A New Method of Extracting Whole Cell Proteins from Soil Microorganisms Using Pre-treatment of Ammonium Hydroxide

Han-Chul Kang* · Jong-Bum Kim · Kyung Hee Roh · Sang-Hong Yoon

Received: 22 October 2012 / Accepted: 30 December 2012 / Published Online: 30 September 2013
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Abstract Efficient extraction of total proteins from soil microorganisms is tedious because of small quantity. In this regard, an improved method for extraction of whole cell proteins is developed from soil microorganisms, *Saccharomyces cerevisiae* and *Pichia pastoris*, of which the cell wall are very strong. Pre-treatment with NH₄OH prior to the final extraction using NaOH/SDS was tried under the basis that ammonium ion was possible to enhance the permeability and/or to weaken the yeast cell walls. The pre-treatment of yeast cells with NH₄OH drastically enhanced the protein extraction when it was compared with control (without NH₄OH pre-treatment). At the pre-treatment of 0.04 N NH₄OH at pH 9.0, about 3 fold of proteins was obtained from *p. pastoris*. Ammonium hydroxide appears to penetrate into the yeast cell walls more readily at basic pH. The effect of NH₄OH pre-treatment was pH dependent. The methods developed in this experiment might be applicable for an effective extraction of yeast proteins for the purpose of biochemical studies, especially proteomic analysis.

Keywords ammonium hydroxide · protein extraction · yeast

Introduction

The yeast strains including *S. cerevisiae* are frequently used as a model eukaryotic organism for studying wide range of biochemical and molecular aspects such as cell cycle regulation, DNA repair, signal transduction, and stress response etc. In general, the cell wall of *S. cerevisiae* consists of two layers. Inner layer includes β -1,3-glucan and chitin, which form skeletal polysaccharides and

are responsible for mechanical strength as well as porous network (for review; Klis et al., 2006). Outer layer is also a complex structure consisting mainly of proteins, mannan oligosaccharides, and β -1,6-glucan. Most of the proteins are associated with the mannan oligosaccharides, hence referred to as mannoprotein complexes (Huang et al., 2005). The two polysaccharides, D-mannan and β -D-glucan in *S. cerevisiae*, are major polysaccharides in yeast cells (Klis et al., 2006). Inner and outer layers architected with these polysaccharides and protein complexes contribute to the strength of the cell walls, showing chemical and physical stability.

For the purpose of various research works, extraction of whole cell proteins is often required and followed by proteomic analysis, which is one of the most commonly used methods. Currently, the widely used methods for protein extraction from microbe, plant, or animal cells include mechanical breakage and chemical extraction. Protein extraction from *S. cerevisiae* was developed by using glass beads (Conzelmann et al., 1988). Recently, a method of extraction from *S. cerevisiae* was tried by cryogenic grinding (Singh et al., 2009). The preparation of yeast proteins has been often tried with other mechanical methods, too. But the mechanical methods can be accompanied with some disadvantages such as tedious work and irregular extent of total proteins released. This problem can provoke a confusion, especially, when a level of protein expression should be analyzed or compared with each other. Other method for protein extraction from *S. cerevisiae* was developed, which included a method of boiling in optimized sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Horwath and Riezman, 1995). The authors noted that the method was rapid, however protein content was not sufficient with cells grown up to stationary phase on minimal media. Especially, yield of protein with higher molecular weight was reduced. Combined method of using laminarinase and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS) was developed from *S. cerevisiae* (Mrsa and Tanner, 1999). In addition to *Saccharomyces*, development of protein extraction from *Pichia* has been steadily studied, which was developed with SDS acetone, or other

H. -C. Kang · J. -B. Kim · K. H. Roh · S-H Yoon
Department of metabolic engineering, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Republic of Korea

*Corresponding author (H. -C. Kang: hckang09@korea.kr)

chemicals such as urea (Kwasiborski et al., 2012).

On the other hand, NaOH solution has been often used in order to weaken the cell wall of plant during various processes of biotechnological purpose. Besides NaOH, other alkaline solution, NH₄OH was tried in order to weaken the soybean fiber and thus enzymatic saccharification increased markedly (Karki et al., 2011). In this experiment, saccharification was more produced from the treatment with NH₄OH than that with NaOH. There was another observation that ammonium ion can penetrate the crystalline structure of sugar cane and cause swelling the plant cell (Aita et al., 2010). Treatment of ammonium ion removed a large proportion of the lignin of sugarcane bagasse without influencing the structural integrity of the plant cell (Beukes et al., 2011), resulting in a drastic increase in enzymatic product. On the other hand, ammonium ion was also used to isolate complex substances from other biomaterial source. The extracellular organic matters in sludge was effectively extracted along with the treatment of NH₄OH and brought about negligible shift in their fluorophore characteristics (Chen et al., 2003). In this experiment, NH₄OH was more effective to extract the extracellular polymeric substances than treatment with NaOH, suggesting that NH₄OH can more efficiently weaken chemical interaction. Similar to these findings, Fujita et al., (2007) showed that ammonium ion can reduce hydrophobic interactions under the basis of molecular dynamics simulations, which calculates the pair potential of mean force as a quantitative measure of hydrophobic interactions.

On the analogy of these findings, it can be expected that NH₄OH can efficiently weaken the microbial cell wall and/or other hydrophobic interactions in the cell. The structure of yeast cell wall is considered to be hard and, thus, more severe conditions might be necessary in order to disrupt the cell for an effective extraction of proteins. We describe an ameliorated method for protein extraction from yeast cells, which involves a pre-treatment with NH₄OH.

Materials and Methods

Yeast strains and growth medium. The yeast strains used in this study were *Scacharomyces cerevisiae* (KACC 30008 and KACC 30044) and *Pichia pastoris* X-33. The strain KACC 30008 is a wild type, baker's yeast. Another *Saccharomyces* strain, KACC 30044, shows a characteristic of its genotype, *his3* and *ura3*. These two strains were obtained from Korean Agricultural Culture Collection (KACC). Another yeast strain, *P. pastoris* X-33, was supplied from Invitrogen (USA, CA). Genotype of the *Pichia* strain is a wild type with phenotype Mut⁺.

All of these yeast cells were grown using 50 mL of liquid YPD medium (1% yeast extract, 2% peptone, and 2% glucose). Cultures were also maintained on solid YPD medium plate supplemented with 1.5% agar. For the purpose of protein extraction, the yeast cells were cultured up to stationary phase at 37°C with agitation of 200 rpm in an orbital incubator. The yeast cells were harvested by centrifugation at 9000 g for 5 min, then

washed with ice-cold Tris-HCl buffer (pH 7.0, 0.1 M). At this stage, the growth of yeast cells was measured spectrophotometrically after replacement of suspension with the same volume of Tris-HCl (pH 9.0, 0.1 M). Each OD₆₀₀ nm measured with 1.0 cm width of cuvette was about 1.0–1.5. Wet weights of yeast cells were about 8–10 mg/mL (culture solution) at the each stationary phase. The glucan structures in yeast cells, which contribute considerably to the strength of cell wall, are much produced and form a microfibrillar like network during stationary growth phase (Klis et al., 2006). Thus, for the purpose of using robust cells, we used yeast cells which were cultured up to stationary phase throughout the experiments.

Pre-treatment of yeast cells with ammonium or sodium hydroxide solution. Yeast pellets were resuspended in a sample buffer consisted of Tris-HCl (0.1 M, pH 7.0), 1 mM phenyl-methylsulfonylfluoride (PMSF) and 0.2 mM ethylene diamine tetra-acetic acid (EDTA). Each suspension (containing over 40 mg of yeast cells by wet weight) was equally redistributed into microtube. For the pre-treatment of yeast cells, NH₄OH or NaOH was added up to 0.15 N (final concentration) to the cell suspension. The cell suspensions were incubated at room temperature for 4 h. After the incubation, the cell suspensions were added with 0.1 N NaOH and 1% SDS (final concentration). For the final step of protein extraction, the suspension mixtures (1 mL of final total volume) were heated at 40°C for 10 min, then centrifuged at 12000 rpm. The supernatant obtained was used for the analysis of protein extraction.

Pre-treatment of ammonium hydroxide at different pH. Pre-treatment of NH₄OH solution was performed at different pH values as follows: pH 5.0, sodium acetate (0.1 M); pH 6.0, sodium phosphate (0.1 M); pH 7.0, 8.0, and 9.0, Tris/HCl (0.1 M). The yeast cells grown up to stationary phase were distributed into each eppendorf tube. The cells were washed with Tris/HCl (pH 7.0, 0.1 M). The resulting pellets were resuspended with each buffer as described above, thus resulting in pH values from 5.0 to 9.0 at this experimental step. Then 0.05 N NH₄OH (final concentration) was introduced into each microtube, then incubated for 4 h at room temperature. The yeast cells were centrifuged and supernatants discarded. The resulting pellets were resuspended with Tris/HCl pH 9.0 (0.1 M) supplemented with 1 mM PMSF and 0.2 mM EDTA. The proteins were finally extracted as described using 0.1 N NaOH and 1.0% SDS.

Other analytical methods. Determination of protein concentration was based on the method of Bradford (1976). Since final step of protein extraction from the yeast cells were performed at alkaline condition throughout the experiments, final solution of protein extracted is under alkaline condition. However, proteins more bind to the Coomassie blue dye at acidic pH condition rather than alkaline pH condition. Therefore, for better analysis, protein extract was supplemented with an acidic buffer, sodium acetate buffer (0.1 M, pH 5.0) before measurement of protein concentration. Bradford solution was added as a freshly prepared mixture just before measurement. Incubation was performed for 10 min with slow agitation in order to minimize a formation of dye-dye

complex, which may hinder the protein quantification. The protein concentrations, which were calculated from mean values of three test samples, were represented as a concentration equivalent to bovine serum albumin as a standard protein.

The efficiencies of protein extraction were also analyzed by protein electrophoresis. SDS-PAGE was performed according to the routine method (Laemmli, 1970) using 5% stacking gel and 12.5% resolving gel, followed by staining of the proteins with Coomassie Brilliant Blue R-250.

Results

Preliminary experiment of protein extraction using a combination of SDS and NaOH. In advance of trying a pre-treatment with NH_4OH , we examined whether protein extraction with SDS alone can be applicable to the yeast cells. For this experiment, the yeast cells, *S. cerevisiae* (KACC 30008 and 30044) and *P. pastoris*, were grown up to stationary phase. The subsequent pellets were suspended with Tris-HCl (pH 9.0, 0.1 M) supplemented with 0.2 mM PMSF and 1 mM EDTA. After addition of SDS up to 2.0%, total proteins were extracted, followed by SDS-PAGE analysis. The protein extraction using SDS solution alone resulted in low concentration of proteins, showing about 0.05 mg/mL from *S. cerevisiae* 30008. And the extents of protein from other two strains were also low (detailed data not shown).

The extraction of proteins was also tested with NaOH (up to 0.3 N) alone for the three yeast strains by the same way. By this extraction, scarce contents of proteins were also released from each yeast strain, showing below 0.03 mg/mL (detailed data not shown). This insufficient release of proteins by NaOH is similar to the previous experiment (Kushnirov, 2000), which was performed with *S. cerevisiae* 5V-H19. However, when NaOH is used in combination with SDS for the three yeast cells in our experiment, the extents of protein release much increased and were roughly proportional up to 0.1–0.15 N of NaOH or 1.0% SDS (detailed data not shown). Therefore, final extraction of protein from the yeast cells was performed with complex solution consisted of 0.1 N NaOH and 1.0% SDS.

Contrary to our result, it was shown that 0.3 M NaOH alone greatly improved the extraction efficiency of proteins from yeast (Matsuo et al., 2006). In this experiment, a fission yeast, *Schizosaccharomyces pombe*, was used, of which the cell wall carries additive cell wall components such as galactomannan (Ishiguro et al., 1998). This demand of higher alkaline solution for the protein extraction appears to be related with a difference in the cell wall composition between the fission yeast and another yeast cells such as *Saccharomyces*.

Protein extraction using ammonium hydroxide pre-treatment. Influence of pre-treatment with NH_4OH on the total protein extraction was examined for the three yeast strains. And the same experiment with NaOH was also simultaneously performed in order to compare the effect of pre-treatment between NH_4OH and

Fig. 1 Protein extraction from *S. cerevisiae* (KACC 30008), which was pre-treated with NH_4OH (A) or NaOH (B) and measurements of protein content (C). Proteins were extracted as described in materials and methods. Fig. (A): M, molecular mass markers; lane 1, no pre-treatment (control); lane 2–6 represent the pre-treatment of NH_4OH 0.02, 0.04, 0.06, 0.08 and 0.15 N, respectively. Fig. (B): lane M and 1 are same as the Fig. (A); lanes 2–6 represent the pre-treatment of NaOH 0.02, 0.04, 0.06, 0.08 and 0.15 N, respectively. Protein concentrations were measured at the pre-treatment of 0.02 and 0.04 N NH_4OH or NaOH.

NaOH. For this experiment, the yeast cells were grown up to stationary phase. The resultant pellets were pre-treated up to 0.15 N of NH_4OH or NaOH and finally the total proteins were extracted using 0.1 N NaOH and 1.0% SDS as described in materials and methods. The efficiencies of protein extraction were assessed by SDS-PAGE analysis and also by quantification of total proteins released (Fig. 1, 2 and 3). For the comparisons of effects between NH_4OH and NaOH, the data were compared at the concentrations of 0.02 and 0.04 N treatment. Fig. 1- a, b and c show the protein extractions from *S. cerevisiae* KACC 3008, which were pre-treated with NH_4OH or NaOH. When the yeast cells was pre-treated with NH_4OH , the content of protein

Fig. 2 Protein extraction from *S. cerevisiae* (KACC 30044), which was pre-treated with NH₄OH (A) or NaOH (B) and measurements of protein content (C). Proteins were extracted as described in materials and methods. Fig. (A): M, molecular mass markers; lane 1, no pre-treatment (control); lane 2–6 represent the pre-treatment of NH₄OH 0.02, 0.04, 0.06, 0.08 and 0.15 N, respectively. Fig. (B): lane M and 1 are same as the Fig. (A); lanes 2–6 represent the pre-treatment of NaOH 0.02, 0.04, 0.06, 0.08 and 0.15 N, respectively. Protein concentrations were measured at the pre-treatment of 0.02 and 0.04 N NH₄OH or NaOH.

extraction considerably increased (Fig. 1A). Under the basis of SDS-PAGE analysis, the optimum concentration of NH₄OH for the pre-treatment is observed around 0.04 N. By the pre-treatment at this concentration, the final amount of protein increased by 67% compared with the control (Fig. 1C). It is notable that proteins with higher molecular weights were more extracted by the pre-treatment with NH₄OH. Figure 1-b shows the protein extraction from the yeast cell, which was pre-treated with NaOH. Pre-treatment of NaOH did not seem to further enhance the efficiency of protein extraction. By the pre-treatment of 0.02 N NaOH, only 11% of proteins increased compared with the control (without pre-treatment) (Fig. 1C). About over 0.06 N of pre-treatment with

Fig. 3 Protein extraction from *P. pastoris* x-33, which was pretreated with NH₄OH (A) or NaOH (B) and measurements of protein content (C). Proteins were extracted as described in materials and methods. Fig. (A): M, molecular mass markers; lane 1, no pre-treatment (control); lane 2–6 represent the pre-treatment of NH₄OH 0.02, 0.04, 0.06, 0.08 and 0.15 N, respectively. Fig. (B): lane M and 1 are same as the Fig. (A); lanes 2–6 represent the pre-treatment of NaOH 0.02, 0.04, 0.06, 0.08 and 0.15 N, respectively. Protein concentrations were measured at the pre-treatment of 0.02 and 0.04 N NH₄OH or NaOH.

NaOH, total proteins appear to be rather decreased, when it compares with the control. The gradual decreases in protein content seem to be resulted from the precipitation of proteins at higher alkaline solution, since NaOH also exist in the final extraction buffer. Despite the decrease in protein content, smaller proteins, about below 15 kDa, occurred more at higher alkaline condition.

Pre-treatment with NH₄OH or NaOH for another yeast strain, *S. cerevisiae* KACC 30044, showed similar results with that of *S. cerevisiae* KACC 30008. When the pre-treatment was performed with NH₄OH, the total amount of protein drastically increased (Fig. 2A). The optimum concentration of NH₄OH was observed

Fig. 4 Protein extraction from *S. cerevisiae* (KACC 30008), which was pre-treated with NH_4OH at different pH values (A) and its quantification of protein concentration (B). After the pre-treatment, proteins were extracted as described in materials and methods. Lane M, molecular mass markers; lane 5–9 represent the pH values of pre-treatment, each corresponding to 5, 6, 7, 8, and 9 of pH, respectively. Protein concentrations were calculated from each corresponding sample of Fig. (A) and represented as the amount equivalent to BSA.

Fig. 5 Protein extraction from *S. cerevisiae* (KACC 30044), which was pre-treated with NH_4OH at different pH values (A) and its quantification of protein concentration (B). After the pre-treatment, proteins were extracted as described in materials and methods. Lane M, molecular mass markers; lane 5–9 represent the pH values of pre-treatment, each corresponding to 5, 6, 7, 8, and 9 of pH, respectively. Protein concentrations were calculated from each corresponding sample of Fig. (A) and represented as the amount equivalent to BSA.

around 0.04 N based upon SDS-PAGE analysis, showing a similarity to the experiment with *S. cerevisiae* KACC 30008. The amount of protein extraction increased by 175% of the control at the pre-treatment of 0.04 N NH_4OH (Fig. 2C). Similar to the experiment with the strain *S. cerevisiae* KACC 30008, it is notable that proteins with higher molecular weights were also more appeared from the strain 30044 on the SDS-PAGE by the pre-treatment with NH_4OH . The pre-treatment of NaOH did not further enhance the protein extraction, too (Fig. 2B). The protein content rather decreased even with the pre-treatment of low concentration of NaOH , 0.02 N (Fig. 2C). In this experiment, smaller proteins were also gradually appeared on the SDS-PAGE along with the increase in the concentration of NaOH .

In addition to *Saccharomyces* strains, an effect of pre-treatment using NH_4OH or NaOH was also studied with *P. pastoris* (Fig. 3A, B, and C) by the same way. Similar to the *Saccharomyces* strains, the extraction of proteins from the *Pichia* strain was markedly enhanced by the pre-treatment of NH_4OH (Fig. 3A). By the pre-treatment of 0.04 N NH_4OH , about 3 fold of proteins were finally extracted (Fig. 3C). However, pre-treatment of NaOH did not further contribute to the enhancement of protein extraction, too (Fig. 3B).

Pre-treatment of ammonium hydroxide at different pH values. In general, ammonia reacts with water molecule at low pH

values, thus generating an ammonium (NH_4^+) and hydroxide ion (OH^-). Above 9.0 of pH, un-ionized ammonia is more predominant and un-ionized ammonia (NH_3) can penetrate cell membranes more easily at higher pH values (Stumm and Morgan, 1981). In this regard, an effect of pH on the ammonium pre-treatment was performed with the three yeast cells. The pre-treatment experiments were conducted with 0.04 N of NH_4OH solution at different pH values ranging from 5.0 to 9.0 of pH values. The suspension of yeast cells were incubated for 4 h with slow inversion agitation. After incubation, the suspension of yeast cells were centrifuged and supernatant discarded. The yeast cells were resuspended with Tris/HCl (pH 9.0, 0.1 M), supplemented with 1 mM PMSF and 0.2 mM EDTA. Therefore, at the moment of final extraction, the suspensions of yeast cells retain the same pH condition, each other. The proteins of yeast cells were extracted with 0.1 N NaOH and 1.0% SDS as described. The liberated proteins were assessed by SDS-PAGE analysis and the protein contents measured (Fig. 4, 5, and 6). The amount of proteins extracted from *S. cerevisiae* KACC 30008 was more enhanced from the pre-treatment at alkaline pH than neutral or weak acidic pH values (Fig. 4A). The protein content after the pre-treatment at pH 9.0 increased by 56% compared with that by the pre-treatment at neutral pH of 7.0 (Fig. 4B), showing a strong dependency of pH values. The similar pH dependency of NH_4OH pre-treatment was also observed from the

Fig. 6 Protein extraction from *P. pastoris* x-33, which was pre-treated with NH_4OH at different pH values (A) and its quantification of protein extracted (B). After the pre-treatment of NH_4OH at different pH values, proteins were extracted as described in materials and methods. Lane M, molecular mass markers; lane 5–9 represent the pH values of pre-treatment, each corresponding to 5, 6, 7, 8, and 9 of pH, respectively. Protein concentrations were calculated from each corresponding sample of Fig. (A) and represented as the amount equivalent to BSA.

experiment for another strain *S. cerevisiae* KACC 30044 or *Pichia* (Fig. 5 and 6). At pH 9.0, the total proteins extracted from *S. cerevisiae* KACC 30044 or *P. pastoris* increased by 25, and 82%, compared with each pre-treatment at neutral pH, respectively (Fig 5A, Fig 6B). At neutral or weak acidic pH of pre-treatment, protein liberation was not considerably obtained.

Protein extraction using ammonium hydroxide alone. We tested whether a treatment of NH_4OH alone (without NaOH and SDS) can liberate proteins from the three yeast strains. For this experiment, NH_4OH was added up to 0.2 N to the yeast cells suspended with Tris/HCl buffer (pH 9.0, 0.1 M), supplemented with 1 mM PMSF and 0.2 mM EDTA. The yeast suspensions were incubated for 1 or 8 h with gentle agitation using a vertical turntable. Then the solution of yeast cells were obtained by centrifugation. The resultant supernatants were analyzed by SDS-PAGE and compared with the control (without treatment). The proteins released from the three yeast cells were scarce, resulting in below 0.05 mg/mL for three yeast strains. The amounts of proteins extracted were scarce regardless of the concentration and incubation time of NH_4OH (detailed data not shown). In combination with the results obtained from the above Figs 1, 2, and 3, it appears that NH_4OH itself does not significantly disrupt yeast cells and might merely contribute to weaken the structure of

yeast cell walls, thus facilitating the subsequent protein extraction using detergent at alkaline pH.

Discussion

The walls of yeast cells can withstand herculean pressure of >300 MPa (Hartman and Delgado, 2004), implying very tight structure of cell wall. Similar to *Saccharomyces* strains, cell walls of *Pichia* strains are also very strong. By reason of the complexity and strong structure of yeast cells, development of cell disruption or mechanical properties of *Saccharomyces* cell itself has been studied until recent years (Stowers and Boczek, 2007; Steson et al., 2011). There are some reports which deals with protein extraction from *Saccharomyces* cells using alkali solutions (Mrsa and Tanner, 1999; Kushnirov, 2000; Matsuo et al., 2006; Zhang et al., 2011). In these experiments, NaOH was used for the extraction of yeast proteins. Recently it was reported that protein extraction from *S. cerevisiae* can be ameliorated by combined use of lithium acetate and NaOH (Zhang et al., 2011). In this experiment, these reagents were pre-treated in order to enhance the permeability of yeast cells prior to protein extraction. For *Pichia* strains, a method of cell permeabilization using *N,N*-dimethyltetradecylamine was developed (Shapard et al., 2002). This method was proved to be more efficient than high-pressure homogenization.

Despite of some reports on the protein extraction from *Saccharomyces* strains, the extraction from *Pichia* strains has been insufficiently studied up to date. Furthermore, *Pichia* strains are widely used for extensive research works as a model of eukaryotic organism as well as *Saccharomyces* strains. Therefore we developed a protein extraction with two *Saccharomyces* strains and also with *P. pastoris*. We tried a pre-treatment of yeast cells prior to protein extraction in an attempt to test the possible weakening of cell wall and the resultant facility of protein extraction. For this experiment, NH_4OH solution was added into the suspension of yeast cells as a pre-treatment step. This pre-treatment is a distinguishing method in our study, which differs from other method of protein extraction. By this pre-treatment, the final extraction of proteins drastically increased from *S. cerevisiae* strains and *P. pastoris* X-33.

It was notable that the optimum condition of protein extraction from *P. pastoris* using the pre-treatment with NH_4OH was much similar to those of two yeast strains, *S. cerevisiae* KACC 30008 and KACC 30044. The pre-treatment with NH_4OH itself dose not appear to considerably disrupt the yeast cell walls under the basis that many of proteins, especially cytosolic proteins, should be readily liberated upon severe disruption of cell wall. But in this experiment, only low contents of proteins were released by the treatment of NH_4OH alone (data not shown). Therefore, the pre-treatment with NH_4OH might mainly contribute to weaken the cell walls of yeast strains, thus making the cells more susceptible to the next step of protein extraction. In general, biological activities of many proteins are lowered during the extraction procedure, especially, under alkaline solution and/or detergent.

The mechanisms of ammonium hydroxide was not clearly elucidated in this paper. But based upon the report of Fujita et al., (2007), it is worthwhile to suggest that ammonium ion might weaken the hydrophobic interactions in the yeast cell wall or interior structure. For the proteomic analysis, enzyme activities are not important and sufficient extraction of protein is more important. In this regard, the methods developed in this experiment might be appropriate for the biochemical studies, especially proteomic analysis.

Collectively, the protein extractions using the pre-treatment with NH₄OH were much effective from two *Saccharomyces* strains and one *Pichia* strain. The method developed in this experiment is not the best method of extracting whole proteins from yeast cells. It would be hopeful if our method is applicable in combination with other methods of extraction through mutual supplementation of advantage and disadvantage, each other.

Acknowledgments This study was performed with the support of “Research program for Agricultural Science and Technology Development (Project No. PJ0086492012)”. National Academy of Agricultural Science, Rural Development Administration, Republic of Korea.

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