Research Article

The Investigation of Virginiamycin-Added Fungal Fermentation on the Size and Immunoreactivity of Heat-Sensitive Soy Protein

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The usage of soy protein for young monogastric animals is restricted due to potential allergens and high molecular weight. The investigation of fungi fermentation effect on soy protein has been interrupted by substrate sterilization. Virginiamycin at 0.05% was added together with *Aspergillus oryzae* for solid state fermentation (SSF) in unsterilized soy meal (SM). When compared to *A. oryzae* SSF alone, virginiamycin did not cause the interference of fungal fermentation but elucidated the protein degradation. SDS-PAGE results showed that both α and α' subunits of β-conglycinin were degraded significantly. In addition, western blot results showed that the immunoreactive signals of soy protein were considerably reduced in virginiamycin-added fermentation with unsterilized SM. Furthermore, fungal fermentation increased total protein and essential amino acid contents, suggesting the value enhancement of SM products. Taken together, this study demonstrated for the first time that virginiamycin could help investigate fermentation effect on heat-sensitive soy protein. Fermented SM has several potential applications in feed industry.

1. Introduction

Soy meal (SM) is the main protein source for monogastric animals in the United States [1]. But its inclusion in newly weaned pigs is limited because of some antinutritional factors and antigenic soy proteins causing hypersensitivity [2]. To date, 34 soybean proteins have been identified as allergens [3]. All three parts of the β-conglycinin, both acidic and basic subunits of glycinin and P34, have been identified as main allergens for young pigs [3–5]. Some studies indicate that incorporation of antigenic soy proteins such as pure glycinin or β-conglycinin to the diet leads to a reduced weight gain and feed efficiency as well as an increased incidence of diarrhea in pigs [4]. Furthermore, SM contains large molecular size proteins that are difficult for digestion, because many digestion enzymes such as pepsin and trypsin cannot perform their entire function until 3 weeks of age in piglets [4].

Fermentation has been applied to improve soy protein immunity and degrade protein molecular size. Song et al. [6]

found that natural fermentation, *Saccharomyces cerevisiae*, and *Bacillus lactis* fermentation of SM reduced in 80%, 77%, and 77% immune response when using 97.5 kUA/l human plasma, respectively. Amnuaycheewa and de Mejia [7] analyzed the profilin in fermented soy products. The reduction in profilin in natto fermented by *Bacillus natto* was 12.8% to 35.4% and for soy paste 12.8% to 46.3%, in comparison to soy flour. But as for the fungal fermentation effect on SM immunity, research is limited. Frias et al. [5] found that *Aspergillus oryzae* or *Rhizopus oryzae* solid state fermentation produced a reduction of immunoreactivity of 68% or 66% of soy meal, respectively. But both fermentations used 121°C 15 min sterilization before and after. Since high heat could denature protein [8] and reduce its immunity [3], it could not elucidate that the deduction of immunity was caused by fermentation. Fungal solid state fermentation of SM has been applied to enhance the nutritional value of SM as monogastric animals' feed [9]. Animal test has already found that feeding fermented SM (FSM) could decrease the immune

response to soy protein in piglets [10]. However, enzymes with different origins have different hydrolytic effect [11]. There is a need to find the proper way to investigate whether and how a specific fungus solid state fermentation affects soy protein immunoreactivity.

Virginiamycin is one of the common antibiotics used in feed industry [12]. Also it has been used in ethanol production to prevent contamination during fermentation [13]. In our research, we applied virginiamycin to inhibit bacteria growth during *A. oryzae* solid state fermentation in unsterilized SMV. In addition, we demonstrated a method to perform fermentation under unsterilized condition and therefore distinctly investigated molecular degradation and immunoreactivity reduction of heat-sensitive soy protein.

2. Materials and Methods

2.1. Microbial Culture. Lyophilized cultures of *A. oryzae* (ATCC 9362) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), revived in potato dextrose broth twice. After revival, culture was inoculated on potato dextrose (PDA) slant, incubated at 30°C for 7 days, and later stored at 4°C for short term preservation. For routine experiments, spore solution was used. Spores were collected from the slants by gently washing with 0.1% Tween 80 to obtain spore suspension of around 10^7 spores/mL. Additionally, spores were suspended in 15% glycerol and stored at −80°C in 1 mL aliquots for long term preservation.

2.2. Substrate Preparation and Fermentation. SM was procured from ADM Alliance Nutrition (Abilene, KS). Solid state fermentation was carried out with three different kinds of substrates, which were original SM with virginiamycin, SM autoclaved at 100°C for 30 min (SM100C), and SM autoclaved at 121°C for 15 min (SM121C). Their corresponding fermented products were characterized as SMV, SM100, and SM121. For SMV, virginiamycin was added at a ratio of 0.05% dry matter base of SM, based on the results of our preliminary experiment. Four milliliters of *A. oryzae* spore solution containing 10^7 spores/mL was inoculated into 20 g substrate. Moisture was adjusted before autoclave. Moisture contributed by inoculation was considered. And final moisture was 50%. The flasks were incubated at 35°C for 36 hr. The fermented samples were then lyophilized and used for analysis.

2.3. Soy Protein Sample Preparation. Lyophilized FSM was milled with mortar and pestle to flour. SM flour was dispersed in distilled water at a ratio of 1:10. The mixture pH was adjusted to pH 8.2 by using 2 N NaOH. After 2 hr of shaking at room temperature, the mixture was centrifuged at 5,000 rcf at 4°C to remove insoluble residues. Soy protein in supernatant was precipitated by adjusting the supernatant pH to pH 4.8.

2.4. Differential Scanning Calorimeter (DSC). The denaturation of soy proteins was assessed with a differential scanning calorimeter (DSC) (DSC7, Perkin-Elmer, Norwalk, CT) calibrated with indium and zinc. Wet soy protein samples were hermetically sealed in a large-volume stainless pan. About 10 mg soy protein (dmb) with moisture around 60% was loaded. Samples were scanned from 10 to 150°C at a heating rate of 10°C/min. Denaturation temperatures (T_d) were determined from the peak temperatures. Denaturation enthalpies (ΔH) were calculated from the areas of the denaturation peaks.

2.5. SDS-PAGE. The precipitated wet protein was diluted with distilled water by adjusting pH to pH 8.2 using 2 N NaOH. Protein concentration was determined with Bradford method. SDS-PAGE of soy protein samples was performed on a 4% stacking gel and 12% separating gel. Fifty milligrams of soy protein was inoculated into the gel for each sample. A prestained standard with molecular weight from 8 kDa to 250 kDa was used. Electrophoresis was performed at 120 V for 2 hr. The gel was stained in 0.1% Coomassie brilliant blue R-250 and destained in a solution containing 10% acetic acid and 40% methanol. Densitometry of the gel was analyzed by the Kodak 1D Image Analysis software, version 4.6 (Kodak, Rochester, NY).

2.6. Western Blot Procedures with Human Plasma. Western blot was performed according to the method of Frias et al. [5] with modifications. Human plasma used had soybean-specific IgE 10 kUA/L provided by PlasmaLab International (Everett, WA). After transferring, the membrane was stained with Ponceau S for 5 min to check the transferring effect. Ponceau S was then washed off with distilled water before proceeding to the next step. For the saturation solution, primary and secondary antibodies were prepared in tris-buffered saline (TBS) instead in 0.01% Tween in TBS (TTBS) buffer, to avoid the dark background. The membrane was exposed to Kodak X-ray film.

2.7. Chemical Analyses. The proximate composition was analyzed by the Agricultural Experiment Station Chemical Laboratories, University of Missouri (Columbia, MO) using the following methods: crude protein (AOAC Official Method 990.03, 2006) [14], crude fat (ether extraction, AOAC Official Method 920.39 (A)) [14], crude fiber (AOAC Official Method 978.10, 2006) [14], acid detergent fiber (ADF) (AOAC Official Method 973.18 (A–D), 2006) [14], neutral detergent fiber (NDF) [14], cellulose (AOAC Official Method 973.18 (A–D), 2006) [14], pepsin digestibility (AOAC Official Method 971.09, 2006) [14], amino acid profile (AOAC Official Method 982.30 E (a, b, c), chp.45.3.05, 2006) [14], and available lysine (AOAC Official Method 975.22, chp.45.4.03, 2006) [14].

3. Results

Figure 1 shows the DSC results of soy protein samples pretreated at different temperatures and their corresponding fermentation products. SM and SMV both had two peaks, with the degradation temperatures (T_d) at around 79°C and 96°C. SM100C and SM100 each had one peak at temperature around 97°C. SM121C and SM121 had one indiscernible peak at around 97°C.
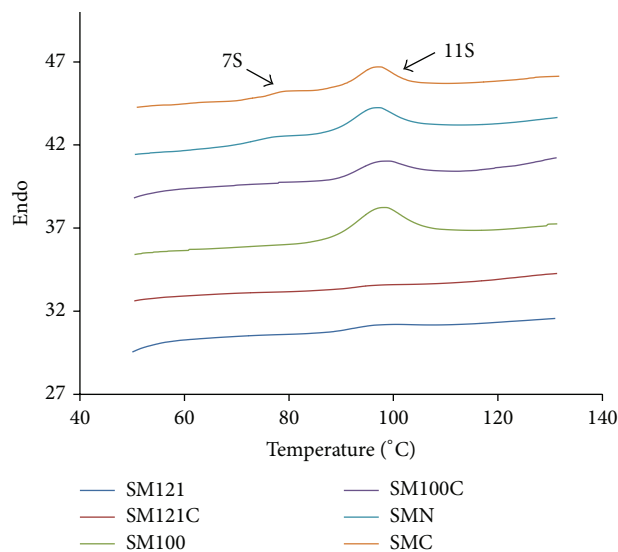
FIGURE 1: DSC thermogram of soy protein and fermented soy protein pretreated at different conditions.

TABLE 1: Proximates of soy meal samples.

Samples	SM	SMV	SM100C	SM100	SM121C	SM121
Crude protein	50.46[b]	58.78[a]	50.74[b]	58.82[a]	50.62[b]	58.47[a]
Crude fat	2.93[a]	3.15[a]	2.06[b]	3.07[a]	2.24[b]	2.94[a]
Crude fiber	3.32[b]	4.14[c]	3.11[c]	4.37[b]	3.02[c]	5[a]
Ash	6.68[d]	8.05[b]	6.93[c]	8.24[a]	6.66[d]	8.31[a]
ADF	5.48[c]	7.7[a]	4.74[c]	7.52[a]	4.67[c]	7.11[b]
NDF	8.18[b]	10.13[b]	12.38[b]	11.41[b]	18.15[a]	12.3[b]
Cellulose	5.41[c]	7.51[a]	4.75[d]	7.42[a]	4.62[d]	6.99[b]
Pepsin digestibility	95.33[a]	93.07[c]	95.18[a]	94.1[b]	94.84[a]	93.33[c]
Available lysine	3.06[b]	3.07[b]	2.95[c]	3.08[b]	2.84[d]	3.26[a]

Mean values in a row with different superscript letters are significantly different according to the least-significant difference (LSD) test at the 0.05 level analyzed by SAS software (2009).

TABLE 2: Amino acids changes of soy protein with different treatment.

Sample	SM	SMV	SM100C	SM100	SM121C	SM121
Hydroxyproline	0[b]	0.11[a]	0.1[a]	0.11[a]	0[b]	0.12[a]
Aspartic acid	5.77[b]	6.27[a]	5.62[c]	6.24[a]	5.66[bc]	6.3[a]
Threonine	1.93[b]	2.09[a]	1.92[b]	2.17[a]	1.85[b]	2.15[a]
Serine	2.14[a]	2.58[a]	2.34[a]	2.57[a]	2.04[a]	2.29[a]
Glutamic acid	8.85[ab]	8.91[ab]	8.42[b]	9.07[a]	8.66[ab]	9.02[a]
Proline	2.53[bc]	3.04[a]	2.45[c]	2.62[bc]	2.51[bc]	2.67[b]
Glycine	2.14[b]	2.28[a]	2.05[b]	2.38[a]	2.07[b]	2.39[a]
Alanine	2.19[b]	2.41[a]	2.13[b]	2.39[a]	2.13[b]	2.45[a]
Cysteine	0.7[b]	0.8[a]	0.66[b]	0.82[a]	0.67[b]	0.82[a]
Valine	2.52[d]	2.96[ab]	2.63[c]	2.89[b]	2.51[d]	3.01[a]
Methionine	0.72[d]	0.75[c]	0.68[f]	0.76[b]	0.7[e]	0.78[a]
Isoleucine	2.39[dc]	2.52[b]	2.32[d]	2.47[bc]	2.33[d]	2.63[a]
Leucine	3.92[c]	4.43[a]	3.95[c]	4.24[a]	3.84[d]	4.24[b]
Tyrosine	1.84[b]	1.94[a]	1.78[b]	1.91[a]	1.77[b]	1.93[a]
Phenylalanine	2.53[bc]	2.75[a]	2.48[c]	2.58[b]	2.48[c]	2.59[b]
Hydroxylysine	0.03[a]	0.02[ab]	0.01[c]	0.02[ab]	0.02[ab]	0.02[ab]
Ornithine	0.03[b]	0.04[b]	0.03[b]	0.05[b]	0.04[b]	0.07[a]
Lysine	3.24[b]	3.22[b]	3.12[b]	3.29[a]	3.1[c]	3.51[a]
Histidine	1.32[b]	1.38[a]	1.27[c]	1.37[a]	1.27[c]	1.4[a]
Arginine	3.64[a]	3.48[c]	3.56[b]	3.48[c]	3.52[bc]	3.57[b]
Tryptophan	0.74[c]	0.81[b]	0.79[b]	0.89[a]	0.71[c]	0.92[a]
Total	49.13[b]	52.73[a]	48.28[b]	52.27[a]	47.84[b]	52.83[a]

Mean values in a row with different superscript letters are significantly different according to the least-significant difference (LSD) test at the 0.05 level analyzed by SAS software (2009).

Figure 2 shows the SDS-PAGE results of soy meal samples with different pretreatment. SM contained 79% bands with molecular weight larger than 60,000 kDa, but SM100C only had 4% bands and SM121C only had 6% bands in that range. For SMV, SM100, and SM121, the three fermented samples, the molecular weight of all peptides was smaller than 60,000 kDa. All the fermented samples had 2% small peptides with molecular weight smaller than 10,000 kDa, which the controlling unfermented samples were not endowed. The α and α' subunits of SM were not shown in SMV, and β-conglycinin was not shown in SM100 and SM121 and their controls.

Figure 3 shows the Ponceau S staining of the membrane after transferring. Comparing with Figure 2, all bands in SDS-PAGE gel had been transferred to the membrane. Figure 4 shows the immunodominant proteins interacting with human plasma 10 kUA/L. SM presented the highest complexity protein profile and plasma immunoreactivity towards α- and α'- (75 kDa) and β- (50 kDa) conglycinin subunits, P34 fraction, and glycinin basic (30 kDa) and acidic (22 kDa) subunits. Its corresponding fermented sample SMV did not have immunoreactive proteins toward α- and α'- (75 kDa) conglycinin subunits. Compared with SM, the immunoreactions signals for β-conglycinin, P34, and acidic (22 kDa) glycinin subunits were weak for SM100C. There was no visible immunoreactivity for β-conglycinin in SM100. SM121 and SM121C had immunoreactive protein towards basic (30 kDa) glycinin only.

The composition changes of SM samples are shown in Table 1. Heat treatment did not significantly influence the content of crude protein, crude fat, crude fiber, total ash, and pepsin digestibility but decreased the content of crude fiber, acid detergent fiber, and cellulose content and increased the neutral detergent fiber. Also, heat treatment decreased the content of available lysine content. The higher the temperature, the lower the available lysine content of heat

pretreated soy meal. The content of all components increased after fermentation, except for the fact that pepsin digestibility decreased. Fermented samples with higher heat treatment had higher available lysine.

Amino acid contents of fermented samples are shown in Table 2. Heat treatment did not significantly affect essential amino acids content, except for lysine. Fermentation
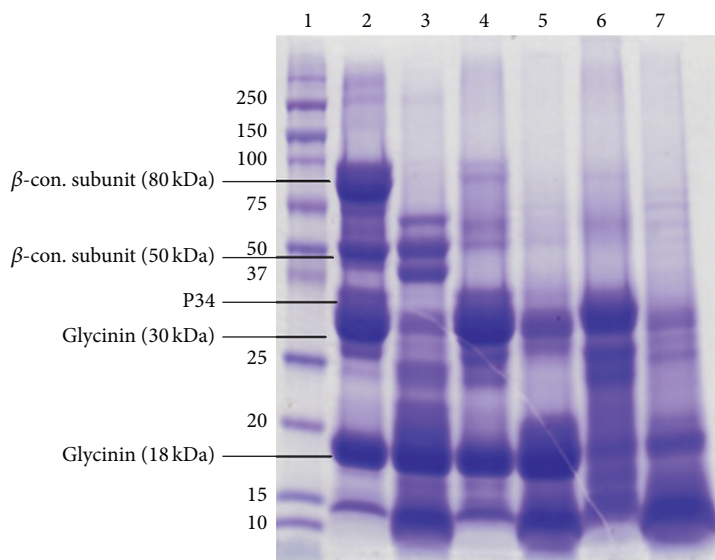
FIGURE 2: SDS-PAGE for soy protein and fermented soy protein pretreated at different conditions. Lane 1: prestained standard; lane 2: SM; lane 3: SMV; lane 4: SM100C; lane 5: SM100; lane 6: SM121C; lane 7: SM121.
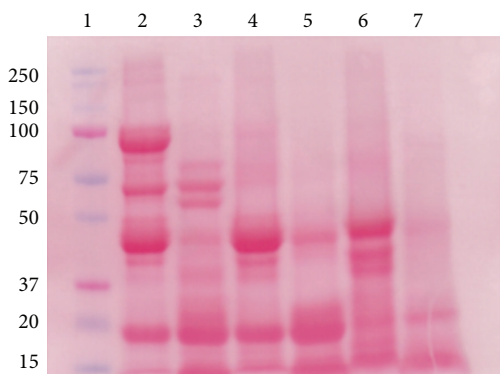

FIGURE 3: Ponceau S staining of the membrane after transferring. Lane 1: prestained standard; lane 2: SM; lane 3: SMV; lane 4: SM100C; lane 5: SM100; lane 6: SM121C; lane 7: SM121.

increased the total amino acids content of SM, SM100C, and SM121C by 7%, 8%, and 9%, respectively. Fermentation also increased all amino acids contents. The higher the temperature, the higher the amino acid content of fermented samples. Methionine, cysteine, and threonine in SM121 increased by 11.4%, 22.39%, and 16.22% after fermentation, respectively. Lysine and tryptophan in SM121 increased by 13% and 30% compared with SM121C.

4. Discussion

Soy protein denaturation is an endothermic process [15], caused by rupture of inter- and intramolecular bonds. Its denaturation degree could be shown by DSC. Undenatured soy protein has two peaks in the DSC curve, which represent the two main soy storage proteins, conglycinin and glycinin [15]. Glycinin is more heat stable than conglycinin [15]. The T_d for glycinin was in the range of 96.3–97.7°C, while the T_d for conglycinin was in the range of 77.1–79.3°C (Figure 1). Protein denaturation is a nonreversible process [15]. The denatured protein would not show peaks on the DSC diagram. According to Figure 1, protein in SM and SMV was nondenatured; 7S subunits in SM100C and SM100 were denatured; in SM121C and SM121, 7S subunits were totally denatured while 11S subunits were almost totally denatured.

SDS-PAGE is a common method to evaluate protein molecular size. But for heat treated samples, it did not work well, according to our result. The denatured 7S subunit of soy protein was not shown on SM100C and SM121C lanes. This did not mean that SM100C and SM121C had less large molecular weight protein. Protein denaturation was the change of the secondary, tertiary, and quaternary structures. Thermal energy input disrupted the weak bonds stabilizing the native conformation, causing protein to unfold [16]. The denatured protein may form large aggregate [17], which may become insoluble and would not be shown on SDS-PAGE. Wang et al. [16] also showed that 100°C heating for 20 min resulted in the loss of protein bands on SDS gel. The addition of virginiamycin avoided the heat pretreatment of soy meal. *A. oryzae* solid state fermentation could degrade large protein molecules into smaller peptides, as shown by comparing the molecular weight of SM and SMV from Figure 2.

About 34 subunits of soy protein have been recognized as allergens [3]. Our result also showed the strong signal of immunoreactive protein in SM. Heat treatment has been shown to affect allergen conformational epitopes and decrease its immunoreactivity [18]. Western blot has been used to illustrate protein immunoreactivity. Like SDS-PAGE, there was still the problem caused by heat induced protein
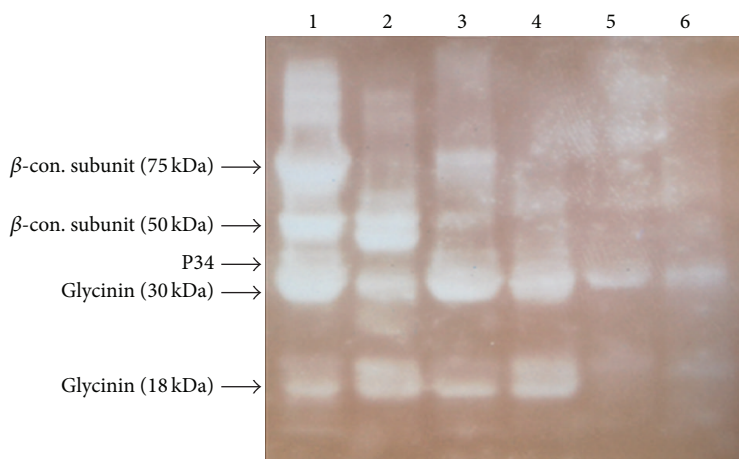
FIGURE 4: Western blot results of soy protein samples. Lane 1: SM; lane 2: SMV; lane 3: SM100C; lane 4: SM100; lane 5: SM121C; lane 6: SM121.

denature. Subunits of soy protein, like β-conglycinin in SM100C and SM121C and acidic glycinin in SM121C, were not visible on western blot. This did not mean that they did not have immunoreactivity, but they were not present on SDS gel. The virginiamycin addition helped investigate soy protein immunoreactivity change during fermentation process. The weaker immunoreactive signals for SMV, comparing with SM, illustrated that *A. oryzae* solid state fermentation could decrease immunoreactive soy protein.

Natural fermentation has been shown to degrade soy protein molecular size with nonheated samples [5]. In the natural fermentation, various kinds of microorganisms have been involved. Different proteases target different protein subunits [19]. In order to achieve better degradation, it is necessary to investigate the fermentation effect of specific microorganism. Virginiamycin could inhibit bacterial growth while the inoculum of desired fungus could inhibit the growth of other contaminating fungi, according to our results. Virginiamycin addition excluded the necessity of heat treatment, which was beneficial to the protein size and immunoreactivity investigation.

A. oryzae secretes acidic and neutral proteases, which could degrade epitopes [18]. According to our research, *A. oryzae* fermentation provides means to degrade soy protein molecular size while decreasing its immunoreactive protein. Soy protein with low molecular weight and weak or no immunoreactive protein have been added to newly weaned piglets' diet to lower the feeding cost [2]. Fermentation parameters, such as temperature, moisture, and fermentation time, need to be optimized to maximize the protein degradation and to produce hypoallergenic fermented soy products for young pigs.

Acid detergent fiber mainly includes cellulose and lignin. Neutral detergent fiber mainly includes hemicelluloses, cellulose, and lignin. Heat treatment may degrade lignin or catalyze complex structure formation between hemicelluloses and lignin or hemicelluloses with other components, like lysine. Lysine is susceptible to react with other compounds, such as reducing sugars to form Maillard compounds, resulting in the loss of available lysine and reduction of nutritional value. *A. oryzae* secretes various kinds of enzymes, such as α-amylase, carboxymethyl cellulase, pectin lyase, protease, and *endo-β*-xylanase (EC 3.2.1.8) [20] to help utilize soy meal components to meet its growth needs. The enhancement of all components by fermentation was mainly because of the dry matter loss resulting from *A. oryzae* consumption, which concentrated the nutritional compounds. Proper heat could unfold protein structure and make protein easier to digest by proteases. The decrease of the pepsin digestibility may be partially contributed by the increased protein content.

Soy protein is rich in lysine and tryptophan but lacking in methionine and threonine, compared with cereals proteins. SM and maize are main ingredients for monogastric animals' diet and provide complementary amino acid profiles. Amino acids deficiency may decrease feed efficiency and feed intake, cause weight loss, and influence animals' growth performance [21]. *A. oryzae* solid state fermentation enhanced the amino acid contents of all samples, especially for the SM121. One reason for the increase of amino acids is the dry matter loss, which has been illustrated for solid state fermentation [22]. The second reason is the hydrolysis of protein by protease secreted from *A. oryzae* [22]. Also, the increase of *A. oryzae* biomass contributes to the amino acids and protein increase [22]. Heat treatment could expose inside peptide bonds and ease enzymatic hydrolysis. Proper heat treatment might benefit the subsequent fermentation process.

A. oryzae solid state fermentation could degrade large soy protein molecular size and decrease soy protein immunoreactivity. *A. oryzae* protease had priority for α and α' components of β-conglycinin. Virginiamycin facilitated the investigation on fermentation degradation of soy protein, by avoiding the interruption from heating. Fermentation enhanced the nutritional value of soy meal, with higher protein content. Essential amino acids contents were also enhanced by fermentation. Proper heat treatment facilitated the fermentation process. The value added FSM should have a wider market than SM, particularly for newly weaned piglets.

Abbreviations

ATCC: American Type Culture Collection
DSC: Differential scanning calorimetry
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide
            gel electrophoresis
ADF: Acid detergent fiber
NDF: Neutral detergent fiber
AOAC: Association of Official Analytical
            Chemists
SM: Soy meal
FSM: Fermented soy meal
SMV: Fermented soy meal with virginiamycin
            added
SM100C: Soy meal autoclaved at 100°C for 30 min
SM100:   Fermented soy meal which was autoclaved
            at 100°C for 30 min
SM121C: Soy meal autoclaved at 121°C for 15 min
SM121:   Fermented soy meal which was autoclaved
            at 121°C for 15 min.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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