*Journal of Chromatography*, 536 (1991) 283–288
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2036

# Determination of 3-methylhistidine in hydrolysed proteins by fluorescamine derivatization and high-performance liquid chromatography

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## ABSTRACT

A method is described for the determination of the 3-methylhistidine content in myofibrilar proteins (myosin and actin) using reversed-phase high-performance liquid chromatography with ultraviolet detection. Proteins were hydrolysed and free amino acids were derivatized with fluorescamine. Elution was performed isocratically with acetonitrile–water (24:76). This method allows the detection of picomole amounts of 3-methylhistidine in myosin and actin.

## INTRODUCTION

The unusual amino acid 3-methylhistidine (3MH) is present in the myofibrillar proteins myosin and actin [1,2]. It has been shown that the methyl group is added after the histidine residue has been incorporated into the polypeptide chains [3]. The 3MH content of actin appears to be much more constant than is the case with myosin. In fact, 3MH is present in about the same amount in all the actins so far analysed [1]. In contrast, 3MH is present in adult white skeletal muscle mysosin and is absent from cardiac and slow muscle myosins and from white skeletal muscle myosin of newborn animals [4]. With the methods so far available, large amounts of protein are necessary for 3MH detection [1] and then it is impossible or very difficult to work with small amounts of material when, for example, human biopsies are to be analysed. For this purpose we have separated and detected 3MH utilizing high-performance liquid chromatography (HPLC) in hydrolysates of nanomole amounts of actin and myosin isolated from rat and rabbit muscles. The method used here was originally described by Wassner and co-workers [5,6] for 3MH determination in plasma and urine and in this work it was adapted to protein hydrolysates. The results obtained for rabbit actin and myosin are in good agreement with those reported in the literature. Further, we have determined for the first time the 3MH content of myosins isolated from rat muscles.

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## EXPERIMENTAL

### *Reagents*

Histidine, 3-methylhistidine, N-methyllysine and fluorescamine were obtained from Sigma (St. Louis, MO, U.S.A.). The amino acid standard was from Bio-Rad Labs. (Richmond, CA, U.S.A.). Acetonitrile (HPLC grade), thiodiglycol, *n*-caprylic acid and perchloric acid were purchased from Carlo Erba (Milan, Italy). Glass-distilled water was filtered through a 0.45-$\mu$m Millipore filter before use.

### *Proteins preparation*

Myosin was prepared from rabbit and rat muscles according to Dalla Libera *et al.* [7]. Actin was prepared from rabbit muscle according to Leadbeater and Perry [8].

About 1 mg of purified protein was precipitated with 10% trichloroacetic acid, washed with ethanol and diethyl ether and hydrolysed for 22 h in 6 *M* HCl at 110°C under vacuum. Then the vials were opened and the HCl was removed with a rotary evaporator connected to a water pump. The hydrolysate was solubilized in a small amount (0.2–0.4 ml) of a buffer which contained 67 m*M* sodium citrate–0.63 m*M* *n*-caprylic acid–48 m*M* thiodiglycol (pH 2.2) and filtered through a 0.22-$\mu$m filter.

### *Preparation of derivatives*

Standard solutions of histidine, 3-MH and N-methyllysine of 1 $\mu$mol/ml were prepared. The amino acid standard was diluted 1:10 with water to a concentration of about 5 $\mu$mol/ml total amino acid. Fluorescamine derivatives were prepared using standards or hydrolysed protein according to Wassner *et al.* [5]. Briefly, to 100 $\mu$l of standards or hydrolysed protein were added 250 $\mu$l of sodium borate solution (0.8 *M* boric acid adjusted to pH 12.2 with NaOH). Then 250 $\mu$l of fluorescamine solution (160 mg per 100 ml in acetonitrile) were added with vigorous agitation. After the addition of 35 $\mu$l of concentrated perchloric acid the sample were incubated at 80°C for 1 h. After cooling at room temperature 100 $\mu$l of 0.5 *M* morpholinopropanesulphonic acid (MOPS) in 3 *M* NaOH were added to the samples just before analysis. Aliquots of 20–30 $\mu$l of the resulting solution were injected into the HPLC system.

### *Apparatus*

A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 3B HPLC system was used. A Sigma 10 data station was employed. Separation was achieved with a $\mu$Bondapak $C_{18}$ (Waters Assoc., Milford, MA, U.S.A.) or RP-8 and RP-18 (Merck, Darmstadt, F.R.G.) of Rosil $C_{18}$ (Alltech, Deerfield, IL, U.S.A.). All columns were 25 cm × 0.46 cm I.D. A guard obtained from Upchurch (Oak Harbour, WA, U.S.A.) was always used to protect the main column. Elution was performed isocratically with acetonitrile–water (24:76) at 1.5 ml/min at room temperature. The derivative can be monitored either at 254 nm (Fig. 1) or 365 nm (Fig. 2); we prefer the latter wavelength as it results in clearer chromatograms, especially in the 3MH area. Peak areas were quantitated by the method of external standardization using 3MH. The molecular weights of actin and myosin were assumed to be 45 and 500 kilodalton, respectively.

RESULTS

Fig. 1A shows a chromatogram of 1360 pmol of 3MH. After a large early-eluting peak and a minor peak at about 10 min, which are also present in all blank runs, a large

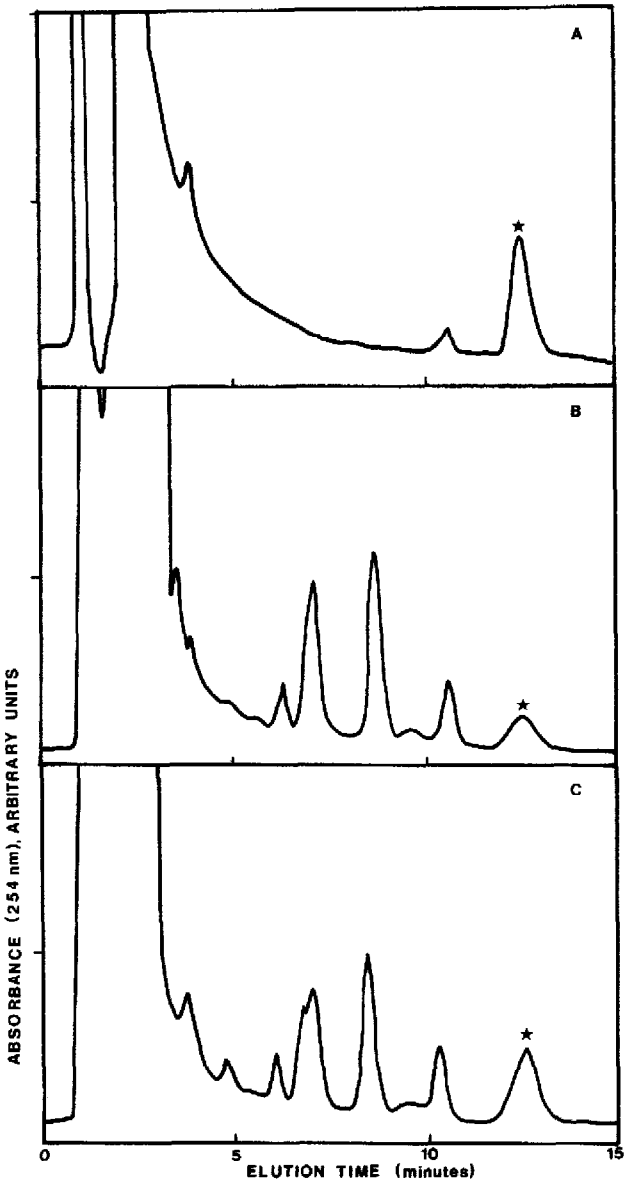


Fig. 1. Chromatograms of 3-methylhistidine from a standard solution and an actin sample. (A) 1360 pmol of 3MH standard; (B) 30 $\mu$l of actin prepared as described in the text, containing 520 pmol of 3MH; (C) 30 $\mu$l of actin (520 pmol of 3MH) and 680 pmol of 3MH standard. The effluents were monitored at 254 nm. The asterisk indicates the 3MH peak.

peak elutes at about 12–13 min. This peak correspond to the 3MH fluorescamine derivative. Peak areas were linear over the range *ca.* 100–1500 pmol. The reproducibility was 1.5%. Fig. 1B shows a chromatogram of an actin sample hydrolysate. In addition to the blank and 3MH peaks, other unidentified peaks are seen. These peaks, which do not correspond to any of the amino acids present in the amino acid standard, may be the result of degradation products of fluorescamine. The identification of the 3MH peak in the actin chromatogram was based not only on retention times but also on standard addition experiments. In Fig. 1C such an elution is show and it is evident that the area of the peak corresponding to 3MH is enhanced by the adding 3MH. We observed that the best separations were obtained using the $\mu$Bondapak $C_{18}$ or Rosil $C_{18}$ columns.

Fig. 2 shows representative chromatograms for the detection of 3MH in myosins from different rat muscles. As with actin hydrolysates, several peaks elute before 3MH. However, the area where 3MH elutes is sufficiently clear for it to be identified. As it is well known that myofibrillar proteins contain large amounts of histidine and also lysines methylated to a variable extent [2,9], hystidine and N-methyllysine were derivatized and chromatographed as described under Experimental section. No peak was visible in the elution time range of 3MH (results not shown). Moreover, to rule out the possibility that artifacts originating during the hydrolysis procedure might affect the 3MH area in subsequent chromatograms, proteins that do not contain 3MH, *viz.*, casein, albumin, trypsin and chymotrypsin, were treated exactly as the myofibrillar protein samples. Again, no peak in the position of 3MH was visible during the chromatographic separation (results not shown).

In Table I are reported the concentrations of 3MH determined in rat and rabbit actin and several myosin of different muscles of rat and rabbit. The values (0.8 residues per mole) for actins determined with the assay here reported are close to those calculated with conventional nynhydrin-based amino acid analysers either from total protein hydrolysates [1,2,4] or in sequence studies [10].

As far as rabbit myosin is concerned, myosin prepared from fast-contracting muscle (adductor) contains about two residues of 3MH per molecule (one residue per heavy chain) whereas myosin prepared from slow-contracting muscle (soleus) does not contain 3MH. The results are in agreement with the data reported previously [2]. With rat myosin we found that the pattern of distribution of 3MH is similar to that observed in the rabbit. In fact, predominantly fast-contracting muscles, *i.e.*, the masseter and the EDL, contain about 1.5 residues of 3MH. Interestingly, the 3MH content of myosin from the diaphragm, which is a mixed muscle (fast and slow), was less than that observed for a pure fast muscle, 0.9 *vs.* 1.4 residues per mole, respectively.

## DISCUSSION

The method described here was first used for the detection of 3MH in urine and plasma [5,6]. In this work it was used for the identification of 3MH in a mixture of amino acids obtained from the acidic hydrolysis of small amounts (less than 1 mg) of the two major myofibrillar proteins, actin and myosin. It is evident that in the case of protein hydrolysates the fluorescamine derivatization is achieved on the condition that the pH of the solution is not acidic. In fact, it is known that the reaction of amino acids with fluorescamine takes place only at alkaline pH [11]. For this reason, it is necessary

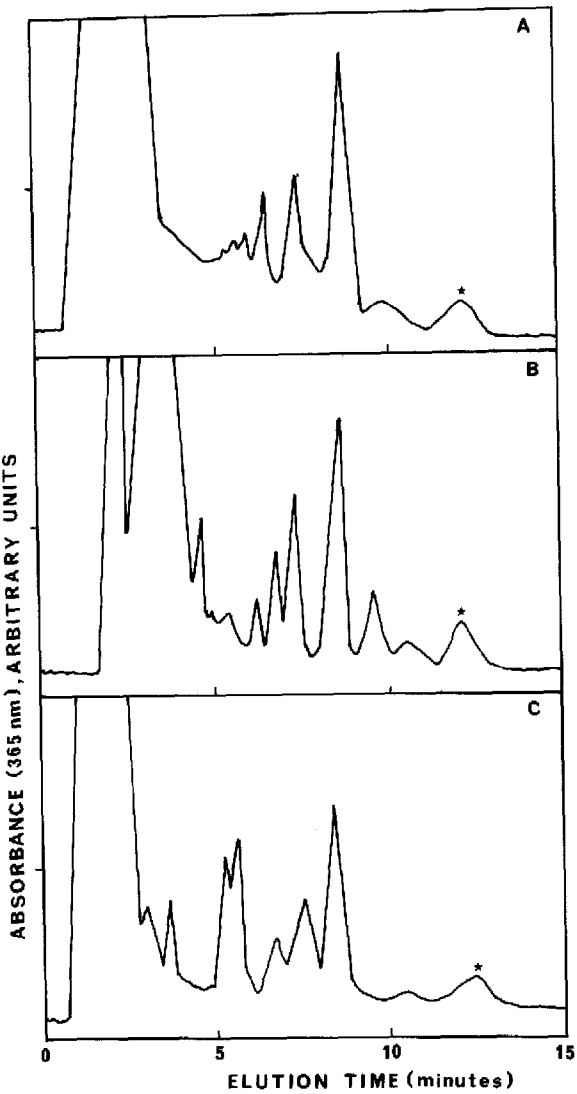


Fig. 2. Chromatograms of 3-methylhistidine from rat myosin samples. (A) Diaphragm muscle; (B) extensor digitorum longus muscle; (C) embryonic muscles. The effluents were monitored at 365 nm. The asterisk indicates the 3MH peak.

to dry the acidic hydrolysates with a vacuum evaporator in order to eliminate all the HCl present.

When less than 30 nmol of actin are available (1 mg), it is possible to perform the hydrolysis and the subsequent derivatization with florescamine and obtain a measurable peak of 3MH on the chromatogram. The values so obtained are in good agreement with the data reported previously [1,2,4,10], indicating that no interfering component, such as histidine, contributes to the 3MH peak area.

We have applied this method to the study of the other major myofibrillar

TABLE I

3MH CONTENTS OF MYOFIBRILLAR PROTEINS

| Sample | 3MH concentration[a] | Literature value |
|---|---|---|
| Actin (rabbit) | 0.75 (6) | 1.0 [1,2,4,10] |
| Actin (rat) | 0.8 (2) | |
| Myosin (rabbit): | | |
| Adductor muscle | 1.6 (1) | 1.5 [2,4] |
| Soleus muscle | 0 (2) | 0 [2,4] |
| Myosin (rat): | | |
| Masseter muscle | 1.4 (2) | |
| Extensor digitorum longus muscle | 1.3 (2) | |
| Diaphragm muscle | 0.9 (3) | |
| Embryonic muscle | 0.8 (2) | |

[a] Values are expressed as residues of 3MH per mole. The number of observations is given in parentheses.

protein, *i.e.*, myosin. We investigated the 3MH content of myosin not only from already studied tissues such as rabbit fast and slow muscles, but also from a yet unstudied species, the rat. In this latter instance the limiting factor is the amount of available starting material, which is too low to perform the analysis of 3MH with conventional ion-exchange chromatographic techniques.

In conclusion, the method appears particularly suitable for the rapid evaluation of 3MH at picomole levels in actin and myosin available in small amounts as in the case of small muscles or biopsies.

ACKNOWLEDGEMENTS

The skilful technical assistance of Mr. S. Belluco is gratefully acknowledged.

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