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On the Splitting-off of the Amide Group from Proteins

The Amides of Zein

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The amide group belongs as an essential part to most of the proteins In connection with the breaking of the peptide bonds by pepsin and trypsin ammonia is also formed. According to Damodaran and Ananta-Narayanan [1] the formation of ammonia is a non-enzymatic reaction whereby the amide groups which are present in peptides formed by proteolytic enzymes are split off at the acid or alkaline reaction. True, Melville [2] has noted that the peptides of glutamine, where the amino group of glutamine is free, are very unstable. The amide group is quantitatively split off from such peptides without any enzymatic effect at pH 1.8 at 37°, *i. e.* in the conditions of peptic hydrolysis.

In examining zein and casein we have noted that at the acid reaction (pH 1.5—1.8) at 37° C or at room's temperature amide nitrogen splits off from these proteins without any preceding pepsin effect. In the following we shall present our experiments pertaining to this as well as our observations on the amide nitrogen in zein.

EXPERIMENTAL

The first experiments were made in Erlenmeyer flasks. In the parallel experiments each flask contained 1 g zein suspended in 50 ml of 0.175 *N* HCl and 2 ml toluene. To one of the flasks was added besides crystalline pepsin (0.6 mg N), to its parallel none. At definite intervals samples were taken from the flasks and ammonia was determined in them. A continuous formation of ammonia was observed even without pepsin. Since, however, in these conditions certain errors are involved in the experimental results owing to the fact that the sample taken from the suspension does not always exactly represent the whole suspension, even be it thoroughly shaken, another method was adopted.

Samples of 100 mg zein, suspended in 5 ml of 0.175 *N* HCl and 0.2 ml toluene,were measured to ampuls. In addition, to the parallel ampuls crystalline pepsin (0.06 mg N) was added. The ampuls were sealed and kept at 37° C shaking occasionally. For each

interruption 1 or 2 ampuls were kept thus the contents of the whole ampul could always be used to determinations of ammonia and amino nitrogen. Frequently 2 parallel analyses were made using 2 ampuls. The results were then regularly in good agreement. The zein used in the experiments (fraction II) was isolated from yellow maize according to the method of Mason and Palmer [3] and fractionated according to the principle of Williams and Watson [4] in the manner modified by Laine [5]. The nitrogen content of the moisture-free zein preparation was 16.2 %. Similar experiments as with zein were made correspondingly with casein (Hammarsten). Ammonia was determined according to Pucher *et al.*[6] using in each receiver 0.01 *N* H_2SO_4 and titrating the excess of acid with 0.01 *N* NaOH. As indicator Tashiro's mixed indicator was used. A quantity of 0.01 *N* acid was equivalent to 0.14 mg ammonia nitrogen. The determination was made as follows.

The contents of the whole ampul were washed to a distilling flask and made alkaline with the Folin's solution. Distillation time was 15 min. Ammonia was determined from the distillate in the above manner. After distillation the residue was made up to 25 ml and amino nitrogen was determined according to Pope and Stevens [7] by the Cu-method.

Glutamic acid was determined according to the principle of Olcott [8] which is based upon a measurement of the loss in amino nitrogen occasioned by the transformation of glutamic acid to pyrrolidonecarboxylic acid at 125° and pH 3.3. pH was adjusted in our experiments to 3.3 by means of acetic acid. The accuracy of the method was tested both with pure glutamic acid and by adding a known amount of glutamic acid to the amino dicarboxylic acid fraction which was obtained by Foreman precipitation from the acid hydrolysate of zein. In the latter case glutamic acid was determined from the Foreman precipitate both before and after addition of glutamic acid. The results of these control experiments are given in the following

No. of expt.	Pure glutamic acid	Glutamic acid added to the hydrolysate of zein
	Found glutamic acid N, % of added	Found glutamic acid N, % of added
1	94.0	94.0
2	95.2	95.0
3	95.3	—
4	93.5	—

According to the above determinations the method in the form employed by us gives on the average 94.5 % of the theoretical value. The values found by us for the glutamic acid in the acid hydrolysate of zein are therefore correspondingly corrected.

Aspartic acid was determined according to Arhimo [9] using the method developed by Pucher *et al.*[6] for determination of malic acid.

RESULTS

Glutamic acid N was found in zein 15.2 % of the total N. Chibnall [10] recently reported an unpublished value of Rees 15.8 %. Taking into account the possible errors in the method used by us the agreement between our values

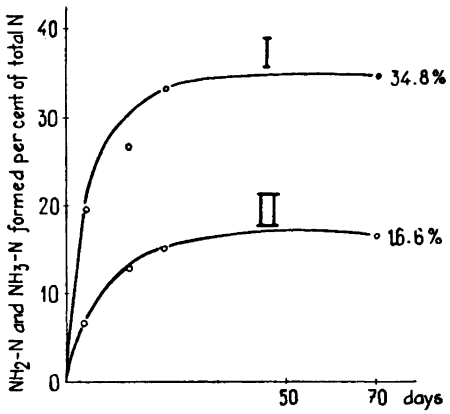

Fig. 1. Formation of amino-N (curve I) and ammonia-N (curve II) in hydrolysis of zein by crystalline pepsin at pH 1.5—1.8 at 37° C.

and those of Rees is fairly good. Laine [5] found in zein glutamic acid N 18.4 %. The method used by him has, however, evidently given too high values.

Aspartic acid N was found by us in zein 2.9 % of total N (2.87—2.99 %). The unpublished value of Rees reported by Chibnall [10] is 3.0 %, the value found by Laine [5] 2.2 %, and that of Dakin [11] 1.18 %. There is a good agreement between Rees's and our values which were obtained by different methods of analysis. The total amount of glutamic acid N and aspartic acid N of zein is according to our findings 18.1 % of total N, according to the values of Rees 18.8 %.

The amount of amide nitrogen found by us in zein was 18.4 % of total N, by Laine [5] 18.6 % and by Chibnall [12] 18.3 %. In regard to amide nitrogen the agreement is thus fairly good. Amide nitrogen corresponds rather accurately the sum total of glutamic and aspartic acid-N, accordingly, the total amide nitrogen in zein can belong to asparagine and glutamine.*

Amino nitrogen was found in zein by the Cu-method 73.2 %, the value of Laine [5] is 74.2 %.

The splitting-off of amide and amino nitrogens from zein *by pepsin* appears from the curves in Fig. 1. The values were obtained by the Cu-method and are too high since the bound NH-groups of lower peptides partly react in this method. The value of 34.8 % found by the Cu-method for amino nitrogen corresponds to about 25 % by the van Slyke method. The average molecular weight in the hydrolysate of zein is of the magnitude of tri-tetrapeptide.

* If a part of the ammonia resulting from acid hydrolysis is split from amino acids, the share of real amide-N is less, being calculated on ammonia. On the basis of the ammonia liberating in peptic hydrolysis it can be concluded that glutamic acid at least is completely amidated.

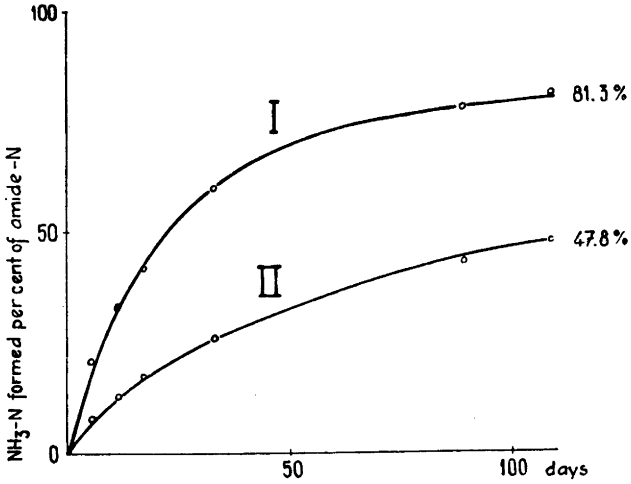

Fig. 2. Splitting-off of ammonia-N from casein (curve I) and from zein (curve II) at pH 1.5—1.8 at 37° C without pepsin.

The ammonia formed in peptic hydrolysis, 16.6 % of total nitrogen, is equivalent to 90.2 % of total amide nitrogen. The entire amide nitrogen is thus not liberated as ammonia in peptic hydrolysis during the experiment (70 days).

Splitting-off of amide nitrogen from proteins as ammonia *without pepsin* is shown by Fig. 2 (zein and casein).

As can be seen from the results large amounts of ammonia have been liberated both from zein and casein at pH 1.5—1.8 without pepsin. From the amide nitrogen contained in zein there have been liberated in 33 days 26 % ammonia-N and in 109 days 48 %. The graph illustrating the velocity of decomposition indicates that the reaction probably ceases at about 50 % unless new decomposition is started by possible breakdowns of the molecule. We have so far followed the ammonia formation in 109 days. In the course of 90 days only 1.8 % amino-N was liberated without pepsin from zein which shows that the peptide bonds are not to any greater extent broken in the zein molecule.

In order to ascertain that the enzymes probably present in the preparation of zein had not caused decomposition of ammonia we heated a water suspension of zein for ½ h at 110° C and made a decomposition experiment with this preparation at pH 1.5—1.8 without pepsin. Ammonia-N was split off in 8 days 10.7 % of the amide nitrogen of zein, hence practically the same quantity of ammonia was set free from heated zein as from unheated.

The amide-N of casein was liberated as ammonia without pepsin in a considerably greater measure than that of zein. Ammonia N amounted in 33 days to 60 % of the amide nitrogen, in 109 days to 81 %. Also the peptide bindings in the casein molecule seem, however, to break down appreciably at pH 1.5—1.8 without pepsin as indicated by the formation of amino nitrogen. After 13 days amino-N was formed 2.2 % and after 109 days 13.6 % of total nitrogen.

These results are in agreement with the results of Carpenter[13] who estimated the soluble and amino nitrogens formed from casein at different temperatures in dilute solutions of casein. Ammonia-N then split off was not determined by him. Osborne and Nolan[14] and Vickery[15] have earlier shown that boiling of gliadin with dilute hydrochloric acid when a relatively small proportion of the peptide bindings are broken yields nearly the same amount of ammonia as is formed during total hydrolysis with concentrated acid.

DISCUSSION

The result obtained show that amide nitrogen is split off at an acid reaction (pH 1.5—1.8) at 37° C from proteins to a very great extent *without enzymatic effect.* Approximately a half of the amide nitrogen of zein seems to be split off in our experimental conditions. The simultaneous increase of amino nitrogen has been very slight, and consequently, no corresponding breaking of peptide bonds has occurred. A small increase in the amino nitrogen was, however, noted.

In the light of the analyses the amide nitrogen in zein corresponds quantitatively to the sum total of glutamic and aspartic acid-N, accordingly, all the amide-N may belong to glutamine and asparagine. Since the share of the amide group of glutamine is about 85 % of the total amide nitrogen and only about a half of the amide nitrogen is split off at pH 1.5—1.8 *without pepsin*, the entire glutamine residue does not give up its amide group in the form of ammonia. Liberation of ammonia is continuously retarded in the course of the experiment. This seems to imply that a part of the amide groups of glutamine is in such a position that its splitting is easily affected at an acid reaction, whereas a part again is located so that the splitting is difficult or impossible. It could be postulated that a part of the amide groups of the glutamine residues is protected in the zein molecule, for instance, by being located between the submolecules united by hydrophobic groups, and consequently, the acid solution has no chance of attacking them, at least not during an experimental period of nearly 4 months.

On decomposition of zein *by pepsin* amide nitrogen is simultaneously split off. The question concerns then, however, the unstability of the glutamine

peptides formed and not the splitting off of amide groups from the zein molecule itself. The entire amide nitrogen is not split off in the peptic hydrolysis. The ammonia formed in about 30 days is approximately equal to the glutamine in zein. This indicates that ammonia is apparently not split off from asparagine peptides, which is in agreement with the findings of Damodaran *et al.*[1] Strange enough, in a period of long duration (70 days) somewhat more ammonia is split off than corresponds to the glutamine residues in the zein molecule, accordingly, ammonia is also formed from some other N-compound besides glutamine. If asparagine peptides were able to give up a little ammonia during prolonged experiment the origin of the excess of ammonia would be explicable. More experiments are needed in this respect. Addition to the proof.

In a later experiment (Virtanen and Kerkkonen), in which after a hydrolysis of 49 days pepsin solution was added to the hydrolysate, total amide-N was split off in 64 days as ammonia (found NH_3-N 18.2 % of total N).

From casein amide nitrogen is split off at pH 1.5—1.8 *without pepsin* even to a much greater extent than from zein. Over a period of nearly 4 months well over 80 % of the amide nitrogen of casein were split off. Since there is no information about whether amide nitrogen in casein belongs entirely to glutamine — the quantity of amide nitrogen (9.1 % of total N) does not by far require the presence of the entire glutamic acid (13.4 % N of total N) in the form of glutamine — it is difficult to say whether ammonia is split off more easily from the glutamine in casein than from that in zein. It must, however, be duly considered that at an acid reaction there is formed appreciably amino nitrogen from casein, and accordingly, the casein molecule seems to break down considerably without pepsin during a longer period. More investigations are needed to explain the breakdown of casein at an acid reaction.

Laine [5] arrived in his studies at the conclusion that pepsin breaks the peptide bonds at the point of glutamine residues in zein, hence, in the peptides formed the amino group of glutamic acid is free and can be oxidized to a hydroxy-group (α-hydroxy-glutaric acid) by means of nitrous acid. The quantity of glutamic acid in zein was according to his determinations so high that it would have recurred (as glutamine residues) as every fourth amino acid in the zein molecule. Since, however, according to the recent determinations the amount of glutamine in zein is lower, this concept does not hold good at least not as such. The sum total of glutamine and asparagine residues, again, is so high that these amino dicarboxylic acids together can replace every fourth amino acid in the peptide chains. The elucidation of this question requires more research.

SUMMARY

It has been shown, that both from zein and casein high percentages of amide nitrogen are liberated as ammonia at an acid reaction (pH 1.5—1.8) at 37° C wthout pepsin. The reaction is non-enzymatic. Over an experimental period of about 4 months about 50 % of the amide nitrogen have been split off from zein, and over 80 % from casein. Since only a few amino groups are liberated from zein during the experiment the amide nitrogen is split off at least to a great extent from undecomposed zein molecule. On the contrary, casein seems to split considerably in these circumstances as indicated by the great increase in the amino nitrogen.

The amide nitrogen of zein (18.4 % of the total nitrogen) well corresponds to the sum of aspartic and glutamic acid N (18.1 % of total N according to our determinations, 18.8 % according to Rees). It is plausible that the amide nitrogen belongs to the asparagine and glutamine residues of zein. In a peptic hydrolysis 90 % of the total amide nitrogen is split off as ammonia (cf. addition on page 852). The glutamine peptides formed in the hydrolysis are first split off.

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