Biochemical characterization and cloning of transglutaminases responsible for hemolymph clotting in *Penaeus monodon* and *Marsupenaeus japonicus*☆

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Abstract

To investigate the shrimp blood clotting enzyme, a transglutaminase in the hemocytes of *Penaeus monodon* (abbreviated as TGH) was purified. TGH is an abundant homodimeric cytosolic protein with 84.2 kDa subunits. It clotted shrimp plasma and incorporated fluorescent dansylcadaverine into succinyl casein upon activation by CaCl_2 in vitro. IC_{50} for the activation was 3 mM, which is below the shrimp plasma Ca^{2+} level. Showing similar properties as other type II transglutaminase, TGH was particularly unstable after activation. MALDI-TOF/TOF mass-analyses of tryptic peptides of *P. monodon* TGH confirmed its identity to STG I (AY074924) previously cloned. A possible allele of the other isozyme STG II (AY771615) has also been cloned from the *P. monodon* cDNA and designated as PmTG. The predicted PmTG protein sequence is 58% similar to that of STG I and 99.2% to that of STG II. Likewise, a novel enzyme Mj-TGH was purified and cloned from *Marsupenaeus japonicus* hemocytes. Results of sequence alignment and phylogenetic analyses of these transglutaminases suggest that STG I and Mj-TGH are 83% identical and orthologous to each other, while PmTG/STG II and a previously cloned *M. japonicus* transglutaminase (AB162767) are their paralogs. Protein of the latter two could not be isolated, their regulated expression was discussed.

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1. Introduction

Transglutaminases (EC 2.3.2.13) are known to play critical roles in blood coagulation and other biochemical process involving protein cross-linking or related super-structures. Enzymes in this family catalyze calcium-dependent acyl-transfer reactions between glutamine residues and lysine residues in protein substrates. The role of Ca^{2+} is to induce enzyme conformational changes essential for substrate-binding and catalysis [1–4]. The formation of intra-molecular or intermolecular 1-(γ -

glutamyl) lysine bonds lead to polymerized or insoluble proteins. In addition, the enzymes mediate other post-translational modification of proteins, e.g., deamidation and amine incorporation [1].

The enzymes are widely distributed in tissues and body fluids of animals. For example, blood coagulation factor XIIIa catalyzes the cross-linking of fibrin monomer during blood coagulation [5], another transglutaminase toughens skin epidermis and hair follicle epithelia [6] in vertebrates. The enzyme is also involved in forming the fertilization plug in female rodents [7]. Up to ten transglutaminases in various invertebrate species have so far been reported [8–11], including those responsible for blood coagulation in Crustacean [9,10]. The enzymes were also found to undergo acetylation [1], phosphorylation, or fatty-acylation [12] that probably regulates their subcellular localization and catalytic activities. Some of them are involved in signal transduction and apoptosis [1,13].

Abbreviations: Mj, *Marsupenaeus japonicus*; Pm, *Penaeus monodon*; TGH, hemocyte transglutaminase; RFE, relative fluorescence enhancement

☆ The cDNA sequences of PmTG and Mj-TGH have been deposited in GenBank with accession No. AF469484 and DQ436474, respectively.

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Tiger shrimp (*Penaeus monodon*) is an economically important species cultured in Taiwan and southeastern Asia. Coagulation of the hemolymph is part of the innate immune response of Crustaceans, it prevents leakage of hemolymph from sites of injury and dissemination of invaders such as bacteria throughout the body. Much earlier, we have purified, characterized and cloned the clottable protein in the hemolymph of *P. monodon* [14]. Previously, two transglutaminases of *P. monodon*, STG I and STG II were cloned and expressed as the shrimp clotting enzymes [8,10]. In the present study, we first reported purification and biochemical characterization of major transglutaminases in shrimp hemocytes (abbreviated TGH) and their stability and regulation. Another transglutaminase from *P. monodon* (designated as PmTG), and a novel hemocyte transglutaminase designated as Mj-TGH from *Marsupenaeus japonicus* (kuruma prawn) were herein cloned and sequenced. Results of peptide sequencing and peptide mass fingerprinting of the purified TGHs from both shrimps enabled us to identify them with previously reported STG I of *P. monodon* [8] and Mj-TGH, respectively. Moreover, transglutaminases from invertebrates were subjected to phylogenetic analysis, and the regulations of their expression were discussed.

2. Materials and methods

2.1. Shrimp hemocytes and the reagents

A total of 90 live specimens of *P. monodon* and 50 live specimens of *M. japonicus* were obtained from local markets. The hemolymph was withdrawn by a syringe of 2.5 ml with a needle of 23 G and 1.25 inch long containing 1/8 volume of anticoagulant (0.1 M sodium citrate, 0.4 M NaCl). It was centrifuged at 1600×g for 5 min at 4 °C, the supernatant was harvested for clotting assay. The pellet was washed twice with a cold buffer (1.5 mM EDTA, 0.4 M NaCl, 0.25 M sucrose in 50 mM Tris–HCl at pH 7.5). Casein and monodansylcadaverin were obtained from (Sigma Chem. Co., USA). Succinyl-casein was synthesized from casein and succinyl anhydride (Pierce, USA) as previously described [15].

2.2. Transglutaminase assay

Succinyl-casein (500 µg/ml) were mixed with 50 µM monodansylcadaverine in 50 mM Tris–HCl (pH 7.6) containing 5–10 mM dithioerythritol and 6 mM CaCl₂. This substrate solution was thermostated at 23 °C in a cuvette before adding hemocyte lysate or purified *P. monodon* TGH. Incorporation of dansylcadaverin to succinyl-casein by TGH was followed by a fluorospectrometer (model MPF-4, Hitachi, Japan) at the excitation/emission wavelengths of 360/500 nm, respectively [15]. Upon adding the TGH, the reaction started immediately in the presence of CaCl₂ and no lag phase was observed. Initial enzymatic rate within 1 min was measured to minimize errors due to enzyme instability, and relative fluorescence enhancement (RFE) was calculated based on the percent fluorescence increase relative to the blank. The shrimp plasma was diluted with equal volume of 50 mM Tris–HCl (pH 7.5) with 10 mM CaCl₂ and then added purified TGH at a final concentration of 14–35 µg/ml. The clotting time was monitored at 23 °C since the shrimps live in natural marine environment at 15–35 °C.

2.3. Purification and characterization of shrimp TGH

After washing, the hemocytes in pellet were lysed by 10 mM Tris–HCl buffer containing 1.5 mM EDTA and 0.1 mM ATP [16] and centrifuged to remove cell debris. The supernatant (i.e., hemocyte lysate) was loaded on a Fractogel TSK DEAE-650(M) column (1.4×7.5 cm) at 4 °C, which had been equilibrated in 50 mM Tris–HCl buffer (pH 7.5) with 1.5 mM EDTA and 0.1 mM ATP. The sample was eluted with a linear gradient of 0–0.3 M NaCl, fractions of 1 ml were collected and assayed for transglutaminase activity. Active fractions were pooled and further purified by a Sephacryl S-300 column (0.9×60 cm) at 4 °C, which was equilibrated and eluted with the 50 mM Tris–HCl buffer containing 1.5 mM EDTA and 0.1 mM ATP.

Molecular weight and purity of *P. monodon* TGH were studied by SDS-PAGE [17]. Native molecular weight of TGH was also estimated by elution volume on a gel filtration column, by interpolation from a calibration plot of “ratios of the elution volume to the void volume” against log molecular weight of the markers. In addition, matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer (Model G2025, Hewlett-Packard, USA) was used to determine its accurate mass, in which 1 µl of purified TGH (0.4 mg/ml) after dialysis against distilled water was added with 1 µl sinapinic acid before crystallized under vacuum.

Fig. 1. Purification of TGH from *P. monodon* hemocyte lysate. (A) About 12 ml hemocyte lysate was separated by a Fractogel TSK DEAE-650 (M) column at 4 °C. The column was eluted with a gradient of 0–0.3 M NaCl in 50 mM Tris–HCl (pH 7.5), containing 1.5 mM EDTA and 0.1 mM ATP. Arrow indicated start of the gradient. (B) partially purified TG was subjected to gel filtration on a Sephacryl S-300 column. Fractions of 1 ml were collected at 4 °C, and transglutaminase activity (○—○) was assayed as detailed in Materials and methods.

Fig. 2. (A) Molecular mass of purified *P. monodon* TGH as determined by MALDI-TOF mass spectrometry. (B) Estimation of the TGH molecular weight by gel filtration. Protein markers used were: β -amylase (β -A, 200 kDa), bovine serum albumin (BSA, 67 kDa) and carbonic anhydrase (CA, 29 kDa). V_e and V_o are elution-volumes for the protein marker and blue-dextran, respectively. (C) SDS-PAGE on 12% acrylamide gel. Lane 1, molecular weight markers; lane 2, hemocyte lysate; lanes 3 and 4 purified TGH with non-reducing and reducing sample buffer, respectively. Molecular weights of the markers were shown at left.

2.4. Protein fragmentation and amino acid sequencing

Likewise, the enzyme Mj-TGH was purified from the hemocytes of another aqua-cultured species *M. japonicus*. Both TGHs (1 mg) of *P. monodon* and *M. japonicus* were denatured in 100 μ l of 6 M guanidine hydrochloride in 0.2 M Tris-HCl (pH 8.5) and reduced by dithiothreitol at 50 °C for 1 h. After adding excess iodoacetic acid and incubated at room temperature for 30 min, the S-carboxymethyl protein was desalted and lyophilized. Amino acid sequences were analyzed by an automatic sequencer (Model 477A, Applied Biosystem, USA).

For fragmentation, *M. japonicus* TGH was dissolved in 100 μ l of 70% formic acid with 0.2 mg of CNBr at room temperature for 24 h. In addition, it was digested with Lys-C endopeptidase (Promega, Madison, WI, USA) in 50 mM Tris-HCl (pH 8.0) at 37 °C for 20 h at an enzyme to substrate ratio of 1:50 (w/w). The reaction was stopped by adding dithiothreitol and heated at 95 °C for 5 min. Then the oligopeptides were purified by reversed-phase HPLC on a C₁₈ column, then N-terminal sequences of three of the fragments.

2.5. Enzyme assay and effect of calcium and other reagents

For the fluorescence assay, CaCl₂ at a final concentration of 10 mM was added to *P. monodon* TGH (77 μ g/ml) in 50 mM Tris-HCl, 1.5 mM EDTA, 0.1 mM ATP (pH 7.5) to start the reaction and initial rate (RFL/min) was

measured [15]. For clotting assay, clotting time was measured after adding the enzyme to a buffer containing 60 μ l supernatant of the centrifuged hemolymph and equal volume of 50 mM Tris-HCl (pH 7.5) containing 20 mM CaCl₂.

To investigate its thermostability, purified TGH in the buffer (32 μ g/ml) were incubated at various temperature up to 50 °C. After a period of time, 30 μ l aliquots were withdrawn for the fluorescence assay. In addition, we have studied effects of pre-incubation of TGH with the chlorides of IIA group metal ion or iodoacetamide which modified active site CysSH of TGH. The remaining enzyme activity was assayed by the fluorescence assay.

2.6. cDNA synthesis and cloning

Standard procedures in molecular biology were used for preparation of plasmid DNA, restriction enzyme digestion, DNA agarose gel electrophoresis, DNA ligation, and the transformation of bacteria [18]. Total RNA was purified from tiger shrimp and kuruma prawn using the RNeasy B kit (Biotecx, Friendswood, TX, USA) [19]. The mRNA was purified using QuickPrep Micro mRNA purification kit (Amersham Pharmacia, Freiburg, Germany). The first strand cDNA was synthesis using oligo(dT)-primer and random primers, and it was used as templates in subsequent polymerase chain reaction (PCR) [20].

To amplify the transglutaminase cDNAs of both species, we have used an antisense oligo(dT) and another degenerate primer designed from the conserved protein sequence (VPAHTIK) of *M. japonicus* TGH, i.e., sense 5'-GTNCCN

Fig. 3. (A) Correlation between clotting and amine-incorporating activities of purified TGH. After adding Tris-HCl buffer containing CaCl₂ to the same volume of shrimp plasma, the clotting was started from the addition of TGH (final TGH concentration ranged from 14 to 35 μ g/ml). In separate experiments, initial rates of dansylcadaverin incorporation were determined by fluorescence assay. (B) Apparent Ca²⁺ dependency of *P. monodon* TGH activity. Purified TGH (18 μ g/ml) was used in the fluorescence assay at various CaCl₂ concentrations in Tris buffer at 23 °C.

Fig. 4. (A) Thermostability of *P. monodon* TGH. The enzyme (0.3 mg protein/ml) in 50 mM Tris–HCl, 1.5 mM EDTA (pH 7.5) was pre-incubated at different temperature (0–50 °C) for 10 min. At indicated time, aliquots were withdrawn for amine-incorporation assay. (B) Stability of *P. monodon* TGH at various pH. The hemocyte lysate was diluted with 0.1 M of the following buffers with 0.4 mM EDTA at 4 °C and incubated 16 h before the amine-incorporation assay at 23 °C; sodium acetate (pH 5.0–5.8) (Δ – Δ), imidazole–HCl (pH 5.8–7.0) ($\blacksquare$ – $\blacksquare$), HEPES (pH 7.0–7.8) ($\circ$ – $\circ$), Tris–HCl (pH 7.9–8.9) ($\blacktriangle$ – $\blacktriangle$), sodium borate (pH 9.0–10) ($\cdots$).

CANCA(TC)ACNAT(TCA) AA-3'. Except for the first and last cycles, each of the 40 cycles was: 94 °C for 30 s, 42 °C for 30 s, and 72 °C for 1 min. The first cycle included an extended denaturation time (2 min) during which polymerase was added while the last cycle had an extended (10 min) elongation period. The cDNAs of 578 bp and 1071 bp were thus produced from tiger shrimp and kuruma prawn, respectively. These cDNA were purified and ligated into pGEM-T (Promega). Each clone was sequenced by using AmpliTaq DNA polymerase and fluorescent dideoxynucleotides on a DNA sequencer (Applied Biosystems model 310) [21].

2.7. Rapid amplification of cDNA ends (RACE)

Total RNA was isolated from both shrimps and poly (A)⁺ RNA was prepared. The first-strand cDNA was synthesized using a modified poly-T primer and 1 μ g of poly (A)⁺ RNA. The second-strand cDNA was obtained using an enzyme cocktail containing RNase H, DNA polymerase, and DNA ligase. Asymmetric adaptor primers (AP primers) were then ligated to both ends of the double-stranded cDNA. An aliquot of this cDNA was diluted 1:100 and subjected to PCR. The 5' ends of mRNAs of PmTG and Mj-TGH were obtained by RACE methods using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). Adaptor primers AP1 (external) and AP2 (internal) were supplied with the kit. RACE was performed with a 27mer sense primer (AP1) and antisense primers, 5'-GTAGTAGTCACCGTACGTCACCTTCC-3' for PmTG/ TGH and 5'-TGATAAGGTCATGGTCCACCAGCTTG-3' for Mj-TGH, respectively. The second round of PCR was carried out with a nested 25mer sense primer (AP2) and nested antisense primers, 5'-CTTCGAGAAGATTCTCCTCAGC-3' for TGH and 5'-GTAGTAGTCAGAGTAGTA

CACCTCC-3' for Mj-TGH, respectively. Except for the first and last cycles, each cycle was: 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 1.5 min. The first cycle included an extended denaturation time (2 min) during which polymerase was added while the last cycle had an extended (10 min) elongation period. After 40 PCR cycles, the products were cloned into pGEM-T vector (Promega) and sequenced [20,21].

2.8. RNA isolation from *P. monodon* tissues and RT-PCR

Total RNA was isolated from gill, heart, hemocyte, hepatopancreas, intestine, lymphoid organ, and muscle of *P. monodon* using the RNeasy reagent (Qiagen, Crawley, UK). RNAase-free DNase (Promega) was added to eradicate the genomic DNA contamination. First-strand cDNA was amplified from 2 μ g total RNA using 10 pmole oligo-dT primer in a 25- μ l reaction containing 30 U RNasin (Promega), 1 mM dNTP, 10 mM dithiothreitol, and 300 U Superscript II (Life Technologies). After being incubated at 42 °C for 1 h in 1.5 mM MgCl₂, 0.25 mM dNTP and 0.5 U ExTaq (Takara Shuzo Co., Japan), 2 μ l of the solution were added to 48 μ l solution of the primers (designed from PmTG; forward, 5'-TGGAAGTGACGTACGGTGAC TACTAC G-3' and reverse, 5'-GTTGTACAGCTGGCTGGAGTTGAAGG-3'). After amplified by PCR at the annealing temperature of 60 °C for 30 s (25–40 cycles), the 390 bp PCR products were examined on a 1.2% agarose gel.

2.9. Molecular phylogenetic analysis

Sequences of invertebrate transglutaminases were retrieved by BlastP [22]. Their amino acid sequences were aligned with the program CUSTAL W [23]. Phylogenetic tree was built by the program PHYLIP (<http://www.evolution.genetics.washington.edu/phyliip.html>) [24]. Degree of confidence of the lineage at each node was determined by the bootstrap analyses of 1,000 replicates [25].

2.10. In-gel digestion of *P. monodon* TGH and peptide mass fingerprinting

After manually excised from the polyacrylamide gel, the electrophoresis band of *P. monodon* TGH was cut into pieces, which were dehydrated with acetonitrile for 10 min, vacuum dried, re-hydrated with 100 mM dithioerythritol in 25 mM NH₄HCO₃ (pH 8.5) at 37 °C for 1 h. They were subsequently

Fig. 5. Effects of metal chlorides on the enzymatic activity of *P. monodon* TGH. The hemocyte lysate (0.4 mg protein/ml) in 50 mM Tris–HCl, 0.1 mM ATP, 0.1 mM EDTA, pH 7.5) was preincubated with 5.3 mM metal chlorides at 4 °C (Δ – Δ , MgCl₂; $\blacksquare$ – $\blacksquare$, BaCl₂; $\square$ – $\square$, SrCl₂; $\blacktriangle$ – $\blacktriangle$, CaCl₂). Aliquots of sample were withdrawn at the time indicated and amine-incorporation activity of TGH was measured at 23 °C.

alkylated with 65 mM iodoacetamide in NH_4HCO_3 at 27 °C for 1 h in the dark, washed twice with 50% acetonitrile in NH_4HCO_3 , dehydrated with acetonitrile for 10 min and dried, then hydrolyzed with 25 ng modified trypsin (Promega, Madison, USA) in 25 mM NH_4HCO_3 (pH 8.5) at 37 °C for 16 h. The oligopeptides were extracted twice with 50% acetonitrile containing 5% formic acid for 15 min and dried under vacuum. Their masses were analyzed with MALDI-TOF/TOF and matched by Mascot Search programs.

3. Results

3.1. Purification and characterization of *P. monodon* TGH

Enhancement of the substrate fluorescence upon incorporating dansylcadaverine into succinyl casein was measured to assay

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M S F F S S F E N F L D N L T D R F R H D 21
1 TCTTGAAGCGGATCGTCTTGGGTTTCTTCAAGATGCTGTTCTCAGTTCCTTGGAGAATTCTCGACACCTGACCGACCGCTTCAGGCATGACC
  L E V N R R E D R E I E I A D E L N D T A N E V D S A T P T P K V L 55
101 TCGAGTCAACCCGCCGAGGACAGGAGATCGAGATTGGGATGAACCTTAATGATACAGCCAATGAAGTCGACTCCGCGACGCGCAACCCGAGGTGCT
  S V D F Q A K P N A V N H F C D K Y E I V E R Q Q A A I V L R R G 88
201 GAGCGTGGACTTCCAGGCCAAACCGAATGCGGTGAACCACTTTTGCACAAAGTACGAAATCGTCGAGCGCAGCAGCGGCTATCGTCTTCGACGGGG
  G S F T I E V E F N L E L E L K K T H L V K L Y F S F G Q R P S A 121
301 GGGACCTTCACCATCGAGTGGATTCAACCTGGAGCTGGAGCTGAAGAAGACCCATTTGGTGAAGCTCTATTTTCTTTTGGTCAGGCTCCGAGCGCG
  A K G T Q A A L Q V S G K K L F D K N H E E W D V R V D G Q N G K K 155
401 CGAAGCGAACCAGCTGCCCTTTCAGGTATCGGGCAAGAACTCTTCGACAAGAACCAGAGGAGTGGACGTCGCGCTGACGCGCAAAACGCAAGAA
  I T F E I Q I P T D A P V G V W N P A V E V S L R A N S D T T R H 188
501 AATCACCTTCGAATTCAGATCCCAACCGACCCCGCTAGTGTTCGAATCCCGCGTGAAGTGAAGTCTCAGAGCTAACTCGGACAGCAGCCGACAT
  L H R S D T H V Y I L F N P W N K H D G T Y M P D E E K R D E Y V 221
601 CTCACCGCTCTGACACGCATGTCTACATCTCTCAATCCCTGGAACAGCAGGATGGGACGTACATGCCGACGAGAGAAACGAGACGAATACGTTT
  L A D V G K I W V G N Y P Y P H G R P W V F G Q F D D A V L P A S V 255
701 TGGCTGACGTAGGAAGATCTGGTGGTAACTACCCCTACCCCAACGGTCCGCCCTGGGTCTTCGGCAGTTCGACGACGCGGTCTCGCCGCGAGCGT
  L L L E K S G V P P E S R G D P I R V S R A I S K M V N S N D D Q 288
801 GCTGCTGCTGAGAAGTCGGCGTCCCGCGGAGTCTCGCGCGACCCATCCCGTGTCCAGAGCCATCTCAAGATGGTGAACAGCAACGACGATCAG
  G V I V G R W D G K Y E D G K S P S S W S G S I K I L E E Y I K S 321
901 GCGTGATCGTGGGTCTGGGACGCAAGTACGAAGAAGGAGTCCCGCTCGTGGTTCAGGCTCCATCAAGATCTCGGAGATACATCAAAAGCA
  K R P V R Y G Q C W V F A G V V N T V C R A L G L P S R V V S N F A 355
1001 AGCGCCCGTCCGTACCGGACGTCTGGGTCTTCGCGGGGTGTCAACACAGTGTGTCTCTCGGTCTGCCAGTTCGAGTGGTCAGCAACTTCGC
  S A H D T N F S L T I D E Y V D E K G D E I D S S Y R G A T P G G 388
1101 CTCAGCACAGACCAACTTCTCCCTCAACATTGACGAATACGTTGACGAGAAGGGGAGAGATCGACTCCTCATACCGGGTGTACCCCTGGAGGC
  L Y D S I W N F H V W N D V W M I R P D L P Q G Y S G W Q V I D A 421
1201 CTGTATGATTCATCTGAACCTTCACGTTTGGATGACGTTGGATGATCGACCTGACCTGCCACAGGTTACAGCGGCTGGCAGGTATCGACGCAA
  T P Q E A S D G S Y Q C G P A S H E A I R R G Q M E Y K Y D V P F V 455
1301 CGCCACAGGAAGCAAGTGTGCTGTACAGTGTGGGCGACGCTCCACAGGCCATCCGACAGGCCAAATGGAATACAAGTACGACGTGCCCTTCGT
  L A E V N A D V V H W Q K D D K A Q D G F K K L T T N K R S V G R 488
1401 GCTGCGGAGGTCAACGCTGACGTCGTGCTGACGAGAGGAGATAGGCACAGGAGGTTCAAGAAGCTCACCAGCAAGAGGAGCGTCCGCCGC
  Q V L T K Q A G P S G D K E D I T L E Y K P K E G T T A E R V T L 521
1501 CAGGTGCTGACGAAGCAGGACGACCTCAGGCGACAGGAGATATCACACTGAGTACAAACCGAAGCAAGGAGACGCGCGAGAGAGTACCGCTAC
  L T A A R R T R A A R H A F R L M S Q G V D D V E F G L E D I E K V 555
1601 TAACAGTCCCGAAGGACCGCGCGCCAGACAGCCCTTCGGTCTCATGTCCCAAGGAGTAGATGACGTTGAGTTCGGCTCGAGGACATCGAGAAAGT
  P I G E G F T V T L N C K N I S E A K R T V S V V M N C T S M Y Y 588
1701 GCCATCGCGGAAGGGTTACCGTGACGCTCAATTGCAAGAACATTAGCGAGGCCAAGAGAACGTTGAGTGGTAAATGAATGCACCATATGTACTAC
  T G A P A Y P I K R R E G E I V L E V G E T K N M T M E V T Y G D 621
1801 ACAGGCCCGCCGCTACCCGATAAAGCGCGTGAGGAGAAATCGTTCTCGAAGTTGGAGAAACCAAGAACATGACCATGGAAGTGACGTACGGTGACT
  Y Y E K L V E H A M V K N V A I C T V A E T S Y A W V G D D G M E I 655
1901 ACTACGAGAACTGTGCAACAGCCATGTAAAGAACTGTATTTGCAACCGTGGCGGAAACCTCTTACCGGTGGTGGCGGAGATGGCATGGAGAT
  Q K P D I K I E I L S E A T G A L P L A L R I S F T N P L P I T L 688
2001 ACAGAAAGCCGATATCAAGATCGAGATCCTGTGGAGGCCACTGCGGGATTACCCCTGCCCTCAGGATATCTTACCAATCCCTCGCGATAACCTC
  R N C E L E V D G P G I L R P K T I P I S N V G P S E N M V H E M 721
2101 AGGAATCGCAGCTGGAGTGGATGTCGCCGATCTCGGCCCAAGACTATACCGATCAGCAACGTTGGGCCCGAGTGAAGATATGTTCCATGAGATGA
  K V F P K K P K H C T I V V T F N S S Q L Y N L T G S T Q V T I N P 755
2201 AAGTGTTCGCAAGAAACCAAGCACTGCACAATAGTGTGACCTTCAACTCCAGCCAGCTGTACAACCTTACGGGGTCAACTCAGGTACCATCAATCC
  A E * 757
2301 GGCAGATGATCGGTGGTGTATTGTGAGCTGCAAGGAAGATTGTGAAGAATGCTATCTACTCTCGGAAAAAAAAAAAA

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Fig. 6. The cDNA and deduced amino acid sequence of PmTG. Sequences homologous to those of Mj-TGH peptides purified by HPLC are underlined. Potential N-glycosylation sites and the RGD motif are doubly underlined.

was about 3 mM (Fig. 3B). Similar level of Ca^{2+} was required for clotting of hemolymph by TGH. This is consistent with the normal Ca^{2+} requirement of other transglutaminases [1]. Other metal chlorides, e.g., 10–100 mM of SrCl_2 , BaCl_2 or MgCl_2 ,

could not substitute CaCl_2 in both TGH assays (data not shown). The strict dependency on Ca^{2+} is different from that for Factor XIII, which can be activated by other divalent metal ions although less effectively [26].

Mj-TGH	MPTVNPYN---ELINRFITNDLEVCRRDRDLEIENELNEITRAFS-ASKEPKIVCVDFQPRLNAAHYHCDRYELVDRTDCVVL	81
STG I	MPTVDAIS---DLANRFITNDLELCRRDRDLEIATELNEIADALS-ASKDPKIVSVDFOPMYNVAVNHCELYELVDRTDCVVL	81
PmTG	MSFFSSFNFDLNTDRFRHDLVNRREDREIEIADELNDTANEVDSATPTPKVLSVDFQAKPNVAVNHCDKYEIVERQQAIVLR	86
Mj-TG	MTFFSHFNFDLNTDRFRSDLEVNRRDRAVEVEDERNEGTNEVDSADTEPKVVSVDFOGKANAVNHRCDKYEIVERQQAIIILR	86
Mj-TGH	RGGMFSMVVLELQQVNLSAEQQLKLYFSFGPRNVKGTQAHLLVSGKTTIEKTHDDWDVKVAKENGKQATIEVQIPTDAPVGVMS	167
STG I	RGGLLSMVVKLQNVALLASQELKLYFSFGSRPNVEKGTQAHLLVTGKTKIEKPHDDWDRLVKEAGKKAIVVQIPTDAPVGVNK	167
PmTG	RGGSFTIEVEFNLELELKKTHLVKLYFSFGQRPAAKGTQAAQLVSGKLFQKNHEEWDVRVDGQNGKKITFEVQIPTDAPVGVN	172
Mj-TG	RGGSFTIEVEFNQALELKKTHLVKLYFSFGKRPTSAKGTQAILQATGKKLFQKNHEEWDVRADGQNGKKITFEVQIPTDAPVGVN	172
Mj-TGH	VAAEVSRRNQPEAEARHLRRFDTHVYILFNPNWENDGTYLPEELMRQEVYLEDVGKVFVGSYPHTRGRHAFGQFDDAVLPACMLLL	253
STG I	VAAEVTLRNQ-AGERHLRRFDTHVYILFNPNWENDGTYLPEELMRQEVYLSAVGTVFVGSYPHTRGRHAFGQFDDAVLPACMLLL	252
PmTG	PAVEVSLRANSDDTHRLHRSDDTHVYILFNPNWKNHGDGTYMPDEEKRDQEVYLDVGKIWVGNYPPHGRPMVFGQFDDAVLPASVLLL	258
Mj-TG	PAIEVSORNPASTARHVRSEMHVYILFNPNWKNHDETYMSDDEKREYVLDVGKIWVGNYPPHGRPMVFGQFDDAVLPASVLLL	258
Mj-TGH	ERSGVKAEARGDPVRSRALSKMVNSNDDSGLLVGKWDGGEYEDGRAPAKWIGSIEIETVLRTRQSVKYGQCVWFAACLNTICRAL	339
STG I	ERSGVSAEARGDPVRSRALSKMVNSNDDSGLLVGKWDGGEYEDGRAPAKWIGSIEILEMVLTRTRQSVKYGQCVWFAACLNTICRAL	338
PmTG	EKSGVPPESRGDPVRSRALSKMVNSNDDSGVIVGRWDGKYEDGKSPSSWISGSIKILEEYIKSKRPVRYGQCVWFAAGVNTVCRAL	344
Mj-TG	ERSGIPSESRGDPVRSRALSKMVNSNDDSGVIVGRWDGKYEDGKSPSSWISGSIKILEAYIKSKRPVRYGQCVWFAAGVNTVCRAL	344
Mj-TGH	GVPORVVTNFASAHDTNLSLIDEYFDEGDKIIEENDRYADPAGIRDSIWNFVWVNDVWMSRPDLPGGFGWQVLDATPQETSQGS	425
STG I	GLPVRVVTNFASAHDTNLSLIDEYFDEGDKIDAADRYVNPAGIRDSIWNFVWVNDVWMSRPDLPGGFGWQVLDATPQETSQGS	424
PmTG	GLPSRVVSNFASAHDTNLSLIDEYVDEKQDEIDSSYRGATPGCLYDSIWNFVWVNDVWMSRPDLPGGFGWQVLDATPQETSQGS	430
Mj-TG	GLPSRVVSNFASAHDTNLSLIDEYVDEKQNEIDSSYTGATPGCLYDSIWNFVWVNDVWMSRPDLPGGFGWQVLDATPQETSQGS	430
Mj-TGH	YQCGPASHEATKQGRVDLKQYDVPFVLAENVADVVWKKDDSVGEFGAKMTTQTSVSGRLILTKKAGPVATGGFFKSDREDITMEYK	511
STG I	YQCGPASHEATROGRVDLKQYDVPFVLAENVADVVWKKDDRAEGGFKLATQTSVSGRLILTKKAGPVATGGFFKSDREDITMEYK	510
PmTG	YQCGPASHEATRRGOMEYKYDVPFVLAENVADVVWKKDDKAQDGFKKLTINKRSVGRQVLTQAGPS-G-----DKEDITMEYK	509
Mj-TG	YQCGPASHEATRRGOMEYKYDVPFVLAENVADVVWKKDDKAKDGFKKLTINKRSVGRQLLTQKPGVDNSGYSGTDKEDITMEYK	516
Mj-TGH	PPECTRAERVSILTAARRTRARQVDMPSAEKEDIVFSTIEDRDQVPIGDNFTVILTTKNTSTEVRTINVMTCASMYTGAHAHT	597
STG I	PPECTRAERVSILTAARRTRARQVDMPSAEKEDVTQIEDRDQVPIGDNFTVITATNTSEVRTINVMTCASMYTGAHAHT	596
PmTG	PKEGTTAERVTLTAARRTRARHARLMSQGVDDVEFTLEDIEKVPICEGTTVLTACKNISEAKRTVSVMNCTSMYYTGAPAYP	593
Mj-TG	PKEGTTAERVTLTAARRTRARHARLMSQEVDDVEFTLEDLERVPICEGTTVLTAKNISSEAKRTISVMNCTSMYYTGAPAFP	600
Mj-TGH	IKRHGDEIHLEPNQTKTSMVEVYSDYYSKLVHDHLSIVTCNVNETTQCGGEDNLEVHKPDIIQIDILSAAVVKKVPPIKISFD	683
STG I	IKRHGDELHLEPSQTKTSMVEVYSDYYSKLVHDHLSIVTCNVNETTQCGGEDNLEVHKPDINIEVLGSAVEGKAVPIKVSEF	682
PmTG	IKRREGELVLEVGETKNTIMEVTYGDYEEKLVHAFVNRVAICTVAETSYAVVGGDGLIEIQKPDIKIEILSEATAGLPLALRSIF	679
Mj-TG	IKRREGELVGVGETKNTIMEVTHGDYEEKLVHAFVRNVAICTVSETSYAVVGGDGLIEIQKPDVKIEILSEAVAGKPLPIRVSEF	686
Mj-TGH	NPILPLMTNOMVILDAPGVMPKRVPIKNIAPKGMTFEMNLNPGRVINTTLVQFNSKOLHNLGAKKVVVSPA-	758 identity
STG I	NPILPLKECCKLKDGCLMRPKTVPLENIAPKGMNFMENLVKKAVERGLIVQFNSRELNLGAKKISVASA-	757 83.6%
PmTG	NPLPITLRNCELEVDGPGILRPKTIPIISVNGPSENMVHEMKVFPKKPKHCTLVVTFNSSQVNLTGSKQVTINPAE	757 58.8%
Mj-TG	NPLPITLRNCELEVDGPGVLRPKTIKISVNGPNEEMVHEMKVFPKKPKHCTLVVTFNSSQVNLTGSKQVTINPPE	764 59.0%

Fig. 8. Amino acid sequence alignment of transglutaminase variants of tiger shrimp and kuruma prawn. Residues identical in all the four sequences are shown in black, and those identical in any three are marked in gray. RGD motifs are doubly underlined and the catalytic triads are shown in bold italics. Dots indicate putative calcium binding sites, and sequences matching those of the purified Mj-TGH oligopeptides are underlined.

Fig. 9. Phylogenetic tree for invertebrate transglutaminases based on the amino acid sequences. Human factor XIIIa (NP-000120) was used as the out-group. Besides PmTG and Mj-TGH, the species and GenBank accession Nos. are: *P. monodon* STG I (AY074924) and STG II (AY771615), *M. japonica*-TG (AB162767), crayfish TG (AAK69205), horseshoe crab TG (A45321), starfish TG (BAB20439), sea urchin (CAD28789), drosophila TG (AAF52590) and grasshopper annulin (P52183). Bootstrap values are shown at the nodes.

P. monodon TGH appeared to be labile at temperature $>25^{\circ}\text{C}$ and pH outside the range 7–9 (Figs. 4A and B). Lyophilization or frozen–thaw could inactivate the enzyme by more than 50%. However, more than 85% of the activity could retain for several days if purified TGH or the hemocyte lysate was stored in the presence of metal chelator (e.g., EDTA) at pH 7–9 at 0–4 $^{\circ}\text{C}$, or kept in buffer in the presence of EDTA and 35% (v/v) glycerol below -14°C .

Like other type II transglutaminases, *P. monodon* TGH was inhibited or inactivated by certain divalent metal ion (e.g., 10–20 μM ZnCl_2) which competed Ca^{2+} binding [3,26,27], thiol-reactive agents (e.g., organomercurials and iodoacetamide), or 2–10 mM histamine and other primary amines (data not shown). Like other transglutaminases [1,28], addition of 5 mM Ca^{2+} to the medium greatly facilitated the inhibition of TGH by 3–10 μM mercurial or 0.1 mM iodoacetamide (data not shown), suggesting exposure of the TGH active site by a Ca^{2+} induced conformational change. Notably, TGH activity was decayed in minutes at mM concentrations of Ca^{2+} . Addition of EDTA could stop the Ca^{2+} -induced decay immediately but could not reverse it, and the presence of dithiothreitol in the enzyme buffer only weakly protected this decay (data not shown). Moreover, pre-incubation with other metal chlorides also inactivated the enzyme although less effectively than CaCl_2 (Fig. 5).

3.3. Cloning, sequencing and phylogenetic analysis

Automatic sequencing of two oligopeptides obtained by HPLC-purification of Lys-C digest of Mj-TGH showed their amino acid sequences as MRQEYVLSVDVGTVFVGSYP and EXLTWEYKPPPEGTRAXRVSILN, respectively. The sequence of another purified CNBr cleavage product was YYTGVPAAH TIKRHDGELHLEPSQT. Degenerate PCR primers were de-

signed based on these Mj-TGH partial sequences. In addition to the cDNA encoding STG I (AY074924), a 578-bp cDNA encoding the C-terminal part of a novel *P. monodon* transglutaminase (designated as PmTG) was produced by PCR. On the other hand, a 1071 bp cDNA encoding the C-terminal part of Mj-TGH was also amplified. The full-length cDNA of PmTG and Mj-TGH were then completed by 5' RACE. PmTG had a total length of 2,387 bp, including 36 bp of the 5'-untranslated region, an open reading frame of 2,271 bp, and 81 bp of the 3'-untranslated

Fig. 10. RT-PCR analysis of tissue expression of PmTG. Total RNA was extracted from various tissues of *P. monodon*. Specific primers were designed to amplify a 390-bp fragment of PmTG cDNA. PCR products at various cycles were analyzed by electrophoresis on 1.2% agarose gel containing ethidium bromide.

region. The putative initiating ATG codon, according to Kozak's rule [29], is at nucleotide 33 (Fig. 6). The open reading frame of PmTG is predicted to encode a protein of 757 residues (pI 5.47) and similar to STG II [10]. Its ORF differs from that of STG II by 15 nucleotides and resulted in the substitution of six amino acid residues, i.e., L158→F, T182→N, Q212→P, V495→A, S499→G, and E541→G. None of them are in the catalytic core domain of the enzymes.

We also cloned Mj-TGH cDNA which contained 2996 bp (included 54 bp of the 5'-untranslated region, an open reading frame of 2274 bp, and 669 bp of the 3'-untranslated region). Its authenticity was confirmed by sequence analyses of the fragments obtained from CNBr cleavage and protease digestion of the purified *M. japonicus* TGH. The putative initiating ATG [29] is at nucleotide 55, and its open reading frame encodes an enzyme of 758 residues (pI 5.76) with seven potential *N*-glycosylation sites and one RGD motif (Fig. 7). The predicted Mj-TGH sequence is 83% and 59% identical to those of STG I and STG II/PmTG, respectively (Fig. 8). PmTG and Mj-TGH probably are both cytosolic, since they do not contain a typical signal-peptide or trans-membrane domains.

Amino acid sequences of all the invertebrate transglutaminases so far solved were aligned and a phylogenetic tree has been

constructed to study their structural relationships (Fig. 9). All the Crustacean enzymes are linked together in the tree and closer to those of other marine arthropods than to those of insects. Apparently, STG I and Mj-TGH are orthologous to each other.

3.4. RT-PCR analyses and tissue expression profile

The expression patterns of PmTG mRNA in various tissues of *P. monodon* were analyzed by RT-PCR. As shown in Fig. 10, a 390-bp DNA fragment could be amplified in contrast to the negative control. The expression levels were highest in hemocyte and muscle, moderate in gill, intestine and lymphoid organ, and lowest in heart and hepatopancreas.

3.5. In-gel digestion and mass analysis of TGH

Purified *P. monodon* TGH band in gel was digested with modified trypsin before analyzed by peptide-mass-fingerprinting. The masses matched nicely with those of the peptides predicted from *P. monodon* STG I [8]. We found at least 34 TGH peptides matching those of STG I (Table 1), representing a total of 396 amino acids or 52% (396/757) of the entire sequence and evenly distributed.

Table 1

Mass match between the tryptic peptides of TGH and STG I (MS_e and MS_c are experimental mass of TGH peptides and calculated mass of STG I peptides, respectively)

Tryptic peptides of STG I	position	MS _e	MS _c
FTNDLELCR	14–22	1167.57	1167.52
EDREFEIA TELNEIADALSASK	24–45	2450.13	2450.18
EFEIA TELNEIADALSASK	27–45	2050.95	2050.01
EFEIA TELNEIADALSASKDPK	27–48	2390.14	2390.19
IVSVDFQPMYNAVNHCELYELV	49–73	3047.36	3048.41
RGGLLSMVVK	82–91	1058.62	1058.63
GGLLSMVVK	83–91	902.47	902.53
LNQNVALASAEQELK	92–105	1497.73	1497.82
FYFSFGSRPNVEK	106–118	1576.77	1576.77
GTQAHLVVTGK	119–129	1109.60	1109.62
IEKPHDDWDIR	132–142	1422.68	1422.69
VAAEVTLR	168–175	857.49	857.50
FDTHVYILFNPWNENDGTYLPEEK	186–209	2940.31	2940.36
MVNSNDDSGLLVGK	275–288	1447.63	1447.70
WDGEYEDGR	289–297	1125.42	1125.44
WDGEYEDGRAPAK	289–301	1492.64	1492.66
WLGSIIEILEMYLR	302–314	1621.82	1621.85
VVTNFASAHDTNISLSIDEYFDEGDQIDAADR	344–376	3642.54	3642.62
YVNPAGIR	377–384	888.41	888.48
YDVPFVLAENVADVVR	444–459	1804.91	1804.94
LATQTSSVGR	473–482	1018.53	1018.54
AGPVATGGFFK	489–499	1050.48	1050.55
SDREDITWEYKPEPTR	500–516	2077.94	2077.97
VSILNAAR	520–527	841.45	842.50
EDVTFQIEDR	544–553	1250.56	1250.58
TINVVMTCASMYTGAK	577–593	1908.83	1909.86
HDGELHLEPSQTK	600–612	1489.70	1489.72
LVDHDLIK	627–634	950.47	951.54
VSFDPNPIPLLLK	679–690	1354.76	1354.79
LDGPGLMRPK	696–705	1081.62	1082.59
MNFEMNLYPK	718–727	1285.55	1285.58
KAVEGTLIVQFNSR	728–741	1560.84	1560.86
AVEGTLIVQFNSR	729–741	1432.74	1432.77
ELCNLTGAK	742–750	1005.52	1005.48

4. Discussion

In shrimps, coagulation is initiated by activation of hyaline cells which release its content including the clotting enzymes [30–32]. *P. monodon* TGH effectively polymerizes shrimp clottable proteins to form stabilized gel (Fig. 3). Notably, the purified *P. monodon* TGH exists as weakly associated dimmers, like the A subunit of mammalian FXIII which is released from megakaryocytes as homodimers and also present as dimmers when crystallized. However, the shrimp TGH is possibly active as monomers like FXIII [1]. Dependent on seawater salinity, the Ca^{2+} concentrations in tiger shrimp hemolymph range from 6.4 to 10 mM [28]. Thus, when TGH is released from hemocytes it would be fully activated to initiate the coagulation (Fig. 3). However, metal ions and high chloride ion [2] in the hemolymph possibly reduce its enzymatic activity within minutes, as a feedback regulation to avoid excess clotting.

The X-ray crystallographic structure [33,34] showed that the catalytic domain (i.e., region 200–550) of FXIIIa contains a hydrogen-bonded Cys–His–Asp triad similar to that found in enzymes of the papain family [35]. The catalytic triad appears to be also conserved in PmTG (and Mj-TGH), i.e., Cys330 (325), His397 (392), Asp420 (415). Their Ca^{2+} -binding sites presumably include the conserved Asn460, Asp462, Glu512, and Glu517 (Fig. 8). With highly conserved region 350–353, His 358 (353) and Glu451 (466) in shrimp TGH may be associated as in FXIIIa [33]. Many conserved Trp residues in the core domain of TGH (Fig. 8) possibly are involved in stabilization of the transition state during the enzymatic reaction [36]. It is known that Ca^{2+} unmask the active site Cys and widens the protein substrate binding site [1,37] and the active site possibly undergoes irreversible conformational changes. Being 35% similar to FXIIIa, TGH is relatively unstable after activation at ambient temperature and in the presence of bivalent metal ions (Fig. 5). Instability of other transglutaminases has been reported before [2,38], but its exact mechanism remains to be explained by 3D structural analyses of both the active and the inactive enzymes or by mutagenesis study [39].

The RGD (or Arg–Gly–Asp) motif, which is also present in fibrinogen, is known to bind to integrins or specific membrane receptors [40]. Positions of RGD motifs are remarkably conserved in all invertebrate transglutaminases (Fig. 8) and human keratinocyte [8]. This motif probably is essential for the shrimp enzyme to bind integrins and form complex with adhesion molecules [1,41], and possibly trigger certain signal pathways, e.g., RhoA activation and cell spreading [42]. Interestingly, the RGD are all flanked by Pro and/or Gly residues (i.e., “proline brackets”) and structurally constrained on the protein surface [43], consistent with their role for protein–protein interaction.

Both STG I and PmTG were transcribed in *P. monodon* hemocytes. However, results of the peptide mass fingerprinting of *P. monodon* TGH confirmed its identity with STG I (Table 1) but not with PmTG or STG II. The molecular mass of *P. monodon* TGH was determined as 84280 ± 168 Da. The predicted *P. monodon* TGH mass would be 84713 Da (based on the STG I sequence, Fig. 8) assuming no disulfide bond was formed and N-terminal acetylation took place after removal of Met 1, as

in the case of FXIIIa [44]. Alternatively, the mass would be 84285 Da if four residues (Met–Pro–Thr–Val) were removed. Thus, the determined mass suggests that TGH possibly is processed by deleting N-terminal residues before acetylation but not glycosylated although they contain seven potential N-glycosylation sites (Figs. 6 and 7).

Differed by merely six amino acid substitutions, PmTG and STG II probably are alleles of the same locus with natural polymorphism. The phylogenetic tree (Fig. 9) shows that the shrimps have at least two paralogous transglutaminases, i.e., PmTG/STG II and STG I in *P. monodon*, and Mj-transglutaminase (AB162767) and Mj-TGH in *M. japonica*. Paralogs are homologous proteins resulted from gene-duplication and they usually co-expressed in the same organism, while orthologs are those from the same gene lineage but expressed in different organisms [45]. Previous immunodiffusion experiments showed that antibodies prepared against of *P. monodon* clottable protein reacted with *M. japonicus* clottable protein [32]. The coagulation systems for this two genetically related species [46] presumably are similar. Results in Fig. 8 confirmed a high structural similarity between the hemocyte enzymes (STG I and Mj-TGH) of both species, but many distinct substitutions between STG I and PmTG at their core regions (residues 200–500) suggesting possible different substrate specificities. Protein sequence of a previously cloned crayfish transglutaminase [9] appears to be more close to that of PmTG or STG II (62%) than that of STG I (55%), whether it is the only isozyme or the real functional TGH of this fresh-water Crustacean remains to be studied.

The recombinant STG I from insect-baculovirus expression system did not show clotting activities [8], and recombinant STG II was postulated to be the clotting enzyme of *P. monodon* [10]. However, STG I was expressed in Sf21 cells cultured at 26 °C and the cell lysate were frozen before assay, the recombinant STG I probably had lost most of its activity under the culture conditions or freeze-and-thaw procedure. The instability of STG I probably lead to the wrong conclusion that STG I is not responsible for the hemolymph coagulation [8]. Moreover, in vitro assays of recombinant STG II may also be misleading, since the clot generated by recombinant STG II was less branched and instable [10], not like the clot formed naturally in crayfish [47] and horseshoe crab [2].

Based on microscopic observation, shrimp hemocytes comprised of hyaline cells, semi-granular cells, and granular cells [48–50]. Coagulation in shrimp is mediated by the circulating hyaline cells [31]. We have cloned more cDNAs encoding PmTG than those encoding STG I although STG I was the only transglutaminase purified from *P. monodon* hemocytes. By RT-PCR, we also found PmTG gene highly expressed in hemocytes and muscle, but less expressed in other tissues (Fig. 10), consistent with the expression patterns of STG II gene [10]. Thus, PmTG mRNA is relatively abundant in the hemocytes, while STG I gene is constitutive expressed at low-level in various tissues [8]. The mRNA of a crayfish transglutaminase (possibly orthologous to those of PmTG/STG II) was also reported to be expressed in semigranular and granular cells [9].

Since shrimp have an open circulatory system and hemocytes are likely to rest onto different tissue surfaces, the Northern blot

results of PmTG (Fig. 10) need to be confirmed by in situ hybridization. Previous in situ hybridization pointed out a low mRNA level of STG I in circulating hemocytes but a high level in mitotic cells of hematopoietic tissue [8], and the RT-PCR experiment also showed that STG I mRNA is decreased when hemocytes matured and released into the hemolymph. Taken together, these results suggest that transcription and translation of STG I in the hemocytes possibly take place in early developmental stages but down-regulated in later developmental stages.

In conclusion, this is the first biochemical study on shrimp hemocyte transglutaminases (TGH) responsible for hemolymph coagulation. The purified shrimp TGHs are shown to be relatively unstable after Ca^{2+} -dependent activation. We also cloned two novel transglutaminases, PmTG from *P. monodon* and Mj-TGH from *M. japonicus*, and confirmed the presence of two transglutaminase genes in both species. By peptide mass fingerprinting (Table 1) or sequencing of purified TGH peptide fragments (Fig. 8), both STG I and Mj-TGH have been identified to be the functional TGH in the two shrimp species. We failed to isolate their paralogous enzymes, PmTG/STG II and another transglutaminase previously cloned from *M. japonicus*, although their mRNAs were expressed in shrimp hemocytes. The specific role and regulated expression of these transglutaminases in different Crustacean remain to be investigated.

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