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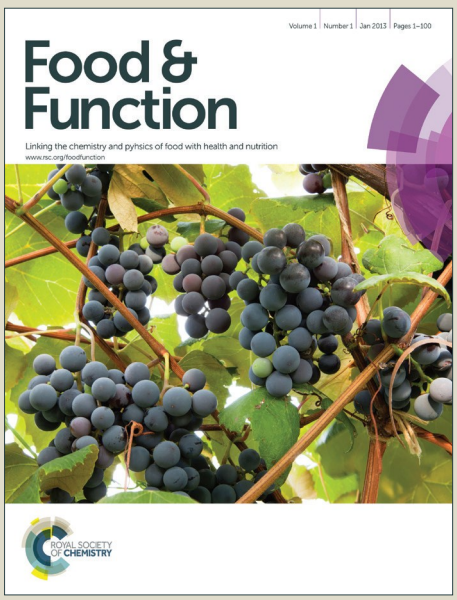

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ARTICLE

Isolation of prolyl endopeptidase inhibitory peptides from a sodium caseinate hydrolysate

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Prolyl endopeptidase (PEP) has been regarded in relation to neurodegenerative disorders, and the PEP inhibitors can restore memory loss caused by amnesic compounds. In this study, we investigated the PEP inhibitory activity of the enzymatic hydrolysates from various food protein sources, isolated and identified the PEP inhibitory peptides. The hydrolysate obtained from sodium caseinate using bromelain (SC/BML) displayed the highest inhibitory activity of 86.8% at 5 mg/mL in the present study, and its IC_{50} value against PEP was 0.77 mg/mL. The F-5 fraction by RP-HPLC (reversed-phase high performance liquid chromatography) from SC/BML showed the highest PEP inhibition rate of 88.4%, and 9 peptide sequences were identified. The synthetic peptides (1245.63-1787.94 Da) showed dose-dependent inhibition effects on PEP as competitive inhibitors with IC_{50} values between 29.8 and 650.5 μM. The results suggest that the peptides derived from sodium caseinate have the potential to be PEP inhibitors.

Introduction

Prolyl endopeptidase (prolyl oligopeptidase, PEP, POP, PREP, EC 3.4.21.26) is a large intracellular serine protease, which cleaves short-length peptides (<30 amino acids) at the carboxyl side of an internal proline [1]. Previous studies indicated that PEP activity is involved in key physiological function, such as learning and memory [2,3], cell division and differentiation [4], signal transduction [5] and protein secretion [6], as well as in some psychiatric disorders. PEP also plays an important role in the degradation of biologically active peptide hormones and neuropeptides that contain proline residues such as oxytocin, vasopressin, substance P, neurotensin and angiotensin [7,8], which has been linked to a variety of neurological disorders, e.g. Alzheimer's disease, amnesia, depression and schizophrenia [9]. A study demonstrated the increased PEP expression in hippocampus of adult transgenic mice before the appearance of β-amyloid plaques but in parallel with the development of memory deficits [10]. Altered serum PEP activity has also been reported in many psychiatric disorders, and abnormal levels of PEP activity have been found to be significantly higher in brains of Alzheimer's patients than normal individuals [11-13]. Furthermore, lowered serum PEP activity was observed in bulimia nervosa and anorexia nervosa patients [14]. PEP-like immunoreactivity has also been detected in the hippocampus of senescence-accelerated mice [15,16]. Therefore, PEP inhibitors are expected to be used as therapeutic agents for progressive memory deficits and cognitive dysfunction related to aging and neurodegenerative diseases of the central nervous system.

Several specific PEP inhibitors have recently been developed, and those may prove valuable to treat various clinical conditions of the brain, as indicated by the neuroprotective and cognition-enhancing effects of PEP inhibitors in experimental animals [17-19]. The PEP inhibitors include pramiracetam [3], baicalin [20], JTP-4819 [21], KYP-2047 [22] and S-17092 [23]. Most of the published inhibitors are chemically synthesized, substrate-like inhibitors that are based on the *N-acyl-L*-prolyl-pyrrolidine structure. But there are only a few studies on PEP inhibitors from proteins and peptides. Proteins and peptides have a wide variety of biological activities that may benefit human health by acting like antioxidant, antihypertensive and antithrombotic agents, among others activities [24,25]. Bioactive peptides are small sequences of amino acids encrypted in food proteins in an inactive form that are released and activated by proteolytic enzymes during food processing or gastrointestinal digestion [26]. These peptides are present in food sources such as milk, meat, eggs, soybean, wheat, maize, rice, and amaranth [24,27,28]. A synthetic peptide, Ile-Tyr-Pro-Phe-Val-Glu-Pro-Ile from human β-casein could inhibit PEP activity *in vitro* (IC_{50}=8.0 μM) [29]. Two γ-zein-related synthetic peptides, His-Leu-Pro-Pro-Pro-Val and His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-Val, have been shown to inhibit PEP with the IC_{50} values of 80 and 30 μM, respectively [30]. A peptide, Met-Pro-Pro-Pro-Leu-Pro-Ala-Arg-Val-Asp-Ala-Leu-Asn, from bovine brain was determined having great PEP inhibitory activity, and its IC_{50} value was 38.4 μM [31]. A previous study has revealed that 6 peptides isolated from sake cake and sake act as PEP inhibitors with IC_{50} values between 11.8 to 42.8 μM [32]. Two peptides isolated from a red wine, and their amino acid sequences and IC_{50} values were Val-Glu-Ile-Pro-Glu (17 μM) and Tyr-Pro-Ile-Pro-Phe (87.8 μM) [33]. These peptides comprised at least one proline residue in their sequences, and the peptide length ranged from 5 to 13 amino acid residues. Therefore, the aim of this study was to investigate the PEP inhibitory activity of the enzymatic hydrolysates from various

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proteins rich in proline content, then isolate and identify the PEP inhibitory peptides.

Materials and methods

Materials and reagents

Wheat gluten, soy protein isolate and sodium caseinate (from cow milk) were purchased from Gemfont Corporation (Taipei, Taiwan). Tilapia fish skins, the processing byproduct recovered from fresh skin-off fillets were supplied by Fortune Life Enterprise Co. Ltd. (Kaohsiung, Taiwan); the milkfish skins were donated by Simmy Seafood Co. Ltd. (Long An Province, Vietnam); the halibut and Atlantic salmon (*Salmo salar*) skins were supplied by Albion Fisheries Ltd. (Vancouver, BC, Canada). The tuna cooking juice was donated by a canned tuna processor in Chiayi County (Taiwan). Gelatin (from porcine skin) was purchased from Sigma-Aldrich, Inc. (St. Louis, Mo, USA). Bromelain (from pineapple) was purchased in dry powder form from St Bio, Inc. (Taipei, Taiwan). Thermolysin (from *Bacillus thermoproteolyticus rokko*), pepsin (from porcine gastric mucosa), pancreatin (from porcine pancreas), trypsin (from porcine pancreas) and Protease XXIII (from *Aspergillus melleus*) were obtained in dry powder form from Sigma-Aldrich, Inc. (St. Louis, Mo, USA). Prolyl endopeptidase (O9515, recombinant, expressed in *E. coli*), Z-Gly-Pro-4-nitroanilide (96286, Z-Gly-Pro-pNA) and bacitracin (PHR1590) were from Sigma-Aldrich, Inc. Alcalase (from *Bacillus licheniformis*) and Flavourzyme (from *Aspergillus oryzae*) were purchased from Neova Technologies Inc. (Copenhagn, Denmark). Orientase 90N (from *Bacillus subtilis*) was obtained in dry powder form from Hankyu Bioindustry Co. (Osaka, Japan). Other chemicals and reagents used were analytical grade and commercially available.

Extraction of gelatin from fish skins

The thawed fish skins were gently washed with running tap water, drained, and cut into pieces (about 5 × 10 cm). The fish skins were soaked in 0.2 M NaOH (1:10; w/v) and stirred in a cold room at 4 °C for 30 min. This procedure was repeated three times to remove noncollagenous proteins and pigments. The skins were washed with running tap water until the pH of the rinsing water was neutral. Afterward, the skins were soaked in 0.05 M acetic acid (1:10; w/v), stirred at room temperature for 3 h, and then washed by running tap water until the pH of the rinsing water was neutral. Almost all of the scales could be removed. The gelatin of the swollen skins was extracted in distilled, deionized water (1:2; w/v) at 70 °C for 3 h. The oil and aqueous layers of the extract were separated by separatory funnels, and the extract was filtered through two layers of cheesecloth, lyophilized, and stored in a desiccator at room temperature until use.

Preparation of protein hydrolysates

Wheat gluten (WG), soy protein isolate (SPI) and sodium caseinate (SC) hydrolysates were prepared using thermolysin (TLN) [E/S (enzyme/substrate) = 3%; pH 8.0; 70℃; 20 min], bromelain (BML) (E/S = 5%; pH 6.7; 45℃; 60 min) or gastrointestinal digestion (GID) [pepsin (E/S = 5%, pH 2.0, 37℃, 3 h) and followed by the mixture of trypsin and pancreatin (1:1 w/w trypsin:pancreatin, E/S = 5%, pH 7.5, 37℃, 3 h)], respectively. Tilapia fish skin gelatin (TSG), milkfish skin gelatin (MSG), halibut skin gelatin (HSG) and porcine skin gelatin (PSG) hydrolysates were prepared using Flavourzyme (FLA) (E/S = 5%, pH 7.0, 50℃, 6 h) or Alcalase (ALC) (E/S = 5%, pH 8.0, 50℃, 6 h), respectively. Atlantic salmon skin gelatin (SSG) was hydrolyzed by Flavourzyme (FLA) (E/S = 3%, pH 7.0, 50℃, 4 h). Tuna cooking juice (TCJ) was hydrolyzed by protease XXIII (PA) (E/S = 2.1%, pH 7.5, 37℃, 60 min) or Orientase 90N (OR) (E/S = 2.1%, pH 7.0, 50℃, 60 min), respectively. After hydrolysis, the hydrolysate solutions were heated in boiling water for 15 min to inactivate enzymes and then cooled in water at room temperature for 20 min. Hydrolysates were adjusted to pH 7.0 with 2 M NaOH and centrifuged (Centrifuge 05P-21, Hitachi Ltd., Katsuda, Japan) at 10,000 *g* and 4℃ for 10 min. The supernatant was lyophilized and stored at -20℃.

PEP inhibitory activity assay

PEP activity determination in this study was performed in 96-well microplates by measuring the increase in absorbance at 410 nm using Z-Gly-Pro-pNA as PEP substrate and according to the method described by the previous study [34] with some modifications. The unit of PEP activity is defined as one unit will hydrolyze 1.0 pmole of Ala-Pro-aminomethylcoumarin per minute at pH 7.5 at 25℃. The lyophilized samples were dissolved in 100 mM Tris-HCl buffer (pH 7.0) at various concentrations (0.1-5 mg/mL). The hydrolysate solution (25 μL) was added with 50 μL of 2.5 mM Z-Gly-Pro-pNA (in 40% 1,4-dioxane) and 100 μL of 100 mM Tris buffer (pH 7.0). The mixture was incubated at 30℃ for 10 min, followed by the addition of 50 μL of 0.5 units/mL PEP. The reaction mixture (total volume of 225 μL) was incubated at 30℃ for up to 60 min and the absorbance of the resulting solution was measured at 410 nm with an ELISA reader (Bio Tek μ QUANT; Bio Tek Instruments, Inc., Winooski, VT, USA). Recorded data were plotted versus time, and the PEP activity was quantified from the linear part of the curve. The % PEP inhibition was defined as the percentage of PEP activity inhibited by a given concentration of hydrolysate. The IC_{50} value corresponds to the concentration of sample needed to inhibit PEP by 50%. Bacitracin was used as positive control. The mode of inhibition of all the isolated peptides was investigated using Lineweaver and Burk kinetic analysis by measuring the initial rate of the reaction at different Z-Gly-Pro-pNA concentrations between 0.5 and 2.5 mM without inhibitors and in the presence of peptides at their IC_{50} concentrations. K_m and V_{max} values were deducted from the Lineweaver and Burk double reciprocal plots. The mode of inhibition was determined by comparing K_m and V_{max} obtained in the presence and absence of the inhibitors.

Ultrafiltration (UF)

The peptides of the hydrolysates were fractionated by ultrafiltration (model ABL085, Lian Sheng Tech. Co., Taichung, Taiwan) with spiral wound membranes having molecular mass cutoffs of 2.5 and 1.0 kDa. The fractions were collected as follows: >2.5 kDa, peptides retained without passing through 2.5 kDa membrane; 1.0-2.5 kDa, peptides permeating through the 2.5 kDa membrane but not the 1.0 kDa membrane; <1.0 kDa, peptides permeating through the 1.0 kDa membrane. All fractions collected were lyophilized and stored in a desiccator until use.

Purification of PEP inhibitory peptides

The fractionated hydrolysates were purified using reversed-phase high performance liquid chromatography (RP-HPLC) (Model L-2130 HPLC, Hitachi Ltd., Katsuda, Japan). The lyophilized hydrolysate fraction (2 mg) was dissolved in 1 mL

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of ddH_2O, and 60 μL of the mixture was then injected into a column (ZORBAX Eclipse Plus C18, 4.6 mm × 250 mm, Agilent Tech. Inc., CA, USA) using a linear gradient of acetonitrile (5-40% in 120 min) in 0.1% TFA under a flow rate of 0.7 mL/min. The peptides were detected at 215 nm. Each collected fraction was then lyophilized and stored in a desiccator until use. The fractions were dissolved in 100 mM Tris buffer (pH 7.0) to the concentration of 1 mg solid/mL, and then the solution was used to determine the PEP inhibitory activity. For obtaining sufficient amount of samples to perform PEP inhibition assay, this process was done repeatedly.

Identification of amino acid sequence by MALDI-TOF/TOF MS/MS

The purified peptides were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), using a delayed extraction source and a 335 nm pulsed nitrogen laser. This analysis was carried out using a MALDI-TOF/TOF (UltraFlexIII, Bruker Daltonics Inc., Billerica, MA, USA). Peptides solution (0.6 μL) was mixed with 0.6 μL of saturated α-cyano-4-hydroxycinnamic acid, and a droplet of the resulting solution was placed on the sample target mass spectrometer. The droplet was dried by evaporation at room temperature and then loaded into the mass spectrometer for analysis. The instrument was operated in positive ion reflection mode with the source voltage set at 20 kV. All spectra were the results of signal averaging of 200 shots. Measurements were determined in the mass range m/z 200-4000 Da, while the peptide sequencing was determined by MS/MS spectra processing, using BioTools (Version 3.2; Bruker Daltonics Inc., Billerica, MA, USA).

Peptide synthesis

Peptides were prepared by the conventional Fmoc solid-phase synthesis method with an automatic peptide synthesizer (Model CS 136, CS Bio Co. San Carlos, CA, USA), and their purity was verified by analytical RP-HPLC-MS/MS.

Statistical analysis

Each data point represents the mean of three samples was subjected to analysis of variance (ANOVA) using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA). A test of comparison of two means was analysed by Duncan's test, and the significance level of $P<0.05$ was employed.

Results and discussion

PEP inhibitory activity of protein hydrolysates

The PEP inhibitory activity of the hydrolysates from wheat gluten (WG/BML; WG/TLN; WG/GID), soy protein isolate (SPI/BML; SPI/TLN; SPI/GID), sodium caseinate (SC/BML; SC/TLN; SC/GID), fish skin gelatin (TSG/FLA; TSG/ALC; MSG/FLA; MSG/ALC; HSG/FLA; HSG/ALC; SSG/FLA), porcine skin gelatin (PSG/FLA; PSG/ALC) and tuna cooking juice (TCJ/PA; TCJ/OR) at the concentration of 5 mg/mL was shown in Fig. 1. The PEP inhibition rates of the wheat gluten, soy protein isolate and sodium caseinate hydrolysates were 57.9-72.4%, 55.5-78.8% and 77.1-86.8%, respectively; the inhibitory activity of fish skin gelatin, porcine skin gelatin and tuna cooking juice hydrolysates were 32.2-64.4%, 24.8-36.1% and 22.1-26.8 %, respectively. To our knowledge, the proteins rich in proline content may have the potential to be the source of PEP inhibitory peptides. Therefore, the hydrolysates from wheat gluten, sodium caseinate, fish and porcine skin gelatin which contain >10 mol% of proline were expected to possess great PEP inhibitory activity [35-37]. From the results shown in Fig. 1, WG/TLN, SPI/BML, SPI/GID, SC/BML, SC/TLN and SC/GID showed relatively high PEP inhibitory activity; but surprisingly, all the skin gelatin hydrolysates performed fair action on PEP inhibition. Furthermore, the hydrolysates from the same protein hydrolyzed by different proteases showed various PEP inhibitory activities. We inferred that the peptide structures determined the PEP-inhibitory activity of hydrolysates. The sodium caseinate hydrolyzed with bromelain (SC/BML) showed significantly higher PEP inhibitory activities (86.8%, $P<0.05$) than the other hydrolysates. Therefore, SC/BML was used for further purification.

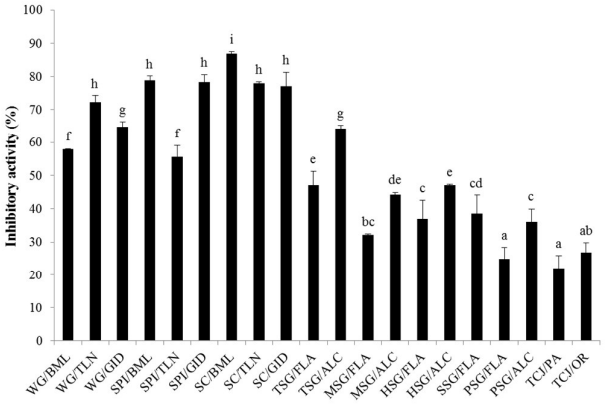

Fig. 1 PEP inhibition rates of various protein hydrolysates. The PEP inhibition rate was determined with the hydrolysates at the concentration of 5 mg/mL. Bars represent standard deviations from triplicate determinations. Different letters indicate significant differences ($P<0.05$). WG: wheat gluten; SPI: soy protein isolate; SC: sodium caseinate; TSG: tilapia fish skin gelatin; MSG: milkfish skin gelatin; HSG: halibut skin gelatin; SSG: salmon skin gelatin; PSG: porcine skin gelatin; TCJ: tuna cooking juice; TLN: thermolysin; BML: bromelain; GID: gastrointestinal digestion; FLA: Flavourzyme; ALC: Alcalase; PA: protease XXIII and OR: Orientase 90N.

PEP inhibitory activity of UF fractions of hydrolysates

The PEP inhibitory activities of the UF fractions (>2.5 kDa, 1.0-2.5 kDa, and <1.0 kDa) of SC/BML at the concentration of 2.5 mg/mL are shown in Fig. 2. The result showed that the <1.0-kDa, 1.0-2.5-kDa fractions and SC/BML (control) had insignificantly different ($P>0.05$) and higher PEP inhibition rates between 77.8 to 82.5% than the >2.5-kDa fraction which displayed the inhibition rates of 72.6% (Fig. 2A). To our knowledge, the majority of the kinetic studies performed on PEP enzymes have been limited to short, synthetic substrates of 2-6 residues or slightly longer neuropeptides of <15 residues with only one or two internal proline residues [38,39]. To date, it is generally thought that the chain length specificity of most PEPs is capped at 30 amino acid residues, that shows PEP can degrade peptides of molecular weight less than 3.0 kDa [8,38]. As reviewed the previous studies, the length of the PEP inhibitory peptides ranged from 3-18 amino acid residues, and these

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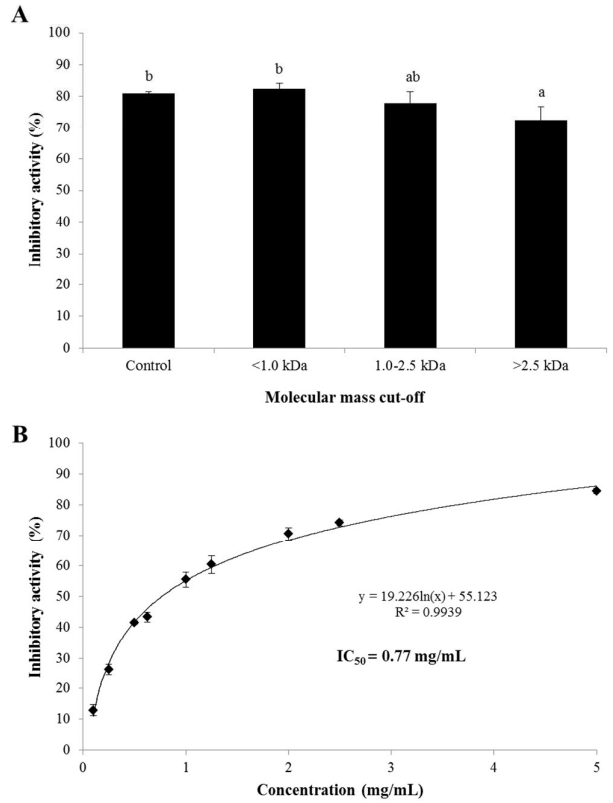

Fig. 2 (A) PEP inhibition rate of SC/BML fractionated by UF at the concentration of 2.5 mg/mL. (B) PEP inhibition rate of the SC/BML at various concentrations. Bars represent standard deviations from triplicate determinations. Different letters indicate significant differences (P<0.05).

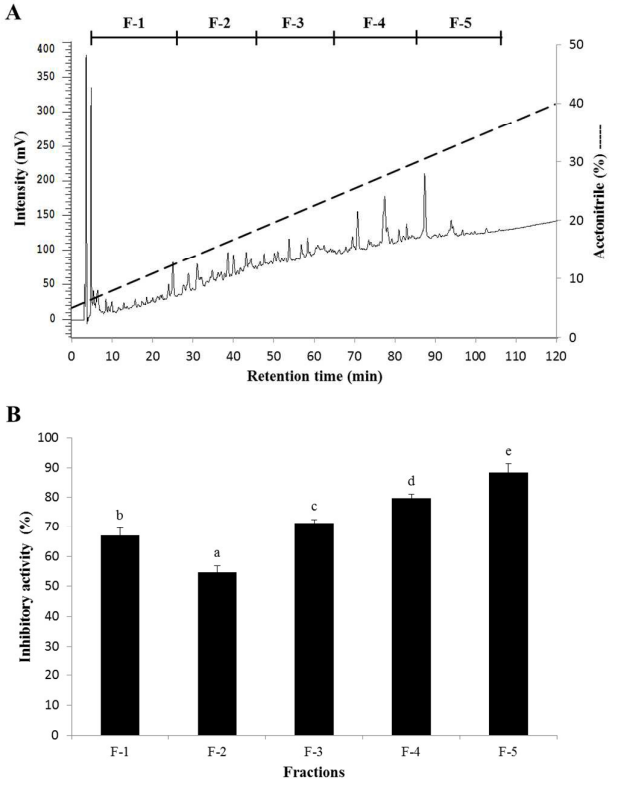

Fig. 3 (A) Elution profile and (B) PEP inhibition rate of the peptide fractions from the SC/BML separated by RP-HPLC. The PEP inhibition rate was determined with each HPLC fraction at the concentration of 1 mg/mL. Bars represent standard deviations from triplicate determinations. Different letters indicate significant differences (P<0.05).

peptides comprised at least one and up to 6 proline residues in their sequences [29,31,33]. Therefore, the PEP inhibitory peptides are proposed to be determined majorly by the appearance of proline residue in the sequence rather than the peptide length. Based on the PEP inhibitory activity of the UF fractions not showing greater than the hydrolysate, SC/BML was used for further purification by RP-HPLC and identification of peptide sequences. The IC_{50} value of the SC/BML was determined and shown in Fig. 2B. The PEP inhibitory activities of SC/BML at various concentrations (0.1-5 mg/mL) ranged from 12.8 to 84.4% in a dose-dependent manner, and the IC_{50} value was 0.77 mg/mL.

Purification of PEP inhibitory peptides by RP-HPLC

The elution profile and PEP inhibitory activity of the fractions from SC/BML separated by RP-HPLC are shown in Fig. 3. To obtain a sufficient amount of purified peptide, chromatographic separations were performed repeatedly. Five fractions (F-1 to F-5) were obtained upon RP-HPLC separation of SC/BML (Fig. 3A), and they were lyophilized and then used to determine their PEP inhibitory activities at the concentration of 1 mg solid/mL. The result showed that F-5 had the highest PEP inhibition rate of 88.4%, as compared to the others which showed the inhibition rates between 54.7 to 79.8% (Fig. 3B). Therefore, F-5 was used to identify the amino acid sequences of the peptides.

The fraction F-5 was used to identify the amino acid sequences of peptides by MALDI TOF/TOF MS. Forty-seven peaks were obtained, and the nine major peaks (m/z ranged from 1246.636 to 1788.943) with strong intensity were selected for MS/MS analysis (Fig. 4). After the analysis by MS/MS spectra processing with BioTools database, the amino acid sequences of the 9 peptides are listed in Table 1. These peptides obtained in this study comprised 13 to 20 amino acid residues, and moreover, they were composed at least one and up to 5 internal proline residues in their sequence. The result therefore is consistent with the hypothesis that the PEP inhibitory peptides exhibit at least one proline demonstrated in the previous study [38].

PEP inhibitory activity of synthetic peptides

The molecular mass, origin and IC_{50} values against PEP of the 9 synthetic peptides are shown in Table 1. Based on the specificity of bromelain with the preference for cleaving the C-terminus of Lys, Ala and Tyr [40], the peptide no. 3, AVPYPQRDMPIQAFLLY, was obtained by bromelain cleavage; however, the other peptides might be obtained by other proteases as the impurity of bromelain used in the present study. The identification by MALDI TOF/TOF MS/MS of these peptides showed homology with the α-S1, β-, and κ-casein molecules as compared to BIOPEP database (www.uwm.edu.pl). The molecular mass of these peptides ranged from 1245.63 to 1787.94, and the IC_{50} values against PEP varied from 29.8 to 650.6 μM. The PEP inhibitory activities of these peptides were not correlated to their molecular mass; peptides with more proline residues, however, showed lower IC_{50} values. For example,

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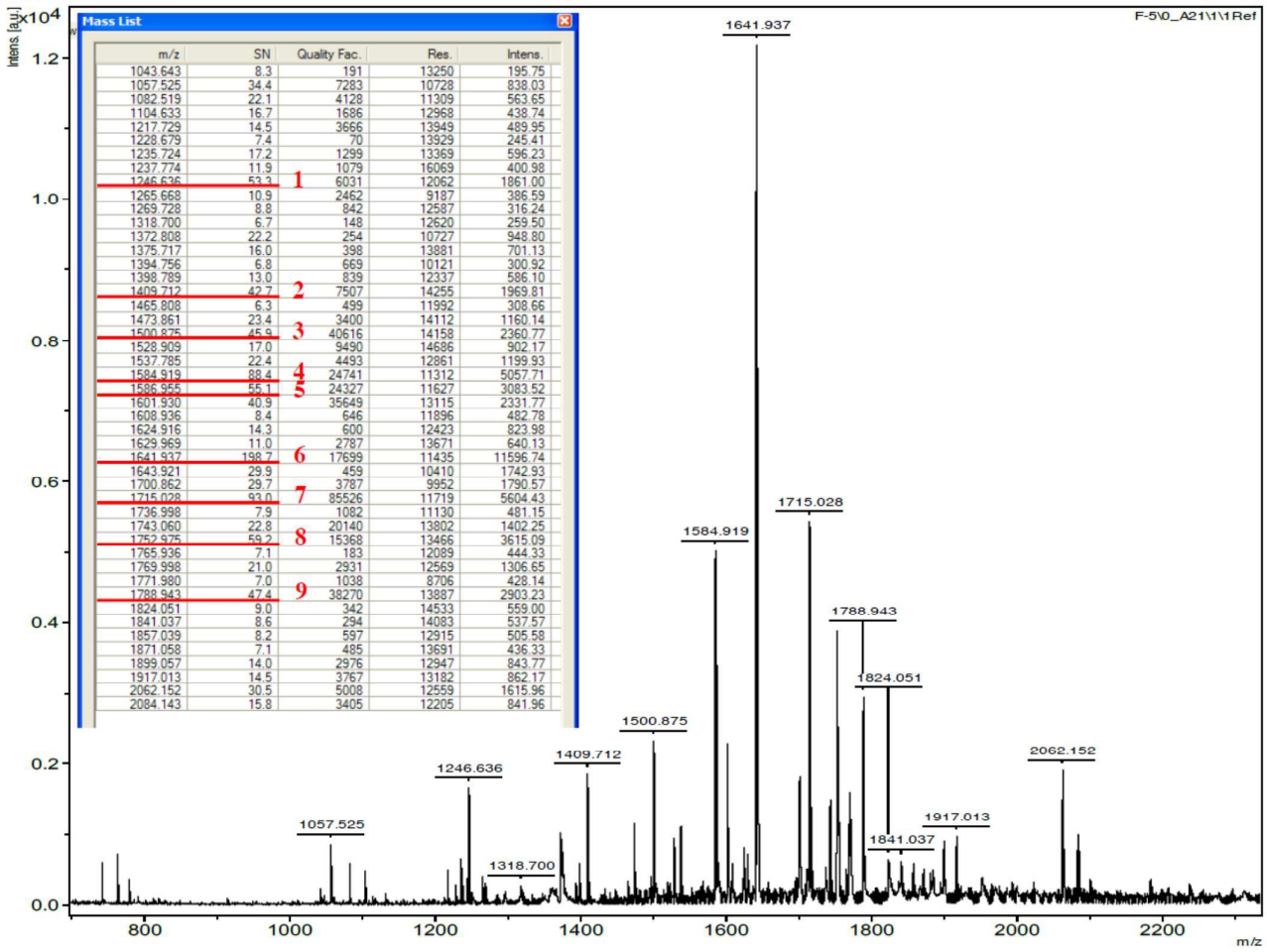

m/z	SN	Quality Fac.		Res.	Intens.
1043.643	8.3	191		13250	195.75
1057.525	34.4	7283		10728	838.03
1082.519	22.1	4128		11309	563.65
1104.633	16.7	1686		12968	438.74
1217.729	14.5	3666		13949	489.95
1228.679	7.4	70		13929	245.41
1235.724	17.2	1299		13369	596.23
1237.774	11.9	1079		16069	400.98
1246.636	53.3	6031	1	12062	1861.00
1265.668	10.9	2462		9187	386.59
1269.728	8.8	842		12587	316.24
1318.700	6.7	148		12620	259.50
1372.808	22.2	254		10727	948.80
1375.717	16.0	398		13881	701.13
1394.756	6.8	669		10121	300.92
1398.789	13.0	839		12337	586.10
1409.712	42.7	7507	2	14255	1969.81
1465.808	6.3	499		11992	308.66
1473.861	23.4	3400		14112	1160.14
1500.875	45.9	40616	3	14158	2360.77
1528.909	17.0	9490		14686	902.17
1537.785	22.4	4493		12861	1199.93
1584.919	88.4	24741	4	11312	5057.71
1586.955	55.1	24327	5	11627	3083.52
1601.930	40.9	35649		13115	2331.77
1608.936	8.4	646		11896	482.78
1624.916	14.3	600		12423	823.98
1629.969	11.0	2787		13671	640.13
1641.937	198.7	17699	6	11435	11596.74
1643.921	29.9	459		10410	1742.93
1700.862	29.7	3787		9952	1790.57
1715.028	93.0	85526	7	11719	5604.43
1736.998	7.9	1082		11130	481.15
1743.060	22.8	20140		13802	1402.25
1752.975	59.2	15368	8	13466	3615.09
1765.936	7.1	183		12089	444.33
1769.998	21.0	2931		12569	1306.65
1771.980	7.0	1038		8706	428.14
1788.943	47.4	38270	9	13887	2903.23
1824.051	9.0	342		14533	559.00
1841.037	8.6	294		14083	537.57
1857.039	8.2	597		12915	505.58
1871.058	7.1	485		13691	436.33
1899.057	14.0	2976		12947	843.77
1917.013	14.5	3767		13182	862.17
2062.152	30.5	5008		12559	1615.96
2084.143	15.8	3405		12205	841.96

Fig. 4 Mass spectrum of the selected peptides in F-5.

HPHPHLSFMAIPP (1245.63 Da) comprised 4 proline residues showed the IC_{50} value of 34.8 μM, while QKEDVPSERYLGYLEQL (1751.97 Da) with 1 proline residue performed the IC_{50} value of 368.7 μM. But VPLGTQYTDAPSFSDIP (1499.87 Da) with 3 proline residues had higher IC_{50} value of 650.5 μM than TIASAEPTVHSTPTTEAIV (1583.91 Da) with only 2 proline residues (123.6 μM). We suggested that the PEP inhibitory activity of peptides might be determined by the number of proline residue and also the next amino acid residue binding to the C-terminal of proline. These peptides all behaved as competitive inhibitors of PEP (Table 1 and Fig. 5). The bacterial PEPs were reported to prefer the cleavage sites on Pro-Gln, Pro-Tyr and Pro-Phe bonds [38,41]. In previous studies, Ile-Tyr-Pro-Phe-Val-Glu-Pro-Ile from human β-casein [29], Tyr-Pro-Ile-Pro-Phe from a red wine [33], Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala, Leu-Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala and Leu-Leu-Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala

Table 1 Amino acid sequence, molecular mass, protein origin, IC_{50} value against PEP and mode of inhibition of the synthetic peptides from sodium caseinate hydrolystes in F-5.

No.	Peptide sequence	No. of proline	Calculated mass /observed mass	Origin	IC_{50} (μM)	Type of inhibition
1.	CLVAVALARPKHPIKHQGLP	3	1787.86/1787.94	ß-casein	265.6	Competitive
2.	QKEDVPSERYLGYLEQL	1	1751.85/1751.97	α-S1-casein	368.7	Competitive
3.	AVPYPQRDMPIQAFLLY	3	1713.93/1714.02	ß-casein	48.2	Competitive
4.	PIHNSLPQNIPPLTQTPV	4	1640.85/1640.93	ß-casein	29.8	Competitive
5.	PVQPFTESQSLTLTDVE	2	1585.80/1585.95	ß-casein	236.3	Competitive
6.	TIASAEPTVHSTPTTEAIV	2	1583.90/1583.91	κ-casein	123.6	Competitive
7.	VPLGTQYTDAPSFSDIP	3	1499.81/1499.87	α-S1-casein	650.5	Competitive
8.	SIITSTPETPTVAVPTT	3	1408.60/1408.71	κ-casein	79.8	Competitive
9.	HPHPHLSFMAIPP	4	1245.67/1245.63	κ-casein	34.8	Competitive
10.	Bacitracin (positive control)				124.6	

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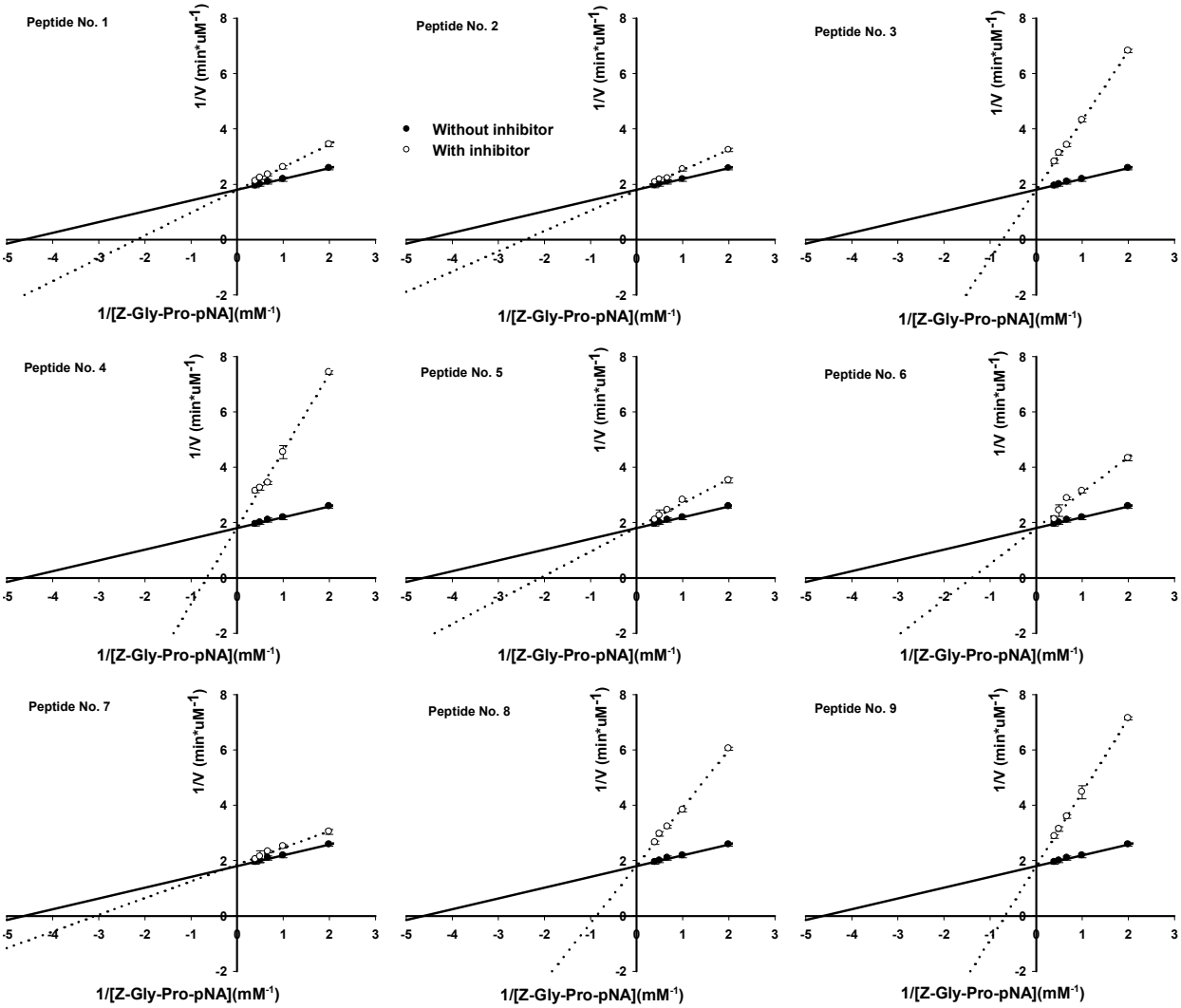

Fig. 5 Lineweaver and Burk double reciprocal plots for PEP inhibition with the 9 synthetic peptides. Values are the mean of three determinations ± SD.

from sake cake [32] were reported to show PEP inhibitory activities with IC_{50} values ranging from 8.0 to 87.8 μM, and all the peptides comprised Pro-Phe in their sequences. While other PEP inhibitory peptides having Pro-Pro, Pro-Val, Pro-Leu, Pro-Ala, Pro-Glu-Pro-Ile, Pro-Ser, Pro-Asn and Pro-Arg in their sequences also showed low IC_{50} values between 11.8 to 80.0 μM [30-33]. In the present study, three peptides AVPYPQRDMPIQAFLLY, PIHNSLPQNIPPLTQTPV, HPHPHLSFMAIPP, having Pro-Tyr, Pro-Gln, Pro-Ile, Pro-Pro, Pro-Val, Pro-His in their sequence, showed greater PEP inhibitory activities than the others with their IC_{50} values below 50 μM. A previous study has demonstrated that the substrate accessibility to the PEP active site, including substrate flexibility and inter-domain dynamics, appears to be the primary factor that limits PEP specificity [38]. In the present study, we have successfully isolated, novel and potent PEP inhibitory peptides, which act as substrate-like inhibitors, from sodium caseinate.

Although the nine peptides isolated in the present study present *in vitro* PEP inhibitory activities, a challenge of the bioavailability of the se peptides is to resist the degradation of digestive enzymes followed by passing through gastrointestinal (GI) epithelium [42]. The long-chain peptides are supposed to be hydrolysed to small peptides or free amino acids and then absorbed by GI tract. In addition, the blood-brain barrier (BBB) is another formidable challenge. Therefore, the further studies on the bioavailability of these peptides and/or the bioavailability improvement of the peptides modified by chemical methods [43] are needed to evaluate the development of these peptides as therapeutic agents of neurological disorders.

Conclusions

Enzymatic hydrolysates from various protein sources were used to determine their PEP inhibitory activity in the present study. SC/BML from sodium caseinate had a superior inhibitory effect on PEP. Nine novel peptides were successfully isolated and identified, and they all acted as competitive inhibitors of PEP. This study indicates that sodium caseinate has the potential to

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be the source of the functional food for prevention of neurological disorders. A future study for the bioavailability of the peptides is needed to evaluate their therapeutic application.

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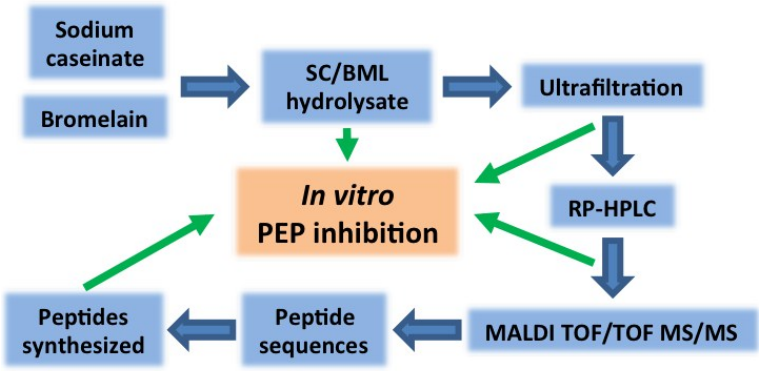
Graphical Abstract



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