International Food Research Journal 32(2): 607 - 620 (April 2025)
Journal homepage: *http://www.ifrj.upm.edu.my*

Harvesting BaCaseinase from *Bacillus altitudinis* 41KF2b for production of bovine casein hydrolysate with potential bioactivities

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Article history

Received:
20 June 2024
Received in revised form:
1 May 2025
Accepted:
5 May 2025

Keywords

angiotensin-converting enzyme inhibitory (ACE-I) capacity, antioxidant activity, Bacillus altitudinis, caseinase, metabolic syndrome

Abstract

Recently, casein-derived hydrolases (BCH) have shown great potential for developing natural alternative treatments to treat metabolic syndrome. The present work aimed to isolate microorganisms that produce an extracellular caseinase from soil samples of rice root systems. The caseinase activity was determined by skim milk agar (SMA) assay. The BLASTn tool was used for phylogenetic analysis. The caseinase was purified by ammonium sulphate precipitation. The caseinase kinetics was determined to produce casein-derived hydrolases (BCH). The BCH revealed the antioxidant and the angiotensin I-converting enzyme inhibitory (ACE-I) activities. Our screening yielded caseinase secreted by a bacterial strain with 99% homology to *Bacillus altitudinis* 41KF2b called BaCaseinase. We then purified and determined the stability and kinetics of the BaCaseinase. It presented a Michaelis constant (Km) of 1.108 mg/mL, and a maximum velocity (Vm) of 21.27 × 10^4 U/mg at 45°C and pH 8. Next, we used the purified BaCaseinase to produce a bovine casein hydrolysate (BCH). The BCH showed three half-maximal inhibitory concentrations, including (IC_{50}) of 0.60 ± 0.003 mg/mL for 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, 0.13 ± 0.003 mg/mL for 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging activity, and 0.43 ± 0.002 mg/mL for ACE-I activity. The purified BaCaseinase is a heat-stable enzyme. This enzyme could digest bovine casein under multiple extreme conditions (an alkaline pH and a wide temperature range) to produce BCH that has both antioxidant and ACE-I activities. This enzyme shows promise for producing new hydrolysates, with the potential to treat metabolic syndrome.

Abbreviations

OD: optical density; DPPH: 1,1-diphenyl-2-picrylhydrazy; ABTS: 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; and IC_{50}: half-maximal inhibitory concentration.

DOI
https://doi.org/10.47836/ifrj.32.2.22

Introduction

The prevalence of metabolic syndrome is increasing throughout the world (Koirala *et al.,* 2023). One of the components of metabolic syndrome is hypertension or high blood pressure; it has been attributed as one of the main risk factors for cardiovascular diseases. A primary solution to control hypertension is to inhibit angiotensin-converting enzyme (ACE) (Stanciu *et al.,* 2023).

Casein-derived hydrolysates are considered natural origins containing bioactive peptides that have been reported to exert antihypertensive, antibacterial, antioxidant, and immunomodulatory activities (Silva and Malcata, 2005; Phelan *et al*., 2009; Marcone *et al*., 2017; Koirala *et al*., 2023). An older report reviewed 18 of 33 peptides in a bovine casein hydrolysate (BCH) containing inhibitory activity to ACE (FitzGerald and Meisel, 2000). Due to these health-promoting properties, casein-derived hydrolases have great potential to produce nutraceuticals and pharmaceuticals in alternative treatments for metabolic syndrome (Koirala *et al*., 2023).

When different enzymes are used for hydrolysis, the reaction products may have different bioactivities. Indeed, researchers have shown that casein hydrolysis with pepsin did not produce

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hydrolysed peptides with antioxidant activity, whereas casein hydrolysed with trypsin, a proteinase, or a combination of enzymes displayed only ACE inhibitory activity (Pihlanto-Leppälä *et al*., 2000; Mohanty *et al*., 2016; Shanmugam *et al*., 2021). The differences in the specific bioactive effects of casein hydrolysates may be attributed to the enzyme potency or the kind of enzymes used for hydrolysis to produce bioactive peptides from different protein sources (FitzGerald and Meisel, 2000; Phelan *et al*., 2009). It is, therefore, essential to discover a novel caseinase that produces bioactive hydrolysates containing bioactivities, including antioxidant activity and the ability to inhibit ACE from different protein sources using a single highly pure caseinase harvested from microbial origin (Tacias-Pascacio *et al*., 2023). Several publications have revealed that tyrosine residues are critical for most bioactive activities in hydrolysates (Pihlanto-Leppälä *et al*., 2000; Mohanty *et al*., 2016; Marcone *et al*., 2017; Shanmugam *et al*., 2021).

Generally, *Bacillus* species are specific producers of extracellular proteases, including those that can degrade casein. The use of the skim milk agar (SMA) assay enables the qualitative determination of protease activity in general, or caseinase activity specifically. Moreover, this technique directly detects the extracellular proteases in the culture medium (Perwendha *et al*., 2020). Enzyme stability and kinetics features may determine the specific activities of enzymes, rendering them more appropriate for industrial applications.

The primary objective of the present work was to isolate an extracellular proteinase from soil samples of rice root systems that could degrade casein. Our screening yielded a BaCaseinase from *Bacillus altitudinis* 41KF2b. We subsequently characterised the optimal conditions for this enzyme. We used it to produce a BCH, and evaluated its antioxidant and antihypertensive activities to assess its potential applications in food, nutritional, and pharmaceutical products. This isolated BaCaseinase could be used in industrial applications to develop novel products to treat metabolic syndrome.

Materials and methods

Screening of microorganisms that produce caseinase

Subsoil samples of rice root systems from three provinces (Tien Giang, BenTre, and LongAn), Vietnam were collected. Subsoil samples were collected at five distinct positions to make a representative sample of each province. Subsequently, representative samples from three provinces were preserved carefully in the container at 4 - 10°C, and transported immediately to the laboratory. Upon arrival, 1 g of the representative sample was resuspended in 9 mL of sterilised distilled water to obtain 10^{-1} dilution. Then, 1 mL of this was added to 9 mL of 0.9% NaCl solution to make 10^{-2} dilution. These dilutions were spread separately onto nutrient agar (NA) plates containing 3 g/L beef extract, 5 g/L peptone, 0.5 g/L NaCl, and 20 g/L agar. The plates were incubated at 37°C for 24 h. Subsequently, 32 colonies on NA were streaked on a caseinase-production medium named SMA containing 28 g/L skim milk, 5 g/L enzymatic casein hydrolysate, 2.5 g/L yeast extract, 1 g/L dextrose, and 15 g/L agar. The SMA plates were incubated at 37°C for 24 h. Following incubation, L9 strain exhibited the highest caseinase activity on SMA, and was selected for subsequent study.

Molecular identification and phylogenetic analysis of L9 strain

The genomic DNA of L9 strain was used as a template for molecular identification through polymerase chain reaction (PCR) amplification. The following primer pair targeting the 16S ribosomal RNA (rRNA) gene was used: 27F (forward), 5′GAGTTTGATCCTGGCTCAG-3′; and 1492R (reverse), 5′-AAGGAGGTGATCCAACC-3′. Each PCR used 1 µL of the template mixture (Dada *et al*., 2013). The PCR products were analysed with 1.5% agarose gel electrophoresis to identify the target band (approximately 1,500 base pairs [bp]). The isolated PCR products were sequenced, and the final sequences were submitted to the GenBank database (National Center for Biotechnology Information [NCBI]). Phylogenetic analysis was conducted using the BLASTn tool to identify similar sequences archived in the NCBI database. The generated phylogenetic tree was constructed with the MEGA 7.0 software, employing the neighbour-joining DNA distance algorithm with a bootstrap of 1,000. During tree construction, positional differences between species pairs were evaluated, attributing bootstrap values at the base of branches with 1,000 resampling iterations. Confidence levels were determined based on the neighbour-joining bootstrap values: > 85%,

high confidence; 65 - 85%, average; and < 65%, low. These values are based on the MEGA 7.0 software criteria.

Effects of temperature, pH, and carbon source on BaCaseinase production

Following molecular identification, L9 strain was identified as *Bacillus altitudinis* 41KF2b, and exhibited the highest caseinase activity in a specialised medium containing 1 g/L bovine casein, 5 g/L peptone, 5 g/L tryptone, 1 g/L glucose, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L KH_2HPO_4, 0.1 g/L $CaCl_2$, and 0.1 g/L KH_2HPO_4, adjusted to pH 8.0; hence, this strain was subcultured in Luria-Bertani (LB) broth containing 10 g/L peptone, 5 g/L yeast extract, 0.5 g/L NaCl, and pH 7.0 to determine the effects of temperature, pH, and carbon source on BaCaseinase production. Subsequently, a 1% proliferation culture was introduced into a 250-mL baffled Erlenmeyer flask containing 100 mL of the caseinase-production media, followed by an incubation for 6 d at 37°C, with constant agitation at 160 rpm. The culture medium was centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatant was utilised to estimate the activity of a crude BaCaseinase. Controls were medium without adding the strain in all experiments. The effect of three factors on BaCaseinase production was performed on the aforementioned medium to evaluate temperature (20, 25, 37, and 40°C), pH (7.0, 8.0, 9.0, 10.0, 11.0, and 12.0), and carbon source (1% w/v glucose, sucrose, lactose, fructose, and maltose). The analysis followed a one-factor strategy to determine the best parameters for BaCaseinase production. Controls were medium without adding the strain in all experiments (Rachanamol *et al*., 2017).

BaCaseinase activity assays

Caseinase or BaCaseinase activity (U/mL) was measured according to Kunitz (1947) with slight modifications. Initially, a reaction mixture was 2.5 mL of a 0.5% casein solution with 250 µL of the caseinase or BaCaseinase solution mixed before conducting the reaction at 37°C for 10 min. After that, this mixture was supplied with 2.5 mL of 110 mM trichloroacetic acid, and continuously incubated at 30 min and 37°C to stop the reaction. Next, this mixture was filtered by a 0.45-µm syringe filter. Finally, 2 mL of the filtrate was transferred to a new tube, and then 500 µL of 0.5 M sodium carbonate and 500 µL of 0.5 mM Folin-Ciocalteu were added. The reacted final solution was incubated continuously at 50°C for 30 min to determine the released products using a spectrophotometer (Sigma-Aldrich) at the absorbance of 660 nm. The amount of enzyme required to release 1 µg of tyrosine/min under standard conditions was assessed as one unit of enzyme activity.

BaCaseinase purification by ammonium sulphate precipitation and concentration

Ammonium sulphate precipitation was used to purify BaCaseinase (Mothe and Sultanpuram, 2016). After growing *B. altitudinis* 41KF2b on a defined medium, the cell-free supernatant containing BaCaseinase was collected by centrifugation at 10,000 rpm for 30 min. Test tubes containing 100 mL of the above solution were added respectively at five different concentrations of ammonium sulphate (40, 50, 60, 70, and 80%), before being softly stirred for 16 h at 4°C. After that, these mixtures were re-centrifuged, and the pellets were suspended in a required volume of phosphate-buffered saline (PBS, 20 mM; pH 8.0). Further purification was carried out using a 3K concentrator (PrierceTM Protein Concentrator-88512) of 70 - 100 mL of BaCaseinase solution, and was concentrated to 12 - 15 mL. The protein content of the BaCaseinase solution was estimated using the Bradford method (Bradford, 1976), with bovine serum albumin (BSA) as the standard. The molecular weight of BaCaseinase was determined with sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) with a 12% (w/v) separating gel and a 5% (w/v) stacking gel, as reported by Laemmli (1970). The SDS-PAGE gel was stained by Coomassie Brilliant Blue R-250 to visualise the protein bands. The PageRuler Prestained Protein Ladder (10 - 250 kDa) was used as a standard protein marker. Purified BaCaseinase was used for subsequent analyses.

Effects of temperature and pH on BaCaseinase activity

All reaction mixtures to assess temperature parameter on the purified BaCaseinase activity were conducted repeatedly as a BaCaseinase activity assay, except for a purified BaCaseinase concentration of 0.01 mg. All reaction mixtures were incubated at nine distinct temperatures between 20 and 60°C for 1 h. To evaluate the effect of pH, bovine casein (0.5%) was added to buffers with a pH of 2 - 12 at 45°C for 1 h. The buffers included 20 mM glycine-HCl for pH 2.0 - 3.0; acetate buffer for pH 4.0 - 6.0; Tris-HCl for pH

7.0 - 8.0; and glycine-NaOH for pH 9.0 - 12.0. The purified BaCaseinase activity was estimated using the BaCaseinase assay described earlier.

BaCaseinase kinetics

Kinetics study of purified BaCaseinase was performed using the same procedure as the BaCaseinase activity assay. All eight treatments at several bovine casein concentrations (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6, and 12 mg/mL) were incubated with the same concentration of a purified BaCaseinase solution (0.01 mg). After treatments were finished, the Michaelis constant (Km) and the maximum velocity (Vmax) were calculated using the Michaelis-Menten equation (Lineweaver and Burk, 1934). The purified BaCaseinase activity was estimated using the assay described earlier.

Effects of BaCaseinase:bovine casein ratio, incubation time, and pH on degree of hydrolysis (DH%) of bovine casein

A 1% (w/v) solution of bovine casein was combined with various enzymes across four purified BaCaseinase:bovine casein ratios (1 - 7%) for incubation times (0, 5, 30, 60, 90, 120, 240, 360, and 480 min) and pH conditions (pH 2 - 12). The reactions were carried out under the previously chosen temperature condition. All hydrolysis reactions were postponed by heating at 90°C for 15 min before centrifuged at 13,000 rpm for 15 min to collect the supernatant containing soluble bovine casein hydrolysate (BCH). Finally, the BCH was stored at -20°C until subsequent analyses.

Degree of hydrolysis (DH%) of bovine casein

The degree of hydrolysis (DH%) of bovine casein was determined by the Ninhydrin colorimetric with slight modifications (Gao *et al*., 2020). Firstly, ninhydrin solution was prepared by dissolving Ninhydrin (1.5 g) in 10 mL of acetate buffer. The reaction mixture contained 60 mL ethylene glycol, 15 mL *n*-propanol, 15 mL *n*-butanol, 3 mL bovine casein, and 1 mL ninhydrin solution. The hydrolysis reaction was carried out by heating 10°C for 20 min. Then, the reaction mixture was cooled immediately in an ice bath before added with 1 mL of ethanol (40% v/v) to develop the colour reaction. The quantity of produced product was determined by measuring the absorbance at 570 nm, with the content of glycine used as the standard product. DH% was calculated using the formula: $DH = [(A_2 - A_1) / A_0] \times 100\%$; where, A_2 = concentration of free amino nitrogen of BCH (mg/mL), A_1 = concentration of free amino nitrogen of bovine casein (mg/mL), and A_0 = concentration of total amino nitrogen of bovine casein (mg/mL).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of BCH

The DPPH radical scavenging activity of BCH was conducted according to Qian *et al*. (2020) with slight modifications. Briefly, a test tube containing 1.0 mL of BCH at one in five concentrations, and 4.0 mL of dissolved 0.076 mM DPPH in methanol was mixed and incubated in the dark at 25°C for 30 min to develop colour reactions. Following incubation, the absorbance was measured at 517 nm with methanol solution as a blank solution. A lower absorbance indicates higher radical-scavenging activity. The percentage of DPPH radical-scavenging activity was calculated using the formula $[(A_3 - A_4) / A_3] \times 100\%$; where, A_3 = absorbance of the control tube (DDPH solution without sample), and A_4 = absorbance of the test tube. A standard curve was constructed using the absorbance of a series of concentrations of ascorbic acid. The experiment on each BCH concentration was repeated in triplicate.

2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity of BCH

The ABTS radical scavenging activity of BCH was performed according to Centenaro *et al*. (2011) with slight modifications. Firstly, the cationic radical ($ABTS^{\bullet+}$) solution was prepared by combining two solutions (7 mM ABTS dissolved in deionised water and 2.45 mM sodium persulphate) at 1:1 ratio, followed by an incubation step in the dark for 16 h at 25°C. Sequentially, the cationic radical $ABTS^{\bullet+}$ solution was diluted with deionised water to an absorbance of 0.70 ± 0.02 at 734 nm. Next, each test tube containing 100 µL of BCH at various concentrations (120, 240, 360, 480, and 600 µg/mL) was mixed with 3 mL of the diluted $ABTS^{\bullet+}$, and then incubated for 6 min to develop a colour reaction. Absorbance was measured at 734 nm. The percentage of ABTS radical scavenging activity was calculated using the formula $[(A_5 - A_6) / A_5] \times 100\%$; where, A_5 = absorbance of the control tube ($ABTS^{\bullet+}$ solution without sample), and A_6 = absorbance of the test tube. A standard curve was constructed using the

absorbance of a series of concentrations of ascorbic acid. The experiment on each BCH concentration was repeated in triplicate.

Angiotensin I-converting enzyme inhibitory (ACE-I) activity assay of BCH

Following a published method (Je *et al*., 2005) with slight modifications, the ACE inhibitory activity of BCH was measured. Initially, a solution of Hip-His-Leu of 8.3 mM was prepared by dissolving 1.8 mg Hip-His-Leu powder in 50 mM sodium borate buffer (pH 8.3). Next, a test tube containing respectively 50 µL at various BCH concentrations (120, 240, 360, 480, and 600 µg/mL) and 50 µL of ACE solution (25 mU/mL) were mixed and incubated at 37°C for 10 min. Then, 150 µL Hip-His-Leu solution of 8.3 mM was added, and the mixture was incubated for 30 min at the same temperature. The reaction was terminated by adding 250 µL of 1 M HCl, centrifuged with 3,450 rpm for 15 min, and then 1.0 mL of the upper layer was transferred to a test tube, and evaporated in a vacuum at room temperature for 3 h. The resulting hippuric acid content in 3.0 mL of distilled water was measured at 228 nm using an ultraviolet spectrophotometer. The ACE inhibitory activity was calculated using the formula $[(A_7 - A_8) / (A_7 - A_9] \times 100$; where, A_7 = absorbance of the mixture without the BCH, A_8 = absorbance of the reaction mixture, and A_9 = absorbance of the buffer solution. A standard curve was constructed using the absorbance of a series of Captopril contents, an ACE inhibitor. The experiment on each BCH concentration was repeated in triplicate.

Statistical analysis

All experiments were performed in triplicate. The data were processed using Microsoft Excel, and Statgraphics Centurion 18 (Stat Graphics Technologies, Inc., The Plains, VA, USA) was used for statistical analysis. The enzyme kinetics parameters were calculated using Prism 8.0.2 (GraphPad Software Inc., San Diego, California, USA).

Results

Screening, molecular identification, and phylogenetic analysis of microorganisms producing caseinase

Extracellular bacterial enzymes are essential proteins occurring in culture medium (Kalisz *et al*., 1988). These enzymes can degrade substrates present in the medium. This ability can screen for microbial enzymes that break down a specific substrate. Initially, we screened 32 microorganisms with various morphological characteristics on sterilised NB media of 100 µL of 10^{-5} of diluted representation samples for soil samples of rice root systems from three provinces (Tien Giang, BenTre, and LongAn). Subsequently, we respectively collected four microorganisms capable of utilising casein, denoted by a white hydrolysis ring on SMA from the secreted caseinase. In a caseinase-producing medium at 37°C for 96 h, and by monitoring caseinase activity every 4 h, L9 strain produced the most noticeable white hydrolysis ring (Figure 1) among the four strains. Thus, we selected L9 strain for in-depth study. We obtained the 16S rRNA sequence of L9 strain after DNA extraction and PCR amplification (Figure 2A). We compared the sequence with other deposited sequences using Bioedit software (Ibis Biosciences, USA), and BLAST analysis indicated 99% similarity with *B. altitudinis* 41KF2b (Figure 2B).

Effects of temperature, pH, and carbon sources on BaCaseinase activity

The effect of temperature (20, 25, 37, and 40°C) on BaCaseinase activity was assessed for 6 d, and the BaCaseinase activity peaked at day 4 (Figure 3A). Table 1 shows significant differences in the BaCaseinase activities at the four surveyed temperatures ($p < 0.05$). It was apparent that the BaCaseinase activity gradually increased from 20 to 37°C to a maximum activity of 52.5×10^4 U/mL at 37°C on day 4. The lowest activity occurred on day 6 at 25°C (Figure 3A). Next, the BaCaseinase activity also varied due to pH values. As shown in Table 1, each pH had a noticeably different effect on the BaCaseinase activities ($p < 0.05$). Similar to temperature, the BaCaseinase activity peaked on day 4 for all pH values, and then decreased. The activity on all days was notably higher at pH 8. Specifically, the highest activity of BaCaseinase was 56.8×10^4 U/mL on day 4 at pH 8. After this day, the activity decreased (Figure 3B).

Among the carbon sources tested, 1.0% glucose (w/v) yielded the highest BaCaseinase activity on day 4 (52.00×10^4 U/mL; Table 1). Similar to temperature and pH, BaCaseinase activity peaked at day 4 (Figure 3C). The results confirmed glucose as the best source for producing BaCaseinase by *B. altitudinis* 41KF2b compared to other carbon sources ($p < 0.05$). Based on the screening results, we chose a

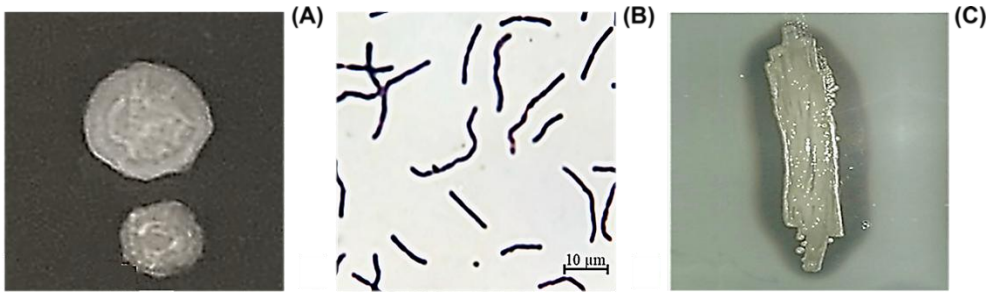

Figure 1. (A) colony morphology, **(B)** Gram-staining, and **(C)** caseinase hydrolysis on SMA of L9 strain.

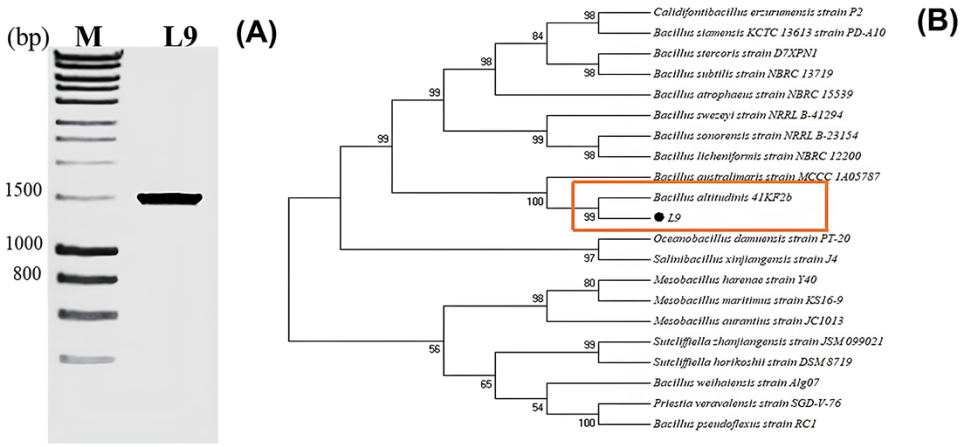

Figure 2. (A) 16S rRNA PCR band from L9 strain, and **(B)** phylogenetic tree based on 16S rRNA sequences of L9 strain compared with GenBank database. L9 strain showed 99% homology with *Bacillus altitudinis* 41KF2b.

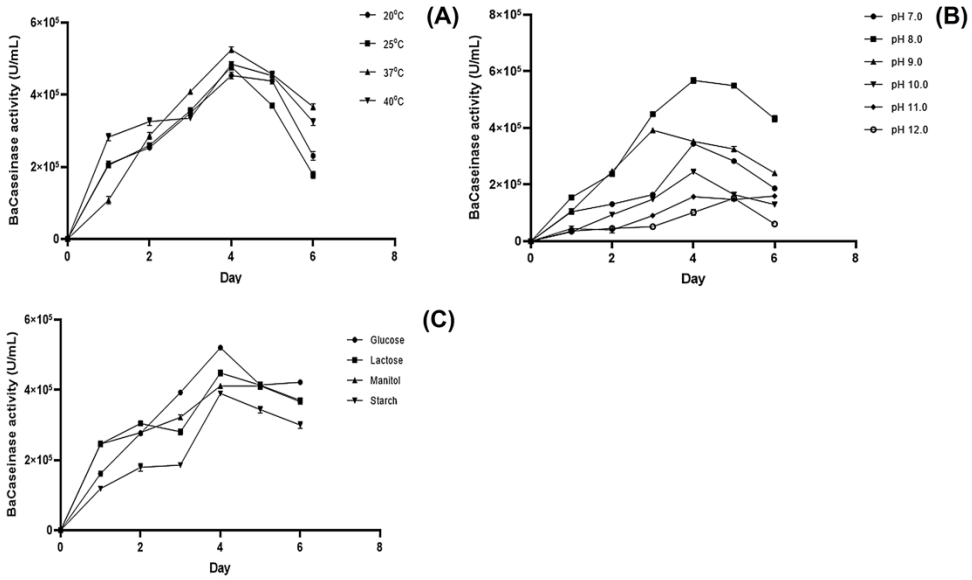

Figure 3. Effect of **(A)** temperature, **(B)** pH, and **(C)** carbon source on *Bacillus altitudinis* 41KF2b BaCaseinase activity. Data are mean of three separate experiments with error bars indicating ± standard deviation.

Table 1. Effect of temperature, pH, and carbon sources on BaCaseinase activity.

Survey factor		Protease activity (U/mL) at survey time interval (day)						
		0	1	2	3	4	5	6
Temperature (°C)	20	0.0 ± 0.00^{aA}	207823.9 ± 8.34^{bB}	253484.3 ± 6.98^{dA}	349561.3 ± 7.84^{eB}	477410.4 ± 9.34^{gA}	438028.3 ± 7.65^{fB}	230654.1 ± 11.76^{cB}
	25	0.0 ± 0.00^{aA}	204589.6 ± 3.71^{cB}	259762.6 ± 4.25^{dA}	355839.6 ± 7.8^{eB}	477410.4 ± 9.26^{gB}	370298.7 ± 5.44^{fA}	177783.2 ± 10.38^{bA}
	37	0.0 ± 0.00^{aA}	107647.8 ± 10.61^{bA}	286588.1 ± 9.41^{cB}	408919.8 ± 6.00^{eC}	525163.5 ± 7.70^{gC}	456863.2 ± 8.02^{fB}	366493.7 ± 8.60^{dD}
	40	0.0 ± 0.00^{aA}	282402.5 ± 9.97^{bC}	325970.1 ± 10.83^{cC}	335292.5 ± 3.15^{cA}	485020.4 ± 5.94^{eB}	453058.2 ± 10.0^{dC}	324828.6 ± 9.50^{cC}
pH	7.0	0.0 ± 0.00^{aA}	103756.3 ± 9.09^{bC}	130962.3 ± 5.91^{cC}	164446.5 ± 4.54^{dD}	344614.8 ± 8.03^{gD}	283163.5 ± 6.82^{fC}	186896.2 ± 6.30^{eD}
	8.0	0.0 ± 0.00^{aA}	154934.0 ± 709^{bD}	238264.2 ± 5.40^{cD}	449253.1 ± 6.84^{eF}	568160.4 ± 10.50^{fE}	549991.4 ± 4.32^{gE}	433081.8 ± 11.12^{dF}
	9.0	0.0 ± 0.00^{aA}	106610.1 ± 8.96^{bC}	247586.5 ± 4.24^{cD}	392177.7 ± 5.766^{E}	352985.8 ± 6.80^{eD}	325779.9 ± 9.20^{dD}	240927.7 ± 3.22^{cE}
	10.0	0.0 ± 0.00^{aA}	3347.7 ± 4.53^{bA}	9346.4 ± 2.31^{cB}	14837.0 ± 6.09^{eC}	24511.3 ± 7.09^{gC}	16452.3 ± 5.37^{fB}	12951.6 ± 8.61^{cB}
	11.0	0.0 ± 0.00^{aA}	4466.4 ± 8.97^{bB}	3994.6 ± 10.52^{bA}	9062.9 ± 4.60^{cB}	15717.9 ± 2.00^{dB}	14728.6 ± 6.18^{eA}	15969.0 ± 5.10^{eC}
	12.0	0.0 ± 0.00^{aA}	3557.0 ± 3.72^{bA}	4517.8 ± 2.58^{cA}	5157.0 ± 6.42^{dA}	10185.4 ± 10.57^{eA}	15208.0 ± 9.45^{fA}	6105.6 ± 2.36^{dA}
Carbon source	Glucose	0.0 ± 0.00^{aA}	161592.8 ± 7.38^{bB}	276504.7 ± 2.00^{cB}	392558.2 ± 5.67^{dD}	520026.7 ± 2.36^{eD}	389894.7 ± 8.7^{dB}	421856.9 ± 6.00^{dC}
	Lactose	0.0 ± 0.00^{aA}	245874.2 ± 7.38^{bC}	304091.2 ± 4.75^{dB}	280500.0 ± 8.50^{cB}	448301.9 ± 8.90^{gC}	413295.6 ± 9.4^{fB}	370108.5 ± 6.22^{eB}
	Mannitol	0.0 ± 0.00^{aA}	245684.0 ± 5.10^{bC}	278026.7 ± 5.38^{cC}	321974.8 ± 7.16^{dC}	411202.8 ± 6.88^{eB}	411393.1 ± 10.0^{eB}	366113.2 ± 4.83^{eB}
	Starch	0.0 ± 0.00^{aA}	118481.8 ± 5.75^{bA}	179286.2 ± 11.12^{cA}	185945.0 ± 6.75^{cA}	389894.7 ± 5.70^{fA}	343663.5 ± 9.2^{eA}	299905.7 ± 9.45^{dA}

Data are mean ± SE of triplicates (n = 3) at significant differences of $p < 0.05$ based on Multiple Range Test. Lowercase superscripts indicate significant difference between periods of each survey factor. Uppercase superscripts indicate significant difference of survey factor of each period.

temperature of 37°C, a pH of 8.0, and glucose as the carbon source to produce the best purified BaCaseinase activity.

Ammonium sulphate fractionation pattern and purification, and determination of BaCaseinase activity

The high quality of BaCaseinase is an initial condition for obtaining accurate kinetics results for the in-depth study. Following the total protein concentrations and the purified BaCaseinase amounts by ammonium sulphate purifications in Table 2, it was revealed that precipitation with 70% ammonium sulphate yielded the highest values in the total protein of 48.9 ± 1.60 mg/mL, and BaCaseinase activity of 711450.3 ± 2.76 U/mL compared to other ammonium sulphate concentrations. These results proved that 70% ammonium sulphate was the best precipitation to harvest high-quality purified BaCaseinase. In addition, a single BaCaseinase band of around 25 kDa on SDS-PAGE gel in Figure 4A combined with a large white ring on SMA (Figure 4B) demonstrated the high purity and enzyme activity of the purified BaCaseinase. The molecular weight of purified BaCaseinase not only resembled the molecular weight of approximately 25 kDa, but also had higher purity than a caseinase produced by *Bacillus subtilis* CSB55 (Hussein *et al*., 2020).

Table 2. Ammonium sulphate fractionation pattern of BaCaseinase produced by *Bacillus altitudinis* 41KF2b.

Ammonium sulphate saturation (%)	Total protein (mg/mL)	BaCaseinase activity (U/mL)
Crude extract	256.9 ± 0.48^{f}	1196993.0 ± 4.31^{g}
40	19.9 ± 0.52^{b}	81893.3 ± 1.13^{a}
50	23.5 ± 0.57^{c}	113317.0 ± 1.35^{c}
60	30.9 ± 1.01^{d}	120700.0 ± 1.00^{e}
70	48.9 ± 1.60^{e}	711450.3 ± 2.76^{f}
80	28.9 ± 1.32^{c}	105627.7 ± 7.00^{b}
3K MWCO	0.9 ± 0.12^{a}	447820.0 ± 5.07^{d}

Data are mean ± SE of triplicates (n = 3). Different lowercase superscripts in similar column indicate significant difference ($p < 0.05$) based on Multiple Range Test.

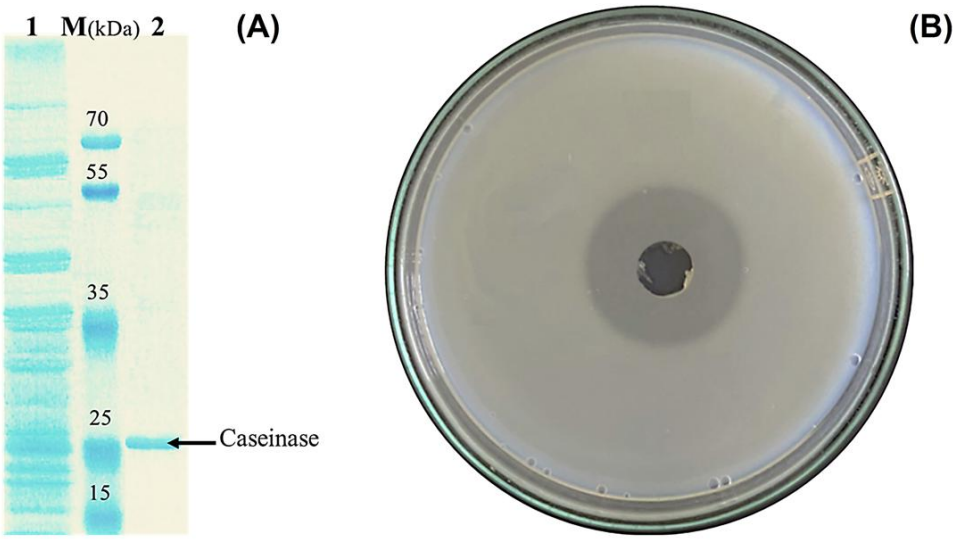

Figure 4. (A) Purification of BaCaseinase produced by *Bacillus altitudinis* 41KF2b on 12% SDS-PAGE. Lane M: PageRuler protein ladder (10 - 250 kDa); and Lane 2: purified BaCaseinase from *Bacillus altitudinis* 41KF2b. **(B)** Evaluation of purified BaCaseinase activity on SMA (protease test).

Effect of temperature, incubation time, and pH on BaCaseinase activity and BaCaseinase kinetics

Most proteases, including caseinase, are sensitive to specific temperatures and pH's. The optimal temperature range for the activity of the purified BaCaseinase was between 35 - 50°C, in which the highest activity was at 45°C (Figure 5A). Regarding pH, purified BaCaseinase activity was notably higher at both pH 8.0 and 9.0, with the highest activity at pH 8.0 (Figure 5B). The ability of purified BaCaseinase to degrade casein in alkaline pH's and at a high temperature makes it compatible with biotechnological and industrial practices.

The kinetics of the reaction catalysed by an enzyme or enzymatic mixture can provide important appropriate parameters for constructing efficient catalyst conditions of BaCaseinase activity for prediction and control of the produced hydrolysate content in applications in industrial scales (Brown *et al*., 2014). Based on the amount of tyrosine product and the standard Michaelis-Menten model, the purified BaCaseinase had a relative Km value of 22.2 × 10^4 U/mg and a relative Vm value of 1.108 mg/mL. The correlation ratio was quite high at 0.989 (Figure 5C).

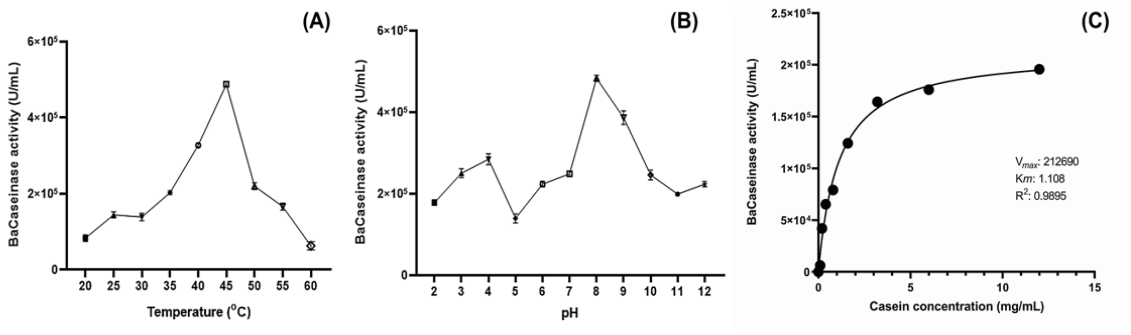

Figure 5. Effect of temperature **(A)** and pH **(B)** on activity of purified BaCaseinase from *Bacillus altitudinis* 41KF2b, and **(C)** BaCaseinase kinetics.

Effect of BaCaseinase:bovine casein ratio, reaction time, and pH on hydrolysis degree (DH%) of casein by BaCaseinase

The purified BaCaseinase:bovine casein ratio, reaction time, and pH are essential for analysing the catalytic parameters of most enzymatic reactions. We found significant differences in DH% at the different purified BaCaseinase:bovine casein ratios, incubation times, and pH's ($p < 0.05$), as summarised in Table 3 and Figure 6. Firstly, among the six surveyed ratios, only the purified BaCaseinase:bovine casein ratio of 1:4 had the highest DH% level of 65.4 ± 1.0% (Figure 6A). Next, the rendered DH% levels at the six incubation times in Figure 6B denoted that the DH% obtained higher values within time ranges from 120 to 480 min than in other incubation times. Besides, the phenomenon in which the DH% increased at an incubation time of 30 min, and then decreased 60 and 90 min later before the DH% reached the highest DH% (67.7 ± 6.19%) at the reaction time of 360 min demonstrated that the prolonged hydrolysis process was more effective in producing the high BCH content. The fluctuations in the DH% during the six incubation times reflected the association and dissociation of the interior exposure of the BaCaseinase structure involving functional groups to perform the casein hydrolyse reaction. Finally, in contrast to the purified BaCaseinase:bovine casein ratio, although the higher DH values were at two pH units of pH 4.0 and 8.0, the highest DH% of 69.4 ± 1.0% was seen at pH 8.0 (Figure 6C).

Characterisation of BCH bioactivities

Casein hydrolysates are involved in bioactive peptides, and can exhibit nutraceutical and pharmacological characteristics, including antioxidant and antihypertensive activities (Balandrán-Quintana *et al*., 2021). Consequently, we investigated the antioxidant capacity of the BCH by assessing its DPPH and ABTS radical scavenging, and ACE inhibitory activities. Table 4 shows that the antioxidant activity of the BCH increased as the concentration increased ($p < 0.05$). Specifically, the DPPH radical scavenging was 53.6% at 600 µg/mL, but for the radical ABTS scavenging activity was 63.3% at 150 µg/mL. Similar to the antioxidant capacity, the ACE inhibitory activity increased by approximately 77% with the higher BCH

Table 3. Effect of BaCaseinase:bovine casein (E:S) ratio, reaction time, and pH on hydrolysis degree (DH) of bovine casein by BaCaseinase.

E:S ratio (%)	%DH	Incubation time (min)	%DH	pH	%DH
0	5.2 ± 0.51[a]	0	3.3 ± 0.58[a]	2	2.4 ± 0.56[a]
1	14.0 ± 2.73[b]	15	22.47 ± 0.25[b]	3	2.8 ± 0.65[a]
2	20.6 ± 5.65[b]	30	57.03 ± 6.24[d]	4	69.4 ± 1.02[f]
3	57.3 ± 5.00[e]	60	39.7 ± 0.29[c]	5	22.7 ± 8.73[b]
4	65.4 ± 1.0[f]	90	39.6 ± 0.40[c]	6	47.5 ± 2.20[d]
5	46.5 ± 5.45[d]	120	57.5 ± 0.58[d]	7	63.2 ± 7.43[f]
6	32.9 ± 5.23[c]	240	65.0 ± 0.61[f]	8	78.5 ± 5.18[g]
7	36.2 ± 1.52[c]	360	67.7 ± 6.19[f]	9	55.8 ± 5.18[e]
		480	65.4 ± 4.01[f]		

Data are mean ± SE of triplicates (n = 3). Different lowercase superscripts in similar column indicate significant difference ($p < 0.05$) based on Multiple Range Test.

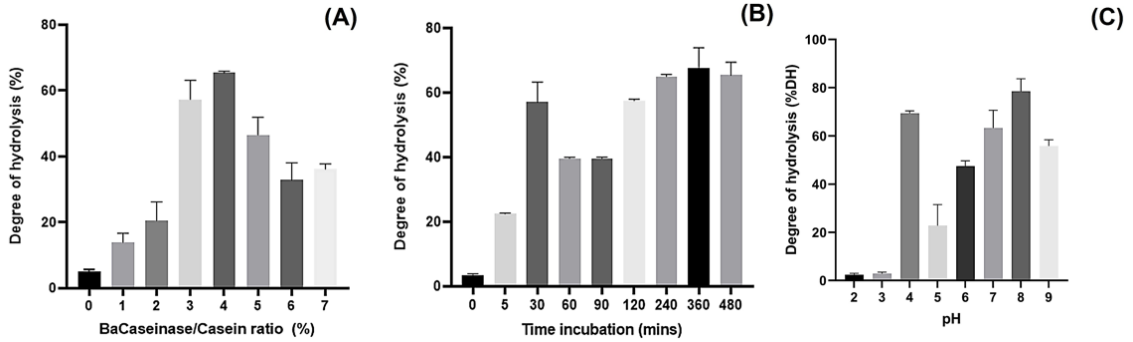

Figure 6. Effect of **(A)** BaCaseinase:bovine casein ratio, **(B)** incubation time, and **(C)** pH on casein hydrolysis. Data are mean of three separate experiments with error bars indicating ± standard deviation.

Table 4. DPPH and ABTS free radical scavenging activities of casein-derived hydrolases (BCH).

BCH (µg/mL)	DPPH activity (%)	BCH (µg/mL)	ABTS• activity (%)	BCH (µg/mL)	ACE inhibitory activity (%)
120	2.9 ± 0.11[a]	30	24.0 ± 0.96[a]	120	12.6 ± 2.05[b]
240	10.4 ± 1.09[b]	60	31.6 ± 0.70[b]	240	25.4 ± 0.71[c]
360	21.9 ± 0.08[c]	90	39.3 ± 0.52[c]	360	38.3 ± 0.36[d]
480	35.1 ± 0.68[d]	120	50.0 ± 1.53[d]	480	50.0 ± 0.62[e]
600	53.6 ± 0.52[e]	150	63.3 ± 1.17[e]	600	76.8 ± 1.06[f]

Data are mean ± SE of triplicates (n = 3). Different lowercase superscripts in similar column indicate significant difference ($p < 0.05$) based on Multiple Range Test.

concentration of 600 µg/mL. We determined the following half-maximal inhibitory concentrations (IC_{50}) of 0.60 ± 0.003 mg/mL for DPPH radical scavenging activity, 0.13 ± 0.003 mg/mL for ABTS radical scavenging activity, and 0.43 ± 0.002 mg/mL for ACE inhibitory activity (Table 5). These values will be valid for future *in vivo* experiments with BCH.

Discussion

Purification and kinetics characterisation of purified BaCaseinase produced from B. altitudinis 41KF2b

Microbial enzymes, which can be more stable and specific than enzymes derived from plants and animals, have been used successfully for industrial

Table 5. Antioxidant and ACE inhibitory activities of bovine casein hydrolysate (this study), Yark casein hydrolysate produced by alcalase and trypsin (Liu *et al*., 2020), whey protein hydrolysate produced by alcalase (Zhang *et al*., 2013), casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790 (YKVPQL) (Maeno *et al*., 1996), and buffalo casein hydrolysate produced by pepsin and trypsin (Shanmugam *et al*., 2021).

	IC_{50} DPPH activity (mg/mL)	**IC_{50} $ABTS^{\bullet}$ activity (mg/mL)**	**IC_{50} ACE- I activity (mg/mL)**
BCH (this study)	0.60 ± 0.003	0.13 ± 0.003	0.43 ± 0.002
Whey hydrolysate (trypsin)			0.34 to 0.45
Whey hydrolysate (pepsin)			1.1
Whey hydrolysate (elastase) (Philanto-Leppala *et al*., 2000)			1.7
Buffalo casein hydrolysate (pepsin and trypsin) (Shanmugam *et al*., 2021)			0.05
Arg-Glu-Leu-Glu-Glu-Leu (Yark casein hydrolysate) (Liu *et al*., 2020)	0.69		
Trp-Tyr-Ser-Leu (whey hydrolysate) (Zhang *et al*., 2013)	1.5		

Data are mean ± SE of triplicates (n = 3).

applications. The diversity and activity of microbial enzymes are associated with microbial adaptation to different habitats (Mohapatra *et al*., 2003). In the present work, we identified caseinase activity based on the SMA assay. We isolated the L9 strain, and its 16S rDNA sequence showed 99% homology to *B. altitudinis* 41KF2b. By ammonium sulphate fractionation, we purified the BaCaseinase produced by *B. altitudinis* 41KF2b with high purity. The best purified BaCaseinase activity was determined at 37°C, pH 8, and 1.0% glucose as a carbon source on day 4. Our findings are very similar to a caseinase isolated from *B. subtilis* CSB55 which acts at the best level at conditions of 40°C and pH 9, and the maximum amount of caseinase was 1.59 µg/mL (Hussein *et al*., 2020). However, the activity of this enzyme has yet to be determined. Hence, the purified BaCaseinase in our study could be more advantageous for industrial applications (Sharaf and Al-Fadel, 2013; Mothe and Sultanpuram, 2016; Hakim *et al*., 2018). In addition, this study also determined the purified BaCaseinase kinetics at 55°C and pH 9, a Vm value of 22.2×10^4 U/mg, and a Km value of 1.108 mg/mL. Thus, to compare to a Vm value of 0.12×10^4 U/mg and a Km value of 0.197 mg/m of a protease (SH21) produced by *Bacillus siamensis* CSB55, it showed that the purified BaCaseinase yielded a 100-fold higher Vm compared with SH21 (Tarek *et al*., 2023).

Bioactive characteristics of BCH

Various BCH include natural bioactive compounds (Díaz and Decker, 2004; Silva and Malcata, 2005; Phelan *et al*., 2009; Hussein *et al*., 2020), and the distinct bioactivities are attributed to key residues and amino acid sequences (Pihlanto-Leppälä *et al*., 2000; Mohanty *et al*., 2016; Shanmugam *et al*., 2021). The present work summarises the antioxidant and ACE inhibitory activities of various hydrolysates reported in the recent literature (Table 5). Among that, an extracted peptide (Arg-Glu-Leu-Glu-Glu-Leu) in Yark casein hydrolysate produced by alcalde and trypsin had an IC_{50} of 0.69 mg/mL for DPPH radical scavenging activity (Liu *et al*., 2020), while another peptide in a whey hydrolysate produced by pepsin and trypsin had an IC_{50} of 1.5 mg/mL. Our BCH produced with single purified BaCaseinase showed a better ability to scavenge DPPH free radicals. Moreover, our BCH had an even better ability to scavenge ABTS free radicals at an IC_{50} of 0.13 mg/mL. Of that, the ABTS activity has not been measured directly from the other

hydrolysates. These findings are consistent with the view that casein hydrolysates produced using different enzymes have diverse peptide lengths and amino acid sequences. The existence of tyrosine after hydrolysis underlies the ABTS radical scavenging activity.

Furthermore, casein hydrolysates have demonstrated antihypertensive activity (Phelan *et al*., 2009). As shown in Table 5, our BCH produced with purified BaCaseinase had an IC_{50} of 0.43 mg/mL to inhibit ACE, approximately equal to the IC_{50} range of 0.34 - 0.45 mg/mL for whey hydrolysates produced by trypsin. Our IC_{50} was noticeably lower than whey hydrolysates produced by pepsin and elastase in the IC_{50} ranges of 1.1 - 1.7 mg/mL (Pihlanto-Leppälä *et al*., 2000). However, another buffalo casein hydrolysate produced by pepsin and trypsin had the lowest IC_{50} of 0.05 mg/mL (Shanmugam *et al*., 2021), representing the best ACE inhibitory activity among the reported hydrolysates.

Using casein hydrolysates that retain bioactive activity may provide a way to prevent food rancidity without affecting food quality (Díaz and Decker, 2004). However, our high-quality BaCaseinase still needs to be tested *in vivo*. Nevertheless, we have provided a comprehensive overview of the potential antioxidant and antihypertensive effects of BCH made by purified BaCaseinase hydrolysis.

Conclusion

We characterised BaCaseinase secreted by *B. altitudinis* 41KF2b. After optimisation with glucose as a carbon source, and bovine casein as a nitrogen source, we successfully mass-produced BaCaseinase in 1 L of optimised media at 37°C, pH of 8.0, and rotary shaker at 140 rpm for four incubation days. After ammonium sulphate precipitation, purified BaCaseinase had a relative Km of 22.2×10^4 U/mg, and a relative Vm of 1.108 mg/mL at 45°C and pH 8.0. Based on the kinetics, BaCaseinase is a heat-stable enzyme, and could have commercial prospects for industrial applications. We also showed that highly pure BaCaseinase could digest bovine casein under multiple extreme conditions (an alkaline pH and a wide temperature range) to produce BCH with antioxidant activity, and the ability to inhibit ACE. These findings pave the way for applying BaCaseinase in various commercial enzyme fields, potentially leading to the development of additional bioactive hydrolysates to treat metabolic syndrome.

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