Purification and Characterization of a Carboxypeptidase Y from *Kluyveromyces fragilis* JSB95

JULIA C. TRANSFIGURACION, BYONG H. LEE,[1] SEUNG Y. PARK,[2] and FRED R. VAN DER VOORT
Department of Food Science and Agricultural Chemistry,
Macdonald Campus of McGill University,
Ste. Anne de Bellevue, QC, Canada H9X 3V9

ABSTRACT

A carboxypeptidase Y was purified to homogeneity from the crude cell extracts of *Kluyveromyces fragilis* JSB95 grown in yeast, peptone, and dextrose broth using an FPLC® system equipped with ion-exchange and gel filtration columns. The enzyme was purified 216-fold over the crude extract; recovery was 18%. The estimated molecular mass was 56 kDa and consisted of two subunits. The maximum activity of the purified enzyme was obtained at pH 6.0 and 25°C and was strongly inhibited by diisopropylphosphofluoridate and phenylmethylsulfonylfluoride as well as by some metal ions. The Michaelis constant and maximum velocity values for *n*-benzoyl-L-tyrosine-*p*-nitroanilide substrate were 5.1 m*M* and 22.58 μM/min per mg of protein; for Cbz-Phe-Ala, values were 2.98 m*M* and 22.58 μM per min/mg of protein. Carboxypeptidase Y showed hydrolytic activity against some hydrophobic peptides of the tryptic digests of α_{s1}- and β-caseins.

(**Key words**: carboxypeptidase Y, *Kluyveromyces fragilis*, cheese ripening, bitterness removal)

Abbreviation key: **BTPNA** = *n*-benzoyl-L-tyrosine-*p*-nitroanilide, **CBPA** = carboxybenzoxy phenylalanylalanine, **YPD** = yeast, peptone, and dextrose.

INTRODUCTION

Carboxypeptidases (E.C. 3.4.12) catalyze the sequential hydrolytic liberation of amino acids from the carboxyl terminal of peptides and proteins. Carboxypeptidases have been found in plants (16), animals (8), and microorganisms (9, 21); carboxypeptidase from yeast has been used extensively for the preparation of food protein hydrolysates in addition to more traditional cheese-making applications. More recently, carboxypeptidase has been gaining popularity for the accelerated ripening of cheese. Carboxypeptidase has the ability to degrade hydrophobic amino acid residues from the C-terminal of peptides and has full activity at low pH and at low ionic strength. During the last 10 yr, several methods have been developed to accelerate the maturation of cheese (11) to meet the increased product demand (11). Enzyme addition has been the most popular method; elevation of the ripening temperature and modification of starter culture also have been used. However, success has been hampered by defects in the final product, such as bitterness, rancidity, soapiness, and poor texture.

Bitterness arises from the hydrolysis of α_{s1}- and β-CN because of the accumulation of peptides containing a large proportion of hydrophobic amino acid residues (5, 12, 14, 17, 20). Recently investigated was the ability of nonstarter lactobacilli to hydrolyze peptides to minimize the accumulation of bitter peptides. Two aminopeptidases, proline-specific aminopeptidases, and esterases from *Lactobacillus casei* have been isolated and extensively studied for their suitability in producing accelerated ripened and enzyme modified cheeses (2, 4, 13, 18). Although these peptidases exhibit strong activities at neutral pH, they lack carboxypeptidase activity, which could be important for their function. Because most lactic acid bacteria are deficient in carboxypeptidases, the C-terminal of caseins is not hydrolyzed, and, thus, accelerated cheese ripening seems to be limited (3). This study was undertaken to isolate, purify, and characterize a carboxypeptidase Y from *Kluyveromyces fragilis* that was selected on the basis of high biomass yield on whey and to investigate its potential as a useful enzyme for the accelerated ripening of cheese without bitterness development in the acidic matrix of cheese.

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[1]Also with Food Research and Development Center, Agriculture and Agri-Food Canada, St. Hyacinthe, QC, Canada J2S 8E3.
[2]Department of Dairy Science, Yonam College of Livestock and Horticulture, Sunghwan, Chonan, Korea 333-800.

MATERIALS AND METHODS

Microorganism and Cultivation

Kluyveromyces fragilis JSB95 was initially selected from the best growth rates among three *K. fragilis* and two *K. lactis* (Agriculture Canada Food Research and Development Center, St. Hyacinthe, QC Canada) in whey permeate containing 0.5% (wt/vol) yeast extract. Stock culture was maintained in YPD broth (1%, wt/vol, yeast extract; 2%, wt/vol, peptone; and 2%, wt/vol, dextrose; Difco, Detroit, MI) containing 5% (vol/vol) glycerol and stored at –40°C. The strain was activated by two successive transfers (0.1%, vol/vol) and grown in YPD broth (1 L) at pH 5.0, and 25°C using a 1.5-L fermentor (Biostat M; Braun Inc., Burlingame, CA). Cell growth was monitored spectrophotometrically at 2-h intervals by following the absorbance at 600 nm. The enzyme production and activity were determined at 4-h intervals using *n*-benzoyl-L-tyrosine-*p*-nitroanilide (**BTPNA**) or carboxybenzoxy phenylalanine (**CBPA**) as model substrates.

Preparation and Purification of Carboxypeptidase Y

The cells were harvested by centrifugation at 10,000 × *g* for 10 min, and the supernatant was assayed for extracellular carboxypeptidase Y activity. The pellet obtained from the centrifugation was washed twice with 250 ml of 0.05 *M* sodium phosphate buffer, pH 7.0, and then subjected to cell disintegration by ultrasonication (Heat Systems Inc., Farmingdale, NY) for 10 min at 1-min intervals with the sample vessel immersed in ice-water and ethanol at 4°C. Crude enzyme extract (20 ml) was precipitated with ammonium sulfate between 50 and 90% saturation with gentle stirring at 4°C. The pellet was collected by centrifugation at 10,000 × *g* for 10 min, resuspended in 10 ml of 0.05 *M* sodium phosphate buffer, pH 7.0, and desalted by passage through a Sephadex G-25 column (Pharmacia, Montreal, QC, Canada) that was previously equilibrated with 0.05 *M* sodium phosphate buffer, pH 7.0.

The FPLC® system (Pharmacia, Uppsala, Sweden) was used for the enzyme purification. The system consisted of two P-500 pumps, LCC 501 controller, FRAC-200 fraction collector, UV-M II monitor, gradient mixer and pH control, REC 102 recorder, P-1 peristaltic pump, two MV-7 valves, MV-8 valve, and two superloops (10 and 50 ml). The fraction collector was programmed to collect 1.5 ml/min of the fraction of interest. The ion-exchange column (Mono Q HR 5/5) was pre-equilibrated with 0.01 *M* sodium phosphate buffer, pH 7.0 (buffer A), and 0.01 *M* sodium phosphate buffer, pH 7.0 plus 0.5 *M* NaCl (buffer B). The localization of carboxypeptidase Y in the chromatographic eluates was performed by injecting 1 ml of the desalted crude extract. The collected fractions were assayed for carboxypeptidase Y activity using CBPA as substrate. When the enzyme was localized, several injections were performed, and active carboxypeptidase Y fractions were collected, combined, and concentrated by ultrafiltration (Centricon-30; Amicon Corp., Toronto, ON, Canada). The concentrated samples were further subjected to gel filtration chromatography using a Superose 12 HR 10/30 column equilibrated with 0.01 *M* sodium phosphate buffer, pH 7.0. Several injections (100- to 200-μl volume) were performed with the same buffer as the eluent at a flow rate of 0.5 ml/min. The active carboxypeptidase Y fractions were pooled, concentrated by ultrafiltration, and assayed for carboxypeptidase Y activity and protein content.

Carboxypeptidase Y Assay

Carboxypeptidase Y activity was measured by using BTPNA and CBPA as substrates. The enzyme activity, using BTPNA as substrate, was determined according to the method of Aibara et al. (1) with minor modifications. The assay mixture was composed of 200 μl of diluted enzyme solution and 1 ml of the substrate solution (3.0 m*M* in 0.1 *M* sodium phosphate buffer, pH 7.0). The mixture was incubated at 25°C for 10 min, and the reaction was stopped by the addition of 0.5 ml of 0.5 *M* of $HgCl_2$. The enzymatic activity for CBPA was determined by estimation of the liberated amino acid at 570 nm using the modified method of Mathesion and Tattrie (15). The reaction mixture was 100 μl of the diluted enzyme solution and 900 μl of the substrate solution (1.5 m*M* in 0.05 *M* sodium phosphate buffer, pH 7.0) and was incubated at 25°C for 10 min. The reaction was terminated by the addition of ninhydrin reagent. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μM of *p*-nitroaniline or L-alanine per minute under the specified conditions of the assay. Specific activity was defined as the enzyme units per milligram of protein.

Protein Assay

Protein content was determined spectrophotometrically at 562 nm using the bicinchoninic protein assay reagent (19) (Pierce Chemicals, Rockford, IL). The reaction mixture was composed of 1 ml of diluted enzyme solution and 2 ml of the working reagent

incubated at 37°C for 30 min. Protein concentration of the samples was estimated by using bovine serum albumin as the standard.

Electrophoresis

A miniaturized Phast electrophoresis system (Pharmacia) was used to examine the purity of the enzyme both by native PAGE and SDS-PAGE at each purification step. The protein samples were applied on polyacrylamide gels (4% stacking; 8 to 25% resolving) and stained with Coomassie blue G-250 (Bio-Rad, Hercules, CA). The relative molecular mass of the pure carboxypeptidase Y was determined on SDS-PAGE by the method of Laemmli (10) using low molecular mass markers: albumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase *b* (94 kDa). High molecular mass markers used for native PAGE were bovine serum albumin (67 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

Properties and Kinetics

The effect of pH on carboxypeptidase Y activity was determined in 0.05 *M* acetate (pH 3 to 5), 0.05 *M* sodium phosphate (pH 6 to 8), and 0.05 *M* glycine-NaOH (pH 9 to 10) buffers at 25°C. The effect of temperature on carboxypeptidase Y activity was determined by incubating the assay mixture at different temperatures ranging from 10 to 70°C. The assay mixture consisted of 900 μl of the buffer containing 1.5 m*M* of CBPA or 3.0 m*M* of BTPNA and 100 μl of the pure enzyme. The kinetic constants for the two substrates were determined by plotting the reciprocals of the reaction velocity against the substrate concentration (Lineweaver-Burk plot), and the kinetic values were computed from the intercept and slope of the regression line. The effects of inhibitors

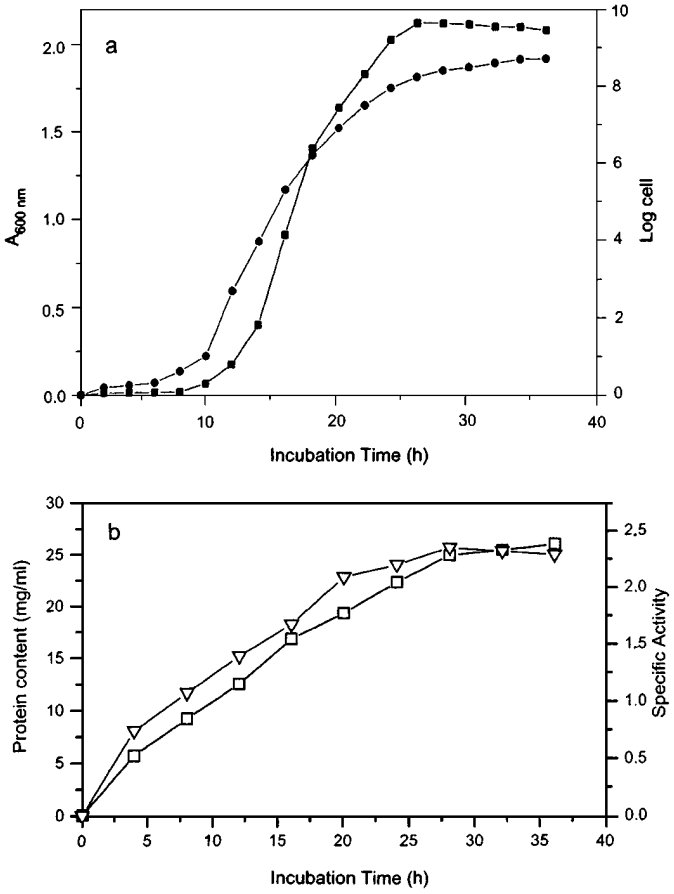

Figure 1. Growth curve of *Kluyveromyces fragilis* JSB95 (a) and carboxypeptidase Y specific activities (b) during 36 h of incubation [absorbance (A) at 600 nm wavelength, ■; $\log_{10}$ cell, ●; protein content, □; and specific activity, ▽].

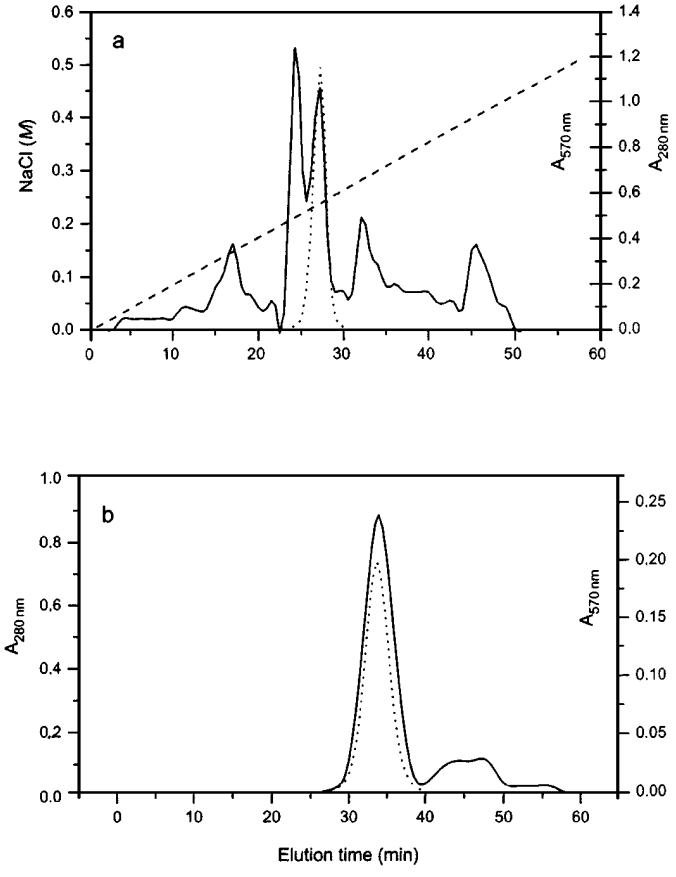

Figure 2. Profiles of carboxypeptidase Y from *Kluyveromyces fragilis* JSB95 after ion-exchange chromatography (a) and after gel filtration chromatography (b). Absorbance at 280 nm ($A_{280\ nm}$, —) or 570 nm ($A_{570\ nm}$, - - -) wavelength.

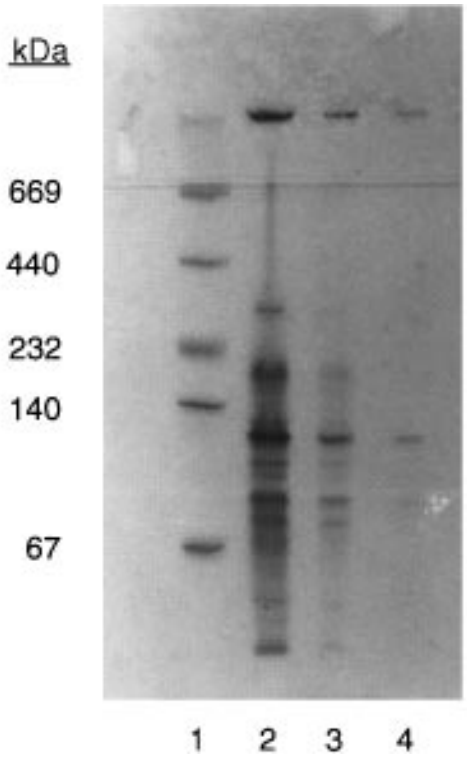

Figure 3. Native PAGE patterns of crude extract and semipurified and purified carboxypeptidase Y from *Kluyveromyces fragilis* JSB95: (lane 1, standard; lane 2, crude extract; lane 3, after ion-exchange chromatography; and lane 4, after gel filtration chromatography).

and metal ions (Table 2) on the enzyme activity were determined by incubating 100 μl of the pure enzyme and 100 μl of the inhibitor or metal ion at final concentrations of 0.1 and 1.0 m*M*. Inhibition was expressed as the percentage of the activity without the effectors. All assays for this study were performed in triplicate from which the means and standard deviations were calculated.

Hydrolytic Activity on Tryptic Digests of α_{s1}- and β-CN

The hydrolytic activity of carboxypeptidase Y on the tryptic digests of α_{s1}- and β-CN was determined by incubation of 500 μl (1 mg/ml) of the respective milk proteins with 4.53 U of the pure enzyme at 37°C for 12 h. All reactions were terminated by heat treatment at 80°C for 10 min. The hydrolyzed samples were then dried (Speed Vac; Savant Ins., Farmingdale, NY), reconstituted with distilled H_2O, and filtered with a 0.45-μm syringe filter (Nyphon, Millipore City, Japan) before injection. Portions (25 μl) were injected into a Delta Pak C18 column (30 × 150 mm; Millipore, Nepean), and the peptides from the hydrolysates were analyzed with a reverse-phase HPLC system (Millipore, Milford, MA). Elution was performed with a binary gradient from 0 to 100% B at a flow rate of 0.5 ml/min. Solvent A was Milli Q H_2O, and solvent B was 60% acetonitrile and 40% H_2O (HPLC grade). Both solvents contained 0.1% and 0.08% trifluoroacetic acid, respectively. The peak absorbances were monitored at 214 nm.

RESULTS

Production and Purification of Carboxypeptidase Y

Figure 1a shows the growth curve of *K. fragilis* in YPD broth during 36 h of incubation. Cell production was at maximum when obtained during 26 h of

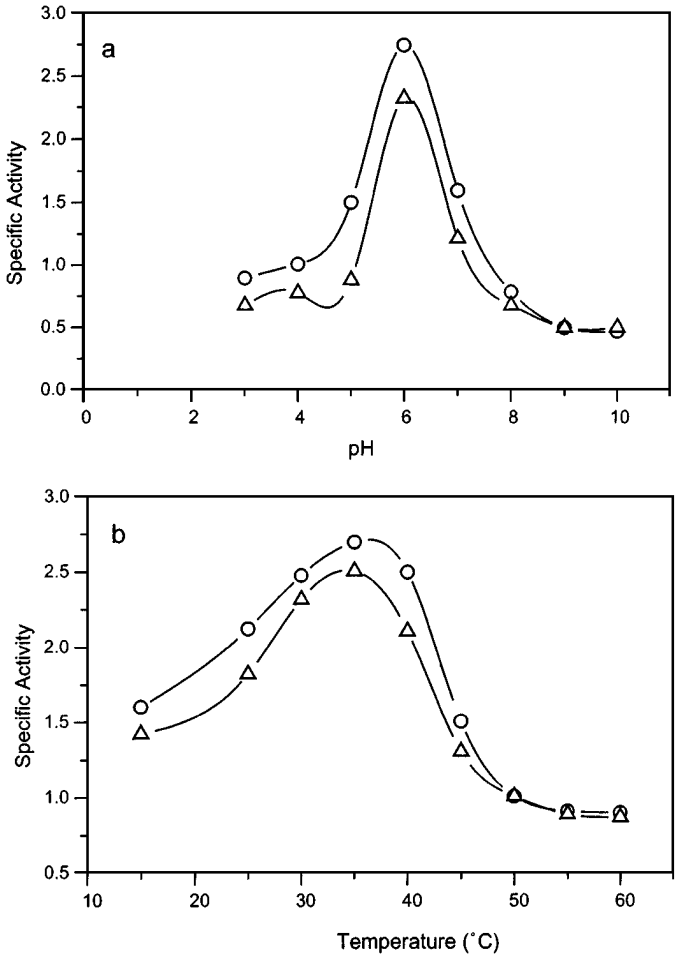

Figure 4. Effects of pH (a) and temperature (b) on carboxypeptidase Y activity (carboxybenzoxy phenylalanylalanine, □; *n*-benzoyl-L-tyrosine-*p*-nitroanilide, o).

TABLE 1. Purification steps of carboxypeptidase from *Kluyveromyces fragilis* JSB95.

Purification step	Total protein	Total activity	Specific activity	Purification	Yield
	(mg)	(U)[1]	(U/mg)[2]	(-fold)	(%)
Crude extract	241.0	510.0	2.1	1	100
Ammonium sulfate precipitation	168.0	507.0	3.0	1.4	99
Ion-exchange chromatography	2.1	275.0	131.0	62.5	54
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 1 μM of *p*-nitroaniline or L-alanine/minute under the specified conditions of the assay.

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

growth at the late logarithmic phase; enzyme production was maximum at 28 h of incubation and had a specific activity of 2.25 U (Figure 1b). A representative purification scheme of carboxypeptidase Y from *K. fragilis* is presented in Table 1. About 99% of the carboxypeptidase Y activity in the crude extract remained in the ammonium sulfate fraction after precipitation (50 to 90%). The elution profile of the crude extract on the ion-exchange column is shown in Figure 2a. The carboxypeptidase Y activity was detected in the fraction eluted at a salt concentration of 0.26 *M*. Approximately 62.5-fold of purification and 54% yield over the crude extract were obtained with ion-exchange chromatography. Several injections were subsequently performed on the ion-exchange column, and the active fractions were combined and concentrated. Figure 2b shows the profile of gel filtration chromatography of carboxypeptidase Y on the Superose 12 HR 10/30 column. A single peak was eluted between 28 and 38 min that had high specific activity (453 U/ml). Consequently, the enzyme was purified approximately 216-fold with 18% recovery in activity over the crude extract.

The purified carboxypeptidase Y appeared to have an apparent molecular mass of 56 kDa on an SDS-PAGE gel (not shown). The molecular mass was estimated to be 120 kDa either on native PAGE gel (Figure 3) or by gel filtration chromatography. These results suggested that carboxypeptidase Y consists of two subunits.

Properties and Kinetics of Carboxypeptidase Y

The optimum pH and temperature for carboxypeptidase Y activities were obtained at pH 6 and 35°C, respectively (Figure 4). Activity decreased sharply after 45°C and was lost completely at 70°C; 50% of its activity was retained at lower temperatures (15 to 40°C; Figure 4b). The pure carboxypeptidase Y (100 μl) was reacted in the presence of 1.0 m*M* of metal cations (100 μl) at 25°C, and the enzyme activity was measured after 30 min under the standard conditions.

Most of the metal ions used in this study inhibited the enzyme activity. The Ni^{2+} ion inhibited enzyme activity most, followed by Mn^{2+}, Mg^{2+}, and Cu^{2+} (Table 2). Among the specific inhibitors (0.1 m*M*) used in the study, diisopropylfluorophosphate and

TABLE 2. Effects of metal ions (1.0 m*M*) and specific inhibitors (0.1 m*M*) on carboxypeptidase Y of *Kluyveromyces fragilis* JSB95.[1]

Agent	Relative activity
	(%)
Control	100
Ag^{2+}	47
Ba^{2+}	55
Ca^{2+}	50
Co^{2+}	46
Cr^{2+}	48
Cu^{2+}	55
Fe^{2+}	52
Hg^{2+}	50
Mg^{2+}	42
Mn^{2+}	40
Ni^{2+}	32
DIPF[2]	32
PMSF	36
EDTA	100
Fe_2SO_4	97
$CoCl_2$	100
$HgCl_2$	62
$ZnCl_2$	84
Iodoacetic acid	84
Iodoacetamide	86
N-Bromosuccinamide	86

[1]Means of triplicate analysis.

[2]DIPF = Diisopropylphosphofluoidates; PMSF = phenylmethylsulfonylfluoride.

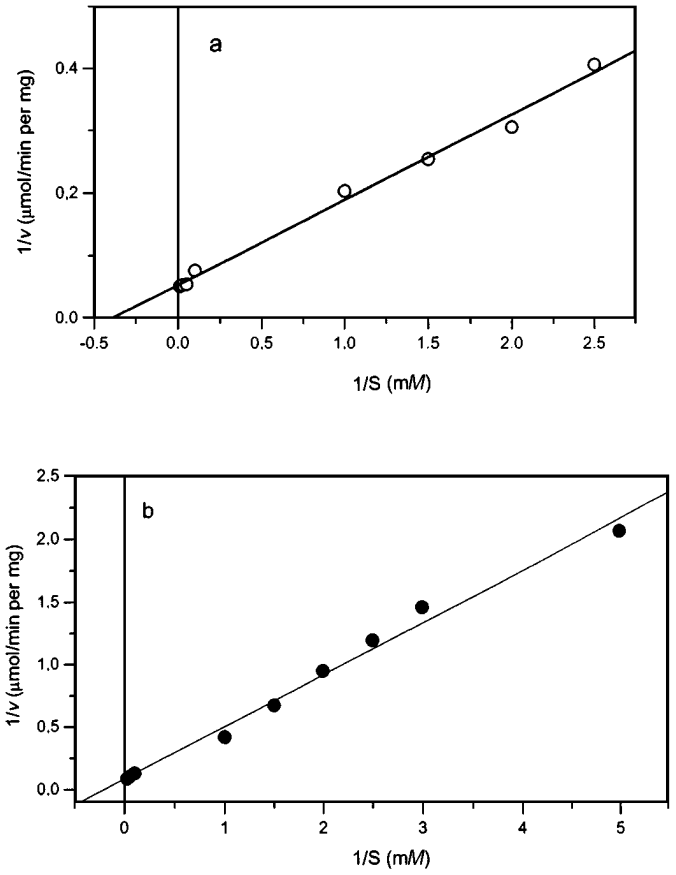

Figure 5. Lineweaver-Burke plot of carboxybenzoxy phenylalanylalanine (CBPA) (a) and *n*-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA) (b) on the purified carboxypeptidase Y (CBPA, □; BTPNA, o).

phenylmethylsulfonylfluoride, a specific inhibitor for serine protease, inhibited enzyme activity; residual activities were 32 and 36%. With other inhibitors, inhibition was slight or nonexistent.

The Michaelis constant and maximum velocity values of the purified carboxypeptidase Y were estimated with both substrates using a Lineweaver-Burke plot. The Michaelis constant and maximum velocity were about 2.98 m*M* and 22.58 μM/min per mg of protein for CBPA (Figure 5a) and 5.1 m*M* and 13.4 μM/min per mg of protein for BTPNA (Figure 5b), respectively. This result indicates that CBPA has stronger affinity to the enzyme than BTPNA.

Hydrolytic Activity on Tryptic Digests of α_{s1}- and β-CN

Figures 6 and 7 show the reverse-phase HPLC chromatograms of the tryptic digests of α_{s1}- and β-CN, and their hydrolysates that were hydrolyzed by carboxypeptidase Y. Many hydrophobic peptides were eluted between 30 and 40 min from the tryptic digests of α_{s1}-CN in Figure 6. When these tryptic digested peptides were further digested with pure carboxypeptidase Y, the amounts of peptides were substantially reduced (Figure 6b). Most of the hydrophilic peptides peak areas and heights were also reduced. Seven hydrophobic peptides from β-CN hydrolysates that were eluted between 37 and 45 min disappeared when they were hydrolyzed further with carboxypeptidase Y (Figure 7). The peak areas of these peptides were reduced to below the detectable level after hydrolysis with the pure enzyme, and the peak areas and heights of many of these peptides were substantially reduced as well.

DISCUSSION

Carboxypeptidase Y activity from *K. fragilis* was mostly detected in the cell-free extract after disintegration of cells, thus indicating the intracellular

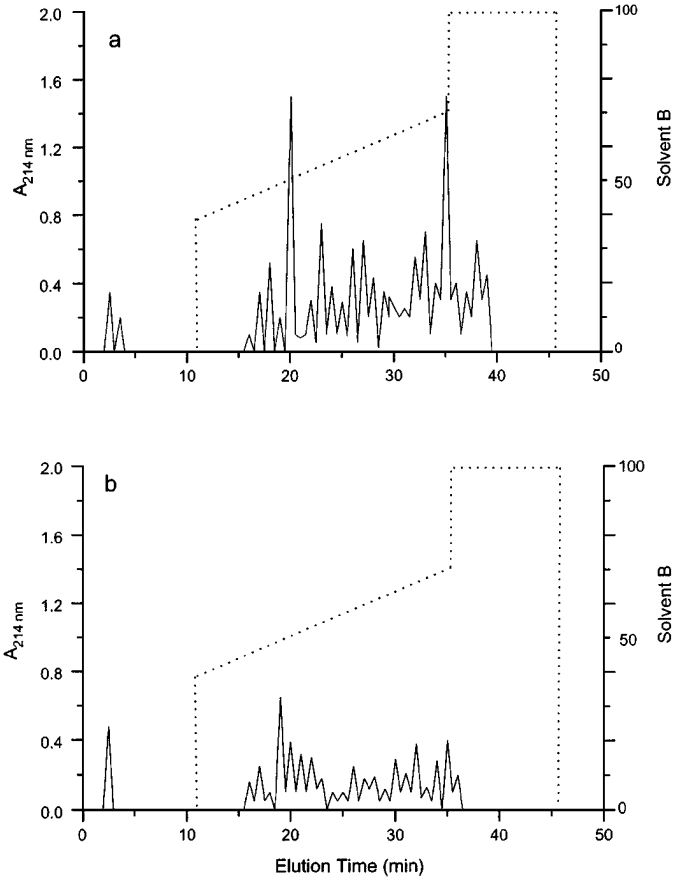

Figure 6. Reverse-phase HPLC profiles of α_{s1}-CN tryptic digest (a) and its hydrolysate digested by carboxypeptidase Y (b). Absorbance at 214 nm wavelength ($A_{214\,nm}$, —); solvent (- - -).

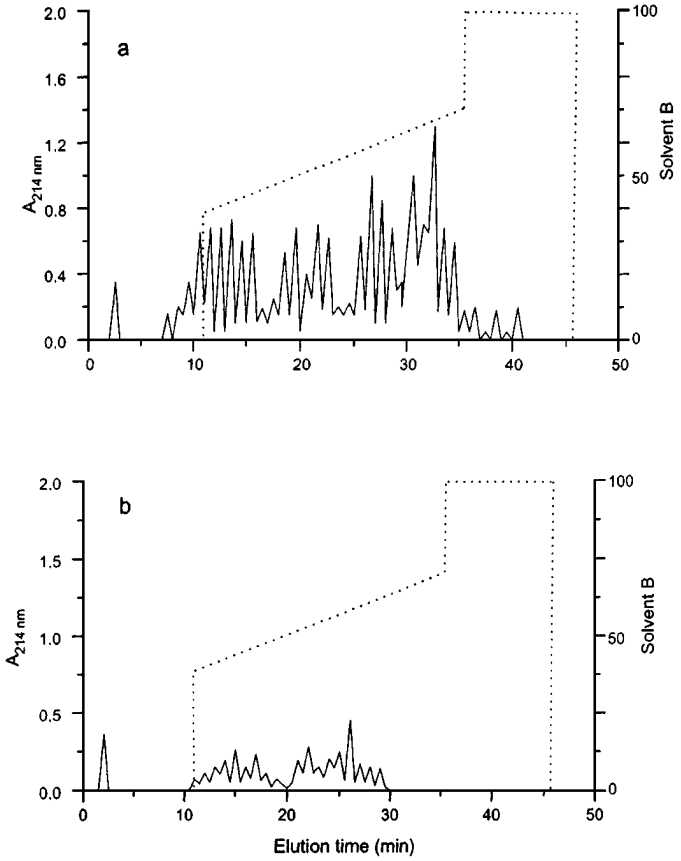

Figure 7. Reverse-phase HPLC profiles of β-CN tryptic digest (a) and its hydrolysate digested by carboxypeptidase Y (b).

nature of this enzyme. The FPLC® system was a more convenient and efficient technique to purify the enzyme than was the conventional chromatographic technique, which requires a longer elution time (5). The molecular mass (56 kDa) and the two subunit structures of this enzyme obtained in this experiment coincide well with the reports of Hayashi et al. (7) and Johansen et al. (9). The temperature optimum of this carboxypeptidase Y was lower than the previously reported carboxypeptidase of *Saccharomyces cerevisiae*. The significant activity at low temperature (15°C) might be useful for the acceleration of cheese ripening.

The inhibition of enzyme activity by some metal ions, such as the mercurials, has been reported for the carboxypeptidase Y from *Saccharomyces cerevisiae*, because of the presence of a histidine residue at its active sites (6). Strong inhibition by diisopropylfluorophosphate and phenylmethylsulfonylfluoride suggests that this enzyme belongs to the category of serine carboxypeptidase. The strong affinity to the enzyme by CBPA is because carboxypeptidase Y specifically cleaves the C-terminal of peptides and proteins. Hence, the rate of release of the free amino acid from peptides blocked at the N-terminal is faster than any other kinds of substrates involved.

Carboxypeptidase Y from *K. fragilis* had the ability to hydrolyze many hydrophobic peptides from the proteolytic digests of α_{s1}- and β-CN, which could have important implications for the cheese industry. Further study of the role of carboxypeptidase Y to the bitter peptides release by trypsin digests of α_{s1}- and β-CN is underway.

In conclusion, a carboxypeptidase Y from *K. fragilis* can be produced in YPD broth and can be purified efficiently with a high yield using an FPLC® system consisting of Mono Q HR 5/5 and Superose 12 HR 10/30 columns.

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