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Germination and extrusion modify ruminal degradation kinetics and protein subunit profiles of soybean seeds

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Abstract

The present study examined how dry matter (DM) and crude protein (CP) from raw, germinated, and extruded whole soybeans are broken down within the rumen over time. Using the *in situ* nylon bag approach alongside SDS-PAGE, we followed how the protein subunits were altered throughout the experiment. Soybean specimens, whether raw or processed, were placed in nylon pouches and incubated within the rumen of three Holstein steers across timepoints spanning 0, 2, 4, 8, 16, 24, and 48 hours. A non-linear modeling approach was applied to the degradation results to compute effective ruminal degradability (ERD). Notable differences ($P < 0.05$) emerged in DM and CP degradation metrics between raw and extruded soybeans. Extrusion processing lowered the rapidly soluble crude protein fraction and increased the slowly degradable fraction, which led to a statistically significant decline ($P < 0.05$) in the effective degradability of crude protein. Densitometric quantification confirmed that *β*-conglycinin and glycinin represent the two dominant protein classes in soy. According to SDS-PAGE analysis of residual protein, the α and α' subunits of *β*-conglycinin were entirely degraded between 2 and 48 hours of ruminal incubation, while the *β* subunit exhibited greater resistance to breakdown. In raw soybeans, glycinin's acidic and basic polypeptides were digested during mid-incubation phases; in contrast, extrusion allowed these subunits to persist largely unaltered for up to 48 hours within the rumen. In conclusion, the electrophoretic evidence indicated that the ruminally undegradable protein fraction of raw soybeans consisted chiefly of glycinin's basic subunit, while in extruded samples it was comprised mainly of *β*-conglycinin alongside both acidic and basic glycinin subunits.

Introduction

Soybean (Glycine max) serves as a cornerstone feedstuff for lactating dairy cattle, delivering a concentrated supply of energy and protein. On an as-fed basis, it typically contains about 38–40% crude protein (CP) along with roughly 17% fat. This dense nutritional composition makes it a preferred ingredient in total mixed rations aimed at high-yielding herds, where it elevates the overall energy content and supports superior productive output. Since the caloric value of fat surpasses that of starch by a factor of approximately 2.25 [1], using whole, full-fat soybeans permits the design of energy-dense diets without relying heavily on rapidly fermentable carbohydrates. As a beneficial outcome this dietary strategy can help avoid metabolic disturbances like subacute ruminal acidosis and milk fat depression that are often triggered by excessive starch loads.

Notwithstanding its excellent nutrient composition, a significant drawback of soybean protein is its vulnerability to rapid microbial breakdown within the rumen [2]. Such swift degradation can create an asynchrony between the ruminal release of nitrogen and the capacity of microbes to capture it for protein synthesis. The consequence is often an inefficient use of dietary nitrogen and a diminished supply of absorbable amino acids reaching the lower gut. Soybean's full nutritional potential is also hampered by inherent anti-nutritional factors (ANFs), with trypsin inhibitors being among the most significant [3]. When present at elevated levels, these inhibitors impair protein digestion [4], provoke excessive pancreatic enzyme secretion, and can ultimately lead to pancreatic enlargement and depressed growth rates in livestock [5, 6]. A range of processing methods has been devised to boost soybean's feeding value by deactivating ANFs and altering its protein's susceptibility to degradation. Thermal treatment, in particular, has gained widespread adoption in dairy cattle feeding as a practical and swift means of destroying or neutralizing ANFs [7] while improving overall nutrient bioavailability [8]. Extrusion technology subjects whole soybeans to a synergistic combination of moisture, high pressure, elevated temperature, and intense physical shear [9]. Documented benefits of this process include a slower rate of ruminal CP breakdown, a larger bypass protein fraction, and subsequently enhanced delivery of amino acids for intestinal uptake in cows. Beyond its impact on protein, extrusion is also highly effective at suppressing the activity of trypsin inhibitors and lowering both urease and lipase activities [1].

Germination offers another processing avenue capable of profoundly reshaping the nutritional and physiochemical attributes of soybeans. The germination process triggers substantial shifts in the seed's chemical profile, enzymatic activity, and ANF levels [10], while also enhancing palatability through the reduction of unpleasant tastes and aromas alongside a decrease in trypsin inhibitor activity [11]. Significant modifications to the physicochemical nature of proteins also occur during sprouting [12], potentially altering their vulnerability to attack by rumen microbes. Such biochemical and structural transformations point to germination as a possible adjunct to thermal methods for controlling ruminal protein breakdown. While prior research has explored the independent impacts of extrusion or germination on soybean quality, data on their comparative and, more importantly, their interactive effects on degradation kinetics and the fate of specific protein subunits remain sparse. Therefore, the objective of this study was to evaluate how germination and extrusion, both individually and in combination, influence the ruminal degradation of soybean proteins. This was done using the *in situ* nylon bag technique along with SDS-PAGE to monitor changes in protein subunit composition over the course of rumen incubation.

Materials and methods

Approval and accordance statements

The study was approved by the Animal Ethics and Environment Committee of Islamic Azad University of Tehran. All experimental procedures were performed in accordance with the guidelines for using experimental animals. The rumen-cannulated Holstein steers were sourced from a private dairy farm in Qazvin Province, Iran, and written informed consent was obtained from the farm owner for the use of the animals and collection of rumen samples for the purpose of this study.

Soybean seed sourcing and germination procedure

Seeds of soybean (*Glycine max* L.) cv. 'Sahar', a commercial cultivar widely planted in Golestan Province, Iran, were utilized in this study. The raw seeds were supplied by local farmers from the region through personal arrangements, and informed consent was obtained from the suppliers for their exclusive use in the present research.

For germination, 2 kg of Iranian soybeans were soaked in distilled water at a 1:5 (wt/vol) ratio for 10 hours at room temperature. Following the soaking period, the water was removed, and the seeds were uniformly distributed over a clean cloth in a flat, sanitary area. The seeds were then covered with another clean cloth and allowed to germinate for 3 days under dark conditions. To maintain adequate moisture throughout the germination period, the seeds were sprayed with water at 6-h intervals. Upon completion of the second and third days of the germination process, the germinated seeds were collected separately and transferred onto a clean cloth to air-dry overnight prior to further processing.

Extrusion of soybean seeds

The extruder used was a self-wiping model (manufactured by Jinan Saixin Machinery Co., Jinan, China), featuring a barrel with a diameter of 65 mm and a length of 1008 mm; the screw speed was adjustable up to 250 rpm. The moisture content of the soybean samples (20%) was selected based on the results reported in previous studies [13–15]. Extrusion parameters were set at a barrel temperature of 140°C and a screw speed of 150 rpm was applied [13, 16]. Following extrusion, the soybean residues were dried at room temperature and subsequently used as substrates for *in vitro* or *in situ* degradation analyses.

Quantification of in situ rumen degradation kinetics

The *in situ* procedure was performed as described by Mehrez and Ørskov [17]. Five grams of each soybean sample were weighed into nylon bags (9 × 21 cm; 45 μm pore size). Three replicate bags per treatment were incubated in the ventral sac of the rumen of three rumen-cannulated Holstein steers for 0, 2, 4, 8, 16, 24, and 48 h. These steers, which were already cannulated and maintained at a private dairy farm in Qazvin Province, Iran, were directly used for the present experiment with the herd owner's cooperation. The animals were fed a diet consisting of 50% forage and 50% commercial concentrate, which was offered daily in two equal meals at 08:00 and 18:00 h. Upon removal from the rumen, the bags were immediately immersed in ice water to terminate microbial activity, followed by thorough washing under running tap water until the rinse water became clear. The washed bags were then dried and weighed to determine dry matter and crude protein disappearance. To estimate the rapidly degradable and readily available fractions of dry matter and crude protein, three additional bags were soaked in a water bath at 20°C for 30 min, after which they were manually washed, rinsed, dried, and weighed.

Curve peeling and calculation of the ruminal degradability

Ruminal degradation kinetic parameters were estimated using the Marquardt iterative algorithm implemented through the NLIN option of the GLM procedure in SAS software (version 9.4; SAS Institute Inc., Cary, NC), according to the model proposed by Ørskov and McDonald [18]:

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

where P represents dry matter (DM) or crude protein (CP) disappearance (%) at incubation time t (h), a is the soluble fraction (%), b is the potentially degradable fraction (%), e is a constant equal to 2.718 and represents the base of the natural logarithm, and c is the fractional degradation rate (/h) of the b fraction.

Effective ruminal degradability (ERD) of DM and CP was calculated using the equation described by Ørskov and McDonald [18]:

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

where ERD is the effective ruminal degradability (%), *k* is the fractional rumen outflow rate (/h), and *a*, *b*, and *c* are as previously defined. Effective degradability values were calculated assuming rumen outflow rates of 0.02, 0.05, and 0.08 /h, as suggested by Bhargava and Ørskov [19].

Monitoring of protein sub-units

Changes in soybean protein subunit composition during ruminal exposure were tracked via SDS-PAGE, following the protocol established by Laemmli [20]. To extract proteins, dried and finely ground samples (particle size ~ 0.1 mm) containing roughly 1 mg of nitrogen were transferred into microcentrifuge tubes. Subsequently, 750 µL of SDS-PAGE extraction buffer consisting of 0.625 M Tris–hydrochloric acid (pH 6.8), 10% sodium dodecyl sulfate, 2.5% β-mercaptoethanol, 7% glycerol, and 4 mg bromophenol blue was added to each tube. Samples were gently mixed and incubated for 15 min at 4°C, after which protein extraction was completed by heating the samples in a water bath at 95°C for 3 min. The extracts were then centrifuged at 10,000 × g for 1 min, and the resulting supernatants were collected. Aliquots (25 µL) of the extracted proteins from both unincubated controls and rumen-exposed residues at different incubation times were loaded onto polyacrylamide gels. The gel system consisted of a 3.75% stacking layer and a 14% resolving layer. Gel electrophoresis was conducted on a Mini-PROTEAN Tetra Cell apparatus (Bio-Rad) at a steady current of 30 mA. After the dye front reached the bottom gels were carefully removed and submerged in a staining solution (0.625 g Coomassie Brilliant Blue R-250, 7% acetic acid, 20% methanol) for 12 hours. Destaining was accomplished with a solution of 7% acetic acid and 5% methanol over a 12-hour period, followed by several rinses in ultrapure water.

A GS-900™ calibrated densitometer (Bio-Rad) operated by Quantity One software (v. 5.2.1) was used for gel imaging and analysis. Scanning was performed with red light in transmissive mode, employing flat-field correction and optical density calibration, at a pixel density of 63.5 × 63.5 µm. After manual lane assignment, band identification was executed with an automated routine and subsequently refined manually. Background was subtracted using a rolling disk algorithm with a radius of 10. A molecular weight standard (10–170 kDa, CinnaGen) was electrophoresed alongside samples in the first lane of every gel to permit molecular weight estimation of soy protein subunits via semi-logarithmic regression of migration distances. For a semi-quantitative structural assessment, several indices were computed from the densitometric traces. The mean molecular weight (kDa) at each time point was calculated as the weighted average of all visible bands, with relative band intensity serving as the weighting factor. The proportion of structurally intact protein was defined as the sum of intensities from bands ≥ 50 kDa, and proteolytic fragmentation was expressed as 100 minus this value. Additionally, the count of discrete bands in each lane was recorded as a proxy for protein heterogeneity. These derived metrics were used descriptively to interpret the mechanistic basis of protein degradation and were complementary to the *in situ* data; therefore, they were not analyzed by inferential statistics.

Chemical analysis

The dry matter (DM) concentration of soy specimens was quantified by freeze-drying duplicate 1-g subsamples. Proximate analysis, encompassing organic matter, ether extract, crude fiber, and total nitrogen, was carried out following the standard protocols of AOAC [21]. The crude protein value was subsequently derived by multiplying the measured nitrogen content by a factor of 6.25. Fibrous fractions, specifically neutral detergent fiber (NDF) and acid detergent fiber (ADF), were quantified according to the procedure of Van Soest et al. [22] with the aid of an automated fiber analyzer (Fibretec System M, Tecator). The spectrum of amino acids present in the samples was characterized via near-infrared reflectance spectroscopy (NIR; Evonik, Germany). Starch content was assessed by applying a modified version of the protocol supplied with the Megazyme total starch assay kit (Megazyme International Ireland, Wicklow, Ireland). Total sugars were measured in accordance with the phenol–sulfuric acid method outlined by Dubois et al. [23]. With regard to anti-nutritional components, urease activity was estimated based on the official AOCS method [24], phytic acid concentration was determined as described by Wheeler and Ferrel [25], and the level of trypsin inhibitor activity was assessed following the procedure established by Hamerstrand et al. [26].

Statistical analysis

All data were subjected to analysis of variance using the General Linear Model (GLM) procedure within the SAS software package (version 9.4; SAS Institute Inc., Cary, NC). The experimental data were fitted to the following statistical model:

$$Y_{ijk} = \mu + Germ_i + Ex_j + (Germ \times Ex)_{ij} + e_{ijk}$$

where Y_{ijk} represents the observed dependent variable, μ denotes the overall population mean, $Germ_i$ corresponds to the fixed effect of germination duration (i = 0, 2, and 3 days), Ex_j signifies the fixed effect of extrusion treatment (j = 0, 1), $(Germ \times Ex)_{ij}$ accounts for the interaction effect between the two processing factors, and e_{ijk} constitutes the random residual error term. Pairwise comparisons among

treatment means were performed using the PDIFF option within the LSMEANS statement of SAS. Statistical significance was declared at $P < 0.05$.

Results

Chemical composition

The results of proximate composition, CNCPS protein fractions, and amino acid composition of untreated, germinated, and extruded soybean seeds are presented in Table 1, while the anti-nutritional factors are shown separately in Table 2. Germination increased DM and CP contents of soybean seeds, whereas no clear trends were observed for ether extract or crude ash contents. Extrusion processing did not exert a marked effect on DM, CP, ether extract, or ash concentrations of germinated soybean seeds, and the minor variations observed among treatments were considered to be within the analytical error range of the applied methods. Germination for four days modified the proximate composition of soybean seeds on a dry matter basis, resulting in increased DM, CP, and ether extract contents, accompanied by slight reductions in crude fiber and ash.

Table 1
Soybean protein CNCPS fractions and amino acid composition.

	Germination (Germ) time					
	Un-germinated		**For 2 days**		**For 3 days**	
Extrusion (Ex):	Un-extruded	Extruded	Un-extruded	Extruded	Un-extruded	Extruded
Chemical composition						
Dry matter, % as is	91.6	95.3	89.9	92.6	93.0	93.1
Crude protein, % DM	35.28	40.51	36.99	41.32	38.28	41.41
Ether extract, % DM	19.16	11.41	19.84	13.87	20.11	12.83
Crude fiber, % DM	8.90	4.80	7.53	4.13	7.47	4.16
Acid detergent fibre, % DM	9.43	5.99	8.30	6.47	6.99	5.75
Neutral detergent fibre, % DM	13.03	10.35	10.40	10.62	9.99	11.03
Starch, % DM	5.20	6.22	4.16	4.53	3.66	4.58
Sugar, % DM	9.73	9.38	8.57	7.92	8.97	7.00
Crude protein CNCPS fractions, % DM						
A	5.28	4.54	5.34	4.77	5.10	4.56
B1	21.72	23.27	24.97	21.86	24.76	24.66
B2	7.05	10.83	5.81	13.03	7.02	11.34
B3	0.49	1.48	0.56	1.10	0.23	0.31
C	0.74	0.70	0.31	0.51	1.18	0.50
Amino acid composition, % DM						
Methionine	0.49	0.60	0.49	0.54	0.51	0.56
Cysteine	0.56	0.63	0.59	0.62	0.61	0.62
Methionine + Cysteine	1.07	1.24	1.09	1.16	1.14	1.18
Lysine	2.21	2.63	2.34	2.56	2.40	2.61
Threonine	1.36	1.70	1.38	1.59	1.45	1.66
Tryptophan	0.46	0.57	0.49	0.55	0.50	0.56
Arginine	2.45	3.02	2.65	3.04	2.81	3.16
Isoleucine	1.51	1.86	1.53	1.79	1.62	1.88
Leucine	2.61	3.20	2.67	3.05	2.80	3.18
Valine	1.61	1.99	1.64	1.88	1.73	1.97
Histidine	0.94	1.12	0.97	1.05	1.01	1.09
Phenylalanine	1.65	2.06	1.73	2.01	1.82	2.10

Table 2
Anti-nutritional factors of different treated soybean seed.

	Germination (Germ) time									
	Un-germinated		For 2 days		For 3 days			*P*-Value		
Extrusion (Ex):	Un-extruded	Extruded	Un-extruded	Extruded	Un-extruded	Extruded	SEM	Germ	Ex	Germ × Ex
Urease activity, ΔpH	2.12^{a}	0.17^{b}	2.25^{a}	0.12^{c}	2.32^{a}	0.11^{b}	0.071	0.62	< 0.0001	0.21
Trypsin inhibitor activity, mg/g	25.8^{a}	6.96^{c}	22.61^{ab}	4.74^{c}	20.97^{b}	3.77^{c}	1.314	0.03	< 0.0001	0.81
Phytic acid activity, mg/g	28.93^{a}	3.17^{c}	21.67^{b}	2.89^{c}	20.81^{b}	2.31^{c}	0.659	< 0.0001	< 0.0001	0.0002
Phosphorus, mg/kg	4,714^{f}	6,977^{a}	5,587^{e}	5,676^{d}	6,873^{b}	5,895^{c}	3.277	< 0.0001	< 0.0001	< 0.0001
Phytic phosphorus, mg/g	2,807^{f}	4,185^{a}	3,353^{e}	3,407^{d}	4,125^{b}	3,534^{c}	13.60	< 0.0001	< 0.0001	< 0.0001
Gross energy, kcal/kg	5,516^{c}	5,136^{f}	5,567^{b}	5,278^{d}	5,589^{a}	5,231^{e}	4.719	< 0.0001	< 0.0001	< 0.0001
Apparent metabolizable energy, kcal/kg	3,370^{b}	3,034^{e}	3,365^{c}	3,057^{d}	3,464^{a}	3,034^{e}	0.854	< 0.0001	< 0.0001	< 0.0001

CNCPS fractions and amino acid composition

As shown in Table 1, the rapidly soluble protein fraction (*A*) decreased following extrusion, whereas germination led to a higher proportion of this fraction. Table 1 also presents the amino acid profiles of raw, germinated, and extruded soybeans. Germination, both alone and in combination with extrusion processing, increased the concentration of amino acids compared with the control treatment.

Anti-nutritional factors

Table 2 displays the anti-nutritional factors of untreated, germinated, and extruded soybean seeds. In the present study, urease activity and trypsin inhibitor activity were significantly reduced ($P < 0.05$) by extrusion and germination processing. Both urease and trypsin inhibitor activities were markedly deactivated by extrusion processing. In contrast, urease activity was not significantly affected by germination, whereas trypsin inhibitor activity increased with increasing germination time. Phytic phosphorus content of soybean seeds was significantly affected by both extrusion and germination processing.

In situ ruminal degradability of DM and CP of untreated and treated soybean

Ruminal DM and CP degradation parameters of untreated, germinated, and extruded soybean seeds are presented in Table 3. Significant differences ($P < 0.05$) were observed in the degradation characteristics of both DM and CP among untreated and processed soybean treatments. Untreated soybeans exhibited the highest rapidly degradable DM fraction (*a*; 45.79%), whereas the lowest value was observed in soybeans germinated for 3 days (41.01%). Germination alone did not significantly affect the rapidly degradable DM fraction in the absence of heat treatment; however, extrusion significantly reduced fraction *a* in germinated soybean seeds ($P < 0.05$), indicating a differential response of this fraction to germination and extrusion processes. For crude protein, the highest water-soluble fraction (*a*) was observed in soybeans germinated for 3 days without extrusion (42.49%), while the lowest value was recorded in extruded soybeans (9.56%). Extrusion markedly decreased ($P < 0.05$) the water-soluble CP fraction (*a*) and numerically increased the potentially degradable fraction (*b*). In contrast, germination tended to increase protein solubility. The degradation rate (*c*) of the potentially degradable fraction for both DM and CP was not significantly affected by germination or extrusion treatments. Effective rumen degradability (ERD) of DM and CP at different ruminal outflow rates was not influenced by germination, whereas extrusion significantly reduced ERD values.

Table 3
Ruminal degradation characteristics of dry matter and crude protein of untreated and treated soybean.

	Germination (Germ) time									
	Un-germinated		**For 2 days**		**For 3 days**			***P*-Value**		
Extrusion (Ex):	Un-extruded	Extruded	Un-extruded	Extruded	Un-extruded	Extruded	SEM	Germ	Ex	Germ × Ex
In situ rumen degradation of dry matter										
a, %	45.79^{a}	41.68^{b}	39.80^{b}	41.10^{b}	44.29^{a}	41.01^{b}	0.647	< 0.001	< 0.01	< 0.01
b, %	50.78^{ab}	39.71^{bcd}	57.83^{a}	37.01^{cd}	45.73^{abc}	33.00^{d}	4.109	0.170	< 0.001	0.47
a + *b*, %	96.58^{a}	81.40^{bc}	97.63^{a}	78.12^{bc}	90.03^{ab}	74.02^{c}	4.082	0.23	< 0.001	0.85
c, h^{-1}	0.0666	0.0700	0.0766	0.0800	0.0733	0.0766	0.0091	0.55	0.88	0.92
Effective rumen degradation (%) of dry matter at different rumen outflow rate (h^{-1})										
0.02 h^{-1}	84.79^{a}	70.89^{b}	85.52^{a}	70.89^{b}	79.96^{a}	70.35^{b}	2.314	0.11	< 0.0001	0.92
0.05 h^{-1}	74.73^{a}	62.93^{b}	74.62^{a}	62.93^{b}	71.18^{a}	63.36^{b}	1.471	0.07	< 0.0001	0.96
0.08 h^{-1}	68.80^{a}	58.49^{b}	67.93^{a}	58.49^{b}	65.90^{a}	59.08^{b}	1.154	0.08	< 0.0001	0.82
In situ rumen degradation of crude protein										
a, %	41.53^{a}	9.56^{d}	40.96^{a}	9.56^{d}	42.49^{a}	24.00^{b}	0.685	< 0.0001	< 0.0001	< 0.0001
b, %	54.59	58.74	55.24	58.74	56.67	43.52	6.855	0.49	0.21	0.37
a + *b*, %	96.19^{a}	68.37^{b}	96.28^{a}	68.37^{b}	99.24^{a}	67.62^{b}	7.128	0.96	< 0.001	0.76
c, h^{-1}	0.0736	0.0665	0.0746	0.0665	0.0755	0.0897	0.0115	0.58	0.68	0.64
Effective rumen degradation (%) of crude protein at different rumen outflow rate (h^{-1}).										
0.02 h^{-1}	84.43^{a}	50.47^{b}	84.45^{a}	50.47^{b}	87.02^{a}	59.34^{b}	3.634	0.49	< 0.0001	0.39
0.05 h^{-1}	74.00^{a}	38.70^{c}	73.96^{a}	38.70^{c}	76.23^{a}	51.59^{b}	2.204	0.04	< 0.0001	0.03
0.08 h^{-1}	67.65^{a}	32.45^{d}	67.55^{a}	32.45^{d}	69.68^{a}	46.64^{b}	1.675	< 0.01	< 0.0001	< 0.01
Values within a row with different superscripts differ significantly at $P \leq 0.05$.										

SDS-PAGE analysis of soybeans proteins

Figures 1 and 2, along with densitometric evaluation in Table 4, provide the molecular weight distribution and proteolysis-related indices of soybean proteins. Across all treatments, *β*-conglycinin and glycinin were identified as the predominant soybean storage proteins, jointly accounting for approximately 70% of total soybean protein. At the start of ruminal incubation (time zero), extruded soybeans, whether previously germinated or not, contained a much larger share of intact proteins (≥ 50 kDa) and a higher average molecular weight compared to non-extruded samples (Table 4). In contrast, germination alone increased protein solubility and accelerated structural breakdown, as evidenced by higher CNCPS fractions A and B1 (Table 2), reduced mean molecular weight, and increased proteolysis indices during early ruminal incubation (Table 4). After 8 h of ruminal incubation, un-extruded treatments—particularly germinated soybeans—exhibited extensive structural proteolysis, with more than 96% of protein mass present as fragments below 50 kDa (Table 4). Extrusion markedly altered this degradation trajectory, even when applied after germination. Germinated–extruded soybeans retained substantially higher proportions of structurally intact protein and higher mean molecular weights at both 8 and 16 h of incubation (Table 4). At 16 and 24 h of

ruminal incubation, un-extruded germinated soybeans showed near-complete structural proteolysis, whereas extruded and germinated–extruded treatments maintained higher molecular weights, a greater number of detectable electrophoretic bands, and lower proteolysis indices (Table 4). The combined application of germination and extrusion resulted in intermediate structural and degradability characteristics.

Table 4
Molecular weight distribution and proteolysis-related indices of soybean seed protein fractions after different ruminal incubation times.

	Germination time					
	Un-germinated		**For 2 days**		**For 3 days**	
Extrusion:	Un-extruded	Extruded	Un-extruded	Extruded	Un-extruded	Extruded
0 h ruminal incubation						
Mean molecular weight, kDa	39.7	71.9	35.8	70.8	38.6	80.9
Structurally intact protein fraction (≥ 50 kDa), %	21.6	37.7	14.7	35.7	28.4	40.1
Extent of structural proteolysis, %	78.4	62.3	85.3	64.3	71.6	59.9
Number of detectable electrophoretic bands	8	8	5	8	6	8
8 h ruminal incubation						
Mean molecular weight, kDa	34.4	65.7	29.4	55.1	50.2	50.2
Structurally intact protein fraction (≥ 50 kDa), %	3.6	30	3.1	19.5	62.8	62.8
Extent of structural proteolysis, %	96.4	70	96.9	80.5	37.2	37.2
Number of detectable electrophoretic bands	7	9	4	9	5	5
16 h ruminal incubation						
Mean molecular weight, kDa	32	110.7	31.1	73.2	30.8	75.7
Structurally intact protein fraction (≥ 50 kDa), %	6.1	58.3	5.1	34	7.9	38.6
Extent of structural proteolysis, %	93.9	41.7	94.9	66	92.1	61.4
Number of detectable electrophoretic bands	3	10	3	7	4	9
24 h ruminal incubation						
Mean molecular weight, kDa	34.1	93.6	65.5	94	63.7	64.6
Structurally intact protein fraction, ≥ 50 kDa, %	4	47.2	100	47.2	100	32.4
Extent of structural proteolysis, %	96	52.8	0	52.8	0	67.6
Number of detectable electrophoretic bands	3	9	2	7	1	8
48 h ruminal incubation						
Mean molecular weight, kDa	32.2	129.8	–	68.8	–	67.9
Structurally intact protein fraction (≥ 50 kDa), %	19.1	69.3	–	3.3	–	28.1
Extent of structural proteolysis, %	80.9	30.7	–	96.7	–	71.9
Number of detectable electrophoretic bands	2	5	–	1	–	3

All values are descriptive, semi-quantitative structural indices derived from densitometric analysis of representative SDS-PAGE profiles; therefore, statistical analysis was not performed, as these electrophoretic data are intended for mechanistic interpretation of protein structural changes rather than inferential comparison among treatments.

	Germination time		
	Un-germinated	**For 2 days**	**For 3 days**

Mean molecular weight (kDa) was calculated as the weighted average molecular weight of all detected protein bands based on their relative densitometric intensity.

Structurally intact protein fraction (≥ 50 kDa, %) was calculated as the cumulative relative intensity of protein bands with molecular weights ≥ 50 kDa.

Extent of structural proteolysis (%) was calculated as 100 minus the proportion of structurally intact protein (≥ 50 kDa).

Number of detectable electrophoretic bands represents the total number of protein bands identified per lane after background subtraction and manual verification.

Discussion

Chemical composition

Increases in seed crude protein concentration and changes in amino acid composition as a consequence of germination have also been reported previously [27, 28]. Mostafa et al. [28] further documented changes in oil content during germination, reporting decreases of 5.37% and 7.69%, respectively. These reductions were likely attributable to the utilization of lipids as an energy source and/or their involvement in the synthesis of structural constituents during early seedling development [29]. The DM concentration of soybean seeds increased following extrusion processing and with increasing duration of germination. These findings are consistent with those of Fathi-Nasri et al. [30] and Wei et al. [31], who reported increased dry matter content in extruded soybean samples, primarily due to enhanced moisture evaporation during thermal processing. Crude protein concentration increased as a result of both extrusion and germination treatments. Extruded soybeans exhibited the highest crude protein content, whereas untreated soybeans showed the lowest values. Similarly, NRC [32] reported approximately a 10% increase in protein concentration in heated soybeans compared with raw soybeans. Chen et al. [33] attributed this increase to reduced moisture content and a concomitant increase in nitrogen concentration following heat treatment.

In contrast, the effects of thermal processing on ether extract content have been reported inconsistently in the literature. Increased ether extract concentrations following heat treatment were observed by Eweedah et al. [34] and White et al. [35], whereas Yalcin and Basman [36] reported a decrease. Prachayawarakorn et al. [37] found that heat processing had little effect on ether extract concentration, and similarly, NRC [32] reported no marked differences between raw and extruded soybeans. Khorasani et al. [38] suggested that changes in the chemical structure of seeds during heating and their effects on fat and fatty acid extractability may explain the reduction in ether extract content observed following extrusion processing.

CNCPS fractions and amino acid composition

The reduction in fraction *A* following heat processing is consistent with previous reports indicating that heat processing reduces non-protein nitrogen (NPN) content in soybeans [30, 39]. The non-protein nitrogen (NPN) pool represents the portion of crude protein that readily dissolves and is conventionally regarded as being swiftly and thoroughly broken down within the rumen environment. From a chemical standpoint, the CNCPS *A* fraction encompasses those nitrogen-containing substances that dissolve in a borate–phosphate buffer yet remain in solution upon treatment with trichloroacetic acid; this category includes free amino acids, oligopeptides, nitrate ions, and ammonia [40]. The measured proportion of this fraction depends on the protein-precipitating agent used, as trichloroacetic acid precipitates peptides of approximately 10 amino acids, whereas tungstic acid precipitates peptides of about three amino acids [41]. Owing to its marked solubility, the A fraction undergoes rapid ruminal breakdown and serves as an immediate precursor to the rumen ammonia reservoir [42]. In soybeans, the bulk of the NPN fraction is composed of free amino acids and short peptides [42]. Consequently, the decline in fraction A content observed after extrusion can be explained by thermal modifications to peptide conformation, which lower solubility and promote precipitation of these nitrogenous constituents. It is plausible that comparable processes also underlie the shifts in NPN levels noted during germination.

Characterization of crude protein fractions was conducted according to the CNCPS [42, 43], in which fraction A represents NPN, fraction B represents true protein, and fraction C corresponds to unavailable protein. Fraction B is further subdivided into B1, B2, and B3 based on differences in solubility and ruminal degradation rates. Fractions A and B1 are soluble in phosphate–borate buffer and are rapidly degraded in the rumen. Fraction B2 is insoluble in buffer but soluble in neutral detergent, whereas fraction B3 is insoluble in both buffer and neutral detergent but soluble in acid detergent. Fraction B3 is associated with the cell wall and is degraded more slowly in the rumen than fractions

B1 and B2, resulting in a greater proportion escaping ruminal degradation. Fraction C, defined as acid detergent insoluble nitrogen (ADIN), is highly resistant to microbial and enzymatic degradation and is considered nutritionally unavailable.

Extrusion processing increased the proportion of buffer-soluble protein in soybeans, whereas germination for two days resulted in a decrease in this fraction. A reduction in fraction B1 accompanied by an increase in fraction B2 is typically associated with decreased rumen-degradable protein (RDP) and increased rumen-undegradable protein (RUP), thereby improving dietary nitrogen utilization efficiency [32, 44]. The observed changes in protein fraction distribution in the present study are in agreement with the findings of López-Barrios et al. [45], Osman et al. [46], and González-Montoya et al. [47].

The increased crude protein content of soybeans following germination has been attributed to losses of soluble carbohydrates and seed coat material during germination, as well as partial synthesis of new proteins [48–50]. The amino acid profiles of raw, germinated, and extruded soybeans are also presented in Table 1. Germination, both alone and in combination with extrusion processing, increased the concentration of amino acids compared with the control treatment. These results are consistent with previous studies evaluating the effects of extrusion and germination on soybean seeds [14, 15, 51]. It has been reported that extrusion processing [52] and germination [49] improve the profile and digestibility of essential amino acids in soybean protein. These improvements are largely attributed to the reduction of anti-nutritional compounds and enhanced accessibility of protein to ruminal microorganisms [42]. Consequently, increased protein hydrolysis and release of amino acids may explain the higher amino acid concentrations observed following processing.

Anti-nutritional factors

The reduction of urease and trypsin inhibitor activities by extrusion agrees with numerous studies [31, 33–36, 52]. Urease activity is commonly used as an indirect indicator of the effectiveness of heat processing in inactivating trypsin inhibitors. The acceptable range for urease activity is 0.2–0.5 mg, with values exceeding 0.5 mg indicating insufficient heat treatment and the presence of active trypsin inhibitors [53]. The results of the present study demonstrated that both urease and trypsin inhibitor activities were markedly deactivated by extrusion processing, which may enhance intestinal protein digestibility and increase amino acid availability in the small intestine [54]. Previous studies have shown that ruminal fermentation alone cannot eliminate the negative effects of trypsin inhibitors on amino acid digestibility of whole soybeans in the small intestine, whereas thermal processing is effective in this regard [52, 55]. Similarly, Ruiz et al. [13] reported that extrusion of soybeans at 140°C reduced trypsin inhibitor activity from 50.80 to 4.70 and urease activity from 1.99 to 0.03. Extrusion at temperatures ranging from 135 to 145°C has also been shown to reduce soybean urease activity and improve animal performance [16].

In contrast to several previous studies [56–58], urease activity was not significantly affected by germination in the present study, whereas trypsin inhibitor activity increased with increasing germination time. This observation is consistent with the findings of Victoria et al. [59] and Tan-Wilson et al. [60]. During legume seed germination, hydrolytic enzymes are activated to degrade starch, storage proteins, and certain anti-nutritional factors. Degradation of storage proteins is essential for generating peptides and amino acids required for seed development and early plant growth [56–61]. Protein-related anti-nutritional factors such as phytic acid, urease, and trypsin inhibitors serve as protective compounds against predation in legume seeds and are therefore subject to enzymatic modification during germination [62–63].

Phytic phosphorus content of soybean seeds was significantly affected by both extrusion and germination processing. In agreement with previous reports [14, 15, 51], extrusion processing decreased phytic acid content. However, the extent of phytic acid reduction during extrusion is influenced by processing conditions such as residence time, moisture content, temperature, and feed rate [64]. It has been reported that extrusion processing at approximately 20% moisture content and 120°C is particularly effective in reducing phytic acid levels in leguminous seeds [14, 15, 51]. Phytic acid and other anti-nutritional factors are known to form complexes with proteins, thereby reducing protein digestibility and mineral bioavailability [65]. During extrusion and germination, protein structures undergo unfolding and structural rearrangement, becoming more porous and fibrous [66]. The disruption of protein–phytate complexes and the release of phosphate groups and associated minerals during these processes likely contribute to the observed reduction in phytic acid content following germination.

In situ ruminal degradability of DM and CP of untreated and treated soybean

The current results support earlier observations that heat treatment reduces the extent of dry matter and crude protein breakdown in the rumen [30, 67–72]. Nowak et al. [68] demonstrated that thermal processing of soybeans at 150°C for 60 min reduced the rapidly degradable fraction (*a*), degradation rate (*c*) of the slowly degradable fraction, and ERD of DM and CP, while increasing the slowly degradable fraction (b). Similarly, Chrenková et al. [40] reported that extrusion of soybean seeds did not significantly alter the rapidly or slowly degradable fractions of CP, but reduced the degradation rate of fraction b and decreased ERD of DM and CP. Khorasani et al. [38] also observed that heating soybeans at 125°C for 15 min decreased CP fraction *a*, degradation rate *c*, and ERD of DM and CP at ruminal passage rates of 2, 5, and 8%/h, while increasing fraction *b*.

The reduction in ruminal degradability of DM and CP following extrusion can be attributed to protein denaturation and the occurrence of Maillard reactions, which involve non-enzymatic interactions between the aldehyde groups of sugars and free amino groups of proteins [69]. These reactions lead to the formation of amino acid–sugar complexes that are more resistant to microbial enzymatic hydrolysis compared with native peptides [73]. Consequently, the degradation rate of fraction *b* and the ERD of DM and CP were reduced in extruded soybeans. Fraction *a* consists mainly of non-protein nitrogen (NPN) and a small proportion of true protein, which rapidly disappears from nylon bags due to high solubility or small particle size and is assumed to be rapidly degraded in the rumen. Fraction *b* represents water-insoluble but potentially degradable protein retained within the nylon bags, whereas fraction *c* is completely undegradable. The degradation of fraction *b* depends on ruminal retention time; theoretically, this fraction would be completely degraded if retained indefinitely in the rumen. It is generally assumed that all of fraction *a* is degraded in the rumen, while fraction c escapes ruminal degradation and is transferred to the small intestine [74]. In extruded soybeans, the decline in the immediately soluble fraction (*a*) and the slower degradation of the slowly degradable fraction (*b*) are likely caused by heat-induced protein insolubilization and obstruction of the sites targeted by rumen microbial enzymes [40]. Protein solubility in ruminal fluid is closely related to its rate of degradation [32]. Because effective rumen degradability of CP is directly influenced by the degradation rate of fraction b, the reduction in ERD observed in extruded soybeans reflects the lower degradability of this fraction. These results suggest that in high-producing dairy cows, where ruminal passage rates can approach 8%/h, heat processing such as extrusion may effectively reduce ruminal degradation of soybean protein and increase the flow of ruminally undegraded protein to the small intestine. Provided that excessive heat damage does not impair intestinal digestibility, this shift may enhance amino acid supply for absorption and utilization by the animal [75].

SDS-PAGE analysis of soybeans proteins

These electrophoretic observations are strongly associated with alterations in CNCPS protein fractions and amino acid composition (Table 2), as well as with changes in *in situ* ruminal degradation kinetics of CP (Table 3). The different rates at which *β*-conglycinin and glycinin are broken down largely dictate the overall extent of ruminal protein degradation. Minor discrepancies in molecular weight values compared with other studies [76–77] likely reflect inherent heterogeneity of soybean proteins, varietal differences, and methodological factors [46, 77], and do not alter the functional interpretation. The structural preservation in extruded treatments provides a mechanistic explanation for the marked reduction in CNCPS soluble protein fraction *A* and the increase in insoluble fractions B2 and B3 (Table 2). Because fractions A and B1 represent rapidly degradable protein pools, their reduction following extrusion is consistent with the lower rapidly degradable CP fraction (a) and reduced ERD of CP (Table 3), confirming that thermal processing alters protein solubility and accessibility to ruminal proteolytic enzymes. The rapid disappearance of *β*-conglycinin subunits in germinated soybeans (α' and α subunits degraded within 2–4 h, β subunit shortly thereafter; Fig. 2b and 2c) indicates partial mobilization of storage proteins during germination, which weakens the protein matrix and enhances susceptibility to ruminal microbial enzymes. Consequently, germinated un-extruded soybeans exhibited higher soluble CP fractions (a), faster degradation rates, and greater ERD of CP (Table 3). The rapid disappearance of high-molecular-weight subunits directly corresponds to the high degradation rate and elevated ERD of CP (Table 3). This demonstrates a close linkage between molecular fragmentation and in situ degradability. Despite this rapid breakdown, glycinin subunits persisted longer than *β*-conglycinin subunits, supporting the concept that glycinin constitutes the primary rumen-undegradable protein fraction in raw and germinated soybeans. The persistence of fractions B2 and B3 in extruded treatments (Table 2) explains the reduced degradation rate of fraction b and lower ERD of CP (Table 3), indicating that extrusion counteracts germination-induced protein degradability through structural stabilization and aggregation. The prolonged persistence of glycinin acidic and basic subunits in extruded samples (Fig. 2d–f) highlights the role of this protein fraction in limiting ruminal degradation following heat processing. The relative resistance of glycinin to ruminal degradation has been attributed to disulfide bonding between acidic and basic subunits [78], increased surface hydrophobicity [79], and reduced accessibility to proteolytic activity of Prevotella ruminicola [80]. In addition, the formation of protein aggregates and crosslinkages during extrusion, via Maillard reactions or iso-peptide bonds, further restricts enzymatic access to peptide bonds [81–83]. The associated increase in surface hydrophobicity following thermal processing further reduces susceptibility to microbial proteases [63]. The complementary interaction between germination (enhanced protein mobilization and increased essential amino acid availability) and subsequent extrusion (partial structural stabilization, limited excessive ruminal proteolysis) explains why germinated–extruded soybeans exhibited lower ERD of CP than germinated-only treatments, but higher ERD than extruded-only soybeans (Table 3), while retaining improved amino acid profiles. From a broader nutritional perspective, these results emphasize that ruminal protein degradability of soybean is governed not only by chemical composition but also by the supramolecular organization and physicochemical properties of its storage proteins. By strategically combining germination and extrusion, it is possible to manipulate protein structure to balance enhanced amino acid availability with controlled ruminal degradation. Such an approach may be particularly relevant for high-producing dairy cows, in which elevated ruminal passage rates can exacerbate nitrogen losses when rapidly degradable protein sources are fed. While the in situ degradation profiles and protein structural analyses reported here offer compelling mechanistic justification for this processing approach, definitive conclusions regarding its practical efficacy await validation through live-animal trials assessing key production parameters, dietary nitrogen capture efficiency, and intestinal amino acid bioavailability.

Conclusions

The primary objective of protein nutrition in dairy cows is to provide sufficient rumen-degradable protein to support microbial activity while ensuring an adequate supply of rumen undegradable protein to optimize amino acid availability and animal performance. The results of the present study demonstrate that germination and extrusion processing, applied either individually or in combination, effectively modify ruminal protein degradation characteristics of soybean seeds through both compositional and structural mechanisms.

The main effect of germination was to increase protein solubility and mobilization, which was evident from larger soluble protein pools and faster breakdown during the initial hours of ruminal incubation. This process improved amino acid availability and promoted rapid degradation of β-conglycinin subunits; however, it exerted only a moderate protective effect against excessive ruminal proteolysis. In contrast, extrusion processing markedly reduced the ruminal degradability of crude protein and dry matter by decreasing water-soluble protein fractions and inducing structural stabilization of soybean proteins. These effects resulted in a significant reduction in effective ruminal degradability of crude protein and increased the proportion of rumen-undegradable protein. These improvements were also reflected in favorable shifts in CNCPS nitrogen fractions, indicating enhanced partitioning of soybean protein toward nutritionally more efficient rumen-degradable and rumen-undegradable pools.

Electrophoretic and densitometric analyses further revealed that changes in ruminal protein degradability were closely associated with differential degradation behavior of soybean storage proteins. β-Conglycinin subunits were rapidly degraded in the rumen, whereas glycinin, particularly its basic subunit, constituted the major fraction of rumen-undegradable protein. Whether applied by itself or after germination, extrusion helped glycinin subunits survive longer in the rumen and limited the overdigestion of *β*-conglycinin, which increased the amount of protein reaching the small intestine. These findings indicate that modification of protein supramolecular structure, rather than changes in crude protein concentration alone, is a key determinant of ruminal protein utilization in processed soybeans.

The combined application of germination and extrusion resulted in intermediate yet nutritionally favorable characteristics, integrating improved amino acid profiles with controlled ruminal protein degradation. Overall, the present study demonstrates that extrusion, particularly when applied after germination, represents an effective processing strategy to improve the nutritional value of soybean seeds by reducing anti-nutritional factors, enhancing amino acid availability, and optimizing the balance between rumen-degradable and rumen-undegradable protein fractions. These structural and kinetic modifications are especially relevant for high-producing dairy cows, in which elevated ruminal passage rates increase the need for efficient nitrogen utilization.

Declarations

Author Contribution Statement

Amir Reza Habibi contributed to the investigation, methodology, and writing of the original draft. Mohammad Chamani and Farhad Foroudi were responsible for the conceptualization, formal analysis, supervision, and writing, review, and editing. Ali Asghar Sadeghi contributed to the formal analysis (statistical analysis) and writing, review, and editing.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request, but restrictions apply to the public availability of these data. The data are not publicly available because they were produced as part of an institutional research project with restricted dissemination policies. However, if access to the raw data is required during the review process for editorial or peer review purposes, the corresponding author may, upon obtaining formal permission from the Department of Animal Science, Islamic Azad University of Tehran, provide the data to the journal in the form of a formal letter or restricted access request.

Conflicts of Interest

The authors declare no conflict of interest.

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Figures

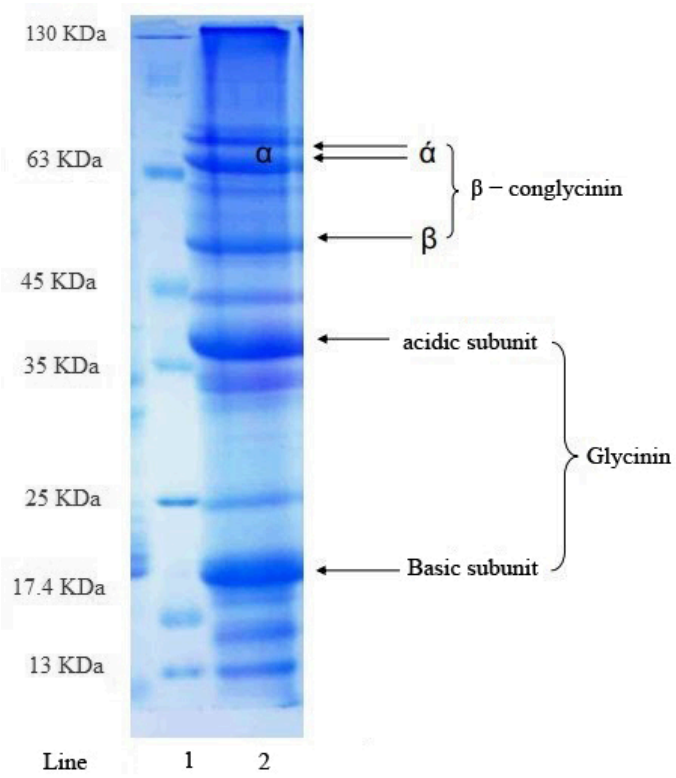


Figure 1

Molecular weights of standard protein (line 1) and untreated soybean protein subunits (line 2).

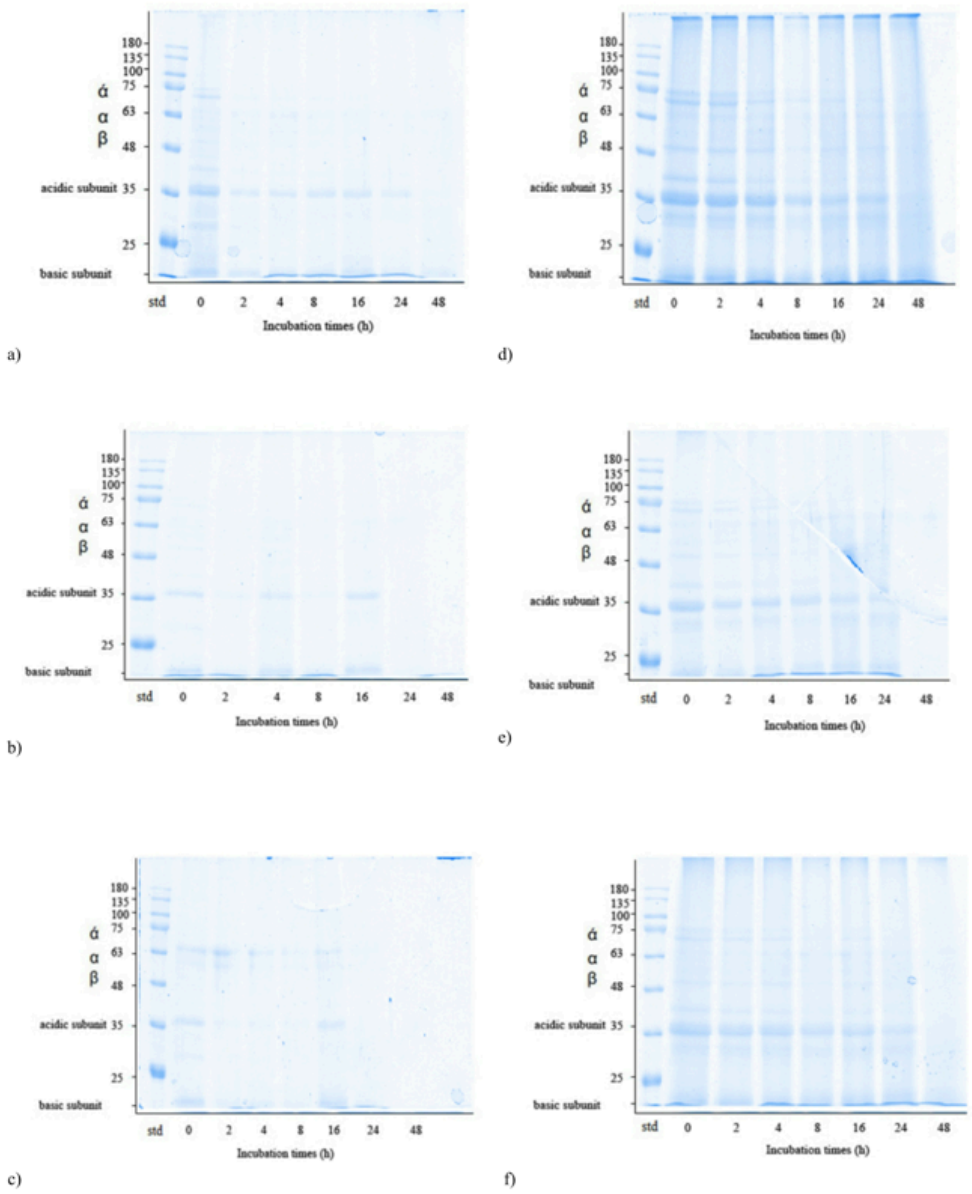


Figure 2

Electrophoretic patterns of soybean seed protein incubated in the rumen: (a) untreated; (b) germinated for 2 days; (c) germinated for 3 days; (d) extruded; (e) germinated for 2 days followed by extrusion; and (f) germinated for 3 days followed by extrusion.