Application of Carboxypeptidase from *Pseudozyma hubeiensis* 31−B in dairy food processing

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Abstract

Application of carboxypeptidase from *Pseudozyma hubeiensis* 31−B (CP31−B) in dairy food processing was investigated. CP31−B reduced the bitterness of the peptide produced by milk casein by a bacterial proteinase. During yogurt production, the addition of CP31−B did not affect pH, acidity or physicochemical properties, but increased free amino acids. Moreover, free amino acids profiles revealed that the content of hydrophobic amino acids clearly increased with the addition of CP31−B. In sensory evaluation, yogurt with CP31−B tended to be preferred in terms of bitterness and overall palatability. In conclusion, carboxypeptidase produced by *P. hubeiensis* 31−B shows potential as a food additive in the dairy industry.

Keywords: *Pseudozyma hubeiensis*, carboxypeptidase, flavor enhancement, food additive

Introduction

Carboxypeptidase (CP) catalyzes the release of amino acid residues from the C-terminal end of peptides and proteins. Various CP have been reported from fungi, bacteria and yeast (Degan *et al*., 1992; Yasuda *et al*., 1992; Hayashi *et al*., 1973), including our previous isolation and subsequent purification of a CP from culture filtrates of *Pseudozyma hubeiensis* 31−B (Mase *et al*. 2017). The purified CP acted as a peptidase with properties similar to those of carboxypeptidase Y (EC 3.4.16.5) from *Saccharomyces cerevisia*e (Hayashi *et al*., 1975). The properties of CP31−B are available in the latest Japanese Published Unexamined Patent Application under number P2017−86A.

CP is a useful enzyme and food additive, being particularly beneficial to the dairy industry. In general, proteolytic enzymes such as proteinase and peptidase are used in dairy food processing, hydrolyzing proteins into peptides or amino acids. However, peptides in the hydrolysates often

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result in a bitter taste. CP can efficiently eliminate this bitterness by hydrolyzing the bitter peptides (Umetsu and Ichishima, 1985). Here, we report a reduction in bitterness with application of CP31−B during yogurt preparation.

Materials and methods

Materials

Prolether FG-F, a bacterial proteinase preparation, was purchased from Amano Enzyme Inc. (Nagoya, Japan). Milk casein was obtained from Wako Co. Ltd. (Osaka, Japan). All other reagents were commercially obtained from other sources.

Carboxypeptidase assay

CP activity was assayed with an acid carboxypeptidase assay kit (Kikkoman Co., Ltd., Noda, Japan), and the unit of CP activity defined using *Z*-Try-Ala according to the manufacturer's protocol. All data represent average values from triplicate experiments unless otherwise noted.

Preparation of purified CP

Purified CP (CP31−B) was prepared from cultural filtrates of *P. hubeiensis* 31−B following four column chromatography. CP31−B was represented by a single band in SDS-PAGE (Mase *et al*., 2017).

Preparation of bitter peptides

A bitter peptide solution was generated by dissolving 2 g of milk casein in 10 mL of 100 mM NaOH and adding 10 mL of 100 mM Tris-HCl buffer (pH 7.0). The pH was adjusted to 7.0 with 100 mM HCl, and the solution diluted to 50 mL with distilled water. Prolether FG-F (100 mg) was then dissolved in the solution followed by incubation at 50° C for 3.5 h. The resulting peptide solution was sterilized by autoclaving at 121° C for 1 min.

Analysis of bitterness

CP31−B (3.22 U) was added to 30 mL of peptide solution adjusted to pH 6.0 with 100 mM HCl, and incubated at 50° C for up to 1 h. Bitterness of the peptide solution was subsequently evaluated with a sensory test by three female students (age: 22 years) of Sugiyama Jogakuen University, Japan, using caffeine solution as a standard to define the ratio of bitterness. The decomposition rate of casein hydrolysis was obtained from the formol-*N* value obtained by formol titration versus total nitrogen measured using the Kjeldahl method (Castillo *et al*., 1962).

Yogurt production and analysis

Yogurt was produced according to the manufacturer's protocol (Rijieru Dore Co., Ltd., Hiroshima Japan). Briefly, CP31−B (0, 16, 32, 64 or 128 U) was added to 150 g of milk ('Meiji Oishi Gyunyu', Meiji Milk Co., Ltd., Tokyo) and 0.3 g of 'Yogurt no Negai' (Rijieru Dore Co.), a

starter containing *Lactococcus lactis* subsp. *cremoris*. The mixture was then fermented at 28° C for 24 h before cooling at 5° C for 1 h.

The pH of the yogurt was measured using a pH meter (LAQUAact, HORIBA Ltd., Kyoto, Japan), and acidity expressed as a percentage of lactic acid according to the 0.1 mol/L NaOH titration method with phenolphthalein as an indicator (Bao *et al*., 2016). Firmness, cohesiveness and adhesiveness of yogurt samples prepared in small cups (diameter: 4 × 2 cm) as described above were measured with a Texturometer TDU (Yamaden Co., Ltd., Tokyo, Japan).

Assays of amino acid content and free amino acid composition were conducted as follows. Ten grams of yogurt was mixed with 40 mL of 0.1 mol/L HCl then homogenized with a mixer (ACE Homogenizer AM−8: NIHONSEIKI KAISHA LTD., Tokyo) at 10,000 rpm for 5 min at 5° C. The resulting supernatants (25 mL) were added to 50 mL of *n*-hexane then, after mixing for 60 min, the hexane layer was carefully aspirated. A 10−mL water layer was then added to 30 mL of acetonitrile and mixed with the solution before storing at 5° C overnight. Insoluble materials were removed by centrifugation at 10,000 rpm for 5 min at 5° C then the total amino acid content assayed using the ninhydrin method. Free amino acid compositions were analyzed by high performance liquid chromatography (HPLC) using an amino acid analyzer (Nexera X2; Shimadzu Co., Kyoto, Japan) equipped with an Inertsil ODS−4 column (75 × 3.0 mm; GL Science Co., Tokyo) and RF−20AXS fluorometric detector (Shimadzu Co.), with excitation set at 350 nm and emission at 450 nm. Only one sample per CP31−B concentration was analyzed.

Sensory evaluation of the yogurt samples

Sensory characteristics of the yogurt samples were ranked by a panel of 10 female students (age: 22 years) of Sugiyama Jogakuen University. The panelists were trained to recognize yogurt taste and flavor. The color, aroma, acidity, bitterness and umami characteristics were determined by discrimination tests, and the overall acceptance determined by a palatability test. Both tests were used to compare the different yogurts. Each sample was given a rating of 1−5, 1 being the most/best with respect to a particular attribute and 5 being the least/worst. Scores for each sample were totaled, and the yogurts numerically ranked from best to worst. Data were analyzed for statistical significance using Kramer's test (Kramer, 1960).

Results

Bitterness of the peptides

The bitterness of the control peptide solution (11.5% hydrolyzed casein) was approximately equivalent to that of 2% caffeine solution. However, after incubation with CP31−B for 1 h, the bitterness of the casein peptide hydrolysate reduced to essentially the threshold equal to that of 0.02% caffeine solution (Fig. 1).

Yogurt characteristics

The results of pH, acidity, amino acid and physicochemical analyses are shown in Table 1.

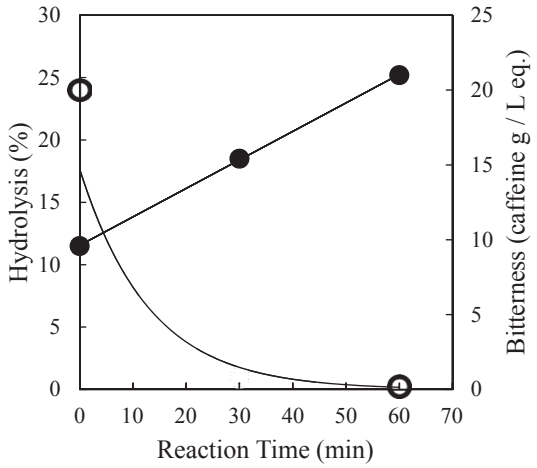

Fig. 1. Effect of carboxypeptidase 31−B (CP31−B) on hydrolysis and bitterness. CP31−B (3.22 U) was added to 30 mL of 4% peptide solution and incubated at 50° C for up to 1 h. The bitterness of the peptide solution was then evaluated with a sensory test by three female students (age: 22 years) of Sugiyama Jogakuen University, Japan. Caffeine solution was used as a standard to define the ratio of bitterness. The decomposition rate of casein hydrolysis was obtained from the formol-*N* value obtained by formol titration versus total nitrogen measured using the Kjeldahl method. Hydrolysis (●) and bitterness (○) were measured as described in the main text.

Table 1. Physico-chemical analysis and amino acid contents of yogurt samples with different carboxypeptidase concentrations.

Analysis	CP31−B concentration (units/150 g of milk)				
	0	16	32	64	128
pH	4.42 ± 0.03	4.38 ± 0.01	4.36 ± 0.02	4.33 ± 0.05	4.31 ± 0.03
Acidity (%)	0.87 ± 0.02	0.86 ± 0.01	0.85 ± 0.03	0.84 ± 0.01	0.85 ± 0.01
Free amino acids (mg/100 g)	77 ± 4	82 ± 3	92 ± 4	107 ± 7	120 ± 6
Rheological properties					
Firmness (N/m^2)	1039 ± 201	926 ± 184	915 ± 216	881 ± 132	971 ± 286
Cohesiveness	0.71 ± 0.04	0.63 ± 0.06	0.68 ± 0.05	0.69 ± 0.03	0.67 ± 0.04
Adhesiveness (J/m^3)	33.2 ± 4.2	48.1 ± 8.6	38.4 ± 5.1	31.6 ± 6.2	39.8 ± 7.8

Each value represents the mean ± SD of n = 3.

Except for the amino acids, values were similar among samples, regardless of the CP31−B concentration. In contrast, amino acid values assayed using the ninhydrin method increased in proportion to the amount of CP31−B added.

Free amino acid profiles The free amino acid profiles of the different yoghurt samples are shown in Fig. 2. Peaks corresponding to cystine and valine overlapped, and hydrophobic amino acids, namely tryptophan (W), phenylalanine (F), leucine (L) and isoleucine (I), exhibited an increased in

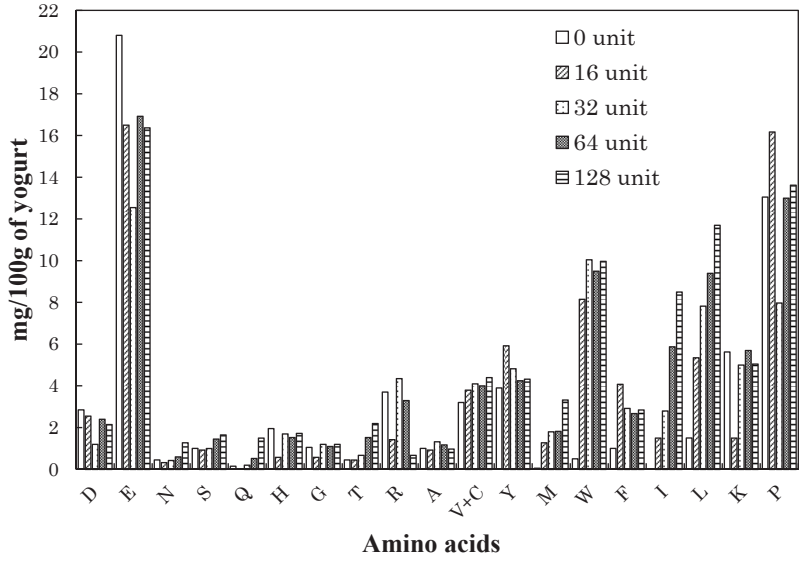

Fig. 2. Effect of different concentrations of carboxypeptidase 31−B (CP31−B) on free amino acid profiles of the yogurt samples.

Free amino acid compositions were analyzed by HPLC using an amino acid analyzer (Nexera X2 system; Shimadzu Co. Kyoto, Japan) equipped with an Inertsil ODS−4 column (75 × 3.0 mm; GL Science Co., Tokyo) and a RF−20AXS fluorometric detector (Shimadzu Co.), with excitation set at 350 nm and emission at 450 nm.

Table 2. Sensory evaluation of yogurt samples containing different concentrations of carboxypeptidase 31−B (CP31−B).

CP31−B (U/150 g of milk)	Palatability attributes					
	Color	Aroma	Acidity	Bitterness	Umami	Overall
0	33	28	29	39	31	36
16	28	36	31	34	30	30
32	27	26	28	28	27	28
64	29	29	27	25	30	29
128	33	31	35	24	32	27

Ten panelists ranked the yogurt samples according to palatability. Differences among total scores were evaluated using Kramer's test. * Significant difference, $p < 0.05$.

yogurt containing CP31−B compared to those without. Other amino acid contents did not differ with the addition of CP31−B.

Sensory evaluation The sensory characteristics of the yogurt samples are shown in Table 2. The color, aroma, acidity and umami scores did not differ among samples. Although the differences in bitterness and overall acceptance were not statistically significant with and without CP31−B, samples preferred by the panel tended to contain CP31−B.

Discussion

The application of CP31−B, purified from filtered culture medium of *P. hubeiensis* 31−B, in dairy product processing was investigated. Milk casein can be extremely bitter when digested with proteolytic enzymes due to peptides containing hydrophobic amino acids (Ichikawa *et al*., 1959; Minamiura *et al*., 1972; Ishibashi *et al*., 1987). Hydrophobic amino acids are generally found in the protein interior and are exposed when a protein is hydrolyzed by proteinase (Li *et al*., 2012). Peptides containing hydrophobic amino acids, particularly C- and N-terminal peptides, result in a particularly bitter taste.

The bitterness of a milk casein peptide solution produced by Prolether FG-F, a *Bacillus* proteinase, was found to be equivalent to that of the 2% caffeine solution standard. In contrast, CP31−B reduced the bitter casein solution to that of 0.02% caffeine solution following 1−h incubation (Fig. 1).

We subsequently investigated yogurt preparations prepared with and without CP31−B since yogurt is one of the most popular fermented dairy products consumed globally. Yogurts prepared with and without CP−31 did not differ in pH, acidity or physicochemical properties, but did differ in both the amount and composition of free amino acids. Free amino acid contents, assayed using the ninhydrin method, increased in proportion to the amount of CP31−B added (Table 1). Moreover, in the free amino acid profiles, the content of hydrophobic amino acids such as tryptophan, phenylalanine, leucine and isoleucine clearly increased in yogurt containing CP31−B compared to samples without (Fig. 2). Moreover, in sensory evaluation tests (Table 2), the differences in bitterness and total acceptance were not statistically significant between yogurt with and without CP31−B; however, those with CP31−B tended to be preferred.

Gordon and Speck (1965) reported isolation of bitter peptides from milk culture of *Streptococcus cremoris*, now referred to as *Lactococcus lactis* subsp. *cremoris* (Vos *et al*., 2005), when used as a yogurt starter. We also used *Lactococcus lactis* subsp. *cremoris* as a starter, so it is possible that this subspecies generated bitter peptides. It is well known that bitter peptides contain hydrophobic amino acids such as leucine and phenylalanine, especially at their C- and N-terminal. Moreover, *Z*-Phe-Leu and *Z*-Phe-Phe were previously found to be good substrates for CP31−B (Mase *et al*., 2017). Together with the sensory evaluation results, whereby the panel favorably evaluated yogurts produced with CP31−B, and based on the free amino acid profiles, it appears that CP31−B released hydrophobic amino acids from the C-terminal of bitter peptides, thereby reducing bitterness.

In conclusion, CP from *P. hubeiensis* 31−B shows potential application in processing of dairy foods such as milk protein hydrolysate, yogurt, enzyme-modified cheese and cheese, by reducing the bitterness of bitter peptides.

References

Bao, Z., Xiong, Jian, Lin, W., and Ye, J. (2016). Profiles of free acids, free amino acids, and volatile

compounds of milk bases fermented by *Lactobacillus casei* GBHM−21 with different fat levels. CyTA *J. Food*, **14**, 10−17.

Castillo, SL., Trimber, WG., Henderson, RC., Herrington, LB., and Turk, LK., (1962). Comparison of orange G dye, formol titration, and kjeldahl methods for milk protein determinations. *J. Dairy Sci*., **45**, 1079−1082.

Degan, D. F., Dumas, R., B., and Breddam, K. (1992). Purification and characterization of two serine carboxypeptidases from *Aspergillus niger* and their use in C-terminal sequencing of proteins and peptide synthesis. *Appl. Environ. Microbiol*., **58**, 2144−2152.

Hayashi, R., and Moore, S. (1973). Serine at the active center of yeast carboxypeptidase. *J. Biol. Chem*., **248**, 8366−8369.

Hayashi, R., Bai, Y., and Hata, T. (1975[b]). Further confirmation of carboxypeptidase Y as a metal-free enzyme having a reactive serine residue. *J. Biochem*., **77**, 1313−1318.

Ichikawa, K., Yamaoto, T., and Fukumoto, J. (1959). Studies on the bitter taste peptides produced by proteinases. I. The formation of bitter taste peptides by "neutral proteinase" of *Bacillus subtilis* and isolation of the peptide (In Japanese). *J. Agric. Chem. Soci. Jpn*., **33**, 1044−1058.

Ishibashi, N., Arita, Y., Kanehisa, H., Kouge, K., Okai, H., and Fukui, S. (1987). Bitterness of leucine-containing peptides. *Agric. Biol. Chem*., **51**, 2389−2394.

Kramer, A. (1960). Rapid method for determing significance of difference from ranking. *Food Technol*., **14**, 576−582.

Li, S. G., Chen, H., and Zhuang, Y. Z. (2012). Preparation of functional oligopeptides by enzymatic hydrolysis of soybean protein. *J. Food Sci.*, **33**, 91−93.

Mase, T., Shitasue, S., Kondo, M., Tanaka, R., Takada, R., Hiwatashi, M., Isshiki, S., Kawai, J. (2017). Purification of a carboxypeptidase from *Pseudozyma hubeiensis* 31−B and its characterization as a food additive. *Jpn. J. Food Chem. Safety*, in press.

Minaniura, N., Matsumura, Y., Fukumoto, J., and Yamamoto, T. (1972). Bitter peptides in Cow milk casein digests with bacterial proteinase. *Agric. Biol. Chem*., **36**, 588−595.

Umetsu, H., and Ichishima, E. (1985). Mechanism of digestion of bitter peptide from a fish protein concentrate by wheat carboxypeptidase. *J. Jpn. Soc. Food Sci.,* **32**, 281−287.

Yasuda, N., Kaneko, M., and Kimura, Y. (1992). Isolation, purification, and characterization of a new enzyme from *Pseudomonas* sp. M−27 carboxypeptidase G3 *Biosci. Biotech. Biochem*, **56**, 1536−1540.