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Purification and characterization of recombinant tropomyosins

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ABSTRACT

The cloning of a cDNA coding for the skeletal human β-tropomyosin in the bacterial expression vector pKK233-2 is reported. Deletion mutants were also constructed. pCF-T1088 was obtained by elimination of exon 9 and pCF-T1089 was built by deleting 2/3 of the first exon. The recombinant tropomyosins were synthesized in *E. coli* after induction by IPTG. The mutant proteins were characterized by western blot using antibodies raised against native tropomyosin. The amount of the human protein synthesized in *E. coli* varies with each mutant, suggesting the involvement of the structure of the protein or of the mRNA on the synthesis or the stability of the recombinant protein. After precipitation of most of the bacterial proteins at 100°C, purification was achieved by high-performance liquid chromatography (HPLC) using TSK-DEAE, hydroxyapatite and reversed-phase columns. The chromatographic behaviour of the mutants were compared. Characterization of the mutated tropomyosins was achieved by tryptic digestion and analysis of the peptide composition by reversed-phase HPLC. A computer program for predicting the retention times of the peptides generated was written. It is shown that it is possible to identify the mutations solely by comparing the chromatogram of the tryptic digest with the profile obtained by computer simulation.

INTRODUCTION

Tropomyosin is a major muscular protein involved in the regulation of muscle contraction by Ca^{2+} [1]. The mechanisms of this regulation are still a matter of debate [1]. Muscle tropomyosin is a two-stranded elongated molecule, having virtually 100% α-helix conformation. It winds along the long-pitch grooves of the F-actin helix. This rod-like fibrillar molecule is considered to be a good model to investigate the nature of protein stability and particularly to understand the respective roles of hydrogen bonds and inter-helix interactions in the formation of the tertiary structure of proteins [2].

Tropomyosin genes from different species and different tissues have already been cloned (for reviews see Colote *et al.* [3] and Sri Widada *et al.* [4]). Muscle tropomyosin gene is constituted of nine exons, three of which arise from alternative splicing. Using an evolutionary approach, we have shown that the alternatively spliced exons are conserved, suggesting an important role in the function of these parts of the molecule. Tropomyosin presents numerous elementary functions such as troponin binding, head-to-tail interactions or actin binding. None of them is defined at the molecular level [1].

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To understand the function of tropomyosin better, it is therefore necessary to dissect the mechanisms that it involves at the submolecular level. Protein engineering permits such an approach. We have cloned and expressed in *E. coli* the human skeletal muscle β-tropomyosin. Two deletion mutants were performed, expressed in *E. coli* and purified using high-performance liquid chromatography (HPLC). In this paper we describe the purification behaviours of these proteins and compare their chromatographic patterns. Tryptic digests were analysed by reversed-phase HPLC. We show that a computer simulation can be used to identify the resulting peptides and thus rapidly characterize the mutants.

EXPERIMENTAL

Materials

HPLC analyses were performed with a Gilson (Paris, France) apparatus. Absorbances at 280 and 220 nm were monitored with a Holochrom spectrophotometer (Gilson).

A DEAE-TSK column (150 × 21.5 mm I.D.) and a TSK column (60 × 21.5 cm I.D.) for gel permeation were obtained from LKB (Uppsala, Sweden). A hydroxyapatite column (Hypatite-C) (100 × 12.5 mm I.D.) was purchased from SFCC (Paris, France). The column containing RP-18 (5-μm spherical particles) (130 × 4.0 mm I.D.) was obtained from Brownlee Labs. (Santa Clara, CA, U.S.A.).

Open chromatography was carried out on an HA-Ultrogel column from IBF (France). Acetonitrile (Chromasol Gradient) was purchased from SDS (Peypin, France).

Recombinant DNA constructions

Construction of the expression vector containing β-skeletal tropomyosin has been described previously [5]. Briefly, the human tropomyosin clone exhibiting an NcoI site at its start codon was inserted into the NcoI site of the PKK 233-2, thus under the control of isopropyl thiogalactoside (IPTG)-inducible trc promoter. This construction produces a complete unfusioned tropomyosin TM1 [5]. A deletion mutant lacking the last 31 amino acids of the C-terminus (TM [C31]) was obtained by taking advantage of a Cla I restriction site at this position. Another deletion mutant of 21 amino acids of the N-terminus (TM [N21]) was obtained after cutting with FnuDII restriction enzyme and addition of initiation codon. TM1, TM[C31] and TM[N21] were produced respectively in HB101, HB101 and JM109 bacteria strains.

Production and extraction of tropomyosin mutants

E. coli containing the different vectors were grown in 20 l of culture medium in a fermenter under controlled conditions. Recombinant protein synthesis was induced by IPTG. Bacteria were centrifuged and then disrupted in a French press, and the supernatant was heated in a boiling water-bath for 5 min. The mixture was centrifuged for 15 min at 10 000 *g*. Tropomyosin present in the supernatant was concentrated by ammonium sulphate fractionation. After precipitation of the nucleic acids at 35% saturation (209 g/l) of ammonium sulphate, β-tropomyosin was precipitated at 60% saturation (addition of a further 164 g/l). The pellet was resuspended and dialysed against buffer D [20 m*M* Tris–HCl (pH 7.5)–0.5 m*M* dithiothreitol (DTT)].

HPLC on DEAE-TSK column

A 250-mg sample of protein in buffer D was applied to the DEAE-TSK column. Elution was performed by increasing the salt concentration to 0.6 *M* ammonium acetate in buffer D with an increase of 10 m*M*/min using a flow-rate of 4.0 ml/min. Absorbance was measured at 220 and 280 nm.

Chromatography on hydroxyapatite column

We employed either open-column chromatography using HA-Ultrogel or HPLC using Hypatite-C. Proteins eluted from the DEAE-TSK column were adjusted to 1 *M* NaCl and 1 m*M* phosphate (pH 7.0)–0.5 m*M* DTT. Proteins were adsorbed on HA-Ultrogel (100 ml) equilibrated with the same buffer. Elution was performed at 100 ml/h with a 400-ml linear gradient from 1 to 300 m*M* phosphate (pH 7) containing 1 *M* NaCl and 0.5 m*M* DTT. Alternatively, the mixture was deposited on a 100 × 12.5 mm I.D. HPLC column containing hydroxyapatite. Elution was performed at 1.0 ml/min using a gradient of the following solutions: solution A, 10 m*M* NaH_2PO_4 (pH 6.9)–0.2 *M* sodium acetate–0.5 m*M* DTT; and solution B, 300 m*M* NaH_2PO_4–0.2 *M* sodium acetate–0.5 m*M* DTT.

Simulation of tryptic digestion pattern and chromatogram

We have written a program that simulates the tryptic digestion pattern of a protein. The retention of peptides on the reversed-phase column was calculated using the algorithm of Meek [6] as modified by Guo *et al.* [7] It nevertheless appears that the correlation between the calculated retention times and the experimental results is good when the peptides are composed of more than six amino acids.

Digestion of 0.2 mg of protein in 0.1 *M* Tris–HCl (pH 8.1) was performed with 10 μg of trypsin (TPCK-treated) (Sigma) for 2 h at 37°C. Reversed-phase chromatography was performed on a C_{18} column using an acetonitrile gradient (1% /min) containing 0.1% trifluoroacetic acid.

Other methods

Immunoblot analysis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli [8], using 12.5% acrylamide. Proteins were transferred from SDS polyacrylamide slab gels to nitrocellulose (Schleicher & Schüll) using a Bio-Rad transfer apparatus in the presence of 0.02% SDS. The nitrocellulose sheets were blocked with 8% dry milk in phosphate-buffered saline (PBS). The nitrocellulose was incubated with anti-tropomyosin antibodies raised against chicken gizzard tropomyosin diluted at 1:100 in PBS supplemented with 5% bovine serum albumin (BSA). The antibody was revealed using a protein A–peroxidase system containing 0.06% of chloronaphthol.

Protein sequence determination. The sequence of the NH_2 terminus was determined with 120 pmol of protein using an Applied Biosystems Model 470A gas-phase sequencer coupled with a Model 12A phenylthiohydantoin analyser.

Amino acid composition. The purified protein was hydrolysed by heating at 110°C in 5.7 *M* hydrochloric acid for 24 h. Composition was determined in a Beckman 7300 system.

RESULTS

Preparation and concentration of recombinant tropomyosins

The same purification scheme was used for the three recombinant tropomyosins, *i.e.*, TM1, TM[C31] and TM[N21]. The heat precipitation step is very interesting because a large amount of material can easily be processed. Most of the bacterial proteins were precipitated whereas the three tropomyosin mutants remained in the supernatant. We did not note any loss of recombinant protein in this step. As tropomyosin is denatured at temperatures above 50°C [9], the solubilization is due to a very rapid and efficient renaturation. The fact that the mutated proteins were resistant to heat treatment implies that the spatial structure is at least partially conserved. Analysis of the purified mutants by circular dichroism confirmed this fact (data not shown).

Although most of the bacterial proteins were removed by heat treatment, the nucleic acid still remained in the supernatant. Some of them were removed by precipitation with 30% ammonium sulphate. Tropomyosin was then precipitated by increasing the ammonium sulphate concentration to 60%. In this step the recovery was generally greater than 90%.

Chromatography on DEAE-TSK

Proteins concentrated by ammonium sulphate precipitation were dialysed against buffer D and quantified using the Bradford reaction. A 250-mg sample of proteins was chromatographed on the DEAE-TSK column as described under Experimental. The chromatogram of each mutant is presented in Fig. 1.

The chromatographic profile is very reproducible for a given preparation. The yield in this step was *ca.* 60%. Nevertheless, the efficiency of the heat precipitation of bacterial proteins varied with the amount of the extract. A better yield was achieved with a smaller amount. The yield of the engineered proteins also depended on the bacterial strain used. Finally, the amount produced varied from one mutant to another.

Fig. 1 shows that mutant TM[C31] was eluted earlier than the other two. This behaviour is in agreement with the number of charges contained within the molecules. TM[C31] possesses only 21 negative charges whereas TM1 and TM[N21] have 26 and 28 negative charges, respectively. Although TM[N21] eluted slightly (but reproducibly) earlier than TM1, this difference is not very important, probably owing to the high number of charges (53 positive and 81 negative charges for TM1 and 43 positive and 69 negative charges for TM[N21]). However, this result also implies that the charges are mainly located at the surface of the molecule. This, in turn, is in good agreement with the spatial model proposed by Phillips *et al.* [10].

Chromatography on hydroxyapatite

Tropomyosin is known to bind Ca^{2+}. We therefore used hydroxyapatite for further purification. Fig. 2 shows the results obtained with the three mutants. The resolution of the column is not as good as with DEAE-TSK. However, because tropomyosin eluted at the end of the gradient (300 m*M* phosphate), it is generally electrophoretically pure after this step. The fact that the three mutants eluted at the same concentration of phosphate strongly suggests that the calcium binding site on the tropomyosin is situated in the middle of the molecule and is not removed from the mutants.

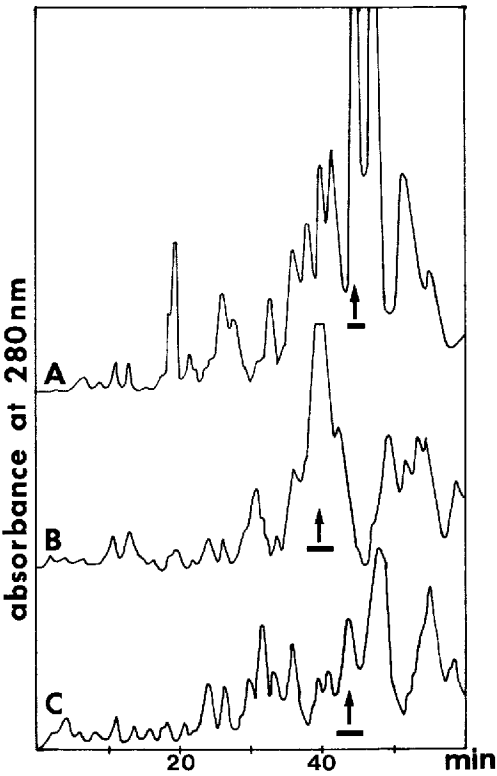

Fig. 1. Chromatographic profile on TSK-DEAE obtained for the three mutants of tropomyosin. Proteins were extracted from *E. coli* containing expression plasmids, heat treated and fractionated by ammonium sulphate precipitation as described. Protein mixtures (250 mg) containing (A) complete tropomyosin (TM1), (B) deletion mutants TM[C31] or (C) TM[N21] (see text) were chromatographed on a TSK-DEAE column as described. Eluted fractions were analysed by SDS-PAGE. The localization of the tropomyosin mutants is shown by bars and the maxima are indicated with arrows.

A further purification was sometimes needed. This was done by gel permeation on a TSK-G300 column. However, the recovery was very low and we carried out this procedure no often than was strictly necessary. We also tried reversed-phase chromatography but the resolution for large molecules was very poor. We performed instead a p*I* precipitation at pH 4.1 to remove the remaining contaminants.

Tryptic digestion analysis

One major problem in site-directed mutagenesis lies in the way one has to control whether the protein produced in *E. coli* is the one desired. As no selective pressure is exerted on this kind of protein, the possibility of mutation or deletion is very high. This makes the control of its sequence essential. To perform this task we used the tryptic digest analysis by taking advantage of the possibility of predicting the elution time of peptides using the algorithm of Meek [6] and the coefficient of Guo *et al.* [7].

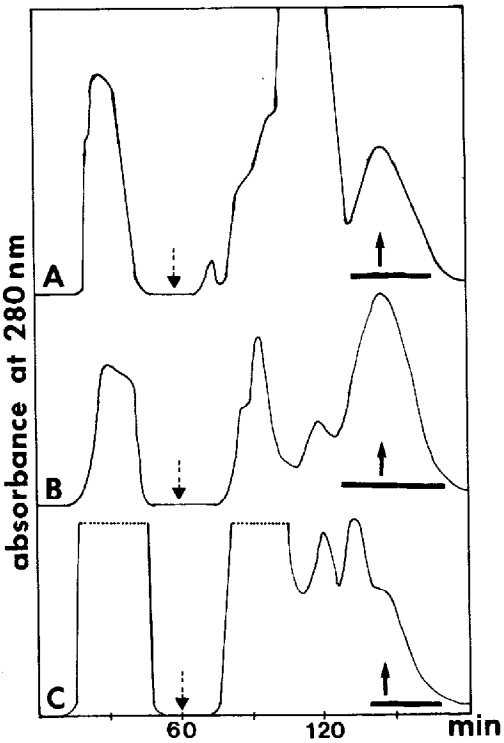

Fig. 2. Hydroxyapatite purification. Fractions from the DEAE-TSK chromatography containing tropomyosin were pooled and purified on hydroxyapatite as described. The eluted fractions were then analysed by SDS-PAGE. The position of the tropomyosin mutants is shown by bars (the broken arows indicate the start of gradient. Chromatograms of (A) TM1; (B) TM[C31] and (C) TM[N21].

Fig. 3 shows the analysis of tryptic digest by reversed-phase HPLC and compares the pattern obtained with that predicted by computer simulation. The presence of tyrosine or phenylalanine in a peptide was monitored by the absorbance at 280 nm and is indicated by black spots in the computer simulation (Fig. 3, A1, B1, C1). First, agreement between the experimental result and the simulation was good for long peptides. This allowed the identification of the peptides that were lost in the TM[C31] mutant. They are missing in the chromatogram of TM[C31] shown in Fig. 3B (these peptides are indicated by black triangles in Fig. 3A, on the chromatogram of TM1). This result clearly demonstrates that the TM[C31] mutant is correct. The case of the TM[N21] mutant was more difficult to resolve by this method. Peptides arising from the deleted region are very small because of the high content of lysine and cannot be identified accurately. Only a new peptide arising from the NH_2-terminus is characteristic of this mutant, and is shown by the black diamond in Fig. 3C.

Further biochemical characterization

Further analyses were carried out to confirm the reality of each mutation. First, the purity and the antigenicity of the protein were checked by western blot analysis.

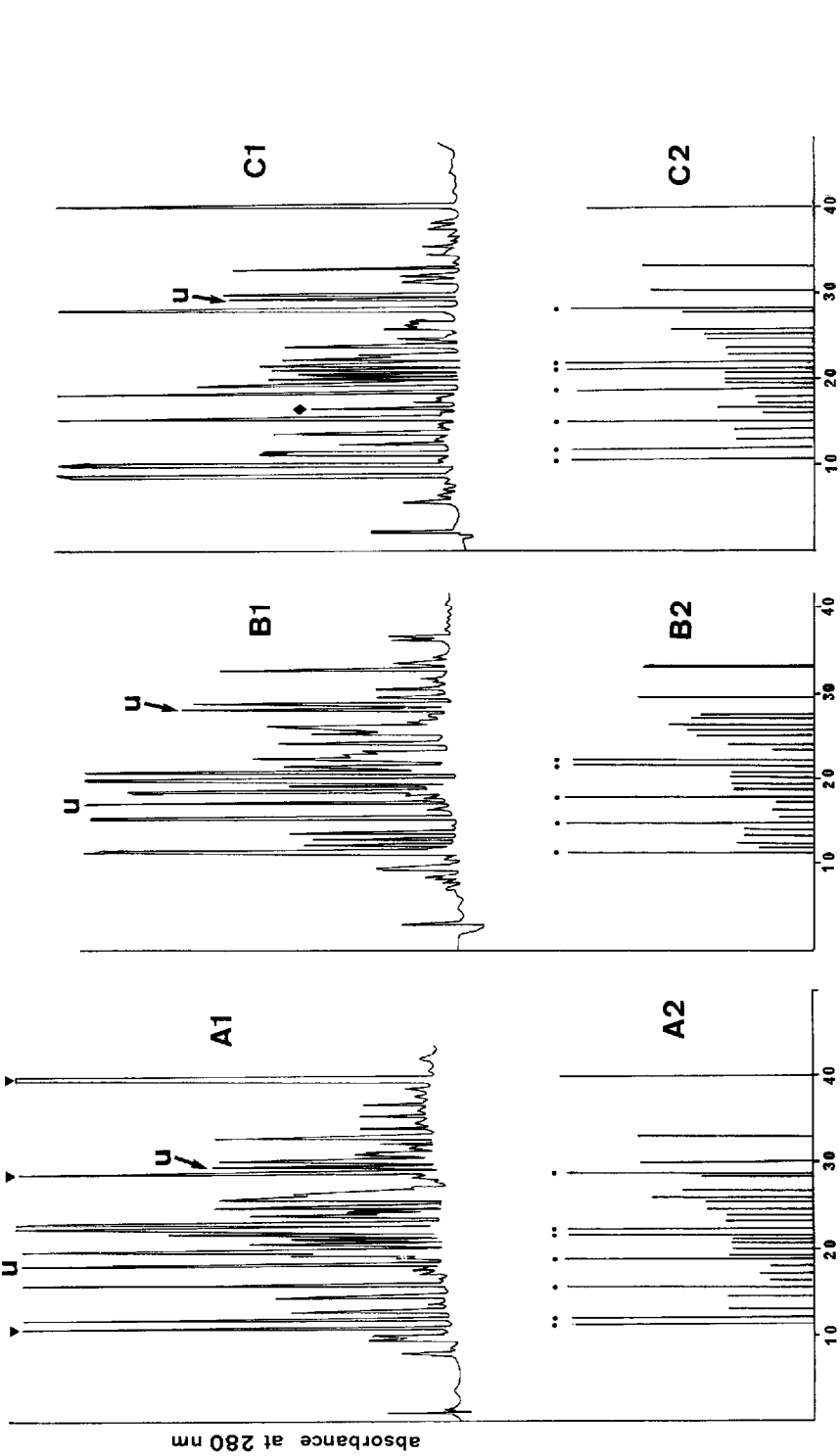

Fig. 3. Comparison of chromatographic behaviours and computer simulation of tryptic digests. After complete purification, as controlled by SDS-PAGE, the tropomyosin mutants were digested by trypsin and the tryptic digests were then chromatographed, as described. The elution profiles (A1, B1, C1 for TM1, TM[C31], TM[N21], respectively) are given in parallel with the results of computer simulation (A2, B2, C2). Peptides not found in TM[C31] deletion mutant are indicated by black triangles on the chromatogram of TM1 (A1) and a new peptide arising from TM[N21] is indicated by a black diamond (C1). On the simulation scheme, the peptides containing a tyrosine or a phenylalanine are indicated by black circles. U represents unidentified major peptides.

The results are shown in Fig. 4. Amino acid analysis was also performed, although the results were difficult to interpret because the sensitivity of the method is very low. However, the amino acid composition coincided with the theoretical results (result not shown). We also sequenced the NH_2-terminus. This is particularly important for the TM[N21] mutant. The results in Table I clearly confirm that the purified protein actually exhibits the desired mutation. The extra alanine at the N-terminus of TM[N21] is due to the cloning procedure. Further, it must be pointed out that methionine was removed only from the TM[N21] mutant. This is due to the size of the amino acid immediately adjacent to the methionine initiator. Methionine is removed by the bacteria if this amino acid is small, as with the TM[21] mutant.

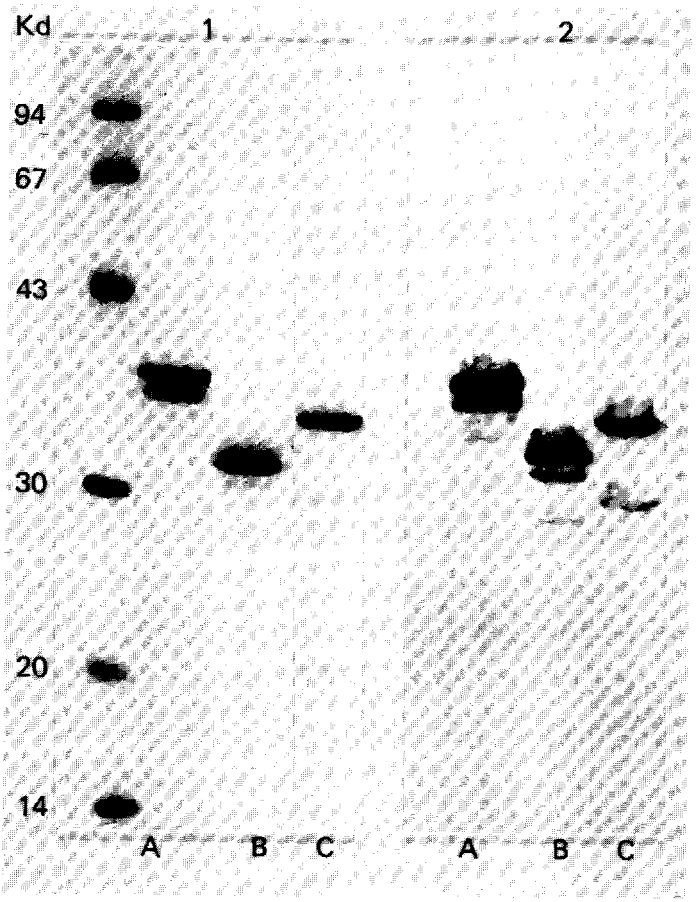

Fig. 4. Purity and antigenicity of proteins. The purified tropomyosin mutants were analysed by SDS-PAGE (1) and controlled by western blotting (2). TM1 is shown as track A, TM[C31] as track B and TM[N21] as track C.

TABLE I

SEQUENCE OF THE NH_2-TERMINUS OF THE PROTEINS

The NH_2 sequences were determined as described under Experimental. The sequence of TM1 and TM[C31] is identical with the N-terminus sequence of genuine tropomyosin. In contrast, as expected, TM[N21] exhibits the sequence of the tropomyosin devoid of the first N-terminal 20 amino acids. Single-letter code used for amino acids; Ac = Acetyl.

Protein	Sequence
Genuine β-tropomyosin	Ac- MDAIKKKMQMLKLDKENAID RAEQAEADKKQAE–
TM1	MDAIKKKMQMLKLDKE–
TM[C31]	MDAIKKKMQMLKLDKE–
TM[N21]	AAEQAEADKKQAE–

CONCLUSION

We have shown that recombinant tropomyosins can be rapidly purified using HPLC. Although the purification was performed at room temperature, very little degradation was observed, probably owing to the very rapid separation. The spatial structure of tropomyosin was conserved. This is worth noting because mutant proteins are often less stable than native proteins. The mutant proteins moreover retained their activity in the regulation of muscle contraction (to be published elsewhere). We have also shown that the use of simulation of tryptic digestion analysis is a powerful tool for rapidly controlling the preparation of proteins. One drawback of this method is that the identification of small peptides is very difficult.

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