Journal of Animal and Feed Sciences, 2, 1993, 67–72

The influence of pH on *in vitro* protein solubility and enzymatic hydrolysis of protein in feedstuffs

J.W. Cone

Research Institute for Livestock Feeding and Nutrition (IVVO-DLO), P.O.box 160, NL-8200 AD Lelystad, The Netherlands

(Received 2 March 1993; accepted 18 May 1993)

ABSTRACT

In vitro protein solubility at pH 3, 6 and 9 in various feedstuffs was determined, by measuring the amount of crude protein (6.25 × N) passing a paper filter after 2 h incubation at 40°C. Also, the enzymatic hydrolysis of the insoluble proteins was determined after incubation (2h, 40°C) with either pepsin, neutrase (protease of *Bacillus subtilis*) or alcalase (endoproteinase of *Bacillus licheniformis*), which are proteolytic enzymes with pH optimum of approximately 3, 6 and 9, respectively. The feedstuffs tested showed a great variation in protein solubility and hydrolysis. Pea and bean samples had a high protein solubility at pH 9 (78 to 85%), which was decisively lower at pH 3 (0 to 28%). On the contrary, for other feedstuffs, such as maize by-products and meat-and-bone meal, the differences in protein solubility at different pH was less pronounced. The enzymatic hydrolysis of pea and bean proteins with alcalase at pH 9 ranged between 0 and 15%, while in most other feedstuffs these values were considerably higher. Neutrase activity (pH 6) was poor in all feedstuffs with the exception of the wheat products. It was concluded that the origin of the proteins had great influence on the variation in protein solubility at different pH and hydrolysis with enzymes with different pH-optimum.

KEY WORDS: enzymes, feedstuffs, hydrolysis, pH, protein, solubility

INTRODUCTION

Accurate and rapid estimates of protein quality in pigs are essential for practical diet formulation by feed manufacturers and can be obtained by measuring protein solubility and enzymatic degradability with *in vitro* methods, which simulate gastro-intestinal processes of proteolysis. Dietary proteins are gradually solubilized and hydrolyset in different sections of the gastro--intestinal tract of monogastric animals. The rate of proteolysis is closely

interrelated with pH, which is lower in the stomach (1 to 4.5) and higher in the small intestine (4 to 7.4) (Chesson, 1987). In the stomach, proteins are hydrolysed by pepsin and in the small intestine by pancreatic and intestinal enzymes, each with its own properties, pH optimum and affinity for food proteins (Longland, 1991).

Proteins which solubilize at o low pH become already available for digestion in the stomach whereas proteins which solubilize at a high pH pass the stomach and become at first available for digestion during passage through the small intestine, depending on passage rate and pattern of gastric emptying (Usry et al., 1991). *In vitro* simulation of the sequence of protein digestion by pigs is usually done with a two step enzymatic method using pH-values of approximately 1 and 7 (Babinszky et al., 1990; Eggum and Boisen, 1991).

In this *in vitro* study, the solubility of proteins of various feedstuffs in a buffering solution at different pH-values (3, 6 and 9) and their hydrolysis *in vitro* by different enzymes were investigated in order to determine differences in protein properties in different feed samples. The solubility of most globular proteins is profoundly influenced by the pH of the system. Proteins are least soluble at the isoelectric pH and solubility rises sharply on either side of this pH. For that reason the different pH-values were chosen, although pH 9 is not a physiological one. Also the capability of enzymes to attack proteins is a measure for the protein quality in the different feedstuffs.

MATERIAL AND METHODS

One gram-samples of each feed, dried and ground to pass through an 1 mm screen, were incubated at 40°C in 25.0 ml of buffer solutions with pH values equal to 3, 6 and 9. After 2 h the mixture was filtered through 2 layers of N-free filter paper (nr 2095, Schleicher & Schüll, Dassel, Germany) and washed out with the same buffer. The amount of total nitrogen in the feedstuffs and in the incubated residue on the filter paper was determined by the Kjeldahl method according to ISO 5983. The amount of N on the filter paper was accounted as insoluble N. The buffer solutions were either a 0.1 M citric acid-phosphate buffer (pH 3 and 6) or a 0.1 M borate, KCl/NaOH buffer (pH 9). Similar incubations were done with addition of 2.0 g of pepsin (Merck, nr 7190, Darmstadt, Germany) per litre of the buffer at pH 3, 2.0 ml of neutrase (protease of *Bacillus subtilis*, NOVO 0.5 L, Novo-Nordisk, Bagsvaerd, Denmark) per litre of the buffer at pH 6 or 1.0 ml of alcalase (endoproteinase of *Bacillus licheniformis*, NOVO 2.4 L, Novo-Nordisk, Bagsvaerd, Denmark) per litre of the buffer at pH 9. The difference in N content in the residue on the filter paper after incubation with the given buffer alone and

TABLE 1

Concentration of crude protein (g kg^{-1} dry matter), its solubility (%) at pH 3, 6 and 9 and hydrolysis in various feedstuffs for pigs

	Protein (g kg^{-1} DM)	Solubility[1] (%) pH 3	pH 6	pH 9	Hydrolysis[2] (%) P	N	A
Pea							
cv poolse I	255	20	51	82	31	14	0
cv finale	268	28	73	85	40	13	15
Bean							
cv Mythos	231	0	51	78	34	4	0
cv Blandine	342	19	72	85	37	16	0
cv Alfred	285	15	61	85	33	12	0
Soy bean meal (solvent extracted)							
batch A	456	11	20	31	37	15	72
batch B	552	12	20	36	40	15	75
Maize by-products							
cornmeal	103	23	24	33	31	1	63
germmeal (se)[3]	240	17	19	22	24	2	60
feedmeal	165	39	39	41	25	2	51
hominy feed (se)	105	13	16	27	34	1	48
gl feedmeal A[4]	212	34	40	40	26	2	16
gl feedmeal B	238	44	47	48	25	0	33
gl feedmeal C	236	61	63	64	45	0	18
Meat-and-bone meal							
batch A	631	32	32	34	21	7	42
batch B	593	32	35	36	23	8	38
batch C	605	28	30	31	28	11	42
Sunflower meal (solvent extracted)							
batch A	333	11	36	43	57	0	74
batch B	377	11	30	40	57	17	75
Wheat by-products							
bran, batch A	171	28	40	50	38	25	32
bran, batch B	156	25	37	45	32	54	22
middlings	184	31	52	74	46	30	28
feedflour	165	38	44	62	64	55	78
Rapeseed meal (solvent extracted)							
batch A	377	12	22	23	35	10	53

[1] Expressed as % of the difference in N-content in the feedstuff and the residue

[2] expressed as a relative amount of N not solubilized in the buffer
P = pepsin in buffer pH 3, N = neutrase in buffer pH 6 and A = alcalase in buffer pH 9

[3] se = solvent extracted

[4] gl = gluten

with added enzymes was accounted as the amount of N solubilized by the activity of the enzymes and was calculated relative to the amount of N not solubilized by the buffer. The amount of crude protein was estimated as 6.25 × N.

RESULTS AND DISCUSSION

Table 1 shows the protein content (N × 6.25) of the feedstuffs, expressed in g kg^{-1} dry matter. Also the percentage of protein solubilized after 2 h of incubation in the buffer is shown and the percentage of protein hydrolysed by the enzymes at different pH. This percentage was calculated relative to the amount of protein not solubilized by the buffer.

There was considerable variation in protein solubility between the different feedstuffs as measured at pH 3, 6 and 9. For most of the feedstuffs, the solubility was highest at pH 9 and lowest at pH 3. The dependency of protein solubility on pH was most remarkable for pea and bean. With these products, about 80% of the proteins were solubilized at pH 9 and less than 30% at pH 3. With bean (cv mythos) no protein was solubilized at pH 3. This means, that when beans and peas are in the diet, the majority of their proteins is not solubilized by the gastric juices containing pepsin. By moving towards the small intestine, pH rises and more proteins are expected to be solubilized. For maize by-products and for meat and bone meal, the differences in protein solubility at different pH-values were less pronounced. The results indicate that the biological origin of the feedstuff caused variation in protein solubility and hydrolysis at different pH.

The various by-products from maize did not differ in protein solubility in relation to the pH of the buffer with the exception of corn meal and hominy feed which had a higher solubility at pH 9 than at pH 6. All the various wheat by-products followed the same pattern (Table 1) with the highest solubility at pH 9 and the lowest at pH 3 suggesting that the differences in protein solubility at different pH were not affected by the methods of their processing in the milling industry.

Although two of the enzymes used are of bacterial origin and one of them has its highest activity at a not physiological pH (9.0) differences in enzymatic hydrolysis of protein in the different feedstuffs gives an indication of the differences in protein properties in the different feedstuffs and alterations in protein properties by technological processing of the feedstuffs. The three enzymes were used in excess to not induce differences in hydrolysation by differences in protein content of the feedstuffs.

The three different enzymes showed great differences in protein hydrolysis. Neutrase had a weak affinity for protein in most of the feedstuffs. However, neutrase hydrolysed 25—55% of the not solubilized proteins in wheat

by-products. In the case of wheat bran, batch B, even more proteins were hydrolysed by neutrase than by pepsin or alcalase.

With alcalase no or minor amounts of protein in bean and pea were hydrolysed. This means that in the buffer with added alcalase as much protein was solubizlied as in the buffer alone. Because most of the bean and pea proteins were already solubilized in the buffer, it can not be stated that alcalase is not capable of hydrolysing the proteins. Probably bean and pea contain a protein fraction which can neither be solubilized by the buffer, nor hydrolysed by alcalase. This fraction may be enclosed in cell wall structures and may only be solubilized and hydrolysed after degradation of the cell wall structure.

The remarkable lack of fluctuation in the degree of protein hydrolysis in the different pea and bean cultivars under the conditions used suggests that the degree of enzymatic hydrolysis is determined by the origin of the feedstuffs. The same was seen for different batches of soya bean meal (solvent extracted), sunflower meal (solvent extracted) and meat-and-bone meal. There were differences between the wheat and maize by-products in the absolute amount of solubilized and hydrolysed protein (Table 1). These may be due to differences in processing in the milling industry, washing out different pools of protein, each with its own properties.

In general the results show that the pH-value in *in vitro* systems is very important for the degree of protein solubility of feedstuffs and also for the sequence of solubilization of protein during passage through the gastro-intestinal tract with a gradually incereasing pH from the stomach to the colon. However, this means, that when the *in vivo* ileal or faecal digestibility of protein is predicted by *in vitro* methods (Eggum and Boisen, 1991) the pH of the *in vitro* system should not beyond the pH actually present in the animal. Presently available *in vitro* techniques (Babinszky et al., 1990; Hsu et al., 1977; Parsons, 1991) use pepsin-HCl with a pH of 1 to 2.2 to simulate the gastric proteolysis. However, the actual pH in the stomach may be considerably higher, depending on the feeding level, daily ration and physiological status of the animal. Moreover, the pH optima of pepsin is between 2 and 3.5 (Longland, 1991). Further research is needed to study the influence of pH between 1.0 and 3.0 on protein solubility and hydrolysis by pepsin and comparison with *in vitro* data.

To simulate acidity in the small intestine *in vitro* pH-values of 6.5 to 8 are used (Eggum and Boisen, 1991), whereas the actual *in vivo* acidity may vary even more. The results show that the pH in an *in vitro* system determines the degree of protein solubility. Further research is needed to evaluate the influence of pH used in *in vitro* system and make the comparison with *in vivo* data obtained under different physiological conditions of the animals in order to predict more accurately the feeding value of feedstuffs.

REFERENCES

Babinszky L., Van der Meer J.M., Boer H., Den Hartog L.A., 1990. An *in-vitro* method for prediction of the digestible crude protein content in pig feeds. J. Sci. Food Agric. 50, 173-178

Chesson A., 1987. Supplementary enzymes to improve the utilisation of pig and poultry diets. In: W. Haresign, D.J.A. Cole (Editors), Recent Advances in Animal Nutrition. Butter-worths, London, pp. 71-89

Eggum B.O., Boisen S., 1991. *In vitro* techniques of measuring digestion. In: M.W.A. Verstegen, J. Huisman, L.A. Den Hartog (Editors), Digestive Physiology in Pigs. Proceedings of the Vth International Symposium on Digestive Physiology in Pigs. Wageningen (Doorwerth), Netherlands, Pudoc, Wageningen, The Netherlands, pp. 213-225

Hsu H.W., Vavak D.L., Satterlee L.D., Miller G.A., 1977. A multienzyme technique for estimation protein digestibility J. Food Sci. 42, 1269-1273

Longland A.C., 1991. Digestive enzyme activities in pigs and poultry. In: M.F. Fuller (Editore) *In vitro* digestion for pigs and poultry. CAB International, Oxon, UK, pp. 3-18

Parsons C.M., 1991. Use of pepsin digestibility, multienzyme pH change and protein solubility assays to predict *in vivo* protein quality of feedstuffs. In: M.F. Fuller (Editor) *In vitro* digestion for pigs and poultry. CAB International, Oxon, UK, pp. 105-115

Usry J.L., Turner W., Stahly T.S., Bridges T.C., Gates R.S., 1991. GI tract simulation model of the growing pig. Transactions of the ASAE. 34, 1879-1890

STRESZCZENIE

Wpływ pH na rozpuszczalność i hydrolizę enzymatyczną białka pasz oznaczone metodą *in vitro*

Rozpuszczalność białka różnych pasz oznaczano metodą *in vitro* przy pH 3, 6 i 9 mierząc ilość białka ogólnego (6,25 x N) przechodzącą przez sączek bibułowy po 2 h inkubacji przy 40°C. Nierozpuszczone białko hydrolizowano inkubując je z pepsyną, neutrazą (proteaza *Bacillus subtilis*) lub alkalazą (endoproteinaza *Bacillus licheniformis*), które są enzymami proteolitycznymi o optymalnym działaniu odpowiednio przy pH 3, 6 i 9. Rozpuszczalność i hydroliza białka badanych pasz charakteryzowały się dużą zmiennością. Białko próbek grochu i fasoli było najlepiej rozpuszczalne przy pH 9 (78 do 85%), a zdecydowanie gorzej przy pH 3 (0 do 28%). Różnice w rozpuszczalności białka innych pasz, takich jak produkty uboczne z kukurydzy, mączka mięsna i kostna, przy różnym pH były mniej wyraźne. Białko grochu i fasoli ulegało hydrolizie enzymatycznej przy pH 9 od 0 do 15%, podczas gdy wartości te dla większości innych pasz były znacząco większe. Aktywność neutrazy była niewielka dla wszystkich pasz z wyjątkiem pochodnych pszenicy. Wnioskuje się, że rodzaj białka ma duży wpływ na stopień jego rozpuszczalności przy różnym pH oraz na jego podatność na hydrolizę enzymami, o zróżnicowanym optimum pH ich działania.