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A NEW TECHNIQUE OF PROTEIN HYDROLYSIS AND ANALYSIS OF THE AMINO ACIDS ON AN AUTOANALYZER

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SUMMARY

A new technique of protein hydrolysis is presented which has been shown to be more efficient than the classical HCl hydrolysis.

The new method consists in hydrolysis with crystallized oxalic acid with some added HCl in a sealed tube. The temperature of hydrolysis seems to be more critical than the time. After hydrolysis the frozen mass is dissolved, filtered, suitably diluted and chromatographed directly without neutralization. No damage to the columns was observed after fifty runs.

Only serine and threonine appear to suffer slight losses due to destruction and the arginine content was lowered somewhat. All the other amino acids show a better recovery.

INTRODUCTION

The hydrolysis of a protein or a polypeptide plays an important role in the determination of its amino acid content. As pointed out by LIGHT AND SMITH[1], this step is one of the weakest links in the entire chain of events leading to a complete analysis of a protein or polypeptide.

Until the present time there has not been a single method described which is able to hydrolyze completely the peptide link without the destruction of one or other of the amino acids. In addition, all the recommended processes for hydrolysis are tedious and time consuming.

So far the refinement of any method necessarily leads to a decrease in yields or to the formation of artifacts (see the excellent review by LIGHT AND SMITH).

Since the introduction of the automatic amino acid analyzer, the micro scale method of MOORE AND STEIN[2] is now employed routinely with or without some modification. This process is considered very good despite the fact that tryptophan is completely destroyed, cystine is reduced to a very low level or completely decomposed, and the threonine and serine levels are lowered to a certain extent.

In order to find a more effective hydrolyzing agent and a less time consuming method we have tried other substances capable of breaking the peptide bond. Some of them have already been mentioned in the literature, *e.g.* monochloroacetic acid

(LIEBEN AND TANDLER[3]) and crystallized oxalic acid (FEIGL[4] and IACHAN *et al.*[5]). Other acidic substances with low melting points (below 100°) have also been used. With the exception of oxalic acid and trichloroacetic acid, no good results were obtained. The method of hydrolysis using oxalic acid as described by IACHAN *et al.*[5] showed more promise, if improved or modified.

EXPERIMENTAL

Both in preliminary and in subsequent experiments, partially purified crystalline bovine albumin was used. In addition, the new technique was tried out on several other crystalline proteins, defatted cocoa beans, and biological fluids.

Acid hydrolysis with 6 *N* HCl was carried out in sealed tubes previously evacuated, using from 1 mg up to 20 mg of material and 5 ml of acid. Hydrolysis conditions were: temperature from 115 to 130° and a hydrolysis time of 16 to 24 h.

After the hydrolysis the tubes were opened, the liquid was diluted, filtered through hard filter paper, and desiccated under low pressure over sodium hydroxide and phosphorus pentoxide, as generally recommended. The residue was redissolved in buffer pH 2.2, filtered again, conveniently diluted and chromatographed in Model 120C amino acid analyzer (Spinco Division of Beckman Instruments, Inc.) using Custom Research Resin type PA-28 and PA-35 for both columns, and a standard programmed operation.

When solid hydrolyzing agents were used they were weighed and one half was introduced into the tube, followed by the protein to be analyzed and finally the rest of the hydrolyzing agent. Some water was added when necessary. The tubes were evacuated and sealed.

All the sealed tubes were of heavy walled borosilicate glass, measuring approximately 18 mm in diameter and 80 mm long; the neck was constructed in order to facilitate the evacuation and sealing operation.

The method finally adopted may be summarized as follows:

Reagents

Crystallized oxalic acid (1.5 g); 6 *N* HCl (0.5 ml).

Protein material

From 1 to 20 mg was used for high protein content. (With defatted cocoa bean, 25% protein, up to 100 mg has been used with good results.)

Procedure

Half of the oxalic acid is placed inside the tube, then the protein, followed by the remaining oxalic acid, and finally, the HCl solution. The tube is evacuated (a few minutes) and sealed, as usual. The sealed tube is immersed in a can containing mineral oil, transferred to an oven regulated at 130° and left overnight (16 h). After cooling and freezing, the tubes are opened and the frozen mass dissolved in water and filtered through a very fine filter paper. The filtrate is suitably diluted and is ready for analysis in the amino acid analyzer.

All the experiments with the new hydrolyzing agents were compared with

those with 6 *N* HCl as described in the experimental part, and chromatographed in a standard operation.

The preliminary hydrolysis with crystallized oxalic acid was made essentially according to IACHAN *et al.*[5], either in a sealed or open tube using 1.5 g of oxalic acid, a temperature of 120° and from 1 mg up to 20 mg of protein.

For the monochloroacetic, trichloroacetic and formic (99%) acid experiments 1.5 g of acid was used and 20 mg of protein. To provide the necessary water for the hydrolysis 0.5 ml of distilled water was added. The temperature was the same as for oxalic acid.

It is very important to note that all the new hydrolyzing agents develop an internal pressure, despite the prior evacuation of the tube. The gas formed contains H_2S (from cystine?). It is important to call attention to the necessity of freezing the ampoule before opening in order to avoid a possible explosion.

RESULTS AND DISCUSSION

The preliminary results obtained are summarized in Tables I and II. Only oxalic and trichloroacetic acids showed some promise with respect to further examination.

TABLE I

HYDROLYSIS WITH NEW HYDROLYSIS AGENTS

Temperature 115°. Time of hydrolysis 24 h, sealed tube. 1.5 g of monochloroacetic, trichloroacetic and formic acids were used and 0.5 ml water.

Reagent	*Moles amino acid/ 100 000 g protein*
HCl 6 *N*	587.8
Monochloroacetic acid	354.7
Trichloroacetic acid	476.4
Formic acid	165.6
Oxalic acid	538.6
Monochloroacetic acid	—no cystine, no valine, no methionine, low serine
Trichloroacetic acid	—no cystine, no methionine, serine preserved
Formic acid	—very low hydrolysis
Oxalic acid	—see Table II

IACHAN *et al.*[5], when describing this method of "dry hydrolysis" with crystallized oxalic acid, claimed a very good recovery of the amino acids. They emphasized the fact that amino acids ordinarily destroyed in the normal acid hydrolysis (such as tryptophan, cystine and serine) were preserved. These authors measured the degree of hydrolysis by the "Sörensen and ninhydrin reactions, and paper electrophoresis". No chromatographic analysis was made.

Our first attempt at using this method, as described by its authors, in an automatic amino acid analyzer, the hydrolysis experiments being made both in a sealed tube and in an open tube, showed low results when compared with normal acid hydrolysis, cystine and tryptophan being completely destroyed. In the open tube

TABLE II

COMPARISON OF HYDROLYSIS WITH HYDROCHLORIC AND OXALIC ACIDS

Moles amino acid/100 000 g protein. Temperature 115°; time of hydrolysis 24 h; crystallized oxalic acid without any addition. Average of two hydrolyses and two chromatographic runs of each.

Amino acids	*6 N HCl*	*Oxalic acid*	
		Sealed tube	*Open tube*
Lysine	78.7	75.3	70.5
Histidine	12.6	12.6	4.3
Arginine	26.3	21.9	15.3
Aspartic acid	57.0	62.8	38.0
Threonine	39.9	26.4	5.2
Serine	23.6	8.8	0.0
Glutamic acid	79.5	80.3	34.8
Proline	31.9	30.3	29.4
Glycine	18.7	18.6	16.6
Alanine	61.3	56.6	67.4
Cystine	0.0	0.0	5.3
Valine	33.4	27.4	29.8
Methionine	3.6	3.3	2.9
Isoleucine	12.9	11.5	8.4
Leucine	63.5	57.7	51.9
Tyrosine	17.6	19.2	15.2
Phenylalanine	31.2	25.9	25.6
Total	591.7	538.6	420.6

results were much lower and serine was also destroyed. However cystine was partially preserved. See Table II.

Furthermore it was observed that neutralization with calcium carbonate lowered the yields of many amino acids. Two hydrolysis experiments were made with the same material, one neutralized with calcium carbonate and diluted with buffer, pH 2.2, and the other without neutralization. Both solutions were chromatographed in the Beckman analyzer. The results obtained showed a decrease of about 15% in the yields of almost all the amino acids for the neutralized solution, showing that calcium oxalate or the excess of calcium carbonate adsorbed some of the amino acids.

As the direct chromatography of the acid hydrolyzate worked so well in the above experiment we were encouraged to repeat this technique, as it could represent a considerable time saving for the overall operation. All the subsequent experiments with oxalic acid were made without neutralization despite its high initial pH. The HCl hydrolyzate was always dried under vacuum and over sodium hydroxide and phosphorus pentoxide. At least fifty runs were made under these conditions without any trouble with the columns.

Apparently addition of water does not decrease the rate of hydrolysis. In experiments not included in this paper the addition of up to 2 ml of water to 1.5 g of crystallized oxalic acid did not alter the rate of hydrolysis. This is very useful for possible analyses of biological fluids.

In order to improve the rate of hydrolysis a higher temperature was tried. A temperature of 130° for 24 h showed the best results, as recorded in Table III.

TABLE III

HYDROLYSIS AT 130° FOR 24 h

Moles amino acid/100 000 g protein; average of two hydrolyses and two chromatographic runs; crystalline oxalic acid without any addition.

Amino acids	*6 N HCl*	*Crystallized oxalic acid*
Lysine	80.4	96.0
Histidine	13.4	12.8
Arginine	27.4	25.4
Aspartic acid	59.2	70.1
Threonine	38.5	28.4
Serine	20.9	7.0
Glutamic acid	84.9	91.1
Proline	32.8	43.3
Glycine	19.1	21.7
Alanine	60.1	68.0
Valine	35.4	37.3
Methionine	3.6	4.1
Isoleucine	13.3	15.2
Leucine	65.5	70.8
Tyrosine	18.3	23.6
Phenylalanine	31.6	35.4
Total	604.3	650.2

Under these conditions a relative decrease in threonine and serine content was observed (*cf.* Table III).

It was also investigated whether the addition of some HCl together with the oxalic acid could improve the hydrolysis rate.

TABLE IV

COMPARISON OF HYDROLYSES AT 130° FOR 16 h

Moles amino acid/100 000 g protein; average of two hydrolyses and two chromatographic runs of each.

Amino acids	*Oxalic acid plus*				*6 N HCl*	
	0.5 ml H_2O	*1 ml H_2O*	*0.25 ml 6 N HCl*	*0.5 ml 6 N HCl*	*16 h at 130°*	*24 h at 115°*
Lysine	78.4	79.1	83.5	93.1	80.4	75.5
Histidine	8.5	11.1	11.7	14.2	13.4	12.1
Arginine	19.1	22.2	21.2	20.3	27.4	27.3
Aspartic acid	61.3	61.0	64.2	64.0	59.2	55.3
Threonine	24.8	26.6	24.3	34.6	38.5	33.6
Serine	8.3	11.1	8.6	14.6	20.9	17.1
Glutamic acid	71.7	70.1	79.3	99.2	84.9	83.8
Proline	35.1	32.0	34.1	36.6	32.8	32.9
Glycine	20.4	20.5	17.9	18.5	19.1	15.8
Alanine	67.7	65.0	65.0	67.2	60.1	66.1
Valine	33.0	32.6	33.8	37.5	35.4	35.8
Methionine	3.8	4.1	3.3	3.5	3.6	3.3
Isoleucine	13.4	13.5	14.5	16.6	13.3	14.9
Leucine	65.6	66.5	66.8	69.0	65.4	66.5
Tyrosine	21.0	20.7	20.5	19.8	18.3	18.3
Phenylalanine	29.7	29.3	29.7	34.0	31.6	30.3
Total	561.7	565.4	578.4	642.7	604.3	588.6

As the temperature of 130° showed better results all the subsequent programmed experiments were made with only 16 h hydrolysis. The results obtained are summarized in Table IV.

The addition of 0.5 ml of HCl to 1.5 g of crystallized oxalic acid proved to be the most effective hydrolyzing agent. In general all the amino acids show higher yields except arginine, threonine and serine which show a relative decrease. Lysine and glutamic acid showed much higher figures. Aspartic acid is also somewhat increased in yield. It is interesting to note that the addition of HCl apparently affords some protection to threonine and serine.

In view of the results obtained we tried to use the new method for the hydrolysis of different materials such as crystalline proteins, defatted cocoa beans and biological fluids.

The hydrolysis of the cocoa bean showed better results than with the normal HCl hydrolysis. Apparently less humin is formed and a larger amount of solid residue is observed probably because inert materials such as fiber or polysaccharides are less hydrolyzed.

It is also interesting that the temperature is more critical than the duration of hydrolysis.

CONCLUSIONS

The hydrolysis of protein material with crystallized oxalic acid together with some added HCl has been shown to be more efficient than the classical hydrolysis method with HCl alone. Only serine and threonine appear to be destroyed to a certain extent, and the arginine content is lowered somewhat. All the other amino acids show a better recovery.

The neutralization step of the hydrolyzate, which is a tedious and time-consuming operation, may be omitted without any danger to the columns of the automatic amino acid analyzer or to the precision of the analysis.

Also, solutions may be analyzed without previous concentration or need only be partially concentrated, provided that the proportion of the remaining water does not exceed very much that recommended, and that concentrated HCl is added to the proportion recommended.

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