

## DIGESTION OF TROPOMYOSIN WITH TRYPSIN

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### 1. Introduction

Tropomyosin is a rod-like  $\alpha$ -helical protein composed of two similar subunits in a coiled-coil configuration. It is situated in the grooves of the F-actin strands and with troponin constitutes the regulatory-complex of vertebrate striated muscle. The sequence of the tropomyosin subunit has been determined by Stone, et al. [1]. As a result of this work it was recognized that a repeat sequence existed [1–3] which correlated to the 7 actin binding sites along the tropomyosin molecule [4]. The location of the troponin binding site has also been established [5]. Since the biological and physical properties of tropomyosin are so well defined and related to its primary structure it seemed to be an ideal protein to study with respect to its fragmentation by trypsin. The object being to examine various tryptic fragments and determine whether these retain any of the properties of the native molecule.

### 2. Materials and methods

#### 2.1. Preparation of proteins

All proteins were obtained from rabbit leg- and back-muscle.

Tropomyosin was prepared using essentially the method of Bailey [6]. Three cycles of purification, consisting of precipitation, at pH 4.5, in 1 M KCl followed by neutralization of the precipitate and subsequent salting-out with  $(\text{NH}_4)_2\text{SO}_4$  between 0.4–0.6 saturation, were applied. At the final step tropomyosin was collected as the precipitate formed between 0.53–0.6 saturation of  $(\text{NH}_4)_2\text{SO}_4$ . Fully

reduced tropomyosin was obtained by exposure of the protein, at 2 mg/ml to 100 mM dithiothreitol for 48 h at 0°C. Tropomyosin was oxidized by bubbling air through the protein solution, at 3 mg/ml, in 20 mM HEPES buffer (pH 8.0) and  $1 \times 10^{-5}$  M  $\text{CuCl}_2$ , i.e., following essentially the method of Stewart [7].

Actin, myosin and troponin were obtained as described previously [8,9].

The purity of all proteins was established by SDS–polyacrylamide gel electrophoresis.

#### 2.2. Digestion of tropomyosin with trypsin

Proteolysis was carried out at 20°C in 20 mM HEPES buffer (pH 8.0) for varying times. The weight ratio of trypsin to tropomyosin used is given in the figure legends. Digestion was stopped either by the addition of a 4-fold weight-excess of soybean trypsin inhibitor or, if the samples were to be analyzed by gel electrophoresis, by heating at 100°C for 5 min in 2% SDS (see below).

#### 2.3. Analytical methods

Polyacrylamide gel electrophoresis was carried out either in 10% polyacrylamide on cylindrical gels or, in 15% polyacrylamide in slab-gels. In both cases the buffer used was 100 mM sodium phosphate and 0.1% SDS, according to Weber and Osborn [10]. Protein samples for electrophoresis were prepared by heating at 100°C for 5 min in 2% SDS containing either 1 mM  $\beta$ -mercaptoethanol (to obtain and/or maintain the reduced state) or, 1 mM NEM (in order to prevent oxidation). Sample loading was approx. 30–50  $\mu\text{g}$  protein. Gels were stained with 0.05% Coomassie Blue in 18% trichloroacetic acid for at least 3 h, followed by destaining in 7% acetic acid and 5% methanol

(v/v). Densitometric scanning of the gels was carried out with a Vitatron spectrophotometer at 560 nm.

Protein concentration was determined by the biuret reaction.

Viscosity was measured using an Ostwald type viscometer with a water out-flow time of about 25 s at 21°C.

### 3. Results and discussion

In the initial experiments tropomyosin was digested with various concentrations of trypsin for different times. It was found that a ratio of trypsin to tropomyosin of between 1:1000 and 1:2000 (w/w) was convenient as this allowed the observation of intermediate (labile) peptides. A representative pattern is shown in fig.1. After digestion for 1 min four fragments were seen, three major components at 19 000, 17 000 and 12 000 daltons and a minor band at 24 000 daltons. The latter was variable and was not a consistent product. The 24 000 and 19 000 peptides were more labile than the smaller two components and were not found after longer digestion times, for example the 20 min sample in fig.1. By scanning the gels and estimating the amount of each component present at varying times it was found that the concen-

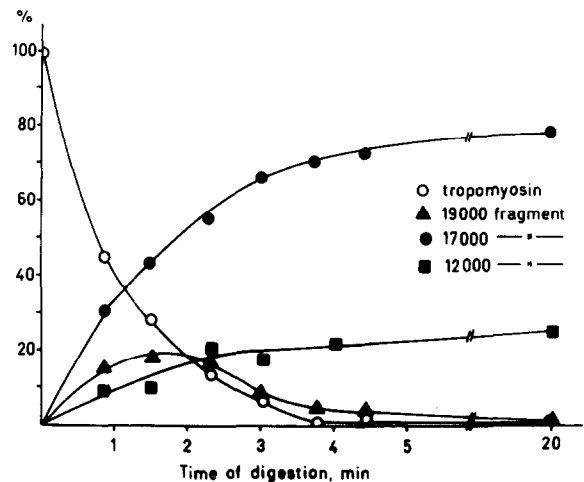


Fig.2. Relative distribution of tropomyosin fragments. Digestion conditions as in fig.1. Populations of various fragments was calculated from scans of SDS-gels.

tration of the two smaller fragments increased as long as undigested tropomyosin existed (fig.2). Illustrated in fig.2 is the lability of the 19 000 component. In contrast the 17 000 and 12 000 fragments were relatively resistant to trypsin and were seen using higher trypsin to tropomyosin ratios, e.g., after digestion for 20 min with a 1:300 trypsin to tropomyosin mixture.

A more critical analysis of the above results was not attempted as it was found that the fragments formed were dependent, to some extent, on the history of the tropomyosin preparation. In older samples the amount of the 12 000 dalton fragment which was produced was much higher than that formed from fresh preparations of tropomyosin. In an earlier observation from this laboratory [11] it was found that storage of tropomyosin resulted in a decrease in the number of free SH-groups. In view of this, the possibility was raised that the variation in digestion profiles may be a reflection of the presence of disulfide-bonds. To check this the digestion by trypsin was carried out using either fully reduced or oxidized tropomyosin (see Materials and methods).

It can be seen from fig.3 that the patterns of trypsin digestion of oxidized and reduced tropomyosin were different. After a 1 min digestion of fully reduced tropomyosin, two fragments were formed of 19 000 and 17 000 daltons. These were present follow-

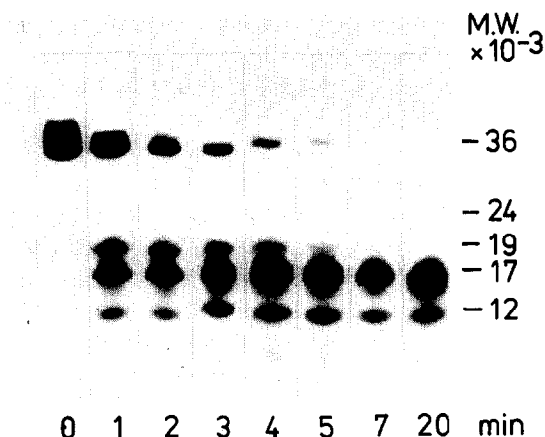


Fig.1. Digestion of tropomyosin by trypsin. Tropomyosin (2 mg/ml) in 20 mM HEPES (pH 8.0) digested with trypsin (1:1000 trypsin:tropomyosin w/w) at 20°C. Time of digestion as indicated. SDS-gels contained 10% acrylamide. Sample loading was 30 µg/gel.

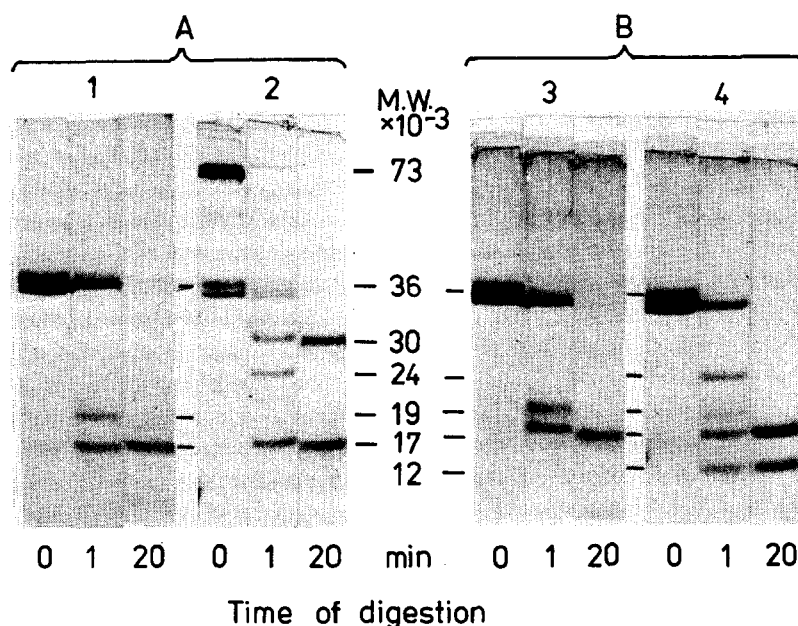


Fig.3. Digestion of reduced and oxidized tropomyosin. Digestion of tropomyosin (1 mg/ml) was carried out as indicated in fig.1. Reduced tropomyosin (1,3) and oxidized tropomyosin (2,4) were digested for different times and applied to SDS-gel electrophoresis (50  $\mu$ g/gel) using either 1 mM *N*-ethylmaleimide (A) or 1 mM  $\beta$ -mercaptoethanol (B) in sample buffer (see Materials and methods).

ing electrophoresis when either NEM or  $\beta$ -mercaptoethanol was used in the gel sample-preparation. The 19 000 component disappeared on longer digestion (see 20 min example, fig.3) and led to an increase in the 17 000 band. With the oxidized tropomyosin and under conditions to preserve the disulfide-bond (i.e. NEM in sample buffer) tropomyosin appeared as the dimer of the 2 subunits, approx. 70 000 daltons and also as the individual subunits. This result is similar to the findings of Stewart [7] and supports the contention that since a disulfide bond was formed between subunits the two chains are in register. The products of tryptic hydrolysis of oxidized tropomyosin were electrophoresed under two conditions: (a) to maintain the disulfide-linkage and in this case NEM was used in the sample preparation and (b) to reduce the disulfide-linkage, using  $\beta$ -mercaptoethanol in the sample preparation. Considering first the former situation: digestion with trypsin for a short period of time (see 1 min sample, fig.3) led to the formation of three major peptides, 30 000, 24 000 and 17 000 daltons. Occasionally traces of a 19 000 component were seen. After 20 min digestion (fig.3) only the 30 000 and

17 000 components were seen. Let us consider now the latter situation where the digestion of the oxidized tropomyosin was identical, but reduction of the products preceded electrophoresis. After 1 min digestion three major fragments were seen, 24 000, 17 000 and 12 000 daltons. Once again the 19 000 component appeared, but as a variable product. It is interesting that the 30 000 fragment was absent and instead a 12 000 band appeared. After longer digestion times only the 17 000 and 12 000 fragments were seen (fig.3).

Without further work it is not possible to establish with any certainty the origin of the above peptides with respect to the native molecule, a contributing complication being that tropomyosin exists in rabbit skeletal-muscle in two forms,  $\alpha$  and  $\beta$  [12]. However, since the  $\alpha$ -form is the dominant species [12] it is possible to make some tentative assignments. The 30 000 fragment probably contains the disulfide-bond and thus represents at least  $2 \times$  the C-terminal third of each chain [1]. Since the 17 000 component was found in all samples it may be a fragment of the chain to the N-terminal side of the cysteine 190 residue [1].

The 19 000 fragment being a precursor of the 17 000 component. An observation that may facilitate future experiments is that the 17 000 fragment can be isolated from the others as the supernatant in 1 M KCl, at pH 4.6.

One of the main reasons for initiating this study was to determine if the fragments possessed any of the properties associated with the native molecule. Consequently a number of assays were carried out to test whether this was the case. For these studies oxidized tropomyosin was used as the substrate at trypsin to tropomyosin ratios similar to that given in fig.3.

The viscosity at low ionic strength decreased rapidly, indicating that the peptides formed did not undergo end-to-end polymerization. These results are similar to those reported earlier [13]. The addition of troponin did not enhance the viscosity. This observation does not exclude the possibility of troponin binding to one of the fragments, but only rules out the situation where troponin might promote end-to-end polymerization of the fragments. The tropomyosin fragments with troponin were also without effect on the  $Mg^{2+}$ -ATPase activity of actomyosin, and did not inhibit activity in the absence of  $Ca^{2+}$ .

The ability to bind to actin was lost also following tryptic digestion. Samples of both oxidized and reduced tropomyosin were digested for short periods of time, so that some of the native molecule remained (see fig.1). This mixture was then mixed with F-actin in 20 mM HEPES, pH 8.0, at a molar ratio tropomyosin: actin of 1:5 and centrifuged at 100 000  $\times g$  for 3 h. The pellet was then examined on SDS-gel electrophoresis. There was no evidence for the binding of either the 17 000 or 12 000 components and only the whole tropomyosin molecule was found in combination with actin. This is interesting as it was reported earlier [4] that each tropomyosin molecule contained 14 potential binding sites for actin. These sites are evenly distributed along the length of the molecule and thus each tryptic fragment would contain several sites. The fact that no binding to actin was detected would suggest that the actin-tropomyosin interaction is cooperative. In this context it is pertinent to mention the recent results of Tawada et al. [14], who found that tropomyosin digested with carboxypeptidase A retained, in combination with troponin, the ability to suppress superprecipitation in the absence of  $Ca^{2+}$ .

Binding of the tropomyosin fragment to actin is therefore implied. The carboxypeptidase A digested tropomyosin had a similar molecular weight to the native molecule and would possess most of its actin binding sites in contrast to the fragments reported above. The fascinating aspect of the work of Tawada et al. is that although biological activity was retained the end-to-end polymerization of the tropomyosin molecules was lost. The importance to the regulatory mechanism of head-to-tail overlap of tropomyosin molecules must therefore be re-examined, and to this end more selective methods of cleaving the tropomyosin chain could be examined.

During the preparation of this report another study on the digestion of tropomyosin by trypsin appeared [15]. The conditions employed however were different from those used by us and a detailed comparison of the two sets of results is not warranted at this time.

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