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The effect of soya protein supplement enriched with three amino acids on duodenal flow of soluble nitrogen fractions and amino acids in dairy cows*

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ABSTRACT

The aim of this study was to confirm the presumption that the duodenal flow of soluble nitrogen fractions: soluble non-ammonia nitrogen (SNAN), soluble protein and long-chain peptides nitrogen (SPLPN), soluble short-chain peptides and free amino acids nitrogen (SSPFAAN) and free amino acids (FAA) will be increased after the application of ruminally protected tablets containing soya protein and amino acids (Met, Lys, His). Control animals received the same mixture but in ruminally non-protected (powder) form. The experiment was carried out on three lactating Holstein cows of average weight of 523 kg fitted with ruminal and duodenal cannulas. The experiment was divided into 4 periods of 14 d (10 d preliminary period and a 4-d experimental period). In the first period one cow received the tablets (T) and the other two received the powder (C) with the same composition. In the subsequent period the rate of animals was antipodal. Cows were fed on diet based on maize silage, lucerne hay and a supplemental mixture. Powder or tablets consisted of purified soya-protein HP 300, Lys, Met and His. The experimental treatment did not significantly ($P>0.05$) influence duodenal flow of SNAN, SPLPN and SSPFAAN. In case of FAA significant ($P<0.05$) increase of flow (in g/day) was obtained in T group for Met (1.25 vs 0.35), Lys (2.07 vs 0.76), His (2.13 vs 0.49) and Arg (0.37 vs 0.15) against the control C.

KEY WORDS: dairy cows, duodenum, soluble nitrogen fractions, free amino acids

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INTRODUCTION

The INRA Protein System (Jarrige, 1989) classifies proteins present in feedstuffs into three principal categories based on the study of Ørskov and McDonald (1979). Category A is water-soluble and can be completely degraded in the rumen. Category B is water insoluble but potentially degradable in the rumen and the degree of its degradation is dependent on the flow rate of digesta and category C is completely rumen non-degradable. A highly sophisticated Cornell System (Russel et al., 1992) uses a chemical method for this fractionation and classifies feed protein into five groups: A, B_1, B_2, B_3 and C. Fractions A and B_1 are buffer-soluble, i. e. degradable in the rumen (Sniffen et al., 1992). This system considers soluble nitrogen fractions as completely rumen-degradable as well. However, recent data indicate that the soluble N-fractions are not degraded at the same rate and that due to their high flow rate the liquid fractions of the digesta can get out of a complete degradation in the rumen. Volden et al. (1998) mentioned that approximately 20% of intraruminally applied free amino acids can escape from the rumen. In an experiment with grass and grass silage eluates it was found out that on average 11.2% of small peptides (with less than 10 amino acid molecules) and 5% of free amino acids were not degraded in the rumen (Volden et al., 2002). These fractions contribute to the total amount of protein digestible in intestines (PDI) due to a good availability of soluble protein in the small intestine.

Nevertheless, the ruminal degradability of soluble nitrogen fractions is high (i. e. more than 80%) and the application of rumen non-protected amino acids and protein concentrates would be uneconomical. The ruminal protection can assure that these important nutritional substances will be transported into the small intestine with only minimal losses.

The aim of this study was to demonstrate that the flow of soluble N-fractions and free amino acids through the duodenum increases after the addition of a ruminally protected soya protein enriched with Met, Lys and His in comparison with non-protected form.

MATERIAL AND METHODS

Animals and procedures

The present experiment is an extension of a previous study (Třináctý et al., 2006) in which three lactating dairy cows were used in the experiment to study the effect of administration of soya-protein enriched with three amino acids (Met, Lys and His) in two forms - either rumen protected or unprotected, added to rumen.

Three lactating Holstein cows (1.-3. lactation, 17.-35. week of lactation) weighing on average 523 kg were fitted with ruminal and duodenal cannulas. The experiment was divided into 4 periods. Each period (14 d) consisted of 10 d preliminary period and a 4-d experimental period. In the first period one cow received the tablets (T group) and the other two received the powder (C group – control) with the same composition. In the subsequent period the rate of animals was antipodal so each animal received both variants in two replications.

Cows were fed individually twice daily (7.00 and 16.35 h) *ad libitum* a mixed diet based on a maize silage (54.7%), lucerne hay (15.0%) and a supplemental mixture (30.3%; composition mentioned in Třináctý et al., 2006). The supplement consisted of purified soya protein concentrate HP 300 enriched with amino acids (Met, Lys, His) in amounts calculated with regard to requirements of experimental animals (Rulquin et al., 2001a,b). The active substance of the supplement (it is, %: purified soya protein concentrate HP 300 93.0, Met 2.4, Lys 1.6 and His 3.0) was mixed thoroughly with the tablet additives (binding materials, modifiers of specific gravity, etc.). The homogenized mass was divided into two parts: control (C) that stayed in this powder form and experimental (T) which was tableted and subsequently enwrapped with a protective layer. As the protective layer a biologically active mixture based on the vinyl-pyridine-styrene copolymer according to patent (Ardaillon et al., 1989) was used. This protective layer is stable at pH>5 and is dissolved at pH<3.5, so the tablets were considered to be rumen-protected. During the whole experimental period paper boluses containing either tablets or powder plus 7.5 g of chromium oxide which was used as a marker of digesta passage were placed into the rumen bottom of each dairy cow through the ruminal cannula twice daily before feeding. The applied amounts of the purified soya bean protein HP 300 and amino acids are presented in Table 1.

Duodenal chymus (500 ml) was sampled from each animal in six-h intervals during the whole four-day experimental period starting on 7.00 a.m. of the first day. On each subsequent day the time of sampling was postponed by 1.5 h so that the four-day experimental period represented a set of chymus samples obtained during the day in 1.5 h intervals (Schwab et al., 1992). The obtained samples were immediately frozen to -20°C.

Feed samples were taken on the third day of each experimental period and the feed refusals were collected and weighed prior to each feeding during the experimental period. These refusals were also kept frozen at -20°C. After the end of the experimental period samples collected from each dairy cow were pooled, homogenized and used for the preparation of a representative sample.

Processing and analyses of chymus and feed samples

Chymus samples obtained within a four-day experimental period were thawed and pooled for each dairy cow and each period. They were continuously

stirred and used for the recovery of four average samples (500 ml). Chymus samples were lyophilized while feed and feed refusals samples were dried at 55°C for 48 h. After the equilibration of water content to laboratory humidity all samples were ground and sifted through a 1-mm screen. In feed and feed refusals samples dry matter (DM), ash (A), crude protein (CP), fat and crude fibre (CF).were estimated according to AOAC (1984). NDF (with α-amylase) and ADF were estimated according to Van Soest et al. (1991). Content of Cr in samples of duodenal chymus were estimated according to Williams et al. (1962). In these samples, ammonia nitrogen was estimated in a water eluate (2 g/100 ml of distilled water) using a gas electrode (manufacturer Radelkis, Hungary). Total soluble nitrogen and soluble nitrogen after trichloroacetic acid precipitation were estimated using a modified method described by Licitra et al. (1996): buffer was replaced by distilled water and the samples were centrifuged at 27,000 *g* and 4°C due to an impaired filterability; nitrogen was estimated in the supernatant using the Kjeldahl method. In these samples, free amino acids (FAA) were estimated as well using the following method: 2 g of the sample were shaked for 30 min in 10 ml of distilled water with a supplement of 5 ml of 10% sulphosalicylic acid. After the filtration the turbidity was removed by centrifugation at 10,000 *g* for 10 min. Detectable free amino acids were estimated in an automatic analyser AAA 400 (Ingos, CR) using a Li citrate buffer system.

Calculations

Soluble non-ammonia nitrogen (SNAN), soluble protein and long-chain peptides nitrogen (SPLPN) and soluble short-chain peptides and free amino acids nitrogen (SSPFAAN) were calculated according the following equations:

$$\text{SNAN} = \text{total soluble N} - \text{ammonia N}$$

$$\text{SSPFAAN} = \text{soluble nitrogen after trichloroacetic acid precipitation} - \text{ammonia N}$$

$$\text{SPLPN} = \text{SNAN} - \text{SSPFAAN}$$

The flow of DM through duodenum was calculated using the following formula:

$$\text{DM flow (kg/d)} = \text{Cr intake (g/d)} / \text{Cr concentration in chymus (g/kg DM)}$$

The flow of all other nutrients (SNAN, SPLPN, SSPFAA and FAA) through duodenum was calculated using the formula:

$$\text{Flow of SNAN, SPLPN, SSPFAA and FAA (g/d)} = \text{DM flow (kg/d)} \times \text{concentrations of SNAN, SSPFAA and FAA in chymus (g/kg DM).}$$

Statistical analysis

Statistical analysis of obtained data was performed using the GLM procedure from the statistic software SYSTAT 11.00.01 (USA). The following equation was used as a model for the comparison of concentrations and flows of nutrients in duodenum:

$$Y_{ij} = \mu + T_i + C_j + T_iC_j + \varepsilon_{ij},$$

where: μ= total average, T_i = effect of the experimental factors (i=2), C_j = effect of the dairy cow (j=3), ε_{ij} = residual error. Unbalanced numbers of animals in each period (1 vs 2) induced lost of degrees of freedom. From this reason the effect of period was propagated to the residual error.

RESULTS

The intake of individual components from tablets (T) or powder (C) was identical in both groups and is presented in Table 1.

Table 1. Consumption of supplemented components, g/day

Component	g/day	Component	g/day
HP 300[1]	306.00	TN[4]	75.76
Met[2]	8.80	TNAN[5]	75.68
Lys[2]	5.70	SNAN[6]	50.28
His[2]	10.60	SPLPN[7]	0.05
Arg_{HP}[3]	0.63	SSPFAAN[8]	50.23
Lys_{HP}[3]	0.50		

[1] soya protein concentrate, [2] added amino acids, [3] free amino acids from HP 300, [4] total nitrogen, [5] total non-ammonia nitrogen, [6] soluble non-ammonia nitrogen, [7] soluble protein and long-chain peptides nitrogen, [8] soluble short-chain peptides and free amino acids nitrogen

The DM intake was significantly higher ($P<0.05$) for cows receiving rumen-protected tablets (T, 16.33 kg/d) than in the control group (C, 15.68 kg/d; Table 2). Increased DM intake was followed by increased consumption of other nutrients as presented in the previous article (Třináctý et al., 2006a). Average milk yield of the T group was higher (17.80 kg/d; $P<0.05$) than that of the control group (16.73 kg/d). Milk yield expressed in 4% FCM did not differ significantly ($P>0.05$) between treatments. Because of the differences in DM intake, milk yield and 4% FCM were expressed in dry matter intake (Table 2) and they were not affected ($P>0.05$) by the treatment.

Table 2. Dry matter intake and milk yield

Item	Unit	C n=24		T n=24		P
		mean	SEM	mean	SEM	
Dry matter intake	kg	15.68[a]	0.325	16.33[b]	0.265	0.032
Milk yield	kg/d	16.73[a]	0.383	17.80[b]	0.281	0.029
4% FCM	kg/d	15.37	0.614	16.04	0.654	0.460
Milk yield/DM intake	kg/kg	1.08	0.034	1.09	0.021	0.675
FCM/DM intake	kg/kg	0.98	0.036	0.98	0.032	0.934

[a,b] means in the same row followed by the different superscripts differ (P<0.05)

Flows of DM, nitrogen fractions and FAA through the duodenum are presented in Table 3. The DM flow through duodenum did not differ significantly (P>0.05) between treatments. Similarly, flows of all nitrogen fractions (TN, TNAN, SNAN, SPLPN and SSPFAAN) were not affected by the treatment (P>0.05) nevertheless duodenal flow of mentioned parameters tended to be higher when tablets (T) were given.

Table 3. Duodenal flows of DM, nitrogen fractions and free amino acids, g/day

Item	C n=6		T n=6		P
	mean	SEM	mean	SEM	
Dry matter, total nitrogen and nitrogen fractions, g/day					
DM	8689.2	658.7	8848.6	224.9	0.816
TN[1]	328.3	20.8	343.0	10.7	0.526
TNAN[1]	309.4	21.9	328.2	9.7	0.452
SNAN[1]	114.4	8.6	122.6	2.8	0.292
SPLPN[1]	5.2	2.5	5.4	2.1	0.945
SSPFAAN[1]	109.1	6.8	117.2	2.2	0.275
Free amino acids, g/day					
essential amino acids					
Arg	0.15[A]	0.03	0.37[B]	0.12	0.009
His	0.49[A]	0.16	2.13[B]	0.35	0.002
Ile	0.07	0.01	0.11	0.02	0.177
Leu	0.71[a]	0.11	1.00[b]	0.21	0.025
Lys	0.76[A]	0.12	2.07[B]	0.33	0.003
Met	0.35[A]	0.08	1.25[B]	0.24	0.002
Phe	1.06	0.15	1.38	0.33	0.185
non-essential and endogenous amino acids					
GABA[2]	1.45	0.20	1.69	0.46	0.566
Gly	11.69	2.11	17.98	5.38	0.391
cysteic acid	0.92	0.12	1.23	0.39	0.448
ornithine	0.27	0.02	0.37	0.08	0.203
taurine	1.57	0.36	3.46	1.26	0.175
Tyr	1.08	0.17	1.39	0.32	0.050

[a,b] means in the same row followed by the different superscripts differ (P<0.05)
[A,B] means in the same row followed by the different superscripts differ (P<0.01)
[1] see Table 1, [2] gamma aminobutyric acid

Flow of Met, Lys and His used for the supplementation of soya protein was significantly higher (P<0.01) in the T group than in the control (C). After usage of the rumen-protected tablets (T) duodenal flow of Lys increased 2.7 times, Met 3.6 times and His 4.4 times in comparison with non-protected powder form. Flow of other two essential amino acids (Arg and Leu) in T group was significantly higher (P<0.01 or P<0.05, respectively) than in the control group C. Flows of Ile and Phe were not affected by the treatment.

The values of the duodenal flow of NEAA and endogenous AA (GABA, Gly, cysteic acid, ornithine, Tau and Tyr) did not differ significantly (P>0.05) between treatments but tended to be higher in the T group in comparison with the control (C).

Because of the differences in DMI between experimental groups values of duodenal flows were converted according to DMI (Table 4) results were

Table 4. Duodenal flows of DM, nitrogen fractions and free amino acids converted according to dry matter intake

Item	C		T		P
	n=6		n=6		
	mean	SEM	mean	SEM	
Dry matter, total nitrogen and nitrogen fractions g/day/kg					
DM	565.96	34.97	571.12	16.82	0.904
TN[1]	21.39	1.01	22.13	0.69	0.584
TNAN[1]	20.14	1.09	21.18	0.69	0.481
SNAN[1]	7.44	0.44	7.92	0.26	0.396
SPLPN[1]	0.33	0.15	0.34	0.13	0.887
SSPFAAN[1]	7.12	0.36	7.58	0.26	0.398
Free amino acids, mg/day/kg					
essential amino acids					
Arg	10[a]	2	24[b]	8	0.011
His	31[A]	10	137[B]	22	0.001
Ile	5	1	7	1	0.164
Leu	47	7	65	14	0.054
Lys	49[A]	7	134[B]	22	0.003
Met	22[A]	5	81[B]	15	0.001
Phe	69	10	90	23	0.265
non-essential and endogenous amino acids					
GABA[2]	95	14	110	32	0.620
Gly	761	136	1166	356	0.400
cysteic acid	60	8	80	27	0.465
ornithine	18	2	24	6	0.257
taurine	103	24	221	76	0.162
Tyr	71	12	91	22	0.149

[a,b] means in the same row followed by the different superscripts differ (P<0.05)

[A,B] means in the same row followed by the different superscripts differ (P<0.01)

[1] see Table 1, [2] gamma aminobutyric acid

similar to unconverted ones. Duodenal flows of DM and nitrogen fractions (TN, TNAN, SNAN, SPLPN and SSPFAAN) were not significantly different between treatments ($P>0.05$). Conversion of flows according to DMI caused a lost of significance in the Leu flow ($P>0.05$) and a decrease of significance level ($P<0.01$ to $P<0.05$) in the case of Arg. Converted flow of Met, Lys and His in T group was significantly higher ($P<0.01$) than in the C group.

DISCUSSION

The aim of this study was to confirm that the flow of soluble N-fractions and free amino acids through the duodenum increases after the addition of a ruminally protected soya protein enriched with Met, Lys and His in comparison with non-protected powder form. In the present experiment the physical form of rumen protection, it is coating with the pH sensitive copolymer based on the patent of Ardaillon et al. (1989) was used. This technology provides a postruminal delivery system that is independent of digestive enzyme function and dependent on the differences in pH between the rumen and abomasum. It has been demonstrated (Schwab, 1995; Robert and Williams, 1997; Třináctý et al., 2006b) that the resulting ruminally inert products have an apparent high coefficient of rumen protection and posses high intestinal release coefficients of the coated substance. This technology used to protect supplemental Met appeared to be the most effective in increasing Met in metabolizable protein as evidenced by the largest increases in blood Met concentrations (Robert et al., 1997; Blum et al., 1999).

The intake of individual components from tablets (T) or powder (C) presented in Table 1 was identical in both groups. The same values for the both forms were assured in such a way that these two forms were exactly weighed according to content of active components and were applied directly to the rumen bottom of experimental animals through the ruminal cannula. Besides of added amino acids (Met, Lys and His) only intakes of Arg_{HP} and Lys_{HP} originating from soya bean protein HP 300 were mentioned because the daily intakes of the other major parts of FAA in HP 300 were very low (100 g/day).

Published results focused on the duodenal flow of soluble nitrogen fractions are scarce even there is no study of the effect of rumen-protected or unprotected form of ruminally supplemented soya-protein + Met, Lys and His on the duodenal flow of the soluble nitrogen fractions.

Values of DM flow are comparable with results obtained in an experiment with dairy cows with similar DMI (Robinson and Kennely, 1990). Duodenal flows of Arg and Leu in the T group were significantly ($P<0.05$) higher. These results are correlated with a relatively high content of these amino acids in soya bean protein (Gonzáles et al., 2000). Due to the rumen protection of mixture duodenal flows of

free Met, Lys, His and Arg increased by 3.6, 2.7, 4.4 and 2.5 times, respectively, against the control.

In dairy cows treated with tablets (T) the intake of DM and other nutrients was higher ($P<0.05$) than with powder (C). This fact was discussed earlier (Třináctý et al., 2006a). From the reason of the mentioned difference in DMI between experimental and control group values of duodenal flows were converted according to DMI. DM flow converted according to DMI can represent apparent DM digestibility expressed as an undigested part of DM. DM flow converted according to DMI was 571.12 and 565.96 g/day/kg DMI in group T and C, respectively ($P>0.05$). These data are comparable with results of apparent DM digestibility obtained in an experiment with dairy cows with similar DMI (Robinson and Kennely, 1990).

If we compare intakes of supplemented amino acids (Met, Lys+Lys_{HP}, His; Table 1) to their duodenal flows in the T group (Table 3), we can see apparently low effectivity of rumen protection (on average 22.5%, ranged from 14.2 to 33.3%). On the other hand increased protein and casein yield ($P<0.05$) in milk from dairy cows fed by ruminally protected tablets (Třináctý et al., 2006a) suggests that the real duodenal flow and availability of mentioned amino acids in this experiment were probably higher. Several possible mechanisms may contribute to the explanation of this discrepancy.

The protective layer of tablets used in the present experiment (vinyl-pyridine/styrene copolymer; Ardaillon et al., 1989) was similar to those commercially used for rumen protection nevertheless our results are different from those published, e.g., by Robert and Williams (1997) for rumen protected methionine - Smartamine™ M (ranged from 75.0 to 97.1%). Smartamine™ M producer reports release of 90% of Met after 2 h incubation in pH 2 buffer. However retention time of feed particles in abomasum can be shorter (Wylie et al., 2000) and that is why part of tablets could escape disintegration in abomasum. Retention time of large and heavy particles is shorter than that of digesta and shape of their passage curve is a narrow peak (Třináctý et al., 2005). In comparison with the Smartamine™ M (2 mm particles) the tablets were lenticular with the diameter of 6.5 mm and density of the tablet, 1.15, is comparable with Smartamine™ M. From this reason part of whole tablets could escaped from sampling and their content was not included in analyses.

Generally, methods for evaluation of ruminally protected amino acid bioavailability using the duodenal sampling provide inaccurate results (Rulquin and Kowalczyk, 2003). The cows in the present experiment were fitted with the T cannulas (Komarek, 1981). Although T-cannulas are less disruptive to intestinal chyme flow than are most re-entrant cannulas major concerns with intestinal cannulation include intestinal dilation proximal to the fistula (Robinson et al., 1985), presumably resulting from deterioration of muscular and intestinal integrity, and excessive scar tissue development around the fistula. Intestinal

dilation may result in digesta pooling, digesta stratification, and unrepresentative sampling.

Furthermore, other detectable FAA: gamma-aminobutyric acid (GABA), glycine (Gly), cysteic acid, ornithin, taurin and Tyr were classified among non-essential and endogenous amino acids. All these amino acids occurred in the supplemented soya protein only in traces and for that reason the metabolic processes are their main source. Taurin and Gly occur above all in bile as taurocholic and glychocholic acids and ornithin participates in the urea metabolism. Increased levels of these AA found in digesta suggest that the T-shaped duodenal cannulas were inserted approximately 60 cm distal to the pylorus, distal to the bile and pancreatic duct. Some deamination of AA may have taken place between the reticulum and the duodenal cannula. However, considerable amounts of AA may have been actively absorbed along the 60-cm stretch between the abomasum and the site of the duodenal cannula.

For the verification of our presumption, that tablets escaped sampling with the contribution of the complex of mechanisms mentioned above, the recovery of marker (chromium oxide) in faeces was determined. We found out that the chromium recovery from faeces was 96.13% (SEM = 3.02, n = 12) which is in agreement with the results published by Hattan and Owen (1970). This finding together with the increase in milk protein and casein yield in T group (Třináctý et al., 2006a) suggests that ruminally protected tablets containing the soya protein enriched with the Met, Lys and His were functional.

CONCLUSIONS

This experiment confirmed the functionality of ruminally protected tablets containing amino-acid-supplemented soya protein by significant increase of duodenal flow of free amino acids: Met, Lys, His and Arg. However, probably because of escape of whole tablets from sampling process, balance between intake and real flow of added amino acids provided non-accurate results.

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