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# PURIFICATION AND CHARACTERIZATION OF CARBOXYPEPTIDASE Y FROM *KLUYVEROMYCES FRAGILIS*

by

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Purification and characterization of Carboxypeptidase.

*Lovingly dedicated to my whole family*

**PURIFICATION AND CHARACTERIZATION OF CARBOXYPEPTIDASE Y FROM *KLUYVEROMYCES FRAGILIS***

Julia de la Cruz Transfiguracion

Dr. Byong H. Lee Thesis Supervisor

## ABSTRACT

Carboxypeptidase Y (E.C. 3.4.12.1) was produced from *Kluyveromyces fragilis* ATCC 28244. The maximum growth and enzyme production were obtained during 24 hr of growth at the late logarithmic phase with optimized conditions (25°C, 300 rpm, pH 5) using YPD (1% yeast extract, 2% peptone, 2% dextrose, w/v) broth medium. A Fast Protein Liquid Chromatography (FPLC) was used for the enzyme purification. The enzyme was purified to 216 fold over the crude extract with a recovery of 18%. The apparent molecular weight of the purified enzyme was estimated to be 120 kDa on Native-PAGE and 56 kDa on SDS-PAGE suggesting that carboxypeptidase Y from *Kluyveromyces fragilis* consists of two subunits. The pH and temperature optima of the enzyme were pH 6.0 and 35°C, respectively. The enzyme activity was strongly inhibited by diisopropylphosphofluoridate (DIPF) and phenylmethylsulfonylfluoride (PMSF), and caused an average 50% loss of activity when incubated with various metal cations.

The apparent $K_m$ and $V_{max}$ values obtained for *n*-benzoyl-*L*-tyrosine-*p*-nitroanilide (BTPNA) and carboxybenzoxyphenylalanylalanine (Cbz-Phe-Ala) were 5.1 mM and 13.4 μmole/min/mg and 2.98 mM and 22.58 μmole/min/mg, respectively. Carboxypeptidase Y hydrolysis on the tryptic digests of $\alpha_{S1}$-and β-casein showed that the enzyme randomly removed five and three hydrophobic peptides, respectively and greatly reduced the size and heights of the other peptides analysed on Reversed Phase-High Performance Liquid Chromatography (RP-HPLC)

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Dr. Byong H. Lee Directeur de thèse

## RÉSUMÉ

Carboxypeptidase Y (E.C. 3.4.12.1) a été produite d' une souche de *Kluyveromyces fragilis* ATCC 28244. La croissance maximum ainsi que la production d' enzyme ont été obtenues lors de la fin de la phase logarithmique d' une croissance de 24 heures selon certaines conditions optimales (25°C, 300 rpm, pH 5.0), et ce en milieu liquide LPD (1% extrait de levure, 2% peptone et 2% dextrose, p/v). Un système de Fast Protein Liquid Chromatography (FPLC) a été utilisé pour la purification de l' enzyme. L' enzyme a été purifié à un niveau 216 fois supérieur à l' extrait primaire avec un taux de récupération de 18%. Le poids moléculaire apparent de l' enzyme sous sa forme native est de 120 kDa et de 56 kDa sous sa forme dénaturée suggérant que l' enzyme est consistuée de 2 monoméres. Le pH et la température optimal de l' enzyme étaient de 6.0 et 35°C, respectivement. L' activité de l' enzyme a été fortement diminué par le diisopropylphosphofluoridate (DIPF) et le phénylméthylsulfonylfluoride (PMSF), et un réduction moyenne de 50% de l' activité a été observé lors de l' incubation avec divers cation bivalent.

Les valeurs apparentes de $K_m$ and $V_{max}$ obtenues pour le *n*-benzoyl-*L*-tyrosine-*p*-nitroanilide (BTPNA) et pour le carboxybenzoxyphenylalanylalanine (Cbz-Phe-Ala) étaient de 5.1 mM et 13.4 µmole/min/mg, et de 2.98 mM et 22.58 µmole/min/mg, respectivement. L' hydrolyse au carboxypeptidase Y sur deux echantillons de $\alpha_{S1}$-caséine et β-caséine obtenus par digestion trypsique a

démontré que l' enzyme pouvait éliminer respectivement au moins cinq et trois peptides hydrophobes constitués respectivement de cinq et trois résidues d' acides aminés tout en réduisant grandement la hauteur et la largeur des pics des autres peptides analysés sur Reversed Phase-High Performance Liquid Chromatography (RP-HPLC).

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## PREFACE

The present investigation consists of two parts. The first phase is related to the production and purification of carboxypeptidase Y from *Kluyveromyces fragilis* and the second phase deals with the characterization of the enzyme. The results of the two studies were presented at the 1993 Institute of Food Technologists (IFT) Annual Conference held in Chicago, IL. and selected as one of the best abstracts in the Phi Tau Sigma Graduate Student Paper Competition. It was also presented orally by the main author during the meeting in the biotechnology and fermentation division.

1. Transfiguracion, J. C. and B. H. Lee. 1993. The purification and characterization of carboxypeptidase Y from *K. fragilis* for accelerated cheese ripening without bitterness. IFT Annual Meeting, Book of Abstracts. p120.

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## 1.0 GENERAL INTRODUCTION

Carboxypeptidases (3.4.12) are enzymes that catalyze the sequential hydrolytic liberation of amino acids from the carboxyl terminal of peptides and proteins. There are two classes of carboxypeptidases, the serine (E.C. 3.4.16) and the metallo (E.C. 3.4.17). Serine carboxypeptidases exhibit optimum activities at acidic pH and are inhibited by diisopropylphosphofluoridate (DIPF) and phenylmethylsulfonylfluoride (PMSF). Metallo carboxypeptidases on the other hand, exhibit optimum activities at neutral pH and are inhibited by metal chelating agents such as ethylenediaminetetraacetic acid (EDTA) and 1,10 phenanthroline. Carboxypeptidase can be found in plants (Kim & Hayashi, 1983; Galleschi & Capocchi, 1986; Mikola & Saarinen, 1986), animals (Dehm & Nordwig, 1970; Headeager-Sørensen & Kenny, 1985), fungi (Ichishima, 1972; Nakadai *et al.* 1972; Takeuchi & Ichishima, 1986) and yeasts (Hata *et al.* 1967; Hayashi *et al,* 1973; Kuhn *et al,* 1974; Johansen *et al,* 1976). From among the non mammalian sources of carboxypeptidases, yeasts has been of highly importance. In the food industry sector, yeasts occupy a significant place in the fermentation division such as in brewing, baking, and wine production. In cheese making, the proteolytic activities of these microorganisms are important in flavor development of numerous soft cheese varieties (Fox, 1989).

Yeasts are known to exhibit strong intracellular caseinolytic activities that might contribute to accelerated Cheddar cheese ripening (Grieve *et al,* 1983). Although lactic and non-lactic starters and their enzymes are known to play major roles in cheesemaking, they do not exhibit a carboxypeptidase activity except for one found in *Lactobacillus casei* (El Soda *et al,* 1978). Since carboxypeptidase activity was only detected on one N-terminal blocked peptide,

no further study was conducted to support that these starters really had carboxypeptidase activity in them.

For the past century, cheese has become an increasingly important food in the diet of the people. Hence, several methods have been used to accelerate cheese maturation and increase production to meet the demand (Law, 1984). The most popular method used is the addition of external food grade proteinases to the cheese curd (Law & Wigmore, 1982; 1983). The indigenous enzymes rather than the viable microfloras are the ones responsible for the maturation of most cheese varieties. Therefore by increasing their concentration could result in reduction of maturation time. Nevertheless, it has not met with much success because of their indiscriminatory and gross proteolysis which result in poor body and often with bitter taste (El Soda & Pandian, 1991). In cheesemaking, gross proteolysis resulting in the accumulation of peptides containing a great amount of hydrophobic amino acid residues (e.g. leucine, phenylalanine, proline) is known to be the major cause of cheese bitterness (Ney, 1981). Much of the on going research in accelerated cheese ripening without bitterness is devoted on peptidases that would hydrolyze the bitter peptides accumulated during cheese manufacture (Baankreis and Exterkate, 1991). Several peptidases from both lactic and non-lactic starters have been tried for accelerated cheese ripening for the purpose of eliminating the bitter taste. Such peptidases are aminopeptidases, x-prolyl-dipeptidyl-aminopeptidases and prolidases (Atlan *et al*, 1990; Tan & Konings, 1990; Arora & Lee, 1992; Habibi & Lee, 1993). These studies showed a maximum 50% reduction in ripening time but success is limited because these enzymes are not fully active at acidic pH. Full activity is of importance because during cheese ripening and depending on the type of starter used and characteristic of the cheese desired, an overnight pH range is

between 4.95 to 5.3 (Nath, 1992). One of the peptidases of great potential for the above purpose which is not found on neither starter or non-starter microflora is carboxypeptidase from yeast. Compared to the other kinds of carboxypeptidases derived from other sources, carboxypeptidase from yeast has the ability to release hydrophobic amino acid residues including proline from the C-terminal of peptides and proteins. It is known to exhibit optimum activity at acidic pH and full functionality even at low ionic strength. These unique properties have made it useful not only in protein sequencing and peptide synthesis studies (Widmer & Johansen, 1979; Svendsen *et al,* 1982) but also in food applications (Grieve *et al,* 1983). The importance of carboxypeptidase produced by *Kluyveromyces fragilis* in cheese ripening was studied by Grieve *et al,* (1983). By addition of the partially purified enzyme in Cheddar cheese curds, they found that the amount of free soluble N was higher compared to the other cheese samples added with other ripening proteinases produced by the same strain. There is no report however, on further purification and detailed study on the properties of carboxypeptidase from *Kluyveromyces fragilis* in order to understand its possible role in accelerated cheese ripening.

*Kluyveromyces fragilis* has been an important species in the aerobic fermentation of undiluted fresh whey for new cell development (Giec & Kosikowski, 1982) and for the production of yeast protein (Castillo & Sanchez, 1978). In spite of the many valuable products derived from whey by yeast fermentation, no studies have been reported on the production of food grade enzymes such as carboxypeptidase Y using whey as substrate.

**The objectives of this work are as follows:**

1) To study the growth kinetics of *Kluyveromyces fragilis* in an enriched medium, YPD and a whey based medium.

2) To compare the carboxypeptidase production and activity on both medium.

3) To purify carboxypeptidase Y using the FPLC system.

4) To characterize carboxypeptidase Y.

5) To study the specificity of carboxypeptidase Y in removing hydrophobic peptides derived from tryptic digests of $\alpha_{s1}$- and β-casein.

## 2.0 LITERATURE REVIEW

### 2.1 Carboxypeptidase Y

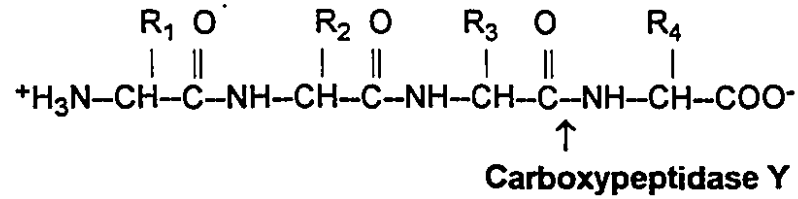
Carboxypeptidase from yeast (E.C. 3.4.12.1) is designated carboxypeptidase Y (CPY) to distinguish it from the other carboxypeptidases derived from other sources. It is an exopeptidase that belongs to the class of serine carboxypeptidases or the so called "acid carboxypeptidases". As the name implies, it has a serine residue at its active site necessary for its activity. Carboxypeptidase from yeast has been isolated from *Saccharomyces cereviseae*

(Hayashi *et al*, 1973; Kuhn *et al*, 1974).

#### 2.1.1 Catalytic Mechanism and Physico-Chemical Properties of Carboxypeptidase Y

The principal action of carboxypeptidase Y is the removal of the COOH-terminal residues of polypeptide chains. A schematic representation on the site of the enzyme cleavage in polypeptide is shown below:

where: R = amino acid; NH = peptide bond.




**Carboxypeptidase Y**

Carboxypeptidase Y is a glycoprotein consisting of a single polypeptide chain with an apparent molecular weight of 61,000 daltons (Hayashi *et al*, 1973). It has a nitrogen content of 12.74%, 2.6 amino sugars and 12.7% neutral hexoses. CPY consists approximately of 430 residues comprising of 13 half cysteine and 16 glucosamine in the carbohydrate moiety (Martin *et al*, 1982;

Svendsen *et al*, 1982). Carboxypeptidase from other sources have demonstrated to consists of three non identical polypeptide chains (Ihle & Dure III, 1972) or composed of two subunits (Headeager-Sørensen & Kenny, 1985; Galleschi & Capocchi, 1986). According to Nielsen *et al*, (1990), CPY is a glycosylated protease located in the vacuole of *Saccharomyces cereviseae*. Overproduction of the enzyme by recombinant DNA technology resulted in two forms of active CPY having molecular weights of 51,000 and 61,000 daltons, respectively. This formation of two active CPY was attributed to the different levels of glycosylation and assumed to have different carbohydrate contents. The low molecular weight CPY species could be due to insufficient capacity of the glycosylation system to process the large amounts of CPY traversing the secretory pathway in CPY overproducing yeast. This phenomena is proposed for a heterologous protein by Green *et al*, (1986).

## 2.2 Methods of Carboxypeptidase Y Assay

### 2.2.1 Colorimetric Method

Chromogenic substrates such as *n*-benzoyl-*L*-tyrosine-*p*-nitroanilide (BTPNA) produces a yellow color when hydrolysed with CPY (Aibara *et al*, 1971). The quantity of liberated *p*-nitroaniline can then be spectrophotometrically measured by absorbance at 410 nm. This concentration is related to the amount of enzyme necessary to convert the substrate into a product at specified conditions.

Carboxybenzoxy (Cbz)-dipeptides derivatives can also be measured colorimetrically by the ninhydrin method (Yemm & Cocking, 1955). Upon hydrolysis with CPY the amount of free amino acid released produces a violet color. This color can be measured by reading the absorbance at 570 nm. The measured optical density of the sample is proportional to the amount of enzyme

required to release the free amino acid at specified experimental conditions. The ninhydrin colorimetric method is not advisable to use in crude extract or in the initial stages of purification because there are many ninhydrin positive compounds (amino acids and peptides) and also ultraviolet absorbing compounds (proteins and nucleic acids) which might interfere with the assay (Hayashi, 1976). Rather, it is more convenient for the analysis of large quantities of samples, as in locating CPY in chromatographic eluates. The use of BTPNA in the first stage of purification gives better and more accurate results because of its specificity.

### 2.2.2 Spectrophotometric Method

The rate of reaction using this method is measured by the decrease in absorbance at 224 nm. Generally, a double beam spectrophotometer is used to accomodate both the cuvette containing the sample and the blank. The reaction is initiated by adding a certain volume of the enzyme solution to the reaction cuvette, and the change in absorbance is recorded for a certain period of time (Pétra, 1976).

### 2.2.3 Titrimetric Method

This method is based on the titrimetric measurement of the release of protons that occurs as a result of CPY hydrolysis on the substrate. The most commonly used substrates for this method are α-*N*-acetyl-*L*-tyrosine-ethyl ester (ATEE) or acetyl-*L*-phenylalanine ethyl ester (APEE) based on the esterase activity of CPY (Wilcox, 1976). The assay is carried out in a pH stat equipped with a thermostatted vessel and a syringe containing the standardized solution. A reaction mixture containing all the reaction solutions is allowed to equilibrate at room temperature for 5 min. Then the desired pH is adjusted (8.0 for ATEE and 7.5 for APEE). The reaction is initiated by addition of the enzyme solution.

The rate of reaction is followed using the rate of consumption of the standardized alkaline solution.

## 2.3 Methods of Enzyme Extraction

The most common method used for CPY extraction is autolysis (Hayashi *et al,* 1973; Kuhn *et al,* 1974). Although mechanical methods such as French pressure cell and ultrasonication can also be used for CPY extraction, there is no report to date using these methods. The autolysis method where the yeast cells is first solubilized with chloroform causes inactivation of the enzyme. To reactivate CPY using this method, dropwise addition of 1.0 N HCl is needed and the pH adjusted to 5. This method is quite tedious since it takes overnight to perform autolysis. Yeast cell free extract can also be obtained by grinding cells in a ball mill (Lee *et al,* 1990).

## 2.4 Methods of Enzyme Purification

CPY has been purified by several enzyme purification techniques such as ammonium sulfate precipitation, ion exchange chromatography, gel filtration chromatography and affinity chromatography.

### 2.4.1 Ammonium Sulfate Precipitation

Ammonium sulfate is the most frequently used salt for protein separation. One major property of ammonium sulfate as a choice is its relatively high solubility (ca. 700g/L) allowing the precipitation of a wide variety of protein molecules.

Ammonium sulfate precipitation has been an important initial step in the purification of CPY (Hayashi *et al,* 1973; Kuhn *et al,* 1974). Precipitation at 50 to 90 % saturation with ammonium sulfate allows the removal of some contaminants. Specific activity of CPY (2.17 units/mg protein) was obtained with a yield of 99.6% when crude enzyme preparation was precipitated with

ammonium sulfate at 90% saturation (Kuhn *et al*, 1974). This value is higher than the value obtained by Hayashi *et al*, (1973) (0.99 units/mg protein) who precipitated the crude enzyme preparation with ammonium sulfate at 50 to 90% saturation. The lower specific activity of CPY obtained could be attributed to the incomplete reactivation of CPY after autolysis.

### 2.4.2 Ion Exchange Chromatography

Biomolecules can be separated according to the difference in their charges by ion exchange chromatography (IEC). IEC is designed specifically for the separation of ionic or ionizable compounds. It has both stationary (column packing) and mobile phases. There are two basic principles involved in the operation. First, the binding of the proteins to the fixed charges involving electrostatic forces interaction. Second, the elution or displacement of the protein from the fixed charges by new counterions which have greater affinity to the fixed charges than the protein (Scopes, 1987). IEC is named on the basis of the sign of these displaceable charges. In anion IEC the fixed charges are positive and in cation IEC, the fixed charges are negative. There are several types of solid phase packings used in IEC of which diethylaminomethyl (DEAE) is the type of resin most commonly used. A CPY from *Saccharomyces cereviseae* was partially purified by first and second IEC using DEAE-cellulose and DEAE-Sephadex columns, respectively (Kuhn *et al*, 1974). Hayashi *et al*, (1973) also purified the enzyme by two ion IEC using DEAE-Sephadex columns.

### 2.4.3 Gel Filtration Chromatography

Gel filtration chromatography is a liquid chromatography technique used for the fractionation of biomolecules based on their relative sizes. This method is normally used as the last step of protein purification basically to remove salts. The most critical factor in this technique is the sample volume rather than the

eluent. Several porous beads are used for the chromatographic support. The most types commonly used are Sephadex G-150, Sephadex G-200, Sepharose CL-2B and Phenyl Sepharose CL-4B. The principle of the method is based on the columns constructed from the porous beads. These columns will have two measurable volumes, the external volume consisting of the liquid between the beads and the internal volume consisting of the liquid within the pores of the beads. Large molecules will only equilibrate with the external volume while small molecules will equilibrate with both the external and internal volumes. When a mixture of protein is applied into the column and allowed to percolate, the larger molecules emerge first from the column because they are excluded from the internal volume. The smaller molecules which can access the internal volume will definitely emerge later.

### 2.4.4 Affinity Chromatography

Affinity chromatography is a type of adsorption chromatography which enables purification of almost any biomolecule on the basis of its biological function or individual structure. The principle of the method is based on the specific and reversible adsorption of a molecule to be purified to a complementary binding substance (ligand) immobilized on an insoluble support (matrix). The most common used ligand is the cyanogen bromide (CNBr) activated Sepharose 4B, a ready-to-use medium for coupling ligands. But more recently, a range of products for immobilizing ligands through different functional groups such as AH and CH Sepharose 4B, Activated Thiol Sepharose 4B and Agarose Adipic Acid Hydrazide has been introduced. Sepharose is the most commonly used matrix for immobilization of the biologically active molecules. CPY was purified to homogeneity by affinity chromatography using *p*-aminobenzylsuccinic acid via azo linkage to Sepharose gly-tyrosine as the

ligand (Johansen *et al,* 1976). This method was described as less tedious than those procedures described by Hayashi *et al,* (1973) and Kuhn *et al,* (1974). Furthermore, yield was higher by approximately 85% and reported to be easily adapted for large scale preparations.

## 2.5 Enzyme Characterization

Characterization of enzymes through their enzymatic properties is important to understand their catalytic efficiency when exposed to surrounding factors. These factors or conditions are pH, temperature, inhibitors and substrate concentration which presumably affects the structural or chemical state of enzyme. Carboxypeptidase from *Saccharomyces cereviseae* has been studied in terms of the above mentioned factors (Hayashi *et al,* 1973; Kuhn *et al,* 1974).

### 2.5.1 pH optimum

For all enzymes, regardless of which class they belong or which source they are derived from, the pH of their environment will greatly affect their catalytic efficiency. Usually there is a pH where they reach maximum activity termed as optimum pH. The optimum pH is due to the presence of charged groups on the enzyme, i.e. the acid-base behavior of enzyme and substrate. The ionization of these groups varies with pH and only one of the many possible ionized forms is enzymatically active. At the optimum pH, it is this ionic form that predominates. Serine carboxypeptidases exhibit optimum activities at acidic pH. Previous reports on the pH dependency of CPY activity revealed a narrow pH profiles (pH 5 to 8) and optimum activity at pH 6.0 (Kuhn *et al,* 1974). Carboxypeptidase from fungi exhibits pH optimum activity at a lower pH (3 to 4) (Ichishima, 1972; Takeuchi & Ichishima, 1986). While for carboxypeptidase derived from plants, pH optimum activity is at 5 (Kim & Hayashi, 1993).

### 2.5.2 Temperature optimum

The effect of temperature accelerates the rate of enzyme catalyzed reactions. However, there is a certain rate at which the enzyme is denatured causing loss of activity. The result of these two opposing effects becomes the temperature optimum depending on the length of time over which the measurements are made. Nearly all enzymes are stable at 37°C but high and sometimes low temperature can lead to their inactivation. Though pH optimum of CPY by previous workers has not been reported, it is known to be stable in the temperature range of 25°C to 55°C. Complete loss of the CPY activity from *Saccharomyces cereviseae* was obtained at 62°C ( Hayashi *et al*, 1973; Kuhn *et al*, 1974).

### 2.5.3 Effect of Inhibitors

CPY has been shown to have a serine and histidine residue at its active site essential for its activity (Hayashi *et al,* 1973, 1975a, 1975b). Evidently, both residues are the reaction sites of diisopropylphosphofluoridate (DIPF) and phenylmethylsulfonylfluoride (PMSF), respectively. DIPF will inhibit CPY activity as it is a phosphorylating agent capable of yielding inactive derivatives in which the hydroxyl group of the serine residue is phosphorylated. Peculiarly, CPY has also one sulfhydryl group near the active site and therefore activity can be inhibited by many kinds of mercurials such as alkylmercurials and $HgCl_2$ (Bai & Hayashi, 1975).

### 2.5.4 Substrate Specificity

CPY preferentially hydrolyses proteins, peptides and synthetic substrates with a free C-terminal residue. Kuhn *et al,* (1974) examined the use of CPY for sequence studies from the β chain of oxidized bovine insulin by Edman degradation. The actual sequence of the sample was Phe-Tyr-Thr-Pro-Lys-Ala-

OH. They found that CPY released free amino acids from the carboxyl terminus in a sequential manner. In addition to aromatic and neutral amino acids, lysine and proline were released in a stoichiometric order. In a similar study performed on RCM RNase (Hayashi *et al,* 1973), five amino acids (Val-Ser-Ala-Asp-Phe) were released in the first ten minutes of incubation. The subsequent release of amino acids were from the COOH terminal according to the actual sequence of RCM RNase, Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp-Ala-Ser-Val (Hirs *et al,* 1960; Smyth *et al,* 1963). It was observed however that the release of glycine and sometimes aspartic acid was slower than most of the amino acid residues. This observation was assumed to be that poly-*L*-aspartic acid is a poor substrate of CPY. This finding is in agreement with that of Smyth *et al,* (1963) who used synthetic substrates having free residue in the C-terminal tail. There are at least three specific sites of the substrates affecting the rate of hydrolysis by carboxypeptidase Y. They are:

1. The amino acid residue occupying the penultimate position. If it is an aromatic or a long chain hydrophobic residue, the substrate concentration necessary to reach half of the maximum velocity is lower than when it is a hydrophilic residue.
2. Variations of the carboxyl terminal also affect the kinetic constants. If the free amino acid occupying the C-terminal is a hydrophobic residue, the substrate concentration necessary to reach half of the maximum velocity is higher than when it is a hydrophilic residue.
3. Variations of the blocking group also affect rate of hydrolysis. Carboxypeptidase Y preferentially hydrolyse the synthetic substrate of the Cbz-N-blocked dipeptides as opposed to the acetyl as blocking group.

## 2.6 The Enzymology of Cheese Ripening and Flavor Development

The basic composition and structure of cheese are determined by the curd manufacturing operations. However, during ripening, unique characteristics of each cheese variety is developed as influenced by temperature, humidity and the biochemical and microbiological composition of the curd. The cheese ripening and flavor profiles are complex and none are characterized sufficiently to permit duplication of their complex flavor by mixture of pure compounds. Cheese ripening is a process involving enzyme systems from at least four or five ripening agents.

### 2.6.1 Rennet chymosin or rennet substitutes

Chymosin (E.C. 3.4.23.4) is the main enzymic component of calf rennet and is an excellent milk clotting enzyme that preferentially cleaves the $Phe_{105}$-$Meth_{106}$ of κ-casein. Because of the shortage of calf rennet, microbial rennets are gaining much importance as substitutes for milk clotting (Emmons, 1990). They are considered to be suitable for soft cheese varieties but not for hard ripened cheeses. Their high proteolytic activity and broader substrate specificities result in the development of undesired effects such as bitterness during ripening.

### 2.6.2 Indigenous milk enzymes

Plasmin and acid milk proteinases are two of the several indigenous enzymes present in milk. They are known to contribute in the ripening of some cheese varieties at different extents (Kaminogawa *et al,* 1980; Fox, 1989). These two enzymes are mainly associated with the casein micelles and are therefore present in the curd. During salting the cheese curd maybe dissociated and the enzymes are lost in the whey. This explains their limited involvement in Cheddar cheese ripening. For brine-salted cheese varieties, the two enzymes are mostly

retained. Hence, plasmin and acid milk proteinase activities are directed mainly towards the breakdown of β- and $\alpha_{s1}$-caseins, respectively. The possible contribution of other indigenous enzymes such as thrombin and milk peptidases in cheese ripening is not well documented. Moreover, their low concentration and activities in milk with regards to proteolysis is regarded to be insignificant.

### 2.6.3 Starter proteinases and peptidases

The major contributory function of lactic starters is for acid production during cheese ripening. These starters eventually die off releasing their intracellular enzymes into the cheese matrix which will then continue to act on the components of the curd (Martley & Lawrence, 1972). The proteolytic system of starter bacteria can be roughly divided into extracellular and intracellular proteinases and peptidases. The extracellular ones are those that are bound to the cell wall or to the cell membrane and released during incubation, while intracellular ones are those that are released after the cells have died and lysed. Both types of enzyme contribute to the whole cheesemaking process.

### 2.6.4 Non-starter bacteria and their enzymes

These are the organisms that either survive pasteurization of the cheese milk or gain access to the pasteurized milk or curd during manufacture. They become the predominant species throughout the ripening period because of their acid tolerance. After death, these cells lyse releasing their enzymes. The *Lactobacillus casei* species are known to be the most metabolically active non-starter bacteria having direct involvement in cheese ripening (Lee *et al,* 1986). They contain strong aminopeptidase activity which in pure form removes the bitterness in enzyme modified cheeses commonly found when commercial proteases are added (Arora & Lee, 1992; Park *et al,* 1993).

### 2.6.5 Enzymes from Secondary Starters

The secondary cheese flora such as *Brevibacterium linens*, yeasts, propionic acid bacteria and penicillia synthesize active proteolytic and lipolytic enzymes that participate in cheese ripening. Yeasts, like the other secondary microorganisms grow on the surface of smear ripened cheese. They predominate during the initial period of ripening reaching maximum numbers within 4 to 5 days. Three proteinases from *Kluyveromyces fragilis* has been known to participate in accelerated Cheddar cheese ripening (Grieve *et al*, 1983). Moreover, crude extracts from *Kluyveromyces fragilis* was found to cause a marked increase in the ripening of Domiati cheese (Hassan & Abozeid, 1988). However, no further studies have ever been reported on the proteolytic enzymes of *Kluyveromyces fragilis* in order to understand their role in accelerated Cheddar cheese ripening.

## 2.7 The Accelerated Ripening of Cheese

The final flavor in cheese is achieved during long ripening process when a bland, elastic curd transforms into a well bodied curve. Ideally, the objective in accelerated ripening should be to accelerate all the desirable reactions involved in ripening in a balanced way while controlling the undesirable ones. Most of the research to date on accelerated ripening has been on internally ripened cheeses such as Cheddar because of their relatively long ripening time (12-24 mo). Essentially, there are four approaches to accelerate cheese ripening (Law, 1984). They are 1) elevated temperature 2) modified starters 3) cheese slurry and 4) enzyme addition.

### 2.7.1 Elevated Temperature

The normal ripening times of most cheese varieties are done at relatively low temperatures (<15°C) although some may be subjected to periods in warmer

environment for the development of specific characteristics. Accelerated ripening of Cheddar cheese by application of elevated temperatures has met with some success. Law & Wigmore (1982) achieved a 50% reduction in ripening time over a period of 24 weeks. However, Aston *et al,* (1985) suggested that the method employed by the previous workers only gives a significant advantage over a short period of time. Otherwise, bitterness could occur if the cheese is kept for more than 24 weeks. Another attempt to accelerate cheese ripening by elevated temperature on Manchego cheese was studied by Nuñez *et al,* (1986). A 90% increase in acid soluble N and FFA were obtained with the cheese ripened at 20°C compared with the cheese ripened at 5°C. In spite of these advantages, the method remains an interesting but not a widely practiced short cut to ripening because of the risks of growth of unwanted microbial contaminants. It will always be a method limited to cheese factories where highly hygienic precautions will be undertaken during production and ripening.

### 2.7.2 Starter Modification

The use of modified starters falls into two categories: 1) The starter bacteria themselves remain unmodified, but the preparation conditions are changed so that they produce more metabolites which contribute to the desirable properties of the cheese. 2) The starter bacteria are modified either physically, chemically or genetically so that their enzyme balance is changed.

Several workers have used freeze and heat shocked cells of *Lactobacillus* species for accelerated ripening of Cheddar, Gouda and Ras cheeses (Abdel-Baky *et al,* 1986; Exterkate, 1987; El Abboudi *et al,* 1991; Ezzat & El-Shafei, 1991). These treatments showed a higher soluble N and total volatile acidity compared to the non treated samples at the end of ripening. Current research is focused on genetic modification of starter bacteria in order to obtain not only an

improved rate of acceleration of the cheese maturation but also develop bacteriophages resistance and mechanism for in *vivo* inhibition of undesirable microorganisms. Genetic engineering or recombinant DNA technology is becoming a popular approach for accelerated cheese ripening (de Vos *et al,* 1989; Kok, 1993). A cloned aminopeptidase have shown to contribute to the acceleration of Cheddar cheese maturation (Robert *et al,* 1993). Moreover, it could also remove the bitterness observed in most cheese and enzyme modified cheeses added with commercial proteinases (Park *et al,* 1993). However, the controversy of genetically modified product has prompted the Food and Drug Administration (FDA) to impose tougher rules until it can be proven that this technology poses no side effects or negative results on the consumer side.

### 2.7.3 Cheese Slurry

Slurry method for acceleration of cheese ripening is done by increasing the moisture content of the cheese curd and incubation at 30°c (Kristoffersen *et al*, 1967). Several studies have been reported on the success of cheese slurry to reduce cheese maturation (Abdel Baky *et al,* 1982a, 1982b). Hofi *et al,* (1989) reported that Ras cheese ripening was reduced by 60% with the addition of full flavor slurry (1 to 2% of cheese milk weight) before hooping. Salam *et al,* (1989) also reported that a slurry added at 10% concentration of Edam cheese, resulted in a good body and flavor at 30 d of maturation. However, the mechanism of this method in relation to accelerated cheese ripening is not clearly understood and controlling the process is quite difficult. Hence, it is not a widely used method for the acceleration of cheese ripening.

### 2.7.4 Enzyme Addition

The largest proportion of the research work relating to the acceleration of cheese ripening deals with the addition of enzymes in one form or another

during manufacturing of the cheese (El Soda & Pandian, 1991). Though the addition of commercial lipases for accelerated ripening of Ras cheese led to a marked increase in the formation of volatile fatty acids (Abdel Salim *et al,* 1976; El Shibiny *et al,* 1978), a strong rancid flavor was also observed (El Neshawy *et al,* 1983). Similarly, Alkhalaf *et al,* (1984) who used the enzymes with higher concentrations reported a bitter flavor formation. Several proteinases have been studied for accelerated cheese ripening (Fedrick *et al,* 1986; Haard & Patel, 1987). These workers have found that with increasing levels of enzyme concentration and storage time, proteolysis and levels of bitterness were also increased. These workers concluded that bitterness is a common defect mostly observed with these preparations and their non-specificity and gross proteolysis have been the main cause of such defect.

## 2.8 Bitterness in Cheese

### 2.8.1 The Q Rule and Hydrophobicity

The most investigated sources of bitter peptides in milk fractions are the $\alpha_{s1}$-and β-caseins (Richardson & Creamer, 1973; Adda *et al,* 1982; Champion & Stanley, 1982; Leadbeater & Ward, 1987). These caseins associate in the ionic environment of the milk to form hydrated particles called the casein micelles. This association is related to the rather unique structure of the casein fractions in which discrete regions of their primary structure contain mostly hydrophobic amino acid residues while in other regions hydrophilic amino acid residues predominate. It is the hydrophobic regions of these proteins that received much attention with respect to bitter peptide formation following proteolysis (Adler-Nissen, 1984).

According to Ney (1979), bitterness is related to the average hydrophobicity (Q value) of the peptide rather than to the specific amino acids or

to peptide chain length. He further hypothesized that a Q value of 1400 cal/mol or higher is necessary for a peptide to be bitter. Q value is calculated from the energy of hydrophobicity (F) and the number of amino acid residues in a peptide (n) according to the following equation:

$$Q = F/n.$$

From this semi-quantitative rule, the prediction of bitterness in any peptide according to its amino acid composition and chain length is possible (Fukui *et al,* 1983; Otagiri *et al,* 1983). However, the intensity of bitterness may not be related directly to the Q-value.

### 2.8.2 Bitter Development in Cheese

The characteristic flavor of ripened hard cheeses is the result of a comp'ex proteo'ytic enzyme system from rennet, starter and non-starter bacteria and the secondary microorganisms involved in cheese ripening. When the delicate balance of this proteolytic reaction is not optimal, bitterness can accumulate to a certain level to detract from the flavor and taste of the cheese (Lawrence & Gilles, 1969).

Bitterness has long been regarded as a major defect in ripened cheeses such as Cheddar and Swiss mainly associated with proteolysis (Edwards & Kosikowski, 1983; Delformo & Parfani, 1986). The accumulation of peptides containing a great proportion of hydrophobic amino acid residues is known to be the major determinant in cheese bitterness (Ney, 1981; Visser *et al,* 1983a & 1983b ). Bitter peptides are released from the casein molecules primarily by the action of rennet and bacterial proteinases. A contribution may be expected from bacterial proteinases which may reduce the size of the peptides initially too large to give a bitter taste (Visser, 1977).

### 2.7.3 Control of Cheese Bitterness

Bitter peptide accumulation in ripened cheese is related to the total balance of the proteinase and peptidase activity regardless of the source (Ney, 1981). The accumulation of these bitter peptides is known to be affected by some factors such as the type of starter culture used, their proteolytic activity and the type or level of coagulant used (Lemieux & Simard, 1991). Therefore, effective method to control bitterness involves the proper selection of starter culture and adjustment of the environmental factors such as pH, temperature, and salt in moisture level. Most importantly, specific peptidases to degrade bitter peptides would pose a major potential for the above purpose.

# 3.0 PRODUCTION AND PURIFICATION OF CARBOXYPEPTIDASE Y FROM *KLUYVEROMYCES FRAGILIS*

## 3.1 Introduction

The ability of *Kluyveromyces fragilis* to ferment lactose in whey for the production of single cell protein has been well documented (Castillo & Sanchez, 1978; Giec & Kosikowski, 1982; Moebus & Teuber, 1983; Moresi, 1983; Moulin *et al,* 1983). However, the production of food grade enzymes such as carboxypeptidase Y by using whey as a substrate seemed to have been overlooked over the years. *Kluyveromyces fragilis* is known to produce at least three aminopeptidase and one carboxypeptidase important in accelerated cheese ripening (Grieve *et al,* 1983). Though carboxypeptidase has been used in protein chemistry studies, only recently its importance in cheesemaking is gaining much popularity because of its peculiar property to remove most hydrophobic amino acid residues from the C-terminal of polypeptide chains. Carboxypeptidase from yeast has been isolated from *Saccharomyces cereviseae* by conventional chromatographic techniques (Hayashi *et al,* 1973; Kuhn *et al,* 1974). The technique involved at least three ion exchange chromatography that resulted in the loss of enzyme activity. The difficulty of isolating proteinase A was also reported because this enzyme has the same physico-chemical properties as carboxypeptidase Y (Hayashi *et al,* 1983). Because of this long technique in isolating carboxypeptidase Y, an affinity chromatography was employed to purify the enzyme (Johansen *et al,* 1976). However, the results presented the isolation of two active carboxypeptidases having molecular weights of 62 and 51 kDa. Moreover, it is not a method used frequently because of the necessity to find a specific ligand to bind with the enzyme of interest prior to its elution.

The objectives of this work were:

(1) To study the growth kinetics of CPY in an enriched medium, YPD and a whey based medium.

(2) To compare the production and activity of carboxypeptidase Y in both media.

(3) To purify the enzyme using the fast protein liquid chromatography (FPLC) system.

## 3.2 Materials and Methods

### 3.2.1 Microorganism and Medium

*Kluyveromyces fragilis* ATCC 28244 was obtained from Agriculture Canada Food Research and Development Center (St. Hyacinthe, PQ, Canada). The strain was maintained and propagated at 30°C in 1% yeast extract, 2% peptone and 2% dextrose (YPD, w/v) broth medium (Difco Laboratories, Detroit, MI). Stock cultures were kept at -20°C with 15% (v/v) glycerol for future use. Culture revival was done in 2-3 subculturing before the actual experiment was performed.

### 3.2.2 Chemicals, Reagents and Equipments,

Unless otherwise specified all substrates, chemicals and reagents used in this study were of analytical grade and were all purchased from Sigma Chemicals (St. Louis, Mo).

#### 3.2.2.1 Fast Protein Liquid Chromatography (FPLC) System

A FPLC system (Pharmacia, Uppsala, Sweden) was used for the enzyme purification. It consisted of two P-500 pumps; a controller, LCC-501 PLUS equipped with a recorder for peak integration; a fraction collector, FRAC-200; a monitor UV-M II; a gradient mixer and a pH control; a recorder, REC-102; a peristaltic pump P-1 for automatic sample injection, 2 MV-7 valves for two columns that can be used simultaneously for one sample run; an MV-8 valve

where eight different samples can be injected at the same time provided the same conditions are applied and two super loops (10 and 50 ml) for sample looping. Pre-packed chromatography columns of Mono Q HR 5/5 (5 cm x 0.5 cm i.d.) and Superose 12 HR 10/30 (30 cm x 1.0 cm i.d.) were used for ion exchange and gel filtration, respectively.

#### 3.2.2.2 PhastSystem

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) and native polyacrylamide gel electrophoresis (Native-PAGE) were performed on the protein samples using the PhastSystem by Pharmacia (Uppsala, Sweden). The system consists of a separation and control unit and a development unit.

##### 3.2.2.2.1 Separation and Control Unit

The separation and control unit contains the microprocessor which controls and monitors both separation and development processes according to the programmed methods, a keyboard to which the methods are programmed and a liquid crystal display (LCD) which shows the method steps during programming. This unit also contains the separation compartment and the power supply. The separation compartment consists of a sample applicator arm, a plunger, eccentric lever, electrode assembly and a separation bed with positions for two gels. Samples are applied to gels with PhastGel Sample Applicators depending on the number and volume of the samples you want to apply, i.e., PhastGel Sample Applicator 8/0.5 will apply eight samples, each approximately 0.5 µl to the gel. A high voltage power supply generates the required electric field for electrophoresis that can be programmed to function in three modes: constant current, constant voltage, or constant power by setting limits on these parameters. For maximum reproducibility, the duration of each method step and the time for sample application is measured in volthours. Volthours indicate the

extent of protein migration in the gel since electrophoretic mobility is proportional to the applied voltage and the time that this voltage is applied. Since the voltage changes continually, the unit is equipped with volthour integrator which integrates volts with time. Table 1 shows the SDS-PAGE method for the denatured sample.

**Table 1. SDS-PAGE Separation Method Using the Phast System.**

| | | | | | | |
|---|---|---|---|---|---|---|
| Sample Application down at | | | 2.1* | | | 1 Vh |
| Sample Application up at | | | 2.1* | | | 10 Vh |
| Separation | 2.1* | 250 V | 10.0 mA | 3.0 W | 15°C | 5Vh |
| Separation | 2.2* | 50 V | 0.1 mA | 0.5 W | 15°C | 0Vh |

*method number

**3.2.2.2.2 Development Unit**

The visible parts of the development unit are: a stainless steel chamber (with a heating foil), a rotating gel holder for one or two gels, a temperature and level sensor on the underside of the lid, and ten ports through which the development chamber can be filled and emptied. Inside the unit there is a pneumatic pump for filling and emptying the chamber, a ten-port valve for the selection of the ports, and a three-port valve for the selection of pump functions i.e., creating vacuum or pressure in the chamber.

**3.2.2.3 PhastGel Media and Chemicals**

Ready to-use gels; 8-25% (Pharmacia, Uppsala, Sweden) were used for the electrophoresis. The gels are about 0.45 mm thick having 13 mm stacking gel zone (4.5% T; 3% C) and 32 mm gradient gel zone. Gradient 8-25 has a

continous 8 to 25% gradient gel zone with 2% crosslinking. The buffer system used in the gels is of 0.112 M acetate (leading ion) and 0.112 M Tris, pH 6.4.

Phast gel SDS buffer strips (Pharmacia, Uppsala, Sweden) are of 0.20 M Tricine (trailing ion); 0.20 M Tris and 0.55% SDS (analytical grade), pH 7.5. The buffer system in the PhastGel native buffer strips is of 0.88 M L-alanine and 0.25 M Tris, pH 8.8. Both buffer strips are made of 2% high quality agarose gels.

A Phast gel Blue R (Coomassie R 350) (Pharmacia, Uppsala, Sweden) was used for sample staining. Coomassie, a triphenylmethane anionic dye, preferentially forms dye complexes with proteins in the gel matrix. The rate of complex formation is limited essentially by the rate of diffusion of the dye into the gel. Phast gel Blue R is in the form of a readily soluble tablet form. Table 2 shows the Coomassie staining development method used for the SDS-denatured sample.

**Table 2. Coomassie Staining Development Method Using the Phast System.**

| Step No. | Solution | In-Port | Out-Port | Time (min) | Temp (°C) |
|---|---|---|---|---|---|
| 1 | stain | 1 | 0 | 8 | 50 |
| 2 | destain | 2 | 0 | 5 | 50 |
| 3 | destain | 2 | 0 | 8 | 50 |
| 4 | destain | 2 | 0 | 10 | 50 |
| 5 | preserving | 3 | 0 | 5 | 50 |

**3.2.2.4 Spectrophotometer.** A Beckman spectrophotometer (model DU-7; Beckman Ins., Irvine, State) was used for all the spectrophotometric assays during the entire course of the study.

### 3.2.3 Cell Growth and Enzyme Production

*Kluyveromyces fragilis* was grown in different concentrations of whey (2 to 10%, w/v) supplemented with 0.5% (w/v) yeast extract. Strain cultivation was conducted with 0.1% (v/v) culture inoculation, batch culture conditions of 25°C, agitation of 300 revolution per min (rpm) for a period of 36 hr using a 1.5 L fermentor (Braun Ins., City, State). Culture volume was 1 L. The strain cultivation on YPD was also performed under the same conditions as described above. The growth was monitored by sampling (1 ml) at 2 hr and 12 hr intervals for YPD and for the different concentrations of whey supplemented with 0.5% yeast extract, respectively. The absorbance of the samples was read spectrophotometrically at 600 nm. In the case of the optical density higher than 1.0, the sample was diluted to fall in the range of 0.2 to 0.8.

For the determination of maximum protein and enzyme production, 100 ml of the culture medium was taken at 4 hr intervals for both YPD and whey supplemented with 0.5% yeast extract. To determine the effect of pH on the growth of *Kluyveromyces fragilis*, strain cultivation on YPD was performed at pH 5, 6 and 7. Cell growth was monitored by reading the absorbance (600 nm) at 4 hr intervals.

### 3.2.4 Protein Determination

Protein content of the crude and purified samples were determined according to the method of Smith *et al,* (1985) using the bicinchoninic acid (BCA) protein assay reagent supplied with the system (Pierce Chem. Co., Rockford, IL). This assay is based on the protein reaction with $Cu^{2+}$ in an alkaline medium yielding $Cu^{+}$ that can be accurately determined using BCA. The purple reaction product formed by the interaction of two molecules of BCA with one cuprous ion ($Cu^{+}$) which is water soluble, sensitive and stable exhibits a strong absorbance

at 562 nm allowing the spectrophotometric quantization of proteins in aqueous solutions. BCA consists of two reagents, A and B. Reagent A consists of an aqueous solution of 1% BCA-$Na_2$, 2% $Na_2CO_3.H_2O$, 0.16% $Na_2$ tartrate, 0.4% $NaHCO_3$. Reagent B consists of 4% $CuSO_4.5H_2O$ in deionized water. The working reagent is made by mixing 50 parts of reagent A and 1 part of reagent B producing an apple green color. The protein assay procedure is done by taking 1 ml of the properly diluted enzyme solution and adding 2 ml of the BCA working reagent. The whole mixture is incubated at 37°C for 30 min and the absorbance taken at 562 nm. A standard curve using bovine serum albumin (BSA) was used for the entire protein assay (Figure 1).

### 3.2.5 Carboxypeptidase Y Assay

Carboxypeptidase activity in the crude extract and at each purification step was measured by using two substrates 1). *n*-benzoyl-*L*-tyrosine-*p*-nitroanilide (BTPNA) and 2). carboxybenzoxy phenylalanylalanine (Cbz-Phe-Ala).

#### 3.2.5.1 BTPNA as Substrate

The amount of liberated *p*-nitroaniline during the enzyme hydrolysis of BTPNA was determined according to the method of Aibara *et al*, (1971) with a modification. The reaction mixture composed of 200 µl of the properly diluted enzyme solution, 1 ml of 0.1 M phosphate buffer (pH 7.0) and 200 µl of the substrate solution (3.0 mM BTPNA). The whole reaction mixture was incubated at 25°C for 10 min and the reaction was stopped by the addition of 0.5 ml of 0.01 M $HgCl_2$. Absorbance were read at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of *p*-nitroaniline per min under the specified conditions of the assay. Specific activity was defined as the enzyme units per mg protein. A standard curve was constructed using

different concentrations of *p*-nitroaniline from which the concentration of the unknown was calculated (Figure 2).

**3.2.5.2 Cbz-Phe-Ala as Substrate**

Estimation of the amount of enzyme required to liberate 1 µmole of *L*-alanine during the enzymic hydrolysis of Cbz-Phe-Ala was determined according to the method of Yemm and Cocking (1955) with a modification. The reaction mixture composed of 100 µl of the properly diluted enzyme solution added with 0.9 ml of the substrate solution (1.5 mM Cbz-Phe-Ala) incubated at 25°C for 10 min. The reaction was stopped by the addition of 0.5 ml of ninhydrin reagent, heated at 100°C for 5 min, cooled and absorbance readings taken at 570 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of *L*-alanine per min under the specified conditions of the assay. Specific activity was defined as the enzyme units per mg protein. A standard curve was constructed with known concentrations of *L*-alanine from which the concentration of the unknown was calculated (Figure 3).

**3.2.6 Enzyme Preparation**

The cells were harvested at the late logarithmic phase by centrifugation at 10,000 x g for 10 min at 4°C (Model J221; Beckman Ins., Irvine State). The pellet was washed twice with 250 ml of 0.05 M phosphate buffer (pH 7.0) and the supernatant was assayed for extracellular carboxypeptidase activity. The obtained pellet was suspended in 10 ml of the same buffer and subjected for cell disintegration using Ultrasonication (model XL2020; Mandel Scientific Co., Montreal, Canada). The whole process of cell disintegration was carried out at 4°C by immersing the sample vessel in ethanol-ice-water-NaCl and disruption was stopped at every minute interval for 10 min to minimize thermal enzyme denaturation. The disrupted cells were subsequently centrifuged at 15,000 x g

**Figure 1. Calibration curve for protein determination**

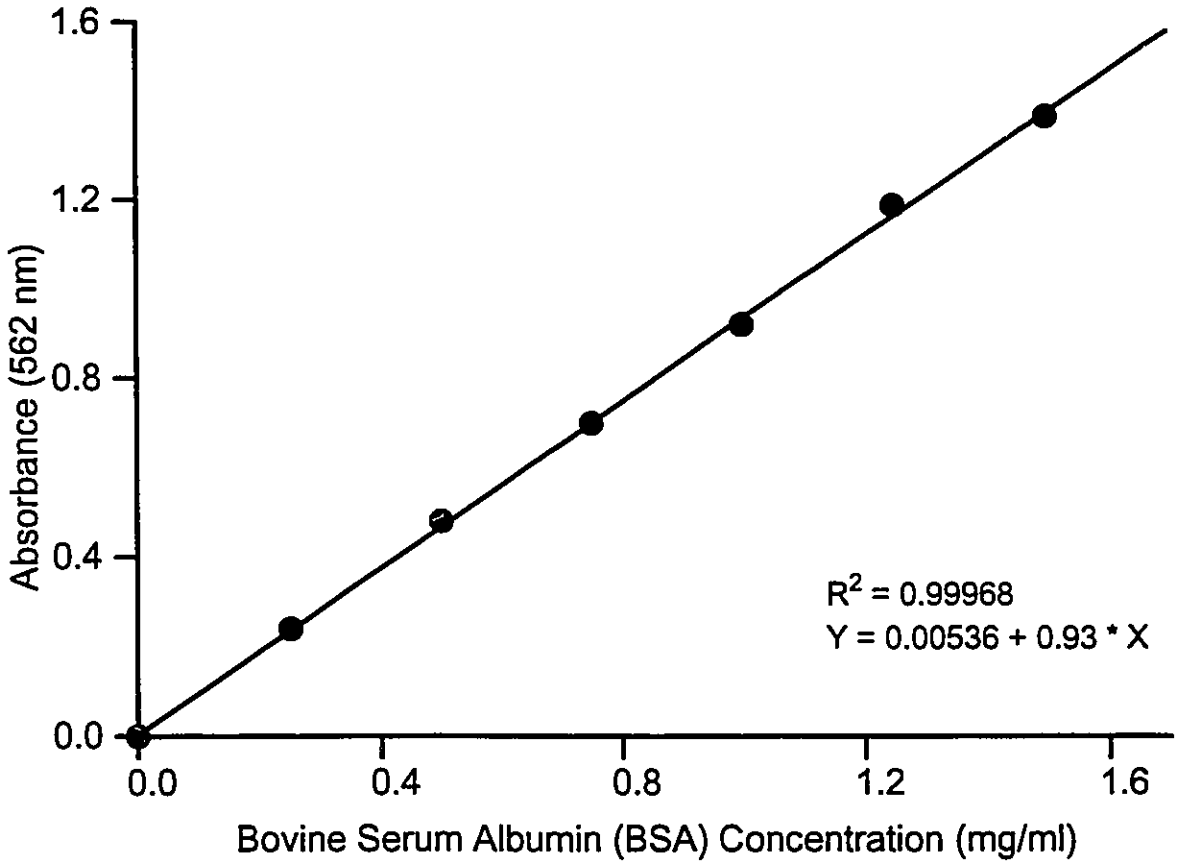

1.6
1.2
0.8
0.4
0.0
Absorbance (562 nm)
0.0
0.4
0.8
1.2
1.6
Bovine Serum Albumin (BSA) Concentration (mg/ml)
R2 = 0.99968
Y = 0.00536 + 0.93 * X

**Figure 2. Calibration curve for carboxypeptidase Y assay using BTPNA as substrate**

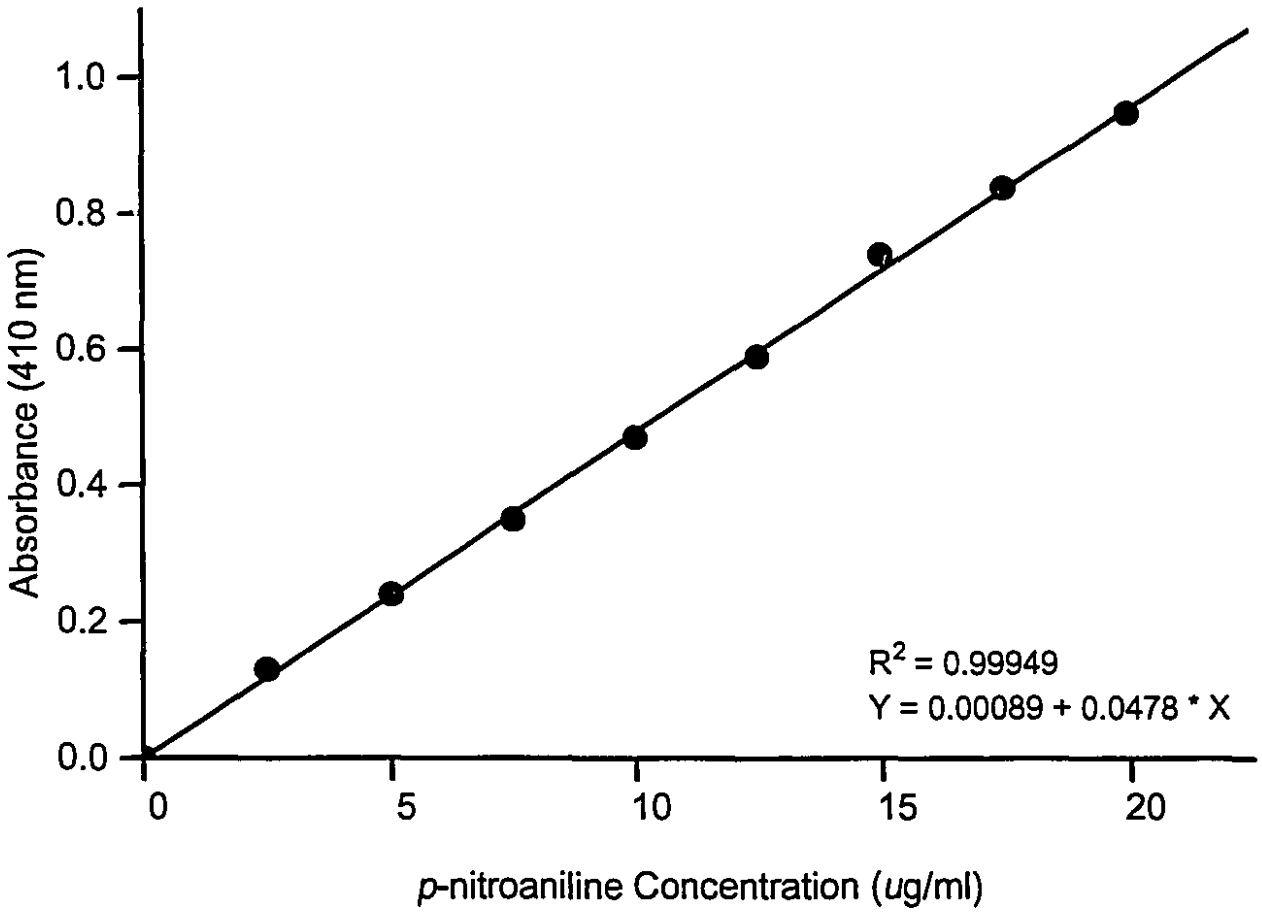
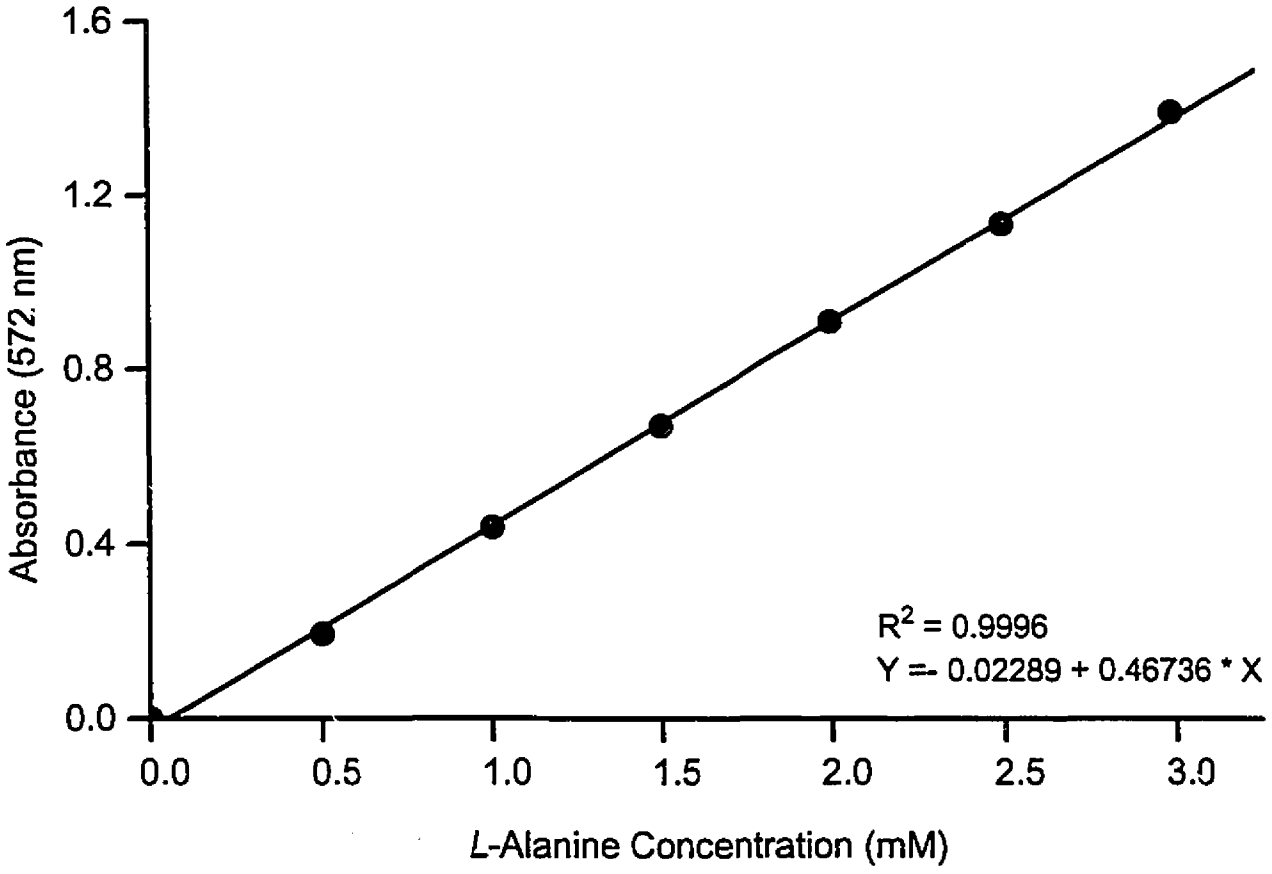

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (410 nm)
0
5
10
15
20
p-nitroaniline Concentration (ug/ml)
R2 = 0.99949
Y = 0.00089 + 0.0478 * X

**Figure 3. Calibration curve for carboxypeptidase Y assay using Cbz-Phe-Ala as substrate**


1.6
1.2
0.8
0.4
0.0
Absorbance (572 nm)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
L-Alanine Concentration (mM)
R2 = 0.9996
Y = 0.02289 + 0.46736 * X

for 30 min at 4°C. The pellet was discarded and the supernatant was directly assayed for protein content and carboxypeptidase activity.

### 3.2.7 Enzyme Purification

The enzyme purification scheme is presented in Figure 4. All purification steps were done at 4°C to preserve the enzyme activity.

#### 3.7.2.1 Ammonium Sulfate Precipitation

The crude extract (20 ml) was precipitated with ammonium sulfate between 50 and 90% saturation with gentle stirring at 4°C. The obtained pellet was collected by centrifugation at 10,000 x g for 10 min ( Model J221, Beckman Ins., Irvine State) and redissolved in 10 ml of 0.05 M phosphate buffer (pH 7.0). The crude sample was desalted by passing through a disposable Sephadex G-25 column (Pharmacia) which was previously equilibrated with 25 ml of 0.05 M phosphate buffer (pH 7.0).

#### 3.2.7.2 Ion Exchange Chromatography

An ion exchange column (Mono Q HR 5/5) was pre-equilibrated with 0.01 M phosphate buffer, pH 7.0 (Buffer A) and 0.01 M phosphate buffer + 0.5 M NaCl, pH 7.0 (Buffer B). The desalted crude extract was loaded into the first superloop (10 ml) and looping (10 times) was programmed to inject 1 ml of the desalted crude sample (containing approximately 10 mg of protein) into the column. The enzyme was eluted at a linear gradient of NaCl (0.1 to 0.5 M) at a flow rate of 0.5 ml/min, sensitivity of 0.5. Preliminary runs were aimed at determining the enzyme location by assaying the activity using Cbz-Phe-Ala as substrate. When the enzyme was located, automatic loading (3 ml; 1 mg protein) into the second superloop (50 ml) was programmed to collect the fraction of interest. The fraction collector was programmed to collect 1.5 ml/min. Carboxypeptidase Y activity and protein content were monitored

**Figure 4. Purification scheme of carboxypeptidase Y from *Kluyveromyces fragilis***

**CRUDE EXTRACT**
(Centrifugation @ 15,000 x g for 10 min)

↓

**AMMONIUM SULFATE PRECIPITATION**
(50 to 90% saturation)
(Sephadex G-25, PD 10 Column)

↓

**ION EXCHANGE CHROMATOGRAPHY**
(Mono Q HR 5/5; Concentration by Ultrafiltration)

↓

**GEL FILTRATION CHROMATOGRAPHY**
(Superose 12 HR 10/30; Concentration by Ultrafiltration)

↓

**PURITY TEST AND MOLECULAR WEIGHT DETERMINATION**
Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and native polyacrylamide gel electrophoresis (Native-PAGE)

spectrophotometrically by reading the absorbance at 570 and 280 nm, respectively. Active fractions were pooled and concentrated by ultrafiltration using CX-30 membrane (exclusion limit 30 kDa; Amicon Corp., Toronto, Canada). The concentrated sample was further subjected to gel filtration chromatography.

### 3.2.7.3 Gel Filtration Chromatography

A Superose 12 HR 10/30 column was used for gel filtration chromatography equilibrated with 0.01 M phosphate buffer (pH 7.0). A looping program (10 times) was installed for automatic injection of 3 ml volume of sample (from ion exchange) into the column. The flow rate was 0.5 ml/min; sensitivity is 0.1 and total elution time of 60 min. The enzyme was located by carboxypeptidase activity assay using Cbz-Phe-Ala as substrate and spectrophotometrically measured at absorbance 570 and 280 nm. The collected active fractions from several injections were then concentrated by ultrafiltration using CX-30 membrane (exclusion limit 30 kDa; Amicon Corp., Toronto, Canada) and assayed for protein content and carboxypeptidase activity.

### 3.2.7.4 Purity and Molecular Weight Determination

The purity of the enzyme at each purification step was examined by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) using the PhastSystem by Pharmacia. The sample was prepared by addition of 2.5% SDS, 5.0% mercaptoethanol, heated at 100°C for 5 min before adding 0.01% of bromophenol blue. The whole mixture was centrifuged for 2 min (Sorvall, Speed Vac, Savant Inc., Farmingdale, NY.) for the precipitation of insoluble materials. Ready to-use gradient gels (8-25%) and SDS-buffer strips as described previously were used. The gels were stained with Coomasie R 350. Low molecular weight markers (Pharmacia, Uppsala, Sweden) were used to construct

a standard curve for the determination of CPY molecular weight (Figure 5). To verify whether CPY composed with a monomeric or subunits, Native-PAGE of the protein sample was similarly run on mini-gels using the PhastSystem. Table 3 shows the method used for Native-PAGE of the non-denatured sample. High molecular weight proteins were used as markers.

**Table 3. Native-PAGE Separation Method Used for Native Protein**

| | | | | | |
|---|---|---|---|---|---|
| Sample Application Down at | | | 1.2* | | 0 Vh |
| Sample Application Up at | | | 1.2* | | 2 Vh |
| Separation 1.1* | 400 V | 10.0 mA | 2.5 W | 15°C | 10 Vh |
| Separation 1.2* | 400 V | 1.0 mA | 2.5 W | 15°C | 2 Vh |
| Separation 1.3* | 400 V | 10.0 mA | 2.5 W | 15°C | 268 Vh |

*method number

### 3.3 Results and Discussions

#### 3.3.1 Cell Growth and Enzyme Production

When the supernatant obtained after cell disruption was assayed for extracellular CPY activity, no activity was found indicating that CPY is an intracellular exopeptidase. Figure 6 shows the growth curve of *Kluyveromyces fragilis* in YPD medium. The maximum cell growth was obtained after 26 hr of growth. The maximum protein and enzyme production were also obtained at the late logarithmic phase during 26 hr of incubation (Figure 7). As for the effect of whey supplemented with 0.5% yeast extract, maximum cell growth was obtained

**Figure 5. Calibration curve for carboxypeptidase Y molecular weight determination on SDS-PAGE**

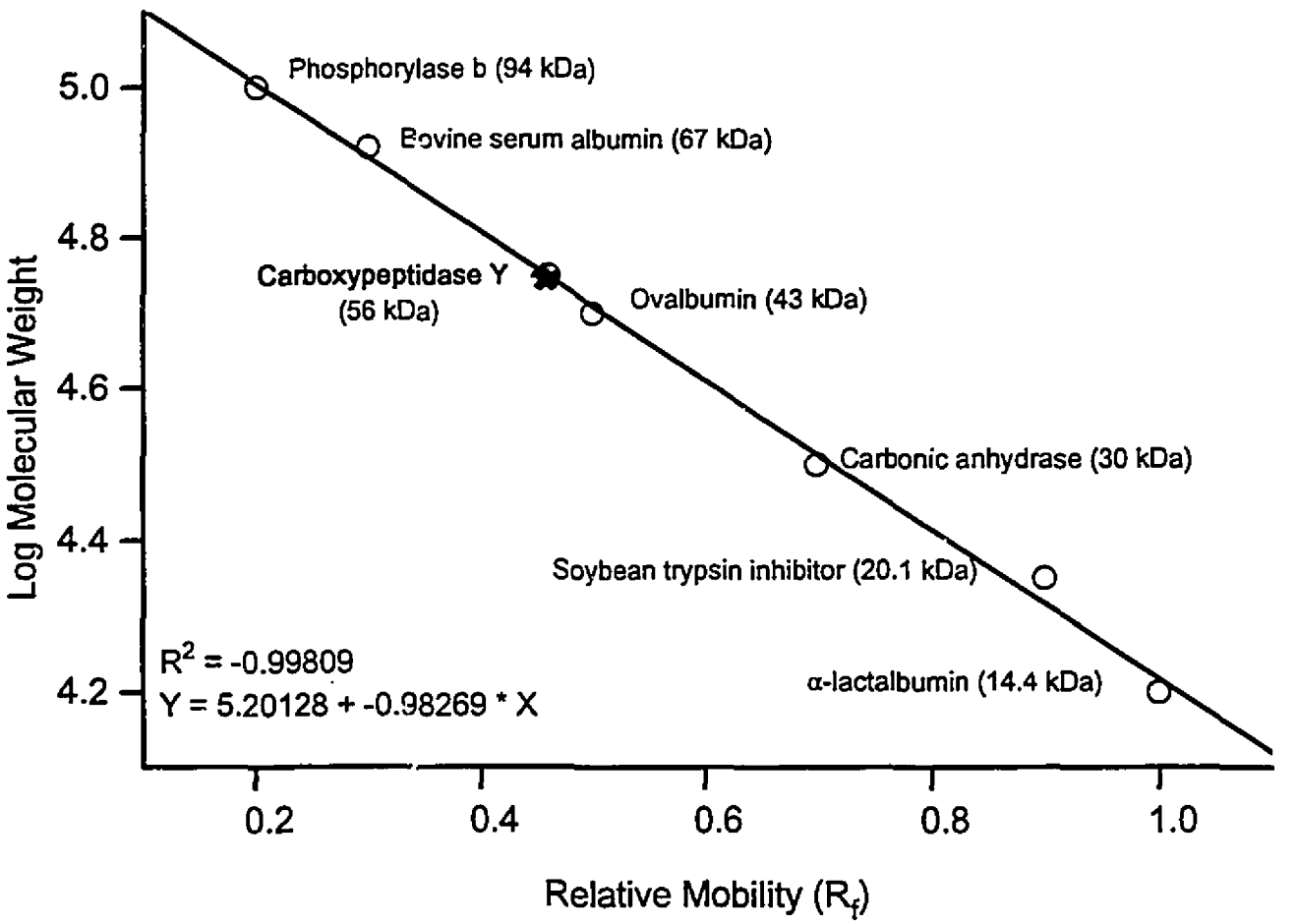

Phosphorylase b (94 kDa)
Bovine serum albumin (67 kDa)
Carboxypeptidase Y
(56 kDa)
Ovalbumin (43 kDa)
Carbonic anhydrase (30 kDa)
Soybean trypsin inhibitor (20.1 kDa)
α-lactalbumin (14.4 kDa)
R2 = -0.99809
Y = 5.20128 + -0.98269 * X
Log Molecular Weight
5.0
4.8
4.6
4.4
4.2
0.2
0.4
0.6
0.8
1.0
Relative Mobility (Rf)

**Figure 6. Calibration curve of Kluyveromyces fragilis in YPD medium**

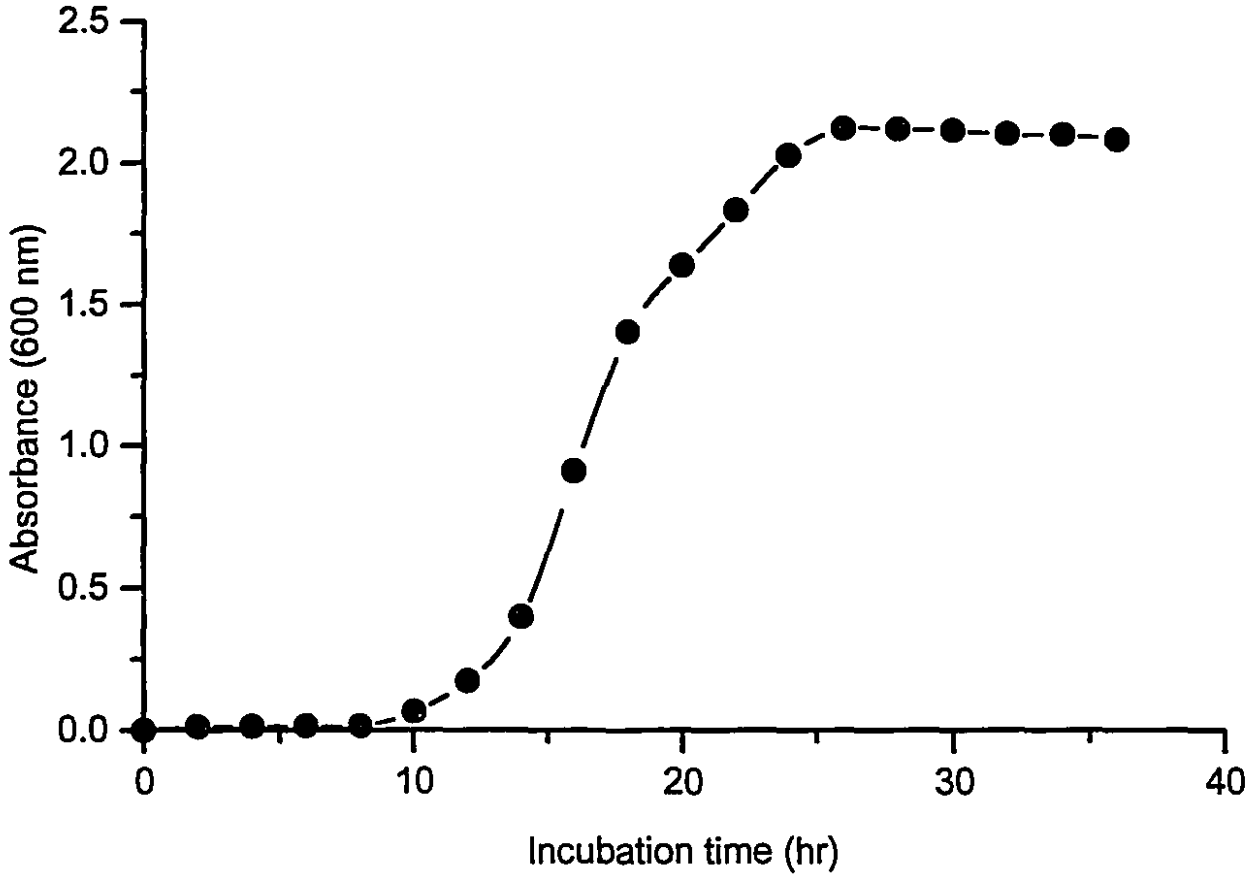
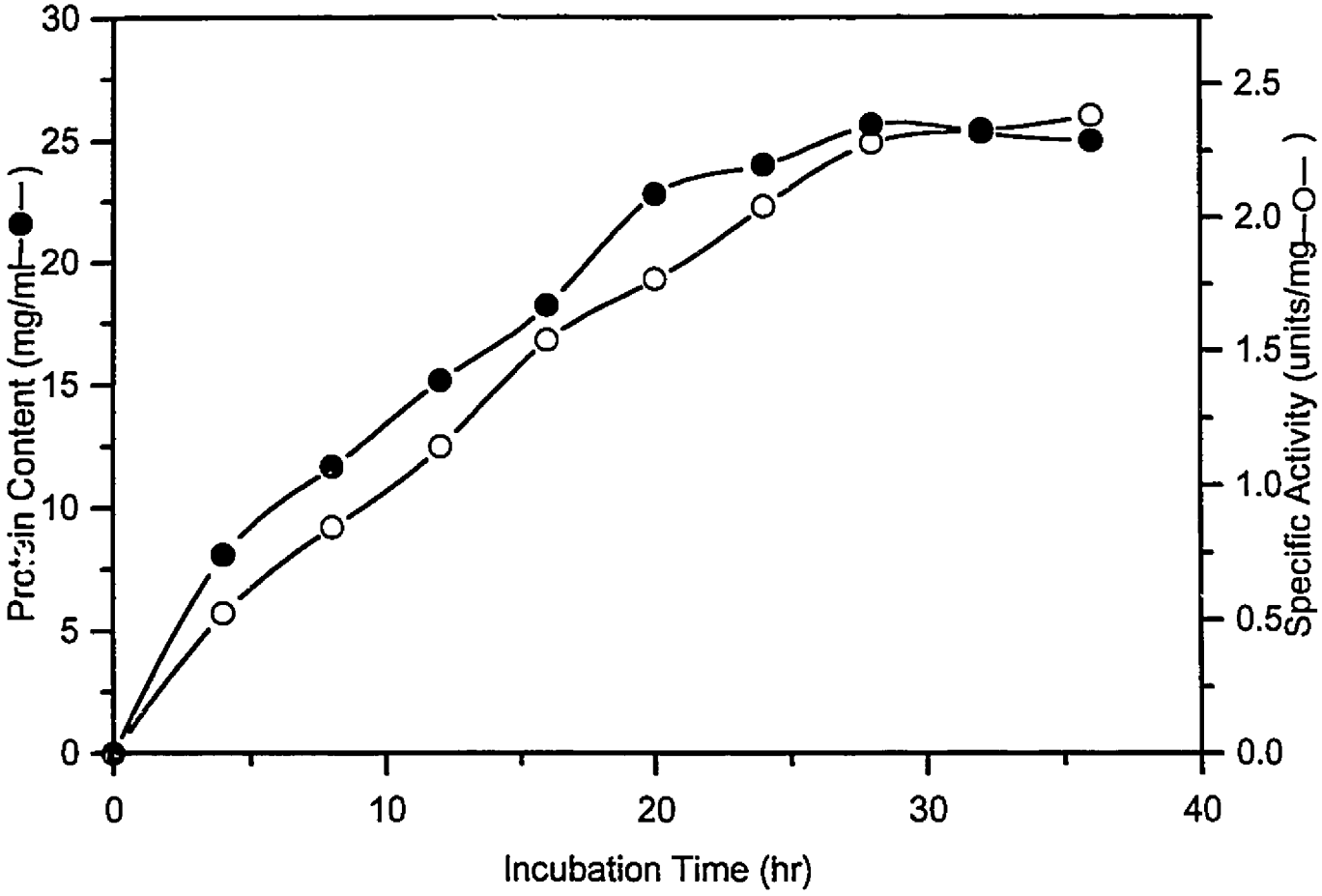

2.5
2.0
1.5
1.0
0.5
0.0
Absorbance (600 nm)
0
10
20
30
40
Incubation time (hr)

**Figure 7. Maximum protein and enzyme production of *K. fragilis* in YPD medium**


30
25
20
15
10
5
0
Protein Content (mg/ml—●—)
2.5
2.0
1.5
1.0
0.5
0.0
Specific Activity (units/mg—○—)
0
10
20
30
40
Incubation Time (hr)

with 4% whey although there was not much difference between 2% and 6% whey. Cell growth decreased when the concentration of whey was above 6% (Figure 8). These results are in agreement with those of Castillo and Sanchez (1978) who found that growth of *Kluyveromyces fragilis* and yield of cells were dependent on whey concentration. This change in growth can be related to the fermentative characteristics of *Kluyveromyces fragilis* being able to produce ethanol, ethyl acetate and volatile compounds that will be degraded in the culture media before they can be assimilated. Hence, 4% whey in 0.5% yeast extract was found to be the optimum concentration for the growth of *Kluyveromyces fragilis*. The protein and enzyme production for the culture grown in 4% whey with 0.5% yeast extract was also determined for a period of 36 hr at 4 hr intervals (Figure 9). The results showed that the protein content was slightly lower than the YPD grown culture but no marked difference in enzyme production was observed. Figure 10 shows the growth of *Kluyveromyces fragilis* in YPD at different pH (5, 6 and 7). With all the other growth conditions maintained i.e., incubation temperature, time and agitation, *Kluyveromyces fragilis* showed the best growth at pH 5.0. Though pH 6.0 showed a good growth, the growth at pH 7.0 was relatively lower.

**3.3.2 Enzyme Purification**

Figure 11 shows the ion exchange elution profile of the crude exract. CPY was eluted at a linear gradient from 0.01 to 0.26 M and an elution time of 25 min. The active fraction eluted at 25 to 27 min were loaded into the second superloop and injected into the gel filtration column. Figure 12 shows the gel filtration elution profile of CPY. The enzyme was eluted at 30 min in a total volume of 60 min. The yield and activity of carboxypeptidase Y is shown in Table 4. The enzyme was purified to 216 fold over the crude extract with a recovery of 18%.

**Figure 8. Effect of substrate concentration (% whey supplemented with 0.5 % yeast extract) on the growth of *K. fragilis***

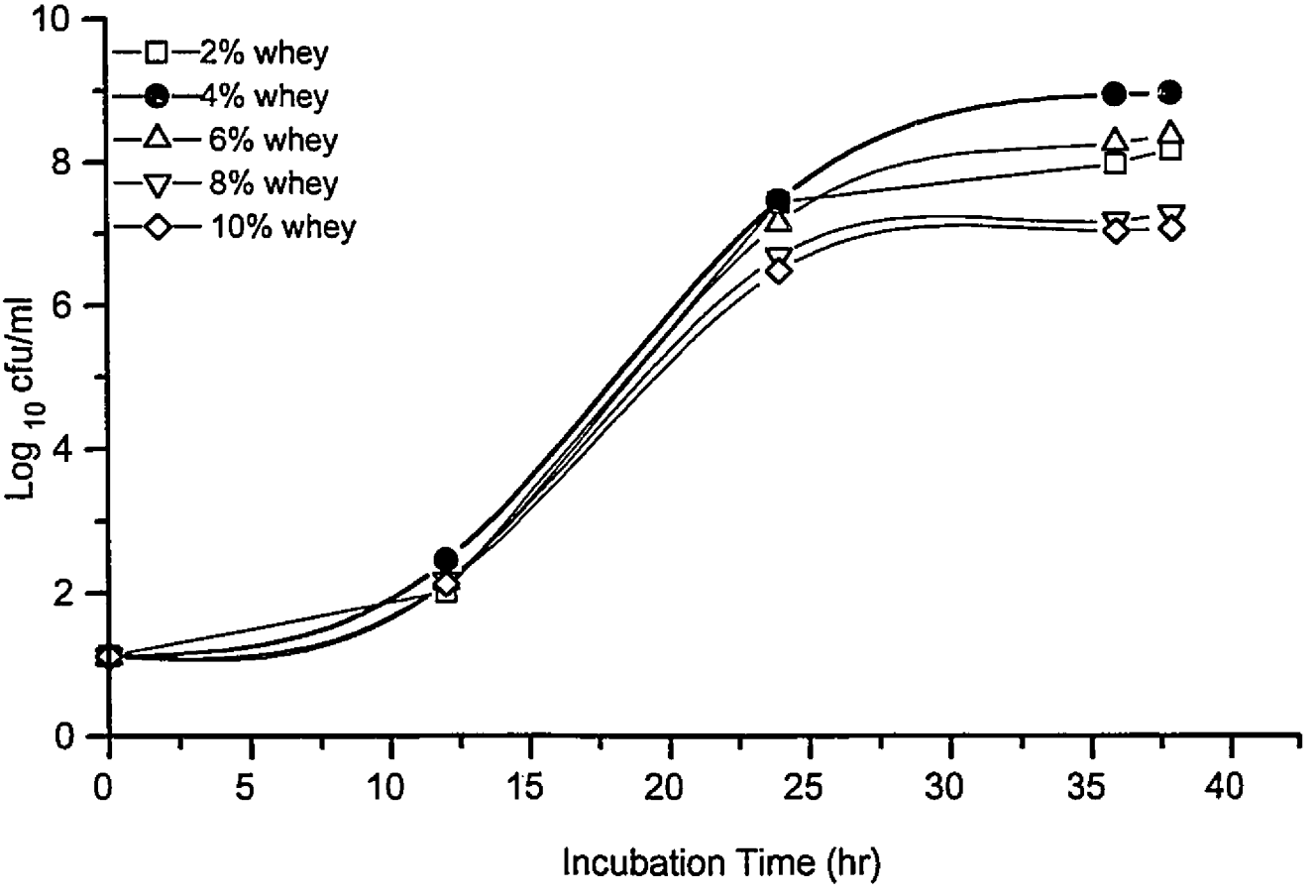

10
8
6
4
2
0
Log 10 cfu/ml
2% whey
4% whey
6% whey
8% whey
10% whey
0
5
10
15
20
25
30
35
40
Incubation Time (hr)

**Figure 9. Maximum protein and enzyme production of *K. fragilis* in 4 % whey supplemented with 0.5 % yeast extract**

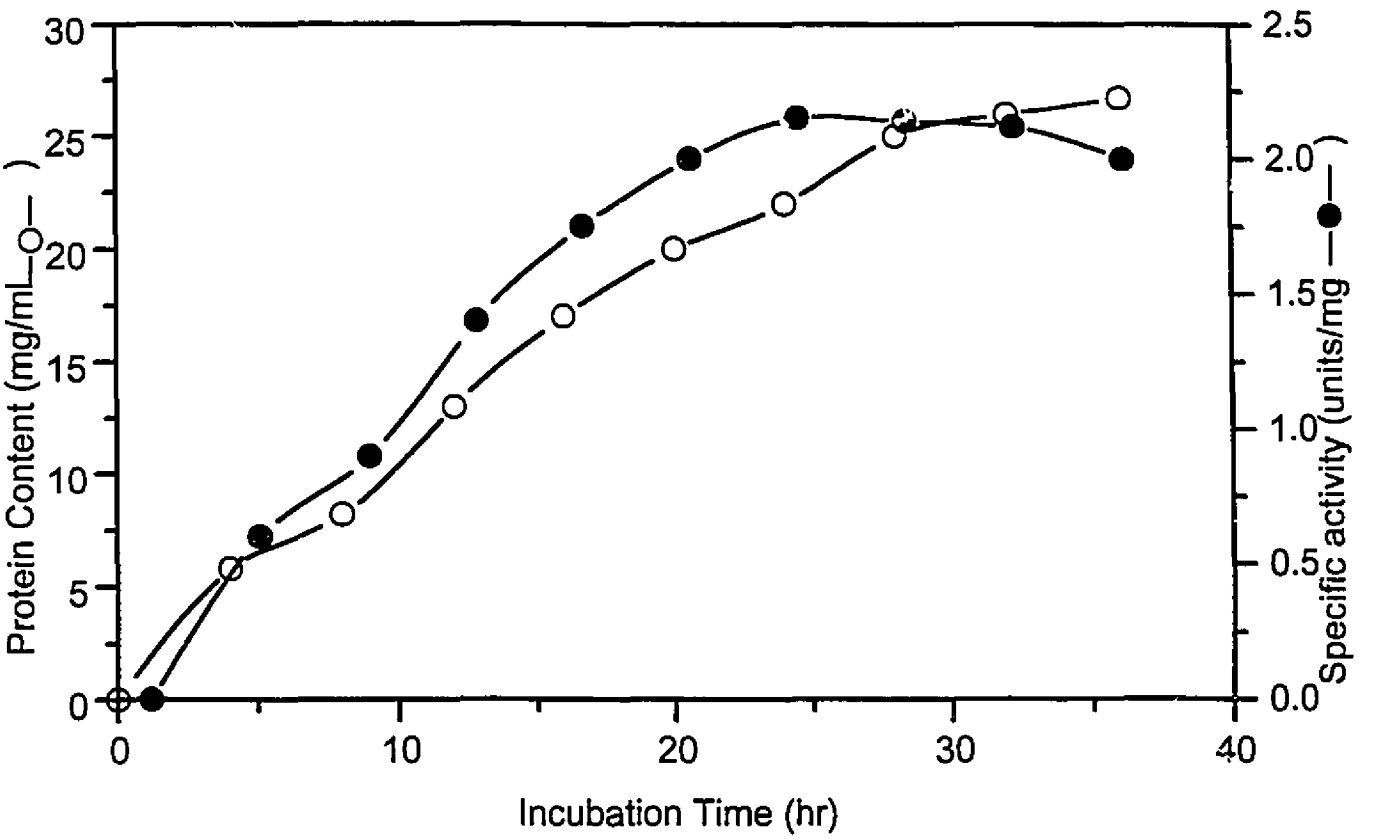

Protein Content (mg/mL—○—)
Specific activity (units/mg —●—)
Incubation Time (hr)
0
5
10
15
20
25
30
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40

**Figure 10. Effect of pH on the growth of *K. fragilis* in YPD medium**

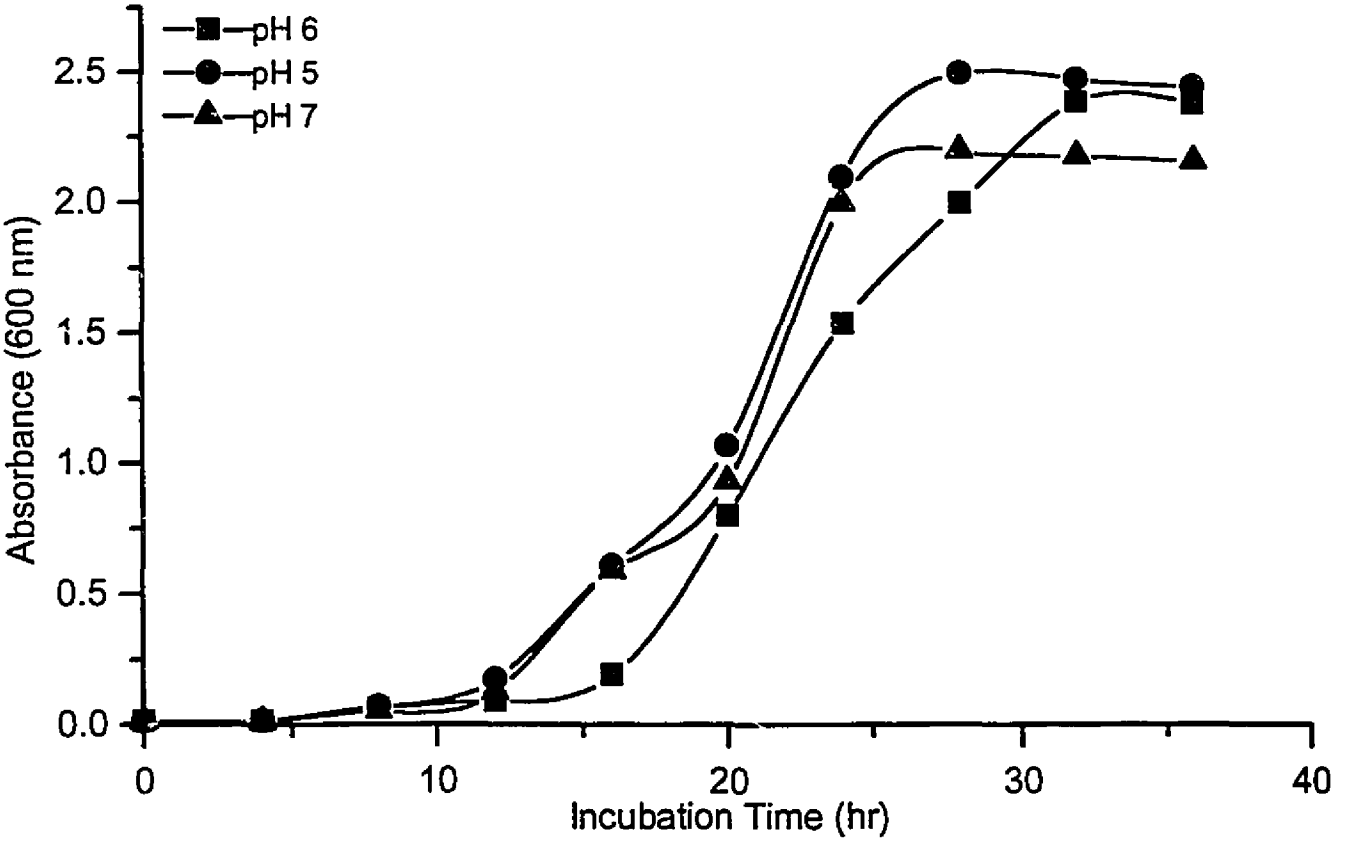

pH 6
pH 5
pH 7
Absorbance (600 nm)
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
Incubation Time (hr)

**Figure 11. Ion exchange elution profile of crude extract in YPD medium after ammonium sulfate precipitation**

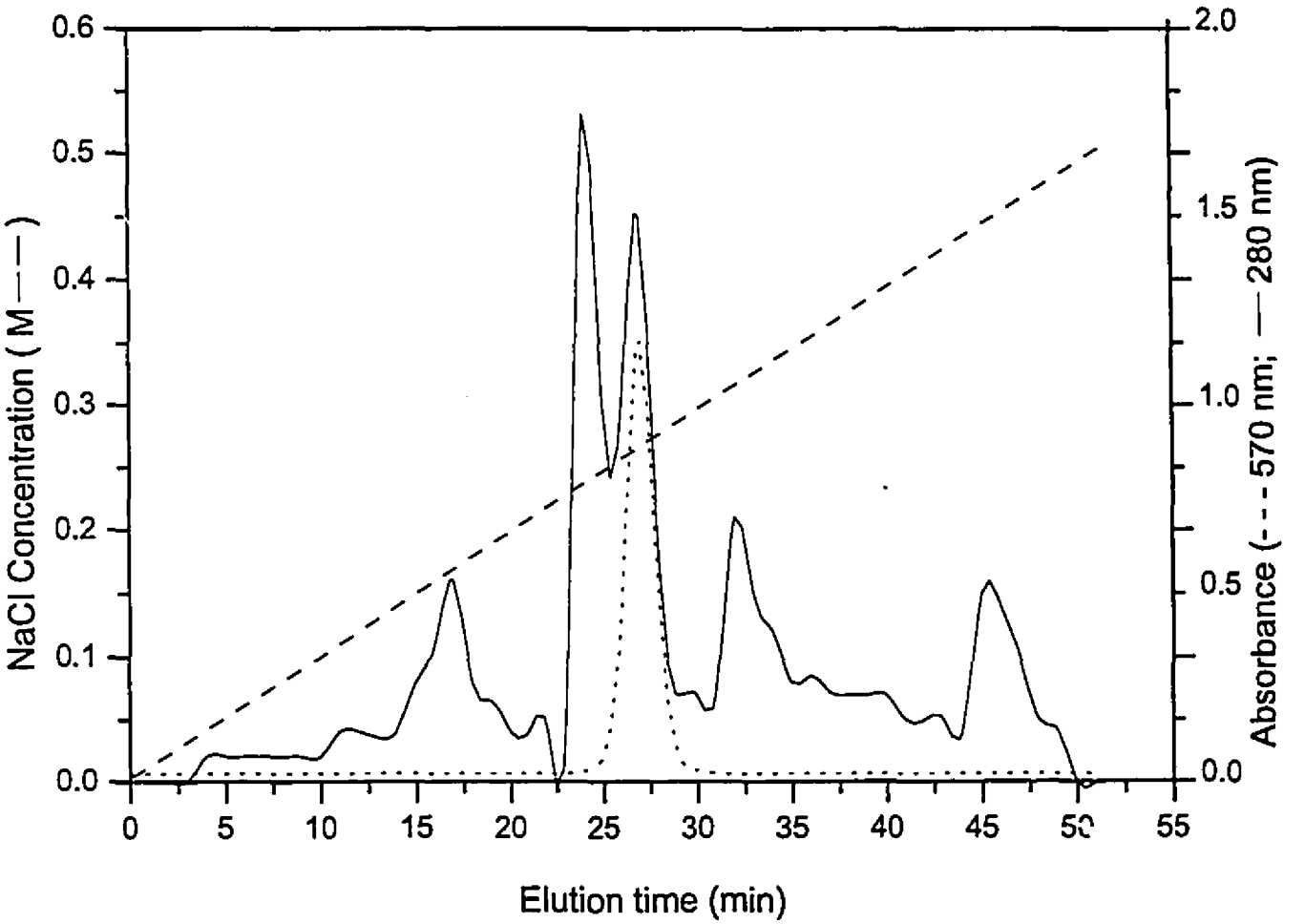

0.6
0.5
0.4
0.3
0.2
0.1
0.0
NaCl Concentration ( M— — )
2.0
1.5
1.0
0.5
0.0
Absorbance (- - - 570 nm; — 280 nm)
0
5
10
15
20
25
30
35
40
45
50
55
Elution time (min)

**Figure 12. Gel filtration elution profile of carboxypeptidase Y active fraction after ion exchange chromatography**

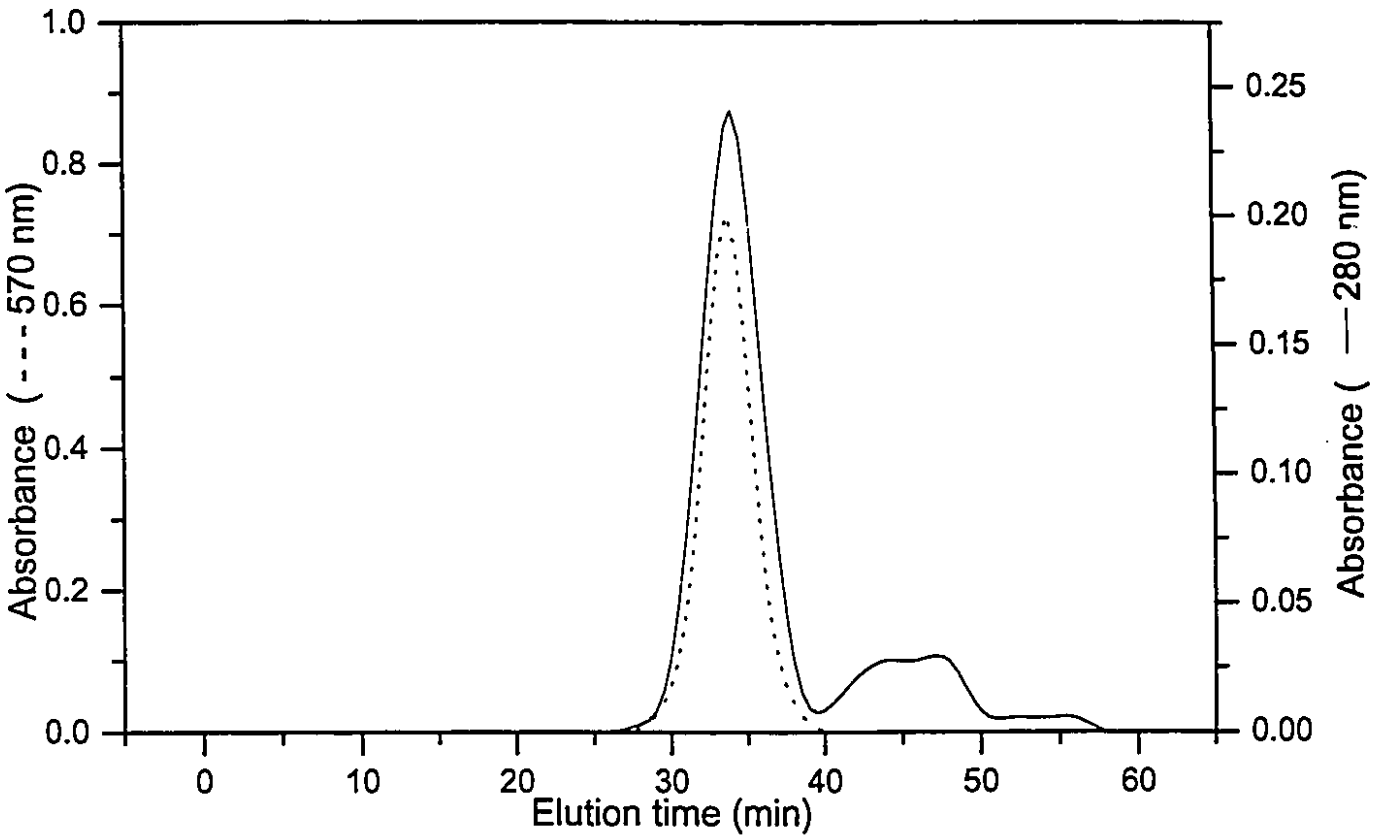

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance ( - - - 570 nm)
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance ( — 280 nm)
0
10
20
30
40
50
60
Elution time (min)

**Table 4. Purification steps of carboxypeptidase Y from *K. fragilis***

Table 4. Purification Steps of Carboxypeptidase Y from *Kluyveromyces fragilis*

| Purification Steps | Total Protein (*mg*) | Total Activity (*units*)[1] | Specific Activity[2] (*units/mg*) | Purification (*-fold*) | Yield (*%*) |
|---|---|---|---|---|---|
| 1. Crude Extract | 241.0 | 510.0 | 2.1 | 1 | 100 |
| 2. Ammonium Sulfate Precipitation | 168.0 | 507.0 | 3.0 | 1.4 | 99 |
| 3. Ion Exchange Chromatography | 2.1 | 275.0 | 131.1 | 62.5 | 54 |
| 4. Gel Filtration Chromatography | 0.2 | 90.5 | 453.0 | 215.7 | 18 |

[1]one unit of enzyme activity is defined as the amount of enzyme required to liberate 1µmole of *p*-nitroaniline or *L*-alanine per min under the specified conditions of the assay.

[2]Specific activity is defined as the enzyme units per mg protein.

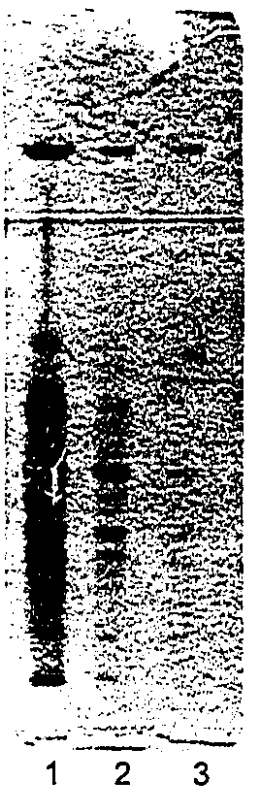
Carboxypeptidase activity obtained from this study after the last purification step was much higher than those obtained by Hayashi *et al*, (1973) and Kuhn *et al*, (1974). Though previous reports mentioned the difficulty of purifying carboxypeptidase Y from *Saccharomyces cereviseae* by conventional chromatographic techniques (Johansen *et al*, 1976), the FPLC system was efficient to purify carboxypeptidase Y from *Kluyveromyces fragilis*. Purity determination and estimation of carboxypeptidase Y molecular weight was determined on SDS-PAGE. The electropherogram of the crude cell extract, partially purified and purified forms of carboxypeptidase Y is shown in Figure 13. The enzyme had an apparent molecular weight of 56 kDa. The molecular weight obtained from this study is similar to those values obtained by previous workers. However, the results of Native-PAGE when plotted showed that CPY composed of two subunits with molecular weight of 120 kDa (Figure 14).

**3.4 Conclusion**

The results presented in this study indicated that *Kluyveromyces fragilis* grown on whey, produced carboxypeptidase Y efficiently as that grown in fully supplemented medium such as YPD. This study also showed that *Kluyveromyces fragilis* produces carboxypeptidase Y that can be conveniently purified by two-step chromatographic procedures with a high recovery using the FPLC system.

**Figure 13. SDS-PAGE patterns of crude extract and carboxypeptidase Y fractions obtained from different purification steps**

Point of Application (-)




Carboxypeptidase Y (56 kDa)

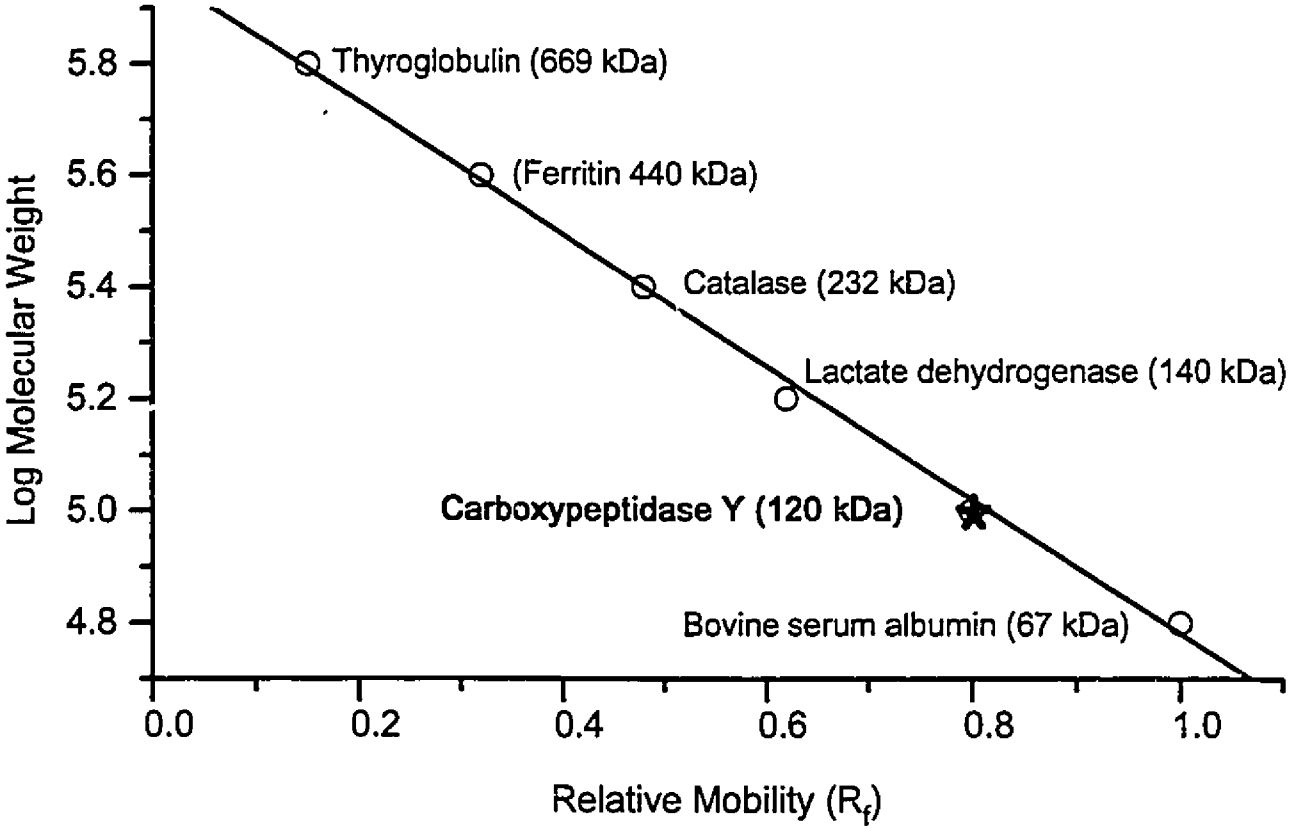
(+)

Lane 1: Crude Extract

Lane 2: After ion exchange chromatography

Lane 3: After gel filtration chromatography
(Purified enzyme)

**Figure 14. Plot of Native-PAGE calibration standards and molecular weight of carboxypeptidase Y under non-denaturing condition**


Thyroglobulin (669 kDa)
(Ferritin 440 kDa)
Catalase (232 kDa)
Lactate dehydrogenase (140 kDa)
Carboxypeptidase Y (120 kDa)
Bovine serum albumin (67 kDa)
Log Molecular Weight
5.8
5.6
5.4
5.2
5.0
4.8
0.0
0.2
0.4
0.6
0.8
1.0
Relative Mobility ($R_f$)

# 4.0 CHARACTERIZATION OF CARBOXYPEPTIDASE Y FROM *KLUYVEROMYCES FRAGILIS*

## 4.1 Introduction

In order to understand the possible role of carboxypeptidase Y in accelerated ripened cheese without bitterness, the enzymatic properties of the enzyme must be studied in terms of prevailing external and internal factors such as pH, temperature, inhibitors and substrate concentrations. The control of these conditions and hence the enzymatic activity is necessary during food processing and preservation. In cheesemaking, and in terms of enzyme activities during ripening, full functionality at acidic pH is important because the cheese matrix can go as low as pH 4.3 depending on the type of cheese being manufactured and the type of starter culture used (Nath, 1992). Although previous reports on carboxypeptidase Y from *Saccharomyces cereviseae* showed a broad pH carboxypeptidase activity between pH 5 and 7 (Hayashi *et al,* 1973; Kuhn *et al*, 1974; Johansen *et al*, 1976), no work has been done to determine its significance in accelerated ripened cheese. Furthermore, the role of this enzyme in removing hydrophobic amino acid residues on peptides and proteins has not been studied with milk proteins such as $\alpha_{s1}$- and β-caseins. These two milk proteins are known to be the main source of bitterness upon hydrolysis with proteases during cheese manufacture (Ney, 1981).

The objectives of this work were:

(1). To study the enzyme kinetics of carboxypeptidase Y, and

(2). To delineate the removal of bitterness mechanism in accelerated ripened cheese of carboxypeptidase Y using $\alpha_{s1}$-and β-caseins as substrates.

## 4.2 Materials and Methods

### 4.2.1 Microorganism, Cell Growth and Enzyme Preparation

*Kluyveromyces fragilis* was cultivated under optimized conditions in YPD medium, the cells harvested by centrifugation and disintegrated by ultrasonication and the cell free extract was purified to homogeneity by using the FPLC system equipped with ion exchange and gel filtration chromatography columns.

### 4.2.2 Chemicals and Reagents

Unless otherwise specified, all chemicals and reagents used in this study were of analytical grade and were purchased from Sigma Chemicals (St. Louis, Mo).

### 4.2.3 Protein Determination

Protein determination of carboxypeptidase Y was measured spectrophotometrically using the bicinchoninic acid (BCA) reagent supplied with the system (Pierce Chem., Rockford, IL) and by the method of Smith *et al,* (1985).

### 4.2.4 Carboxypeptidase Y Assay

Carboxypeptidase activity throughout the entire study was determined by measuring the quantity of *L*-alanine released from the enzymatic hydrolysis of Cbz-Phe-Ala, and the amount of *p*-nitroaniline released from the enzymatic hydrolysis of *n*-benzoyl-*L*-tyrosine-*p*-nitroanilide (BTPNA) by the methods of Yemm and Cocking (1955) and Aibara *et al,* (1971), respectively. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of *L*-alanine or *p*-nitroaniline per min under the specified conditions of the assay. Specific activity was defined as the enzyme units per mg protein.

### 4.2.5. Hydrolysis of $\alpha_{S1}$-and β-Caseins with Trypsin and Carboxypeptidase Y

$\alpha_{S1}$-and β-caseins (1mg/ml) were digested with 20 µg of trypsin (.234 units) at 37°C for 18 hr. After digestion, 500 µl from each sample were taken out and hydrolyzed with 10 µl of carboxypeptidase Y (4.53 units) at 37°C for 12 hr. Enzyme inactivation of all the samples were performed by heating at 80°C for 10 min. The hydrolysed samples were dried (Speed Vac, Savant Ins. Farmingdale, NY.) and reconstituted with distilled water, filtered with a 0.45 µm nyphon syringe filter (Millipore City, Japan) before injection. Portions (25 µl) were injected in a Delta Pak C18 column (30 mm x 150 mm) and the peptides from the hydrolysates were analyzed with reversed-phase high performance liquid chromatography system (Millipore, Milford, MA) eluted with a binary gradient from 0 to 100% B at a rate of 0.5 ml/min for a period of 60 min. Solvent A was Milli Q water and solvent B was 60% acetonitrile and 40% water (HPLC grade). Both solvents contained 0.1% and 0.08% trifluoroacetic acid, respectively. The peaks were monitored by absorbance at 214 nm.

### 4.2.6 Enzymatic Properties and Kinetic Studies

#### 4.2.6.1 Determination of pH Optimum

The effect of pH on the activity of carboxypeptidase Y was measured in 0.05 M acetate buffer (pH 3.0 to 5.0); 0.05 M phosphate buffer (pH 6.0 to 8.0) and 0.05 M glycine-NaOH buffer (pH 9.0 to 10.0). The reaction mixture composed of 900 µl of the substrate solution (1.5 mM Cbz-Phe-Ala or 3.0 mM BTPNA, suspended in the different buffers described) incubated with 100 µl of carboxypeptidase at 25°C for 10 min. The absorbance of product released is read at 570 nm for Cbz-Phe-Ala and 410 nm for BTPNA.

#### 4.2.6.2 Determination of Temperature Optimum

The effect of temperature on the activity of carboxypeptidase Y was performed by incubating 100 µl of the pure enzyme with 900 µl of the substrate solution (1.5 mM Cbz-Phe-Ala or 3.0 mM BTPNA suspended in 0.05 M phosphate buffer, pH 7.0) for 10 min at temperatures ranging from 15°C to 60°C. The amount of product released is read at absorbance 570 nm for Cbz-Phe-Ala and 410 nm for BTPNA.

#### 4.2.6.3 Effect of Specific Inhibitors and Metal Ions

The reaction mixture composed of 100 µl of carboxypeptidase Y and 100 µl of the cations or inhibitors (final concentration: 0.1 or 1.0 mM) with 800 µl of the substrate solution (1.5 mM Cbz-Phe-Ala in 0.05 M phosphate buffer, pH 7.0) incubated at 25°C for 10 min. The enzyme activity was assayed under the standard conditions described for the enzyme assay procedure. Inhibition was expressed as a percentage of the activity without the effectors.

For the determination of the type of inhibition caused, a Lineweaver-Burke plot was constructed with initial velocity versus the substrate concentration. Three concentrations of the inhibitors were used: $I_0$ = control (no inhibitor); $I_1$ = 0.1 mM of inhibitor and $I_2$ = 1.0 mM of inhibitor. Carboxypeptidase activity was measured according to the standard procedure described in the enzyme assay.

#### 4.2.6.4 Effect of Substrate Concentration

The effect of substrate concentration was performed on both Cbz-Phe-Ala and BTPNA. Final concentration of the substrates ranged from 0.4 to 2.0 mM for Cbz-Phe-Ala and 0.2 to 100 mM for BTPNA. The reaction mixture composed of 900 µl of the substrate solution added with 100 µl of carboxypeptidase Y

incubated at 25°C for 10 min. Carboxypeptidase activity was measured according to the standard procedure described in the enyzme assay.

The kinetic constants ($K_m$ and $V_{max}$) of the pure enzyme were determined for the two substrates used. A Lineweaver-Burke plot was constructed by plotting reciprocals of the initial velocity versus substrate concentration and $K_m$ and $V_{max}$ were computed from the slope and intercept of the regression line. Different dipeptides were also tried for the peptidase assay activity: Cbz-Glu-Tyr; Cbz-Gly-Ala; Cbz-Pro-Phe; Cbz-Phe-Leu; Cbz-Gly-Leu and Cbz-Gly-Pro. The hydrolysis of the substrates was monitored spectrophotometrically by reading the absorbance at 570 nm. All activity assays for the Cbz-dipeptides derivatives were performed in triplicates and the mean and standard deviations were calculated.

## 4.3 Results and Discussions

### 4.3.1 pH Optimum

The effect of pH on the enzyme activity is shown in Figure 15. Results indicated that carboxypeptidase Y from *Kluyveromyces fragilis* has a pH optimum of 6.0. This finding is in agreement with the results obtained by other workers on the pH optimum of carboxypeptidase Y from *Saccharomyces cereviseae* which had either pH 5.0 or 6.5 (Hayashi *et al,* 1973; Kuhn *et al,* 1974).

### 4.3.2 Temperature Optimum

The temperature optimum of carboxypeptidase Y from *Kluyveromyces fragilis* is 35°C and had a broad range of activity at lower temperature (15°C to 40°C) (Figure 16). The enzyme activity completely lost its activity at 70°C. Although there is no study indicating the temperature optimum of carboxypeptidase Y from *Saccharomyces cereviseae*, stability at a wider range

**Figure 15. Effect of pH on carboxypeptidase Y activity**

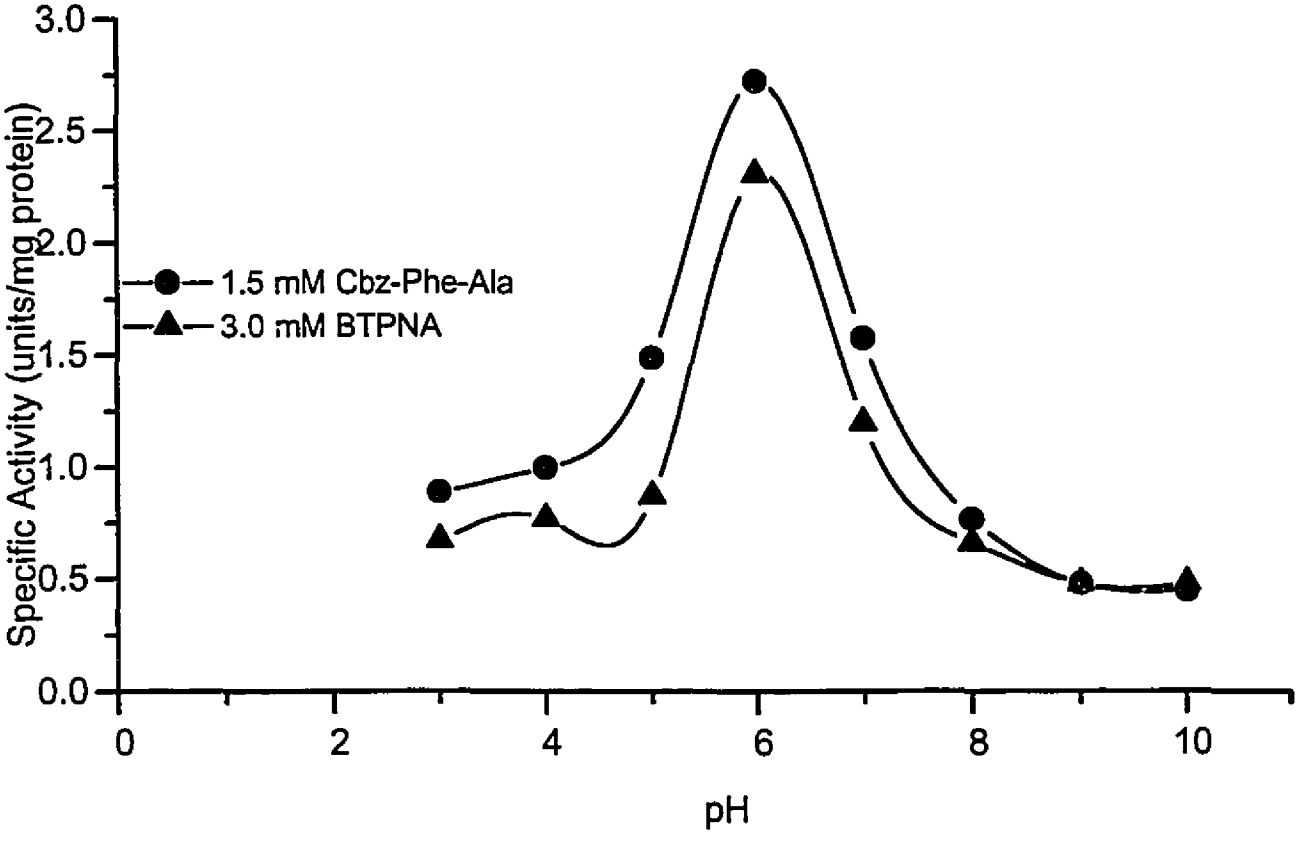
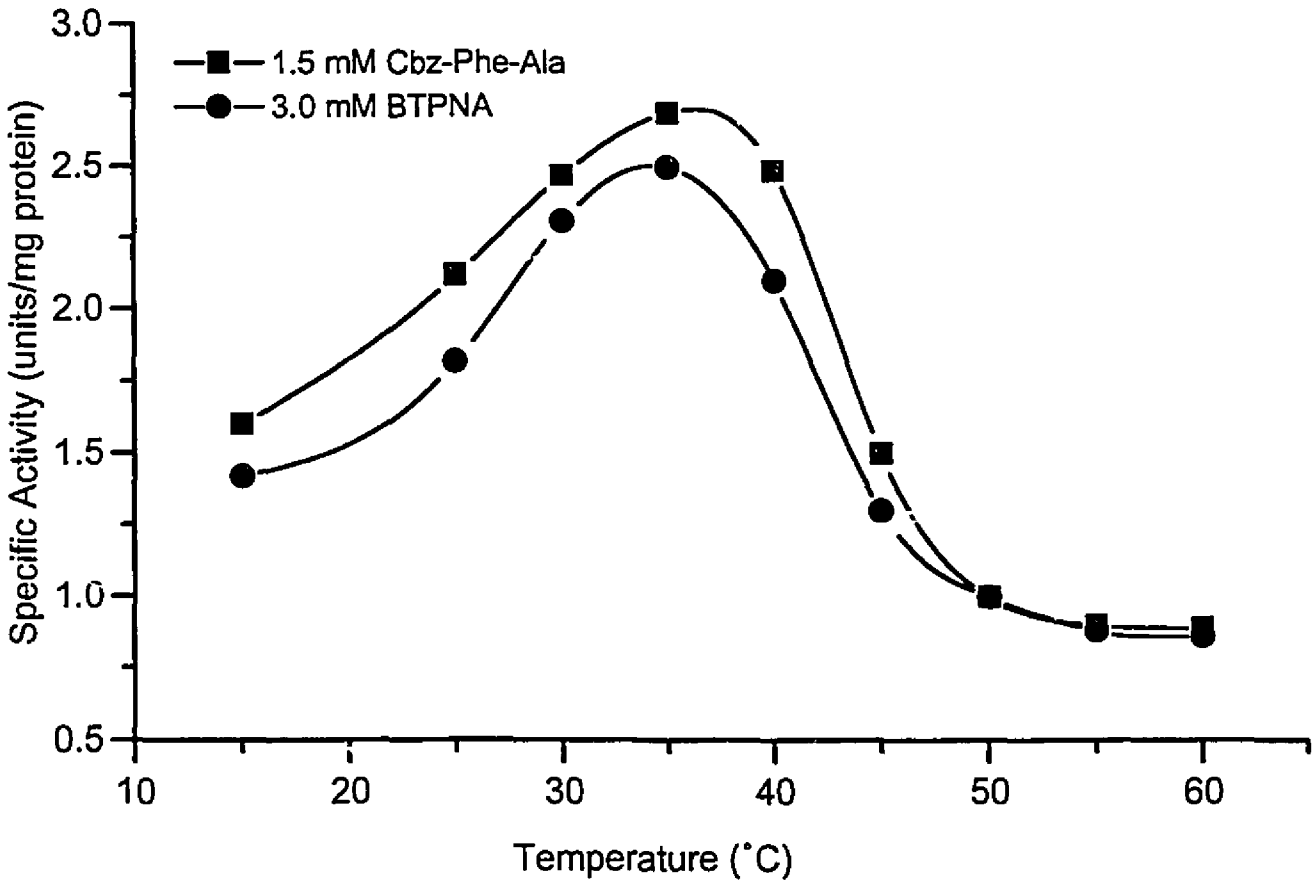

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Specific Activity (units/mg protein)
1.5 mM Cbz-Phe-Ala
3.0 mM BTPNA
0
2
4
6
8
10
pH

**Figure 16. Effect of temperature on carboxypeptidase Y activity**


1.5 mM Cbz-Phe-Ala
3.0 mM BTPNA
Specific Activity (units/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
10
20
30
40
50
60
Temperature (°C)

of temperature (20°C to 60°C) was reported (Hayashi *et al,* 1973; Kuhn *et al,* 1974).

### 4.3.3 Effect of Metal Ions and Specific Inhibitors

Figure 17 shows the effect of metal ions on carboxypeptidase Y activity. The enzyme showed about 50% inhibition on all the metal ions studied. This finding is in agreement with the findings obtained by Hayashi (1976) on the effect of metal ions on carboxypeptidase Y activity from *Saccharomyces cereviseae.* The effect of different specific inhibitors studied on carboxypeptidase Y activity is shown in Figure 18. Diisopropylphosphofluoridate (DIPF) and phenylmethylsulfonylfluoride (PMSF) showed a strong inhibition with about 25% retained activity with a concentration of 0.1 mM inhibitor. For the other inhibitors, slight or no inhibition at all was observed.

Lineweaver-Burke plots on the type of inhibition showed a type of competitive for DIPF (Figure 19) and uncompetitive for PMSF (Figure 20). For DIPF, the $K_m$ and $V_{max}$ values obtained were 0.9 mM and 8.0 µmole/min/mg for no inhibitor ($I_0$); 0.63 mM and 3.0 µmole/min/mg for 0.1 mM ($I_1$) and 0.57 mM and 1.9 µmole/min/mg for 1.0 mM ($I_2$). For PMSF, $K_m$ and $V_{max}$ values obtained were 0.84 mM and 7.4 µmole/min/mg for no inhibitor ($I_0$); 0.24 mM and 2.2 µmole/min/mg for 0.1 mM inhibitor ($I_1$); and 0.22 mM and 2.0 µmole/min/mg for 1.0 mM inhibitor ($I_2$).

### 4.3.4 Effect of Substrate Concentration

The affinities of the two substrates used in the assay is shown in Figures 21 and 22 for BTPNA and Cbz-Phe-Ala, respectively. As the enzyme concentration increases, the hydrolysis increased linearly up to a saturation point. These results were further illustrated via Lineweaver-Burke plot as shown in Figures 23 and 24. The $K_m$ and $V_{max}$ values for BTPNA are 5.1 mM and 13.4

µmole/min/mg and for Cbz-Phe-Ala, $K_m$ and $V_{max}$ values are 1.9 mM and 28.0 µmole/min/mg. From the values obtained, Cbz-Phe-Ala has a stronger affinity with the enzyme than BTPNA. This phenomenon can be attributed to the fact that carboxypeptidase Y specifically cleaves the C-terminal of peptides and proteins. Thus, the rate of release of the free amino acid from the N-terminal blocked dipeptides are faster than the other kind of substrate involved.

The summary of the kinetic parameters obtained for the different Cbz-dipeptides derivatives used for the peptidase assay is presented in Table 5. Apparent $K_m$ and $V_{max}$ values obtained were: for ***Cbz-Pro-Phe*: 2.5** mM and **17.5** µmole/min/mg; for ***Cbz-Glu-Tyr*: 8.1** mM and **22.7** µmole/min/mg; for ***Cbz-Gly-Ala:* 1.6** mM and **9.9** µmole/min/mg; for ***Cbz-Gly-Leu:* 2.1** mM and **11.9** µmole/min/mg; for ***Cbz-Phe-Leu:* 1.4** mM and **4.8** µmole/min/mg; for ***Cbz-Gly-Pro:* 1.1** mM and **10.0** µmole/min/mg. The validity of the assay is quite reproducible as shown with the relative standard deviations except for Cbz-Pro-Phe and Cbz-Phe-Ala on their $V_{max}$ values. These values obtained from this study were slightly higher than those values obtained by Hayashi *et al,* (1973), Johansen *et al,* (1976), Hayashi (1976) on carboxypeptidase Y from *Saccharomyces cereviseae*. However, these results are not comparable since the two enzymes came from different strains and studied at different conditions. Nevertheless, it showed that carboxypeptidase Y from *Kluyveromyces fragilis* has the ability to remove hydrophobic amino acid residues including proline from the C-terminal of peptides. Among the Cbz-dipeptides derivatives studied, Cbz-Glu-Tyr had the greatest affinity with the enzyme as shown by the lowest $K_m$ value obtained.

### 4.3.5 Hydrolysis of $\alpha_{s1}$-and β-Caseins with Tyrpsin and CPY

Figure 25 shows the HPLC chromatographic profile of $\alpha_{s1}$-casein hydrolyzed with trypsin, and Figure 26 shows the similar chromatographic profile of the tryptic digest of $\alpha_{s1}$-casein hydrolyzed with carboxypeptidase Y. At least two hydrophobic peptides which were eluted at 35 and 40 min with the $\alpha_{s1}$-tryptic digest disappeared when hydrolyzed with carboxypeptidase Y. It can also be noted that the heights of all the peptides from the tryptic digest of $\alpha_{s1}$-casein were greatly reduced when hydrolyzed with carboxypeptidase Y. Figure 27 and 28 show the HPLC chromatographic profiles of β-casein hydrolyzed with trypsin, and β-casein tryptic digest hydrolyzed with carboxypeptidase Y, respectively. Results obtained showed that at least four hydrophobic peptides which were eluted at 40, 42, 44 and 45 min and two more peptides which were eluted at 22 and 28 min with the tryptic digest of β-casein disappeared when hydrolyzed with carboxypeptidase Y. Moreover, the sizes and heights of all the peptides were also greatly reduced when hydrolyzed with carboxypeptidase Y.

## 4.4 Conclusion

The results obtained from this study showed that carboxypeptidase Y from *Kluyveromyces fragilis* belongs to the class of serine carboxypeptidases being strongly inhibited by DIPF and PMSF, as well as exhibiting optimum activity at pH 6.0. It has the ability to remove hydrophobic peptides from the hydrolysates of the milk proteins studied. Moreover, with different peptides tried for peptidase assay, it was shown that CPY can hydrolyse and release the hydrophobic amino acid at the C-terminal site. These results obtained indicated the significance of carboxypeptidase Y in accelerated ripened cheese without bitterness. However, further study is needed for the identification of these peptides to confirm the exact role and specificity of carboxypeptidase Y for such purpose.

**Figure 17. Effect of metal ions on carboxypeptidase Y activity**

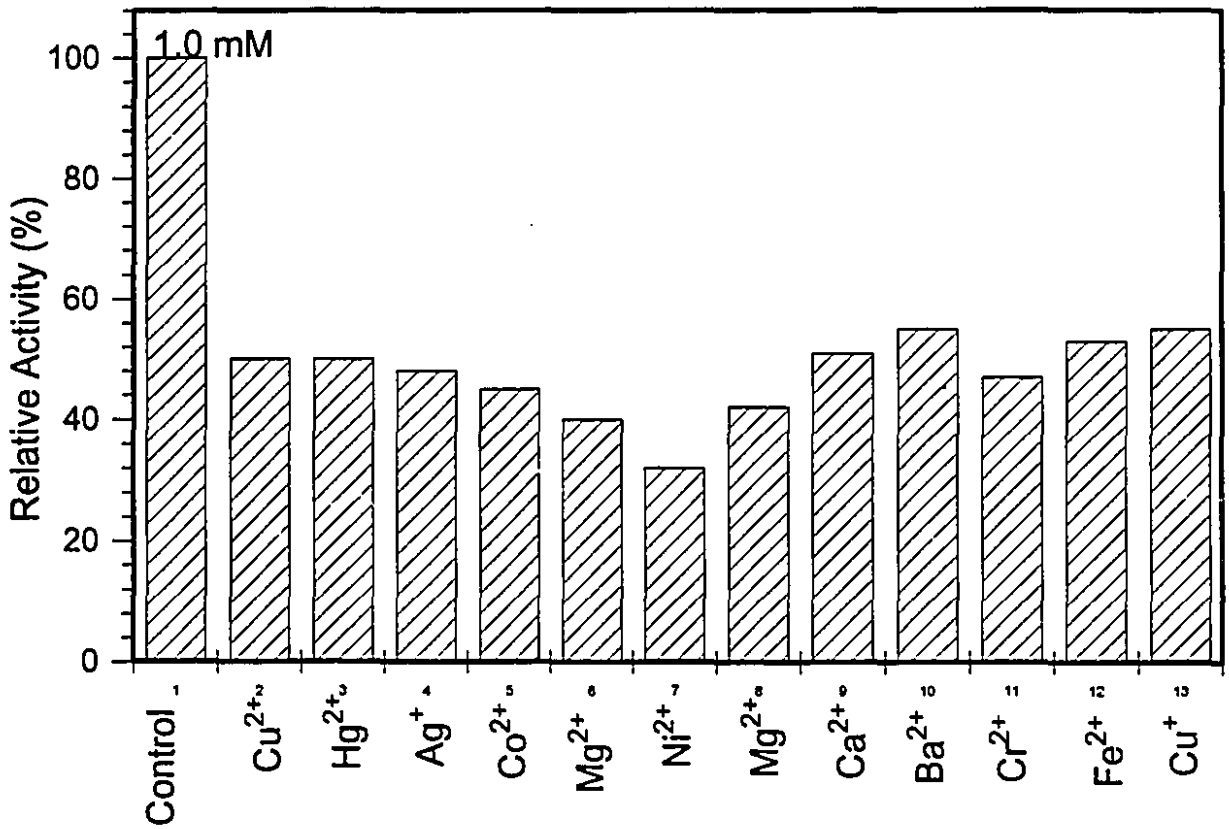

1.0 mM
Relative Activity (%)
0
20
40
60
80
100
Control 1
Cu2+2
Hg2+3
Ag+4
Co2+5
Mg2+6
Ni2+7
Mg2+8
Ca2+9
Ba2+10
Cr2+11
Fe2+12
Cu+13

Figure 18. Effect of inhibitors on carboxypeptidase Y activity

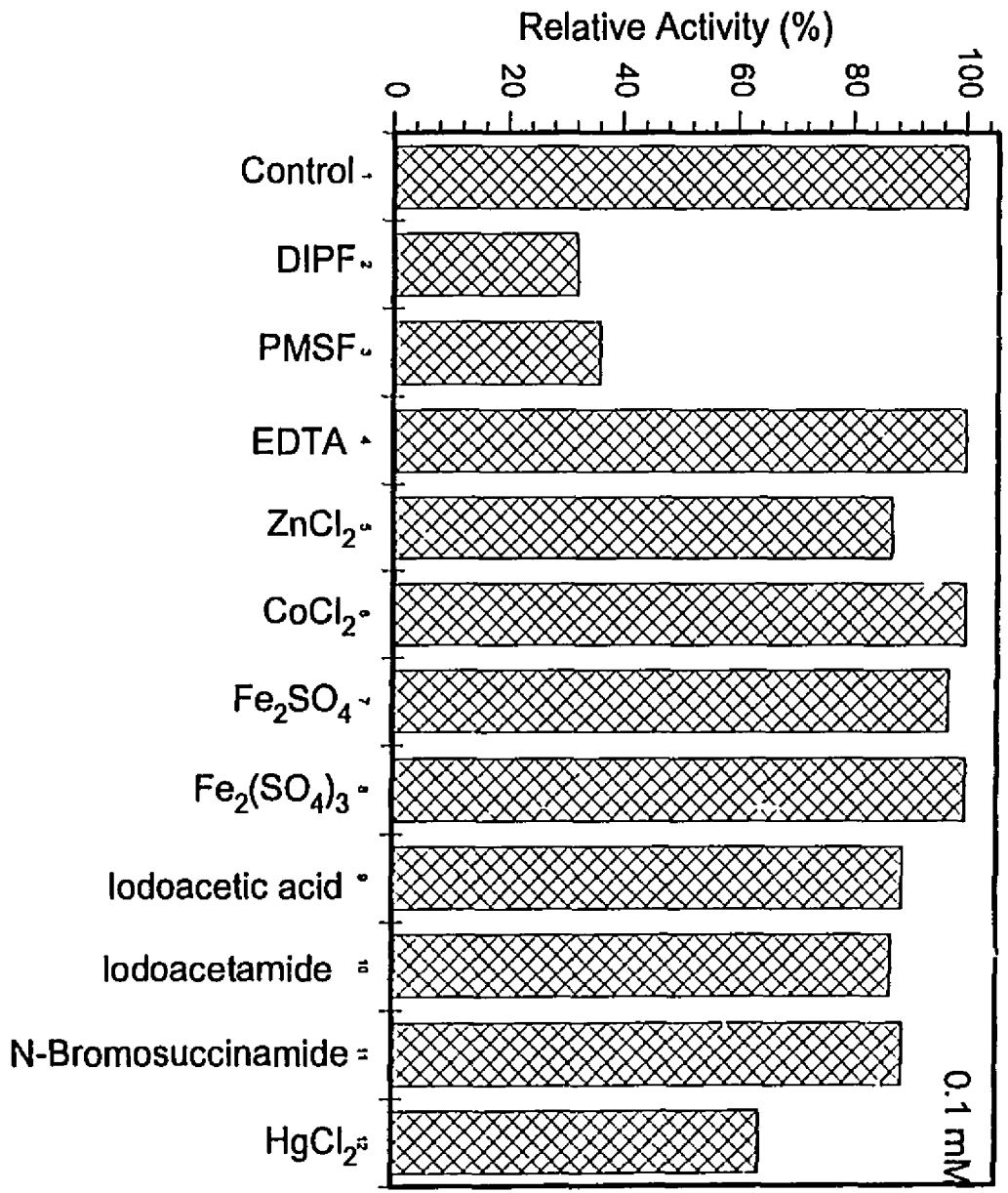

Relative Activity (%)
0
20
40
60
80
100
Control
DIPF
PMSF
EDTA
$ZnCl_2$
$CoCl_2$
$Fe_2SO_4$
$Fe_2(SO_4)_3$
Iodoacetic acid
Iodoacetamide
N-Bromosuccinamide
$HgCl_2$
0.1 mM

**Figure 19. Lineweaver-Burke plot showing competitive inhibition by DIPF on carboxypeptidase Y activity**

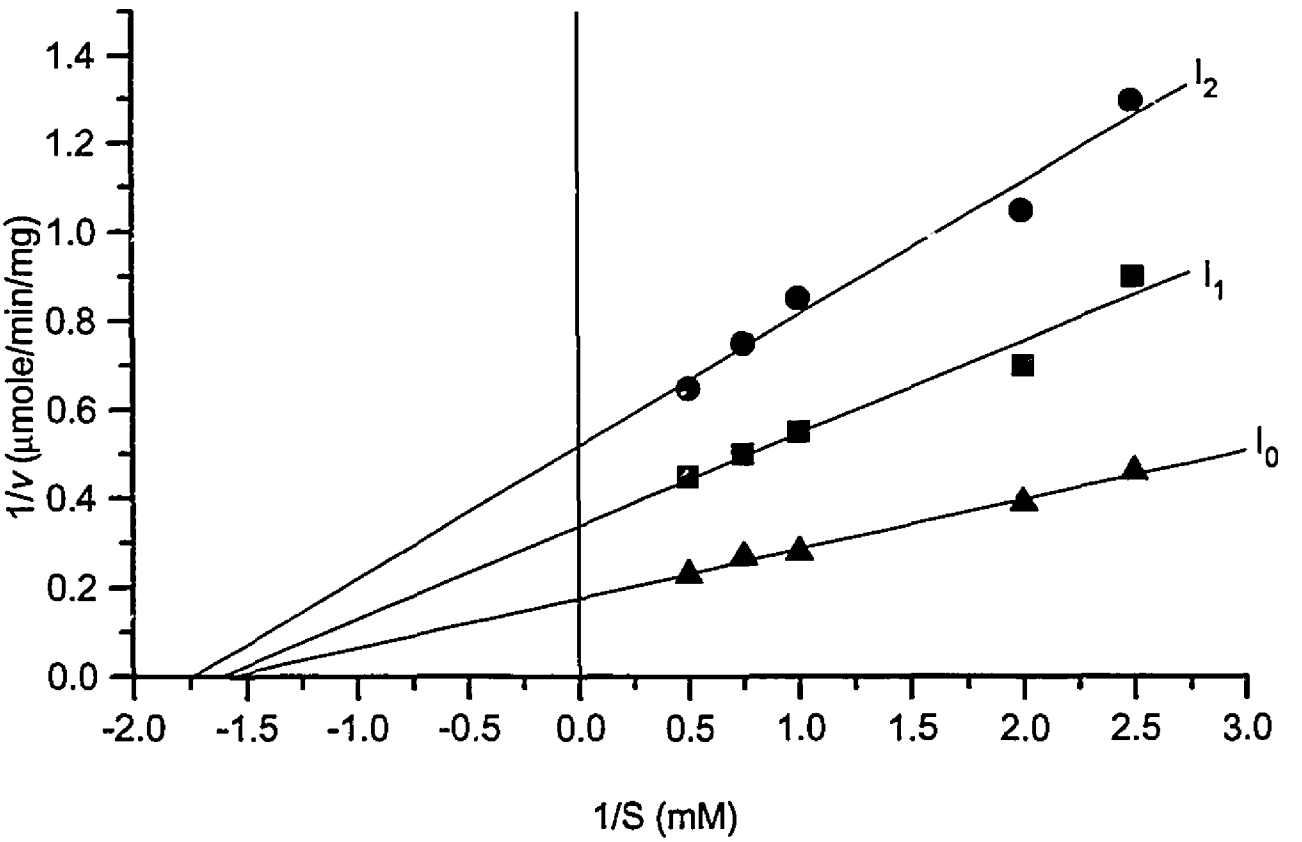

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1/v (µmole/min/mg)
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
1/S (mM)
I2
I1
I0

**Figure 20. Lineweaver-Burke plot showing uncompetitive inhibition by PMSF on carboxypeptidase Y activity**

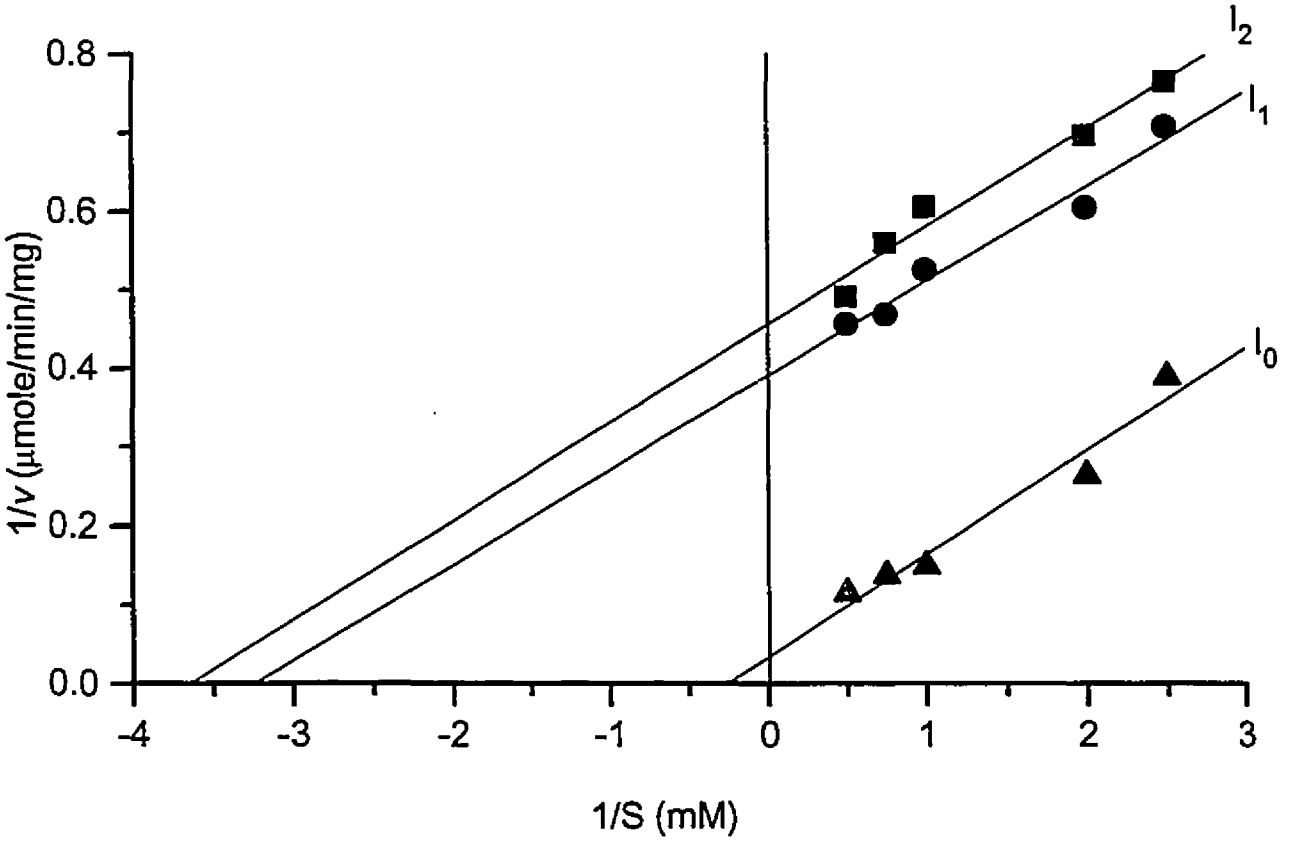

I2
I1
I0
0.8
0.6
0.4
0.2
0.0
1/v (µmole/min/mg)
-4
-3
-2
-1
0
1
2
3
1/S (mM)

Figure 21. Effect of substrate concentration (BTPNA) on carboxypeptidase Y activity

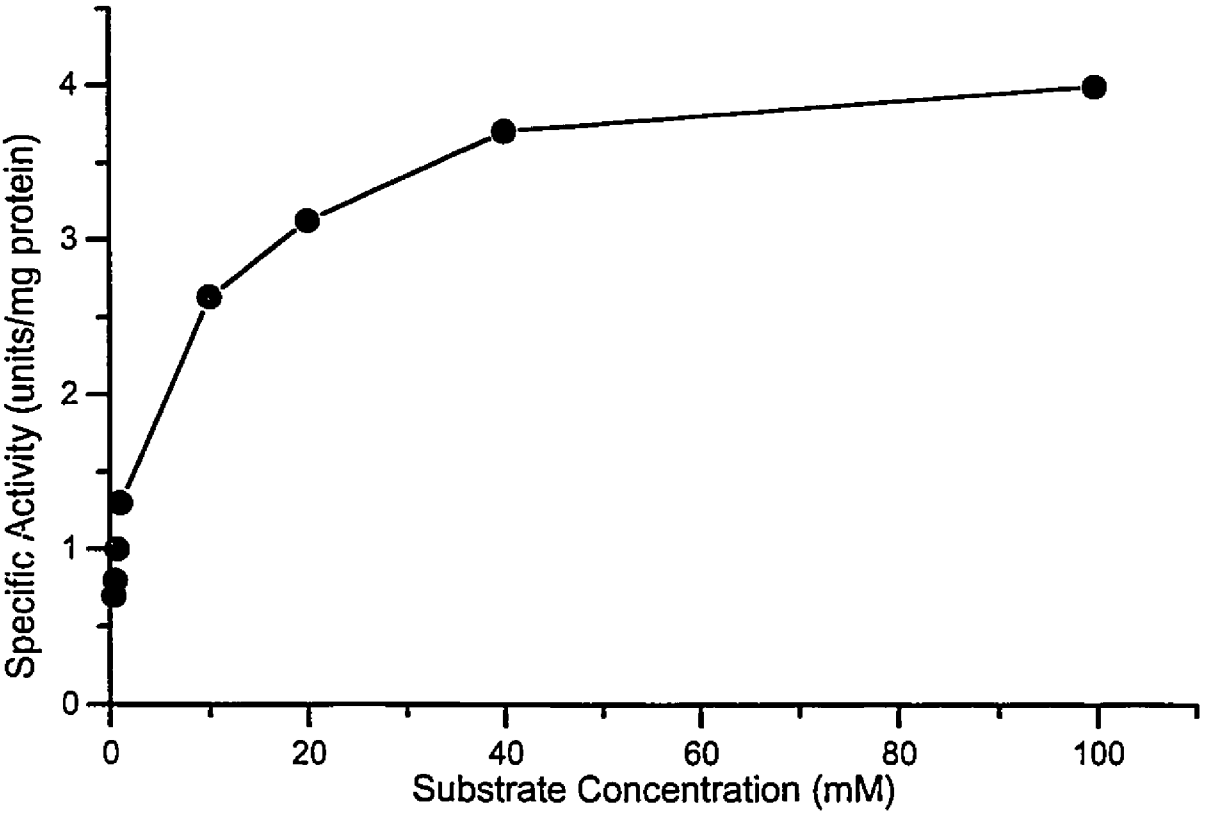

Specific Activity (units/mg protein)
0
1
2
3
4
0
20
40
60
80
100
Substrate Concentration (mM)

**Figure 22. Effect of substrate concentration (Cbz-Phe-Ala) on carboxypeptidase Y activity**

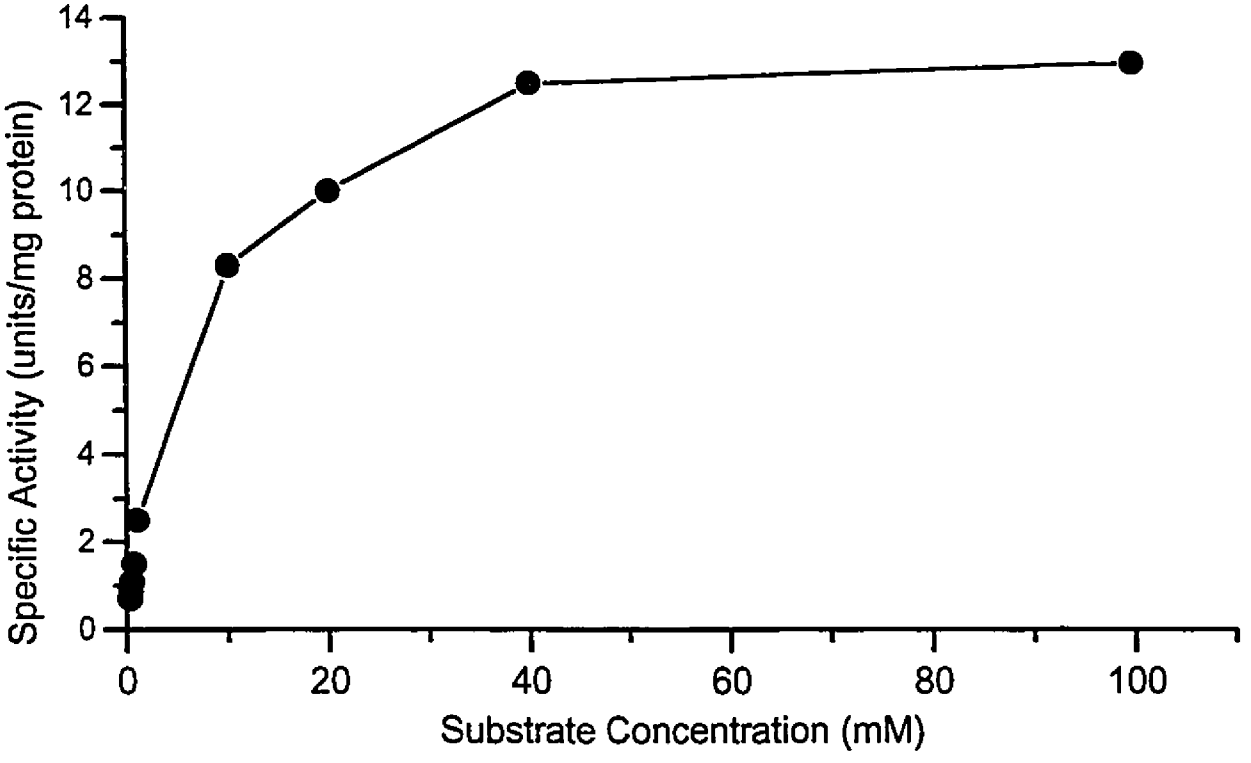

Specific Activity (units/mg protein)
14
12
10
8
6
4
2
0
0
20
40
60
80
100
Substrate Concentration (mM)

**Figure 23. Lineweaver-Burke plot of carboxypeptidase Y with BTPNA as substrate**

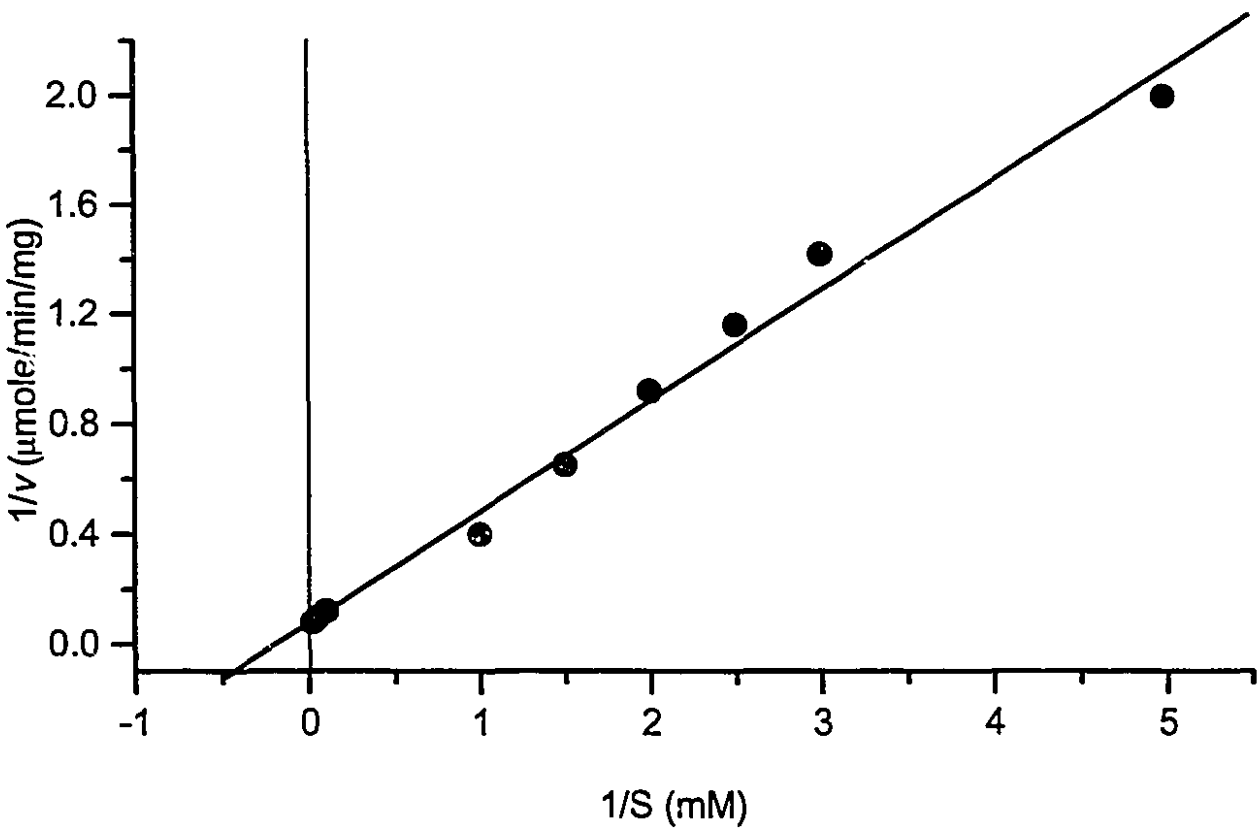

2.0
1.6
1.2
0.8
0.4
0.0
1/v (µmole/min/mg)
-1
0
1
2
3
4
5
1/S (mM)

**Figure 24. Lineweaver-Burke plot of carboxypeptidase Y with Cbz-Phe-Ala as substrate**

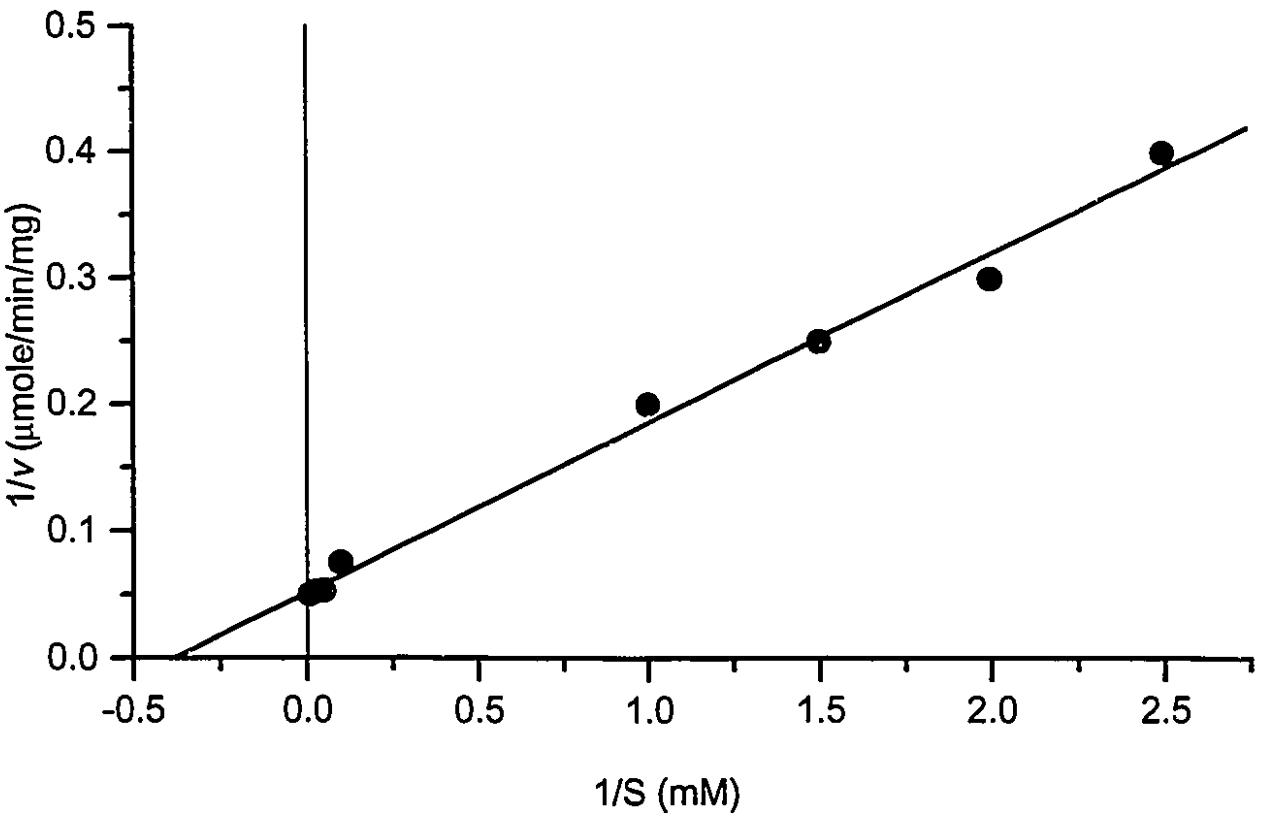

0.5
0.4
0.3
0.2
0.1
0.0
1/v (µmole/min/mg)
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
1/S (mM)

Figure 25. RP-HPLC profile of $\alpha_{S1}$-casein hydrolysed with trypsin

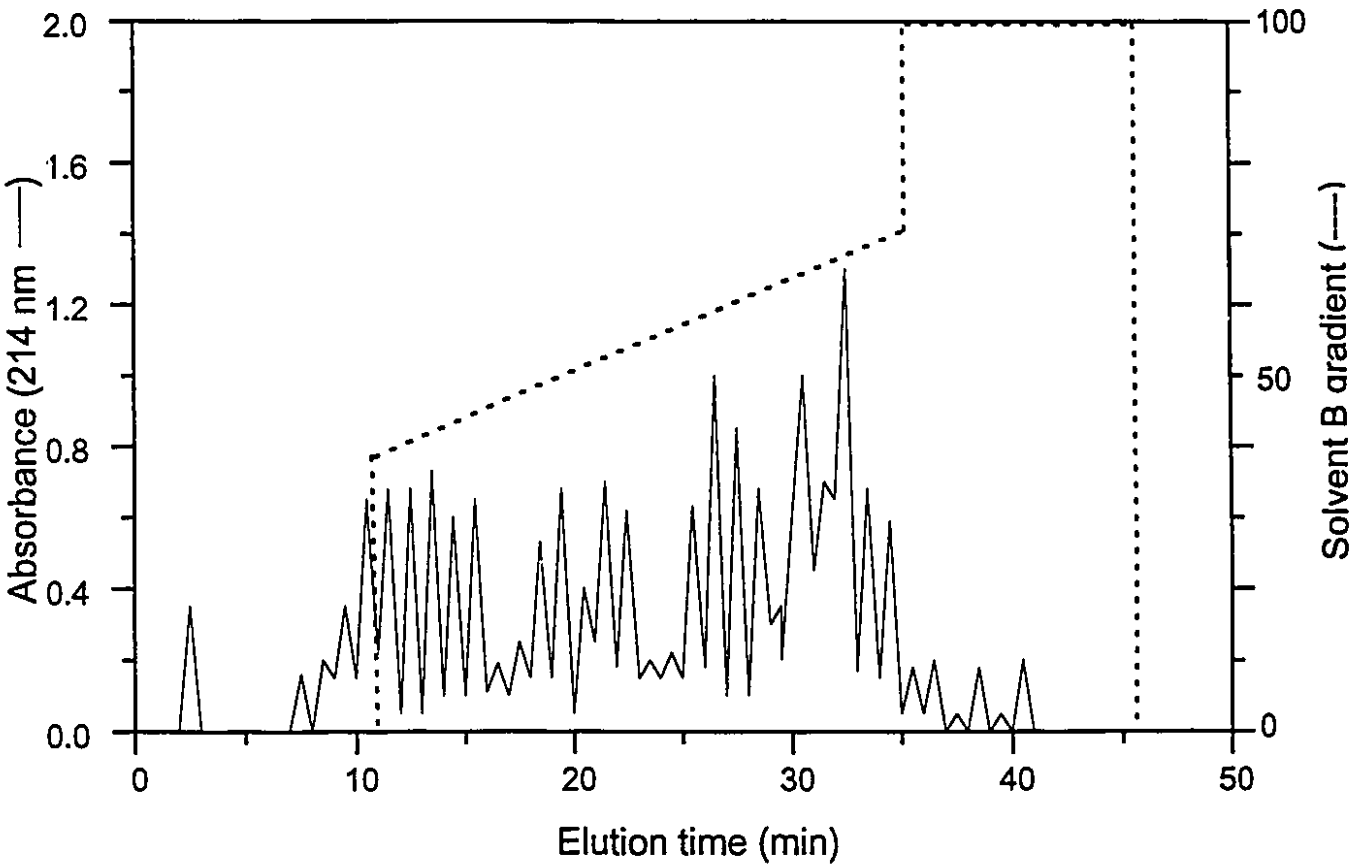

2.0
1.6
1.2
0.8
0.4
0.0
Absorbance (214 nm ——)
100
50
0
Solvent B gradient (----)
0
10
20
30
40
50
Elution time (min)

Figure 26. RP-HPLC profile of $\alpha_{S1}$-casein tryptic digest hydrolysed with carboxypeptidase

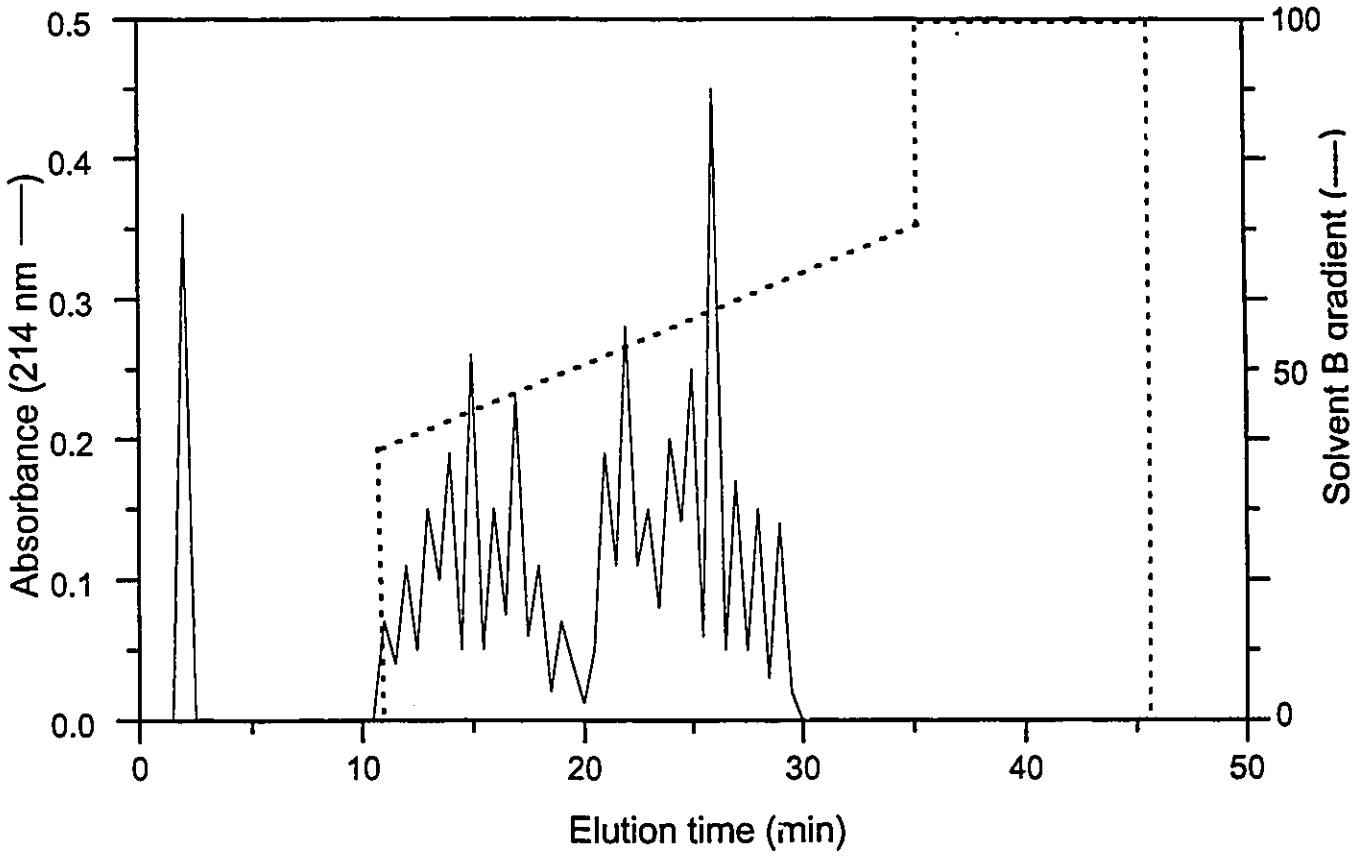

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance (214 nm —)
100
50
0
Solvent B gradient (—)
0
10
20
30
40
50
Elution time (min)

**Figure 27. RP-HPLC profile of β-casein hydrolysed with trypsin**

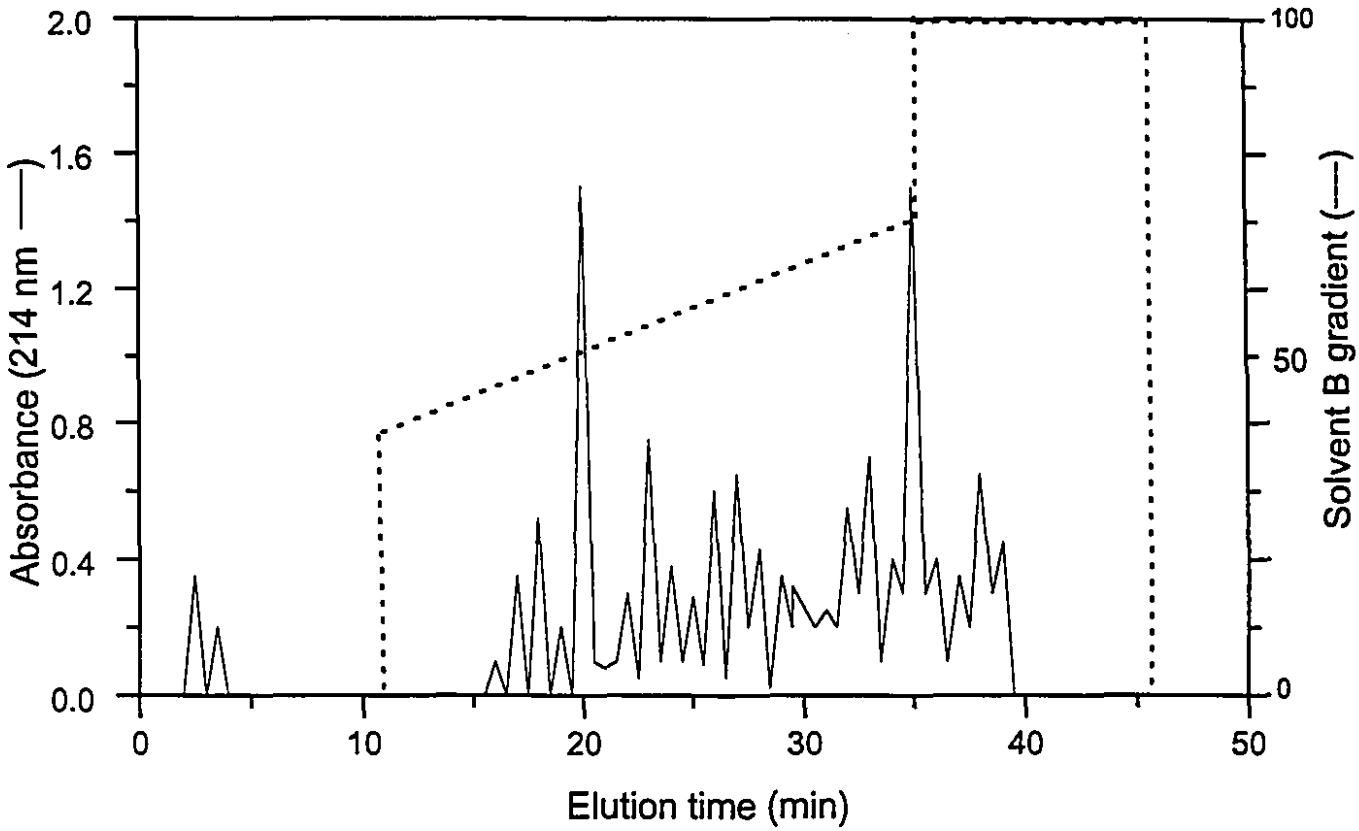

2.0
1.6
1.2
0.8
0.4
0.0
Absorbance (214 nm —)
100
50
0
Solvent B gradient (—)
0
10
20
30
40
50
Elution time (min)

**Figure 28. RP-HPLC profile of β-casein tryptic digest hydrolysed with carboxypeptidase**

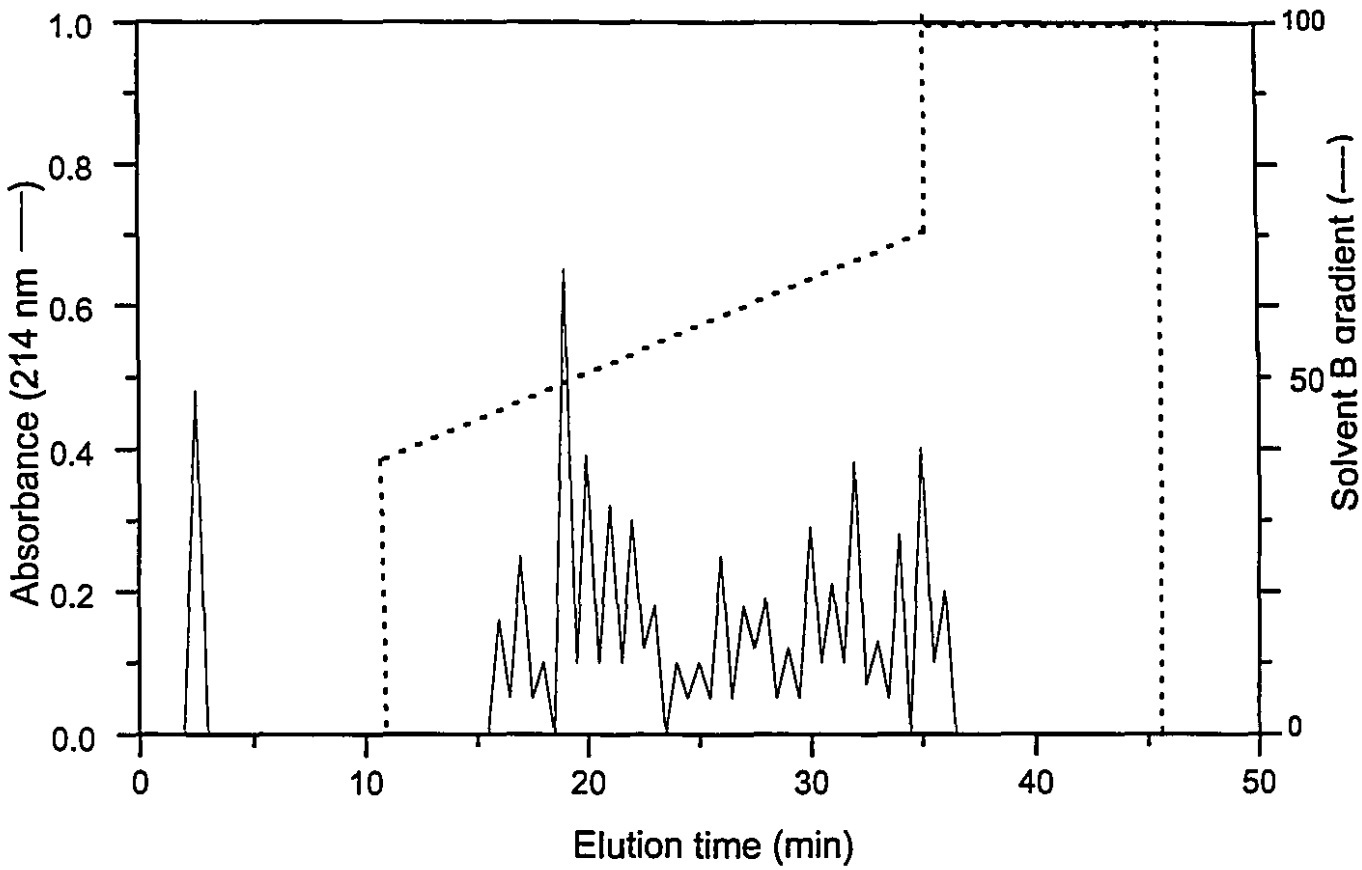

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance (214 nm —)
100
50
0
Solvent B gradient (—)
0
10
20
30
40
50
Elution time (min)

**Table 5. Kinetic values of the different Cbz-dipeptides derivatives assayed with carboxypeptidase Y**

**Table 5. Kinetic values of the different Cbz-dipeptides derivatives assayed with carboxypeptidase Y**

| Substrates | $K_m$ (mean ± SD) | $V_{max}$ (mean ± SD) |
|---|---|---|
| 1. Cbz-Pro-Phe | 2.5 ± 0.01 | 17.5 ± 2.3 |
| 2. Cbz-Glu-Tyr | 8.1 ± 0.01 | 22.7 ± 0.02 |
| 3. Cbz-Phe-Ala | 1.9 ± 0.01 | 28.0 ± 0.13 |
| 4. Cbz-Gly-Ala | 1.6 ± 0.1 | 9.9 ± 0.01 |
| 5. Cbz-Gly-Leu | 2.1 ± 0.01 | 11.9 ± 0.01 |
| 6. Cbz-Phe-Leu | 1.4 ± 0.03 | 4.8 ± 0.07 |
| 7. Cbz-Gly-Pro | 1.1 ± 0.03 | 10.0 ± 0.03 |

## 5.0 GENERAL CONCLUSIONS

Based on the results obtained from this study, a general conclusion can be drawn that carboxypeptidase Y from *Kluyveromyces fragilis* could be of importance in accelerated cheese ripening without bitterness. Its functionality at acid pH and low temperature is of great significance in accelerated ripening of hard cheeses such as Cheddar and Emmental. Normal ripening temperature is < 15°C and pH is between 4.3 to 5.5. Low temperature prevents the growth of unwanted microorganisms which could otherwise leads to spoilage. Low pH maintains the oxidation reduction potential of ripening enzymes during the long storage time. Furthermore, as shown by the ability of carboxypeptidase Y to remove hydrophobic peptides from the hydrolysates of $\alpha_{s1}$- and β-caseins, bitterness in cheese which is mostly observed with accelerated ripened cheese and enzyme modified cheese with added proteases could disappear.

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## 7.0 APPENDIX

**7.1** YPD Broth Media: Culture Media for Yeast Growth

| | |
|---|---|
| 1 % yeast extract | 10 g |
| 2 % peptone | 20g |
| 2 % dextrose | 20g |

Dissolve the appropriate g of nutrients into 1L of distilled water. Autoclave for 15 min at 121°C.

**7.2** YPD Agar Media: for plate count

| | |
|---|---|
| 1 % yeast extract | 10 g |
| 2 % peptone | 20g |
| 2 % dextrose | 20 g |
| 1 % agar | 10 g |

Dissolve the appropriate g of nutrients in 1L of distilled water. Autoclave for 15 min at 121°C

**7.3** Coomassie Staining Solutions (*Pharmacia Phast Coomassie Staining Manual*)

1. Fixing solution: 20 % trichloroacetic acid

2. Washing Solution: 30 % methanol and 10% acetic acid in distilled water (3:1:6)

3. Staining Solution: 0.02 % Phast Gel Blue R solution in approximately 30 % methanol and 10 % acetic acid in distilled water and 0.1 % (w/v) $CuSO_4$.

Stock solution: dissolve 1 Phastgel Blue R tablet in 80 ml of distilled water by stirring for 5-10 min. Add 120 ml of methanol and stir for 20 min. This makes 0.2 % solution.

Final solution: mix 1 part of filtered stock solution with 9 parts of methanol: acetic acid: distilled water (3:1:6) solution listed in step 2 above. Add $CuSO_4$ to 0.1 % (w/v). The $CuSO_4$ is added to decrease the background staining.

4. Destaining Solution: 30 % methanol and 10 % acetic acid in distilled water (3:1:6).