PYRROLID-2-ONE-5-CARBOXYLIC ACID AS AN *N*-TERMINAL GROUP OF THE LOW-SULPHUR PROTEINS OF WOOL*

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The detection of *N*-terminal pyrrolid-2-one-5-carboxylic acid (pyroglutamyl) residues in proteins is complicated by the ready formation of pyroglutamyl peptides from peptides with *N*-terminal glutaminyl residues which may be liberated during enzymic digestion of proteins. Recently, several methods for overcoming these problems have been described (Ikenaka *et al.* 1966; Wilkinson, Press, and Porter 1966; Doolittle and Armentrout 1968) and this communication describes the results obtained when two of these methods were applied to component 8, one of the two major low-sulphur proteins extracted from reduced and carboxymethylated wool (Thompson and O'Donnell 1967).

Interest in the question of whether pyroglutamyl terminal groups are present in wool arises from the lack of stoichiometry in the yield of acetylated amino terminal residues. Previously it has been reported that acetylated peptides *N*-acetylSer.Phe.Asp.Phe and *N*-acetylSer.Tyr.Asp.Phe have been isolated from component 8 (O'Donnell and Thompson 1968) in yields totalling 0·45–0·5 mole/mole of component 8 (M.W. 45,000). The low recovery could be due to the presence of other sequences with blocked amino groups which would not be amenable to the isolation of acidic peptides using the method of Narita (1958). It was concluded that either sequences such as *N*-acetylSer.Arg were present or that the remaining *N*-terminal groups could be pyroglutamyl residues.

Recently, Jeffrey (1968) reported evidence which suggests that component 8 consists of two subunits each of molecular weight approximately 22,500 (cf. DeDeurwaerder and Harrap 1965). If this is correct amino terminal residues in addition to acetyl groups are required, since total acetyl contents (O'Donnell, Thompson, and Inglis 1962) are unlikely to exceed 1 mole per 30,000 g.

To prevent pyroglutamyl peptide formation after the enzymic cleavage of the protein Wilkinson, Press, and Porter (1966) allowed the free amino groups of the peptides in a 3-hr digest to react with 1-fluoro-2,4-dinitrobenzene before passage through sulphonated polystyrene, while Ikenaka *et al.* (1966) (cf. Prahl 1967) poured the digest through a cold column (4°C) of sulphonated polystyrene. Glutamic acid found in acid hydrolysates of eluates from these columns was assessed to have been present as *N*-terminal pyrrolid-2-one-5-carboxylic acid groups in the original protein. Both of these methods have been applied to component 8.

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Experimental

In a previous paper (O'Donnell and Thompson 1968) the methods of enzyme digestion (using 200–400 mg of component 8 per digestion) and isolation of acidic peptides (with no positively charged side-chains) were described. In addition to the separation of acidic peptides on Dowex 50 at room temperature (*c.* 22°C) separations were carried out at 4°C (Ikenaka *et al.* 1966).

The method of Wilkinson, Press, and Porter (1966) for preventing pyroglutamyl peptide formation in the enzymic digest of component 8 was used as described by them for heavy chains of immunoglobulin G. In a second experiment (using 160 mg) attempts were made to extract the last traces of dinitrophenyl derivatives from the hydrolysates by increasing the number of extractions with ethyl acetate.

Results and Discussion

(i) *Method of Wilkinson, Press, and Porter (1966).*—Table 1 gives the results of experiments carried out by this method together with those from a similar digestion where no dinitrophenylation, ethyl acetate extractions, or column separations on

TABLE 1

AMINO ACID CONTENTS OF HYDROLYSATES OF ELUATES FROM DOWEX 50 CHROMATOGRAPHY OF PRONASE DIGESTS OF COMPONENT 8

Component 8 was digested for 3 hr with pronase at pH 8–8·5 at 37°C. Fluorodinitrobenzene was added (Wilkinson, Press, and Porter 1966) and the pH kept constant for 3 hr in a pH-stat. Several extractions with ethyl acetate and one passage through a column of talc were necessary to remove fluorodinitrobenzene and dinitrophenyl peptides before passage through the Dowex 50 column

Amino Acid	Moles of Amino Acid/Mole of Component 8 (M.W. 45,000)		
	40 mg Protein Digested	160 mg Protein Digested	Control*
Aspartic acid	0·10	0·05	0·30
Threonine†	0·11	0·06	0·27
Serine†	0·57	0·28	0·94
Glutamic acid	0·25	0·14	0·39
Proline	trace	trace	0·27
Glycine	0·20	0·11	0·33
Alanine	0·09	0·05	0·16
Valine	trace	trace	0·19
Isoleucine	trace	trace	0·10
Leucine	0·04	trace	0·18
Tyrosine	trace	trace	0·13
Phenylalanine	trace	trace	0·06
S-Carboxymethylcysteine	0·04	trace	0·17

* No treatment with fluorodinitrobenzene or ethyl acetate extraction.

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

talc were used. It is seen that when fluorodinitrobenzene is used to prevent pyrrolidone carboxylic acid formation the serine and glutamic acid values are lower than when it is not used and also that the values in the experiment in which 160 mg is used, which had more extraction with ethyl acetate than the one in which 40 mg is used, are lower than those in the latter experiment. This is probably due to the

formation of *O*-dinitrophenyl derivatives of serine and serine peptides and their subsequent extraction into the ethyl acetate. The main conclusion from this experiment is that glutamic acid is present in the hydrolysates of the eluates from the Dowex 50 column. This suggests that there are in fact pyroglutamyl groups in component 8. The results cannot be interpreted on a quantitative basis because of the presumed loss of *O*-dinitrophenyl peptides. It is interesting that in both cases the glutamic acid value is half the serine value.

(ii) *Method of Ikenaka* et al. (*1966*).—Serine and glutamic acid were the only two amino acids present in significant amounts in the hydrolysate of the eluate obtained on passing a pronase digest of component 8 through a Dowex 50 column. The temperature (4 or 25°C) of the column had no significant effect on the quantitative values. After a digestion period of 3–4 hr the glutamic acid content was *c.* 0·4 mole/mole of component 8 (M.W. taken as 45,000). This value increased to 1–1·5 and 2·2 after digestion for 24 and 48 hr respectively. Paper ionophoresis showed that these eluates contained acetylserine and pyroglutamic acid as major bands. There was also evidence of a much smaller amount of ninhydrin-negative peptide whose acid hydrolysate contained serine and glutamic acid. This could be *N*-acetyl-Ser.Glu (or $GluNH_2$) or pyroglutamylSer. The presence of the latter compound can be used to interpret the serine and glutamic acid contents obtained using the method of Wilkinson, Press, and Porter (Table 1). Thus it is possible that the eluate from the Dowex 50 column contained equal amounts of acetylserine and pyroglutamyl-serine. Only trace amounts of other pyroglutamyl peptides could be found in bands on the paper.

Wilkinson, Press, and Porter (1966) and Ikenaka *et al.* (1966) used the cyclization of glutamine to pyroglutamic acid as a control in their experiments. In the present work the stability of this compound was confirmed using pronase digestion for a period of 24 hr. Additional peptide controls [$Glu(NH_2).Asp(NH_2)$ and $Glu(NH_2).Glu(NH_2).Asp(NH_2).Asp(NH_2)$] showed no cyclization of their *N*-terminal glutamine residues in 6 hr and less than 10% in 45 hr. If these control experiments be accepted as valid controls for the behaviour of all peptides, the results given here suggest that there is at least 0·4 mole of amino-terminal pyroglutamyl groups per mole of component 8 and it is hence a major end-group.

The larger value of 1–1·5 obtained for a 24-hr pronase digest may be partially artifactual (due to ring closure of any internal glutaminyl peptides released during the pronase digestion) but, if not, pyroglutamyl groups together with *N*-acetylseryl groups could account satisfactorily for the *N*-terminal groups of component 8 if the molecular weight is 22,500.

In addition to the heavy chain of immunoglobulin G and α_1-acid glycoprotein, pyrrolidonylamino terminii have been reported in other peptides and proteins (for summary see Blombäck 1967). To date component 8 is the only protein in which both acetyl- and pyrrolidonylamino terminii have been reported but the unambiguous demonstration of pyroglutamyl *N*-terminii in any peptide or protein would only seem possible by the use of the recently isolated enzyme pyrrolidonyl peptidase (Doolittle and Armentrout 1968).

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References

Blombäck, B. (1967).—In "Methods in Enzymology". Vol. XI. p. 398. (Ed. C. H. W. Hirs.) (Academic Press, Inc.: New York and London.)

DeDeurwaerder, R., and Harrap, B. S. (1965).—*Makromolek. Chem.* **86,** 98.

Doolittle, R. F., and Armentrout, R. W. (1968).—*Biochemistry* **7,** 516.

Ikenaka, T., Bammerlin, H., Kaufmann, H., and Schmid, K. (1966).—*J. biol. Chem.* **241,** 5560.

Jeffrey, P. D. (1968).—An associating low-sulphur protein from wool. *Biochemistry.* (In press.)

Narita, K. (1958).—*Biochim. biophys. Acta* **28,** 184.

O'Donnell, I. J., and Thompson, E. O. P. (1968).—*Aust. J. biol. Sci.* **21,** 385.

O'Donnell, I. J., Thompson, E. O. P., and Inglis, A. S. (1962).—*Aust. J. biol. Sci.* **15,** 732.

Prahl, J. W. (1967).—*Nature, Lond.* **215,** 1386.

Thompson, E. O. P., and O'Donnell, I. J. (1967).—*Aust. J. biol. Sci.* **20,** 1001.

Wilkinson, J. M., Press, E. M., and Porter, R. R. (1966).—*Biochem. J.* **100,** 303.