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Full Length Research Paper

Consecutive treatment with phytase and arazyme influence protein hydrolysis of soybean meal

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Soybean meal (SBM) is the main protein supplement used in animal feed worldwide. The degree of hydrolysis (DH) of SBM treated with two enzymes viz. phytase and arazyme was investigated for the first time in this study. The DH of SBM in the treatment with arazyme increased significantly as compared to the control without enzyme application. About 1.5-times and 10-fold higher DH were observed in phytase treatment when compared to the control treatments containing no enzyme. At the end of 24 h, enzymatic hydrolysis was done through consecutive treatment with 0.5% (w/v) phytase and 0.02% (w/v) arazyme, and the protein in the hydrolysate were mostly degraded free amino acids and peptides (<6 KDa) when SDS-PAGE and fast protein liquid chromatography (FPLC) techniques used. Free amino acids contents of the soybean meal treated with phytase-arazyme increased by 2 to 14 fold as compared to products without enzyme. These results suggested that soybean meal proteins continuously treated with phytase and arazyme can be used as commercial feed additive for accelerated livestock growth.

Key words: Soybean meal, phytase, arazyme, hydrolysis.

INTRODUCTION

Phytate or phytic acid is a hexaphosphate form of myo-inositol (Ins P6). It is commonly found in soymilk (about 2 g per 100 g of protein) and soy protein isolates (1 to 2 g per 100 g of protein) (Anderson et al., 1995). Phytate presence causes regulatory effects on mineral uptake in humans and animals (Lopez et al., 2001). It is active in broad pH regions and negatively charged ions which give it stronger affinity for minerals, trace elements and proteins having positive charge(s) (Lopez et al., 2001; Sandstorm et al., 1992; Hurrell et al., 2003). Upon reaction with such minerals, a phytate complex is formed which can reduce mineral bioavailability and hence decrease solubility (Lapvetelainen et al., 1991). It would be worthwhile to obtain reduced-phytate proteins for making high-quality foods and feedstuffs. To hydrolyze phytate is difficult due to lack of endogenous phytate-degrading enzymes (Iqbal et al., 1994). It is therefore necessary to treat the foodstuff with exogenous phytase, which can help to hydrolyze phytate to partially phosphorylated myo-inositol phosphate esters to eliminate the adverse effect of phytate on mineral absorption (Sandberg et al., 1989; Brune et al., 1992). Similarly, arazyme has been reported as a novel protease produced by the strain of *Arnicola proteolyticus* (a gram-negative aerobic bacterium) (Baumann et al., 1993; Bersanetti et al., 2005). Its size is 51.5 kDa. Arazyme is commercially produced as detergent additive, leather treatment and food processing (Bersanetti et al., 2005). Some work has been done on function of arazyme, but still more understanding is needed.

Phytate hydrolysis has dual advantages of (i) eliminating antinutrient compound phytate while improving the

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absorption of minerals and (ii) having partially low levels of phosphorylated myo-inositol phosphates. Ways to reduce phytate from food include the addition of exogenous phytate-degrading enzymes such as phytase or arazyme; changes in breeding; agronomic conditions; genetic engineering or changes in food processes such as prolonged process time or change in pH of the product (Lopez et al., 2001; Bohn et al., 2008). Although ample information is available in relation to the use of phytase treatments of food/feed stuff, however, little or no information are available on arazyme's effect on protein hydrolysis.

Soybean meal (SBM) is the main protein supplement used in animal feed worldwide. In recent years, there has been an increasing use of SBM as a vegetable protein substitute for fishmeal because of its high quality, cheaper cost and safety. For its successful use in animal feed, about 20% SBM of yellowtail feeds (Shimeno et al., 1993), 26% of red drum and *Sciaenops ocellatus* (Reigh et al., 1992), and 100% of broiler feed were substituted (Lee and Lee, 1971). SBM diet contributes to growth and lysine utilization of broilers (Kim et al., 1995). SBM is also a potentially valuable protein source in animal diets, because of its high protein content and relatively well-balanced amino-acid pattern, but has poor digestibility and absorption *in vivo* with economical and environmental concerns.

Therefore, the present experiments were conducted to investigate the enhancement of SBM-nutrients utilization in feeds. It was found that application of enzymes to soybean-meal can enhance oligosaccharides and compound carbohydrates utilization (Kang et al., 2001) and binding of phosphate (P) in vegetable feedstuffs (Eeckhout et al., 1994). The ability of poultry to utilize the P bound in the phytase is generally assumed to be poor (Richardson et al., 1985; Kornegay, 1996). The efficacy of microbial phytase in enhancing the P availability in poultry diets and in decreasing water pollution is well-documented (Cromwell et al., 1993). As far as the authors are concerned, phytate digestion and protein-digestibility enhancement in vegetable feedstuffs have not been investigated. The objectives of this study were to investigate the effect of consecutive treatment of phytase and arazyme on protein hydrolysis of SBM.

MATERIALS AND METHODS

Enzymes, chemicals and SBM

Soybean meal was purchased from the local market. The protein content (Nx6,25) was 46% on a moisture-free basis. Phytase (activity 3.5 units/mg) was purchased from Sigma Chemical Co. (St, Louis, Mo), and arazyme (activity 5,000 units/g) was purchased from Insect Bio Tech Co. (Daejeon, Korea). All the biochemicals were used without further purification. Other chemicals were of commercial guaranteed grade.

Enzyme treatment of SBM and enzymatic hydrolysis

Soybean meal samples were divided into control (CON; the treatment containing no enzyme), 0.5% (w/v) phytase (PHY) alone, and 0.5% (w/v) phytase treatment and 0.02% (w/v) arazyme (PAT) by consecutive method.

To compare the two different methods (arazyme and phytase-arazyme treatment) for enzymatic hydrolysis reactions of soybean meal, 3 g of the soybean meal were re-suspended in 90 ml of distilled water and the pH was adjusted to 8.0 with 2.5 M NaOH; 0.01, 0.02 and 0.03% (w/v) enzyme solution in water (9, 18 and 27 mg of arazyme) were added to the samples and digestion was allowed to proceed at 37°C for 24 h. For the phytase-arazyme treatment, 3 g of the meal were mixed in 90 ml of distilled water and the pH was maintained at a range of 5.0 to 5.5 using 2 M HCl; 0.05 to 0.5% solution (45, 90, 270 and 450 mg of phytase) was added, and the solution was incubated at 50°C for 10 h. The reaction was stopped, followed by adjustment of pH to 8.0 with 2.5 M NaOH; 0.02% (w/v) was then added, and the solution incubated at 37°C for 24 h. The suspension was centrifuged at 3000 rpm for 10 min. The supernatant was used to estimate the degree of hydrolysis.

Degree of hydrolysis (DH)

The DH was determined using the modified method of Alder-Nissen (1979). An amount of 250 µl of the supernatant was added to 1.25 ml of 1% sodium dodecyl sulfate (SDS) from Sigma Chemical (St Louis, MO, USA) and the mixture was heated to 75°C for 15 min. Properly diluted samples (25 µl) were mixed thoroughly with 200 µl of 0.2125 M phosphate buffer (pH 8), followed by the addition of 200 µl of 1% 2,4,6-trinitrobenzenesulfonic acid (TNBS) (from Sigma Chemical, St Louis, MO, USA) solution. The resulting mixture was shaken and incubated in the dark at 50°C for 1 h. After that time, 400 µl of 0.1 N HCl was added, and after 30 min, absorbance was measured with a spectrophotometer at 340 nm. The calibration curve was prepared using a 1.5 mM solution of L-Leucine (L-Leu) from Sigma Chemical (St Louis, MO, USA), which was diluted to yield the different points along the calibration curves. The results were expressed as mmols of L-Leu per 1 g of protein of soybean meal.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was run according to the method of Laemmli (1970) using 4% stacking and 12.5% resolving polyacrylamide gel. Molecular mass markers were obtained from Sigma Chemical Co. (MW-GF-70 mixture): phosphorylase B (113 KDa), bovine serum albumin (92 KDa), ovalbumin (52.3 KDa) carbonic anhydrase (35 KDa), soybean trypsin inhibitor (28.9 KDa) and lysozyme (21 KDa). Gels were visualized with Coomassie brilliant blue R 250 staining.

Fast protein liquid chromatography (FPLC) analysis

The FPLC of soybean meal hydrolysates was performed on a Pharmacia (Milford, MA) protein pak superdex 200HR 10/30 column of 7.5 x 300 mm. The column was fitted to an amersham pharmacia PC 3.2/30, ÄKTA^{TM} FPLC^{TM} (Amersham pharmacia biotech) apparatus, equipped with a P-920 pump model. Proteins were run in 50 mM phosphate buffer (pH 7.0) 0.15 M NaCl, at a flow rate of 0.5 ml/min. A standard curve was obtained by using molecular weight protein markers including blue dextran (2,000 KDa), albumin bovin serum (66 KDa), carbonic anhydrase (29 KDa) and aprotinin (6.5 KDa).

Amino acid analysis

Amino acid composition of the hydrolysates was determined after

Table 1. Degree of hydrolysis profiles from soybean meal by incubation with arazyme at 37℃ for 24 h.

Arazyme [%(w/v)]	DH[1] (mmol/g-protein)	Protein relative percentage
0.00	0.22±0.02[a2]	100.0
0.01	0.38±0.04[b]	172.7
0.02	0.45±0.02[c]	204.5
0.03	0.49±0.01[d]	222.7

[1]DH, Degree of hydrolysis; [2]quoted values are means of duplicate experiments. Values presented in the relative percentage column are in comparison with the control (the treatment containing no enzyme). Means with different superscripts within a row indicate significant differences ($p < 0.05$).

Table 2. Degree of hydrolysis profiles from soybean meal by incubation with phytase at 50℃ for 10 h after arazyme treatment at 37℃ for 24 h.

Phytase (w/v)	Arazyme [%(w/v)]	DH[1] (mmol/g-protein)	Protein relative percentage
0.00	0.00	0.22±0.03[a2]	100.0
0.05	0.02	0.59±0.05[b]	268.2
0.10	0.02	1.16±0.04[c]	527.3
0.30	0.02	1.59±0.06[d]	722.7
0.50	0.02	1.95±0.07[e]	886.4
0.50	0.00	1.26±0.07[c]	572.7

[1]DH, Degree of hydrolysis; [2]quoted values are means of duplicate experiments. Values presented in relative percentage column are in comparison with the control (the treatment containing no enzyme). Means with different superscripts within a row indicate significant differences ($p < 0.05$).

hydrolysis under vacuum with 6 N HCl at 110℃ for 24 h. The hydrolysates were concentrated to dryness under vacuum and dissolved in 100 µl of 0.2 M citrate buffer (pH 2.2). Amino acids were analyzed with a SYKAM Co, S433 (Germany) amino acid analyzer.

Statistical analysis

Data were subjected to one-way or two-way ANOVA when required. Differences between means at $p < 0.05$ were analyzed using the Tukey test. The Statistic version 4.0 package (Analytical Software, AZ, USA) was used.

RESULTS AND DISCUSSION

Degree of hydrolysis of SBM

The effect of arazyme on the degree of hydrolysis (DH) of SBM was assessed (Table 1). The results showed that gradual application of arazyme significantly enhanced the DH of SBM. The DH of SBM treated with 0.02 and 0.03% arazyme increased two-fold than that of the treatments containing no enzyme. Similarly, when the combination of different concentration of phytase and arazyme was applied, the DH of SBM was improved significantly than the control. Time increase in the DH of soybean meal proteins was observed for 0.5% (w/v) phytase and 0.02% (w/v) arazyme treatment when compared with the control treatment (Table 2). However, using higher concentration (0.50%) of sole phytase resulted in lesser amount of DH of SBM than the phytase and arazyme treatments.

Previously, it was revealed that nitrogen (N) digestibility of broiler chicks was improved by SBM pre-treated (50℃ for 2 h) with protease P_3 (isolated from *Aspergillus* species) than the untreated ones (Ghazi et al., 2003). Similarly, it was also observed that N digestibility by SBM treated with protease P_2 from *Aspergillus* sp. in tube-feeding experiments increased (Ghazi et al., 2002). Other studies have also suggested that the application of exogenous enzymes to the feedstuffs can enhance not only the mineral composition but also the hydrolysis of essential biochemicals (Cromwell et al., 1993). These results suggested that the DH of SBM by arazyme was effective. For positive effect on protein digestion and utilization of SBM, SBM was mixed with phytase from *Aspergillus* sp. SM-15 and also with protease from *Aspergillus* sp. MS-18. Thus, a higher increase in protein extraction rate of SBM was observed for the enzyme mixture with phytase and protease when compared with the treatment containing the enzyme alone (Cho and Chun, 2000). The addition of such enzymes in the feedstuffs partially prevents the formation of phytate-protein complexes by the prior hydrolysis of phytate and thus increases the digestibility of protein (Liu et al., 2007). Similar observa-tions were also found in this study as both the enzymes, that is, phytase and marazyme had

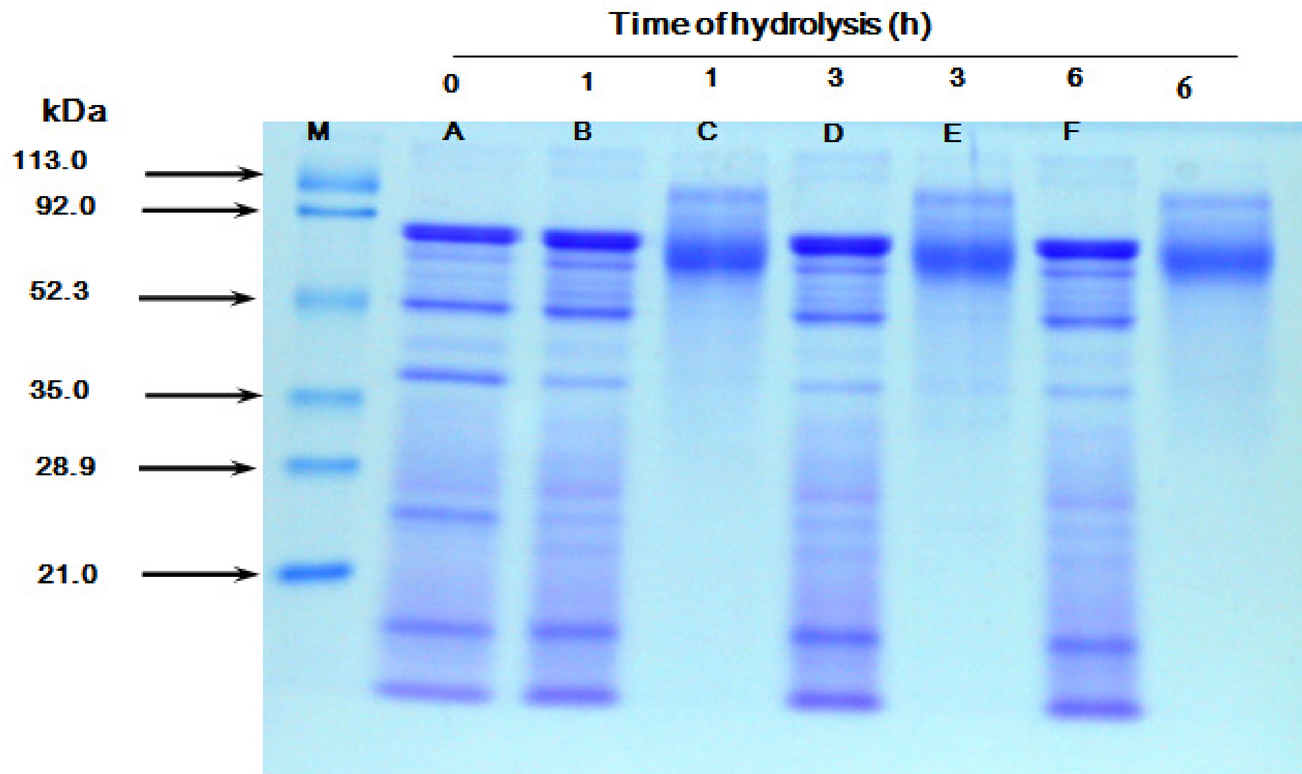

Figure 1. SDS-PAGE (12.5%) profiles of hydrolysates from soybean meal treated with 0.5% (w/v) phytase at 50℃ for 10 h after 0.02% (w/v) arazyme at 37℃ for 24 h. Lane M, molecular weight markers; Lane A, non-enzyme treatment; Lanes B, D and F, phytase treatment; Lanes C, E and F, phytase and arazyme treatment. Vertical numbers indicate molecular weight markers in KDa. Horizontal numbers refer to time of hydrolysis

higher degree of hydrolysis.

Hydrolysates of soybean meal

SDS-PAGE analysis were performed for the SBM treated with phytase and arazyme patterns of hydrolysates from SBM treated with 0.5% (w/v) phytase at 50℃ for 10 h after 0.02% (w/v) arazyme treatment at 37℃ for 24 h (Figure 1). The composition of SBM proteins was almost degraded by the phytase-arazyme and phytase alone. As shown in Figure 2, most of the protein degradation was observed in the SBM proteins applied with phytase-arazyme through FPLC analysis. Similar results were obtained by Lapvetelainen et al. (1991), Yu et al. (2002) and Liu et al. (2007) who applied enzymes to enhance digestion of soybean protein (SPC). From these results, SBM proteins treated with protease would have higher protein usefulness in feedstuff, while in combination with arazyme the effects can be more ameliorative.

Amino acid composition

The amino acid profiles of soybean meal by incubation with 0.5% (w/v) phytase at 50℃ for 10 h after 0.02% (w/v) arazyme treatment at 37℃ or 24 h are shown in Table 3. The results showed significant influence of both the enzyme's application on the amino acid composition of SBM (Table 3). During analysis of amino acid composition, a 3, 5, 6 and 14 fold higher quantities of cystein, aspartic acid, threonine, methionine and leucine were observed in phytase treatment and 0.02% (w/v) arazyme (PAT) treatment when compared with the controls (CON). On the other hand, about 1.5 times higher increase in proline, valine and cystein were observed in phytase (PHY) alone when compared to CON. Thus, the total amino acid content of SBM treated under PAT treatment was 46.10%. So, protein in SBM was shown to be dehydrated. Similar results were found by Francisco and Laurent, (2001); Jalal et al. (1999) reported that the digestibility of the coefficients of Met, Cys, Ala and Glu were significantly improved by phytase in a corn-soybean meal diet. These results indicated that SBM treated with arazyme would be important to supply a high quality feedstuff.

In conclusion, the phytase and arazyme application to SBM protein enhanced DH. Thus, the results suggests that continuous treatment of phytase and arazyme of SBM proteins can be used as commercial feed additive for accelerated livestock growth.

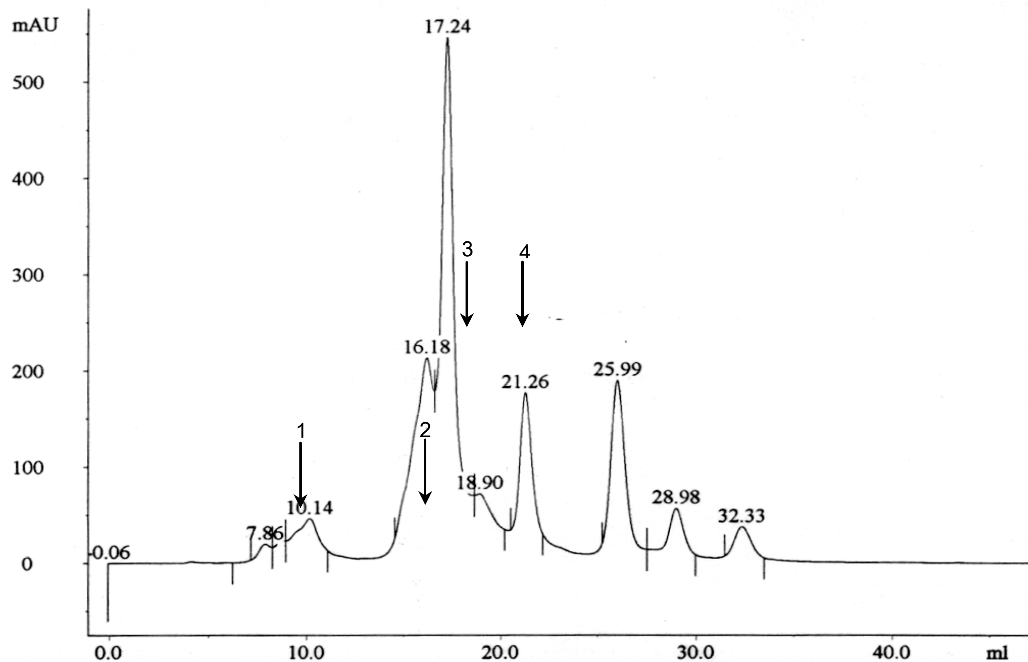

Figure 2. FPLC patterns of soybean meal treated with 0.5% (w/v) phytase at 50°C for 10 h after 0.02% (w/v) arazyme treatment at 37°C for 24 h. Peak 1, blue dextran (2,000 KDa); 2, albumin bovin serum (66 kDa); 3, carbonic hydrase (29 kDa); 4, aprotinin (6.5kDa).

Table 3. Amino acid content[1] of the soybean meal by incubation with 0.5% (w/v) phytase at 50°C for 10 h after 0.02% (w/v) arazyme treatment at 37°C for 24 h.

Amino acid	Content (g/100 g)			
	Control	Arazyme	Phytase	Phytase-Arazyme
Indispensable				
Arg	0.81±0.012^{a}	2.62 ±0.04^{c}	2.50±0.03^{b}	3.29±0.02^{d}
His	0.41±0.02^{a}	1.69 ±0.03^{d}	1.11±0.02^{b}	1.41±0.01^{c}
Ileu	0.20±0.01^{a}	1.79 ±0.02^{c}	1.39±0.03^{b}	2.01±0.03^{d}
Leu	0.24±0.01^{a}	2.76 ±0.01^{c}	2.32±0.04^{b}	3.37±0.02^{d}
Lys	0.76±0.03^{a}	3.36 ±0.03^{d}	2.32±0.02^{b}	2.79±0.01^{c}
Met	0.08±0.01^{a}	0.48 ±0.01^{c}	0.37±0.01^{b}	0.51±0.01^{d}
Phe	0.27±0.02^{a}	1.94 ±0.02^{c}	1.48±0.02^{b}	2.16±0.03^{d}
Thr	0.41±0.02^{a}	1.90 ±0.02^{c}	1.67±0.01^{b}	2.10±0.02^{d}
Val	0.23±0.01^{a}	1.92 ±0.01^{c}	1.56±0.03^{b}	2.37±0.03^{d}
Dispensable				
Ala	0.66±0.02^{a}	2.00 ±0.02^{c}	1.58±0.02^{b}	2.05±0.01^{d}
Asp	0.98±0.03^{a}	5.15 ±0.03^{c}	3.82±0.07^{b}	5.42±0.04^{d}
Cys	0.21±0.01^{a}	0.56 ±0.01^{c}	0.37±0.01^{b}	0.59±0.01^{d}
Glu	2.17±0.06^{a}	9.48 ±0.04^{d}	6.40±0.06^{b}	8.99±0.09^{c}
Gly	0.48±0.02^{a}	2.15 ±0.02^{d}	1.46±0.01^{b}	1.96±0.02^{c}
Ser	0.32±0.01^{a}	1.98 ±0.02^{c}	1.93±0.01^{b}	2.59±0.03^{d}
Tyr	0.44±0.02^{a}	1.37 ±0.01^{c}	1.30±0.01^{b}	1.80±0.02^{d}
Pro	0.57±0.03^{a}	2.52 ±0.02^{d}	1.41±0.02^{b}	2.23±0.01^{c}
Try	0.10±0.01^{a}	0.52 ±0.01^{c}	0.45±0.01^{b}	0.46±0.01^{b}
Total	9.34±0.02	25.73 ±0.04	33.44±0.02	46.10±0.02

[1]Dry matter basis; quoted values are means of duplicate experiments. Means with different superscripts within a row indicate significant differences ($P < 0.05$).

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