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Original Article

Finding of polysaccharide-peptide complexes in *Cordyceps militaris* and evaluation of its acetylcholinesterase inhibition activity

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ABSTRACT

Acetylcholinesterase (AChE) inhibition enhances learning and cognitive ability for treatment of Alzheimer's disease. Polysaccharide–peptide complexes were identified in *Cordyceps militaris* (CPSPs) and characterized for their AChE inhibitory properties. Three polymers (CPSP-F1, -F2, and -F3) were extracted and separated by ultrasound-assisted extraction and diethylaminoethanol (DEAE)–Sepharose CL-6B column chromatography. Polysaccharide–peptide complexes were identified by DEAE–Sepharose CL-6B column chromatography and high-performance gel-filtration chromatography, Fourier transform infrared spectra, amino sugar composition analysis, and β-elimination reaction to identify polysaccharide–peptide bond categories. Separation of CPSP can increase AChE inhibitory activity from the crude polysaccharide of *C. militaris*. CPSP-F1 and CPSP-F2 exhibited half maximal inhibitory concentrations of 32.2 ± 0.2 mg/mL and 5.3 ± 0.0 mg/mL. Thus, we identified polysaccharide–peptide complexes from *C. militaris* and suggest CPSP has great potential in AChE inhibition bioassay.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that impairs memory and behavior in elderly people [1,2]. Reduced acetylcholine in the brain is the most remarkable biochemical change in AD patients [3]. Acetylcholinesterase (AChE) terminates nerve impulse transmission by rapid hydrolysis of acetylcholine; thus, AChE inhibition serves as a mechanism for the treatment of AD [4]. AChE inhibitors such

as tacrine, donepezil, rivastigmine, and galanthamine have been used for clinical AD therapy [5].

Several screening methods for AChE inhibitory activity in natural sources have been reported based on Ellman's reactions [4]; a rapid method of colorimetric determination of AChE inhibitory activity [6]. In this inhibitory mechanism, the inhibitor binds to the same active site as the enzyme substrate, and this is suggested to be a nonmetabolizable reaction [7]. Polysaccharide (PS) have been extracted or modified and assessed for their ability to inhibit AChE. *Flammulina velutipes*

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polysaccharides were extracted by ultrasonic extraction and reported to be the main AChE-inhibiting component of this organism; 18.51% AChE inhibition was obtained at 0.6 mg/mL *Flammulina velutipes* polysaccharide [8]. Gallic acid-grafted chitosans produced at different grafting ratios exhibited potent AChE inhibition with half maximal inhibitory concentration (IC_{50}) of 138.5 ± 0.25 μg/mL to 397.6 ± 5.2 μg/mL [9]. *Cordyceps militaris* possesses antioxidant, immunopotentiation, antitumor, anti-inflammatory, hypoglycemic and hypocholesterolemic activities [10–12]. In our continued search for bioactive materials, we explored AChE inhibitory activity in *C. militaris*.

Polysaccharides are present in several mushrooms as polysaccharide–peptides (PSPs) and polysaccharide–triterpenoids [13]. Several studies have reported PSP complexes in mushrooms such as *Tricholoma lobayense*, *Coriolus versicolor*, *Lentinus edodes*, and *Grifola frondosa* [14–16]. Many *C. militaris* polysaccharides have been structurally characterized and analyzed for bioactivity [10,17], but PSP complexes have not been reported in *C. militaris*. In previous studies of *Cordyceps* sp., three different PSP complexes of *Cordyceps sphecocephala* were fractionated and their anticancer activities were investigated in human hepatocarcinoma (HepG2) and neuroblastoma (SK-N-SH) cells [18,19]. A protein-bound polysaccharide HS002-II was produced from *Hirsutella sinensis* Liu, Guo, Yu & Zeng, which is the anamorph of *Cordyceps sinensis*, and its immunomodulatory activity was investigated in murine macrophage cell line RAW264.7 cells [20]. The protein-bound polysaccharides were extracted from *Cordyceps ophioglossoides* and their anticancer activities were investigated in mice [21]. We explored *C. militaris* for the presence of PSP complexes. *C. militaris* polysaccharide–peptide complexes (CPSPs) were fractionated and identified by column chromatography and high-performance gel-filtration chromatography (HPGFC), Fourier transform infrared (FTIR) spectra, amino sugar composition analysis, and β-elimination reaction to identify polysaccharide–peptide bond categories. We also studied the AChE inhibitory activity of CPSPs to understand their potential utility as AChE inhibitors.

2. Materials and methods

2.1. Materials

C. militaris fruiting bodies (BCRC 32219, deposited at the Bioresource Collection and Research Centre, Food Industry Research and Development Institute, Hsinchu, Taiwan) were provided by Xin-Run Biotechnology Co. (Changhua, Taiwan). Glucose and glucosamine hydrochloride standards were purchased from Sigma-Aldrich (St Louis, MO, USA). 5,5′-dithio-bis-(2-nitrobenzoic) acid, acetylthiocholine iodide, AChE type VI-S, bovine serum albumin (BSA), and galanthamine hydrobromide were obtained from Sigma-Aldrich. All reagents and organic solvents were analytical grade. Absorbance was recorded with a UV-Vis Spectrophotometer V-530 (Jasco, Tokyo, Japan).

2.2. Extraction and preparation of crude PS

Crude PS was extracted by ultrasound-assisted extraction (UAE) (Model: MS-600, Min Shin Machinery Co., Changhua, Taiwan) from *C. militaris* fruiting bodies. Powdered *C. militaris*

fruiting bodies (3.0 g) were extracted in 45 mL reverse osmosis (RO) water. Extractions were performed at 100°C for 30 minutes at 300 W ultrasonic power. After extraction, 45 mL RO water was added to the residual powder for a second extraction to maximize yield. The extract was collected and concentrated under reduced pressure. The concentrated extracts were mixed with three volumes 95% ethanol at 4°C overnight. The crude PS was precipitated by centrifugation at 6000 rpm (3864*g*) for 20 minutes.

2.3. Chemical analysis

Total carbohydrate content was determined by the phenol sulfuric acid method [22]. Reducing sugars were measured by the dinitrosalicylate method [23]. Glucose was used for the standard curve. The crude PS content was calculated as the difference between the contents of total carbohydrate and reducing sugars [24]. Total protein content was determined by the Lowry assay [25] with BSA for the standard curve.

Total amino sugar content was estimated by the method of Rondle and Morgan [26]. In this study, we referred to the method of Chen and Johnson [27] to estimate total amino sugar of CPSP with a glucosamine hydrochloride standard. Prior to analysis, samples were hydrolyzed by heating in a sealed tube with 4N HCl at 100°C for 10 hours. After hydrolysis, excess acid was removed by adjusting to neutral pH with a minimum quantity of 1N NaOH and the final volume was adjusted to 10.0 mL with RO water.

2.4. Chromatographic fractionations of CPSP

The concentrated crude PS solution was filtered through a 0.45-μm nylon membrane. The concentrated filtrate (212.5 mg/mL in 10.0 mL) was loaded onto a DEAE–Sepharose CL-6B anion-exchange column (2.5 cm × 10.0 cm; GE Healthcare, Uppsala, Sweden). Fractions were eluted with a stepped NaCl gradient (from 0.1M to 0.5M to 1.0M) at 0.6 mL/minute (0.7 rpm, Chrom Tech TP-T100T peristaltic pump; Taipei, Taiwan). The eluate was collected in 6.0 mL fractions (Chrom Tech FC-100A). Eluates were analyzed by measuring the absorbance at 490 nm for carbohydrates after using the phenol sulfuric acid method and at 280 nm for proteins [18]. Each CPSP fraction was concentrated and desalted using a dialysis membrane (Molecular Weight Cut-Off (MWCO) 12–16 kDa) with distilled/deionized water.

2.5. Infrared spectral analysis of CPSP

The IR spectrum of CPSP was recorded with an FTIR spectrometer (FTIR-8400S; Shimadzu, Kyoto, Japan) equipped with Shimadzu IR solution 1.30 software. The CPSP sample was ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4000/cm to 400/cm.

2.6. Molecular characteristics of CPSP

The molecular characteristics of CPSP were determined by an HPGFC system (Jasco) consisting of a PU-2080 Plus pump, UV-2075 Plus UV detector at 280 nm, RI-2031 Plus RI detector, and a PolySep-GFC-P5000 column (300 mm × 7.80 mm;

Phenomenex, Torrance, CA, USA). The mobile phase was distilled/deionized water and the flow rate was 0.8 mL/minute at room temperature. The sample injection amount was 20 μL.

2.7. Characterization of monosaccharide and amino acid compositions

Monosaccharides and amino acids in each CPSP fraction were identified and quantified by gas chromatography/mass spectrometry (Agilent 6890; Wilmington, DE, USA) [28] with a capillary column (30 m × 0.32 mm; Restek Rtx-225; Bellefonte, PA, USA) and a capillary column (30 m × 0.25 mm; HP-5MS; Agilent), respectively, which was commissioned at the testing and analysis center for food and cosmetics at Hungkuang University (Taichung, Taiwan).

2.8. Analysis of glycan−peptide bond

CPSP linkages were analyzed by β-elimination reaction [29,30]. The CPSP sample was incubated with 0.2 mol/L NaOH containing 1.0 mol/L $NaBH_4$ for 30 minutes at 45°C, scanned from 210 nm to 270 nm on a UV-Vis spectrophotometer, and compared to the non-alkali-treated sample [31].

2.9. AChE inhibitory assay

AChE inhibitory activity was assessed as described by Sacan and Yanardag [5] with modifications. Sample aliquots of varying concentrations (100 μL), 325 μL Tris−HCl buffer (50mM, pH 8.0), and 25 μL 0.28 U/mL AChE in 50mM Tris−HCl buffer containing 0.1% (w/v) BSA were incubated for 15 minutes at room temperature. Then, 75 μL of 15mM acetylthiocholine iodide in water and 475 μL of 3mM 5,5′-dithio-bis-(2-nitrobenzoic) acid in Tris−HCl buffer (50mM, pH 8.0) were added, and the final mixture was incubated for 30 minutes at room temperature. Absorbance was measured at 405 nm. Galanthamine hydrobromide was used as a positive control.

3. Results and discussion

3.1. Extraction and preparation of crude PS

Crude PS extracts were prepared. To maximize yield, 3.0 g powdered *C. militaris* fruiting bodies was extracted five times by continuous ultrasound-assisted extraction. The maximum yield of crude PS was achieved by extracting three times, for a total yield of 27.3 ± 0.3% (w/w) (Table 1). The accumulated extraction rate was over 95% after the second extraction; therefore, the *C. militaris* powder was extracted twice for every crude PS extract preparation.

3.2. Chromatographic fractionations of CPSP

CPSP was separated from crude PS on a DEAE−Sepharose CL-6B anion-exchange column to yield fractions CPSP-F1, CPSP-F2, and CPSP-F3 (Fig. 1). The recovery of each fraction was 88.6%, 3.7% and 0.3%, relative to the total amount of crude PS. The recovery ratio was calculated from the following Equation (1). These fractions were eluted with an increasing NaCl

Table 1 − The five continuous ultrasound-assisted extractions for polysaccharide.

Extraction times	Extraction yield (%)	Accumulated extraction rate (%)
No. 1	23.2 ± 0.2	84.8
No. 2	3.4 ± 0.0	97.3
No. 3	0.8 ± 0.0	100
No. 4	N.D.	
No. 5	N.D.	
Total extraction yield (%)	27.3 ± 0.3	

All experimental data are expressed as mean ± SD ($n \geq 3$).
N.D. = not detected.

gradient. Anion-exchange column chromatography revealed coincident peaks for carbohydrates and proteins, indicating that PS and protein were linked in CPSP-F1, CPSP-F2, and CPSP-F3 [32]. Then, CPSP-F1, CPSP-F2 and CPSP-F3 were collected, concentrated, characterized, and evaluated for AChE inhibitory activity.

$$\text{Recovery ratio (\%, w/w) of each fraction} = \text{CPSP} / \text{Crude PS} \times 100 \tag{1}$$

3.3. Characterization of CPSP-F1, CPSP-F2 and CPSP-F3

The carbohydrate and protein components of the concentrated CPSP-F1, CPSP-F2, and CPSP-F3 solutions are listed in Table 2. CPSP-F1 contained a larger amount of carbohydrate than protein, but CPSP-F2 and CPSP-F3 contained more protein than carbohydrate. After hydrolysis, amino sugars in the CPSP-F1, CPSP-F2, and CPSP-F3 hydrolytes were measured by colorimetric analysis. All fractions contained amino sugar, with all reactions yielding a light red color [26]. Table 2 shows CPSP-F1 and CPSP-F2 contained 5−6‰ (w/w) amino sugars,
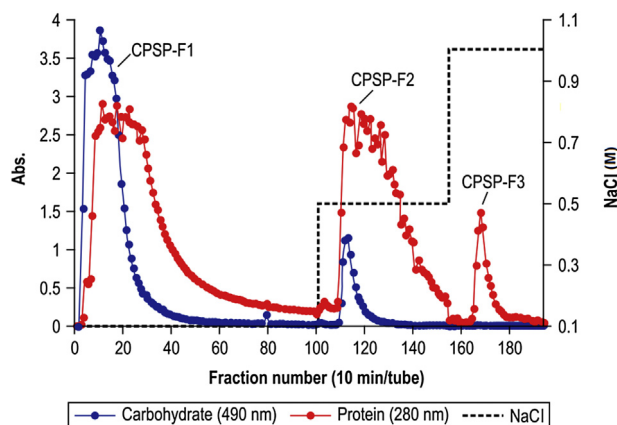

Fig. 1 − Elution profiles of crude polysaccharide in DEAE−Sepharose CL-6B anion-exchange column chromatography. The column was eluted with a step gradient of NaCl (0.1−0.5−1.0M) at a flow rate of 0.6 mL/minute. Eluates were analyzed by measuring the absorbance at 490 nm for carbohydrates after using the phenol sulfuric acid method and at 280 nm for proteins.

Table 2 – Total carbohydrate, total protein, and total amino sugar contents of concentrated CPSP-F1, CPSP-F2, and CPSP-F3.

Polymer	Total carbohydrate content (%, w/w)	Total protein content (%, w/w)	Total amino sugar content (‰, w/w)
CPSP-F1	72.7 ± 1.4	36.9 ± 3.5	5.7 ± 0.8
CPSP-F2	4.9 ± 0.4	95.6 ± 1.1	5.0 ± 0.3
CPSP-F3	3.0 ± 0.1	91.7 ± 0.1	N.C.

All experimental data are expressed as mean ± SD ($n \geq 3$).
CPSP = *C. militaris* polysaccharide-peptide; N.C. = absorption was too small to be calculated.

but the absorption of CPSP-F3 was too low to be calculated; thus, amino sugar residues were present in all three CPSP fractions.

3.4. FTIR analysis of CPSP-F1, CPSP-F2, and CPSP-F3

The functional groups of CPSP-F1, CPSP-F2, and CPSP-F3 were investigated by FTIR analysis at 4000–400/cm (Fig. 2). All samples displayed a broadly stretched, intense peak at around 3400/cm characteristic of a hydroxyl group, and a weak C–H stretching band near 2960/cm for constituent sugar residues [33,34]. The absorption band between 1440/cm and 1395/cm was associated with the stretching vibration of C–O bonds [35]. The absorption bands of CPSP-F1, CPSP-F2, and CPSP-F3 were at 1395/cm, 1400/cm, and 1420/cm, respectively. Near 1660/cm and 1560/cm, the absorption showed protein feathering, corresponding to the carbonyl bond of an amide group and bending vibration of the N–H bond [36]. The absorption peak at 825/cm indicated an α-glycosidic linkage [37]. The absorption at 893/cm suggested a β-glycosidic linkage, but the bands at 930/cm, 1038/cm, 1078/cm, and 1161/cm were also indicative of β (1→3) glycosidic bonds [38].

The PSP was one form of PS linking peptide. Spectroscopy revealed characteristic functional groups of PS, peptide and glycosidic linkages in CPSP-F1, CPSP-F2, and CPSP-F3, suggesting they are PSP complexes.

3.5. Molecular characteristics of CPSP-F1, CPSP-F2, and CPSP-F3

In a previous study, size exclusion chromatography was used to separate PSP complexes of *Pleurotus geesteranus*, in which the UV peaks for protein had the same retention time as the polysaccharide peaks detected by Refractive Index (RI), suggesting the proteins were bound to the polysaccharide components [39]. The polysaccharopeptide of *C. versicolor* was separated by gel permeation HPLC and monitored with UV and RI detectors, which showed the protein and polysaccharide moieties were tightly linked, with coincident UV and RI peaks [32].

We used HPGFC to separate CPSP-F1, CPSP-F2, and CPSP-F3. The UV and RI superimposed chromatograms are shown in Fig. 3. As shown in the RI chromatogram for carbohydrates, CPSP-F1 contained two different polymer distributions at 11.8
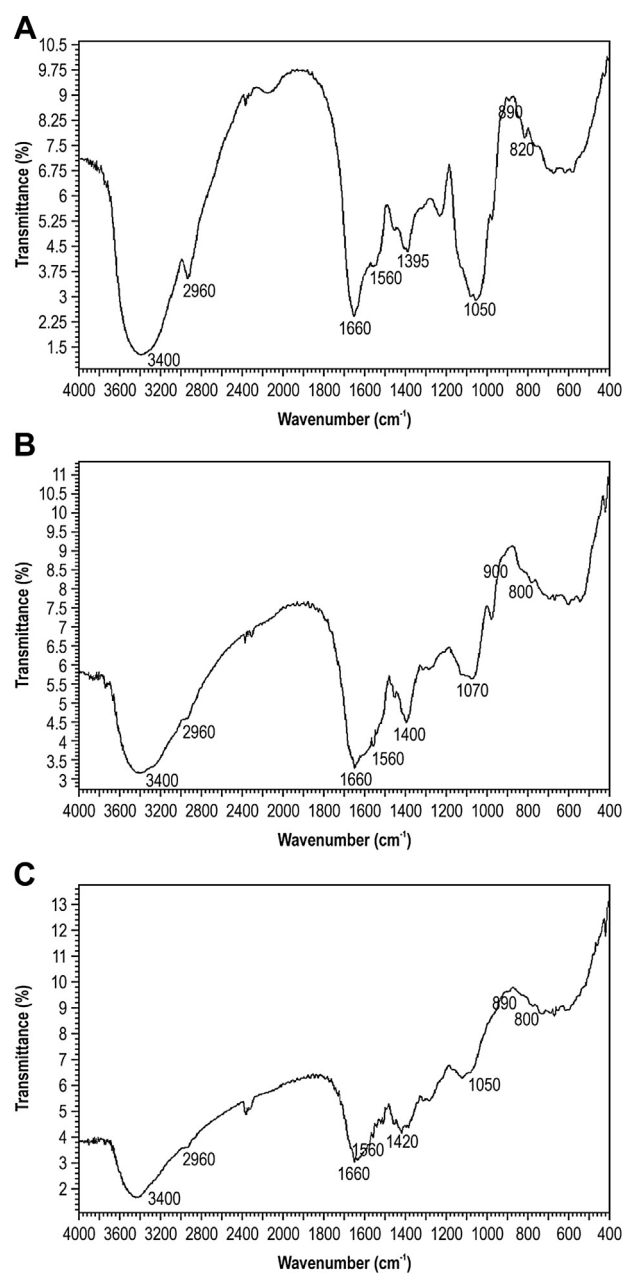
Fig. 2 – FTIR spectra of CPSP-F1, CPSP-F2, and CPSP-F3. CPSP samples were ground with KBr powder and pressed into pellets for FTIR measurement at 4000–400/cm. (A) CPSP-F1; (B) CPSP-F2; and (C) CPSP-F3. CPSP = *C. militaris* polysaccharide-peptide; FTIR = Fourier transform infrared.

minutes and 12.2 minutes with protein signals in the UV chromatogram (Fig. 3A); CPSP-F2 contained polymer distributions at 10.3 minutes and 11.1 minutes with protein signals in the UV chromatogram (Fig. 3B); CPSP-F3 included polymer distributions at 7.7 minutes, 12.2 minutes, and 15.7 minutes, but the protein signals in the UV chromatogram appeared only at 7.7 minutes and 12.2 minutes (Fig. 3C). We conclude that PSP complexes were present in CPSP-F1, CPSP-F2, and CPSP-F3.
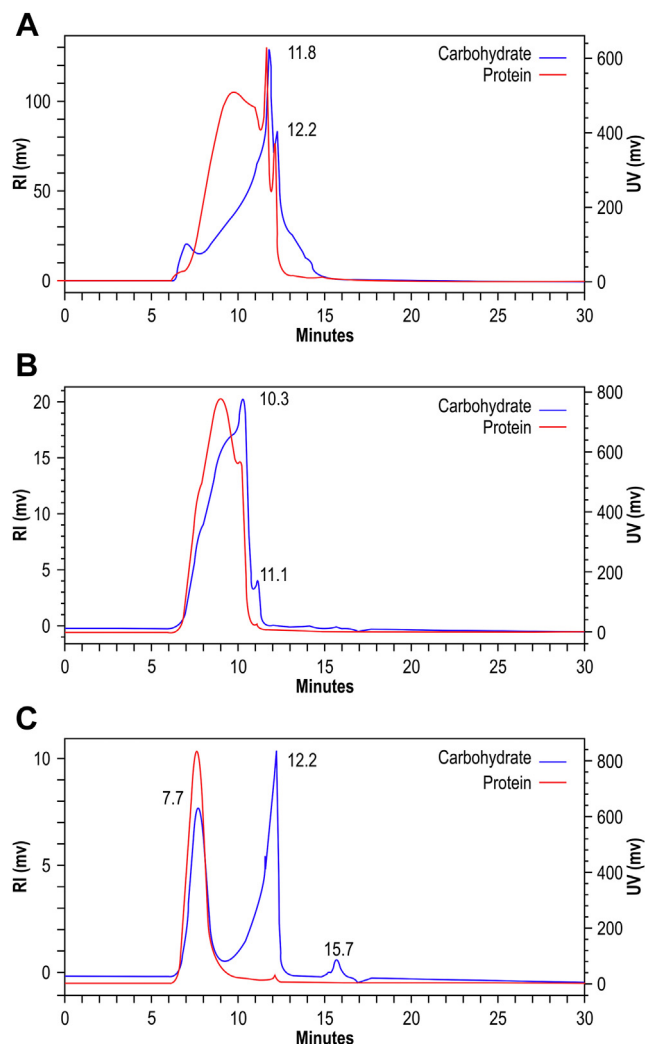
Fig. 3 – HPGFC spectra of CPSP-F1, CPSP-F2, and CPSP-F3. The HPGFC system consisted of a UV detector at 280 nm, an Refractive Index (RI) detector, and a PolySep-GFC-P5000 column. The mobile phase was distilled/deionized water with flow at 0.8 mL/minute at room temperature. (A) CPSP-F1; (B) CPSP-F2; and (C) CPSP-F3. CPSP = _C. militaris_ polysaccharide-peptide; HPGFC = high-performance gel-filtration chromatography.

3.6. Characterization of monosaccharide and amino acid compositions

The monosaccharide and amino acid compositions have been characterized in _C. sphecocephala_ and _H. sinensis_ [18,20].

Table 4 – Amino acid compositions of CPSP-F1, CPSP-F2, and CPSP-F3.[a]

Amino acid	Composition (%, w/w)		
	CPSP-F1	CPSP-F2	CPSP-F3
Alanine	12.5	0.6	0.5
Glycine	10.4	0.8	0.2
Valine	7.3	2.5	0.3
Leucine	9.3	2.4	1.2
Isoleucine	6.0	1.6	0.6
Proline	23.4	12.4	13.0
Glutamic acid	7.2	19.8	7.5
Methionine	1.2	8.8	8.3
Asprtic acid	3.9	2.5	2.0
Hydroxyproline	4.4	5.1	1.3
Phenylalanine	2.1	0.8	0.7
Cysteine	2.2	0.6	0.5
Lysine	7.6	39.0	61.4
Histidine	0.4	0.3	1.8
Tyrosine	2.0	2.8	0.8

CPSP = _C. militaris_ polysaccharide-peptide.
[a] Amino acid compositions were analyzed by gas chromatography/mass spectrometry.

In our studies, the CPSP fractions were composed of mainly mannose, galactose, and arabinose sugar moieties (Table 3). The polymers consisted of acidic and nonpolar amino acids such as glutamic acid and proline. Furthermore, nonpolar amino acids alanine, glycine, and leucine were higher in CPSP-F1, while basic and nonpolar lysine and methionine were higher in CPSP-F2 and CPSP-F3 (Table 4).

3.7. Glycan–peptide linkage analysis

Glycan–peptide linkages are classified by their stability under alkaline, such as O-glycosidic and N-glycosidic conditions. The alkali-sensitive O-glycosidic linkages are readily cleaved under relatively mild conditions by a β-elimination mechanism that releases the carbohydrate moiety. This method has been widely used to analyze glycoprotein linkages [40].

To analyze the linkages in our PSP complexes, UV scanning spectra of CPSP-F1, CPSP-F2, and CPSP-F3 with and without alkali treatment were obtained (Fig. 4). Alkali-treated samples had distinctly higher absorbance at 240 nm, showing that the β-elimination reaction had taken place, and that CPSP-F1 contained O-glycosidic linkages but CPSP-F2 and CPSP-F3 did not. Thus, CPSP-F2 and CPSP-F3 have only N-glycosidic linkages.

Table 3 – Monosaccharide compositions of CPSP-F1, CPSP-F2, and CPSP-F3.[a]

Polymer	Monosaccharide contents (molar ratio)					
	Mannose	Galactose	Glucose	Arabinose	Fucose	Xylose
CPSP-F1	2.2	1.4	0.2	3.0	0.2	1.2
CPSP-F2	2.1	2.2	0.1	1.9	0.9	1.0
CPSP-F3	2.3	1.7	0.1	3.0	0.1	1.0

CPSP = _C. militaris_ polysaccharide-peptide.
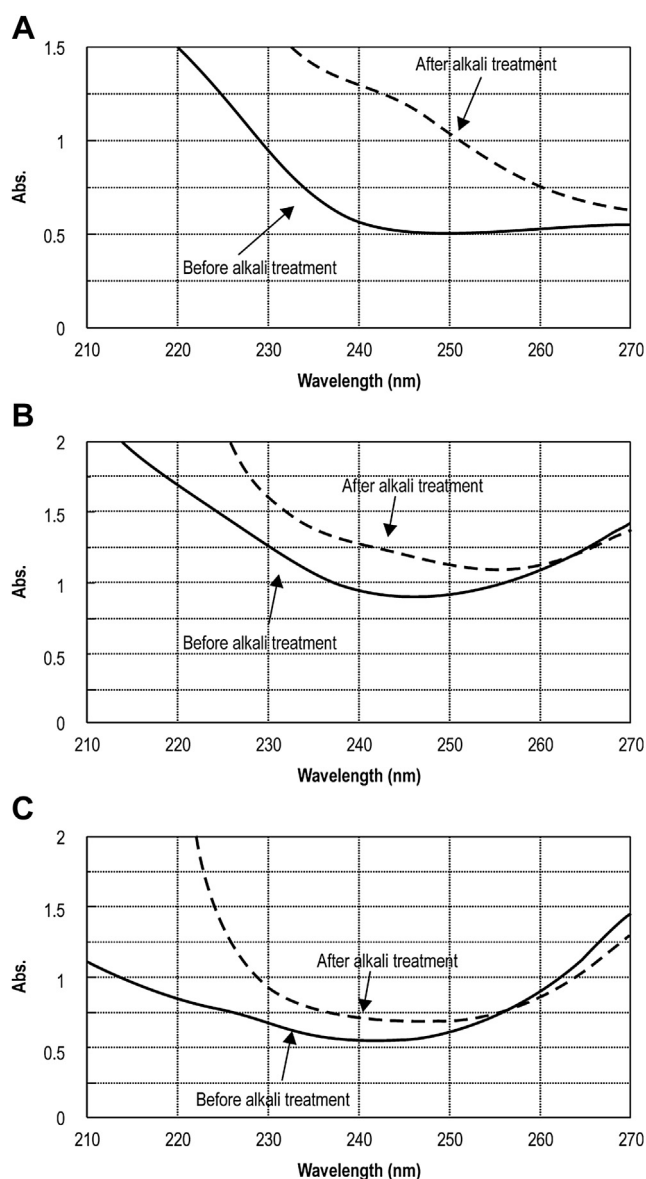[a] Monosaccharide compositions were analyzed by gas chromatography/mass spectrometry.

Fig. 4 – UV spectra of β-elimination before and after alkali treatment for CPSP. The sample was incubated with 0.2 mol/L NaOH containing 1.0 mol/L NaBH₄ for 30 minutes at 45°C, scanned the UV spectrum from 210 nm to 270 nm, and compared to the non-alkali-treated sample. (A) CPSP-F1; (B) CPSP-F2; and (C) CPSP-F3. CPSP = C. militaris polysaccharide-peptide.

Table 5 – AChE inhibitory activity of different CPSPs.

Processes/sample name	IC$_{50}$ value[d] Solid (mg/mL)
Precipitation[a]	
Crude PS	48.9 ± 0.2
DEAE–Sepharose CL-6B[b]	
CPSP-F1	32.2 ± 0.2
CPSP-F2	5.3 ± 0.0
CPSP-F3	Not active[e]
Positive control	
Galanthamine hydrobromide[c]	0.0187 ± 0.0004

AChE = acetylcholinesterase; CPSP = C. militaris polysaccharide-peptide; DEAE = diethylaminoethanol.
* AChE inhibition = $(A_0 − A_1)/A_0 × 100\%$. A_0 is the absorbance of the blank. A_1 is the absorbance of sample − absorbance of sample color.
[a] Crude polysaccharides were precipitated by 3 volumes of 95% ethanol.
[b] CPSP-F1, CPSP-F2 and CPSP-F3 were NaCl-eluted fractions separated by DEAE–Sepharose CL-6B column chromatography.
[c] Purity of galanthamine hydrobromide was ≥ 94%.
[d] IC$_{50}$ was defined as the concentration that yielded 50% AChE inhibition, and the results are means ± standard deviation ($n ≥ 3$).
[e] Sample content was too small to reach 50% AChE inhibition.

3.8. AChE inhibitory activity

AChE inhibition by CPSP-F1, CPSP-F2, and CPSP-F3 and crude PS extracts of C. militaris was measured; the IC$_{50}$ values are shown in Table 5. Although the active content of CPSP-F3 was too low to reach 50% AChE inhibition, crude PS, CPSP-F1, and CPSP-F2 exhibited significant AChE inhibitory activity. IC$_{50}$ values of crude PS, CPSP-F1 and CPSP-F2 were 48.9 ± 0.2 mg/mL, 32.2 ± 0.2 mg/mL, and 5.3 ± 0.0 mg/mL, respectively. Further, the AChE inhibitory activity of CPSP can be increased by separation from crude PS. The most potent inhibition appeared to be present in CPSP-F2. We are believed to be the first to report *in vitro* inhibition of AChE by CPSP and our results suggest CPSP-F2 may have great potential to provide a chemical framework for the development of new AChE inhibitors.

4. Conclusion

PSP complexes were identified in CPSP-F1, CPSP-F2, and CPSP-F3 from C. militaris. The PSP complexes produced coincident PS and protein peaks by DEAE–Sepharose CL-6B column chromatography and HPGFC. FTIR spectra revealed characteristic functional groups for PS, peptide, and glycosidic linkages. The polymers also contained amino sugar residues. β-Elimination showed O-glycosidic linkages in CPSP-F1 and N-glycosidic linkages in CPSP-F2 and CPSP-F3. We analyzed the monosaccharide and amino acid compositions as well as the total carbohydrate and protein contents.

Inhibition of AChE, the key enzyme in the breakdown of ACh, is considered a promising method for the treatment of neurological disorders such as AD and senile dementia. Here, we found that CPSP is a strong inhibitor of AChE and significant activity was observed in the crude PS, CPSP-F1, and CPSP-F2. Activity was enhanced by separation and CPSP-F2 displayed the best AChE inhibitory activity. CPSP from C. militaris fruiting bodies are a potential source of AChE inhibitors. In the future, CPSP may be used as a nutraceutical product for preparation of functional foods.

Acknowledgments

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