STUDIES ON REDUCED WOOL

VIII.* N-ACETYL PEPTIDES ISOLATED FROM A MAJOR COMPONENT

By I. J. O’DONNELL† and E. O. P. THOMPSON‡

[Manuscript received July 27, 1967]

Summary

Two amino terminal acetyl peptides, N-acetyl Ser.Phe.Asp.Phe (A) and N-acetyl Ser.Tyr.Asp.Phe (B), have been isolated from component 8, one of the two major components present in extracts of reduced and carboxymethylated wool. These peptides could not be eluted from columns of Dowex 1 but could be satisfactorily fractionated by DEAE-cellulose chromatography with a gradient of formic acid and by paper ionophoresis. The yield of peptide A was approximately twice that of peptide B. If the molecular weight of component 8 is 45,000 then these acetyl peptide sequences account for only approximately half of the theoretical amount of terminal peptides. This low recovery and the presence of two different but related terminal peptides provides additional evidence for heterogeneity of component 8, and support the idea that a family of related proteins is present in keratin.

I. INTRODUCTION

In a previous paper (Thompson and O’Donnell 1967) the yield of chemical degradation products of the two major components (called components 7 and 8) of extracted wool proteins was studied. These components arise from the “low-sulphur” fraction of wool, which is associated with the “crystalline” or regular portion of the fibre. It has previously been shown (O’Donnell, Thompson, and Inglis 1962) that acetylated residues comprise the major terminal amino groups in wool proteins. Attempts to identify N-acetyl peptides in partial acid and enzymic digests of wool proteins (O’Donnell and Thompson 1964) were unsuccessful, only N-acetylalanine being detected. The N-acetylalanine was present only in small amounts, mainly in the “high-sulphur” fraction of wool proteins, and fractionation of acidic peptides from the low-sulphur fraction was complicated by the presence of large amounts of pyroglutamyl peptides. In the present work purified components of the low-sulphur fraction have been studied. Initially the N-terminal fragment from the cyanogen bromide degradation of component 8 was used since on tryptic digestion it gave an N-acetylated peptide fraction containing only one residue of glutamic acid and consequently was less likely to give acidic pyroglutamyl peptides. The study was extended to component 8 itself which was more readily available, and a quantitative estimation of the acidic peptides released by several different enzymes was carried out. The results cannot be interpreted in terms of a single unique sequence of amino acid residues in component 8 and provide further evidence of its chemical heterogeneity.

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Preparation of Proteins

Component 8 and the cyanogen bromide fragments from it were prepared as described previously (Thompson and O’Donnell 1967). Merino wool from a single fleece (MW 138) was used.

Enzyme Digestions

Digestions with Pronase-P (Kaken Chemical Co. Ltd., Tokyo, Japan) and trypsin and chymotrypsin (Worthington Biochemical Corp., Freehold, New Jersey) were carried out at 37°C on protein solutions (c. 1%), using a weight ratio of enzyme to protein of 1 to 100. The times of digestion varied from 3 to 24 hr and the pH was kept in the range 8–8.5 by the intermittent addition of 1N ammonia. For the longer periods of digestion, the solutions contained 0·10% phenol to prevent bacterial growth. Digestions on small peptides were carried out with Nagarse (Teikoku Chemical Industry Co., Osaka, Japan) for 7 hr at 37°C in 0·2M ammonium acetate at pH 8·5 (cf. Ambler 1963). Digestions with pepsin (Armour) were carried out for 24 hr at 37°C in 5% formic acid (Harris and Polgár 1965).

Isolation of Acetyl Peptides

Acetyl peptides were isolated from enzyme digests of component 8 (400 mg) by two passages through a regenerated column (15 x 0·9 cm diameter) of Dowex 50-X2 (50–100 mesh) in the hydrogen form (Narita 1958a). The eluate which contained acetyl peptides, pyroglutamyl peptides, and cysteic acid peptides, together with small amounts of ninhydrin-positive material, was then subjected to paper ionophoresis at pH 3·5. The peptides were detected by the chlorine–tolidine–iodide method (Reindel and Hoppe 1954) and the required (unstained) bands eluted for amino acid analysis and further study. Alternatively, the eluate was loaded in water onto a column (20 x 0·9 cm diameter) of DEAE-cellulose (Serva Entwicklungslabor, Heidelberg; 0·69 m-equiv/g) in the formate form at 25°C and eluted with a linear gradient of formic acid. An attempt to fractionate the Dowex 50 eluate on a column (15 x 0·9 cm diameter) of Dowex 1-X8 (100–200 mesh) in the formate form and eluted with 0·2N HCl was not satisfactory in this work though it has been for several other proteins (cf. Offer 1965; Alving and Laki 1966). It was found that although acetylsarine was readily eluted under these conditions, the longer peptides containing the aromatic residues phenylalanine or tyrosine or both were not efficiently removed even with 6N HCl. Andersen (1963) has found that di-tyrosine and tri-tyrosine, the cross-linking compounds in resilin, were firmly held by sulphonated polystyrene resins. Alkaline hydrolysies of fractions prior to their determination with ninhydrin reagent were carried out as described previously (Thompson and O’Donnell 1967).

Ionophoresis and Staining

High-voltage paper ionophoresis under toluene at pH values of 3·5 and 1·9 was carried out as described previously (Thompson and O’Donnell 1967). Papers were stained with ninhydrin or the chlorine–tolidine–potassium iodide reagent. Acetyl serine did not stain well [cf. Titani, Narita, and Okunuki (1962) and O’Donnell and Thompson (1964) for staining of acetyl amino acids].

It is of interest that contaminant urea in the protein preparations was unadsorbed by the columns of Dowex 50, Dowex 1, or DEAE-cellulose and moved slowly towards the positive electrode at pH 3·5. It stains readily with the chlorine–tolidine–potassium iodide reagent.

Amino Acid Analyses

Amino acid analyses were carried out using a Beckman Spinco amino acid analyser as described previously (Thompson and O’Donnell 1967). Analyses of small peptides were carried out assuming that the average amounts of the predominant amino acids were integral. Trace amounts were ignored. With a range card fitted to the analyser, satisfactory analyses were carried out on 0·01 µmole peptide. In the later experiments the addition of 10 µl 0·1M phenol to the 1 ml
constant-boiling HCl used for hydrolyses was helpful in preventing severe destruction of tyrosine (Sanger and Thompson 1963).

**Purification of Peptides**

Peptides eluted from DEAE-cellulose were further purified on a column (2·3 by 125 cm) of Sephadex G-25 in 0·01M ammonia. Sometimes purification was carried out using paper chromatography with pyridine–isoamyl alcohol–water (7 : 7 : 6 v/v) (Schroeder et al. 1962) as solvent. N-acetylserylserine was made by the method of Narita (1958b). N-acetyltyrosine was prepared from O,N-diacetyl-L-tyrosine (Mann Research Labs. Inc., New York) as described by Greenstein and Winitz (1961). Pyroglutamic acid was made as described by Ellfolk and Synge (1955).

**Partial Acid Hydrolyses**

Partial acid hydrolyses of peptides were carried out either with 6N HCl at 100°C for 30 min (Ambler 1963) or with 10·5N HCl at 39°C for 3 days. The peptides after removal of acid were separated by paper ionophoresis at pH 1·9.

**Hydrazinolysis**

This was carried out on 0·1 μmole of peptide according to the method of Bradbury (1956), applying the benzaldehyde-extracted solution directly to the amino acid analyser at pH 2·2.

### III. Results

When the insoluble fraction (fraction S2 of Thompson and O'Donnell 1967) of a tryptic digest of CNBr3 (an N-terminal fragment of component 8) was digested with pronase, chymotrypsin, or pepsin, there was an acidic fraction not absorbed by Dowex 50. Paper ionophoresis of these fractions at pH 3·5 gave two bands of similar mobility which were ninhydrin-negative but chlorine–tolidine–potassium iodide positive. These peptides did not contain glutamic acid and were therefore not pyroglutamyl peptides. In view of the interest in their quantitative yields it was essential to isolate them by column procedures. The preparation of CNBr3 is a time-consuming operation and the purity and yield of CNBr3 is variable. For quantitative estimates of the acetyl peptides, digests of component 8 were studied.

(a) **Acidic Peptides from Columns of Dowex 50**

Table 1 lists the amino acid composition of the fraction of the digest unadsorbed on Dowex 50 in the acid form after component 8 was digested with pronase for 4 hr, or alternatively with trypsin for 2 hr followed by chymotrypsin for 16 hr. If the time of pronase digestion was increased to 24 hr the values were similar except for glutamic acid which was markedly increased to 1·4 moles per mole of component 8. It was found that in the 24-hr pronase digests there were large amounts of free pyroglutamic acid which was not the case with 3-hr digests.

(b) **Paper Electrophoresis of Pronase Digest of Component 8**

When the unadsorbed fraction from the Dowex 50 column separation of the pronase digest of component 8 was subjected to paper ionophoresis at pH 3·5, a weakly staining spot running in the same position as N-acetylserylserine (mobility relative
to aspartic acid = 1·5) was observed. This spot on elution and amino acid analysis after hydrolysis yielded serine exclusively and therefore is N-acetylserine. Its yield from a 4-hr pronase digest was 0·42 mole per mole of component 8 and for a 24-hr digest the yield was 0·47 mole. A control experiment in which a standard amount of N-acetylserine was subjected to paper ionophoresis and then eluted with water gave a recovery factor of 100%. There are probably small amounts of peptides containing N-acetylserine remaining in the digest even after 24 hr of digestion and hence the proportion of N-acetylserine in component 8 could be somewhat higher, of the order

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Pronase</th>
<th>Trypsin + Chymotrypsin</th>
<th>Amino Acid</th>
<th>Pronase</th>
<th>Trypsin + Chymotrypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0·28</td>
<td>0·81</td>
<td>Valine</td>
<td>0·13</td>
<td>0·41</td>
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<tr>
<td>Threonine*</td>
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<td>0·28</td>
<td>Isoleucine</td>
<td>0·09</td>
<td>0·14</td>
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<tr>
<td>Serine</td>
<td>0·93</td>
<td>1·12</td>
<td>Leucine</td>
<td>0·17</td>
<td>0·37</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0·37</td>
<td>1·00†</td>
<td>Tyrosine*†</td>
<td>0·13</td>
<td>0·53</td>
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<tr>
<td>Proline</td>
<td>0·24</td>
<td>0·29</td>
<td>Phenylalanine</td>
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<td>0·79</td>
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<td>Glycine</td>
<td>0·34</td>
<td>0·37</td>
<td>S-Carboxymethylcysteine</td>
<td>0·40</td>
<td>0·34</td>
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<tr>
<td>Alanine</td>
<td>0·28</td>
<td>0·16</td>
<td>Cysteic acid§</td>
<td>0·18</td>
<td>0·08</td>
</tr>
</tbody>
</table>

* No correction factor has been applied for the destruction of these amino acids during hydrolysis.
† Tyrosine values varied somewhat (being as low as 0·05 for pronase and 0·21 for trypsin plus chymotrypsin digests) from experiment to experiment and this is assumed to be due to a non-reproducible destruction of tyrosine.
‡ With shorter digestion times values were smaller but somewhat erratic.
§ These values probably arise from the cysteic acid initially in component 8 and not from destruction of S-carboxymethylcysteine on hydrolysis.

of 0·5–0·6 mole per mole, but it is unlikely to approach 1 mole per mole of component 8. In a previous paper (O'Donnell and Thompson 1964) it was stated that we could find no evidence for the presence of N-acetylserine in enzyme digests of a low-sulphur wool protein fraction S-carboxymethylkeratine A (SCMKA). This was no doubt due to the weakly staining properties of N-acetylserine with the chlorine–tolidine–potassium iodide reagent, to the contamination with pyroglutamyl peptides, and to the fact that only approximately one-third of SCMKA is component 8, the other two-thirds being component 7 and its aggregates (Thompson and O'Donnell 1965). If there is no N-acetylserine in component 7, then that arising from component 8 would tend to be concealed by pyroglutamyl peptides in digests of SCMKA. The evidence to date suggests that there is no acetylserine released by pronase from component 7.
(c) Paper Ionophoresis of Peptides from Component 8 after Digestion with Trypsin and Chymotrypsin

When those peptides unadsorbed by Dowex 50 in a combined trypic–chymotryptic digest of component 8 were subjected to paper ionophoresis at pH 3.5 there were two acidic peptides which reacted negatively to ninhydrin but positively to the chlorine–tolidine–potassium iodide reagent. In some experiments they moved at a speed of 0.63 (peptide B) and 0.64 (peptide A) relative to aspartic acid (distances being measured from the position of the neutral amino acids) but the ease of separation varied for unknown reasons. It was probably related to the buffering capacity of the toluene-dissolved buffer during ionophoresis. When eluted from paper, peptide A had the analysis (Ser,Asp,Phε₂) and peptide B had the approximate analysis (Ser,Asp, Phε,Tyr) but with some other contaminating amino acids. Digestion of CNBr₃ with pepsin or for 1–2 hr with pronase yielded a mixture of the acetylated tripeptides (Ser,Phε,Asp) and (Ser,Tyr,Asp).

**Table 2**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Amino Acid</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0.35</td>
<td>0.33</td>
<td>Isoleucine</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Threonine*</td>
<td>0.05</td>
<td>0.10</td>
<td>Leucine</td>
<td>0.07</td>
<td>0.12</td>
</tr>
<tr>
<td>Serine*</td>
<td>0.33</td>
<td>0.43</td>
<td>Tyrosine*</td>
<td>0.09</td>
<td>0.15</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>0.08</td>
<td>0.15</td>
<td>Phenylalanine</td>
<td>0.52</td>
<td>0.41</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.08</td>
<td>0.13</td>
<td>S-Carboxymethylcysteine</td>
<td>0.02</td>
<td>0.15</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.03</td>
<td>0.04</td>
<td>Cysteic acid</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td>Valine</td>
<td>0.07</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Phenol (which minimizes tyrosine losses) was not added in these experiments; no correction factor was applied for destruction of these amino acids during hydrolysis.

When peptide A and peptide B from a trypic–chymotryptic digest of component 8 were eluted together after electrophoresis, the mixed bands were analysed (Table 2). If the serine and aspartic acid values are taken to be indicative of the amount of N-acetyl tetrapeptides present, it is seen there are about 0.35 mole per mole of component 8. This agrees fairly well with the amount of acetylserine released by pronase digestion. Note that there is some variation between the two analyses, as might be expected for incompletely purified mixtures, but that the phenylalanine plus tyrosine is approximately double the aspartic acid value.

(d) Isolation of N-Acetyl Peptides from Component 8 on DEAE-cellulose

Figure 1 shows the elution pattern from DEAE-cellulose of the acidic peptides (i.e. unadsorbed by Dowex 50) present in a trypic–chymotryptic digest of component 8. The high ultraviolet absorption indicates the location of the tyrosine-containing peptide B. Analyses of these fractions are given in Table 3. Again if the
serine and aspartic acid values are taken as indicative of the total amount of tetrapeptides present, then 0·4–0·5 mole tetrapeptide per mole of component 8 is present. Of the total tetrapeptides, peptide A comprised two-thirds and peptide B one-third.

Peptide A gave a single spot when chromatographed in pyridine–isoamyl alcohol–water. Partial acid hydrolysis liberated a peptide which moved slower than serine during ionophoresis at pH 1·9 and on analysis gave serine and phenylalanine. Hydrazinolysis gave a 40% yield of phenylalanine and the sequence of peptide A is therefore acetyl Ser.Phe.Asp.Phe.

Peptide B was purified by passage through Sephadex G-25 but different preparations varied in the degree of purity obtained. When chromatographed with pyridine–isoamyl alcohol–water, it ran predominantly as a single spot with the same R_F as peptide A. There was a small amount of peptide which was found to contain

| Table 3 |
|------------------|------------------|-----------------|------------------|
| Amino Acid       | Peptide A        | Peptide B       | Amino Acid       | Peptide A | Peptide B |
| Aspartic acid    | 0·31             | 0·11            | Valine + 1/2 cystine | 0         | 0·05      |
| Serine*          | 0·31             | 0·16            | Leucine          | 0         | 0·02      |
| Glutamic acid    | 0·03             | 0·03            | Tyrosine*        | 0         | 0·14      |
| Glycine          | 0·03             | 0·02            | Phenylalanine    | 0·59      | 0·14      |
| Alanine          | 0                | 0·01            |                  |           |           |

* No correction factor has been applied for the destruction of these amino acids during hydrolysis.

serine and tyrosine. Digestion of 0·2–0·3 μmole of this peptide B with Nagarse gave an acidic fraction which, when isolated by passage through a small (1·5 ml) column of Dowex 50-X2 (100–200 mesh), on analysis after hydrolysis was shown to contain also serine and tyrosine. The material retained by the Dowex 50 after elution with ammonia followed by hydrolysis contained aspartic acid and phenylalanine. Hydrazinolysis gave a 100% molar yield of phenylalanine and therefore the sequence of peptide B is acetyl Ser.Tyr.Asp.Phe. Paper chromatography did not give good separations of peptide A from peptide B. Kilmartin and Clegg (1967) could only separate peptides with a tyrosine substituted for a phenylalanine in the α-chains of horse haemoglobin by prolonged chromatography in butanol–acetic acid–water–pyridine (15 : 3 : 12 : 10 v/v). Ionophoresis was not satisfactory.

It was found that acetylserine and acetyltyrosine are eluted from the DEAE-cellulose column in the same positions as peptides A and B respectively. Pyroglutamic acid is eluted in the same position as peptide B. Acetylalanine is eluted about five tubes earlier than peak A.

Using 1400 as the molar extinction coefficient for tyrosine, the calculated value for the tyrosine content of peak B of Figure 1 agrees with that obtained analytically
in the hydrolysate. So there was little or no destruction of tyrosine during hydrolysis in this particular experiment. In other experiments, however, there was a substantial loss of tyrosine which was prevented in the later experiments by the addition of phenol.

(e) **Behaviour of Peptides A and B on Sephadex G-25**

Both peptides gave two incompletely separated peaks on Sephadex G-25 which had identical amino acid analyses and the most probable explanation is that one has the C-terminal Phe on the α-aspartyl carboxyl group and one has the C-terminal Phe on the β-aspartyl carboxyl group (cf. Groskopf et al. 1966).

![Fig. 1. — Elution curve from a column (20 by 0.9 cm diameter) of DEAE-cellulose of the acidic peptides (i.e. unadsorbed by Dowex 50) from a tryptic-chymotryptic digest of 400 mg component 8. A linear gradient from water (100 ml) to 0.07M formic acid (100 ml) was used. Fraction size c. 2.4 ml. Flow rate 10–12 ml/hr. 200-μl aliquots taken for alkaline hydrolysis before ninhydin colour development and measurement at 570 mμ (●). The remainder of the fraction was diluted with 1 ml water before measurement of ultraviolet absorption at 276 mμ (×).](image)

**IV. DISCUSSION**

A knowledge of the N-terminal residues of a protein can provide useful information regarding possible heterogeneity of the preparation both from a qualitative and a quantitative point of view. In the case of masked (e.g. acetyl) terminal amino groups, quantitative estimation can be difficult because it relies on the separation of an acidic peptide fraction not bound to sulphonated polystyrene resin which, besides the acetyl peptide, can also contain pyroglutamyl or cysteic acid peptides.

If it is assumed that the enzyme has split the bonds quantitatively and that no basic amino acids are attached to the acetylated amino acid residues in the peptides produced, then an analysis of the material present in enzyme digests which was not adsorbed on the ion-exchange resin Dowex 50 gives a maximum permissible value for possible masked terminal amino acids. If acetylserine is the sole terminal residue then the estimate will be high, to the extent that serine-containing pyroglutamyl and cysteic acid peptides are present.
Our results show that N-acetylserine is a major N-terminal residue, the maximum amount of which from such analyses (calculated from the serine value) is of the order of 1 mole per mole of component 8. However, direct isolation of N-acetylserine by paper ionophoretic techniques yielded only 0.45 mole per mole of component 8 while isolation on DEAE-cellulose yielded approximately 0.50 mole. This value is somewhat low due to the presence of larger peptides containing N-acetylserine but would probably not exceed 0.6 mole per mole of component 8. If the enzyme digestion is not complete then the value could be higher.

Similarly, the analysis of the acidic peptides identified in a tryptic–chymotryptic digest of component 8 show a maximum amount of serine to be 1 mole per mole of component 8 (some serine could be formed from contaminating non-terminal peptides). However, fractionation of the peptide mixture by paper ionophoresis on DEAE-cellulose shows that the amount of purified N-acetylserine peptides corresponds only to 0.47 mole per mole of component 8 in agreement with the acetylserine value from pronase digests.

Thus, on the facts available, we have been unable to find enough N-terminal residues for there to be one residue per mole of component 8 of molecular weight 45,000. It is always possible that there are other N-terminal sequences with basic residues at (e.g. N-acetylgarginine) or near (e.g. N-acetylserylarginine) the terminal acetyl group which would not be detected by our present methods. An accurate determination of total acetyl would help to decide this. Alternatively pyroglutamyl terminal residues could be present as have been found in other proteins (cf. Wilkinson, Press, and Porter 1966; Ikenaka et al. 1966). If either of these possibilities do exist in component 8 it is indicative of heterogeneity among the protein molecules. Moreover, our results show that two different N-acetylserine sequences are present. The major one is N-acetyl Ser.Phe.Asp.Phe, but there is also present the related sequence N-acetyl Ser.Tyr.Asp.Phe in an amount approximately half that of the major peptide. Some of our results suggest that some of the terminal phenylalanine in this peptide may also be replaced by tyrosine.

The results reported here confirm the idea previously deduced from a study of the degradation products of component 8 using cyanogen bromide or trypsin (Thompson and O'Donnell 1967) that there is more than one different polypeptide chain in this component. The similarity in peptide sequences of the first tetrapeptide is consistent with the idea of a family of related proteins.

In comparison with other peptide chains with masked N-terminal residues, it is of interest that so far only single acetylpeptide sequences (but see Moczar, Alving, and Laki 1966) have been found in actin (Alving and Laki 1966; Gaetjens and Bárány 1966), myosin (Offer 1965), and tropomyosin (Alving, Moczar, and Laki 1966). However, in the peptide chains of immunoglobulins different pyroglutamyl terminal sequences have been reported (Wilkinson, Press, and Porter 1966). The variations reported in other proteins were discussed in Part VII.

V. Acknowledgments

We wish to thank Dr. H. Lindley for helpful discussion and for suggesting the use of DEAE-cellulose to avoid the disadvantages we encountered with Dowex 1.
VI. References
