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Purification and characterization of paralytic shellfish toxin-transforming enzyme, sulfocarbamoylase I, from the Japanese bivalve *Peronidia venulosa*

Yuko Cho [a,*], Noriyuki Ogawa [b], Miyako Takahashi [b], Hsi-Pin Lin [b], Yasukatsu Oshima [b,1]

[a] Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori - Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan
[b] Graduate School of Life Sciences, Tohoku University, 1-1 Tsutsumidori - Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan

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ABSTRACT

The Japanese bivalve *Peronidia venulosa* contains paralytic shellfish toxin (PST)-transforming enzymes that convert the weakly toxic C-toxins to the more potent decarbamoyl toxins. The enzyme was purified 154-fold with a yield of 0.26% and was named sulfocarbamoylase I. It was found to be a protein with an estimated molecular weight of 300 kDa by gel filtration column chromatography. Observation of a single band equivalent to 150 kDa on SDS-PAGE with or without reducing agents suggested it to be a homodimer with ionically bound subunits. The enzyme catalyzes the hydrolysis of the carboxyl bond in the *N*-sulfocarbamoyl moiety of PSP-toxins. The sulfonyl moiety in the carbamoyl side chain of substrates is essential for enzyme recognition. The N-terminal amino acid sequences of nine tryptic peptides were determined by the Edman degradation method. In a database search using the BLAST program, no protein that shows remarkable homology was retrieved. Several characteristics of the enzyme were also compared with those of another PST-transforming enzyme, carbamoylase I, which was previously isolated from the Japanese clam *Mactra chinensis*.

1. Introduction

Paralytic shellfish poisoning (PSP) is one of the most frequent types of human poisoning caused by seafood. The causative toxin first isolated from the siphon of the Alaska butter clam *Saxidomus giganteus* was designated saxitoxin and it was found to act as a voltage-gated sodium channel blocker [1–3]. More than 30 analogues have been reported to date and they are generally referred to as paralytic shellfish toxins (PSTs) [4]. Several species of dinoflagellate, such as *Alexandrium tamarense* [5,6], *A. catenella* [7], *Pyrodinium bahamense* var. *compressum* [8], and *Gymnodinium catenatum* [9] are known to produce PSTs and these toxins accumulate in marine animals. Seafood, particularly shellfish, contaminated by PSTs has posed serious health problems in many areas of the world. In recent years, the wider distribution of the causative organism, dinoflagellates, has led to increases in PSP events, even in areas where no PSP event had previously occurred [10].

Paralytic shellfish toxins exist as a mixture of analogues and primarily comprise three groups of toxin that possess different specific toxicities: 1) *N*-sulfocarbamoyl toxins (C1–C4, GTX5, and GTX6); 2) carbamates (GTX1-4, STX, and neoSTX); and 3) decarbamoyl toxins (dcGTX1-4 and dcSTX). Various toxin profiles have been found among causative dinoflagellate species and strains, even among members of the same species [11]. The toxicity of contaminated shellfish is in principle thought to reflect the toxin profile and biomass of the toxic dinoflagellates ingested; however, differences in toxin profile and content among shellfish species collected at the same time and in the same areas of algal bloom, and discrepancies in the toxin profiles of causative dinoflagellates and those of certain shellfish species are observed [11–13]. This indicates that the subsequent effects of PSTs in shellfish differ among species and that the dynamics of PSTs *in vivo* are complex. Differences in toxicity are associated with differences in ingestion, distribution, metabolism (chemical and enzymatic transformation), accumulation, and excretion of each shellfish species. With regard to metabolism among these processes, chemical conversion of PSTs has been investigated and the influence of pH, temperature [14], and reductants, such as glutathione and cysteine, has been determined [15–20].

Despite their importance, the characteristics of enzymes involved in the metabolism of PSTs are poorly understood, largely because of difficulties in isolation due to instability and low yield. The presence of PST-transforming enzyme was first recognized in the little neck clam *Protothaca staminea in* 1981 [21]. In 1995, two bivalve species, *Mactra chinensis* and *Peronidia venulosa*, among eighteen Japanese bivalves [14] and surf clams of *Spisula solidissima* from the Atlantic coast of North America [22], were found to possess such enzymes. In 2004, we obtained pure enzyme from the digestive gland of the Japanese clam *Mactra chinensis* and designated this enzyme carbamoylase I [13]. Incubation studies using this pure enzyme revealed that it hydrolyzes the carbamoyl moiety of both *N*-sulfocarbamoyl toxins and carbamate toxins, but that it has no influence on any other functional moieties,

* Corresponding author. Tel.: +81 22 717 8973; fax: +81 22 717 8922.
E-mail address: choyuko@biochem.tohoku.ac.jp (Y. Cho).
[1] Present address: School of Marine Biosciences, Kitasato University Sanriku-cho, Ofunato-shi, Iwate Prefecture 022-0101, Japan.

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such as the *N*-1 hydroxyl group. Recently, *Spisula solida* and *Scrobicularia plana* from the northwest coast of Portugal were found to have similar enzymatic activity [12].

In the present study, another Japanese bivalve species, *P. venulosa*, was used as the source of a novel PST-transforming enzyme. We showed that this organism possesses an enzyme (sulfocarbamoylase I) that catalyzes the hydrolysis of *N*-sulfocarbamoyl moieties (Fig. 1). The differences between the two known PST-transforming enzymes by comparison of their properties are also discussed.

2. Materials and methods

2.1. Materials

Live specimens of *P. venulosa* for enzyme purification were collected at Tomakomai, Hokkaido, Japan, in July 2003. Tissues of *P. venulosa* after dissection were stored at −80 °C until use.

Gonyautoxin (GTX)1, GTX2, GTX3, GTX4, GTX5, GTX6, C1, C2, C3, C4, STX, and neoSTX for use as substrates and high-performance liquid chromatography (HPLC) analytical standards were prepared from contaminated shellfish collected in Japan, Australia, and Palau.

Centrifugal concentrators (Ultra free-MC, Ultra free 0.22 μm, and Centricon Plus-20) were purchased from Millipore (Bedford, MA, USA). SnakeSkin^{R}Pleated Dialysis Tubing 10000MWCO was from Thermo Fisher Scientific (Rockford, IL, US). Apollo 20 ml ultrafiltration devices were purchased from Orbital Biosciences LLC (Topsfield, MA, USA). Sep-pak Vac C18 cartridges were obtained from Waters (Milford, MA, USA). Spectra/Gel^{TM} absorbent was purchased from Spectrum (Los Angeles, CA, USA). Octyl sepharose 4 Fast Flow, Superdex 200 pg 16/60, and Con A sepharose were obtained from Pharmacia Biotech (Uppsala, Sweden). The Micro BCA Protein Assay Reagent Kit was from Pierce (Rockford, IL, USA). SYPRO RUBY protein gel stain and 0.2-μm PVDF membranes were obtained from Bio-Rad Laboratories (Melville, NY, USA). DEAE Toyopearl-650M, DEAE 5PW, and TSK-gel G-3000 SWxL ion exchange materials were purchased from Tosoh (Tokyo, Japan). Aprotinin, E-64, bestatin, leupeptin, and AEBSF protease inhibitor were from Sigma (St. Louis, MO, USA), and benzenesulfonyl fluoride (BSF) was from Aldrich (Milwaukee, WI, USA). Amounts of protein were assayed using Micro BCA Protein Assay Reagent Kits with bovine serum albumin as a standard.

2.2. Analytical methods for sulfocarbamoylase activity

A reaction mixture consisting of 3 μl of substrate dissolved in 50 mM acetic acid and 27 μl of sample or purified enzyme solution was incubated at 20 °C for 60 min. During purification and determination of optimal temperature and pH as well as the effects of chemical substances, an equilibrated mixture of C1 and C2 (51.4 and 47.2 μM, respectively) was used as the substrate solution. Because ammonium sulfate and high concentrations of NaCl inhibit sulfocarbamoylase activity, sample solutions containing either of these were desalted using Ultra-free MC microcentrifuge filters prior to assay. The reaction mixture was filtered using Ultra free-MC microcentrifuge filters (10,000 Da cut-off, 11000 ×*g* for 25 min at 4 °C) and the reaction products (dcGTX2 and dcGTX3) in the filtrate were quantitated by the HPLC-fluorometric method (HPLC-FLD) of Oshima et al. [23,24]. One unit of activity was defined as the amount of enzyme that produces 1 μmol of reaction product (total amount of dcGTX2 and dcGTX3) in 1 min.

To determine the effects of chemical substances, we individually added the following to the reaction mixture: ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt (EDTA 2Na) at a final concentration of 1 mM; aprotinin, 5 μg/ml; *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64), 3 μg/ml; bestatin, 3 μg/ml; leupeptin, 3 μg/ml; 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 1 mM; or benzenesulfonyl fluoride (BSF), 1 mM.

2.3. Anatomical distribution of sulfocarbamoylase activity in P. venulosa

Three live specimens were dissected into eight parts (digestive glands, foot, gills, adductor, siphon, mantle, crystalline style, and others) and were stored at −80 °C until use. To minimize individual variations, homogenates from three specimens were prepared for each tissue and 300 mg of homogenate was extracted with 1 ml of 100 mM potassium phosphate buffer (pH 7.0). After centrifugation, the supernatant was passed through Sep-Pak Cartridges Vac C18 50 mg/1 cc^{R} and a 0.22-μm membrane filter to remove lipophilic compounds and insoluble particles that interfere with HPLC analysis. An aliquot of filtrate was analyzed as in section 2.3 above. Because bivalve specimens contain trace amounts of toxin, tissue extracts without substrate toxins were tested as blanks and amounts of toxin detected in the blanks were subtracted from those obtained for sample solutions.

2.4. Purification of sulfocarbamoylase I

Crystalline style (107 g wet weight) was homogenized and extracted with 930 ml of 50 mM potassium phosphate buffer containing 500 mM of ammonium sulfate, 5 μg/ml of aprotinin, 5 μg/ml of bestatin, and 0.1 mM of EDTA at 4 °C. After centrifugation at 550 ×*g* for 30 min and 7500 ×*g* for 30 min at 4°C, the supernatant was filtered through a

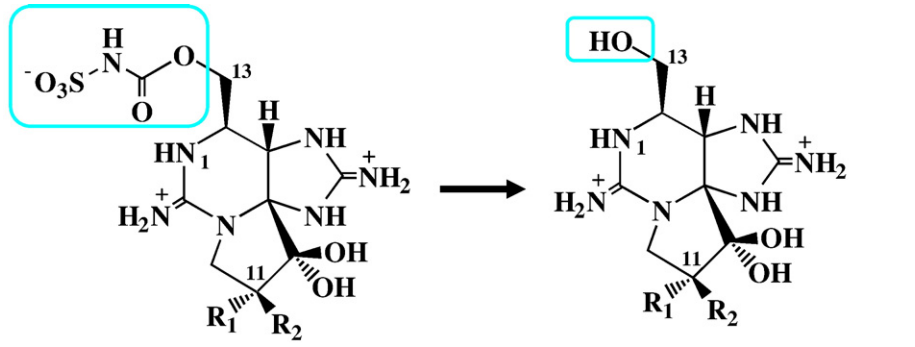

Fig. 1. Structural transformation by sulfocarbamoylase I can increase toxicity. Specific toxicities (MU/μ mol) are indicated in parentheses.