Studies on Peptidases of Cheddar Cheese-Associated *Lactobacillus casei* Species

by

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Dedicated to my mom
meree mañ ko samarpit

STUDIES ON PEPTIDASES OF CHEDDAR CHEESE-ASSOCIATED *LACTOBACILLUS CASEI* SPECIES

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ABSTRACT

Preliminary experiments by API ZYM enzyme system showed that *Lactobacillus casei* (*Lb. casei*) subspecies contained low proteinase and high aminopeptidase and esterase-lipase activities, which are the desirable traits of microorganisms to be used as starter adjuncts in Cheddar cheese-making. Six strains of *Lb. casei* (ssp. *casei*, ssp. *rhamnosus*, and ssp. *pseudoplantarum*), selected from superior peptidase and esterase-lipase profiles, were further studied for their amino-, di-, tri-, and carboxy-peptidase activities using thirty synthetic substrates. This study revealed useful information towards improving our understanding of the peptidase profiles and probable role of *Lb. casei* in Cheddar cheese ripening. Although individual strains varied in their specific activities against different substrates, *Lactobacillus* subspecies generally exhibited high amino- and di-peptidase, relatively weak tripeptidase, but no carboxypeptidase activities. The knowledge gained from these studies helped us selecting two strains (*Lb. casei* ssp. *casei* LLG and *Lb. casei* ssp. *rhamnosus* S93) with highest amino- and di-peptidase activities for further research. In order to study their enzymatic characteristics and kinetics, aminopeptidase of these two strains were purified to homogeneity by Fast Protein Liquid Chromatography (FPLC). A single monomeric enzyme was shown to be responsible for the entire aminopeptidase activity of the cell-free extracts. This investigation provided new insights and revealed fundamental knowledge about the peptidases of *Lb. casei* group. In addition, new methodologies were developed for rapid enzyme purification using FPLC system, and evaluation of peptidases by API ZYM enzyme system.

ETUDES DES PEPTIDASE D'ESPACES DE *LACTOBACILLUS CASEI* ASSOCIES AU FROMAGE CHEDDAR

GULSHAN ARORA

Dr. Byong H. Lee Directeur de thèse

RESUME

Des expériences préliminaires sur le système enzymatique API ZYM ont démontré que le *Lactobacillus casei* (*Lb. casei*) sous-espèce possède une faible activité protéinase et de fortes activités aminopeptidase et esterase-lipase. Ces caractéristiques sont désirables pour son utilisation comme démarreur dans la fabrication du fromage Cheddar. Six souches de *Lb. casei* (ssp. *casei*, ssp. *rhamnosus*, et ssp. *pseudoplantarum*) sélectionnées pour leurs profiles peptidase et esterase-lipase, ont été étudiées pour leur activité amino-, di-, tri- et carboxy-peptidase sur 30 substrats synthétiques. Cet étude a révélé des informations très utiles qui nous permettrons de faciliter notre compréhension en ce qui concerne les profiles peptidase et leur rôle attribué dans la maturation du fromage Cheddar. Bien que certaines souches varient dans leurs activités spécifiques avec des substrats différents, l'espèce *Lactobacillus* contient en général une haute activité amino- et di-peptidase, une faible activité tripeptidase et aucune activité carboxypeptidase. Deux souches de *Lb. casei* (ssp. *casei* LLG et ssp. *rhamnosus* S93) ayant les plus hautes activites amino- et di-peptidase ont alors été sélectionnées. Les aminopeptidases de ces deux souches ont été purifiées par Fast Protein Liquid Chromatography (FPLC), afin d'étudier leurs caractéristiques enzymatiques et cinétiques. Une seule enzyme monomère a été identifiée comme responsable de toute l'activité aminopeptidase obtenue à partir des extraits cellulaires frais. Cette recherche nous a permis d'aquérir des connaissances fondamentales sur les peptidases du groupe *Lb. casei*. De plus, de nouvelles méthodes ont été développées pour une purification enzymatique rapide par le système FPLC et une évaluation des peptidases par le système enzymatique API ZYM.

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CONTRIBUTIONS TO KNOWLEDGE

The objective of this study was to select *Lb. casei* strains with desirable peptidase profiles, which could be used as cheese starters or starter adjuncts, for improved flavor and accelerated ripening of Cheddar cheese. Since very little was known about the peptidase activities of *Lb. casei*, extensive work was required to understand their peptidase complex. Our study contributed to the knowledge in many aspects, briefly mentioned below:

1) A comprehensive review of cheese microbiology and role of peptidases during cheese ripening was completed. The review also served to bring into focus the potential of lactobacilli as cheese starters or as starter adjuncts.

2) The study is a first successful attempt to purify and characterize an aminopeptidase from *Lb. casei* species. A rapid purification protocol was developed by which aminopeptidase from two strains was purified to homogeneity. The procedure developed uses advantages of monobead columns and Pharmacia Fast Protein Liquid Chromatography, requiring only 3 working days to get a good yield of highly active homogeneous aminopeptidase from bacterial biomass. The information obtained on characteristics of the purified aminopeptidase will help food scientists to explore its role in the food industry.

3) A rapid, simple and reproducible method (API ZYM) was developed to evaluate and localize various hydrolytic enzymes of cheese starters. This method will help starter companies to assess pertinent enzyme activities of starter strains they provide for industries. An extensive study was done on the hydrolytic enzyme profiles of *Lb. casei* species by using this enzyme system. This primary information will be useful

to enzymologists in designing future experiments for the characterization of their enzymes.

4) A systematic study was carried out on growth, biomass, and cell disintegration of *Lb. casei* species. Critical parameters were determined which may be helpful for efficient and uniform growth, and lysis of *Lb. casei* species.

5) A detailed comparative study was carried out on the specificities and specific activities of different peptidases of *Lb. casei* species. This study supported the application of lactobacilli peptidases to accelerate cheese ripening, and also proved useful in assessing their role in eliminating bitterness during ripening.

6) The investigation resulted in the selection of two strains with superior peptidase activities. These strains or their enzymes can be used by the cheese industry to improve and accelerate cheese flavor production without bitterness.

In summary, through a combination of fundamental research and practical approach, a potential for the utilization of peptidases of *Lb. casei* species has been explored. Taken as a whole, the investigation is a major contribution to cheese technology and starter enzymology.

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1 GENERAL INTRODUCTION

Cheese has evolved from its humble beginning since 6000 years B.C. as a simple means of conserving milk constituents. Today it is accepted as a highly nutritious food of *haute cuisine* with epicurean qualities. The mechanism by which fresh curds mature from a relatively bland elastic mass to well-bodied cheese with a distinct flavor is still not well understood and has been the subject of much interest. Owing to its fabrication being limited to farms and monasteries, cheesemaking remained an art rather than a science until recently. However, due to enormous world-wide increase in cheese production (20 million metric tonnes in 1987; Sliter, 1988), today's highly competitive cheese industry is striving hard to control and accelerate the biochemical mechanisms involved in the development of mature cheese flavors to meet the increasing demand of the consumer.

On the basis of currently available scientific information, it is generally agreed that all the major milk components (protein, carbohydrate and fat) contribute to the development of cheese flavor through enzymatic degradation. While degradation of carbohydrate and fat is seen as fundamental process in cheese ripening, their importance in directly influencing the Cheddar cheese flavor is not well understood (Law, 1984). On the other hand, extensive protein breakdown (25-30%) has an obvious role in determining the texture, background flavor intensity, and the availability of flavor precursors in all matured cheese varieties (Law, 1987).

Because of their fastidious nutritional requirements, lactic acid bacteria have developed complex enzymatic systems which allow them to efficiently utilize the abundant nutrients present in milk (Law & Kolstad, 1983). Using a combination of proteinases, peptidases and specialized transport systems, the cultures are able to

utilize protein-bound amino acids. Qualitative data suggest that lactobacilli have the most extensive amino acid requirements (Morishita *et al*, 1981). Studies concerning proteolytic capabilities of lactic acid bacteria have focused primarily on the Group N streptococci, due to their economic significance as the starter bacteria in Cheddar cheese (Law *et al*, 1974; Exterkate, 1975 & 1977; Mills & Thomas, 1978; Kaminogawa *et al*, 1984b; Law, 1984). However, similar studies focused on lactobacilli, indicate that most species of this genus exhibit greater peptidase activities than the lactococci (Pettersson & Sjostrom, 1975; Hickey *et al, 1983;* Lee *et al*, 1986).

Both modern and traditional methods of manufacture of Cheddar cheese aged longer than three months rely upon the chance presence of lactobacilli to facilitate the latter stages of ripening. They are one of the few genera capable of growth to high cell densities ($> 10^7$/g cheese) under selective conditions of cheese ripening (Peterson *et al*, 1989). This is in contrast to the lactococci, the primary starter used in cheesemaking, which readily decline in number during early stages of cheese ripening (Chapman & Sharpe, 1981; Thomas, 1986). The role played by lactobacilli in development of desirable Cheddar cheese flavor is equivocal. Some studies suggest that certain *Lactobacillus* spp. are typically associated with either good or poor quality cheese (Sherwood, 1937, 1939a & b). Intentional addition of various *Lactobacillus* strains at concentrations normally associated with cheese or at levels well in excess of the normal amounts have proven successful in the production of more intense and desirable flavor attributes in some instances (Pettersson & Sjostrom, 1975; Law *et al*, 1976a; Bartels *et al*, 1985; Abdel Baky *et al*, 1986; Puchades *et al*, 1989; Laleye *et al*, 1990; Lee *et al*, 1990a &b) but not in others (Reiter & Sharpe, 1971). Most of these studies looked only at differences in gross composition and general sensory characteristics of the control and experimental cheeses.

Other workers have focused attention on the proteolytic and peptidolytic activities of various cellular fractions isolated from species of lactobacilli using selected synthetic substrates (El Soda *et al*, 1978a, 1978b; Eggimann & Buchmann, 1980; Hickey *et al*, 1983; Abo-Elanga & Plapp, 1987). Consequently, it was shown (Lee *et al*, 1986) that *Lb. casei* group (ssp. *casei*, ssp. *rhamnosus* and ssp. *pseudoplantarum*) were more active in various peptidase activities than any other lactic acid bacteria. Further studies on the pilot scale demonstrated that the use of *Lb. casei* in conjunction with the starter lactococci shortened the ripening period, with an improvement in Cheddar flavor (Puchades *et al*, 1989; Lee *et al*, 1990b). Other workers (Lemieux *et al*, 1989) demonstrated the use of *Lb. casei* species for the elimination of bitterness during cheese ripening. Although these positive attributes were mainly related to the proteolytic systems, there is lack of information on the characteristics of the responsible enzymes.

Therefore, the objectives of this research were as follows:

- Firstly, to evaluate the enzyme profiles of various cheese-associated strains of *Lb. casei* group to obtain an insight to the presence and localization of desirable enzymes.

- Secondly, to investigate the substrate specificities of their peptidase activities (amino-, di-, tri-, carboxy-peptidase *etc.*).

- Finally, to study the characteristics of pure aminopeptidase preparations of strains selected from their superior peptidase profiles.

2 REVIEW OF LITERATURE

2.1. Chemistry and Microbiology of cheese maturation

2.1.1 Cheese, an overview

In simplest terms, cheese may be defined as a consolidated form of milk curd which may be consumed as such or after moderately or even extremely modifications through the introduction of different microorganisms or additives and/or by aging (or curing or ripening) for varying periods of time (Vedamuthu & Washam, 1983). In general practice, cheese is a generic name given to a group of fermented-milk-based products produced in at least 800 varieties throughout the world, many of which are similar, differing in shape, size, degree of ripening, type of milk or condiments used, packaging and locality of its manufacture.

With the exception of some soft cheese varieties, the production of a vast majority of cheeses can be subdivided into two well-defined phases namely, manufacture and ripening. Although the manufacturing protocols for individual varieties differ in detail, the basic steps are common to most varieties. These steps are acidification, coagulation, dehydration (cutting the coagulum, cooking, stirring, pressing *etc.*), shaping (moulding and pressing), and salting (Fox, 1987). The nature and the quality of the finished cheese are determined to a large extent by the various manufacturing steps. However, it is during the ripening phase that the characteristic flavor and texture of the individual cheese varieties develop, primarily, through the action of its microflora and their enzymes.

Cheese ripening refers to the storage of cheese under controlled conditions of time, temperature and humidity, during which various physical, chemical and microbiological changes transform the fresh, tasteless and rubbery curds of different cheese varieties to smooth textured, finished products with the characteristic flavors. Biochemically, cheese flavor production is a dynamic process and represents a finely orchestrated series of successive and concomitant biochemical events over a period of time which in tune leads to products with highly desirable aromas and flavors, but if out of balance, off-flavors and odors result.

It is impossible to review the biochemistry involved in the ripening of all individual cheese varieties. However, it is generally accepted that both texture and flavor are influenced by the type and extent of lactose metabolism, proteolysis and lipolysis catalysed by coagulant and bacterial and milk enzymes. The type and rate of these and other enzymatic and nonenzymatic reactions (responsible for flavor development) depend upon the composition and micro-environment within the product (Adda *et al*, 1982). The texture depends mainly on the manner in which casein coagulates and the extent of proteolysis and the flavor is determined primarily by the pathways of degradation of lactose, protein and fat.

2.1.2. Biochemistry of flavor generation

The Danish poet and Nobel Laureate Johannes Jensen (1921) once proclaimed that the flavor of cheese reminded him of decay and smouldering erotic passion. Scientists have tried to describe the flavor of cheese in more prosaic terms. Their efforts have been summarized in a number of reviews (Langsrud & Reinbold, 1973; McGugan, 1975; Adda *et al*, 1978 & 1982; Law, 1980, 1981 & 1982; Aston & Dulley,

1982; Cuer, 1982; Green & Manning, 1982). From a food chemist's perspective, cheese flavor is a blend of numerous compounds mainly derived from controlled slow hydrolysis and decomposition of principle milk constituents such as fat, protein and lactose, by the enzymes present in milk, coagulant and of starter as well as non-starter bacteria. Over 180 compounds have been isolated and identified from Cheddar and over 125 from Swiss cheese, yet few of them characterize the typical flavor of the particular cheese (Moskowitz & Noelck, 1986).

A great deal of research, on identification of important flavor compounds, mechanisms by which these compounds are produced in cheese, and systems to control and accelerate flavor development, has been devoted in the last 25 years (Fryer, 1969; Wong, 1974; Manning, 1979; Aston & Creamer, 1986; Barlow *et al*, 1989). However, the precise nature of complex enzymatic and nonenzymatic reactions during cheese maturation leading to cheese flavor compounds, and the way in which their relative rates are controlled is poorly understood. Recognition of the fact that vast number of compounds in cheese could influence cheese flavor led to the component balance theory (Mulder, 1952). This theory suggests the presence of a background flavor common to most cheese varieties, and the specific varietal flavor results from a blend of specific components in proper proportions.

Much of the research on cheese flavor has been directed in two principal directions: (a) the chemical isolation and identification of compounds which constitute flavor, and (b) the microbiological and biochemical agents, and the mechanisms capable of producing such compounds. Sensitive analytical methods such as gas chromatography and mass spectrometry have led to the isolation and identification of a large number of compounds such as ketones, aldehydes, alcohols, fatty acids and volatile sulfur compounds which could contribute to Cheddar flavor (Fryer, 1969; Liebich *et al*, 1970; Adda *et al*, 1982; Aston & Dulley, 1982; Law, 1982). However, attempts to

reproduce Cheddar flavor by combining defined components have failed so far (Law, 1984). Recent studies on flavor chemistry are mainly focused on separate fractions of cheese flavor distillates which contain greatly reduced number of potential flavor compounds and yet contain the Cheddar flavor and aroma. Head space and distillate analyses has indicated the presence of protein based volatile sulfur compounds such as hydrogen sulfide, methanethiol and dimethyl sulfide as predominant components of Cheddar flavor (McGugan *et al*, 1979; Green & Manning, 1982). In all cases, the formation of -SH group at low redox potential has been related to the development of desirable Cheddar flavor (Law, 1987).

There has been considerable interest in accelerating flavor development as a means of reducing cheese curing cost. Kristofferson *et al* (1967), Law (1979) and Harper and Wang (1980) have investigated cheese slurries as a means of producing flavor more efficiently. A water-cheese slurry is formed and held under carefully controlled conditions at 30°C for 7 days (Chapman & Sharpe, 1981). Such slurries when added to curds, result in more flavor development. Although many workers have been able to use slurried curd to accelerate cheese ripening (Dulley, 1976) and to produce intense cheese flavors (Sood & Kosikowski, 1979), the mechanism by which flavor is so quickly produced has remained unclear. Besides, the process is also difficult to control. Microencapsulation of enzymes and substrates to produce cheese flavor compounds also has been suggested as a means of intensifying and directing cheese flavor development (Braun *et al*, 1982; Braun & Olson, 1986; Kirby *et al*, 1987). By this technique, the substrates and enzymes are packed in hydrophobic capsules which ensure their homogeneous mixing in cheese matrix and allow a greater control on flavor intensity.

2.1.2.1. Influence of lactose hydrolysis

Changes in lactose occur largely during the cheesemaking process and the initial stages of ripening, as most of the lactose disappears within 3 days (Fox, 1987) Lactose metabolism has less obvious but important effect on the course of cheese maturation. Lactic acid has a stabilizing effect due to its antibacterial properties (Babel, 1977) and also lowers the redox potential and pH of cheese. This ensures that enzymatic reactions proceed slowly, over long storage times, to develop full flavor, while inhibiting the growth of undesirable organisms such as *coli aerogenes* group. Low redox potential also ensures that flavorful sulfur compounds, having characteristic Cheddar flavor, remain in reduced form (Law, 1984).

The fermentation of residual lactose in freshly-pressed cheese curd by secondary lactic acid bacteria (lactobacilli, pediococci) has been directly related to the quality of Cheddar cheese (Fryer, 1982). However, heterofermentation of lactose by certain species of these organisms has been implicated in the development of undesirable flavors and other defects in matured cheeses (Thomas *et al*, 1979).

2.1.2.2. Role of Lipolysis

The role of fat is important in the perception and formation of flavor. It has been well documented that skim milk cheeses lack the typical flavor, as they have considerably fewer free fatty acids (FFA) than normal cheeses (McGugan, 1963; Ohren & Tuckey, 1965; Dulley & Grieve, 1974; Aston & Dulley, 1982) The typical aroma of Cheddar cheese develops only when fat content is at least 40-50% on dry basis (Fox, 1987). Patton (1963) first claimed that volatile fatty acids (C_1-C_8) were the backbone of Cheddar aroma. However, Manning and Price (1977) showed that removal of volatile fatty acids from Cheddar cheese headspace did not affect its aroma at all, and concluded that these acids were only important in the background

taste of the cheese. Reviews on cheese flavor by Harper (1959) and Day (1966) have highlighted the importance of lipolytic release of fatty acids in the development of flavor in aged cheeses. Schormuller (1968) have summarized free fatty acid concentrations of various cheeses.

Lipolytic activity indigenous to the milk or that produced by lactic acid bacteria or by psychrotrophic bacteria may all contribute to hydrolysis of milk triglycerides and subsequent release of free fatty acids (Fryer, 1969; Reiter & Sharpe, 1971; Law *et al*, 1976a; Law, 1979). Since lactic starter lipases seem to be active mainly on mono- and di-glycerides (Stadhouder & Veringer, 1973), milk triglycerides are first degraded by native lipases of milk as well as lipases of adventitious microflora of raw milk (Reiter & Sharpe, 1971) during storage and processing of milk. Excessive lipolysis by native and microbial lipases during prolonged storage has been implicated in rancid and other off flavors in Cheddar cheese (Law *et al*, 1976b; Law, 1982; Law & Wigmore, 1985).

The role of lipolysis and importance of free fatty acids in flavor development is well accepted in case of mould ripened soft cheeses such as Romano, Parmasen and Blue veined cheese (Moskowitz, 1980). However, the effect of lipolysis is difficult to assess in most hard and semi-hard varieties which rely on their lactic flora for flavor development as these organisms are capable of only limited lipid hydrolysis (Stadhouders & Veringer, 1973; Umemoto & Sato, 1975; Paulsen *et al*, 1980). Studies on Cheddar cheese indicate that fatty acids liberated during ripening are further degraded to short chain fatty acids like butyric, caproic, caprylic and capric acid. These fatty acids may further be hydrolysed to methyl ketones by oxidative decarboxylation. Esterification of short chain fatty acids with methanethiol (normally present in cheese as a degradation product of methionine) by secondary cheese microflora (lactobacilli and pediococci) during late ripening stages generates thio-

esters with cheesy aroma (Law, 1984). Other fat derived flavor compounds that are implicated in Cheddar flavor included lactones. Although lactones have been shown to improve blue cheese flavor (Jolley & Kosikowski, 1975), their contribution to Cheddar flavor is less clear due to their low concentration (below threshold level) in matured cheese (Wong *et al*, 1975).

Characterization of unique properties of various lipolytic enzymes, and recognition of flavor potential of milkfat have led to the development of numerous applications of controlled lipolysis for flavor development. Consequently many patents involving the application of various lipolytic enzymes for specific processes have been issued (Fukumoto, 1971; Kraftco Corp.. 1971; Roberts & Kraftco Corp , 1971 & 1972). Also a number of commercial lipolytic enzyme preparations and lipolysed products are currently being marketed (Arnold *et al*, 1974).

2.1.2.3. Role of Proteolysis

Vakaleris and Price (1959) found that the degree of cheese ripening could be assessed by measuring the tyrosine content. With most of whey proteins lost with cheese-whey during cheesemaking process, proteolysis in cheese ripening is mainly confined to the degradation of casein, the principal milk protein. Proteolytic cleavage of casein forms peptones and peptides, which are further broken down to amino acids. The formation of amino acids commences during cheesemaking and continues during cheese ripening. Eighteen different amino acids have been reported (Wong, 1974). Peptones, peptides, amino acids alongwith other derived proteolytic products as a result of deamination, transamination, decarboxylation *etc.* have been found in different cheeses, giving each cheese its specific flavor (Hemme *et al*, 1982; Irvine & Hill, 1985).

Proteolysis contributes to cheese ripening in many ways: 1) by a direct contribution to taste and flavor via formation of amino acids and peptides, some of which may cause off flavors such as bitterness (Lowrie & Lowrence, 1972; Lowrie, 1977; Visser *et al*, 1983). It also indirectly contributes to cheese flavor and texture development via catabolism of amino acids to amines, acids, thioesters *etc.* (Kristofferson & Olson, 1955; Law, 1987). 2) by change in pH via formation of ammonia, and 3) by changes in texture from breakdown of protein network, increase in pH and greater water binding by newly formed amino and carboxyl groups (Lawrence *et al*, 1987). Although the ripening of some soft and semi-soft varieties (*e.g.* Blue and Romano) is dominated by lipolytic reactions, proteolysis is more or less important in all varieties. In case of Cheddar and Dutch type cheeses, proteolysis is regarded the principal biochemical pathway for their characteristic flavor (Fox, 1989). Consequently, many authors have related the intensity of Cheddar cheese flavor and the degree of cheese ripening with the rate and type of proteolysis as well as with the concentration of proteolytic products and byproducts (Wong, 1974; Ney, 1981; Aston *et al*, 1983a & b). Besides, considerable information has been compiled in many recent reviews on the level and type of proteolysis in principal cheese groups (Castberg & Morris, 1976; Desmazeaud & Gripon, 1977; Grappin *et al*, 1985; Rank *et al*, 1985; Law, 1987).

2.2 Proteolytic agents in cheese

Fox (1989) distinguished three categories of proteolytic agents that are involved in the ripening of cheese: 1) rennet or rennet substitutes (*i.e.* chymosin, pepsin, or microbial proteinases, 2) indigenous milk proteinases (particularly important in raw milk cheeses), and 3) enzymes from cheese microflora.

2.2.1. Rennet or rennet substitutes

Although the prime function of chymosin and other rennets is to initiate the formation of milk gel, about 5-6% of the rennet added to cheesemilk is retained in the curd (Fox, 1989). Since most of the rennets used are highly bond specific, they result in low level of proteolysis, but contribute significantly in releasing large peptides (> 1400 molecular weight) which are easy targets of microbial proteinases and peptidases. The proteolytic specificities of chymosins and pepsins on individual caseins (αs, ß & γ) in solution and in cheese have been established and is documented in many recent reviews and books (Desmazeaud & Gripon, 1977; Grappin *et al*, 1985; Thomas & Pritchard, 1987; Fox, 1989).

2.2.2. Indigenous milk proteinases

Cow's milk contains many indigenous proteinases as well as microbial proteinases secreted by its microflora (Humbert & Alais, 1979; Law, 1979; Visser, 1981; Grufferty & Fox, 1988). Plasmin, the principal milk proteinase is almost exclusively associated with the casein micelles (Humbert & Alais, 1979), and is therefore present in rennet cheese curd. It is an alkaline and heat-stable serine proteinase which appears to contribute to the breakdown of ß-casein in surface ripened cheese (Trieu-Cuot & Gripon, 1982; Grappin *et al*. 1985). Milk also contains acid and neutral proteinases and aminopeptidases which are relatively heat-labile (Kaminogawa *et al*, 1980). Many of the characteristics of milk proteinases have been reviewed recently by Fox (1989).

Microbial proteinases in raw milk are mainly derived from its psychrotrophic

microflora. As they multiply during storage, they produce heat-resistant proteinases and lipases which survive high temperature short time (HTST) pasteurization and may adversely influence the manufacture (lower cheese yield) and ripening (bitterness and soapiness) of some cheese varieties. Most proteinases of this class are neutral or alkaline metallo-proteinases requiring divalent cations for activity and stability (Law, 1979). Detailed specificities and their influence on cheese flavor have recently been reviewed (Fairbairn & Law, 1986).

2.2.3. Role of cheese microflora

The absence of any Cheddar flavor in aseptically produced glucuno-δ-lactone acidified cheese, and the development of typical balanced flavor in "starter added" cheese, indicated that starter has a definitive role in the development of cheese flavor (Reiter *et al*, 1966 et 1967; O'Keeffe et al, 1976). Initial research by workers of New Zealand and England (Lowrie *et al*, 1974; Law & Sharpe, 1977; Law, 1981) introduced the concept of indirect contribution of microorganisms to the production of flavor compounds. They felt that the main role of starter and nonstarter bacteria was limited merely to providing a suitable environment such as the required redox potential, pH and moisture content in cheese that allows enzymatic reactions to proceed favorably. However, it seems more reasonable at this stage to assume that lipolytic and proteolytic enzymes of both viable and dead cells are involved in cheese maturation, and hence significantly contribute to final sensory attributes of finished cheeses.

Currently, lactic acid bacteria are believed to serve the following functions in cheese manufacturing and maturation, that directly or indirectly influence cheese flavor: 1) fermentation and depletion of fermentable sugars which control growth and

composition of adventitious bacteria; 2) creating a low oxidation reduction potential during early stages of cheese maturation; 3) competition and synergism with adventitious bacteria during cheese manufacturing and early stages of maturation; 4) protein hydrolysis; and 5) synthesis of flavor compounds (Olson, 1990). During the past few years, several general reviews and books have been written on the role of cheese starter bacteria (Law, 1984; Kamaly & Marth, 1989).

Microorganisms involved in cheese manufacture and ripening are conventionally divided into two groups, namely starter flora and nonstarter or adventitious flora. While starter bacteria are deliberately added to cheesemilk for acidity development and to bring about other desirable changes, nonstarter bacteria (*e.g.* lactobacilli, pediococci, and micrococci *etc.*) mainly gain access to the pasteurized cheesemilk or curd through environment and equipments.

2.2.3.1. Starter bacteria

Because of their key role in Cheddar cheese production, from fermentation through ripening, the lactic streptococci are commonly referred as the "primary" Cheddar cheese microflora. Most of these strains are homofermentative, producing L(+) lactic acid from lactose metabolism. The mesophilic group N streptococci, such as *Lactococcus lactis* (*Lc. lactis*) and *Lc. lactis* ssp. *diacetylactis*, and thermopilic *Lc. salivarius* ssp. *thermophilus* strains are the primary strains used in Cheddar cheesemaking. However, some heterofermentative leuconostocs are often used in starter cultures for their aroma-producing properties.

Today, starter bacteria in cheesemaking refer to carefully selected microorganisms that are deliberately added to milk or cream to initiate and carry through the desired cheese fermentation. Starters are now added not only to bring specific changes to

improve rheological and organoleptic characteristics of the final product, but also to accelerate cheese maturation.

The primary function of starter bacteria is acid production which has secondary effects in coagulation, expulsion of whey, texture formation, initiation of taste and flavor generation, and providing protection against pathogens and longer shelf stability (Vedamuthu & Washam, 1983). In commercial practice the lactococci are often used as combined cultures. While *Lc. lactis* ssp. *lactis* and *Lc. lactis* ssp *cremoris* are used primarily for acid production, *Lc. lactis* ssp. *diacelylactis* and *Leuconostoc* spp. are used to provide aroma (*e.g.* cottage cheese, cream cheese) or to produce CO_2 giving desired open texture for mould growth (*e.g.* Stilton) or eye formation (*e.g.* Gouda). *Lc. lactis* ssp. *diacetylactis* is used as acid producer in cheeses requiring high cooking temperatures (Sandine *et al*, 1972; Law & Sharpe, 1978)

2.2.3.2. Nonstarter bacteria

Non-starter microflora is the term used to refer to microorganisms that gain access to the cheese vat accidentally and are retained in the cheese. Reiter *et al* (1967) suggested that non-starter microflora of Cheddar cheese, comprised of heat resistant bacteria of raw milk (surviving pasteurization) and post-pasteurization contaminants from the creamery environment, also contribute to ripening. Although widely variable, the non-starter lactic acid bacteria (NSLAB) population consists primarily of *Lactobacillus* spp. (*Lb. casei*, *Lb. plantarum* and *Lb. brevis*), and to a lesser extent, species of pediococci and micrococci (Fryer, 1969; Reiter & Sharpe, 1971; Chapman & Sharpe, 1981). Since the starter lactococci die out during the early ripening period, the non-starter lactic acid bacteria are the dominant flora in the cheese for the greater part of its storage life (Law, 1980; Chapman & Sharpe, 1981; Thomas, 1986).

These microorganisms and their enzymes, therefore, contribute significantly to the maturation of cheeses.

As early as 1892, it was reported that "*Bacillus* acid lactici" was the predominant bacterium in English Cheddar cheese, and based on limited cheese trials, appeared to be responsible for the changes which occurred during maturation (Fryer, 1969). Additional reports by Evans and his group (1914) on the microbial composition of Cheddar cheese indicated that Cheddar microflora consisted of lactococci, lactobacilli and micrococci. Hucker (1922) studied the relationship of cheese microflora with quality of 39 commercial Cheddar cheese samples from 25 factories and reported that the flora in poorer grades was composed largely of spore formers and gram-negative rods, whereas the better quality cheeses contained, primarily, lactobacilli. Since then, a great amount of research work has been devoted to lactobacilli, particularly on their impact on Cheddar flavor.

2.3 Role of lactobacilli in cheese ripening

2.3.1. Lactobacilli, an overview

Lactobacilli are Gram-positive, nonsporing, mesophilic, rod shaped cells which share the unique characteristic of utilizing lactose as carbon source. Based on their mode of lactose fermentation, the genus *Lactobacillus* is divided into three groups (Kandler & Weiss, 1986). Group I constitutes obligate homofermenters such as *Lb. delbrueckii*, *Lb. acidophilus* and *Lb. helveticus*. Some strains of *Lb. delbrueckii* (ssp. *lactis* and ssp. *bulgaricus*) are unable to ferment galactose (gal^-) resulting in the accumulation of galactose in some bacterial ripened cheeses (Turner & Mortley, 1983). The accumulated galactose acts as an energy source for undesirable starter bacteria

causing potential off-flavors and late gassing. The presence of galactose is also a cause of digestive disorders in some lactose intolerant consumers (Alms, 1982; Houts, 1988). Group II is represented by *Lb. casei* and *Lb. plantarum* which are characterized as facultative heterofermenters. In all types of cheeses with ripening period longer than 14 days, mesophilic lactobacilli (*Lb. casei*, *Lb. plantarum*, *Lb. brevis etc.*) originating from milk or the dairy environments, reach levels as high as 10^6-10^8/g during ripening (Naylor & Sharpe, 1958a & b). Based on DNA/DNA homology and physiological characteristics, *Lb. casei* has been further subdivided into four subspecies; ssp. *casei*, ssp. *pseudoplantarum*, ssp. *rhamnosus*, and ssp. *tolerans*. Group III includes obligate heterofermenters, gas forming lactobacilli (*e.g. Lb. brevis* & *Lb. fermentum*), which are considered responsible for off-flavors and other defects in Cheddar cheese (Sherwood, 1939b; Marth, 1963; Laleye *et al*, 1989).

Most of *Lactobacillus* spp. present in cheeses, being mesophiles are totally destroyed by high temperature short time (HTST) pasteurization of raw milk (Reiter *et al*, 1967). Hence, they reenter cheese mainly via post-pasteurization contamination, usually through contact with air or equipment (Reiter & Sharpe, 1971; Thomas, 1986, 1987).

2.3.2. Growth and predominance

Sherwood (1939a) carried out detailed systematic studies on the bacterial flora of New Zealand Cheddar cheese and found that it consisted almost exclusively of *Lb casei* and *Lb. plantarum*. Further experiments on Cheddar cheesemaking using pure and mixed cultures of lactobacilli showed that more desirable flavor was obtained in cheeses to which *Lb. casei* and *Lb. plantarum* had been added. On the other hand, more off flavors, openness in texture, and discoloration was observed in cheeses containing *Lb. brevis* (Sherwood, 1939b). However, cheeses appear to develop

atypical Cheddar flavor at higher counts of even desirable strains. The optimum level of cell number appeared to be that present in raw milk (approximately 12 x 10^3 organisms/ml). Sherwood's work also demonstrated that in some instances, mixed cultures of lactobacilli produced better cheese and suggested symbiotic effects in the mixed cultures. In a similar study, Franklin & Sharpe (1963) indicated that strains of *Lb. casei* occurred most frequently in both experimentally- and commercially-manufactured cheeses.

Although these and other workers clearly established the predominance of lactobacilli in nonstarter bacterial population of Cheddar cheese (Davis, 1935; Sherwood, 1937, 1939a & b; Johns & Coles, 1959), their enumeration at various stages of cheese ripening was complicated for the lack of selection medium for lactobacilli. The usual media supporting the growth of both lactococci and lactobacilli led to anomalous results, particularly at early stages of ripening when lactococci were the predominant flora. Hence, many earlier reports led to the assumption that lactobacilli grew little until the lactococci started to decrease in number (Naylor & Sharpe, 1958a & c). Rogosa *et al* (1951) and De Man *et al* (1960) developed a medium that favored the growth of lactobacilli over other lactic acid bacteria. Mabbit and Zielinska (1956) developed a selective medium (*Lactobacillus* selection agar), containing sodium acetate and acetic acid at levels inhibitory to starter lactococci.

Using the selective medium, Naylor and Sharpe (1958a) observed 10 to 10^4 lactobacilli/g after 10 days and increasing to 10^6 to 10^7/g at 20 days, and reaching a plateau between 10^7 and 10^8/g at 60 days. In a separate study, they found $3x10^6$/g at 180 days in cheese made from milk containing less than 1 *Lactobacillus*/ml and 10^8/g at 15 days in cheese from milk containing 200 lactobacilli/ml (Naylor & Sharpe, 1958b). Among the lactobacilli, homofermentative types (*Lb. casei* and *Lb.*

plantarum) present consistently throughout the ripening, were partially replaced by heterofermenters (*Lb. brevis*) during late ripening stages (Naylor & Sharpe, 1958a). Davis (1935) recovered both *Lb. plantarum* and *Lb. casei* between 1 to 5 months of age, but was able to detect only *Lb. casei* beyond that point. Since then, many reports indicating varying counts of lactobacilli during different stages of cheese maturation have been published (Dawson & Feagan, 1957; Johns & Coles, 1959; Chapman & Sharpe, 1981; Peterson *et al*, 1989). In a more recent study, Laleye *et al* (1989) reported that undesirable heterofermentative lactobacilli levels in late ripening stages can be controlled by the intentional addition of homofermentative lactobacilli.

It is now generally believed, that lactobacilli are the only lactic acid bacteria to increase significantly in number ($> 10^7$/g) under the selective conditions (low Eh, low pH, limited carbohydrates *etc.*) of Cheddar cheese maturation, except for the less frequently occurring pediococci. A number of physiological and biochemical studies indicate that lactobacilli may utilize metabolites such as lactate, amino acids, ribose, and N-acetyl amino sugars, as nutrient sources more effectively than either the lactococci or other NSLAB (Fryer, 1969; Nath & Ledford, 1973; Thomas, 1986 & 1987), which may partly explain their dominance during ripening.

2.3.3. Role of proteolytic enzymes of lactobacilli in cheese ripening

Proteolysis is of fundamental importance in the ripening of cheese both in the positive and negative sense. Various aspects of this subject have been extensively reviewed, *e.g.* flavor (Law, 1984) and especially bitterness development (Visser, 1981; Law & Kolstad, 1983; Stadhouders *et al*, 1983), texture development (Lawrence *et al*, 1987) and accelerated ripening (Law, 1984).

The role of proteolytic enzymes of starter and nonstarter bacteria does not end with the cessation of bacterial growth. These enzymes, along with the rennet added as coagulant and with indigenous milk enzymes continue to act during cheese ripening on diffe.ent milk components. The complex nature of these simultaneous multiple reactions makes the proteolytic pathways difficult to define. However, the relative roles of the different proteolytic agents in Cheddar cheese has been partially defined (O'Keefe *et al*, 1976 & 1978; Visser, 1977 & 1981; Law, 1984).

Most of the studies on the proteolytic systems of lactobacilli indicate that these strains possess weak proteinases and lipases, but have strong peptidase and esterase activities (Lee *et al*, 1986; Arora *et al*, 1990). Investigations where Cheddar was made using lactobacilli cells or their cell free extracts showed that proteases inherent to lactobacilli were responsible for increased levels of small peptides and free amino acids (Lloyd *et al*, 1980; Lee *et al*, 1990b) which are considered important in Cheddar cheese flavor (McGugan *et al*, 1979; Aston & Creamer, 1986). Lactobacilli proteolysis thus contributes significantly to flavor development.

Casein molecules contain a high proportion of hydrophobic residues (e.g. leucyl, prolyl, phenyl-alanyl) so that their hydrolysates have a marked propensity to bitterness (Sullivan & Jago, 1972). When the concentration of bitter peptides in cheese exceeds the taste threshold a bitterness defect is detected (Visser *et al*, 1983). The level of bitter peptides depends on their rate of formation (which involves primarily rennet and some other proteinases) relative to their rate of degradation (which mainly involves starter and nonstarter peptidases). Peptide accumulation and bitterness were first correlated during research in Gouda cheese (Raadsveld, 1953) and later, with Cheddar cheese (Harwalkar & Elliot, 1965 & 1971; Hamilton *et al*, 1974) All strains of cheese associated lactobacilli showed the presence of peptidases that are capable of degrading bitter peptides. However, initial trials using lactobacilli

cells as adjunct starter produced conflicting results in terms of elimination of bitterness, leading to the classification of strains into bitter or non-bitter lactobacilli (Lemieux *et al*, 1989).

In addition to the type, number and proteolytic ability of lactobacilli, there are number of other factors which influence the rate of starter proteolysis in cheese. These include the stability of starter enzymes (Cliff & Law, 1979), the salt-in-moisture level (Stadhouders *et al*, 1983; Lawrence *et al*, 1987) and the ripening temperature (Stadhouders & Hup, 1975). In Cheddar and similar hard cheeses proteolysis is slow, and hence, limits the rate of maturation. As discussed before, lactobacilli possess higher concentrations of desirable proteolytic enzymes, and are able to grow during the selective conditions of cheese ripening. Therefore, various successful attempts have been made to reduce the ripening period by the addition of selected lactobacilli cells (Laleye, 1986; Puchades *et al*, 1989; Lee *et al*, 1990b) or by direct addition of free enzymes having specific roles (El Soda *et al*, 1981a & 1982; Barach & Talbot, 1985). Recent studies on subjecting whole cells to sublethal conditions (freeze-shock and heat-shock) showed enhanced proteolytic ability in lactobacilli strains (Abdel Baky *et al*, 1986; Frey *et al*, 1986b; Ardo & Petterson, 1988). However, future strategies for accelerated ripening may involve genetic manipulation of lactobacilli strains to increase the expression of rate-limiting proteolytic enzymes (Gasson & Davies, 1984).

One of the major problem associated with the addition of enzymes to cheesemilk is their considerable subsequent loss in cheese-whey. A promising system, involving the use of liposome-encapsulated enzymes (commercial proteinase and starter peptidases) has been shown to reduce the ripening time to half (Braun *et al*, 1982; Piard *et al*, 1986; Kirby *et al*, 1987). The action of these enzymes in cheese will not be limited by the availability of substrate.

2.4. Lactobacilli as adjunct cultures

Early studies on cheese maturation microbiology clearly demonstrated the dominance of lactobacilli at a later stage of cheese ripening, leading to the general conclusion that in order to produce full Cheddar flavor it is essential to ensure certain levels of NSLAB (non-starter lactic acid bacteria) during cheesemaking. However, the role played by lactobacilli in Cheddar cheese flavor development was not fully understood, and many studies had been conducted in the past, utilizing NSLAB as adjuncts to the starter lactococci, to enhance cheese flavor (Law *et al*, 1976a; Rabie *et al*, 1986).

As early as 1939, Lane and Hammer inoculated pasteurized milk with eight different strains of *Lb. casei* plus a lactic starter and followed chemical and sensory changes in cheese. Six of the eight strains brought about more and extensive protein decomposition than occurred in control cheese. Flavor scores of cheeses made from pasteurized milk inoculated with *Lb. casei* were generally more uniform and higher than those of control cheeses. Improvement in flavor of Cheddar made from milk inoculated with *Lb. casei* were also noted by Sherwood (1939b). In a similar study McDonald (1945) reported more pronounced improvements in flavor when the culture was added directly to cheese curd rather than cheesemilk.

At 4 months of age, cheese inoculated with *Lb. casei* showed free amino acid levels similar to the control cheese (Bullock & Irvine, 1956). However, by 8.5 months, a higher level of free amino acids was detected in the inoculated cheese. Titsler *et al* (1947 & 1948) compared the effect of three *Lactobacillus* spp. added to cheesemilk on its quality. They noted that the cheese produced from inoculated *Lb. casei* was

generally more acid, and had higher flavor score but a poor body. However, *Lb. plantarum* inoculated cheese showed improvement in flavor without concurrent increase in the acidity. On the other hand, Cheesemilk inoculated with *Lb. brevis* resulted in poor quality cheese and developed objectionable flavors. Similar results were also reported by Decre (1953). Evaluation of cheesemaking trials in which viable strains of thermophilic lactobacilli (*Lb. delbrueckii* ssp. *bulgaricus*, *Lb. helveticus*, *Lb. delbrueckii* ssp. *lactis*, and *Lb. fermentum*) were added to the cheesemilk indicated that these species contributed little to the natural development of Cheddar cheese flavor. While some strains failed to grow under ripening conditions, others produced off-flavors and gassiness (Tittsler *et al*, 1947 & 1948; Lloyd *et al*, 1980).

Johns and Cole (1959) investigated the effects of adding lactobacilli to the pasteurized cheesemilk at varying rate of inoculation. After 6 months and one year of ripening, cheese made from factory raw milk or from the same milk pasteurized and inoculated with lactobacilli showed enhanced Cheddar flavor over the cheese made from pasteurized milk only. The conclusion drawn was that flavor intensity correlated with numbers of lactobacilli present at the start of cheesemaking and at subsequent stages of ripening.

In another study on the use of lactobacilli to accelerate maturation of Cheddar cheese, Puchades *et al* (1989) and Lee *et al* (1990a & b) noted that amino acid concentrations were higher in experimental cheeses than the control during the first four months of maturation. The flavor intensity was the highest in cheese in which a strain of *Lactobacillus casei* was added. Kowaleska *et al* (1985) isolated a number of aroma bearing compounds in cultures of *Lb. helveticus* which were also detected in aromatic extracts from Swiss, Cheddar and Parmesan cheeses.

Many other researchers have suggested a generally positive effect of lactobacilli in terms of improved flavor (Kristofferson *et al*, 1967; Law *et al*, 1976a), increased proteolysis (Di Palma *et al*, 1987), reduction of redox potential (Hickey *et al* 1983), and the production of sulfide and methionine rich flavor compounds (Green & Manning, 1982). However, the effect was not consistently observed in many other reports. For example, Mabbit *et al* (1955), in an attempt to generate more typical flavor in non-starter cheese, added strains of *Lb. casei* and *Lb. brevis* to acidified cheesemilk. Although adjunct cultures grew relatively well in the ripening cheeses, they invoked minimal improvement in cheese flavor. The author speculated that lack of flavor development was related to presence of comparatively high quantities of lactose in the maturing cheese. The lactobacilli presumably utilized lactose in preference to carbon sources normally metabolised in the absence of lactose.

Most of the earlier work was confined to studies of the role of lactobacilli towards flavor development. However, variations in the results were observed in some studies because strains were selected from the quality characteristics of cheese rather than being selected based on physiological characteristics. Studies in the last decade have therefore focused more on enzymes (El Soda *et al*, 1985; Frey *et al*, 1986a; Abo-Elanga & Plapp, 1987; Peterson *et al*, 1989; Arora & Lee, 1990), genetic systems (Lee-Wickner & Chassy, 1985; Baldi & Warner, 1987; Kok & Venema, 1988) as well as on their other positive attributes, such as acceleration of cheese maturation (El Soda *et al*, 1981a & 1982; Abdel-baky *et al*, 1986; Frey *et al*, 1986b; Ardo & Pettersson, 1988; Vafopoulou *et al*, 1989), and removal of certain cheese defects (Laleye *et al*, 1989; Lemieux *et al*, 1989). The information generated through enzymology and genetic studies has been further used to improve certain traits of NSLAB (Scheirlinck *et al*, 1989; Vos *et al*, 1990).

2.5. Proteolytic systems of lactobacilli

Numerous amino acids are either stimulatory or essential for the growth of lactic acid bacteria. Lactobacilli have been considered as having the most extensive amino acid requirement of the lactic acid bacteria (Morishita *et al*, 1981). It has been shown that at least one species of lactobacilli, *Lb. casei*, can actively transport amino acids and peptides across the cell membrane into the cell (Leach & Snell, 1960). The native concentration of free amino acids and low molecular weight peptides is low in milk, and is rapidly consumed by the starter lactococci during Cheddar cheese manufacture (Mills & Thomas, 1980). However, both lactococci and lactobacilli seem to possess a number of proteolytic enzymes which act in concert to hydrolyse milk proteins to free amino acids (Thomas *et al*, 1974; Exterkate, 1975; Mills & Thomas, 1978).

The proteolytic system of lactic acid bacteria is usually considered to possess two functionally distinct classes of enzymes - proteinases, which catalyse the hydrolysis of native or denatured protein molecules, and peptidases, which further catalyse the degradation of the smaller peptides produced by proteinase action (Thomas & Mills, 1981; Law & Kolstad, 1983).

2.5.1. Proteinases

Unlike many other Gram-positive bacteria (*e.g. Bacillus, Proteus, Pseudomonas etc.*), lactic streptococci and lactobacilli possess weak proteinases (Ducastelle & Lenoir, 1969; Law & Kolstad, 1983; Geis *et al*, 1986), and apparently do not secrete significant levels of them into the growth medium (Thomas & Mills, 1981; Hugenholtz *et al*, 1984 & 1987; Ezzat *et al*, 1985; Thomas & Prichard, 1987).

However, there is evidence that starter lactococci may also release one or more extracellular proteinases into the growth medium (Exterkate, 1976). In studies of *Lc. lactis*, at least four proteinases have been isolated, all of which are cell-wall associated (Law & Kolstad, 1983).

Synthesis of cell-wall proteinases appears to be regulated by amino acids or peptides at the level of mRNA translation, such that low concentrations of casein derived amino acids (CDAA) do not prevent proteinase synthesis. Moreover, cells respond to higher concentration of CDAA by reducing the rate of enzyme synthesis until the cell has exhausted the external nitrogen source (Law & Kolstad, 1983). Early reports of spontaneous loss of proteinase synthesis ability in lactococci suggested that the enzymes are plasmid encoded (Davies & Gasson, 1981). The proteinase gene has been mapped on a 33 M dalton plasmid of *Lc. lactis* 712 (Gasson, 1983) and on a 17.5 M dalton plasmid in *Lc. lactis* ssp. *cremoris* Wg2 (Kok *et al*, 1985).

Proteinase systems of lactobacilli, however, have not received the amount of research attention as the lactococci. Searle *et al* (1970) first reported that lactobacilli probably have cell-wall bound proteinases as evidenced by the hydrolysis of milk protein by whole cell suspensions. Many further experiments with *Lb. casei* (Neviani *et al*, 1984; El Soda *et al*, 1985 & 1986), *Lb. plantarum* (El soda *et al*, 1986), *Lb. delbrueckii* ssp. *bulgaricus* (Argyle *et al*, 1976; Chandan *et al*, 1982; Ezzat *et al*, 1986), and *Lb. helveticus* (Vescovo & Betazzi, 1979; Ezzat *et al*, 1985; Zevaco & Gripon, 1988) have reconfirmed the presence of cell-wall associated proteinases.

Frey *et al* (1986a), while comparing the proteolytic efficiency of different cheese-associated lactobacilli, found that *Lb. casei* exhibited proteolytic activity at only one-half the level of *Lb. delbrueckii* ssp. *bulgaricus* and one-third that of *Lb. helveticus*. However, the growth and proteinase assay were carried out at higher temperature

(40 °C) than used for *Lb. casei* or related mesophiles. Hickey *et al* (1983) observed that species of lactobacilli, including *Lb. casei* and *Lb. plantarum* were more proteolytic and released amino acids more rapidly from sodium caseinate than did two strains of *Lc. lactis* ssp. *cremoris*, especially in combination with rennet treatment. More recent study on *Lb. casei* and *Lb. plantarum* showed that strains of both species possessed proteinases capable of hydrolysing α_{s1}- and ß-casein. However, *Lb. casei* was found to exhibit significantly greater proteolytic activity than *Lb. plantarum*. It was also observed that cells grown on milk were more proteolytic than the cells cultivated on MRS medium (El Soda *et al*, 1986). This suggests the probable existence of a regulatory mechanism for lactobacilli proteinase synthesis similar to that proposed for lactococci (Exterkate, 1983).

2.5.2. Peptidases

Information on the peptidase activities of starter bacteria has been accumulating since the early 1970s and has been reviewed on several occasions (Thomas & Mills, 1981; Law & Kolstad, 1983; Van Boven & Koning, 1986; Thomas & Pritchard, 1987; Peterson & Marshall, 1989; Olson, 1990; Kok, 1990). The study of peptidases has involved the isolation and biochemical characterization of the (partially) purified enzymes, and by now a number of different peptidase activities have been described.

The location of peptidases, especially aminopeptidases in cells is controversial. Many of the published studies on peptidases in starter bacteria have involved disruption of cell suspensions by sonification (Kaminogawa *et al*, 1984a), French press (Hickey *et al*, 1983) or grinding (El Soda *et al*, 1978b; Ezzat *et al*, 1986). Peptidase activities of such preparation include intracellular peptidases plus those released from cell wall or cell membrane by the disruption procedures used, thus making it difficult to

establish the exact location of these enzymes. However, attempts have been made in this regard by using more gentle techniques of cell fractionation, such as enzyme lysis (El Soda *et al*, 1978a; Exterkate, 1984; Kolstad & Law, 1985) or solvent extraction (Exterkate, 1984). Consequently, peptidases have been detected in and isolated from several subcellular fractions of starter lactic acid bacteria (Kolstad & Law, 1985; Ezzat *et al*, 1986).

Apart from the difficulty in establishing the cellular location of particular peptidase activities, the number of different peptidase enzymes in different starter and non-starter organisms is not yet known. Several attempts have been made to investigate the peptidase complexes by fractionation of cell extracts using ion-exchange chromatography (El Soda *et al*, 1978b; Kaminogawa *et al*, 1984b), gel filtration (Mou *et al*, 1975; El Soda *et al*, 1978b) or polyacrylamide electrophoresis (El Soda & Desmazeaud, 1982; Abo-Elanga & Plapp, 1987), and then assaying for peptidase activity using a variety of substrates. The existence of a wide range of different types of peptidases - aminopeptidases, di- and tripeptidases, aryl-peptidyl amidase, aminopeptidase P, proline iminopeptidase, prolinase and prolidase, X-prolyl-dipeptidyl aminopeptidases, endopeptidases, and carboxypeptidases has been described in literature cited. Most of these enzymes have also been shown to play a significant role in peptide degradation during cheese ripening.

As with proteinases, peptidases of lactococci have been more widely investigated than those of lactobacilli. Mou *et al* (1975) detected five different peptidases, based upon type of substrate hydrolysed, in crude cell-free extracts of *Lc. lactis* ssp. *diacetylactis* and *Lc. lactis* ssp. *cremoris*. These included di- and tri-peptidases, an aminopeptidase-P, a proline iminopeptidase, and a general aminopeptidase. Based on their data, they have suggested that the specificities of these enzymes are wide enough to completely hydrolyse all polypeptides (derived from casein after

proteolysis) to free amino acids. Other researchers have reported four to nine peptidases for species of starter lactococci (Sorhaug & Kolstad, 1981; Kaminogawa *et al*, 1984b).

Brandsaeter and Nelson (1956b) appear to have been the first to focus attention upon lactobacilli peptidases. Using synthetic dipeptides and tripeptides, they found that the highest peptidase activity of cell-free extract of one *Lb. casei* strain occurred between 45-50 °C and pH 7.0-8.0. Enzyme activation by Co^{2+} or Zn^{2+} at pH 5.0-5.5 and pH ≥7.0, respectively was also observed. Mn^{2+} and Mg^{2+}, however, were found to have little effect.

Over a period from 1956 to 1978, little work occurred in this area until El soda *et al* (1978a) chose to continue studies on *Lb. casei* (NCDO 151). Using cell-free extracts, they demonstrated the presence of amino- (leucyl *p*-NA), di- (glycyl-tyrosine), carboxy- (N-benzyloxycarbonyl-glycyl-L-arginine) and endo- (succinyl-phenylalanine *p*-NA) peptidase activities. This was the first and perhaps the only strain identified until today to exhibit the presence of carboxypeptidase activity in lactococci and lactobacilli. These results implied that as with the lactococci, most peptidase activity was associated with the cytoplasmic fraction, a conclusion further substantiated in subsequent work (El Soda *et al*, 1986). Also noted was the influence of the cellular growth phase on the synthesis of peptidases, which appeared to reach its maximal level at the early stationary phase. Dipeptidase activity did not seem to be influenced significantly by N source (casein, peptone or amino acids) in the growth medium, while carboxypeptidase activity was measured higher in cells grown in the casein or peptone medium, as compared to the amino acid media. Aminopeptidase and endopeptidase activities were highest when free amino acids had been the end source.

Following further purification via gel chromatography, El Soda *et al* (1978b) partially characterized a dipeptidase, a carboxypeptidase and an aminopeptidase from the same bacterium. The temperature maxima and pH optima of different peptidases were 30°, 40°, 45°C, and 7.6, 7.2, and 6.5 respectively. All three peptidases were strongly inhibited by EDTA and 1,10-phenanthroline but were reactivated by Co^{2+} and Mn^{2+}.

In a more recent study (Abo-Elanga & Plapp, 1987), enzyme activity staining on polyacrylamide gel electrophoresis (PAGE) of *Lb. casei* (NCDO 151) cell-free extracts allowed the separation of two dipeptidases, a carboxypeptidase, and a tripeptidase. Both dipeptidases were identical in substrate specificity. Out of 120 dipeptides tested, 109 were hydrolysed to some degree by each. The dipeptidases were unable to hydrolyse substrates having a C-terminal proline, due to steric factors introduced by the imino group in this position. However, proline in N-terminal position did not appear to block activity. The carboxypeptidase exhibited widest specificity, having the ability to hydrolyse various dipeptides and tripeptides, in addition to various carbobenzoxy-peptides. Growth studies indicated that all the peptidases mentioned above were constitutive enzymes. However, they discovered the presence of two additional dipeptidases in cells grown on skim milk, suggesting an induction mechanism governing their synthesis. Comparative studies of peptidases of strains of *Lb. casei* and *Lb. plantarum* demonstrated a notable difference in activity and substrate specificity among the strains.

Aminopeptidase and carboxypeptidase activities of four *Lactobacillus* spp., with emphasis on the thermophiles, were evaluated by Hickey *et al* (1983). The pH optima for aminopeptidase activity in all species, including *Lb. casei* and *Lb. plantarum*, were found to be between 6.5-7.0. Activity toward alanyl-, leucyl- and arginyl-derivatives was substantially higher in strains of *Lb. helveticus* and *Lb.*

delbrueckii ssp. *bulgaricus* than in strains of *Lc. salivarius* ssp. *thermophilus*. *Lb. helveticus* also possessed higher proline iminopeptidase and aminopeptidase-P activities. Carboxypeptidase activity was not detected in any of the strains under the conditions employed. The studies also demonstrated the complementary effect of rennet on the peptidase activity in overall release of amino acids from sodium caseinate. Results of another study (Frey *et al*, 1986a) showed that at least some strains of *Lb. casei* exhibit greater aminopeptidase activity toward lysyl and leucyl derivatives than strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lb. delbrueckii* ssp. *lactis*. The authors speculated that cells with low protease and high aminopeptidase activities should accelerate cheese ripening without development of excessive bitterness, a theory substantiated through actual production of Gouda cheese (Bartel *et al*, 1987).

El Soda and Desmazeaud (1982) made an attempt to characterize peptidases of 14 strains of different *Lactobacillus* species by activity staining on disc gel electrophoresis. By this method, the presence of one general aminopeptidase was demonstrated in all the strains. Although the enzyme of different strains had the same electrophoretic mobility on disc gels, it differed significantly in its substrate specificities. *Lb. delbrueckii* ssp. *lactis* possessed an additional aminopeptidase having different specificity and greater electrophoretic mobility. A distinct dipeptidase having higher electrophoretic mobility than aminopeptidase was also located in each bacterium. *Lb. delbrueckii* ssp. *lactis*, however, possessed two additional dipeptidases. Later, Ezzat *et al* (1986) determined the properties of partially purified amino- and di-peptidases of three of the above strains. Peptidases of these strains showed higher amino- and di-peptidase activities near neutral pH at 30 to 40°C. However, contrary to most aminopeptidases characterized up to now, the aminopeptidase of *Lb. helveticus* and *Lb. delbrueckii* ssp. *bulgaricus* were not inhibited by EDTA and were characterized as serine proteases, whereas, aminopeptidase of *Lb. delbrueckii* ssp.

lactis and dipeptidases of all the strains were metal-activated enzymes.

The high proline content of the caseins has prompted several investigators to investigate specifically for peptidases acting on proline containing substrates. While most studies on lactic starter peptidase profiles indicate very weak activity of general aminopeptidase and dipeptidases for the hydrolysis of peptide bond containing proline, a separate cluster of peptidase enzymes, showing specificity for peptide bonds involving proline, has been elucidated in both lactococci and lactobacilli, and is described in literature. Casey and Meyer (1985) demonstrated the presence of a X-prolyl dipeptidylpeptidase (X-PDP) in a wide range of *Lactobacillus* and *Lactococcus* species. All X-PDPs specifically degrade peptides of the structure X-Pro-Y...(X and Y can be any amino acid) but the rate of hydrolysis depends on the type of amino acids surrounding the proline. Polyacrylamide gel electrophoresis showed this to be an enzyme distinct from prolyl iminopeptidase which was also present in most of the strains, but at much lower levels of activity. While all the 16 strains of lactobacilli used in the study showed the presence of X-prolyl dipeptidyl amino- and prolyl amino-peptidase, the latter activity was absent in lactic streptococci. Hickey *et al* (1983) showed the presence of proline aminopeptidase and aminopeptidase-P activities in wide range of lactobacilli. In a comparative study, they concluded that *Lb. helveticus* had the highest activities of all the peptidases when compared to other species of lactobacilli and lactococci.

X-PDP has recently been purified and characterized from *Lb. helveticus* (Khalid & Marth, 1990), *Lb. delbrueckii* ssp. *bulgaricus* (Atlan *et al*, 1990) and *Lb. delbrueckii* ssp. *lactis* (Meyer & Jordi, 1987), as well as from several lactococci (Kiefer-Partsch *et al*, 1989; Zevaco *et al*, 1990). In general, X-PDP of both lactococci and lactobacilli showed maximum activities between 40-50°C and at neutral pH. However, in contrast to general aminopeptidase of lactic acid bacteria, which is in most cases a metal-

dependant enzyme, X-PDP was found to be a serine protease.

The presence of this enzyme in many plasmid-free lactococcal strains suggested that the enzyme might be coded on the chromosome (Kiefer-Partsch *et al*, 1989). A recent study on mutant stains of *Lactobacillus delbrueckii* ssp. *bulgaricus* deficient in X-PDP showed a decrease in the growth rate with simultaneous increase in cell wall proteinase activity in such strains than in control samples (Atlan *et al*, 1990). Similar previous studies carried out on aminopeptidase-deficient strains of the same species also displayed an increase in cell wall proteinase and X-PDP activities (Atlan *et al*, 1989). Based upon their findings, the authors suggested the existence of common regulatory mechanisms controlling the biosynthesis of aminopeptidase, X-PDP and cell wall proteinases.

While peptidases acting on proline-containing peptides were easy to categorize, based on their substrate specificities, such was not the case with most other amino- as well as di-peptidases. An unusually wide spectrum of specificity has been observed in purified aminopeptidase enzyme from *Lb. delbrueckii* ssp. *lactis* (Eggimann & Buchmann, 1980) and *Lb. acidophilus* (Machuga & Ives, 1984). In addition to its usual N-terminal exopeptidase activity, the enzyme from both of these strains was active with a wide range of aminoacyl *p*-nitroanilide derivatives as well as on certain dipeptides, tripeptides and tetrapeptides. On the contrary, the aminopeptidase from *Lc. lactis* was not active with any of the dipeptides tested nor was dipeptidase fraction able to use aminoacyl *p*-nitroanilides as substrates (Kaminogawa *et al*, 1984b). However, a separate study on purified aminopeptidase from a different strain of the same species showed broad spectrum aminopeptidase activity, degrading N-terminal amino acids of dipeptides, tripeptides, oligopeptides along with *p*-nitroanilide amino derivatives (Tan & Koning, 1990). It appears that aminopeptidases of broad specificity are common in lactic acid bacteria, but they did

not show carboxypeptidase and endopeptidase activities.

Dipeptidase activities showing a broad specificity have been detected in several lactic acid bacteria (El Soda *et al*, 1978a; Kaminogawa, *et al*, 1984b; Abo-Elanga & Plapp, 1987). Dipeptidases of two strains of *Lc. lactis* ssp. *cremoris* have been purified and characterized (Hwang *et al*, 1981; Van Boven *et al*, 1988). Both enzymes were metallopeptidases and were able to catalyse hydrolysis of a wide range of dipeptides. However, the enzyme could not degrade dipeptides containing proline or glycine at the N-terminal.

Virtually all the information on the peptidase activity in starter bacteria relates to various types of exopeptidases, *i.e.* enzymes catalysing the cleavage of one or two amino acid residues from the free end of the peptide chain. There are rare reports on the existence of endopeptidases capable of hydrolysing oligopeptide substrates at bonds distant from the N- or C-terminal ends. The first report came from Exterkate (1984) who demonstrated the presence of two cell membrane-associated endopeptidases (distinct from proteinase activity) from *Lc. lactis* ssp. *cremoris* strain. The endopeptidase nature of these enzymes was assumed from their ability to hydrolyse aminoacyl nitroanilides with the N-terminal group blocked by a glutaryl group. However, hydrolysis of C-N bond in aromatic amide derivatives of amino acids is diagnostic of an arylamidase but not necessarily of an endopeptidase (El Soda & Desmazeaud, 1981). This enzyme has recently been purified and characterized (Yan *et al*, 1987).

El Soda *et al* (1981b) detected a similar enzyme in all the strains of *Lb. casei* ssp. *casei*, *Lb. casei* ssp. *rhamnosus*, *Lb. casei* ssp. *alactosus* and *Lb. casei* ssp. *pseudoplantarum*. The enzyme was also detected in some of the strains of *Lb. brevis* and *Lb. fermentum*, while no activity was found in some closely related species, such

as *Lb. plantarum*, *Lb. helveticus*, *Lb. delbrueckii* ssp. *lactis* , *Lb. acidophilus* and *Lb. delbrueckii* ssp. *bulgaricus*.

Considerable evidence has now been documented for the existence and cellular location of different types of peptidases in lactobacilli. While nothing can be concluded at this stage on the status of endopeptidases, a reasonably consistent picture of exopeptidase complex of lactobacilli is beginning to emerge from these various investigations. The complex appeais to comprise of perhaps five or six distinct enzymes: a general aminopeptidase, a dipeptidase of broad specificity, two or more exopeptidases acting on bonds involving proline and a distinct tripeptidase. Some of these intracellular enzymes may be localized in or outside the cell membrane possibly in isoenzymic forms. More knowledge on peptidase profiles of different *Lactobacillus* strains, as well as purification and characterization of each of these enzymes is necessary to establish the distinct number of enzymes, the range of substrates that they are capable of hydrolysing and the contribution that each enzyme makes to the proteolytic degradation pathway of milk proteins.

2.6. Assay of Peptidase activity

Much of the work with the intracellular peptidases of lactic acid bacteria has focused upon dipeptidase and aminopeptidase activities (Exterkate, 1975; El Soda *et al*, 1978b; Hickey *et al*, 1983; Frey *et al*, 1986a; Abo-Elanga & Plapp, 1987). Synthetic substrates have typically been employed for measurement of activity. The goal in all cases was to quantify the number of peptide bonds hydrolysed by an enzyme of interest. Spectrophotometric methods offer the simplest, most rapid and least expensive means of quantifying soluble components. However, such methods are applicable only when the components of interest absorb light within the ultra violet

or visible spectrum in accordance with Beer's law. While some aromatic amino acids, such as tyrosine and tryptophan, do meet this criteria and have been used as indicators for cheese ripening (Vakaleris & Price, 1959), free amino- or carboxyl-groups exposed by an enzyme during peptide hydrolysis do not. Consequently, a chromophore which, through association or dissociation with free amino or carboxyl groups, produces a soluble colored product adhering to Beer's law, must be included in the reaction at some point prior to quantification.

In case of dipeptides, that chromophore may only be introduced after hydrolysis has occurred. The original and most extensively used reagent for this purpose is ninhydrin. Moore and Stein (1948) were the first to employ ninhydrin as a means of determining amino acid concentrations. The ninhydrin reacts with free amino groups of amino acids to form a blue color complex which can be measured spectrophotometrically. Several researchers, including Yemm and Cocking (1955), have since introduced modifications to improve reaction efficiency as well as end product stability. However, even with the modifications, the method was not ideally suited for peptidase assays. It produced color yields for peptides (*i.e.* blank values) nearly equal to those of free amino acids, making it difficult to detect changes in free amino group concentration caused by hydrolysis. However, Matheson and Tattrie (1964) were able to lower the color yield, or molar extinction coefficient, of peptides through reduction of buffer concentration and heating time prescribed by Yemm and Cocking.

Fields (1971) developed an alternative method which replaced ninhydrin with 2,4,6-trinitrobenzenesulfonic acid (TNBS). Reaction of L-amino groups with TNBS produces a trinitrophenylated amino-sulfite complex which absorbs strongly at 420 nm. Both TNBS and ninhydrin are acknowledged as extremely sensitive reagents for amino acid detection (Rowlett & Murphy, 1981; Samples *et al*, 1984). The latter is

still considered the reference method to which all subsequently developed amino acid assays are compared. However, both methods suffer at least two disadvantages. These include heating and cooling steps or extended incubation periods, and require construction of standard calibration curves each time when the assays are performed. In addition, the ninhydrin reagent must be frequently prepared and stored under inert atmosphere due to oxygen and nitrogen sensitivity.

One technique which achieves equal or greater sensitivity than ninhydrin or TNBS, without the associated disadvantages, is the fluorescamine assay (Schwabe, 1973). As with ninhydrin and TNBS, fluorescamine reacts with free primary amino groups, to yield products which not only absorb but also fluoresce. Although this method is far more sensitive than spectrophotometric methods, very few laboratories are equipped with fluorometers, limiting its applicability. In addition, it has been reported that fluorescamine may often fail to react completely with all primary amines, especially tryptophan, even when the reagent is present in excess (Chen *et al*, 1979).

Yet another fluorogenic reagent which, as its predecessors, reacts with primary amino groups, is *o*-phathaldialdehyde (OPA). The properties of this compound as well as its applicability to amino acid quantification and proteolytic assays have been extensively investigated (Benson & Hare, 1975; Chen *et al*, 1979; Goodno *et al*, 1981; Porter *et al*, 1982). The reaction of OPA with primary amines occurs only in the presence of a thiol, typically ß-mercaptoethanol, and is enhanced at basic pH. Under these conditions, 1-thioalkyl-2-alkylisoindoles are formed, which, following excitation at 340 nm, fluoresce at 450 nm. Simons and Johnson (1976) were the first to identify the structure of OPA-thiol-primary amine adduct.

The OPA reagent was found to meet many of the criteria of an ideal reagent for routine analysis of peptide hydrolysis reactions. These include sensitivity in the

micro-molar range, stability of the reagent under ordinary atmospheric conditions, rapid and complete reactivity, and simplicity to use (Rowlett & Murphy, 1981). As a fluorogenic agent, OPA still suffers several significant drawbacks, including poor yields for some amino acids, namely lysine, cysteine, cystine, and all peptides, as well as poor stability of the OPA-mercaptoethanol adduct (Simons & Johnson, 1976). Recent work has circumvented the instability problem via substitution of ethanethiol for mercaptoethanol in the reagent mixture (Rowlett & Murphy, 1981). Although recent development of a spectrophotometric OPA assay method has increased its potential for a method of choice, one major drawback still associated with this assay is its lower sensitivity (than spectrophotometric method) as well as its inability to measure cysteine and proline concentrations.

The assays for aminopeptidase activity are considerably less complicated than for dipeptidase activity. The most commonly used methods employ a substrate composed of an amino acid with a chromophore attached at the N-terminal position. When intact, the derivative is colorless. However, hydrolysis by an aminopeptidase causes release of the colored chromophore. Intensity of the colored product is proportional to the amount of substrate hydrolysed, in accordance with Beer's law. Derivatives of *p*-nitroaniline and ß-naphthylamide have been used extensively in studies of lactic acid bacteria.

Growth and Disintegration of *Lactobacillus casei* Cultures

KEY WORDS

Lactobacillus casei, Physiology, Lactic acid bacteria, Growth, Cell lysis.

ABSTRACT

Growth of *Lactobacillus casei* in MRS medium resulted in a gradual decrease in medium pH, which greatly influenced its growth curve. Although the duration of lag and exponential phases differed by the levels of inoculation, it was possible to relate various growth phases with the growth parameters such as viable count (CFU), pH and absorbance (A_{600}) of the culture medium. A close interrelationship was observed among these parameters in the exponential phase of cell growth, irrespective of the level of initial cell count.

The effectiveness of three different cell disruption methods was also evaluated. Mechanical grinding of cells with glass beads in a mixer mill produced >95% disruption of cells without any loss of enzyme activity. The disruption efficiency was influenced most by the operating time, bead size and mixing frequency. In contrast to mechanical grinding, enzymatic methods involving lysozyme and commercial lytic enzyme proved ineffective in the lysis of *Lb. casei* ssp. *casei* LLG. The method using lytic enzyme interfered with the API ZYM test which was applied to evaluate intracellular enzymes in crude extracts. Cell lysis by enzymatic methods also showed significant variation ($P>0.05$) among the replicates.

INTRODUCTION

Starter bacteria must possess very limited metabolic diversity in order to produce cheeses with acceptable organoleptic characteristics. The two most essential features of the starter lactic acid bacteria are the possession of an efficient proteolytic system and the ability to rapidly ferment lactose, almost exclusively to lactic acid (Lawrence *et al*, 1984). Earlier studies on *Lactobacillus casei* have clearly demonstrated their superiority in these traits (Carini & Lodi, 1974; Law & Sharpe, 1977; Arora *et al*, 1990; Laleye *et al*, 1990). The lengthy maturation period of cheese offers varied environmental conditions in terms of acidity, redox potential as well as availability of different nutrients to which the microorganisms must adapt in order to grow in sufficient numbers. In spite of their complex nutrient requirements, lactobacilli are well adapted to grow in the selective conditions of cheese ripening (Thomas, 1987; Peterson *et al*, 1989; London, 1990).

For assessing the manifold influences of environmental conditions on a microbial culture, it is essential to study the growth pattern of the organism in defined conditions. Cell growth is generally monitored by direct methods such as cell number, cell mass or cell volume, or indirectly by relating cell growth with any change in chemical and physiological parameters (Meyer *et al*, 1985). Since the production of lactic acid from lactose is the major and essential energy yielding pathway of *Lactobacillus casei* species, a drop in pH of the medium has been used and correlated in growth measurement studies (Roy *et al*, 1987). Optical density (extinction, opacity) is the other fast indirect method used for batch as well as on-line measurement of microbial cultures. The possibilities, problems and the limits of this method has been well known (Wyatt, 1973).

Besides obtaining an insight into the cell metabolism, growth studies also constitute an essential initial step in the production of higher cell biomass and its enzymes. The present investigation was designed with these objectives.

Almost all studies related to localization and characterization of enzymes of microorganisms involve an efficient cell breakage procedure. A number of biological (*e.g.* enzymatic and osmotic lysis) and mechanical (sonification, pressure shearing and ballistic disintegration) methods are available for cell fractionation (Hughes *et al*, 1971; Schnaitman, 1981), and most of these have been attempted on various lactobacilli with varying degree of efficiency (Ishiwa & Yokokura, 1971; Chassy & Giuffrida, 1980; Hummel & Kula, 1989). In general, enzymatic lysis and ballistic disintegration are the two preferred methods for the disintegration of lactobacilli. However, the operating conditions of each method need optimization for higher enzyme yield and to minimize the loss of enzyme activity.

MATERIALS AND METHODS

Strains, maintenance and growth

Lactobacillus casei ssp. *casei* LLG used in this study was isolated from a high quality Cheddar cheese. The strain was maintained on lactobacilli MRS agar medium (Difco Laboratories, Detroit, MI)(De Man *et al*, 1960) at 4°C, and at -30°C in reconstituted sterile 20% (vol/vol) skim milk powder (Difco) diluted equally with MRS medium, and were transferred triweekly. The strains were also regularly examined microscopically and by API ZYM test for determination of purity and physiological activity.

Culture was revived by 2-3 consecutive subculturing in MRS broth before growth of the cells for actual experiments. The cultivation of the strain was conducted in batch culture conditions at 30°C for up to 24 hours without any agitation, and portions of cultures were removed at various time intervals. The samples were analyzed for pH, CFU/ml, and optical density (A_{600}).

Disintegration of cells

Unless otherwise stated, Cells from late logarithmic phase were harvested by centrifugation (10,000 x g for 15 minutes). 0.05*M* sodium phosphate buffer (pH 7.0) was used to wash and suspend the cells, which were subjected to the following procedures.

1). Cell lysis by lysozyme

Lysozyme used for the cell lysis experiment was obtained from Sigma Chemicals (St. Louis, USA). Cell lysis was carried out by the method of Exterkate (1984). The cells were washed once with sterile sodium phosphate buffer (0.05*M*, pH 7.0) and once with distilled water. Cell suspension was adjusted to an absorbance of 0.5 at 600 nm, and the pH was adjusted to 7.0. An equal amount of lysozyme solution (6 mg/ml in distilled water) was added to the cell suspension, and the mixture was stirred gently for up to 60 minutes at 30°C. Portions (10 ml) of the assay were drawn at different time intervals, centrifuged (47,000 x g for 15 minutes) and the supernatant was tested for the enzyme activities by API ZYM method. Samples absorbance was also monitored by a Beckman spectrophotometer (Model DU-7; Beckman Instruments Inc., Irvine, USA) at 600nm.

2). Cell lysis by lytic enzyme

A commercial lytic enzyme (Type SP249) specific to Gram-negative organisms was procured from Novo industries, Montreal, Canada. 10 ml of cell suspension in 0.05*M* sodium phosphate buffer (pH 6.0) was used for the experiment, and the lysis was carried out by the method of Ishiwa and Yokokura (1971). The washed cells were treated with chloroform for 5 minutes at room temperature and then suspended in phosphate buffer at pH 6.0. Reaction was started by the addition of lytic enzyme to the cell suspension (previously adjusted to O.D. 1.0 at 600nm), and the lysis was measured by decrease in optical density of cell suspension. Enzyme activities in the cell-free extract were also analyzed by the API ZYM system.

3). Cell lysis by ball mill

250 ml of the washed cell suspension (adjusted to O.D. 1.0 at 600nm) was centrifuged and resuspended in 6.0 ml (approximately 25% cell slurry) of the same buffer. A Brinkmann ballmill type MM2 (Brinkmann Instr. Ltd., Rexdale, Canada) was used to break the cells (Appendix III). The mixing vessel was fastened to one of the vibrating arms, then 11.0 ml glass beads, representing a loading volume of 90%, were filled in with the aid of a small funnel through an opening in the grinding vessel. The cell suspension was then injected into the grinding vessel by a syringe, and the opening closed by a hand screw. The disruption procedure was started by the timer, and the vibration frequency set as desired. At periodic time intervals, the experiment was stopped, and the sample was drawn by a syringe for analytical determination after removing the screw. The whole process of disintegration and fractionation was carried out at 4°C. Crude extract was centrifuged at 47,000 x g for 15 minutes, and analyzed for protein content and enzyme activity.

At the end of the experiment, glass beads were washed, and sterilized by a hot air oven. The grinding vessel was also washed and sterilized with alcohol before next use. The efficiency of cell lysis was measured from CFU/ml of cell slurry before and after the disintegration. Cells debris were allowed to stand in MRS medium for 30 minutes at 30°C for the possible resuscitation of damaged cells before agar plating.

API ZYM assay

The API ZYM kits were obtained from API Laboratory Products Ltd. (St. Laurent, Qué, Canada) and the method of Arora *et al* (1990) was used for this experiment. 25 μl of cell suspension or cell-free extract was added to each of the 19 cupules containing dehydrated chromogenic enzyme substrates (Table 3), and the strips were incubated for 4 hours at 37°C. The manufacturer's instructions were followed for the development of color (Appendix II). The activity was measured by comparing the color developed in 5 minutes to the color chart provided by the manufacturer, and expressed on a scale of 0 (no activity) to 5 (maximum activity; >40 n*M* of chromophore released).

Protein assay

Protein was determined spectrophotometrically by the BCA (bicinchoninic acid) assay reagent supplied with the system (Pierce Chemical Ltd., Rockford, USA)(Smith *et al*, 1985). Bovine serum albumin (Sigma Chemical Company, St. Louis, USA) was used for the calibration curve (Fig.1).

Statistical analysis

All experiments were performed in triplicate. Growth data of different experiments was analyzed by exponential least square regression analysis. All regression coefficients of individual experiments were greater than 0.997.

RESULTS AND DISCUSSION

The experiment carried out for growth curve included the cultivation of *Lactobacillus casei* ssp. *casei* LLG in the MRS medium at 30°C for 24 hours. A stationary phase culture was inoculated at different rates (0.1%, 0.25%, 0.5% and 0.75%), and the growth was measured at different time intervals. The CFU/ml, optical density and pH of cultures are shown in Figure 3, 4, and 6, respectively. As expected, the time required to attain the stationary phase was proportionally lowered with the increase in inoculation size (Fig. 3). Under the conditions of the experiment, the cultures attained the stationary phase at 1 x 10^{10} CFU/ml after 16-24 hours of incubation, depending upon the initial cell count of the cultures (Fig. 3). Although the growth curves of cultures inoculated at different levels showed similar slope in the exponential phase of growth, a lower lag phase was noticed in cultures at higher initial inoculum (Fig.3). The slope of logarithmic phase depicted a generation time of 127 minutes for the strain.

The optical density of the growth medium was also measured for the strain during various growth phases (Fig. 4). Similar to the results of viable count, the optical density of the medium was increased with the growth time. However, the absorbance

profiles differed significantly ($P<0.05$) during the initial stages of growth, when compared to viable count curve (Fig. 3). A close correlation (R^2 value of 0.99) was observed between the logarithms of cell count and the medium absorbance in the exponential growth phase at all levels of inoculation (Fig. 5). Statistical analysis of growth data of individual plots revealed a higher degree of correlation (R^2 value) with the increase in the level of inoculation. The pH measurements during various growth phases demonstrated that *Lactobacillus* growth in batch culture gradually lowers the pH of the medium (Fig. 6), which in turn becomes the major limiting factor to its subsequent growth. The initial slow response to pH change was primarily due to the buffering action of the medium, and this also accounted for an increase in apparent lag phase in curves with lower rates of inoculation. Consequently, the pH of the medium decreased exponentially to a steady level (around pH 4.0), which corresponded to the stationary phase of the growth curve. Since both pH and CFU/ml are directly related to cell metabolism, a high correlation (R^2 value of 0.98) was observed between cell number and pH of the medium in the exponential phase of the growth (Fig. 7).

In addition to providing information about cell physiology, the above study was also helpful in selecting growth parameters that were used in the further experiments to evaluate and compare the biomass production and enzyme activities of different *Lactobacillus* strains (Arora & Lee, 1990).

Disintegration of cells

Both enzymatic (using lysogenic enzymes) and mechanical methods (french press, ball mill, homogenizer *etc.*) have been used with varying success in the study of intracellular enzymes of lactic starters. Three different methods of cell lysis, involving both enzymatic (using egg lysozyme and lytic enzyme) and mechanical means were

evaluated for their lysis efficiency. The experiment was conducted with the objective to find an efficient cell disruption method for further studies on the characterization of intracellular enzymes of different *Lactobacillus* species.

1). Lysozyme treatment

Disintegration efficiency of lysozyme treatment of *Lactobacillus casei* ssp. *casei* LLG was continuously monitored spectrophotometrically, and the results are listed in Table 1. There was no apparent decrease in absorbance under the conditions of the assay after 60 minutes of incubation with lysozyme. However, API ZYM results of cell-free extracts obtained after lysozyme treatment indicated slight leakage of cell material (Fig. 8). This was also apparent from 15% reduction in CFU/ml of cell suspension after the enzyme treatment. While some previous workers have obtained efficient disruption of some *Lactococcus* (Exterkate, 1984; Kruese & Hurst, 1972) and *Lactobacillus* (Chassy & Giuffrida, 1980) strains by a similar lysozyme treatment, the enzyme was not found effective in the cell lysis of *Lb. casei* strain under the conditions of the assay.

Lytic enzyme treatment

Cells were incubated with the lytic enzyme and the lysis was monitored for up to 60 minutes. Cell lysis was measured by decrease in absorbance, CFU/ml and enzyme activities (API ZYM system). Lytic enzyme resulted in relatively poor cell lysis (~27%), when compared to mechanical grinding of cells. In addition, the API ZYM test could not be used for the evaluation of enzyme profile of *Lactobacillus casei*. The impurities present in the crude lytic enzyme preparation were mainly

responsible for higher overall readings of API ZYM. The lysis efficiency also varied significantly ($P>0.05$) within the replicates.

Disintegration by ball mill

Cells were disrupted by shear forces generated during vibration and enforced motion of glass beads in a mixer mill. Initial trials were conducted by the method of Schutte and Kula (1987), which resulted in ~80% of cell disruption after 60 minutes of grinding. Further experiments were carried out to optimize various parameters, such as buffer pH (Table 2A), size of glass beads (Table 2B), grinding time (Fig.9), which were reported to greatly influence disruption efficiency and enzyme activity. In order to minimize contamination during preparation of cell slurry, sterilized equipments and buffers were used.

As shown in Table 2B, a higher degree of disruption could be achieved with relatively smaller glass beads size. This relates to the previous findings for cell disintegration in a Dyno-Mill (Marffy & Kula, 1974). However, there was no significant difference in disintegration efficiency between bead size of 0.25 mm and 0.5 mm (diameter). pH of the cell suspensions also did not have any significant effect on the disruption efficiency (Table 2A). Therefore, further trials were conducted at neutral buffer pH and with glass beads of ~0.5 mm diameter.

The disintegration kinetics of the strain (25% suspension) was followed by measuring the decrease in viable count, release of intracellular protein, and the activity of various enzymes (by API ZYM system). A minimum of 45 minutes grinding time was required for optimum cell disruption (Fig. 9). However, further grinding up to 60 minutes did not cause any loss in the activity of enzymes. Similar studies by previous

workers have reported varying grinding time periods (from 2 to 60 minutes) for bacterial strains (Hummel & Kula, 1989). Under these conditions, ball mill disintegration produced 90-95% reduction in the viable cell count. Statistical analysis of replicates revealed good reproducibility in cell disruption by ball mill. API ZYM results of crude cell-free extracts showed higher activities for all the enzymes tested, when compared to lysozyme (Fig. 8).

CONCLUSION

The results from the growth study indicate that the cultivation of *Lactobacillus casei* in MRS batch culture follows a characteristic growth profile, which is largely a function of nutrient availability and pH of the medium. Studies also showed a direct relationship of cell growth in the exponential phase with the increase in medium absorbance as well as a decrease in medium pH, which can be efficiently employed for quick monitoring of the cell numbers. Among the various methods tried for cell disruption, mechanical grinding of cells in a mixer mill was found to be a more efficient method, as compared to the other enzymatic methods of lysis. Further research is needed in this area to search for delicate methods of cell lysis which could be used on different strains of lactobacilli and result in more precise fractionation of cell organelle.

Table 1. Effect of lytic enzymes treatment on cell absorbance of *Lb. casei* ssp. *casei* LLG.

Incubation period (minutes)	Absorbance[1] ($Abs._{600}$)	
	Lysozyme	Lytic enzyme
0	0.50	1.00
20	0.50 (0.01)	0.95 (0.02)
40	0.49 (0.01)	0.83 (0.07)
60	0.49 (0.01)	0.78 (0.08)

[1] All means represent duplicate analysis made on cell-free extracts of three experiments (± S.E. in parentheses).

Table 2. Standardization of cell disintegration by ball mill

A. Effect of buffer pH on cell disintegration[1]

Buffer pH	lysis[2] (%)	Protein (mg)
7.0	96.2 (2.5)	42.75 (1.8)
7.5	93.1 (3.2)	40.62 (1.5)
8.0	96.7 (2.9)	42.31 (1.7)

[1] All means represent duplicate analysis made on cell-free extracts of three experiments (± S.E. in parentheses).
[2] Measured from decrease in viable count during disintegration (± S.E. in parentheses).

B. Effect of bead size on cell disintegration[1]

Bead size	lysis[2] (%)	Protein (mg)
0.25	96.9 (1.9)	46.75 (0.9)
0.45	95.6 (2.3)	47.25 (1.2)
0.75	89.3 (2.1)	44.39 (1.7)

[1] All means represent duplicate analysis made on cell-free extracts of three experiments (± S.E. in parentheses).
[2] Measured from decrease in viable count during disintegration (± S.E. in parentheses).

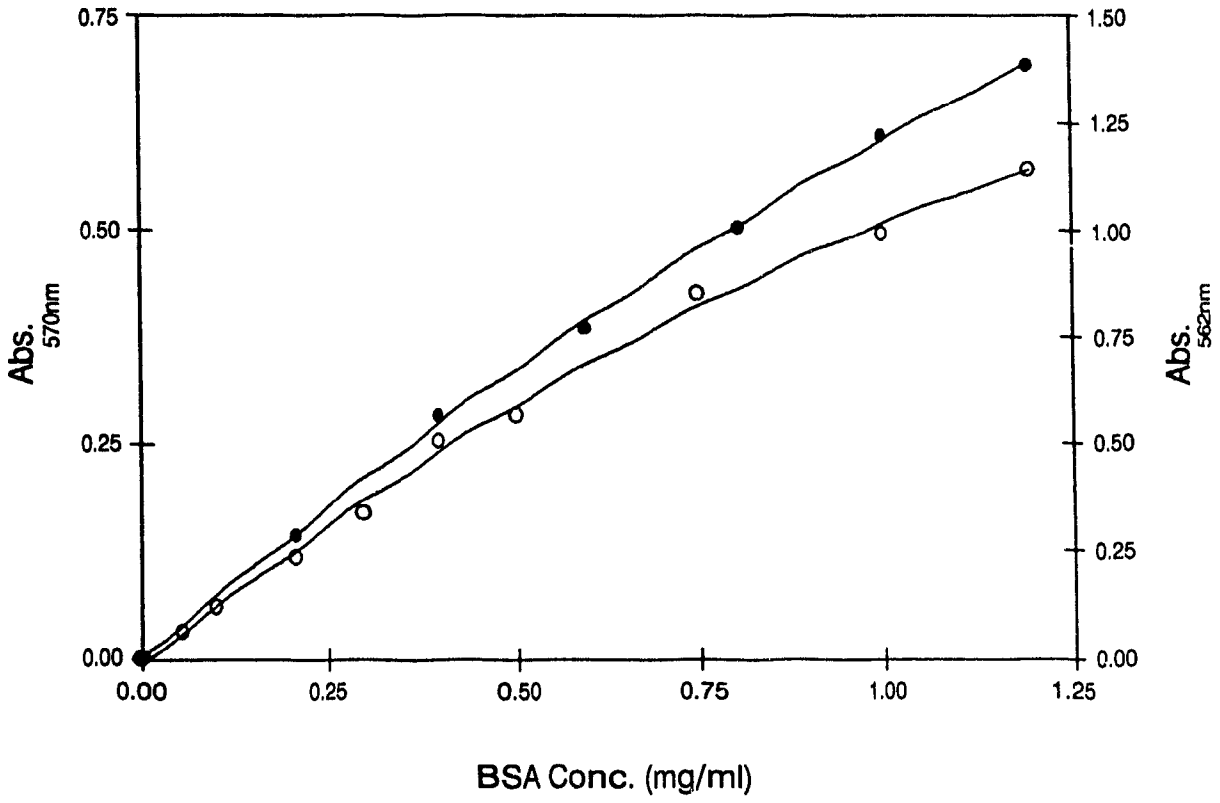

Figure 1. Calibration curve of bovine serum albumin using BCA protein assay reagent on Beckman spectrophotometer (●), and on Lambda plate reader (○). A_{562} (●): $R^2 = 0.9994$, $Y = 0.00283 + 1.462X - 0.2539X^2$. A_{570} (○): $R^2 = 0.9976$, $Y = -0.00311 + 0.6768X - 0.1661X^2$.

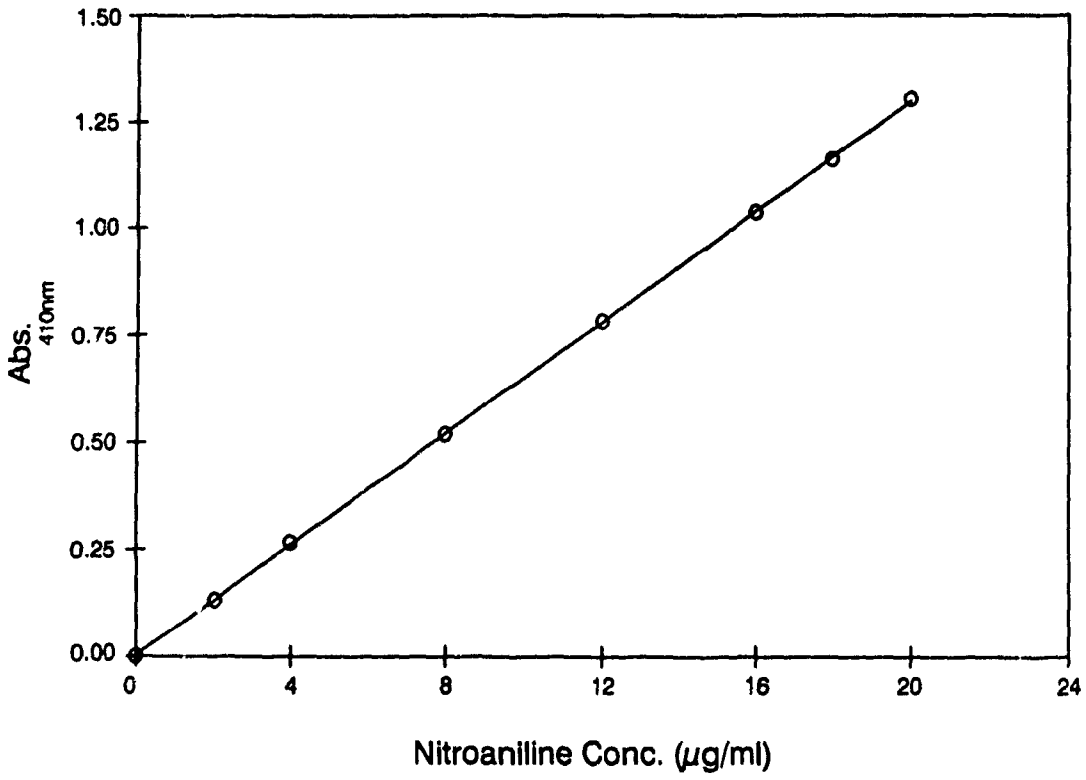

Figure 2. Calibration curve of *p*-nitroaniline for aminopeptidase assay. $R^2 = 0.9998$, $Y = 0.00271 + 0.06479X$.

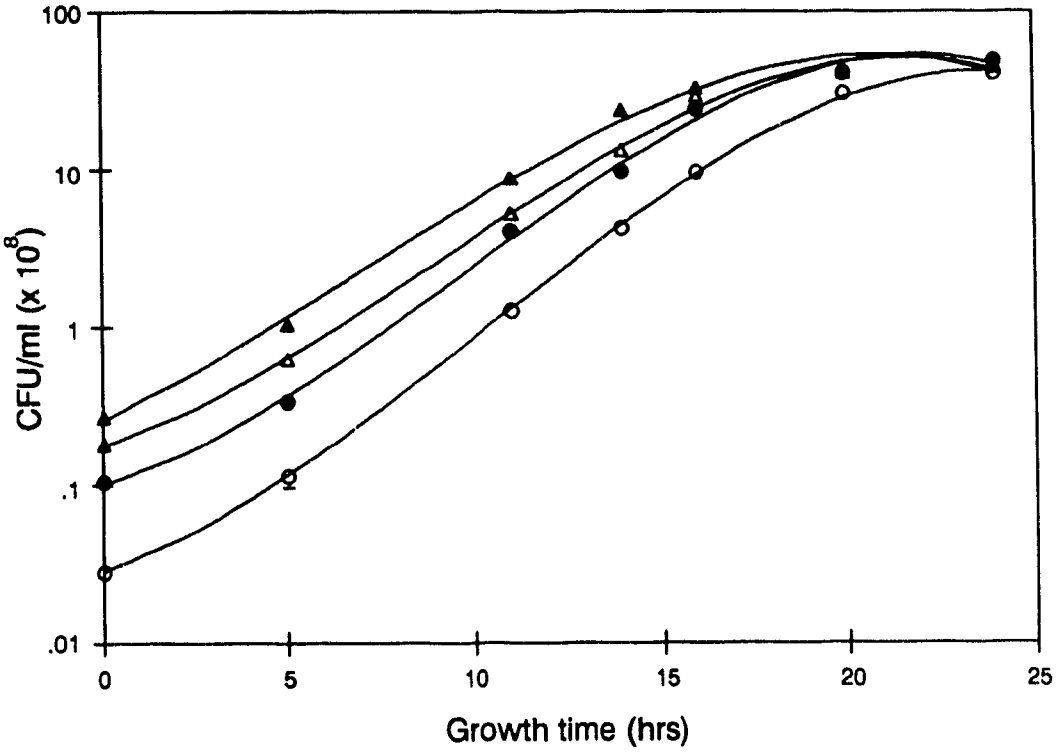

Figure 3 . Growth curve of *Lb. casei* ssp. *casei* LLG at different levels of inoculation.

0.10% (○): R^2 = 0.9996, Y = -1.5587 + 0.0788X + 0.0103X^2 - 0.00034X^3
0.25% (●): R^2 = 0.9976, Y = -1.0090 + 0.0675X + 0 0108X^2 - 0 00038X^3
0.50% (△): R^2 = 0.9976, Y = -0.7658 + 0.0753X + 0.0089X^2 - 0 00033X^3
0.75% (▲): R^2 = 0.9964, Y = -0.6040 + 0.1102X + 0.0054X^2 - 0.00026X^3

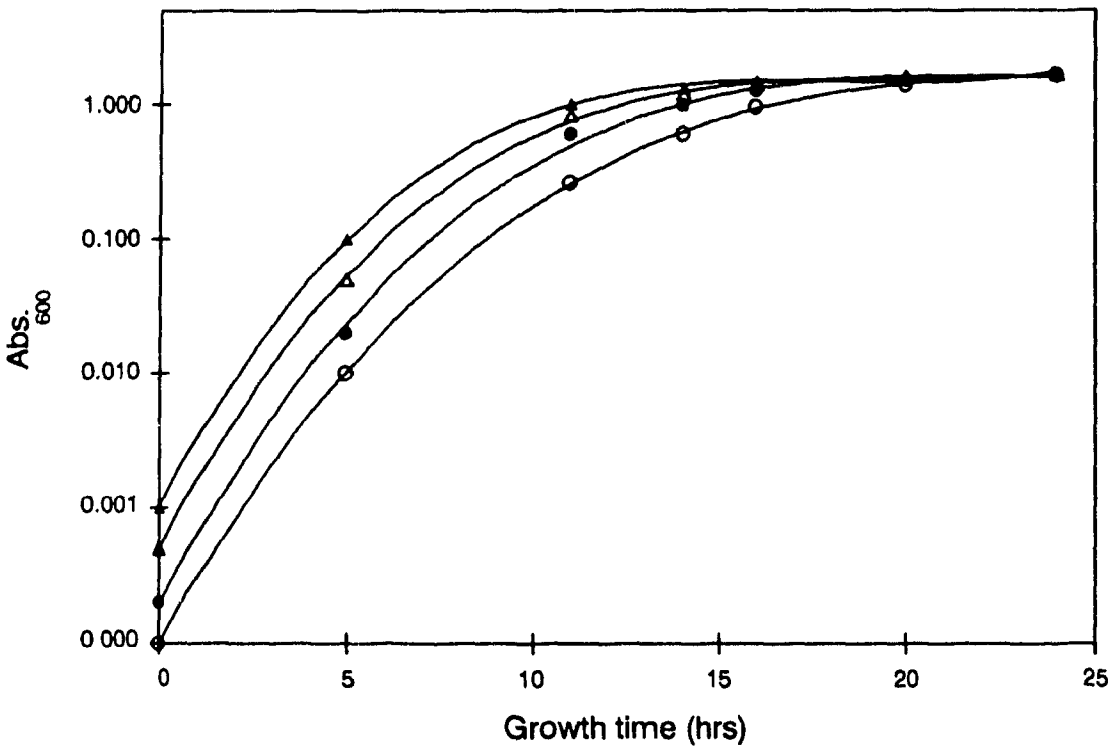

Figure 4. Effect of growth phases of *Lb. casei* ssp. *casei* LLG on the medium absorbance (A_{600}) at different levels of inoculation.

0.10% (○): R^2 = 0.9998, Y = -4.0015 + 0.4916X + 0.0194X^2 - 0.00026X^3
0.25% (●) R^2 = 0.9990, Y = -3.7185 + 0.5246X + 0.0233X^2 - 0.00034X^3
0.50% (△) R^2 = 0.9998, Y = -3.3070 + 0.5271X + 0.0265X^2 - 0.00045X^3
0.75% (▲) R^2 = 0.9996, Y = -2.9932 + 0.5273X + 0.0291X^2 - 0.00053X^3

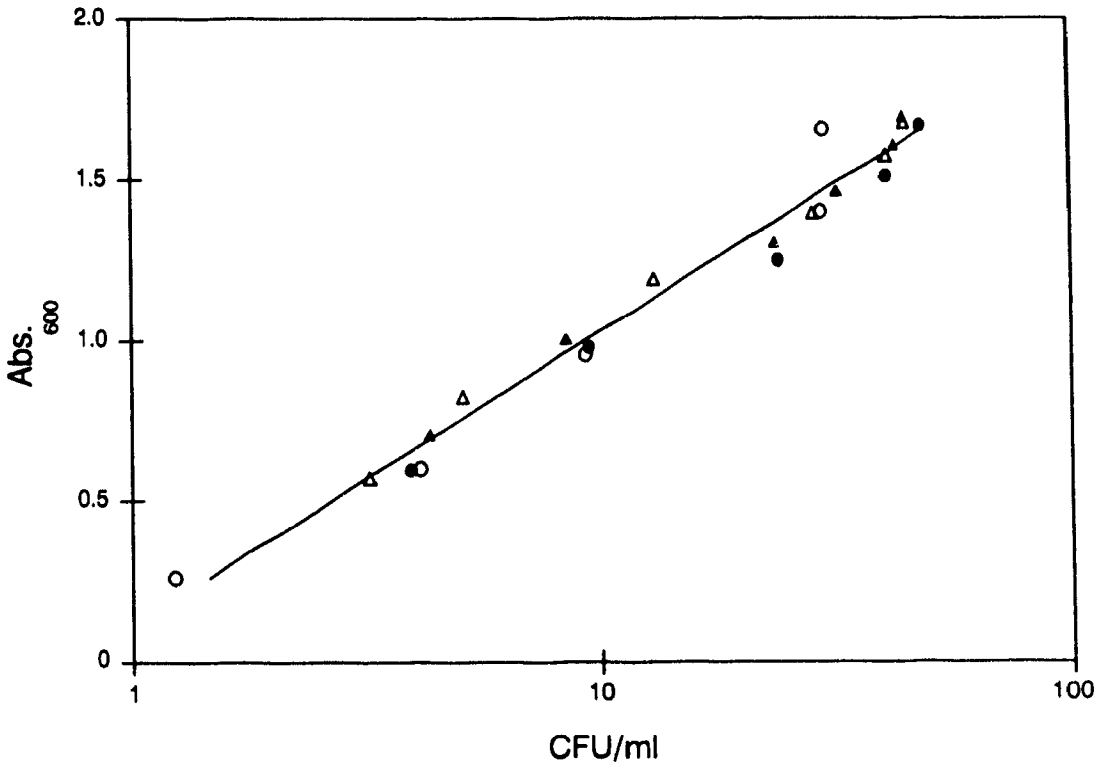

Figure 5. Relationship between viable cell count (CFU/ml) and medium absorbance (A_{600}) during the exponential growth phase of *Lb. casei* ssp. *casei* LLG. Levels of inoculation: 0.1% (○), 0.25% (●), 0.5% (△) and 0.75% (▲). $R^2 = 0.9694$. $Y = 0.11645 + 0.91252X$

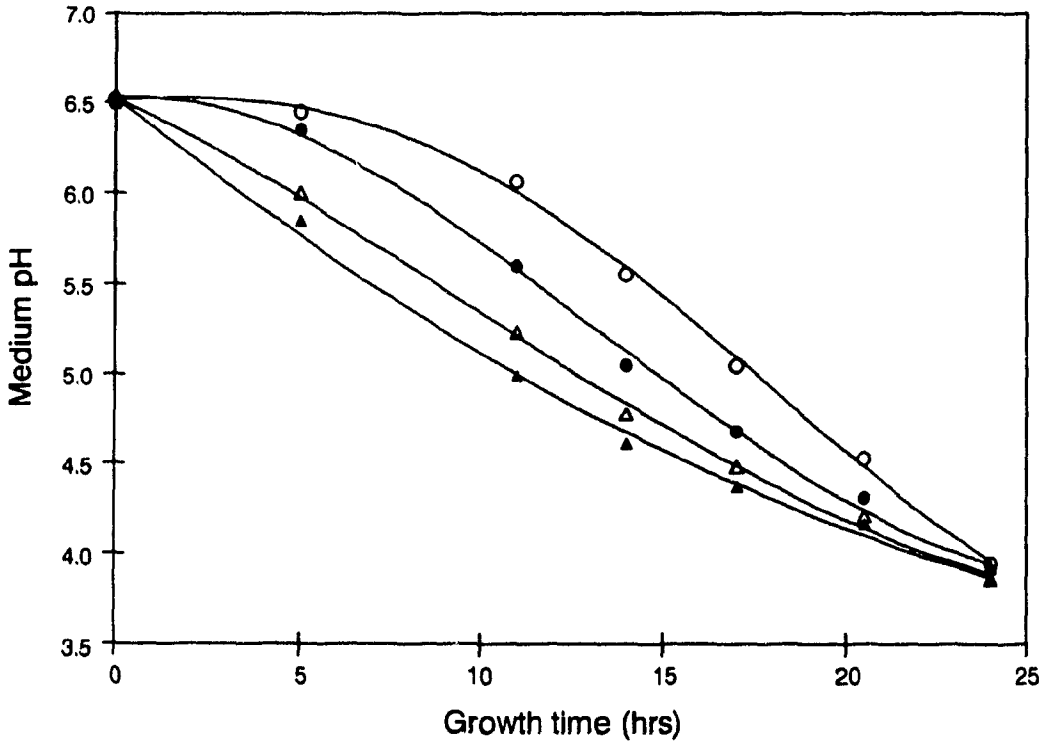

Figure 6. Effect of growth phases of *Lb. casei* ssp. *casei* LLG on the medium pH at different levels of inoculation.

0.10% (○): $R^2 = 0.9944$, $Y = 6.5080 + 0.0771X - 0.0145X^2 + 0.00028X^3$
0.25% (●): $R^2 = 0.9950$, $Y = 6.5365 + 0.0378X - 0.0158X^2 + 0.00041X^3$
0.50% (△): $R^2 = 0.9964$, $Y = 6.5281 + 0.0890X - 0.0047X^2 + 0.00016X^3$
0.75% (▲): $R^2 = 0.9966$, $Y = 6.5030 + 0.1509X + 0.0006X^2 + 0.00005X^3$

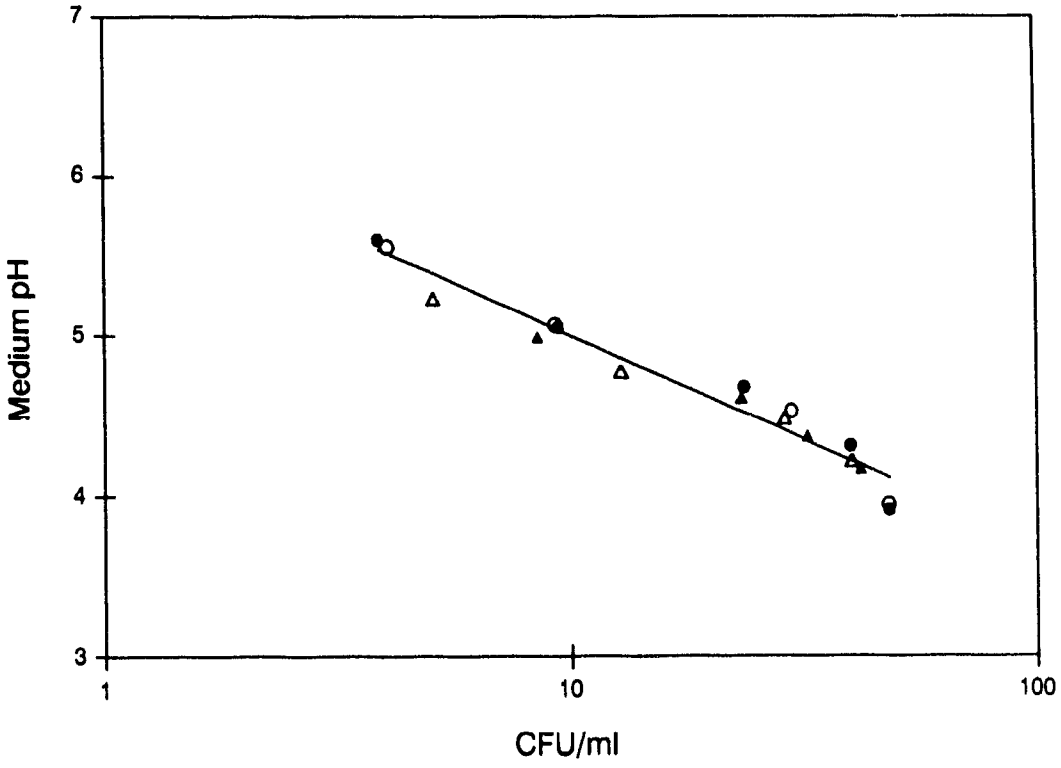

Figure 7. Relationship between viable cell count (CFU/ml) and medium pH during the exponential growth phase of *Lb. casei* ssp. *casei* LLG. Levels of inoculation: 0.1% (○), 0.25% (●), 0.5% (△) and 0.75% (▲). $R^2 = 0.9555$, $Y = 6.3112 + 1.3081X$.

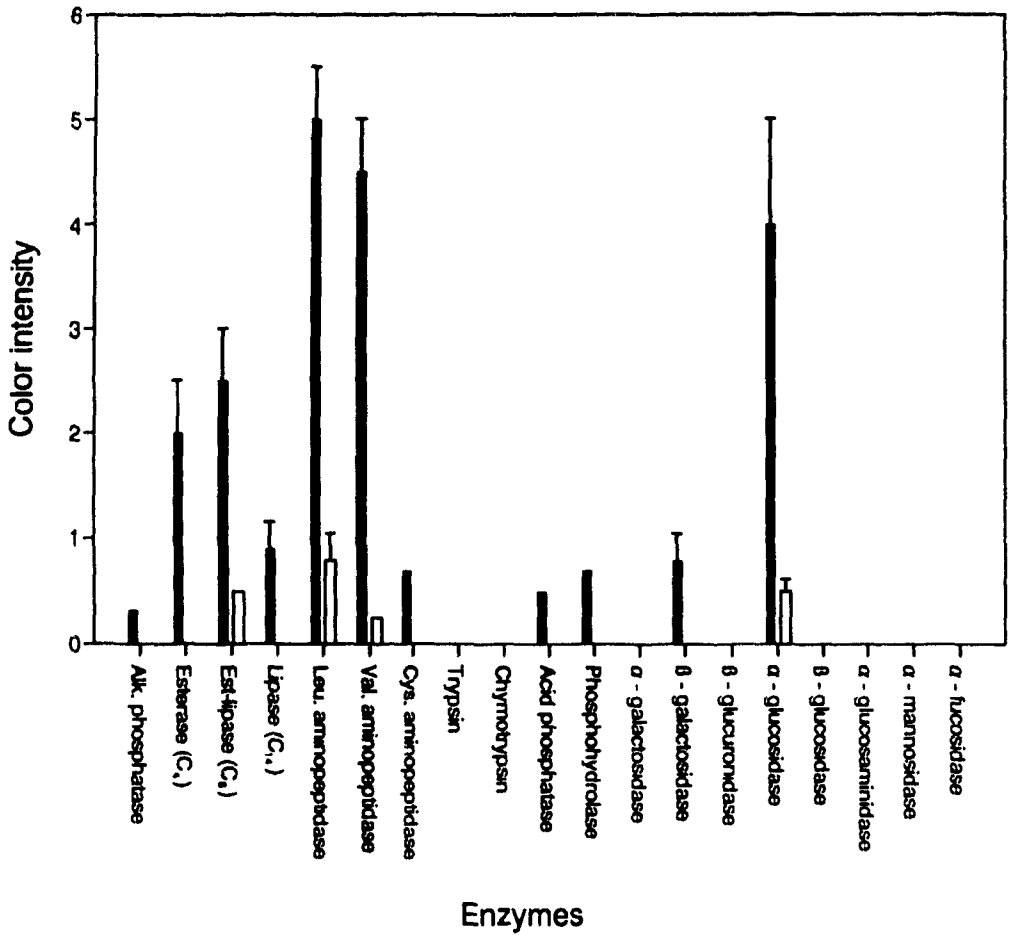

Figure 8. API ZYM profile of intracellular enzymes after cell lysis of *Lb. casei* ssp. *casei* LLG by lysozyme (□) and ball mill (■).

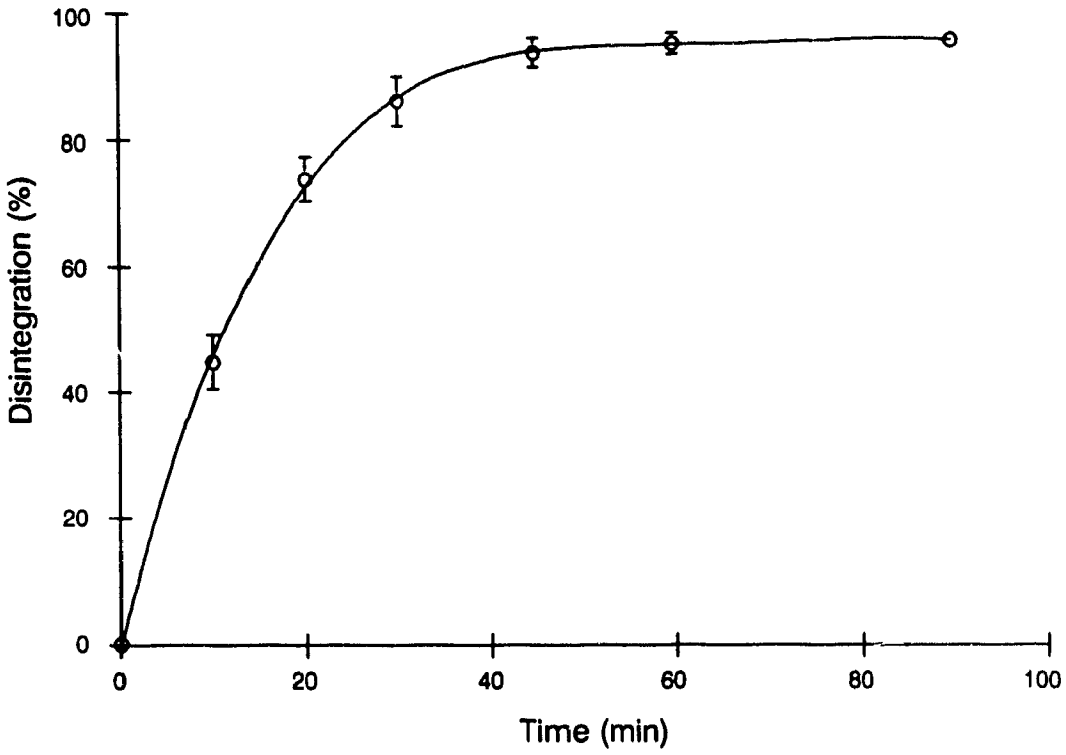

Figure 9. Disintegration of *Lb. casei* ssp. *casei* LLG cells by ball mill (bars denote standard deviations among replicates).

Charaterization of Enzyme Profiles of *Lactobacillus casei* Strains by API ZYM Enzyme System

KEY WORDS

Lactic acid bacteria, *Lactobacillus casei*, API ZYM method, Cheese, Peptidase, Esterase, Lipase, Dairy Microbiology, Enzymes.

ABSTRACT

Lactobacilli have gained much attention recently due to their strong peptidase and esterase activities , which are of great importance in the accelerated maturation and enzyme modification of cheese. Twenty strains of *Lactobacillus casei* (ssp. *casei*, ssp. *rhamnosus* and ssp. *pseudoplantarum*) were compared for their enzyme profiles. Specificity and specific activity studies were carried out on 19 hydrolytic enzymes for each strain using the microenzyme API ZYM system. Typical *Lactobacillus casei* enzyme profile indicated the presence of weak proteinase, and high peptidase and esterase-lipase activities. The API ZYM method has been modified to evaluate and compare closely related *Lactobacillus casei* strains, and also to identify the location of their enzymes (extracellular, cytoplasmic, and cell wall and membrane bound). The peptidases, and most of the esterases and carbohydrases were intracellular, while the phosphatases were mostly cell wall and membrane associated. The method offers a simple and convenient way for quick assessment of the hydrolytic enzymes of starter strains at both the industrial and research level.

INTRODUCTION

Most varieties of ripened cheese undergo a maturation period of 9-12 months. The process involves sequential breakdown of milk components, such as fat, protein and lactose, by the enzymes of starter bacteria (Law, 1984). Therefore, a fundamental understanding of starter enzymes is of prime importance in evaluating their suitability, and in predicting their influence on the final cheese quality.

At present, studies of enzyme profiles of individual strains involve lengthy, expensive and skillful operations and hence these studies are mainly confined to research laboratories. The industrial approach of evaluating strains is still traditional, based on their influence on the final cheese quality. The process is slow, random and privetized.

The Analytab Products API ZYM method has been developed for the semi-quantitative analysis of specific hydrolytic enzymes (Table 3), most of which are of much interest to cheese manufacturers. The system is based on Buissiere's observation of 1967 (Buissiere *et al*, 1967), who suggested that specific enzymes of microorganisms can be detected by adding a heavy inoculum of organisms on specific substrates, even in the absence of growth. The idea has been commercialized into a handy kit of 19 chromogenic substrates dried on absorbent microcupules.

The method first found its use in clinical microbiology for the identification of pathogenic bacteria (Humble *et al*, 1977; Tharagonnet *et al*, 1977). Its first application in food systems was reported by Lee *et al* (1986), who used the system for the differentiation of homofermentative and heterofermentative lactic acid

bacteria. They also reported that *Lactobacillus casei* species exhibit higher number and greater activities of peptidases and esterases, when compared to other starter strains, including the primary strain used in Cheddar cheese industry (*Lactococcus* spp.).

The present investigation explores the possibility of using the API ZYM technique as a rapid and simple mean to evaluate and localize nineteen different hydrolases of *Lactobacillus casei* species, and also to select strains with superior enzyme profiles, especially their peptidases and esterases, for accelerated maturation and enzyme modification of Cheddar cheese.

MATERIALS AND METHODS

Strains and Maintenance

A total of 20 strains of *Lactobacillus casei* ssp. *casei* (7), ssp. *rhamnosus* (6) and ssp. *pseudoplantarum* (7) were used in this study. *Lactobacillus casei* ssp. *casei* (ATCC 334, ATCC 393, ATCC 25302, and ATCC 25303), ssp. *pseudoplantarum* (ATCC 25598) and ssp. *rhamnosus* (ATCC 7469, ATCC 9595, ATCC 15820, ATCC 12116 and ATCC 11982) were obtained from American Type Culture Collection, Rockville, MD. While *Lactobacillus casei* ssp. *casei* (ANO-108, LLG, and S119), ssp. *pseudoplantarum* (L2F, L3E, 11C, 137, 83.4 and S81) and ssp. *rhamnosus* (S93) were isolated from a high quality Cheddar cheese. All strains were maintained on lactobacilli MRS agar medium (Difco Laboratories, Detroit, MI)(De Man *et al*, 1960) at 4°C, and at -30°C in reconstituted sterile 20% (vol/vol) skim milk powder (Difco) diluted equally with MRS medium, and were transferred triweekly.

Cultures were revived by 2-3 consecutive subculturing in MRS broth before growth of cells for actual experiments. Fresh early stationary phase cells were prepared by inoculation of 0.01% of active culture in 300 ml of MRS broth and were incubated at 30°C. Growth was arrested by cooling at pre-standardized growth parameters indicating late logarithmic phase (when optical density reaches 1.0 at A_{600} (Spectronic 20) with concurrent arrival of medium pH to approximately 5.0 and the viable count (measured as colony forming units/ml) to approximately $1x10^9$/ml.

Disintegration and Fractionation of cells

The subcellular fractionation scheme is outlined in Figure 10. Cells from the late logarithmic phase were harvested by centrifugation (10000 x g for 15 min). 0.05*M* sodium phosphate buffer (pH 7.0) was used to wash and suspend the cells. 250 ml of washed cell suspension (adjusted to O.D. 1.0 at A_{600}) was centrifuged and resuspended in 6.0 ml (approximately 25% cell slurry) of the same buffer. A Brinkmann ballmill Type MM2 (Brinkmann Instruments Ltd., Rexdale, Ont., Canada) was used to break the cells (Appendix III). The whole process of disintegration and fractionation was carried out at 4°C. Both the cell free extract and the pellet of cell wall and membrane fraction were diluted to 250 ml by sodium phosphate buffer, and a portion (25 μl) of which was used for the API ZYM assay. All experiments were performed in triplicate.

The disintegration efficiency was monitored by measuring protein content of cell free extracts and by counting colony forming units of cell suspensions before and after the disintegration. The process resulted in >90% disintegration of cells for all the strains.

API ZYM Assay Procedure

The API ZYM kits were obtained from API laboratory Products Ltd. (St. Laurent, Qué., Canada). 25 μl of cell free extract or cell suspension was added to each of the 19 cupules containing dehydrated chromogenic enzyme substrates, and the strips were incubated for 4 h at 37°C. The addition of the aqueous sample rehydrates the substrates and initiates the reactions. Hydrolytic action of respective enzymes on naphthyl derivatized substrates results in the release of ß-naphthol, which was detected by the detector reagents (A and B) supplied with the system. A humid atmosphere was provided during incubation by dispensing approximately 5 ml of tap water in the incubation tray provided with the enzyme kit. The reaction was terminated by the addition of one drop of reagent A (tris (hydroxymethyl) aminomethane; 250g, hydrochloric acid; 110 ml, sodium dodecyl sulfate; 100g, distilled water; 1000 ml q.s.), and the color was developed by the addition of one drop of reagent B (fast blue BB;3.5g, 2-methoxyethanol;1000 ml q.s.).

The Activity was measured by comparing the color developed in 5 min to the color chart provided by the manufacturer, and was expressed on a scale of 0 (no activity) to 5 (maximum activity; ≥40n*M* of chromophore released).

Protein assay

Protein was determined spectrophotometrically by the BCA (bicinchoninic acid) assay reagent supplied with the system (Pierce Chemical Ltd., Rockford, IL)(Smith *et al*, 1985). The method combines the well known reaction of protein with Cu^{2+} in an alkaline medium (yielding Cu^{+}) with a highly sensitive and selective

detection of Cu^{+} with bicinchoninic acid. Bovine serum albumin (SIGMA Chemicals, St. Louis, MO) was used for the standard curve (Fig. 1).

RESULTS AND DISCUSSION

Although *Lactobacillus casei* strains showed variations in specific activities, all showed similiarity in the presence or absence of the type of enzymes. Figure 11 shows the typical enzyme profile of *Lactobacillus casei*. The presence of weak proteinases (trypsin and chymotrypsin with mean activities of 0.1 and 0.2 respectively), and high peptidases (leucine- and valine-aminopeptidase with respective means of 4.7 and 4.4) and esterase-lipases (C_4 and C_8 with mean activities of 1.4 and 2.4) are the desirable traits for their use in accelerated ripening of cheese and production of cheese flavorings. Cheese starters with low proteinase and strong peptidase activities are also found useful in reducing bitterness and improving body and texture defects, which are often caused by most of the microbial preparations when used as rennet substitutes (Law, 1984). In addition, "soapiness" defect caused by the accumulation of long chained fatty acids in many ripened cheese varities can be removed by using strains with strong esterase-lipase activities.

The API ZYM technique was also applied on cell free extracts and cell wall and membrane fractions, in an attempt to localize enzymes in these subcellular portions. Although the severe disintegration conditions resulted in varying loss of activity of certain enzymes, all enzymes of whole cell profile could be recovered in either of the two fractions. ß-galactosidase and phosphoamidase showed greater loss (95% and 70% respectively) and wide variations among replicate experiments, whereas activity of α-fucosidase was increased upon disintegration.

Figure 12 depicts the relative proportion of activity of different enzymes in cytoplasmic and cell wall and membrane fractions. While all aminopeptidases were predominantly found in the intracellular fraction (>80%), phosphatases were mainly detected in the cell wall and membrane fraction. Esterases and lipase activities were observed in both cytoplasmic and cell wall and membrane fraction. Among the various carbohydrases tested, galactosidases and glucosidases were intracellular, whereas α-fucosidase, α-mannosidase and ß-glucuronidase were cell wall and membrane associated. No activity was detected in the extracellular fraction.

In order to study the influence of mechanical disintegration, two other methods of enzymatic lysis were tried. While lysozyme treatment (Exterkate, 1984) failed to lyse cells of all the twenty strains, the other method of using a lytic enzyme (Ishiwa & Yokokura, 1971) interfered with the results of API ZYM technique due to the presence of contaminating enzymes in the preparation itself (Chapter 1).

The peptidase and lipase profiles of the 20 strains were compared to screen the ones with superior traits for our ongoing project of production and utilization of these enzymes for accelerated cheese maturation and flavor production (Fig. 13 and 14). All the 20 strains showed relatively weak esterases and lipase activities, as compared to peptidases (Fig. 11). However, when compared to other cheese strains; *Lactococcus* spp. (Lee *et al*, 1986) and *Pediococcus* spp.(Tzanetakis & Litopoulou-Tzanetaki, 1989), *Lactobacillus casei* species contain superior esterase and lipase activities. Since leucine and valine aminopeptidases showed maximum activities (≥5) for most of the strains, separate experiments were performed for these enzymes at lower cell concentration (O.D. 0.2 at A_{600}). Overall profiles showed that *Lactobacillus casei* ssp. *rhamnosus* strains contained greater activities for both lipases and aminopeptidases followed by *Lactobacillus casei* ssp. *casei* strains (Fig. 13 and 14).

All strains showed higher aminopeptidase activity against leucine followed by valine and cystine. Likewise higher esterase-lipase activity was observed in all the twenty strains for caprylate (C_8) followed by butyrate (C_4) and myristate (C_{14}). This similiar relative responses of all the strains to the various substrates of lipases and peptidases suggest that either a single aminopeptidase as well as lipase may be responsible for the activity or *Lactobacillus casei* species contain same number of enzymes.

During the course of investigation *Lactobacillus casei* ssp. *rhamnosus* strain ATCC 15820 showed abrupt loss of activity for peptidases, esterases and many other hydrolytic enzymes, whereas, the fresh revived cells from its lyophilized mother culture exhibited the original enzyme profile. Similar loss of enzymes activity has also been reported in a previous API ZYM study carried out on proteinase-deficient mutant of *Pseudomonas fluorescens* (McKeller, 1985). Thus, the method may also be useful for quick assessment of physiological activity of starter strains in industrial quality control laboratories which is often influenced by various reasons, such as microbial contamination, phage attack and loss of plasmid(s).

The presence of high ß-galactosidase (mean activity of 3.7)(Fig. 17) and α- and ß-glucosidase activities (respective means of 2.0 and 2.5)(Fig. 16), and relatively low activities toward other carbon sources such as mannose, fucose and glucuronides (<1)(Fig. 11) suggest that *Lactobacillus casei* species prefer lactose and glucose sugars for their carbon and energy requirement. Although fast lactose utilization by many lactobacilli is a desirable character in yogurt and many other fermented milk products including many cheese varieties, excessive acid production by lactobacilli restrict their use as primary starters in cheese making. The problem can, however, be solved by genetic development of strains with desired lactose metabolic activity. All the strains showed weak activities for α-galactosidic bond. The presence of strong ß-galactosidase activities in all the twenty strains (Fig. 17) is also indicative of the

occurrence of lactose permease system as one of the possible efficient mechanism for lactose transport in *Lactobacillus casei* species. Strong glycosidases help them hydrolyse all glucose polysugars linked by either alpha or beta linkages, with the exception of *Lactobacillus casei* ssp. *rhamnosus* strains, which showed relatively weak α-glycosidase activities (Fig. 17). High acid phosphatase and acid phospho-hydrolase activities (Fig. 15) associated with the cell wall and membrane (Fig. 12) are useful in metabolizing phosphates in the acidic external environment prevalent in cheese maturation.

Although in earlier reports, the API ZYM method was found suitable to distinguish among various species of lactic acid bacteria (Lee *et al*, 1986), it was difficult to differentiate strains within a species due to the close homology in their enzyme profiles.

CONCLUSION

The results from this study indicate that the API ZYM test provides a rapid, simple and reproducible method, which may be used for the identification and evaluation of starter strains for their specific physiological activities. The enzyme system is also useful for a quick and reliable assessment of localization of enzymes in different subcellular fractions. The information obtained from API ZYM enzyme profiles will help starter companies to assess pertinent enzyme activities of starter strains they provide for industry. With more research, strains may be selected which will provide optimum enzyme activities to catalyse desirable reactions in food systems. API ZYM enzyme profile studies on *Lactobacillus casei* strains indicate the presence of many desirable traits for its use in enzyme modification of ripened cheese varieties.

Table 3. Enzymes detected through API ZYM system.

Enzyme	Substrate	Cupule Number
Esterase (C_4)	2 naphthyl butyrate	E3
Esterase-lipase (C_8)	2 naphthyl caprylate	E4
Lipase (C_{14})	2 naphthyl myristate	E5
Leu. aminopeptidase	L-leucyl-2-naphthylamide	E6
Val. aminopeptidase	L-valyl-2-naphthylamide	E7
Cys. aminopeptidase	L-cystyl-2-naphthylamide	E8
Trypsin	N-benzoyl-DL-Arg-2-naphthylamide	E9
Chymotrypsin	N-Glu-Phe-2-naphthylamide	E10
Alk. phosphatase	2 naphthyl phosphate	E2
Acid phosphatase	2 naphthyl phosphate	E11
Phosphohydrolase	Naphthyl ASBI-phosphate	E12
α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	E13
β-galactosidase	2-naphthyl-βD-galactopyranoside	E14
β-glucuronidase	Naphthyl-ASBI-βD-glucuronide	E15
α-glucosidase	2-naphthyl-2-D-glucopyranoside	E16
β-glucosidase	6-Br-2-naphthyl-βD-glucopyranoside	E17
α-glucosaminidase	1-naphthyl-N-acetyl-βDglucosaminide	E18
α-mannosidase	6-Br-2-naphthyl-2-D-mannopyranoside	E19
α-fucosidase	2-naphthyl-αL-fucopyranoside	E20

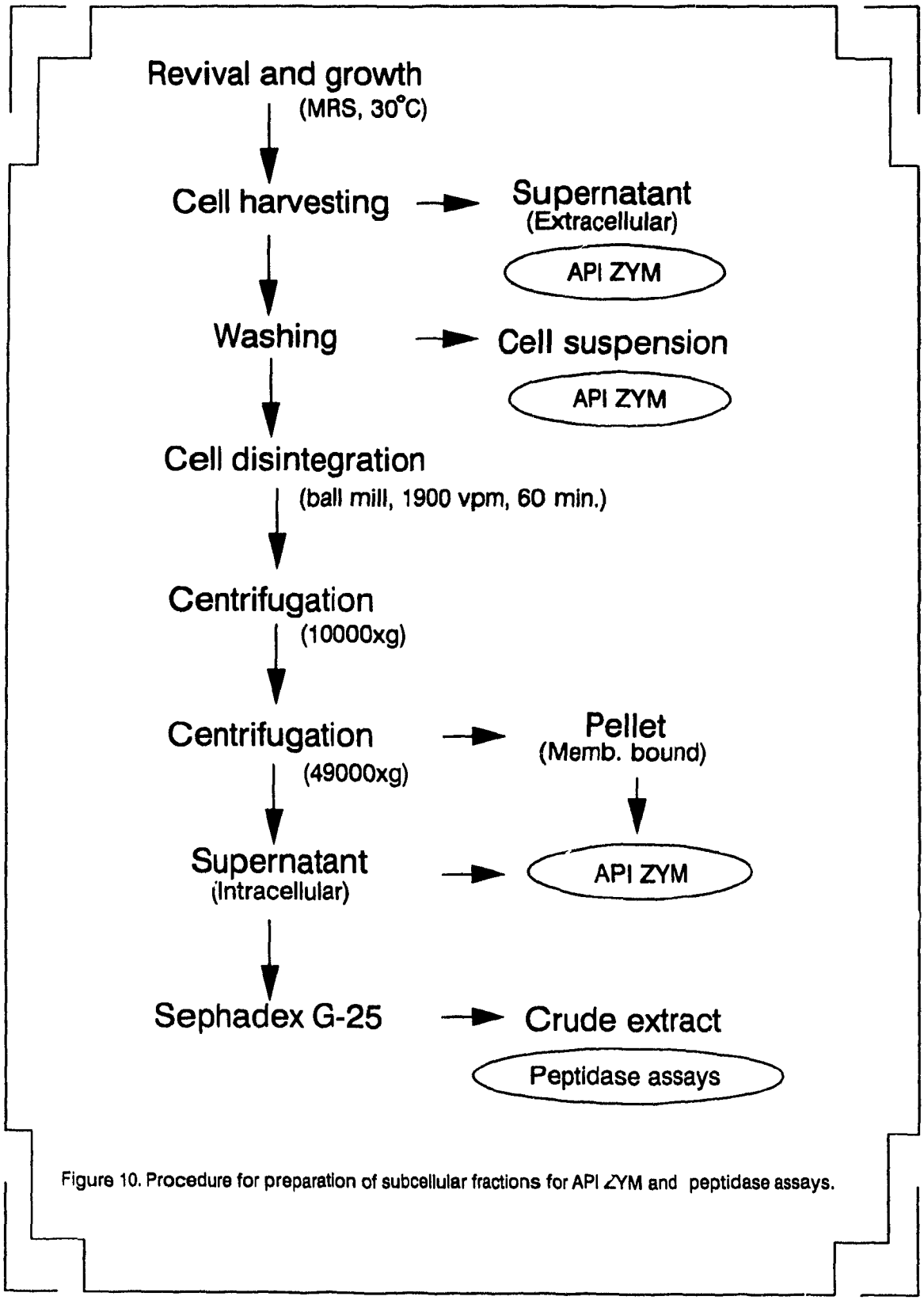

Figure 10. Procedure for preparation of subcellular fractions for API ZYM and peptidase assays.

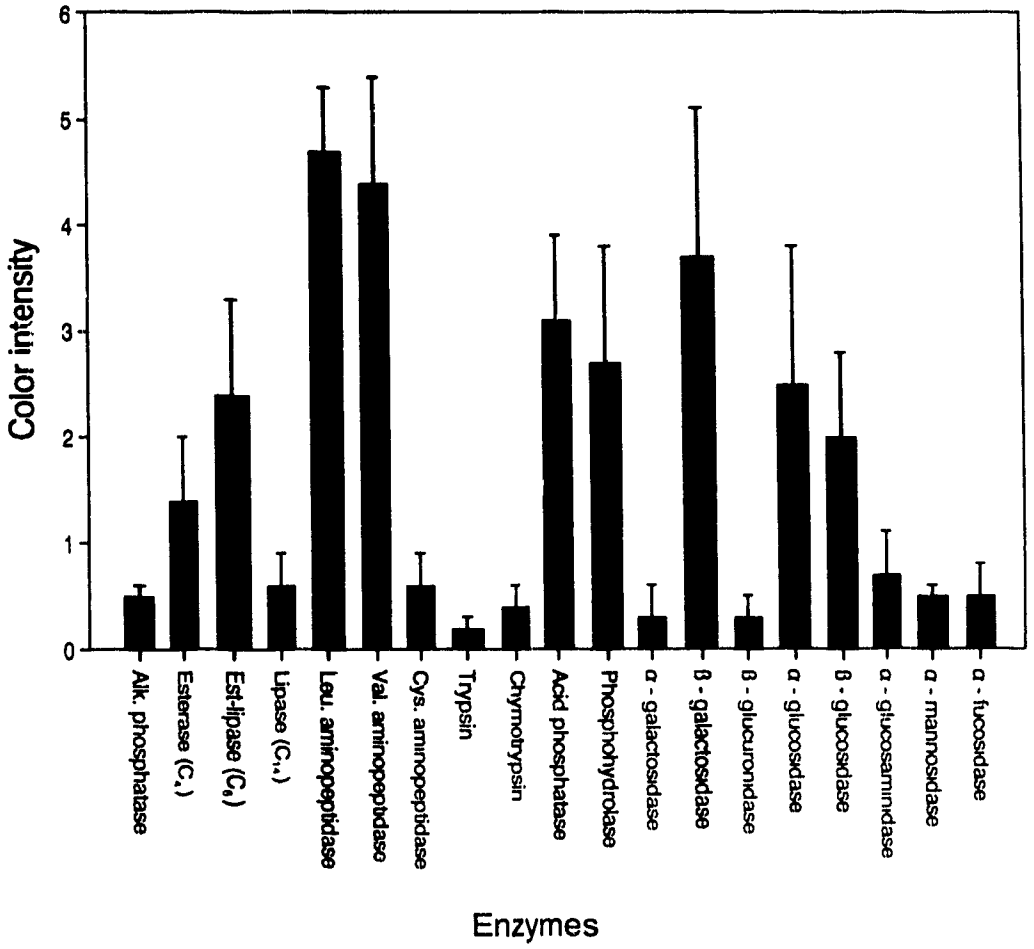

Figure 11. A characteristic enzyme profile of *Lb. casei*. Results are the average values of API ZYM results (in triplicate) of 20 strains (bars denote standard deviations). E2 to E20 represent 19 enzymes of API ZYM kit. Color intensity: 0 = no activity, 1-4 = intermediate activity, and 5 = maximum activity (>40 n*M* of chromophore released).

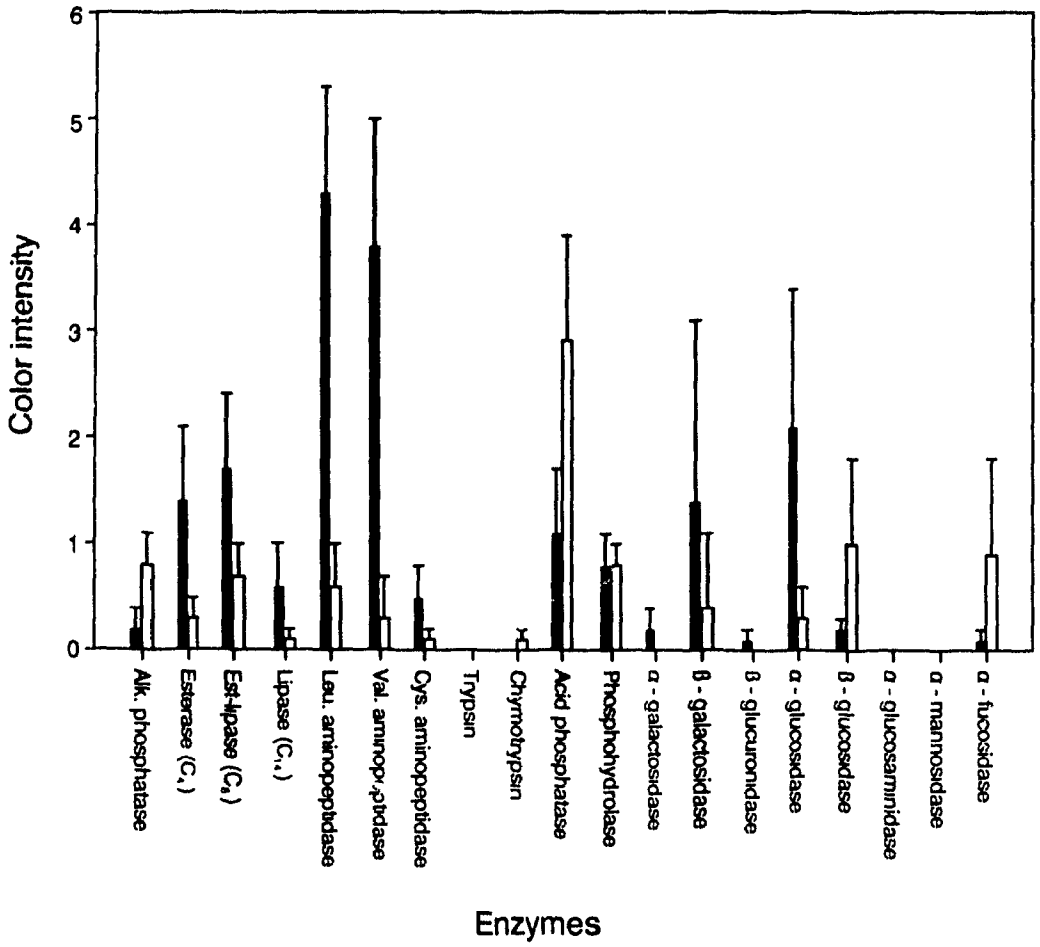

Figure 12. Localization of *Lb. casei* enzymes in subcellular fractions. (■) intracellular, (□) Membrane bound. Results are the average values of API ZYM results (in triplicate) of 20 strains (bars denote standard deviations). E2 to E20 represent 19 enzymes of API ZYM kit. Color intensity: 0 = no activity, 1-4 = intermediate activity, and 5 = maximum activity (≥40 n*M* of chromophore released).

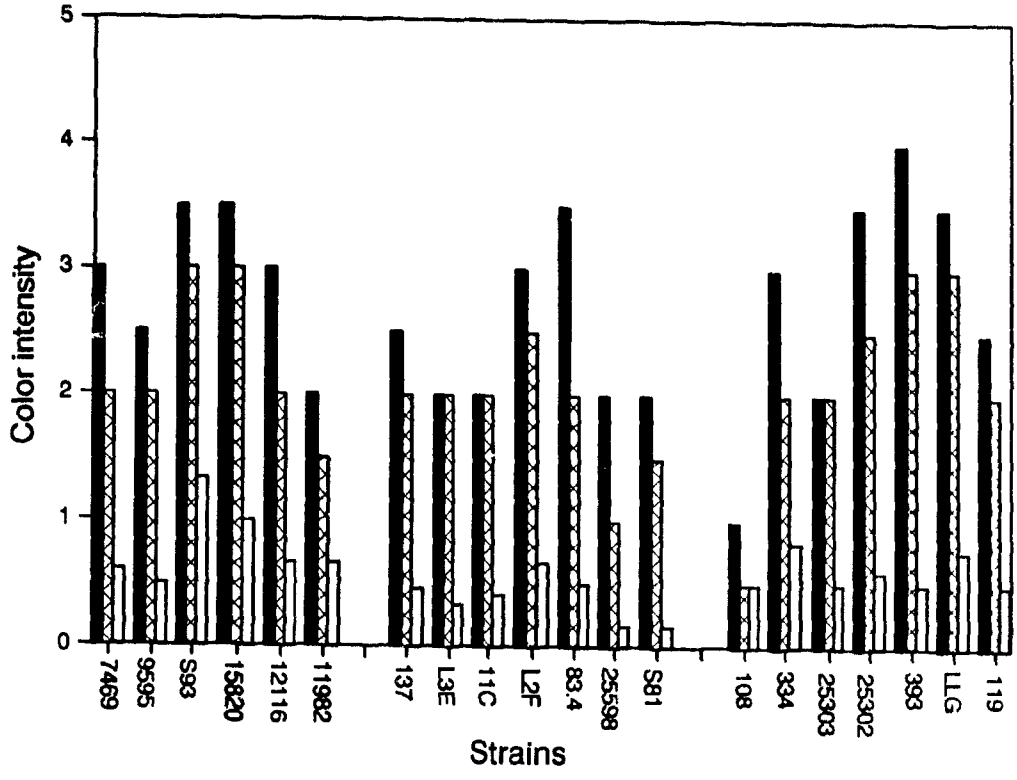

Figure 13. Aminopeptidase profiles of *Lb. casei* ssp. *rhamnosus* (7469 to 11982) ssp. *pseudoplantarum* (137 to S81) and ssp. *casei* (108 to 119). Leu. aminopeptidase at O.D. 0.2 (■), Val. aminopeptidase at O.D. 0.2 (□), Cys. aminopeptidase at O.D. 1.0 (□). Color intensity: 0 = no activity, 1-4 = intermediate activity, and 5 = maximum activity (≥40 n*M* of chromophore released).

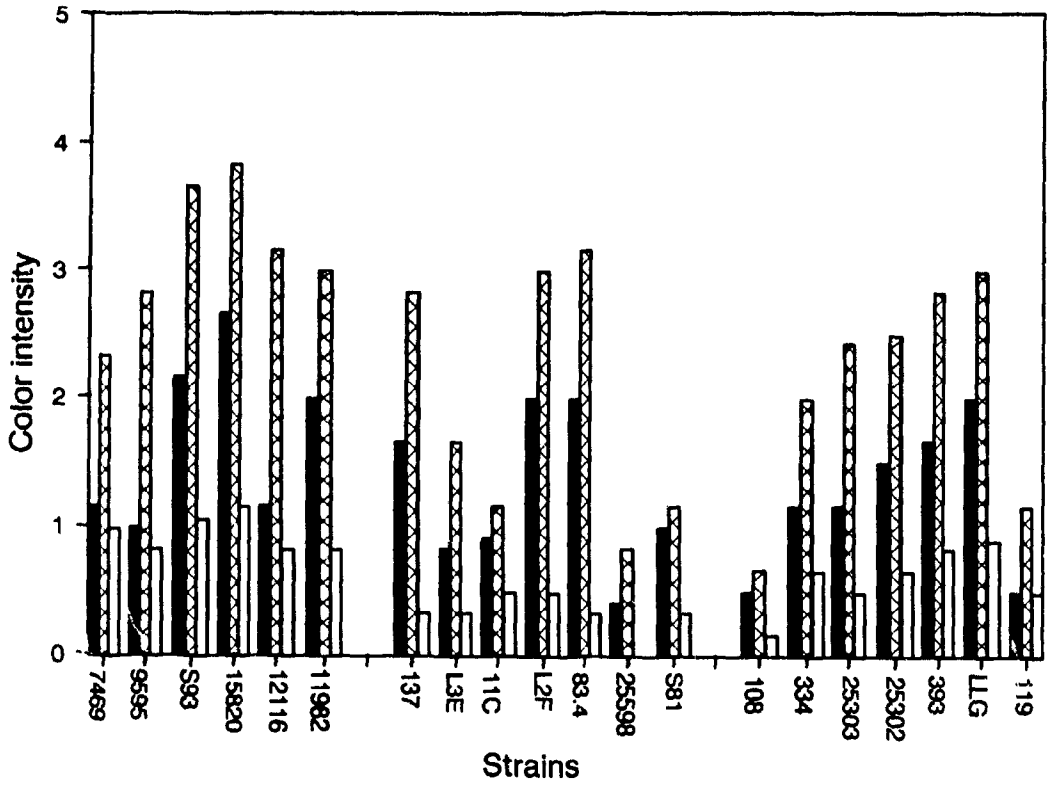

Figure 14. Esterase-lipase profiles of *Lb. casei* ssp. *rhamnosus* (7469 to 11982) ssp. *pseudoplantarum* (137 to S81) and ssp. *casei* (108 to 119). C_4-esterase (■), C_8-esterase-lipase (□), C_{14}-lipase (□). Color intensity: 0 = no activity, 1-4 = intermediate activity, and 5 = maximum activity (≥40 n*M* of chromophore released).

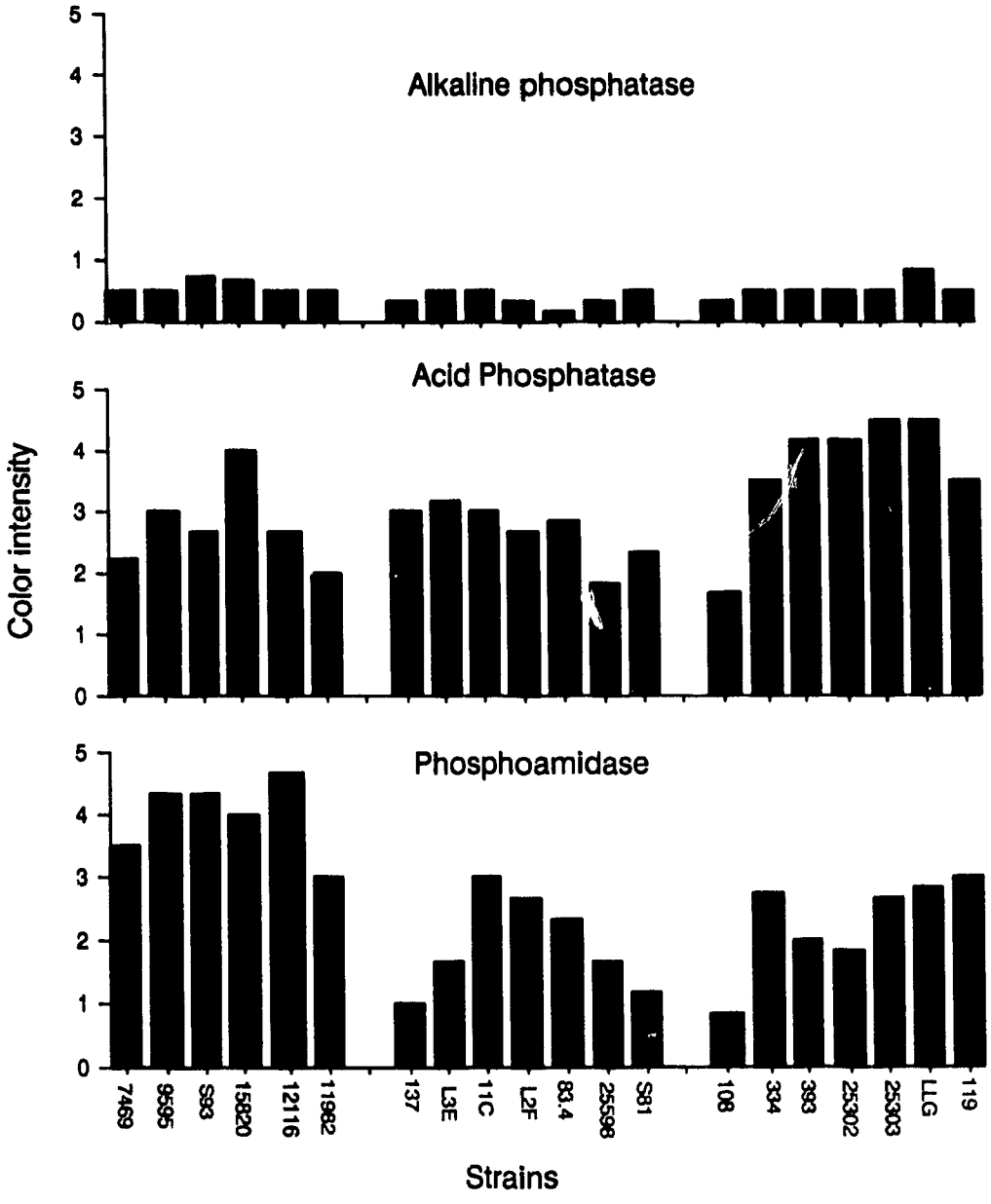

Figure 15. Phospho-hydrolases profiles of *Lb. casei* ssp. *rhamnosus* (7469 to 11982) ssp. *pseudoplantarum* (137 to S81) and ssp. *casei* (108 to 119). Color intensity: 0 = no activity, 1-4 = intermediate activity, and 5 = maximum activity (≥40 n*M* of chromophore released).

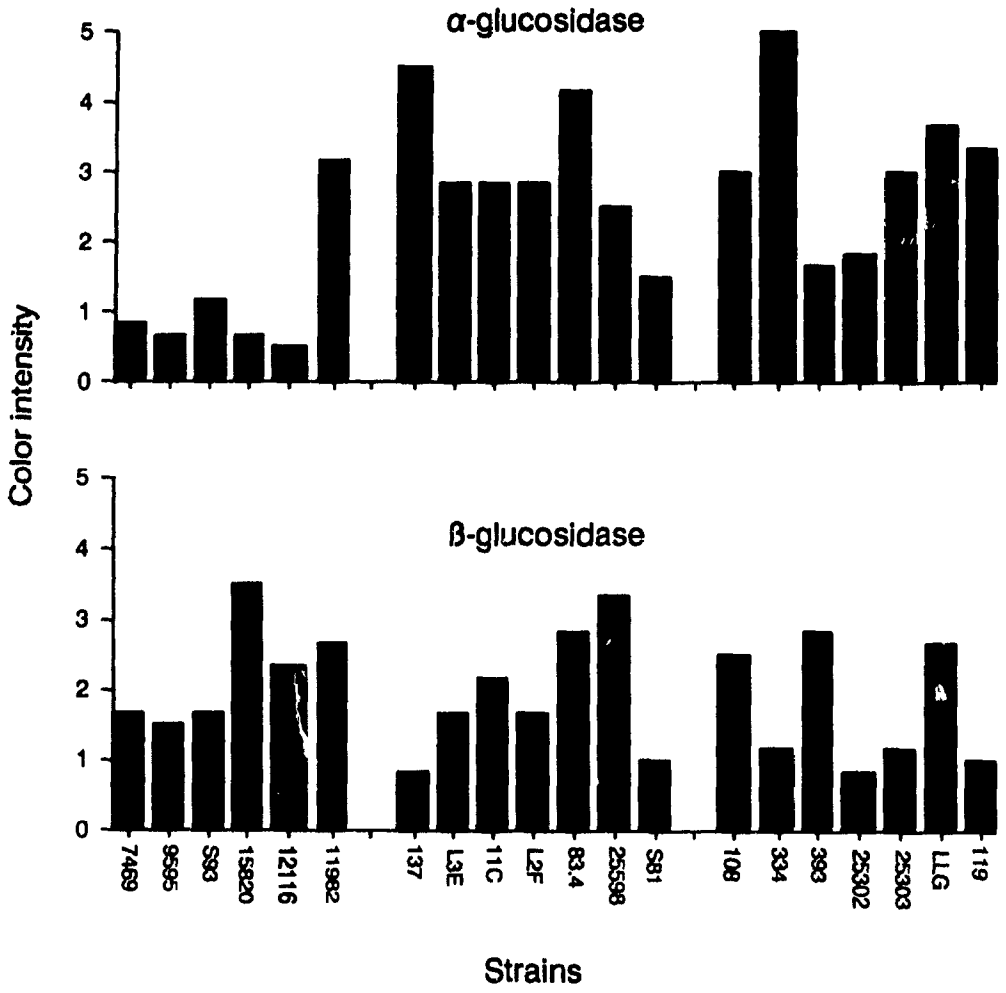

Figure 16. α and ß glucosidase profiles of *Lb. casei* ssp. *rhamnosus* (7469 to 11982) ssp. *pseudoplantarum* (137 to S81) and ssp. *casei* (108 to 119). Color intensity: 0 = no activity, 1-4 = intermediate activity, and 5 = maximum activity (≥40 n*M* of chromophore released).

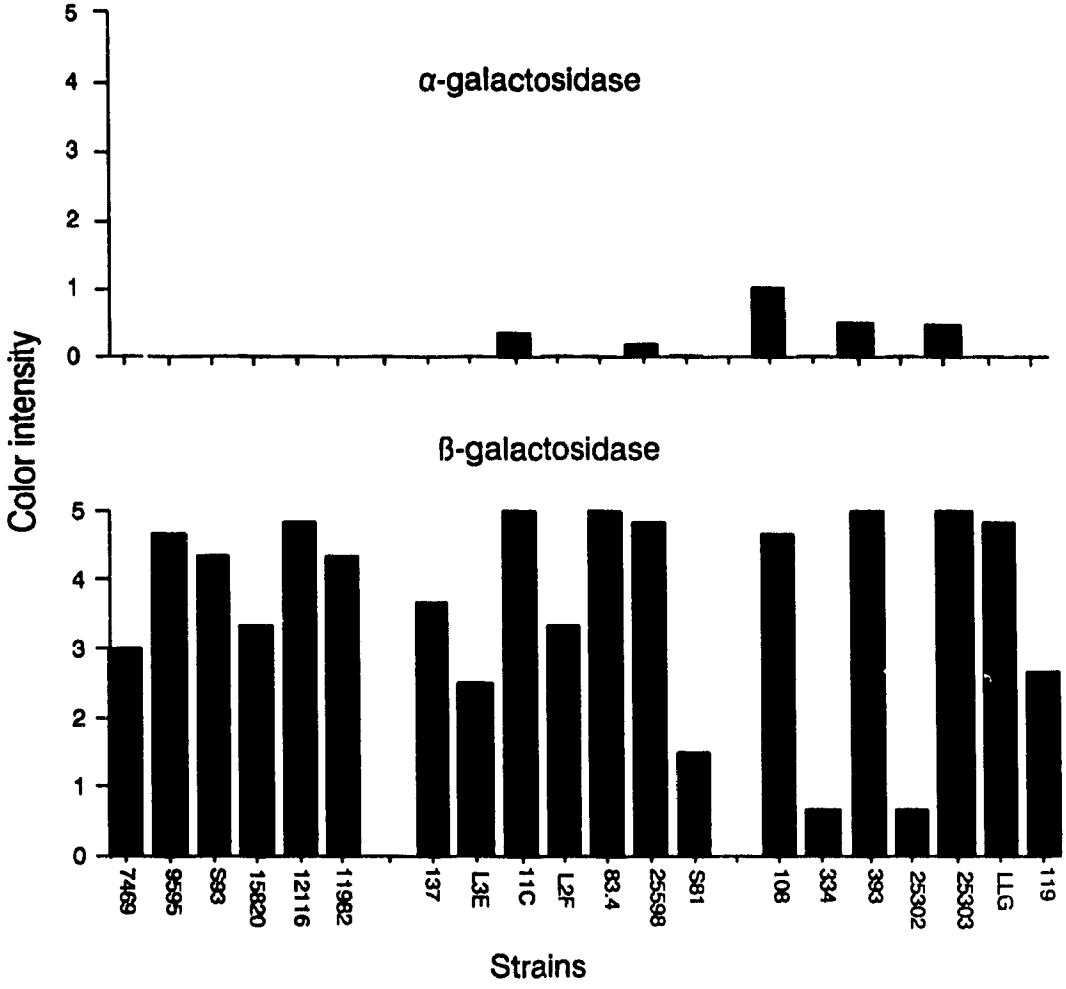

Figure 17. α and ß galactosidase profiles of *Lb. casei* ssp. *rhamnosus* (7469 to 11982) ssp. *pseudoplantarum* (137 to S81) and ssp. *casei* (108 to 119). Color intensity: 0 = no activity, 1-4 = intermediate activity, and 5 = maximum activity (≥40 n*M* of chromophore released).

Comparative Studies on Peptidases of *Lactobacillus casei* Species

KEY WORDS

Lactobacillus *casei*, Dairy Microbiology, Peptidase, Cheese, Starter, Enzymes, Lactic acid bacteria.

ABSTRACT

The objective of this study was to isolate strains of lactobacilli containing peptidases of high specific activities and wide spectrum specificities for the enzyme-modification and accelerated ripening of Cheddar cheese. Six selected strains of *Lactobacillus casei* (ssp. *casei*, ssp. *pseudoplantarum* and ssp. *rhamnosus*) were evaluated for their amino-, di-, tri- and carboxy-peptidase activities using 30 different synthetic peptides and peptide derivatives. All six strains showed similar substrate specificities towards various peptides, except for Phe-Pro, which was only hydrolysed by *Lactobacillus casei* ssp. *casei* strains. Analysis of variance revealed significant differences among strains with respect to their specific activities. Overall profiles showed that *Lactobacillus casei* subspecies contained high amino- and di-peptidase, with relatively weak tri-peptidase activities. *Lactobacillus casei* ssp. *casei* strains LLG and ATCC 393 had the highest di- and tri-peptidase activities respectively, whereas *Lactobacillus casei* ssp. *rhamnosus* S93 contained the highest activities for leucine-, lysine, alanine-, valine- and methionine-aminopeptidases, and proline-iminopeptidase. The dipeptidases of all the six strains exhibited 8-10 times higher affinity for Ala-X dipeptides. Highest activities for amino- and di-peptidases were observed with

leucine *p*-nitroanilide (256 μmoles/min/mg of protein) and Ala-Met (4976 absorbance units/min/mg of protein) as substrates. *Lactobacillus casei* ssp. *casei* strains showed strong activities against bitter peptides (Phe-Pro, Pro-Phe, Pro-Ile) and sulfur containing amino- and peptide- derivatives (methionine *p*-nitroanilide, cysteine *p*-nitroanilide, and Ala-Met), which are of great importance in debittering and ripening of cheeses. None of the strains showed any detectable carboxypeptidase activity, when tested on seven carboxyl-end-substituted peptides.

INTRODUCTION

Proteolytic enzymes of cheese starters have a major contribution to rheological attributes of ripened cheese varieties such as body and texture, as well as the color and flavor of the finished cheese. Hence many attempts have been made in the past decade to modify or enrich cheese flavors, and to accelerate cheese ripening by supplementing this biological system with exogenous food grade proteinases (Law & Wigmore, 1982). The major problem associated with these microbial proteolytic preparations is their indiscriminatory and gross proteolysis, which results in poor body, and often a bitter taste. With the scientific information at hand, cheese starters with low proteinase and high peptidase activities are believed to give desirable body and flavor with no bitterness (El Soda *et al*, 1978a).

Both modern and traditional methods of the manufacture of Cheddar cheese, aged longer than three months, rely upon the chance presence of lactobacilli to facilitate the later stages of ripening (Johns & Coles, 1959). Preliminary results obtained from our laboratory demonstrated that *Lactobacillus casei* subspecies produce a greater range of enzymes than do the lactococci starters, and that the enzymatic system (peptidases and esterases) appears to be responsible for the

development of the desirable flavors (Lee *et al*, 1986). El Soda *et al* (1981a) in a similiar study indicated that *Lactobacillus casei* peptidases cause an acceleration of cheese ripening without any significant difference in the overall composition of cheese, when compared to the control sample. Though some authors have studied the peptidases of different lactobacilli (Brandseater & Nelson, 1956b; El Soda & Desmazeaud, 1982; Hickey *et al*, 1983; Frey *et al*, 1986a; Abo-Elanga & Plapp, 1987), very little information is available on the enzyme systems of *Lactobacillus casei* subspecies.

The paper reports on specificities and specific activities of peptidases of six strains of *Lactobacillus casei* selected from their superior enzyme profiles (Arora *et al*, 1990). The study provides a better understanding for their role in acceleration of cheese maturation and enzyme modification of cheeses.

MATERIALS AND METHODS

Preparation of crude cell-free extracts

The following six strains of *Lactobacillus casei* were used for the study: *Lactobacillus casei* ssp. *casei* (ATCC 393) and ssp. *rhamnosus* (ATCC 15820) were obtained from American Type Culture Collection, Rockville, MD, while *Lactobacillus casei* ssp. *casei* (LLG), ssp. *pseudoplantarum* (L2F and 83.4) and ssp. *rhamnosus* (S93) were isolated from a high quality Cheddar cheese.

Strain maintenance, propagation and the preparation of crude cell-free

extracts were accomplished as previously described by Arora *et al* (1990). Cell growth was measured by counting colony forming units on lactobacilli MRS agar medium (Difco Laboratories, Detroit, MI). Crude enzyme was passsed through disposable G-25 (Pharmacia PD 10) column to remove amino acid residues and thus avoid high blank values with Yemm and Cocking (1955) reagent used in di-, tri- and carboxy-peptidase assays (Fig 10).

Aminopeptidase activity

Aminopeptidase activities were measured on chromogenic substrates (*p*-nitroanilide derivatives of L-anomers of leucine, lysine, alanine, valine, arginine, proline, methionine, and glutamine) by the method of El-soda *et al* (1982). The assay mixture contained 0.25 ml of the substrate (16.4 m*M* in methanol), 6.0 ml of sodium phosphate buffer (0.05 *M*, pH 7.0) and 0.25 ml of enzyme solution (1.2mg protein/ml). Samples were drawn at different time intervals of incubation, and the enzyme activity was measured by absorbance at 410 nm. The concentration of p-nitroaniline was calculated from the derived value of molar absorption coefficient (ϵ = 9024 $M^{-1}cm^{-1}$) (Fig. 2). Under the conditions of the assay, a linear correlation was observed for enzyme activity/time with all the substrates. Dipeptidyl-aminopeptidase activities were assayed using acetyl-alanine-, glutamyl-phenyl alanine- and alanyl-alanine- *p*-nitroanilide as substrates.

One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmole of *p*-nitroaniline per min under the conditions of the assay. Specific activity was expressed as units/mg of protein.

Di-, Tri- and Carboxy-peptidase activities

Dipeptidase activities were measured on Ala-Met, Ala-Phe, Ala-His, Leu-Tyr, Pro-Ile, Pro-Phe, DLeu-Leu, Phe-Pro, and Leu-Leu, tripeptidase activities on Gly-Ala-Tyr, Gly-Glu-Phe and Gly-Leu-Phe, and carboxypeptidase activities on the following carbo-benzoxy (CBZ) peptides; CBZ-threonine, CBZ-tyrosine, CBZ-leucine, CBZ-arginine, CBZ-glycyl-alanine, CBZ-phenyl alanyl-alanine, and CBZ-prolyl-D-leucine. The hydrolysis of synthetic substrates was carried out at 30°C and the activities were determined by the estimation of the liberated amino acids by modified Yemm and Cocking reagent (Matheson & Tattrie, 1964). The reaction mixture (500 μl) contained 50 μl of peptide solution (5 m*M* in sodium phosphate buffer), 400 μl of sodium phosphate buffer (0.05 *M*, pH 7.0) and 50 μl of crude enzyme (1.2 mg protein/ml). Aliquotes (0.1 ml) were taken at different time intervals and the reaction was arrested by the addition of 50 μl of 0.1 M acetic acid.

One unit of the enzyme activity was defined as the amount of enzyme required to cause an increase of 0.01 unit of absorbance at 570 nm per min under the conditions of the assay.

Chemicals and reagents

Unless otherwise specified, all amino- and peptide- derivatives used in this study contained L-anomers of aminoacids, and were purchased from SIGMA Chemical Company, St. Louis, MO.

Protein assay

The protein content of the crude cell-free extract was determined by the BCA (bicinchoninic acid) assay reagent supplied with the system (Pierce Chemical Ltd., Rockford, IL)(Smith *et al*, 1985). Bovine serum alburnin and *p*-nitroaniline (SIGMA) were used for the standard curves, (Fig.1).

Statistical analysis

The experimental design was of complete randomized type. Analysis of variance was performed by Duncun's multiple range test using SAS computer program (Snedecor & Cochran, 1980), (Appendix I).

RESULTS AND DISCUSSION

Cell-free extracts of six *Lactobacillus casei* species were tested for their peptidase activities on 30 synthetic peptides and peptide derivatives. Overall profiles indicated the presence of high amino- (Table 4) and di-peptidase (Table 5) activities and low imino, dipeptidyl amino- (Table 4) and tri-peptidase (Table 6) activities in all the strains. Similar observations have been reported for other *Lactobacillus* species by previous investigators (Hickey *et al*, 1983; Frey *et al*, 1986a). All the six strains also showed similarity in substrate specificities for different peptidases, which could be attributed to their close taxonomical relationship. However, they differed significantly in relative biomass production, intracellular protein yield (Fig. 18), and in their specific activities against different peptides.

As shown in Table 4, *Lactobacillus casei* species had wide-spectrum aminopeptidase activity profiles by being active against eight out of eleven substrates used in the study. Highest aminopeptidase activities were observed against leucine *p*-nitroanilide for all the strains ($P<0.05$). These results differ with the findings of Frey *et al* (1986a), who reported higher activities for lysine than those of leucine aminopeptidase. None of the strains contained glutamine aminopeptidase activity. While *Lactobacillus casei* ssp. *rhamnosus* S93 showed the greater aminopeptidase activity against most of the substrates ($P<0.05$), highest arginine aminopeptidase activities were observed in *Lactobacillus casei* ssp. *casei* strains (LLG and ATCC 393). Both strains of *Lactobacillus casei* ssp. *pseudoplantarum* had relatively poor aminopeptidase activity profiles when compared to the other two subspecies. All the strains contained low but distinct iminopeptidase activity against proline *p*-nitroanilide. When compared to lactococci, however, lactobacilli were reported to possess superior iminopeptidase activity (Hickey *et al*, 1983). Dipeptidyl aminopeptidase activity assays results showed the ability of all the six strains to cleave Ala-Ala *p*-nitroanilide, whereas they failed to hydrolyse the other two peptide derivatives. Both imino- and dipeptidylamino-peptidase activities were significantly higher ($P<0.05$) for *Lactobacillus casei* ssp. *rhamnosus* S93 (Table 4).

Dipeptidase activities were assayed using nine dipeptides (Table 5). Overall dipeptidase profiles indicated close resemblance among the strains in their substrate specificities, with the exception of their action against Phe-Pro dipeptide, which was only hydrolysed by *Lactobacillus casei* ssp. *casei* strains (LLG and ATCC 393). *Lactobacillus casei* ssp. *casei* strains also contained superior activities against the other two bitter peptides (Pro-Phe and Pro-Ile) used in the study. This is a desirable character for their use as cheese starters to prevent bitterness in ripened cheeses. Bitter tastes are often caused by most of the commercial proteinases used for the acceleration of cheese maturation.

All the strains exhibited higher dipeptidase affinity (8-10 times) for dipeptides having alanine on its carboxyl end (Ala-Phe, Ala-Met and Ala-His). Among the various dipeptides tested, maximum activity was observed against Ala-Met in five out of six strains. Release of methionine during cheese maturation through the action of strong methionine aminopeptidase and Ala-Met dipeptidase activities has an important contribution for the development of Cheddar cheese flavor (Law, 1984). *Lactobacillus casei* ssp. *casei* strains LLG and ATCC 393 showed the higher dipeptidase activities ($P<0.05$) against all the dipeptides used in this study.

While all the six strains showed the ability to cleave the three tripeptides assayed in this study, highest activities were observed against Gly-Leu-Phe ($P<0.05$)(Table 6). When compared to dipeptidase activities, *Lactobacillus casei* species seemed to have weaker tripeptidase activities. Similar to dipeptidase profiles, *Lactobacillus casei* ssp. *casei* strains (ATCC 393 and LLG) cleaved tripeptide more rapidly than ssp. *rhamnosus* and ssp. *pseudoplantarum* strains.

No carboxypeptidase activity was detected under the conditions of the assay during the two hours of incubation period, when tested on seven carboxyl end substituted peptides. Reports from no carboxypeptidase (El Soda *et al*, 1978a; Hickey *et al*, 1983) to weak carboxypeptidase activity (El Soda & Desmazeaud, 1982) have been reported in various other lactobacilli. More recently, Abo-Elanga & Plapp (1987) showed the presence of a carboxypeptidase with broad specificity in a *Lactobacillus casei* strain by activity staining on gel electrophoresis, but failed to isolate the enzyme.

All the enzyme assays were performed with fixed protein levels of crude enzyme. We also measured the release of intracellular protein and the relative

biomass of each strain grown, harvested and disintegrated under identical experimental conditions (Fig. 18). Preliminary growth studies of *Lactobacillus* species indicated the end of the logarithmic growth phase, when the viable count (measured as CFU/ml) reaches approximately $1x10^9$/ml with corresponding values of optical density of approximately 1.0 (A_{600}) and medium pH of approximately 5.0. Among the six strains tested, *Lactobacillus casei* ssp. *rhamnosus* S93 resulted in highest biomass with the lowest protein yield, whereas *Lactobacillus casei* ssp. *pseudoplantarum* L2F produced maximum protein from lowest cell mass.

CONCLUSIONS

The results of this study indicate the presence of broad peptidase specificities and high peptidase specific activities in *Lactobacillus casei* species. Among the six strains studied, *Lactobacillus casei* ssp. *rhamnosus* S93 showed the highest amino- and imino-peptidase activities, whereas *Lactobacillus casei* ssp. *casei* strains (LLG and ATCC 393) had superior di- and tri-peptidase activities. In addition, *Lactobacillus casei* species showed promising ability to hydrolyse bitter peptides and produce high free N, which have important bearing on Cheddar cheese flavor.

Table 4. Aminopeptidase specific activities[1] of different strains of *Lactobacillus casei*.

	Strains[2]					
	ssp. *rhamnosus*		ssp. *pseudoplantarum*		ssp. *casei*	
Substrate[3]	S93	15820	L2F	83.4	LLG	393
	specific activities					
Leucine	256(2)^{aA}	66(1)^{dA}	95(3)^{cA}	89(5)^{cA}	118(5)^{bA}	109(2)^{bA}
Lysine	91(5)^{aB}	56(1)^{cB}	23(1)^{dC}	5[illegible]^{cB}	91^{aB}	82(1)^{bB}
Methionine	99(1)^{aB}	23(1)^{dD}	30(1)^{cB}	31^{cC}	42(2)^{bD}	38(1)^{bD}
Arginine	63(9)^{aC}	42(4)^{bC}	8(3)^{cD}	30(2)^{bC}	74^{aC}	69(1)^{aC}
Alanine	54(3)^{aCD}	19(2)^{dDE}	17(1)^{dC}	22^{dD}	34(1)^{cE}	39(1)^{bE}
Valine	39(4)^{aD}	15(1)^{bE}	19(2)^{bC}	17(1)^{bD}	20(1)^{bF}	15(1)^{bF}
Proline	16(3)^{aE}	5(1)^{bF}	3^{bE}	3^{bE}	3^{bG}	4^{bF}
Glutamine	0	0	0	0	0	0
Ala-Ala	5^{aE}	1^{cF}	1^{cE}	2^{bcE}	2^{bG}	1^{cF}
Ace-Ala	0	0	0	0	0	0
Glu-Pne	0	0	0	0	0	0

[1]All means represent duplicate analyses made on cell-free extracts of three fermentations (± S.E. in parentheses).

[2]Subspecies (ssp.) of *Lactobacillus casei*.

[3]All substrates are *p* nitroanilide derivatives of L anomeric amino acids.

[a,b,c,d]Means with the same letter within the row are not significantly different ($P<.05$).

[A,B,C,D,E,F,G]Means with the same letter within the column are not significantly different ($P<.05$).

Table 5. Dipeptidase specific activities[1] of different strains of *Lactobacillus casei*.

	Strains[2]					
	ssp. *rhamnosus*		ssp. *pseudoplantarum*		ssp. *casei*	
Peptide[3]	S93	15820	L2F	83.4	LLG	393
	specific activities					
Ala-Met	2236^{cA}	2148^{cdA}	2149^{cdB}	1816^{dA}	4976^{aA}	3950^{bA}
	(98)	(126)	(87)	(150)	(55)	(163)
Ala-Phe	1359^{bB}	1099^{cB}	2025^{aB}	1425^{bB}	2225^{aB}	1459^{bB}
	(31)	(142)	(62)	(96)	(74)	(81)
Ala-His	1212^{cB}	1140^{cB}	2535^{aA}	1397^{cB}	1989^{bB}	1606^{bcB}
	(70)	(44)	(265)	(235)	(215)	(22)
Leu-Tyr	259^{bC}	157^{bcC}	223^{bcC}	138^{cC}	404^{aC}	462^{aC}
	(1)	(41)	(9)	(9)	(38)	(59)
Pro-Ile	133^{bcCD}	58^{dC}	72^{cdC}	65^{cdC}	267^{aCD}	160^{bD}
	(3)	(16)	(12)	(29)	(33)	(21)
Pro-Phe	40^{cD}	44^{cC}	79^{bcC}	44^{cC}	142^{aCD}	124^{abD}
	(4)	(12)	(4)		(35)	(8)
D Leu-Leu	38^{bcD}	22^{cdC}	22^{cdC}	7^{dcC}	59^{abD}	77^{aD}
	(7)	(7)	(7)		(15)	(11)
Phe-Pro	0	0	0	0	21^{bD}	126^{aD}
					(6)	(73)
Leu-Leu	0	0	0	0	0	0

[1]All means represent duplicate analyses made on cell-free extracts of three fermentations (± S.E. in parentheses).

[2]Subspecies (ssp.) of *Lactobacillus casei*.

[3]Otherwise indicated all peptides constitute L anomeric amino acids.

[a,b,c,d]Means with the same letter within the row are not significantly different ($P < .05$)

[A,B,C,D]Means with the same letter within the column are not significantly different ($P < .05$).

Table 6. Tripeptidase specific activities[1] of different strains of *Lactobacillus casei*.

	Strains[2]					
	ssp. *rhamnosus*		ssp. *pseudoplantarum*		ssp. *casei*	
Peptide[3]	S93	15820	L2F	83.4	LLG	393
	specific activities					
Gly-Leu-Phe	79^{bA} (7)	45^{bA} (8)	42^{bA} (18)	28^{bA} (1)	219^{aA} (6)	214^{aA} (35)
Gly-Ala-Tyr	36^{bB} (3)	16^{bB} (1)	9^{bA}	27^{bA} (3)	126^{aB} (42)	128^{aB} (3)
Gly-Glu-Phe	20^{bB} (4)	14^{bB} (4)	32^{bA} (2)	18^{bB}	17^{bC} (1)	59^{aB} (18)

[1]All means represent duplicate analyses made on cell-free extracts of three fermentations (± S.E. in parentheses).

[2]Subspecies (ssp.) of *Lactobacillus casei*.

[3]All peptides constitute L anomeric amino acids.

[a,b]Means with the same letter within the row are not significantly different ($P<.05$).

[A,B]Means with the same letter within the column are not significantly different ($P<.05$).

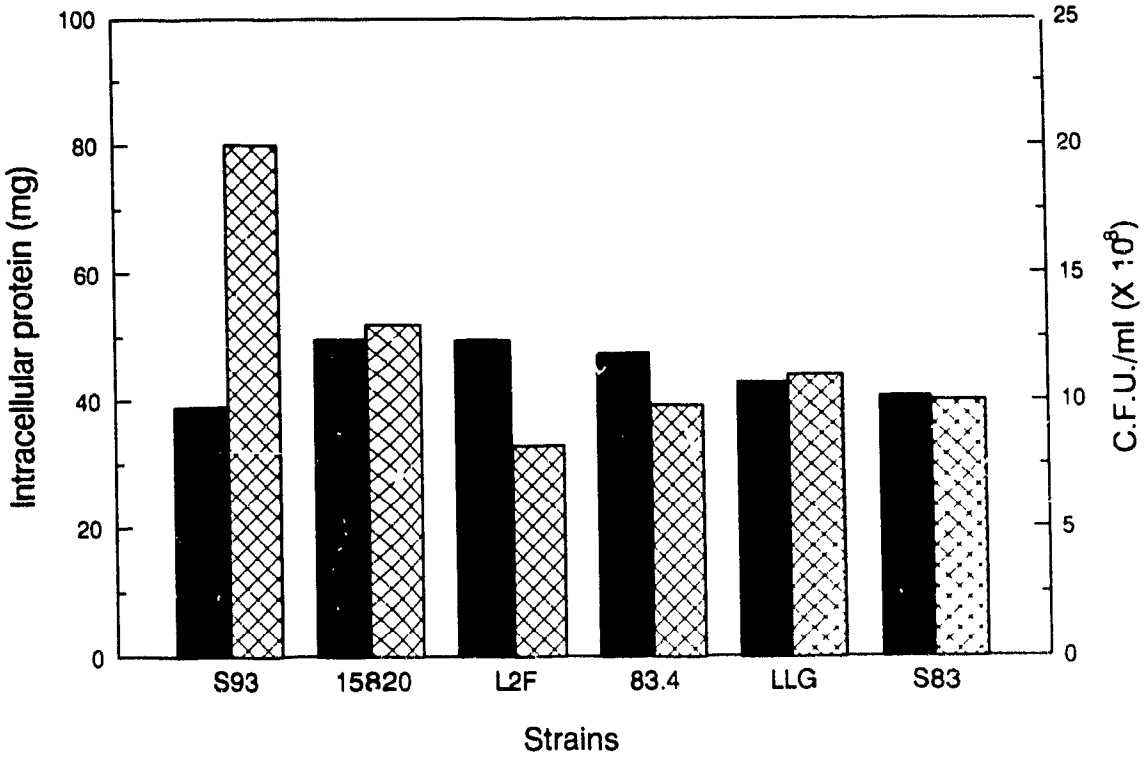

Figure 18. Relative cell growth in MRS medium and intracellular protein production by *Lb. casei* species. CFU/ml at A_{600} = 1.0 (□), protein content in mg (■).

Purification and Partial Characterization of Aminopeptidase from *Lactobacillus casei* ssp. *casei* LLG and *Lb. casei* ssp. *rhamnosus* S93.

KEY WORDS

Lactobacillus casei, Aminopeptidase, Cheddar cheese, Enzyme purification.

ABSTRACT

An aminopeptidase of broad specificity has been purified to homogeneity from *Lactobacillus casei* ssp. *casei* LLG and ssp. *rhamnosus* S93 by Fast Protein Liquid Chromatography (FPLC). The desalted crude enzyme from ammonium sulfate fraction was purified on Mono Q ion-exchanger and by chromatofocusing on ampholyte exchanger PBE-94. The low molecular weight impurities were further removed by gel-exclusion chromatography on Superose12 column. The purified enzyme of each strain appeared as a single band on native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purity of the enzyme was further confirmed by the activity staining on substrate-coated blotted membrane. Aminopeptidase of both strains which constituted of a single monomeric form, had a molecular weight of about 85 kd, an isoelectric point near 4.75. The paper also describes a convenient and fast method of purification by FPLC system which was used for the purification of the aminopeptidase of both strains.

INTRODUCTION

Lactobacilli are the predominant flora during Cheddar cheese maturation (Sherwood, 1937, 1939a & b; Johns & Coles, 1959; Fryer, 1969; Chapman & Sharpe, 1981; Peterson, 1990) and play an important role in proteolysis and lipolysis, which are considered to be the principal pathways of flavor and texture generation during cheese ripening. The characteristic flavor of Cheddar cheese has been closely related to proteolytic activities in the cheese matrix (McGugan *et al*, 1979; Aston *et al*, 1983a & b; Aston & Creamer, 1986). In addition to their significant role in generation of cheese flavor, peptidases from lactobacilli have also been credited with the elimination of bitterness (Peterson, 1990). The bitter tastes are usually caused due to hydrophobic accumulation of peptides (molecular weight 2 - 1.4 kd)(Law & Kolstad, 1983) by extensive proteolysis by rennet and other proteinases (Lowrie & Lawrence, 1972; Mills & Thomas, 1980; Visser *et al*, 1983). The breakdown of bitter peptides into non-bitter or some flavorful short peptides and amino acids by bacterial peptidases appear to be the principle mechanism involved in the removal of bitterness (Sullivan *et al*, 1973; Jago, 1974; Beeby, 1980; Cliffe & Law, 1990). Since carboxypeptidase activities are virtually absent in lactobacilli (Hickey *et al*, 1983; Arora & Lee, 1990), they mainly rely on their strong aminopeptidases for various amino acid requirements.

Most of the studies on peptidase profiles were done on lactococci due to their importance as primary strain used in Cheddar cheesemaking. However, the growth pattern studies on cheese maturation clearly demonstrated the gradual decline and replacement of lactococcal flora with non-starter flora, constituting primarily of *Lactobacillus* spp. (Chapman & Sharpe, 1981). Further studies on proteolytic systems reported that lactobacilli possessed more activity of different peptidases than other

lactic acid bacteria, including lactococci (Pettersson & Sjostrom, 1975; Hickey *et al*, 1983; Ezzat *et al*, 1986; Lee *et al*, 1986). *Lb. casei* group showed the highest exopeptidase activities against wide range of substrates (El Soda *et al*, 1978b; Abo-Elanga & Plapp, 1987; Arora *et al*, 1990). Many investigations on cheesemaking with selected *Lactobacillus* strains or their enzymes as adjunct to normal starter showed improvement in desirable flavor with no bitter taste (Sherwood, 1939b; Lloyd *et al*, 1980; Law & Wigmore, 1982; Abdel Baky *et al*, 1986; Laleye *et al*, 1990, Lee *et al*, 1990a & b).

Attempts have been made in the past to detect the number of peptidases in crude cell-free extracts from different cheese-associated *Lactobacillus* strains by cell fractionation and activity staining (El Soda & Desmazeaud, 1982; El Soda *et al*, 1983; Abo-Elanga & Plapp, 1987), and a few of them have also been purified and characterized (Eggimann & Buchmann, 1980; Machuga & Ives, 1984; Atlan *et al*, 1989). Although one dipeptidyl aminopeptidase, with highly selective specificity has been characterized from *Lb. casei* ssp. *casei* strain, complete purification was not successful (El Soda & Desmazeaud, 1981). This report is probably the first to demonstrate the presence of a single enzyme in *Lb. casei* species, which accounts for most of the aminopeptidase activities found in their crude extracts. The paper describes the purification and partial characterization of this enzyme from two strains of *Lb. casei* (ssp. *casei* LLG and ssp. *rhamnosus* S93). These strains were chosen because their enzyme studies exhibited superior peptidase profiles (Arora *et al*, 1990; Arora & Lee, 1990).

MATERIALS AND METHODS

Organisms and preparation of cell extracts

Lb. casei ssp. *casei* LLG and *Lb. casei* ssp. *rhamnosus* (S93) used for this study were isolated from a high quality Cheddar cheese. Strain maintenance, propagation and the preparation of cell-free extracts were accomplished as previously described (Arora & Lee, 1990). Crude enzyme preparations were stored at -18°C in the presence of stabilizers (10% glycerol, 100 m*M* ammonium sulfate, 1 m*M* dithiothreitol) until further use (Neviani *et al*, 1989).

Chemicals, Reagents and Equipments

Unless otherwise specified, all chemicals used in this study were purchased from Sigma Chemicals (St. Louis, USA). A Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Upppsala, Sweden) was used for purification of the enzyme. It consisted of two P-500 pumps, an injection valve MV-7 with appropriate loops and superloops for sample injection, a G-250 controller, UV monitor UV-1 (having 1 cm optical path) set at 280 nm, a fraction collector Frac-100, a pH gradient monitor, and a REC-482 dual pen recorder. Pre-packed chromatography columns of Mono Q HR 5/5 (5 cm x 0.5 cm I.D.), Mono P HR 5/20 (20 cm x 0.5 cm I.D.) and Superose12 HR 10/30 (30 cm x 1.0 cm I.D.)(Pharmacia) were used for ion-exchange, chromatofocusing and gel-filtration, respectively. Gel electrophoresis was performed on Phast system (Pharmacia) using native (8-25%), SDS (12.5%, 8-25%) and Isoelectricfocusing (pH 4 - 6.5) mini-gels. Gels, staining dye (Coomassie G-250) and molecular weight markers for the electrophoresis were supplied by Pharmacia

(Montreal, Canada). Deoxyribonuclease (DNase) and ribonuclease (RNase) used for digestion of nucleic acids from crude extract were purchased from Amersham (Canada) Ltd. (Oakville, Canada). Zeta-positive membrane used for enzyme blotting was obtained from Bio-Rad Laboratories (Montreal, Canada). All other chemicals were of analytical reagent grade.

Protein assay

Protein was determined spectrophotometrically by the BCA (bicinchoninic acid) assay reagent supplied with the system (Pierce Chemical Ltd., Rockford, USA)(Smith *et al*, 1985). The method combines the well known reaction of protein with Cu^{2+} in an alkaline medium (yielding Cu^{+}) with a highly sensitive and selective detection of Cu^{+} with bicinchoninic acid. Bovine serum albumin (SIGMA) was used for the standard curve (Fig. 1).

Aminopeptidase activity

Aminopeptidase activity was measured by assaying the quantity of *p*-nitroaniline produced from the substrate (leucine *p*-nitroanilide) by the method of El Soda and Desmazeaud (1982). The method was adapted to measure the enzyme activity of eluted fractions on micro-assay plate and was read in Lambda plate reader (Perkin-Elmer Ltd., Montreal, Canada). The micro-assay mixture contained 10 μl of the substrate (16.4 m*M* leucine *p*-NA in methanol), 140 μl of sodium phosphate buffer (0.05*M*, pH 7.0) and 50 μl of the appropriately diluted enzyme solution. The plate was incubated at 30°C, and the enzyme activity was measured by absorbance at 405 nm after 15 and 30 minutes of incubation.

One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmole of *p*-nitroaniline per minute under the conditions of the assay. Specific activity was expressed as units/mg of protein.

Purification of enzyme

Figure 19 outlines the experimental protocol used for the purification of aminopeptidase. All purification steps were done at 4°C to preserve the enzyme activity. The active fractions during purification were stored at -18°C in the presence of stabilizers (10% glycerol, 100 m*M* ammonium sulfate, 1 m*M* dithiothreitol)(Neviani *et al*, 1989).

1). Removal of nucleic acids and ammonium sulfate fractionation

In the preliminary attempt, the precipitation of nucleic acids by streptomycin sulfate (Khalid & Marth, 1990) as well as protamine sulfate resulted in the loss of about 40-50% of the aminopeptidase activity. However, DNAse and RNAse efficiently hydrolysed nucleic acids in 30 minutes of storage at the concentrations prescribed, with minimal loss of enzyme activity. DNase (10 mg/ml) and RNase (10 mg/ml) were added in the presence of $MgCl_2$ (5 m*M*) to the cell-free extract to a final concentration of 2.5 μg/ml and 5.0 μg/ml of crude extract, respectively. The enzyme was stored overnight at 4°C after the addition of ammonium sulfate.

Ammonium sulfate addition to the crude extracts was helpful in preserving the aminopeptidase activity. Initial studies showed that 85% of enzyme activity was

salted out between 57% and 70%. Ammonium sulfate was added to the crude extracts of previous step, stored overnight with gentle stirring, and the fraction precipitating between 50-75% saturation was dissolved in minimum quantity of 0.02 *M* Tris-HCl buffer (pH 8.0 at 4°C). The redissolved precipitate was applied to disposable Sephadex G-25 (Pharmacia) column (5 cm x 1.6 cm), previously equilibrated with the same buffer. Sample (2.5 ml) applied to the column was eluted in 3.5 ml of the Tris-HCl buffer.

2). Ion-exchange Chromatography

The ion-exchange (Mono Q) column was equilibrated with 0.02*M* Tris-HCl buffer (pH 8.0). Portions (500 μl) of desalted crude extract containing between 15-20 mg of protein were injected into the column, and the column was washed with 6.0 ml of 0.02*M* Tris-HCl buffer containing 0.1*M* NaCl (pH 8.0). The enzyme was eluted with a linear gradient of NaCl (0.1 to 0.25 *M*) at a flow rate of 1.0 ml/minute. Fractions (1.5 ml) were collected and tested for the aminopeptidase activity. Active fractions were pooled and stored at -18°C in the presence of stabilizers. Pooled fractions of different chromatographic runs were combined and concentrated by ultrafiltration using CX-30 membrane (exclusion limit 30 kd; Amicon Corp., Toronto, Canada). Concentrated enzyme was desalted by the G-25 column, as described in the previous step, and tested for protein concentration and enzyme activity.

The concentrated enzyme was further subjected to the second ion-exchange chromatography, but with a linear NaCl gradient of 0.1 to 0.2*M* at a flow rate of 0.5 ml/minute. Active fractions against leucine *p*-NA were pooled, concentrated and equilibrated in 0.025*M* piperazine-HCl buffer (pH 5.7).

3). Chromatofocusing

Enzyme was applied to the chromatofocusing Mono P column (20 cm x 0.5 cm), after equilibration of the column with 0.025*M* piperazine-HCl buffer (pH 5.7), and the pH of the eluate was measured by an in-line pH monitor assembly. The column was washed with 10 ml of the piperazine-HCl buffer, and the proteins were eluted with a linear pH gradient of 5.0 to 4.0, obtained with a mixture of ampholytes (polybuffer 7-4, Pharmacia) previously adjusted to pH 4.0 by 6*M* HCl. at a flow rate of 0.4 ml/minute. Elution was performed by the method recommended by the manufacturer. The pH value of the fractions was immediately adjusted to pH 7.0 by the addition of Tris-HCl buffer to a final concentration of 0.1*M*. Polybuffer was removed from the active fractions by the passage through G-25 column after equilibration with 0.1*M* Tris-HCl (pH 7.5), and the enzyme was concentrated by the ultrafiltration using CX-30 membrane (Amicon).

4). Gel-Filtration

Molecular-sieve chromatography was performed with Superose12 column (30 cm x 1.0 cm) which separates protein with molecular weight 40 kd - 300 kd under a pressure of 15 bars. Portions (100 to 200 μl) of concentrated enzyme were injected into the column, and eluted with 0.1*M* Tris-HCl buffer (pH 7.5) at a flow rate of 0.3 ml/minute. Active fractions were pooled and tested for protein and enzyme activity.

Purity determination

The purity of the enzyme at each purification step was examined by native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE by the method of

Laemmli (1970) with a 4% stacking gel and 8-25% gradient running gel. Mini-gels (5 cm x 4 cm) were run on a Phast electrophoresis system (Pharmacia) for 25-30 minutes at 15°C and 30 V/cm.

The purity of the enzyme was confirmed on 12.5% acrylamide gels (with a 4% stacking gel), with or without sodium-dodecyl-sulfate (SDS), and the proteins in the gels were stained by Coomassie blue R-250 (Pharmacia) as described in the manufacturer's manual. The protein samples were mixed 1:1 with the sample buffer (20 m*M* Tris-HCl, 2 m*M* EDTA, 5% SDS, 10% ß-mercaptoethanol, pH 8.0), and boiled for 5 minutes before applying to SDS-polyacrylamide gels. Tris-glycine-HCl buffer of pH 8.8, (with or without SDS sample buffer) was used as the counter-ion.

Determination of Molecular size

The molecular weight of the purified enzymes was estimated by gel-filtration on Superose12 column (30 cm x 1.0 cm). The enzyme was loaded onto the column previously equilibrated with 0.1 *M* Tris-HCl buffer (pH 7.5). The following standard proteins were used to calibrate the column: ribonuclease (13.7 kd), chymotrypsin (25 kd), ovalbumin (43 kd), bovine serum albumin (67 kd), and alcohol dehydrogenase (150 kd). Void volume (V_o) of the column was determined by eluting blue dextran (approx. 2,000 kd) through the column. The enzyme was eluted from the column with the same buffer under the conditions recommended by the manufacturer.

Molecular size was also determined on 12.5% SDS-PAGE (with 4% stacking gel), according to the method described in the previous step. The low Molecular weight proteins (α-lactalbumin, soyabean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase b) were used for the estimation of

molecular weight of the pure protein. The gels were stained with Coomassie blue R 250, and preserved according to the manufacturer's instructions. The corresponding R_f values (relative mobility) were calculated and a calibration curve was constructed by plotting R_f values against the logarithms of the molecular weights of the standard proteins (Fig. 33). The molecular weights of the enzyme was then determined from this standard curve.

Determination of isoelectric point

Isoelectricfocusing of the purified enzyme was carried out on 5% acrylamide slab gels at 2000 V, 5.0 mA, 3.5 W and 15°C for 25-30 minutes using the Phast system. Pharmalytes in the pH range of 4.0 - 6.5 were used as carrier ampholytes, which form a stable pH gradient across the gel between two electrodes. The proteins applied to the gel migrate under high current until they reach the pH corresponding to their isoelectric point (pI). pI of the pure protein was estimated from migration profile of the following reference proteins: glucose oxidase (pI 4.15), soyabean trypsin inhibitor (pI 4.55), ß-lactalbumin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), and human carbonic anhydrase B (pI 6.55). A calibration curve was constructed by plotting the migration distance of different proteins against their pIs, and the isoelectric point of the pure protein was estimated from this curve (Fig. 35 & 36).

Activity staining

The purified enzyme was electrophoresed on 8-25% gradient acrylamide gels at 400 V, 20 mA, 2.5 W and 15°C for 30-35 minutes. Two parallel gels were run at the

same time in the Phast system. While one of the gel was stained in the phast staining chamber, other gel was re-run in the same unit for electrophoretic transfer of proteins on a zeta-positive blotting membrane, according to the method prescribed by the manufacturer. The blotted membrane was washed in the assay buffer (0.1*M* Tris-HCl, pH 8.0) and the enzyme activity against leucine *p*-NA (16.4 m*M* in methanol) was detected by the method of Sock and Rohringer (1988). By this procedure, it was possible to associate enzyme activity with the defined protein band seen with Coomassie blue staining on the parallel gel (Fig. 28).

RESULTS AND DISCUSSION

Enzyme Purification

Following ammonium sulfate precipitation, the crude enzyme fractions of *Lb. casei* ssp. *casei* LLG and ssp. *rhamnosus* S93 were subjected to four subsequent column chromatography steps. The aminopeptidase of each strain was purified by the same purification protocol (Fig. 19). The yields and activity of aminopeptidase during purification for the strain LLG and S93 are summarized in Table 7 and 8, respectively. The enzyme was purified approximately to 153-fold for *Lb. casei* ssp. *casei* LLG and 195-fold for *Lb. casei* ssp. *rhamnosus* S93. Although the purified enzyme of both strains showed high specific activities, their relatively lower yield could be attributed to the deactivation of the enzyme during the purification steps.

Ammonium sulfate precipitation step removed 28-30% of protein from the crude extract without any significant loss in enzyme activity. This step offered a convenient mean of concentration and preservation of the crude enzyme. Overnight storage of

enzyme also provided sufficient time for nucleases (DNAse and RNase) to digest almost all the nucleic acids of crude extract. Nucleases were added to eliminate viscous nucleic acids which otherwise bind to the columns, thereby lowering the column separation efficiency and the enzyme yield (Scopes, 1987).

The respective elution profiles of the aminopeptidase by ion-exchange chromatography of strain LLG and S93 are shown in Fig. 20 and 21. After elution with NaCl gradient, the highest leucine *p*-NA hydrolysing activities were obtained between 0.12 to 0.18 *M* concentration of NaCl. The major activity fractions after concentration and equilibration were applied again to ion-exchange column, and eluted with a narrow NaCl gradient (0.1 to 0.2*M*). Unlike the first eluate, the second eluate showed only one activity peak corresponding to leucine *p*-NA hydrolysing activity from both strains (Fig. 22 & 23). The highest aminopeptidase activity of strain LLG and S93 was eluted at 0.16 *M* and 0.14 *M* NaCl, respectively. This two step ion-exchange chromatography at the initial stages of purification resulted in >99% removal of crude extract protein with good enzyme recovery for each strain.

Active fractions after ion-exchange chromatography were combined, concentrated, equilibrated and further subjected to chromatofocusing column in a narrow pH gradient of 5.0 to 4.0. The enzyme peak of each strain was eluted at around pH 4.5 (Fig. 24 & 25). Native-PAGE patterns of proteins at various stages of purification and the activity staining of the purified enzyme on the blotted membrane are shown in Fig. 28 & 29. PAGE electrophoretic profile of chromatofocusing eluate of each strain showed in a single major protein band corresponding to the enzyme activity band, and a minor band of relatively low molecular weight. Finally, gel-filtration resulted in complete homogeneity as both protein and enzyme peak were resolved into a single peak (Fig. 26 & 27). Both PAGE and SDS-PAGE also revealed one protein band after staining with Coomassie blue (Fig. 28 and Fig. 29). Under non-

denaturating conditions, a single enzyme form was confirmed for both strains by the activity staining on the blotted membrane. This histochemical staining involved a coupling reaction of the product with amino acid oxidase and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)(Fig. 28). These results showed that the enzyme in each case was purified to homogeneity. This is in agreement with the aminopeptidase of broad substrate specificity purified from *Lb. acidopholus* (Machuga & Ives, 1984) and *Lc. lactis* (Neviani *et al*, 1989; Tan & Koning, 1990).

All further experiments on characterization of the enzyme were carried out with the purified enzyme preparations.

Molecular weight and isoelectric point

The molecular weight of aminopeptidase was estimated by gel-filtration by plotting the log molecular weight of the standard proteins against K_{av} and extrapolating the corresponding ratio of the enzyme protein (Fig. 30). Aminopeptidase of strain LLG and S93 had an apparent molecular weight of about 87 kd and 83 kd (Fig. 30) respectively.

On SDS-PAGE the purified enzymes of both LLG and S93 gave a single band, even in the presence of ß-mercaptoethanol (Fig. 31). The molecular weights computed by this method also yielded the same values as obtained by the gel-filtration (Fig. 32 & 33). The identical results obtained under denaturating (SDS-PAGE) and native (gel-filtration) conditions indicate that the enzyme is probably a monomer consisting of a single polypeptide chain. This enzyme differed substantially from the aminopeptidase isolated from *Lb. acidophilus* which was found to be a tetramer of molecular weight of 150 kd (Machuga & Ives, 1984), However, it greatly resembled

to the monomeric aminopeptidases from *Lb. lactis* (Eggimann & Buchmann, 1980; Atlan *et al*, 1989).

The pI of the enzyme was estimated by 5% PAA isoelectrofocusing gel (Pharmacia) by relating the mobility of enzyme protein with that of standard proteins of known pI (Fig. 34). The isoelectric point of LLG and S93 aminopeptidase, as computed by this method, was 4.73 and 4.77 respectively, which are in the same range as reported on aminopeptidases from the other lactic acid bacteria (Meyer & Jordi, 1986; Abo-Elanga & Plapp, 1987; Exterkate & Veer Gerrie, 1987).

The presence of a single monomeric aminopeptidase of broad specificity in these strains seem necessary for the degradation of peptides in a substrate starving and non-homogeneous cheese matrix. This, in turn has a greater importance in release of large number of amino acids during cheese ripening, thereby, enriching cheese flavor.

CONCLUSION

The procedure described above uses the advantages of Pharmacia chromatography columns (Mono Beads ion-exchanger, Mono Q; ampholyte exchanger, PBE-94; and Superose12; gel-filtration), and the FPLC system, which takes 2 working days to obtain a highly active homogeneous enzyme preparation from the bacterial biomass. This investigation demonstrated that *Lactobacillus casei* subspecies contain a highly active aminopeptidase, constituting a single monomeric form. The aminopeptidase of strain LLG and S93 had molecular weight of 87 and 83 kd, and isoelectric point of 4.73 and 4.77, respectively. This enzyme is distinctively different from the dipeptidyl aminopeptidase and proteinases purified earlier from *Lb. casei* species.

Table 7. Summary of the purification steps of aminopeptidase from *Lb. casei* ssp *casei* LLG

Fraction step	Total protein (mg)	Total activity (units)[1]	Specific activity (units/mg)	Purification (fold)	Yield (%)
1. Crude extract	178.5	856	4.8	1.0	100
2. Ammonium sulfate	127.9	812	6.3	1.3	95
3. Ion-exchange I	4.90	228	46.4	9.7	27
4. Ion-exchange II	1.69	184	109.0	22.7	22
5. Chromatofocusing	0.15	81	556.2	115.9	10
6. Gel-filtration	0.10	74	734.9	153.2	9

[1]One unit of enzyme is defined as the amount of enzyme required to release one μmole of *p*-nitroaniline per minute under the conditions of the assay. Specific activity is defined as enzyme units per mg of protein.

Table 8. Summary of the purification steps of aminopeptidase from *Lb. casei* ssp *rhamnosus* S93

Fraction step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
1. Crude extract	170.9	1392	8	1.0	100
2. Ammonium sulfate	120.3	1399	12	1.4	100
3. Ion-exchange I	3.48	653	188	23.0	47
4. Ion-exchange II	0.46	221	482	59.2	16
5. Chromatofocusing	0.12	121	1051	129.1	9
6. Gel-filtration	0.06	99	1584	194.5	7

[1]One unit of enzyme is defined as the amount of enzyme required to release one μmole of *p*-nitroaniline per minute under the conditions of the assay. Specific activity is defined as enzyme units per mg of protein.

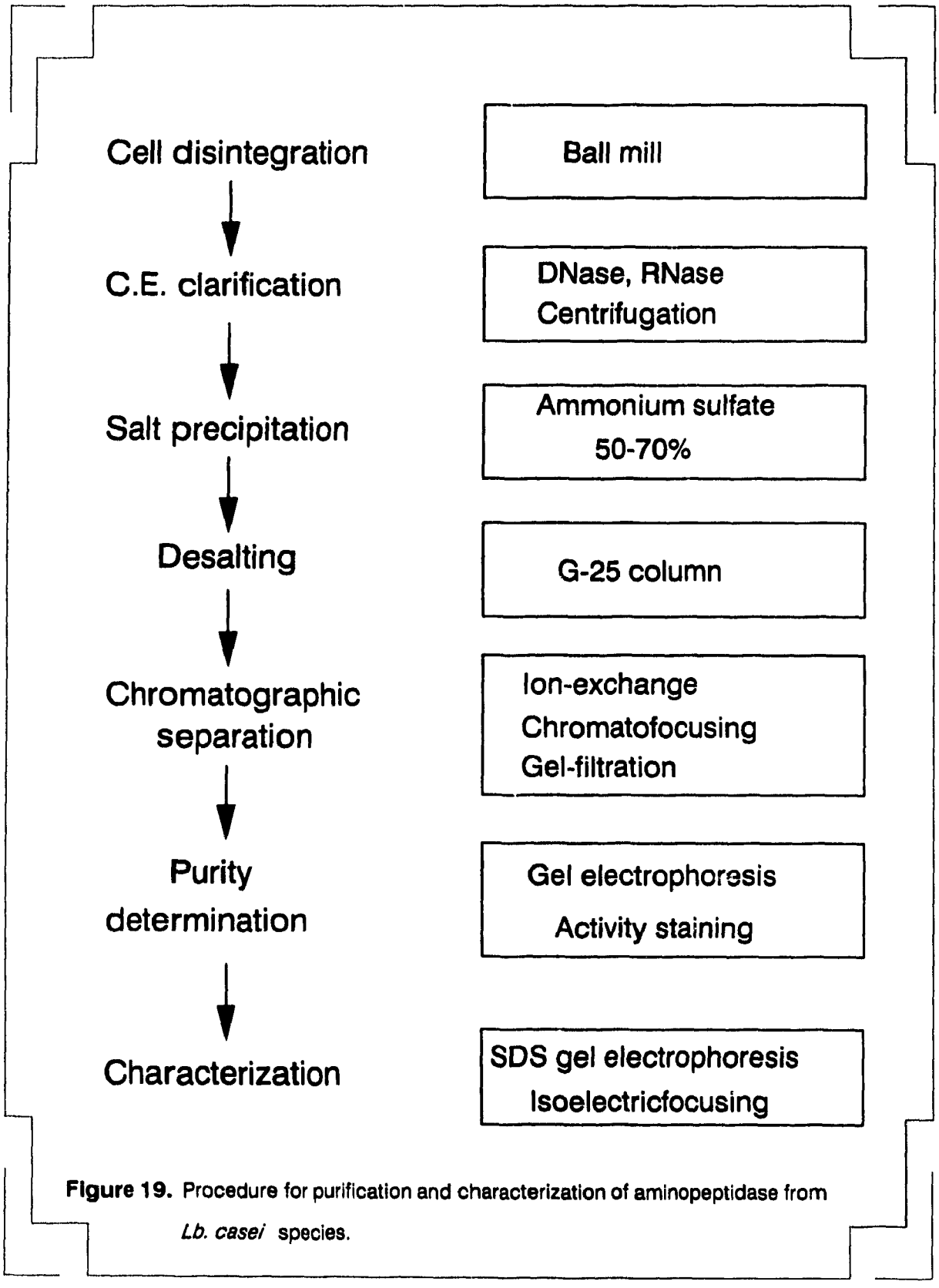

Figure 19. Procedure for purification and characterization of aminopeptidase from *Lb. casei* species.

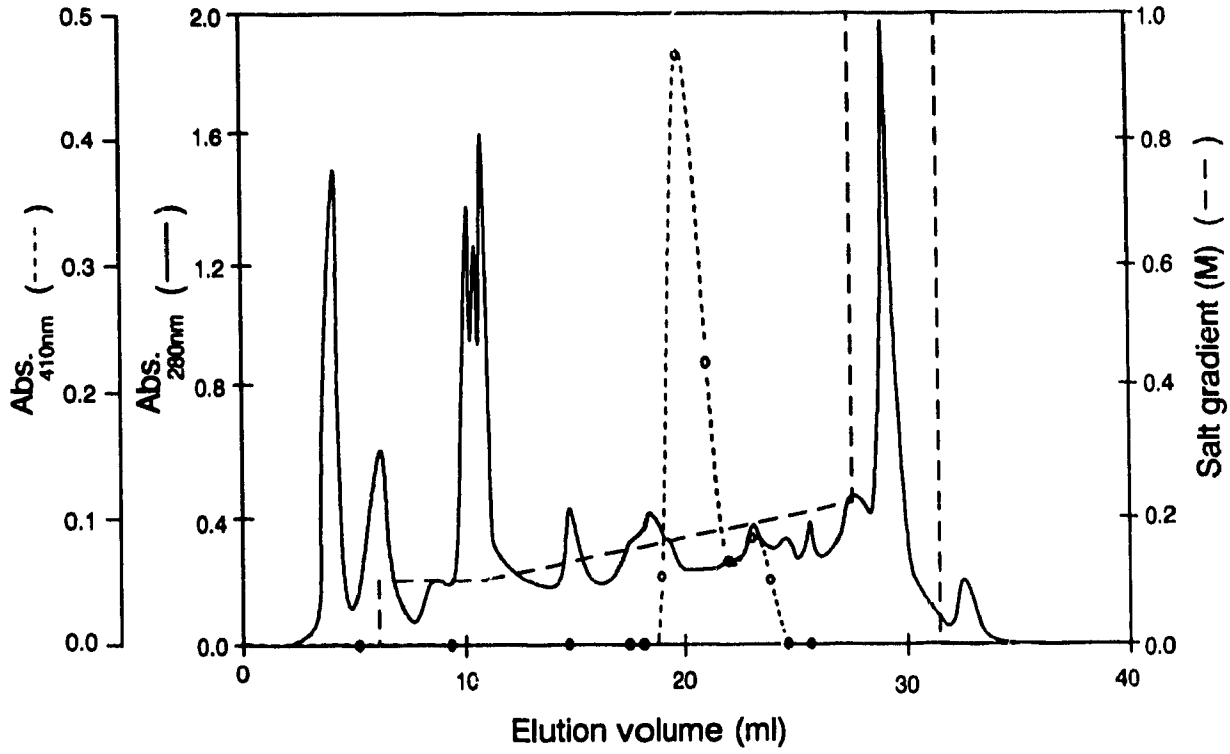

Figure 20. First ion-exchange elution profile of crude extract of *Lb. casei* ssp. *casei* LLG on Mono Q ion-exchange column (5 x 0.5 cm). Enzyme activity at A_{410} (-----), Protein at A_{280} (——), Salt gradient (– – –).

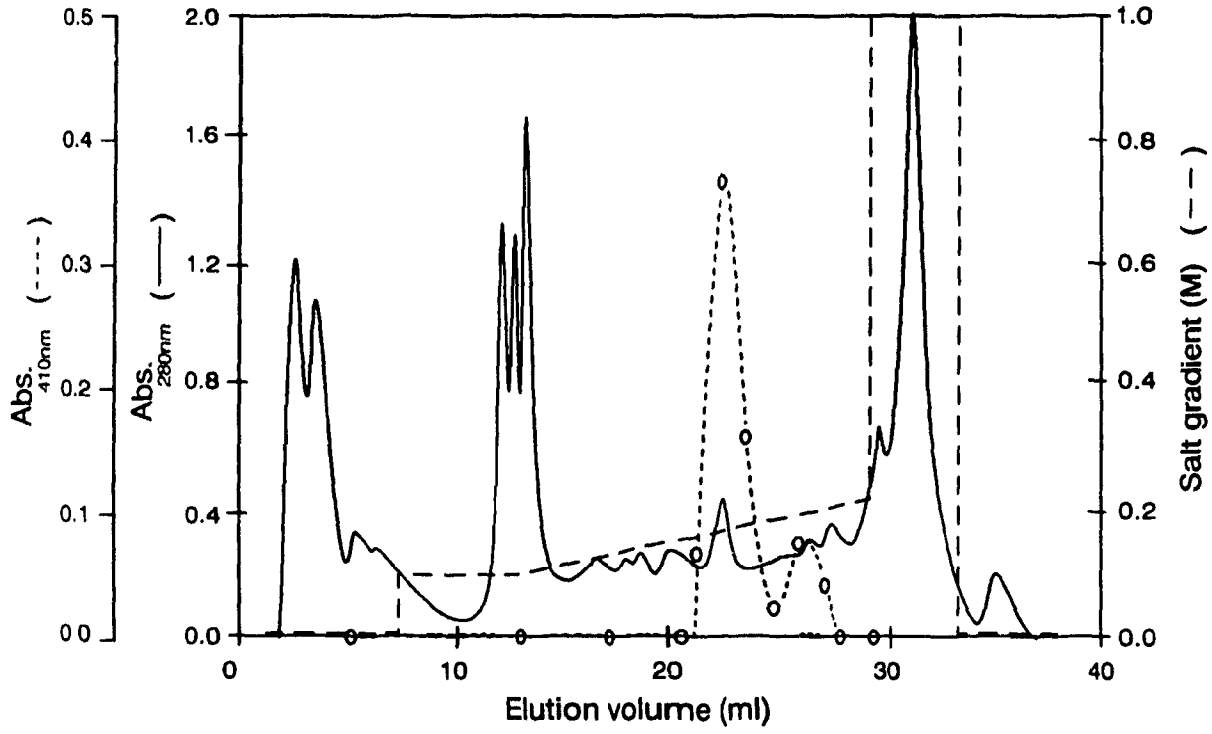

Figure 21. First ion-exchange elution profile of crude extract of *Lb. casei* ssp. *rhamnosus* S93 on Mono Q ion-exchange column (5 x 0.5 cm). Enzyme activity at A_{410} (-----), Protein at A_{280} (——), Salt gradient (– – –).

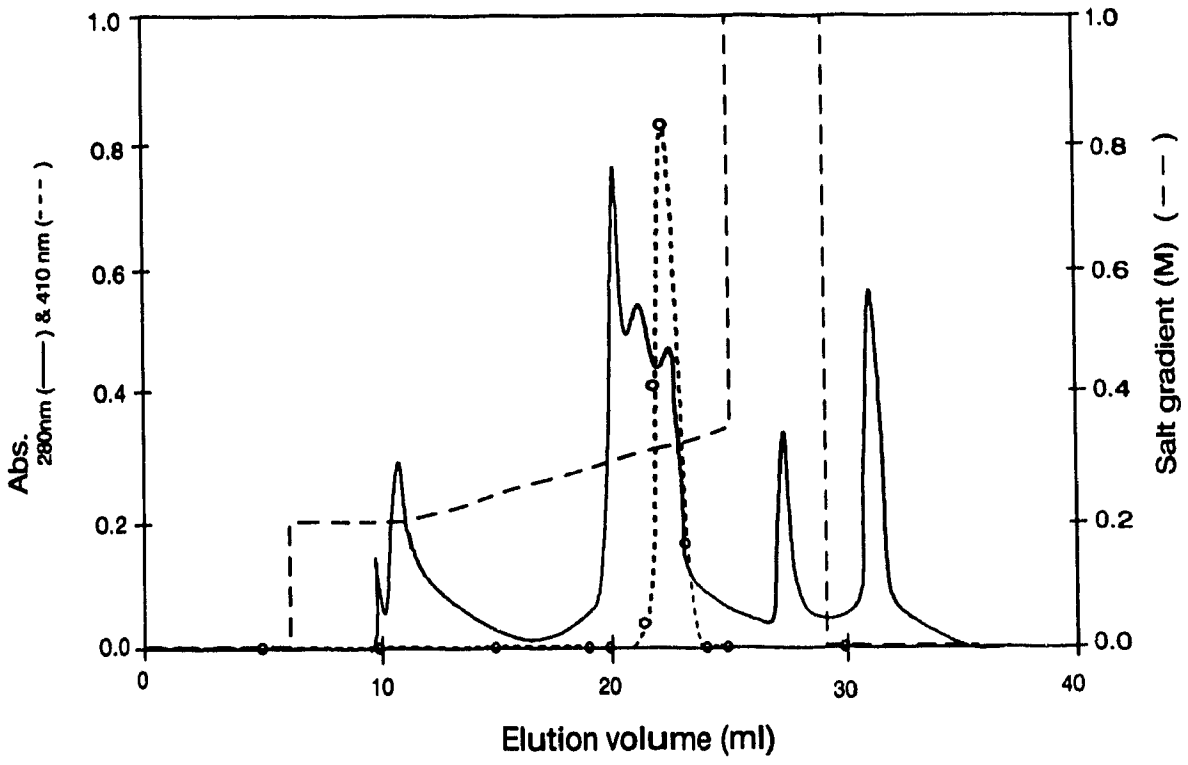

Figure 22. Second ion-exchange elution profile of active aminopeptidase fractions of *Lb. casei* ssp. *casei* LLG on Mono Q ion-exchange column (5 x 0.5 cm). Enzyme activity at A_{410} (-----), Protein at A_{280} (---), Salt gradient (---).

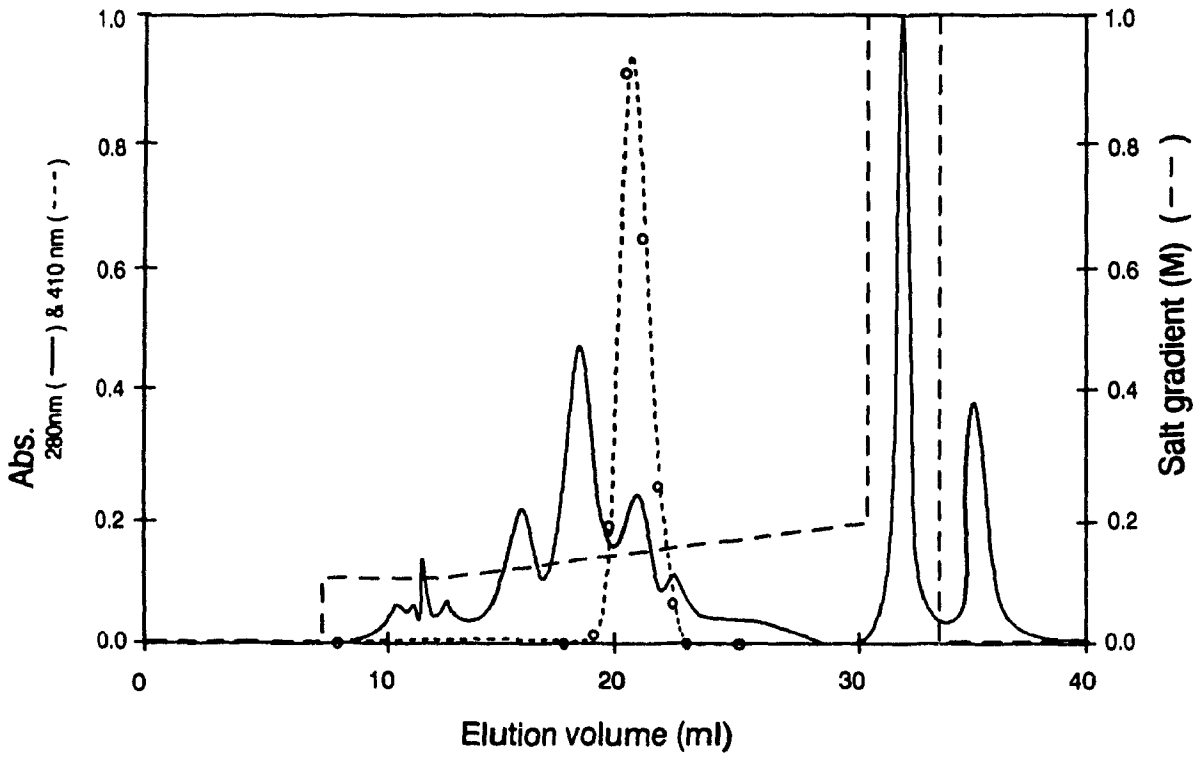

Figure 23. Second ion-exchange elution profile of active aminopeptidase fractions of *Lb. casei* ssp. *rhamnosus* S93 on Mono Q ion-exchange column (5 x 0.5 cm). Enzyme activity at A_{410} (-----), Protein at A_{280} (——),Salt gradient (- - -).

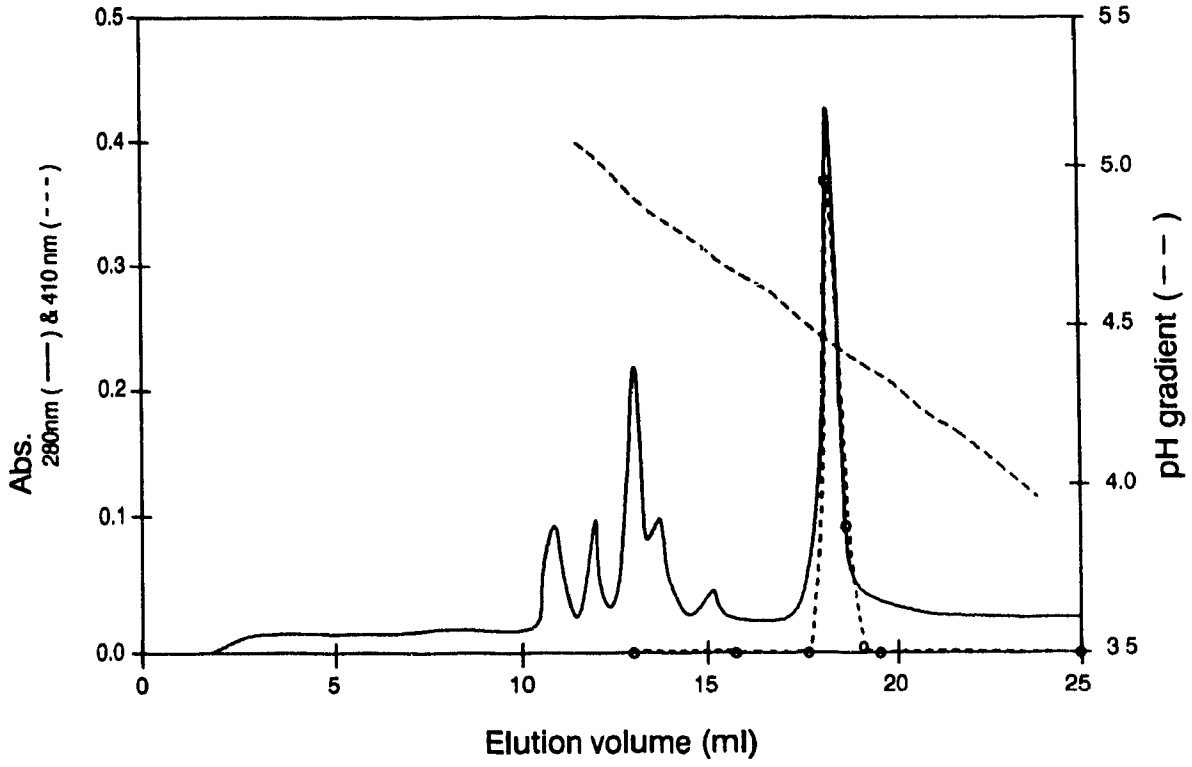

Figure 24. Elution profile of active aminopeptidase fractions of *Lb. casei* ssp. *casei* LLG on PBE-94 chromatofocusing column (20 x 0.5 cm). Enzyme activity at A_{410} (-----), Protein at A_{280} (——), pH gradient (---).

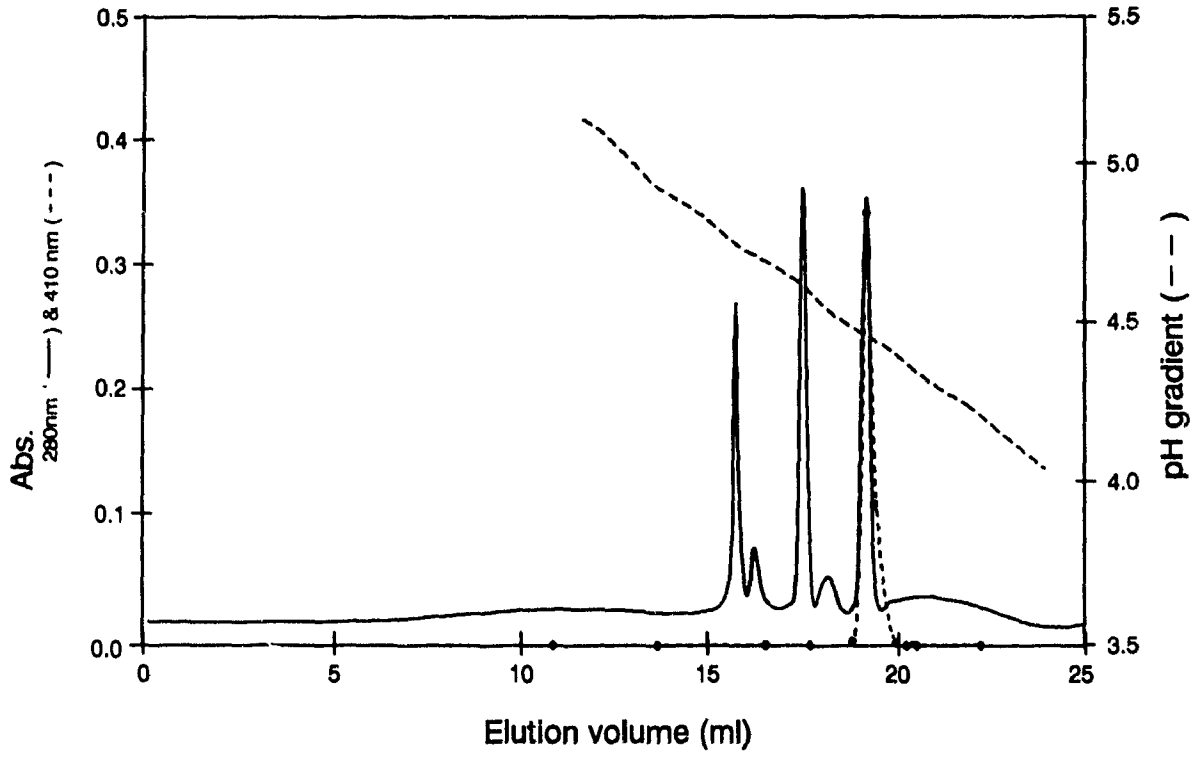

Figure 25. Elution profile of active aminopeptidase fractions of *Lb. casei* ssp. *rhamnosus* S93 on PBE-94 chromatofocusing column (20 x 0.5 cm). Enzyme activity at A_{410} (-----), Protein at A_{280} (—), pH gradient (---).

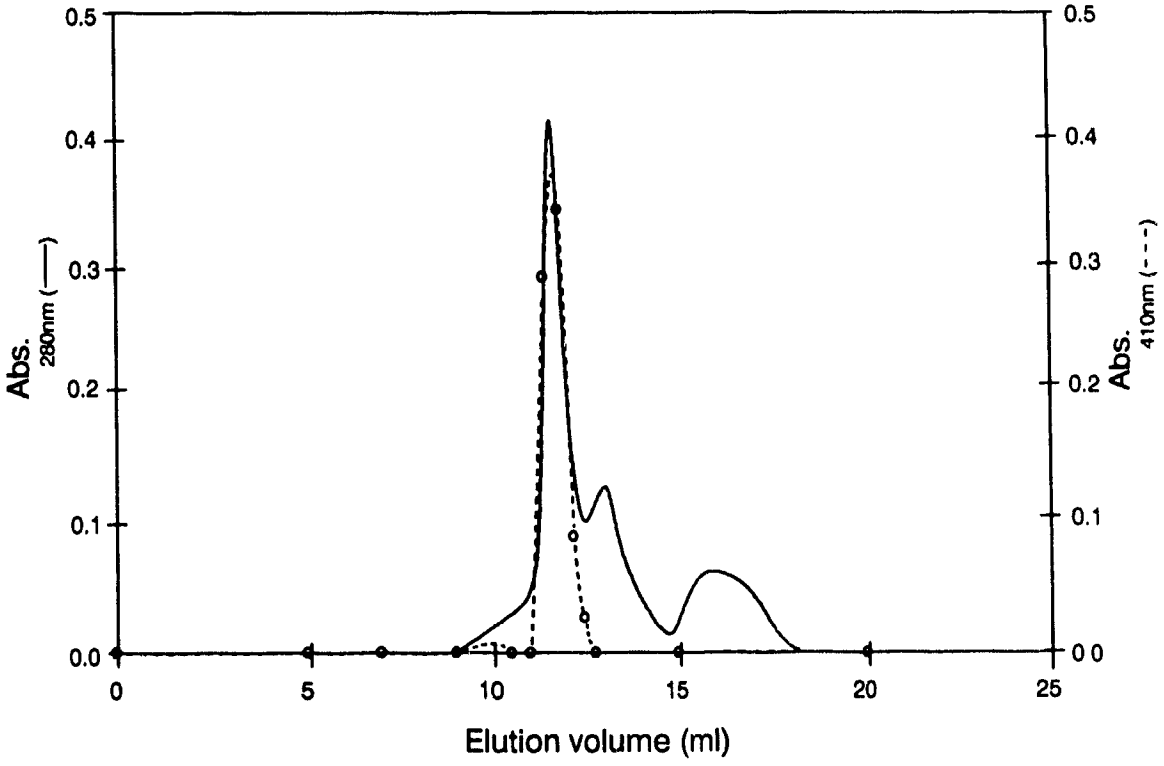

Figure 26. Elution profile of active aminopeptidase fractions of *Lb. casei* ssp. *casei* LLG on Superose-12 gel-filtration column (30 x 1 cm). Enzyme activity at A_{410} (-----), protein at A_{280} (—).

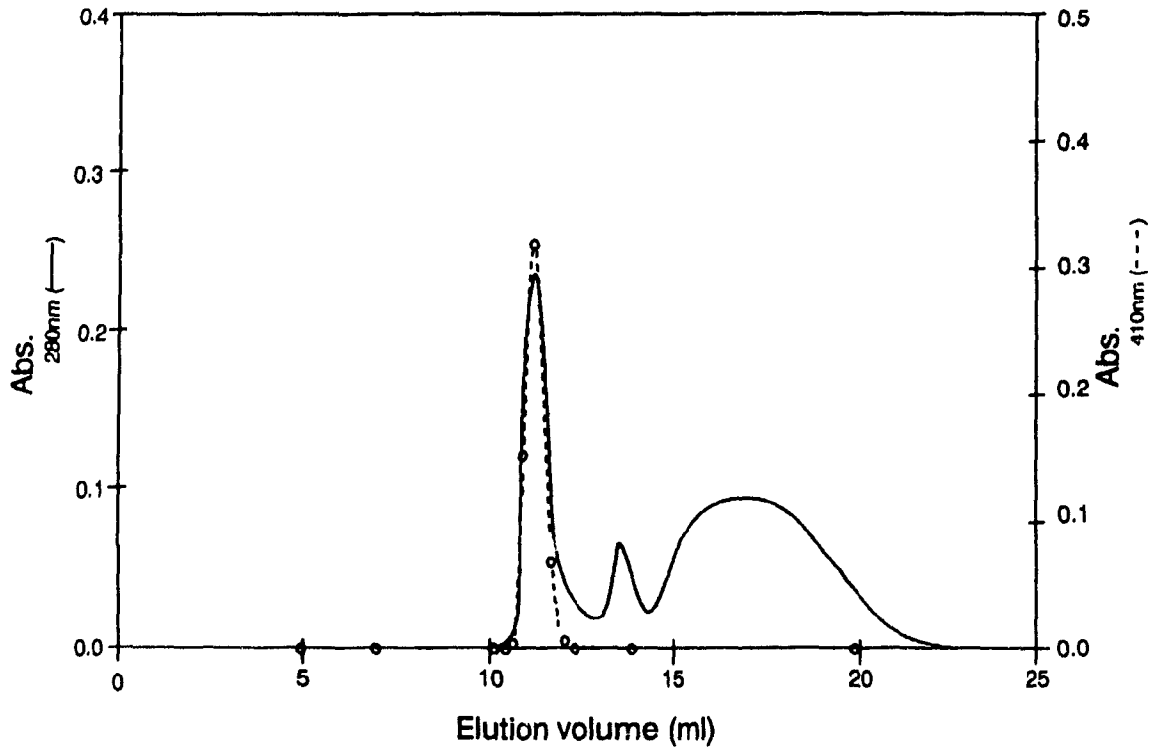

Figure 27. Elution profile of active aminopeptidase fractions of *Lb. casei* ssp. *rhamnosus* S93 on Superose-12 gel-filtration column (30 x 1 cm). Enzyme activity at A_{410} (-----), Protein at A_{280} (—).

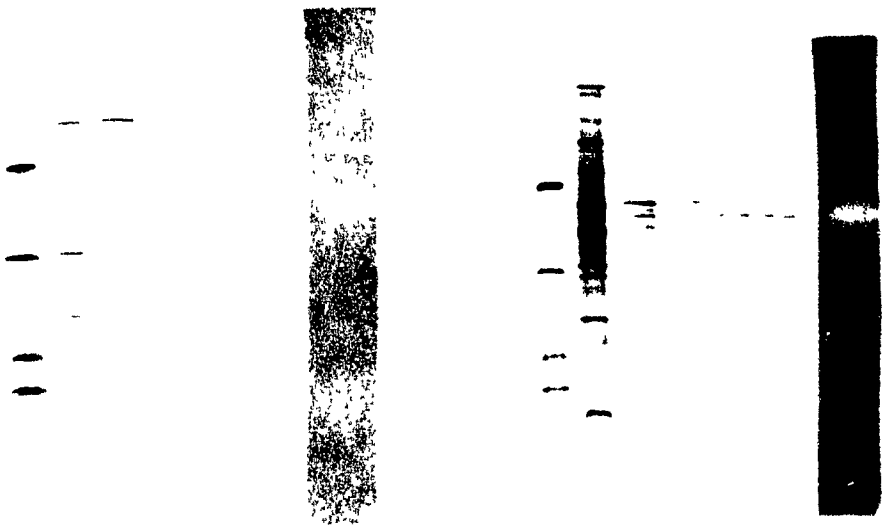

Fig. A (LLG)

Fig. B (S93)

Figure 28. Native-polyacrylamide gel electrophoresis (Native-PAGE) patterns of cell extract and aminopeptidase fractions obtained from different purification steps of *Lb. casei* ssp. *casei* LLG (Fig. A) and ssp. *rhamnosus* S93 (Fig. B). 8-25% gradient acrylamide gel (Coomassie blue stain). Lanes: 1, marker proteins; 2, crude cell extract; 3, after first ion-exchange chromatography; 4, after 2nd ion-exchange chromatography; 5, after chromatofocusing; 6, after gel-filtration; 7, activity staining of purified enzyme. Molecular weight markers in kilodaltons (top to bottom): bovine serum albumin (67), lactate dehydrogenase (140), catalase (232), ferritin (440), thyroglobulin (669).

Fig. A (LLG)

Fig. B (S93)

Figure 29. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of cell extracts and aminopeptidase fractions from *Lb. casei* ssp. *casei* LLG (Fig. A) and ssp. *rhamnosus* S93 (Fig. B) at different stages of purification. 8-25% gradient acrylamide gel (Coomassie blue stain). Lanes: 1, marker proteins; 2, crude extract; 3, after first ion-exchange chromatography; 4. after 2nd ion-exchange chromatography; 5, after chromatofocusing; 6, after gel-filtration. Molecular weight markers in kilodaltons (top to bottom): α-lactalbumin (14.4), soyabean trypsin inhibitor (20.1), carbonic anhydrase (30.0), ovalbumin (43.0), bovine serum albumin (67.0), and phosphorylase *b* (94.0).

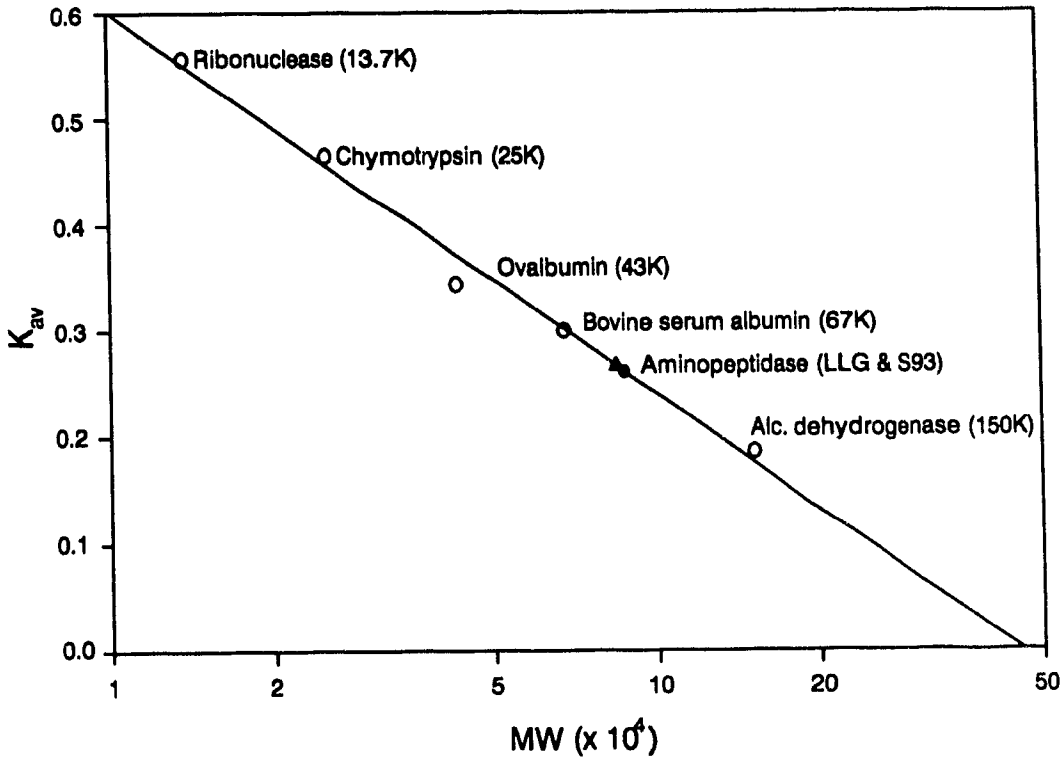

Figure 30. Molecular weight determination of native aminopeptidase from *Lb. casei* ssp. *casei* LLG (Δ) and ssp. *rhamnosus* S93 (●) on Superose12 gel-filtration column (30 x 1 cm). $K_{av} = V_e - V_o / V_t - V_o$. $R^2 = 0.9886$, $Y = 2.0207 - 0.3571X$.

Fig. A (LLG)

Fig. B (S93)

Figure 31. SDS-PAGE of purified aminopeptidase from *Lb. casei* ssp. *casei* LLG (Fig. A) and ssp. *rhamnosus* S93 (Fig. B) on 12.5% acrylamide gels. Lanes: 1, marker proteins; 2, purified aminopeptidase. Molecular weight markers in kilodaltons (top to bottom): α lactalbumin (14.4), soyabean trypsin inhibitor (20.1), carbonic anhydrase (30.0), ovalbumin (43.0), bovine serum albumin (67.0), and phosphorylase *b* (94.0).

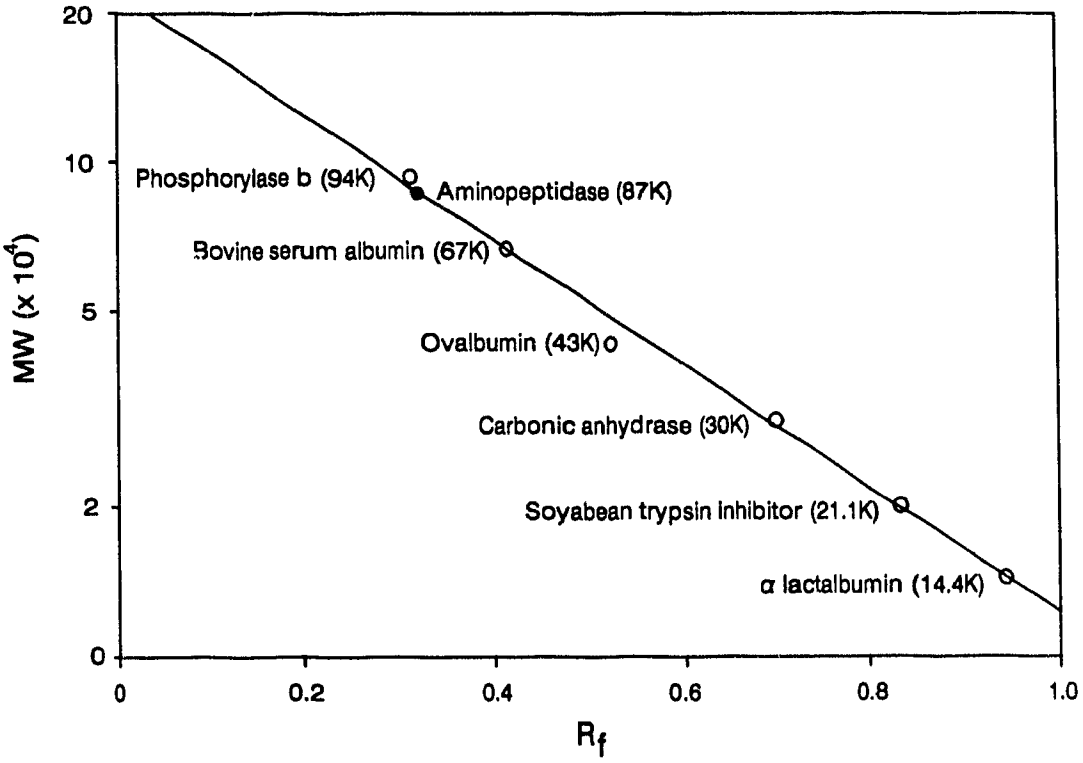

Figure 32. Estimation of molecular weight of aminopeptidase of *Lb. casei* ssp *casei* LLG (•) by SDS-PAGE (12.5% polyacrylamide). $R^2 = 0.9928$, $Y = 5.351 - 1.2635X$.

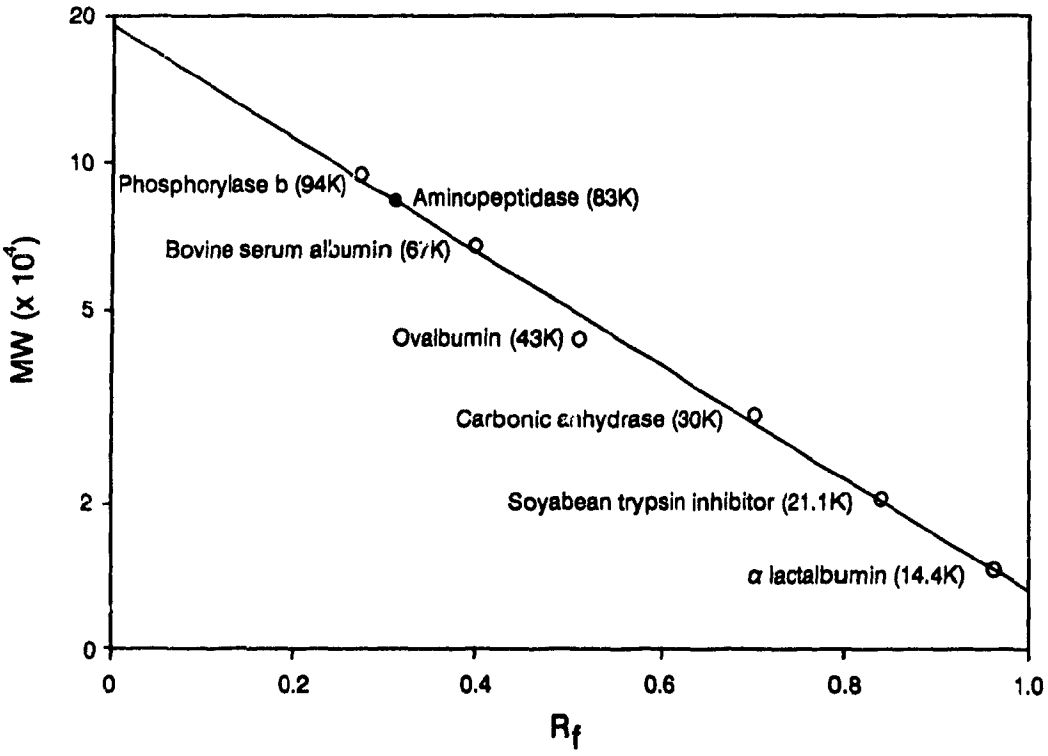

Figure 33. Estimation of molecular weight of aminopeptidase of *Lb. casei* ssp. *rhamnosus* S93 (●) by SDS-PAGE (12.5% polyacrylamide). R^2 = 0.9934, Y = 5.2807 - 1.1653X.

Fig. A (LLG) Fig. B (S93)

Figure 34. Isoelectricfocusing (IEF) electrophoresis pattern of purified enzyme from *Lb. casei* ssp. *casei* LLG (Fig. A) and ssp. *rhamnosus* S93 (Fig. B) on PhastGel® (pH range 4 to 6.5). Lanes: 1, reference proteins; 2, purified enzyme. Reference proteins with isoelectric point (top to bottom): glucose oxidase (4.15), soyabean trypsin inhibitor (4.55), ß-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), and human carbonic anhydrase B (6.55).

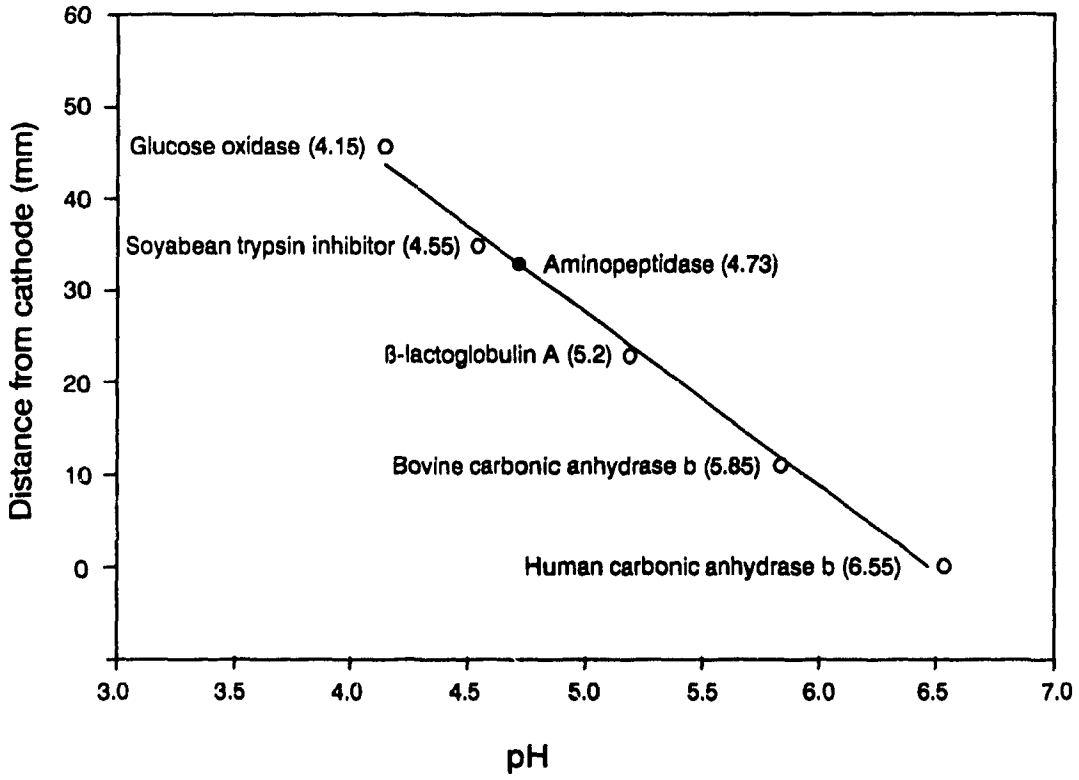

Figure 35. Estimation of isoelectric point (pI) of native aminopeptidase of *Lb. casei* ssp. *casei* LLG (●) by IEF-PAGE (pH 4.0 to 6.5). R^2 = 0.9924, Y = 122.226 - 18.87X.

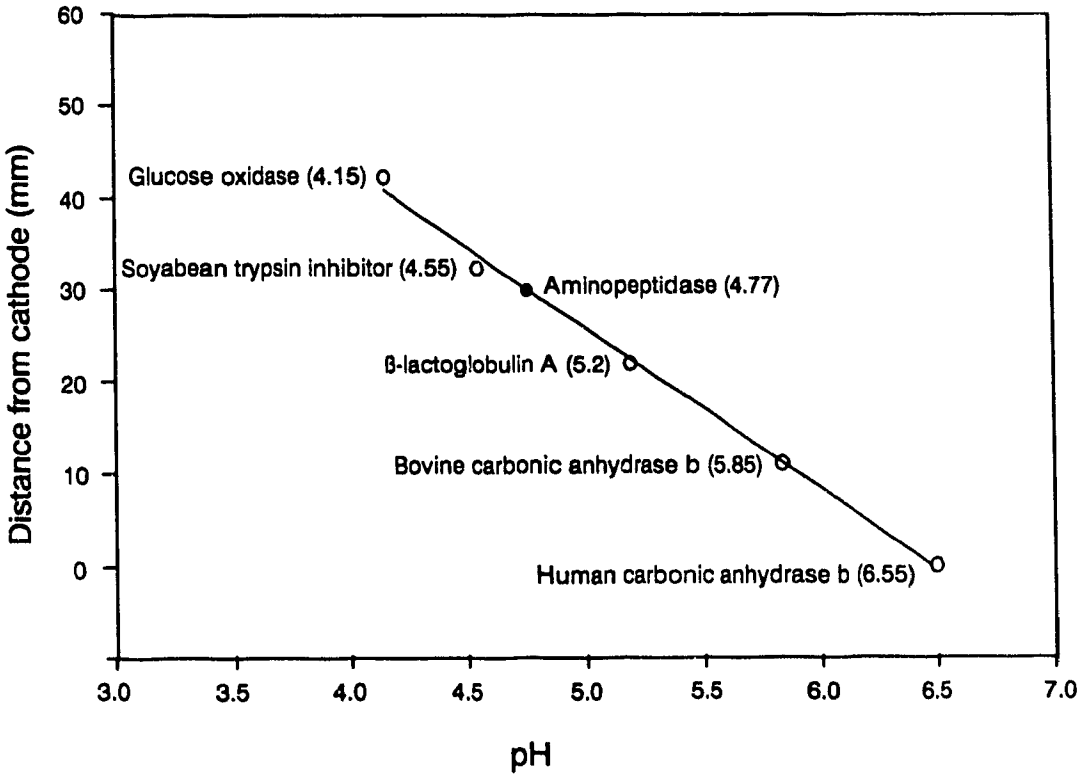

Figure 36. Estimation of isoelectric point (pI) of native aminopeptidase of *Lb. casei* ssp. *rhamnosus* S93 (●) by IEF-PAGE (pH 4.0 to 6.5). R^2 = 0.9924, Y = 113.15 - 17.424X.

Properties of Aminopeptidase from *Lactobacillus casei* ssp. *casei* and *Lb. casei* ssp. *rhamnosus*

KEY WORDS

Aminopeptidase, *Lactobacillus casei*, Properties, Lactic acid bacteria, Cheese Enzymes

ABSTRACT

A single cytoplasmic aminopeptidase purified from *Lb. casei* ssp. *casei* LLG or *Lb. casei* ssp. *rhamnosus* S93 was able to hydrolyse a range of nitroanilide-substituted amino acids, as well as dipeptides, and accounted for all the aminopeptidase and some of the dipeptidase activities found in cell-free extracts. The kinetic studies indicated that the enzyme has a low affinity for leucine *p*-nitroanilide (K_m 0.22 m*M* and 0.08 m*M* for strain LLG and S93, respectively) but can hydrolyse the substrate at very high rates (respective V_{max} 16.3 and 12.6 mmol/minute/mg of protein for strain LLG and S93). The aminopeptidase of both strains was shown to be a metal-dependant enzyme with an optimal activity at pH 7.0 and 6.9 at 39 and 37°C for strain LLG and S93. Both EDTA and 1,10 Phenanthroline resulted in complete inhibition of aminopeptidase activity. While Co^{2+} and Ni^{2+} could fully restore the activity lost by the treatment of 1,10 Phenanthroline, EDTA caused an irreversible inhibition of the enzyme activity. Higher concentration of substrate and hydrolysis products also inhibited the activity of the enzyme. Inhibitors specific for serine and thiol proteases had little effect on the aminopeptidase activity. Both enzymes were fairly stable over the pH range of 5-9 and below 45°C. However, the enzyme from *Lb. casei* ssp. *casei* LLG was found more sensitive to heat and pH change than the corresponding enzyme from *Lb. casei* ssp. *rhamnosus* S93.

INTRODUCTION

The coagulating enzymes (rennet or rennet substitutes) and the proteolytic enzymes of starter and non-starter cheese-associated lactic cultures play an important role in protein breakdown during the manufacturing and ripening process of cheeses (Visser, 1977; Fox, 1989; Kamaly & Marth, 1989). Although widely variable, the non-starter lactic acid bacteria (NSLAB) of good quality Cheddar cheese consist predominantly of *Lb. casei* and *Lb. plantarum* (Fryer, 1969; Puchades *et al*, 1989; Peterson, 1990). Many groups have reported an improvement in sensory and physical characteristics of Cheddar cheese manufactured by utilizing *Lb. casei* species as adjuncts to starter lactococci (Lane & Hammer, 1939; Laleye, 1986; Puchades *et al*, 1989), and have related these positive attributes to their proteolytic activities. However, there is little information available concerning the specific characteristics of proteolytic enzymes produced by *Lb. casei* subspecies.

Lee *et al* (1986) reported that *Lb. casei* species contained higher peptidase and esterase activities against a wide range of substrates, when compared to lactococci, the primary strain used in Cheddar cheesemaking. In another similar study, El Soda *et al* (1983) reported higher peptidase activities in *Lb. casei* strains when compared to *Lb. plantarum* strains. Experimental trials on cheesemaking using *Lb. casei* species as adjunct culture with lactococci redu ed the ripening period to a third, with more intense Cheddar flavor (Lemieux *et al*, 1989; Puchades *et al*, 1989; Laleye *et al*, 1990). During a comparative study on peptidase profiles, it became evident that *Lb. casei* ssp. *rhamnosus* S93 and *Lb. casei* ssp. *casei* LLG had the highest respective activities of aminopeptidase and dipeptidase (Arora & Lee, 1990). Subsequently, aminopeptidase of these two strains was purified to homogeneity (Chapter IV). The present investigation was undertaken to study and compare different properties of the purified aminopeptidase from these two strains. The effect of amino-acid modifying agents on enzyme activity was used to identify the active-site ionizable group.

MATERIALS AND METHODS

Organism and preparation of purified enzyme

Strain maintenance, propagation and the preparation of crude cell-free extracts were previously described (Arora & Lee, 1990). Aminopeptidase of *Lb. casei* ssp. *casei* LLG and *Lb. casei* ssp. *rhamnosus* S93 was purified by a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Upppsala, Sweden)(Fig. 19).

Chemicals, Reagents and Equipments

Unless otherwise specified, all chemicals used in this study were purchased from Sigma Chemicals (St. Louis, USA) and were of analytical reagent grade. FPLC system and chromatography columns used for the purification are described before (Chapter IV). Lambda reader (Perkin Elmer Ltd., Montreal, Canada) was used for all spectrophotometric measurements.

Protein and peptidase assays

Protein was determined spectrophotometrically by the BCA (bicinchoninic acid) assay reagent supplied with the system (Pierce Chemical Ltd., Rockford, USA)(Smith *et al*, 1985). Aminopeptidase activity was measured on leucine *p*-nitroanilide (*p*-NA) throughout the experiment by the micro-assay method described in Chapter IV. Specific activities of the aminopeptidase were studied using various chromogenic substrates (*p*-nitroanilide derivatives of L-anomers of leucine, lysine, alanine, valine, arginine, proline, methionine and glutamine). Similarly, dipeptidyl-aminopeptidase activities were assayed on acetyl-alanine, glutamyl-phenyl alanine and alanyl-alanine *p*-nitroanilides. One unit of enzyme activity was defined as the amount of enzyme

required to release 1 μmole of *p*-nitroaniline per minute under the conditions of the assay.

Dipeptidase hydrolysing ability of the purified aminopeptidase was measured using Ala-Phe, Ala-Met, Ala-His, Leu-Tyr, Pro-Ile, Pro-Phe, DLeu-Leu, Phe-Pro and Leu-Leu as substrates. The hydrolysis of synthetic substrates was carried out at 30°C and the activities were determined by the estimation of the liberated amino acids by Yemm and Cocking reagent (Matheson & Tattrie, 1964). The micro-assay mixture contained 60 μl of sodium phosphate buffer (0.05*M*, pH 7.0), 20 μl of the substrate (1 m*M* in 0.05M sodium phosphate buffer) and 20 μl of the appropriately diluted enzyme solution. After 15 and 30 minutes of incubation, the reaction was stopped with 0.1*M* acetic acid. The dipeptidase activity was measured from the release of amino acids which were quantified by measuring the absorbance (A_{570}) of the color developed by the ninhydrin reagent. One unit of the enzyme activity was defined as the amount of enzyme required to cause an increase of 0.01 unit of absorbance per minute at 30°C.

Effect of pH and temperature

The effect of pH on the activity of the purified enzyme was measured in 0.1*M* acetate (pH 4.0 and 5.0), 0.1 *M* phosphate (pH 6.0 and 7.0), 0.1 *M* Tris-HCl (pH pH 8.0) and 0.1*M* glycine-NaOH (pH 9.0 and 10.0) buffers at 30°C. The appropriate buffer (140 μl) was incubated with the enzyme (50 μl) and the reaction was initiated by the addition of 10 μl of 16.4 m*M* leucine *p*-NA. The release of *p*-nitroaniline was measured after 10 and 20 minutes of incubation. To estimate the stability of the enzyme, aminopeptidase from each strain was incubated at 15 and 30°C at pH 4 to 10 for a period of one hour, and the residual activity was determined as described above.

The influence of temperature from 15 to 70°C on the enzyme activity was determined in 0.05*M* phosphate buffer, pH 7.0. The leucine *p*-NA alone showed no variation in absorbance with change in temperature and pH. To estimate the thermal stability of the enzyme, it was pre-incubated at pH 7.0 in sodium phosphate buffer (0.05*M*, pH 7.0) at different temperatures (from 15 to 70°C) for 30 minutes. The solution was then cooled and the remaining activity was measured at 30°C in the same buffer with leucine *p*-NA as substrate.

Determination of kinetic parameters

The K_m and V_{max} of the purified enzyme were determined for the substrate leucine *p*-NA. The enzyme solution was incubated with various concentrations of substrate (leucine *p*-NA in methanol), yielding a range of final concentrations of the substrate from 0.01 to 2.0 m*M*. The hydrolysis of the substrate was continuously monitored spectrophotometrically in Lambda plate reader (Perkin-Elmer). The Lineweaver-Burk plots were constructed by plotting reciprocals of the reaction velocity against substrate concentration (Fig. 42), and the kinetic constants (K_m and V_{max}) were computed from the slope and intercept of the regression line.

Effect of divalent cations, metal chelators, and other inhibitors

A mixture containing 50 µl of the purified enzyme solution and 50 µl of bivalent cations or inhibitors (final concentration 0.1 and/or 1.0 m*M*) was incubated for 30 minutes at 25°C. The reaction was initiated by the addition of 10 µl of substrate (leucine *p*-NA), and the enzyme activity was assayed under standard conditions. Inhibition was expressed as a percentage of the activity without effectors.

Reactivation of enzyme activities

The purified enzyme was pre-incubated for 30 minutes at 25°C with EDTA or 1,10 phenanthroline (final concentration 0.1 m*M*) in 0.05*M* sodium phosphate buffer (pH 7.0). Portions (50 μl) of the enzyme was again incubated for 30 minutes at 25°C with 0.1 and 1.0 m*M* (final concentration) of various divalent salts prior to addition of leucine *p*-NA. The enzyme activity was determined as previously described.

RESULTS AND DISCUSSION

Effect of temperature and pH

The effect of pH on aminopeptidase activity was examined at pH values ranging from 4.0 to 10.0. The maximum activity was obtained at pH 7.0 and 6.9 for strain LLG and S93, respectively (Fig. 37). The pH stability at 15 and 37°C was also determined by pre-incubation of the enzyme at different pH values (Fig. 38 and Fig. 39). Aminopeptidase of both LLG and S93 were fairly stable in the pH range of 4.5 to 9.0 at both temperatures, but strain S93 tends to be more stable at acidic pHs. In general, higher activities were observed when enzyme was stored in slightly alkaline medium (pH 8.0 to 9.0). This stimulatory effect was more pronounced for the aminopeptidase of strain LLG, but there was a sharp decrease in enzyme activity, when stored at pH 10. The enzyme stability under acidic environment (pH 5.0) is of significant importance in their role in Cheddar cheese ripening. In a recent report, aminopeptidase from *Lactobacillus casei* strain has been shown to retain almost all of its activity during four weeks of cheese maturation (El Abboudi *et al*, 1990b)

The aminopeptidase of strain LLG and S93 showed the highest activities at 37°C and 39°C respectively (Fig. 40). However, activity was sharply decreased after 45°C,

probably due to its thermal inactivation. The enzyme activity was also measured at 15°C and pH 5.0, to assess their residual hydrolysing efficiency under cheese ripening micro-environment. Under these conditions, both enzymes had 10-15% residual activity, when compared with that stored at 37°C and pH 7.0. The enzyme from both strains appeared to be stable up to 40°C, but retained less than 50% of the activity at 50°C, and then lost completely at 60°C (Fig. 41). Enzyme stability for up to 40°C is beneficial for its use in high-cooking-temperature cheeses as well as for cheese slurries.

Substrate specificity

The hydrolytic action of the enzyme towards various amino derivatives and dipeptides is summarized in Table 9 and Table 10. Since the purified enzyme of both strains hydrolysed all the amino acyl-derivatives used in the study, it could account for almost all the aminopeptidase activity found in their crude extracts (Arora & Lee, 1990). We could designate this enzyme as a general aminopeptidase. Aminopeptidases of similar nature have been isolated previously from other strains of lactobacilli (Eggimann & Buchmann, 1980; Machuga & Ives, 1984; Abo-Elanga & Plapp, 1987) and lactococci (Neviani *et al*, 1989; Tan & Koning, 1990). However, these results substantially differ from that of Atlan *et al* (1989), who isolated three different aminopeptidases with variable activity from *Lb. delbrueckii* ssp. *bulgaricus*.

As shown in Table 9 & 10, aminopeptidase of each strain exhibited similarity in substrate specificities, which could be attributed to their close taxonomical relationship. In general, the enzyme showed higher affinities for positively charged amino acids such as lysine and arginine, and very low activity toward glutamine, indicating the nucleophilic nature of the active site. However, very little activity toward proline indicated that this enzyme is unable to accommodate the imino group at its active site. Recently a separate dipeptidyl aminopeptidase of high specific

activity for X-Pro *p*-NA has been isolated from *Lactobacillus* species (Atlan *et al*, 1990; Khalid & Marth, 1990). Lactobacilli also hydrolysed hydrophobic dipeptides preferentially over the hydrophilic ones (Arora & Lee, 1990; Peterson *et al*, 1990).

In addition to broad N-terminal hydrolytic ability, aminopeptidase from each strain also showed specific activity against some of the dipeptides (Table 10), a characteristic common to aminopeptidases of lactic acid bacteria (Kaminogawa *et al*, 1984b; Thomas & Pritchard, 1987; Kok, 1990). Both enzymes had the highest activities against Leu-Tyr. This was in contrast to our earlier findings on crude cell-free extracts that showed relatively 10-12 times more dipeptidase activities against Ala-X peptides when compared to Leu-Tyr as well as all other peptides used in the study (Arora *et al*, 1990). *Lb. casei* species thus may contain an another exoenzyme, probably of dipeptidase nature, capable of efficient hydrolysis of Ala-X and other dipeptides. Dipeptidases and tripeptidase specific only to dipeptide and tripeptide hydrolysis, respectively have recently been purified from some lactococcal strains (Van Boven *et al*, 1988; Bosman *et al*, 1990).

Enzyme kinetics

The apparent Michaelis-Menten constant (K_m; expressed as m*M*) and maximum velocity (V_{max}; expressed as enzyme units/minute/mg of protein) of aminopeptidase for the hydrolysis of leucine *p*-NA at pH 7.0 and 25°C were calculated from Lineweaver-Burk plots (Fig. 42). The respective K_m and V_{max} of the enzyme were estimated to be 0.22 m*M* and 16.3 for strain LLG, and 0.06 m*M* and 12.6 for strain S93 (Fig. 42). In addition to weak affinity for the substrate (low K_m values), both enzymes were also strongly inhibited by higher substrate and end-product concentration. Similar substrate and endproduct inhibition have been reported by other workers for the aminopeptidase purified from *Lb. delbrueckii* ssp. *lactis* (Eggimann & Buchmann, 1980) and *Lb. acidophilus* (Machuga & Ives, 1984).

However, the higher maximal velocity of hydrolysis (high Vmax) of these enzymes is mainly responsible for their extensive exopeptidase activity. Aminopeptidases of low Km and high Vmax have also been purified from other lactic acid bacteria (Hwang *et al*, 1982; Van Boven *et al*, 1988).

Effect of metal ions and enzyme inhibitors

The remaining enzyme activities after pre-incubation of the enzyme with various divalent salts is shown in Table 11. The enzyme activity was strongly activated (approximately two-fold) by 1.0 m*M* $CoCl_2$. Except for Ca^{2+} ions, which had an activating effect on aminopeptidase of strain S93, all other metal ions caused slight to marked inhibition of the enzyme. While Cu^{2+} and Fe^{2+} ions showed strong inhibition of the enzyme, its activity was not significantly affected by Mn^{2+} and Ni^{2+}.

Of the enzyme inhibitors tested, only metal-complexing reagents gave marked inhibition. The enzyme from both strains was totally inhibited by EDTA and 1,10 phenanthroline at 1 m*M* concentration. While at 0.1 m*M* concentration, only EDTA caused total inhibition of enzyme activity (Fig. 43). Relative lower sensitivity of the enzyme for phenanthroline has also been reported for metallo-aminopeptidases of other lactic starters (Yan *et al*, 1987; Tan & Konings, 1990).

EDTA showed an irreversible inhibition of the aminopeptidase from strain LLG, as none of the cations was able to recover any enzyme activity (Table 12). This peculiar characteristic of the enzyme has also been reported for the aminopeptidase of *Lc. lactis* ssp. *cremoris* (Tan & Konings, 1990). However, in case of strain S93, Co^{2+}, Mn^{2+} and Ni^{2+} were able to reverse the EDTA inhibition of aminopeptidase with significant increase (two-to-five fold) in the activity as compared with control sample (Table 12).

Phenanthroline inhibition could be reversed partially by the addition of several metal ions (Table 13). Co^{2+} ions stimulated the enzyme activity of both strains. This indicates that some heavy metal ions, especially Co^{2} may have some affinity for the structure of the enzyme's active center. Stimulatory action by Co^{2+} may also be attributed to promotion of alignment of the enzyme on the substrate molecule (Chopra *et al*, 1982). The presence of some of these ions in cheese (Jarrett, W.D., 1979) might contribute to the stability of this enzyme.

The enzyme from strain LLG was more sensitive to SH-blocking agents than that of S93, while aminopeptidase of S93 was more sensitive to PMSF than LLG aminopeptidase. The structures of the aminopeptidase of LLG and S93 thus appeared to be different. The partial inhibition of the LLG enzyme with N-ethyl malemide and sulfydryl inhibitors such as iodoacetate, iodoacetamide and PCMB suggests the possible involvement of a functional sulfydryl group(s) at or near its active site. Incubation of the enzyme with disulfide reducing agents, such as dithiothreitol and mercaptoethanol did not affect the enzyme activity (Fig. 43). This enzyme was also not inhibited by cysteine. These results suggest that an intact disulfide group(s) was not essential for the mechanism of action by this enzyme.

The inhibition studies clearly indicate that the aminopeptidase purified from each strain is a metal-dependant enzyme. However, these enzymes differed in the mode and extent of inhibition when incubated with various types of inhibitors, suggesting a marked difference in their structure

Aminopeptidase from both strains showed higher activity at or near neutral pH and in the temperature range of 37-40°C, but they differed in some other properties. In general, aminopeptidase from strain S93 showed superior stability to pH and temperature variations, and contained higher specific activities towards all the substrates. While both enzymes clearly demonstrated their dependence on metal ion(s) for the activity, an irreversible inhibition of LLG aminopeptidase by metal

chelator suggested the role of metal ions in its structural integrity.

This study on characterization of the purified aminopeptidases from *Lb. casei* species provides information on the likely role of this enzyme in the overall complex cheese ripening process, which depends on bacterial enzymes functioning in proper sequence. The physiological function of this multi-substrate specific enzyme probably is to participate in the release of various amino acids during overall proteolysis of milk proteins. These amino acids are not only essential for bacterial growth, but also are the major precursors or components of cheese flavor. The study is also useful in further genetic investigations leading to increased production of this enzyme by the use of a multicopy vector, thereby intensifying its action during ripening.

Conclusions

This investigation demonstrated that *Lb. casei* species contain a wide spectrum general aminopeptidase enzyme which accounted for most of their N-terminal exopeptidase activity. Although the enzyme showed low affinity for the substrate, it could cause extensive degradation of the substrate due to its high maximal rate of hydrolysis. In general, enzyme of both strains contained higher activities against positively charged amino acids which is indicative of the nucleophilic nature of their active site. The enzyme showed optimum catalysis at neutral pH and ambient temperatures. However, the enzyme retained part of its activity at low temperature and acidic environment suggesting their contribution to proteolysis of cheese ripening. Complete inhibition of the enzyme by metallic inhibitors, and reactivation by cobalt and nickel salts demonstrated that one of these metal ions is essential for the structural integrity of the aminopeptidase. Further studies on characterization of purified peptidases of lactobacilli are needed to unfold the overall peptide degrading complex in these species.

Table 9. Aminopeptidase and dipeptidyl aminopeptidase activities of *Lb. casei* (ssp. *casei* LLG and ssp. *rhamnosus* S93).

Substrate	Relative activity (%)	
	S93	LLG
Lysine	100	100
Arginine	133	99
Leucine	48	54
Alanine	28	22
Methionine	18	20
Valine	2	1
Proline	1	1
Ala-ala	2	2
Ace-Ala	0	0
Glu-Phe	0	0

Table 10. Dipeptidase activities of *Lb. casei* (ssp. *casei* LLG and ssp. *rhamnosus* S93).

Substrate	Relative activity (%)	
	S93	LLG
Leu-Tyr	100	100
Ala-Phe	20	8
Ala-His	17	10
Ala-Met	14	7
DLeu-Leu	10	0
Pro-Phe	0	0
Pro-Ile	0	0

Table 11. Effect of metal ions on aminopeptidase activities of *Lb. casei* (ssp. *rhamnosus* S93 and ssp. *casei* LLG).

Substrate	Relative activity (%)	
	S93	LLG
Blank	100	100
Ni^{2+}	95	90
Fe^{2+}	22	62
Fe^{3+}	12	62
Co^{2+}	235	192
Cu^{2+}	26	28
Zn^{2+}	83	73
Mn^{2+}	95	98
Mg^{2+}	86	95
Ca^{2+}	121	89

Table 12. Reactivation of enzyme inhibited by EDTA

Substrate	Relative activity (%)	
	S93	LLG
Blank (+ve)	0	0
Blank (-ve)	100	100
Ni^{2+}	539	0
Fe^{2+}	4	0
Fe^{3+}	4	0
Co^{2+}	229	0
Cu^{2+}	8	3
Zn^{2+}	16	0
Mn^{2+}	195	0
Mg^{2+}	2	0
Ca^{2+}	6	0

Table 13. Reactivation of enzyme inhibited by 1,10 Phenanthroline

Substrate	Relative activity (%)	
	S93	LLG
Blank (+ve)	0	7
Blank (-ve)	100	100
Ni^{++}	14	53
Fe^{++}	21	8
Fe^{+++}	25	8
Co^{++}	49	89
Cu^{++}	7	14
Zn^{++}	13	38
Mn^{++}	26	12
Mg^{++}	3	6
Ca^{++}	9	10

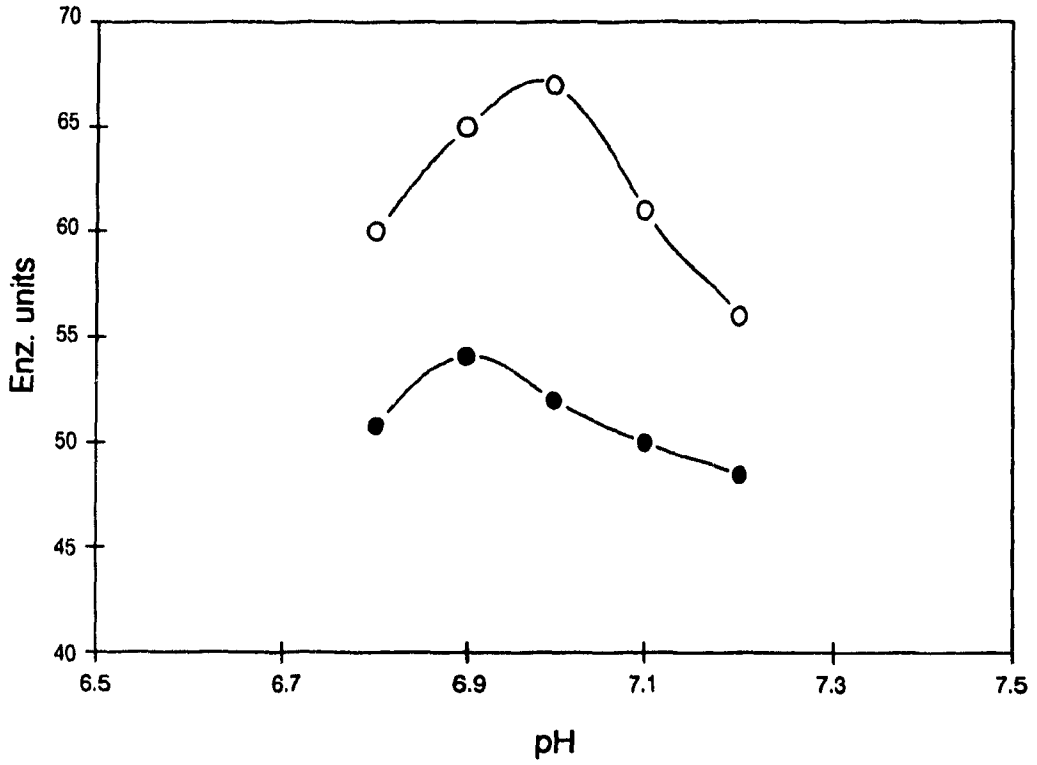

Figure 37. Effect of pH on aminopeptidse activity of *Lb. casei* ssp. *casei* LLG (○) and ssp. *rhamnosus* S93 (●).

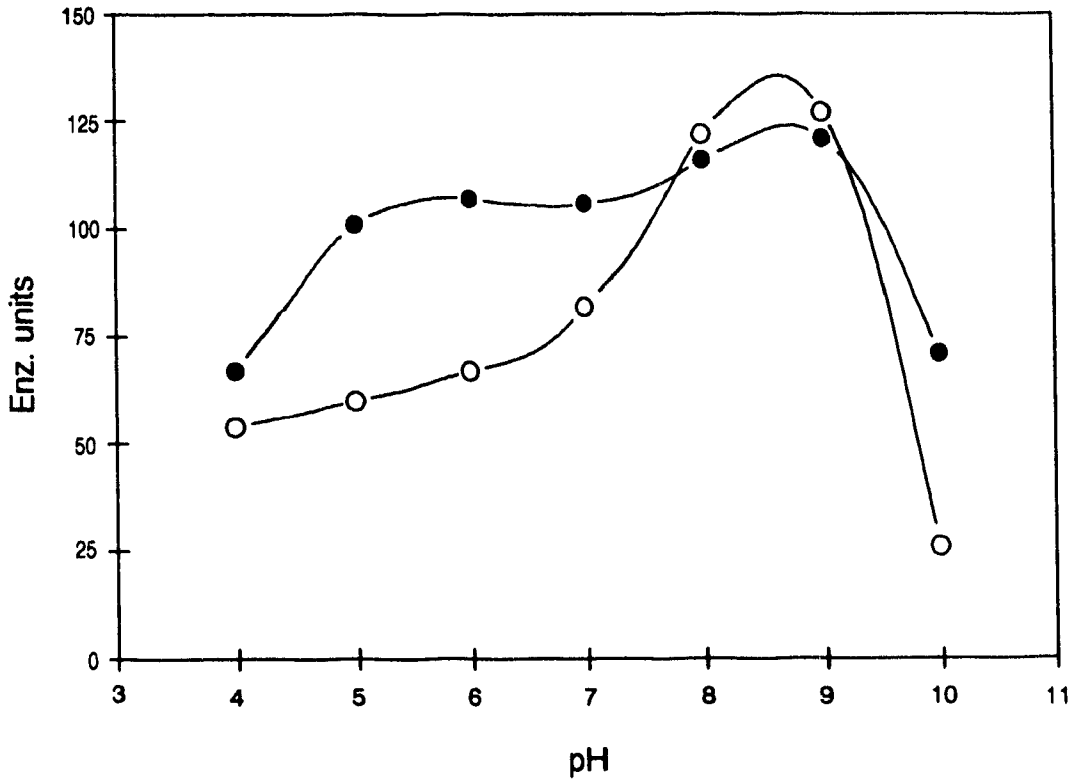

Figure 38. pH stability of aminopeptidase of *Lb. casei* ssp. *casei* LLG (○) and ssp. *rhamnosus* S93 (●) at 15°C.

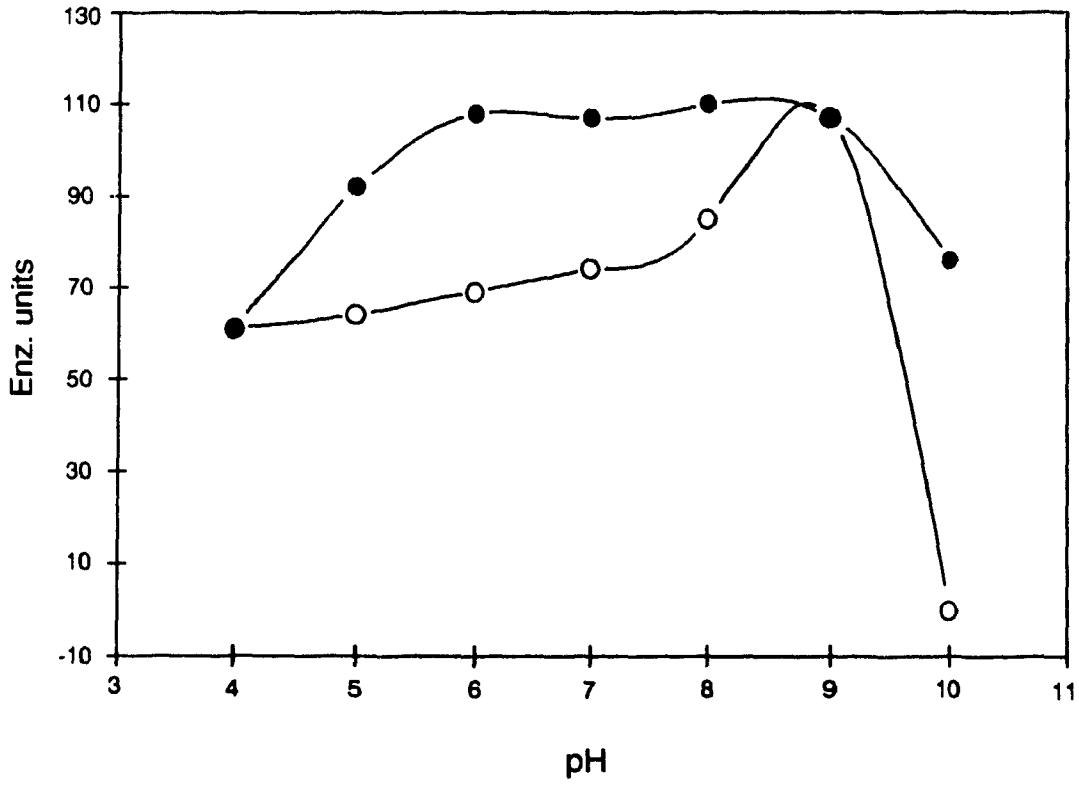

Figure 39. pH stability of aminopeptidase of *Lb. casei* ssp. *casei* LLG (○) and ssp. *rhamnosus* S93 (●) at 37°C.

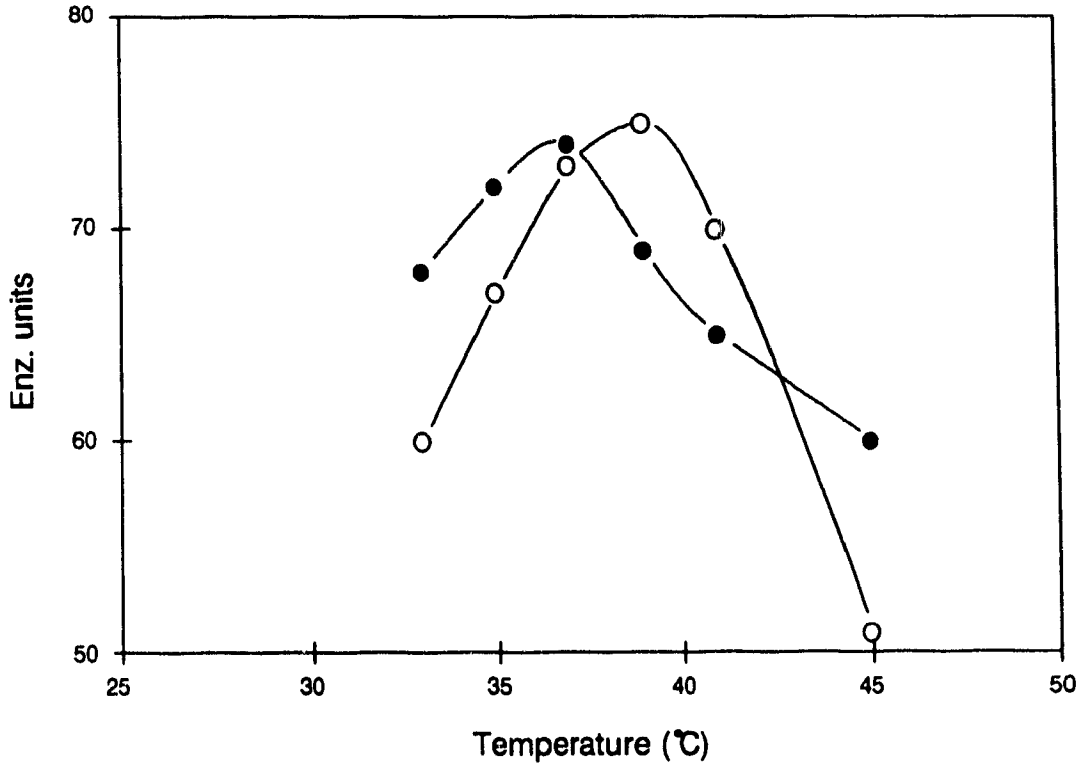

Figure 40. Effect of temperature on aminopeptidse activity of *Lb. casei* ssp. *casei* LLG (○) and ssp. *rhamnosus* S93 (●).

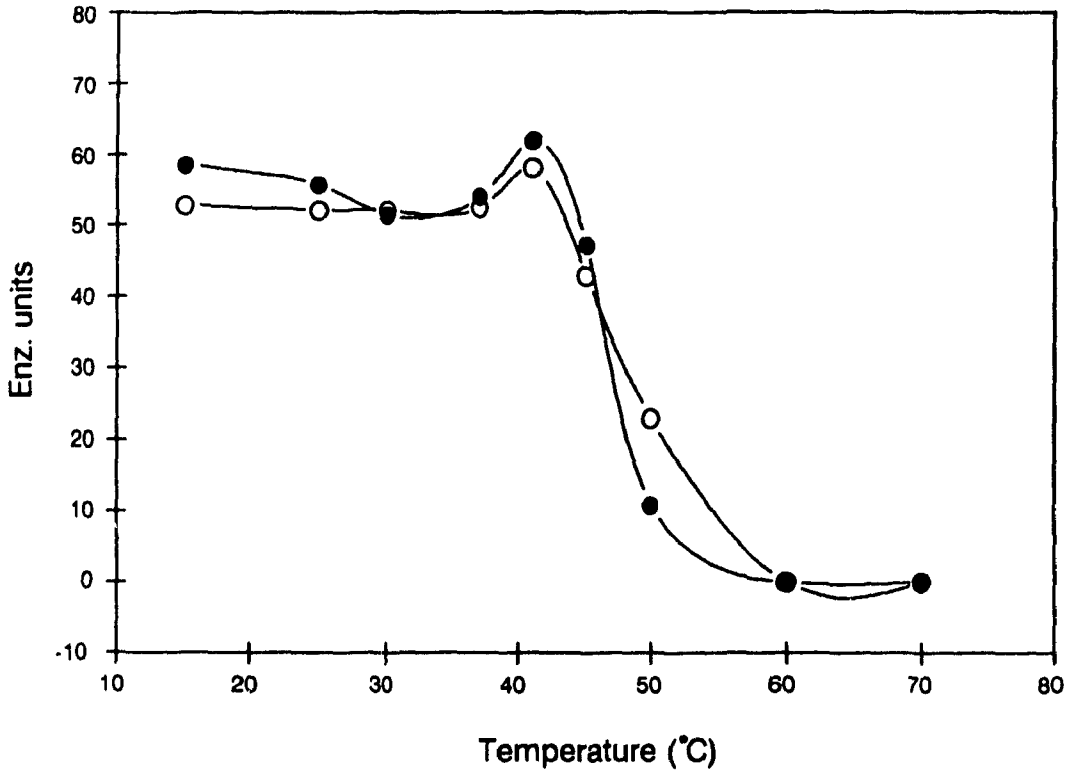

Figure 41. Stability of aminopeptidase of *Lb. casei* ssp. *casei* LLG (○) and ssp. *rhamnosus* S93 (●) at different temperatures.

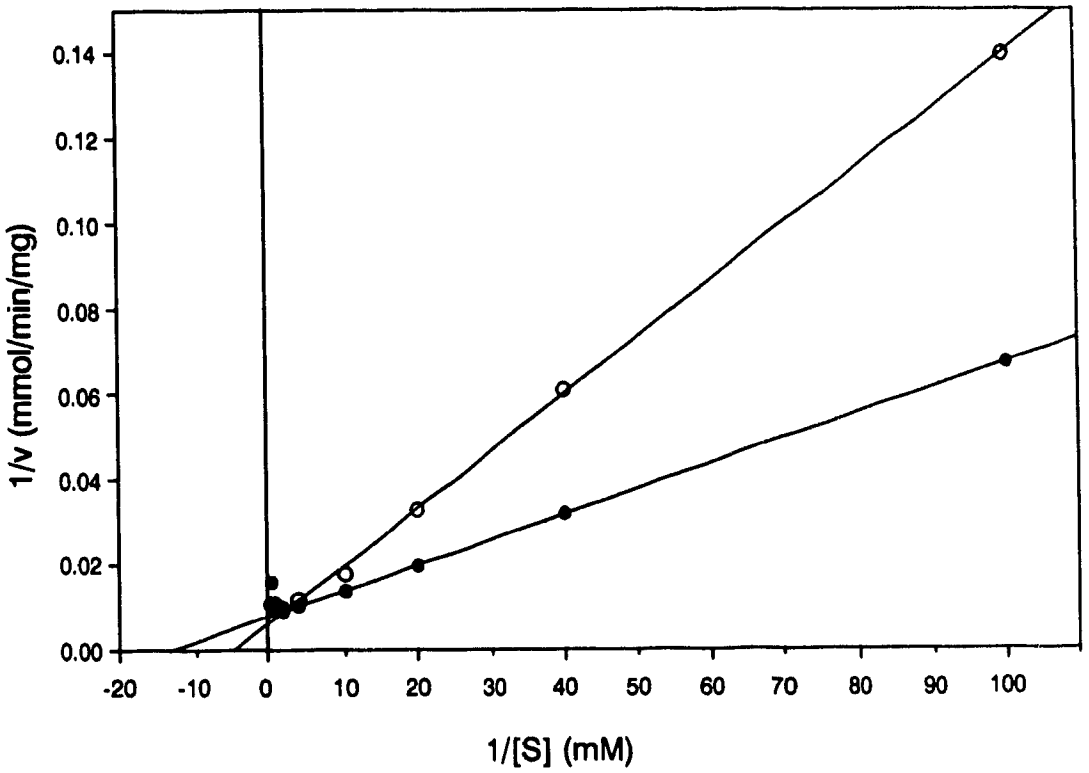

Figure 42. Lineweaver-Burk plots of aminopeptidase activity of *Lb. casei* ssp. *casei* LLG (○) and ssp. *rhamnosus* S93 (●) with leucine *p*-nitroanilide as substrate. Strain LLG: $R^2 = 0.9952$, $Y = 0.01585 + 0.00104X$. Strain S93: $R^2 = 0.9952$, $Y = 0.00705 + 0.001323X$.

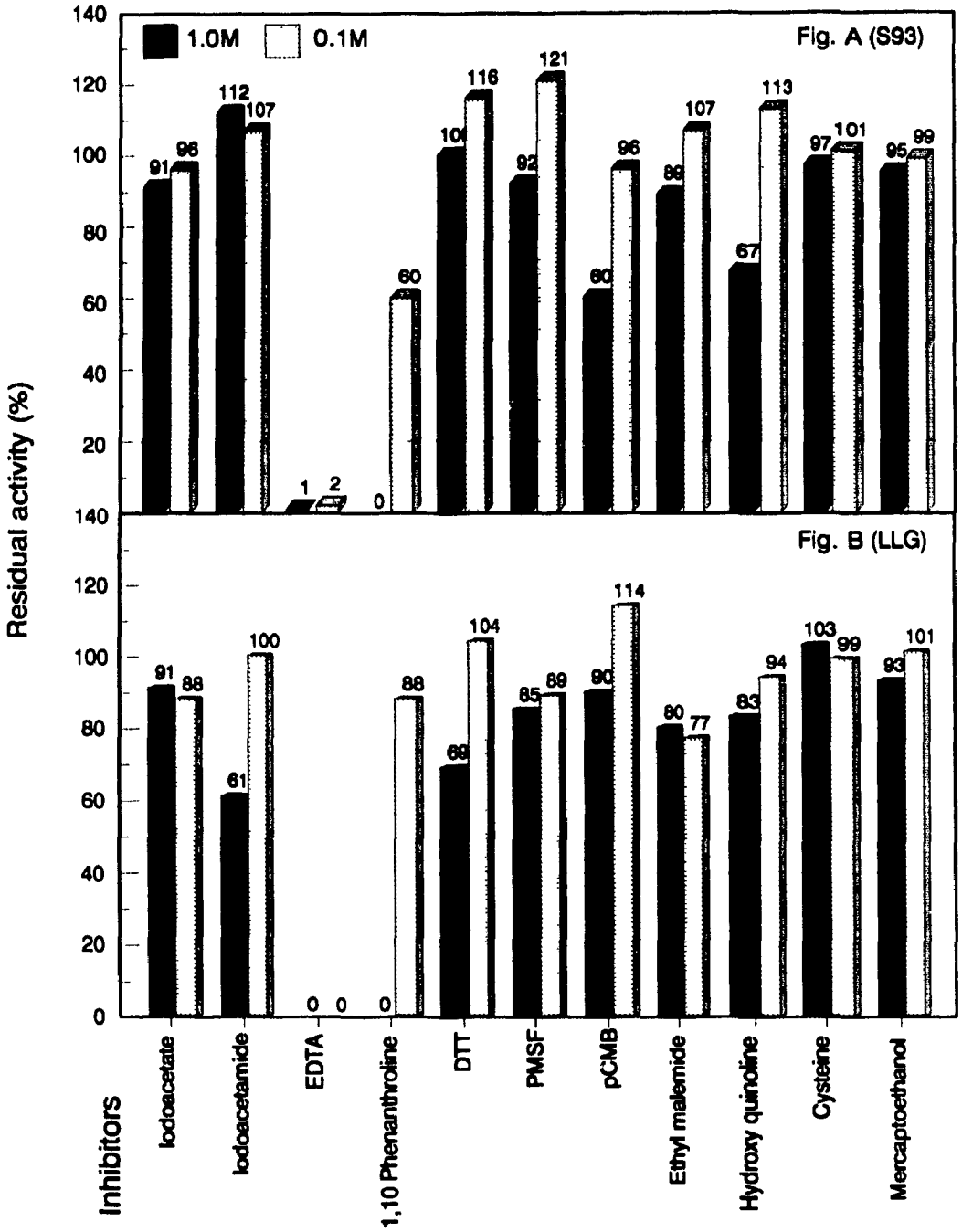

Figure 43. Effect of inhibitors on aminopeptidase activities of *Lb. casei* ssp. *rhamnosus* S93 (Fig. A) and ssp. *casei* LLG (Fig. B).

GENERAL CONCLUSIONS

A comprehensive study was carried out on the peptidase profiles of *Lb. casei* subspecies. A range of different peptidases have been isolated from lactic acid bacteria which include - aminopeptidase, dipeptidyl aminopeptidase, dipeptidase, tripeptidase and carboxypeptidase. All these peptidases were quantified and their specificities and specific activities were studied in details by using synthetic peptides as substrates. In addition to peptidases, strains were also evaluated for the presence of other desirable enzymes such as esterases, lipases, carbohydrases *etc*. by API ZYM technique. These two different approaches provided an insight into the mechanism of peptide degradation in this species. The results indicated that the careful assessment of these enzymes can yield useful information relative to improving our understanding of the proteolysis mechanism occurring in cheese maturation which greatly influences the organoleptic characteristics of ripened cheeses. The use of new API ZYM technique permitted a rapid and extended analysis of 19 different hydrolytic enzymes in various subcellular fractions of 20 strains of *Lb. casei* (ssp. *casei*, ssp. *rhamnosus* and ssp. *pseudoplantarum*). This study concluded that *Lb. casei* species contain high aminopeptidase and dipeptidase, relatively low tripeptidase, endopeptidase and dipeptidyl aminopeptidase, and no carboxypeptidase activities. The peptidases of *Lb. casei* species also demonstrated the ability to hydrolyse bitter peptides and to produce high free N, which have important bearing on Cheddar cheese flavor.

Although the primary objective of this investigation was to gain a more complete understanding of the nature of peptidases and their possible role during ripening of cheese, these studies were also helpful in selecting two strains (*Lb. casei* ssp. *casei* LLG and *Lb. casei* ssp. *rhamnosus* S93) which showed the highest overall aminopeptidase and dipeptidase specific activities. The knowledge gained from these studies was further extended to purify and characterize the aminopeptidase of these strains by Fast Protein Liquid Chromatography. The purification scheme developed

yielded a highly active homogeneous aminopeptidase in good yield from each strain. On the basis of electrophoretic analysis and activity staining, the aminopeptidase of each strains was designated as a monomeric enzyme of molecular weight of around 85kd. Further kinetic studies of the enzyme revealed its low affinity for the substrate but high maximal rate of hydrolysis. The purified enzyme accounted for most of the aminopeptidase activities found earlier in crude extract. In particular, the enzyme had higher activities towards positively charged amino acids, indicating nucleophilic nature of its active site. Although it could hydrolyse some of the dipeptides, the studies strongly suggested the presence of another enzyme, specific mainly to dipeptides in *Lb. casei* species. The single monomeric form, high maximal rate of hydrolysis, and broad substrate specificity are all important characteristics to release sufficient amino acids in substrate limiting environment such as milk or cheese. Through the action of divalent cations and inhibitor studies, it was concluded that either cobalt or nickel ion is essential for its structural integrity.

From the results of our investigations, and from the established predominance of *Lb. casei* species during ripening of Cheddar cheese, we conclude that the activity of peptidases of *Lb. casei* certainly is an important factor in the degradation of casein derived small peptides including the bitter hydrophobic peptides to amino acids, resulting in changes in texture and flavor of the cheese. In light of this, it is a priori consistent that it would be of value to increase the production of this enzyme by the use of a multicopy vector, thereby intensifying its action during ripening. The study has mainly explored the role of *Lb. casei* for cheese ripening. However, these studies could also be used for the production of enzyme-modified cheese flavorings for snackfood ingredients and hydrolysates of nutritionally valuable milk proteins, which may be used as food additives, and as nutritional or dietetic aids.

This study provides a basic understanding on the existence and catalytic ability of different peptidase of *Lb. casei* species. However, further studies on purification and characterization of other individual peptidases as well as on regulation of their synthesis and activity, will be important from both scientific and commercial aspects.

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APPENDICES

Appendix [I]

SAS program used for evaluation of peptidase profiles of *Lb. casei* species (Chapter II)

```
OPTION LINESIZE=72
       PAGESIZE=64

PDISK;
DATA PEPTIDASE;
INPUT PEPTIDE $ REP S93 S95 L2F S834 LLG S83 @@;
DROP S93 S95 L2F S834 S95 LLG S83;


       ST=1; ACT=S93; OUTPUT;
       ST=2; ACT=S95; OUTPUT;
       ST=3; ACT=L2F; OUTPUT;
       ST=4; ACT=S834; OUTPUT;
       ST=5; ACT=LLG; OUTPUT;
       ST=6; ACT=S83; OUTPUT;


CARDS;
*PEPTIDE REP S93 S95 L2F S834 LLG S83;


PROC SORT;
BY PEPTIDE;
PROC MEANS N MEAN STD STDERR MAXDEC=0;
BY PEPTIDE;
TITLE "PEPTIDASE ACTIVITY OF LB. CASEI SPECIES";


PROC SORT;
BY PEPTIDE;
PROC ANOVA;
CLASS ST; MODEL ACT=ST;
MEANS ST / DUNCAN;


PROC SORT;
BY ST;
PROC ANOVA;
CLASS PEPTIDE; MODEL ACT=PEPTIDE;
MEANS PEPTIDE / DUNCAN;
```

Appendix [II]

The principle and procedure of API ZYM enzyme system used for enzyme profiles of *Lb. casei* species (Chapter III).

The API ZYM is a semi-quantitative micromethod system designed for the detection of enzyme activities. The technique allows the systematic and rapid study of 19 enzymatic reactions using very small sample quantities (25 μl).

The system consists of a series of microcupules containing dehydrated chromogenic enzyme substrates. The addition of an aqueous suspension of the sample to be tested rehydrates the components and initiates the reaction. The API ZYM strip is incubated for the desired interval (usually 4 to 6 hrs.). Reactions are visualized after the addition of the detector reagent, and the color intensity of the individual cupule is measured on a scale of 0 (negative reaction) to 5.0 (reaction of maximum intensity).

Reagent A

Tris (hydroxymethyl) aminomethane	250 g
Hydrochloric acid (37%)	110 ml
Laurylsulfate (sodium dodecyl sulfate)	100 g
Distilled water	q.s. 1000 ml

Reagent B

Fast Blue BB	3.5 g
2-methoxyethanol	q.s 1000 ml

Procedure used:

API ZYM strips previously stored in at 4°C were equilibrated at room temperature for 5 to 10 min in humid incubation tray supplied with the system. 25 μl of clear microbial suspension (A_{600} 1.0) or crude extract (at fixed protein concentration) was added to each cupule, and the strips were incubated at 37°C for a period of 4 hrs. The color was developed with the addition of one drop of reagent A and B, previously equilibrated at room temperature. The color was allowed to develop for 5 min and strip was read in bright day light.

Appendix [III].

Description of mixer mill:

The mixer mill type MM2, from Brinkmann Instruments (Rexdale, Canada) is shown in the figure. The vibrating arms onto which the two grinding vessels are fastened are driven by a 110-V universal motor at 50 Hz. The motor rotation is translated in to a vibratory movement via a belt to a camshaft. The grinding time is adjusted by a timer in the range of 0 to 60 min, and the mixing frequency can be continuously regulated from 150 to 1800 min^{-1} (0-100%). For our experiments, we used a 12.5 ml stainless steel mixing vessel. The mixing vessel is composed of two parts (as shown in the figure), which are put together. A sealing ring between both parts prevent leakage of cell suspension. The opposed motions of the vibratory arms, keeps the mixer mill in firm position on the table. The machine uses glass beads of different diameters and can be operated efficiently between 4 to 40°C.