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Characterization of a transglutaminase from scallop hemocyte and identification of its intracellular substrates

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Abstract

Scallop hemocytes contain a transglutaminase (TGase) that is electrophoretically different from the TGase in the adductor muscle. The optimum temperature of the hemocyte TGase was lower (about 15 °C), compared with the muscle TGase (35–40 °C). Other properties, such as the high sodium chloride (NaCl) and $CaCl_2$ concentrations required for activation, instability in salt solutions, and the Km values against monodansylcadaverine (MDC) and succinylated casein, were similar for both enzymes. When hemocyte homogenate was incubated with MDC at 10 °C, MDC was incorporated into the 230 k and 100 k proteins of the hemocytes. The 100 k protein was only detected in the supernatant, the 230 k protein was insoluble, and the 210 k protein was detected in both fractions. In the absence of MDC, the 230 k, 210 k, and 100 k proteins were cross-linked by endogenous transglutaminase. The 230 k protein was most quickly cross-linked and formed huge polymers within 5 min. These results suggest that if scallop tissues are injured, hemocyte transglutaminase may be activated, initially cross-linking the insoluble hemocyte 230 k protein, followed by the 210 k and 100 k proteins, to form a cross-linked protein matrix with inter cross-linking of hemocyte sheets, to stop the bleeding.

Keywords: Adductor muscle; Cross-linking; Hemocyte; Hemolymph; Mollusks; Scallop; Transglutaminase; Wound healing

1. Introduction

Transglutaminases (protein–glutamine: amine γ-glutamyltransferase, EC 2.3.2.13, TGases), which catalyze the Ca^{2+}-dependent cross-linking of proteins via an isopeptide bond between the γ-carboxamide group of peptidyl glutamine and an ϵ-amino group of peptidyl lysine, are classified into several groups depending on their distribution, structure, and biological function (Folk, 1980; Lorand and Conrad, 1984; Ichinose et al., 1990; Wilhelm et al., 1996). The physiological role of the common tissue-type TGases is still not entirely understood, but recent studies suggest that the enzyme is involved in apoptosis, cell differentiation, and cell growth regulation (Greenberg et al., 1991; Taresa et al., 1992; Aeschlimann et al., 1995; Autuori et al., 1998; Aeschlimann and Thomazy, 2000).

Nozawa et al. (1997) prepared TGases from dorsal muscle of the scallop, *Patinopecten yessoensis*, botan shrimp, *Pandalus nipponensis*, squid, *Todarodes pacificus*, carp, *Cyprinus carpio*, rainbow trout, *Oncorhynchus mykiss*, and atka mackerel, *Pleurogrammus azonus*, and compared their physicochemical and enzymatic properties. The most interesting finding was that the activities of marine invertebrate TGases were dramatically increased at a sodium chloride (NaCl) concentration similar to normal seawater, which was very different from the salt requirement of fish and mammalian enzymes. Kumazawa et al. (1997) have reported a similar finding for the Japanese oyster *Crassostrea gigas*. It has recently been reported that NaCl-induced activation of the enzyme was restricted to marine inverte-

Abbreviations: Coomassie brilliant blue R-250, CBB; Dithiothreitol, DTT; Ethylenediamine tetraacetic acid, EDTA; *O*,*O*′-bis(2-aminoethyl) ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid, EGTA; 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, HEPES; monodansylcadaverine, MDC; polyacrylamide gel electrophoresis, PAGE; sodium dodecyl sulfate, SDS; Transglutaminase, TGase.

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brates because NaCl did not enhance the enzyme activities of freshwater invertebrates (Nozawa et al., 2001). Based on these findings, it is postulated that shellfish TGases may function in extracellular fluid or may be activated by contact with natural seawater (Nozawa and Seki, 2001).

Marine bivalve mollusks such as scallops, oysters, and clams are widespread in coastal waters. As they are osmoconformers and have an open blood vascular system, all their organs are bathed in hemolymph liquid containing about 470 mM Na^+, 550 mM Cl^-, and 10 mM Ca^{2+}, which is isosmotic to seawater (Prosser, 1973).

A well-known transglutaminase in mammalian blood is factor XIIIa, which functions in the terminal stage of the blood coagulation cascade, wherein it catalyzes the formation of cross-linked fibrin repeating units that comprise the fibrin matrix of blood clots (Lorand and Conrad, 1984). Crustaceans have an instant coagulation mechanism to prevent substantial blood loss from their circulatory system upon wounding. The formation of stable clots in the hemolymph of crustaceans requires both hemocytic and plasmatic factors, and transglutaminase-catalyzed cross-linking of proteins is an important reaction in the clotting mechanism (Needham, 1970; Osaki and Kawabata, 2004). Since bivalves and gastropods have no fluidal coagulation system, their hemolymph cannot clot on bleeding. Instead, bleeding is stopped by forming sheets of hemocytes at the wound site (Suzuki and Awaji, 1995; Awaji and Suzuki, 1998). In this wound-healing system, it remains unresolved as to whether TGase plays an important role in the process of sheet formation. In addition, it is still unclear whether TGase exists in scallop hemolymph. In order to clarify the mechanisms responsible for the arrest of bleeding through hemocyte sheets formation, a TGase was partially purified from scallop hemolymph and its enzymatic properties were compared to muscle TGase. The second part of the study involved the identification of the substrate proteins for the hemolymph TGase.

2. Materials and methods

2.1. Materials

Live cultured scallops, *P. yessoensis*, were purchased from a wholesale market in Hakodate, Japan.

Coomassie brilliant blue R-250 (CBB) and monodansylcadaverine (MDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediamine tetraacetic acid (EDTA), *O*,*O*′-bis(2-aminoethyl)ethylene-glycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were obtained from Dojindo Laboratories (Kumamoto, Japan). Succinylated casein was prepared from Hammarsten's casein (E. Merck) by the method of Franzen and Kinsella (1976). A TSK guard column SW, which is used for high-performance liquid chromatography (HPLC) analysis and a TOYOPEARL DEAE-650 M were purchased from the Tosoh (Tokyo, Japan). All other chemicals were of analytical grade and were obtained from Wako (Osaka, Japan).

2.2. Preparation of scallop hemocyte and plasma

Hemolymph was collected from sinuses in the adductor muscle of live scallops using a 20 G-needle and immediately diluted in 10 v of 20 mM HEPES (pH 7.5)–10 mM EGTA–1 mM dithiothreitol (DTT; buffer A). After centrifugation at 200×*g* for 3 min, the precipitate was washed with 20 v of buffer A and centrifuged again at 200×g for 3 min. The precipitate obtained was then used as scallop hemocyte. In order to obtain plasma, the hemolymph was directly centrifuged at 200×g for 3 min and the supernatant was used as the plasma fraction. All preparation procedures were carried out at 4 °C.

2.3. Partial purification of transglutaminase

Transglutaminase was extracted by homogenizing a sample of scallop hemocyte with five volumes of 20 mM HEPES (pH 7.5)–1 mM EGTA–1 mM DTT and centrifuging it at 80,000×*g* for 30 min. The supernatant was applied on a DEAE-TOYOPEARL column (2.8×11.0 cm) and eluted by a NaCl linear gradient of 0–0.8 M. A single active peak was eluted at 0.15 M NaCl and was used as scallop hemocyte TGase. The muscle enzyme was also purified as described by Nozawa et al. (1997). All purification procedures were carried out at 4 °C.

2.4. Assay of transglutaminase activity

The enzyme activity was assayed at 10 °C and pH 7.5 as described by Nozawa et al. (1999). The standard reaction mixture contained 1.0 mg/mL succinylated casein, 0.1 mM MDC, 10 mM $CaCl_2$, 2 mM DTT, 0.6 M NaCl, 0.1 M HEPES buffer (pH 7.5), and scallop TGase in a total volume of 0.5 mL. The reaction was terminated by adding 0.1 M EDTA and the MDC bound to the succinylated casein was analyzed using a TSK guard column SW (7.5×75 mm) on HPLC. One unit of TGase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of MDC into succinylated casein for 1 min.

2.5. Activity staining

The fluorescent activity staining of TGase was conducted according to the method of Stenberg and Stenflo (1979). The enzymes were applied on native polyacrylamide gel electrophoresis (PAGE) using 6% gel at 6 °C. After electrophoresis, the gel was incubated in the standard reaction mixture at 25 °C for 1 h. The fluorescent pattern of the MDC-bound proteins was detected by ultraviolet illumination.