Characterization and expression of sweetfish (*Plecoglossus altivelis*) cathepsin D

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Abstract: Cathepsin D (CTSD) is a lysosomal acidic endoproteinase that plays an important role in immune response. In this study, we obtained sweetfish (*Plecoglossus altivelis*) CTSD (PaCTSD) via de-novo transcriptome sequencing of sweetfish macrophages. The full length cDNA sequence of PaCTSD was 1955 bp encoding a propeptide of 397 amino acids. The deduced protein had a calculated molecular weight of 43.17×10^3. Multiple alignment with other known CTSD amino acid sequences revealed amino acid conservation through the teleosts. Phylogenetic tree analysis showed that PaCTSD grouped tightly with other fish CTSD, and was close to that of Atlantic salmon and rainbow trout. Subsequently, PaCTSD was prokaryotically expressed and refolded by the urea gradient method on a nickel-nitrilotriacetic acid column. Enzyme activity analysis showed that PaCTSD exhibited pH-dependent proteolytic activity. Quantitative real-time PCR showed that PaCTSD mRNA was expressed in all detected tissues in healthy sweetfish. The highest expression was observed in the spleen and white blood cells, followed by liver, head-kidney, kidney, intestine, gill, and muscle. After *Listonella anguillarum* infection, PaCTSD transcripts were up-regulated significantly in liver, spleen, white blood cells, and head-kidney of sweetfish. In summary, PaCTSD has proteolytic activity and is closely involved in the immune response of sweetfish.

Keywords: Cathepsin D; *Plecolossus altivelis*; Bacterial infection; Prokaryotic expression; qRT-PCR

Cathepsin D (CTSD) is an aspartic protease widely distributed in a variety of organisms (Cho et al, 2002). CTSD plays a key role in lysosomal digestive activity, protein synthesis and activation (Diment et al, 1989; Morales et al, 2004; Pan et al, 2011; Huber-Lang et al, 2012). Besides these functions, CTSD also plays a role in immune response. Severe tissue injury induces the up-regulation of CTSD, resulting in the cleavage of C5 and subsequent generation of functional C5a (Huber-Lang et al, 2012). CTSD also increases proliferation, metastasis and progression of breast cancer (Ohri et al, 2008). Because of its importance, CTSD has subsequently been identified in various fish species, including rainbow trout (*Oncorhynchus mykiss*) (Brooks et al, 1997), turbot (*Scophthalmus maximus*) (Jia & Zhang, 2009), grass carp (*Ctenopharyngodon idella*) (Dong et al, 2012), channel catfish (*Ictalurus punctatus*) (Feng et al, 2011), miiuy croaker (*Miichthys miiuy*) (Liu et al, 2012) among others. Several investigations have revealed that CTSD participates in immune response to bacterial infection in fish (Jia & Zhang, 2009; Feng et al, 2011; Dong et al, 2012; Liu et al, 2012).

Sweetfish (*Plecoglossus altivelis*), an amphidromous fish, is the sole member of the Osmeriformes family Plecoglossidae, distributing in East Asian countries including China, Japan, and Korea. Sweetfish are popular and highly valued edible fish because they possess a special smell and taste. However, recently sweetfish have become severely affected by pathogens, predominantly *Listonella anguillarum* (Li et al, 2009), in part due to increased culture and requirements. Accordingly, in this study we investigated the immune response of sweetfish for guiding the culture of sweetfish. Here, we

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cloned CTSD in sweetfish and studied its expression pattern after being infected with *L. anguillarum*.

MATERIALS AND METHODS

Sweetfish and materials

All fish in the present study were obtained from Aquatic Product Market of Great World, Ningbo, China. Fish were acclimatized to laboratory conditions for 7 days before experiments and all fish used in this study were healthy and without any pathological signs. Ex Taq DNApolymerase, RNAiso, AMV-reverse transcriptase, Hind III and Xho I enzyme, T4 DNA polymerase, Oligotex-dT30<super>mRNA Purification Kit, SYBR Premix Ex Taq Kit, and cDNA Library Construction Kit were purchased from TaKaRa (Dalian China). Gel Extraction Kit was purchased from Omega (Shanghai, China). SMART RACE cDNA Amplification Kit was purchased from Clontech (Palo Alto, CA, USA). *Escherichia coli* TG1, BL21 (DE3) pLys E, vector pET-28a(+) and *L. anguillarum* (Li et al, 2009) were preserved in our lab. Sequencing and primer synthesis were performed by Invitrogen (Shanghai, China).

PaCTSD cDNA analysis

The cDNA sequence of PaCTSD gene was obtained from transcriptome analysis of sweetfish monocytes/ macrophages (Lu et al, 2013). The authenticity of PaCTSD cDNA was confirmed by further cloning and sequencing (JP725490). Signal peptide prediction was performed by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). Glycosylation was predicted by NetCGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetCGlyc/). Multiple alignments were analyzed by BLASTP (http://www.ncbi. nlm.-nih.gov/BLAST/). Multiple sequence alignment was produced by ClustalW (http://clustalw.ddbj.nig.ac.jp/). Phylogenetic and molecular evolutionary analyzes were performed by MEGA 4.0 (Tamura et al, 2007).

Prokaryotic expression of PaCTSD

The mature protein of PaCTSD was amplified using primers: pET-PaCTSD(+): 5'-*C*AAGCTTCCCTTATTC GAATTCCGTTAAAG-3' and pET-PaCTSD(-): 5'-*C*CT CGAGTTATTTGGATTTGGCAAAGC-3' (underline means the site of Hind III and Xho I, and italic type means protective bases). After being digested with Hind III and Xho I, the amplicon of 1 147 bp was inserted into the multiple cloning. Prokaryotic expression of PaCTSD was induced in BL21. The recombinant PaCTSD was further resolved by SDS-PAGE.

Purification and refolding of PaCTSD

After induction, cultures were collected by centrifugation for PaCTSD purification and refolding as previously described (Hwang & Chung, 2002). The cell pellet was resuspended in sonic buffer (50 mmol/L KH_2PO_4, 300 mmol/L KCl). Sonication was carried out to extract protein using an ultrasonicator. The inclusion bodies were harvested by centrifugation and resuspended in 8 mL solubilization buffer (20 mmol/L Tris-HCl, 8 mol/L urea, 500 mmol/L NaCl, 500 mmol/L imidazole, pH 8.0). The supernatant is collected by centrifugation at 12 000 r/min for 30 min. The 2 mL soluble fraction was applied onto a Ni-nitrilotriacetic acid column (QIAGEN) to purify and refold PaCTSD. Urea was removed slowly using a gradient from 8 to 0 mol/L urea in 20 mmol/L NaH_2PO_4, 0.5 mol/L NaCl, 10 mmol/L 2-mercaptoethanol, and 20% glycerol, pH 8.0. The column was then washed with wash buffer (20 mmol/L NaH_2PO_4, 0.5 mol/L NaCl, 10 mmol/L 2-mercaptoethanol, 20% glycerol, 20 mmol/L imidazole, pH 8.0). The bound protein was eluted with elution buffer (20 mmol/L NaH_2PO_4, 0.5 mol/L NaCl, 10 mmol/L 2-mercaptoethanol, 20% glycerol, 250 mmol/L imidazole, pH 8.0). The PaCTSD containing solution was desalted and concentrated by ultrafiltration device (Millipore Corporation, Bedford, MA) before being stored at −80 ºC until use.

PaCTSD enzymatic activity

Proteolytic activity of PaCTSD was evaluated as described by Barrett (Barrett, 1970). Briefly, hemoglobin was denatured in 100 mmol/L formic acid (pH 3.5). 3 μg recombinant PaCTSD was mixed with 25 μL hemoglobin in 800 μL reaction volume with sodium citrate buffer of different pH (3, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0). After incubation at 37 ºC for 30 min, reaction was terminated by adding trichloroacetic acid and incubating for 10 min. The hemoglobin was removed at 15000 rpm for 10 min. The supernatant was collected and the absorbance at 280 nm was measured. All reactions were performed in triplicate.

Tissue preparation

For health samples, we selected 4 sweetfish to harvest tissues including liver, spleen, head-kidney, kidney, gill, muscle, and intestine. For white blood cell isolation, blood samples were collected from sweetfish and gently mixed with PBS. The mixture was transferred onto the top of Ficoll (Sigma), and isolated by centrifugation at 400 rpm for 30 min. White blood cells were separated on the interface layer of Ficoll gradient. The cells were collected for subsequent experiments. For *L. anguillarum* infected samples, sweetfish were infected by intraperitoneal injection

of *L. anguillarum* (1.0×10^4 CFU/fish). The tissue was obtained at 6, 12, 24, 48, and 72 h post infection. The fish tissues were collected, immediately snap-frozen in liquid nitrogen, and preserved in at −80 ºC until examined.

Quantitative real-time PCR (qRT-PCR) detection

We employed qRT-PCR to detect the expression of PaCTSD mRNA in different sweetfish tissues. Total RNA was extracted from sweetfish tissues using RNAiso reagents as described by Huang et al (2011). The first-strand cDNA was synthesized using M-MLV. Primers of PaCTSD were designed to amplify a 138 bp fragment, PaCTSDtest(+): 5'-AGGAACCGACCCCAAATACT-3' and PaCTSDtest(-): 5'-ACGATAGCCTCACAGCCACT-3'. The primers of β-actin were used to amplify a 231 bp fragment, pActin(+): 5'-TCGTGCGTGACATCAAGGA G-3' and pActin(-):5'-CGCACTTCATGATGCTGTTG-3' (Huang et al, 2011). RT-PCR reaction was carried out in triplicate. Ct values of PaCTSD for all samples were normalized to β-actin using the ΔCt method (Livak & Schmittgen, 2001).

Data analysis

The OD value and qRT-PCR data were described as means±*SE*. We employed SPSS 13.0 to analyze data by one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

PaCTSD sequence analysis

PaCTSD cDNA with the GenBank accession number JP725490 consisted of 1 955 bp that translated in an open reading frame from 131 to 1 321 to give a predicted 397 aa. The estimated molecular weight (MW) was 43.17×10^3. The N-terminal 18-residue sequence of PaCTSD was predicted as signal peptide. Sequence analysis showed that PaCTSD had three conserved disulfide bonds (Cys107−Cys114, Cys272−Cys276, and Cys315−Cys352). One N-glycosylation site was predicted to exist at Asn^{131}. Catalytic sites included two critical aspartic residues Asp^{94} and Asp^{281} (Figure 1).

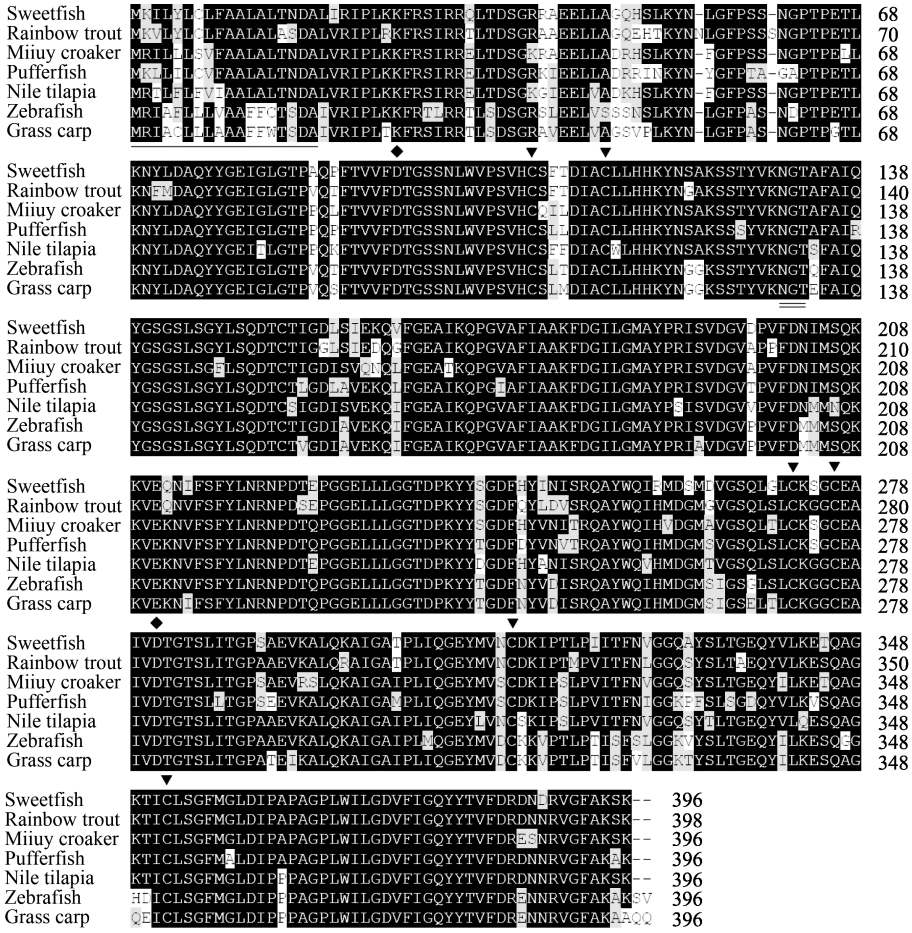

Figure 1 Multiple alignment of PaCTSD amino acid sequences with those of other species

The signal peptides are shown with single underlines. Double underline means N-glycosylation site. The cysteine residues are shown with "▼". The catalytic residues are marked with diamonds. Shading threshold was > 60%. Accession numbers are as follows: zebrafish (*Danio rerio*), BX000347; grass carp (*Ctenopharyngodon idella*), JX255676; rainbow trout (*Oncorhynchus mykiss*), NM_001124711; miiuy croaker (*Miichthys miiuy*), HM628578; pufferfish (*Takifugu rubripes*), NM_001078584; Nile tilapia (*Oreochromis niloticus*), XM_003452585; sweet fish (*Plecoglossus altivelis*), JP725490.

Amino acid sequence comparisons showed that PaCTSD shared the highest identity (90%) with Atlantic salmon (*Salmo salar*) and rainbow trout (*O. mykiss*), followed by miiuy croaker (*M. miiuy*) (89%), pufferfish (*Takifugu rubripes*) (86%), zebrafish (*Danio rerio*) (82%), and grass carp (*C. idella*) (82%). The phylogenetic tree analysis showed that all fish CTSDs formed a fish cluster distinct from frog CTSDs (Figure 2). The PaCTSD was closest to Atlantic salmon and rainbow trout.

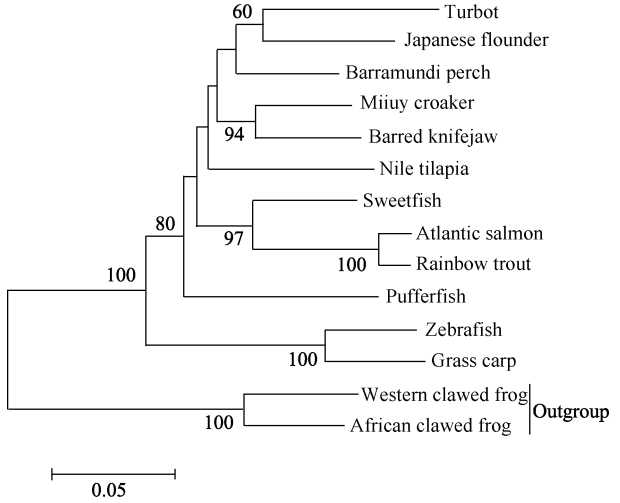

Figure 2 Phylogenetic tree of amino acid sequences of sweet fish and other animal CTSDs

Neighbour-Joining Method. The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping the data (1 000 replicates; shown only when ≥60%). The scale bar shows the number of substitutions per site. GenBank accession numbers of sequences used are listed below. Miiuy croaker (*Miichthys miiuy*), HM628578; Barramundi perch (*Lates calcarifer*), EU143237; Barred knifejaw (*Oplegnathus fasciatus*), AB597935; Nile tilapia (*Oreochromis niloticus*), XM_003452585; Pufferfish (*Takifugu rubripes*), NM_001078584; Turbot (*Scophthalmus maximus*), EU077233; Atlantic salmon (*Salmo salar*), BT043515; Rainbow trout (*Oncorhynchus mykiss*), NM_001124711; Japanese flounder (*Paralichthys olivaceus*), FJ172450; Zebrafish (*Danio rerio*), BX000347; Grass carp (*Ctenopharyngodon idella*), JX255676; Western clawed frog (*Xenopus tropicalis*), BC123962; African clawed frog (*Xenopus laevis*), NM_001091934; Sweetfish (*Plecoglossus altivelis*), JP725490.

Prokaryotic expression of PaCTSD

The pET-28a-PaCTSD vector was confirmed by sequencing. The recombinant PaCTSD was overexpressed after IPTG treatment in BL21 pLys E. The recombinant protein was resolved in SDS-PAGE. The results showed that purified PaCTSD was approximately 42.5×10^3, which was identical with the expected size (Figure 3).

Enzymatic activity of PaCTSD

Recombinant PaCTSD was refolded on the nickel-nitrilotriacetic acid column (Figure 3) to measure hemoglobin hydrolytic activity. The result showed that

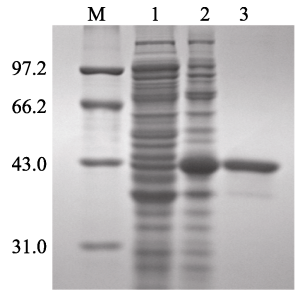

Figure 3 SDS-PAGE analysis of recombinant PaCTSD protein

M: low mass proteins ruler ($\times10^3$); 1: pET-28a-PaCTSD/BL21 without IPTG induction; 2: pET-28a-PaCTSD/BL21 with IPTG induction; 3: purified recombinant PaCTSD protein.

recombinant PaCTSD possessed hemoglobin hydrolytic activity within pH range 3.0−6.0 (Figure 4). The hydrolytic activity at pH 3.5 was highest, suggesting that PaCTSD was highly active in acidic conditions.

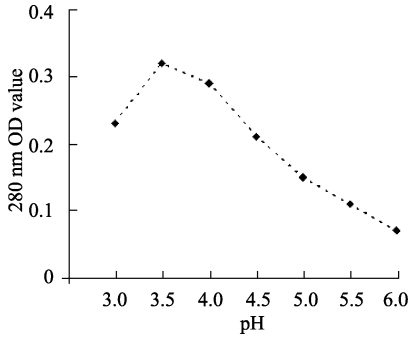

Figure 4 Proteolytic activity of PaCTSD at different pH

Tissue distribution of PaCTSD mRNA

We used qRT-PCR to measure the expression of PaCTSD mRNA in liver, spleen, white blood cells, head-kidney, kidney, intestine, muscle, and gill of health sweetfish. Our results showed that PaCTSD was expressed in all tested tissues (Figure 5), highest in spleen, followed by white blood cells, liver, head-kidney, kidney, intestine, muscle. The mRNA expression of PaCTSD in spleen is 18.7 times greater than in muscle.

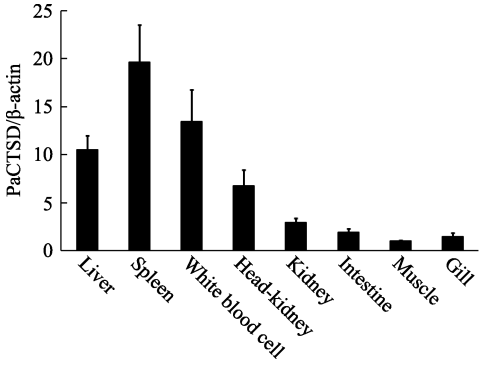

Figure 5 mRNA expression pattern of PaCTSD in different tissues

Relative expression of PaCTSD/β-actin in muscle is defined as 1. n=3.

PaCTSD mRNA expression after *L. anguillarum* infection

After *L. anguillarum* infection, liver, spleen, white blood cells, and head-kidney of sweetfish were used to measure PaCTSD mRNA expression by qRT-PCR. The data showed that PaCTSD mRNA expression in liver was upregulated at 12 h and 24 h (Figure 6A), and PaCTSD mRNA in spleen was upregulated at 24 h (Figure 6B). The PaCTSD mRNA expression was increased at 12 h in white blood cells (Figure 6C), and was increased at 12 h and 24 h in head-kidney tissue (Figure 6D).

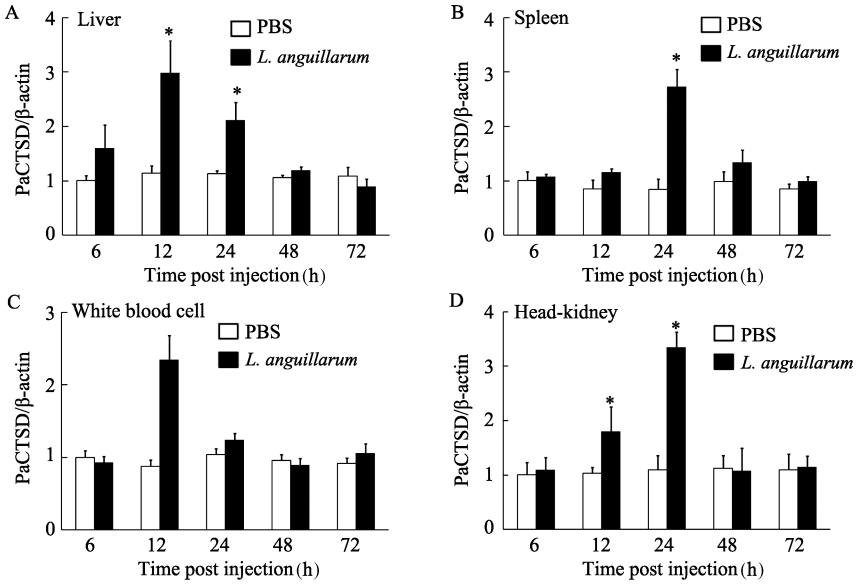

Figure 6 PaCTSD mRNA transcript changes in four tissues of sweetfish upon *L. anguillarum* infection

*: significant difference (n=3, P< 0.05), compared with the control group. Relative expression of PaCTSD/β-actin at 6 h in PBS group is defined as 1.

DISCUSSION

We obtained the CTSD cDNA sequence from sweetfish macrophages. Sequence analysis showed that the structure of PaCTSD protein was similar with other fish, mainly including three conserved disulfide bonds and two aspartic residues that form catalytic sites. PaCTSD showed the highest identity with the CTSDs of Atlantic salmon and rainbow trout. The phylogenetic tree analysis revealed that the PaCTSD amino acid sequence clustered within the fish CTSD group. PaCTSD formed a small cluster with the CTSDs from Atlantic salmon and rainbow trout. These results suggest that PaCTSD is conserved in sequence with other fish CTSDs.

Several investigations suggest that CTSD has proteolytic activity in acidic condition. In mammals, the most appropriate pHs were 3.5 and 2.5−3.0 for proteolytic activity of human and pig CTSDs (Barrett, 1970; Canduri et al, 1998). In fish, the most appropriate pHs were 3.0 and 3.5 for proteolytic activity of Atlantic cod and tilapia CTSDs (Wang et al, 2007; Jiang et al, 1991). In our study, PaCTSD had proteolytic activity at pH 3.0−6.0, and highest proteolytic activity was at 3.5. Our result suggests that the expression pattern of PaCTSD is similar with other fish. Together, these results may indicate that fish CTSDs also function in acidic conditions.

Besides proteolytic activity, CTSD plays an important role in immune response. CTSD is released after severe tissue injury to cleave C5 for subsequent generation of functional C5a (Huber-Lang et al, 2012). In fish, several reports suggest that CTSD is related with infection. For example, *Vibrio harveyi* infection induces CTSD mRNA expression in head-kidney, liver, and spleen of turbot (Jia & Zhang, 2009). *Aeromonas hydrophila* infection induces CTSD mRNA expression in liver, spleen, head kidney, and intestine of grass carp (Dong et al, 2012). In our study, PaCTSD mRNA expression was upregulated after *L. anguillarum* infection in liver, spleen, white blood cells and head kidney at different time points. The peak level of PaCTSD expression was at 24 h in spleen and kidney, and at 12 h in liver. Our results suggest that PaCTSD plays an important role in response to infection.

In summary, we identified CTSD cDNA sequence from sweetfish, measured the enzymatic activity, and detected the mRNA expression in different tissues after

L. anguillarum infection. Our results reveal that PaCTSD participates in immune response to infection, and provides the basis for further researching the structure, function, and mechanisms of PaCTSD.

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