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Full Length Research Paper

Feasibility of wood pulping black liquor for treatment of soybean meal as a source of rumen protected protein

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This study was carried out to determine the effects of neutral black liquor and moisture heating treatments of soybean meal (SBM) on *in situ* rumen degradability characteristics in cow and its proteins sub-units fractions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) discontinuous system. SBM were treated with xylose, neutral black liquor and moisture heating. Neutral black liquor, xylose and moisture heated treatment significantly (P<0.05) reduced degradability values of crude protein (CP) for SBM. Treating could effectively decrease *a* fraction and increase *b* fraction in experimental treatments. Electrophoretic patterns of untreated and treated SBM protein shows that β-conglycinin α, ά and β subunit were degraded completely within 4 h of incubation in the rumen, whereas the acidic and basic subunits of glycinin were degraded after 48 h incubation in treated SBM with xylose and black liquor treated same as the xylose group. It is concluded that SBM proteins can be effectively and economically protected from degradation in the rumen.

Key words: Soybean meal; degradability; black liquor; moisture heat; SDS-PAGE.

INTRODUCTION

Black liquor is the spent cooking liquor from the kraft process when digesting pulpwood into paper pulp, removing lignin, hemicelluloses and other extractives from the wood to free the cellulose fibers (Stenius, 2000). The black liquor is an aqueous solution of lignin residues, hemicelluloses and the inorganic chemicals used in the process. The black liquor contains more than half of the energy content of the wood fed into the digester. It is normally concentrated to 65 to 80% by multi-effect evaporators and burned in a recovery boiler to produce energy and to recover the cooking chemicals. Tall oil is an important byproduct separated from the black liquor with skimming before it goes to the evaporators or after the first evaporator stage. Approximately 7 tones of black liquor (15% solids by weight of which 10% are inorganic and 5% are organic) are produced in the manufacturing of one tone of pulp (Biermann, 1993). New waste-to-energy methods to recover and utilize the energy in the black liquor have been developed but black liquor is alkaline and if not exactly, management could create threat for the environment and animal life. High-producing dairy cows require sufficient protein in the diet to optimize microbial growth and fiber digestion in the rumen, and adequate amounts of essential amino acid (AA) should be available in the small intestine to provide for their increased metabolic and lactation demands (NRC, 2001; Cant et al., 2003).

Recently, treatment of dietary proteins source with various agents to decrease ruminal degradation has received considerable attention. One of the primary reasons for manipulating nitrogen transactions in the rumen either by protein protection or other means is to increase the outflow and balance of amino N to the duodenum of high producing ruminant. The most commonly used methods of protecting protein include heat and formaldehyde treatments, although other agents such as sodium hydroxide, alcohol, acids and xylose have been used successfully (Xu, 1995; Folman et al., 1981; Stern, 1984, 1985). More recently, treatment of soybean meal with xylose was successful in decreasing rumen degradation of soybean protein (Mehrez and Ørskov, 1977) and thus cloud visualize waste with xylose can affect soybean meal degradation kinetic. Cleale et al. (1986) found that treatment of soybean meal with xylose (3 mol xylose/mol lysine) was effective in reducing degradation of soybean protein by rumen micro-

organisms. It was concluded that controlled non enzymatic browning improved efficiency of soybean protein utilization by ruminants. Chalupa (1975) suggested that if the Millard reaction between sugar aldehyde groups and free amino groups can be controlled to decrease protein degradability in the rumen without adversely affecting intestinal protein digestibility, animal performance evaluated either by N retention or production would be increased. The objectives of this study were to (1) treat soybean meal with wood pulping waste (black liquor) as source of xylose and (2) reduce sugar and stimulate Millard reaction and determine protein degradation in the rumen and protein digestion of treated soybean meal (a source of rumen protected protein).

MATERIALS AND METHODS

Sample preparation and treatment

The SBM samples (soybean meal imported from Brazil) were obtained from commercial sources in Iran. SBM was treated with 3 and 6% neutral black liquor which was added together with 20% additional water to solvent-extracted soybean meal and heated (121°C and 117 KPa) via an autoclave for 30 min. The mixture was then cooled to room temperature and air dried to approximately 10% moisture.

In situ evaluation of dry matter and crude protein

Nylon bag technique was used to measure disappearance in the rumen of untreated and treated SBM. Nylon bags (45-m pore size; 9 × 14 cm bag size) containing 5 g of SBM samples were incubated in the rumen of each cow. Two bags of each type of treated SBM were removed after 2, 4, 8, 16, 24 and 48 h of incubation in the rumen. Then, individual bags with contents were washed in running tap water until the bags were free of rumen matter. Bags were then dried to a constant weight at 60°C for 48 h and weighed. The solubility or washing loss was determined by soaking samples of each material in water at 37 to 40°C for 1 h followed by the washing procedure earlier mentioned. Digestion kinetics of CP was determined according to the equation of Ørskov and McDonald (1979):

$$P = a + b(1 - e^{-ct})$$

Where, *p* is the amount degraded at *a* time; *a,* the rapidly soluble fraction (g/kg); *b*, the potentially degradable fraction (g/kg); *c,* the constant rate of disappearance of *b*; and t, the time of incubation (h).
The effective rumen degradability of CP was estimated using the equation of Ørskov and McDonald (1979):

$$P_e = \frac{a + bc}{K + C}$$

Where, P_e is the effective degradation; k, the fractional ruminal outflow rate; *a, b* and *c* are as defined earlier.
Effective degradability was calculated with an estimated solid outflow rate from the rumen (k) of 0.02, 0.05 and 0.08 h^{-1} (Bhargava and Ørskov, 1987).

Determination of rumen degradability

In the procedure of ruminal incubation, the method of Mehrez and Ørskov (1977) was followed. For this, 5 g of different samples of SBM were weighed in duplicate into nylon bags. Each group included 42 samples (two replicates × seven incubation periods × three cows for each treatment) prepared into individual nylon bags for assay. Bags were incubated in the ventral sac of the rumen of three Iranian Taleshi native cows for 0, 2, 4, 8, 16, 24 and 48 h. Diet was offered at 20 g/kg of body weight daily in two equal portions (08:00 and 16:00 h). Immediately after removal from the rumen, bags were put in ice water to stop microbial fermentation and washed under tap water until the rinsing water became colorless, then dried out and weighed.

SDS-PAGE

Protein sub-units were fractionated by a SDS-PAGE discontinuous system (Laemmli, 1970). All ruminal undegradable fractions from each incubation period were ground and replicate samples were pooled. 20 µl of untreated or treated SBM was placed into 750 µl SDS-PAGE sample buffer. After 30 min of mixing (vortex and inverse), samples were immersed at 90°C for 3 min, and then centrifuged at 10000 ×*g* for 1 min. A 25 µl aliquot of each sample was loaded into the sample well. Electrophoresis of proteins was on 12.5% resolving gel (1.0 × 110 × 140 mm) with 3.75% acrylamide stacking gel. The gels were kept at a constant current of 30 mA until the bromophenol blue marker dye reached the bottom of the gel. Protein fixation and staining were completed simultaneously using a solution of Coomassie brilliant blue. Gel destaining was accomplished by using a 300 ml/l methanol and 70 ml/l acetic acid solution. One standard protein mixture including β-galactosidase (116 kDa), bovine plasma albumin (66.0 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), soybean trypsin inhibitor (21.5 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) was used.

Calculation of the ruminal degradability

Digestion kinetic parameters of DM and CP were determined according to the equation of Ørskov and McDonald (1979) as:

$P = a + b\,(1 - e^{-ct})$

Where: P is CP disappearance (g/kg) at time t (h), a is the soluble fraction (g/kg), b is the potentially degradable fraction (g/kg) and c is the rate of degradation (/h) of b fraction. Effective rumen degradability of DM and CP was estimated using the equation of Ørskov and McDonald (1979) as:

$$ERD = a + \frac{bc}{c + k}$$

Where, ERD is the effective rumen degradability, k is the rumen outflow rate, and *a, b* and *c* are as defined earlier. Effective degradability was calculated with estimated rumen outflow rates of 0.02, 0.05 and 0.08/h (Bhargava and Ørskov, 1987).

Statistical analysis

The degradability parameters for the *in situ* nylon bags and *in vitro* CP digestibility data were analyzed as a completely randomized design by a variance analysis GLM procedure of SAS (1996) according to this model: $Y = \mu + Ti + e_{ij}$. Where µ is the overall

Table 1. The rumen degradation characteristics of crude protein in untreated and treated soybean meal.

Parameter	Degradation characteristic (g/kg)				ERD (g/kg) at outflow rate (/h)		
	a	***b***	***a+b***	***C***	**0.02 h^{-1}**	**0.05 h^{-1}**	**0.08 h^{-1}**
Control	8.55^{a}	88.81^{c}	97.38^{a}	0.082	80.00^{a}	63.77^{a}	53.57^{a}
Xylose (5 gkg^{-1})	2.53^{bc}	90.90^{b}	93.43^{c}	0.089	76.83^{ab}	60.83^{ab}	50.53^{ab}
Xylose (10 gkg^{-1})	3.28^{bc}	90.43^{b}	93.71^{c}	0.085	76.40^{ab}	60.23^{ab}	49.87^{ab}
Neutral black liquor (3%)	2.58^{bc}	90.83^{b}	93.41^{c}	0.093	77.60^{b}	61.93^{ab}	51.70^{ab}
Neutral black liquor (6%)	1.71c	93.86^{a}	95.46^{b}	0.083	77.27^{b}	60.30^{ab}	49.53^{ab}
SEM	0.2631	1.1032	1.1725	0.0016	1.0054	0.8809	0.7850
P value	0.04238	0.0326	0.0126	0.0645	0.0421	0.0722	0.0644

'*a*' The water soluble fraction (g/kg), 'b' the potentially degradable fraction (g/kg), 'c' the rate of degradation (h^{-1}) of 'b' fraction, ERD, effective rumen degradability of DM or CP (g/kg DM or CP) measured at outflow rate k = 0 02, 0.05 and 0.08/h.

Table 2. Molecular weights (kDa) of major subunits of soybean meal proteins.

Subunit	This study	Romagnolo et al. (1990)	Mujoo and Trinh (2003)
β-Conglycinin			
Ά	86.26	93.2	80.0
A	70.59	72.4	75.0
B	43.41	48.4	50.0
Glycinin			
Acidic	30.76	36.3-38.9	34.0
Basic	18.01	20.7	15.0

average, T_i is the treatment effect and e_{ij} is the residual error. When a significant difference was found, means were separated using Tukey test (Steel and Torrie, 1980). Differences were considered to be significant if $P<0.05$.

RESULTS

Ruminal CP degradability

Ruminal CP degradation parameters of untreated and treated black liquor of SBM are shown in Tables 1 and 2. Coefficients *a*, *b* and *a+b* for CP were affected ($P<0.05$) by the 30 min autoclaving processing time and levels of xylose and black liquor. Moisture heating with xylose and black liquor significantly affected *a* and *b* fractions as compared to the control group. Application of xylose 5 and 10 g/kg decreased *a* fraction as compared to the control group and from 8.55%, it significantly reached 2.53 and 3.28%, respectively, but usage of neutral black liquor of 3 and 6% had better effect on the decrease of *a* fraction and significantly reached 2.58 and 1.71%, respectively. Results show that reducing sugar such as xylose with stimulation of Millard reaction could decrease solubility of proteins of soybean meal.

The *b* fraction increased with treatment. The levels of xylose including 5 and 10 g/kg affected *b* fraction content and significantly were increased from 88.81% in the control group to 90.90 and 90.43%, respectively. Also, application of 3% neutral black liquor had the same result with xylose but 6% level of neutral black liquor had good effect on the increase of *b* fraction and significantly reached 93.86%.

Electrophoretic profile of soybean meal protein sub-units

The SDS-PAGE analysis of different treated soybean meal protein is presented in Figure 1. Two major components were observed: β-congylcinin and glycinin. Three components of β-congylcinin α, ά, and δ were separated with estimated molecular weights of 86.26, 70.59 and 43.41 kDa, respectively. Two main polypeptide bands were identified as acidic and basic sub-units of glycinin with estimated molecular weights of 30.76 and 18.01 KDa, respectively. The estimated molecular weights for acidic and basic glycinin sub-units are in agreement with those previously reported (Kella et al.,

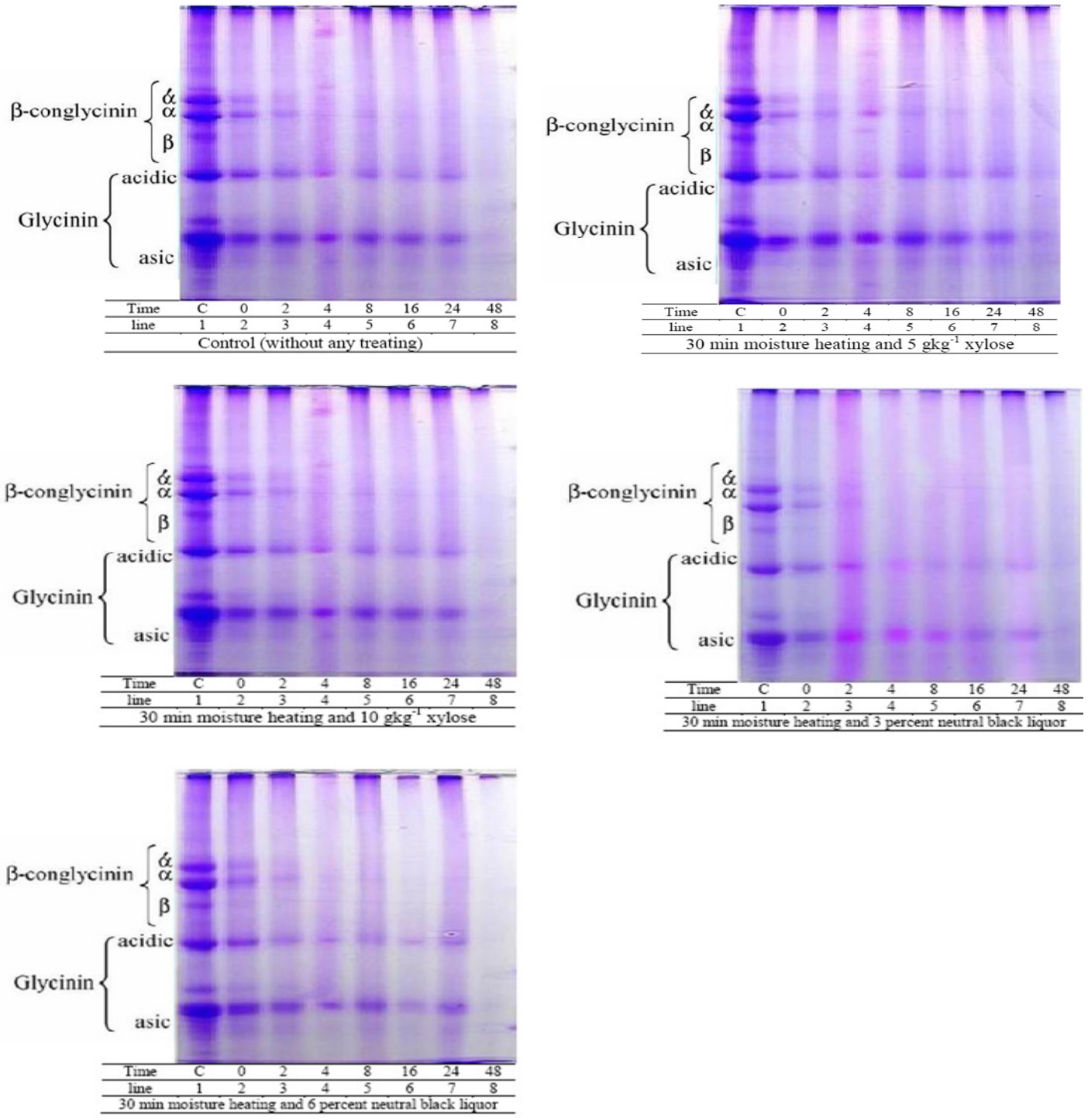

Figure 1. 12% SDS-PAGE slab gel analysis of different treated soybean meal proteins. α, ά`, and β: Sub-units of ά β-congylcinin, acidic and basic sub-units of glycinin.

1989; Van der Aar et al., 1983). β-Congylcinin was more susceptible to rumen degradation than the glycinin sub-units (Figure 1).

The resistance to ruminal degradation of glycinin when compared with β-conglycinin is probably associated with its chemical and physical structure. Its acidic and basic subunits are associated through intermolecular disulfide bridges and most of the S-S links are buried in the interior part of the glycinin molecules (Langan, 1972). In addition, electrostatic and hydrophobic associations are involved in

maintaining the tertiary structure of glycinin.

Molecular weights of SBM protein sub-units

The 12% SDS-PAGE slab gel analysis of SBM proteins is shown in Figure 1. Molecular weights of the principal components of the SBM proteins are shown in Table 2. The two major components observed were β-conglycinin and glycinin. The approximate molecular weights of the β- conglycinin ά, α and β subunits were 86.26, 70.59 and 43.41 kDa and glycinin acidic and basic subunits were 30.76 and 18.01 kDa, respectively.

DISCUSSION

Protein degradation of untreated and treated SBM in the rumen

According to the results, neutral black liquor effectively stimulated Millard reaction and this product when compared with xylose, is very cheap and is economical for increase in soybean meal nutritional value for high producing ruminants. These results are in agreement with other studies using SBM (McAllister et al., 1993; Stanford et al., 1995). Potential of degradability *(a+b)* was related with *a* and *b* contents. Treating SBM decreased *a* fraction and this condition affected *(a+b)* contents, and from 97.38% in control group, it reached 93.43 to 95.46% in experimental treatment. Rate of degradation (*c*) changed from 0.081 to 0.093 fraction h^{-1} but it was not significant. Crude protein in untreated SBM had a degradability curve characterized by an instantaneous soluble fraction “a” of 8.55; fraction “b” of 88.81; fraction “c” of 0.082 (Table 1). These values are approximately in the normal range for SBM (Ørskov, 1992). The predicted effective degradability values of untreated SBM CP at rumen outflow rates of 0.02, 0.05 and 0.08/h were 80.0, 63.77 and 53.57, respectively. The value of soybean meal at rumen outflow rate of 0.05/h is similar to the Ganesh and Grieve (1990) value of 0.656. Romagnolo et al. (1990) obtained a degradability of 0.738, which was equal to the value obtained at a 0.04/h outflow rate in this study. Xylose treatment decreased effective rumen degradability values of CP at three outflow rates. Windschitl and Stern (1988) reported that Lysine is the primary amino acid involved in the Maillard reaction, which is responsible for the decrease in CP degradation after xylose treatment. SBM samples treated with neutral black liquor like xylose could stimulate Millard reaction and protect SBM protein against microorganism degradation and increase the proportion of RUP in SBM considerably (Windschitl and Stern, 1988; Waltz and Stern, 1989). Black liquor is one of wood and paper factory waste and is hazardous to the environment, but when neutralized, this product is converted to good and cheap xylose source for treating protein supplements. Autoclaving decreased the immediate soluble CP fraction and increased the potentially degradable fraction. The degradation rate of the *b* fraction decreased with this treatment. As a consequence, the effective degradability of CP at rumen outflow rate of 0.05/h had difference as compared to untreated SBM. The mechanisms causing the protection of protein against ruminal degradation in heat-treated SBM are complex. However, it is likely that chemical reactions occurring during heat processing are responsible for the reduction in ruminal degradation. To predict the effect of heat treatment, the processing history of the feedstuff, processing temperature and time, moisture content, site and temperature of steam/water added, pressure, energy input, and variety and growing conditions should be known. However, black liquor as source of reducing sugar helps in increasing the rate of formation of Millard reaction.

Conclusion

It is concluded that SBM proteins can be effectively and economically protected from degradation in the rumen by black neutral liquor and moisture heating treatment. SDS-PAGE is useful in studying the fractional protein degradation of soybean meal proteins in the rumen.

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