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AMINO ACID ANALYSIS: THE HUMIN PROBLEM

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SUMMARY

To minimize the development of coloured condensation products (humin) from proteins (especially glycoproteins) undergoing acid hydrolysis, the inclusion of mercaptosuccinic acid or oxalic acid in the hydrolysate is proposed. The presence of either of these compounds was shown to retard the production of dark brown precipitates and the development of highly coloured hydrolysates due to tryptophan degradation. Amino acid analyses of lysozyme and fetuin were obtained after hydrolysis in the presence of mercaptosuccinic acid or oxalic acid (thioglycollic acid and mercaptopropionic acid were also tested). The clarification of coloured hydrolysates with a resin–Norit mixture was also investigated.

INTRODUCTION

With the development of ion-exchange amino acid analysis procedures of increased speed and sensitivity, it has become more important to remove from the sample substances that can either clog the ion-exchange resin or otherwise interfere with the required course of the analysis. Such a substance is humin, a collective name for a mixture of coloured compounds produced during the acid hydrolysis of many proteins. Depending on their solubility in the buffers used, these compounds can either form a layer on top of the resin bed and give rise to sudden increases in back-pressure from the ion-exchange column, or can move down the column and lead to the occurrence of base-line irregularities during the running of the chromatogram. A major contributory factor in the formation of humin is the presence of tryptophan and amino sugars such as glucosamine and galactosamine in the protein[1]. These compounds undergo extensive degradation during acid hydrolysis, resulting in the production of humin. The sialic acids, which are widely distributed components of glycoproteins, decompose readily in mineral acids to yield dark-coloured material[2].

Humin can be removed from acid hydrolysates by the addition of activated charcoal (Norit), although the process requires that the hydrolysate be neutralized before the addition[3]. A procedure has also been described[3] wherein a mixture of Dowex-1 ion-exchange resin and Norit is used to remove coloured condensation products from acid hydrolysates without prior neutralization. Other workers have attempted to minimize the destruction of tryptophan and consequent production of humin by the addition of thioglycollic acid to the protein before hydrolysis[4].

This compound had previously been used to prevent the oxidation of methionine, tyrosine and carboxymethylcysteine during acid hydrolysis[5,6].

This paper describes an investigation concerning the use of ion-exchange resin–Norit mixtures to remove humin from protein hydrolysates before determining their amino acid contents and the effectiveness of certain compounds (hydrolysate additives) in preventing degradation of tryptophan during acid hydrolysis. The compounds used were thioglycollic acid (TGA), 2-mercaptopropionic acid (MPA), mercaptosuccinic acid (MSA) and oxalic acid. Two proteins were used in the study. The first, chicken lysozyme, was selected because it is known to contain six tryptophan residues but no amino sugar[7,8], and the second, fetuin, an α-globulin found in foetal calf serum, was selected because it contains tryptophan, glucosamine, galactosamine and sialic acid[9,10].

MATERIALS AND METHODS

The glycoprotein, fetuin, was a gift from Dr. E. R. BRUCE GRAHAM of the Australian National University, Canberra. The lysozyme (recrystallized twice) and the 2-mercaptopropionic acid were supplied by Fluka, AG, Switzerland. The thioglycollic acid was obtained from Pierce Chemical Co., Rockford, Ill., U.S.A. The mercaptosuccinic acid was obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. and the oxalic acid (AnalaR) from BDH, Poole, Dorset, Great Britain. The sources of the other reagents used for amino acid determinations have been listed previously[11] and the buffers used were prepared to the formulation of SPACKMAN *et al.*[12]. The amino acid analyses were carried out with a modified Technicon Auto-Analyser[11,13].

Samples of fetuin and of lysozyme were hydrolysed by the method of HIRS *et al.*[14] and the weight of protein used in each case was 2.5 mg. The various sample tubes contained: 1, protein plus thioglycollic acid (20 μl); 2, protein plus mercaptopropionic acid (20 μl); 3, protein plus mercaptosuccinic acid (20 μl of a 1 mg/ml solution); and 4, protein plus 1 ml of oxalic acid solution (1 mg/ml). Tubes numbered 5 and 6 contained no additives. All the samples were hydrolysed for 22 h at 110° in 6 *N* hydrochloric acid. After hydrolysis, samples 1 to 5 were rotary-evaporated to dryness and dissolved in 3 ml of citrate buffer of pH 2.2. A 1-ml volume of each of these solutions was further diluted to 3.5 ml with the same buffer prior to analysis. Sample 6 was rotary-evaporated to dryness and dissolved in 2 ml of de-ionized water. About 10 mg of Norit and 50 mg of Dowex-1 (200–400 mesh) (Cl^- form) were added. The mixture was centrifuged and the liquid phase evaporated to dryness. The sample was subsequently dissolved in 3 ml of citrate buffer of pH 2.2 and further diluted as described for the other preparations. A sample of each protein was also oxidized by performic acid by the method of HIRS[15] to obtain an accurate determination for half-cystine as cysteic acid and for proline.

RESULTS AND DISCUSSION

Table I indicates the colour development and the presence or absence of solid material (humin) in the differently treated acid hydrolysates of lysozyme and fetuin.

When thioglycollic acid, mercaptopropionic acid, mercaptosuccinic acid or

oxalic acid was added to lysozyme (before the addition of mineral acid and sealing under vacuum), it was found that after completion of hydrolysis there was no precipitate or colour development in the hydrolysate. All hydrolysates that did not contain an additive developed a yellow colour but no precipitate. When fetuin was hydrolysed in the presence of thioglycollic acid, mercaptopropionic acid, mercaptosuccinic acid or oxalic acid, no precipitate was formed but there was some yellow colour development, although very little occurred with hydrolysates containing mercaptosuccinic acid or oxalic acid. All the hydrolysates of fetuin that did not contain an additive developed a brown colour and contained a precipitate.

TABLE I

COLOUR DEVELOPMENT IN THE DIFFERENTLY TREATED HYDROLYSATES

Hydrolysate treatment	*Fetuin hydrolysate*		*Lysozyme hydrolysate*	
	Colour	*Humin precipitate*	*Colour*	*Humin precipitate*
TGA	Yellow	—	Clear	—
MPA	Yellow	—	Clear	—
MSA	Faint yellow	—	Clear	—
Oxalic acid	Faint yellow	—	(Very faint yellow)	—
Prior to Norit–resin	Brown	+	Yellow	—
No treatment	Brown	+	Yellow	—

TABLE II

EFFECT OF DIFFERENT ADDITIVES ON THE YIELD OF AMINO ACIDS (residues/mole) FROM THE HYDROLYSIS OF LYSOZYME

Amino acid	*Additive*						
	MPA	*TGA*	*MSA*	*Oxalic acid*	*Norit–resin*	*Untreated*	*Oxidized with performic acid*
Try	4.0	4.9	3.6	Trace	—	—	—
Lys	6.9	5.86	7.2	6.3	7.1	6.8	6.5
His	1.1	1.2	1.2	1.1	1.3	1.1	1.2
Arg	11.8	10.7	11.0	11.1	10.6	11.1	11.5
Asp	21.4	21.7	21.2	20.8	20.3	18.9	20.2
Thr	6.5	6.9	7.1	7.1	6.5	6.8	7.2
Ser	9.2	10.0	9.1	9.5	8.7	9.1	9.6
Glu	5.3	5.3	5.7	5.3	5.2	5.2	5.9
Pro	7.2	7.4	5.7	4.4	3.5	3.7	2.4
Gly[a]	12.0	12.0	12.0	12.0	12.0	12.0	12.0
Ala	12.0	12.0	12.4	12.0	12.1	12.3	12.0
Val	6.1	6.7	6.4	6.3	6.1	6.5	6.3
Met	2.2	2.3	2.3	2.2	1.9	2.2	—
Ile	6.1	6.3	6.0	5.6	5.6	6.1	6.4
Leu	8.1	8.6	8.5	8.3	7.9	8.0	8.9
Tyr	3.1	3.1	3.3	3.2	1.2	3.3	—
Phe	2.73	3.2	3.3	3.3	1.6	3.3	3.1

[a] Glycine taken as twelve residues.

To confirm the role of tryptophan in the development of colour in acid hydrolysates, samples of lysozyme and of a synthetic mixture of amino acids (not containing tryptophan) were hydrolysed in the presence of N-acetylneuraminic acid (sialic acid). The lysozyme hydrolysate was dark brown in colour and the synthetic amino acid mixture was colourless. There were, however, small black specks of solid material present in the latter hydrolysate. Thus, as expected, tryptophan and carbohydrate, when present together in the protein being hydrolysed, give rise to the most highly coloured solutions, whereas in the absence of tryptophan, even when carbohydrate (sialic acid) is present, no colour is produced.

Tables II and III show the amino acid analyses of acid hydrolysates of the two proteins when a mercaptan or oxalic acid additive was either present or absent during hydrolysis. Chromatograms obtained from the lysozyme hydrolysates containing mercaptan (Table II) showed the presence of tryptophan, as a peak preceding lysine, when the dual-column procedure of analysis of SPACKMAN *et al.* was used[12]. However, complete recovery of tryptophan was not achieved, an average of two of the six residues present in the protein[7,8] being lost during hydrolysis. Subsequent hydrolyses of lysozyme in the presence of increased amounts of mercaptans showed only slightly improved recoveries of tryptophan. The lysozyme hydrolysate that contained oxalic acid, although yielding no precipitate and being almost without colour, gave a low recovery of tryptophan. Also, the disappearance of tryptophan was accompanied by the appearance on the chromatogram of a new ninhydrin-positive substance that was eluted just before tryptophan. It has been reported previously that artefacts arising from the breakdown of tryptophan are located in this position on the chromato-

TABLE III

EFFECT OF DIFFERENT ADDITIVES ON THE YIELD OF AMINO ACIDS (residues/mole) FROM THE HYDROLYSIS OF FETUIN

Amino acid	*Additive*						
	MPA	*TGA*	*MSA*	*Oxalic acid*	*Norit–resin*	*Untreated*	*Oxidized with performic acid*
Try	—	—	—	—	—	—	—
Lys	17.8	17.5	17.5	17.2	16.8	16.6	17.5
His	8.3	12.4	7.2	7.4	7.4	7.3	5.8
Arg	13.6	13.2	12.3	12.3	12.3	11.8	11.6
Asp	29.4	29.4	30.0	30.0	30.0	29.5	27.0
Thr	21.0	21.3	19.7	20.6	20.4	20.0	16.3
Ser	23.5	24.0	22.0	22.6	23.0	23.0	21.0
Glu	38.8	39.4	35.0	36.2	40.0	37.2	37.0
Pro	30.0	30.0	29.5	25.0	26.0	24.5	27.0
Gly	21.5	20.0	20.0	19.6	20.2	20.8	19.4
Ala	26.0	25.6	24.0	24.8	25.8	25.0	22.2
Cysteic acid	—	—	—	—	—	—	12.3
$^1/_2$ Cys	—	—	—	5.0	7.5	6.9	—
Val	32.8	33.4	30.0	30.0	31.8	30.0	29.0
Met	3.8	3.8	3.6	3.6	3.3	3.5	—
Ile	12.8	13.2	13.3	13.4	12.8	13.5	12.3
Leu[a]	27.0	27.0	27.0	27.0	27.0	27.0	27.0
Tyr	9.9	10.0	10.4	9.7	2.9	9.2	—
Phe	13.0	13.0	12.8	12.6	5.7	12.3	—

[a] Leucine taken as twenty-seven residues.

gram[16]. When mercaptosuccinic acid is used as the hydrolysate additive, the recovery of tryptophan was slightly lower than when mercaptopropionic acid or thioglycollic acid was used as the additive. Traces of tryptophan found in lysozyme hydrolysates that did not contain an additive could be explained by the presence of hydrogen sulphide as a contaminant in the hydrochloric acid used for hydrolysis.

No tryptophan was detected in any hydrolysate of fetuin, even when the hydrolysis was carried out in the presence of a mercaptan or oxalic acid (Table III). The inability of these compounds to reduce the degradation of tryptophan in this instance can be attributed to the presence of sialic acid and amino sugar residues in fetuin.

Hydrolysates of fetuin and lysozyme that did not incorporate an additive were also treated with a Dowex-1–Norit mixture. The hydrolysates were rapidly decolorized and the analysis of the resulting clear solutions gave values for the concentrations of amino acids that were in good agreement, except for tyrosine and phenylalanine, with the values obtained when hydrolysate additives were used. The chromatograms showed that losses of tyrosine and phenylalanine had occurred through adsorption to the decolorisation agents.

When fetuin hydrolysates that did not contain an additive and had not been decolorised by the resin–Norit treatment were analysed, the resin surface of the ion-exchange columns became darker in colour and the back-pressures from each column were found to increase. The back pressure from the 57-cm column was increased by 25 % and the pressure from the 7-cm column by 200 %. Repeated washings with sodium hydroxide solution restored the back-pressure from the 57-cm column to normal but failed to do so with the 7-cm column. Thus it is concluded that only a limited number of analyses of "untreated" samples of fetuin can be carried out before the top layer of the ion-exchange resin needs to be replaced or the columns re-poured.

Examination of the chromatograms obtained when mercaptan additives were used in the hydrolysates reveals certain complications that arise from their use. These are: (1) the recovery of proline is always greater than that obtained from hydrolysates that do not contain the mercaptans; (2) no cystine is recovered — this is of minor importance as cystine is normally determined independently as cysteic acid and (3) a spurious peak is introduced into the chromatogram that can interfere with the determination of carboxymethylcysteine.

In the system of amino acid analysis of SPACKMAN *et al.*[12], cysteine and proline are eluted together from the 57-cm column and recorded as a single peak. When lysozyme was hydrolysed in the presence of mercaptopropionic acid or thioglycollic acid, the size of the "proline" peak indicated the presence of seven residues of proline and six residues when mercaptopropionic acid was used. With no additives, the proline content was estimated to be four residues. As the accepted value for proline in lysozyme is two residues[7], it seems likely that the excessive number of proline residues indicated was due to contamination of the proline peak by cysteine. Examination of the 570/440 nm ratio over the peak supported this interpretation. MATSUBARA AND SASAKI[4] have also suggested contamination of the proline by cysteine in the amino acid analysis of three proteins, including lysozyme; when thioglycollic acid was used as an additive, the contamination was considerably increased, especially in the case of lysozyme. As all the cysteine in lysozyme is present in the form of cystine, any

cysteine that appears in the hydrolysate must have been formed by splitting of the S–S bridge, presumably by reduction either by the additive used in the hydrolysis or, where no additive was present, possibly by hydrogen sulphide present as a contaminant in the hydrochloric acid used.

A separate determination was made of the cystine content of lysozyme through oxidation of the molecule with performic acid and eight residues of cysteic acid were found. Eight residues of half-cystine have previously been cited for this molecule[7]. A determination of proline was also carried out on the performic acid-oxidized sample and was found to agree with the accepted value of two residues. Fetuin was also subjected to the performic acid oxidation. Twelve residues of half-cystine and twenty-seven residues of proline were found to be present in this molecule.

Under the conditions of analysis used in this work, all the acidic and neutral amino acids except tryptophan are eluted from the 57-cm ion-exchange column; tryptophan is eluted from the 7-cm column along with lysine, histidine and arginine. In the case of the smaller column, no interference with the normal development of the chromatogram was observed due to the presence of the mercaptan in the hydrolysate. However, with the 57-cm column, a peak appears on the chromatogram in a position preceding carboxymethylcysteine and aspartic acid. This peak is recorded in the manner of proline and hydroxyproline, with the 440-nm wavelength cuvette giving the greater optical density deflections. The normal baseline is regained before the print-out for carboxymethylcysteine occurs. This spurious peak arises only when mercaptopropionic acid or thioglycollic acid is used. When mercaptosuccinic acid is used, a small peak located at the position of carboxymethylcysteine appears on the chromatogram. MATSUBARA AND SASAKI[4] have previously reported the presence of a large peak in this position on the chromatogram, arising from the use of thioglycollic acid during acid hydrolysis. These workers hydrolysed smaller amounts of protein (0.1–0.2 mg) than in the present work (2.5 mg) and obtained higher recoveries of tryptophan. They also reported an inaccurate determination for arginine when thioglycollic acid was used in hydrolysates of Hg-papain. In the present work an inaccurate determination for histidine was obtained when this mercaptan was used in fetuin hydrolysates.

Because of the complications that arise from their use, thioglycollic acid and mercaptopropionic acid cannot be recommended as additives in the hydrolysis of fetuin and lysozyme. On the other hand, it is clear that hydrolysates of these proteins prepared in the absence of additives are unsuitable for direct application to the analyser ion-exchange columns. Resin–Norit treatment of these hydrolysates, although removing the danger of column contamination, results in a considerable loss of tyrosine and phenylalanine. The use of mercaptosuccinic acid and oxalic acid as hydrolysate additives appears to effectively overcome these difficulties and is the procedure to be recommended. With the use of these additives, however, a separate performic acid oxidation and subsequent hydrolysis of the protein must still be carried out to enable accurate values for half-cystine and proline to be obtained. This difficulty with the proline determination had previously been encountered by BELL *et al.*[17] in their work with α-lactalbumin.

Table IV shows a comparison between the values obtained in the present study for the amino acid residues of fetuin and lysozyme and those obtained by other workers. The current values shown are those given in column 2 (oxalic acid additive)

of Table II and Table III, adjusted to the nearest whole number. The values for half-cystine and proline in each case were obtained from column 4 of Tables II and III.

TABLE IV

AMINO ACID COMPOSITION OF FETUIN AND OF LYSOZYME

Amino acid	*Fetuin*			*Lysozyme*	
	Calculated no. of residues per mole of protein to nearest whole number			*Calculated no. of residues per mole of protein to nearest whole number*	
	Residues from Table III (Column (2))	SPIRO AND SPIRO[9]	FISHER *et al.*[10]	*Residues from Table II (Column (2))*	CANFIELD[8]
Try	—	2[a]	2[a]	4[b]	6
Lys	17	17	16	6	6
His	7	10	11	1	1
Arg	12	12	16	11	11
Asp	30	33	27	21	21
Thr	21	25	16	7	7
Ser	23	26	21	10	10
Glu	36	34	31	5	5
Pro	27	34	35	2	2
Gly	20	24	18	12	12
Ala	25	33	28	12	12
$^1/_2$Cys	12	12	$<(18)$	8	8
Val	30	40	33	6	6
Met	4	—	—	2	2
Ile	13	15	12	6	6
Leu	27	27	28	8	8
Tyr	10	7	8	3	3
Phe	13	11	9	3	3

[a] Determined spectrophotometrically.
[b] Value taken from lysozyme hydrolysate containing MSA.

It can be seen that the values obtained for the composition of lysozyme are in excellent agreement with those obtained by CANFIELD[8]. For fetuin there is also good agreement, apart from the presence of methionine being detected and the values for histidine, alanine and valine being lower than those previously reported.

From the work carried out on fetuin and lysozyme, the best procedure to follow to determine the amino acid composition of a protein that contains tryptophan and sialic acid, etc., is to hydrolyse four samples of the protein: the first sample to include oxalic acid, the second to include mercaptosuccinic acid, the third to be resin–Norit-treated after hydrolysis, and the fourth to be oxidized with performic acid before hydrolysis.

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