ASPARTIC ACID, ASPARAGINE, GLUTAMIC ACID, AND GLUTAMINE CONTENTS OF WOOL AND TWO DERIVED PROTEIN FRACTIONS

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Abstract

A method is described for the hydrolysis of wool which entails successive reduction, carboxymethylation, and digestion with the three enzymes, Pronase, prolidase, and leucine aminopeptidase. The reliability of the method has been checked using two proteins of known composition, viz. ribonuclease A and insulin.

Amino acid analyses of enzyme digests of wool and of the low- and high-sulphur wool proteins, *S*-carboxymethylkerateines A and B, are presented. The individual contents of aspartic acid, asparagine, glutamic acid, and glutamine, which cannot be determined by analysis of acid hydrolysates, are presented for the first time.

I. Introduction

The acid hydrolysis of proteins followed by automated ion-exchange chromatography is used routinely for amino acid analysis. Because asparagine and glutamine are converted to aspartic acid and glutamic acid during acid hydrolysis, determination of the individual contents of these four amino acids is not possible. However, direct determination of these amino acids is possible if the protein is hydrolysed by an enzymic method which does not convert asparagine to aspartic acid or glutamine to glutamic acid. Hill and Schmidt (1962) have described the application of such a method to a number of soluble proteins but not to wool.

The most satisfactory procedure found by Hill and Schmidt entailed rupture of disulphide bonds by oxidation, followed by digestion with papain and then with a mixture of prolidase and leucine aminopeptidase. However, when this procedure was applied to wool we obtained only moderate yields of many amino acids, presumably due to incomplete peptide bond fission. This paper describes in detail an improved enzyme digestion procedure and its application to ribonuclease A, insulin, wool, and two soluble wool proteins, *S*-carboxymethylkerateines A and B.† Amino acid analyses of enzyme digests of the last three proteins provide, for the first time, individual values for their aspartic acid, asparagine, glutamic acid, and glutamine contents.

II. Materials and Methods

Merino wool top (MW148), cleaned by successive extraction with light petroleum, ethanol, and water, was reduced with 2-mercaptoethanol in the presence of 8M urea, alkylated with iodoacetate, and dialysed against water according to the method of O'Donnell and Thompson (1964). The soluble portion of this crude *S*-carboxymethylkerateine (SCMK) was fractionated into low- and high-sulphur proteins (SCMKA and SCMKB, respectively) by fractional precipitation

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† A preliminary account of this work was presented at the Fourth International Wool Textile Research Conference (Milligan, Holt, and Caldwell 1971).

(Harrap and Gillespie 1963). Ribonuclease A (Worthington Biochemical Corp., U.S.A.) and bovine insulin (Commonwealth Serum Laboratories, Australia) were reduced and carboxymethylated by the same procedure.

Pronase, purified by fractional precipitation from aqueous acetone followed by chromatography on diethylaminoethylcellulose, was kindly donated by Dr. J. R. Yates. Leucine aminopeptidase and prolidase were obtained from porcine kidney. Leucine aminopeptidase was purified by the method of Hill *et al.* (1958) as far as step C; its proteolytic coefficient (C_1) using L-leucinamide as substrate was 0·8. C_1 for prolidase, purified to step 2 (Davis and Smith 1957), was 20 when glycyl-L-proline was used as substrate.

The following digestion procedure was found to give best results. The *S*-carboxymethyl protein (50 mg) was incubated with Pronase (1 mg) in 0·04M borate buffer (20 ml, pH 8·0) at 40°C. In the case of SCMKA and SCMKB, L-norleucine (20 μmoles) was also added as an internal standard. Manganous chloride (0·025M, 0·1 ml) and a solution (1 ml) containing leucine aminopeptidase (5 mg) and prolidase (2 mg) (freshly dialysed against deionized water to remove autolysis products) were added after 48 hr. Incubation at pH 8·0, 40°C was continued for a further 24 hr. A control experiment, in which the wool protein was omitted, was carried out simultaneously. The enzyme digests were finally dialysed against water (4 vol.) at 4°C for 24 hr and the dialysates were evaporated to dryness at 40°C under reduced pressure. The residues were stored at −20°C in the dry state prior to analysis. Acid hydrolyses were carried out with 6N HCl in sealed glass tubes at 105°C for 24 hr. If necessary the enzyme hydrolysis could be carried out on one-tenth the scale using these procedures.

Amino acid compositions were determined with a Beckman Spinco amino acid analyser, model 120B. A modification of the lithium citrate buffer system of Benson, Gordon, and Patterson (1967), which permits resolution of asparagine and glutamine from the other amino acids, was used for the analysis of enzyme hydrolysates. A column (60 by 0·9 cm) of Aminex A4 spherical resin was used, with a buffer flow rate of 68 ml/hr and ninhydrin flow rate of 40 ml/hr. A buffer change from pH 2·95 to 4·15 was made at 150 min, and a temperature change from 38 to 57°C was commenced at 90 min; back pressure was 190 lb in^{-2}. The analysis for acidic and neutral amino acids was completed in 250 min.

The extent of peptide bond cleavage during enzyme digestion was determined by a colorimetric ninhydrin method. The enzyme digest and an acid hydrolysate of the digest were first freed from ammonia and then treated with ninhydrin in pyridine–citrate buffer (pH 5·5). The colour yield of the digest, relative to that of the hydrolysate, provides a measure of the extent of digestion.

III. Results and Discussion

In order to facilitate digestion of proteins with enzymes it is desirable to cleave any disulphide bonds present (Hirs 1956). This is particularly important for proteins with a high cystine content. For example, Hill and Schmidt (1962) found it necessary to oxidize the disulphide bonds of ribonuclease in order to achieve complete proteolysis. We have found in the case of wool that more complete proteolysis occurs if the disulphide bonds are cleaved by reduction and carboxymethylation rather than by oxidation (Milligan, Holt, and Caldwell 1971). The former procedure has the added advantage that it does not destroy tryptophan; a determination of all of the amino acid residues originally present is therefore possible.

Maximum yields of amino acids were obtained from reduced and carboxymethylated wool (SCMK) by digesting first with Pronase and then with a mixture of prolidase and leucine aminopeptidase. We preferred to use Pronase instead of papain as it caused SCMK to dissolve much more rapidly and hydrolysed peptide bonds more extensively than did digestion with papain. Pronase was not inactivated

TABLE 1

AMINO ACID COMPOSITION OF ACID AND ENZYME HYDROLYSATES OF WOOL PROTEINS

Hydrolysate values are given as μmoles/g dry protein. Enzyme hydrolysate yield expressed as a percentage of the amount present in the acid hydrolysate. All values are the means from two replicate experiments; the variability between replicates for the three proteins was 4%. A.H., acid hydrolysate; E.H., enzyme hydrolysate

Amino Acid	SCMK			SCMKA			SCMKB			Autolysis Correction†
	A.H.	E.H.*	E.H. Yield (%)	A.H.	E.H.*	E.H. Yield (%)	A.H.	E.H.*	E.H. Yield (%)	
Lys	241	236	98	293	273	93	56	36	64	67
His	60	68	113	55	52	95	60	48	80	22
Ammonia	1330	—	—	992	—	—	859	—	—	7
Arg	562	565	100	620	605	98	527	448	85	39
Trp	0	36	—	0	27	—	0	30	—	7
SCMC	908	840	93	590	543	92	1764	1748	99	0
Thr	558	521	93	417	404	97	861	920	107	60
Ser	942	929	99	791	801	101	1076	1202	112	64
Pro	609	510	84	365	301	83	1110	964	87	13
Gly	737	574	78	792	663	84	527	456	87	76
Ala	462	404	87	535	477	89	252	197	78	90
Val	480	476	99	501	486	97	475	464	98	66
Met	30	31	103	40	36	90	0	2	—	8
Ile	260	279	107	287	291	101	246	246	100	44
Leu	650	613	94	812	809	100	298	290	97	91
Tyr	310	311	100	364	370	102	158	182	115	39
Phe	225	211	94	259	231	89	144	140	97	42

* Corrected for the contribution due to autolysis.

† Expressed as μmoles of amino acid released by autolysis of the amount of enzymes which would be used for digestion of 1·0 g of wool protein.

prior to addition of the other enzymes and so it was necessary to carry out a "blank" digestion in order to correct the analyses for the amounts of amino acids produced by autodigestion of the enzyme mixture (autolysis).

Table 1 shows the amino acid composition of both acid and enzyme hydrolysates of SCMK, SCMKA, and SCMKB together with the yields of amino acids in enzyme digests, relative to those in the corresponding acid hydrolysates.* These yields averaged 95%. Yields less than 100% are probably due to incomplete peptide bond fission although they may be due partly to the use of invalid autolysis corrections, especially when these corrections are proportionately large. The amounts of amino acids produced by autolysis of the enzyme mixture in the absence of wool may well be larger than those produced when wool proteins and their digestion products are present; hence the values for autolysis correction which we have used may be too high. Yields greater than theoretical probably arise as the result of decomposition of some amino acids during acid hydrolysis in addition to the use of invalid autolysis correction factors.

TABLE 2

GLUTAMIC ACID, GLUTAMINE, ASPARTIC ACID, AND ASPARAGINE CONTENTS (EXPECTED AND EXPERIMENTALLY DETERMINED,* RESIDUES/MOLE) OF RIBONUCLEASE A AND INSULIN

Amino Acid	Ribonuclease A			Insulin		
	Expected†	Found	Yield (%)	Expected‡	Found	Yield (%)
Glu	5	4·88	98	4	4·00	100
Gln	7	4·80	69	3	2·22	74
Asp	5	4·34	87	0	0·16	—
Asn	10	8·02	80	3	2·75	92

* By amino acid analysis of enzyme hydrolysates.

† Smyth, Stein, and Moore (1963).

‡ Ryle *et al.* (1955).

In order to check the yields of glutamic acid, glutamine, aspartic acid, and asparagine produced using our modified enzyme digestion procedure, it was necessary to use proteins in which the contents of these amino acids were well known. Ribonuclease A and insulin were chosen for this purpose. Table 2 shows the theoretical contents of glutamic acid, glutamine, aspartic acid, and asparagine in these two proteins together with the contents determined by amino acid analysis of enzyme digests. The other constituent amino acids were obtained in yields averaging 96% (range 81–102%). Although glutamic acid was obtained in virtually theoretical yield from both proteins, the yield of glutamine averaged only 72%, probably due to loss by cyclization to pyrrolid-2-one-5-carboxylic acid (*cf.* Melville 1935). The relatively low yields of aspartic acid and asparagine are almost certainly due to incomplete peptide bond fission.

* The contents of glutamic acid, glutamine, aspartic acid, and asparagine are shown separately (Table 3) because individual yields of these amino acids cannot be calculated.

The glutamic acid, glutamine, aspartic acid, and asparagine contents of SCMK, SCMKA, and SCMKB determined by amino acid analysis of enzyme digests, are shown in Table 3.* Although individual yields cannot be calculated, it is obvious that the sum of the glutamic acid and glutamine contents is considerably less than the glutamic acid content determined after acid hydrolysis. Similarly the aspartic acid and asparagine contents, when added, are appreciably less than the aspartic acid content of an acid hydrolysate. If we assume, on the basis of the results obtained with ribonuclease A and insulin, that glutamic acid is released in quantitative yield by enzyme digestion, then it is possible to calculate the glutamine content by subtracting the glutamic acid content determined by analysis of an enzyme digest

TABLE 3

GLUTAMIC ACID, GLUTAMINE, ASPARTIC ACID, AND ASPARAGINE CONTENTS FOUND BY ANALYSIS OF ACID AND ENZYME HYDROLYSATES OF SCMK, SCMKA, AND SCMKB

Hydrolysate values are given as μmoles/g dry protein; all values are the means from two replicate experiments; the variability between replicates for the three proteins was 12%. A.H., acid hydrolysate; E.H., enzyme hydrolysate

Amino Acid	SCMK		SCMKA		SCMKB		Autolysis Correction†
	A.H.	E.H.*	A.H.	E.H.*	A.H.	E.H.*	
Glu	985	563	1238	696	690	177	77
Gln	0	330	0	244	0	347	24
Asp	537	168	708	252	203	66	49
Asn	0	312	0	274	0	61	28

* Corrected for the contribution due to autolysis.

† Expressed as μmoles of amino acid released by autolysis of the amount of enzymes which would be used for digestion of 1·0 g of wool protein.

from that determined in the corresponding acid hydrolysate. This method cannot be applied to aspartic acid and asparagine analyses because experiments with ribonuclease A and insulin indicate that neither amino acid is obtained in quantitative yield. Instead, we have assumed that the individual yields of aspartic acid and asparagine in enzyme digests of wool proteins are the same as the average yield of these two amino acids and used this value to correct the experimentally determined values for the occurrence of incomplete peptide bond fission. These calculated values are shown in Table 4, together with independently determined values for the side-chain amide and carboxyl contents of the original proteins. The contents of glutamic acid, glutamine, aspartic acid, and asparagine for wool were derived from those for SCMK by allowing for the weight increase due to reduction and carboxymethylation.

* The relatively poor replication of the analyses for these four amino acids is due partly to the fact that corrections for autolysis are proportionately large and to variable loss of glutamine by cyclization to pyrrolid-2-one-5-carboxylic acid. Greater variability in yield is to be expected in the case of an amino acid which is released from proteins with difficulty.

There is reasonable agreement between the sums of asparagine and glutamine contents and the amide contents and also between the sums of aspartic acid and glutamic acid contents and the carboxyl contents. However, it would be unwarranted to claim that the results are more than semiquantitative in view of the assumption made in calculating the values.

TABLE 4

CORRECTED CONTENTS OF GLUTAMIC ACID, GLUTAMINE, ASPARTIC ACID, AND ASPARAGINE IN SCMK, WOOL, SCMKA, AND SCMKB

Values given as μmoles/g dry protein

Amino Acid	SCMK	Wool*	SCMKA	SCMKB
Glu	563	594	696	177
Gln	422	445	543	513
Asp	188	198	339	105
Asn	349	368	369	98
Gln+Asn	771	813	912	611
Amide†	—	795	827	561
Glu+Asp	751	792	1035	282
Carboxyl‡	—	809	1119	332

* Derived from the contents in SCMK by allowing for the weight increase due to reduction and carboxymethylation.

† Determined by estimation of the ammonia released by short-term acid hydrolysis (Leach and Parkhill 1956).

‡ Derived from the sum of glutamic and aspartic acids in an acid hydrolysate minus the amide content.

It is interesting to note that the carboxyl groups in wool and in SCMKA are present mainly as glutamic acid rather than aspartic acid residues. The distribution of carboxyl and amide side chains is quite different in SCMKB, which has a relatively high glutamine content.

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