Effect of Heat or Formalin Treatment of Proteinaceous Feed on Protein Degradation with *in vitro* Rumen Fermentation

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Abstract The effect of heat or formalin treatment of proteinaceous feed on protein degradability in the rumen was studied in two trials, adopting an *in vitro* culture method. Protein degradation in the rumen was calculated from the measurement of ammonia nitrogen ($\text{NH}_3\text{-N}$) and bacterial nitrogen. Bacterial nitrogen was estimated from the value of diaminopimelic acid in the medium. Soybean meal (SBM) and casein were treated at 150°C for 2 hours (trial 1) or with formalin (HCHO) at 1% CP for 72 hours (trial 2). They were incubated in a medium comprising 1 part of rumen liquor and 1 part of McDougall's artificial saliva for 24 hours. The total VFA concentration was higher in untreated substrates. No increase in VFA concentration was observed as the incubation time proceeded in the HCHO treated casein. $\text{NH}_3\text{-N}$ was higher in untreated substrates, especially in untreated casein. There was little change with the time in HCHO treated casein, while a decrease in $\text{NH}_3\text{-N}$ was found in HCHO treated SBM. The protein degradabilities of heat treated SBM, heat treated casein, untreated SBM and untreated casein after 24 hours' incubation in trial 1 were 6.7%, 13.3%, 34.3% and 42.3%, respectively. Those of HCHO treated SBM, HCHO treated casein, untreated SBM and untreated casein in trial 2 were 0.6%, 0.0%, 32.6% and 58.0%, respectively.

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Ruminants have two kinds of amino acid source from the upper digestive tract, namely dietary protein (bypass protein) and microbial protein, excepting endogenous protein. Obviously, extra energy is necessary to build up microbial protein. However, the conversion of dietary poor quality protein to microbial protein in general means improvement of the quality of protein as regards amino acid composition. Meanwhile, it might be better to protect the dietary protein from degradation by microbes in the rumen when the animals are fed a diet containing high quality protein like soybean meal or casein.

Many attempts have been conducted to increase the quantity of protein reaching the lower digestive tracts, by means of protecting dietary protein from degradation in the rumen by physical or chemical procedures^{1,2,3)}. Such treatment of dietary protein may restrict rumen fermentation and influence the utilization of feed protein.

No report has documented the state of rumen fermentation to ascertain whether such treatments limit bacterial activity in the rumen or not. Moreover, only ammonia nitrogen ($\text{NH}_3\text{-N}$) concentration has been measured to discuss the effect of heat treatment or formalin (HCHO) treatment of proteinaceous feed on the protein degradation in some *in vitro* experiments^{4,5,6}. In those reports, protein degradation might be underestimated as the result of microbial proliferation.

The present investigation was undertaken to anticipate the effect of heat or HCHO treatment of proteinaceous feed on rumen fermentation and protein degradation from the value of $\text{NH}_3\text{-N}$ and bacterial nitrogen. The bacterial nitrogen was estimated by diaminopimelic acid (DAPA) as the bacterial marker.

Materials and Methods

Commercial, solvent-extracted soybean meal (SBM) and casein (from milk, Wako Pure Chemical Industries, Ltd., Tokyo) were used as protein source. Corn starch (Wako Pure Chemical Industries, Ltd., Tokyo) was used as carbohydrate source. Heated SBM and casein were prepared by dry-heating for 2 hours in a circulation air oven at 150°C . The HCHO treatment of both SBM and casein was carried out by adding 1 g HCHO to 100 g crude protein, mixing well, storing in polyvinyl bags for 72 hours and then air-drying until no smell of HCHO was detected.

Ruminal fluid for incubation was obtained just before the morning feeding from a fistulated steer fed a diet of 15.6% hay, 38.1% wheatbran, 20.4% alfalfa meal, 20.4% crushed barley, 5.1% SBM and 0.4% salt and minerals. The fluid was strained through threefold cheesecloth and then centrifuged at 1000 rpm for 5 minutes. The same quantities of the supernatant solution and McDougall's artificial saliva⁷ were mixed and adjusted to pH 7.1 with Na_2CO_3 . Fifty ml of this inoculum was added to each of 100 ml glass test tubes containing the appropriate substrate. The tubes were immediately saturated with carbon dioxide, capped with Bunsen valves, and incubated in a water bath at 39.5°C . Three tubes for each treatment were taken out after 6, 12 and 24 hours' incubation. The pH values of samples were measured immediately, and stored in a freezer at -20°C until analysis.

In trial 1, the effect of heat treatment was examined using four kinds of substrates, untreated SBM, heated SBM, untreated casein and heated casein. Exactly 50 mg of nitrogen in the form of those nitrogen sources was added to each tube, and 0.5 g of corn starch was also added as carbohydrate source. Except the control which contained inoculum alone, all tubes contained 100 mg additional nitrogen per 100 ml inoculum, so that degradability (%) of added protein could be obtained by the sum of $\text{NH}_3\text{-N}$ and bacterial N increase after incubation per 100 ml culture medium.

Trial 2 was conducted in the same way to examine the effect of HCHO treatment using HCHO treated SBM and casein instead of heated substrates in trial 1.

Total VFA concentration was determined by PACKETT and McCUNE's method⁸. $\text{NH}_3\text{-N}$ concentration was determined by the phenol-hypochloride reaction⁹. DAPA as the marker for bacterial protein in the samples after 24 hours' incubation was

determined by the method of ion exchange chromatography using an automatic amino acid analyzer (Model KLA-5, Hitachi Co. Ltd., Tokyo). Bacterial N was calculated by the equation $N = \text{DAPA} \times 18$ of HUTTON *et al*⁽⁹⁾.

Results

The effects of heat treatment on the periodical changes of average pH, VFA concentration and $\text{NH}_3\text{-N}$ concentration are shown in Figure 1. DAPA concentration and increments of $\text{NH}_3\text{-N}$ and bacterial N in the culture medium after 24 hours' incubation and the estimated degradability (%) of added protein are shown in Table 1.

VFA concentration was increased by N substrate addition, the increase being most remarkable in untreated SBM. VFA production in the tubes containing heated SBM or heated casein was restricted compared with untreated ones. An inverse relationship between VFA concentration and the ruminal pH is apparent. The $\text{NH}_3\text{-N}$ concentration increased in untreated protein substrates, most remarkably in the untreated casein. The $\text{NH}_3\text{-N}$ concentration was depressed by heat treatment in SBM. The DAPA concentration after 24 hours' incubation was greater in amount with untreated protein substrates than with heat treated substrates. This was especially great in the SBM.

The effects of HCHO treatment on the pH and chemical values are shown in

Table 1. Effects of heat treatment on DAPA concentration and nitrogen status with *in vitro* rumen fermentation (24 hrs)

	DAPA ^a concentration (mg/dl)	Nitrogen status		
		Increased $\text{NH}_3\text{-N}$ (mg/dl)	Increased bacterial N (mg/dl)	Degradation of protein (%)
Untreated SBM	1.73	10.2	24.1	34.3
Heated SBM	1.05	-5.1	11.8	6.7
Untreated casein	1.14	28.8	13.5	42.3
Heated casein	0.99	2.6	10.7	13.3

^a DAPA concentration was 0.39 mg/dl at 0 hour.

Table 2. Effects of HCHO treatment on DAPA concentration and nitrogen status with *in vitro* rumen fermentation (24 hrs)

	DAPA ^a concentration (mg/dl)	Nitrogen status		
		Increased $\text{NH}_3\text{-N}$ (mg/dl)	Increased bacterial N (mg/dl)	Degradation of protein (%)
Untreated SBM	1.48	13.9	18.7	32.6
HCHO treated SBM	0.89	-7.4	8.0	0.6
Untreated casein	1.18	44.8	13.2	58.0
HCHO treated casein	0.45	-0.2	0.1	0.0

^a DAPA concentration was 0.44 mg/dl at 0 hour.

Fig. 1. Effects of heat treatment of protein source on pH, VFA and $\text{NH}_3\text{-N}$ concentration in *in vitro* rumen fermentation.

Fig. 2. Effects of formalin treatment of protein source on pH, VFA and $\text{NH}_3\text{-N}$ concentration in *in vitro* rumen fermentation.

Figure 2. DAPA concentration and the nitrogen status after 24 hours' incubation are shown in Table 2.

The pH values began to decline after the start of incubation in each substrate. This tendency was more pronounced in SBM than in casein. It was observed that HCHO treatment of each protein source apparently kept pH values higher than in untreated substrate. The VFA concentration increased rapidly after the start of incubation in untreated SBM and untreated casein. The tubes which contained the HCHO treated substrates showed lower VFA levels than the untreated substrates; no increase of VFA was observed in HCHO treated casein.

Discussion

Heat or HCHO treatment obviously depressed the production of VFA and $\text{NH}_3\text{-N}$, regardless of source of protein. However, an appreciable change can be seen in VFA and $\text{NH}_3\text{-N}$ concentration for heat treated SBM, heat treated casein and HCHO treated SBM. Also, there was no increase in VFA and $\text{NH}_3\text{-N}$ concentration in the control. It is considered that added starch and protein, and carbohydrates contained in SBM seem to be the most probable sources for VFA production. The increases in both VFA and $\text{NH}_3\text{-N}$ concentration in untreated SBM show that all of these three were principal sources for VFA production. However, in the case of heat treated SBM, the VFA concentration increased, but the values were not higher than those for untreated SBM; and the $\text{NH}_3\text{-N}$ concentration showed a lower value in heat treated SBM. These suggest that the protein in SBM which was one of the sources of VFA production was limited by heat treatment. And it could be assumed that the principal sources for VFA production were the starch and the carbohydrate in SBM. It is well known that lysine rich in SBM interacts with glucose by heat treatment and they make a compound which is not easily attacked by enzymatic digestion. This suggests that the heat treatment of SBM might affect not only the degradation of protein but the availability of carbohydrate by enzyme. Therefore, it might be expected that the principal source for VFA production was only starch in the case of heat treated SBM, and it induced the lower VFA production than the untreated SBM.

On the other hand, it is considered that the depression of VFA production would also occur in cases where nitrogen sources for microbial proliferation were limited. The $\text{NH}_3\text{-N}$ concentration at 6 hours showed no increase in heat and HCHO treated casein, and even a decrease in both treatments of SBM. At the start of incubation, the $\text{NH}_3\text{-N}$ concentration was approximately 6 mg/dl in trial 1 and approximately 8 mg/dl in trial 2. These values are sufficient for microbial proliferation¹¹⁾. Therefore, the lower value of VFA production in heat treated SBM at the early stage of incubation might be attributed exclusively to the depressed supply of easily available carbohydrate and protein from heat treated SBM. More frequent measurements at the early stage of incubation are necessary to make this point clear.

There are some reports on the effect of heat or HCHO treatment. CHALMERS *et al.*¹²⁾ reported that the feeding of heated casein reduced rumen $\text{NH}_3\text{-N}$ concentra-

tion. A similar result with heated SBM was also reported by HUDSON *et al.*¹³⁾ THOMAS *et al.*⁵⁾ reported that the denaturation by heat treatment at 138° or 149°C for 4 hours protected the SBM from degradation by rumen microbes. On the other hand, SHARMA *et al.*²⁾ reported that 0.7 g HCHO per 100 g CP was necessary to reduce the degradation of casein by rumen microbes. SCHMIDT *et al.*¹⁴⁾ and THOMAS *et al.*⁵⁾ reported that more than 0.4 g HCHO per 100 g CP was necessary to inhibit the release of $\text{NH}_3\text{-N}$ from degradation of SBM protein with *in vitro* rumen fermentation. In the present experiment, higher temperatures of more HCHO than in those experiments were adopted for protein denaturation. Therefore, the results obtained were clearly in the same direction.

Fairly high VFA production could be seen in heated SBM and casein, and a considerable decrease and transitory decrease in $\text{NH}_3\text{-N}$ in heated casein and SBM respectively occurred at the same time. This would indicate that the VFA production in heated SBM and casein was not so remarkable as compared with in untreated ones, however the considerably active proliferation was carried out in the case of heated SBM and casein. On the other hand, there was no apparent increase of VFA production and $\text{NH}_3\text{-N}$ at any time of incubation in HCHO treated casein nor in the control. But a fairly large increase in VFA production and decrease in $\text{NH}_3\text{-N}$ could be found in HCHO treated SBM. The difference in results between SBM and casein in HCHO treatment would be considered to be owing to the difference in sensitivity to HCHO. It can be assumed that the HCHO treated casein obtained almost perfect resistivity to degradation by rumen microbes.

The above mentioned presumptions would be supported also by the results of increased $\text{NH}_3\text{-N}$, increased bacterial N and the degradability of protein after 24 hours' incubation. In other words, in the case of heat treatment, the amount of increased bacterial N was lower than untreated ones and these values were closely related to the VFA levels at 24 hours. This clearly indicated that microbial proliferation was closely associated to VFA production. The reduction in $\text{NH}_3\text{-N}$ and lower value for increased bacterial N in the heated SBM might show that the deficiency of an easily available N source through heat treatment would restrict microbial proliferation and result in lower levels of VFA production. The same pattern can be seen in the case of HCHO treatment.

In the case of untreated casein both in trial 1 (heat treatment) and trial 2 (HCHO treatment), the degradability of protein was high, especially the value for the untreated casein which was the control in trial 2, at 58.0%. However, this value was obviously lower than the degradability of casein N in actual feeding. Part of the explanation for the unexpected value may lie in the difference between the actual N content of bacteria and the N content calculated from the equation of HUTTON *et al.*¹⁰⁾ and/or in the difference in the ratio of the amount of nitrogen source to bacterial population between *in vitro* experiment and in actual feeding. It is considered that the equation to calculate bacterial N from DAPA should be obtained in every experiment, and that the addition of smaller amounts of protein N than 100 mg/dl might be

desirable to obtain high protein degradability with *in vitro* experiments. In the case of untreated SBM as control for the heated SBM and HCHO treated SBM, 10.2 (trial 1) and 13.9 mg/dl (trial 2) of $\text{NH}_3\text{-N}$ were increased in the medium after 24 hours. In the case of untreated casein, 28.8 and 44.8 mg/dl of $\text{NH}_3\text{-N}$ were accumulated in 24 hours' incubation. If $\text{NH}_3\text{-N}$ arising from added protein was absorbed via rumen wall and excreted as urea into the urine, there must be resigned the considerable amount of high quality amino acid source.

It can be recognized that heat or HCHO treated casein and SBM became resistant to attack by rumen microbes. In actual feeding, if these proteins escaped into the post reticulo-rumen without being degraded, it would induce a shortage of readily available nitrogen for microbial proliferation in the rumen. In that case, it would be necessary to satisfy the nitrogen requirement of rumen microbes with some methods. Urea-N added to the diet as a vicarious nitrogen source might assume a great role when protein protected by some treatment from microbial attack in rumen was given as feed. WALLER *et al.*¹⁵⁾ reported that better nitrogen utilization would be found with a diet containing distillers' byproducts, which degrade with difficulty, when urea was added to the diet as a vicarious nitrogen source. What happens to vicarious nitrogen sources in the digestive tract of ruminants must be studied, and thereby, information about how to use it in actual feeding for maximum production must be obtained.

It is not clear in the present study whether the treated protein escaping into the post reticulo-rumen is attacked readily by the animal's own enzyme. Further study would be necessary to clarify these points.

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蛋白質飼料の加熱処理またはホルマリン処理が、反すう胃微生物による *in vitro* での蛋白質分解に及ぼす影響

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バイパス蛋白質を増加させる手段として，蛋白質飼料に加熱処理またはホルマリン処理を行なった場合の，反すう胃内発酵および細菌態蛋白質量の変化について予備的な知見を得るために2回の *in vitro* 発酵試験を行なった。試験1では通風乾燥器内で150℃ 2時間の加熱処理をした大豆粕およびカゼインを，また，試験2ではCPの1%に相当するホルマリンで72時間処理をした大豆粕およびカゼインを，牛第一胃液を1に対して McDougall 人工唾液を1混合した培養液にて24時間培養し，それぞれ無処理の場合と比較した。

VFA 濃度は大豆粕，カゼインともに無処理で高くなった。ホルマリン処理カゼインでは時間の経過にともなうVFA 濃度の増加が認められなかった。アンモニア態窒素濃度は無処理区で高く，とくに24時間後の無処理カゼ

インが著しく高かったが，ホルマリン処理カゼインでは時間の経過による変化はほとんどみられず，ホルマリン処理大豆粕では時間の経過とともに低くなった。

ジアミノピメリン酸を指標物質として用い，細菌態N量を算出し，その値とNH₃-N量から24時間培養後の蛋白態Nの分解率(%)を求めた結果，試験1では加熱処理大豆粕，無処理大豆粕，加熱処理カゼインおよび無処理カゼインでは，それぞれ6.7，34.3，13.3および42.3であった。また，試験2ではホルマリン処理大豆粕，無処理大豆粕，ホルマリン処理カゼインおよび無処理カゼインの分解率は，それぞれ，0.6，32.6，0.0および58.0であった。

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