PURIFICATION AND ANALYSIS OF THE CYANOGEN BROMIDE PEPTIDES OF TROPONIN T FROM RABBIT SKELETAL MUSCLE

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Summary. Troponin T was cleaved at its methionine residues by digestion with cyanogen bromide. Seven peptides were isolated by chromatography on Sephadex G-50 and SP-Sephadex, using volatile solvents. Amino acid analysis shows that one of the peptides represents about 60% of the protein, and arises from partial cleavage at one of the methionine residues. The sum of the amino acid compositions of the other six fragments is in good agreement with the composition of troponin T, and gives a calculated molecular weight for the polypeptide chain of 31,950.

1. Introduction

Troponin is a complex of three proteins (1) which, together with tropomyosin, confers calcium sensitivity to the interaction of actin and myosin. The complex consists of a calcium-binding protein (TnC)[1], an inhibitory protein (TnI) and a protein which has a strong affinity for tropomyosin (TnT). The amino acid sequences of TnC (2,3) and TnI (4) from rabbit skeletal muscle have been determined.

2. Materials and Methods

The rabbit skeletal muscle TnT used in this study was donated by Dr. James D. Potter. It was purified as described previously (6) on DEAE-Sephadex using buffers containing 6 M urea. The protein was desalted on a column of Sephadex G-25 equilibrated with 25% acetic acid, concentrated by rotary evaporation, diluted and lyophilized. The resulting material appeared homogeneous on electrophoresis in 10% polyacrylamide gels in the presence of

[1]Abbreviations: TnC, troponin C; TnI, troponin I; TnT, troponin T; CB-, cyanogen bromide peptide; SDS, sodium dodecyl sulfate; Hse, homoserine.

SDS, with an apparent mol wt of 37,000. Conditions for cyanogen bromide cleavage, subsequent treatment of the digest and chromatography on Sephadex G-50 were essentially the same as described by Elzinga (7). Ninhydrin monitoring of column fractions was done by spotting 5 µl of each fraction on paper and staining with cadmium-ninhydrin reagent (8); a visual estimate of relative staining intensity was plotted. To ensure that no weak-staining or ninhydrin-negative peptides were overlooked, fractions were also tested by ninhydrin after alkaline hydrolysis (9). Electrophoresis on 10% polyacrylamide gels in SDS (10) was useful for characterizing the large cyanogen bromide peptides.

3. Results and Discussion

Amino acid analysis of the cyanogen bromide digest of TnT indicated a 95% conversion of methionine to homoserine. Analysis without prior hydrolysis yielded no free homoserine, showing the absence of any Met-Met peptide bonds in TnT. One would therefore expect to find 6 or 7 cyanogen bromide peptides to account for the 5-6 residues (per 37,000 g) of methionine in TnT. Seven

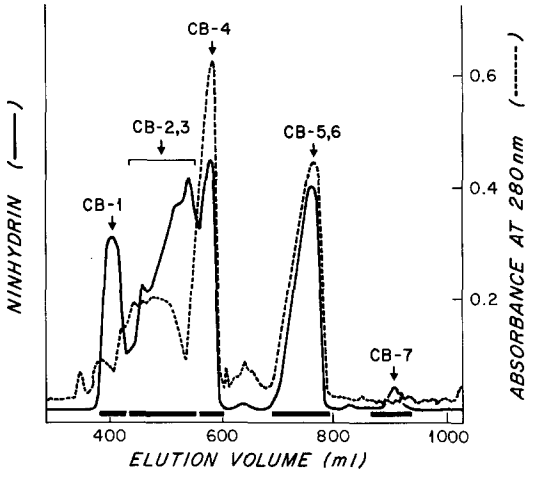

FIGURE 1. Chromatography of a cyanogen bromide digest of 4.4 µmoles of TnT on a 1.9 x 400 cm column of Sephadex G-50 (fine) equilibrated with 25% acetic acid. Horizontal bars indicate fractions pooled. The column was run at room temperature at a flow rate of 9 ml/hr and 6 ml fractions were collected.

	CB-1	CB-2	CB-3	CB-4	CB-5	CB-6	CB-7	Total CB2-7	Troponin-T CNBr Digest	Troponin-T Greaser et al. (12)
Lys	20.7 (18)	7.01 (7)	10.8 (11)	10.0 (10)	4.44 (4)	6.03 (6)		38	42.6	38.5
His	4.13 (5)	3.96 (4)	1.07 (1)	.94 (1)				6	6.06	6.42
Arg	18.1 (16)	2.05 (2)	14.0 (14)	4.02 (4)	2.02 (2)	.97 (1)	2.14 (2)	25	24.0	23.6
Asp	10.5 (10)	6.47 (6)	4.16 (4)	7.05 (7)	1.74 (2)		.85 (1)	20	22.2	21.2
Thr	.87 (1)	.80 (1)		2.05 (2)	.85 (1)	1.75 (2)		6	6.19	6.42
Ser	3.30 (5)	1.83 (2)	2.75 (3)	.98 (1)	1.80 (2)	.88 (1)		9	8.76	9.31
Glu	47.8 (50)	25.3 (25)	24.7 (25)	9.02 (9)	3.02 (3)		1.06 (1)	63	58.6	60.8
Pro	14.7 (13)	12.9 (13)		.89 (1)				14	11.2	12.8
Gly	1.47 (1)	1.00 (1)	.28	1.22 (1)	2.00 (2)	3.82 (4)		8	9.71	8.65
Ala	18.5 (18)	5.83 (6)	12.1 (12)	1.90 (2)	4.06 (4)	3.29 (3)	1.05 (1)	28	27.4	26.7
Val	7.43 (9)	7.96 (8)	.93 (1)			.98 (1)	2.05 (2)	12	11.2	11.9
Met	.72								.21	4.51
Hse	.73 (2)	.76 (1)	.78 (1)	.70 (1)	.81 (1)		.83 (1)	5	4.32	
Ile	4.83 (5)	2.01 (2)	2.98 (3)	2.82 (3)				8	8.08	8.71
Leu	9.86 (9)	2.05 (2)	7.49 (7)	7.89 (8)	.96 (1)	.87 (1)		19	21.2	20.6
Tyr	.35		.10	1.71 (2)	1.61 (2)			4	4.18	4.51
Phe	1.92 (2)	.98 (1)	.96 (1)	1.92 (2)		.88 (1)		5	4.56	5.45
Trp				(1)		(1)		2	(2)	1.8
Total	164	81	83	55	24	21	8	272	272	272
Yield, %	34	49	47	80	83	60	95			

major peptides (CB-1 to CB-7) were isolated in yields of 34-95%. There were also several minor (less than 10% yield) components present in the digest which were not characterized. Initial chromatography of the digest on Sephadex G-50 (Fig. 1) yielded three essentially pure peptides and two mixtures. CB-1, which eluted near the void volume of the column, contained small amounts of aggregated material and only traces of higher mol wt fragments (see Fig. 4). Approximately 85% (as judged by ultraviolet absorption) of the CB-4 present was obtained nearly pure by appropriate pooling of the fractions. CB-7 was subjected to automatic sequence analysis by the method of Hoan and Laursen (11). The sequence, Asn-Val-Arg-Ala-Arg-Val-Glu-Hse, is consistent with its amino acid composition (Table 1). CB-2 and CB-3 were separated by chromatography on SP-Sephadex (Fig. 2). A small amount of impure CB-4 was also recovered from this column. The relatively weak ninhydrin staining intensity of CB-2 may be due to its high proline content (Table 1). On SDS-polyacrylamide gel electrophoresis (Fig. 4), CB-2 was homogeneous, while CB-3 appeared about 95% pure. CB-5 and CB-6 were also separated on SP-Sephadex (Fig. 3). CB-5 appeared homogeneous when subjected to automatic sequence analysis (amino terminal sequence: Gly-Ala-Asn-Tyr-Ser-Ser-Tyr-Leu-Ala). The purity of CB-6 is established by its amino acid composition; it is the only peptide which lacks homoserine, aspartic acid and glutamic acid. Since CB-6 lacks homoserine, it must represent the carboxyl-terminal 21 residues of TnT.

In Table 1 the amino acid compositions of cyanogen bromide-treated TnT and the peptides CB-1 to CB-7 are presented. The sum of the compositions of CB-2 to CB-7 agrees very well with the composition of TnT, while CB-1

TABLE 1. Amino acid analyses of cyanogen bromide-treated troponin T and the peptides isolated from the digest. Samples were hydrolyzed for 20 and 96 hr in 6 N HCl at 110°C; values for Thr, Ser, Tyr and Hse are from 20-hr hydrolysates and are not corrected; 96-hr values are used for Val and Ile. Ratios are in most cases the averages of three or more analyses. Numbers in parentheses are nearest integers, except in the case of CB-1. For the purpose of comparison, CB-1 was normalized to 164 residues and the integers in parentheses are the sums of CB-2 and CB-3. Homoserine (Hse) was determined after incubating hydrolysates for a few minutes at 50°C and pH 11-12 prior to analysis.

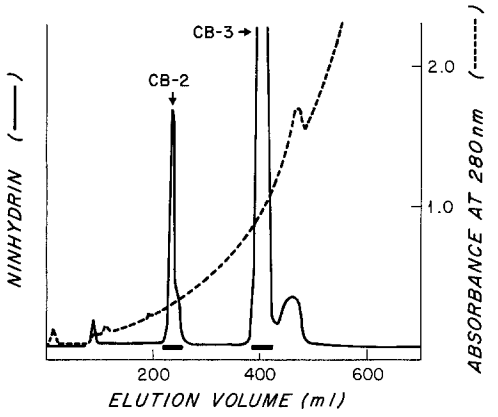

FIGURE 2. Chromatography of the fractions containing CB-2 and CB-3 (see Fig. 1) on a 0.9 x 20 cm column of SP-Sephadex C-25 (acid form) equilibrated with 25% acetic acid. Following sample application, the column was eluted with a concave gradient made up of 600 ml of 25% acetic acid and 230 ml of pyridine:acetic acid:water (25:25:50, v/v). Increasing absorbance at 280 nm is due to increasing pyridine concentration. The column was run at room temperature at a flow rate of 15 ml/hr and fractions of 5 ml were collected. Horizontal bars indicate fractions pooled.

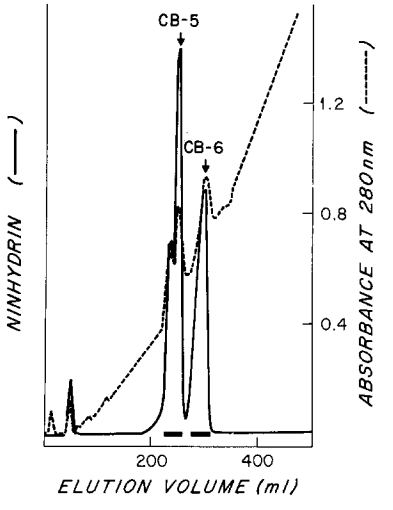

FIGURE 3. Separation of CB-5 and CB-6 on SP-Sephadex. Details are the same as for Fig. 2 except that the final pyridine concentration was 15%.

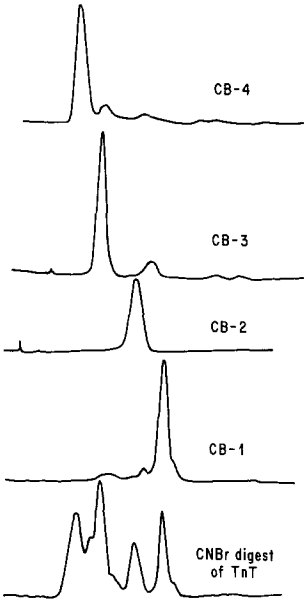

FIGURE 4. Densitometer scans of cyanogen bromide-treated TnT and peptides CB-1 to CB-4 after electrophoresis on 10% polyacrylamide gels in the presence of SDS. Direction of migration is from right to left. Identity of peptides with bands in the original digest was confirmed by coelectrophoresis of combined samples.

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appears to be derived from partial cleavage of the bond linking CB-2 and CB-3. The mol wt of TnT calculated from the sum of the amino acid compositions of CB-2 to CB-7 is 31,950.

Pearlstone et al. (13) have also isolated the cyanogen bromide peptides of TnT, and are determining their amino acid sequences. The results they have obtained so far agree (within experimental error) with those reported here.

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