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**PRODUCTION OF ENZYME-MODIFIED CHEESE AND
BIOACTIVE PEPTIDES BY *LACTOBACILLUS* AND
COMMERCIAL ENZYMES**

by

Seble Sereke Berhan Haileselassie

**Department of Food Science and Agricultural Chemistry
Macdonald Campus of McGill University
Montreal, Quebec, Canada**

**A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Masters of Science**

February, 1999

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Suggested short title:

PRODUCTION OF ENZYME-MODIFIED CHEESE

Dedication

To my parents Sereke Berhan and Work Abeba and my husband Mekonnen.

ABSTRACT

To optimize conditions to prepare a good quality of enzyme-modified cheese (EMC), EMC samples were prepared by using combinations of Neutrase®/*Lactobacillus casei* enzymes, Neutrase®/Debitrase™, Neutrase®/Flavorzyme™ and Neutrase®/Palatase® M. Based on the results obtained from sensory and RP-HPLC analysis, the optimal combinations to prepare a good quality of EMC were found to be: Neutrase® with (I) *L. casei* enzymes (aminopeptidase activity 86.4 LAPU/g and esterase activity 110.0 U/g), (II) Debitrase™ (aminopeptidase activity 22.0 LAPU/g), (III) Flavorzyme™ (aminopeptidase activity 6.5 LAPU/g), and (IV) Palatase® M (lipase activity 200 LU/g).

The water-soluble fractions of EMCs prepared with different enzyme combinations were subjected to RP-HPLC on a Delta Pack C₁₈ column, and selected peaks were purified on the same column using a binary gradient. One peak from Neutrase® digest, five peaks from Neutrase®/Debitrase™ digest, and two peaks from Neutrase®/*L. casei* enzyme digest were purified and identified by API mass spectrometry. All the purified peptides contained active sites within their sequences.

The volatile compounds in a series of EMCs prepared by *L. casei* and commercial enzymes as well as Cheddar cheese (mild, old, extra old) were also identified by using Pyrolysis/GC/MS and dynamic headspace techniques. Overall, 5 ketones, 8 fatty acids, 3 alcohols and 2 aldehydes were detected in most of the samples using Py/GC/MS. Propanoic, hexanoic, octanoic, decanoic, dodecanoic and tetradecanoic acids were found to be the major fatty acids present in EMC prepared by *L. casei* enzymes. Dynamic headspace analysis revealed the presence of 17 compounds including fatty acids, ketones, alcohols, aldehydes, and hydrocarbon in most of the sample analyzed. The flavor of EMC seems to depend not on any particular key component, but rather on a critical balance of all components present.

RÉSUMÉ

Dans le but d'optimiser les conditions de production d'un fromage modifié enzymatiquement (FME) de bonne qualité, des échantillons de FME ont été préparés en utilisant les combinaisons de protéase et aminopeptidase/estérase suivantes: Neutrase®/enzymes de *L. casei*, Neutrase®/Debitrase™, Neutrase®/Flavorzyme™, et enfin Neutrase®/Palatase®. Sur la base des résultats obtenus lors d'analyses sensorielles et de chromatographie par HPLC, les conditions optimales pour la préparation d'un FME de Cheddar frais sont les suivantes: Neutrase® suivi soit de (I) enzymes de *L. casei* (activité aminopeptidasique 86.4 LAPU/g et activité estérasique 110.0 U/g), ou (II) Debitrase™ (activité aminopeptidasique 22.0 LAPU/g), ou (III) Flavorzyme® (activité aminopeptidasique 6.5 LAPU/g), ou encore (IV) Palatase® M (activité lipasique 200 LU/g).

Les fractions hydrosolubles de FME préparés avec différentes combinaisons enzymatiques ont été analysées sur RP-HPLC avec colonne Delta-Pak C₁₈, et certains pics sélectionnés ont été purifiés sur la même colonne par un gradient binaire. Ainsi, un pic d'un digestat de Neutrase®, cinq pics d'un digestat de Neutrase®/Debitrase™, et deux pics d'un digestat de Neutrase®/enzymes de *L. casei* ont été purifiés et identifiés par spectrométrie de masse API. Tous ces pics contenaient des sites actifs dans leurs séquences.

Lors d'une autre étape, les composés volatils présents dans une série de Cheddars (doux, mi-fort, extra-fort) ainsi que dans une série de FME préparés par enzymes de *L. casei* et enzymes commerciaux, ont été identifiés via Pyrolyse/GC/MS et analyse en *dynamic headspace*. Au total, 5 cétones, 8 acides gras, 3 alcools et 2 aldéhydes ont été détectés dans la plupart des échantillons. Les acides propanoïque, hexanoïque, octanoïque, decanoïque, dodecanoïque et tétradecanoïque ont principalement été retrouvés dans les FME préparés avec les enzymes de *L. casei*. L'analyse *en dynamic headspace* a révélé la présence de 17 composés incluant acides gras, cétones, alcools, aldéhydes, dans la plupart des échantillons. Dès lors, la saveur des FME semble dépendre

non pas d'un composé spécifique, mais plutôt d'un équilibre particulier entre tous les composés présents.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
α -La	α -lactalbumin
BAP	Bioactive peptides
BCA	Bicinchoninic acid
β -Lg	β -lactoglobulin
β -CM	β -casomorphin
CPP	Caseinophosphopeptides
EMC	Enzyme-modified cheese
FFA	Free fatty acids
FID	Flame ionization detector
FPLC	Fast protein liquid chromatography
GI	Gastrointestinal
GC	Gas chromatography
HPLC	High performance liquid chromatography
LAB	Lactic acid bacteria
MWCO	Molecular weight cutoff
PBL	Peripheral blood lymphocytes
SHR	Spontaneously hypertensive rats
SFE	Super fluid extraction
SPME	Solid phase microextraction
TFA	Trifluoroacetic acid
WSF	Water-soluble fractions

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FOREWORD

This thesis is presented in the form of original papers suitable for journal publications. The introduction presents the rationale and objectives of the study, followed by Chapter 1, a general literature review of the background information on the subject of the thesis. Chapters 2 to 4 contain the integral part of papers to be published in appropriate journals, and the last section is a general conclusion. This thesis format has been approved by the Faculty of Graduate Studies and Research, McGill University, and follows the conditions stated in the guidelines concerning thesis preparation, Section C.

“Candidates have the option of including as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts providing logical bridges between the different papers are mandatory. The thesis must still conform to all other requirements of the “Guidelines for Thesis Preparation”. The thesis must include: (a) a table of contents, (b) a general abstract in English and French, (c) an introduction which clearly states the rationale and objectives of the research, (d) a comprehensive review of the literature, and (f) a final conclusion.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of examiners is made more difficult in these cases, it is in candidate’s interest to make perfectly clear the responsibilities of the different authors of co-authored papers.”

Although all the work reported in this thesis is the responsibility of the candidate, the project was supervised by Dr. Byong H. Lee, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

CONTRIBUTION OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATIONS

Dr. Byong H. Lee, my supervisor, is co-author on all publications presented in this thesis and contributed in a supervisory role. Dr. Lee fully reviewed the manuscripts. This work was supported by NSERC strategic grant awarded to Dr. Lee.

Mr. Bernard F. Gibbs carried out analysis of bioactive peptides on the mass spectrometer. He is co-author on one of my publication.

Dr. Anahita Keyhani and Mr. Brian Stewart carried out analysis of flavor compounds on the GC/MS.

A. Part of this work has been accepted for publication.

- **Haileselassie, S. S., Gibbs, B. F. and Lee, B. H. (1998). Purification and identification of potentially bioactive peptides from enzyme-modified cheese. J Dairy Sci. (In press).**

B. Part of this work has been prepared for publications

- **Haileselassie, S. S. and Lee, B. H. (1999). Milk protein-derived bioactive peptides. CRC Rev. Food Sci. Technol.**
- **Haileselassie, S. S., Yaylayan, V. A., and Lee, B. H. (1999). Analysis of volatile flavor compounds in enzyme-modified cheese and Cheddar cheese by using different methods. J. Agri. Food Chem.**

C. Part of this work has been presented at scientific meetings

- **Haileselassie, S. S., Gibbs, B. F., and Lee, B. H. (1998). Purification and identification of potentially bioactive peptides from enzyme-modified cheese. IFT Annual Meeting, Atlanta-oral.**

- **Haileselassie, S. S. and Lee, B. H. (1997).** Production of enzyme-modified cheese and bioactive peptides by *Lactobacillus* and commercial enzymes, 40th CIFST Conference, Montreal-poster.
- **Haileselassie, S. S., Yaylayan, V. A., and Lee, B. H. (1997).** Identification of volatile flavor components in enzyme-modified cheese produced by *Lactobacillus* and commercial enzymes, 92nd ADSA Annual Meeting, Guelph-oral.
- **Haileselassie, S. S. and Lee, B. H. (1997).** Optimization of conditions to prepare a good quality of enzyme-modified cheese by *Lactobacillus* and commercial enzymes, IFT Annual Meeting, Orlando-poster.

GENERAL INTRODUCTION

Each year, about 2.6 billion lb and 58.8 million lb of Cheddar cheese is manufactured in the United States (USDA, 1998) and in Canada (The Dairy Review, 1998), respectively. Cheese is widely used by a consumer in many forms. For instance, cheese itself is consumed as an end product, but also high-intensity cheese concentrate may be in a dried form or contained in convenience products. The production of matured Cheddar cheese, however, represents considerable costs of cheese industry. Up to 2 yr may be needed to produce extramature varieties, and suitable storage facilities cost between \$1 to \$2 metric ton per day (Fox, 1988-1989). Therefore, any development leading to accelerated cheese ripening and production of enzyme-modified cheese (EMC) would represent considerable savings to the manufacturer. Also due to a consumer demand for a wider choice of processed and low-fat products that possess cheese flavor, there is a great need in developing such products. The basis of EMC technology is the use of specific enzymes such as proteinases, peptidases, lipases, and esterases acting at optimum conditions to produce typical cheese flavors from suitable substrates. A wide ranges of these enzymes are now commercially available from mainly microbial and animal sources. Studies on the enzymatic activities of different strains of lactobacilli also demonstrated that these strains possess strong peptidase and esterase activities. Consequently the crude enzyme extract from *Lactobacillus casei* species was successfully used in acceleration of Cheddar cheese ripening and production of EMC with intense cheese flavor (Trepanier *et al.* 1992 a, b; Park *et al.* 1995).

High-intensity cheese flavor concentrates, such as EMC, cheese powder and cheese flavor are the main alternatives to the use of natural cheese in convenience foods requiring a cheese flavor. The flavor and quality of the product that possess cheese flavor depend on the amount and type of cheese used. The use of natural cheese, however, can result in insufficient flavor strength, increased lactose/fat levels, and higher production costs. Presently, the most common method for producing economic and consistent cheese flavor is through EMC production. The principal difference between a mature cheese and an EMC is that the body and texture are not factors in the quality of the latter. EMCs,

however, contain almost all flavor compounds that are produced during ripening of natural cheese, but their relative concentrations may vary, depending on the conditions used to manufacture the product. EMCs are generally used in processed foods to intensify an existing cheesy taste or to give a specific cheese note to a more bland-tasting cheese product.

Apart from that EMC process which is mainly designed to produce cost-effective cheese flavor ingredients may simultaneously produce nutraceuticals such as bioactive peptides, which are considered to be of physiological importance. Due to the growth of health conscious consumers demanding natural products rather than chemically synthesized ones, nutraceuticals and functional foods have been gaining popularity since the mid 90s. In fact, the total market for functional foods reached \$171.1 million in 1997 and is expected to reach \$314.1 million in 2002 (Giese and Katz, 1997). Japan is the world largest nutraceutical market, a total nutraceutical market is estimated to reach \$200 billion by the year 2005 (Japan's Nutraceuticals, 1998). Currently nutraceuticals constitute \$4 billion market in Japan, and play a major role in that country's health revolution (Food Labeling News, 1994). It is predicted that food and pharmaceutical companies' nutraceutical products will boom in the North America market, mainly because of demographic and health care-trends. Nutraceuticals are defined as any substance that is a food or food ingredient which provides medical or health benefits, including prevention and treatment of disease (Mullally *et al.* 1996). Milk-derived bioactive peptides are known to have opioid, angiotensin converting enzyme (ACE) inhibitory, immunostimulating, antimicrobial, and antithrombotic effect. However, their physiological significance as exogenous regulatory substances is not yet fully understood. Bioactive peptides have been identified from enzymatic digest of several food proteins, but no information is available on the identification of bioactive peptides from EMC.

The objective of this study were:

- (i) to investigate optimum conditions to prepare commercially viable EMC by *Lactobacillus* and commercial enzymes and to asses the effect of different treatments on the rate of flavor formation;
- (ii) to purify and identify bioactive peptides from EMC prepared by *Lactobacillus* and commercial enzymes;
- (iii) to identify volatile flavor compounds from EMC prepared by *Lactobacillus* and commercial enzymes and their impact on the overall EMC flavor

CHAPTER 1.0

LITERATURE REVIEW

ENZYME-MODIFIED CHEESE (Section 1.1) and MILK PROTEIN DERIVED BIOACTIVE PEPTIDES (Section 1.2)

This chapter was summarized in the form of publication suitable for journal publication. The manuscript entitled "Enzyme-modified cheese and milk protein-derived bioactive peptides" was co-authored by Seble S. Haileselassie and Byong H. Lee. This paper was written by Seble S. Haileselassie and supervised by Dr. Byong H. Lee, who acted in an editorial capacity, evaluating the manuscript. This chapter serves as an introduction to the thesis, showing the background for the research that was done and the reasons why this project was significant. One manuscript will be submitted to CRC Rev. Food Sci. Technol.

1.1.1 ABSTRACT

Consumer demand for novel nutritious convenience foods with a cheese flavor is driving the development of customized cheese flavor products, which has highlighted the requirement for further research into cheese flavor pathways and the identification of specific cheese flavor compounds. The mechanism of flavor development in enzyme-modified cheese may be related to the curing of cheese. Although many of the mechanisms for flavor development in cheese are not well understood, carbohydrates, proteins and fat undergo enzymatic degradation during cheese ripening and these reactions are important in the development of flavor in cheese and enzyme-modified cheese. A review on EMC describes the formation of cheese flavor and the use of different enzymes to effect proteolysis and lipolysis in cheese in order to produce high-intensity cheese flavor concentrates without bitterness.

Key words: EMC, Flavor, enzyme, proteolysis, lipolysis, bitterness

1.1.2 ENZYME-MODIFIED CHEESE

Cheese that has been treated enzymatically to enhance its flavor or a significant portion of its flavor profile is considered to be enzyme-modified cheese (EMC) and it provides the food manufacturer with a strong cheese note in a form that is cost effective, nutritious and natural (Moskowitz and Noelck, 1987). The biggest market for EMCs is the USA where much of the cheese eaten is of the processed type and there is a very large snack industry (West, 1996). EMCs are added in products such as cheese analogues, cheese spreads, salad dressings, dips, soups, sauces, snacks, biscuits, pizza toppings, frozen foods, microwave meals, ready-made meals, canned foods, crackers, cake mixes, fillings, pasta products, quiches, gratins, low-fat and no-fat cheese products and cheese substitutes/imitations (West, 1996). The main advantages of adding small quantities (0.1 - 2 % W/W) of EMCs to food products are to (i) boost an intensive cheese flavor and (ii) reduce product costs by as much as 40-80 % (Kilcawley *et al.* 1998). Talbott and McCord (1981)¹ described a guide to determine the dosage of EMC for replacement of natural cheese as percentage of natural cheese in the product multiplied by the desired replacement percentage and divided by the flavor intensity of the EMC. Certain high-intensity products can replace up to 50 % of the cheese used in some applications (Buhler, 1996). The addition of EMCs to food products creates the desired flavor without an increase in fat content; the addition of EMC at levels of 0.1 % for instance, contributes less than 0.07 % fat (2.28 calories) per 100 g (Buhler, 1996). The flavor profile of EMCs may be quite different than that of a natural cheese and yet on dilution with a suitable bland or nearly bland base, provide the desired cheesy note in the final product. EMCs have approximately 15-30 times the flavor intensity of natural cheese and are available as pastes or spray-dried powders (Moskovitz and Noelck, 1987 ; Freund, 1995).

Production of EMCs is an important industrial activity, which has been increasing due to a continual demand of food manufacturers for cheese flavor with improved

¹ Dosage of EMC for replacement = $\frac{\% \text{ of natural cheese in the product} \times \text{desired replacement \%}}{\text{flavor intensity of EMC}}$

technical, flavoring and organoleptic properties for use in convenience foods (Kilcawley *et al.* 1998). EMCs are ideal in frozen cheese-type products as the proteins from natural cheese tend to coagulate and produce a grainy texture; since the proteins in EMCs have been hydrolyzed to more soluble peptides and amino acids, thus these problems are overcome (Missel, 1996). Other important parameters are improved functionality, batch to batch consistency, reduced storage space and ease of handling. Production of EMCs also results in increase in production capacity and product stability (Kilcawley *et al.* 1998). EMC flavors available include Cheddar, Mozzarella, Romano, Provolone, Feta, Parmesan, Blue, Gouda, Swiss, Emmental, Gruyere, Colby and Brick.

1.1.2.1 Formation of cheese flavor

Cheese flavor develops as a result of proteolysis, lipolysis and glycolysis, the extent of which varies according to the cheese variety (Fox 1989, 1993; Wilkinson, 1993). Proteolysis is probably the most important biochemical event during the ripening of most cheese varieties. It is responsible for the development of the desired texture and has a direct contribution to aroma and background flavor intensity in most cheese varieties and facilitates the availability of flavor precursors in all matured cheese varieties (Fox, 1988-1989; 1989; Fox *et al.* 1993, Fox *et al.* 1996). It contributes to cheese ripening in at least four ways: (1) directly via amino acids and peptides, and indirectly via catabolism of amino acids; (2) by a release of sapid compounds during mastication; (3) by a change in pH via the formation of NH_3 ; (4) by a change in texture arising from breakdown of the protein network. The main proteolytic agents in cheese are: (1) indigenous milk proteinases, especially plasmin, (2) chymosin or rennet substitutes, (3) starter proteinases and peptidases released from lysed cells, (4) enzymes from non-starter bacteria and (5) proteinases and peptidases from secondary micro-organisms added to certain varieties of cheese (Fox, 1981, 1989; Gripon *et al.* 1991; Fox *et al.* 1993).

Products of proteolysis, small peptides and amino acids are found in the water-soluble fraction (WSF) of cheese. The water-soluble fraction of cheese contributes significantly to the intensity of cheese flavor (McGugan *et al.* 1979). It contains both

non-volatile and volatile compounds (Cliffe *et al.* 1993; Engles and Visser, 1994; Aston and Creamer, 1986). Fraction studies of WSF from several types of cheese revealed that cheese flavor components are of low molecular weight (< 500 Da) (Engles and Visser 1996). WSF contains mainly free amino acids and small peptides, which have considerable contribution to the basic flavor of cheese (e.g. savory, brothy and bitter) (Engels and Visser, 1994). However, since the flavor of cheeses such as Cheddar and Gouda results not only from peptides and amino acids, other ripening products such as degradation products of free amino acids and fatty acids formed via enzymatic/or chemical pathways seem essential for the actual cheese flavors (Schrodter, 1990; Crow *et al.* 1993; Visser, 1993; Urbach, 1993; Engels and Visser, 1994).

Lipolysis refers to the hydrolysis of triglycerides, diglycerides, and monoglycerides to yield FFAs and flavor precursors by lipolytic enzymes (Fox, 1981; Aston and Creamer, 1986; Birschbach, 1994). The degree of contribution of lipolysis to cheese flavor varies considerably between varieties (Fox, 1993). Lipolysis is a major contributor, directly or indirectly in flavor development in strong-flavored cheeses such as hard Italian and Blue cheese varieties. About 20% of the fat may be hydrolyzed to medium and long chain fatty acids, between C_8 and C_{14} which are oxidized to methyl ketones and in turn are reduced to secondary alcohols (Moskowitz and Noelck, 1987). Based on the results of a study on the contribution of lipolysis in other cheese varieties, Moskowitz and Noelck (1987) concluded that for some EMC types, the flavor profile or intensity was proportional to the degree of lipolysis and release of low molecular weight FFAs, as with Romano and Provolone-type EMCs. However, for Cheddar and Swiss EMCs, the authors found that even though the relative ratios of FFA were similar, yet one tasted like Cheddar and the other like Swiss. Therefore, it would appear that the relative proportions of FFAs are critical for Roman and Provolone EMC flavor but not for Cheddar and Swiss EMCs.

Glycolysis refers to the conversion of lactose to lactic acid by the action of starter bacteria. Lactic acid makes a major contribution to the flavor of acid-coagulated cheeses (Fox and Wallace, 1997). Fresh cheese curd contains 0.8 to 1.5 % lactose, which is

fermented, mainly to L-lactic acid, in all cheese varieties to give a pH of ~5.0 (Fox *et al.* 1996). Lactose is usually completely fermented within at most, a few weeks; however, a high level of salt may cause its incomplete fermentation (Thomas and Pearce, 1981). The complete and rapid metabolism of residual lactose and its component monosaccharides is essential for the production of good quality cheese (Fox, 1988-1989; Fox *et al.* 1993). A number of known flavor components such as diacetyl, acetic and propionic acid are thought to be produced, at least in part, from carbohydrates (Moskovitz and La Belle, 1981).

1.1.2.2 Production of EMC

Production of EMC involves incubating mature or immature cheese with specific exogenous enzymes (proteinases, peptidases, lipases and esterases) and/or microorganisms in slurry system, terminating the process by pasteurization and standardizing the final product to a desired flavor intensity. Two approaches can be used for the production of EMC: a one step process where the fat and protein hydrolysis occur simultaneously and a component approach where several different flavor components are created and then blended in a desired ratio (Kilcawley *et al.* 1998). The component approach is very flexible and allows a very wide variety of flavors to be produced. In addition, each enzyme process can be run under optimum conditions using the most appropriate substrate, pH, and temperature (West, 1996). Different procedures for the production of EMC are used, depending on the EMC type, manufacturer's preference, product application and appearance of the end product. Several authors described various industrial processes for the production of EMC and there are excellent reviews on technology and production of EMCs by Moskovitz and Noelck (1987) and Kilacwely *et al.* 1998. A commercial procedure developed by Imperial Biotechnology for the production of EMC is outlined in Fig. 1.1.

In general, most EMC types are produced from cheese pastes made from an immature cheese of the same type to give the most authentic flavor (Kilcawley *et al.* 1998). Flavor enhancers, such as monosodium glutamate, yeast extract, diacetyl and

some compounds associated with specific cheese flavor may also be added, although some may have to be declared on the labeling of the final product into which EMC is added (Anon, 1993; West, 1996). Apart from the consistency and the quality of the initial substrate, process parameters such as time and temperature are also critical in obtaining a consistent product (Vafiadis, 1996). Care must be taken not to destroy the developed flavor by over-cooking the final product during inactivation of the enzyme by heat treatment. The major problems associated with the production of EMC are bitterness, which is due to the action of proteolytic enzymes on casein and microbial spoilage, as optimum conditions exist for their growth. West (1996) demonstrated that microbial spoilage is a major problem in systems where lipase is not used, as high levels of short chain FFA tend to have bacteriostatic effect. However, inclusion of potassium sorbate has a major effect on controlling levels of contaminants, such as yeast and coliforms (Dulley, 1976). Other bacterial inhibitors such as nitrates, sorbic acid, and nisin are also commonly used (Mann, 1981).

1.1.2.3 Bitterness in EMC

Bitterness is a common defect in EMC and results from the production of bitter peptides by the action of proteolytic enzymes on casein (West, 1996). Limiting the protein hydrolysis can reduce bitterness. In many types of cheese flavor, however, protein breakdown is essential for the development of a full rounded flavor. Bitter peptides isolated from cheese originate mostly from α_{s1} -casein and β -casein proteins of a high average hydrophobicity (Q) (Gomez *et al.* 1997). According to Ney (1979), Q value can be calculated by dividing the sum of the amino acid chain hydrophobicities by the number of amino acid residues ($Q = \Sigma \Delta f / n$), where Q = average hydrophobicity of a peptide; Δf = hydrophobicity value of individual amino acid; and n = number of amino acid residue. A hydrophobicity value, Δf (free energy of transfer of the side chains of amino acids), exists for each amino acid, based upon its solubility properties (Table 1.1). Peptides with a Q value < 1300 cal/residue tend to be non-bitter and those with a Q > 1400 bitter, whereas no prediction can be made on the bitterness of peptides with a Q value in the range 1300-1400 (Ney, 1979).

Although many researchers have generally accepted the Q rule, the theory does not address the mechanism by which peptide structure imparts bitterness. Thus, several researchers have attempted to study the specific physicochemical properties of peptides that impart bitter taste. Study of various portions and analog of the sequence of the fragment H-Arg²⁰²-Gly-Pro-Ile-Ile-Val²⁰⁹-OH, revealed that for a peptide to exhibit strong bitterness at least six amino acids were necessary (Kanehisa, 1984). According to the results of this study, a basic amino acid at the N-terminal position and a hydrophobic amino acid at the C-terminal position significantly enhance bitterness. Ishibashi *et al.* (1987), in working with leucine containing peptides, found that the hydrophobicity of leucine residues markedly caused the bitterness of peptides, and the number of leucine residues in a peptide determined its bitterness. Ishibashi *et al.* (1988) studied the influence of side chain of amino acids on bitterness. As reported, for peptides to exhibit bitterness, the side chain skeleton of the amino acid should consist of at least 3 carbons. Peptides are bitter if hydrophobic amino acids (Trp, Ile, Tyr, Phe, and Leu) are at the C-terminus or if basic amino acids (Arg, Lys, and His) are at the N- terminus (Phelan *et al.* 1973; Ishibashi *et al.* 1987; and Ishibashi *et al.* 1988). Alder-Nissen (1988) proposed that the molar concentration and chain length of the most hydrophobic peptides also are important properties responsible for bitter sensation. In a more recent work, Lee *et al.* (1996) also confirmed that the average hydrophobicity of a peptide might be an indication of whether a peptide would be bitter but could not be the sole determinant factor.

1.1.2.4 Methods for controlling bitterness in EMC

The control of bitterness in EMC involves methods for decomposing bitter peptides by adding a correct balance of endopeptidase and exopeptidase enzymes. Endopeptidases cleave susceptible peptide bonds within the polypeptide chain, whereas exopeptidases break down bitter peptides by cleaving one or two amino acids from either the N-terminus (aminopeptidase) or the C-terminus (carboxypeptidase) (Alder-Nissen, 1993). The amino acids and small peptides produced play an important role in the

development of cheese flavor through further reactions that give rise to amines, thioesters, and other thiol compounds (Varnam and Sutherland, 1994). Proline-containing peptides at the N-terminal residue, however, are less susceptible to the action of general aminopeptidase and the broadly specific di- and tri-peptidases that remove most of the other amino acid residues (McDonald *et al.* 1969). Thus, proline-specific peptidases appear to degrade proline residue (Casey and Mayer, 1985 and Booth *et al.* 1990). Casey and Meyer (1985) were the first to report the presence of proline-specific peptidase from starter and non-starter lactic acid bacteria. Several research groups have further studied proline-specific peptidases from lactic acid bacteria. An aminopeptidase from *Lactobacillus casei* ssp. *casei* LLG that has also specificity for proline was purified and characterized by Arora and Lee (1992) and this enzyme is considered to play a significant role in degradation of bitter peptides during cheese ripening. Habibi-Najafi and Lee (1994) also studied proline-specific peptidases of two subspecies of *Lactobacillus casei*, ssp. *casei* LLG and *rhamnosus* S93, who concluded that these enzymes could be used for accelerating cheese ripening without bitterness. Proline-specific peptidases are composed of several exopeptidases and endopeptidases. Proline aminopeptidase or aminopeptidase P (E. C 3.4.11.9), imidopeptidase (prolidase; E. C 3.4.13.9), iminopeptidase (prolinase; E. C 3.4.13.8), proline iminopeptidase (E. C 3.4.11.5) and post- or x-prolyl dipeptidyl peptidase (E. C 3.4.14.5) have all been detected in various species of lactic acid bacteria (Mou *et al.* 1975; Hickey *et al.* 1983; Kaminogawa *et al.* 1984; Baankries and Exterkate, 1991).

Several enzymes from starter and non-starter microorganisms have been purified and their debittering effect of bitter peptides derived from α - and β -casein was studied. Park *et al.* (1995) found that addition of cell-free extract from *Lactobacillus casei* subsp. *casei* LLG with wide range of peptidolytic activities (aminopeptidase, x-prolyldipeptidyl peptidase and proline-iminopeptidase) resulted in debittering of EMC prepared from Cheddar by Neutrase (a neutral proteinase). Lee *et al.* (1996) also evaluated the effect of peptidases from *Lactococcus lactis* subsp. *cremoris* SK11 on bitter peptides extracted from Cheddar. In this study five bitter peptides, rich in proline, identified as α_1 -CN f(1-7), f(1-13), f(11-14), α_2 -CN f(191-197), and β -CN f(8-16) were degraded to less bitter or

non-bitter peptides and amino acids. Currently, commercial enzyme preparations obtained from *Lactococcus lactis* (e.g. Savorase™, Debitrase™ and Accelase™ from Imperial Biotech.) and *Aspergillus oryzae* (e.g. Flavorzyme™ from Novo Nordisk) are available on the market for debittering of protein hydrolysates.

1.1.3 CONCLUSION

Since consumer demand for healthier and natural products has created a demand for enzyme-produced flavors, the mechanism through which enzymes intervene in cheese flavor development has been under investigation from different perspectives. A number of key factors are involved in EMC production: (i) the flavor intensity required, (ii) quality of starting material, (iii) type and specificity of enzyme, cultures used, and their concentration, (iv) processing parameters (pH, temperature, agitation, aeration, and incubation time), and (v) use of processing aids (emulsifiers, bacteriocins, flavor enhancers and precursors). The flavor intensity required, raw material quality as well as processing time and temperature are the major factors for the dosage and selection of enzyme and/or starter culture for EMC production.

As more is learned about cheese flavor pathways and cheese flavor compounds, a myriad of cheese flavors can be produced through EMC technology. However, a detailed knowledge of the enzymatic reactions under the conditions used must be fully understood before production can be achieved on a consistent basis.

SECTION 1.2
MILK PROTEIN-DERIVED BIOACTIVE PEPTIDES

1.2.1 ABSTRACT

Milk proteins have always been regarded as foodstuff for adults and an essential nutriment for the neonate. However, during the last two decades, attention has been drawn to the functional significance of milk proteins i.e., caseins, α -lactoalbumin, β -lactoglobulin, and lactoferrin and their biologically active fragments apparently displaying a variety of effects. The physiological significance of bioactive peptides as exogenous regulatory substances is not yet fully understood. Nevertheless, several bioactive peptides derived from milk proteins have been shown to exert beneficial physiological effects. Milk-derived peptides are already produced on an industrial scale and have been considered for application both as dietary supplements in “functional foods” and as drugs. This paper reviews a functional significance of milk proteins as angiotensin converting enzyme (ACE) inhibitors, opioid receptor ligands, immunomodulators, antimicrobial agents, antithrombotic agents, and phosphopeptides. Biochemical properties, the mechanism of their formation, and biological activities and structure-activity relationship of milk protein-derived bioactive peptides are also discussed.

Key words: ACE, opioid peptides, immunomodulators, antimicrobial and antithrombotic peptides, phosphopeptides

1.2.2 INTRODUCTION

Peptides derived from milk proteins have been shown to possess significant biological functions. *In vitro* opioid activity of bovine β -casein hydrolysate was first reported by Brantl *et al.* (1979). Precursors of biologically active peptides have been demonstrated *in vivo* after digestion of milk (Meisel and Frister, 1989; Scanff *et al.* 1992). Peptides that inhibit angiotensin I converting enzyme (ACE) have been isolated from bovine casein hydrolysate (Maruyama and Suzuki, 1982; Kohmura *et al.* 1990; Yamamoto *et al.* 1994; Nakamura *et al.* 1995; Maeno *et al.* 1996). Peptides that inhibit platelet aggregation (Fiat *et al.* 1989), stimulate the immune system (Migliore-Samour *et al.* 1989), and are involved in solubilization and absorption of intestinal Ca (West, 1986) have also been isolated from bovine casein hydrolysates.

Bioactive peptides derived from milk proteins are inactive within the sequence of the parent protein and can be released by enzymatic hydrolyses. To exert physiological effects *in vivo*, these peptides must be released from the precursor protein and then reach their target sites in peripheral organs or at the luminal side of the intestinal tract. These peptides are potential modulators of various regulatory processes in the body. With respect to their mode of action, bioactive peptides may reach target sites at the luminal side of the intestinal tract or, after absorption, in peripheral organs (Meisel, 1993).

1.2.2.1 Angiotensin I Converting Enzyme (ACE) inhibitory Peptides

ACE (peptidyl dipeptide hydrolase, EC 3.4.15.1) is a multifunctional enzyme, which catalyzes both the production of the vasoconstrictor angiotensin II and the inactivation of bradykinin (a vasodilating nonapeptide), and enkephalin (Mullally *et al.* 1997). ACE has been associated with the renin-angiotensin system regulating peripheral blood pressure. The enzyme can increase blood pressure by converting angiotensin I (a decapeptide) to the potent vasoconstrictor, angiotensin II (an octapeptide). Therefore, inhibition of ACE can result in a lowering of blood pressure (Meisel, 1993). Because of its multifunctional activity in the organism, the inhibition of ACE by ACE inhibitors may

influence different regulatory systems. Sequential formation of angiotensins I, II, and III (Adams, 1995) is summarized in Fig. 1.2.

The first competitive inhibitors to ACE were first discovered in snake venom and had a sequence of Ala-Pro or Pro-Pro at the COOH-terminal (Maruyama and Suzuki, 1982). Oshima *et al.* (1979) were the first to report ACE inhibitors from food proteins. Since then, many ACE inhibitors have been isolated from enzymatic hydrolysates of tuna muscle (Kohama *et al.* 1988), human casein (Kohmura *et al.* 1989, 1990), bovine casein (Maruyama and Suzuki, 1982; Kohmura *et al.* 1990; Fiat *et al.* 1993; Yamamoto *et al.* 1994a,b; Kuwabara *et al.* 1995; Nakamura *et al.* 1995a,b; Maeno *et al.* 1996), sake and sake lees (Saito *et al.* 1994), whey proteins (Mullally *et al.*, 1997), and other food proteins. Some are chemically synthesized (Koike *et al.* 1980). Many works also suggest that fermented dairy products exhibit antihypertensive property (Nakamura *et al.* 1995a,b; Okamoto *et al.* 1995; Hata *et al.* 1996). Several studies have reported an antihypertensive effect, following administration of casein hydrolysates or peptides derived thereof to human volunteers and to rats (Karaki *et al.* 1990; Yamamoto *et al.* 1994a,b; Nakamura *et al.* 1995a,b; Maeno *et al.* 1996). However, limited studies have been carried out on whey protein-derived ACE inhibitory peptides. Chiba and Yoshikawa (1986) reported that albutensin A, a serum albumin-derived peptide was an ACE inhibitor. Mullally *et al.* (1997) also reported that di- and tetrapeptides corresponding to sequences on α -lactalbumin (α -La) and β -lactoglobulin (β -Lg), were inhibitors of ACE. The most active milk protein-derived ACE inhibitors are summarized in Table 1.2. Some of these peptides exhibit strong antihypertensive effect in spontaneously hypertensive rats (SHR) with a low dose of peptides following oral administration. However, some peptides may undergo a rapid degradation by peptidases and might lose their ACE inhibitory *in vivo*. In fact, casokinin-5 (α_{s1} (f23-27)), which is a potent ACE inhibitor *in vitro* had no antihypertensive effect *in vivo* (Meisel, 1993). Casokinins corresponding to α_{s1} -casein (f23-24, f194-199) and β -casein (f177-183) (Schlimme *et al.* 1988) and (f193-202) (Meisel and Schlimme, 1994) also inhibit ACE. Other antihypertensive peptide are located in the primary sequence of bovine β -lactoglobulin (β -lactorphins) (Mullally *et al.* 1996) and human β - and κ -casein (Fiat *et al.* 1993) and in proteins from food (e.g.

gelatin), vegetables (zeine from maize), or fish (Ariyoshi, 1993). Since the ideal antihypertensive treatment does not yet exist, the production of effective and safer antihypertensive agents may provide a better treatment in the management of hypertension.

The structure-activity relationship of ACE inhibitory peptides has not yet been established but these peptides show some common features. Meisel (1993) demonstrated that the binding of ACE inhibitors to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate. It was found that the presence of hydrophobic (aromatic or branched side chains) amino acid residues at each of the three C-terminal positions of the substrate contribute substantially to the inhibitory potency. The mechanism of ACE inhibition also involves interaction to subsites not normally occupied by substrates or to an anionic inhibitor-binding site that is different from the catalytic site of the enzyme.

1.2.2.2 Opioid Peptides

A number of milk protein fragments has been shown to behave like opioid receptor ligands and able to address opiodergic systems in the adult's or in the neonate's organism. Peptides with opioid activity derived from a bovine β -casein hydrolysate were first reported by Brant *et al.* (1979). Besides analgesic activity, opioid peptides have various physiological effects such as control of gastrointestinal function (Froetschel, 1996), central nervous functions such as stress and adaptation (Przewlocki, 1993), reproductive mechanism (Petraglia, 1993), and may have immunological significance (Sibinga and Goldstein, 1988). Opioid peptides have an affinity for opioid receptor that is most abundant in the central nervous system (Mansour and Waston 1993) and in many peripheral tissues of the mammalian organism (Witter *et al.* 1996). There are several types of receptors, μ -, δ -, and κ -opioid receptors, which again can be divided into subtypes, i.e., μ_1 - μ_2 -receptors etc (Witter *et al.* 1996).

With respect to the proteins, which they are derived off, opioid peptides have been named α -casein exorphins or casoxin D (α -casein), β -casomorphins or β -casorphins (β -casein), casoxin or casoxin A, B, or C (κ -casein), α -lactorphins (α -lactoalbumin), β -lactorphin (β -lactoglobulin) or lactoferroxins (lactoferrin). β -Casomorphin (β -CM) is a particular group that has received more research attention than other biologically active peptides (BAP). Various sizes of β -CM have been isolated from enzymatic digests of casein (Brantl *et al.* 1979; Henschen *et al.* 1979) and synthesized chemically. The primary sequence of these β -CM corresponds to the 60 to 77th amino acid residues (YPFPGPIPNSL) of β -casein (Schlimme *et al.* 1988). A similar amino acid sequence appears in the β -casein of sheep milk (Richardson and Mercier, 1979) and water buffalo milk (Petrilli *et al.* 1983). Opioid peptides also arise from fragments: 90-96 of bovine α_1 -casein (exorphines) (Loukas *et al.* 1983), 40-44 of human β -casein (β -casorphins) (Chiba and Yoshikawa, 1986), 50-53 of human and bovine α -lactalbumin, 102-105 of bovine β -lactoglobulin (lactorphins) (Chiba and Yoshikawa *et al.* 1986) and serum albumin (serorphins) (Tani *et al.* 1994). The N-terminal sequence YPF, is present in all the peptides and is critical for their opioid activity (Muehlenkamp and Warthensen, 1996). Origin and properties of β -CM have been reviewed by several researchers (Scanff *et al.* 1992; Teschemacher *et al.* 1994; Froetschel, 1996; Meisel, 1997), and elsewhere. Only the selected β -CM sequences are given in Table 1.3. According to Daniel *et al.* (1990), the high content of proline residues in β -CM confers resistance to many proteolytic enzymes and the β -CM sequence is highly conserved from β -casein of ovine, bovine, and human species (Richardson and Mercier, 1979; Petrilli *et al.* 1984; Svedberg *et al.* 1985).

Some works suggest that dairy products contain natural β -CM. More recently, β -CM precursors have been identified in Parmesan (Froetschel, 1996) and enzyme-modified cheese (Park *et al.* 1996; Haileselassie *et al.* 1999). However, it is not known whether β -casomorphins are formed or degraded in dairy products as a result of enzymatic hydrolysis by native milk enzymes, bacterial starter enzymes, or exogenous enzymes used to enhance the cheese flavor.

1.2.2.3 Immuno peptides

The bioactivity of immuno peptides was characterized by different *in vitro* and *in vivo* test systems. Migliore-Samour *et al.* (1989) and Gattagno *et al.* (1988) reported that enzymatic digests of human caseins contained immuno peptides which stimulate the phagocytosis of sheep red blood cells (SRBC) by murine peritoneal macrophages, and exert a protective effect against *Klebsiella pneumoniae* infection in mice after intravenous treatment. Two active peptides, a hexapeptide VEPIPY, originated from human β -casein (f54-59) and a tripeptide GLF, not localized in a known sequence of human β - or κ -caseins but present in α -lactalbumin (f51-53) have been reported to show immunomodulatory activity (Fiat *et al.* 1993). Both peptides (VEPIPY and GLF) protected mice against *K. pneumoniae* infection after intravenous treatment and the hexapeptide was significantly active at a dose as low as 0.5 mg/kg (Fiat *et al.* 1993).

Fiat *et al.* (1993) also reported that among the immuno peptides isolated from bovine caseins, the tripeptide (LLY, β -casein (f191-193)) and the hexapeptide (TTMPLW, C-terminal part of α s1-casein) stimulated murine peritoneal macrophages at doses of 1 mg/kg and 0.5 mg /kg, respectively. Another immuno hexapeptide (PGIPN, β -casein (f63-68)) represents a C-terminal part of β -casomorphin-11 (Meisel, 1986). The C-terminal sequence of β -casein (f193-209), containing β -casokinin-10 obtained from a pepsin-chymosin digest of bovine casein induced a significant proliferative response in rat lymphocytes. The dipeptide, YG and tripeptide, YGG corresponding to fragments of bovine α -lactalbumin and κ -casein, respectively, significantly enhanced the proliferation of human peripheral blood lymphocytes (PBL) at concentrations ranging from 10^{-11} to 10^{-4} mol/L (Kayser and Meisel, 1996). The opioid peptide (β -casomorphin-7) has also been found to inhibit the proliferation of human colonic lamina propria lymphocytes (LPL) (Meisel, 1986). Recent study examined the immunogenicity of casein phosphopeptides (CPP) that were derived from tryptic hydrolysis of β -casein and the findings indicated that CPP thereof are significantly less immunogenic than the β -casein (Heddeleson *et al.* 1997). More recently, *in vivo* antibacterial activity against *Staphylococcus aureus* and

Candida albicans has been described for isracidin, the 1-23 fragment of α_{s1} -casein obtained from the action of chymosin (Lahov and Regelson, 1996). Some examples of milk protein-derived immunomodulators are given in Table 1.4.

Although the structure-activity relationship and the mechanism by which milk protein derived peptides exert their immunomodulatory effect is not well understood, some works suggest that opioid peptides may affect immunoreactivity of lymphocytes via the opiate receptor (Elitsur and Luk, 1991).

1.2.2.4 Antimicrobial Peptides

Antimicrobial peptides have been mostly derived from the minor whey protein, lactoferrin. Lactoferrin is an iron-binding glycoprotein present in most biological fluids of mammals including milk. Although the antimicrobial mechanism of lactoferrin is more complex than binding of iron, it is widely considered to be an important component of the host defense against microbial infection. The existence of an antimicrobial sequence near the N-terminus of lactoferrin in a region distinct from its iron-binding sites has been also reported (Bellamy *et al.* 1993). The peptide fragment, lactoferricin, having one intramolecular disulphy bond (FKCRRWNRMKKLGAPSITCVRRAF, lactoferrin (f17-41) generated upon enzymatic cleavage of lactoferrin with pepsin has bactericidal properties more potent than undigested lactoferrin (Bellamy *et al.* 1992). Low molecular weight peptides generated by pepsin cleavage of lactoferrin showed broad-spectrum antibacterial activity, inhibiting the growth of a number of Gram-negative and Gram-positive species, including strains that are resistant to native lactoferrin (Tomita *et al.* 1991). Hence, it is assumed that much smaller size of lactoferrin may facilitate access to target sites on the microbial surface.

The antimicrobial activity of lactoferricin seems to be correlated with the net positive charge of the peptides, which have been shown to kill sensitive microorganisms by inducing an increase in cell membrane permeability (Bellamy *et al.* 1993). Antibacterial activity of casocidin-I, cationic α_{s2} -casein fragment (f165-203) has been

also demonstrated towards the growth of *Escherichia coli* and *Staphylococcus carnosus* (Zucht *et al.* 1995). Research on recombinant lactoferrins in microorganisms and milk cow are actively underway (Wang *et al.* 1998)

1.2.2.5 Antithrombotic Peptides

A large number of molecular similarities have been reported between clotting of blood and milk (Jollès and Caen, 1991). Casoplatelins, peptides originated from the C-terminal part of bovine κ -casein, inhibit the aggregation of ADP-induced platelets as well as binding of the human fibrinogen γ -chain to specific receptor site on the platelet surface (Jollès *et al.* 1986).

The main antithrombotic peptides originated from bovine κ -casein are undecapeptide (MAIPPCKKNQDK, (f106-116)), (MAIPPCK, (f106-112)), and (NQDK, (f113-116)), however, the two smaller tryptic peptides contained in the undecapeptide have a much lower effect on the platelet aggregation and do not inhibit fibrinogen (Fiat *et al.* 1993). The undecapeptide originated from κ -casein contains three amino acid residues (isoleucine, lysine, and asparagine), which are in homologous position with γ -chain sequence of human fibrinogen (Fiat *et al.* 1989). These residues seem to be important for the inhibitory effect, which is due to the competition between antithrombotic peptides and the γ -chain for the platelet receptors (Meisel, 1997).

1.2.2.6 Caseinophosphopeptides

Phosphopeptides are derived from proteolysis of α_{s1} -, α_{s2} , β -, and κ -casein. The phosphorylated residues are commonly grouped in sequences of three or more (West, 1986). It has been reported that caseinophosphopeptides (CPP) may function as carriers for minerals specially calcium and can form soluble organophosphate salts (Saito *et al.* 1986). Several mineral-binding caseinophosphopeptides corresponding to different phosphorylated regions of α_{s1} -, α_{s2} -, and β -casein have been isolated from enzymatic hydrolysates of casein (Migliore-Samour, *et al.* 1989). Fragments 43-45, 59-79, 66-74

from α_{s1} -casein, 46-70, 1-21, 1-28, 33-48, from β -casein have been reported to contain phosphoserine residues in their sequences (Kitts *et al.* 1992). Due to their resistance to enzymatic proteolysis, CPPs containing multiple phosphoserine residues can be found in the gut where they form stable complexes with calcium phosphate (Reynolds, 1994). These complexes are very soluble and increase the calcium absorption by hindering calcium phosphate precipitation. In particular CPPs-Ca complexes may enhance calcium absorption in the distal small intestine where passive transport occurs. The passive transport is the main calcium absorption route under physiological conditions and it is effective in bone calcification and treatment of rickets (Kitts *et al.* 1992). As CPPs have not shown allergenicity their addition to toothpaste formula has been suggested in order to prevent enamel demineralization and to obtain anticariogenic effect (Reynolds, 1994). CPP have been shown to increase calcium bioavailability in rats and to alter temporal systolic blood pressure in spontaneously hypertensive rats (Kitts *et al.* 1992). The binding sites of these peptides for minerals are represented by the negatively charged phosphate groups of the amino acid residues (serine phosphate cluster, glutamyl residues, and glutamic acid) (Berrocal *et al.* 1989). However, further amino acids around the phosphorylated binding sites can also result in significant difference in calcium binding activity of phosphopeptides.

1.2.2.7 Other Potential Bioactive Peptides

Some bioactive peptides that are not released under physiological conditions could be produced commercially and used as nutraceuticals. In fact, casein-derived peptides were already produced on industrial-scale and have been considered both as dietary supplements in functional foods and as pharmaceutical preparations (drugs) (Meisel, 1997). Functional food has been defined as foods derived from naturally occurring substances that can be consumed as part of the daily diet (Schmidl, 1993). Several bioactive peptides may be used as highly active drugs with a well-defined pharmacological effect, for example, in the treatment of diarrhea (casomorphin), hypertension (casokinins), thrombosis (casoplatelins), dental and bone diseases, as well as malabsorption (casein phosphopeptides), and immunodeficiency (immunopeptides).

1.2.1 CONCLUSION

Milk is traditional source of high-quality proteins in the human diet. In recent years research attention has been focused on the fractionation of both caseins and whey proteins with the objective of producing proteins better suited for particular applications than the crude protein mixtures. Bioactive peptide fragments originating from milk proteins are now considered as potential modulators of various regulatory processes in the body. Many biologically active peptides of food origin have been found by using sensitive *in vitro* assay systems. Although animal as well as plant proteins also contain potential bioactive sequences, milk proteins are currently the main source of a range of biologically active peptides. However, not all the peptides are effective following oral administration. Therefore, methods to increase their potency should be explored and additional work is needed to obtain further data on the structure-activity relationship. Genetic engineering may be a very significant method by which the functionality and perhaps biological activity of milk proteins can be improved and the overproduction can be achieved in microorganisms or animal bioreactor after cloning these peptides.

Table 1.1: The hydrophobicity values (Δf)* of the side chain of amino acids

Amino acid	Three-letter code	One-letter code	Δf value (cal/mole)
Glycine	Gly	G	0
Serine	Ser	S	40
Threonine	Thr	T	440
Histidine	His	H	500
Asparatic acid	Asp	D	540
Glutamic acid	Glu	E	550
Arginine	Arg	R	730
Alanine	Ala	A	730
Methionone	Met	M	1300
Lysine	Lys	K	1500
Valine	Val	V	1690
Leucine	Leu	L	2420
Proline	Pro	P	2620
Phenylalanine	Phe	F	2650
Tyrosine	Tyr	Y	2870
Isoleucine	Ile	I	2970
Tryptophan	Trp	W	3000

***Q = $\frac{\sum \Delta f}{n}$** (Modified from Ney, 1979).

Where,

Q = average hydrophobicity

$\sum \Delta f$ = hydrophobicity value of individual amino acid

n = number of amino acid residue

Table 1.2: Angiotensin I- converting enzyme (ACE) inhibitory activity of milk protein- derived peptides.

Peptides	Source	Preparation	IC ₅₀ (μM) ^a	Reference
SKVLPVPE	β-Casein	Proteinase	39	(Yamamoto <i>et al.</i> 1994a,b)
PFQSVLSLSQSKVLPVPE	β-Casein	Proteinase	25	>>
LLYQQPVLPVVRGPFPIIV	β-Casein	Proteinase	21	>>
DELQDKIHFFAQTQSLVYFPGPIPN	β-Casein	Proteinase	4	>>
VPP	β-Casein	Fermentation	9	(Nakamura <i>et al.</i> 1995a,b) [*]
IPP	β-and κ-Casein	Fermentation	5	(Yamamoto, 1997) [*]
AVPYPQR	β-Casein	Trypsin	15	>>
KVLPVP	β-Casein	Digestive enzyme	5	>>
VYP	β-Casein	Synthesis	44	(Kohmura <i>et al.</i> 1990)
LVYP	β-Casein	Synthesis	170	>>
SLVYP	β-Casein	Synthesis	40	>>
QSLVYP	β-Casein	Synthesis	41	>>
TQSLVYP	β-Casein	Synthesis	73	>>
AQTQSLVYP	β-Casein	Synthesis	76	>>
FAQTQSLVYP	β-Casein	Synthesis	25	>>
HPFAQTQSLVYP	β-Casein	Synthesis	26	>>
IHPFAQTQSLVYP	β-Casein	Synthesis	19	>>
KIHFFAQTQSLVYP	β-Casein	Synthesis	39	>>
MKPWQPK	α ₁ -Casein	Proteinase	22	(Yamamoto, 1997) [*]
FFVAPFPEVFGK	α ₁ -Casein	Trypsin	77	(Yamamoto, 1997) [*]
FFVAP	α ₁ -Casein	Peptidase	6	(Koike <i>et al.</i> 1995)
TTMPLW	α ₁ -Casein	Trypsin	16	(Yamamoto 1997) [*]
PLW	α ₁ -Casein	Synthesis	36	>>
VAP	α ₁ -Casein	Synthesis	2	>>
FVAP	α ₁ -Casein	Synthesis	10	>>
YLLF	β-Lactoglobulin	Hydrolysis	171.8	>>
—	^b WPC	Trypsin	195.1	>>
—	^b WPC	Trypsin	201.1	>>
—	β-Lactoglobulin	Trypsin	130.0	>>
—	β-Lactoglobulin	Trypsin	160.4	>>

^a The concentration of an ACE inhibitor required to inhibit 50% of ACE activity.

^{*} Peptides exhibiting strong antihypertensive activity in SHR with a low dose of peptides following oral administration.

^b Whey protein concentrate.

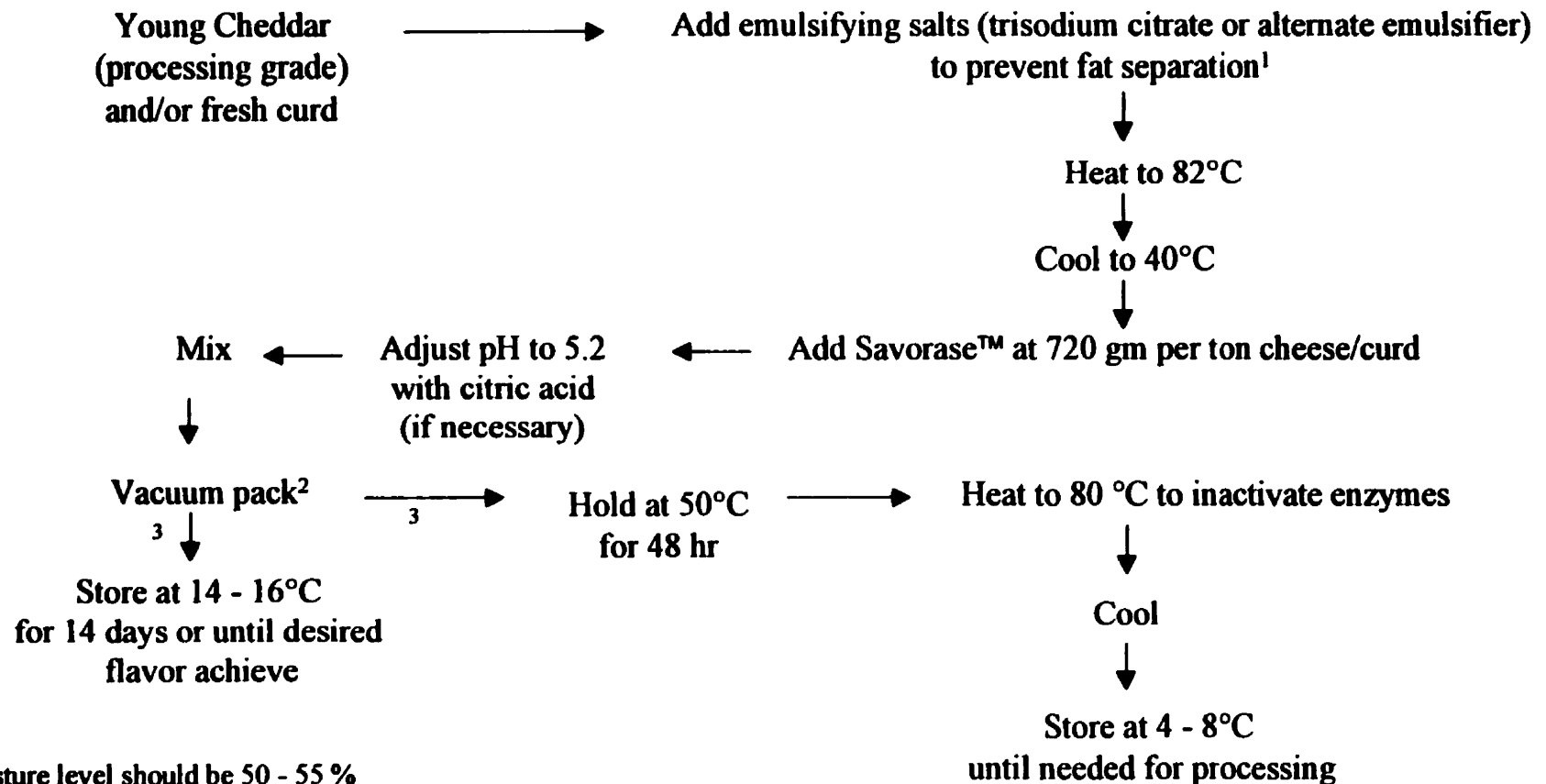
Table 1.3: Amino acid sequences of some natural β -casomorphins.

Bovine β-Casomorphin (1-4) amide	H-Tyr-Pro-Phe-Pro-NH₂
Bovine β-Casomorphin (1-7)	H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH
Bovine β-Casomorphin (1-11)	H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-OH
Human β-Casomorphin (1-4) amide	H-Tyr-Pro-Phe-Val-NH₂
Human β-Casomorphin (1-8)	H-Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-OH
Ovine β-Casomorphin (1-8)	H-Tyr-Pro-Phe-Thr-Gly-Pro-Ile-Pro-OH
Water buffalo β-Casomorphin (1-8)	H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-OH

(Adapted from Teschemacher *et al.* 1997)

Table 1.4: Examples of some milk protein-derived immunomodulators and antithrombotic peptides.

Peptide Sequence	Fragment	Name	Immunomodulatory % of control	Reference
YPFPGPI	β -Casein (f60-66)	β -Casomorphin-7	-21/+26	(Kayser and Meisel, 1996)
TTMPLW	α_{s1} -Casein (f194-199)	α_{s1} -Casokinin-6	+162	(Maruyama <i>et al.</i> 1987)
YQQPVLGPVR	β -Casein (f193-202)	β -Casokinin-10	-28/+14	(Meisel and Schlimme, 1994)
PGPIP	β -Casein (f63-68)	Immunopeptide	+139	(Meisel, 1993)
LLY	β -Casein (f191-193)	Immunopeptide	+148	(Meisel, 1993)
YG	α -Lactalbumin (f50-51) (f18-19)			
	κ -Casein (38-39)		+101	(Kayser and Meisel, 1996)
YGG	α -Lactalbumin (f18-20)		+35	>>



¹Moisture level should be 50 - 55 %

²Vacuum packaging reduces risk of bacterial contamination

³Alternate ripening procedure

Fig. 1.1. Procedure for the production of high flavored curd (EMC) using Savorase™ (Adapted form Imperial Biotech)

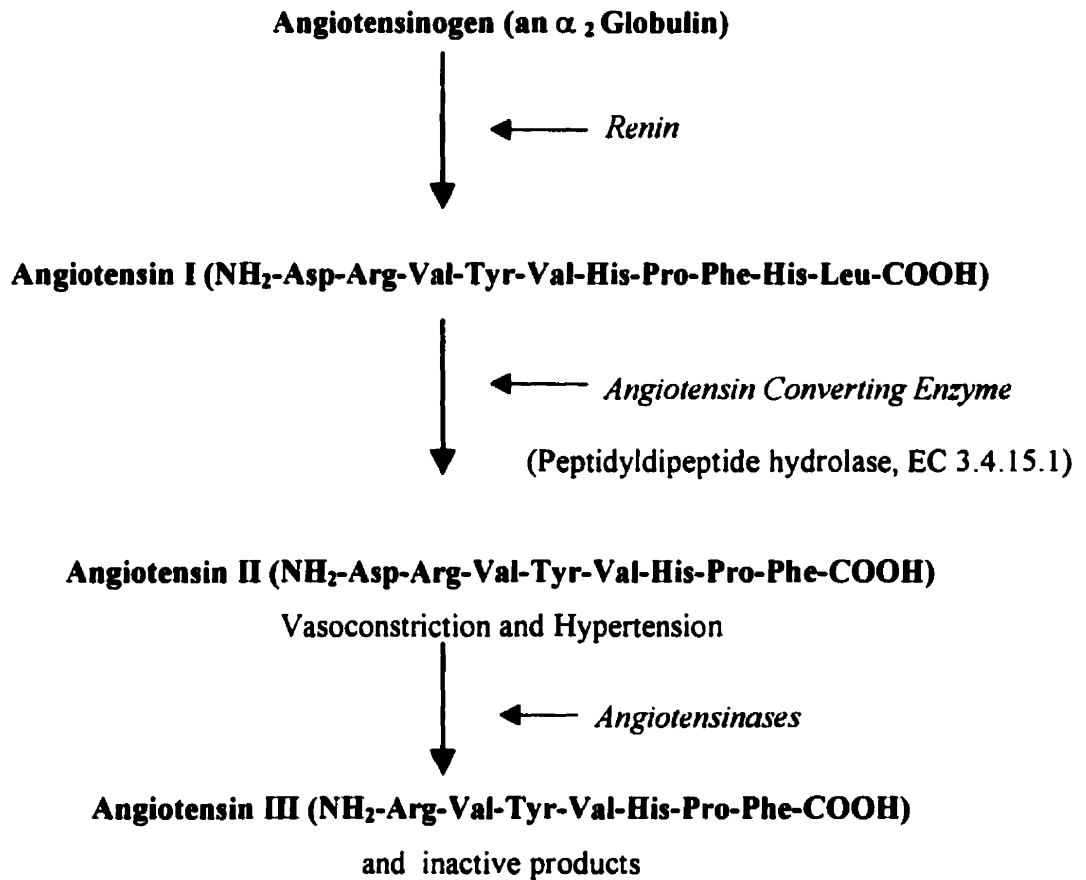


Fig. 1.2. Sequential formation of bovine angiotensins I, II, and III (Adapted from Adams, 1995).

CHAPTER 2.0

PRODUCTION CONDITIONS OF A GOOD QUALITY OF EMC BY *LACTOBACILLUS* AND COMMERCIAL ENZYMES

In order to prepare a good quality of EMC by *Lactobacillus casei* and commercial enzymes, EMC samples were prepared by combinations of different enzymes under different incubating conditions. Optimum conditions for preparation of a good quality of EMC were established. These conditions were used to prepare EMC for further studies.

The major results of this study were summarized as a manuscript suitable for journal publication. The manuscript entitled "Production conditions of EMC by *Lactobacillus* and commercial enzymes" was co-authored by Seble S. Haileselassie and Byong H. Lee. The project was supervised by Dr. Byong H. Lee, while the actual experimental work, and writing the manuscript were done by Seble S. Haileselassie.

2.1 ABSTRACT

To establish optimum conditions to prepare a good quality of EMC, EMC samples were prepared by using combinations of Neutrase®/*L.casei* enzymes, Neutrase®/Debitrase™, Neutrase®/Flavorzyme™, and Neutrase®/Palatase® M at different concentrations under controlled conditions. EMC samples were withdrawn at 4, 8, or 12 hrs for sensory evaluation and RP-HPLC analysis. The bitterness of EMC prepared by Neutrase® alone was decreased significantly after treatment with Debitrase™ and the crude enzyme of *Lactobacillus casei* and these two EMCs developed an intense flavor in 3 and 4 days, respectively. EMC prepared by Neutrase®/Flavorzyme™ remained bitter and EMC prepared by Neutrase®/Palatase® developed cheese flavor rapidly, but with rancidity.

Key words: *Lactobacillus*, Neutrase®, Debitrase™, Flavorzyme™, Palatase®, EMC

2.2 INTRODUCTION

The development of an improved technology for Cheddar cheese flavor development started with the discovery of novel fungal proteinases and lipases (Sood and Kosikowski, 1979). Kosikowski and Iwasaki (1974) showed that addition of combinations of microbial enzyme preparations into cheese curds hastened ripening and improved cheese flavor. In general, *Bacillus* and plant proteinases are the most cost-effective for protein hydrolysis but their use can eventually lead to high levels of bitterness (West, 1996). Fungal proteinases are known to produce lower amounts of bitter peptides than alkaline proteases, and some of them may contain high levels of both carboxy- and amino-peptidases which have a role in debittering (West, 1996). However, the bacterial proteinase, Neutrase (Novo Nordisk) tends to induce the accumulation of intensely bitter peptides, along with a minor enhancement of the aroma (Law and Wigmore, 1982; Law, 1986; Cliffe and Law, 1990). Thus, a mixture of exopeptidases and endopeptidases with broad specificity is desirable for extensive hydrolysis of protein and debittering of hydrolysate in the production of EMC.

The peptidolytic systems of starter (Cliffe and Law, 1990) and non-starter (El Aboudi *et al.* 1991; Trépanier *et al.* 1992a,b) lactic acid bacteria (LAB) have been successfully used for accelerated cheese ripening. Due to their wide ranges and higher activities of peptidolytic and esterolytic enzymes, *Lactobacillus casei* subspecies (*L. casei* ssp. *casei* LLG and *L. casei* ssp. *rhannosus* S93) isolated from matured Cheddar cheese were also reported as potential strains for the acceleration of cheese ripening (Lee *et al.* 1986; Arora and Lee, 1990, 1992). Consequently, incubation of bitter EMC prepared by Neutrase 0.5L with the crude enzyme extract of *Lactobacillus casei*, under controlled conditions resulted in the development of an intense cheese flavor in three days without bitterness or rancidity.

A wide range of protein-hydrolyzing enzymes is now commercially available from two sources, *Aspergillus oryzae* and lactic acid bacteria (LAB). However, *Aspergillus oryzae* preparations may also contain low levels of endopeptidase activity

(Pawlett and Bruce, 1996). The use of lipolytic enzymes to generate an intense cheese flavor can eliminate the need for an extremely hydrolysed protein substrate, reducing the possibility of off-flavor generation. Moskowitz and Noelck (1987) suggested that after enzyme hydrolysis during EMC production, the resulting fat phase is more capable of dissolving flavor compounds, thereby increasing the perception of flavor. Revah and Lebault (1989) also found that the fat phase also acts as a solvent for the liposoluble compounds produced during ripening of cheese and it can be assumed that this is the case for EMC types. The effects of lipase on cheese flavor formation has been evaluated by a number of workers and a wide range of lipases are commercially available from a number of sources, mainly animal and microbial. The correct choice of lipase for the production of EMC is extremely important, since the free fatty acids (FFA) and flavor profiles vary significantly with type of lipase used (Kilara, 1985). Thus, knowledge of the biochemical pathway leading to flavor production would be necessary to make the right choice of enzymes for enzymatic flavor production.

We undertook the present study to investigate the optimum conditions (enzyme activity, temperature, and time) to prepare a good quality of EMC by *Lactobacillus* and commercial enzymes.

2.3 MATERIALS AND METHODS

2.3.1 Strain and preparation of enzyme extract

Lactobacillus casei isolated from a good quality Cheddar cheese was grown in MRS broth for 18 h at 30°C. The cells were harvested by centrifugation (Beckman, J2-21, Paolo Alto, California) at 8,000 x g for 15 min at 4°C. The cell pellet was washed three times with 0.05 M phosphate buffer (pH 7.0) and then suspended in an appropriate amount of the same buffer and disintegrated at 2 s interval for 1 min using an Ultrasonic Processor XL (Heat System, New York, NY). Cell debris was removed by centrifugation at 10,000 x g for 30 min at 4°C and the supernatant was used for assaying aminopeptidase and esterase activities and for the preparation of EMC.

2.3.2 Enzyme purification

The prepared crude cell-free extract was fractionated by salting out with solid ammonium sulfate to 80% saturation. The precipitate was collected by centrifugation at $10,000 \times g$ for 20 min, dissolved in a minimum amount of 0.05 M phosphate buffer, pH 7.0 and dialyzed overnight against the same buffer using a Spectrapor membrane (30,000 MWCO; Spectrum Co., Houston, TX). The dialyzed fraction was analyzed for protein concentration, aminopeptidase, and esterase activities. Figure 2.1 shows the procedure for preparation and purification of aminopeptidase and esterase. Aminopeptidase and esterase were purified using FPLC system equipped with ion-exchange column (Mono Q 16/10; Pharmacia, Montreal) and gel-filtration column (Superose, HR 10/30; Pharmacia), according to the methods described by Arora and Lee (1992).

2.3.3 Protein and enzyme assays

Protein was determined spectrophotometrically at 562 nm using the BCA (bicinchoninic acid) protein assay reagents supplied with the system (Pierce Chemical Ltd., Rockford, IL), (Smith *et al.* 1985). Bovine serum albumin (Sigma Chemical, St. Louis, MO) was used as a standard. The assay mixture containing 180 μL of 0.05 M buffer (pH 7.0), 10 μL of substrate and 10 μL enzyme fraction was incubated at 32°C for 30 min. Activity was determined spectrophotometrically using Lamda Plate (Bio-Rad) at 410 nm by measuring the amount of *p*-nitroaniline produced from leucine-*p*-nitroanilide. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitroaniline per min from leucine *p*-nitroanilide. Esterolytic activity was measured by assaying the quantity of *p*-nitrophenol produced from the substrate *p*-nitrophenyl derivatives of fatty acids mixture ($\text{C}_4\text{-C}_{12}$) at 410 nm. The assay mixture containing 180 μL of 0.01 M phosphate buffer (pH 7.1), 10 μL of 1.5 mM of the substrate and 10 μL of enzyme solution was incubated at 37°C , for 30 min. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min from *p*-nitrophenyl derivatives of fatty acids mixture ($\text{C}_4\text{-C}_{12}$). Specific activity was expressed as the enzyme unit per mg of protein.

2.3.4 Commercial enzymes

Four commercial enzymes used in this experiment are: (1) Neutrase® 0.5L, a bacterial protease produced from *Bacillus subtilis* (1,883 U/g, pH 5.5-7.5), (2) Palatase® M 1000, a fungal lipase derived from *Rhizomucor miehei* (1,000 LU/g, pH 5.0-7.0), (3) Flavorzyme™, a fungal protease/peptidase cocktail produced from *Aspergillus oryzae* (1,000 LAPU/g, pH 5.0-7.0) (Novo Nordisk A/S, Bagsvaerd, Denmark), and (4) Debitrase™ DBP20, a blend of peptidase extracted from *Lactococcus lactis* and *Aspergillus oryzae* (220 LAPU/g, pH 5.0-7.0) (Imperial Biotech, London, UK). Each commercial enzyme was suspended in 0.05 M phosphate buffer (pH 7.0) and incorporated to 100 g of cheese slurry prepared by Neutrase.

2.3.5 Preparation of EMC

EMC was prepared according to the method of Park *et al.* (1995). Shredded mild Cheddar cheese (600 g) was mixed with 2.5% (Na₂HPO₄) and 195 ml of distilled water. The mixture was heated at 90°C for 3 min to emulsify the cheese slurry and to inactivate endogenous enzymes, and then cooled down to 50°C. Neutrase® 0.5L (1,883 U/g) was filtered through a 0.45 µm membrane filter and added to the cheese slurry. The slurry was incubated for 8 h under vacuum at 45°C to supplement the starter proteinase activity. Resulting Cheddar cheese slurries (100 g) were then treated separately with: (i) crude extracts of *Lactobacillus casei* containing aminopeptidase 86.4 LAPU/g at 34°C for 72 h (EMC-NL72) and esterase 110.6 U/g at 45°C for 24 h (EMC-NL24), (ii) Debitrase (22.0 LAPU/g) at 40°C for 96 h (EMC-ND96), (iii) Flavorzyme (6.5 LAPU/g) at 50°C for 36 h (EMC-NF36), and (iv) Palatase (600 LU/g) at 35°C for 16 h (EMC-NP24). Individual slurries were placed in airtight sterilized plastic containers and incubated according to the optimum conditions of each enzyme. The enzymes were then inactivated by heat treatment at 80°C and the samples stored at -20°C for further analysis.

2.3.6 Experimental design

To establish optimum conditions to prepare a good quality of EMC, different combinations of enzyme concentration, incubation time and temperature (Table 2.1) were tested. EMCs were prepared by (1) Neutrase alone, (2) Neutrase/*L. casei* enzyme, and (3) Neutrase® and other commercial enzymes (Debitrase™, Flavorzyme™, and Palatase®). Three experiments were carried out with duplicates. EMC samples were withdrawn at 4, 8, and 12 hrs for sensory evaluation and RP-HPLC analysis. The optimum conditions were determined based on the results obtained from sensory and RP-HPLC analysis. Significant effects of each treatment was determined by analyzing the sensory data by using Duncan's Multiple range Taste (Duncan, 1955).

2.3.7 Extraction of water-soluble peptides

The modified method of McGugan *et al.* (1979) was used to extract the water-soluble peptides of EMCs. The procedure for extraction of water-soluble fractions (WSF) is represented in Fig. 2.2. Portions (5 g) of EMC samples were centrifuged (25,000 x g, 50 min, 20°C). The aqueous layer (1 mL) was mixed with 1 mL methanol, 1 mL methylene chloride and 0.6 mL of water and shaken vigorously. After centrifugation (25,000 x g for 30 min, 20°C), 1mL methanol-water fraction was concentrated by a Speed Vac (Savant Instrument, NY) for 3 h and dissolved with 50 µL of distilled water.

2.3.8 RP-HPLC analysis

The waters HPLC system (Millipore, Milford, MA) consisted of a 600 E system controller, U6K Injector, 486 Tunable Absorbance Detector and a Millenium 2010 Chromatography Manager, was used to separate peptides. Portions (25 µL) of WSF were injected on a Delta Pak C18 column (100 Å, 30 x 150 mm). The peptides were eluted at a flow rate of 0.5 mL/min with a binary gradient of acetonitrile/water. Gradient of solvents at a flow rate of 0.5 mL/min is shown in Table 2.2. Solvent A was 0.1 % TFA in Milli Q water and solvent B was 0.08% TFA in a mixture of acetonitrile/water at 40:60

ratio. The elution was monitored at A_{214} nm. All samples were filtered through 0.45 μ m syringe filter before injection.

2.3.9 Sensory evaluation

EMC samples prepared by *L.casei* and commercial enzymes were assessed for bitterness, flavor acceptability, and texture. The sensory attributes of EMC samples were evaluated by 8 panelists, who were accustomed to tasting cheese, but were not specially trained. EMCs prepared with different enzyme combinations were coded with three-digit numbers and presented in a randomized order to panelists. Samples were held at room temperature for 1 hr before their presentation to panelists. Quinine sulfate was used as bitter standard according to Minagawa *et al.* (1989) by which a range of solutions (0.8 to 100 μ M) with increasing concentrations were prepared. Evaluations were made using a numerical scoring system of 0 for non-bitter to 8 extremely bitter. EMC prepared by Neutrase (EMC N8) was used as a standard and all EMC samples were compared to the standard. Flavor acceptability scale was ranging from (0-1) unacceptable to 8-excellent. All sensory tasting was conducted in duplicate and sensory data were analyzed by using Duncan's Multiple Range Test (Duncan, 1955), where $\alpha = 0.05$.

2.4 RESULTS AND DISCUSSION

2.4.1 Enzyme Activity

The summary of proteolytic, peptidolytic, esterolytic, and lipolytic activities of the crude enzyme extract and commercial enzymes is presented in Table 2.3. The crude enzyme extract of *Lactobacillus casei* ssp. *casei* LLG (18.50 mg protein/ml) contained low proteolytic and lipolytic activities, but high aminopeptidase activity (86.4 LAPU/g). The results were comparable to those of Arora and Lee (1992), Habibi and Lee (1994), and Park *et al.* (1995). The crude extract of *L. casei* ssp. *casei* LLG was also found to contain high esterolytic activity (110.0 U/ml). Neutrase®0.5L (28.48 mg protein/ml)

contained high proteolytic activity (1,883 U/g protein) with low peptidolytic activity (8.01 LAPU/g). The results obtained in our experiment were comparable to those of Park *et al.* (1995). Debitrase™ contained high peptidolytic (210.01 LAPU/g) but low proteolytic activity (0.81 U/g). Flavorzyme™ contained high peptidolytic (908.02 LAPU/g) and relatively low proteolytic activity (108.1 U/g). Palatase® M possessed high lipolytic (1,000 U/g) but low esterolytic activity (2.50 U/g).

2.4.2 Bitter flavor intensity and flavor development in EMC

Sensory evaluation of EMCs prepared by *L. casei* and commercial enzymes is shown in Table 2.4. Taste panel results indicated that incubation of the cheese slurry for 8 hr with Neutrase resulted in significant increase in bitterness ($P < 0.05$). This is probably due to the formation of bitter peptides by the action of proteolytic enzyme (Neutrase) on casein. The bitterness of EMC-N8, however, was significantly reduced ($P < 0.05$) by further treatment of the sample with *L. casei* enzymes and Debitrase. This clearly demonstrated the debittering effect of *Lactobacillus* enzymes and Debitrase on bitter EMC. Consequently EMC-NL72 and EMC-ND96 developed an intense cheese flavor within 3 and 4 days, respectively. Contributions by *Lactobacillus* enzymes and Debitrase to palatable peptide conversions in EMC were evident from comparison between flavor acceptability of these EMCs versus EMC prepared by Neutrase. The panelist's comments indicated that EMC-NL72 and EMC-ND 96 were of a better quality than other EMCs, but both EMCs displayed watery texture.

Although EMC-NF36, prepared by combination of Neutrase and Flavorzyme developed relatively intense flavor in two days, its flavor was impaired by bitterness. This effect may be due to the proteolytic activity of the enzyme (Flavorzyme), which is effective in releasing hydrophobic bitter peptides from casein. RP-HPLC analysis also confirmed that EMC-NF36 contained late-eluting hydrophobic peptide with RT of 44 min. This peptide was purified and identified to be a tripeptide with a sequence of Val⁸⁰-Thr-Pro⁸² originating from β -casein and the Q value of this peptide was calculated using Δf value of each amino acid. Because of its high Q value ($Q = 1583$ cal/mole) the

tripeptide Val⁸⁰-Thr-Pro⁸² is considered to impart bitterness to EMC-NF36. However, no quantitative analysis was performed to determine the effect of location of the amino acids on the bitterness of the peptide. A prolonged incubation of EMC-NF36 did not help in debittering, but rather resulted in development of unpleasant off-flavor. EMC-NF36 possessed grainy texture that was found to be due to uneven incorporation of the enzyme in the slurry.

EMC-NP16, prepared by combination of Neutrase and Palatase developed an intense cheese flavor rapidly in 12 h, but thereafter deteriorated after 16 hr. This is probably due to the formation of butyric acid at high concentration by lipolysis from milk fat in the slurry. Although fatty acids are important in the flavor of many cheese types, large amount of butyric acid is undesirable, since it results in the formation of rancidity or unclean flavor (Engels *et al.* 1997).

2.4.3 Treatment of EMC

Figure 2.3 shows the elution profiles of EMC-N8 prepared by Neutrase[®]0.5L (A) and EMC-NL72 (prepared by combination with Neutrase and *L. casei* enzymes) (B). The RP-HPLC chromatogram of bitter EMC-N8 showed that this EMC contained mainly peptides with long retention time eluted in hydrophobic region (second half). Bitter peptides found in cheese and other foods are relatively rich in hydrophobic amino acids e.g. phenylalanine, leucine, isoleucine, and proline (Matoba and Hata, 1972; Richardson and Creamer, 1973; Guigoz and Solms, 1976), and peptides containing these amino acids would exhibit long retention times on a reverse-phase column (Hodges and Mant, 1991). The presence of late eluted peptides, in the second half of the chromatogram in EMC-N8 indicates the bitter peptides from the cheese being hydrophobic.

The increase in peak heights and areas of hydrophilic peptides (first half) in EMC-NL72 suggests that peptidolytic enzymes from *L. casei* could hydrolyze hydrophobic peptides released by Neutrase into early-eluting hydrophilic peptides. Similar effect has been observed in EMC-ND96 prepared by combination of Neutrase

and Debitrase in Fig. 2.4, but the elution patterns of both EMCs showed different hydrolytic patterns. In addition, both EMC-NL72 and EMC-ND96 had savory tastes. This supports the concept that early-eluting components are likely to be either palatable peptides or derivatized amino acids, as the UV-absorbing free amino acids elute later on the reverse-phase column.

The elution profile of EMC-NF36, prepared by combination of Neutrase and Flavorzyme, is shown in Fig. 2.5. Although most of the late-eluting hydrophobic peptides released by Neutrase were hydrolyzed to hydrophilic peptides and derivatized amino acids, there was one peak appearing in hydrophobic region (second half) that tends to be bitter. Thus the enzymatic treatment with Flavorzyme (commercial peptidase/proteinase cocktail) proved to be less effective than the *Lactobacillus* and Debitrase treatments. However, a combination of Flavorzyme with other peptidolytic enzymes in a proper balance might give acceptable cheese flavor without bitterness. The results obtained from this study support the findings of Cliffe and Law (1990) that bitterness in Neutrase-treated Cheddar cheese curd slurries was associated with late-eluting peptides and that treatment of these slurry with peptidase-containing lactococcal extract removed the bitterness and established a normal Cheddar flavor which was associated in early-eluting, more hydrophilic peptides on RP-HPLC.

2.4.4 Optimum conditions for the production of a good quality of EMC

The effects of enzyme concentration, temperature, and incubation time on the formation of intense cheese flavor was compared (Table 2.4). Flavor of EMCs varied from bland taste to rancid off-flavor and bitterness varied from 0.6 (not bitter) to 6.0 (very bitter). To produce EMCs with intense flavor without bitterness and rancidity, combinations of neutral proteinase, peptidase and esterase were necessary.

According to the sensory evaluation and RP-HPLC analysis the optimum concentrations of the enzymes to prepare a good quality of EMC were found to be; *L. casei* enzymes (aminopeptidase activity 86.4 LAPU/g and esterase activity 110.0 U/g)

and Debitrase (aminopeptidase activity 22.00 LAPU/g). The development of flavor in EMCs was influenced by temperature and time of incubation. Incubation of the slurry with *L. casei* aminopeptidase for 72 h at 34°C and with *L. casei* esterase for 24 h at 45°C resulted in more flavor and savory taste than those of other conditions. After 96 h at 35°C, EMC prepared by combination of Neutrase/Debitrase displayed cheese flavor and savory taste.

EMC-NF36, prepared by combination of Neutrase/Flavorzyme developed medium cheese flavors with bitterness after 24 h at 36°C. Increasing the concentration of the enzyme, temperature, and time, however, resulted in development of unpleasant flavor with low bitterness. After 12 h at 34°C, EMC prepared in combination of Neutrase/Palatase developed noticeable cheese flavors but was characterized by slight rancidity with bitterness. After 20 h at 34°C, the same EMC developed intense cheese flavor with an increased rancidity.

2.2 CONCLUSION

An alternative process to prepare EMCs by *Lactobacillus* and commercial enzymes suggests that high intensity cheese flavors can be produced in a matter of days. The addition of Debitrase (22.0 LAPU/g) and the crude enzyme extract (86.4 LAPU/g and 110.0 U/g) of *L. casei* to Cheddar cheese slurry prepared by Neutrase produced a good quality of EMC. A reduction of bitterness and development of savory taste indicated the ability of these enzymes to convert bitter peptides into palatable peptides. Esterolytic enzymes of *L. casei* have also contributed to the formation of desirable cheese flavor. Although EMCs prepared by combination of Neutrase/Flavorzyme and Neutrase/Palatase developed bitter and rancid-off flavor, respectively, a correct cocktail of these enzymes with other peptidases and/or esterases may be used to prepare non-bitter intense cheese flavor.

Table 2.1: Experimental design for the preparation of EMC.

Treatment	Enzyme activity (g protein)	Temperature (°C)	Time (h)
Neutrase®	1,883 U ^a	40 - 45	8 - 24
Neutrase® + <i>L. casei</i> (aminopeptidase)	86.40 LAPU ^b	30 - 40	12 - 96
Neutrase® + <i>L. casei</i> (esterase)	110.01 U ^a	40 - 45	12 - 36
Neutrase® + Debitrase™	22.0 LAPU ^b 110.0 LAPU ^b 220.0 LAPU ^b	30 - 40	24 - 108
Neutrase® + Flavorzyme™	2.0 LAPU ^b 6.5 LAPU ^b 10.0 LAPU ^b	45 - 55	6 - 48
Neutrase® + Palatase® M	200.0 LU ^c 600.0 LU ^c 1,000.0 LU ^c	30 - 40	4 - 24

^aunit

^bleucine aminopeptidase unit

^clipase unit

Table 2.2. HPLC gradient of solvents at 0.5 mL/min flow rate.

Time (min)	Water (%)	Acetonitrile/ Water (%) (40:60)	Ratio
0	80	20	Gradient
10	80	20	Gradient
15	60	40	Gradient
30	40	60	Gradient
45	0	100	Isocratic
55	80	20	Gradient
60	80	20	Gradient

Table 2.3: Proteolytic, peptidolytic, and esterolytic activities in the crude extract of *L. casei* and commercial enzymes.

Enzymes	Proteinase	Aminopeptidase	Esterase	Lipase
	(U/g) ₁	(LAPU/g) ₂	(U/g) ₁	(U/g) ₁
Neutrase® 0.5L	1,833.0	8.01	--	--
Crude extract	0.5	86.4	110.01	0.85
Debitrase™	0.8	201.02	--	--
Flavorzyme™	108.1	908.01	--	--
Palatasc® M	--	--	0.5	1,000

^{1,2}Unit: amount of enzyme required to release 1 μmol of chromophore from the substrate under specified conditions in Materials and Methods (means of two samples for each duplicates).

Table 2.4: Panel scores of EMCs prepared by Neutrase® (EMC-N8), by combination of Neutrase®/*L. casei* enzymes (aminopeptidase, EMC-NL72; esterase, EMC-NL24), Neutrase®/Debitrase™ (EMC-ND96), Neutrase®/Flavorzyme™ (EMC-NF24), and Neutrase®/Palatase® M (EMC-NP16).

Treatments	Bitterness*	Flavor	Flavor** acceptability	Texture
EMC-N8	6.0 ^{***}	No noticeable taste	2	Pasty
EMC-NL72	1.6 ^b	Savory taste	6	Watery
EMC-NL24	2.8 ^c	Cheesy butter milk	6	Soft
EMC-ND96	0.6 ^d	Cheesy pleasant	7	Watery
EMC-NF36	4.8 ^e	Cheesy	5	Lumpy
EMC-NP16	5.8 ^f	Rancid	3	Lacks uniform consistency

*Bitterness score was scaled from 0 = not bitter, 2 = slightly bitter, 4 = distinctly bitter, 6 = very bitter, and 8 = extremely bitter.

**Flavor acceptability was scaled from 0-1 = unacceptable, 2-3 = poor, 4 -5 = acceptable, 6 - 7= good, and 8 = excellent.

***means the different letters are significantly different at the level of $p < 0.05$ in two samples each in duplicate.

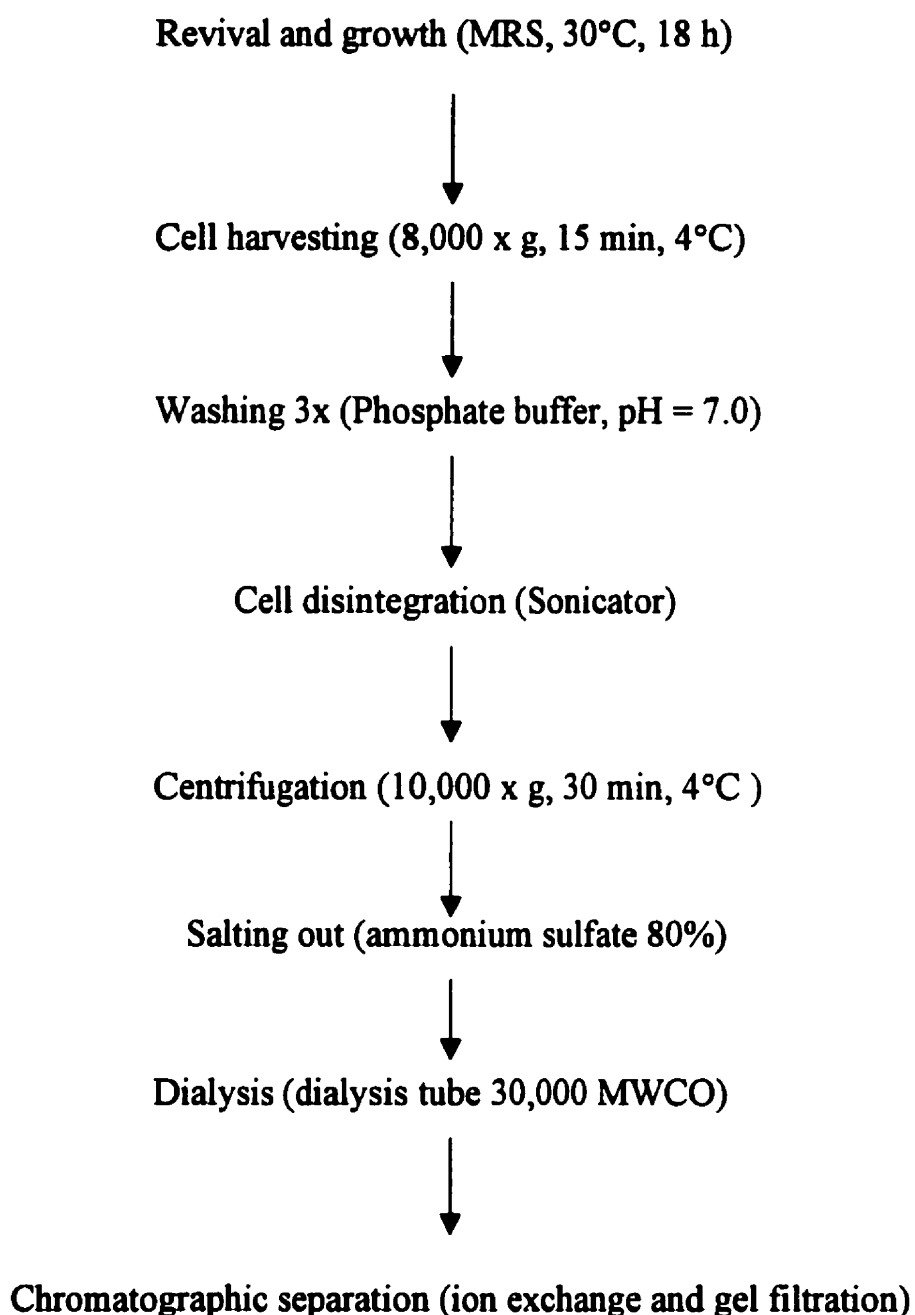


Fig. 2.1. Procedure for preparation and extraction of the crude extract from *Lactobacillus casei* ssp. *casei* LLG.

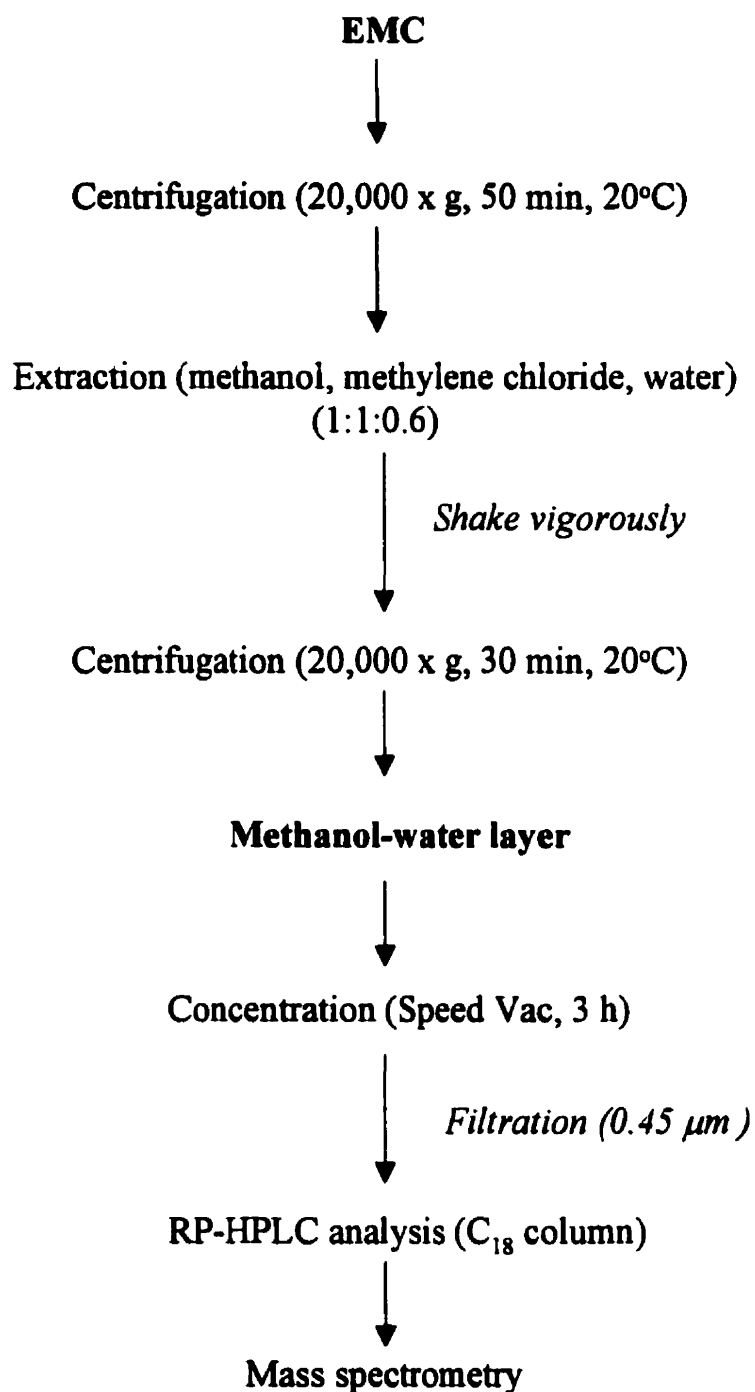


Fig. 2.2. Procedure for extraction of water-soluble peptides from EMC.

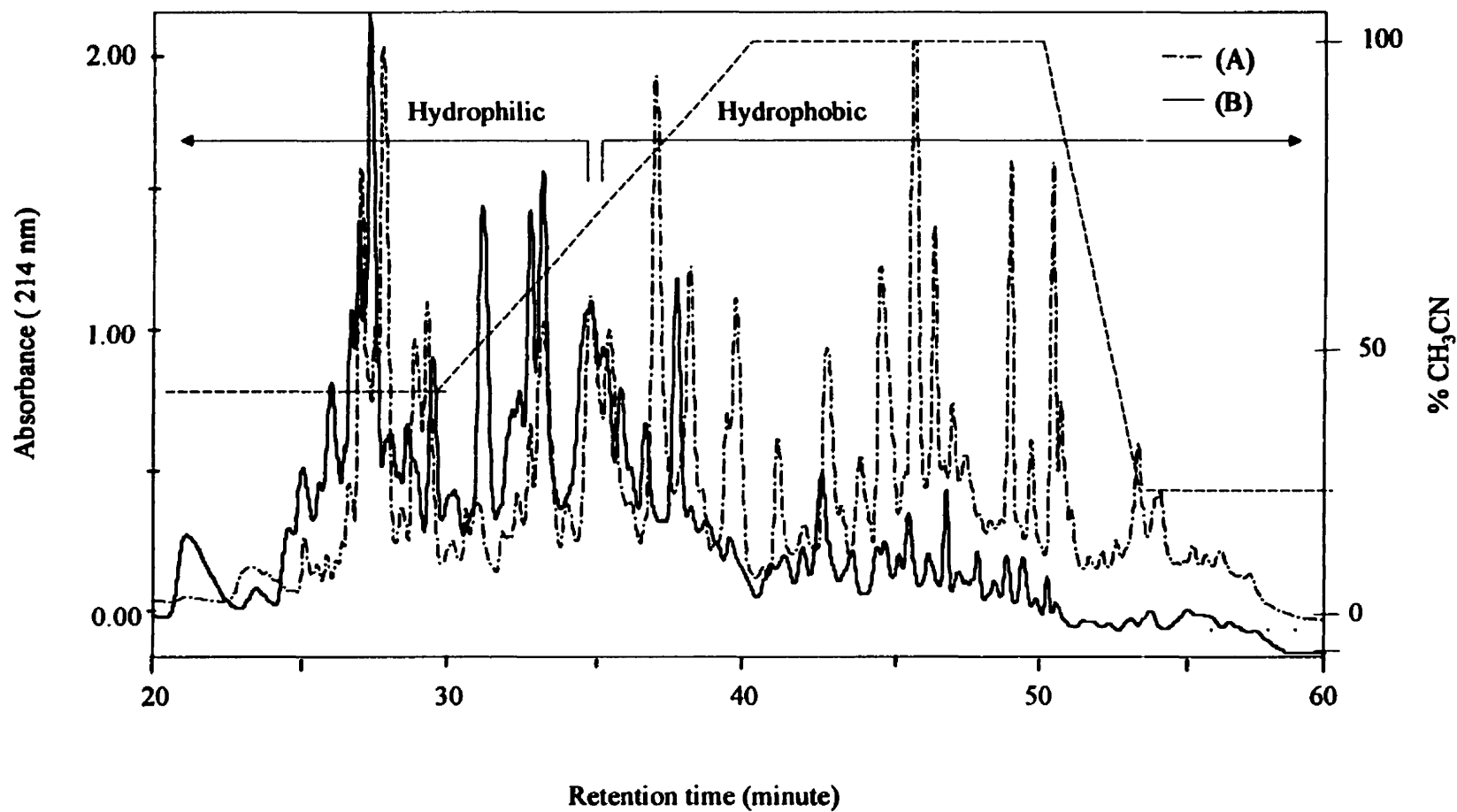


Fig. 2.3. Elution profile of water-soluble peptides from EMCs prepared by Neutrase® (EMC-N8) (A) and by combination of Neutrase®/*L. casei* enzymes (EMC-NL72) (B).

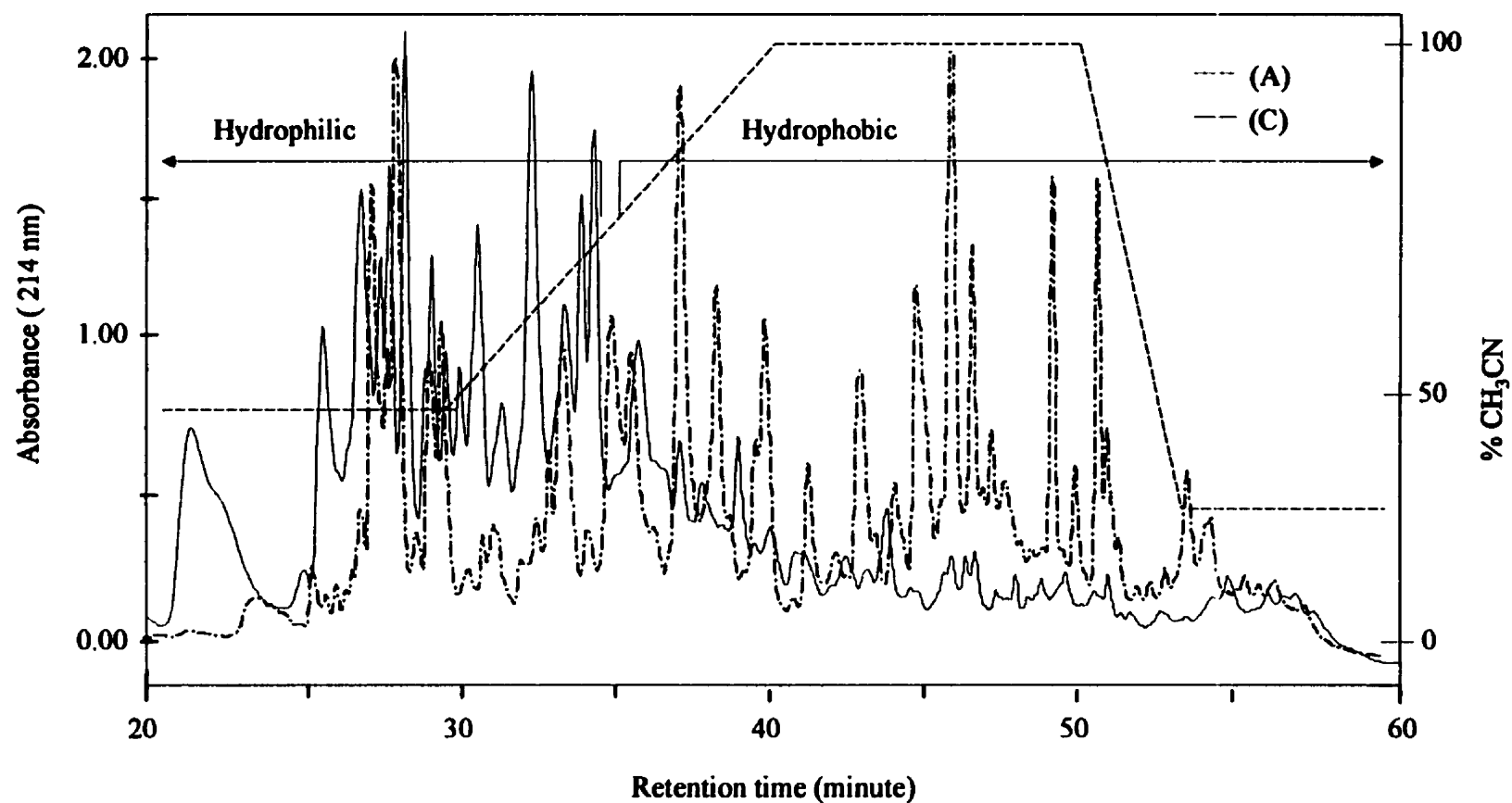


Fig. 2.4. Elution profile of water-soluble peptides from EMCs prepared by Neutrased® (EMC-N8) (A) and by combination of Neutrased®/Debitrase™ (EMC-ND96) (C).

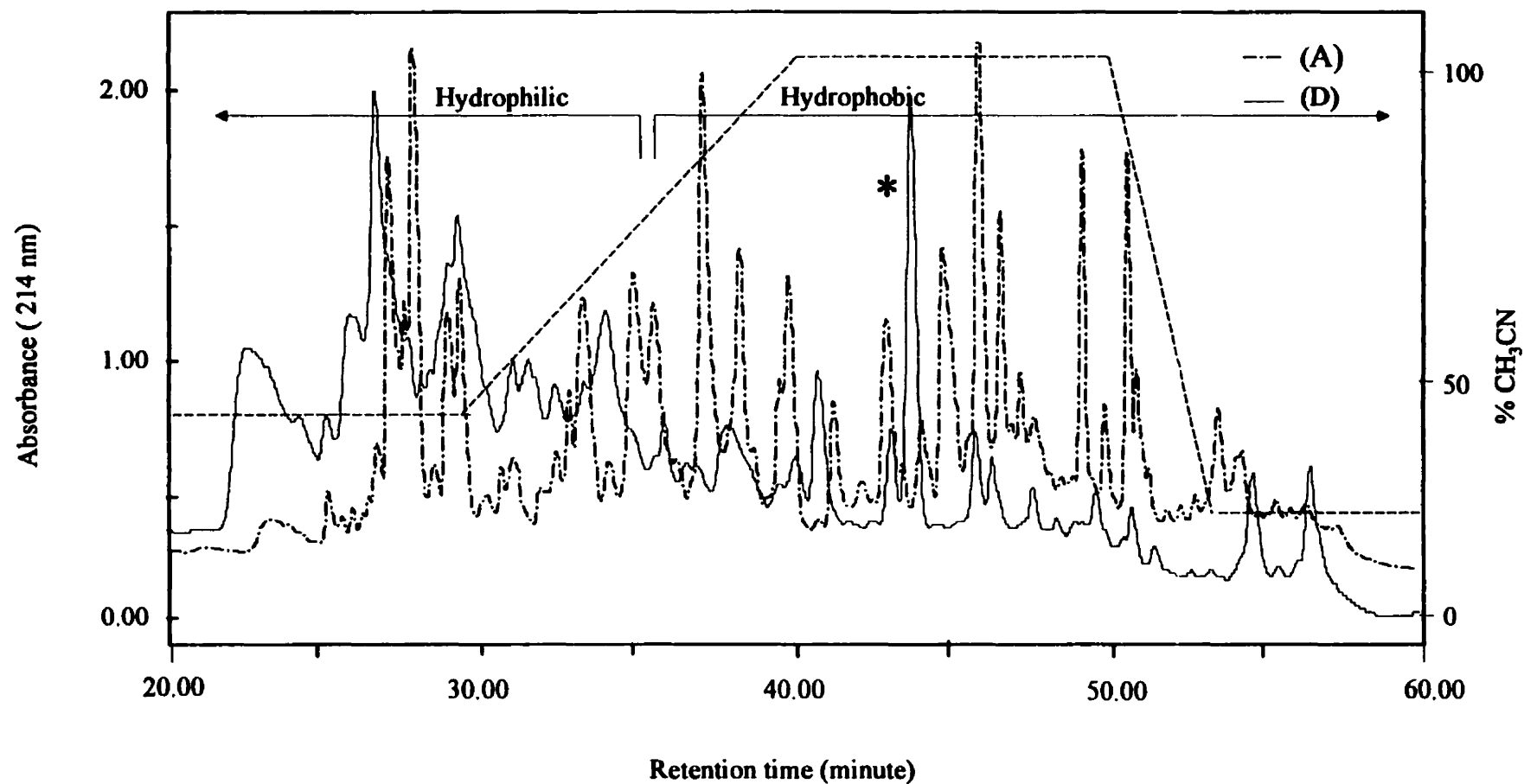


Fig. 2.5. Elution profile of water-soluble peptides of EMCs prepared by Neutrase® (EMC-N8) (A) and by combination of Neutrase®/Flavorzyme™ (EMC-NF36) (D).

CHAPTER 3.0

PURIFICATION AND IDENTIFICATION OF POTENTIAL BIOACTIVE PEPTIDES FROM ENZYME-MODIFIED CHEESE PREPARED BY *LACTOBACILLUS* AND COMMERCIAL ENZYMES

The mechanism of formation of potential bioactive peptides during preparation of EMC was studied. Potential bioactive peptides were purified and identified and the sequence of amino acids for these peptides was proposed.

The results of this study were summarized and published in Journal of Dairy Science. The manuscript entitled "Purification and identification of potential bioactive peptides from EMC prepared by *Lactobacillus* and commercial enzymes" was co-authored by Seble S. Haileselassie, Bernard F. Gibbs and Byong H. Lee. The mass spectrometry analyses were conducted by Bernard F. Gibbs. Co-authors Bernard F. Gibbs and Byong H. Lee edited the manuscript prior to submitting it for publication. The project was supervised by Dr. Byong H. Lee, while the actual experimental work and writing of the manuscript were done by Seble S. Haileselassie.

3.1 ABSTRACT

Antihypertensive peptides inhibiting angiotensin I-converting enzyme have been isolated from enzymatic hydrolysates of various food materials, but no information is available on the isolation of antihypertensive peptides from enzyme-modified cheese. In this study, several bioactive peptides mainly potential antihypertensive peptides from enzyme-modified cheese prepared by commercial and *Lactobacillus casei* enzymes, were purified and identified. Enzyme-modified cheese samples were prepared by combination of Neutrase® (1883.0 U/ml), *Lactobacillus casei* enzymes (aminopeptidase activity 86.4 leucine aminopeptidase U/g), and Debitrase™ (22.0 leucine aminopeptidase U/g). The water-soluble fractions of the enzyme-modified cheese that were prepared by combination of Neutrase®/*L. casei* enzymes and Neutrase®/Debitrase™ were subjected to reverse phase HPLC on a Delta Pak C₁₈ column, and each peak was purified on the same column using a binary gradient. One peak from the Neutrase® digest, five peaks from the Neutrase®/Debitrase™ digest, and two peaks from the Neutrase®/*Lactobacillus* enzyme digest were purified and identified by API mass spectrometry. On the basis of their molecular masses, amino acid sequences of purified peptides were identified. β -Casomorphin with a sequence like that of β -casein (YPFPGPI f 60-66) was found after the Neutrase® digest. All the seven peptides purified from the digests of Neutrase®, Neutrase®/*Lactobacillus* enzymes, and Neutrase®/Debitrase™ contained potentially active sites in their sequences. The presence of sites containing potential antihypertensive peptides suggests that the purified peptides may have antihypertensive properties. Thus, the enzyme-modified cheese process, mainly designed to produce flavor ingredients, may simultaneously produce bioactive peptides, which are considered to be of physiological importance.

Key words: bioactive peptide, antihypertensive peptide, enzyme-modified cheese, *Lactobacillus casei*

3.2 INTRODUCTION

Milk protein is a rich source of bioactive peptides (BAP) such as antihypertensive peptides [angiotensin converting enzyme (ACE) inhibitory peptides], opioid peptides, immunostimulating peptides, antimicrobial peptides, and cholesterol lowering peptides (Meisel, 1997). Possible roles of casein hydrolysates as antihypertensive agents, immunostimulants, and exorphins have recently been suggested by several groups (Harwalkar and McMahon, 1993; Yamamoto *et al.* 1994a,b; Kuwabara *et al.* 1995; Nakamura *et al.* 1995; Maeno *et al.* 1996; Yamamoto, 1997; and Mullally *et al.* 1997). β -Casomorphins are particular peptide sequences in β -CN that have received more research attention than other BAP. Various lengths of β -casomorphin have been isolated from enzymatic digests of casein or have even been synthesized. The primary sequence of these β -casomorphins corresponds to amino acid residues of β -CN (f 60-70) (Park *et al.* 1996). The N-terminal Tyr residue found in all β -casomorphins is critical to their bioactivity (Schlimme *et al.* 1988). Because of the high content of proline residues, these peptides are apparently resistant to proteolytic attack (Kreil *et al.* 1983). During digestion of β -CN, these BAP are absorbed intact, and they inhibit gastrointestinal (GI) motility, as well as the emptying rate of the stomach by direct interaction with opioid receptors (Daniel *et al.* 1990).

The ACE is a dipeptidyl carboxypeptidase that catalyzes the production of the vasoconstrictor angiotensin II, and the inactivation of the vasodilator bradykinin plays an important role in blood pressure regulation and hypertension (Yamamoto *et al.* 1994a,b). Recently some ACE were produced by the enzymatic hydrolysis of caseins. Nakamura *et al.* (1995a,b) reported that two peptides with amino acid residues of VPP and IPP, isolated from sour milk fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, exhibited ACE inhibitory and antihypertensive activity. The concentrations of those peptides required to inhibit 50% of angiotensin I converting enzyme activity (IC₅₀) were 9 and 5 μ M, respectively.

Several peptides derived from α_{s1} -CN (f 24-31, f 170-199) and β -CN (f 168-175, f 183-190, f 113-127, f 193-210, f 70-97, f 191-210, and f 16-97) by *L. helveticus* CP790 proteinase also exhibited ACE inhibitory and antihypertensive activities on spontaneously hypertensive rats (SHR) after oral administration (Yamamoto *et al.* 1994a,b). Among those peptides, a peptide (f 43-69) derived from β -CN showed the highest ACE inhibitory activity; $IC_{50} = 4 \mu M$. A potent antihypertensive heptapeptide (KVLPVPG; f 169-175) from β -CN also identified in casein hydrolysate was produced using proteinase from *L. heleveticus*, but this peptide had lower ACE inhibitory activity; $IC_{50} = 1000 \mu M$ Maeno *et al.* (1996). The antihypertensive effect of the peptide was found to be dose dependent in that study.

The purposes of the present work were (1) to purify bioactive peptides from EMC produced by *Lactobacillus casei* and commercial enzymes and (2) to identify the peptides by comparing their sequences with sequences of known bioactive peptides.

3.3 MATERIALS AND METHODS

3.3.1 Strain and Preparation of Enzyme Extracts

Lactobacillus casei, isolated from a good quality Cheddar cheese, (Arora and Lee, 1990), was grown in MRS broth for 18 h at 30°C. The cells were harvested by centrifugation (8,000 x g, 15 min, 4°C) and washed with 0.05 M phosphate buffer (pH 7.0). The harvested cells were then disintegrated at 2 s intervals for 1 min using an Ultrasonic disintegrator (Sonifier 450, Branson, Danbury, Connecticut, USA), and the crude extract was obtained after centrifugation (10,000 x g, 30 min, 4°C).

3.3.2 Enzyme Purification

The crude extract was precipitated using ammonium sulfate fractionation (45 to 80%). The precipitate was dissolved in a minimum volume of 0.05 M phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer using a Spectrapor membrane

(30,000 Da molecular mass cutoff; Spectrum Co., Houston, TX). Aminopeptidase was purified using a FPLC[®] system equipped with an ion-exchange column (Mono Q 16/10; Pharmacia, Montreal, Canada) and a gel-filtration column (Superose, HR 10/30; Pharmacia), according to the methods described by Arora and Lee (1992).

3.3.3 Protein and enzyme assays

Protein was determined spectrophotometrically at 562 nm using the bicinchoninic acid (BCA) assay reagents supplied with the system (Pierce Chemical Ltd., Rockford, IL). Bovine serum albumin (Sigma Chemical, St. Louis, MO) was used as a standard. The assay mixture containing 180 µl of 0.05 M buffer (pH 7.0), 10 µl of substrate and 10 µl of enzyme fraction was incubated at 32°C for 30 min. Activity was determined spectrophotometrically at 410 nm by measuring the amount of *p*-nitroaniline produced from leucine-*p*-nitroanilide. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitroaniline/min from leucine *p*-nitroanilide. Specific activity was expressed as the enzyme unit per milligram of protein.

3.3.4 Preparation of EMC

The EMC was prepared according to the method of Park *et al.* (1995). Shredded mild Cheddar cheese (600 g) was mixed with 2.5% Na₂HPO₄ and 195 ml of distilled water. The mixture was heated at 90°C for 3 min to emulsify the cheese slurry and to inactivate endogenous enzymes and then was cooled to 50°C. Neutrase [0.5L (1883 U/g)] was filtered through a 0.45 µm membrane filter and added to the cheese slurry. The slurry was incubated for 8 h under vacuum at 45°C. Resulting Cheddar cheese slurries (50 g) were then treated separately with 1) crude extracts from *L. casei* (86.4 LAPU/g) at 34°C for 72 h and 2) Debitrase (22.0 LAPU/g) at 40°C for 96 h.

3.3.5 Extraction of water-soluble peptides

The modified method of McGugan *et al.* (1979) was used to extract the water-soluble peptides of the EMC. Portions (5 g) of EMC samples were centrifuged (25,000 x g, 50 min, 20°C). The aqueous layer (1 ml) was mixed with 1 ml of methylene chloride and 0.6 ml of water and shaken vigorously. After centrifugation (25,000 x g for 30 min), 1 ml of the methanol-water fraction was concentrated by a Speed Vac (Savant Instruments, New York, NY) for 3 h and dissolved with 50 µl of distilled water.

3.3.6 RP-HPLC analysis

The Waters HPLC system (600 E System Controller, U6K Injector, 486 Tunable Absorbance Detector, and a Millennium 2010 Chromatography Manager; Millipore, Milford, MA) was used to separate peptides. Portions (25 µl) of the concentrated water-soluble fractions were injected on a Delta Pak C18 column (100 Å, 30 x 150 mm). The peptides were eluted at a flow rate of 0.5 ml/min with a binary gradient from 20 to 40% of solvent B (0.08 % TFA in a mixture of acetonitrile/water at 40:60 ratio) for 15 min, from 40 to 60% for 15 min, from 60 to 100% for 5 min, and from 100 to 20% for 35 min using a system controller. Solvent A was 0.1% TFA in deionized water. The elution was monitored at an absorbance of 214 nm. All samples were filtered through a 0.45-µm high syringe filter before injection.

3.3.7 Identification of peptides

To identify bioactive peptides from EMC samples, selected peaks were collected and purified (three times) by reverse-phase HPLC on the same column with the same gradient. Collected samples were then analyzed by API-mass spectrometry to determine their molecular masses and their amino acid sequences. Identification of peptides from enzymatic digests was based on the amino acid composition; the nearest integer was determined by the molar ratios of amino acids of the peaks, and that of Neutrase® was determined by the ion-mass of individual peptides within the range of ± 0.5 to 1.0 atomic

mass unit (amu). to the actual mass of the peptide with 100% or at least 75% relative intensity.

3.3.8 Mass spectrometry

Mass spectra were obtained in the positive mode on a triple stage mass spectrometry (Model API III; Scix, Toronto, Canada). Samples were dissolved in 10% acetic acid and infused through a stainless steel capillary (100- μ m i.d.). A stream of air (pneumatic nebulization) was introduced to assist the formation of submicron droplets (Covey *et al.* 1988). These droplets were evaporated at the interface by nitrogen gas producing highly charged ions, which were detected by the analyzer. The calibration of the system was performed using ammonium adduct ions of polypropylene glycol with known mass to charge (m/z) ratio throughout the range of 0 to 2470 amu. Mass charges were used throughout the range of the instrument (0 to 2470 amu). Simple algorithms were used to correlate the charges produced by these compounds to their molecular masses. After determining the molecular mass of different fragments, sequences of amino acids for peptides 1, 2, 3, 4, 5, 6, 7, and 8 were proposed.

3.4 RESULTS

Figure 3.1 shows the peptide profiles of the Neutrase digest (EMC-N8), Neutrase[®] and *L. casei* enzyme digest (EMC-NL72), and Neutrase[®] and Debitrase[™] digest (EMC-ND96). Most of the peptides from EMC N8 were eluted in a wide range of acetonitrile concentrations (from 100% to 20%) and more peptides appeared in the hydrophobic region (the second half). In contrast, peptides from EMC NL72 and EMC ND96 appeared in the hydrophilic region (the first half). This elution profile indicates that hydrophobic peptides that were eluted late produced by Neutrase were hydrolyzed by peptidases from *L.casei* and commercial enzymes. This finding is similar to those of Minagawa *et al.* (1989); Cliffe and Law, (1990); and Park *et al.* (1995).

Because of the similarity of the retention time (48 min) to the standard β -casomorphin, one peak that was eluted from EMC N8 at 48 min was purified and analyzed by API-MS. Figure 3.2 shows the purified peptide from EMC prepared by Neutrase only (peptide 1) and the standard β -casomorphin. The purified peptide was identified as β -casomorphin and had the same sequence as (YPFPGPI, f 60-66) of β -casein as the standard β -casomorphin. A total of 5 peaks from EMC ND96 (Fig. 3.3) were collected and analyzed by API-Mass Spectrometry: peak 2, 3, 4, 5, and 6. Two peaks (Fig. 3.4) 7 and 8, from EMC NL72 were also purified and analyzed by API-Mass Spectrometry. All the purified peptides were identified to be potential antihypertensive peptides.

Table 3.1 shows the sequence of amino acids for the fragments obtained by mass spectrometry. As β -CN is the most hydrophobic of the caseins that undergo strong temperature dependent association, all identified amino acid sequences obtained after fragmentation by API-Mass Spectrometry were based on the sequence of β -CN. In Table 4.1 the sequence of amino acids for peaks 1, 2, 3, 4, 5, 6, 7, and 8 were identified as follows: YPFPGPI for peak 1, LTLTDVE for peak 2, YPQRDMPIQAFLLYQEPV for peak 3, EMPFPKYPVEPFTESQSLTL for peak 4, SLVYPFPGPIPNLSLPQNIPPLT for peak 5, LVYPFPGPIPNLSLPQNIPPLT for peak 6, PGPIP for peak 7, and PKHKEMPFPKYPVEPFT for peak 8. For identification of amino acids, see appendix I.

3.5 DISCUSSION

In this study, β -casomorphin with a sequence of YPFPGPI (f 60-66 of β -CN) was detected in the EMC digest with Neutrase only (EMC N8). However, the opioid activity was not determined. The detection of β -casomorphin in Neutrase digest suggests that Neutrase (a neutral proteinase) from *Bacillus subtilis* has specificity to break V-Y (59-60) and I-P (66-67) bonds of β -casein. β -Casomorphin was absent in EMC-NL72 and EMC-ND96. The absence of β -casomorphin could be the result of two factors. Firstly, β -casomorphin was not resistant to proteolysis and might be degraded by proteolytic and peptidolytic system of *Lactobacillus* and commercial enzymes. Secondly, β -casomorphin

might be present in a concentration below the HPLC detection threshold value. A more sensitive analytical method such as radioimmunoassays might be appropriate to detect lower concentrations. However, if no physiological benefits could be gained by trace amounts, further analysis of lower concentrations might not be necessary. In addition to the loss of β -casomorphin, we also observed the increase in the peak in hydrophilic region.

Potential antihypertensive peptides were detected in EMC-ND96 and EMC-NL72. These results suggest that β -casomorphin and/or other hydrophobic peptides released by Neutrase might serve as a precursor for antihypertensive peptides. The residues found in peak 7 and 8 (corresponding to PGPIP and PKHKEMPFPKYPVEPFT) suggest that aminopeptidases from *L. casei* have specificity to break F-P (60-61), P-N (67-68), A-P (103-104), and T-E (120-121) bonds of β -casein. The detection of peaks (2, 3, 4, 5, and 6) as indicated in Table 3.1 suggests that Debitrase, a blend of peptidases extracted from *Lactococcus lactis* and *Aspergillus oryzae* has specificity to break S-L (124-125), E-N (131-132), P-Y (179-180), V-L (197-198), K-E (107-108), L-T (127-128), Q-S (56-57), T-Q (78-79) and S-L (57-58) bonds of β -casein. Although the identified peptides are longer than the antihypertensive peptides reported on literature and be hidden by the peptides configuration, they may be degraded upon digestion and may exert antihypertensive activity.

The formation of peptides containing active sites suggests that EMC production may be an alternative process for the production of antihypertensive agents. However, it is important to determine the physiological benefits and application of these peptides by measuring their ACE inhibitory activity (IC_{50}) *in vitro* and *in vivo*.

3.6 CONCLUSION

The present study demonstrates that EMC process simultaneously produce bioactive peptides that may provide savory, nutritional, medical, and health benefits. The stability of β -casomorphin may be dependent on the type of enzyme present in the

system. As β -casomorphin can be hydrolyzed by proteolytic enzymes in the living body, it might produce fragments with potent inhibitory activity *in vivo*. However, further studies are necessary to elucidate the formation mechanism and physiological significance of biologically active peptides.

Table 3.1. Sequences of amino acids for the peptides derived from EMCs prepared by Neutrase® (EMC-N8), by combination of Neutrase®/*L. casei* enzymes (EMC-NL72), and Neutrase®/Debitrase™ (EMC-ND96) after fragmentation by MS.

Treatment	Peak No.	Ion mass of actual peak	Fragment	Peptides sequence	Peptide MW
EMC-N8	1	790.94	(f60-66)	<u>Y</u> PFP <u>GPI</u>	791.00
EMC-ND96	2	790.89	(f125-131)	<u>LTL</u> TDVE	791.00
	3	2209.50	(f180-197)	<u>Y</u> PQR <u>D</u> MP <u>I</u>	2211.00
	4	2341.68	(f108-127)	AFL <u>LYQ</u> EPV	2343.00
				<u>EM</u> PFP <u>KYP</u> VEP	
	5	2362.77	(f57-78)	<u>F</u> TESQ <u>SL</u> TL	2263.00
	6	2275.50	(f58-78)	SL <u>V</u> <u>Y</u> PFP <u>GPI</u> P	
NSLPQ <u>NIP</u> PLT				2275.70	
EMC-NL72	7	480.58	(f63-67)	<u>P</u> G <u>PI</u> P	482.00
	8	2073.46	(f104-120)	PKH <u>K</u> EM <u>P</u> F <u>P</u> P	2074.00
				<u>KYP</u> VEP <u>FT</u>	

* Fragments of main peptides are bolded, bioactive sites are underlined and regions in the main peptides that contain overlapping bioactive sites are italicized.

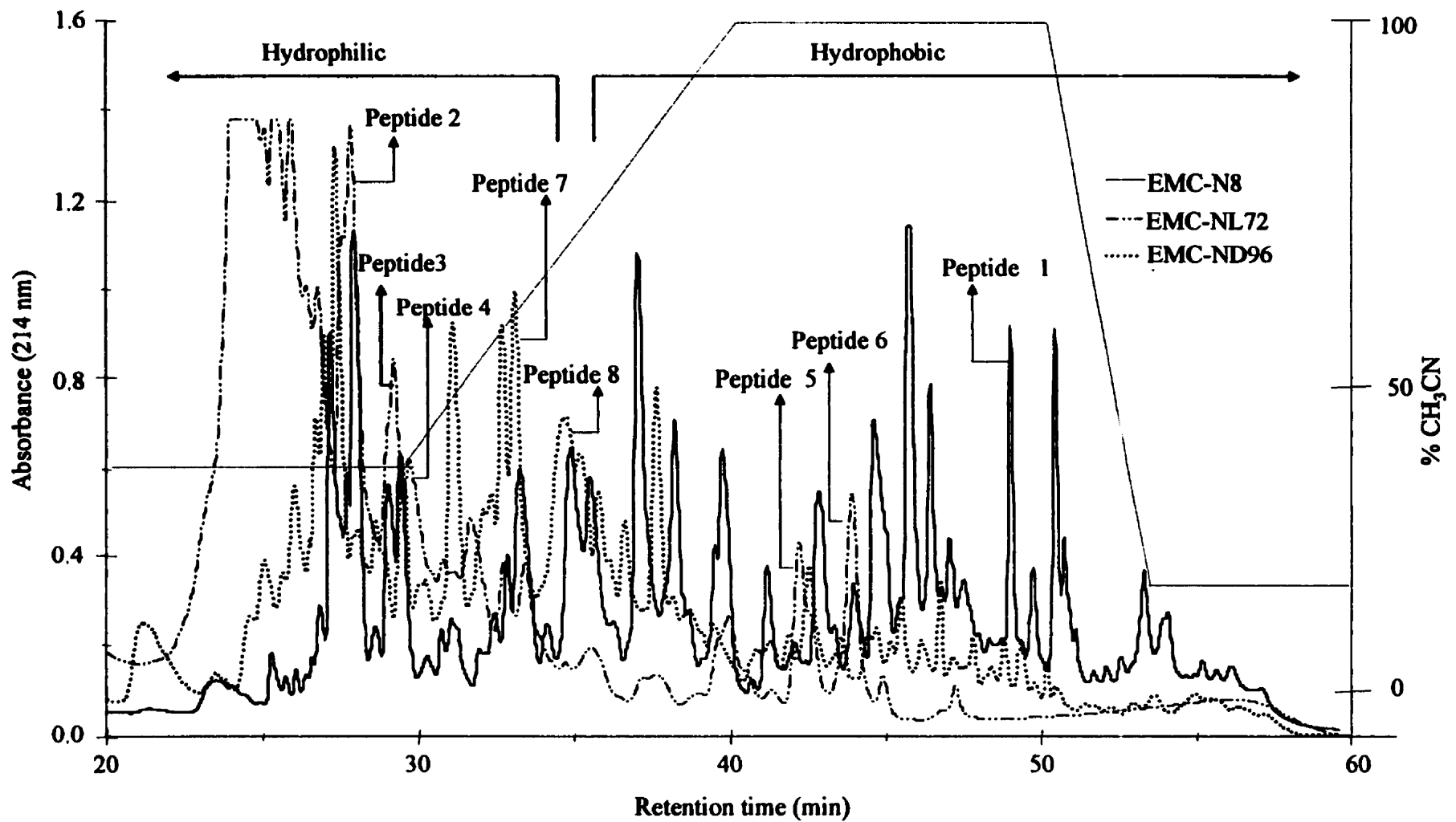


Fig. 3.1. Peptides profile of EMCs prepared by Neutrase (EMC-N8), combination of Neutrase®/*L. casei* enzymes (EMC-NL72), and Neutrase®/Debitrase™ (EMC-ND96).

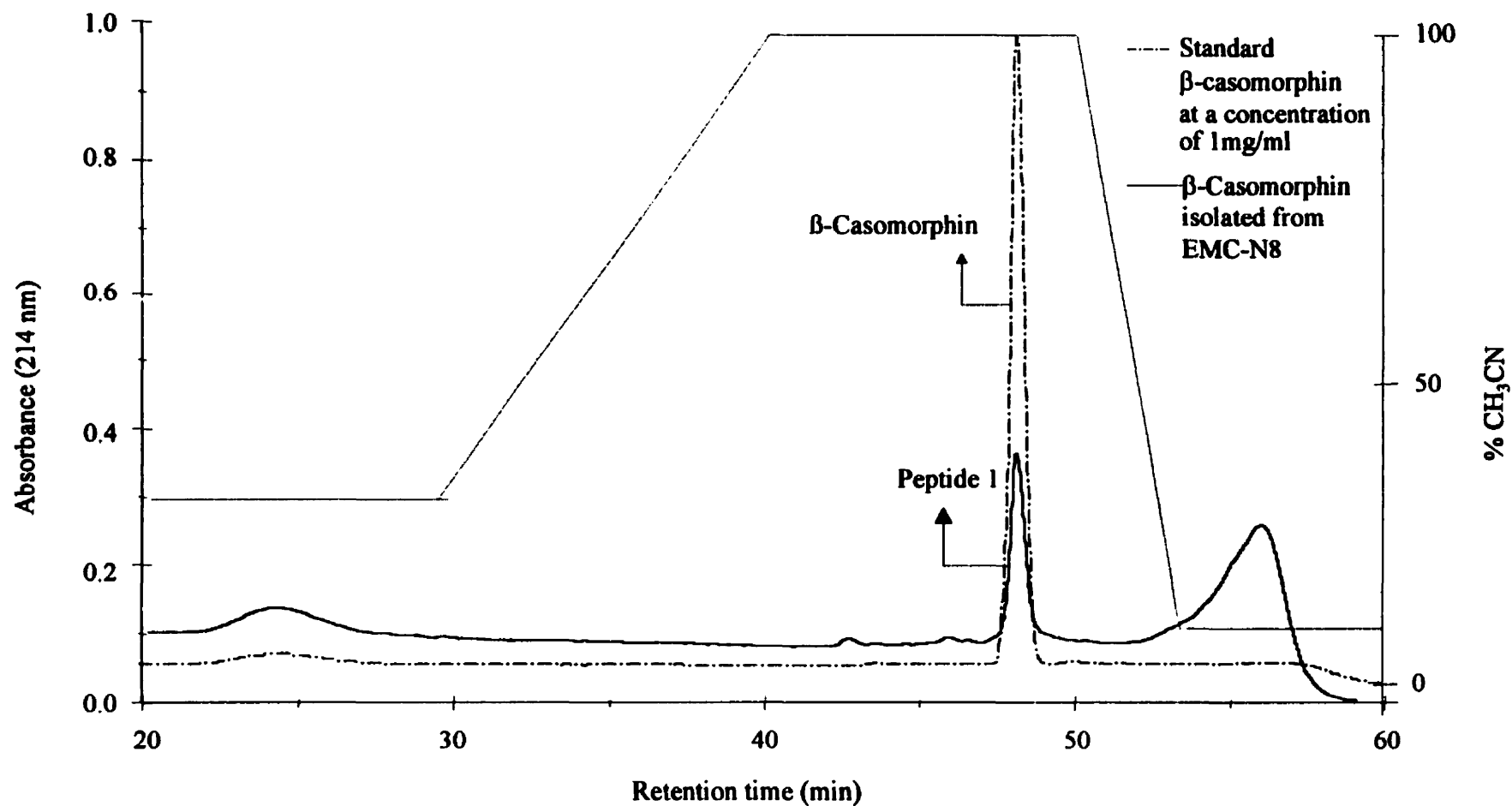


Fig. 3. 2. HPLC chromatogram of standard β -casomorphin (1 mg/mL) and β -casomorphin purified from EMC prepared by Neutrase[®] (EMC-N8).

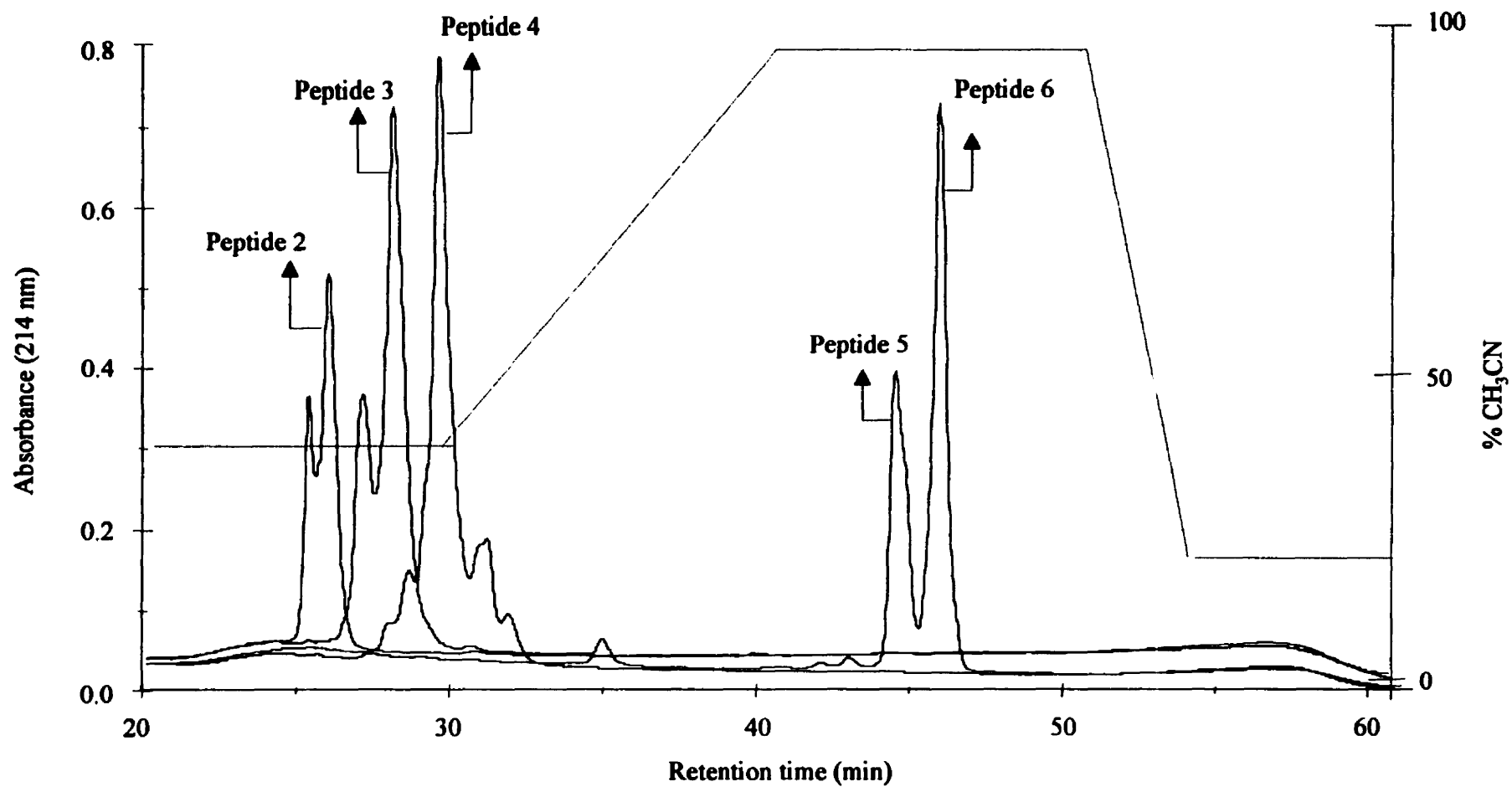


Fig. 3.3. Potential bioactive peptides purified from EMC prepared by combination of Neutrase®/Debitrase™ (EMC-ND96).

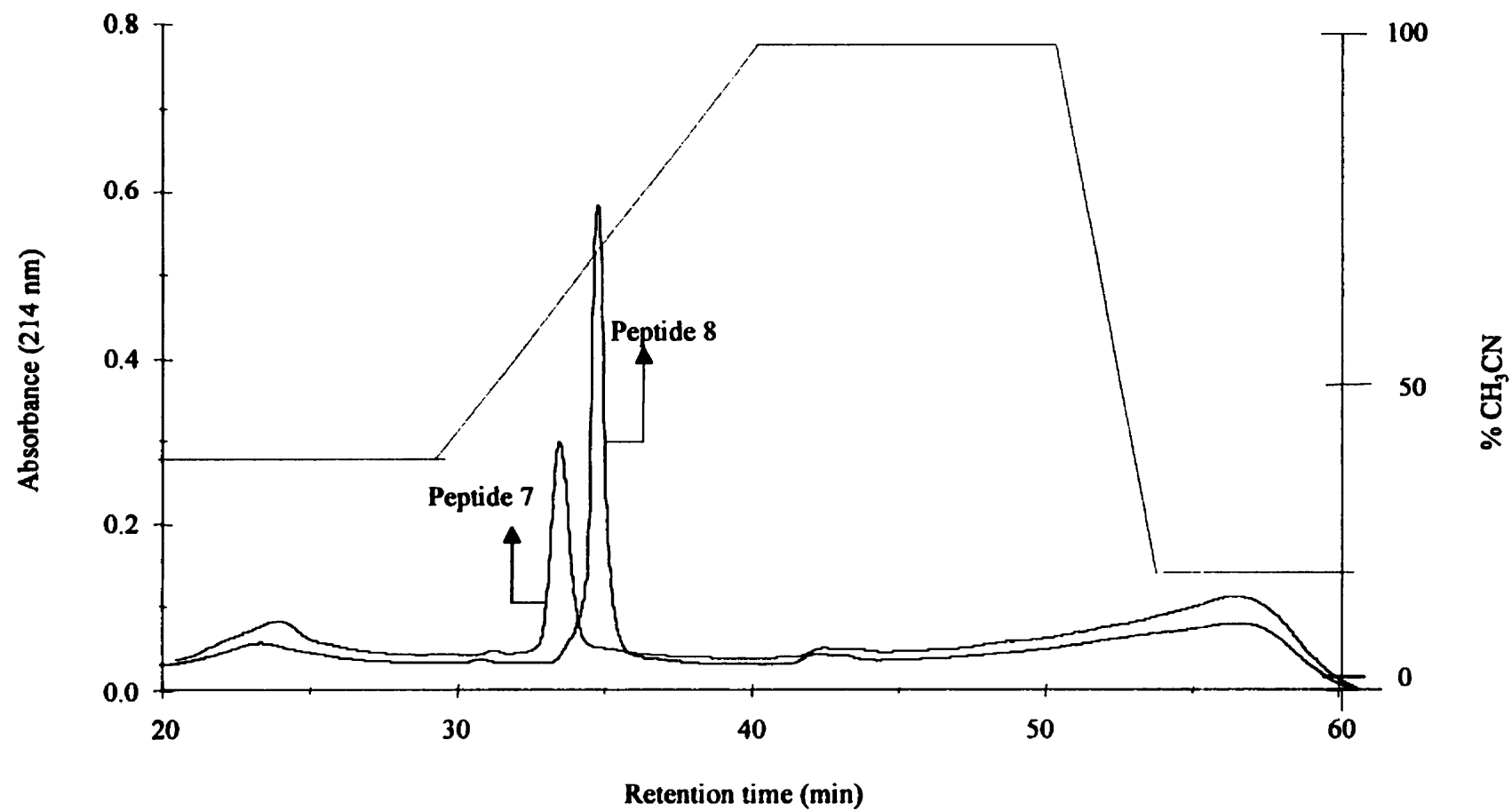


Fig. 3. 4. Potential bioactive peptides purified from EMC prepared by combination of Neutrase®/*L.casei* enzymes (EMC-NL72)

CHAPTER 4.0

ANALYSIS OF VOLATILE FLAVOR COMPOUNDS IN ENZYME-MODIFIED CHEESE AND CHEDDAR CHEESE BY USING DIFFERENT METHODS

This chapter is a complement to Chapter 2.0 where the optimum conditions for the production of EMC were established. The volatile flavor compounds in EMCs prepared by *Lactobacillus* and commercial enzymes were identified.

The results of this study were summarized in the form of publication suitable for journal publication. The manuscript entitled "Analysis of volatile compounds in enzyme-modified cheese and Cheddar cheese by using different methods" was co-authored by Seble S. Haileselassie, Varoujan A. Yaylayan and Byong H. Lee. The GC/MS analysis was conducted by Anahita Keyhani and Brian Stewart. The project was supervised by Dr. Byong H. Lee, while the actual experimental work and writing of the manuscript were done by Seble S. Haileselassie. The manuscript was revised by Drs. Byong H. Lee and Varoujan A. Yaylayan.

4.1 ABSTRACT

Two different techniques (Pyrolysis/GC/MS analysis and headspace GC/MS analysis) were applied for the identification of volatile flavor compounds in EMC as well as Cheddar cheese. For Pyrolysis/GC/MS analysis solid samples (1- 4 mg) of EMC and Cheddar cheese were pyrolyzed inside the coil probe at 200°C for 20 s and were then analyzed by GC/MS. In dynamic headspace GC/MS analysis, volatile compounds were separated from EMC samples headspace by purge and trap extraction, enriched by cryofocusing, and analyzed by GC/MS. Twenty compounds including ketones, fatty acids, alcohols, aldehydes, and lactone were detected in EMCs and Cheddar cheese (mild, old, and extra old) by using Py/GC/MS. Dynamic headspace analysis revealed the presence of 17 compounds including aldehydes, ketones, fatty acids, alcohols, and hydrocarbon in EMCs prepared by combination of different enzymes and mild Cheddar cheese. However, the overall impact of these compounds on cheese flavor is not known.

Key words: Pyrolysis, dynamic headspace GC/MS, purge and trap, EMC, Cheddar, volatile compounds, flavor

4.2 INTRODUCTION

Flavor is one of the most important factors affecting consumer acceptance and preference of dairy products. Cheddar cheese flavor has been extensively investigated and more than 180 compounds are identified, however, after 5 decades of research, both the origin and the composition of Cheddar cheese flavor remain puzzling questions (Aston and Dulley, 1982). The breakdown of milk proteins, fat, lactose, and citrate during ripening gives rise to a series of volatile and non-volatile flavor compounds (Engels *et al.* 1997). Free fatty acids, esters, aldehydes, alcohol, ketones, and sulfur compounds, that are generated from microbial, enzymatic, and chemical transformations were shown to be the major contributors of Cheddar cheese flavor (Moskovitz and Noelck, 1987).

Recent research on flavor profiling of headspace and vacuum distillate by GC/MS (Dimos *et al.* 1996), head space by GC/MS/sniffing (Arora *et al.* 1995), and vacuum distillate, dialysate, and solvent-extract by GC/MS/sniffing (Vandeweghe and Reineccius, 1990) of cheese samples provided an extensive list of compounds in Cheddar cheese. Although no one of these compounds represented the characteristic aroma of Cheddar, Dimos *et al.* (1996) observed that there was a correlation between the intensity of Cheddar flavor and the concentration of a combination of methanethiol and decanoic or butanoic acid. Apart from that, the weaker Cheddar flavor in reduced-fat cheese was also found to be due to the lower level of methanethiol. Arora *et al.* (1995) reported that lipid-derived aldehydes, methyl ketones, and esters were the principal aroma-bearing components present in the volatiles isolated from Cheddar cheese, on the other hand, alcohols and hydrocarbons were shown to contribute little to the aroma of Cheddar cheese. Vandeweghe and Reineccius (1990) were the first to identify 2-propanol, 1,3-butandiol, δ -undecalactone, and γ -decalactone as flavor components of Cheddar cheese. These authors point out that great many compounds are no doubt involved in the flavor and aroma of Cheddar and the authors also support the component balance theory for Cheddar cheese flavor (Kosikowski and Mocquot, 1958). In addition, some odor-active compounds, in concentration below the threshold level of analytical instrument were also found to contribute to Cheddar cheese flavor and aroma.

Studies suggest that no single free fatty acid (FFA) is predominant contributor to cheese flavor, however, free fatty acids are significant contributors to the Cheddar cheese aroma and play an important role in terms of organoleptic quality (Wood and Lindsay, 1982; de Jong and Badings, 1990). Ohren and Tuckey (1969) reported that typical Cheddar flavor was related to the balanced proportion of free fatty acids. Large amount of butyric acid, which might originate from butyric acid fermentation is nevertheless, undesirable (Engels *et al.* 1997). Excess concentrations as well as unbalanced proportions of the free fatty acids cause the rancid off-flavor in cheese (Woo and Lindsay, 1982). In spite of the many compounds, which have been isolated from Cheddar cheese, so far no one was able to isolate a compound or compounds with the flavor impact of Cheddar. Various methods have been applied for the extraction, isolation and identification of volatile compounds from food products. They can be divided into five main groups: (1) solvent extraction, (2) supercritical fluid (superfluid) extraction (SFE) using CO₂, (3) steam distillation, (4) high-vacuum distillation and (5) headspace techniques (Bosset and Gauch, 1993). Direct injection of Cheddar cheese oil obtained by centrifugation (Liebich *et al.* 1970) and dialysis (Benkler and Reineccius, 1979) were also used for obtaining gas chromatographic profiles of Cheddar cheese.

The ideal technique, however, should be not only reproducible and sensitive but also simple, inexpensive, and able to process a large number of samples in a short period of time. Headspace techniques (Bosset and Gauch, 1993; Wood *et al.* 1994; Yang and Min, 1994; Arora *et al.* 1995) are often used in recent years because distillation methods, or other extraction procedures, tend to suffer from artifact formation and decomposition of components (Manning *et al.* 1984; Wood *et al.* 1994). Mindrup (1998) demonstrated that solid phase microextraction (SPME) is very effective for analysis of flavor and fragrance compounds in liquid or solid samples. Pyrolysis coupled with gas chromatography/mass spectrometry (Py/GC/MS) has also been demonstrated to be fast and convenient technique for the analysis of Maillard reaction products (Huygheus-Despointes *et al.* 1994; Keyhani and Yaylayan, 1996), but no information is available on the use of this technique for analysis of flavor components in food.

The objectives of this work were to (1) identify flavor compounds in EMCs and Cheddar cheese by Pyrolysis/GC/MS and headspace for the analysis of volatile flavor and (2) understand the respective contribution of each flavor compound to the overall EMC flavor.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals and reagents

Unless otherwise specified, all standards were purchased from Chemservice Inc. (West Chester, PA, USA) and other chemicals used in this study were purchased from Sigma-Aldrich Canada (Oakville, ON).

4.3.2 Enzymes

The four enzymes used in this experiment are: (1) the crude extract from *Lactobacillus casei* ssp. *casei* LLG (esterase activity 110.06 U/g), (2) Neutrase[®] 0.5, a bacterial protease produced from *Bacillus subtilis* (1,883 U/g, pH 5.5-7.5), (3) Palatase[®] M 1000, a fungal lipase derived from *Rhizomucor miehei* (1,000 LU/g, pH 5.0-7.0), (4) Flavorzyme[™], a fungal protease/peptidase cocktail produced from *Aspergillus oryzae* (1,000 LAPU/g, pH 5.0-7.0) (Novo Nordisk A/S, Bagsvaerd, Denmark), and (5) Debitrase[™] DBP20, a blend of peptidase extracted from *Lactococcus lactis* and *Aspergillus oryzae* (220 LAPU/g, pH 5.0-7.0) (Imperial Biotech, London, UK).

4.3.3 Preparation of EMC

Mild, old and extra old Cheddar cheese of the same brand used in this study was obtained from local grocery. EMCs were prepared according to the method of Park *et al.* (1995). Fresh Cheddar cheese (600 g) was shredded and mixed with 2.5% phosphate salt (Na_2HPO_4) and 195 ml of distilled water. The mixture was heated at 90°C for 3 min, and

then cooled down to 50°C. Neutrase 0.5 L (1,883.0 U/g) was filtered through 0.45µm membrane filter and added to the cheese slurry. The slurry was incubated for 8 h under vacuum at 45°C. Cheddar cheese slurries (50 g) were then treated separately with: (i) crude extracts from *Lactobacillus casei* (esterase activity 110.6 U/ml) at 45°C for 24 h, (ii) Palatase (lipolytic activity 200 LU/g) at 34°C for 16 h, (iii) Debitrase (22.0 aminopeptidase LAPU/g) at 40°C for 96 h, and (iv) Flavorzyme (6.5 aminopeptidase LAPU/g) at 45°C for 36 h.

4.3.4 Pyrolysis/GC/MS analysis

A Hewlett-Packard GC/mass selective detector (5890 GC/5971BMSD) interfaced to a CDS pyroprobe 2000 unit was used for the Py/GC/MS analysis. Solid samples (1-4 mg) of EMCs and Cheddar cheese were introduced inside a quartz tube (0.33 mm thickness) plugged with quartz wool and inserted inside the coil probe. The pyroprobe was set at 250°C at a heating rate of 50°C/min and with a THT (total heating time) of 20 s. The GC column flow rate was 0.8 ml/min for a split ratio of 92:1 and a septum purge of 3 ml/min. The pyroprobe interface temperature was set at 200°C, capillary direct MS interface temperature at 180°C and ion source temperature at 280°C, respectively. The ionization voltage was 70 eV and the electron multiplier was 1682 V. The mass range analyzed was 30-300 amu. The column was a fused silica DB-5 column (30 mm x 0.25 mm; Supelco, Inc.). Unless otherwise specified the column initial temperature was -5°C for 2 min and was increased to 50°C at a rate of 30°C/min; immediately, the temperature was further increased to 250°C at a rate of 8°C/min and kept at 250°C for 5 min.

4.3.5 Headspace/GC/MS analysis

Frozen EMC and cheese samples (approximately 0.5 g) were thawed at room temperature (20°C) and weighed into a 55 mL tube and internal standard, 10 µL of D10-n-butanol, (9.2 µg/mL) was added for retention time adjustment. The tube headspace was purged with 20 mL/min helium gas at room temperature for 15 min. The volatiles emitted under these conditions were swept onto a stainless steel trap (4.5 mm i.d. x 105 mm long)

containing tenax TA 60/80 mesh (Supelco Inc., Toronto, ON). The excess water was then removed by dry purging the trap with 20 mL/min helium for 5 min. Desorption and enrichment of trapped volatiles was carried out with a Tekmar model LSC 2000 purge and trap (Tekmar Co., Cincinnati, OH) under the following conditions. The traps were preheated rapidly to 225°C, and the volatiles were enriched onto glass beads in a glass tube by cryofocusing at -80°C with liquid nitrogen. The volatiles were desorbed by heating the cryofocusing unit to 230°C for 5 min and injected into the column for 1 min. Temperature of transfer line was 225°C. Tenax traps were reconditioned after each run at 225°C in a stream of helium (80 mL/min) and stored in a desiccator at 4°C.

A Varian 3,400 GC (Scientific Instrument Services, Ringoes, NJ, USA) equipped with DB-5 (30 x 0.32 i.d, Supelco, Inc) was used for purge and trap GC/MS analysis. The column was operated with helium carrier gas at a flow of 0.7 mL/min. The oven temperature was programmed from 40°C with 5 min hold to 150°C at rate of 5°C/min; immediately the temperature was further increased to 240°C at a rate of 30°C/min with a 2 min hold at a final hold. The GC column was connected directly to the ion source (180°C) of Finnigan Incos 50 GC/mass spectrometer (Finnigan Corporation, Sunnyvale, CA, USA). Mass analyzer was set at 35-350 m/z at 0.31 sec/scan.

4.4 RESULTS AND DISCUSSION

4.4.1 Flavor compounds identified in EMC and Cheddar cheese by Py/GC/MS

Py/GC/MS has the advantage of minimal manipulation of the sample, which avoids undesirable contaminants and artifacts. Huygheus-Despointes *et al.* (1994), Keyhani and Yayalayan, (1996), and Huygheus-Despointes and Yaylayan, (1996) demonstrated that Py/GC/MS is a fast and convenient technique for the analysis of Maillard reaction products, but no information is available on the use of this method for the analysis of flavor compounds in dairy products. Preliminary trials on flavor analysis by Py/GC/MS demonstrated the presence of only 20 compounds in EMCs prepared by using combinations of Neutrase®/*L.casei* (EMC-NL72), Neutrase®/Palatase® M

(EMCNP-16) in Fig. 4.1. The volatile compounds identified from EMCs and Cheddar (mild, old, and extra old) include ketones, fatty acids, alcohols, aldehydes, and lactone. All the volatile compounds detected in this study have been previously reported for other cheeses, but with different concentrations. Among the 20 compounds detected, seven components were identified in both EMCs and Cheddar cheese. Qualitative differences between EMCs and Cheddar cheese (mild, old, and extra old) are shown in the flavor profiles presented in Fig. 4.1 and 4.2, respectively. All the compounds detected by this method were also identified (Table 4.1). The results indicate that most of the components were present in all cheese types (mild, old and extra old), but at different concentrations estimated by areas of peaks.

Figure 4.3 shows the comparison of aldehydes, ketones, alcohols, and lactone detected in Cheddar (mild, old, and extra old), EMC-NL24 and EMC-NP16. EMC-NL24 was found to contain all aldehydes, ketones, alcohols, and lactone identified in this study. An important factor for the cheesy pleasant flavor (taste panel results, Section 2.4.2) of EMC-NL24 appeared to be the presence of all the components in the correct ratio. 2-Propanol, which could be originated from acetone was the major alcohol present in EMC-NL24 and mild Cheddar. This finding was in agreement with those from Vandeweghe and Reineccius (1990) and Urbach (1993). However, 2-propanol was absent in old and extra old Cheddar. This is probably due to its interaction with other components in cheese or loss during maturation. It is widely accepted that alcohols have little influence on cheese flavor, however, they may indirectly contribute to flavor because of their ability to form esters with fatty acids.

Fatty acids are important components in flavor of many cheese types, however, the presence of butyric acid at high concentration is reported to confer undesirable flavor to cheese though butyric acid is important to cheese flavor at a balanced proportion (Engles *et al.* 1997). The fatty acids contribute to desirable flavor but too high on FFA concentration may induce a rancid off-flavor in the cheese. The rancid off-flavor of EMC-NP16 was also probably attributed to the formation of longer chain fatty acids ($>C_{12}$) at high concentration by the action of Palatase[®]M (lipolytic commercial enzyme)

on milk fat. However, the use of this enzyme at low concentration, in conjunction with other peptidolytic enzymes might help in producing an intense cheese flavor in a short period of time. Figure 4.4 shows the comparison of selected fatty acids in EMC-NL24, EMC-NP16 and Cheddar (mild, old, extra old). EMC-NL24 and extra old Cheddar have similar profile of fatty acids except for acetic and butyric acid. The former was present in EMC-NL24 whereas the latter was present in extra old Cheddar.

Except for butyric acid, propanoic acid, 2-heptanone, and benzenacetaldehyde, EMC-NL72 (prepared by combination with Neutrase® and *L. casei* enzymes in 72 h) contained most of the flavor components identified in extra old Cheddar. This suggests that *L. casei* enzymes can produce intense cheese flavor, comparable to that of extra old Cheddar in a matter of days. This finding was similar to those reported by Park *et al.* (1995).

Although Urbach (1993) concluded that there was no Cheddar flavor without sulphur compounds, flavor profiles using Py/GC/MS did not reveal the presence of sulphur compounds. Headspace study based on direct cryofocusing of volatiles also failed to detect sulphur compounds, mainly hydrogen sulfide and methanethiol, and probably due to degradation of these compounds during sample preparation (Wood *et al.* 1994) and during pyrolysis at high temperature (250°C for 20 s). The presence of sulphur compounds and their contribution to various cheese flavor was also reported by many researchers (Manning, 1979; Hemme *et al.* 1982; Adda, 1986; Barbieri *et al.* 1994).

4.4.2 Flavor compounds identified in EMC and Cheddar cheese by dynamic headspace GC/MS

Dynamic headspace GC/MS analysis has been shown to be reliable, and highly sensitive for identification of volatile compounds in the headspace of dairy products. In the present study, 17 compounds including 3 alcohols, 4 ketones, 3 acids, 4 aldehydes, 1 each of hydrocarbon, acetonitrile, and chloroform were detected using dynamic headspace GC/MS analysis. As no quantitative data can be obtained only qualitative data

on all the compounds were presented in Table 4.2. One possible explanation for the presence of acetonitrile and chloroform in some of the samples is their contamination during sample preparation, storage or analysis. Typical GC chromatograms of volatile compounds identified in EMC-NL24, EMC-NP16, EMC-ND96, EMC-NF36 and mild Cheddar cheese are shown in Fig. 4.5, 4.6, 4.7, 4.8, and 4.9, respectively. Ethanol, 2-propanol, 2,3 butanedione, acetic acid, 2-butanone, nonanal, and decanal were detected in all samples. Butyric acid was present in EMC-ND96, EMC-NF36, and EMC-NP16 but was not detected in EMC-NL24 and mild Cheddar cheese. Similar results were obtained by using Py/GC/MS analysis. EMC-NF36 was found to contain 13 compounds identified in this study. Taste panel results also confirmed that EMC-NF36 had cheesy taste, which might be due to the presence of all the components in the EMC, however, this EMC was distinctly bitter (Section 2.4.2). Therefore, the use of Flavorzyme™ in combination with other peptidases may be used to produce commercially viable EMC. All samples contained 2,3 butanedione, which has a buttery, nut-like flavor (Welsh *et al.* 1989), and considered being one of the most important ketone flavor compounds in cheese.

Although long chain fatty acids and ketones were detected in EMC-NL24, EMC-NP16 and mild Cheddar cheese in the previous analysis using Py/GC/MS, these compounds were not detected in any of the samples using dynamic headspace GC/MS analysis. However, all the volatile compounds identified in this study have been previously reported to be the major contributors to cheese flavor. The number of volatile flavor compounds identified in EMC-NL24 by purge and trap technique was lower than those identified by Py/GC/MS. This difference may occur due to: (i) preparation of the samples for analysis, (ii) mode of analysis, and (iii) analysis of samples at different time.

The presence of sulphur compounds in the headspace of Cheddar cheese and their contribution to Cheddar cheese flavor was reported by Arora *et al.* (1995), Dimos *et al.* (1996), and Engles *et al.* (1997). It has also been reported that the presence of sulphur compound is essential for Cheddar cheese flavor (Urbach, 1993). However, no sulphur compounds were detected in this study. The absence of sulphur compounds in the cheese headspace was likely attributed to: (i) degradation of sulphur compounds during storage

or sample preparation, (ii) poor absorption of sulphur compounds on the Tenax trap or their absorption at a concentration below the detection threshold value of MS, or (iii) their interaction with other compounds present in the cheese. The purging of the cheese samples in headspace was achieved at room temperature for only 15 minutes, less volatile compounds might not be emitted from the matrices of the samples. Therefore, purging the samples at higher temperature (50 - 60°C) for longer period could have been better to identify most of the volatile compounds present in all samples.

4.5 CONCLUSION

The results of this study indicate that the flavor of EMC and Cheddar cheese is indisputably due to the presence of numerous compounds; fatty acids, aldehydes, alcohols, ketones, hydrocarbon, and lactone. The contribution of each component identified by using Py/GC/MS and dynamic headspace was not determined. However, there was a strong correlation between the level of long chain fatty acids and rancid-off flavor (Section 2.4.2) detected in EMC prepared by combination of Neutrase®/Palatase®M. Py/GC/MS is proved to be relatively simple and rapid approach to the analysis of flavor compounds in cheese. However, this is the first study on the use of Py/GC/MS for the analysis of flavor compounds in cheese, and thus further studies are required to standardize the method to identify and elucidate the contribution of each flavor compounds to cheese flavor. Dynamic headspace analysis and mass spectrometry detection was proved to be effective for analysis of volatile flavor compounds in cheese, but certain variables need to be standardized to make the system quantitative.

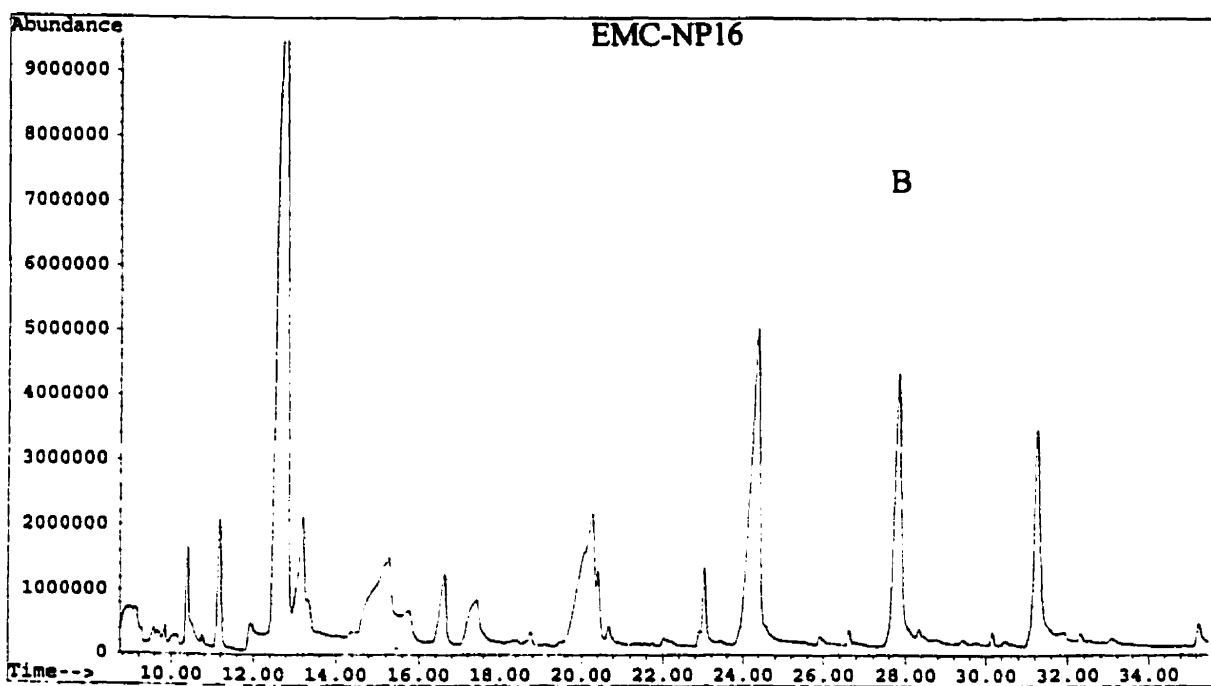
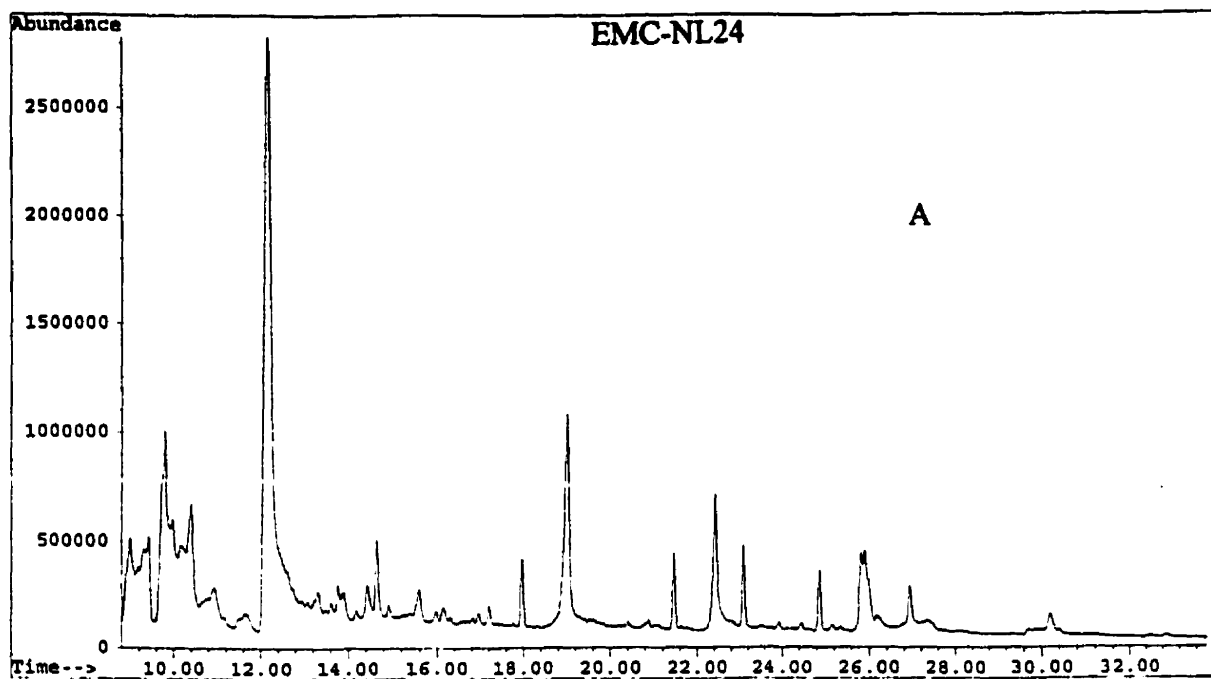
Table 4.1: Flavor compounds identified in EMC-NL24, EMC-NP16, and Cheddar (mild, old, and extra old) by

Compound	Treatment				Cheddar		
	<i>L. casei</i> (EMC-NL24)		Palatase (EMC-NP16)		Mild	Old	Extra old
	RT (min)	Area/mg	RT (min)	Area/mg	Area/mg	Area/mg	Area/mg
Acetic acid	8.7	1.50E+05	11.18	8.20E+06		4.90E+06	
2-Propanol	12.18	1.50E+07			4.30E+06		
n-Butyric acid			9.1-10.75	3.00E+07		1.87E+07	1.41E+07
2,3-Butanedione	10.4	2.20E+06				2.70E+06	
2-Heptanone							2.30E+06
Hexanoic acid	11.65	6.00E+06	11.94	2.20E+07	3.80E+06	4.80E+06	5.80E+06
1,2-Propanediol			12.79	1.10E+08			
Benzenacetaldehyde	14.44	4.60E+05			1.20E+06	3.90E+05	5.20E+05
2-Nonanone	14.66	7.80E+05	18.76	1.70E+06	9.40E+05	5.30E+05	7.60E+05
Nonanal	14.92	1.60E+05			2.80E+05	3.50E+05	2.30E+05
Octanoic acid	15.59	4.70E+06	20.27-20.42	3.49E+07	2.30E+06	2.30E+06	1.10E+06
Benzoic acid			20.68	2.00E+06			
2-Decenal					2.50E+05	3.00E+05	
2-Undecanone	17.97	5.70E+05			9.70E+05	3.90E+05	5.40E+05
Decanoic acid	19.02	4.60E+06	24.39	3.60E+07	6.40E+06	3.60E+06	5.60E+06
Undecanoic acid			25.91	3.60E+07			
2-Dodecenal					3.50E+05		
2-Tridecanone	21.46	6.00E+05	26.63	7.40E+05	8.90E+05	2.20E+05	4.80E+05
Dodecanoic acid	22.43	1.90E+06	27.84	3.20E+07	3.00E+06	1.20E+06	2.30E+06
Propanoic acid			28.34	1.80E+06	1.40E+06	1.10E+06	1.10E+06
2-pentadecanone	24.87	5.20E+05	30.16	6.80E+05	6.60E+05	1.40E+06	2.90E+05
Tetradecanoic acid	25.83	6.20E+05	31.28	2.30E+07			6.70E+05
Hexadecanoic acid			35.26	2.40E+06			
Tridecanlactone	25.9	1.10E+06					

Table 4.2: Flavor compounds identified in EMC-NL24, EMC-NP16, EMC-N96, EMC-NF36, and mild Cheddar cheese by dynamic headspace GC/M

Compound	Treatment				Mild Cheddar
	<i>L. casei</i> (EMC-NL24)	Palatase (EMC-NP16)	Debitrase (EMC-ND96)	Flavorzyme (EMC-NF36)	
Ethanol	+	+	+	+	+
Acetonitrile		+	+		
Acetone				+	
2-Propanol	+	+	+	+	+
2,3 Butanedione	+	+	+	+	+
Hexane					+
Acetic acid	+	+	+	+	+
Chloroform	+				+
2-Butanone	+	+	+	+	+
Butyric acid		+	+	+	
Heptanone				+	+
2,5 Furandione		+			
Hexanoic acid				+	
2 Furanone			+	+	
Octanal		+		+	+
Nonanal	+	+	+	+	+
Decanal	+	+	+	+	+

+ = detected



Retention time (min)

Fig. 4.1. Pyrograms of EMC prepared by combination of Neutrase®/*L. casei* enzymes (EMC-NL24) (A) and Neutrase®/Palatase® M (EMC-NP16) (B).

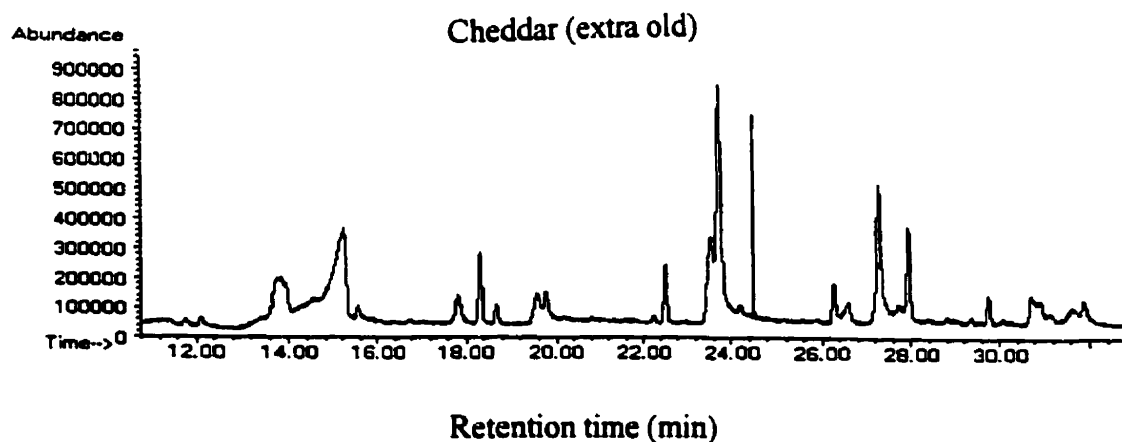
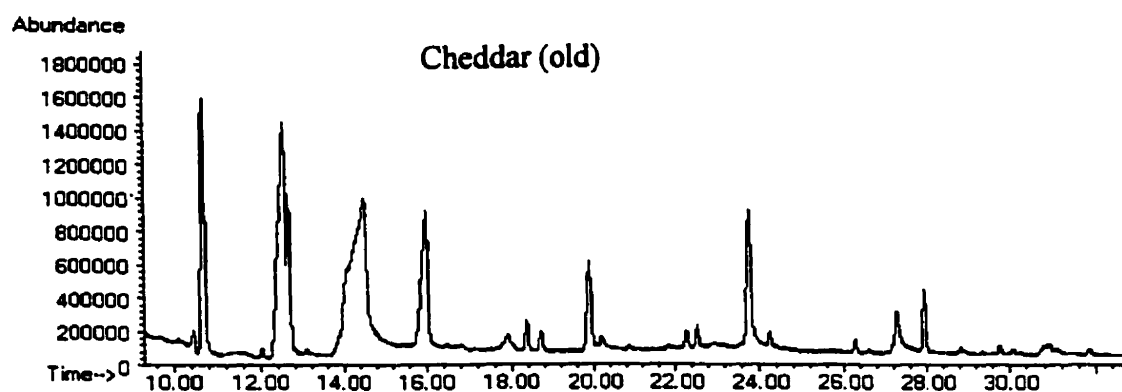
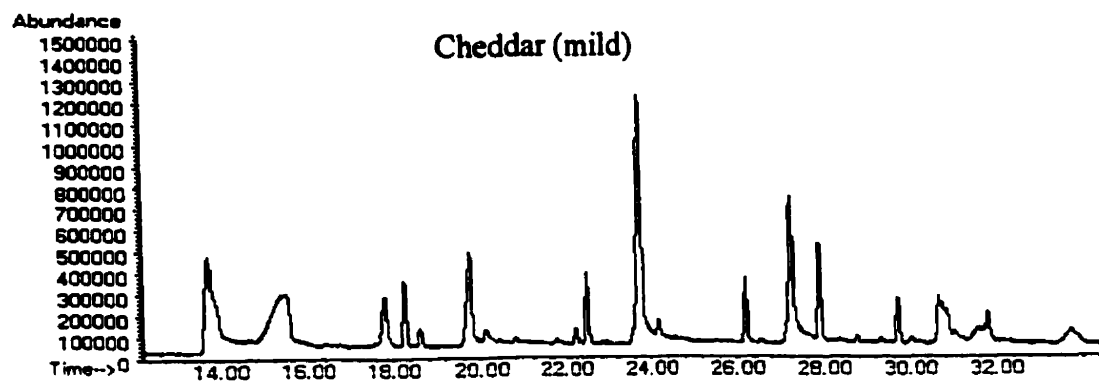


Fig. 4.2. Pyrograms of Cheddar cheese (mild, old, and extra old).

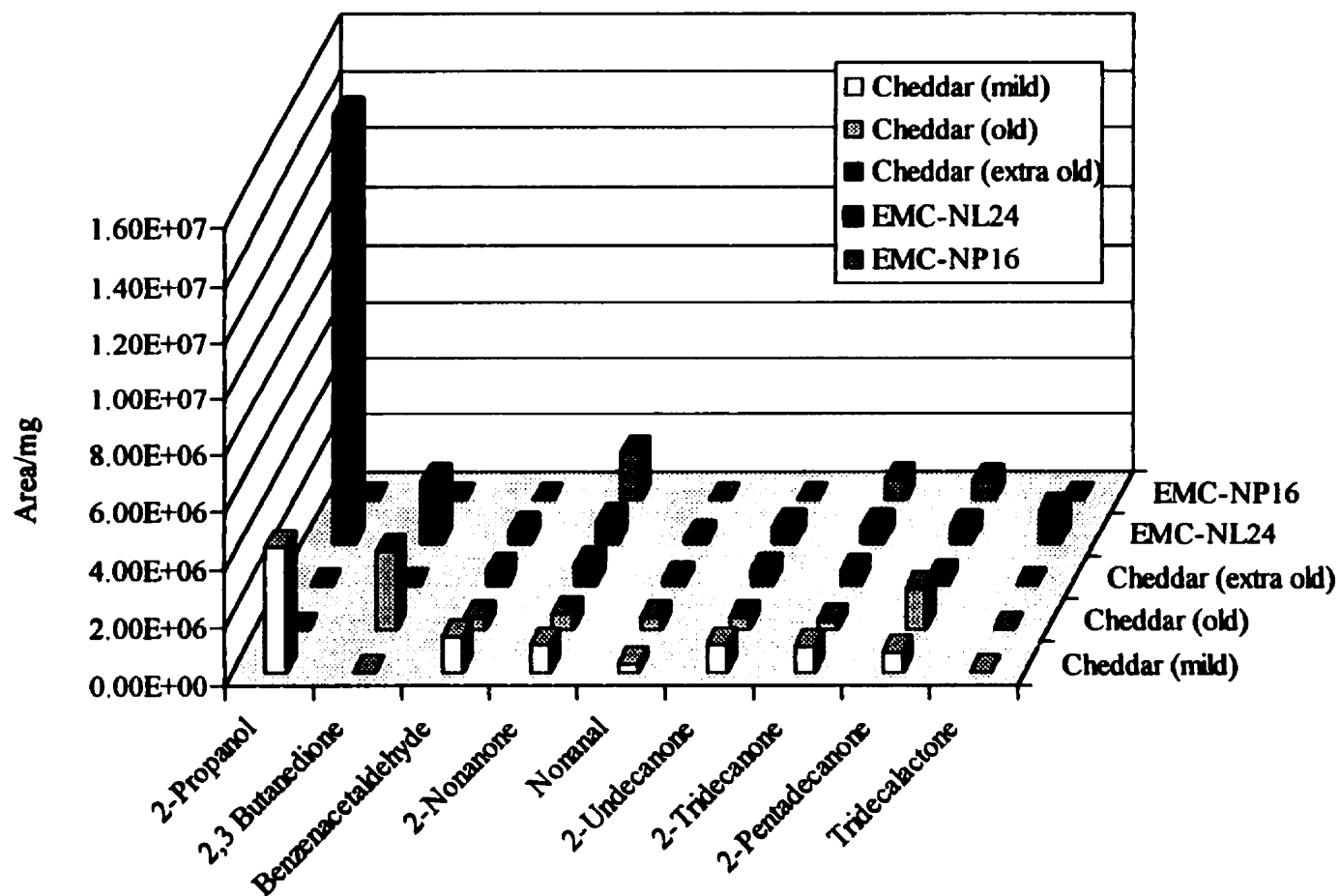


Fig. 4.3. Comparison of aldehydes, ketones, and alcohols in EMC-NL-24, EMC-NP16, and Cheddar cheese (mild, old, and extra old).

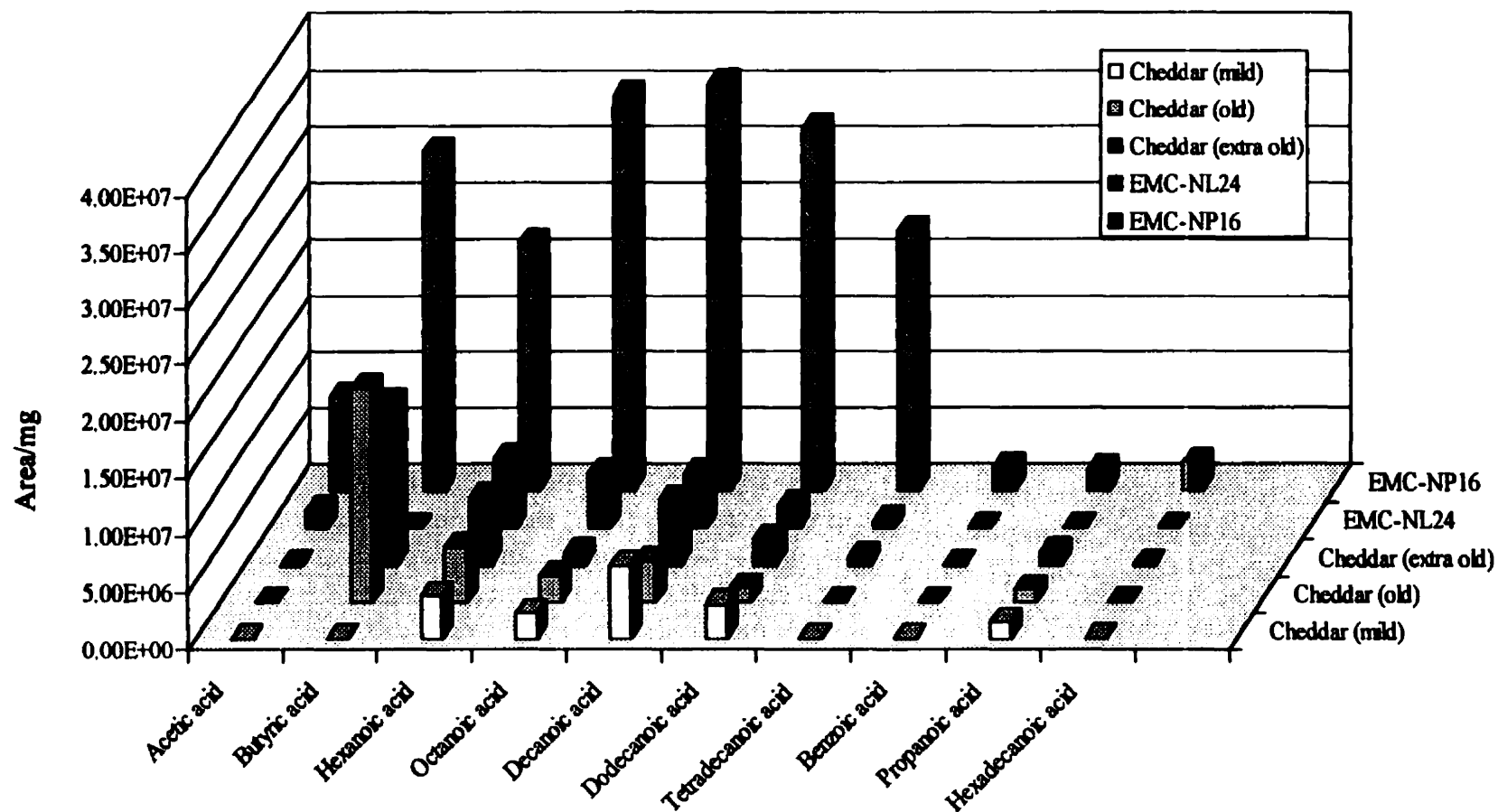


Fig. 4.4. Comparison of fatty acids in EMC-NL24, EMC-NP16, and Cheddar cheese (mild, old, and extra old).

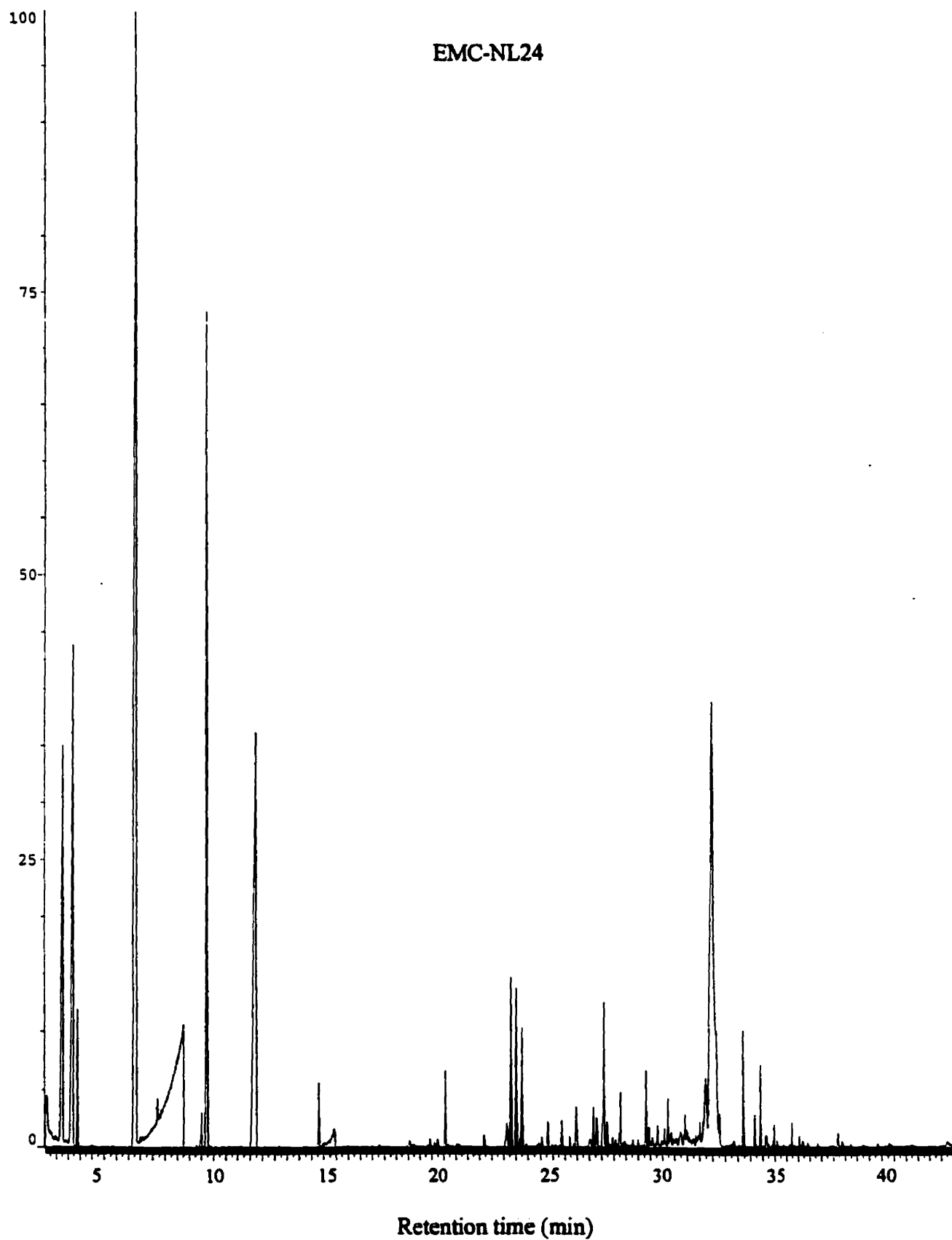


Fig. 4.5. Headspace GC/MS chromatogram of EMC prepared by combination of Neutrase®/*L.casei* enzymes (EMC-NL24).

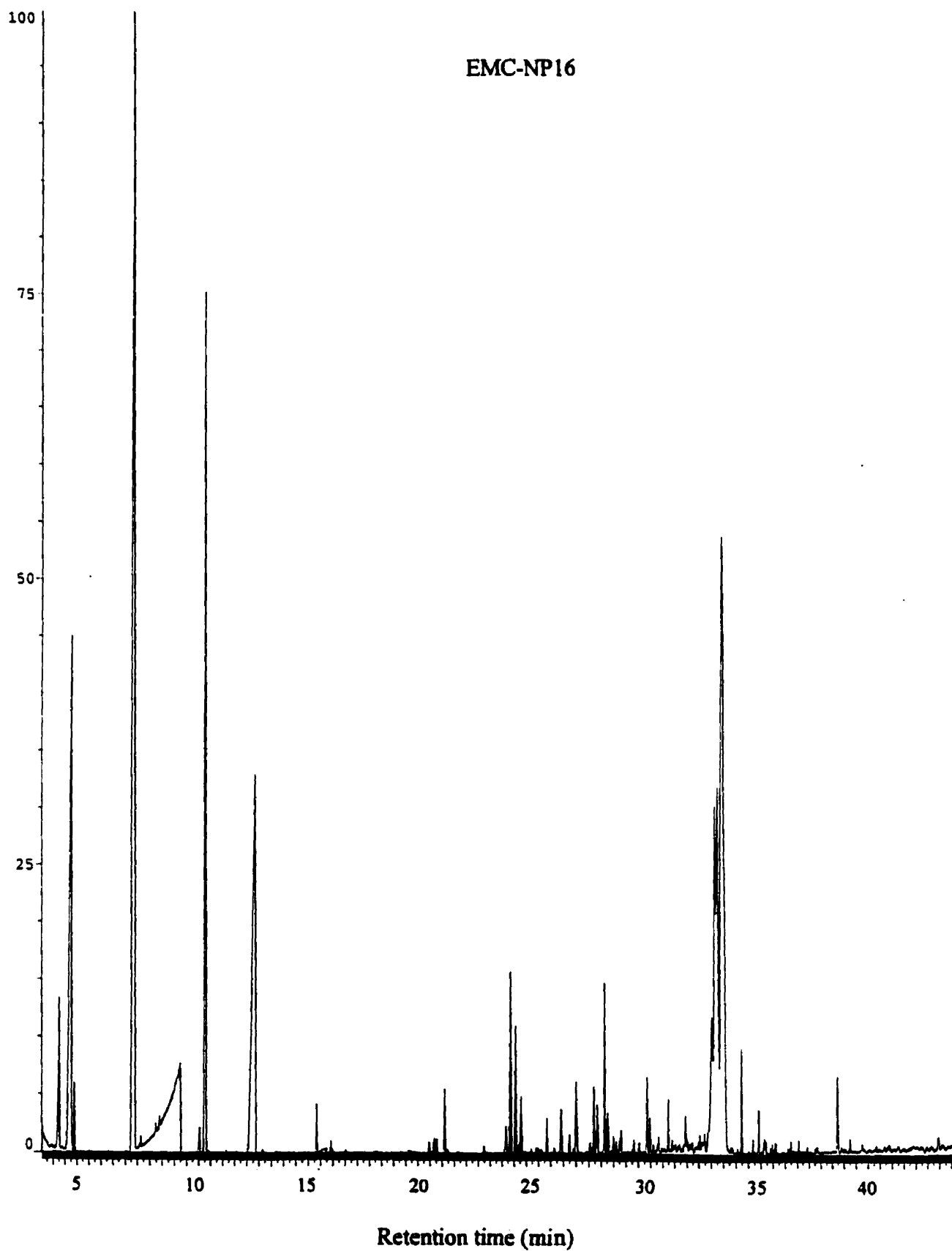


Fig. 4.6. Headspace GC/MS chromatogram of EMC prepared by combination of Neutrase[®]/Palatase[®] M (EMC-NP16).

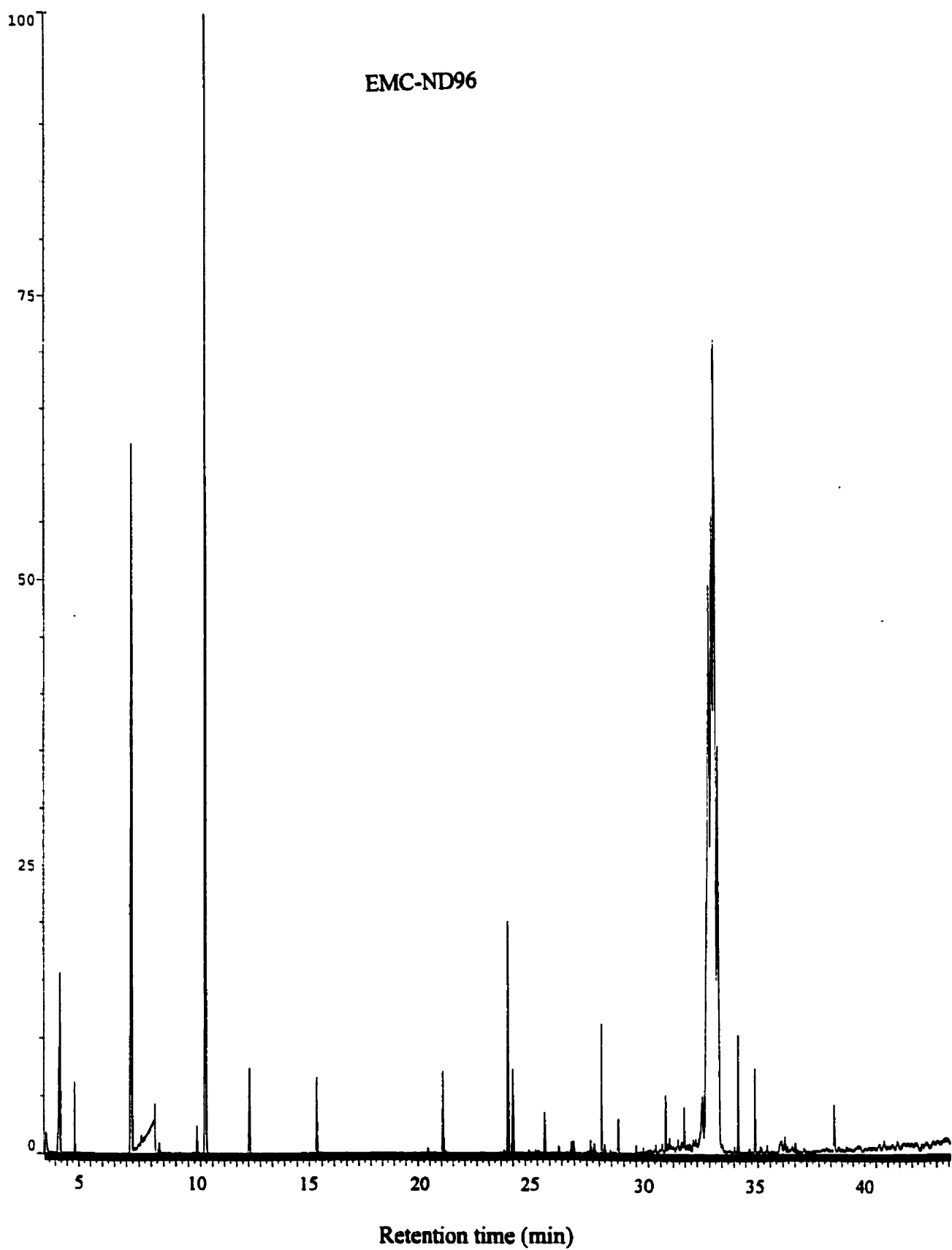


Fig. 4.7. Headspace GC/MS chromatogram of EMC prepared by combination of Neutrase®/Debitrase™ (EMC-ND96).

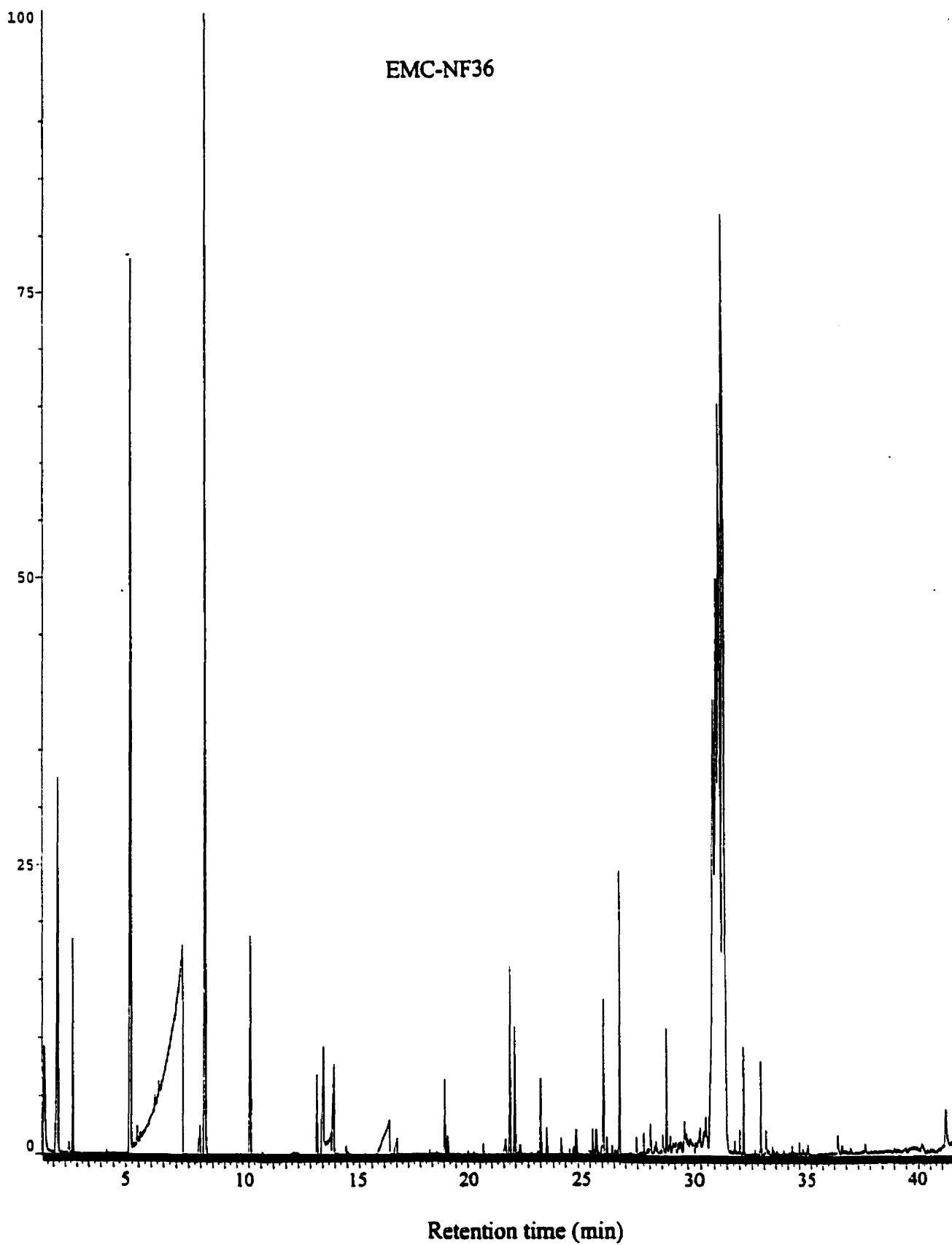


Fig. 4.8. Headspace GC/MS chromatogram of EMC prepared by combination of Neutrase®/Flavorzyme™ (EMC-NF36).

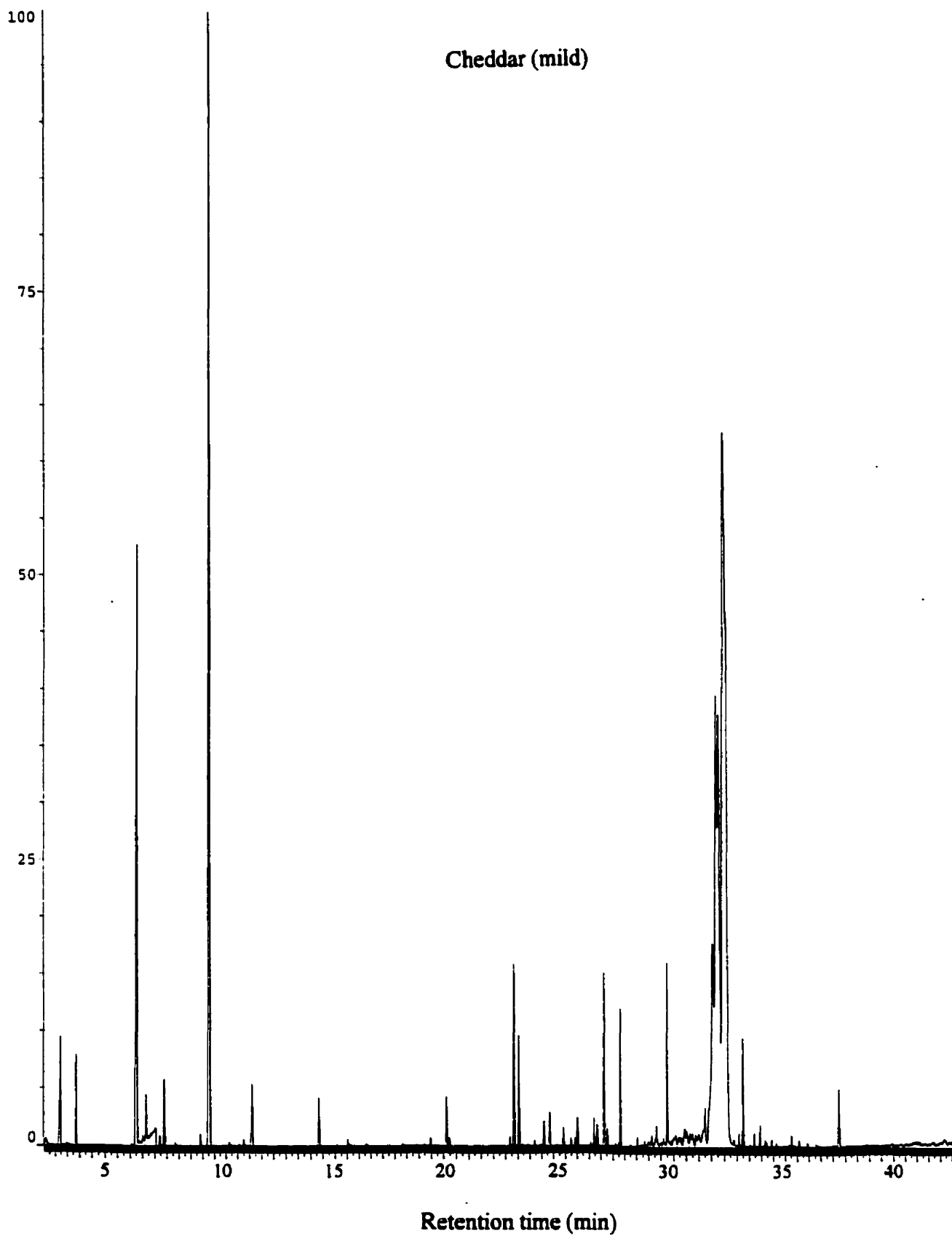


Fig. 4.9. Headspace GC/MS chromatogram of mild Cheddar cheese.

GENERAL CONCLUSION

The production of EMC either with *Lactobacillus casei* or other commercial enzymes was feasible to produce intensive cheese flavors within 24-96 h and simultaneously potentially bioactive peptides, which might serve as pharmacologically active agents.

To investigate the optimum conditions to produce a good quality of EMC, EMC samples were prepared by combinations of different enzymes under different conditions (temperature and time). An intense cheese flavor was developed when Cheddar cheese slurry treated with Neutrase® 0.5L was further incubated with *L. casei* enzymes (aminopeptidase activity, 86.4 LAPU/g, 34°C, 72 h; esterase activity, 110.0 U/g, 45°C, 24h) and Debitrase™ (22.0 LAPU/g, 35°C, 96 h). The results of sensory analysis and RP-HPLC analysis indicated that both enzymes *L. casei* and Debitrase™ showed a debittering effect on EMC and were able to hydrolyze the bitter hydrophobic peptides to smaller palatable peptides. Meanwhile EMC-NF36 (prepared by combination of Neutrase® /Flavorzyme™), remained significantly bitter.

Apart from the debittering effect of *L. casei* and Debitrase on EMC, the cleavage of milk protein by these enzymes led to the formation of potentially bioactive peptides. Overall, seven potentially bioactive peptides mainly angiotensin converting enzyme inhibitory peptides and immunostimulating peptides were detected in Neutrase®/*L.casei* and Neutrase®/Debitrase™ digests. β -Casomorphin with a sequence of YPFPGPI was detected in EMC-N8 (prepared by Neutrase). However, the physiological benefits of these peptides were not determined. Due to the high content of proline (iminopeptide) β -casomorphin can be adsorbed intact through the intestine and can exert opioid activity. Thus, consumption of EMC containing potentially bioactive peptides at a certain concentration may have a physiological effect. Further investigation by using *in vitro* and *in vivo* assays with animal and human systems will be very useful to elucidate the physiological benefits of these peptides.

The results of isolation and identification of volatile flavor compounds from EMCs and Cheddar cheese by using Py/GC/MS and purge and trap techniques revealed the presence of 20 compounds including ketones, alcohols, aldehydes, fatty acids, hydrocarbon, and lactone. EMC prepared by combination of Neutrase®/*L. casei* enzymes and extra mature Cheddar cheese contained most of the flavor components identified by Py/GC/MS analysis technique. In contrast, EMC prepared by combination of Neutrase®/Flavorzyme™ was found to contain most of the compounds identified by using dynamic headspace analysis technique but the impact of each component on the overall EMC and Cheddar flavor was not identified. EMC and Cheddar cheese flavors were likely the synergistic effects of several components in a correct balance.

Comparisons of aldehydes, alcohols, ketones, lactones and selected fatty acids indicated the presence of long chain fatty acids at high concentrations and the absence of aldehydes, alcohols, and lactones in EMC-NP16 (prepared by combination of Neutrase®/Palatase® M). The rancid-off flavor detected in EMC-NP16 is likely due to the incorrect balance of the components present.

Py/GC/MS analysis proved to be a simple and rapid technique to analysis of flavor compounds in cheeses. Further studies are needed to standardize the method to identify and elucidate the contribution of each flavor compounds to cheese flavor. Dynamic headspace analysis proved to be effective technique to analysis of volatile flavor compounds from cheeses. However, to perform quantitative analysis certain variables in this technique need to be standardized.

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APPENDIX I

Amino acid symbols

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Asparatic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Termination	End	U
Asn or Asp	Asx	B
Gln or Glu	Glx	Z
Unknown	Xxx	X