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In vitro evaluation of protein digestibility in the abomasum and small intestine of ruminants

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Abstract. The investigation included studies with microbial protein, 0,5 % formaldehyde (HCHO) treated protein in soybean meal, and ruminally undegradable feed proteins in cotton seed and sunflower seed cakes. Microbial mass was separeted with centrifugations from the rumen contents of roughage-fed sheep. Undegradable feed proteins were prepared with the nylon bag technique.

The proteins were digested in solutions of pepsin-HCl and trypsin-chymotrypsin. The average digestibility for microbial protein was 80.7 %. The corresponding values for the undegradable proteins in cottonseed cake and sunflower seed cake were 83.7 % and 82.7 %. In the incubation with pepsin-HCl, pH in the solution affected significantly ($P < 0.001$) the digestibility of HCHO-treated protein in soybean meal. The average digestibilities at pH 1.5—2.0, pH 2.5—3.0, and pH 3.1—4.0 were 97.2, 76.0, and 71.0 %, respectively.

Introduction

In vitro tests of protein digestibility have mainly been used for non-ruminants. In the studies for ruminants KOWALCZYK *et al.* (1977, 1978 a, b) used abomasal fluid from a fistulated animal and they also evaluated protein digestibilities with rats. Multi-enzyme tests were used when the digestibilities of ruminally undegradable feed proteins (*in sacco*) were analyzed. In nylon bag studies *in vitro* methods would most obviously be relevant, because the digestibility of undegradable protein has to be estimated from a very small sample. The digestibility of microbial protein separated from the rumen contents could also be determined (e.g. BERGEN *et al.* 1967).

The aim of the present study was to investigate possibilities to use in digestibility analyses a method which included protein treatments with pepsin-HCl and trypsin-chymotrypsin. This method was chosen, because it

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was reported e.g. by MOZERSKY and PANETTIERI (1983) that the methods based on a pH drop in the incubations (e.g. HSU *et al.* 1977), would not be applicable to proteins of which the *in vivo* digestibility is not known. The present study included investigations mainly with the protein in microbial mass, which was separated from the rumen contents of sheep. The digestibility of ruminally undegradable protein in sunflower seed cake, cottonseed cake, and formaldehyde treated protein (0.5 % treatment level) in soybean meal was also studied.

Materials and methods

In vitro technique

The technique was modified using procedures described in the literature, mainly those by AKESON and STAHMAN (1964), and ANON (1970). The procedure was divided into two phases, incubations with pepsin-HCl and with trypsine and chymotrypsine. In the first phase samples of 500 mg were incubated at +39°C in pepsin-HCl solution for 16 hours. Pepsin (Merck 7190, 1 : 10000, 2000 FIP-U/g) concentration in the solution of 0.05 N HCl was 0.1 %. The ratio of the solution to the sample protein was 0.7 ml/mg protein. The amount of pepsin-HCl was hence changed according to the amount of protein in the sample, because Van BRUCHEM and Van't KLOOSTER (1980) suggested that proteins stimulated abomasal secretions of acid *in vitro*. Incubations were made in glass tubes (volume 100 ml) with rounded bottoms and the tubes were carefully shaken at the beginning of the incubations. The pH in the incubations was always kept under pH 2.0.

In the second phase contents of the tubes were centrifuged at the speed of 400 g for 10 min. Supernatant in the tube was carefully discarded and the sediment was neutralized (pH 7.0) using washings with phosphate buffer (pH 7.0). The contents of the tubes were centrifuged as described above and the sediment was taken for further analyses.

Solutions of bovine chymotrypsine (Merck 2307, 45 U/mg) and bovine trypsine (Merck 24579, 3.5 U/mg) were prepared in phosphate buffer (pH 7.0) using 50 mg of chymotrypsine, or 5 mg of trypsine for 50 ml of the buffer.

The solutions of trypsine and chymotrypsine were added into the tubes so that the amounts were 10 ml and 1 ml, respectively. In addition, 9 ml of the buffer was transferred into the tubes.

The incubations in the second phase were made (at pH 7) at +39°C for 24 hours. After this the samples were centrifuged as described earlier. The supernatants in the tubes were carefully discarded and the sediments (undigestible protein of the sample) were transferred for the analysis of protein N.

Analysis of protein N in the samples

The sample was transferred into the Kjeldahl bottle and incubated in 80 ml of distilled water, at +70°C for 1.5 hours. The contents of the bottles were carefully stirred 4—5 times during the incubation.

After this incubation 1.5 ml of $Al_2(SO_4)_3K_2SO_4 \cdot 12H_2O$, 15 ml of the solution of copper sulphate (156.4 g $CuSO_4 \cdot 5H_2O$ in 1000 ml of distilled water), and 15 ml of 2.5 % NaOH were added into the bottles. When the contents of the bottles were cool and clear they were squeezed through an N-free filter paper, and the sample on the filter paper was washed with 750 ml of warm, distilled water. The sample and the paper were carried to N determination which was made using the Kjeldahl method but excluding $CuSO_4$ as a catalyst. Blank tests including filter paper and reagents were used in all determinations. The method described is based on the procedure used at the Department of Animal Husbandry, University of Helsinki.

Separation of microbial mass from the rumen contents

Microbial mass was separated from the rumen contents of fistulated sheep in the hay or hay and grass silage (65 : 35 on DM basis) diets. Rumen contents were collected from different parts of the rumen, squeezed through a cheese cloth, and the filtrate was centrifuged at 400 g for 5 min. The sediment was discarded and the filtrate was further centrifuged over 30 000 g at +2°C for 20 min. The sediment was carefully separated and stored frozen for the analyses.

Samples of feed proteins

The samples of the formaldehyde treated protein in soybean meal, and ruminally undegradable (*in sacco*) proteins in sunflower seed cake, and cotton seed cake were prepared by Dr. Torben Hvelplund, National Institute of Animal Science, Denmark (see Hvelplund 1983).

Results and discussion

Studies with microbial protein

Composition of the microbial mass

Fresh, separated microbial mass was used in incubations. Samples were not dried before incubation, because WALLACE (1983) among others suggested that for instance freeze drying might affect digestibility. Crude protein (N × 6.25) content in the random-sampled (n = 7) mass was 37.1 ± 1.0 % in fresh weight. The proportion of protein N in total N was 86.7 ± 0.7 % (n = 7).

Effect of HCl and the enzymes on digestibility

According to our results HCl alone gave a poor digestibility value for microbial protein (Table 1). Moreover, the combination of HCl and pepsin was not sufficient either, and it could be suggested that one of the enzymes in the pancreatic fluid is needed for a proper protein digestion. However, pepsin-HCl might have more importance in the digestion of the protein of plant origin when the sample is less digestible owing to the fibrous structure etc. On the other hand, ROJAHN and WAGNER (1961) observed the difference of 9 %-units only in the digestibility of barley protein when the estimation was made either with pepsin-HCl (lower values) or with pepsin-HCl and pancreatin.

Those incubations in which a stronger HCl was used, tended to give higher digestibilities for microbial protein. KORTE (1979) wondered whether results of this kind could be caused by a better digestibility of nucleic acids in microbial cells. However, our results emphasized the importance of the enzymes of the pancreatin in the digestion of microbial protein.

Digestibility of microbial protein

When the digestibility of microbial protein was determined with the pepsin-HCl and trypsin-chymotrypsin, the mean of the digestibility was 80.7 % (see Table 1). This value is the same average value which could be calculated (e.g. 80.4 %) from the papers of TAS *et al.* (1977), HAGEMEISTER *et*

Table 1. The effect of different factors on the digestibility-% of microbial protein *in vitro*.

	Digestibility-%		
	$\bar{x}$	SEM	N
0.05 N HCl	20.5	0.9	2
0.05 N HCl + pepsin	69.2	1.0	2
0.05 N HCl + pepsin, chymotrypsin	86.1	0.3	2
Trypsin + chymotrypsin	81.2	1.4	4
0.05 N HCl + pepsin, chymotrypsin and trypsin	80.7	0.8	14
0.14 N HCl + pepsin, chymotrypsin and trypsin	86.5	1.1	4

N = number of incubations

al. (1980), STORM & ØRSKOV (1982), HVELPLUND (1983), STORM *et al.* (1983), and WALLACE (1983). In some of the incubations digestibility of microbial protein differed quite clearly from the mean value. This type of variation was also found by BUCHMANN (1979) between *in vitro* incubations. In the present study the coefficient of the variation (CV-%) of the results between the incubations was 3.7 %, being higher than reported by BUCHMANN (1979).

In the experiments reviewed from the literature there was also a great variation in the results between different investigations. This can be partly explained by the different experimental techniques. However, BERGEN *et al.* (1967) and WALLACE (1983) showed great differences in protein digestibility between bacterial strains and therefore part of the differences between different studies and incubations (present study) might be explained by the different composition of the bacterial mass digested. Ecpecially the increase in the proportions of Gram + (WALLACE 1983) or cellulolytic bacteria (BERGEN *et al.* 1967) in microbial mass can decrease digestibility of microbial protein. In our technique, occasional difficulties in squeezing the small sample in different phases of the work contributed also most evidently to the variation of the results.

Studies with microbial protein and feed protein

Effect of pH and incubation period

During the incubations the pH in the contents of the tubes was carefully followed. The corresponding values in pepsin-HCl incubations and in trypsin-chymotrypsin incubations were pH 1.6 ± 0.02 and pH 6.9 ± 0.01.

It is doubtful, however, whether pH in the contents of the abomasum and small intestine remains so stable *in vivo*. In the experiment with goats von ENGELHARDT and HAUFFE (1975) found pH 2.3 ±0.3 in the aboma-

Table 2. Effect of incubation period in trypsin-chymotrypsin incubations and pH in pepsin-HCl incubations on the digestibility-% of microbial and feed proteins.

	Microbial protein		Protein in HCHO-soybean meal		Undegradable protein			
					Sunflower seed cake		Cottonseed cake	
	18 hours	24 hours	18 hours	24 hours	18 hours	24 hours	18 hours	24 hours
pH 1.5—2.0	83.0 85.0	84.0. 83.0	98.0 97.0	97.0 97.0	84.0 83.0	85.0 83.0	85.0 85.0	86.0 86.0
	x̄ 84.0	x̄ 83.5	x̄ 97.5[a]	x̄ 97.0[a]	x̄ 83.5	x̄ 84.0	x̄ 85.0	x̄ 86.0
pH 2.5—3.0	80.0 82.0	86.0 86.0	75.0 75.0	76.0 78.0	83.0 82.0	82.0 83.0	81.0 82.0	83.0 84.0
	x̄ 81.0	x̄ 86.0	x̄ 75.0[b]	x̄ 77.0[b]	x̄ 82.5	x̄ 82.5	x̄ 81.5	x̄ 83.5
pH 3.1—4.0	81.0 81.0	81.0 82.0	69.0 69.0	73.0 73.0	83.0 82.0	83.0 81.0	83.0 82.0	84.0 84.0
	x̄ 81.0	x̄ 81.5	x̄ 69.0[b]	x̄ 73.0[b]	x̄ 82.5	x̄ 82.0	x̄ 82.5	x̄ 84.0

$P < 0.001$, a—b; mean between pH treatments with different letters differ significantly

sum. McALLAN (1981) reported an average value, pH 2.9, for steers and the pH varied form 2.50 to 3.17. Moreover, he also suggested that pH in the small intestine can vary being 5.69 at the beginning of the intestine and approaching pH 7.3 at the terminal end of the small intestine.

In order to study the pH effect, the pH in pepsin-HCl incubations was adjusted to three different levels (Tables 2 and 3). However, any clear changes in the digestibility of undegraded feed proteins and microbial protein were not observed when the pH varied from 1.5 to 4.0. AMBROSE and SNYDER (1964) also found only a limited effect on protein digestibility caused by a change in the pH from 1.2 to 2.5. The digestibility of the formaldehyde treated feed protein was significantly ($P < 0.001$) decreased when a pH higher than 1.5—2.0 was used. These results demonstrated the importance of acidic conditions for the release of formaldehyde-protein complex as suggested for instance by FERGUSON *et al.* (1967) although on the basis of their results as low a pH as in our study would not be necessary.

The effect of the pH on the incubations with trypsin-chymotrypsin was not studied. JOHNSON *et al.* (1983) reported that a pH higher than 6.9 could change the activity of some enzymes of the pancreatin. At least this was the case with α-amylase. However, according to the paper of McALLAN (1981) the effect of pH *in vivo* may not be significant in controlling the activity of trypsin and chymotrypsin.

When protein digestibilities *in vitro* are applied to *in vivo* a critical point might be the length of the incubation period, e.g. the retention time of the feed particles in the abomasum and in the small intestine of the ruminant. In pepsin-HCl incubations length of the incubation may not be important if the activity of pepsin is reasonably high (e.g. AOAC-standard). In that case AMBROSE and SNYDER (1964) did not report clear differences in digestibilities evaluated after the periods of 2, 4, 6, or 16 hours.

In the literature there is not very much information available about the retention time of feed particles in the abomasum and small intestine. In one of the publications COLUCCI *et al.* (1982) reported retention times from 11 to 17 hours for concentrate and roughage in the omasum, abomasum, and small intestine. According to these findings an incubation period of 24 hours in our study was too long. When the incubation period was shortened to 18 hours, which is still reasonable regarding the practical work, there were very small and non-significant changes in the digestibility values of microbial and feed proteins (Table 2).

The digestibilities of undegradable proteins in cotton seed cake and sunflower seed cake were, however, generally higher than reported *in vivo* by HVELPLUND (1983). It is

Table 3. Statistical parameters of the data in table 2 (analysis of variance, factorial experiment).

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-value
Incubation	0.0083	1	0.0083	
S (substrate)	33.4167	3	11.1389	
P (period)	18.7500	1	18.7500	
pH	588.2917	2	294.1458	36.2168***
S × pH	1005.7083	6	167.6181	20.6380***
P × pH	9.1250	2	4.5625	
S × pH × P	19.2083	6	3.2014	
Error	186.8017	23	8.1218	

*** $P < 0.001$

possible that the *in vitro* conditions created optimum circumstances for the activity of enzymes and therefore higher digestibilities were obtained.

In conclusion, the *in vitro* technique is a reasonable method in evaluation of protein digestibility in the lower digestive tract of a ruminant when it is used in studies with microbial protein or nylon bags. However, the analysis has to be carefully made if HCHO-treated feeds are studied.

It is clear that the described technique is relatively complicated and laborous, but it is more simple than the *in vivo* techniques generally available. However, direct comparisons between *in vitro* and *in vivo* determinations have to be made, before *in vitro* results can be applied to *in vivo* conditions.

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SELOSTUS

Proteiinin sulavuuden määrittäminen märehtijän juoksutusmahassa ja ohutsuolessa in vitro

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Tutkimuksessa selvitettiin pötsin sisällöstä sentrifugoimalla erotetun mikrobiproteiinin sekä nailonpussimenetelmällä tuotetun pötsissä hajoamattoman rehuproteiinin sulavuutta *in vitro* -menetelmällä. Mikrobiproteiini eristettiin lampailta, jotka olivat joko heinä- tai heinä-säilörehu -ruokinnalla. Tutkittavat rehut olivat auringonkukkakakku ja puuvillasiemenkakku. Lisäksi tarkasteltiin formaldehydi-käsittelyn (0,5 % -käsittely) vaikutusta soijarouheen valkuaisen sulavuuteen.

In vitro -menetelmässä tutkittava substraatti inkuboitiin pepsiini-HCl- ja trypsiini-kymotrypsiini -liuoksessa. Mikrobiproteiinin keskimääräinen sulavuus oli 80,7 % ja aurinkokukkakakun sekä puuvillasiemenkakun pötsissä hajoamattoman proteiinin sulavuus vastaavasti 83,7 % ja 82,7 %.

Pepsiini-HCl -inkubaatiossa käytetty pH vaikutti merkitsevästi ($P < 0.001$) formaldehydi-käsitellyn soijarouheen valkuaisen sulavuuteen. Sulavuudet pH-alueilla 1.5—2.0, 2.5—3.0 ja 3.1—4.0 olivat vastaavasti 97,2 %, 76,0 % ja 71,0 %.

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