of a calf. Arguably, the use of human milk as a reference for protein quality may undermine the quality of amino acid requirements for adults, because human milk is a neonatal diet per se.

Table 1.2 Essential amino acid content in skim-milk powder, whey proteins, Cn and human milk, amino acid pattern in FAO/WHO/UNU(1985)<sup>a</sup>

Amino acids	Skim-milk Powder	Cn	Whey Protein	Human Milk	FAO amino acid Scoring Pattern 1985)
Isoleucine	52	54	76	49	46
Leucine	97	95	118	91	93
Lysine	71	81	113	65	66
Methionine + Cysteine	34	32	52	37	42
Phenylalanine + Tyrosine	96	111	70	76	72
Threonine	41	47	84	44	43
Tryptophan	14	16	24	NA <sup>b</sup>	17
Valine	63	75	72	52	55
Total Essential Amino acids	468	511	609	-	424

<sup>a</sup> The values refer to mg amino acid per g protein

<sup>b</sup> NA, not analyzed

Adopted with modifications from Hambraeus (1992)

It has been postulated that the role of Cn is the nutrition of the neonate as a source of amino acids as well as calcium and inorganic phosphate. The ratio of methionine/cystine is reportedly 2-3 times higher than that in other animal proteins and more than seven times higher than in human milk, whose ratio is close to 1.0. The high content of whey proteins in human milk (Table 1.3) are a major contributor to this disparity as is the low Cn content. Metabolically and nutritionally, this is significant since the infant cannot convert methionine to cysteine which makes either cystine or cysteine a limiting amino acid in the infant. The use of Cn in infant formula presents a nutritional disadvantage from this point of view, with the need to supplement for the sulphur-rich amino acids (Hambraeus, 1992).

The ratio of calcium and phosphorous in milk is reported as 1.5 (Schmidt, 1982) which is present in the same ratio as reported from bone implying some physiological significance because it may be a source of the elements for bone growth and remineralization. Additionally, calcium phosphate-containing Cn micelles make the content of calcium and inorganic phosphate in milk far higher than would be expected from their physicochemical solubility in milk (Hambraeus, 1992). Furthermore, curd formation may be of physiological relevance because this precedes specific enzyme hydrolysis meaning they help in gastric enzyme recognition of the substrate (Hambraeus, 1992), although such claims are not buttressed by any data. Cn is a thermo-stable protein but is precipitated by both low pH and chymosin, which conditions present in the stomach, resulting in curd formation.

Table 1.3 Protein Composition in mature human milk and cow's milk

Component	Human Milk (mg/mL)	Cows' milk (mg/mL)
Total Nitrogen	1.54	5.2
Whey nitrogen	1.20	0.7
Non-Protein Nitrogen	0.31	0.3
Cn	2.33	27
Whey Proteins		
◇ $\alpha$ -Lactalbumin	1.73	0.9
◇ $\beta$ -Lactoglobulin	-	3.0
◇ Lactoferrin	1.48	0.0012
◇ Secretory Ig A	1.15	
◇ Lysozyme	0.07	0.0001
◇ Serum Albumin	0.38	0.3

Adopted from Hambraeus, (1992)

Although the curd characteristics between both human and bovine Cn differ, there is a possibility that Cn plays a role in the supply of phosphorous in the human adult also. It is known that Cn derived peptides rich in phosphorous (Cn phospho-peptides, CPP) play a significant role in the mineral bioavailability through their ability to sequester divalent and trivalent trace elements such as calcium, magnesium, and iron thereby enhancing mineral transport. The negatively charged peptide side chains, especially of their esterified phosphate groups represent the binding sites for these cations with the formation of CPP in the intestine reportedly increasing the concentration of soluble calcium (Lee *et al*, 1983). This is because in the human intestine where the pH is neutral or slightly alkaline, CPP can retard the formation and deposition of calcium phosphate while keeping the calcium in solution thereby making it bio-available and consequently increasing calcium absorption and utilization.

Clusters of phospho-serine peptides in the Cn binds iron with high affinity which is in contrast to whole Cn that is demonstrably inhibitory to iron absorption in humans. Yeung *et al* (2001) showed that partial hydrolysis of Cn resulting in CPP reveals the peptides that can bind the iron, thus diminishing this inhibition and enhancing iron bioavailability. This property may find useful applications in iron supplementation of infant formulas because of the low levels of iron in human milk. In some populations, the high values up to 75% of such minerals as calcium requirements in the human diet are sourced from milk and milk products (Cn).

Table 1.4 Amino acid content in Cn and whey protein compared to wheat and soybean protein (mg amino acid per g total nitrogen)

Amino Acid	Human Breast Milk	Cn	Whey protein	Egg	Beef	Wheat (Whole grain)	Soybean
Isoleucine	254	345	476	393	301	204	284
Leucine	471	607	736	551	507	417	486
Lysine	337	518	704	436	556	179	399
Methionine	78	178	151	210	169	94	79
Cystine	114	23	174	152	80	159	83
Phenylalanine	171	334	224	358	275	282	309
Tyrosine	223	371	214	260	225	187	196
Threonine	228	297	527	320	287	183	241
Tryptophan		103	147	93	70	68	80
Valine	296	430	449	428	313	276	300
Arginine	171	239	175	381	395	288	452
Histidine	114	186	144	151	213	163	158
Alanine	166	196	341	370	365	226	266
Aspartic acid	451	455	766	601	562	308	731
Glutamic acid	1000	1406	1231	796	955	1866	1169
Glycine	98	126	126	207	304	245	261
Proline	513	738	450	260	236	621	343
Serine	228	385	374	478	252	287	320

Adopted from Hambraeus (1992)

It is contestable whether whole Cn has been added in food formulations for more than its functional properties since it is not possible to estimate the direct impact of Cn in the human diet and Hambraeus (1992) argues that Cn is by no means the optimal protein from a nutritional point of view. Indeed, several animal proteins such as egg and whey proteins as well as a few vegetable proteins, such as rapeseed and soy proteins show superior nutritive value to Cn as a source of essential amino acids (Table 1.2 and Table 1.4). Nevertheless, Cn has been used as reference protein for many years in the evaluation of the nutritive value of proteins especially in biological assays. It would be naïve indeed, to expect bovine Cn to be an ideal human food because it is a specific nutrient source for the calf and it should be viewed first in terms of its adequacy in meeting the calf nutrient requirements.

## 1.2 REVIEW OF RELEVANT LITERATURE

### 1.2.1 Industrial Processing of Casein

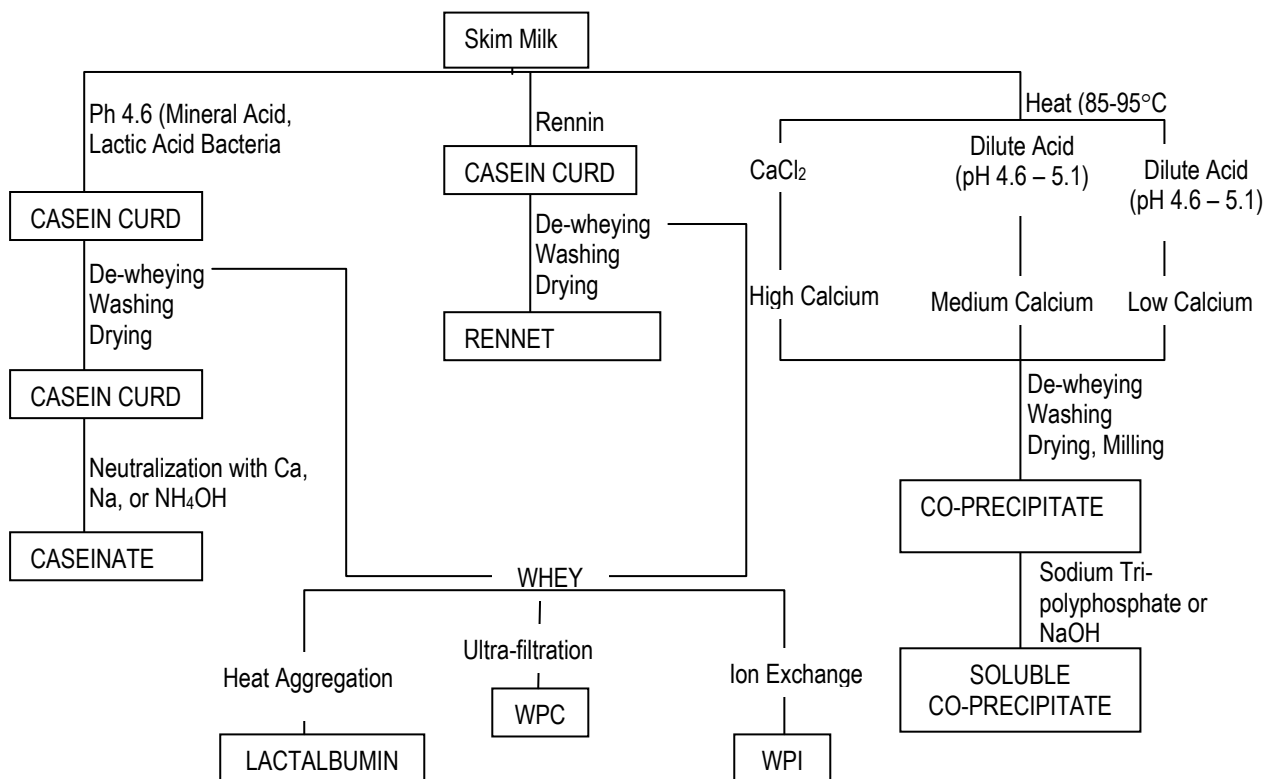


Fig 1. 5 Processing of Skim Milk into Milk Protein Products, *Adapted from Wong et al (1996):*

*WPI = Whey Protein Index, WPC = Whey Protein Concentrates.*

Unique properties of Cn have been exploited to process Cn and whey protein fractions of milk industrially because it can be isolated in skim milk from whey by use of its isoelectric precipitation at pH 4.6 and 20°C. Other fractionation means include size exclusion chromatography as well as limited proteolysis of micellar  $\kappa$ -Cn by chymosin resulting in the coagulation of the altered Cn micelles and separation of the whey fraction (Swaisgood, 1992). Besides, lactic acid Cn may also be produced by inoculating pasteurised skim milk with a mixed or multiple defined strain starter followed by incubation at 22-26°C (Mulvihill, 1992). The coagulation results from the formation of lactic acid during incubation because of conversion of lactose. The resulting Cn gel has a good water-holding capacity. Major processing techniques are summarized in Fig 1.5.

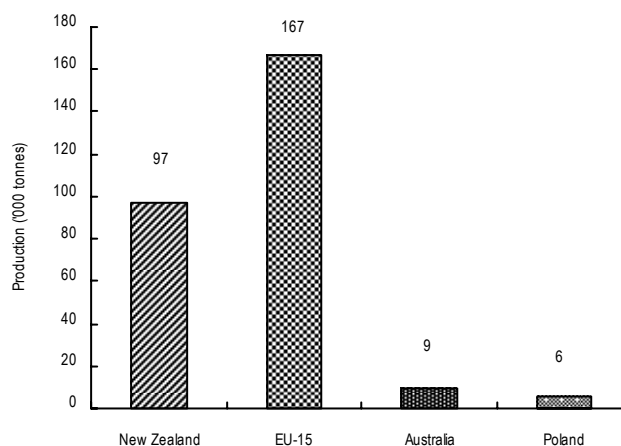


Fig 1. 6 World Production of Cn in 1999 ('000 tonnes)-Source EU- Directorate of Agriculture, 2003

Data on how much milk is converted annually into the milk protein products are not up to date although figures neighbouring 300, 000 tonnes (EU, 2001) have been broached. The production of milk proteins is at present possible and the innovations in the dairy industry have ensured production of food-grade Cn which has catapulted its uses in both the industrial and food applications. The increasing sophistication of end-user requirements for industrial and higher still for food-grade Cn meant the need by manufacturers to produce milk protein products with improved functional properties coupled with controlled quality is increased. This inevitably has led to improvements in the quality of Cn as well as emergence of a wide range of Caseinates and co-precipitates of both Cn and whey proteins (Muller, 1982). Industrial manufacture of Cn in its various forms peaked at close to 300, 000 tonnes with New Zealand (Fig 1.6) given as the single largest producer dwarfed only by the conglomeration that is the 15-bloc of European Union countries.

## 1.2.2 Uses of Casein

### 1.2.2.1 Industrial Applications of Casein

Initially, it would seem that industrial manufacture of Cn was inspired because of extra-nutritional applications into which it was widely put. During the Second World War Cn gained in use because glue which had been manufactured earlier in Europe from Cn was used as a water-resistant adhesive in the construction of aircraft, which were then made of wood (Muller, 1982). Apart from the manufacture of adhesives, Cn has had traditional applications in paper industry where it is used in paper-coating and polishing and sizing. Fibre and plastic production (billiard or snooker balls for example), paints and leather finishing products as well as animal foods are cited too as some of the applications to which Cn has been put. Fancy coloured ornaments and pens as well as buttons were manufactured from Cn 'plastic' because of the ease with which it is embossed with colour through dyeing. It is probable that

some of these applications are now out-competed by cheaper sources of raw materials as Cn has increasingly found diverse uses in the food and much recently pharmaceutical industry applications.

### 1.2.2.2 Food Applications of Casein

Mulvihill (1992) reviews comprehensively the wide applications into which Cn is put in either the food or pharmaceutical industry. Applied as either Cn, sodium Caseinate or as co-precipitates, the applications are summarized in Table 1.5 below.

Table 1.5 Applications of Cn in the Food Industry

Applications	Desirable Properties
Confectionery/Baking	Nutritional, Sensory, Emulsification, Dough Consistency, Texture, Volume
Imitation Cheeses	Fat & water binding, texture enhancement, melting properties, shredding properties
Coffee Creamers	Emulsification, whitener, gives body and texture, promotes resistance to feathering, sensory properties
Milk Beverages, Imitation milks, liquid milk fortification, milk shakes	Nutritional, emulsification, foaming properties
Cultured milk products (yoghurt)	Increase gel firmness, reduces syneresis
High Powders, Shortening, whipped toppings and butter-like spread	Emulsification, texture enhancing, sensory properties.
Drinking Chocolate, fizzy drinks and fruit beverages	Stabilizer, whipping and foaming
Cream Liqueurs, wine aperitifs	Emulsification

Adopted from Mulvihill (1992)

By mid 1980s, more than three-quarters (Muller, 1982) of the world production of Cn was consumed in the food ingredients sub-sector. It would be expected that this figure has continued to fluctuate either way, with the recent excitement that Cn has aroused in the functional food industry because of the discovery of bioactive peptides deriving from it following enzymatic hydrolysis. However, much of the applications would appear to have been intended to exploit the functional properties of Cn.

### 1.2.2.3 Enzymatic Modification of Casein

A number of methods are applied to modify food proteins; physical, chemical or enzymatic following which there are changes in protein conformation, structure and consequently their physicochemical and functional properties (Whitaker, 1977; Fox *et al*, 1982). Chemical modification by acid hydrolysis may permit release of free amino acids while destroying some of them. This is a burden on the body transport system as well as taxing the osmotic balance in the body and may lead to such upsets as diarrhoea (Fujimaki *et al*, 1977; Mahmoud, 1994).

Cns are acquiescent to enzymatic modification inspiring enormous utilizations in academic, technological as well as functional applications. Improvements on the functional properties of Cn

through enzymatic attack have received increasing attention from research over time with the advantage that the side-effects of chemical hydrolysis are reduced, if not greatly avoided, because the conditions are relatively moderate and therefore preservation of the nutritive value is equally high. The controlled enzymatic hydrolyses at low or high degrees of hydrolysis (DH) have conferred improved functional properties (Chobert *et al*, 1988). Such functional properties as solubility (Adler-Nissen, 1976; Slattery and FitzGerald, 1998), water holding, solubility and emulsifying properties (Chobert *et al*, 1988; Lee *et al*, 1987; Slattery and FitzGerald, 1998) and whipping properties (Gunther, 1979) as well as flavour improvement (Fujimaki *et al*, 1977) have been reported by investigators targeting optimization of the protein functional properties. Solubility of peptides even at pH values close to native protein iso-electric precipitation points have been increased with concomitant reduction in viscosity and significant changes in foaming, gelling and emulsifying properties completely dissimilar to those of the native protein following enzymatic modifications (Chobert *et al*, 1988).

### 1.2.2.3.1 Uses of Casein Hydrolysates

Cn hydrolysates have found applications in infant formula as well as defined formulas for clinical nutritional formulations for patients suffering from malnourishment and problems of protein digestion or absorption. They are also used in functional foods ingredients, nutritional fortifications, and pharmaceutical as well as nutraceutical applications (Slattery and FitzGerald, 1998). Cn hydrolysates used in hypoallergenic infant formulas removed the impediment of the infant's hypersensitivity to cow's milk (Mahmoud *et al*, 1992) with an extensive hydrolysis regime usually being employed as opposed to when functional properties are targeted in which case the hydrolysis is only limited. Mahmoud *et al* (1992) found a correlation between the peptide chain length and reduction in immunogenicity and report that with increasing DH and lowering of peptide length, there is a concomitant reduction in antigenicity of peptides.

Although it would be technologically easy to incorporate free amino acids to supplement deficiencies, this is limited by their hypertonic nature, such that hydrolysates are administered as replacements because they present less osmotic problems, making peptide mixtures a viable alternative (Fujimaki *et al*, 1977). This is because peptides are superior to amino acids of which they are constructed, in increasing assimilation rate at the brush border membranes (Hara *et al*, 1984). Besides, an amino acids mixture may have a strong taste and sometimes gives off an objectionable odour and as such is not always acceptable as food.

De-phosphorylated Cn hydrolysates were suggested for alimentary feeding or medical applications being soluble as well as similar to soybean and fish protein hydrolysates (Brule *et al*, 1994). Cn hydrolysates have further been suggested for applications as improved emulsifying agents in protein fortified low pH drinks and as whipping agents in high pH confectionery products (Slattery and FitzGerald, 1998). Additionally, they are reputed as a source for increased calcium and protein enrichment and besides being a source of the limiting amino acids lysine, tryptophan and methionine in fortified bakery products (Crowley *et al*, 2002).

### 1.2.2.3.2 Tackling Hydrolysate Bitterness

Inevitably, enzyme hydrolysis of proteins leads to liberation of bitter peptides as well as alteration in flavour attributes. There is, indeed, a positive correlation between degree of hydrolysis and hydrophobicity and there exists a close correlation between peptide bitterness and hydrophobicity, the so-called Q-rule (Fujimaki *et al*, 1977; Adler-Nissen, 1986; Saha and Hayashi, 2001). Furthermore, Mahmoud *et al* (1992) showed that peptide hydrophobicity (and hence bitterness) rises concomitant with increasing DH initially and begins to fall with advancing DH beyond 24%. It is therefore inevitable that the Cn hydrolysates are hampered in their wide applications to some extent by bitterness whence debittering is achieved *inter alia* by hydrolysis to very low DH, through masking or extensive hydrolysis by use of an exo-peptidase (Fujimaki *et al*, 1977; Saha and Hayashi, 2001) among other technological manoeuvres.

Further use of specific enzymes to catalyze formation of an insoluble proteinaceous substance, plastein, at a pH different from that used for hydrolysis by a given enzyme has been used to tackle hydrolysate bitterness (Fujimaki *et al*, 1977; Adler-Nissen, 1986). Plastein formation was claimed to be successful not only in debittering peptides by use of papain catalyzed transpeptidation to form 'longer' peptides. Free amino acid esters were also covalently incorporated into proteins (Saha and Hayashi, 2001) leading to improved functional and nutritional properties. Although some methods have been used to remove the bitter peptides, including use of activated carbon and hydrophobic interaction chromatography and chromatography on silica gel as well as extraction with alcohol (Kanekanian *et al*, 2000), the loss in amino nitrogen as a result of these bitter fragments being discarded is uneconomical (Saha and Hayashi, 2001) sometimes resulting in unacceptable loss of essential amino acid tryptophan which is hydrophobic.

Furthermore, extensive solubilization and debittering with an exo-peptidase leads to an inevitable increase in content of free amino acids resulting in a product that is a burden for the body system for amino acids transport at the intestinal brush border barrier as well as undermining the nutritional and physiological efficiency of the hydrolysates. Morato (2000) reported that di- and tri-peptides present the most usable form of the essential amino acids tryptophan and tyrosine and it would be desirable to have a low solubility amino acid like tyrosine presented in a mixture with highly soluble tyrosine-containing short chain peptides rather than as a free amino acid.

### 1.2.2.4 Biological Activity and Uses of Casein Derived Peptides

The role of diet and specific foods in the prevention and treatment of diseases and improving body functions has become more prominent and active recently, leading to the emergence of the new area of functional foods. Most proteins have biological activity following enzymatic hydrolysis, although only the hydrolysates of native Cn will be reviewed here. Hydrolysis of Cn with a number of enzymes such as trypsin, chymotrypsin and pepsin released peptides that were discovered to possess some structure-function relationship(s) with remarkable physiological characteristics not associated with native Cn

(Schlimme and Meisel, 1995; Meisel, 1997; Meisel and Bockelmann, 1999). It is teleological that being derived from food sources, the peptides are innocuous and that further processing to improve safety may not be required. The need for preventive medicine acknowledges that the bioactive peptides can help delay or effectively prevent the onset of disease.

Great strides in advanced analytical procedures as well as enzyme technology and protein chemistry offered by the countless panoply of modern analytical instrumentation makes it possible to enzymatically release and identify bioactive peptides sequestered in inert native proteins, which become trophically activated by proteolytic activities during digestion or during processing (Meisel and Bockelmann, 1999).

Thus increasing attention has been given to Cn peptide fragments shown *in vitro* to have immune enhancing and antithrombotic peptides (Fiat *et al*, 1993), opiate-like peptides (Loukas *et al*, 1983) as well as mineral binding (Ono *et al*, 1994) and antimicrobial functions (Lahov and Regelson, 1996; Recio and Visser, 1999). Furthermore, within Cn have been characterized some peptides varying in length and amino acid composition which have properties that inhibit angiotensin-I converting enzyme, ACE (Maruyama, 1987a; 1987b). There are antioxidant peptides too within the sequence of native Cns and these may contribute to reduction in free radical injury to the soft tissues and DNA thus helping to combat cancer, atherosclerosis and ageing (Suetsuna *et al*, 2000). Additionally, the hydrolysis of  $\kappa$ -Cn liberates two fragments- the phosphopeptide-containing para-kappa-Cn and what has variously been referred to as  $\kappa$ -Caseinoglycomacropeptide (CGMP), Caseinomacropeptide and Caseinoglycopeptide, with a Bifidobacteria growth promoting factor as well as binding and therefore elimination of *Escherichia coli* toxins (Brody, 2000). It may be deduced from Table 1.6 that bioactive peptides from proteins have multifunctional properties unlike the endogenous peptides with some 'strategic' regions in the primary structure of Cn containing overlapping peptide sequences that exert different physiological functions (Schanbacher *et al*, 1997).

There is inevitably, increasing focus on probable role of Cn as a source of hitherto unknown physiologically active peptides which help stave off disease conditions. Whether this has been the main and unknown function of Cn remains a grey area. Nevertheless, researchers are now satisfied that these functional peptides are generated and have been isolated from lactic acid bacteria-fermented milk (Nakamura, 1995), cheese (Smacchi and Gobetti, 1998) as well as *in vivo* in rats following ingestion of a Cn or milk containing diet (Masuda *et al*, 1996). There are proprietary concerns already and industrial exploitations are in operation currently to produce nutraceuticals (Han and Shin, 1998; Guan *et al*, 2001; van der Veerdonk *et al*, 2003) so as to turn over the benefits of physiological activities for general and upgraded production and consumption.

Table 1.6 Bioactive Peptides derived from Cn

Fragment	Name	Biological Activity
$\beta$ -Cn (f60-70)	$\beta$ -Casomorphin-11	Opioid Agonist
$\beta$ -Cn (f60-66)	$\beta$ -Casomorphin-7	Opioid Agonist/ ACE Inhibition/ Immune modulating
$\beta$ -Cn (f60-64)	$\beta$ -Casomorphin-5	Opioid Agonist
$\beta$ -Cn (f1-25)	Caseinophosphopeptide	Mineral binding/ transport
$\alpha_{s1}$ -Cn (f194-199)	$\alpha_{s1}$ -Immunocasokinin	ACE Inhibition/ Immune modulating
$\alpha_{s1}$ -Cn (f90-96)	$\alpha$ -Cn Exorphin	Opioid Agonist
$\alpha_{s1}$ -Cn (f1-23)	Isradicin	Antibacterial/ Antifungal
$\alpha_{s1}$ -Cn (f158-164)	Casoxin D	Opioid Antagonist
$\alpha_{s1}$ -Cn (f23-27)	$\alpha_{s1}$ -Casokinin-5	ACE Inhibition
$\alpha_{s1}$ -Cn (f59-79)	Caseinophosphopeptide	mineral binding/ transport
$\alpha_{s1}$ -Cn (f43-58)	Caseinophosphopeptide	mineral binding/ transport
$\alpha_{s2}$ -Cn (f)	Casocidin	Antibacterial
$\kappa$ -Cn (f106-169)	$\kappa$ -Cn CGMP	Bifidobacteria growth factor
$\kappa$ -Cn (f112-116)	$\kappa$ -Cn - CGMP	Thrombin Inhibitory peptide
$\kappa$ -Cn (f106-116)	Casoplatelin	Antithrombotic
$\kappa$ -Cn (f25-34)	Casoxin C	Opioid Antagonist/ Immune modulating

Most of the peptides reported were shown *in vitro* to possess bioactive properties. The parent protein was hydrolyzed by animal proteinases trypsin, chymotrypsin, pepsin etc.

Functional foods with formulations incorporating CPP have been used in a number of innovative applications including the dental care chewing gums, toothpaste and confectionery products (Luo and Wong, 2000), in pharmaceuticals (FitzGerald, 1998) as well as in fowl feed and skin and hair care products (Han and Shin, 1998). The avidity of CPP for iron (Fe) binding, and its resistance to dissociation, was shown to solubilize and make it bioavailable as well as alleviating Fe deficiency in study animals (Ait-oukhatar *et al*, 1997; Yeung *et al*, 2001). Cn was reported to facilitate for increased Fe solubility in its native form although the absorption was poor, which absorption was greatly enhanced by Cn derived CPP peptides. Indeed, FitzGerald (1998) suggested that CPP may be used to 'humanize' bovine milk owing to the supply of a good calcium/phosphorous ratio from bovine milk. Naito and Suzuki (1974) showed that CPP is generated *in vivo* and can resist further hydrolytic degradation in the gut, surviving until isolation from the faeces of experimental animals (Schlimme and Meisel, 1995).

Solubilization and consequently, bioavailability of calcium was reported and the application of CPP in oral care products deems it a source for remineralization of the enamel (Reynolds *et al*, 1995) as well as prevention of formation of insoluble hydroxyapatite, and presents opportunities and challenges for infant food as well as formulas for lactating mothers. CPP is available in a range of products such as fruit juices, chewing gums as well as sports drinks and may be a good source for calcium stressed

populations such as menopausal, pre-term infants and the elderly who have a declining vitamin D-dependent  $\text{Ca}^{2+}$  absorption system because CPP favours a vitamin D-free  $\text{Ca}^{2+}$  absorption (FitzGerald, 1998).

Elliott and Laugesen (2001) teach that  $\beta$ -casomorphins may be applied in combating diabetes and diarrhoea because they modulate postprandial metabolism as well as amino acid uptake (Meisel, 1997). The  $\beta$ -casomorphins have been offered in animal feeds (Carnie *et al*, 1989) because they favour increase in milk secretion or lean body weight gain in slaughter animals. ACE inhibitory peptides are suggested for inclusion in functional food formulations so as to ward off incidences of myocardial arrest and cardiac infarction (Guan, *et al*, 2001; van der Veerdonk *et al*, 2003) since continued uptake in preventive medicine rationale far outweighs treatment of the disease. Antioxidant peptides are suggested as natural sources of protection against auto-oxidation of fat containing foods, as well as contributing to quenching and reduction of deleterious free radicals injurious to DNA and the smooth tissues, although the potency of peptides in this regard is in doubt compared to the existing phenolic and other antioxidants.

### 1.3 ANGIOTENSIN-I CONVERTING ENZYME AND ITS INHIBITION

Hypertension, generally defined as abnormally increased blood pressure, is a disease which is estimated to affect more than 170 million people worldwide. Hypertension is also implicated in more than one third of the deaths from stroke (Mark and Davis, 2000). One regulator of hypertension, the renin-angiotensin system, is fairly well understood (Ondetti and Cushman, 1982).

In this system (Fig 1.7), angiotensinogen is secreted by the liver and is cleaved by renin to yield the biologically active decapeptide, angiotensin-I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). Angiotensin-I converting enzyme, ACE (peptidyl dipeptide hydrolase, EC 3.4.15.1), an unusual dipeptide liberating exopeptidase, is classically associated with the renin-angiotensin system and plays a major role in cardiovascular homeostasis by catalyzing the conversion of angiotensin-I to the potent vasopressor octapeptide, Angiotensin-II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) by cleaving off a dipeptide from the carboxy-terminal of angiotensin-I. It also hydrolyzes (hence inactivating) the natriuretic nona-peptide bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), a vasodilator and mediator of inflammation (Cheung *et al*, 1980). The human and rabbit forms of ACE are glycosylated zinc metalloenzymes approximating 146KDa which are activated by chloride ions and form an integral part of the cell membrane. ACE is found in a large variety of cells, tissues and biological fluids including semen, intestinal epithelial cells and brain among others. Specifically, angiotensin-II causes an increase in blood pressure by contracting the smooth muscle of the blood vessel walls while promoting release of aldosterone by acting on the adrenal cortex (Ondetti and Cushman, 1982).

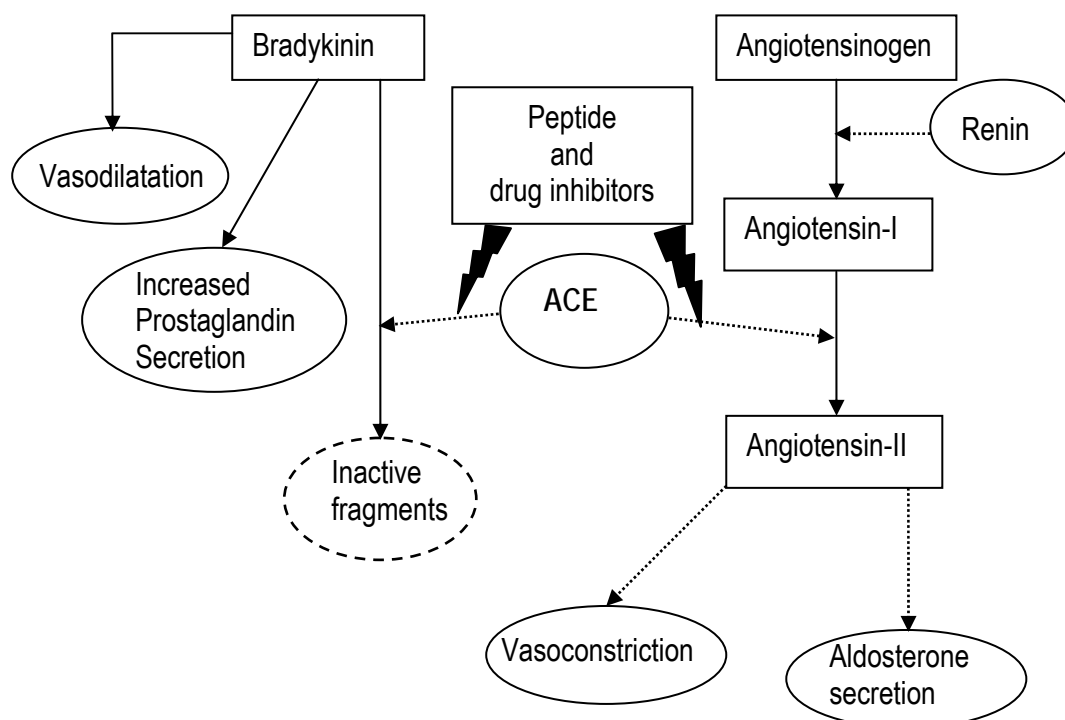


Fig 1.7 Renin–Angiotensin System (RAS). The release of renin stimulates the conversion of angiotensinogen to angiotensin-I that is converted to angiotensin-II by ACE. Angiotensin-II leads to vasoconstriction and the release of aldosterone. ACE also converts bradykinin to inactive peptide metabolites.

The activity of ACE in the aorta, brain, lung and kidney is thought to be responsible for the hypertension of spontaneously hypertensive rats (SHR), while antihypertensive effect of ACE inhibitors may be related to sustained ACE inhibition in the aorta and kidney (Masuda *et al*, 1996). Since ACE not only produces a peptide that causes high blood pressure but also decomposes the vasodilator peptide bradykinin, inhibiting it could be an effective means to depress hypertension. There is a large body of clinical data demonstrating the beneficial effects of ACE inhibitors in reducing mortality, rate of recurrent myocardial infarction and subsequent development of heart failure in patients after myocardial infarction and in patients with chronic congestive heart failure.

Several site-directed inhibitors of ACE (Fig 1.8) are effective in the treatment of systemic hypertension and congestive heart failure (Ondetti and Cushman, 1982) and in this regard various prophylactic drugs have been formulated with the aim of blocking the action of ACE in the renin-angiotensin system. Currently, pharmaceutical anti-hypertensive products such as calcium channel blockers,  $\beta$ -blockers, diuretics,  $\alpha$ -blockers, central  $\alpha$ -agonists, angiotensin-II antagonists and ACE inhibitors such as Captopril and Enalapril manage hypertension. Whereas these cocktails of pharma-drugs are effective in depressing hypertension, they are beset by undesirable side effects, which include increased cholesterol levels, cough, hypotension and development of skin rashes. Furthermore, withdrawal

symptoms associated with cessation of the administration of the drugs are also likely (Mark and Davis, 2000).

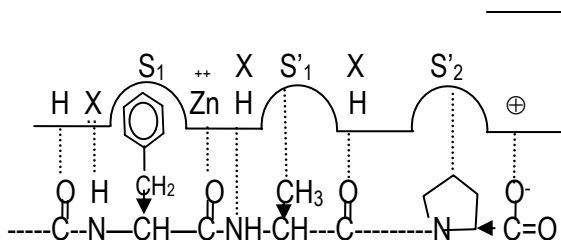


Fig 1.8 Schematic diagram of proposed binding of a peptide substrate or competitive inhibitor to ACE, the peptide and drug inhibitors like Captopril interact with the enzyme sub-sites thus preventing access of angiotensin-I. Adopted from Ondetti and Cushman, (1982)

Initially, peptide ACE inhibitors were reported from snake venom oligopeptides (Ondetti and Cushman, 1982). However, recently it was shown that various food proteins released peptides sequestered in their native structures following hydrolysis by trypsin or pepsin or during processing, that have some degree of ACE inhibition and may provide hurdles to delay the pathological development of the disease condition (Yamamoto, 1997), although the inhibition is still a far cry from the potency of the prophylactic drugs, with the most potent tri-peptides Val-Pro-Pro and Ile-Pro-Pro being 100 times weaker than Captopril (Pihlanto-Leppälä *et al*, 1998). However, milk protein hydrolysates inhibit ACE *in vitro*, irrespective of their molecular weight distribution and therefore possess diverse antihypertensive potentials *in vivo* (Yamamoto and Takano, 1999). ACE inhibitors from milk proteins hydrolyzed with trypsin (Maruyama *et al*, 1987b) as well as from milk fermented by lactic acid bacteria (Gobbetti *et al*, 2000; Yamamoto *et al*, 1994) from various cheeses (Smacchi and Gobbetti, 1998; Saito *et al*, 2000) and sour milk produced by *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Nakamura *et al*, 1995) have been characterized for ACE inhibition and antihypertension. However, Pihlanto-Leppälä *et al* (1998) reported that there was no significant release of ACE inhibitors when different strains of micro-organisms were used to ferment milk.

The characterization of antihypertensive effects of the various purified peptides as well as the fermented milks did implicate ACE inhibition in suppression of elevated blood pressure (Yamamoto *et al*, 1994). However, the relationship between potent ACE inhibition and antihypertension was not straight-forward and the peptide lengths were thought to restrict the success of antihypertension by the ACE inhibiting peptides (Maruyama *et al*, 1987b). Longer peptides with high ACE inhibition did not necessarily translate to potent antihypertension while shorter peptides with relatively lower ACE inhibition were potentially antihypertensive. This may be accounted for by the restriction of uptake of the longer peptides in the enteral brush border regions (Hara *et al*, 1984; Nakamura *et al*, 1995) or further cleavage preceding uptake to release peptides that are less potent (Maruyama *et al*, 1987b) at the enteral brush border regions by the myriads of endo-peptidases and dipeptidases. The ACE inhibitors from milk hydrolysates show an interactive physiological effect; peptides such as  $\beta$ -casomorphin-5 with opioid agonist properties showed ACE inhibition in a typical case of multifunctional peptides (Meisel, 1997).

Typically, most of the ACE inhibitors interact with the sub-sites (Fig 1.8) of the enzyme in a characteristically competitive inhibition. Generally, the peptide inhibitors were shown to exert their action via specific C-terminal dipeptide (Cheung *et al*, 1980) or tri-peptide (Maruyama *et al*, 1987a) residues with preference for imino amino acid proline-rich hydrophobic residues. The most favourable C-terminal amino acids are aromatic amino acids, tryptophan, tyrosine and phenylalanine (Meisel, 1997). Meisel (1997) implicated the positive charge of the guanidino group in arginine for inhibition since most of the reported potent inhibitors have arginine flanking the C-terminal residues. The most potent Cn derived inhibitors, the tri-peptides Val-Pro-Pro and Ile-Pro-Pro survived post ingestion hydrolysis (Nakamura *et al*, 1995), were isolated in the aorta following consumption of a milk diet containing them (Masuda *et al*, 1996) and did mediate lowering of hypertension in spontaneously hypertensive rats in a study (Nakamura *et al*, 1995).

Most of the ACE inhibitory peptides from Cn have been characterized and isolated following hydrolysis by the animal proteinases trypsin, pepsin, and chymotrypsin (Maruyama *et al*, 1987a; 1987b; Pihlanto-Leppälä *et al*, 1998) as well as from fermented milk and dairy products produced following action by fermentative action of micro-organisms (Nakamura *et al*, 1995). While the characterization of ACE inhibitors from fermented milk and dairy products is evidence that food processing techniques lead to liberation of peptides inhibitory to ACE, the amounts produced are subject to various factors including the extent and length of fermentation as well as the types and number of microbial strains involved in the processing (Gobbetti *et al*, 2000; Nakamura *et al*, 1995; Saito *et al*, 2000; Smacchi and Gobbetti, 1998; Yamamoto *et al*, 1994; Yamamoto *et al*, 1999) and the amounts released remain inconsistent, with the antihypertensive effect still largely controversial (Pihlanto-Leppälä *et al*, 1998; Yamamoto *et al*, 1994).

The food-protein originating peptides with inhibitory potential enjoy the advantage that they are perceived to be innocuous. It would be desirable to have a consistency in production of these peptides, which may not be feasible with food processing methods that produce the reported antihypertensive peptides per se owing to variations in production conditions (Guan *et al*, 2001). Nevertheless, fermentation is currently exploited at proprietary level to produce a product rich in the tri-peptides Val-Pro-Pro and Ile-Pro-Pro (Kitamura and Ueyama, 2001) with claims that it is antihypertensive.

## 1.4 CURRENT PROBLEMS AND GENERAL OBJECTIVES

The review of literature vividly brings out the fact that Cn has and continues to play a central role both in the academic development of protein chemistry as well as innovations in industrial and food production applications. There is substantive evidence that food proteins serve much more than provide nutrients and, when in excess supply, energy. Indeed at the close of the last century, Cn received immense attention owing to the presence of bioactive peptides in its rich reservoir of amino acids enabling release of functional peptides in countless permutations of structure-function relationships. We may as well expect that as scientific consumerism catches up with the dinner table, the knowledgeable consumer will not so much choose essential amino acids sourced from any protein foods. Such consumers may want to consider the possibility of these amino acids being presented in several bioactive permutations which may elicit functional properties before further digestion to liberate them. The academic and commercial potentials and benefits are numerous; the challenges for tapping the production are equally gigantic.

Most of the initial attempts at Cn hydrolysis for the study of bioactive peptides were done using the animal pancreatic proteinases; trypsin and to some extent pepsin as well as chymotrypsin. Animal proteinases are expensive (Adamson and Reynolds, 1996) and attempts at reaching a wider consumer base with the benefits of bioactive peptides ought to be preceded by deliberate reductions in the cost of production. In order to lay out further functional and bioactive properties of Cn peptides, to show their true potential as well as promote their large-scale utilization in the functional foods, nutraceuticals and pharmaceutical industry, it is imperative to reinforce the research in progress and undertake investigations on the potentials of various cheaper microbially sourced enzymes for modifications of this unique protein.

Adamson and Reynolds (1996) used Alcalase 2.4L (EC 3.21.62), an industrial and food-grade subtilisin of microbial origin to hydrolyse Cn to produce CPP. Characteristics of the enzyme specificity were studied as were the issuing Cn phosphopeptides (CPP) found in the hydrolysates and the functional fragments were shown to exist and have bioactive properties. CPP is industrially produced (Fig 1.9 below) and will continue to find innovative applications meaning that Cn-NPP (its industrial by-product), which are of little interest to the investors in the CPP industry are likely to pose greater environmental concerns besides leading to losses in essential amino acid and protein nitrogen as well as some bioactive peptides. Besides, the use of alcohol in precipitating out CPP also serves the purpose of debittering it because the alcohol soluble Cn-NPP peptides are both hydrophobic and bitter (Kanekanian *et al*, 2000). There is need, therefore, to study the bioactive properties of Cn-NPP and to determine means of production that can optimize these properties as well as conserving the essential nitrogen contained therein.

To date, various food protein sources have been hydrolyzed by Alcalase 2.4L and the resulting peptides studied for their ACE inhibition with promising potencies. Yust *et al* (2003) have recently employed Alcalase 2.4L to hydrolyze chickpea and purified peptides with potent ACE inhibition. Byun and Kim

(2001) hydrolyzed Alaska Pollack (*Theragra chalcogramma*) skin and reported that although a number of proteinases were tried out, Alcalase 2.4L released peptides with higher inhibition (lower IC<sub>50</sub> values) towards ACE. Indeed, Kristinsson and Rasco (2000) showed during studies on the kinetics of hydrolysis of Atlantic salmon (*Salmo salar*) by various alkaline proteinases that Alcalase 2.4L had the highest activity per gram compared with Flavourzyme 1000L, Corolase PN-L and 7089. Although other enzymes have been employed to produce ACE inhibitors from Cn, so far no reports have been encountered on the application of Alcalase 2.4L in their production from Cn and it would be desired that this enzyme is studied for its potential to produce the same.

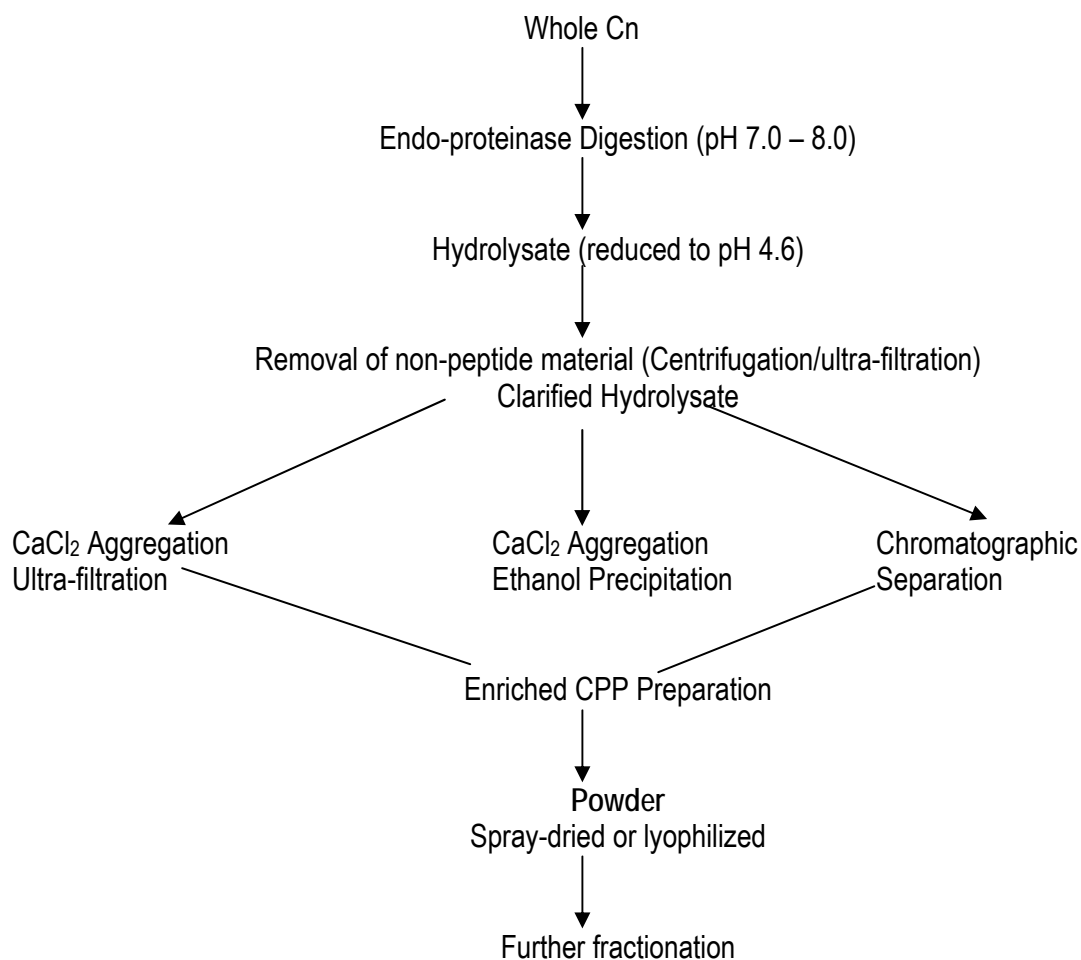


Fig 1. 9 Generalized scheme for the production of CPP, *Adopted from FitzGerald (1998)*- No reports of the fate of Cn-NPP which is expected to carry more nitrogen than the CPP fraction unless the process is optimized to recovery more of it in CPP

Morato (2000) reported that compared to trypsin hydrolysates of Cn, subtilisins produced less free amino acids and more of the di- and tri-peptides probably because the short chain peptides are not substrates for further subtilisin degradation. This was shown, however, to relate to the enzyme to substrate (E/S) ratio, with increasing E/S leading to a concomitant decrease in both peptide length and free amino acid content as well as an increase in medium length and contents of di- and tri-peptides. It would be physiologically desirable, therefore, to present a peptide mixture that is not laden with

problems associated with free amino acid uptake, and subtilisins present such an avenue. Furthermore, the short peptides may elicit some bioactivity in the body system prior to further hydrolysis to free amino acids in the body system, however transient this benefit may be. It would be desired to draw the doors open for further research aimed at optimizing the use of Cn as a source of bioactive peptides, for this may indeed shed some light in the long run on the apparent over-expression and supply of certain amino acids in Cn. Brule *et al* (1994) reported that the essential amino acid tryptophan is preserved during hydrolysis in alkaline pH, but lost in acidic pH. Use of an alkaline or neutral enzyme like Alcalase 2.4L is therefore desirable to preserve this essential amino acid.

The need to commercially industrialize the nutraceutical industry has led to a scramble to produce peptides that are purified to varying degrees targeting high-end nutraceutical consumers and pharmaceutical applications. For some consumers, the benefits remain only a mirage because the ambitious purification protocols employed are not too inexpensive leading to a prohibitive shelf price of the end-product. Applications of cheaper resin alternatives to purify the hydrolysates taking advantage of their various physico-chemical properties are invited. Hydrolysis of proteins involves real-time scouting of the DH and the most popularly applied means in alkaline pH hydrolysis is the pH-stat method. The pile-up of ash material following the use of an alkali to regulate pH and estimate DH demands its complete removal immediately after hydrolysis and prior to the product being packaged and offered in the various forms for food-grade applications. Although dialysis and the various filtration protocols are attractive, small-scale manufacturers grappling with cost cutting may not afford these besides some of them being too slow thus cheaper alternatives are called for.

Additionally, there are varying degrees of bioactivities of Cn hydrolysates with varying DH as well as within a given peptide mixture as demonstrated by various studies regarding for example, ACE inhibition (Maruyama *et al*, 1987a; van der Ven *et al*, 2001). This means that the molecular sizes as well as structures of bioactive peptides are diverse as is their ACE inhibition potential. The sum effect of the peptide mixture may be synergistic, meaning therefore that isolation of one or a few peptides with ACE inhibitory activity is wasteful, expensive and unjustified (van der Ven *et al*, 2002). Absolute purity of the peptide mixtures, therefore, raises the spectre of cost of the final product and this curtails access to the benefits of functional foods by some consumers that should benefit from these innovations.

Although the complete catalogue of bioactive peptides isolated *in vitro* are yet to be proven to exist or survive further exposure to human or study animal gut enzymes, they may be introduced after commercial manufacture and incorporated as active ingredients into the human dietary supplements or any one of the many functional foods. The natural non-pharmaceutical antihypertensive peptides which are effective in oral dosage and have low toxicity and great safety are greatly free from the side-effects associated with the current antihypertensive pharmaceutical products. Means of producing bioactive peptides are limited only by the innovativeness of the food technologist. Genetic engineers may invoke transgenic expressions of peptides in various genetic bioengineering expressions followed by various purification procedures (Carnie *et al*, 1989). Indeed in May of 2003, Chinese scientists reported that the ACE inhibitory peptide CEI<sub>12</sub> whose sequence was identified by Maruyama *et al* (1987a) from Cn was

successfully expressed in *Escherichia coli* JM109 using DNA recombinant technology with a harvest of 580µg/L culture (Lu *et al*, 2003). Prospects exist for investigations on the viability of DNA recombinant technology as a huge resource to further bring down the cost of the final product, if controlled proteolysis is costly. Genetic engineering will, however, have to surmount the hurdles of socio-cultural and sometimes political resistance whereby enzymatically modified proteins are widely accepted.

Whereas bioactive peptides are liberated during postprandial hydrolysis of Cn and/ or during food processing, the variations in food processing conditions do not necessarily guarantee production of physiologically significant amounts to elicit the effects associated with them. Indeed, it remains unclear how much milk or dairy products one needs to consume to have a threshold of bioactive peptides concentration to cause the effect to be physiologically significant. The meagre amounts of peptides liberated as well as the lack of consistency during the release of bioactive peptides *in vivo* and during food processing necessitates a steady means of commercial mass-production. The assumption is that the peptides so characterized are absorbed in the gut, taken up to the target organs without further alteration and can excite the bioactivity in the local organs. Again this is difficult to ascertain owing to the limitations for uptake of peptides longer than a couple or so amino acids, necessitating deliberate commercial production of peptide mixtures for the purpose of meeting consumer demands.

The brief of this study was to investigate the angiotensin –I converting enzyme inhibitory properties of Cn non-phosphorylated peptides (Cn-NPP) following hydrolysis by Alcalase 2.4L at varying DH values and to provide guideposts for purification protocols employing competitively priced resins available. It was also designed to provide a product which may be formulated into functional foods or with further purification applied in nutraceutical and/ or pharmaceutical uses as part of prophylactic drugs to manage and prevent hypertension. We have used Cn as an abbreviated form for casein and we therefore use the abbreviation Cn-NPP to show that the peptides are originated from Cn.

## 1.5 SPECIFIC OBJECTIVES OF THIS RESEARCH

The specific objectives of this study were as hereunder:

1. To investigate the relationship between degree of hydrolysis (DH) and release of Cn non-phosphorylated peptides (Cn-NPP) inhibitory to angiotensin-I converting enzyme (ACE).
2. To evaluate cheaper desalting (desalination) resin alternatives using commercially available macroporous adsorption resins (MAR) used in water purification, study the adsorption/desorption mechanisms of Cn-NPP and the resins so chosen and discuss the effects of various adsorption/desorption conditions with the aim of optimizing the recovery of Cn-NPP during desalting.
3. To investigate the elution conditions for Cn-NPP in gel filtration chromatography and study various conditions for optimum resolution of the peptides fractions as well as study the ACE inhibition properties and potency of each fraction as related to relative molecular weights of the peptides.
4. To carry out further purifications using anion exchange resins (IEXR) and to study the effects of various conditions on the adsorption/desorption of Cn-NPP onto/from IEXR. Further, to analyze the relationships between Cn-NPP adsorbed onto and desorbed from IEXR at various adsorption/desorption conditions and ACE inhibition.
5. Propose probable uses and applications of Cn-NPP in the functional foods and nutraceutical and/or pharmaceutical industries.

## CHAPTER TWO:

### PART A: CASEIN HYDROLYSIS & Cn-NPP PRODUCTION

#### 2.1.0 INTRODUCTION

Degree of hydrolysis (DH) is defined as percent of peptide bonds cleaved in a protein. When approximated by the pH-stat method, as expounded in the treatise by Adler-Nissen (1986) it offers a simple but reliable method for estimation of the percentage of peptide bonds broken down during enzymatic hydrolysis of a protein although it doesn't offer a means to predict the products of such a reaction. The maintenance of a constant pH (pH-stat method) as afforded by titration of a base against the acid end-groups in the reaction hydrolysate offers a good estimate when the titre is plotted against time. DH control by pH-stat method, therefore, offers a rapid and useful tool for real-time monitoring of protein hydrolysis at laboratory level. The concept of degree of hydrolysis (DH) postulates that a protein in its native form has a DH equivalent to 0% while a protein completely degraded at its peptide bonds to completely liberate free amino acids has a DH of 100%. In-between the two DH extremes lies a regime within which is a versatile tool employed by researchers to achieve, *inter alia*, a variety of products and an improvement of protein functionalities.

Several methods for tracking DH have been reported in literature including the pH-stat, osmometry, soluble protein methods as well as the colorimetric - tri-nitro-benzene-sulfonic acid (TNBS) method (Adler-Nissen, 1979). The pH-stat method is more suited for alkaline (pH > 7) conditions and is reportedly effective in single-enzyme hydrolysis and in situations where the anticipated DH is lower than 30%. However, it is difficult to attain higher DH values with single enzymes and when an enzyme mixture is used; it is not practical to have all such enzymes operating optimally at alkaline conditions. The colorimetric (TNBS) method is tedious, slow and involves reagents that have been criticized as being environmentally unfriendly besides the risk of explosion if mishandled (Nielsen *et al*, 2001).

It was desired in this study to produce hydrolysates at various DH values (10%, 15% and 20%) using Alcalase 2.4L and later to characterize the same for ACE inhibition so as to establish a relationship between DH and ACE inhibition.

## 2.1.1 MATERIALS AND METHODS

### 2.1.1.1 Materials

Casein (86.7% w/w protein) obtained from Inner Mongolia, China, manufactured June 1999 brand name Hairu; Alcalase 2.4L (EC 3.4.21.62) obtained from Novo Nordisk (Bagsvaerd, Denmark); clamp stirrer model JB300D rating 220V, 50Hz, 50-1500rpm; pH-meter model pHS-2 (mV x 100, range 0-60°C, pH 0-14.0) all made in Shanghai, China. All other chemicals were of reagent grade available from the university chemical store.

### 2.1.1.2 Methods

#### *2.1.1.2.1 Dissolution and Hydrolysis of Casein Powder*

In each case, ca. 75g of Casein (86.7% protein) was dissolved in deionized water at 60°C (initially 20%, w/v) in a 2L batch enzyme reactor with a variable speed stirrer and the pH maintained at 8.5 by manual addition of 2M NaOH. When the Cn was dissolved and pH stabilized (final substrate concentration 15%), 1mL Alcalase 2.4L solution (E/ S ratio 36AU/Kg) was added and the time recorded.

#### *2.1.1.2.2 Maintenance of Constant pH & Selective Precipitation of CPP*

Following addition of the enzyme the pH fell rapidly and it was kept constant at pH  $8.5 \pm 0.2$  by manual titration of 2M NaOH each time the pH fell to  $8.3 \pm 0.04$  to raise it to  $8.7 \pm 0.04$ . When the pH hit the 8.5 mark on the drop the time was recorded and the corresponding amount of base used to correct pH was recorded against the time. A time course hydrolysis graph of Cn for the respective DH values was then plotted. This was done for DH 10%, 15% and 20% until the calculated base volumes were depleted. When the amount of calculated base to attain that DH was added, the pH was allowed to fall to 8.5 before the hydrolysis was rapidly terminated by addition of 1.15% (w/v)  $\text{CaCl}_2$  (4.3g in each case), followed by addition of 95% food-grade ethanol (1:20, w/v) to achieve a final strength of 70% alcohol concentration. The temperature was then raised to 80°C, held for 15 minutes to arrest the enzyme activity followed by cooling. The temperature took about ten minutes to reach 80°C. After standing overnight at refrigeration temperatures, the supernatant containing the Cn non-phosphorylated peptides (Cn-NPP) was decanted and the precipitate Caseinophosphopeptide (CPP) fraction was lyophilized and preserved for recovery calculations.

The volume of base required to attain a particular DH was worked out using the relationship according to Adler-Nissen (1986):

$$DH(\%) = V_b \times N_b \times \frac{1}{\alpha} \times \frac{1}{W_p} \times \frac{1}{h_{tot}} \times 100$$

Where:  $V_b$  = volume of NaOH required to maintain constant pH in mL  
 $N_b$  = normality of the NaOH (2mol/L in this study)  
 $\alpha$  = Average degree of dissociation of the  $\alpha$ -NH groups  
 $W_p$  = mass of protein in sample (given by N (%) x 6.38) in g (65.03g in this case)  
 $h_{tot}$  = total number of peptide bonds in the protein substrate (meqv/g protein).

In the conditions for the experiment, pH 8.5 and temperature 60°C, the values for  $1/\alpha = 1.03$ , while  $h_{tot} = 8.2$  meqv/g

#### *2.1.1.2.3 Centrifugation and Low-Pressure Concentration and Lyophilisation of Cn-NPP*

The supernatant (Cn-NPP) was decanted and centrifuged at 32000 rpm, (220V, 60W, 6.5A, 50 Hz), followed by distillation to remove and recover alcohol at a vacuum pressure of <0.09MPa at 30 to 35°C in a rotary evaporator. It was further concentrated to remove excess water at 40-45°C as a pre-treatment in preparation for freeze-drying. The samples were then frozen and later freeze dried at -18 to -20°C at a vacuum pressure of 15-20 Pa for 24 hours.

#### *2.1.1.2.4 Molecular Weight Determination of the Cn-NPP*

The average molecular weight of the Cn-NPP was estimated using high performance size exclusion chromatography (HP-SEC) with the following conditions:- the molecular weight ( $M_r$ ) of Cn-NPPs were estimated by comparing the retention times with those of standard peptides in a TSKgel2000 SWXL 7.8ID x 300mm column in Waters 600 HPLC (allocating 2487 UV detector and M32 work station). The following standard peptides or proteins were used; cytochrome C ( $M_w$  12500), aprotinin ( $M_w$  6500), bacitracin  $M_w$  1450), Gly-Gly-Tyr-Arg ( $M_w$  451) and Gly-Gly-Gly ( $M_w$  189). A buffer of ratio acetonitrile: water: acetocastin 45:55:0.1 (V/V) was used as the elution solvent with a flow rate 0.5mL/min. The temperature of the column was 30°. The peptides in the effluent were monitored with a UV detector set at 220nm.

#### *2.1.1.2.5 Quantitative Amino Acid Analysis of Cn-NPP Hydrolyzed to DH 15%*

The quantitative amino acids content of Cn-NPP as determined by RP-HPLC using Hitachi 835 amino acid analyzer following digestion at with 6N HCl 110°C for 24hrs *in vacuo* and the high performance chromatogram compared to those of standard amino acids.

#### 2.1.1.2.6 Statistical Analysis

The analyses were done in duplicate except for HPLC analysis and the mean values calculated using a CASIO® fx-500A Calculator produced by CASIO Computer Co. Ltd., Japan and significance among the data values determined by the Student's *t* test ( $p < 0.05$ ).

## 2.1.2 RESULTS AND DISCUSSIONS

The time course hydrolysis curve for Cn by Alcalase 2.4L at 60°C, pH 8.5 and DH controlled by pH-stat method is presented in Fig 2.1-1. Fig 2.1-2 is the RP-HPLC chromatogram of amino acid analysis in Cn-NPP whereas Fig 2.1-3 presents our schematic processing of Cn-NPP and CPP. The amino acid analysis results for Cn-NPP produced at DH 15% are presented in Table 2.1-1

#### 2.1.2.1 Characteristics of Alcalase 2.4L Enzyme

Most bioactive peptides from food proteins, chiefly milk, have been obtained following hydrolysis with trypsin, pepsin or chymotrypsin (Maruyama *et al*, 1987a; 1987b; Adamson and Reynolds, 1996), animal pancreatic proteinases that are beset by the high cost while considering applications on a wider industrial-scale. Subtilisins are a group of enzymes that are of considerable interest in both academic and industrial applications where they are applied in such diverse uses as meat tenderizing, laundry detergents and proteolytic medicines. Alcalase 2.4L (EC 3.4.21.62), a subtilisin, is a proteinase of molecular weight approximating 30,000Da and is active in alkaline conditions although its activity curve shows that it is fairly active and stable down to pH 5 (Adler-Nissen, 1986) meaning it can also be applied as a neutral pH proteinase.

Alcalase 2.4L is obtained from a food-grade bacterium, *Bacillus licheniformis*; it is cheaper than the enzymes of animal origin, has high activity (Kristinsson and Rasco, 2000) and should provide great potential in industrial applications (Adamson and Reynolds, 1996). Like the digestive enzymes chymotrypsin and trypsin it is a serine endopeptidase that hydrolyzes both basic and neutral ester substrates, which have a low degree of association with the enzyme. It exhibits a higher activity towards esters of aromatic amino acids than those of aliphatic ones (Blackburn, 1976).

The quantity  $\alpha$  is the calibration factor in pH-stat method; it is dependent on temperature because of the influence on pK by temperature, although it (pK) is relatively independent of substrate. At higher pH values the value of  $\alpha$  is high and falls with falling pH. At pH values above pK, the  $\alpha$ -amino group will be less than half protonated but the  $\alpha$ -carboxyl group fully dissociated which leads to a net decrease in pH if uncontrolled (Adler-Nissen, 1986).

In studies on the optimum enzyme conditions for Alcalase 2.4L, Zhao *et al* (2002a) reported that the activity of the enzyme was higher at pH 9.5 while an E/ S concentration of 36AU/kg was optimum to achieve DH values approaching 20% in reasonable time (about 3hrs). Further, the enzyme showed a higher activity at 60° than 50 °C. Indeed at this activity, the enzyme hydrolyzed Cn to attain DH 10% in

less than 30 minutes, while the DH 15% and 20% were reached after one and three hours, respectively. From their study, it was possible to relate the activity of the enzyme required to hydrolyze Cn to a required DH value in a specific time under experimental conditions, which may be used to evaluate the cost-effectiveness of the process.

$$\alpha = \frac{10^{\text{pH} - \text{pK}}}{1 + 10^{\text{pH} - \text{pK}}}$$

However, when considering practical applications, it was decided that pH 9.5 may be beset by exposure of the enzyme to higher base needed to regulate pH at this level and the likelihood of the enzyme salting out would be a menace, thus necessitating the use of a lower working pH. It was decided that pH 8.5 and 60°C be used because it is above the pK<sub>a</sub> of the Cn α-NH group and could therefore allow for their sufficient dissociation so as to allow efficient use of the pH-stat method. The value of pK<sub>a</sub> for Cn is reported to be 6.85 (Adler-Nissen, 1986) but the calculated value at 60°C, pH 8.5 from the value of α<sup>-1</sup> = 1.03 was found to be about 6.98.

Alcalase 2.4L is a thermostable enzyme and Adler-Nissen (1986) contends that pH shock should be sufficient to inactivate it. However, we found it sufficient to use temperature (80°C for 15 minutes) because of the synergistic effect of high base and calcium salts as well as alcohol in inactivating it. Alcalase 2.4L was, therefore, found to be a suitable candidate for use in production of Cn-NPP from Cn

#### *2.1.2.2 Effect of Degree of Hydrolysis on Hydrolysis Behaviour of the Enzyme*

First order kinetics were observed in the initial stages of the hydrolysis, with an initial rapid increase in DH against time as recorded in the frequency of use of NaOH required to maintain pH. This is because of the 'ideal' conditions presented by the optimum E/S ratio whereby the enzyme is completely saturated with the substrate. This leads to a large number of peptide bonds being broken down. This was followed by a decreased rate of hydrolysis finally approaching a stationary phase where little apparent hydrolysis takes place, especially for higher DH values approaching 20%. This may be accounted for by the reduction in E/ S ratio because of reduction in substrate concentration (substrate limiting) or change in the nature of substrate for hydrolysis (Fig. 2.1-1). It is probable that Alcalase 2.4L has a reduced hydrolytic action on the resulting peptides as opposed to the native Cn as corroborated by Morato *et al* (2000).

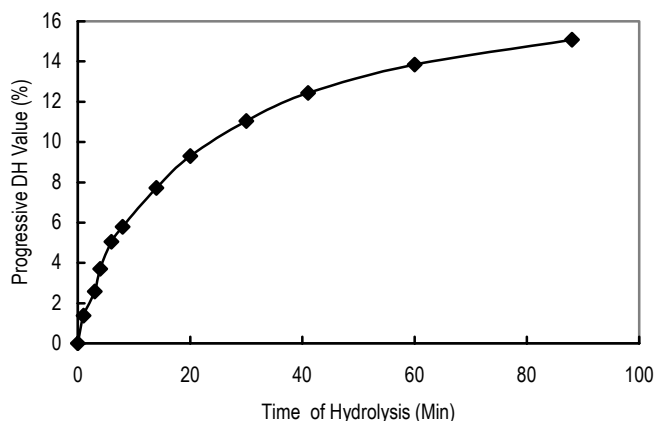


Fig 2.1- 1 Time course of Cn hydrolysis curve for DH = 15% (E/ S = 36AU/kg, pH = 8.5, 60°C). The pH was maintained constant by manual addition of 2M NaOH, the volume of base recorded against time on the pH drop to 8.5.

At higher DH, however, there may be a combination of other factors such as product inhibition or autolysis (Kristinsson and Rasco, 2000). What we cannot understand for certain, nor can it be ruled out entirely however, is the probable interference from the dramatic increase in the ash content within Cn-NPP with increasing DH. It is possible that the altered ionic concentration has an inhibitory impact on the enzymatic activity as do some resulting peptides, although it is at this point speculative, at best. The hydrolysis curve relates well with published ones (Adler-Nissen, 1986; Mahmoud *et al*, 1992).

#### *2.1.2.3 Total Amino Acid Composition of Cn-NPP from Casein hydrolyzed to DH 15%*

The RP-HPLC chromatogram of amino acids in Cn-NPP produced by hydrolysis to DH 15% is presented in Fig 2.1-2. A cursory look at Table 2.1-1, leads to the conclusion that almost all the essential amino acids (FAO/WHO/UNU, 1985) are present in amounts that should justify the conservation and utilization of Cn-NPP as an invaluable source of the same as well as nutritional nitrogen. Furthermore, the statistical appreciation of the individual groups of amino acids according to Sarkar *et al* (2002) reveals a high level of the nine important essential amino acids, including histidine (49.73% w/w), which is higher than 33%, the recommended index of essential amino acids according to the nutritional recommendations (Lee *et al*, 1978).

Barrantes (1973) proposed several groupings of amino acids and treatment of such categories with statistical tools. The ratio of hydrophobic amino acids to total amino acids was important for this study, with a high proportion of 51.63% (w/w) captured in the Barrantes statistic. Tryptophan was the limiting amino acid in this analysis, although this may be because it is not captured in the assay method since routine estimation of tryptophan involves alkaline derivatization (Alegría *et al*, 1999). However, Cn is equally low in tryptophan content. There was a high ratio of the acidic amino acids, with glutamic acid being the most prevalent amino acid (17.93%), which may be exaggerated by additive conversion of glutamine during the acid hydrolysis.

Inevitably, the presence of hydrophobic amino acids in high proportion means the peptide mixtures in Cn-NPP are hydrophobic. This may present a problem of bitterness as has been corroborated in literature with the so-called Q-rule, relating average peptide hydrophobicity to bitterness (Fujimaki *et al*, 1977; Adler-Nissen, 1986; Saha and Hayashi, 2001). The organoleptic properties of Cn-NPP were not determined although as a rule of thumb, average amino acid hydrophobicities exceeding a threshold neighbouring 5.43 kJ/mol may be bitter (Adler-Nissen, 1986). However, because of the numerous possible permutations of the amino acids within the peptides in Cn-NPP, it is probable that individual amino acids with high hydrophobicity are expressed bound to those with less hydrophobicities such that the result is a greatly reduced average hydrophobicity such that some of the peptides may have low hydrophobicity values while others may be highly hydrophobic and objectionably bitter.

It would be anticipated therefore, that although there is a significant amount of essential amino acid nitrogen in the Cn-NPP, the bitterness that may be inferred from the apparently high amount of hydrophobic amino acids (51.63% w/w) would have to be tackled while considering Cn-NPP's use in various consumable forms.

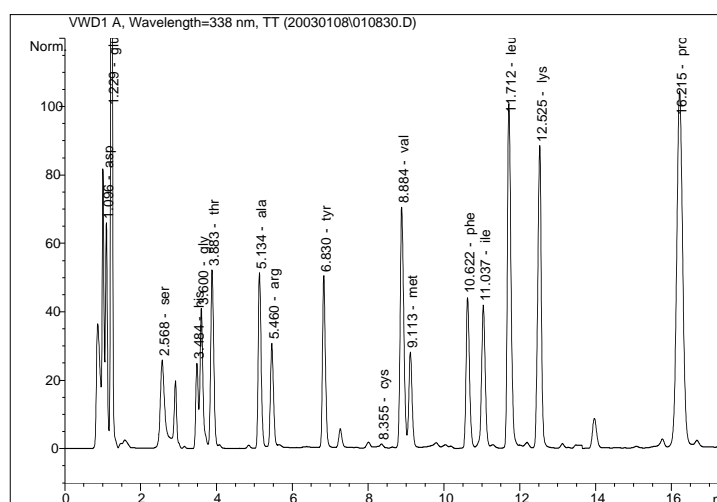


Fig 2.1-2 RP-HPLC chromatogram of amino acids of Cn-NPP hydrolyzed to DH 15%, analysed by Hitachi 835 high speed amino acid analyzer following digestion with 6N HCl at 110°C for 24hrs *in vacuo* and compared to elution profiles of standard amino acids monitored at 328nm. Peaks are labelled with three-letter abbreviations for amino acids.

Table 2.1-1 Amino Acid composition of Cn-NPP (DH = 15%)

Amino Acid	Amount (g/100g)	% (w/ weight total amino acid)	Hydrophobicity kJ/mole <sup>a</sup>
Histidine	2.32	2.97	2.09
Isoleucine	3.26	4.18	12.54
Leucine	8.14	10.43	9.61
Lysine	5.44	6.97	6.25 <sup>b</sup>
Methionine	2.32	2.97	5.43
Phenylalanine	4.42	5.66	10.45
Threonine	3.13	4.01	1.67
Tyrosine	4.73	6.06	9.61
Valine	5.06	6.48	6.27
<b>Totals (EAA<sub>9</sub>)</b>	<b>38.82g/100g</b>	<b>49.73%</b>	
Alanine	2.30	2.95	2.09
Arginine	2.76	3.54	3.10 <sup>b</sup>
Aspartic Acid	5.47	7.01	2.09
Glutamic Acid	13.99	17.93	2.09
Glycine	1.48	1.90	0
Proline	10.07	12.90	10.87
Serine	3.15	4.04	-1.25
<b>Totals nEAA</b>	<b>39.22g/100g</b>	<b>50.27%</b>	-

EAA = essential amino acids                      nEAA = non-essential amino acids

<sup>a</sup> Adopted from Damodaran (1996)

<sup>b</sup> Adopted from Cheftel *et al* (1985)

<sup>c</sup> EAA<sub>9</sub> = (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine and valine); 49.73%

<sup>c</sup> Hydrophobic (alanine, isoleucine, leucine, methionine, phenylalanine, proline, tyrosine and valine); 51.63%

#### 2.1.2.4 Scheme for producing Cn-NPP

We have demonstrated that it is possible to achieve fractionation of CPP and Cn-NPP using selective precipitation of enriched CPP (Fig 2-1.3, below) with CaCl<sub>2</sub> and alcohol. Our process obviates an isoelectric precipitation procedure at pH 4.6 ostensibly to remove un-hydrolyzed Cn, which must have been entrained in the calcium-phosphate complex containing peptides resulting in precipitation with CPP. It is anticipated that this procedure simplifies the manufacturing process for both CPP and Cn-NPP and flexible modifications to industrial scale applications are tenable.

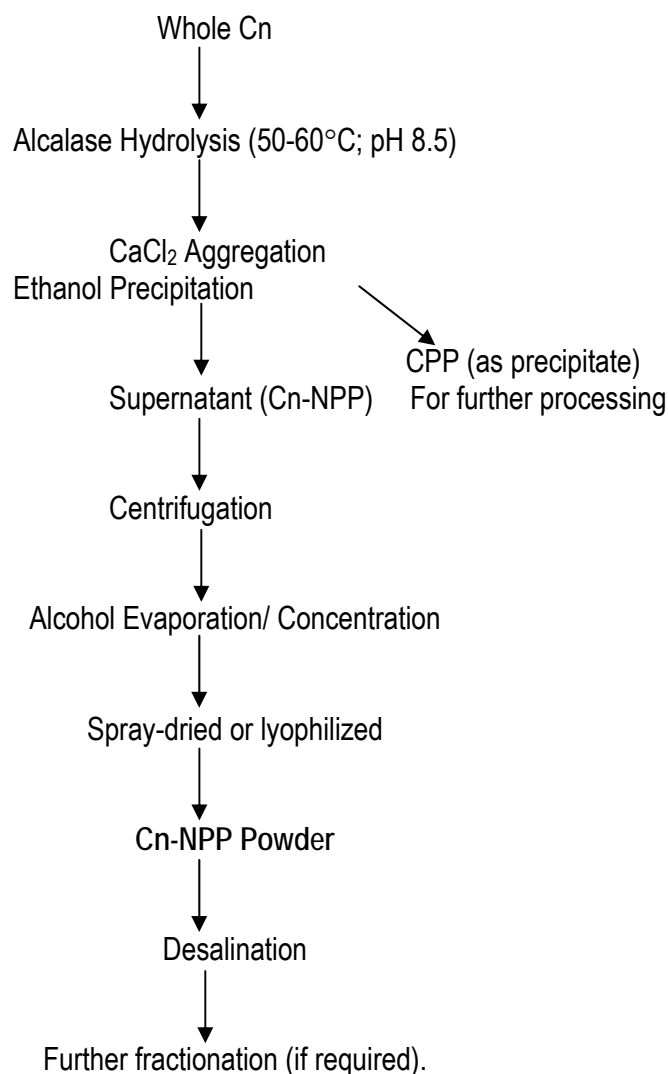


Fig 2.1- 3 Our Scheme for producing Cn-NPP with Alcalase 2.4L, the iso-electric precipitation at pH 4.6 to remove un-hydrolyzed Cn is obviated (Cf. Fig 1.9 page 22) as it is entrained with the CPP, if any significant amounts remain intact at the DH values under study. Use of alcohol for industrial application needs studies on economy compared with membranes, while desalination may be achieved by use of macroporous adsorption resins.

## 2.1.3 CONCLUSIONS

From this section we can conclude that Cn hydrolysis by Alcalase 2.4L follows the trend established for subtilisins and generates peptides of varying lengths depending on DH. Additionally, the amino acids contained in the Cn-NPP show superior nutritional quality, contain the essential amino acids in proportions that are significant and aside from the bioactivities that are latent therein, they may serve as an invaluable source of essential protein nitrogen. Cn-NPP should be considered as good source of essential amino acids although it presents challenges to overcome bitterness, which is probably high owing to the high proportion of hydrophobic peptides.

## **PART B: Cn-NPP ANALYSIS**

### **2.2.0 INTRODUCTION**

Milk is a source of several essential nutrients as well as being a carrier of various forms of specific minerals. It is the main nutritional source of calcium and at least at parturition, it services the needs for growth of the mammalian neonate. The mineral content of milk may be exploited either in whole milk, in its variety products or following enzymatic manipulation, in presentations that are both enriched and improved. As such it would be desirable to conserve as much as is practically possible, of the precious contributions of milk to dietary requirements of man.

Confronted by the emerging techniques in enzyme modification of food proteins either to obtain bioactive peptides, as is now in vogue or to improve the functional properties, it is imperative to present the nutrient composition of milk.

### **2.2.1 MATERIALS & METHODS**

All the materials used in this section were of analytical grades unless otherwise specified.

#### *2.2.1.1 Proximate Analysis*

The proximate scores (protein, ash, moisture) for Cn, CPP and Cn-NPP were determined according to the AOAC (1995) official methods.

#### *2.2.1.2 Phosphorous*

The modified procedure of Morrison (1964) was used to quantify phosphorous.

#### *2.2.1.3 Statistical Analysis*

The samples were analyzed in duplicates and in the case of spectrophotometric analysis, duplicates with two blanks. The values shown are mean values of the same with significance tested using the Student's *t* test ( $p < 0.05$ ). The calibration curves and the regression analysis were generated with Microsoft Excel software (Microsoft Corporation, USA).

## 2.2.2 RESULTS AND DISCUSSIONS

The proximate analysis data is presented in Table 2.2-1 while a plot of the nitrogen recovery and N/P molar ratio (% N/ % P) x 31/14 is presented in Fig 2.2-1 each plotted against the respective DH. Fig 2.2-2 presents the molecular weight distribution of the hydrolysates in Cn-NPP at DH = 15%.

### 2.2.2.1 Effect of Degree of Hydrolysis on Nitrogen and Phosphorous Recovery in Cn-NPP

Recovery of nitrogen was evaluated by the relationship

$$N_R = N_{CPP} / N_{Cn}$$

Where

$N_R$  = % nitrogen recovery

$N_{CPP}$  = Nitrogen in CPP (g) at the given DH

$N_{Cn}$  = Nitrogen in Casein (g)

The nitrogen recovered in Cn-NPP was evaluated using the relationship:

$$\%N_{Cn-NPP} = (100 - N_{CPP \text{ recovery}}).$$

This eliminated the cumbersome evaluation of the same in Cn-NPP because the many steps involved in processing inevitably led to losses, which were minimized, with the use of CPP in the calculations owing to the minimum handling processes involved. The phosphorous concentration was a deductive inference relating the concentration in Cn initially and in the CPP, since direct determination of the same in Cn-NPP was not tenable owing to the trace amounts contained in it.

Table 2.2-1 Proximate Analysis Data for Cn-NPP

DH Value	<sup>a</sup> N (%)	<sup>b</sup> N/P (%) Molar ratio	<sup>c</sup> N <sub>Recovery</sub> (%)
Cn	13.59	32.34	-
10 %	13.36	149.00	69.53
15 %	13.00	152.36	72.64
20 %	12.55	189.57	80.81

Notes: <sup>a</sup>N = Nitrogen, <sup>b</sup>N/P = molar ratio (% N/ % P) x 31/14,

From Table 2.2-1 and Fig 2.2-1, we notice that the index N/P molar ratio, which was intended to monitor the extent to which protein nitrogen and phosphorous ‘migrated’ between Cn and the two fractionated products, CPP and Cn-NPP, did point to a concomitant increase in recovery within Cn-NPP with a decrease in recovery in the CPP fraction. With respect to nitrogen there was an increase in recovery in Cn-NPP while the same fell in the CPP with increasing DH; the converse obtained with phosphorous

through the three DH values under investigation. If indeed the target of the investor industry is CPP per se, then there is substantial loss of essential nitrogen with a whopping 80% being entrained in the effluent. It would be logical even from this point of view to conserve the nitrogen migrating in the Cn-NPP by devising industrial means of co-producing CPP and Cn-NPP.

Horne (1992) has eloquently reviewed alcohol stability of milk and demonstrated the relationship between alcohol concentration and calcium-phosphate complex precipitation. Alcohol precipitation is a powerful tool that has been exploited to achieve selective precipitation of the calcium-phosphate-complex containing peptides (CPP) as well as providing a debittering procedure for CPP when used at 70% concentration with the alcohol soluble fraction being made up mainly of bitter, hydrophobic peptides (Kanekanian *et al*, 2000). Generally, the higher the alcohol concentration, the faster and the greater is the extent of precipitation.

Intuitively, at the lower DH values the enzyme attacks the parent protein into large fragments and as the native Cn (substrate) concentration falls, thus leading to an alteration of the substrate characteristics, the enzyme begins to attack the peptides leading to shorter chains. The longer peptides containing the enriched calcium-phosphorous-peptide complex are unstable to alcohol and therefore precipitate out of the hydrolysate mixture. With advancing DH, the enzyme acts on these longer peptides releasing shorter, calcium-phosphorous-complex free peptides, which are recovered in the Cn-NPP.

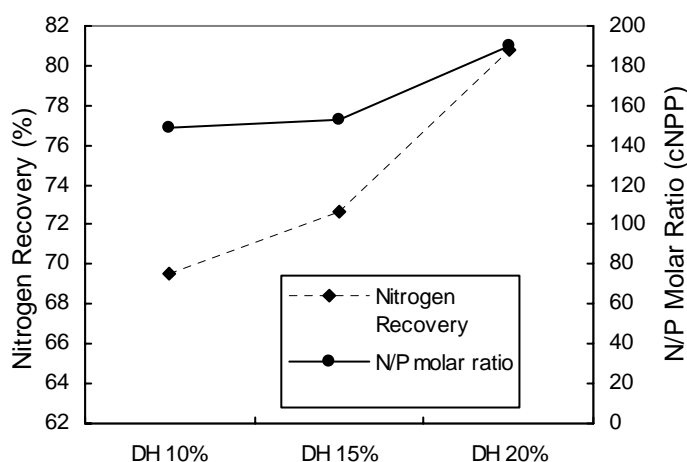


Fig 2.2- 1 Recovery of Nitrogen and N/P molar ratio in Cn-NPP. The nitrogen recovered and N/P ratio increased in Cn-NPP with increasing DH (%) because of liberation of shorter Ca-Phosphorous free peptides.

Further, from Table 2.2-1 we see that there is a fall in the recovery of phosphorous in Cn-NPP, as is apparent from the N/P molar ratio. Indeed, at DH 20%, it is possible that most of the practically recoverable phosphorous is contained in CPP, with the amount calculated to be in Cn-NPP being the phosphorous in colloidal form which is probably not precipitated as the calcium-phosphorous-peptide complex entrainment (Schmidt, 1982).

From Fig 2.2-2, the resulting peptide mixture in the Cn-NPP at DH 15% agrees well with the report by Morato *et al* (2000) that subtilisins give di- and tri-peptides rather than free amino acids. The

physiological significance of the presence of these short chain peptides cannot be over-emphasized. At the enzyme substrate ratio used in our study, we were able to obtain to a limited extent a few free amino acids.

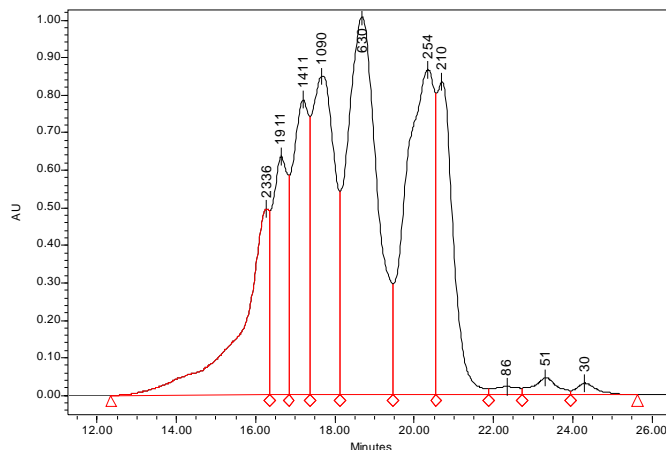


Fig 2.2- 2 Molecular weight distribution on HP-SEC of Cn-NPP (DH = 15%). Performed using TSKgel2000 SWXL 300mm× 7.8mm column in Waters 600 HPLC (allocating 2487 UV detector and M32 work station). Standard peptides or proteins used; cytochrome C ( $M_w$  12500), aprotinin ( $M_w$  6500), bacitracin  $M_w$  1450), Gly-Gly-Tyr-Arg ( $M_w$  451) and Gly-Gly-Gly ( $M_w$  189). A buffer acetonitrile: water: acetocastin 45:55:0.1 (V/V) at a flow rate of 0.5mL/min

## 2.2.3 CONCLUSIONS

The action of Alcalase 2.4L on Cn led to liberation of peptides with varying lengths and the amino acid analysis indicated that the Cn-NPP fraction contains some invaluable essential amino acids and in ratios that are physiologically important. Furthermore, the release of phosphorous by Alcalase 2.4L hydrolysis as indicated by the N/P molar ratio shows that there is insignificant phosphorous in the Cn-NPP while most of it may be entrained in the CPP. Neither the nutritional significance of the Cn-NPP nor the probability of bitter peptides being in high concentration owing to alcohol precipitation of CPP which also retained the hydrophobic peptides in the alcohol soluble fraction need to be overemphasized. However, this by-product of active CPP production should not be discarded, if on the basis of the amino acid composition.

## CHAPTER THREE:

### DESALTING OF Cn-NPP ON MACROPOROUS ADSORPTION RESIN (MAR)

#### 3.0 INTRODUCTION

Caseins are among the most hydrophobic proteins with an average hydrophobicity value of 4.74 kJ/mole (Adler-Nissen, 1986) as compared with other proteins (whey 4.32 kJ/mole, gelatine 3.71kJ/mole, haemoglobin 3.96 kJ/mole and soya flour 3.75 kJ/mole). Furthermore, there is a distinct difference within its genetic products with respect to hydrophobicity with  $\beta$ -Cn being the most hydrophobic (invariably close to 6 kJ/residue) irrespective of the polymorphism within it. It is followed by  $\kappa$ -Cn (5.12 kJ/residue),  $\alpha_{s1}$ -Cn (4.89 kJ/residue) and  $\alpha_{s2}$ -Cn (4.64 kJ/residue) in order of decreasing average hydrophobicities. Besides, there is clear delineation within the structure of the  $\beta$ -Cn with the anionic phosphoserine clusters conglomerated at the highly charged N-terminal domain clearly distinct from a highly hydrophobic C-terminal domain (Swaisgood, 1992). The case is replicated in the case of the other Cns as well, with patches of highly hydrophobic clusters striating the molecular chain. In a scenario as obtained in the present study where the CPP was precipitated out by alcohol, it would be anticipated that some remarkable amounts of the hydrophobic residues are entrained in the alcohol-soluble Cn-NPP, thus raising its average hydrophobicity.

Maintenance of a constant pH in the pH-stat procedure involves inevitable consumption of high amounts of base, which is commensurate with the DH. This becomes a nuisance when the product is to be purified to an acceptable degree for various products of consumption and/ or subsequent analysis. In addition salts are formed and they need to be removed while ensuring minimum losses of the product. Traditionally, desalting is performed in specific columns with high efficiency but which are costly, thus raising the cost of desalination. Alternatively, desalination may be done with dialysis, which is pretty slow.

The column processes employed are either hydrophobic interaction chromatography or desalting in special gel filtration columns with the desired product being adsorbed while the salts are washed out. Cheaper resin alternatives with good hydrophobic ligands have been used to separate organic products by adsorption from fermentation liquors containing inorganic salts (Zhigang *et al*, 2002). The separation effectively adsorbed the biological products while little adsorption of the salts occurred. The macroporous resins are so-called because they have large interstitial pores, ranging in size from 8-25nm, sometimes even larger and they have been used in desalination processes.

Screening experiments showed the resins to have good hydrophobic interaction properties, being influenced by temperature and  $(\text{NH}_4)_2\text{SO}_4$  as well as pH. Zhao *et al* (2002b) screened adsorption of Cn

hydrolysates on a wide range of macroporous resins available in the Chinese market and found that the one designated DA201-C had superior adsorption recovery rates. This particular resin has properties similar to those of Amberlite XAD-3. The nature of adsorption of the resin to the hydrolysates was shown to be subject to flow rate and pH while alcohol was important for desorption. Zhigang *et al* (2001) reported that the adsorption behaviour of macroporous adsorption resins obeyed a typical Langmuirian adsorption isotherm. Being entropy driven, hydrophobic interactions are encouraged by an increase in temperature, while low temperatures vitiate them.

This study was commissioned to study the effects of different conditions on the adsorption of Cn-NPP on macroporous adsorption resin (MAR) during desalination, determine the conditions for optimum adsorption and desorption as well as perform proximate analysis of the recovered Cn-NPP following desorption to provide evidence of desalination.

## 3.1 MATERIALS AND METHODS

### 3.1.1 Materials

DA201-C resin was obtained from Jianying Organic Chemical Plant (Jianying, China), bovine serum albumin (BSA) was purchased from Shanghai Biochemistry Co. (Shanghai, China). All the other chemicals were of analytical grade available from the Southern Yangtze University Chemical store.

### 3.1.2 Methods

#### *3.1.2.1 Adsorption Behaviour of Cn-NPP on MAR*

Pre-swollen resin slurry (10mL) submerged in a further 10mL of deionized water was put in 150mL Erlenmeyer flask and 50mL of the solution of peptides were added. The flasks were incubated in a constant temperature shaking water bath operated at 15, 25 and 37°C and agitated at 180-190 rpm. After 24hrs, the flasks were removed and after allowing the contents to settle, 1mL from the top clear solution was skimmed off and diluted as appropriate and analyzed for protein content according to the method of Lowry *et al* as simplified by Peterson (1977), using BSA as standard. The concentration of peptides before adsorption and after 24 hrs was related as percent adsorption recovery. This procedure was repeated for various weights of peptides from 0.5g through 9.0g at 0.5g intervals. A plot of concentration of peptides in solution (non-adsorbed),  $C_s$ , against concentration of peptides adsorbed,  $C_m$ , per mL resin was then plotted to obtain the adsorption isotherm and from the isotherm was obtained a Langmuir transformation curve to calibrate for the peptide adsorption properties for MAR.

#### *3.1.2.2 Effect of $(NH_4)_2SO_4$ on Adsorption of Cn-NPP on MAR*

1g, 5g and 10g Cn-NPP was dissolved in 100mL water containing 1%  $(NH_4)_2SO_4$  (w/v) and 50mL transferred to 150mL Erlenmeyer flasks each containing 10mL resin submerged in 10mL water. The

contents were transferred to a constant temperature water bath operated at 15, 25 and 37°C. After 24 hour, 1mL of the contents were skimmed off the clear top, diluted as appropriate and analyzed for protein concentration. The process was repeated and averages were obtained from the duplicates.

### *3.1.2.3 Relationship Between Desorption of Cn-NPP from MAR and Alcohol Content*

In each case, ca. 2g Cn-NPP was dissolved to make a 100mL solution, containing from 0% to 80% ethyl alcohol at incremental intervals of 10%. Resin slurry equilibrated in water (10mL) was measured out using a 25mL-measuring cylinder and transferred to a 150mL Erlenmeyer flask. The resin slurry was then pre-equilibrated with 6 volumes of the respective alcohol strength solution. Next 50mL of the Cn-NPP solution was transferred to the flasks and their mouths covered with polythene foil and then secured with another plastic wrapping and rubber band. This was to ensure minimum leakage of the alcohol at the operating temperature. The flasks were then placed in a constant temperature shaking water-bath at 20°C and shaken at 180-190 rpm for 12hrs. The solutions were allowed to settle then 3 or 1 mL of the clear solution was taken and analyzed for peptide concentration using the method of Lowry *et al* with BSA as a standard. Desorption efficiency was calculated as ratio of protein concentration in solution after adsorption during 12hrs, divided by the original concentration before adsorption and expressed as a percentage. This is because the hindrance of adsorption by alcohol relates well with its ability to desorb the peptides from MAR.

### *3.1.2.4 Desalting of Cn-NPP with MAR*

Pre-swollen resin slurry was packed into a 150mL (2.6cm x 30cm) column and Cn-NPP dissolved in deionized water in the ratio 1:250 (w/v). The Cn-NPP solution was pumped through the column at one bed volume per hour, equivalent to a linear flow rate of 30cm/hr. The assembly was connected to a UV detector locked to 220nm in series with a recorder. The amount of peptides in the eluent was monitored at intervals of 30 minutes by collecting aliquots of 1-3mL to analyze for peptide concentration using the method of Lowry *et al* according to the procedure of Peterson (1977). The peptides solution was desalted until the adsorption rate fell to about 90%. The elution of the peptides was then halted and three bed volumes of deionized water was pumped through the column to rinse off the remaining salt solution or until the conductivity was zero. The eluent was also monitored for peptides. After this 70% alcohol was also pumped through the column to desorb the peptides until the eluent recording on the recorder attached to the UV detector was back to the base-line, and the peptide content as determined by method of Lowry *et al* was less than 5%. Washing with 1mol/L NaOH followed by 1mol/L HCl solution then regenerated the resins.

### *3.1.2.5 Proximate Analysis*

The proximate composition (ash, moisture, protein) was performed as prescribed in AOAC (1995) standard procedures with expedient modifications.

### 3.1.2.6 Analytical Assays during Desalting of Cn-NPP on MAR

The peptide concentration during the purification was analyzed by the method of Lowry *et al* according to Peterson (1977), using bovine serum albumin (BSA) as a standard and done in duplicates using two blanks.

### 3.1.2.7 Statistical Analysis

The values reported are means of duplicates determined using 2 blanks with the method of Lowry *et al*. Sample data standard deviation ( $\sigma_{n-1}$ ) was calculated using a CASIO® fx-500A Calculator produced by CASIO Computer Co. Ltd., Japan and significance among the data was determined by the Student's *t* test ( $p < 0.05$ ).

## 3.2 RESULTS AND DISCUSSION

The properties of the DA201-C macroporous adsorption resin are summarized in Table 3.1 while Table 3.2 summarises the Langmuir transformation values for Cn-NPP adsorption isotherm at 15, 25 and 37°C. Table 3.3 summarizes desorption of Cn-NPP from MAR by alcohol of various concentrations. The adsorption isotherm of Cn-NPP on MAR is presented on Fig 3.1, while Fig 3.2 is the Langmuir transformation of the adsorption isotherm.

### 3.2.1 Adsorption Behaviour of Cn-NPP on MAR

Table 3.1 Properties of DA201-C Macroporous Adsorption Resin

Polarity	None
Pearl Size	0.4-1.25mm
Surface Area	1000-1300m <sup>2</sup> g <sup>-1</sup>
Average Pore Diameter	30-40nm
Pore Volume	1.0-1.1cm <sup>3</sup> g <sup>-1</sup>

Adopted from the manufacturer's declarations

From the results in this study, it is clear that the adsorption of the Cn-NPP to the MAR follows a typical Langmuirian adsorption isotherm (Fig 3.1). Because the interactions between peptides are weak, the increase in adsorption of Cn-NPP per unit quantity of resins ceases with the formation of the Langmuir-Stern monolayer and the amount adsorbed remains constant even though the concentration of peptides in the solution is increased further (Sewell *et al*, 1987). This is probable since there are minimum peptide-peptide interactions, therefore limiting further layering of the peptides. The case may be different at higher temperatures, however, whence there may be stronger peptide-peptide hydrophobic interaction because high temperature favours hydrophobic interactions and this may explain why the recovery of peptides at 37°C was higher than at 25°C.

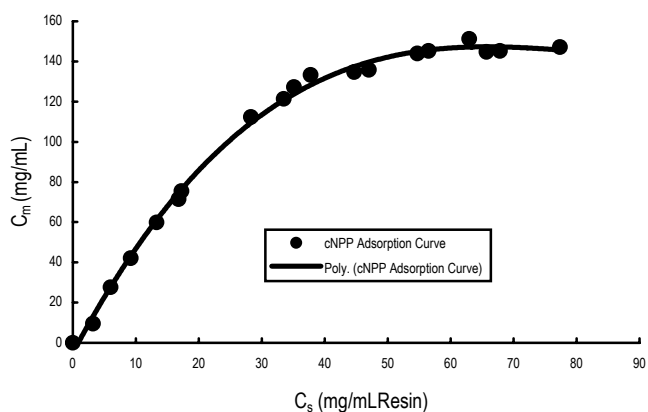


Fig 3. 1 Adsorption Isotherm of Cn-NPP on Macroporous Adsorption Resin (0.5g through 9.0g at 0.5g intervals of Cn-NPP dissolved in de-ionized water and shaken in a constant water bath at 180-190rpm, 25°C for 24hrs followed by analysis for peptide concentration by use of the method of Lowry *et al.* C<sub>s</sub> = concentration (mg/mL) of Cn-NPP in solution, C<sub>m</sub> = concentration (mg/mL) of Cn-NPP adsorbed per mL resin).

When fitted to the Langmuir equation derived according to Brockington *et al*/(1985), a relationship of the type  $C_m/C_m^{\max} = K.C_s / (1 + K.C_s)$ , is derived when all the possible sites available on the resins are taken up by Cn-NPP. In this case C<sub>m</sub> and C<sub>s</sub> represent Cn-NPP concentrations adsorbed on the resin and in solution, respectively. C<sub>m</sub><sup>max</sup> is the maximum adsorption capacity in mg Cn-NPP adsorbed per mL resin whereas K is the Langmuir constant for the conditions.

When this relationship is converted to the form;

$$C_m = \frac{C_m^{\max} + K \times C_s}{K \times C_s}$$

Thus the equation below is obtained

$$\frac{1}{C_m} = \frac{1 + K \times C_s}{C_m^{\max} \times K \times C_s} = \left( \frac{1}{K \times C_m^{\max}} \right) \frac{1}{C_s} + \frac{1}{C_m^{\max}}$$

Which is in the form  $y = mx + c$ .

A plot of 1/C<sub>m</sub> Vs 1/C<sub>s</sub> (Fig 3.2) gives the intercept equivalent to the value of 1/C<sub>m</sub><sup>max</sup>, thus C<sub>m</sub><sup>max</sup> is 434.78mg/mL, while the value of K is calculated from the relationship as 4.76 x 10<sup>-4</sup> at 25°C (Table 3.2 below).

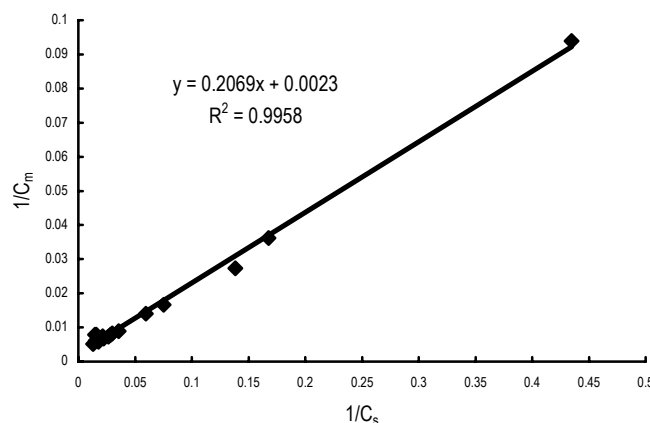


Fig 3. 2 Langmuir Transformation of the Cn-NPP adsorption isotherm at 25°C plotted from inverse values obtained in Fig 3.1 above and the values of K,  $C_s$  and  $C_m$  calculated.

It has been argued that more than one ligand on the adsorbing material is involved in hydrophobic interaction adsorption, i.e. multipoint attachment (Eriksson, 1998). Furthermore, Tanford (1962) showed that hydrophobic forces were important in stabilizing proteins, with non-polar amino acid groups avoiding contact with water thus resulting in protein association, which may play a role in Cn micelle stability.

### *3.2.2 Effects of Temperature and 1% $(NH_4)_2SO_4$ on Adsorption of Cn-NPP to MAR*

It was found in this study that adsorption was affected by temperature (Table 3.2), being depressed at 15°C while being almost independent of peptide concentration at 37°C. The effect of  $(NH_4)_2SO_4$  on adsorption was marginal at 15°C but highest at 37°C. This is a mixture of the entropy and salting out as promoted by both temperature and the salt thus creating an environment conducive for hydrophobic interaction between the peptides and the hydrophobic ligand. When the  $(NH_4)_2SO_4$  was added to the peptide solution before being applied to column desalting, however, the peptides eluted with the rinsing water, owing to a reduction in the ionic strength further confirming that the nature of interaction between the resin and the peptides to be hydrophobic. Lowering of ionic strength has been used to elute proteins from hydrophobic interaction ligands (Wilchek and Miron, 1976). It was not feasible to apply the effect of temperature on the column owing to the need for lagging, which would necessitate the use of expensive column materials.

It would suffice, therefore, that although higher temperature and  $(NH_4)_2SO_4$  favoured increased hydrophobic interaction, resulting in increased adsorption, they limit the handling and recovery of the peptides at rinsing. This limits these conditions to tests and this may not be tenable during desalination in practice in this case because of losses due to leaching of peptides while rinsing as a result of reduced ionic concentration environment of the column. In our case the peptides had a high ash content (>13%) before desalting so that use of  $(NH_4)_2SO_4$  was not of any practical use.

Table 3.2 Values for Langmuir Transformation

Temperature °C	$C_m^{\max}$ Mg/mL	K $\times 10^{-4}$
15	390.64	6.78
25	434.78	4.46
37	504.27	3.44

Hjertén (1973) reviewed the effect of pH and temperature as well as ionic strength on hydrophobic interaction chromatography (HIC) and reported that lower pH, higher temperature and high buffer ionic strength favoured hydrophobic interaction, while the converse suppressed. The effect of temperature in promoting hydrophobic interaction is entropy driven,  $\Delta G = \Delta H - T\Delta S$ , implying that the interaction increases with an increase in temperature. Eriksson (1998) argues that the increase in entropy ( $\Delta S > 0$ ) originating from water molecules leaving the more ordered structure around the non-associated solutes for the more unstructured bulk water is the main driving force for increasing hydrophobic interaction.

### *3.2.3 Relationship Between Desorption of Cn-NPP from MAR and Alcohol Content*

Desorption of Cn-NPP from MAR by alcohol may be regarded as elution by displacement, with the alcohol acting as a detergent (Hjertén, 1973); further proof that the interaction involved was indeed hydrophobic in nature (Wilchek and Miron, 1976). This is because of the presence of both the hydrophobic zone (hydrocarbon chain) and a hydrophilic zone (the hydroxyl group) in alcohol. In this study it was shown (Table 3.3) that with about 60% alcohol concentration, desorption approached 90% with the appearance of a slight plateau at higher alcohol concentrations than these.

It is therefore feasible to desorb Cn-NPP with 60% alcohol, although in practice 70% alcohol was employed in our study. It was not quantified in this study whether there was any denaturation of the peptides, which may, however, be minimal as opposed to when native proteins are involved, because peptides are relatively more stable under denaturing conditions. After desorption, ash level in the Cn-NPP found was  $1.67 \pm 0.22\%$  of the total solid, from a pre-desalting high of  $13.37 \pm 0.14\%$ . This indicates the effectiveness of MAR in desalting the Cn hydrolysates with good recovery and minimal wastage.

Hydrophobic interaction occurs in proteins in order to minimize the free energy by exclusion of water whereby the polypeptide in the protein folds to achieve an energetically favourable configuration. This is achieved by the non-polar amino acid residues avoiding contact with the water. This folding in proteins allows the polar side chains to point out towards water whereas the apolar sides point inwards to exclude water, which forms a structural layer on the hydrophobic protein (Tanford, 1962). The addition of an organic solvent will prevent this exclusivity tendency and thus discourage hydrophobic interaction by disrupting the association because of reduced the 'phobia' between the non-polar peptides and the solvent (Wilchek and Miron, 1976), thus the use of alcohol in desorption.

Table 3.3 Desorption of Cn-NPP from MAR with varying concentrations of alcohol

Alcohol (%)	Conc. Before Adsorption (mg/mL)	Conc. After 12hrs Adsorption (mg/mL)	Desorption (%)
0	12.26	3.09	25.20
10	15.26	6.85	44.89
20	13.61	7.04	51.76
30	14.74	8.77	59.50
40	16.05	10.75	66.97
50	14.83	11.43	77.07
60	13.50	11.88	88.00
70	13.76	12.05	87.57
80	14.10	12.86	91.21

Resins pre-equilibrated in alcohol of varying concentrations and Cn-NPP dissolved in the same and shaken in a water bath at 25°C for 12 hrs, followed by analysis of adsorbed peptides by use of the method of Lowry *et al.*

### 3.3 CONCLUSIONS

This study confirms temperature as the paramount factor influencing the adsorption of Cn-NPP on MAR, together with the effect of  $(\text{NH}_4)_2\text{SO}_4$ . Further it is concluded that the application of  $(\text{NH}_4)_2\text{SO}_4$  in the desalination process is not feasible owing to the already high ash content in Cn-NPP. Our results yielded further proof of hydrophobic interactions when peptides were eluted at reduced ionic concentration during rinse-washing. These findings are in agreement with others, which found that temperature, pH and flow rate influenced the recovery of peptides on MAR as well as the behaviour of proteins in HIC. Besides alcohol was found to be important for desorption. The MAR resins, therefore, offer a viable option to desalt protein hydrolysates following a hydrolysis process involving estimation of DH by the pH-stat method.

## CHAPTER FOUR:

### SIZE EXCLUSION CHROMATOGRAPHY OF Cn-NPP ON SEPHADEX G-15

#### 4.0 INTRODUCTION

Gel filtration (GF) or as is alternately referred to as size exclusion chromatography (SEC) is a chromatographic separation process, which separates biomolecules based on their size (Hagel, 1998; Irvine, 1997). The two terms, SEC and GF shall be used interchangeably with ease in the present paper wherever expedient. Theoretically, in GF molecules that do not enter the interstices of the gel matrix are eluted in the void volume because they pass through the column at the same speed as the flow of the buffer. Those molecules with partial access into the pores of the matrix elute from the column in their order of decreasing size. The separation is therefore basically regarded as being due to the differences in residence times of solutes within the liquid phase that is entrapped in the gel matrix (Hagel, 1998), which times are related to the fraction of pores available for the solute to access. Being a resolution process, which is (assumed to be!) devoid of adsorption, it is a volume sensitive chromatographic technique whereby sample concentration is only limited by its contribution to viscosity. Besides, it is mild and apart from the elution buffer, the sample may not suffer any exposure to denaturing conditions, as is the case when adsorbed molecules have to be desorbed, sometimes by use of harsh conditions.

The litanies of GF purification protocols encountered in literature make reference to protein purification (see for example Hagel, 1998). Although similar in nature and closely related, there are remarkable differences between peptides and proteins with respect to the influence for example of pH, among other conditions, on protein conformational stability, with little or insignificant effect on peptide stability. These differences, however subtle, may influence the ease with which the purification process may be optimised. In addition, a mixture of peptides presents other challenges because unlike native proteins whose molecular conformations may permit distinct delineation from each other and/ or from a group with near homogenous molecular weight characteristics in elution profiles in GF, the molecular sizes of peptide resulting from a hydrolysis process are contiguous over a given range so that clear base-line separations may become increasingly elusive. This is aggravated if the mixture of peptides is highly hydrophobic as is the case when alcohol precipitation is involved at high concentrations to fractionate hydrolytic products with the alcohol soluble fraction being more hydrophobic (Kanekanian *et al*, 2000).

Many factors influence the degree of peak separation in the GF of molecules. They include, sample volume, the ratio of sample volume to column volume, column dimensions, particle size packing density, pore size of the particles, flow rate and viscosity of both the sample and buffer. Moreover, the sample may require special pH, solvent, additives, or pre-treatment before GF. The choice of the gel is therefore

restricted by these factors and nature of sample with respect to exclusion-permeation range of the gel matrix (Hagel, 1998).

Besides, ideal SEC is not always achievable in practice because of the nature of the protein and/or the matrix and the interactions arising hence. All SEC matrixes have some ionogenic groups which produce a negative surface charge within the working pH ranges besides favouring hydrophobic interactions to some degree (Dubin and Principi, 1989; Golovchenko *et al*, 1992), unless the pH is too low not to allow for the matrix to be ionized (Kopaciewicz and Regnier, 1982). In Sephadex (a cross-linked dextran) the hydrophobic interactions are possibly instigated by interactions with ether bridges introduced by the cross-linker, while the ionogenic groups are due to carboxylic acid groups in the dextran (Hagel, 1998).

At lower eluting buffer ionic strength such charged groups favour electrostatic interactions between the molecules and the column packing, either double layer repulsion if the pH is higher than the pI of the molecules or attraction if the pH is lower owing to increased density of positive charges on the molecules (Golovchenko *et al*, 1992; Kopaciewicz and Regnier, 1982). At higher ionic strength, there is induction of solvophobic effect which results in protein hydrophobic adsorption on the matrix, as well as involving themselves in peptide-peptide self (hydrophobic) interaction because of mobile phase ionic strength 'antagonism'. This leads to the so-called nonideal SEC (nSEC). Furthermore, charge interactions elicit possible Donnan effects resulting in an increase of the electrolyte concentration in the pore interstices caused by the presence of macroions in the void volume such that the interstitial microenvironment pH in these regions is varied from the buffer pH

To overcome these impediments, one would require prior knowledge of the pI of the protein(s) and/ or peptide(s) which case is not possible in a morass of peptide mixtures as presented by hydrolysates that are yet to be fractionated. Arriving at ideal conditions, therefore, is fortuitous and involves tinkering with the mobile phase pH and ionic strengths so as to attain the working environmental conditions which attenuate both the peptide-matrix ionic and the hydrophobic interactions (Dubin and Principi, 1989) to allow for optimal, if exclusive SEC.

Some GF purification protocols of bioactive lacto (milk)-peptides have involved Sephadex LH-20 as an initial GF media followed by Sephadex G-25 as well as HP-SEC (Saito *et al*, 2000) to achieve the intended purification. Although rapid and largely unrestricted by the high hydrophobicity of the hydrolysates, both RP-HPLC and HP-SEC bring about the spectre of the inflated cost of the purification process besides being destructive, especially their analytical variations. Furthermore, the use of a two-stage GF increases the handling as well as raising the problem of losses besides being unnecessarily expensive. It would be desirable, therefore, to develop cheap single-step GF procedures for fractionating protein hydrolysates using conventional column chromatography during a purification process. Confronted by highly hydrophobic peptides (Table 2.1-1, page 33) that are sensitive to both buffer pH and concentration, methods to vitiate the hydrophobic forces would be imperative for any meaningful elution peak separation.

It was the intention of this investigation to study the elution buffer environmental factors (pH and ionic concentration) that influence satisfactory peak resolution of highly hydrophobic peptide hydrolysates in GF as well as the investigating means to surmount the same so as to optimize the separation protocol. Further it was desired to discuss the effects of various elution solutions containing some percentage of alcohol, as a condition to attenuate hydrophobic interactions so as to provide a road map for future purification strategies.

## 4.1 MATERIALS AND METHODS

### 4.1.1 Materials

Sephadex G-15 gel was from Amersham-Biosciences (Uppsala, Sweden), all the other chemicals and materials used in this study have been declared in previous reports unless otherwise stated.

### 4.1.2 Methods

#### *4.1.2.1 Evaluation of Factors Affecting Peptide Separation on SEC*

The effects of buffer pH and ionic strength as well as alcohol concentration on peak resolution were investigated using the Sephadex G-15. The glass column (1.6i.d X 100cm) was washed with 3-bed volumes of degassed, de-ionized water followed by equilibration with the buffer (in most cases 3-bed volumes or until the pH and/ or conductivity of the eluent was similar to that of the buffer). In each case Cn-NPP was dissolved in the elution buffer or solution and filtered through a 0.45 $\mu$ m acetate membrane using a syringe filter. 2mL (1% of bed volume) of the filtered solution was injected manually using a disposable pipette. Each time, the surface of the gel was allowed to just drain and the solution added gently without disturbing the surface of the gel. The elution buffer (or 2% alcohol in deionized water) was then used to wash down the top of the column previously in contact with the Cn-NPP solution. When just drained, the outlet was clipped to prevent further drainage and more wash buffer was added to the rim. The column was closed and pumping by use of a pulse pump commenced, in each case at 0.28~0.32mL/min or equivalent to a linear flow rate of 8.35~9.55cm/min. The elution was monitored by an online assembly of a UV detector operating at 220nm and connected to a potentiometer recorder.

#### *4.1.2.2 Analytical Assays during Gel Filtration.*

The method of Lowry *et al* according to the modifications of Peterson (1977) was used to determine downstream peptide concentration, with BSA as the standard.

#### *4.1.2.3 Statistical Analysis*

The peptide concentrations were determined in duplicate with two (2) blanks and the mean values used in calculations. The values were tested for significance with the Student's *t* test ( $p < 0.05$ ). The elution profiles recorded on the potentiometer recorder were transformed and regenerated using Microsoft Excel software (Microsoft Corporation, USA).

## **4.2 RESULTS & DISCUSSIONS**

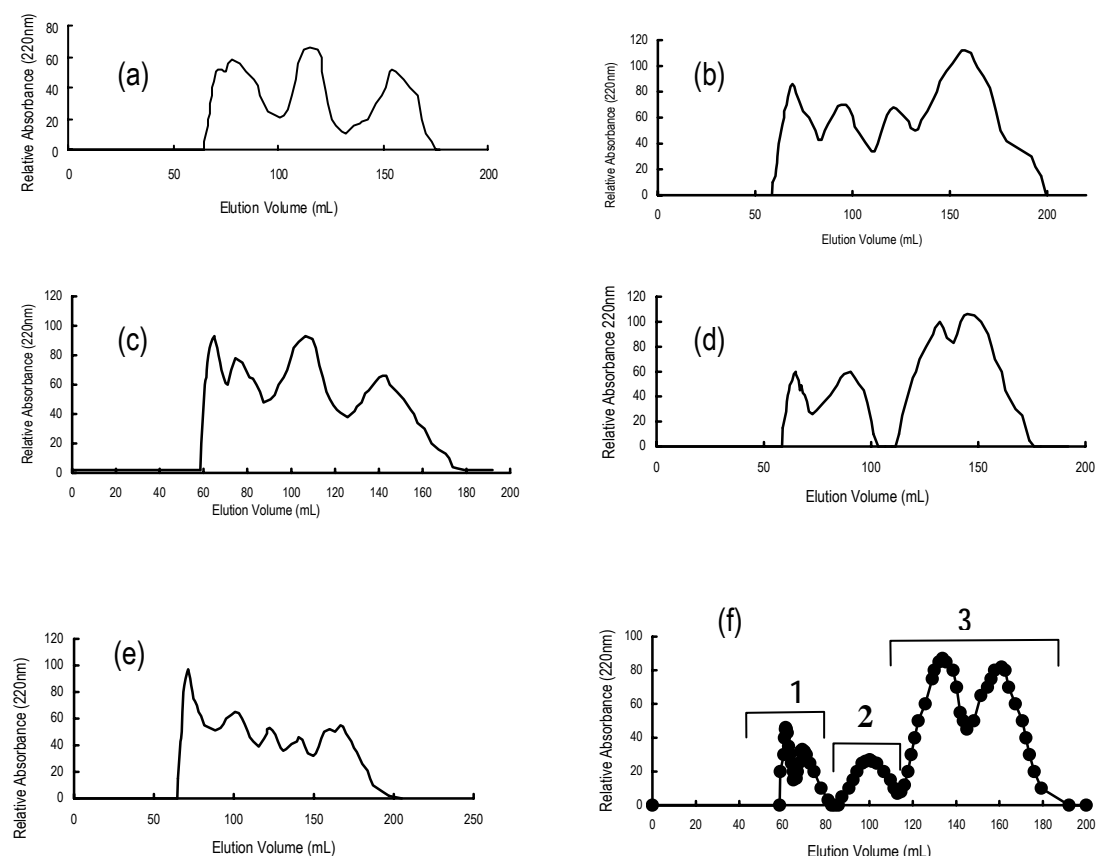
### *4.2.1 Factors Affecting Peak Separation*

The buffer pH and ionic strength on peak separation and resolution are shown in Fig 4.1a, Fig 4.1b, Fig 4.1c, Fig 4.1d and Fig 4.1e. The effect of alcohol and its concentration is presented in Fig 4.1f.

#### *4.2.1.1 Effect of Buffer pH 5.5 on SEC of Cn-NPP on Sephadex G-15*

From Fig 4.1a it is clear that one peak which appears in Fig 4.1b and Fig 4.1f is lost because of pH induced hydrophobicity between peptides at low elution buffer pH. Besides, Cn hydrolysis may have released peptides with pI values (slightly) lower than neutral in which case these peptides have a high density of positive charges at the pH 5.5. This would result in attractive ionic interaction with the (negatively) charged matrix surface. At low pH, therefore, there is hydrophobic association of peptides as well as attractive ionic interaction between the peptides and charged matrix surface especially at such low buffer ionic strength (Dublin and Principi, 1989) as the one used in this investigation (0.02mol/L). However, the association was reversed with an increase in pH to 7.5 (Fig 4.1c) at the same ionic strength, implicating both hydrophobic and ionic interaction at lower pH (5.5).

The effect of pH and ionic strength in either favouring or attenuating hydrophobic interaction has been reviewed by Hjertén (1973), who concludes that pH and temperature play an important role in determining the nature and strength of hydrophobic interactions of proteins. It is postulated that as proteins and peptides become more hydrophilic with increasing pH, there is a concomitant decrease in affinity for hydrophobic interaction and this has been attributed to the titration of the hydrophilic groups, which makes the peptides more hydrophilic (Hjertén, 1973).



**Fig 4. 1** Sephadex G-15 chromatograms of Cn-NPP from a 1.6ID x 100cm glass column eluted by a pulse pump, flow rate 20.4mL/hr under various influences of buffer pH and ionic strength. (a): 107 mg/mL, 0.02mol/L Na-Acetate buffer pH 5.5, (b): 107 mg/mL, 0.01mol/L Na-Phosphate buffer at pH 7.5, (c): 110 mg/mL, 0.02mol/L Na-Phosphate buffer pH 7.5, (d): 100 mg/mL, 0.02mol/L borate buffer at pH 8.5, (e): 105 mg/mL 0.5mol/L Na-Phosphate buffer pH 7.5, (f): 109 mg/mL, 2% alcohol in Deionized Water, no salt buffer, pH  $\approx$  6.8. The sample was filtered with a 0.45 $\mu$ m acetate membrane using a syringe filter followed by manual injection of 2mL equivalent to 1% column bed volume and the eluent monitored with a UV detector at 220nm and 2mL fractions collected. Peak 3 was further fractionated by anion exchange resin (section 5.1.2.3, page 58).

#### 4.2.1.2 Effect of Buffer pH 7.5 and 8.5 on SEC of Cn-NPP on Sephadex G-15

Dubin and Principi (1989) and Golovchenko *et al* (1992) argue that since most matrixes in SEC are negatively charged besides being hydrophobic to some degree (Adachi *et al*, 1991), at pH values above the pI of the peptides (or in this case above the pI of the majority of peptides in the mixture) the matrix charge is of the same sign as the charge density of the peptides. This results in dominance of double layer repulsive forces between the matrix surface charge and the high negative charge density of the peptides. This repulsion would be expected to increase with an increase of pH far from pI owing to augmentation of the (negative) charge density on the peptides. It would seem, therefore, that apart from higher pH favouring a lowering in the hydrophobicity of the peptides, thus reducing the peptide-peptide interaction as well as attenuating the peptide-matrix hydrophobic interaction at low buffer ionic strength, the double layer repulsive forces also favour a near ideal SEC although it is not possible to tell in this case which aspect is more important than the other, thus vitiating an attempt to attribute one factor to

the better SEC profiles attainable at high pH. However, at lower ionic strength the profile is rather less diffuse even when the buffer pH is sufficiently high to favour low hydrophobic interaction (Fig 4.1b).

Nonetheless, the better peak separation in Fig 4.1c may be attributable to contributions of the effects of higher buffer pH favouring low peptide-peptide as well as peptide-matrix surface hydrophobic interaction (Adachi *et al*, 1991). This is in addition to the double layer repulsion between the negatively charged ionogenic groups on the Sephadex surface and the negative charge density on the peptides whose pI is lower than the operating buffer pH (Dublin and Principi, 1989; Golovchenko *et al*, 1992; Kopaciewicz *et al*, 1982). Striking a compromise between conditions that attenuate hydrophobic interactions (low ionic strength and higher pH) as well as vitiating matrix-peptide ionic interactions (pH higher than pI and high ionic strength) is inevitable to achieve exclusive SEC conditions (Kopaciewicz and Regnier, 1982).

It is not practical in a situation like this one where the peptide mixtures are hydrophobic, besides the pI being unknown to manipulate pH and/ or pI without involving trial and error. However, it is underscored that buffer pH and ionic strength contribute significantly to the peak elution characteristics due to their influence, either separately or in concert, on the peptide-peptide and peptide-matrix surface interaction. Ion exclusion reportedly occurs when the mobile phase pH of low ionic strength is above the 'average' pI of the peptides (Kopaciewicz and Regnier, 1982), giving way to a SEC-dominated mechanism at pH 7.5 which possibly shifted to a mixture of the two phenomena (SE-ionic sorption mechanism below the average pI). A caveat, however, must be sounded here with regard to the use of the average pI as this should not be taken to mean net pI but rather the point of high pI density- that is where most peptides have their pI range falling within. In our case we evaluated the quantitative concentrations of the amino acids in Cn-NPP and found that more than half are in the acidic pH range (Table 5.1, page 61). How significant this value is we cannot authoritatively say, although it serves as rough guide on the probable see-saw point around which pH could influence the behaviour of peptides.

Indeed, whereas in the case of SEC of native proteins it would be critical to consider the buffer concentration from the point of view of protein stability, the case of highly hydrophobic peptides is unique. This is because highly hydrophobic peptides are not so much influenced by buffer concentration in terms of conformational stability. However, hydrophobic interactions amongst peptides and between the peptides and the GF matrix are the most important consideration. It can be deduced, therefore, that buffer pH aside, its concentration is so crucial in a situation such as the one obtaining in our study- where the peptides are highly hydrophobic that a delicate balance ought to be struck between good buffering while being cautious not to induce hydrophobic interaction which would cause a loss of the resolution of the GF process.

Conversely the reduction in ionic strength should not compromise the peptide elution by permitting peptide-matrix surface ionic interaction because of reduced matrix surface screening. In any case, where pH is sufficiently high to attenuate peptide-peptide as well as peptide-matrix hydrophobic interaction and the ionic strength is low enough to provide ionic screening of the matrix surface, the size

excluded and ion excluded peptide elution occurs and the peaks are fairly sharper than in the case where there is interference from the solvent or matrix characteristics (Golovchenko *et al*, 1992).

Although higher pH is favoured for good separation, Kopaciewicz and Regnier (1982) showed that a pH not too high above pI was suitable, with 1-pH value above pI being recommended as the region of maximum exploitation of electrostatic effects in nSEC. Indeed using a 0.02M-borate buffer of pH 8.5 (Fig 4.1d) gave indistinct separation, with the behaviour of the profile seemingly implying association, which is a reversal of the achievements of high pH in surmounting hydrophobic interactions. Kopaciewicz and Regnier (1982) found a similar scenario whereby at too high pH as well as too low ionic concentration, the elution of the proteins became heterogeneous. This was attributed to mutual ion-exclusion (over-repulsion by the matrix surface) resulting in a loss of the resolution. This is aggravated at low buffer concentration where the shielding effect of the buffer is weakened. This seems to set a definite narrow window of operation, which, together with the stability range of the gel matrix, curtails over-tinkering with the conditions for elution of the hydrolysates and indeed any protein in SEC.

#### *4.2.1.3 Effect of Buffer Ionic Strength on SEC of Cn-NPP on Sephadex G-15*

Selective alcohol precipitation (to 70% concentration) of CPP resulted in Cn-NPP peptides that are highly hydrophobic as well as bitter (Kanekanian *et al*, 2000). The high hydrophobicity of the resulting peptides proved an impediment to good peak resolution when attempts at separation using Sephadex G-15 were done with 0.5mol/L Na-Phosphate buffer pH 7.5 (Fig 4.1e). This was due to a solvophobic effect favouring peptide-peptide hydrophobic interactions as well as hydrophobic interactions between hydrophobic matrix and the peptides (Kopaciewicz and Regnier, 1982).

The poor separation of peptides with an appearance of a shallow range of peaks is easily attributable to a solvophobic effect on the peptides, which resulted in a probable peptide-matrix as well as peptide-peptide hydrophobic interaction induced by a high buffer ionic strength. This could be eliminated by low ionic strength as may be deduced from Fig 4.1c and Fig 4.1d above. The changes in the ionic strength of the mobile phase affect the double-layer potential owing to the screening of the matrix surface charges from interaction with the peptides by binding of the counter-ions (Golovchenko *et al*, 1992). However, too high an ionic strength induces hydrophobic interaction between the peptides as well as hydrophobic interaction between peptides and even the weakest of the hydrophobic moieties on the column packing matrix (Kopaciewicz and Regnier, 1982).

Obviously the chemical identity of the buffer ions determines the extent to which they bind to the charged surface and consequently reduces the double-layer interaction. It has been recommended that exploitable nSEC for proteins of pI between 5 and 8 are achieved at ionic strengths between 0.005 and 0.025mol/L (Kopaciewicz and Regnier, 1982). Cn hydrolysates are probably in this pI range and 0.02mol/L buffer ionic concentration was found satisfactory for good peak elution. It would seem, from this profile and behaviour of the peptide elutions, that this means could also afford a rough map of the pI

'density' of the peptides, which would place it in the region below 7 although this, again, is not conclusive.

#### *4.2.1.4 Effect of Alcohol eluent on Peak Separation on SEC of Cn-NPP on Sephadex G-15*

The hydrophobic interaction free energy in ethanol is less than in water and as such alcohol has been used to disrupt the hydrophobic interaction and thus allow separation of peptides enabling them to elute with less interference from these forces (Yamashita *et al*, 1976). It may be deduced from Fig. 4.1f, that alcohol leads to a lowering in the free energy and thus permits the peptides to be delineated into distinct groups, whereby the group designated Peak 1 is completely separated from the second group with about 10 minutes of baseline separation. The attenuation of hydrophobic forces by alcohol may be attributed to the reduction in 'animosity' between the hydrophobic peptides and the solvent because of the presence of a 'friendly' environment (afforded by alcohol).

Sephadex is stable to alcohol up to a concentration of 25% (Amersham-Biosciences Handbook No. 18-1022-18) although the gel initially shrunk as the alcohol was used. Nevertheless this had no adverse effects on the GF. Yamashita *et al* (1976) used alcohol to elute Soya Protein Isolate (SPI) with hydrophobic aromatic amino acids and obtained good delineation with Sephadex G-15. The attenuation of hydrophobic interactions (both peptide-peptide and peptide-matrix) by use of alcohol was achieved. Coupled with the manipulation of the ionic strength of the buffer at pH 7.5, use of alcohol underlines the presence and interaction of both hydrophobic as well as ionic interactions in interfering with good peptide separation in SEC. When alcohol was used with buffer at pH 7.5, however, there was no improvement on the resolution underlining the finite range of the resolution.

This condition (alcohol) afforded an improved separation of peptides into groups that could further be polished by subsequent separation in ion exchange chromatography (IEX) to separate the two shallow peaks that were collected together in peak 3. Furthermore, the ease with which alcohol can be removed through vacuum evaporation, and the obviation of a further desalination stage as would be mandatory if a salt buffer were used guided the decision to adopt this as the working condition during the gel-filtration of Cn-NPP.

### **4.3 CONCLUSIONS**

All SEC columns present (negatively) charged ionogenic groups as well as possessing hydrophobic properties. Hydrophobic Cn hydrolysates are interfered with in SEC by a concert of buffer pH and/ or ionic strength acting either in isolation or in unison to thwart the separation and the factors may be exploited to achieve good separation of Cn-NPP in SEC. Taking a trial and error approach in attempting to attain good, if satisfactory, separation is unavoidable in a case where the ionic properties of the peptide mixtures is unknown. The delicate balance between peptide charge, support charge and mobile phase pH and ionic strength cannot be emphasized enough, since successful manipulation of these

factors is only possible over a very finite range of conditions. With a low enough buffer ionic strength to screen the matrix surface while at the same time not instigating peptide-peptide and peptide-matrix hydrophobic interaction the elution is SEC. We have shown that when these factors are optimized, column selectivity is greatly enhanced such that it is possible to achieve clear base-line delineation of Cn-NPP peptides eluting from GF. The chromatographic process, therefore, was found to be strongly dependent not only on buffer ionic strength but also on its pH and alcohol provided a suitable condition for separation as well as giving attractive ease of application owing to the ease with which it is removed as well as obviating a post-GF desalination process, its cost notwithstanding.

## CHAPTER FIVE:

### PURIFICATION OF Cn-NPP ON ANION EXCHANGE RESIN

#### 5.0 INTRODUCTION

Proteins are amphoteric by nature, so too are the peptides they release following hydrolysis. However, unlike proteins where some charged side chains might be buried in some conformations, peptides are likely to have most of their amino acid moieties exposed to the working buffer. Both bind to ion exchangers by electrostatic forces between their surface charges and clusters of charged groups on the ion exchange (IEX) matrix surfaces. Because the charges on the matrix are balanced by counter-ions from the buffer, the peptides must displace them to be bound- with the net charge of the peptides being the same sign as the counter-ions displaced- hence ion exchange. IEX has been reported as being either reversible or irreversible, with the former being the desired interaction (Scopes, 1982).

In practice, the desired peptides are adsorbed and following desorption are characterized- or both the unadsorbed as well as the adsorbed peptides are characterized (Scopes, 1982). Purification of bioactive peptides using ion exchangers is an almost consistent protocol, being varied only in the step at which it is hyphenated in the purification protocol. Where the hydrolytic process does not involve a pile-up of ash, it is almost the immediate process proceeding hydrolysis (Recio and Visser, 1999). Other investigators use IEX after gel filtration and of course following a complete desalting procedure to remove encumbrances provided by the ions in the hydrolysate ash. With proteins, the IEX mechanism is almost straight forward, with separation of proteins from one another requiring a few pH regimes. It is even easier when the proteins in the mixture are few and their pI characteristics are known. However, when presented with a morass of peptide mixtures as in our case, there will be the probability that whole arrays of peptides differing in their pI values and adsorption properties in tenths of units are present.

Buffer pH and ionic strength are two of the most critical factors influencing IEX chromatography owing to the influence on the ionic pedigree present on the peptides relative to the adsorbing matrix charge. Where the ionic strength is too high, the matrix surface charge is 'shielded' and the peptides are not 'exchanged'- with the buffer ions being exchanged instead. Additionally, when resins are used, the high ionic strength may induce hydrophobic interactions (Karlsson *et al*, 1998). Conversely, when the ionic strength is too low or the pH is too far away from pI, the peptides are adsorbed so intimately that desorption is achievable under harsher conditions. Striking a balance between what is acceptable or not involves, as is the case in each chromatography, a practical trial and error method. Even then arriving at suitable conditions is fortuitous, nay sometimes serendipitous.

The difference in ionization properties of amino acid side chains at different pH values have been exploited to discriminate between proteins within 0.1 pH units during polishing stages in purification protocols. Proteins are characteristic in their pH solubility and/ or stability range and this may limit the

extent to which they may be subjected to adsorption in ion exchangers in varying pH values. However, peptides display remarkable stability over a wide pH range and are only limited by solubility in a given pH range in attempts to manipulate the separation characteristics based on ionization properties (Scopes, 1982). There is, however, a close relationship between protein pI and a suitable pH such that a pH value too far from the pI leads to high charge densities and inevitable increase in avidity for binding, whereby higher salt ionic strengths are required to desorb. Too close a pH to the pI leads to weak adsorption because of less net charge but desorption may be relatively easy. However, cases of asymmetric charge distributions may occur whereby the protein binds strongly although the pH is only a few (sub) units from the pI (Karlsson *et al*, 198).

Classical IEX media are expensive whereas resin based IEX media based on polymers of styrene and divinylbenzene are a cheaper alternative. The IEX groups in the resins are attached to the matrix and X8 for example, denotes the percentage of cross-linker in the polymerization mixture, with anion IEX resins (positive surface charge) having quaternary amines (Karlsson *et al*, 1998) as the exchanger ion species. Polystyrene IEX resins have been criticized for having a low capacity for proteins due to small pore sizes, although they are suited for small molecules like peptides and amino acids. Additionally, the presence of hydrophobic interaction may lead to irreversible binding to proteins.

This study was designed to investigate the adsorption characteristics of Cn-NPP on anion exchange resins designated 001X8, with a view to understanding the influence of various operating buffer conditions on the fractionation of the Cn-NPP. Further it was undertaken to study the conditions suitable for desorption as well as characterization of the adsorbed peptides for ACE inhibition following desorption as well as attempting to explain the inhibition characteristics in terms of the ionic nature of the peptides.

## 5.1 MATERIALS AND METHODS

### 5.1.1 Materials

The anion exchange resin 001X8 was purchased from Jianying Organic Chemical Plant, (Jianying, China) while all the other reagents used and equipment were as declared in previous reports unless otherwise specified.

### 5.1.2 Methods

#### *5.1.2.1 Effect of pH on Adsorption of Cn-NPP on Anion Exchange Resin*

To determine the effect of pH on adsorption of Cn-NPP peptides on anion exchange resin, the test tube scheme outlined in Amersham-Biosciences (Handbook No. 18-1114-21) manual for ion exchange was adopted with modifications. The peptides were dissolved to make between 90-100mg/mL in each case.

Resin slurries (10mL) in 150mL Erlenmeyer flasks were pre-equilibrated with excess 0.5mol/L Na-acetate buffer, at pH 5.5 and 6.5; and 0.5mol/L Na-Phosphate buffer at pH 7.5 equivalent to washing six times each time with 30mL buffer. This was followed by equilibration by similar buffer at 0.02mol/L by washing a further 6 times with 30mL, or until the conductivity was similar to that of the buffer. The peptide solution (30mL), dissolved in 0.02mol/L of the working buffer was added and the flask mouth secured with polythene and immediately put in a shaking water bath operated at 25°C and shaken at between 180~190rpm for 12 hours. At the end of this time, the vibration was stopped and the clear supernatant skimmed off and after appropriate dilutions, assayed for unadsorbed peptides concentrations. Adsorption was expressed as percentage of the adsorbed peptides concentration over the original concentration in the flask. The peptides concentrations were determined in duplicate with 2 blanks using the method of Lowry *et al* with BSA as the standard and the results presented are mean values of the same.

#### *5.1.2.2 Effect of Buffer Concentration on Adsorption of Cn-NPP on Anion Exchange Resin*

At pH 7.5, which was found to have highest adsorption for the peptide mixture in anion exchange resins, the resin slurries were equilibrated as in Section 5.1.2.2 above with 0.02mol/L sodium phosphate buffer of the following concentrations: 0, 0.005, 0.01, 0.015, 0.02, 0.025 and 0.03mol/L. The peptides were dissolved in similar buffer and added as stated in Section 5.1.2.2. After incubation in the water bath at 25°C for 12 hours, the supernatant was assayed for adsorption using the method of Lowry *et al* with BSA as standard. Adsorption was calculated by relating the peptides concentrations after 12 hours and before and expressed as a percentage. The concentration of buffer to just allow adsorption of peptides was chosen as the working buffer ionic strength. The determinations were done in duplicate with 2 blanks containing buffer instead of peptide solution and the results presented are average values of the same.

#### *5.1.2.3 Fractionation of Cn-NPP from the Sephadex G-15 Peak 3 (Fig 4.1f) on Anion Exchange Resin*

It was desired to further resolve the peak designated 3 in Fig 4.1f (on page 51) by use of anion exchange resins with 0.02mol/L sodium phosphate buffer of pH 7.5. The fraction was desalted with MAR as outline in Section 3.1.2.4 and desorbed with 70% alcohol, concentrated, lyophilized and re-dissolved in the IEX buffer. The resin slurry packed in a 2.6ID x 30cm glass column was equilibrated with the buffer until the pH and conductivity reached equilibrium. Next the filtered peptides were manually added as outlined in Section 4.1.2.1 above. 10mL of the peptide (95mg/mL) was added and the wash buffer pumped through at 30cm/hr linear flow rate. An online assembly including a UV detector set to 220nm and a recorder monitored the eluent. When the peak returned to the baseline, the eluent was analyzed for peptides by the procedure of Lowry *et al* with BSA as a standard. The adsorbed peptides were isocratically eluted with 1.5mol/L NaCl, collected, and after desalination, were lyophilized and kept for characterization for ACE inhibition activity.

#### *5.1.2.4 Fractionation of the Cn-NPP on Anion Exchange Resin*

Desalted Cn-NPP was dissolved in 0.02mol/L sodium phosphate buffer pH 7.5 in the ratio 1:200 (w/v), filtered with an acetate membrane using a nylon filter and 10mL injected manually into a glass column (2.6i.d X 30) packed with anion exchange resin pre-equilibrated with the buffer. Next, the buffer was pumped through the column at 30cm/hr to elute the unadsorbed peptides and the eluent monitored with a UV detector set to 220nm and connected to a detector. The amount of peptides in the eluent was also monitored at intervals of 30 minutes by collecting aliquots of 1-3mL to analyze for peptide concentration using the method of Lowry *et al* according to the procedure of Peterson (1977). When the detector recording was back to the baseline and the peptides concentration was below 5% in the eluent, 1.5mol/L NaCl was pumped through the column and the eluent collected. The collected peptides were either lyophilized or desalted immediately depending on the availability of the facilities. The resulting desorbed peptides were kept for analysis of ACE inhibition according to section 6.1.2.3 (page 66).

#### *5.1.2.5 Effect of NaCl Concentration on Desorption of Adsorbed Peptides*

At pH 7.5 which was found to have highest adsorption for the peptide mixture, the resin slurries were equilibrated as in Section 5.1.2.2 above with 0.02mol/L sodium phosphate buffer containing NaCl of the following concentrations: 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0mol/L. The peptides were dissolved in similar buffer and added as stated in Section 5.1.2.2. After incubation in the water bath at 25°C for 12 hours, the supernatant was assayed for adsorption using the method of Lowry *et al* with BSA as standard. Desorption was calculated by relating the peptide concentration after 12 hours and before and expressed as a percentage. The concentration of NaCl to desorb peptides was chosen as that which just thwarted adsorption. The lowest possible such concentration was chosen. The determinations were done in duplicate and the results presented are average values of the same.

#### *5.1.2.6 Statistical Analysis*

The determinations were done in duplicate and their means were calculated using the statistic mode of a CASIO® fx-500A Calculator produced by CASIO Computer Co. Ltd., Japan. Significance among the data was determined by the Student's *t* test ( $p < 0.05$ ). The elution profiles recorded on the potentiometer recorder were transformed and plotted using Microsoft Excel software (Microsoft Corporation, USA).

## **5.2 RESULTS AND DISCUSSION**

The amino acid composition of the Cn-NPP is presented in Table 5.1; Fig 5.1 shows the distribution of the pI values of the amino acids and their concentrations in the mixture. Fig 5.2 shows the desorption characteristics of Cn-NPP from anion exchange resin with various concentrations of NaCl. Fig 5.3 is a typical isocratic elution profile for Cn-NPP from anion exchange resin by 1M NaCl.

5. 2.1 Effect of Buffer pH and Concentration on Adsorption of Cn-NPP on Anion Exchange Resin

Presented in Table 5.1 below are the analysis results for the amino acid composition of the Cn-NPP from DH 15%. From the table we can deduce that most of the peptides in the mixture fall within the acidic pH range as plotted from the Fig 5.1.

Admittedly, it is not easy to point out the point of pl index of the peptides in Cn-NPP. However, from the comparison of the concentration and pl, it is possible that the average lies fairly in the range below 6 (50.15% w/w) for most of the peptides in the mixture. In fact when the trials were done for a suitable pH, a remarkable precipitation of the peptides was visible at pH 4.5. This value, however, could be lower with the peptides precipitating because of the Donnan effect occurring which may have pushed the effective pH to 5.5 (one unit higher in anion exchangers), and this should be around the point where the pl for most of the peptides falls, since it is reported that the Donnan effect is favoured by low ionic strengths of the buffer (Scopes, 1982).

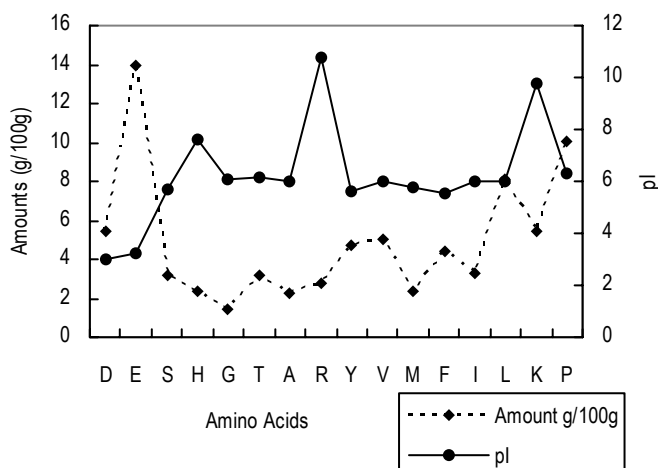


Fig 5. 1 The distribution of the amino acids in the Cn-NPP relating pl and amount (single letter amino acid codes used). The amino acids were analyzed by Hitachi high-speed amino acid analyzer (Table 5.1) following hydrolysis with 6N HCl at 110°C for 24hrs *in vacuo*. pl values were adopted from Cheftel *et al*(1985).

Barrantes (1973) introduced various statistical tools to analyze quantitative and qualitative amino acid composition of a protein. The ratio of neutral and basic amino acids (pI > 6.0) in Cn-NPP obtained from casein hydrolyzed to DH 15% was is 49.85% (w/w) while acidic amino acids (pI < 6.00) are present at 50.15% (w/w). Being a statistic, however, it doesn't give any indication of how far up or below on the pI scale the peptides fall. Furthermore, this value may be undermined by the contribution of low molecular weight amino acids whose contribution is significant at a glance knowing their molecular weight nature as contrasted with high molecular weight amino acids like tyrosine whose weight may exaggerate the amounts present in the Cn-NPP.

Table 5.1 The distribution of the amino acids in the Cn-NPP, relating pl & amounts (g/100g)

Amino Acid	Molecular Weight (g/mol)	Amount (g/100g)/	pI <sup>a</sup>
Alanine	89.1	2.30	6.02
Arginine	174.2	2.76	10.76
Aspartic Acid	133.1	5.47	2.97
Glutamic Acid	147.1	13.99	3.22
Glycine	75.1	1.48	6.06
Histidine	155.2	2.32	7.58
Isoleucine	131.2	3.26	6.02
Leucine	131.2	8.14	6.00
Lysine	146.2	5.44	9.74
Methionine	149.2	2.32	5.75
Phenylalanine	165.2	4.42	5.53
Proline	115.1	10.07	6.30
Serine	105.1	3.15	5.68
Threonine	119.1	3.13	6.16
Tyrosine	204.2	4.73	5.65
Valine	117.1	5.06	5.97

<sup>a</sup> Adopted from Cheftel *et al*(1985);  
 Basic amino acids (pI > 7.00) = 13.48%  
 Between pI 6.00~6.99 = 36.37%%  
 Below pI 6.00 = 50.15%

However, peptides were adsorbed at pH 7.5 than at both 5.5 and 6.5 when either the un-fractionated Cn-NPP or peak 3 from Sephadex G-15 (Fig 4.1f, page 51) was eluted through the column. This for anion exchange means that pH 7.5 is above the pI of most of the peptides resulting in high negative charge density such that they are exchanged at the anion exchangers. The peptides that were still positively charged at pH 7.5 could not be exchanged at this pH and remained in solution. The ionic strength that just allowed adsorption was 0.015mol/L, although 0.02mol/L was chosen. This is because too low an ionic strength results in more avid adsorption with desorption being ever more difficult or only achievable under harsh conditions. From this experiment it was shown that peptides could be recovered at any pH although the amounts involved at a given pH varied with pH above 6 giving fairly good adsorption recovery. The adsorption at pH 7.5 was however beset by a requirement to use a higher concentration of NaCl to desorb, indicating possible strong adsorption tendency particularly for peptides whose pI was way below this working pH. This may also have been contributed by the weak ionic strength of the buffer corroborating the findings of Karlsson *et al*(1998).

It was found that buffer pH and ionic strength were crucial to good adsorption although the practical implications when the column elution was used were slightly different with less than anticipated peptides being adsorbed and with the flow rate playing an important role as well. Indeed when water alone (pH 6.8) was used to elute the peptide there was adsorption with the peptides being desorbed by elution with 1.5mol/L NaCl. Although the ionization nature of the peptides in the Cn-NPP mixture was not known for certain, the statistical tools employed gave a rough idea where to place emphasis while using anion exchangers.

### 5. 2.2 NaCl Concentration on Desorption of Cn-NPP from Anion Exchange Resin

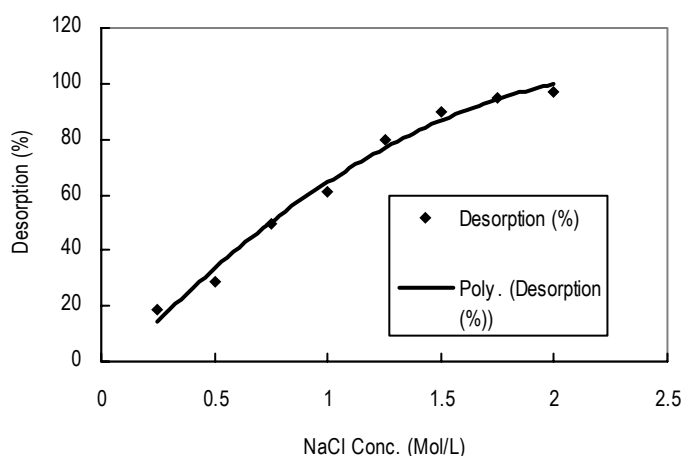


Fig 5. 2 Desorption of Cn-NPP from anion exchange resin by NaCl. Cn-NPP was dissolved in the elution buffer containing NaCl concentrations from 0 to 2mol/L at pH 7.5, put into Erlenmeyer flasks containing anion exchange resin and shaken in a constant water bath kept at 25°C for 12 hrs. Peptide concentrations were evaluated by use of the method of Lowry *et al* with BSA as a standard and desorption expressed as ratio of concentration in solution after 12 hrs over the concentration before adsorption as a percent.

The NaCl used in the buffer prevented exchange of peptides by screening the charged resin surfaces. The amount of salt to cover all the sites of the resins and prevent this adsorption was found to be 1.5mol/L and interpreted as the concentration to effect complete desorption. There was reduction in adsorption through all the NaCl concentrations under study, indicating a potential for stepwise and/ or gradient elution although when gradient elution was attempted it was found to take too long to attain any appreciable elution. When studied for possible hydrophobic interaction by washing the resins with alcohol and analysing the eluent for peptides, the resins were found to be weakly hydrophobic, such that the adsorption may be a mixture of both ionic as well as hydrophobic interactions, consistent with the criticism encountered for resins in IEX (Karlsson *et al*, 1998).

It would have been desired to employ the better peak sharpening properties associated with gradient elutions; but Isocratic elution with 1.5mol/L NaCl was adopted to elute the peptides. The eluted peptides were lyophilized then re-dissolved, desalted and lyophilized or desalted and lyophilized straight away depending on the availability of the desalting column.

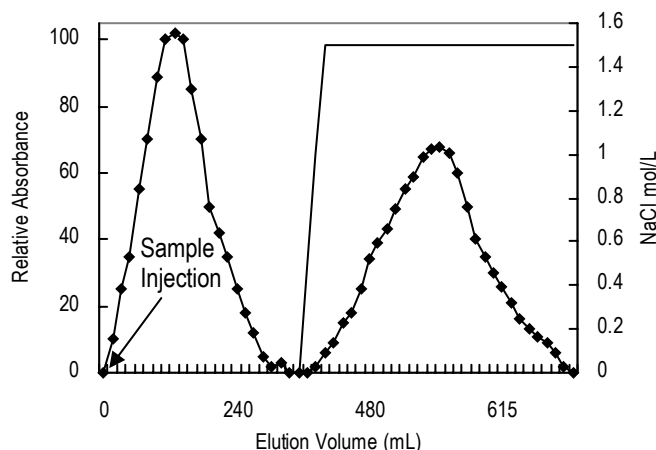


Fig 5. 3 Typical isocratic elution profile for Cn-NPP with 1.5M NaCl from a 2.6ID x 30cm glass column packed with anion exchange resin (001X8). Peptides solution was manually injected equivalent to 5% bed volume and pumped with a pulse pump at 30cm/hr. The eluent was monitored by a UV detector clocked at 220nm and fractions collected at 2mL. This is representative of several trials made.

### 5.3 CONCLUSIONS

The involvement of ion exchange in protein and peptide purification is the rule rather than the exception and evolution of newer ligands to optimize the IEX separation makes it a must-use step in any purification protocol. It was proven in this experiment that the resins were able to provide adsorption to the peptide mixtures and afford a means of fractionation. Furthermore, pH and ionic strength of the buffer were the most crucial determinants of the adsorption. Temperature was not investigated to ascertain its influence on adsorption (owing to the presence of hydrophobic interaction forces as well as) being an adsorption process whereby temperature would be expected to influence it. The peptides were desorbed with good recovery by use of 1.5mol/L NaCl.

## CHAPTER SIX:

### ANGIOTENSIN-I-CONVERTING ENZYME (ACE) INHIBITION CHARACTERISTICS OF Cn-NPP

#### 6.0 INTRODUCTION

The fact that ACE inhibitors have been characterized from Cn and indeed all lactoprotein hydrolysates obtained by use of other enzymes (Pihlanto-Leppälä *et al*, 1998) and processed in various chromatographic techniques is evidence that the ACE inhibitory nature of the hydrolysates is diverse (van der Ven, 2002) and procedures to optimize the ACE inhibitory effect of the hydrolysates are called for.

Earlier ACE assays were based on physiological responses, either contractile or haemodynamic to Angiotensin-II generated by ACE from its native substrate Angiotensin-I. The development of simple chemical and radiometric methods to assay for ACE activity by utilizing selective amino-substituted tripeptide substrates greatly facilitated both ACE purification and studies of the role of ACE in cardiovascular haemostasis (Ondetti and Cushman, 1982). Simple tripeptide artificial substrates have largely replaced assays, which use natural substrates Angiotensin-I and bradykinin. The artificial substrates are easily synthesized, inexpensive, and not susceptible to hydrolysis by angiotensinases (endo- or aminopeptidases) as well as having  $K_m$  values for ACE comparable to or greater than the natural substrates (Cheung *et al*, 1980).

The method promulgated by Cushman and Cheung (1971) has been widely used with modifications for the assay of ACE activity. The modifications are aimed at optimizing the tracking of the amount of hippuric acid released as a result of the cleavage of the dipeptide from the artificial substrate. Among these is the recent modification by Wu *et al* (2002), which obviates the extraction of the hippuric acid by ethyl acetate while hyphenating HPLC in the quantification of the acid released thus offering a powerful tool to rapidly assay the enzyme activity. The theory of the method involves treatment of a chromogenic substrate, Hip-His-Leu (HHL) with rabbit lung ACE whereby the cleavage of the dipeptide His-Leu leads to formation of hippuric acid (HA). The amount of the acid released is in proportion with the geometry of peak absorbance at 228nm. Wu *et al* (2002) reported that in the traditional spectrophotometric method, there was always interference from the HHL entrained as a co-extract with HA (12% of peak area) since it also absorbs strongly at 228nm. This was reduced drastically or eliminated entirely in reversed-phase high performance liquid chromatography (RP-HPLC) assaying especially when geometries of the substrate and hippuric acid peaks were clearly delineated at the baseline with no self-interference.

This study was initiated to investigate the ACE inhibitory potential of Cn-NPP peptides hydrolyzed to various degrees of hydrolysis, as well as to determine the relationship between their molecular-weight

distributions to their inhibitions to ACE; to characterize fractions purified in anion exchange resin and to study the relationship of the same with ACE inhibition. Furthermore, use of the modernized ACE activity assays by RP-HPLC procedures were done to provide empirical evidence for the ACE inhibition by the peptide mixtures so obtained.

## 6.1 MATERIALS AND METHODS

### 6.1.1 Materials

Substrate- Hippuryl-L-histidyl-L-leucine (Hip-His-Leu, HHL) and ACE (EC 3.4.15.1), purified from pig lung were from Sigma Chemical Company (St. Louis, MO, USA). Chemicals and reagents used in RP-HPLC were of HPLC grade. All other chemicals and reagents were of analytical grade obtainable from the chemical store of Southern Yangtze University

### 6.1.2 Methods

#### *6.1.2.1 Relationship between DH of Casein and Inhibition to ACE by Cn-NPP Peptides*

The hydrolysates from the hydrolysis stage as outlined in Section 2.1.1.2 (page 27 above) corresponding to degree of hydrolysis (DH) 10, 15 and 20% were dissolved in 100mL of 200mM borate buffer (containing 300mM NaCl, pH 8.3). The solutions were then centrifuged at 8000 rpm for 30 minutes and the supernatant was skimmed off for use in ACE inhibition studies.

For the direct RP-HPLC assay of ACE activity a total of 75 $\mu$ L reaction volume was used. It was made up of 10 $\mu$ L buffer, 10 $\mu$ L peptide solution, 40 $\mu$ L of 6.5mol/L HHL and 15 $\mu$ L ACE. The peptide solution and HHL substrate were combined in a micro-centrifuge polythene tubes, vortexed and pre-equilibrated for 10 minutes at 37°C in a constant temperature water bath. ACE was then added and immediately vortexed, followed by incubation at 37°C for a further 30 minutes- with vortexing every ten minutes, at the end of which the enzyme reaction was arrested by addition of 85 $\mu$ L 1mol/L HCl. The reaction mixture was then directly analyzed for ACE inhibition with a blank containing 10 $\mu$ L buffer in place of the peptide solution. The RP-HPLC conditions were done after Wu *et al*, (2002) as follows:

Model: Waters 2690 Separation Module

Detector: Waters 996 Photodiode Array Detector allocating 200 – 770nm

Column: Lichrospher C<sub>18</sub>, 5 $\mu$ m 2.6 x 250mm

Injection Volume: 10 $\mu$ l

Flow rate: 0.3ml/min

Detection: 228nm

Column Temperature: 20°C

Mobile phase: Solution A: 90% Acetonitrile + 10% water + 0.1% formic acid

Solution B: 100% Water + 0.1% formic acid

Solution C: 100% Acetonitrile

Gradient: The column was calibrated with 10% A + 90% B.

10% - 66% A: 90% - 34% B from 0- 20 minutes, followed by 90% A in 1 minute and finally 100% C in 1 min followed by calibration.

The inhibition is related to the elution peak geometry of Hippuric acid (HA) liberated by the action of ACE using the relationship

$$\text{ACE Inhibition (\%)} = \frac{P_B - P_S}{P_B} \times 100 \%$$

Where  $P_B$  is the peak area of blank

$P_S$  is the peak area of the reaction mixture with peptide solution.

To calibrate for ACE inhibition, peptide solutions of concentration 0.1-3.5mg/mL were analyzed for inhibition. A plot of inhibition (%) against concentrations was generated and regression analysis performed using Microsoft Excel software (Microsoft Corporation, USA).  $IC_{50}$  was defined as the mass concentration of the peptide mixtures to inhibit 50% ACE activity calculated from regression analysis of the ACE inhibition versus peptide concentrations the curve.

#### *6.1.2.2 Relationship between Relative Molecular Weight of Cn-NPP and Inhibition to ACE*

The fractions obtained from gel filtration chromatography section were handled as outlined in section 6.1.2.1 above and the ACE inhibition by each fraction determined.

#### *6.1.2.3 Relationship between Anion Exchanged Cn-NPP Peptides and Inhibition to ACE*

The fractions desorbed from the anion exchange resin as outlined under methods in section 5.1.2.3 and 5.1.2.4 were characterized for ACE inhibition according to the scheme outlined in section 6.1.2.1 above. Only peptide from 0.02mol/L sodium phosphate buffer of pH 7.5 was characterized since the amount was fairly higher compared with the Cn-NPP fractions lyophilized from the elutions at 0.02mol/L sodium acetate buffer of pH values 5.5 and 6.5.

#### *6.1.2.4 Statistical Analysis*

The peptide concentrations were determined in duplicate and the mean values used in calculations. The values were tested for significance with the Student's *t* test ( $p < 0.05$ ). The elution profiles recorded on the potentiometer recorder were transformed and regenerated using Microsoft Excel software (Microsoft Corporation, USA).

## 6.2 RESULTS AND DISCUSSIONS

The summary of the IC<sub>50</sub> values for respective DH values are presented in Table 6.1, while Table 6.2 presents the IC<sub>50</sub> for the elution fractions from SEC eluted with 2% alcohol. Fig 6.1 is the typical elution profile for ACE assay using gradient elution in RP-HPLC while Fig 6.2 is the structure of Hippuric acid.

### 6.2.1 Relationship between DH of Casein and Inhibition to ACE by Cn-NPP

Table 6.1 Relationships between DH (%) and ACE Inhibition

DH (%)	10	15	20
ACE Inhibition IC <sub>50</sub> (mg/mL)	1.87	0.90	0.7

It is apparent from Table 6.1 that there was a concomitant increase in ACE inhibition with increasing DH. It is possible to relate the trend to the release of shorter, more hydrophobic peptides (Mahmoud *et al*, 1992) with increasing DH. These peptides are more potent in inhibition towards ACE as implied by Meisel (1997) who suggests that the hydrophobic residues tryptophan, proline and tyrosine as well as arginine are responsible for ACE inhibition when present at the C-terminal of the peptides. Furthermore, subtilisins of which Alcalase 2.4L is a member, reportedly hydrolyze both basic and neutral ester substrates with higher activity towards esters of aromatic amino acids than those of aliphatic ones (Blackburn, 1975) and this may lead to the release of the hydrophobic peptides with terminal aromatic amino acid residues.

The DH 15% was chosen for further processing based on these results because there was little difference in ACE inhibition from DH 20%, although the hydrolysis regimes in terms of both time and energy costs are enormous. The difference in ACE inhibition largely demonstrates a relationship to DH and agrees well with the reported works (van der Ven, 2002), and there is need to optimize the ACE inhibition vis-à-vis DH. Indeed our results suggest that extensive hydrolysis, resulting in the formation of many low molecular weight peptides results in high ACE inhibition. Indeed this would be desirable owing to the physiological significance of shorter peptides that are absorbed at enteral brush-border regions (Hara *et al*, 1984) and the reported resistance of the same to further degradation by gut enzymes (Nakamura *et al*, 1995) post-prandially or when released during digestion.

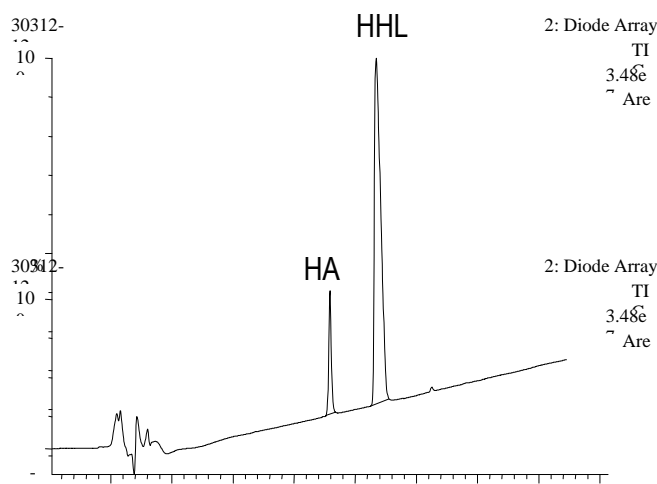


Fig 6. 1 A Typical RP-HPLC elution profile for ACE Inhibition Assay showing the substrate (HHL) and Hippuric acid (HA) eluting at 11.30 and 11.47 minutes respectively with complete (2minutes) baseline separation achieved. The ACE reaction mixture was analyzed with a gradient elution as in section 6.1.2.1 above without extraction of the enzyme activity product. The total run time in RP-HPLC was 40 minutes.

#### *6.2.2 Relationship between Relative Molecular Weight of Cn-NPP and Inhibition to ACE*

The results tabulated in Table 6.2 strongly confirm the assertion that ACE inhibition is favoured by shorter peptides, although longer peptides have more potent inhibition sometimes (Maruyama *et al*, 1987a; 1987b). These characteristics also underscore the diversity of ACE inhibitory peptides with respect to size and should encourage conservation of peptides of whatever length since they are either precursors of shorter peptides or exert the physiological effects themselves. There was a steady increase in ACE inhibition with decreasing relative molecular weight when fractions from Sephadex G-15 were analyzed for ACE inhibition. This is obviously attributable to the separation into various groups with characteristic average molecular weights. Again the lower molecular weight peptide groups are more potent for ACE inhibition.

This result agrees yet again with some published works (van der Ven, 2002) and it would be desirable to confirm the antihypertensive effects of the peptide groups *in vivo* so as to further elucidate the relationship between ACE inhibition and antihypertension on the one hand and peptide length/molecular weight, on the other, in a bid to optimize the same. Furthermore, the modernized method of assaying for ACE activity eliminates interference from the substrate, which also absorbs strongly at 228nm just like hippuric acid (Fig 6.2). Wu *et al* (2002) showed that extraction of hippuric acid by use of a detergent prior to spectrophotometric method gave values that were exaggerated by contamination with the substrate when the extract was subjected to RP-HPLC. This is eliminated by the use of RP-HPLC analysis of the enzyme reaction mixture without extraction with good baseline separation of the HHL and HA peaks. The peak geometry in our determination showed good baseline separation to totally eliminate detection of the substrate peak in the hippuric acid peak (Fig 6.1)

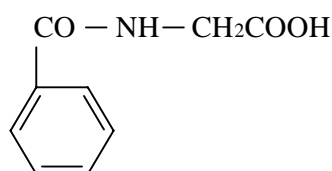


Fig 6. 2 Hippuric Acid;  $\lambda_{\text{max}} = 228\text{nm}$

Table 6.2 Inhibition Characteristics of Cn-NPP Fractions Obtained from Sephadex G-15 Column (Fig 4.1f, page 51) and IEX fractions

	<sup>a</sup> IC <sub>50</sub> (mg/mL)
Blank	-
Peak 1	1.357
Peak 2	0.841
Peak 3	0.5
Anion Exchanged Peak 3	1.17
Anion exchanged Cn-NPP	0.6

<sup>a</sup>IC<sub>50</sub> is the peptide concentration (mg/mL) to attain 50% inhibition.

### 6.2.3 Relationship between Anion Exchanged Cn-NPP Peptides and Inhibition to ACE

The peptides adsorbed on anion exchange resin at 0.02mol/L Na-phosphate buffer pH 7.5 and obtained following isocratic desorption with 1.5mol/L NaCl and desalted on MAR were characterized for ACE inhibition. The fraction showed high inhibition with an IC<sub>50</sub> value of 0.6mg/mL (Table 6.2). The peptides were not characterized further for molecular weight distribution although it would be desirable to estimate the molecular weight. However, the ion exchange did fractionate peptides that showed some ACE inhibition and this could be combined with the other chromatographic techniques to further resolve the purity if need be.

### 6.2.4 Characterization of Cn-NPP Peak 3 from Sephadex G-15 fractions for Inhibition to ACE after IEX

The peak 3 from Sephadex G-15 (Fig 4.1f, page 51) following anion exchange resin (section 5.1.2.3 page 58) was characterized for ACE inhibition. The adsorbed peptides showed good ACE inhibition with an IC<sub>50</sub> value of 1.17mg/mL (Table 6.2). It is possible that purification with anion exchange resin resulted in pruning off of more potent peptides (Yust *et al*, 2003), which were not characterized because we were interested in the peptides adsorbed and desorbed from the anion exchanger.

### 6.3 CONCLUSIONS.

Peptides soluble in 70% alcohol concentration are highly hydrophobic and the hydrophobicity of the Cn-NPP may be implicated in potent ACE inhibition. Cn hydrolysates showed diverse ACE inhibition properties irrespective of the purification protocols employed and the inhibition varied according to molecular weight and possibly pI values as may be deduced from the SEC and IEX fractions. Furthermore, DH affected inhibition of ACE in more or less the same way as molecular weight does, the higher the DH (shorter, more peptides) the higher the inhibition and vice versa. Cn hydrolysis by Alcalase 2.4L liberates peptides with varying characteristics; which inhibit ACE potently and diversely. Alcalase 2.4L may therefore be employed in industrial production of non-phosphorylated peptides with ACE inhibitory properties from Cn. Furthermore, this study underscored the relevance of RP-HPLC in day to day operations in the laboratory and more improvements on the assay procedures are called for. Additionally, the study did point to the fact that gradient elution is superior to isocratic elution when assaying for ACE activity.

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## GENERAL CONCLUSIONS AND RECOMMENDATIONS

### GENERAL CONCLUSIONS

The present investigation reported the ACE inhibition properties of Cn-NPP obtained from Cn following hydrolysis with Alcalase 2.4L, a subtilisin. A close monitoring of the hydrolysis process, evaluation of desalting and gel-filtration procedure as well as ion exchange on the ACE inhibition potentiation of the Cn-NPP led to the following conclusions:

1. The Hydrolysis of Cn by Alcalase 2.4L liberates bioactive CPP as well as Cn-NPP with ACE inhibitors of varying inhibitory potential. It would be possible to optimize the degree of hydrolysis (DH) to achieve high ACE inhibition by Cn-NPP by varying the DH. Cn-NPP was shown to have an invaluable pool of essential amino acids.
2. Monitoring of DH with the pH-stat method during casein hydrolysis and desalting of Cn-NPP following thereafter could be achieved completely by use of macroporous adsorption resins (MAR). It is possible to obtain a controlled desalting process based on the principles of hydrophobic interaction chromatography (HIC) whence the Cn-NPP is adsorbed with little if any salts being adsorbed, while the adsorbed peptides may be eluted with alcohol of varying concentrations.
3. The SEC chromatography of Cn-NPP is nonideal, being affected by buffer pH and ionic concentration. With proper control of these variables, good delineation of Cn-NPP elution peaks could be obtained for satisfactory fractionation. Alcohol as used to elute the peptides attenuates the hydrophobic interaction amongst peptides and between peptides and the matrix with good separation.
4. The RP-HPLC characterization of peptides for ACE inhibition was rapid and economical, requiring only  $\mu\text{L}$  amount of peptide solutions besides being reproducible. It is not to say, however, that is inexpensive.
5. Cn-NPP has potential for application in the functional food and nutraceutical industry, as well as in the pharmaceutical industry following further purification. However, grappling with bitterness is a reality that must be resolved if objectionable properties are to be reduced.
6. Finally, if the procedure adopted in this study may be improved upon and scaled up to industrial level it may provide potential for small scale industries that could use cheaper desalting and purification resins to achieve a competitively priced product which should help to place nutraceuticals on the dinner tables of average consumers as well as being presented in functional foods with various formulations.

## RECOMMENDATIONS

1. The present work has revealed that Cn-NPP has biological activity, with ACE inhibition as the determined desirable physiological effect. This study has laid a foundation for further works on the applications of Cn-NPP in functional food formulations, which should help reduce wastage and effluent from CPP manufacture since it provides for a means of tapping the potential of the same. It is hoped that some people will be spurred to venture into the formulation of the same into functional foods, nutraceuticals and pharma-drugs.
2. Further works are required to empirically demonstrate *in vivo* antihypertensive effects of the ACE inhibitory peptide mixtures. Furthermore, the requirement for comprehensive animal studies should be inspired to demonstrate other bioactivities in the Cn-NPP.
3. Further work is required to tackle the probable bitterness of the peptide mixtures in the Cn-NPP so as to reduce the objectionable nature of bitter peptides.
4. Cost effective means of production requires model studies so as to evaluate the viability of the projects that will emanate from Cn-NPP and associated industrialization.

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3. Cheison S.C., Z. Li, Z. Wang and S-Y Xu. Relationship between degree of hydrolysis and ACE inhibition by Casein non-phosphorylated peptides following hydrolysis by Alcalase 2.4L. '*Journal of Food Science and Technology*', (in Chinese). Status. Under Review