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Quantification of rumen undegradable protein fractions of conventional and non conventional protein supplements by SDS-PAGE

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ABSTRACT

Conventional [solvent extracted GNC and mechanically extracted mustard cake (MC)], and non-conventional protein supplements [machanically extracted castor oilseed cake (COSC), un-decorticated solvent extracted neem seed cake (NSC) and corn gluten meal (CGM)] before and after rumen incubations for different intervals were subjected to sequential fractionation into globulin, albumin, prolamin and glutelins on the basis of solubility. The polypeptides in globulin, prolamin and glutelin fraction of groundnut cake with molecular weight 35, 15, 16 and 19 KDA, respectively, were highly resistant to microbial degradation even after 24h or rumen incubation. The degradation pattern of mustard cake revealed that subunits of globulin, prolamin and glutelins with MW of 14–33, 14 and 31 KDA, respectively, were highly resistant to microbial degradation. In NSC subunits with MW 124 (in globulin), 30 (in albumin), 66 (in prolamin) and 54 kDa (in glutelin) were resistant to microbial degradation. The subunits in prolamin fraction of CGM with MW 18, 24 and 43 kDa and that in glutelin fraction with MW of 22 and 60 kDa were found to be highly resistant to microbial degradation. The results conclusively revealed that in majority of the cases, the low molecular weight polypeptides in the tested protein supplements showed resistance to rumen microbial fermentation, even after 48h of rumen incubation.

Key words: Conventional, *In-sacco* rumen degradability, Non-conventional, Protein fractions, Protein supplements, SDS-PAGE

The relative proportion of rumen degradable (RDP) and undegradable (RUDP) protein plays an important role in productive and reproductive performance of high yielding dairy cattle. RUDP content of groundnut cake, mustard cake and sunflower cake varies between 23 and 26% of CP, while that of cottonseed cake (CSC), corn gluten meal (CGM) between 46 and 78%. The higher concentration of RUDP in the CSC and CGM is mainly because of higher prolamins and glutelins (Wadhwa *et al.*1993). The groundnut and mustard cake had significantly higher intestinal digestion as compared to rumen un-degradable fraction of these supplements. However, no such differences were observed between ruminally exposed or unexposed samples of neem seed cake and corn gluten meal, indicating higher potential of these non-conventional protein supplements as rumen bypass proteins (Wadhwa *et al.*2007). Irrespective of protein supplements, the degradability of globulin was highest, followed by albumin, glutelin and prolamin (Wadhwa *et al.*2010). However, little information is available on the quantitative degradability/resistance of peptide/polypeptide fractions of such proteins in the rumen. Therefore, the present study was conducted to assess the quantitative degradation of these protein fractions, before and after microbial exposure in the rumen by SDS-PAGE and densitometeric analysis.

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MATERIALS AND METHODS

Protein supplements: Conventional [solvent extracted groundnut cake (GNC) and mechanically extracted mustard cake (MC)] and non-conventional protein supplements [mechanically extracted castor oil seed cake (COSC), un-decorticated solvent extracted neem seed cake (NSC) and corn gluten meal (CGM)] were ground in a Willey mill and passed through screen of 1 mm pore size.

Rumen incubations: Three rumen fistulated male buffalo calves (332±17 kg body weight) were used for evaluation of protein supplements. Each animal was offered 2 kg concentrate mixture (wheat 15, maize 15, solvent extracted groundnut cake 10, de-oiled mustard cake 20, rice bran 10, solvent extracted rice bran 27, mineral mixture 2 and salt 1%) supplemented with 2 kg available green fodder and 5 kg wheat straw, so as to meet the nutrient requirements (NRC 2001). Nylon bag (8 cm × 17cm with pore size 50±10µm) technique (Mehrez and Orskov 1977) was used to assess the rumen degradability of protein supplements. The bags

containing 5 g of the test sample in triplicate were incubated in the rumen of buffalo calves. One bag was taken out at 2, 4, 6, 8, 12, 24, 36 and 48 h after incubation from the rumen of each buffalo calf. All the bags were washed under running tap water, till the outgoing fluid became colourless. The bags containing sample as such were washed in running tap water to assess the loss at 0 h. The bags were squeezed gently and dried in a forced air oven at 55°C for 48 h. The residue was used for protein fractionation.

Protein fractionation: The protein supplements and the residue obtained after *in-sacco* degradation were fractionated into 4 protein fractions based on the solubility (Monteiro *et al.*1982). 0.5 g defatted samples in duplicate were extracted thrice with 5 ml of 0.5M sodium chloride for 60, 30 and 30 min each. The samples were centrifuged for 15 min at 5000g after each extraction. The supernatant obtained was pooled and labeled as salt soluble fraction (globulin). The residue obtained after extraction was washed thrice with 5 ml distilled water for 15 min each and centrifuged for 15 min at 5000g after each washing. The supernatant of all the 3 washings was pooled and labeled as water soluble fraction (albumin). The residue was stirred with 5 ml of 70% isopropanol containing 0.6% mercaptoethanol, thrice for 30 min each and centrifuged at 5000g for 15 min. The supernatant was pooled and labeled as alcohol soluble fraction (Prolamin). The residue was extracted with 5 ml of 0.05M borate buffer (pH10) containing 0.5M sodium chloride 0.6%, mercapto ethanel and 0.5% sodium dodicyl sulphate, three times for 60, 30 and 15 min each and centrifuged for 15 min at 5000g after each extraction. The supernatant was pooled and labeled as buffer soluble (glutelin). The protein content of fractions was estimated (Lowry *et al.*1951).

Separation and quantification of protein fractions: Different polypeptides in the protein fractions of the test sample and residue left after rumen incubation were separated by sodium dodecyl polyacrylamide gel electrophoresis, SDS-PAGE (Laemmli 1970) and the intensity of individual bands was measured by densitometer. 20 µl protein fraction (globulin, albumin, prolamin and glutelin) and 5 µl buffer [0.6 ml of 1M Tris-HCl (pH 6.8) + 5 ml of 50% glycerol and 2 ml of 10% lithium dodecyl sulphate + 0.5 ml mercaptoethanol + 1 ml of 1% bromophenol blue + 0.9 ml distilled water] were mixed in 1 ml eppendorf tube and heated at 100° C for 3 min. The samples were centrifuged for 5 min at 10 000g and the supernatant (25µg protein) was loaded on gels. The molecular weights (MWs) of protein sub-fractions were determined by plotting log MW of test samples verses log relative mobility of standard protein markers. The relative mobility (Rm) was calculated as:

Rm = (Distance moved by protein/Distance moved by tracking dye)*100

The standard protein markers used were cytochrome C (12.3 kDa), myoglobin (17.23 kDa), carbonic anhydrase (303 kDa), ovalbumin (42.73 kDa), albumin (66.33 kDa) and ovatransferrin (76–783 kDa). The gels were scanned on a 'laser gel scanner' at chart speed 0.5 mm/s, scan speed 20 and absorbance range 1.0. The peak area was calculated as a product of peak height and width at the midpoint of total height.

Chemical and statistical analysis: The finely ground samples of protein supplements were analysed for total ash, nitrogen, ether extract (AOAC 1995) and cell wall constituents like neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent insoluble nitrogen (ADIN) and acid detergent lignin (ADL), according to the methods of Robertson and Van Soest (1981). The residue, obtained after incubations in the rumen of buffalo calves, was analysed for total-N only. The data were analyzed in a completely randomised and factorial design (Snedecor and Cochran1994) by using SPSS (2007) version 16 and differences in mean were assessed by using Tukey's b test.

RESULTS AND DISCUSSION

Chemical composition of protein supplements: The chemical composition revealed that the GNC, MC, COSC, NSC and CGM contained 38.9, 35.4, 44.5, 18.9 and 58.9% CP, 0.93, 1.17, 1.24, 2.32 and 9.61% ADIN, 1.36, 8.50, 7.20, 1.04 and 3.02% EE and 39.3, 26.2, 28.4, 62.2 and 32.9% NDF, respectively. The CGM had the highest ($P<0.05$) CP and ADIN and NSC had the lowest CP, while the ADIN was lowest in GNC. The ether extract was highest ($P<0.05$) in mustard cake and lowest in neem seed cake, which was comparable to that of groundnut cake.

Protein fractions and their degradability: The sequential fractionation of protein supplements revealed that COSC had the highest ($P<0.05$) amount of globulin and albumin (20.4 and 6.3%), while CGM had the highest ($P<0.05$) prolamin and glutelins (13.5 and 11.7%), respectively (Wadhwa *et al.*2010). The degradability of the globulin and albumin fractions was highest ($P<0.05$) in COSC (89.8 and 86.5%), while it was lowest ($P<0.05$) in CGM (60.2 and 9.6%). The prolamin of corn gluten meal was highly resistant and did not degrade at all. The degradability of glutelin fraction of corn gluten meal was the lowest (23%). The albumins and globulins, being cytoplasmic proteins are little protected, so are degraded rapidly, whereas prolamins and glutelins are the reserve proteins, which are present mainly in albumen so are protected against degradation. Irrespective of the protein supplements, the globulin fraction was the most degradable (83%) and prolamin the least (50%) degradable fractions.

Quantification of rumen undegradable protein fractions

Degradation of groundnut cake: The globulin fraction of groundnut cake contained 16 bands with MW ranging from 16 to 164 kDa (Table 1, Fig.1). The major fractions corresponded to the MW of 19, 30 and 67 kDa. The unusual association-disassociation characteristics of 12S globulin

Table 1. Quantitative degradation of protein fractions of solvent extracted groundnut cake (kDa)

	Without incubation	Incubation period (h)		
		12	24	48
Globulin				
	15.9 (8.1)	16.9 (27)	16.3 (30)	34.9
	17.5 (3.9)	20.6 (23)	20.4 (30)	
	18.2 (2.7)	31.7 (29)	30.5 (22)	
	19.3 (15.2)	34.9 (12)	34.2 (9)	
	21.3 (6.7)	36.7 (18)	36.0 (8)	
	22.8 (7.2)			
	24.6 (5.7)			
	25.8 (2.9)			
	28.1 (3.8)			
	29.8 (15.2)			
	30.9 (2.1)			
	34.5 (3.9)			
	38.1 (6.3)			
	41.3 (4.5)			
	67.2 (10.0)			
	164.2 (1.8)			
Prolamin				
	15.3	11.1 (25)	11.9 (12)	14.9
	12.4 (21)	12.2 (14)		
	15.1 (54)	15.6 (74)		
Glutelin				
	17.2 (4)	15.8 (52)	15.1 (13)	15.6 (68)
	20.9 (12)	19.6 (48)	16.3 (50)	19.4 (32)
	23.3 (3)		20.1 (37)	
	24.0 (8)			
	26.5 (4)			
	32.1 (13)			
	36.9 (17)			
	41.1 (18)			
	43.6 (19)			
	66.8 (2)			

Values in parenthesis indicate relative proportion.

have also been reported in almost all the oilseeds (Norton 1989). Most of the fractions disappeared within 12 h of incubation in the rumen, except the fractions with MW of 17 to 37 kDa. With increase in the rumen incubation periods, all the subunits were degraded except with MW of 35 kDa which appeared as major band even after 48 h of rumen incubation.

The prolamin constituted of a single polypeptide with MW of about 15 kDa, which was resistant to microbial degradation even after 48h incubation. The glutelin contained 10 sub-fractions with MW ranging from 17 to 67 kDa. Most of these subunits disappeared within 12 h of rumen incubation, except the ones with MW 16 and 20 kDa, which constituted 52 and 48% of the total protein fraction, respectively. Further, exposure to rumen microbes degraded the polypeptide with MW 20 kDa partially to smaller fraction with MW 16 kDa. The fractions with MW 16 and 19 kDa which constituted 68 and 32% were highly resistant to microbial degradation, even 48 h of rumen incubation. Overall in GNC, the protein fractions with MW of 15, 16, 19 and 35 kDa were resistant to microbial degradation.

Degradation of mustard cake: The globulin fraction of mustard cake showed the presence of 4 fractions with MW ranging from 16 to 31 kDa (Table 2, Fig. 1). Laroch *et al.* (1984) reported the presence of cruciferin-12S protein (20–33 kDa), napin-2S protein (12–14 kDa) from seeds of different varieties of cruciferous oil seeds. Each cruciferin had 6 subunits (29, 32, 33 kDa and 21–23 kDa) which showed association and disassociation characteristics. In 0 h samples, washing with water eliminated most of the fractions except those with MW of about 15 and 18 kDa constituting 60 and 40% of 20 kDa. Disappearance of 20 kDa component on washing in zero time samples was also observed by Spencer *et al.* (1988). The aggregation of smaller fractions resulted in presence of polypeptides with MW 38 and 43 kDa, constituting 27 and 39% at 6 h incubation. These fractions

Table 2. Quantitative degradation of protein fractions of mechanically extracted mustard cake (kDa)

	Without incubation	Incubation period (h)				
		0	6	12	24	48
Globulin						
	16.3 (55)	14.8 (60)	14.3 (34)	13.1 (77)	16.3 (100)	17.3 (29)
	20.4 (26)	17.9 (40)	38.4 (27)	36.4 (08)		21.4 (44)
	27.9 (08)		43.0 (39)	39.3 (15)		30.4 (09)
	30.6 (11)					32.9 (17)
Prolamin						
	12.1 (04)	14.2 (100)	12.2 (03)	13.1 (09)	11.5 (10)	14.2 (100)
	12.7 (16)		12.7 (04)	15.0 (91)	12.7 (17)	
	15.1 (80)		13.0 (09)		14.3 (73)	
		15.0 (84)				
Glutelin						
	14.6 (53)	16.0 (55)	16.8 (27)	15.2 (51)	31.1 (38)	30.5 (100)
	30.3 (21)	33.5 (21)	34.4 (36)	32.5 (32)	53.3 (62)	
	74.6 (26)	74.4 (24)	66.7 (37)	65.2 (17)		

Values in parenthesis indicate relative proportion.

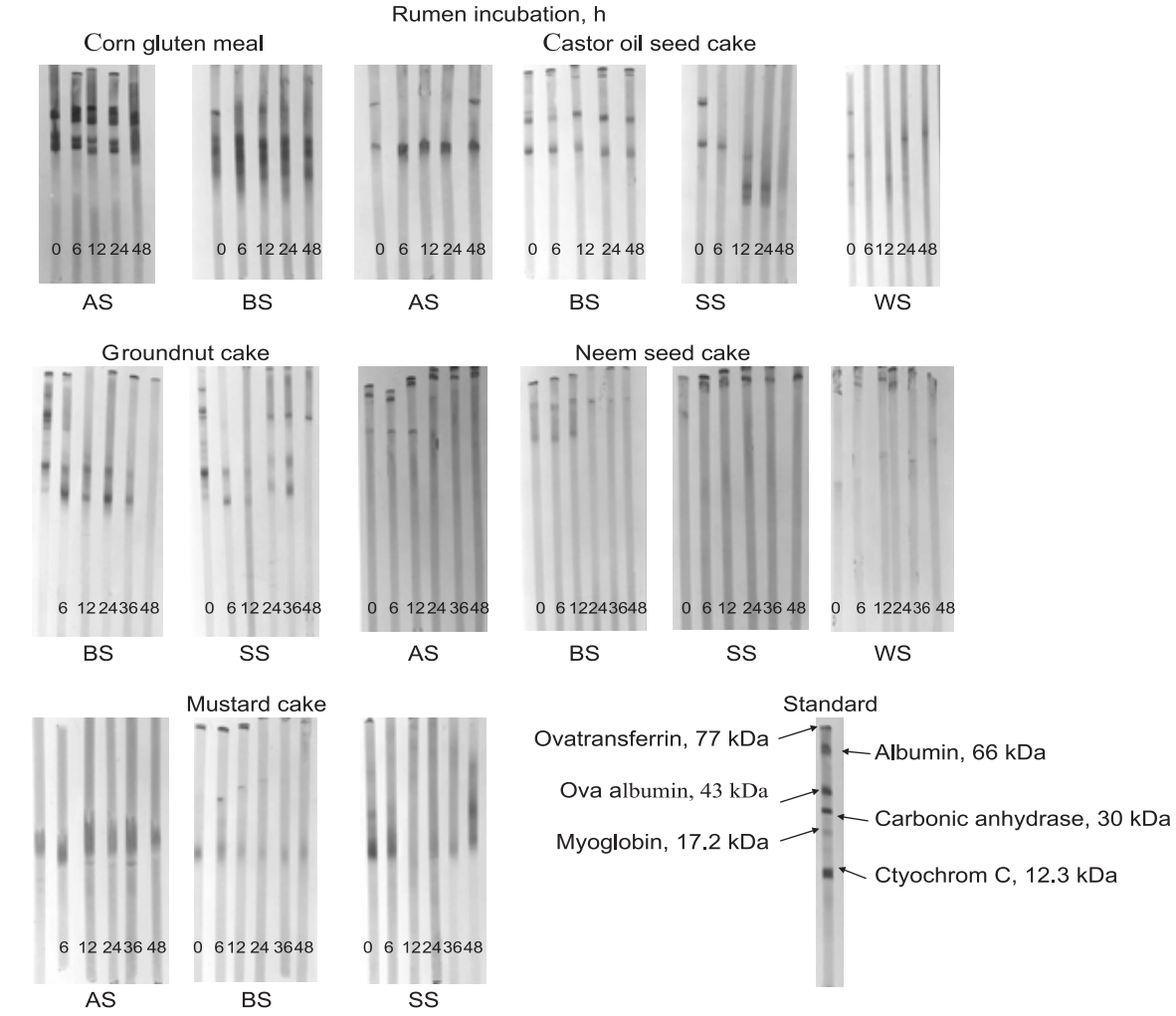

Fig. 1. Degradation (SDS-PAGE) pattern of some protein fractions of protein supplements.

were degraded to smaller fractions and their relative proportion decreased from 27 to 8% and 39 to 15% within 12 h whereas the subunit with MW 14 kDa constituting 34% in 6h was degraded to 13 kDa and constituted 77% of the total protein in 12 h sample. The polypeptides with MW 17, 21 and 33 kDa were resistant to rumen micro-organisms as they constituted about 44, 29 and 17% of globulin fraction.

The prolamins contained 3 polypeptides, 2 with MW 12, 13 and 1 with MW 15 kDa constituting about 20 and 80%, respectively. These fractions with MW 12–13 kDa were degraded within 48 h, whereas, the subunit with 15 kDa was degraded slowly to smaller fraction of 14 kDa, which was highly resistant to microbial degradation, as it was present even after 48 h of rumen incubation.

The glutelins contained 3 polypeptides with MW 15, 30 and 75 kDa constituting 53, 21 and 26%, respectively. The low MW fraction (15 kDa) was completely degraded within 24 h rumen incubation. The subunit with MW 75 kDa was degraded to 53 kDa in 24 h and may be to a smaller subunit of 30.5 kDa in the next 24 h rumen incubation. The subunit with MW 30.5 kDa appeared even after 48 h rumen incubation. The degradation pattern of mustard cake revealed that subunits with MW 14 (in prolamin), 17, 21 and 33 (in globulin) and 30 (glutelins) kDa were resistant to microbial degradation. Youle and Huang (1981) showed the presence of high amount (62% of total seed protein) of low weight 2S protein in mustard cake. The amount of S-containing amino acids in 2S protein of mustard cake was more than that in groundnut cake and this could be responsible for higher rumen undegradable protein in mustard cake.

Degradation of castor oil seed cake: The degradation pattern of globulin fraction of castor oilseed cake showed the presence of 4 major bands with MW 15, 19, 29 and 31 kDa constituting 22, 30, 27 and 20% of the total protein fraction and a minor protein with MW 70 kDa (Table 3, Fig.1). On washing with water, this 70 kDa protein fraction

Table 3. Quantitative degradation of protein fractions of mechanically extracted castor oilseed cake (kDA)

	Without incubation	Incubation period (h)				
		0	6	12	24	48
Globulin						
	15.3 (22)	15.2 (38)	14.3 (74)	13.9 (12)	13.0 (37)	12.1 (01)
	19.4 (30)	18.3 (45)	17.7 (24)	14.4 (46)	13.9 (54)	14.4 (99)
	29.0 (27)	18.9 (16)	41.3 (02)	15.4 (29)	16.9 (09)	
	31.4 (20)	27.9 (01)		18.6 (10)		
	70.3 (01)	37.8 (0.2)		20.3 (03)		
Albumin						
	12.5 (12)	12.6 (36)	17.3 (80)	12.9 (19)	18.1 (100)	19.4 (100)
	13.0 (15)	14.0 (23)	93.5 (20)	13.5 (19)		
	13.8 (17)	17.2 (30)		14.3 (28)		
	16.8 (18)	26.5 (01)		17.7 (34)		
	24.9 (12)	62.6 (10)				
	26.7 (06)					
	58.5 (21)					
Prolamin						
	19.2 (77)	17.0 (72)	18.38 (100)	18.35 (100)	18.6 (100)	17.9 (91)
	32.3 (23)	31.5 (28)				
Glutelin						
	19.6 (42)	19.0 (55)	18.0 (53)	19.4 (53)	18.8 (54)	18.3 (77)
	29.9 (37)	29.6 (31)	27.9 (39)	29.9 (40)	29.0 (42)	28.5 (04)
	34.7 (18)	34.5 (14)	86.8 (08)	165.2 (07)	103.3 (04)	99.5 (19)
	64.0 (03)					

Values in parenthesis indicate relative proportion.

Table 4. Quantitative degradation of protein fractions of un-decorticated solvent extracted neem seed cake (kDA)

	Without incubation	Incubation period (h)				
		0	6	12	24	48
Globulin						
	38.6	39.6	42.0	130.3	123.5	124.3
Albumin						
	18.8	19.4	23.9	38.7	22.4	29.6
Prolamin						
	43.0	47.2	34.7	56.7	63.6	66.0
Glutelin						
	30.7	30.9	31.0	54.2	56.4	53.9
	(46)	(46)	(48)	(100)	(100)	(100)
	71.2	58.5	57.4			
	(27)	(22)	(22)			
	86.2	63.9	62.8			
	27)	(32)	(30)			

Values in parenthesis indicate relative proportion.

disappeared. The fraction with MW 15 and 19 kDa were degraded slowly to 13–14 and 17 kDa in 24 h incubation in rumen whereas other fractions undergo some association of dissociation and finally disappeared as only one fraction with MW about 14 kDa appeared after 48 h exposure, showing resistance to rumen micro-organisms. Youle and Huang (1981) reported that 2S (ricin), 7S and 11S protein constituted approximately 44, 14 and 42% of total globulin seed protein in castor seed. The 2S protein of castor bean was rich in sulphur amino acids (3% methionine and 8.5% cysteine).

Castor oilseed cake was the only protein supplement amongst the selected ones which showed the presence of albumin to a considerable extent. The albumin protein fraction showed the presence of 7 polypeptides with MW ranging from 12 to 58 kDa. Only 4 polypeptides of 13, 14, 17 and 64 kDa constituting approximately 36, 23, 30 and 10% of total protein were resistant to water washing. Exposure to rumen micro-organisms for 6 h revealed aggregation of some of the small fractions to only 2 polypeptides with MW 17 and 94 kDa, which constituted 80 and 20%. With further exposure to rumen micro-organisms, most of the proteins were degraded but fraction with MW 18–19 kDa showed significant resistance and appeared even after 48 h incubation.

The prolamin fraction of castor oilseed cake contained 2 polypeptides with MW 19 and 32 kDa constituting 77 and 23%. The polypeptide with MW 32 kDa was eliminated within 6 h of rumen incubation whereas the other fraction with MW 18–19 kDa was highly resistant to microbial degradation in the rumen even after 48 h.

The alkali soluble fraction showed the presence of 3 major fractions with MW about 20, 30 and 35 kDa constituting 42, 37 and 18% of total protein fraction, another polypeptide with MW 64 kDa which constitutes a minor fraction (3%)

Table 5. Quantitative degradation of protein fractions of corn gluten meal (kDA)

	Without incubation	Incubation period (h)				
		0	6	12	24	48
Prolamin						
	18.01 (20)	20.1 (29)	20.4 (21)	22.0 (29)	21.3 (46)	17.7 (46)
	20.43 (37.5)	22.8 (32)	22.9 (23)	24.9 (20)	23.5 (26)	24.0 (25)
	22.95 (42.5)	30.4 (39)	30.4 (55)	35.9 (51)	35.3 (28)	42.7 (29)
Glutelin						
	26.27 (100)	15.8 (15)	22.6 (69)	20.4 (74)	22.9 (33)	21.9 (45)
	19.9 (38)	28.1 (31)	27.8 (26)	33.2 (67)	60.2 (55)	
	39.7 (47)					

Values in parenthesis indicate relative proportion.

was eliminated by washing in water. On exposure to rumen micro-organisms, the fractions with MW 20 kDa (42%) and 30 kDa (37%) undergo little change in size and appeared as such with great change in their relative proportion (4 and 77%), respectively. The fraction with MW 35 kDa showed association-disassociation characteristics and appeared as an aggregate of high MW of about 100 kDa (19%) after 48 h exposure to rumen micro-organisms. Overall, the protein fractions of COSC with MW of 14, 18, 19 and 100 kDa were highly resistant to rumen micro-organisms. Earlier reports also indicated the presence of resistant protein of castor bean in the range of 13–29 kDa (Sharief and Steven 1982).

Degradation of neem seed cake: The neem seed cake had a peculiar picture in the sense that globulin, albumins as well as prolamins fractions were constituted by single polypeptide with MW 39, 19 and 43 kDa, respectively (Table 4, Fig. 1). All the 3 polypeptides appeared to aggregate resulting in polypeptides with high MW i.e. 30–32, 124 and 66 kDa, respectively, which were resistant to microbial degradation.

About 46% of glutelins was constituted by protein fraction with MW 31 kDa which was completely eliminated within 12 h of rumen incubation whereas the other 2 fractions with MW 71 and 86 kDa were degraded to smaller units with MW 54 kDa which was highly resistant to microbial degradation even after 48 h of rumen incubation. Overall in NSC polypeptides with MW 30 (in albumin), and 54 (in glutelins), 66 (in prolamin), 124 kDa (in globulin) were resistant to microbial degradation.

Degradation of corn gluten meal: The prolamin fraction of corn gluten meal, zein was made up of 3 fractions with MW 18, 20 and 23 kDa contributing 20, 38 and 42% (Table 5, Fig. 1). All the 3 polypeptides appeared to aggregate resulting in fractions with MW 18, 24 and 43 kDa, which were resistant to microbial degradation even after 48 h rumen incubation. Esen (1986) reported that in addition to α-zein (20–24 kDa), the corn gluten meal had 17–18 kDa methionine-rich polypeptide and 27 kDa proline rich polypeptide. Similarly, Hamaker *et al.* (1995) have shown the presence of α-zein (20–24 kDa) and β-zein (14, 22 and 14 kDa). The 14 kDa component of β-zien was shown to have high amount of a sulphur containing and non-polar, basic amino acids.

The polyacrylamide gel electrophoresis revealed the presence of only one fraction with MW 26 kDa in the glutelins of corn gluten meal. Landry *et al.* (1983) reported that polypeptide with MW 25–27 kDa (major constituent of glutelins) was rich in proline histidine, arginnine and cysteine, which form intermolecular disulphide bonds with each other and may yield large oligomers. The exposure to rumen micro-organisms degraded the glutelins fraction (26kDa) to smaller fraction with MW ranging from 16 to 23 kDa, some of which aggregated to constitute fraction with high MW varying between 40 and 60 kDa. Two fractions with MW 22 and 60 kDa were found to be highly resistant to rumen microbial degradation. Overall in CGM fractions with MW 18, 24 and 43 kDa (in prolamin) and, 22 and 60 kDa (in glutelins) were resistant to microbial degradation even after 48 h rumen incubation. The results conclusively revealed that the low MW polypeptides showed resistance to rumen micro-organisms.

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