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**Article**

# Development of Methods for Protein Extraction from Three Major Korean Fermented Soy Foods for 2-Dimensional Gel and Mass Spectrometric Analyses

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**Three different protein extraction methods-phenol extraction, trichloroacetic acid (TCA) precipitation, and desalting/TCA precipitation-were compared to determine the optimal reproducible high resolution 2-dimensional (2-D) electrophoresis for each chungkugjang, doenjang, and kochujang samples. The soluble proteins from Chungkugjang extracted by phenol were separated with high reproducibility and resolution, and gained 1.75- to 3-fold more protein spots on 2-D gel than those from the other methods. On the contrary, the extracted proteins from doenjang and kochujang treated by desalting/TCA precipitation method showed about 1.5- to 3.3-fold more protein spots on 2-D gel. Using the established methods, the changes in the protein profiles of the fermented soy foods were monitored during the fermentation period by 2-DE. One of the major proteins in soy, β-conglycinin α-subuint, and some proteins with unknown functions were localized on 2-D gel as the protease-resistant proteins throughout the fermentation period of doenjang. Changes in the protein profile monitored by the established methods can provide basic information on unfolding the mechanisms of the generation of biofunctional activity in the fermented soy foods.**

**Key words :** *2-dimensional gel, chungkukjang, doenjang, kochujang, protein profile, soluble protein, soy*

Among the Korean fermented soybean products, chungkugjang, kochujang, and doenjang are three of the most favored traditional soy foods widely consumed by the Korean people due to their unique flavors and nutritional values. As the recipes for these fermented soy products differ from one region to another, the nutritional values and the contents of the bio-functional substances also differ among the products. Thus, it is necessary to evaluate and optimize the recipes to make more favorable, bio-functional, and nutritious fermented soy foods.

Because the soy proteins are digested by the microbial enzymes during fermentation, analysis of the protein profiles in the fermented soy food can be beneficial to understanding the mechanism of the generation of bio-functional molecules. However, very limited studies have been reported on the changes of protein profiles in the fermented soy products. For this purpose, proteomic approaches to analyze the protein profile changes in the fermented soy products is promising.

Proteomics enables the investigation of the protein profiles in various samples obtained from different conditions and the selection of the "absent/present" or "increased/decreased" proteins, allowing the identification of the separated and isolated proteins possible by separation techniques, such as, 2-D SDS-PAGE or multidimensional liquid chromatography in combination with the state-of-the-art mass spectrometric analysis. Protein spots separated on 2-D gels are digested with a site-specific enzyme, trypsin, to obtain reproducible and

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**Abbreviations:** CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; 2-D, two-dimensional; 2-DE two-dimensional gel electrophoresis; DTT, dithiothreitol; IEF, isoelectric focusing; IPG, immobilized pH gradient; MALDI-TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; MS/MS, tandem mass spectrometry; MW, molecular weight; PMF, peptide mass fingerprinting; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoroacetic acid

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unique peptides from each protein. The molecular masses of the peptides are accurately determined using MALDI-TOF. The empirical mass data from the mass spectrometric analysis are fed to search the *in silico* digested protein databases for statistical and significant matches [Yates *et al.*, 1993; Wilkins *et al.*, 1995]. This high throughput identification approach depending on the accurate peptide masses of proteins is named as PMF [Pappin *et al.*, 1993; reviewed in Cottrell, 1994]. Because the protein sample preparation methods affect the reproducibility and resolution of 2-D separation on gels due to the contaminated interfering molecules that affect the resolution of 2-DE, an improved sample preparation method will enhance the resolution of 2-D gels and facilitate the clear spot picking for protein digestion by proteolytic enzymes including trypsin for better protein identification by PMF.

Due to the complexity of the matrices and interfering molecules in the fermented soy foods, studies on protein extraction, and changes in and identification of proteins have been very limited. Changes in water-soluble protein concentration and amino acid profiles during the fermentation periods of chungkukjang are the best available reports [Lee *et al.*, 1971; Seok *et al.*, 1994]. Recent studies show that the organic solvent extraction, such as extraction with phenol or ethanol [Mooney *et al.*, 2004], and the removal of interfering molecules by precipitation with TCA can improve the resolution of 2-D gel [Jiang *et al.*, 2004; Natarajan *et al.*, 2005; Natarajan *et al.*, 2007]. Our group showed the protein profiles in chungkukjang during the fermentation period by proteomic analysis [Santos *et al.*, 2007]. Modification of these methods and optimization for 2-D gel analysis on the proteins from the fermented soy foods was necessary for the removal of salts and interfering molecules to achieve reproducible profiles of proteomes in the fermented soy foods.

In this study, the protein extraction methods were optimized to show the profile changes of the soluble proteins during the fermentation periods of the fermented soy foods and localize some protease-resistant soluble proteins in doenjang on 2-D gel, which may need to be analyzed for the biofunctional activity.

## Materials and Methods

**Preparation of proteins from the fermented soy foods.** Chungkugjang, doenjang, and kochujang made using the Korean traditional recipes were obtained from Moon-Ok-Rae Food in Soonchang, Korea. Samples were taken during the fermentation period, dried by lyophilization, and kept at −80°C until used. Soluble proteins from each fermented soy food were extracted by agitating and soaking the samples in 0.1× phosphate buffered saline for 1 h on ice in the presence of protease inhibitors including antipain, bestatin, chymostatin, E-64, leupeptin, pepstatin, phophoramidon, pefabloc, and aprotinin (Roache, Indianapolis, IN). The soluble fraction was separated from the insoluble materials by centrifugation at 10000×g for 1 h at 4°C. The soluble proteins from chungkugjang, kochujang, and doenjang for 1-D and 2-D gel analyses were prepared by either precipitation with 10% TCA or extraction with 0.1 M Tris-buffer-saturated phenol, pH 8.0, and precipitation with 5 volumes of acetone, or desalting through PD-10 Sephadex G-25 columns (GE Healthcare, Uppsala, Switzerland) followed by TCA precipitation. The precipitated protein pellet was washed three times with cold ethanol and dried under vacuum. The dried pellets were solubilized in the rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 0.1% DTT, and 0.2% ampholyte with a pH range of 3 to 10.

**1- and 2-DE, and image analyses.** Over 1 mg of proteins were dissolved in the rehydration buffer and loaded onto the IPG strips. The strips were rehydrated passively for 1 h and actively for 15 h at 50 V at 22°C, followed by IEF at 250 V for 15 min, ramping to 10,000 V for 4 h, and focusing at 10,000 V up to 70,000 volt h (Vh). Subsequently, IPG strips were equilibrated for 10 min each in the equilibration buffer I containing 6 M urea, 2% SDS, 20% glycerol, 130 mM DTT in 0.375 M Tris-HCl, pH 8.8, and finally in the equilibration buffer II containing 135 mM iodoacetamide before the second-dimensional gel electrophoresis. The equilibrated IPG strips after the first-dimensional electrophoresis were placed onto a disc-gel that consisted of 12% acrylamide, pH 8.8, for separating the gel, and 4% acrylamide, pH 6.8, for stacking the gel. Second-dimensional separation was run at 10 mA per gel at 15°C overnight. At the end of each run, the gel was stained with 0.1% Coomassie Brillian Blue R-250 in 50% methanol and 10% acetic acid and destained in 40% methanol and 10% acetic acid. The stained gels were scanned using a Powerlook 1100 scanner (UMAX, Dallas, TX), and the images were exported to the image analysis software program, PDQuest (Bio-Rad, Hercules, CA). Spots over a certain level of intensity were detected and counted by automatic spot-detection. The intensities of spots on the gel were normalized and compared with the imaginary standard gel made by merging the gel images being compared.

**In-gel digestion.** The protein spots were excised from the gel, sliced into about 1 mm in thickness, and transferred to the reaction tubes containing distilled water. The gel particles were incubated for 20 min in a washing

solution (50% acetonitrile ($CH_3CN$) in 50 mM $NH_4HCO_3$) to remove the staining dye, and the gel particles were dried in a vacuum centrifuge for 40 min. Subsequently, 20 μL of trypsin solution (10 ng/μL in 50 mM $NH_4HCO_3$) was added to tubes with the gel particles, which were incubated at 37°C for 16~20 h. After digestion, the peptides were extracted in the extraction buffer (5% TFA in 60% $CH_3CN$), concentrated by a Speed-Vac centrifugation for 3 h, and resolubilized in the resuspension solution (0.1% TFA in 50% $CH_3CN$).

**Mass spectrometric analysis and protein identification.** α-Cyano-4-hydroxy-*trans*-cinnamic acid (40 mg/mL) and nitrocellulose (20 mg/mL) were separately dissolved in acetone and mixed with isopropanol each at the ratio of 2:1:1 (v/v). The matrix solution was mixed with the sample at 1:1. Then, 1 μL of the mixture was spotted onto the target and dried. Bradykinin, angiotensin I, and neurotensin were used as internal and/or external calibrants for spectra calibration.

**MALDI-TOF MS and PMF.** Samples were analyzed using a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA). The spectra were acquired in the delayed extraction-reflector mode, and standard conditions of 20,000 V acceleration voltage and 150 ns delay time. Peptides were selected in the mass range of 800-3500 Da. The spectra obtained by averaging 300 individual laser shots were calibrated internally and/or externally with the calibrants and trypsin autolytic products at 842.5, 1045.6, and 2211.1 *m/z*. For identification of the protein fragments, the MS-Fit program in ProteinProspector of University of California (San Francisco, CA) (http://prospector.ucsf.edu/) and Mascot by Matrix Science (www.matrixscience.com) were used.

## Results and Discussion

**Comparison of protein preparation methods for the fermented soy foods for 2D gel analysis.** Three different protein purification methods were compared for the preparation of soluble proteins from the fermented soy foods for 2D-SDS PAGE analysis. As soy is the major ingredient of doenjang, chungkugjang, and kochujang, the protein extraction methods for these foods were optimized by employing and modifying the published protein purification methods for soy [Mooney *et al.*, 2004; Jiang *et al.,* 2004; Natarajan *et al.*, 2005; Natarajan *et al.,* 2007]. In addition, depending on the regional and manufacturing recipes, the salt concentrations of doenjang and kochujang varied from 10 to 18% [Kang *et al.*, 1997; Kim *et al.*, 1993; Kim *et al.*, 2003]. Therefore, pretreatment of the sample to remove salts and interfering molecules from the fermented soy foods is necessary for the high throughput analysis of the proteins by proteomic approaches using 2-D SDS-PAGE and LC-MS analyses. Interfering molecules for 2-D SDS-PAGE and mass spectrometry analysis were present in the soluble soy proteins extracted from soy [Santos *et al.*, 2007]. To remove the salts in doenjang and kochujang, phenol extraction/acetone precipitation (hereafter, phenol extraction), TCA precipitation, and desalting via desalting columns, followed by TCA precipitation were performed. Soluble proteins from chungkugjang, which do not contain salts, were separated well on 2D gel. Neither TCA precipitation method nor desalting/TCA precipitation method appears to be appropriate for 2D gel analysis in terms of the first-dimensional separation of proteins on the IEF strips. Interestingly, protein preparation methods sensitively affected the 2D gel profile and resolution of the soluble proteins from chungkugjang (Fig. 1). The total spot numbers on 2-D gels detected by an image analysis program, PDQuest, depicted the efficiency of the protein preparation methods for 2D gel analysis (Table 1). Phenol extraction method showed 3-fold more spots (435 spots) on the gel, whereas 145 and 256 spots were detected by TCA and desalting/TCA methods, respectively, from the soluble proteins of chungkugjang.

TCA precipitation has been known to help enhance the resolution of individual protein spot on 2D gel by removing the salts [Jiang *et al.*, 2004]. In the present study, however, TCA precipitation alone in the presence of high salts or interfering molecules from the soy foods did not work well in terms of the protein yield and the resolution on 2-D gel. The addition of desalting step prior to TCA precipitation enhanced the protein yield and the resolution as well. In addition, the drying step was also important to obtaining higher yield of proteins from TCA precipitation. A substantial amount of undissolved proteins remained when air-dried or vacuum-centrifugated proteins were dissolved in the rehydration buffer. Moreover, the TCA-precipitated protein pellet dried by lyophilization at high-vacuum easily dissolved in the rehydration buffer without leaving any undissolved materials, as similarly observed in the urine protein preparation; proteins in urine were barely precipitated by TCA [Pyo *et al.*, 2003]. However, desalting by dialysis or desalting column, followed by TCA precipitation enabled the preparation of good quality proteins for IEF [Pyo *et al.*, 2003; Oh *et al.*, 2004].

**Protein profile changes in doenjang and kochujang.** Using the selected desalting/TCA precipitation method, the soluble proteins from doenjang and kochujang were prepared to run 2D-SDS PAGE for the time course of the fermentation periods to analyze the changes in protein profiles (Figs. 2 and 3). Due to the high concentration of

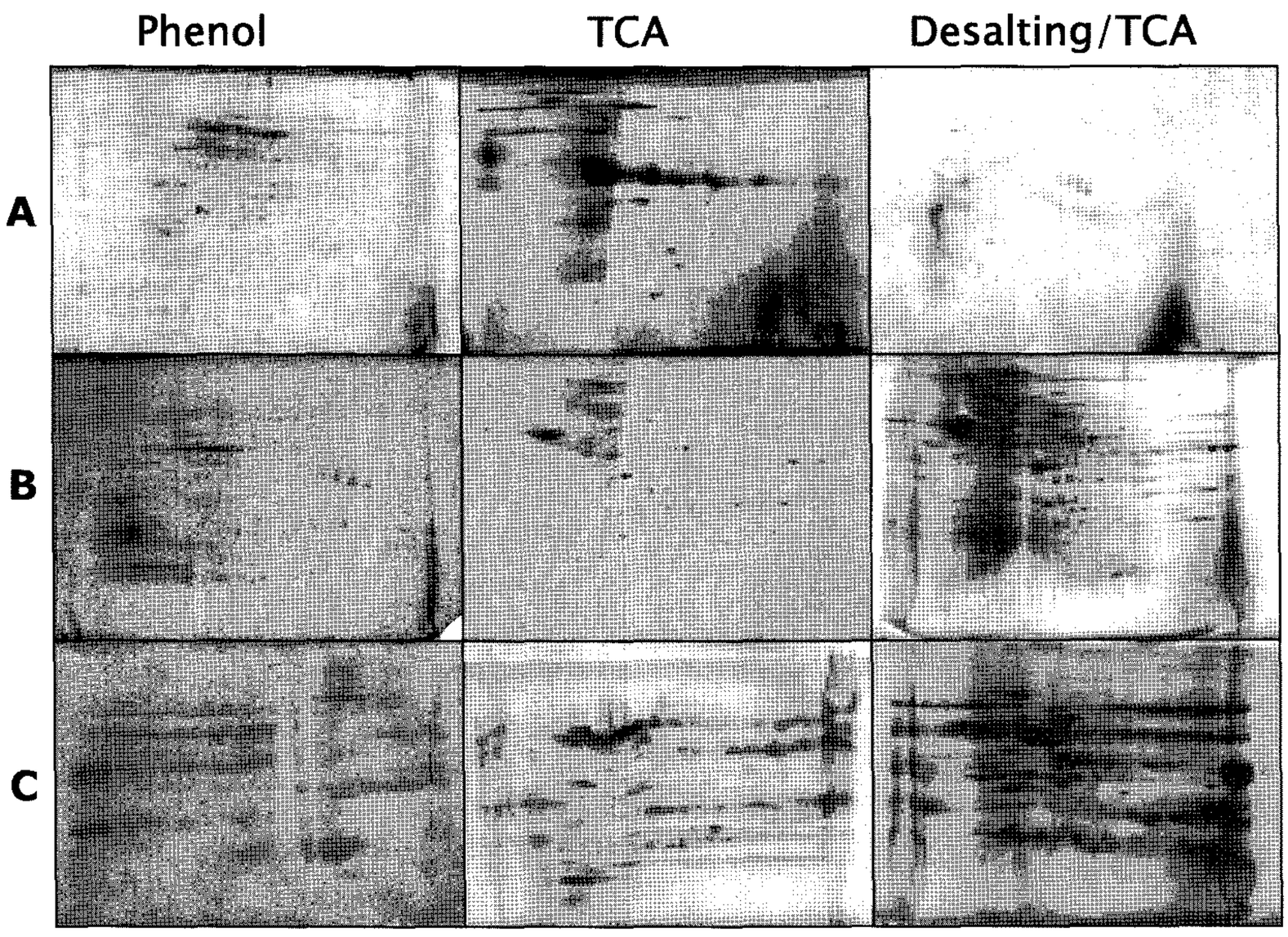


**Fig. 1. Comparison of protein preparation methods for chungkugjang (A), doenjang (B), and kochujang (C) on 2-D SDS PAGE.** Soluble proteins were prepared by phenol extraction (Phenol), TCA precipitation (TCA), and desalting followed by TCA precipitation (Desalting/TCA).

**Table 1. Spot numbers detected on 2D SDS-PAGE by PDQuest analysis**

| Soluble Proteins from | Protein purification method | | | Ratio[a] |
|---|---|---|---|---|
| | Phenol | TCA | Desalting/ TCA | |
| Chungkugjang | 435 | 145 | 256 | 3 |
| Doenjang | 151 | 203 | 493 | 3.3 |
| Kochujang | 187 | 345 | 512 | 2.7 |

[a]highest number/lowest number

salts, the progression of protein degradation in doenjang and kochujang went much slower than in chungkugjang.

The amount of soluble proteins extracted from doenjang was much less than that from kochujang (Fig. 4A and B). The soluble proteins present in soy are degraded into low molecular weight peptides during the fermentation of meju, the raw material for doenjang preparation, and leached into the saline water during the preparation of soy sauce [Im *et al.*, 1998; Park *et al.,* 2002]. The residual soluble proteins in soy beans could be dissolved into the extraction buffer, concentrated, and dissolved into solubilization buffer. However, kochujang gave much more soluble protein mass compared to doenjang. The complexity of the protein profile of kochujang comes from the ingredients, such as hot pepper and powdered rice [Yeo and Kim, 1978] (Fig. 3). As expected, the 2-D gel separation pattern was also very different from that of doenjang and the protein profile was not changed significantly during the fermentation period. The proteins from kochujang were evenly spread out over the pH range of 3 to 10, whereas most of the proteins from doenjang were mostly acidic, below pH 7. The interfering molecules from kochujang affected the performance of IEF and the second-dimensional separation. 2-D SDS-PAGE showed poor resolution and separation with horizontal streaks even under the most optimized conditions (Figs. 1 and 3). Although the streaks and abundant proteins obscured the low abundant proteins on 2-D gels, some minor spots could be monitored for the degradation during the fermentation.

For doenjang and kochujang, the phenol extraction method gave the least spot numbers with 151 and 187 compared to 203 and 345 for TCA precipitation, and 493 and 512 for desalting and TCA precipitation, respectively, showing about 3-fold difference in the spot numbers between the protein preparation methods. This finding suggests that the resolution of 2-DE is significantly affected by the preparation methods (Fig. 1).

**Identification of the decreased and unchanged proteins during the fermentation periods.** Significantly decreased and unchanged proteins during the fermentation period were selected by image analysis of the protein

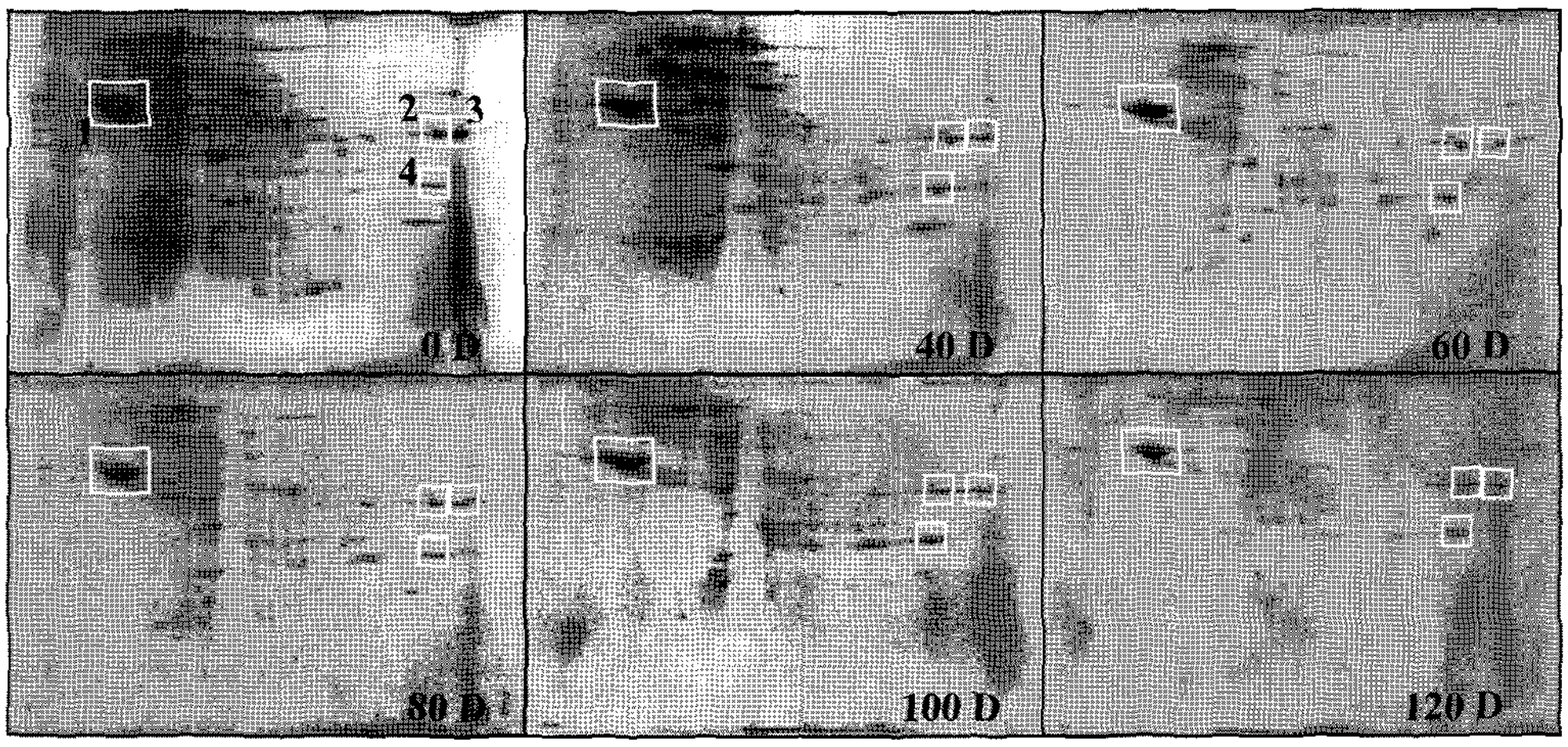


**Fig. 2. Protein profile changes in doenjang during the fermentation period on 2-D gels.** The protease-resistant proteins are boxed and numbered. The pH range of the gel is 4 to 7.

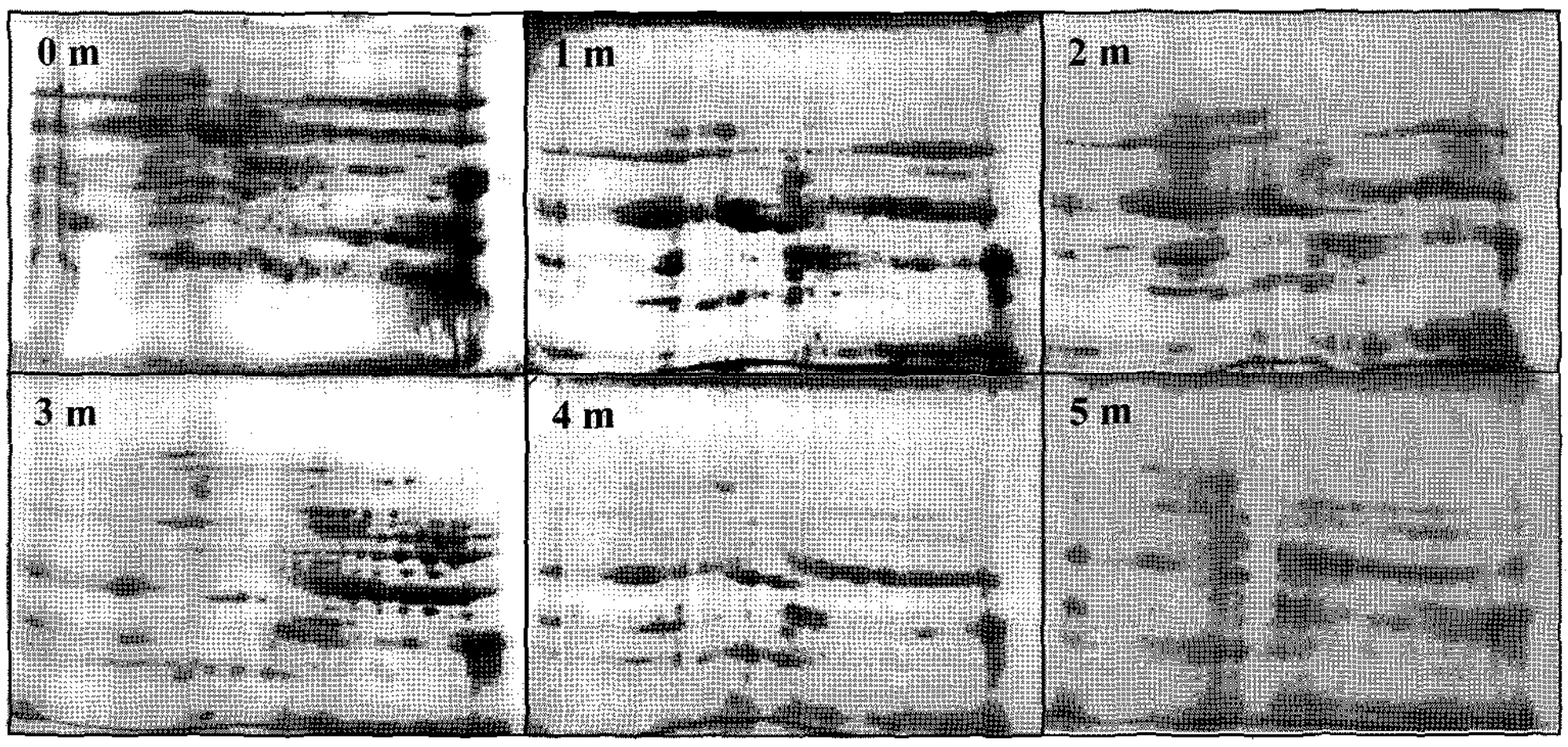


**Fig. 3. Protein profile changes in kochujang during 5 month (0 to 5 month) fermentation period (0 to 120 days) on 2-D gels.** The pH range of the gel is 4 to 7.

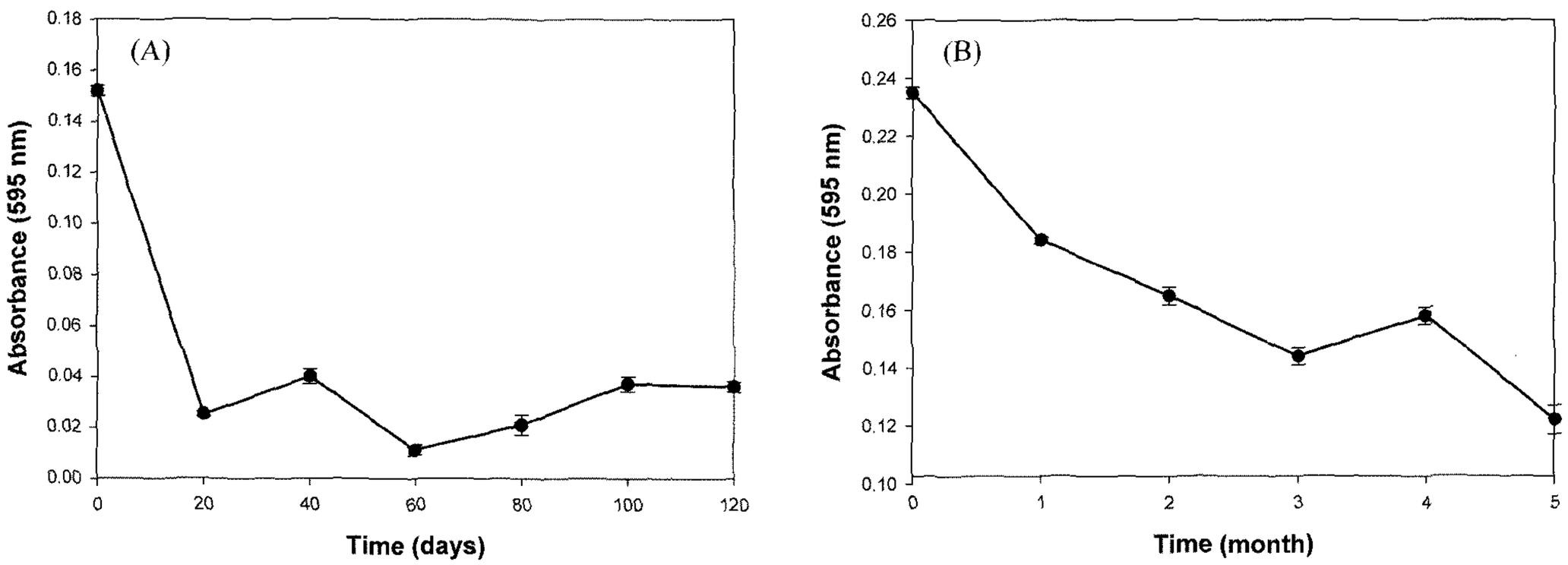


**Fig. 4. Extraction of soluble proteins from doenjang (A) and kochujang (B) sampled during the fermentation period.**

spots on 2-D gels using an image analysis software, PDQuest (BioRad, Hercules, CA). Majority of the proteins gradually decreased as the fermentation proceeded. Some of the proteins decreased more dramatically and some were not significantly changed during the fermentation period. The poor resolution of the 2-D gel images of

**Table 2. Identified protease-resistant proteins in doenjang by PMF**

| Spot ID | Sequence coverage (%) | MOWSE score | NCBI Accession number | Protein identification |
|---|---|---|---|---|
| 1 | 34 | 225 | gi\|9967357 | α-subunit of β-conglycinin |
| 2 | 42 | 51 | gi\|18410807 | unknown protein |
| 3 | 77 | 54 | gi\|122892558 | PAX7 |
| 4 | 33 | 52 | gi\|21223563 | hypothetical protein |

kochujang proteins made it difficult to monitor the changes in the protein profiles during the fermentation period. However, the protein profiles of doenjang on 2-D gels provided a good comparison for localizing the four unchanging protease-resistant proteins (Fig. 2).

Table 2 shows the list of the decreased and unchanged proteins from doenjang and kochujang. The acidic protein (number 1 on Fig. 2) was identified as an α-subunit of β-conglycinin, otherwise known as 7S globulin of soy. Conglycinin is one of the major storage proteins in soy bean. This protein consists of three subunits, α (~65 kDa), α' (~62 kDa), and β (~47 kDa) [Thanh and Shibasaki, 1977; Natarajan *et al.*, 2007]. β-Conglycinin stimulates the expression of low density lipoprotein [Sirtori *et al.*, 1993] and reduces plasma cholesterol [Lovati *et al.*, 2000]. This protein itself or the digested peptides interacts with the intestinal tracts and influences the lipoprotein metabolism [Lovati *et al.*, 2000; Gianazza *et al.*, 2003].

Due to the limitation of soy protein databases, protein numbers 2 to 4 on Fig. 2 were identified as proteins with unknown functions or known functions with very low reliability (low score). Further analysis by MS/MS or *de novo* sequencing can clarify the function of the protease-resistant proteins, and provide information on biofunctionality of the proteins in the body.

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