STUDIES ON THE PROTEIN HYDROLYSIS FOR THE LYSINE ESTIMATION IN FEED

By

Fumio KONDO and Tadashi HATANO

Department of Animal Husbandry, Faculty of Agriculture, Tohoku University, Sendai, Japan

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Introduction

As the first step to investigate the effect of lysine for the milk cows, we have reported on the use of 1 (+)-lysine decarboxylase for the estimation of lysine in feeds (1). It has been said that certain amino acids are destroyed by the acid hydrolysis, especially protein is hydrolysed with carbonyl compounds, aldehydes and carbohydrates; tryptophane is completely destroyed by the action of boiling acid (2-3); a small amount of hydroxyamino acid, serine and threonine, undergoes destruction during the acid hydrolysis (4-7), although basic amino acids are resistant to acid hydrolysis (8), there are some reports stating that both arginine and histidine are destructed during the process (9-10). Thus it is evident that there are many problems on the hydrolysis of feeds before estimation.

It was recently reported (11) that when casein, to which ten folds of starch were added, was hydrolysed, lysine in this protein was almost destroyed.

Considerable amounts of carbohydrates usually are contained in feeds and it is very difficult to extract all the protein in pure form therefrom. Therefore it is doubtful whether we can obtain an adequate value of lysine in feeds even by the use of the precise quantitative method for the hydrolysates of feeds hydrolysed by the mineral acid.

As a proper method for such cases, Ernst Kofrányi suggested that the samples be pre-treated by 5-N formic acid and then hydrolysed by HCl after the nitrogeneous compounds have been collected. It is necessary to establish a precise method for estimating lysine including the hydrolysis procedure. Therefore, we attempted to clarify the quantitative and qualitative differences between the two hydrolysates which were gained by HCl hydrolysis and E. K.'s hydrolysis, and to investgate the effect of time in acid hydrolysis.

Experimental

I. Samples used

Defatted rice-refuse, wheat bran on the market in Sendai, and the green mowed barley which was cultivated on our school farm were examined. Their chemical compositions are given in Table 1, in which pure protein/crude protein and starch/crude protein of the samples are also shown.

Table 1. Chemical composition of the sample (%)

Constituent / Feed	moisture	crude protein (1)	pure protein (2)	starch (3)	(2)/(1)	(3)/(1)
Rice-refuse	12.93	21.06	20.19	34.94	95.85	166
Wheat bran	12.66	17.56	15.55	70.16	88.61	402
Green mowed barley	9.07	17.50	9.50	34.74	54.25	199

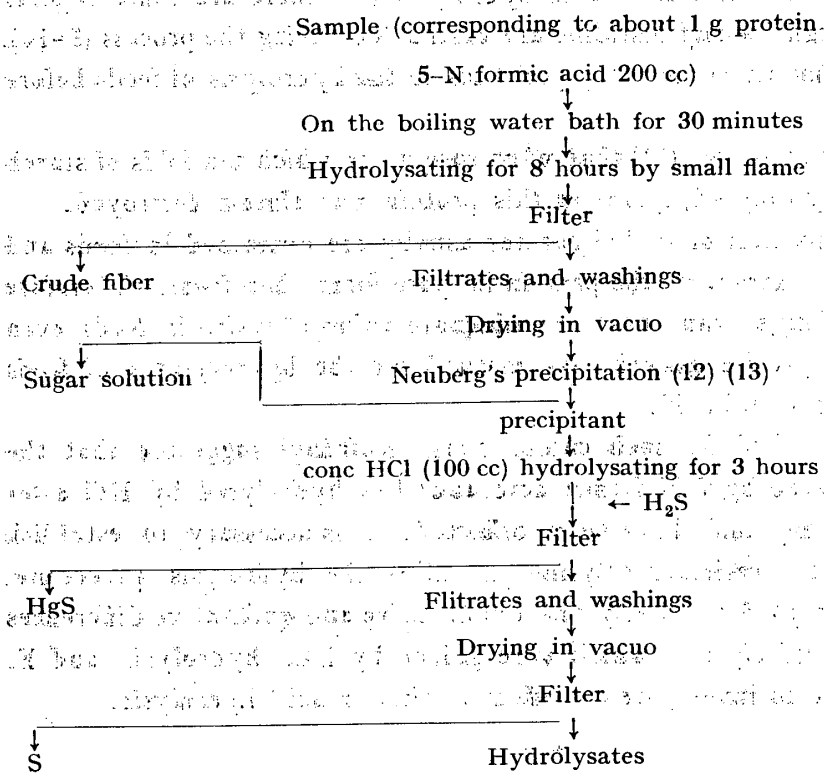
(these values are calculated as starch after the total sugar in the samples were determined by the Bertrand method)

II. Feed hydrolysis

1) 20 percent HCl hydrolysis

The flask containing 10 g of sample and 20 percent HCl 200 cc, was attached with a reflux condenser and then placed respectively for 12 and 24 hours on the

Table 2. The outline of E. K.'s hydrolysis



heater after being on the boiling water bath for 30 minutes. To maintain the same condition, in our experiment two electric heaters (500 W), series conjuncted and covered with asbestos, were used. The hydrolysating flasks were also covered with asbestos.

After hydrolysis, the hydrolysates were filtered, thoroughly washed with hot water and then the filtrates and washings were evaporated together to dryness in vacuo successively for three times. Then the dry residue was disolved in about 15 cc of water and the volume made up to 25 cc. These were used for analysis.

2) Ernst Kofrányi's hydrolysis

We followed E. K.'s method. The outline of this method is shown in Table 2.

The nitrogen loss of each fraction in this method until the final solution was gained, are shown in Table 3.

Table 3. Nitrogen loss of the each fraction in E K's hydrolysis (% for total-N of the sample)

Fraction / Feed	crude fiber	suger solution	HgS
Rice-refuse	15.18	4.12	2.23
Wheat bran	8.55	5.88	2.14
Green mowed barley	10.60	12.56	7.18

We measured the total-N, amino-N, NH_3-N of each hydrolysate gained by the methods described above. Total -N was determined by the Kjeldahl method, amino-N by Van Slyke's method and NH_3-N by the usual method using MgO.

Table 4. The percentage of total-N (1), amino-N (2), NH_3-N (3) in the hydrolysates to the total-N in the sample (4)

	Feed / Method	Rice-refuse	Wheat bran	Green mowed barley
(1)/(4)	E. K.'s hydrolysis	74.52	78.00	53.47
	HCl hydrolysis for 24 hrs	92.18	86.02	82.13
	HCl hydrolysis for 12 hrs	92.89	79.14	79.09
(2)/(4)	E. K.'s hydrolysis	48.11	48.64	31.06
	HCl hydrolysis for 24 hrs	74.03	56.94	61.58
	HCl hydrolysis for 12 hrs	83.59	60.13	67.18
(3)/(4)	E. K.'s hydrolysis	7.28	10.07	12.05
	HCl hydrolysis for 24 hrs	9.96	15.25	9.86
	HCl hydrolysis for 12 hrs	7.55	14.00	7.83

Table 4 shows the percentage of total-N, amino-N, NH_3-N in the hydrolysates to the total-N in the sample.

The percentage of amino-N, NH_3-N to the total-N in the hydrolysates are shown in Table 5.

Table 5. The percentage of amino-N (1), NH_3-H (2) to total-N (3) in the hydrolysate

Method \ Feed		Rice-refuse(%)	Wheat bran(%)	Green mowed barley(%)
(1)/(3)	E. K.'s hydrolys	64.56	62.30	58.08
	HCl hydrolysis for 24 hrs	80.30	66.18	74.99
	HCl hydrolysis for 12 hrs	89.99	75.97	84.94
(2)/(3)	E. K.'s hydrolysis	9.77	12.92	22.54
	HCl hydrolysis for 24 hrs	10.81	17.72	11.99
	HCl hydrolysis for 12 hrs	8.13	17.74	9.90

III. Chromatographic procedure

1 cc of the test hydrolysates solution was neutralized by 1–N NaOH, and made up 5 cc. 0.01 cc of each solution was spotted on the filter paper (Toyo No. 2) on which was measured an area of 30×30 cm^2. The sample was placed at a point which was 3 cm distant from both side of the measured area. It was developed first with phenol, 0.1 percent NH_3 (8 : 2) on the one side for about seven hours. After the solvent was completely evaporated after development, n-butanol-acetic acid-water (4 : 1 : 1) run was employed on the other side for about six hours. After the second development, the sheet was dried thoroughly to remove the solvent at room temperature.

0.2 percent ninhydrin solution in butanol was sprayed on the paper and placed in the oven at 95°C for five minutes.

Each amino acid was determined from the R_f value of the known amino acid and the relative position of each amino acid.

The amino acids detected are shown in Table 6.

Table 6. The amino acids detected in the hydrolysates

	E. K.'s hydrolysis			HCl hydrolysis for 24hr			HCl hydrolysis for 12hr		
	Rice-refuse	Wheat bran	Barley	Rice-refuse	Wheat bran	Barley	Rice-refuse	Wheat bran	Barley
Glycine	+	+	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	+	+	+	+
Threonine	−	−	−	+	+	+	+	+	+
Valine	−	−	−	+	±	±	+	+	+
Asparatic acid	+	+	+	+	+	+	+	+	+
Glutamic acid	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+	+	+
Proline	+	+	+	+	+	+	+	+	+

Though identification was not done, there was another spot in every chromatogram.

IV. Lysine determination

We followed Gale's decarboxylase method which may be outlined as follows. The specific decarboxylase preparation for the estimation of l (+)-lysine used in this experiment corresponds to the acetone powder from *Bact. cadaveris* no. 6578 described by Gale (14).

Bact. cadaveris was grown for 24 hours at 25°C in a medium containing

3 percent tryptic digest of casein and 2 percent glucose and this was made into acetone powder. The powder was kept in an ice chest for a few days. It was then rubbed up in M/5 phosphate buffer of pH 6.0 (20 mg powder/ cc buffer) and 0.5 cc of the suspension was used for each estimation.

Warburg manometers were used to measure the CO_2 liberated from lysine by specific lysine decarboxylase at the optimum pH 6.0 and 30°C. The "acid-tip" method was also applied to estimate the total CO_2.

The results gained by this method are shown in Table 7.

Table 7. Lysine content of the feed (lysine-N/total-N ×100)

Method \ Feed	Rice-refuse	Wheat bran	Green mowed barley
E. K.'s hydrolysis	6.37	6.06	3.04
HCl hydrolysis (24hr)	7.53	5.99	6.55
HCl hydrolysis (12hr)	6.96	6.78	10.38

Discussion

The nitrogen content of the curde fiber in E. K.'s hydrolysis was different from each other and this value of rice-refuse is higher than that of the others. Thus it becomes an interesting problem if the nitrogen is not valuable for the nutrition as once said by Ernst Kofrányi (11). We wish to test this view by the feeding trials.

In the case of the green mowed barley, the amount of nitrogen retained in the sugar solution and HgS exceed that of the others.

It was shown in Table 4 that the NH_3 content of hydrolysates prepared by E. K's method is lower than that hydrolysed by HCl except in the case of barley. We are in the opinion that this may be due to the destruction of non-protein nitrogeneous compounds, which was more abundant in the barley, to NH_3 in the presence of Hg.

Comparing the HCl hydrolysis of 12 hours and 24 hours, we found that the quantities of the total-N transferred from the feed to hydrolysates is greater for 24 hours than for 12 hours, but the amino-N of the solution decreased and NH_3–N increased for 24 hours. With regard to the formation of NH_3 in the hydrolysating procedure, it was said that the formation of NH_3 from amide was completed within four hours and then NH_3 was slowly formed probably from the monoamino acid by deamination (15). Therefore, we think that the hydrolysating of these feeds for 24 hours is too long to obtain the proper hydrolysates.

The percentage of total–N, amino–N of the hydrolysates prepared by E. K.'s hydrolysis per total-N of the feed, showed values lower than those gained

by HCl hydrolysis. However, in E. K's method, as the loss of N in the procedure exceeds that by HCl hydrolysis, we showed NH_3-N, amino-N/total-N in the hydrolysates in Table 5. These values by E. K's hydrolysis was also lower than by HCl hydrolysis.

Amino acids detected by the two dimensional paper chromatography are shown in Table 6. Although the spots corresponding to valine and threonine were clearly detected from an examination of the hydrolysates by the conventional method, we failed to find those spots by an examination of the hydrolysates by E. K'.s method.

Hopkins-Cole's reaction for tryptophane, Pauli's reaction for tyrosine and histidine, and the Sakaguchi's reaction for arginine were also performed. All the hydrolysates gave the same reaction. Tryptophane was destroyed by both hydrolysis. Arginine, histidine and lysine existed in the hydrolysates but we could not find any spots corresponding to histidine and lysine, it is probable that the quantity of these amino acids were too small to give the color with ninhydrin.

The lysine content of the hydrolysates are shown in Table 7, from which it will be noticed that the values obtained by E. K.'s method is lower than those obtained by the conventional method.

Conclusion

We found the conventional method to be more advantageous than E. K.'s method which is rather more complicated, for the estimation of lysine in these samples which contain carbohydrate as is shown in Table 1. However, the hydrolysating period should be selected adequately to the nature of the sample.

The lysine content of the feed shown in Table 7 was higher as was already mentioned in the previous paper. This may either depend upon the difference of the samples or to the difference of analytical method.

To study the effect of lysine for the milk cow, we compared some methods of estimating the lysine and hydrolysating feed, in previous and this papers. We expect to progress our study by use of the decarboxylase method and HCl hydrolysis for lysine estimation.

Summary

To study the effect of lysine for milk cow, we compared E. K'.s method and the conventional method using HCl and obtained the following results.

1) NH_3 content of the hydrolysate prepared by E. K.'s hydrolysis is lower than when hydrolysed by HCl except in the case of green mowed barley.

2) The quantities of total-N of the feeds transferred to hydrolysates is greater for 24 hours than 12 hours hydrolysis but amino-N of hydrolysates

decreased and NH_3-N increased for 24 hours hydrolysis.

3) Two spots corresponding to valine and threonine were not detected in the two dimensional paper chromatogram of the hydrolysate by E. K's hydrolysis though these were found in that of the hydrolysate by HCl hydrolysis.

4) The same results were obtained by Hopkins-Cole's reaction, Pauli's reaction and Sakaguchi's reaction for all of the hydrolysates.

5) Lysine content of hydrolysates obtained by E. K.'s hydrolysis is lower than those obtained by HCl hydrolysis.

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