Journal of Chromatography, 268 (1983) 306–310
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,993

Note

Specific radiolabelling of keratin proteins by amidination

A. STRÜSSMANN*, R. C. MARSHALL* and H. ZAHN
Deutsches Wollforschungsinstitut, Veltmanplatz 8, Aachen D-5100 (F.R.G.)
(Received May 16th, 1983)

Wool and hair keratins consist of a complex mixture of proteins[1]. The method most often used to examine the components initially involves a fractionation of the proteins into three constituent classes (low-sulphur, high-sulphur and high-tyrosine) which are then separately investigated by electrophoresis[1]. Alternatively, the unfractionated total protein extract can be examined directly by one- or two-dimensional polyacrylamide gel electrophoresis[2].

Proteins in polyacrylamide gel may be located by conventional staining procedures, but in some cases, *e.g.*, to increase sensitivity, radiolabelling is advantageous. In many biological systems either intrinsic or extrinsic radiolabelling procedures may be used, but so far attempts to radiolabel hair cortical proteins intrinsically in cell culture have not been successful[3]. Extrinsic radiolabelling of the cysteine residues of reduced keratin proteins by ^{14}C- or ^{3}H-labelled iodoacetate has found wide application[2–5], but is not suitable when the proteins are devoid of cysteine or cystine[6], or contain unreactive cysteine derivatives, *e.g.*, cysteic acid after performic acid oxidation[7]. We were therefore interested in developing a method for radiolabelling such proteins.

Hair and wool keratins contain relatively large amounts of serine, threonine and arginine[1], but methods for modification of these residues under mild conditions were not found. On the other hand, the commonly-used iodination procedure for tyrosine suffers from non-specific side reactions. Lysine and amino terminal residues can be modified by treatment with acetic anhydride, sodium borohydride or acetimidates[8]. Although wool and hair contain only small amounts of lysine, it was decided to attempt the modification of this residue since the reactions generally are quite specific and are carried out under fairly mild conditions[8,9]. In this paper we report some results obtained by amidination of S-carboxymethylated wool proteins, this method being chosen so that the properties of the modified and unmodified proteins will be similar in many respects.

MATERIALS AND METHODS

Preparation of amidinated wool proteins

Proteins were extracted from Buenos Aires wool according to the procedure

* Present address: CSIRO, Division of Protein Chemistry, 343 Royal Parade, Parkville 3052, Australia.

0021-9673/83/$03.00

of O'Donnell and Thompson[10]. Methylene chloride-washed wool top was treated with 0.15 *M* 2-mercaptoethanol in 8 *M* urea at pH 10.4 for 3 h. The cysteine residues of the solubilized proteins were then S-carboxymethylated with iodoacetate, and after dialysis the proteins were recovered by freeze drying.

In order to amidinate the S-carboxymethylated wool proteins as completely as possible, the modification reaction was carried out at a succession of different pH values (9.5, 10.0, 8.8, 9.5), as recommended by Ludwig and Hunter[11,12]. In preliminary experiments non-radioactive ethyl acetimidate was used. Nine mg of ethyl acetimidate hydrochloride (Aldrich) (approximately a twenty-fold molar excess) were added with stirring to 20 mg of wool proteins dissolved in 2 ml of 0.1 *M* borate buffer (pH 9.5). The pH was maintained at 9.5 for 1 h by additions of 1 *M* sodium hydroxide. Second and third additions of the imido-ester (each of 9 mg) were performed at pH 10.0 and 8.8, and the pH was again kept constant at the respective pH values for 1 h. Finally, 18 mg of the imido-ester were added at pH 9.5. Changes in pH values were effected with 1 *M* sodium hydroxide or 1 *M* hydrochloric acid.

In the radiolabelling experiments, both radioactive and non-radioactive ethyl acetimidate were added at the first three pH values, and only non-radioactive material at the final pH. The amounts of 1 *M* sodium hydroxide and 1 *M* hydrochloric acid added to maintain a constant pH or to change the pH were calculated on the basis of the observed values in the non-radioactive experiments. All operations were carried out with shaking. To 500 μg of wool proteins dissolved in 50 μl of 0.1 *M* borate buffer were added 5 μl of a [1-^{14}C]ethyl acetimidate solution [500 μCi of the imido-ester (57.5 mCi/mmol, Amersham) dissolved in 100 μl anhydrous methanol]. After 30 min, 163 μg of ethyl acetimidate hydrochloride and 3 μl of 1 *M* sodium hydroxide were added. After a further 30 min the pH was raised to 10 by the addition of 3 μl of 1 *M* sodium hydroxide, and the procedure repeated. The pH was then lowered to 8.8 by the addition of 7 μl of 1 *M* hydrochloric acid, and labelled and non-labelled imido-ester added as described above. The pH was maintained with only 1 μl of 1 *M* sodium hydroxide. A further 5 μl of 1 *M* sodium hydroxide changed the pH to 9.5, where upon 550 μg of ethyl acetimidate hydrochloride and 3 μl of 1 *M* sodium hydroxide were added. After 1 h, the sample was stored at −20°C.

Analysis of amidinated proteins

Because acid hydrolysis converts amidinolysine into lysine, it is necessary to use special procedures for the determination of the extent of amidination. In this study, the amino acid compositions of protein preparations were determined after enzymatic digestion. This procedure suffers from the problem that some combinations of amino acids show resistance to complete digestion by enzymes, thus the amino acid composition may not be strictly quantitative. Nevertheless, a reliable estimate of the extent of modification can be obtained from a comparison of the lysine contents in the modified and unmodified protein preparations. The digestibility of lysine-containing peptides was not expected to be significantly altered after the modification because the introduction of the amidino group does not change the charge and results in only a small increase of the residue size.

Five mg of protein substrate were digested at 39°C for 24 h with 0.1 mg Pronase E (Merck) in 1 ml of 0.05 *M* Tris-HCl (pH 8.25) buffer containing 2.3 mg dithiothreitol. Digestion was then continued for a further 24 h with a mixture of 0.1 mg

Aminopeptidase M (Merck) and 25 μl Prolidase (Sigma)[13]. The amino acid composition was determined with a Biotronik LC 6000 E analyser, and corrected for autolysis of the enzymes.

Polyacrylamide gel electrophoresis

The proteins were examined by polyacrylamide gel electrophoresis in slab gels measuring 170 × 130 × 0.75 mm (Pharmacia Model GE-2/4). For electrophoresis at pH 8.9, the procedure of Davis[14] was used except that 8 *M* urea was incorporated in the gel. Electrophoresis was also carried out in the presence of sodium dodecyl sulphate (SDS) using the method of Laemmli[15]. In both procedures, electrophoresis was continued until the bromophenol blue tracking dye almost reached the bottom of the separating gel. After electrophoresis the proteins were located by either staining with Coomassie Blue G250 or the fluorographic method of Bonner and Laskey[16].

RESULTS AND DISCUSSION

In the initial experiments where non-radioactive ethyl acetimidate was used,

TABLE I

AMINO ACID COMPOSITIONS DETERMINED AFTER ENZYMATIC DIGESTION OF UNMODIFIED AND AMIDINATED S-CARBOXYMETHYLATED BUENOS AIRES WOOL PROTEINS

Compositions are expressed as residues per 100 residues, each value being the average of the analyses of two digestions.

Amino acid	*Unmodified*	*Amidinated*
Cysteic acid	0.2	0.2
Aspartic acid	1.8	1.5
Threonine	6.2	6.8
Serine	10.9	11.6
Glutamic acid	4.2	4.2
Proline	5.3	5.6
Glycine	8.6	8.1
Alanine	6.5	6.3
Valine	6.1	6.1
Methionine	0.2	0.2
Isoleucine	3.5	3.6
Leucine	9.1	9.5
Tyrosine	5.9	5.7
Phenylalanine	3.8	3.9
Lysine	2.7	0.1
Amidinolysine*	–	2.6
Histidine	0.7	0.7
Arginine	8.3	8.3
Glutamine	4.0	3.4
Citrulline	0.1	0.0
Asparagine	4.4	3.2
Ornithine	0.0	0.3
S-Carboxymethylcysteine	7.7	8.2

* Estimated by the difference between the lysine contents of the amidinated and unmodified preparations.

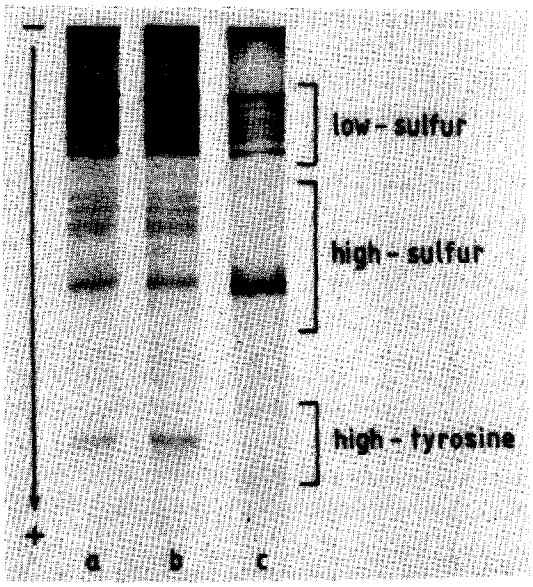

Fig. 1. SDS polyacrylamide gel electrophoretic patterns (15% gel) of S-carboxymethylated protein preparations from Buenos Aires wool: a, unmodified; b, amidinated; c, [^{14}C]amidinated. In a and b the proteins were located by Coomassie Blue staining, and in c by fluorography.

the amino acid composition and the electrophoretic properties of the amidinated proteins were compared with those of the unmodified proteins. The amino acid compositions, determined after enzymatic digestion, were closely similar (Table I) except for the expected smaller lysine content in the amidinated preparation. From these results the extent of modification was calculated to be about 96%. The electrophoretic properties of the proteins after amidination were not significantly changed, as shown by electrophoresis at pH 8.9 in the presence of SDS (Fig. 1a and b) and 8 *M* urea (patterns not shown). After SDS electrophoresis, the three major protein fractions (low-sulphur, high-sulphur, high-tyrosine) were located in different areas of the pattern (Fig. 1), and in the low-sulphur and high-sulphur protein regions a number of bands were observed which correspond to well characterized proteins[4].

On the basis of the above results, other wool protein preparations were radiolabelled with [1-^{14}C]ethyl acetimidate and then examined by electrophoresis. The bands in the SDS electrophoretic pattern detected by fluorography of the radiolabelled proteins (Fig. 1c) corresponded to many in the unmodified preparation (Fig. 1a). As expected, the largest discrepancy occurred in the high-sulphur protein region since many of the components contain no lysine residues or free amino terminals[17]. Only one high-sulphur band was extensively modified. It was shown by two-dimensional electrophoresis[4] to correspond to the IIIB family of components which is known from amino acid sequence studies to contain lysine[17].

CONCLUSIONS

If intrinsic radiolabelling of proteins *in vivo* or *in vitro* is not possible, extrinsic

modification reactions may be applied. Unfortunately only a few of these procedures are specific and can be carried out under mild conditions. One such case is the modification of cysteine residues of keratin proteins by S-carboxymethylation. The results given in the present paper show that keratin proteins can be radiolabelled by amidination of amino groups in high yields. Work is in progress to extend this method to the examination of highly anionic keratin protein fractions which are difficult to locate by conventional staining techniques.

ACKNOWLEDGEMENTS

We thank Drs. K. Röper and J. Föhles for the enzymatic digestions and the determination of the amino acid compositions. R. C. Marshall would like to thank the Alexander von Humboldt Foundation, West Germany, for the receipt of a Research Fellowship.

REFERENCES

1 W. G. Crewther, in K. Ziegler (Editor), *Proc. 5th Int. Wool Textile Res. Conf., Aachen, Sept. 2–11, 1975*, Vol. I, 1976, p. 1.
2 R. C. Marshall, *Text. Res. J.*, 51 (1981) 106.
3 D. R. Hewish and R. C. Marshall, *Aust. J. Biol. Sci.*, 35 (1982) 103.
4 R. C. Marshall and J. M. Gillespie, *Proc. 2nd Natl. Conf. Wool Harvesting Research and Development, Sydney, Australia, Aug. 10–13, 1981*, p. 117.
5 R. C. Marshall, *J. Invest. Dermatol.*, 80 (1983) in press.
6 H. Zahn, *Proc. 6th Quinquennial Int. Wool Textile Res. Conf., Pretoria, Aug. 26–Sept. 3, 1980*, Vol. I (Addendum), p. 1.
7 J. H. Buchanan, *Biochem. J.*, 167 (1977) 489.
8 A. N. Glazer, *The Proteins*, Vol. II, Academic Press, New York, 3rd ed., 1976, p. 2.
9 M. J. Hunter and M. L. Ludwig, *J. Amer. Chem. Soc.*, 84 (1962) 3491.
10 I. J. O'Donnell and E. O. P. Thompson, *Aust. J. Biol. Sci.*, 17 (1964) 973.
11 M. L. Ludwig and M. J. Hunter, *Methods Enzymol.*, 11 (1967) 595.
12 M. J. Hunter and M. L. Ludwig, *Methods Enzymol.*, 25B (1972) 585.
13 M. Hubbuch, *Dissertation an der Rheinisch-Westfälischen Technischen Hochschule*, Aachen, 1981.
14 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
15 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
16 W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.*, 46 (1974) 83.
17 L. S. Swart, F. J. Joubert and D. Parris, in K. Ziegler (Editor), *Proc. 5th Int. Wool Textile Res. Conf., Aachen, Sept. 2–11, 1975*, Vol. II, 1976, p. 254.