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Stereoselective enzymatic synthesis of an aspartame precursor of *N*-CBZ-L-Asp-L-PheOMe

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Abstract

A stereoselective protease produced by *Bacillus amyloliquefaciens* KCCM 12091 was isolated. The enzyme catalyzed the synthesis of *N*-CBZ-L-Asp-PheOMe from *N*-CBZ-L-Asp and L-PheOMe, but not *N*-CBZ-L-Asp-D-PheOMe from *N*-CBZ-L-Asp and D-PheOMe. More than 50% of added L-PheOMe was consumed when eutectic mixtures of *N*-CBZ-L-Asp, racemic L- and D-PheOMe were used for synthesis of an aspartame precursor of *N*-CBZ-L-Asp-L-PheOMe. D-PheOMe was not involved in the reaction and did not affect synthesis of *N*-CBZ-L-Asp-L-PheOMe.

Keywords: Aspartame precursor; Stereoselective protease; Eutectic substrate mixture

1. Introduction

Numerous peptides with physiological activities have been developed in recent years from microorganisms, plants, and animals. Peptides that constitute structural proteins inside cells play an important role in controlling metabolic functions. Other peptides are used as sensory products, food and feed additives, pharmaceuticals, and fine chemicals [1,2].

Chemical methods [2], which are widely used in the synthesis of peptides, have advantages in large scale production. However, these methods also present problems, such as protection of amino acid side chains and a requirement for excessive amounts of coupling agents and solvents. Although high yields can be obtained in chemical reaction processes, resolution or racemization processes are required due to formation of racemic mixtures. Enzymatic methods [2] have the advantages of low temperature reactions, a reduced number of reaction steps, less protection required for amino acid side chains due to regio-selectivities, and no need for optical resolution. Proteases have been used in peptide synthesis [3]. Reactions are often carried out in organic phases in which the equilibrium is favorable to synthesis rather than hydrolysis [4]. A high substrate solubility, simple purification procedures for products, and reduced contamination with organic solvents can be achieved. However, in many cases the substrate solubility is still limited. Enzymes can be denaturated due to conformational changes in solvents. As a means to solve these problems, enzymatic reactions using eutectic substrate mixtures have been developed [3,5–7].

Aspartame (L-Asp-L-PheOMe) is a low calorie peptide sweetener (approximately 200 times sweeter than sucrose), and is currently used in foods and

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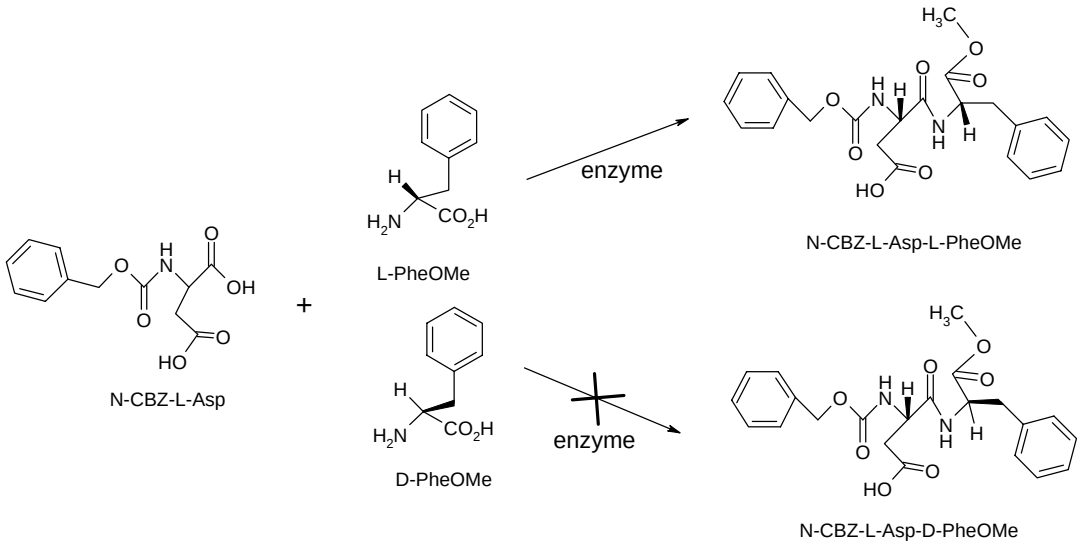

Fig. 1. An enantio-selective reaction scheme for synthesis of *N*-CBZ-L-Asp-L-PheOMe from *N*-CBZ-L-Asp and racemic L- and D-PheOMe.

beverages in more than 90 countries [8]. Aspartame is synthesized from L-Asp and L-PheOMe both chemically and enzymatically. The compounds L-Asp and L-Phe are currently produced by fermentation, but they can also be produced by chemical methods. In the latter case, since Asp and Phe are usually formed in racemic mixtures, optical resolution of the mixtures is required for synthesis of aspartame. These extra processes can greatly increase the expense. However, if L-PheOMe-specific protease is available, racemic L and D-PheOMe, which is chemically synthesized, can be used instead of optically pure L-PheOMe. In the racemic mixtures, only L-PheOMe will be involved in the synthesis of L-Asp-L-PheOMe.

We screened an enantio-selective protease from microorganisms that can catalyze reactions with L-Phe OMe but not with D-PheOMe (Fig. 1). Eutectic mixtures of the substrates *N*-CBZ-L-Asp and racemic L- and D-PheOMe were prepared and selective synthesis of the aspartame precursor *N*-CBZ-L-Asp-L-PheOMe was attempted.

2. Materials and methods

2.1. Microorganisms

Standard strains obtained from the Korean Culture Center of Microorganisms (KCCM) in Seoul and microorganisms isolated from soy sauce were used for screening of a stereoselective protease.

2.2. Enzymes and chemicals

Pepsin (EC 3.4.23.1), α-chymotrypsin (EC 3.4.21. 1), subtilisin Carlsberg (EC 3.4.21.62), thermolysin (EC 3.4.24.27), and papain (EC 3.4.22.2), and *N*-benzyloxycarbony-L-aspartic acid (*N*-CBZ-L-Asp) were purchased from Sigma Chemical Co. L-Phenylalanine methyl ester hydrochloride (L-PheOMe·HCl) and D-phenylalanine methyl ester hydrochloride (D-PheOMe·HCl) were products of Aldrich Chemical Co. MEA (2-methoxy ethyl acetate), DMSO (dimethyl sulfoxide), and other solvents of HPLC grade were obtained from Hayman Co.

2.3. Preparation of N-CBZ-L-Asp-L-PheOMe and N-CBZ-L-Asp-D-PheOMe

N-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-PheOMe were synthesized using thermolysin [9]. Substrates consisted of 1 mmol of *N*-CBZ-L-Asp and 1 mmol of L-PheOMe, or 1 mmol of *N*-CBZ-L-Asp and 1 mmol of D-PheOMe. After substrates were added to 10 ml round bottom vials, water (5%), MEA (6%), and DMSO (6%) were added as adjuvants. Eutectic mixtures were formed by incubation for 10 min

in a water bath at 60 °C. After cooling, thermolysin was added in 10% (w/w) to the mixtures. Reactions were performed for 3 h on a water bath shaker at 37 °C with an agitation speed of 160 rpm. The reaction products were confirmed with TLC. The reacted solutions were mixed with chloroform:methanol (4:1), then were loaded onto a silica gel column. After the solutions were eluted with the same solvent solution, the *N*-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-PheOMe fractions were separated and confirmed using TLC with *N*-CBZ-L-Asp-L-Phe OMe as a standard.

2.4. The screening method for PheOMe-selective protease producers

Four media with different N-sources were prepared (Table 1). Medium 1 and 2 contained *N*-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-PheOMe, respectively, as the sole nitrogen source. Medium 3 contained no nitrogen source and Medium 4 included NH_4 NO_3.

Standard strains of KCCM and strains isolated from soy sauce were tested for their ability to produce protease. The strains were inoculated into 250 ml flasks containing 20 ml of nutrient broth, then cultivated for 24 h on a shaking incubator at 30 °C and 150 rpm. One milliliter of culture broth was placed into an eppendorf tube, then centrifuged for 15 min at 12,000 rpm. Precipitates were suspended in 1 ml of 0.9% saline solution, then centrifuged again to separate the cells. One milliliter of 0.9% saline solution was added to the cells, followed by vortex. Twenty microliter of the cell suspension was spread on four different plates (Table 1), then cultivated for 48 h in an incubator at 35 °C. Colonies formed on plates of either Medium 1 or 2 were selected as PheOMe-selective strains.

Table 1
Media with different N-sources

Nitrogen source (0.5%, w/v)	
Medium 1	*N*-CBZ-L-Asp-L-PheOMe
Medium 2	*N*-CBZ-L-Asp-D-PheOMe
Medium 3	None
Medium 4	NH_4NO_3
Other composition (%, w/v)	
Glucose	1.0
KH_2PO_4	0.1
$MgSO_4$	0.05
KCl	0.05
$FeSO_4$	0.001
Agar	1.5

2.5. Production of proteases by the first selected strains

Strains selected from the first screening were inoculated into 500 ml flasks containing 100 ml of nutrient broth and 0.5% yeast extract for use as seed cultures. Strains were cultivated for 24 h on a reciprocal water bath shaker at 35 °C and 150 rpm. Main cultures used 10 ml of the cultivated broth transferred to 2 l flasks containing 500 ml of fermentation medium. Then, broths were cultivated for 48 h on a reciprocal water bath shaker at 35 °C and 150 rpm. After cultivation, the fermentation broths were centrifuged for 20 min at 6000 rpm to produce crude enzyme solutions.

Protease activities were measured with substrate solutions made by dissolving milk casein (0.6%) in a 50 mM phosphate buffer (pH 7.0) [10]. After 1 ml of the crude enzyme solution was mixed with 1 ml of the substrate solution, reactions were performed for 30 min at 30 °C. Reactions were stopped by adding 2 ml of 1% trichloroacetic acid. The solutions were then filtered through Whatman paper (No. 42). After 1 ml of a solution consisting of 0.5 M Na_2CO_3 and 0.2 M Folin–Ciocaiteu phenol reagent was added to 2 ml of the supernatants, the resulting solutions were incubated for 20 min at 37 °C. Absorbance values were measured on a spectrophotometer at 660 nm and converted into activities. One unit of activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine/1 ml from casein.

2.6. Partial purification of enzymes

Ammonium sulfate was added in 80% saturation to the crude enzyme solutions produced by *B. amyloliquefaciens* KCCM 12091. The solutions were then stirred for 1 h at 4 °C. After the solutions were centrifuged for 30 min at 6000 rpm, they were dissolved in small volumes of 50 mM phosphate buffer (pH 7.4). The solutions were placed into dialysis tubes (Sigma D-9652), then incubated in 50 mM phosphate buffer solutions for 24 h at 4 °C. After dialysis, solutions were stored in a refrigerator.

2.7. Procedure for reactions with L-PheOMe-specific protease

MEA (6%), DMSO (6%), and water (5%) were added as adjuvants to 10 ml vials filled with *N*-CBZ-L-Asp (0.5 mmol) and L-PheOMe (0.5 mmol), or *N*-CBZ-L-Asp (0.5 mmol) and D-PheOMe (0.5 mmol). The resulting solutions were then incubated for 10 min in a water bath at 60 °C to produce eutectic substrate mixtures. After cooling, 0.5 ml of the enzyme solution was added to the mixtures followed by incubation in a water bath shaker at 37 °C for reaction. Reactions were stopped by adding 0.4 ml of 5% (v/v) trifluoroacetic acid and 10 ml of acetonitrile.

2.8. Analysis of reaction products

Thin layer chromatography was used to analyze *N*-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-Phe OMe. Samples were spotted onto a plate (25 DC-Alufolien Kiesegel 60 F_{254}, Merck), then developed with chloroform:methanol (4:1). The R_f-values of the samples were measured by detection with a ninhydrin solution. *N*-CBZ-L-Asp-L-PheOMe purified with a semi-prep HPLC was used as a standard.

Quantitative analysis of products was performed by HPLC (Waters, Deltaprep-4000) with a carbohydrate column (4.6 mm × 250 mm), a UV-detector, an auto sampler, and a pump. Elution was performed at a flow rate of 0.5 ml/min with a gradient of acetonitrile:water of 30:70–0:100 containing 0.1% (v/v) trifluoroacetic acid. The amounts of *N*-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-PheOMe were calculated from the peak areas estimated from ε_{280}.

2.9. Separation and structural analysis of N-CBZ-L-Asp-L-PheOMe

N-CBZ-L-Asp-L-PheOMe was separated from the reacted solutions using semi-prep HPLC (Merck). HPLC and operation conditions were the same as specified previously. The structure of *N*-CBZ-L-Asp-L-PheOMe was confirmed by analysis of ^{13}C NMR and ^{1}H NMR (JNM-LA 500, JEOL).

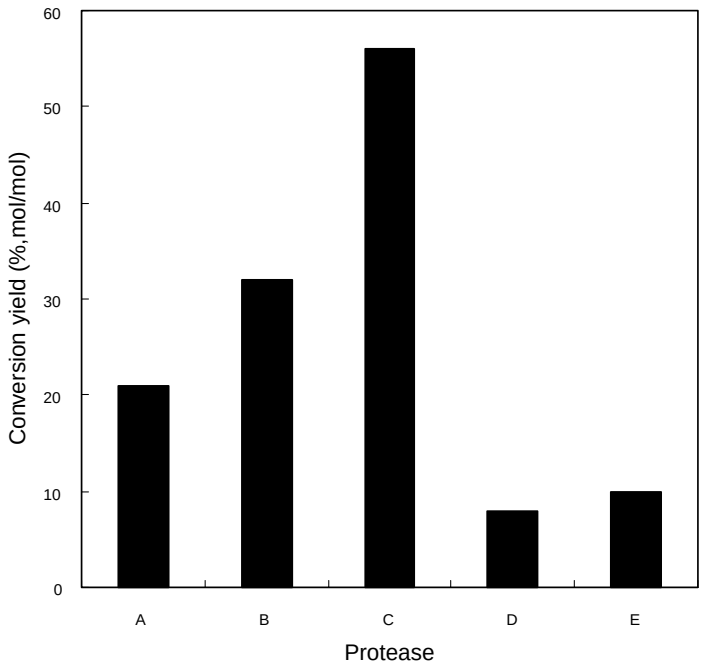

Fig. 2. Conversion yields in the synthesis of *N*-CBZ-L-Asp-D-PheOMe from *N*-CBZ-L-Asp and D-PheOMe by commercial proteases: (A) pepsin; (B) subtilisin Carlsberg; (C) thermolysin; (D) papain; (E) α-chymotrypsin.

2.10. Synthesis of N-CBZ-L-Asp-L-PheOMe in the presence of D-PheOMe

Eutectic mixtures of *N*-CBZ-L-Asp and L-PheOMe were prepared as specified previously. D-PheOMe in 20% (mol/mol) was added to the eutectic substrate mixtures. Then, 0.5 ml of the enzyme from *B. amyloliquefaciens* KCCM 12091 was added to the mixtures, which were then incubated in a reciprocal water bath shaker for 3 h at 37 °C and 160 rpm. After reaction, the *N*-CBZ-L-Asp-L-PheOMe content was measured.

3. Results and discussion

3.1. Synthesis of N-CBZ-L-Asp-D-PheOMe by commercial proteases

Thermolysin is often used in production of the aspartame precursor *N*-CBZ-L-Asp-L-PheOMe [11–14]. Reactions using thermolysin are performed primarily in organic solvents [11–13]. Enzymatic formation of *N*-CBZ-L-Asp-L-PheOMe in ionic liquids has also been reported [14].

We used commercial proteases that synthesize *N*-CBZ-L-Asp-L-PheOMe from *N*-CBZ-L-Asp and L-PheOMe [3] to synthesize *N*-CBZ-L-Asp-D-PheOMe from *N*-CBZ-L-Asp and D-PheOMe. After eutectic mixtures of *N*-CBZ-L-Asp and D-PheOMe were prepared, five commercial proteases were separately added to the mixtures. Synthesis of *N*-CBZ-L-Asp-D-PheOMe resulted with all the enzymes (Fig. 2). The highest conversion yield of 56% was obtained with thermolysin. The commercial enzymes, including thermolysin, showed no difference in selectivity between L-PheOMe and D-PheOMe. Both *N*-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-PheOMe were synthesized with thermolysin. In contrary to our results, there are reports [15,16] that *N*-CBZ-L-Asp-L-PheOMe is synthesized from *N*-CBZ-L-Asp and racemic L and D-PheOMe using thermolysin in a water phase, but

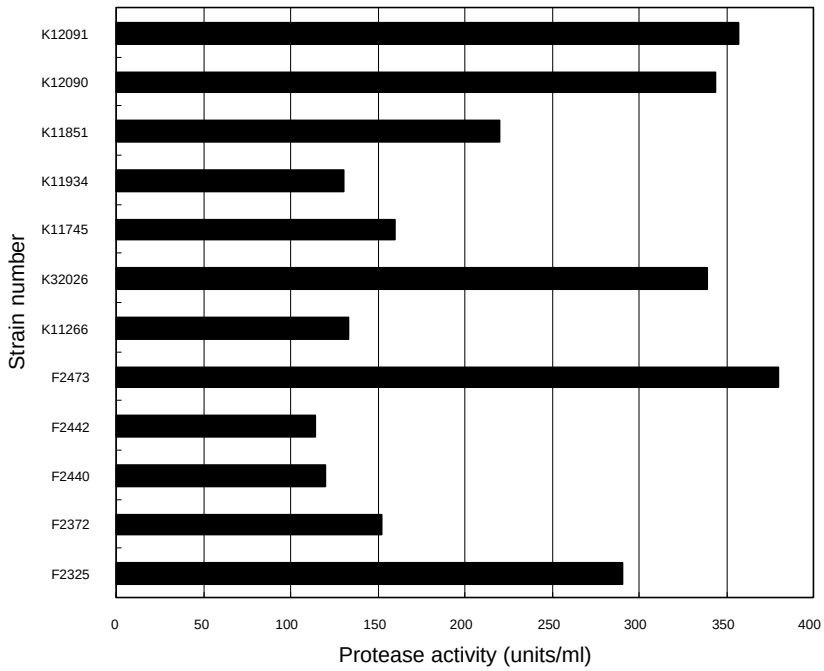

Fig. 3. Protease activities of the enzymes produced by the first 12 screened strains. K(strains of KCCM): *Pseudomonas aeruginosa* KCCM 11266, *Bacillus amyloliquefaciens* KCCM 12090, *Bacillus amyloliquefaciens* KCCM 12091, *Bacillus megaterium* KCCM 11745, *Bacillus megaterium* KCCM 11934, *Bacillus licheniformis* KCCM 11851, *Pseudomonas* sp. KCCM 32026; F(strains isolated from soy sauce): *Bacillus megaterium* F2325, *Bacillus meganterium* F2372, *Bacillus megaterium* F2440, *Enterococcus faecalis* F2442, *Bacillus laterosporus* F2473.

N-CBZ-L-Asp-D-PheOMe is not. There was a big difference in that we used eutectic substrate mixtures in which the substrate concentration levels are up to 100 times greater than concentration in a water phase.

At industrial scales of reaction, synthesis of an aspartame precursor using eutectic substrate mixtures can be significant with high product yields. However, there will be problems if *N*-CBZ-L-Asp and racemic L- and D-PheOMe are used as substrates with the commercial proteases because both *N*-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-PheOMe are synthesized. Therefore, L-PheOMe-selective proteases need to be screened.

3.2. Isolation of PheOMe-enantioselective protease producers

L-PheOMe-enantioselective proteases are required in order to synthesize *N*-CBZ-L-Asp-L-PheOMe from *N*-CBZ-L-Asp and racemic L- and D-PheOMe without also synthesizing *N*-CBZ-L-Asp-D-PheOMe. We isolated microorganisms that produce L-PheOMe-enantioselective proteases. Plates were made with four different agar media (Table 1). Previously synthesized *N*-CBZ-L-Asp-D-PheOMe was used as a nitrogen source of the Medium 2. Nine *Bacillus* strains of KCCM and 29 strains isolated from soy sauce were inoculated onto the plates, which were then cultivated for 48 h. Colonies grown on either Medium 1 or 2 were selected. Twelve strains, which used either *N*-CBZ-L-Asp-L-PheOMe or *N*-CBZ-L-Asp-D-PheOMe as a nitrogen source, were selected.

Fermentations using the 12 strains were conducted. Crude enzyme solutions were prepared from culture broths and protease activities were measured (Fig. 3). The enantio-selectivities of the enzymes produced by the 12 strains were tested. Eutectic substrate mixtures of *N*-CBZ-L-Asp and L-PheOMe or *N*-CBZ-L-Asp and D-PheOMe were made by addition of adjuvants (water 5%; MEA 6%; DMSO 6%) [6]. After the 12 enzyme solutions were separately added to the mixtures, reactions were performed at 37 °C. Both *N*-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-PheOMe were synthesized by most of the enzymes (Fig. 4), indicating no enantio-selectivity to PheOMe. However, the enzyme

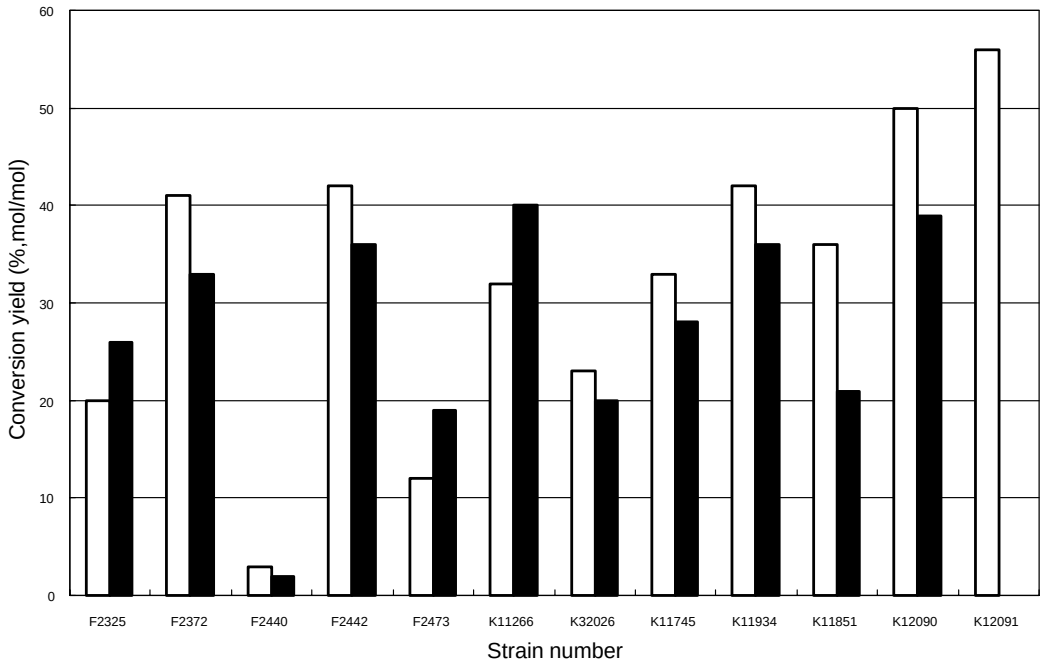

Fig. 4. Enantio-selectivities of the proteases produced by the first 12 screened strains in synthesis of *N*-CBZ-L-Asp-(L,D)-PheOMe and *N*-CBZ-L-Asp-D-PheOMe.(□), *N*-CBZ-L-Asp-L-PheOM; (■), *N*-CBZ-L-Asp-D-PheOMe. Strains are same as those in Fig. 3.

from *B. amyloliquefaciens* KCCM 12091 caused synthesis of *N*-CBZ-L-Asp-L-PheOMe, but not *N*-CBZ-L-Asp-D-PheOMe, indicating that this enzyme is selectively reactive with L-PheOMe but not with D-PheOMe.

3.3. Effect of D-PheOMe on the synthesis of *N*-CBZ-L-Asp-L-PheOMe

N-CBZ-L-Asp-L-PheOMe was synthesized from *N*-CBZ-L-Asp and L-PheOMe by the partially purified protease of *B. amyloliquefaciens*. The conversion yield increased gradually with the reaction time, reaching 53% at 4 h (Fig. 5). Thereafter, the yield did not increase further. The effect of D-PheOMe on synthesis of *N*-CBZ-L-Asp-L-PheOMe was investigated. The conversion yield of *N*-CBZ-L-Asp-L-PheOMe was estimated in the presence of different mole fractions of D-PheOMe. *N*-CBZ-L-Asp and D-PheOMe did not react. The final yield of *N*-CBZ-L-Asp-L-PheOMe was

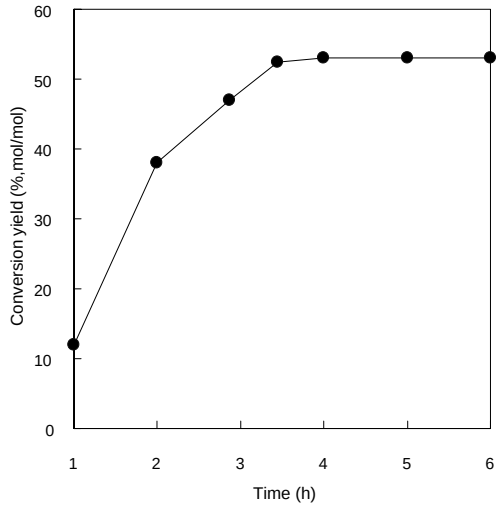

Fig. 5. Conversion yields of *N*-CBZ-L-Asp-L-PheOMe vs. time by the protease of *B. amyloliquefaciens* KCCM 12091.

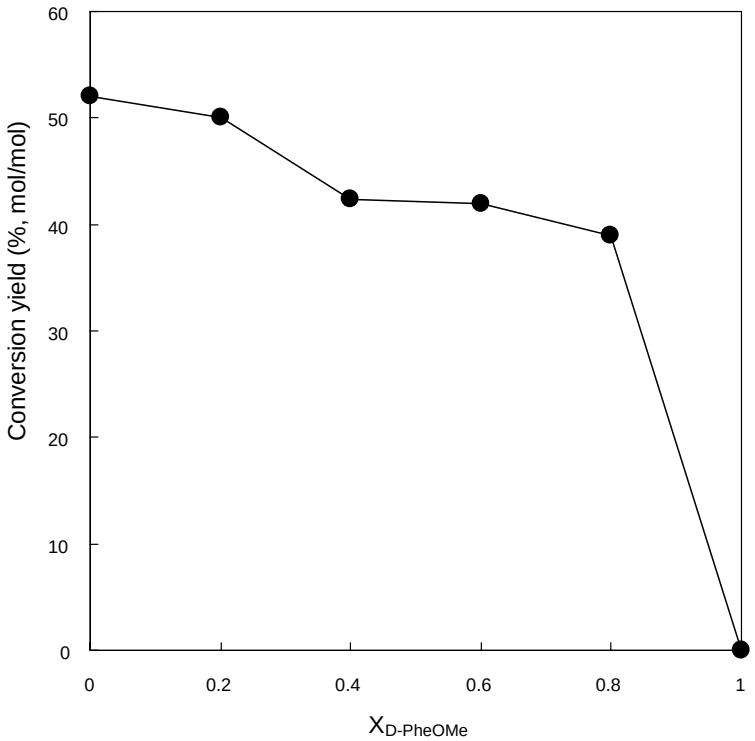

Fig. 6. Conversion yields of *N*-CBZ-L-Asp-L-PheOMe and *N*-CBZ-L-Asp-D-PheOMe in the presence of D-PheOMe with different mole fractions by the protease of *B. amyloliquefaciens* KCCM 12091.

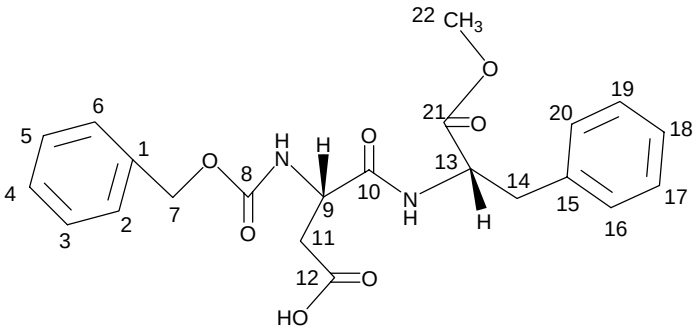

Fig. 7. Carbon numbers in structure of *N*-CBZ-L-Asp-L-PheOMe.

approximately 40–50% (Fig. 6), similar to the yield in the absence of D-PheOMe, indicating that the protease of *B. amyloliquefaciens* is L-PheOMe-selective, and that D-PheOMe does not affect the reaction between *N*-CBZ-L-Asp and L-PheOMe.

3.4. Confirmation of N-CBZ-L-Asp-L-PheOMe

The product *N*-CBZ-L-Asp-L-PheOMe was separated from reacted solutions of *N*-CBZ-L-Asp and L-Phe OMe using prep-HPLC. The second peak fractions were collected and analyzed by ^{13}C NMR. The reaction product was confirmed as *N*-CBZ-L-Asp-L-Phe OMe (Fig. 7). *N*-CBZ-L-Asp-L-PheOMe ^{13}C NMR (DMSO-d_6, 600 MHz, ppm): δ 137.003 (C-1), 127.840 (C-2), 129.585 (C-3), 128.190 (C-4), 129.585 (C-5), 127.840 (C-6), 65.525 (C-7), 156.147 (C-8), 52.897 (C-9), 171.123 (C-10), 36.450 (C-11), 172.822 (C-12), 48.965 (C-13), 36.222 (C-14), 134.506 (C-15), 128.575 (C-16), 128.829 (C-17), 127.518 (C-18), 128.829 (C-19), 128.575 (C-20), 169.377 (C-21), 50.924 (C-22).

4. Conclusion

A protease specific to the L-form of PheOMe was used to synthesize *N*-CBZ-L-Asp-L-PheOMe selectively from a mixture of *N*-CBZ-L-Asp and racemic L- and D-PheOMe. Synthesis of an aspartame precursor (*N*-CBZ-L-Asp-L-PheOMe) was only achieved with racemic L- and D-PheOMe. Substrate costs can be reduced because chemical synthesis of racemic L- and D-PheOMe is less expensive than production of L-PheOMe by fermentation.

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