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Preparation and Characterization of Anti-CeTNT-1 and Anti-CeTNT-4 Antibodies

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Abstract

Among the four CeTNT isoforms, CeTNT-1, CeTNT-2 are body wall types, CeTNT-4 is pharynx type and CeTNT-3 is expressed in both the body wall and pharyngeal tissue. In our previous study, we used body wall and pharynx type anti-CeTNI, anti-CeTNC and anti-CeTM antibodies to observe the tissue specific interaction of the TNI isoforms with others TN subunits and tropomyosin isoforms. To extent the interaction study of CeTNT isoforms, in this study, we prepared and characterized the body wall type anti-CeTNT-1 and pharynx type anti-CeTNT-4 antibodies. For the preparation of the anti-CeTNT-1 and anti-CeTNT-4 antibodies, in this study we constructed the pCTNT-1 and pCTNT-4 expression vectors. The sub-cloned of the pCTNT-1 and pCTNT-4 expression vectors were verified by DNA sequencing. These expression vectors were used to generate fusion proteins of the body wall, TNT-1 and pharyngeal TNT-4 isoforms in *Escherichia Ecoli*. The expression of these fusion proteins were confirmed by SDS-PAGE analysis. The anti-CeTNT-1 and anti-CeTNT-4 antibodies were prepared in the rabbit by using the gel cut of the CeTNT-1 and CeTNT-4 fusion proteins. The antibody specificity of the CeTNT-1 and CeTNT-4 fusion proteins was also judged by Western-analysis using prepared anti-CeTNT-1 and anti-CeTNT-4 antibodies. The antibody specificity results indicated that anti-sera against each of both the body wall type TNT-1 and pharynx type TNT-4 isoforms had tissue specificity.

Key words: Troponin T, Caenorhabditis elegans, Body wall, Pharynx

Introduction

Antibody, a protein produced by lymphoid cells (plasma cells) in response to foreign substances (antigens) and capable of coupling specifically with its homologous antigens (the one that stimulated the immune response) or with substances that are chemically very similar to that same antigen (Tiselius and Kabat, 1939). The antibody is response to produce a wide range of affinity reagent. For example, specific antibodies can be used to determine the precise subcellular location of an antigen, to isolate an individual antigen from a complex mixture of competing molecules, to find other macromolecules that interact with antigen, and even to determine the exact concentration of the antigen. These types of methods have been made antibodies one of the most useful reagents for studying molecules of interest (Harlow and Lane, 1998).

As antibodies used as highly specific reagents in the identification of the molecule, therefore antibody based approached is predicted to be a powerful tool in developing and understanding of the functional and structural properties of the molecule. It may be also useful for the identification of functionality of unknown protein identified in the proteome of organism after genome sequencing. The detection of TNI isoforms by specific antibody staining provides a convenient and reliable method of muscle cell typing (Dhoot *et al.*1978) and presence of TNT in serum can be used for detection of disease in skeletal and cardiac muscle (Cummins and Perry, 1978).The immunological detection of serum cardiac TNI is widely used in cardiology as an index of myocardial damage (Maria, 1976).

Muscle contraction is the result of a series of protein-protein interaction. In striated muscle, the thin filament complex troponin (TN) and tropomyosin (TM) regulates contraction. The troponin complex components includes the tropomyosin binding protein troponin T (TNT), calcium binding protein troponin C (TNC), the troponin I (TNI) which is involved in inhibition of the actomyosin ATPase activity (Ohtsuki *et al.* 1986 and Gordon *et al.* 2000). The interaction study of the thin filament component is important to know the mechanism of muscle contraction. In our previous study, we observed the interaction of the CeTNI isoform with CeTNC

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and CeTM isoforms and the prepared anti-CeTNI, anti-CeTNC and anti-CeTM antibodies were used in this interaction study (Amin et al. 2007).To extend the interaction study of CeTNT isoforms with other troponin subunits and tropomyosin isoforms in *C. elegans*, it would be necessary to prepared and characterized the anti-CeTNT antibody. There are four TNT isoforms in *C.elegans*, among them CeTNT-1-2 are expressed in body wall, CeTNT-3 expressed in both the body wall and pharynx and CeTNT-4 expressed only in the pharynx (T. Allen, 2000). In this study, we focused on the preparation of body wall anti-CeTNT-1 and pharyngeal anti-CeTNT- 4. The antibodies prepared in this study could be useful for immunostaining and interaction study of TNT with other TN subunits and tropomyosin isoforms.

Material and Method

Construction of the TNT-1 and TNT-4 expression vectors

pCTNT-1 was constructed by inserting an EcoRI restriction fragment corresponding to the 1215-bp full length tnt-1/mup-2 cDNA from yk1354g3 into the EcoRI site of pET-28a(+) vector. This restriction fragment was prepared by PCR using yk1354g3 as a template with TNT-1-s(5'-TTG-AAT TCA TGT CCG ACG AGG AGGAGG TAT AC-3') and TNT-1 as (5'-TTG AAT TCA TTC, GAC AAC GAC CTC TTC TCC-3') primer followed by EcoRI digestion. pCTNT-4 was constructed by inserting a BamHI restriction fragment corresponding to the 1044-bp full length tnt-4 cDNA from yk1174g10 into the BamHI site of the pET-28a (+) vector. This restriction fragment was prepared by PCR using yk1174g10 as a template with TNT-4s (5'-CCT GGA TCC TCG ATG AAC ATG TCT GAC GAG GAA TAC TCC G-3') and TNT-4 as (5'-TCT GGA TCC TTA ATA GTC TTC CTC TTC CTC GGC -3') primers.

Anti-CeTNT-1 and anti-CeTNT-4 anti-sera preparation and processing

Anti-CeTNT-1 and anti-CeTNT-4 were prepared in this study by immunizing rabbits with 1mg of bacterially expressed CeTNT isoforms. The bacterially expressed CeTNTs were prepared by using the standard protocol in Molecular cloning (Sambrook and Russell 2001). The anti-CeTNT-1 and anti-CeTNT-4 were produced by immunizing rabbits with gel homogenates containing 1mg of His-tag CeTNT-1 and CeTNT-4 fusion peptide which was isolated from the Coosmassie Blue stained gel. The immunization injection sample (antigen) of CeTNTs was prepared as follows: 8% SDS-PAGE gel was prepared for separating proteins. The stacking gel pours at the top of the 8% SDS-PAGE and one blade comb inserted into the top. The sample solution applied on the top of the stacking gel and about 100-200µl of bacterially expressed (CeTNT) protein samples were processed by running the gel around 2 hours (10mA were selected for each gel plate). After running, the gel was stained by using CBB for 1 hour and then the gel was de-stained approximately 6-8 hours. The target band was excised from the de-stain gel by using a sharp blade and scaled the weight of the excised band. The excised band was cooled into the mortar under liquid nitrogen and grind. The grinded gel was transferred into a 10ml beaker and equal volume of the complete Freund's adjuvant (GIBCO) were mixed with grinded gel (for the first injection complete Freund's adjuvant should be used but for 2nd and 3rd injection incomplete should be used) and homogenated by sonication and kept it at $4^{O}C$ before injection into rabbit. Step 2: Bleeding of the rabbits: The rabbits were anesthetized by injecting the Nembutal injection (Dainippon pharmaceutical co., ltd.) (approximately 1.5 ml of Nembutal should be injected to the each of the 2 kg weighted rabbit). The whole blood was collected into the sterilized conical flask.

Step 3: Processed the anti-sera: The collected whole blood were keep at room temperature for 2 hours for coagulation and transfered the sample at 4 ^{O}C for over night. The anti-sera were collected by centrifugation at 5,000 rpm, 4 ^{O}C for 15 minutes. The collected anti-sera were kept at -20 ^{O}C for future use.

Results and Discussion

The pCTNT-1 and pCTNT-4 expression vectors were constructed for the preparation of anti-CeTNT-1 and anti-CeTNT-4 antibodies. The sub-cloned of the pCTNT-1 and pCTNT-4 expression vectors were verified by DNA sequencing as shown in figure 1 and figure 2. These expression vectors pCTNT-1 and pCTNT-4 were expressed CeTNT-1 and CeTNT-4 fusion proteins in E. coli. The expression and the molecular size of the fusion proteins were judged by SDS-PAGE and analyzed to in Figure 3a & b. The anti-CeTNT-1 and anti-CeTNT-4 antibodies were raised in rabbit against the gel cut of CeTNT-1 and CeTNT-4 fusion proteins (see details in Materials and methods).These antibodies were characterized by Western analysis. The anti-CeTNT-1 antibody cross-reacted specifically with body wall type CeTNT-1 but did not cross-react with pharyngeal CeTNT-4 (Fig. 4a). Similarly, anti-CeTNT-4 also cross-reacted with only the pharyngeal CeTNT-4 but did not cross-reacted with body wall type CeTNT-1 (Figure 4b). The smaller band of CeTNT-4 may have come from limited proteolysis of CeTNT-4 in bacteria or from a second initiation

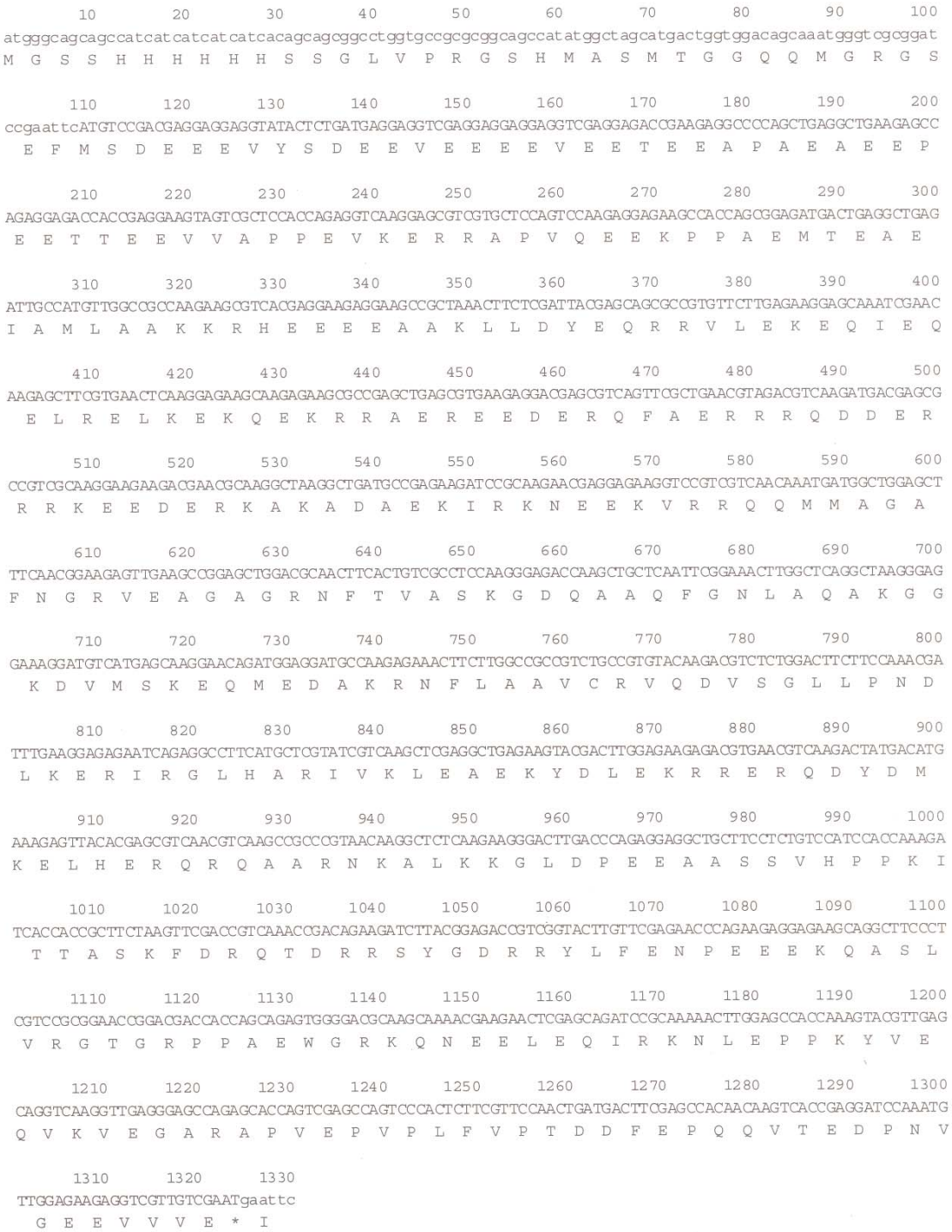

Fig. 1. The sequence of pCTNT-1 expression vector for body wall CeTNT-1 and its respective protein.

product of the construct. The results obtained by Western analysis indicated that both the body wall and pharyngeal anti-CeTNT-1 and anti-CeTNT-4 antibodies are tissue specific. This result is consistent with our previous study that the anti-CeTNC-1 and anti-CeTNC-2 cross-reactivities are tissue specific (Ruksana *et al.* 2005). Ruksana *et al.* (2005) also reported that the cross-reactivity of the pharyngeal anti-CeTNI-4 antibody with others CeTNI isoforms are tissue

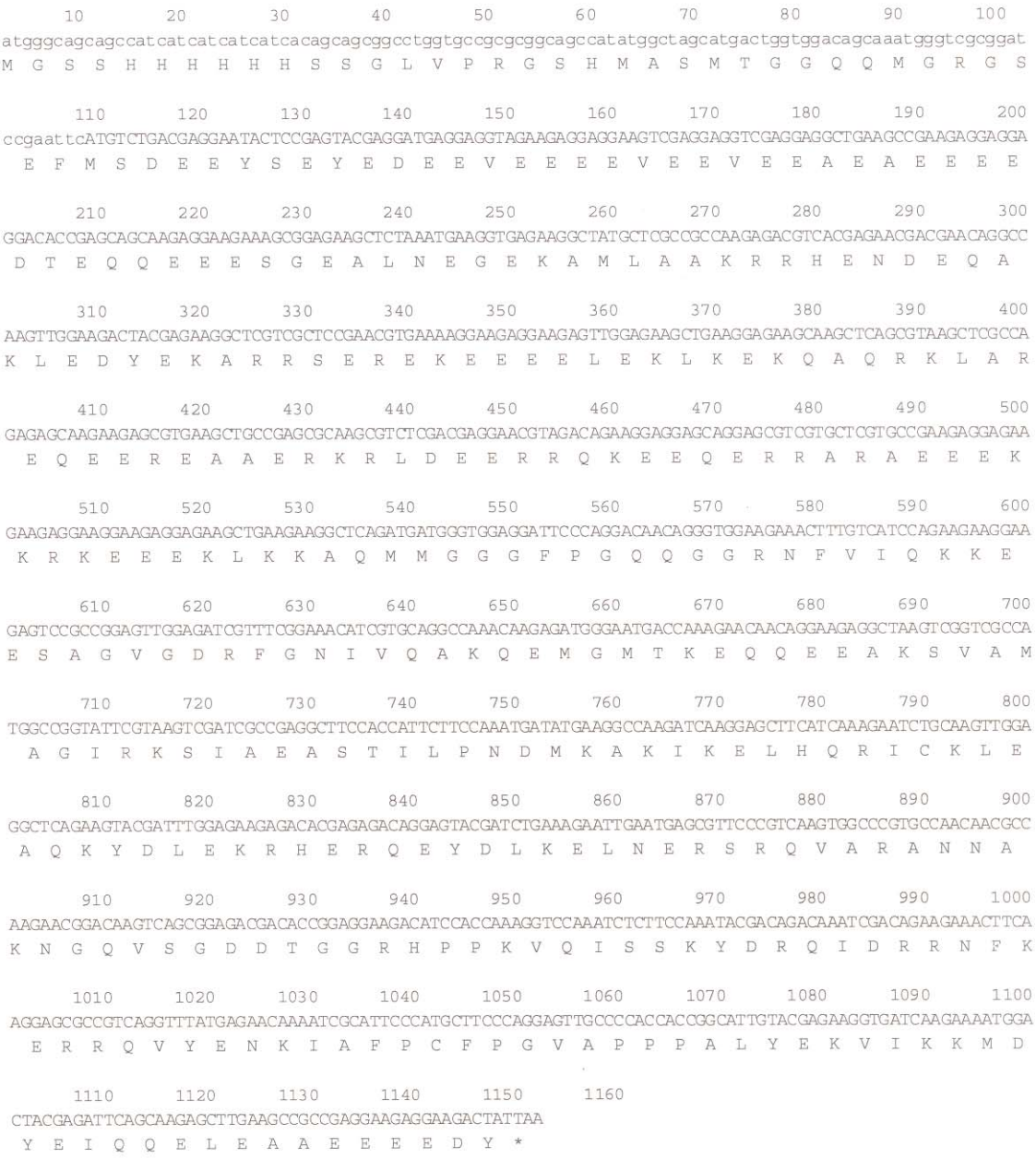

Fig. 2. The sequence of pCTNT-4 expression vector for pharyngeal CeTNT-4 and its respective protein.

specific, i.e anti-CeTNI-4 antibody specifically react with only CeTNI-4.But the cross-reactivity of the body wall anti-CeTNI-2 antibody with both the body wall and pharyngeal CeTNI isoforms are not tissue specific. The similar results were observed that the anti-CeTM antibody cross-reacted with its corresponding fusion protein (Amin *et al.* 2007). Anyanful *et al.*(2001) reported that the cross-reactivity of the pharyngeal anti-CeTMIII antibody with others tropomyosin isoforms are non specific but the cross-reactivity of the body wall anti-CeTMI antibody with other CeTM isoforms are tissue specific.

In this study, we found that anti-CeTNT-1 and anti-CeTNT-4 are tissue specific but it is unknown why these interactions are tissue specific. To answer this question, the comparison of the DNA sequence of both the body wall pCTNT-1 and pharyngeal pCTNT-4 expression vector from figure 1 and 2 shown that the C-terminal end of the pharyngeal CeTNT-4

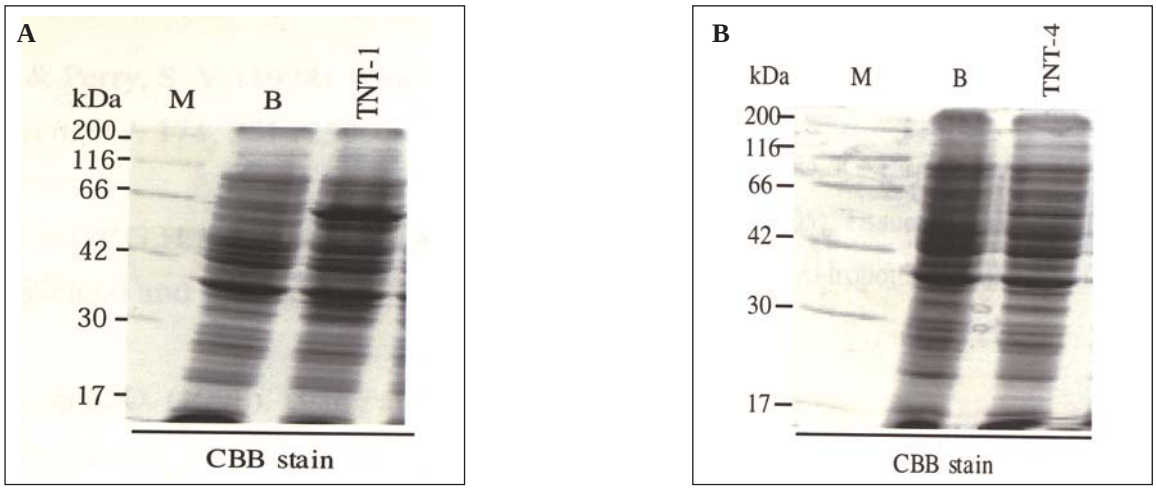

Fig. 3. SDS-PAGE analysis of body wall CeTNT-1 and pharyngeal CeTNT-4 isoforms

(a) Coomassie Blue stain of body wall type expression fusion protein TNT-1. Extracts of *E.coli* BL 21 (DE3) cells harbouring the pCTNT-1 expression vector.

(b) Coomase Blue stain of pharyngeal expressed fusion protein TNT-4. Extracts of *E. coli* BL21(DE3) Cells harbouring the pCTNT-4 expression vector. Each of the bacterial expressed fusion proteins were fractionated by SDS-PAGE using 8 % acrylamide gels. The positions are indicated by the molecular size of marker proteins in kiloDaltons (kDa). M indicates the molecular marker protein. Lane B contained the total protein in *E.coli* BL21 (DE3). The arrow head indicate the 52 kDa TNT-1 and a 45 kDa TNT-4.

Fig. 4. Western analysis of body wall CeTNT-1 and pharyngeal CeTNT-4 isoforms.
(a) Cross-reactivity of two TNT isoforms with the anti-CeTNT-1 antibody.
(b) Cross-reactivity of two TNT isoforms with the anti-CeTNT-4 antibody.

isoform rich in glutamic acid as compare to body wall CeTNT-1 isoform. Therefore, it could be the one reason for the tissue specific interaction of these isoforms. Another thing, the CeTNT-1 and CeTNT-4 isofcms could be evolved from the individual ancestral gene. At present the location of the antibody detection sites of either antibodies (anti-CeTNT-1 and anti-CeTNT-4) are not known. Molecular dissection techniques with epitope mapping of TNT will help to determine, which part of the molecule interact with other molecules (Hamada *et al.* 2002). To, know clearly about the tissue specific interaction of these isoforms more details in vivo or in vitro study is needed. Finally, the results obtained with tissue-specific antibodies might be available not only as an analytical tool for the study of TNT but will also be used

for in *vitro* or *vivo* study of other TN subunits and tropomyosin (TM) isoforms in C. elegans.

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