Primary Structure of Rabbit Skeletal Muscle Troponin-T

PURIFICATION OF CYANOCEN BROMIDE FRAGMENTS AND THE AMINO ACID SEQUENCE OF FRAGMENT CB2*

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Troponin-T was cleaved by cyanogen bromide (CB) to produce the seven fragments CB1 (151 residues), CB3 (70 residues), CB2 (81 residues), CB5 (24 residues), CB4 (54 residues), CB7 (8 residues), and CB6 (21 residues). The NH2-terminal fragment CB1, composed of CB3 plus CB2, had an internal homoserine which was not completely cleaved. The amino acid sequence of CB2 was determined by a combination of automated and manual Edman degradation techniques. Peptides suitable for the latter method were derived from trypsic, a-chymotryptic, a-lytic protease, and thermolytic digestions. Fragment CB2 contains 81 of the 259 residues of troponin-T.

The troponin complex in conjunction with tropomyosin constitutes the calcium-sensitive regulatory system of contraction and relaxation in rabbit skeletal muscle. The following three proteins exist in a 1:1:1 molar ratio in the troponin complex; troponin-C (molecular weight of 17,900) which binds calcium, troponin-I (20,900) which, in the presence or absence of calcium, inhibits actin-myosin interaction, and troponin-T (M = 30,500) which binds to tropomyosin. Recent studies have indicated that Tn-T interacts, in addition, to troponin-C (1-6) and possibly to troponin-I (7). In order to understand the interactions of these muscle proteins as well as their functional relationship to the actomyosin ATPase system, their primary, secondary, and tertiary structures must be known. To date, the amino acid sequences of rabbit skeletal a-tropomyosin (8), troponin-C (9), and troponin-I (10) have been determined. In this paper as well as the two following (11, 12), the strategy and experimental evidence used to document the complete sequence of Tn-T is reported. The first section of this paper describes the cyanogen bromide cleavage of the protein and the subsequent purification of six unique fragments. The sequence determination of CB2, an 81-residue fragment, is also included. A preliminary report of some of this work has been published (13).

METHODS

Isolation of Troponin-T - The troponin complex was prepared from rabbit skeletal muscle according to the method of Ebashi et al. (14) as modified by Strauss et al. (15). Troponin-T was subsequently isolated from the troponin complex as described by Greaser and Gergely (16).

Cyanogen Bromide Cleavage - Cyanogen bromide fragments were obtained using a 200-fold molar excess of cyanogen bromide over methionine at 20° in 70% formic acid. After 24 h, the reaction mixture was diluted 10-fold with water and lyophilized to remove the excess reagents.

Column Chromatography - The cyanogen bromide digest (600 mg) was initially applied to a Sephadex G-75 column (5 x 250 cm) and eluted with 10% formic acid. Fractions were collected in 13-ml aliquots and the elution profile determined by alkaline hydrolysis followed by ninhydrin reaction (17). In some cases, enzyme digests were also subjected to this method as a first step in the purification procedure.

Fragments CB5 and CB6, co-eluted off the Sephadex G-75 column, were further separated on a phosphocellulose column (2.5 x 40 cm) equilibrated with 1 mM sodium acetate buffer (pH 3.8). The amount of sample applied to the column was 37.4 mg. A linear gradient of sodium chloride from 0.25 to 1.0 M was used and 10-ml fractions collected. Absorbance measurements at 230 nm determined the position of the peaks. Similarly, the purification of CB2 and CB3 was achieved on a DEAE-cellulose column (2.5 x 40 cm) equilibrated with 1 mM Tris buffer, pH 8.0, using a linear gradient of NaCl from 0 to 0.4 M. The amount of sample mixture applied was 185 mg. Fractions 2-4 in Tables II through VI were found to contain the A subunit.

In all three papers of this series, peptides were designated according to the methods used for cleavage as follows: CB, cyanogen bromide; C, a-chymotrypsin; TC, TLCK-chymotrypsin; AP, a-lytic protease; T, TPCK-trypsin on citraconylated fragment; television, trypsin on unmodified fragment (CB3 and CB7); Th, thermolysin; H, partial acid hydrolysis; P, pepsin; B, BNPS-skatele. To avoid confusion, some peptides have been assigned a combination of these letters in chronological order of the enzymes or reagents used.

Some of the data are presented as a miniprint supplement immediately following this paper. Fig. 4 and Tables II through VI are found on pp. 976-977. For the convenience of those who prefer to obtain supplementary material in the form of 15 pages of full size photocopies, these same data are available as JBC Document No. 76M-913. Orders for supplementary material should specify the title, authors, and references to this paper, the JBC Document Number, and the number of copies desired. Orders should be addressed to the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $2.25.

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† The abbreviations used are: Tn-T, troponin-T; SDS, sodium dodecyl sulfate; TPCK, tosyl phenylalanine chloromethyl ketone; PTH, 3-phenyl-2-thiohydantoin; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; TLCK, N,N'-p-tosyl-L-lysine chloromethyl ketone HCl; Quadrol, N,N',N'-tetakis(2-hydroxypropyl)ethylenediamine; DMAA, 3-methyl-3'-bromoindolienine; TLC, thin layer chromatography.
High Voltage Paper Electrophoresis—The further purification of the shortest CNBr fragment, CB7, was accomplished by high voltage paper electrophoresis (18) at pH 6.5 (3000 V for 45 min). Side strips were cut off and stained with ammonium thiocyanate. The band was cut out of the fragment diluted off the paper with water. The preparative purification of smaller peptides from enzymic digestes was achieved by a combination of high voltage paper electrophoresis at pH 6.5 and 1.8. In order to identify the tryptophan-containing cyano- gen bromide fragments, purified samples were run at pH 6.5 as described previously and the paper stained with Ehrlich reagent (19).

SDS-Polyacrylamide Gel Electrophoresis—To determine the purity and size of fragments CB1, CB2, CB3, and CB4, polyacrylamide (8%, w/v) gel slab electrophoresis (20) was carried out in the presence of sodium dodecyl sulfate in 100 mM sodium phosphate buffer, pH 7.0, as described by Weber and Osborn (21).

Enzymic Digestions—Trypsin (toys) phenylalanine chloromethyl ketone and chymotrypsin were purchased from Worthington, thermolysin from Calbiochem; α-lytic protease was prepared in the laboratory of Dr. D. R. Whitaker, University of Ottawa. Tryptic, α-chymotrypsin, and α-lytic protease digestions were carried out at 37° for 3 h in 0.2 M ammonium bicarbonate buffer (pH 8.1). The enzyme/substrate molar ratio was 1:50 in all cases. Thermolysin was used in a molar ratio of enzyme to substrate of 1:100 in 0.2 M ammonium acetate, 1 mM calcium chloride buffer, pH 8.2, at 37° overnight.

Citraconylation of Peptides—The method of Gibbons and Perham (22) was modified slightly. The peptide was dissolved in 0.05 M sodium phosphate buffer (pH 8.0). A 40-fold molar excess of citraconic anhydride (Eastman) over lysine residues was added at 25° with continuous stirring. The pH of the reaction was maintained at 8.0 by the addition of 3 M sodium hydroxide. After completion of the reaction, the citraconylated peptide was desalted by column chromatography using Sephadex G-25 equilibrated with 0.2 M ammonium bicarbonate buffer, pH 8.0. The citraconyl groups were removed, after cleavage of the peptide, by incubation in 10% formic acid at 25° for 6 h.

Determination of Acids and Amides—The electrophoretic mobilities of peptides relative to aspartic acid at pH 6.5 were used to calculate the net charge (23). In peptides containing several glutamyl and aspartyl residues, the acids and amides were assigned by removing a sample after each step of the subtractive Edman procedure and determining the mobility of the resultant peptide. After automated sequence analysis, some assignments were confirmed by gas-liquid chromatography of the PTH-derivatives. A summary of the evidence for acid and amide assignments is given in Table VI.

Degradation—Manual Edman degradations were carried out according to the method of Gray (24). The NH₂-terminal residues were analyzed by dansylation (25).

Amino Acid Compositions—Amino acid analyses were performed on a Durrum model D500 or Beckman model 120C analyzer. Purified peptides were hydrolyzed in 6 N HCl containing 0.1% phenol at 110° for 6 h. The hydrolysates were dried over NaOH pellets in a vacuum desiccator. For homoserine analyses, the dried HCl hydrolysates were incubated at 110° in pH 6.5, water/pyridine/acetate (897/100/3, v/v/v) buffer for 1 h to convert homoserine lactone to homoserine. The samples were then dried and freeze-dried. Samples from each peak were submitted to SDS-polyacrylamide gel electrophoresis to determine the size and purity. The last peak off the column contained no peptide material when amino acid analysis was used as the criterion. Fragments CB1 and CB4 were pure at this stage and suitable for direct sequence determination. CB7 was subjected to high voltage paper electrophoresis at pH 6.5. CB2 and CB3 as well as CB5 and CB6 were cross-contaminated so that further purification was necessary. Fig. 2 shows the fractionation of CB2 and CB3 on a DEAE-cellulose column. The two major peaks, well separated from minor impurities, were desalted on a Sephadex G-25 column equilibrated with 10% formic acid. The complete resolution of CB5 from CB6 was achieved on a phosphocellulose column (Fig. 3). Peaks 1 to 4 inclusive had identical amino acid compositions. Their partial separation might be attributed to minor differences in charge, possibly due to the removal of some amide groups and the occurrence of both homoserine and homoserine lactone at the COOH-terminal position. This group was design

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Gel filtration of cyanogen bromide digest of troponin-T on a Sephadex G-75 column (5.0 x 250 cm) equilibrated with 10% formic acid. Fractions (0.3 ml/tube) were collected and monitored by alkaline hydrolysis followed by ninhydrin reaction. The horizontal bars indicate fractions which were pooled.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Chromatography of CB2 + CB3 on a DEAE-cellulose column (2.5 x 40 cm) equilibrated with 1 mM Tris buffer, pH 8, using a linear gradient of NaCl from 0 to 0.4 M. Fraction size, 5 ml. ---, absorbance at 280 nm; ----, NaCl gradient. The horizontal bars indicate fractions which were pooled.
TABLE I

| Amino Acid | CB1 | CB2 | CB3 | CB4 | CB5 | CB6 | CB7 | Sum CB2-7 | Troponin-T |
|------------|-----|-----|-----|-----|-----|-----|-----|-----------|------------|
| Asp        | 12.0| 10.7(10) | 4.5(4) | 6.2(6) | 1.2(1) | 2.0(2) | 1.0(1) | 20.9(20) | 20.7       |
| Thr        | 1.8 | 1.0(1) | 1.0(1) | 2.1(2) | 1.1(1) | 1.5(3) | 1.9(2) | 6.1(6)  | 6.4        |
| Ser        | 4.2 | 4.5(5) | 2.8(3) | 1.7(2) | 1.1(1) | 1.8(2) | 0.9(1) | 8.3(9)  | 8.7        |
| Glu        | 38.4| 43.9(45) | 24.0(24) | 19.2(21) | 8.7(8) | 3.0(3) | 1.0(1) | 56.0(57) | 53.7       |
| Pro        | 8.3 | 7.1(8) | 7.1(8) | 1.0(1) | 1.3(3) | 3.9(4) | 8.1(9) | 9.6      |
| Gly        | 2.0 | 1.2(1) | 1.2(1) | 1.2(1) | 2.3(2) | 3.9(4) | 8.6(8) | 9.0      |
| Ala        | 17.5| 16.6(16) | 11.1(11) | 5.2(5) | 2.4(2) | 4.1(4) | 3.1(3) | 9.0      |
| Val        | 7.5 | 7.3(8) | 0.9(1) | 6.4(7) | 1.1(1) | 2.2(2) | 10.6(11) | 11.7     |
| Ile        | 4.9 | 4.6(5) | 2.8(3) | 1.8(2) | 2.9(3) | 7.7(8) | 0.9(1) | 8.1      |
| Leu        | 10.2| 9.3(9) | 7.2(7) | 2.1(2) | 7.8(8) | 0.9(1) | 1.0(1) | 10.9(19) | 19.6      |
| Tyr        | 1.9 | 1.0(1) | 1.0(1) | 2.1(2) | 1.0(1) | 5.0(5) | 3.4(3) | 5.6      |
| Phe        | 1.8 | 2.0(2) | 1.0(1) | 1.0(1) | 2.0(2) | 1.0(1) | 5.0(5) | 5.3      |
| His        | 4.9 | 4.3(5) | 0.9(1) | 3.4(4) | 1.0(1) | 5.0(3) | 3.4(3) | 5.6      |
| Lys        | 19.7| 17.9(18) | 10.8(11) | 7.1(7) | 10.0(11) | 4.1(4) | 5.7(6) | 37.7(39) | 36.5      |
| Trp        | 1.7 | 1.5(15) | 14.2(14) | 2.1(2) | 3.9(6) | 2.0(2) | 1.0(1) | 25.1(25) | 24.0      |
| Arg        | 1.5 | 2.0(2) | 1.0(1) | 1.0(1) | 0.9(1) | 0.8(1) | 1.0(1) | 4.7(5)  |
| Met        | 0.4 |       |       |       |       |       |       | 4.6      |
| Total      | 152.3| (151) | (81) | (70) | (55) | (24) | (21) | (8) | (259) | 258.2     |
| Yield (%)  | 40  | 39  | 57  | 78  | 34  | 26  | 28  |        |
| Mr         | 17,899| 17,893 | 9,852 | 8,059 | 6,789 | 2,702 | 2,217 | 974  | 30,503 | 30,254 |
| NH₂-terminus | Glx | Lys | Gly | Leu | Asx |       |       |       |

*Residues per molecule. Integral values in parentheses were determined from sequence analysis.

Fig. 3. Chromatography of CB5 + CB6 on a phosphocellulose column (2.5 x 40 cm) equilibrated with 1 mm sodium acetate buffer, pH 3.8, using a linear gradient of NaCl from 0.25 to 1.0 M. Fraction size, 10 ml. ---, absorbance at 230 nm; ---, NaCl gradient. Arrow indicates start of gradient. The horizontal bars below the peaks indicate fractions which were pooled. The brackets above the peaks identify the CB fragments.

Characterization of Cyanogen Bromide Fragments – A summary of the amino acid compositions, NH₂ termini, molecular weights, and overall yields of the CNBr fragments is given in Table I. The compositions obtained from amino acid analyses show good correlation with those determined by sequence analysis (enclosed by parentheses). The sum of CB2 to CB7 inclusive is 259 residues as compared to 258.2 obtained from the hydrolysis of whole troponin-T. We can conclude, therefore, that all the amino acids of Tn-T have been accounted for in the fragments CB2 to CB7. The molecular weight of the protein, found to be 30,503 by sequence determination, is much smaller than the value of 37,000 previously estimated by SDS-polyacrylamide gel electrophoresis. CB6 was assigned the COOH-terminal position of Tn-T, since it alone lacked homoserine. A total of two tryptophan residues was found in the protein by the method of Liu and Chang (30). These residues were located in fragments CB4 and CB6 by subjecting samples from each CNBr fragment to high voltage paper electrophoresis, then staining with Ehrlich reagent (19).

The following experimental evidence identified CB1 as consisting of CB3 + CB2 with incomplete cleavage at methionine 704: (a) the amino acid compositions of the first two columns of Table I were in close agreement; (b) both CB1 and CB3 had blocked NH₂ termini; (c) peptic cleavage of CB1 produced a fragment which stretched from residues 58 (in CB3) through 72 (in CB2); (d) when CB1 was subjected to automated sequence analysis, the first cycle was blank while steps 2 to 30 were identical with the sequence of residues 71 to 99 in CB2; (e) when a sample of CB1 after one cycle through the sequenator was subjected to SDS-polyacrylamide gel electrophoresis, two additional bands with mobilities identical with CB2 and CB3 were seen. The last two results indicated that the conditions of the first Edman cycle could effect partial cleavage of the bond between residue 70 and glutamic acid 71 in CB1 to produce CB3 plus CB2. Since the NH₂ terminus of CB3 is blocked, the subsequent cycles involved the sequencing of CB2.

Amino Acid Sequence of Fragment CB2 – The amino acid sequence of CB2 was determined by a combination of automated sequence analysis of the intact CB2 fragment and manual Edman degradation of peptides derived by proteolytic digestion of CB2. Table II shows the result obtained after each step through the sequenator. Summaries of electrophoretic mobility, molecular weight, net charge, yield, and amino acid composition of CB2 were given in the text. In all three papers of this series, the amino acids are numbered according to their position in the completed sequence of troponin-T.
composition of the peptides are given in Tables III, IV, and V for each type of secondary cleavage. Fig. 5 summarizes the peptides used to document the complete sequence of CB2. Glutamine 73 was assigned by gas-liquid chromatography of the phenylthiohydantoin derivative (see Table II). All other acids and amides were determined from the mobilities, after high voltage paper electrophoresis at pH 6.5, of smaller peptides directly after the run and/or during consecutive cycles of the manual Edman procedure.

The first 44 residues were obtained from automated Edman degradations. Proceeding from the NH₂ terminus of CB2, the peptides providing a minimum of 2-residue overlaps were as follows: T4(71-83), C3(81-93), T3(84-96), C8(94-109), automated sequence results for residue up to 114, ClO-Thl(110-119), ClO-AP2(113-123), T13(122-128), C11(123-148), ClO-APii(128-148), and T4(147-151). Several smaller peptides stretching through the same regions as these overlapping peptides have been included in Fig. 5 to provide details of the manual sequencing results. Larger peptides from portions of CB2 whose sequence had already been established were subjected to only a few cycles of manual degradation until their identities were implicit from their amino acid compositions.

Several tryptic and chymotryptic peptides (T4, C1, C4, C5 of Tables III and IV) derived from the NH₂ terminus of CB2 had a higher net negative charge than could be accounted for on the basis of their amino acid compositions. The reason for this is presently unclear and is under further investigation to determine whether the Glu-71 residue is modified or has a prosthetic group attached to it. The incomplete cyanogen bromide cleavage of the Met-Glu bond between residues 70 and 71 could possibly be related to the extra negative charge at this position, although an incomplete cleavage by cyanogen bromide at a Met-Glu bond has been recently reported in the sequence of flagellin of Bacillus subtilis 168 (31).

Fig. 5. Summary of peptides used to establish the complete amino acid sequence of CB2. →, automated Edman degradation; −−−, subtractive manual Edman degradation; −−−−−, regions inferred from amino acid composition and comparison with analogous peptides.
CB2 (residues 71 to 151) contains 81 of the 259 amino acid residues of troponin-T. It is the second cyanogen bromide fragment in the sequence, placed after the NH₂-terminal CB3. Recent studies (32) have implicated CB2 in the binding of Tn-T to tropomyosin. Fragment CB1 (residues 1 to 70) gave no such increase while the viscosity change with CB2 (residues 71 to 151) was intermediate. Jackson et al. (32) have concluded that the tropomyosin binding site on Tn-T is between residues 71 and 151, although one cannot exclude the possibility of a role for the COOH-terminal region of CB3 in this binding as well as other parts of the molecule.

As previously pointed out (13), predictions of secondary structure by the method of Chou and Fasman (33) indicate that fragment CB2 has by far the largest proportion of α-helical content in the Tn-T molecule, with two long α-helical segments from residues 80 to 102 and residues 122 to 146. This fragment is also highly ionic with 59% of the residues either positively or negatively charged at neutral pH. It is tempting to speculate that it is one or both of these highly charged regions, predicted to be in the α helical conformation, which are involved in the interaction with tropomyosin over a limited region of its coiled-coil structure.

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Sequence of Rabbit Skeletal Troponin-T

The following sequence of rabbit skeletal troponin-T was isolated from the myofibrillar protein fraction of rabbit skeletal muscle. The sequence is presented in the table below.

| Peptide | Field | pH | Net Charge | Number of Amino Acids | Amino Acid Composition | Source, Reference |
|---------|------|----|-----------|-----------------------|----------------------|------------------|
| 51-84   | -0.27| 1486| 0         | 34                    | Ala-Glu-Glu-Val-Glu   | 51-84            |
| 82-111  | -0.38| 1331| -1        | 30                    | Asp-Glu-Asp-Val-Glu   | 82-111           |
| 100-113 | -0.17| 1599| -1        | 14                    | Glu-Ala-Leu-Asp-Val   | 100-113          |
| 32-72   | -0.37| 1908| -2        | 41                    | Glu-Asp-Asp-Val-Glu   | 32-72            |
| 75-78   | -0.26| 1538| -1        | 16                    | Ala-Glu-Val-Asp-Val   | 75-78            |
| 22-25   | -0.26| 1908| -2        | 23                    | Ala-Val-Asp-Val-Glu   | 22-25            |
| 15-18   | -0.38| 1171| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 15-18            |
| 7-10    | -0.37| 1908| -2        | 23                    | Ala-Val-Asp-Val-Glu   | 7-10             |
| 1-4     | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 1-4              |
| 28-31   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 28-31            |
| 6-9     | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 6-9              |
| 11-14   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 11-14            |
| 16-19   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 16-19            |
| 21-24   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 21-24            |
| 26-29   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 26-29            |
| 32-35   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 32-35            |
| 36-39   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 36-39            |
| 40-43   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 40-43            |
| 44-47   | -0.38| 1446| -1        | 14                    | Ala-Glu-Val-Asp-Val   | 44-47            |

The table above shows the sequence of rabbit skeletal troponin-T isolated from the myofibrillar protein fraction of rabbit skeletal muscle. The sequence is presented in the table above. The table includes the peptide sequence, the field, pH, net charge, number of amino acids, amino acid composition, and the source and reference for each peptide.
Sequence of Rabbit Skeletal Troponin-T

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### Table V

| Peptide | Identity of Peptide | Mass (kDa) | Number of Residues | Mass Compositional Error (kDa) | Mass of Each Amino Acid Component of Peptide (kDa) |
|---------|---------------------|------------|--------------------|--------------------------------|-----------------------------------------------|
| C15-AQ1 |                |            |                    |                                |                                               |
| C15-AQ11 |              |            |                    |                                |                                               |
| C15-AQ2 |              |            |                    |                                |                                               |
| C15-AQ3 |              |            |                    |                                |                                               |
| C15-AQ4 |              |            |                    |                                |                                               |
| C15-AQ5 |              |            |                    |                                |                                               |
| C15-AQ10 |             |            |                    |                                |                                               |
| C15-AQ11 |           |            |                    |                                |                                               |

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### Table VI

Summary of evidence for acid and basic assignments in fragments (R1)

| Residue | Net Charge of Peptide | Mass Compositional Analysis of Peptide |
|---------|-----------------------|---------------------------------------|
| C15-21 |                        |                                       |
| C15-22 |                        |                                       |
| C15-23 |                        |                                       |
| C15-24 |                        |                                       |
| C15-25 |                        |                                       |
| C15-26 |                        |                                       |
| C15-27 |                        |                                       |

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**Figure 4**

**DEAE Chromatography of C15 Peptide:** A DEAE-cellulose column was equilibrated with 50 mM Tris-HCl, pH 7.5, and fractions were collected and monitored at 220 nm. The shaded areas correspond to those described in Fig. 1.
Primary structure of rabbit skeletal muscle troponin-T. Purification of cyanogen bromide fragments and the amino acid sequence of fragment CB2.

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