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Article

Isolation and Characterization of Edestin from Cheungsam Hempseed

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Edestin, a major hempseed storage protein from Cheungsam, was isolated to apparent homogeneity by acid precipitation and gel filtration chromatography. The native molecular weight of purified edestin was approximately 300 kDa by Sephacryl S-300 gel filtration. Upon adding 2-mercaptoethanol, the isolated edestin of 56.7 kDa on the non-reduced sodium dodecyl sulfate polyacrylamide gel was converted into subunits, suggesting that the protein might be composed of subunits linked by disulfide bond. Cheungsam edestin was rich in essential amino acids and it has 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity. The results suggest that Cheungsam edestin might be utilized as a superior antioxidative nutrient.

Key words: characterization, Cheungsam, edestin, isolation

Hemp, non-drug varieties of *Cannabis sativa* L., has provided an important source of industrial fiber. Hemp fiber has been used in the production of specialty paper, ropes, fabrics, automotive and building insulation, construction materials, and many other durable goods. The use of hemp as a source of food, fiber and medicine is widespread in the old world, and the whole seed continues to be used as important agricultural commodities in Canada, USA, and China [Tang *et al.*, 2006; Wang *et al.*, 2009; Lua *et al.*, 2010]. A variety of hempseed has been known to contain high quality oil and protein, with considerable amounts of dietary fiber, vitamins, and minerals [Callaway, 2004]. The hempseed is recognized as an important source of essential fatty acids [Lua *et al.*, 2010]. The hempseed oil showed significant health benefits, such as reducing Low-density lipoprotein (LDL)-cholesterol, platelet aggregation and high blood pressure, wound healing, and alleviating atopic dermatitis [Callaway *et al.*, 2005]. Hempseed and hempseed meal are known to be excellent sources of digestible protein, especially hempseed lacks the anti-nutritional trypsin-inhibiting factors that are found in soy and most other vegetable products. Thus, a greater proportion of the protein in hempseed seems to be digested and available for absorption.

Hempseed protein was found to mainly consist of high-quality storage protein, named edestin, which accounts for about 60-80% of the total protein content, with albumin making up the balance. Edestin was easily digested and contained nutritionally significant amounts of all essential amino acids [Callaway, 2005]. As with the soy protein glycinin, edestin was a kind of hexameric legumin [St Angelo *et al.*, 1968; Patel *et al.*, 1994; Tang *et al.*, 2006]. Edestin was reported to be similar to serum globulins, and the biologically active protein of edestin was metabolized in the human body to biosynthesize immuno-globulins, hormones, haemoglobin, and enzymes [Tombs, 1960].

In Korea, a new variety of industrial fiber hemp, named "Cheungsam", was developed by crossing IH3 Netherland variety and Korea local variety [Moon *et al.*, 2002]. The new variety of "Cheungsam" is regarded as non-drug type hemp, with a low level of δ-9 tetrahydrocannabinol (THC). However, the information on the edestin from Cheungsam is not available, although we characterized hempseed protein isolate of Cheungsam in our previous report [Kim and Lee, 2011]. In this investigation, we described the isolation and characterization of edestin from Cheungsam by using gel filtration chromatography following acid precipitation. Some neutrochemical properties of edestin were characterized.

Materials and Methods

Hempseed preparation. Dehulled hempseeds from industrial fiber hemp, named "Cheungsam", were ground in liquid nitrogen and then dispersed in deionized water at room temperature.

Isolation of edestin from Cheungsam. In order to isolate edestin from Cheungsam hempseeds, acid precipitation of hempseed protein isolate was followed by chromatographic separation. One gram of hempseed flour was mixed with 100 mL deionized water, and the mixture was adjusted to pH 10.0 with 1.0 N NaOH. After continuously stirring for 1 h, the

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suspensions were centrifuged at 8,000 g for 30 min and the precipitates were discarded. Then, the pH of the supernatants was adjusted to pH 5.0 at 4°C with 1 N HCl, and the precipitates were collected by centrifugation at 6,500 g for 25 min. The obtained precipitates were washed with pre-cooled deionized water, and dispersed in the deionized water. The dispersions were subjected to dialysis with 10 mM Tris buffer (pH 10.0) at 4°C for overnight. The dialysate was applied to Sephacryl S-300 column pre-equilibrated with 10 mM Tris buffer (pH 10.0). After washing with buffer, hempseed protein isolate was then further purified through Sephacryl S-300 gel filtration chromatography to an electrophoretically homogeneous form [Downes and Hall, 1998; Barbarino and Lourenço, 2005]. The absorption spectrum and absorbances were recorded on a UV-visible spectrophotometer. All spectra were recorded at room temperature.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed with slight modifications of discontinuous buffer system of Laemmli [1970]. For SDS-PAGE under reduced condition, the protein samples were directly dissolved in the sample buffer, namely 0.125 M Tris-HCl buffer (pH 8.0) containing 0.1% (w/v) SDS, 0.05% (w/v) bromophenol blue, 30% (v/v) glycerol and 5% (v/v) β-mercaptoethanol (2-ME). The SDS-PAGE under non-reduced conditions was carried out as mentioned above, except using the samples dissolved in the sample buffer without 2-ME [Tang *et al.*, 2006; Kim *et al.*, 2011].

Periodic acid-Schiff (PAS) staining for carbohydrate. After finishing SDS-PAGE, gels were fixed overnight in 25% isopropanol, 10% acetic acid and 65% water. Gels were washed for 1 h in running water, soaked in 1% $NaIO_4$, 3% acetic acid for 1 h and then washed for 2 h in running water and twice for 0.5 h with distilled water. The gels were then stained for 2 h with Schiff's reagent prepared from Basic Fuchsin [Kim and Lee, 2011]. Excess Schiff's reagent was removed by soaking in 0.5% sodium metabisulphite. The gels were then soaked for two times 3 h in 7% acetic acid.

Amino acid analysis. The amino acid composition of edestin subunits, which were eluted from the SDS-PAGE gel, were determined by an automatic amino acid analyzer (Waters M510, Milford, MA), using PICO, TAG column [Wang *et al.*, 2008]. The determination was carried out 38°C, and the detection wavelength 254 nm and flow rate 1.0 mL per min. The samples were hydrolyzed with 6 N HCl for 24 h at 110°C in sealed tube. The amino acid composition was calculated as relative peak area of high-pressure liquid chromatography (HPLC) chromatogram against peak area of standard amino acid.

N-terminal amino acid sequence analysis. After SDS-PAGE, the purified protein on the gel was transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting and stained with Coomassie blue. The stained material was excised and used for N-terminal sequencing by the automated Edman degradation method at the Korea Basic Science Institute (KBSI).

1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. The DPPH radical scavenging activity was determined by the method described earlier [Seo *et al.*, 2010]. 2 mL of the sample solution was mixed with freshly prepared 2 mL of 0.2 mM DPPH ethanolic solution and vortexed for about 10 s. The resulting solution was then left to stand for 30 min, prior to reading the absorbance at 517 nm. Ethanol was used as a blank. Reduction of absorbance at 517 nm indicates an increase in DPPH scavenging activity. Each experiment was performed at least three times in triplicate.

Results and Discussion

Isolation of edestin from Cheungsam hempseed. Cheungsam edestin was isolated by acid precipitation and Sephacryl S-300 gel filtration chromatography. Table 1 shows the purification profile of edestin from hempseeds of Cheungsam. The overall isolation yield of edestin was about 48.6% and total amount of edestin (142.9 mg) was isolated from 1 g of hempseeds.

The isolation profile of edestin on the SDS-polyacrylamide gel under reduced and non-reduced conditions were shown in Fig. 1. On the SDS-polyacrylamide gels under the reduced condition with 2-ME, the edestin was composed of

Table 1. The isolation of edestin from Cheungsam hempseeds

Purification step	Total protein (mg)	Yield (%)
Crude extract	293.95	100
Acid precipitation	144.35	49.1
Gel-filtration (Edestin)	142.9	48.6

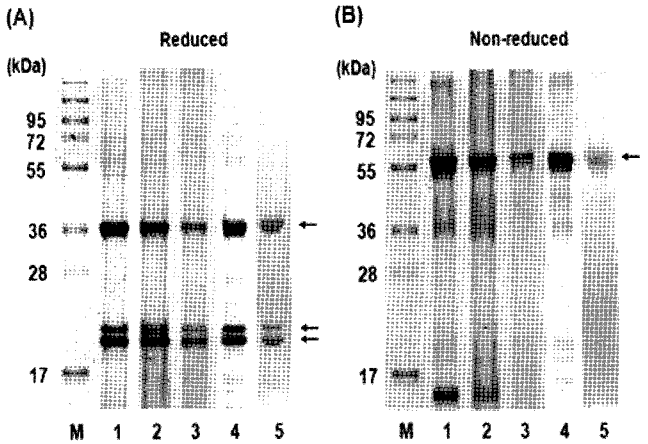

Fig. 1. SDS-polyacrylamide gel electrophoretic profile of edestin in the presence (A) and absence (B) of β-mercaptoethanol. M: Molecular weight markers, 1: Crude extract, 2: Acid precipitates, 3: Purified edestin through gel filtration, 4: Commercial edestin (MP Biomedicals), 5: Carbohydrate staining for purified edestin by periodic acid-Schiff staining

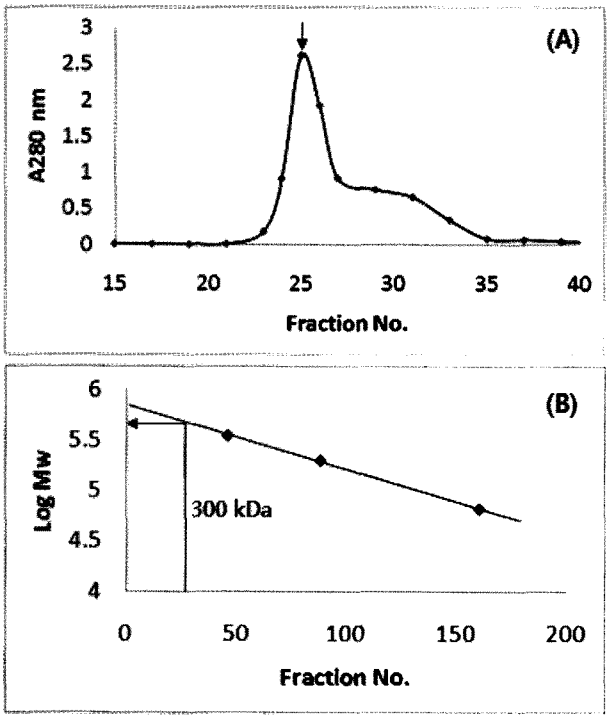

Fig. 2. Gel filtration chromatographic profile (A) and native molecular weight determination (B) of edestin from Cheungsam.

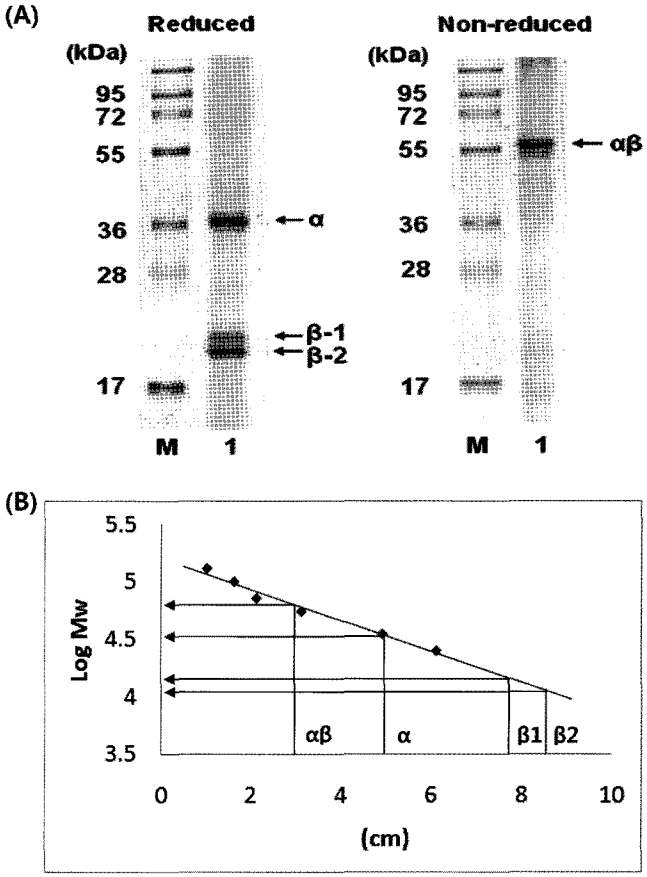

Fig. 3. SDS-polyacrylamide gel electrophoretic profile of edestin (A) and subunit molecular weight determination for edestin (B). M: Molecular weight markers, 1: Edestin

multisubunits. However, only single protein band was found in the absence of 2-ME, showing the protein band under non-reduced condition has different size from that under reduced condition because disulfide bonds between subunits detected on the reduced gel were not disrupted on the non-reduced gel. Moreover, the small subunits were heterogeneous on the reduced gel. Edestins from other hempseeds including commercial edestin (MP Biomedicals, Solon, OH) were also reported to be composed of acidic (AS) and basic (BS) subunits [Tang *et al.*, 2006; Wang *et al.*, 2008] and the small subunits were more heterogeneous than large subunit like Cheungsam edestin [Tang *et al.*, 2006]. Edestin had carbohydrate moiety in the structure as judged by the carbohydrate staining by periodic acid-Schiff staining on the SDS- polyacrylamide gel.

Molecular weight determination for edestin. Edestin was purified as electrophoretically homogenous form by the Sephacryl S-300 gel filtration chromatography with 10 mM Tris buffer (pH 10.0) as shown in Fig. 2. The native molecular weight of edestin was determined on the Sephacryl S-300 column. The molecular weight of the purified edestin around fraction 25°C, determined by comparison of relative elution volumes on the gel filtration, was approximately 300 kDa (Fig. 2). SDS-PAGE profiles of protein constituents of edestin in the presence and absence of 2-ME were presented in Fig. 3. On the SDS-polyacrylamide gels, only single protein band of about 56.7 kDa was found in the absence of 2-ME, and the edestin showed similar protein constitutions such as α and β subunits. By comparison with standard proteins, the molecular weights of α, β-1 and β-2 subunits were determined to be about 36.4, 20.4, and 18.3 kDa, respectively (Fig. 3). In this investigation, α subunit band of about 36.4 kDa was homogeneous, while β subunit bands consisted of two bands of β-1 and β-2. As with commercial edestin, other reported edestin small subunits that were detected on the reduced SDS-polyacrylamide gel were also heterogeneous [Tang *et al.*, 2006], although whole edestin consisted of identical six subunits. The reason for the detection of multiple bands for small subunit of edestin on the reduced SDS-polyacrylamide gel is unclear, although the two bands of β-1 and β-2 might result from the oxidative damage or proteolysis during extraction for SDS-PAGE is plausible.

Patel *et al.* [1994], using crystallographic techniques, showed that edestin molecules is composed of six identical subunits, and each subunit consists of an acidic subunit and basic subunit linked by one disulfied bond [Tang *et al.*, 2006], like the hexamer of soy glycinin. Taken together, Cheungsam edestin seems to have hexameric form in comparison native molecular size with subunit molecular size. The molecular weight of Cheungsam edestin was about 300 kDa, which is close to that of 290 kDa reported by Hall [1950] and much higher than that of

Table 2. Amino acid compositions of Cheungsam edestin subunit

Amino acids	Subunit		
	α	β-1	β-2
ASX[b]	174.52	78.06	118.99
GLX[b]	280.94	92.04	128.81
Serine	153.75	79.45	100.78
Arginine[a]	227.60	101.00	133.53
Threonine[a]	80.31	44.34	53.86
Alanine	132.88	102.02	125.48
Proline	644.23	582.66	506.28
Tyrosine	10.11	6.55	6.89
Valine[a]	100.57	69.11	74.12
Methionine[a]	27.95	18.84	35.97
Isoleucine[a]	90.96	43.55	67.01
Leucine[a]	129.74	72.12	107.94
Phenylalanine[a]	73.50	47.54	71.61
Cysteine	9.85	5.21	6.39
Total	2,136.91	1,342.49	1,537.66

[a]Essential amino acid
[b]ASX, GLX means the sum of asparagine & aspartic acid and glutamine & glutamic acid, respectively.
*Tryptophan was excluded from the analysis because it is destroyed by acid hydrolysis.
**Gly, His, and Lys were excluded in this report in order to prevent data errors found in gel eluted proteins due to the presence of Gly in SDS-PAGE buffer system.

Table 3. N-terminal sequences of Cheungsam edestin subunits

Edestin	N-terminal sequences
α subunit	I-S-R-S-A-V-Y
β subunit-1	G-L-E-E-T-F-X
β subunit-2	G-L-E-E-T-F-X

X: Not detected

212 kDa reported by Svedberg and Stamm [1929] in other hempseeds.

Amino acid compositions of α and β subunits of Cheungsam edestin. The amino acid compositions of α and β subunit of edestin, eluted from the SDS-polyacrylamide gel, are shown in Table 2. Notably, essential amino acids such as arginine, threonine, valine, methionine, isoleucine, leucine and phenylalanine were rich in Cheungsam edestin. The amino acid composition result shows that Cheungsam edestin is rich in essential amino acids and might be used as a superior nutrient.

N-terminal sequence analysis of edestin. Table 3 indicates the amino terminal sequences of the edestin α subunit and β subunits. α subunit had the seven amino acid residues of Isoleucine-Serine-Arginine-Serine-Alanine-Valine-Tyrosine in the N-terminus. Two constituents of β subunits had an identical N-termius of Glycine-Leucine-Glutamic acid-Glutamic acid-Threonine-Phenylalanine-X.

DPPH free radical scavenging assay. The DPPH free radical scavenging ability of Cheungsam edestin was presented in Fig. 4. The free radical scavenging ability of the edestin gradually

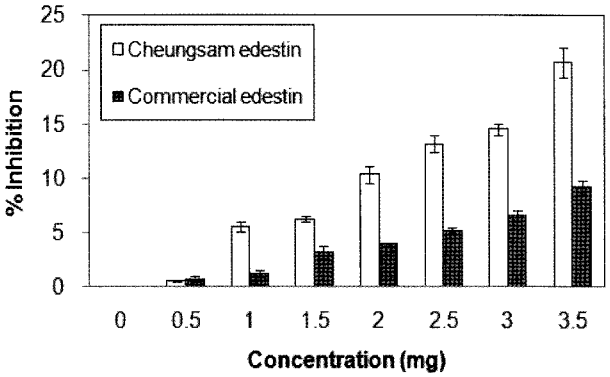

Fig. 4. DPPH free radical scavenging activity of edestin

increased with increasing edestin concentrations. By comparison with commercial edestin (MP Biomedicals), Cheungsam edestin has a higher level of free radical scavenging activity than that of commercial edestin (Fig. 4). A variety of studies reported that various food proteins including soy proteins, whey proteins and wheat seeds have radical scavenging abilities [Galleschi *et al.*, 2002; Moure *et al.*, 2006; Bayram *et al.*, 2008]. Therefore, edestin from Cheungsam might be utilized as a superior source of antioxidative nutrient in food.

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