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Kiwifruit Actinidin: A Proper New Collagenase for Isolation of Cells from Different Tissues

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Abstract Actinidin is a cysteine protease abundant in Kiwifruit. This enzyme is known as a meat-tenderizing protease. In this project, actinidin was purified from kiwifruit by salt precipitation and ion exchange chromatography. Collagenolytic effect of the purified enzyme was tested in four different buffer systems. Thereafter, the enzyme was used for isolation and culture of cells from three different tissues: endothelial cells from human umbilical vein, hepatocytes from rat liver, and thymic epithelial cells from rat thymus. Our results revealed that actinidin can hydrolyze collagen types I and II at neutral and alkaline buffers. Furthermore, actinidin compared with type II or IV collagenase isolated intact human umbilical vein endothelial cells, hepatocytes, and thymic epithelial cells with viability more than 90%. These results address a novel and valuable collagenase, which can be used efficiently for hydrolysis of collagen and isolation of different cell populations from various solid tissues.

Keywords Actinidin · Collagenase · Hepatocytes · Kiwifruit · Thymic epithelial cells · Umbilical vein endothelial cells

Introduction

Isolation of cells from various tissues essentially relies on disintegration of extracellular matrix, which consists of various fibrillar proteins, glycoproteins, and proteoglycans. Collagen is the main component of extracellular matrix that has important roles in maintaining the adhesion and growth of cells [1, 2]. Fibers of collagens consist of three chains that are wounded into a triple helical structure. The fibers provide the major

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biomechanical scaffold for cell attachment and anchorage of macromolecules, allowing the shape and form of tissues to be defined and maintained [3]. Due to its structure, collagen is resistant to the action of ordinary proteases. Collagenases are the only enzymes that able to cleave peptide bonds in the triple-helical regions of collagens [4, 5]. Two types of collagenases are known: (1) microbial such as *Clostridium histolytica* collagenase that generally split each polypeptide chain of collagen at multiple sites and (2) tissue collagenases, the other type, have been found in vertebrate tissues undergoing growth or remodeling. The vertebrate collagenases are distinguished by their ability to dissolve collagens by making a single scission across all three α chains at a specific sensitive site [6].

Actinidin (EC 3.4.22.14) is a thiol protease, first characterized by Arcus [7]. This enzyme is the major protein in most *Actinidia* fruits [8]. Actinidin is known as a good meat-tenderizing enzyme. However, there are few studies on the collagenolytic activity of this protease [9–11]. These studies did not report the hydrolytic effect of actinidin on collagen; however, Morimoto et al. [11] reported that atelocollagen (pepsin-hydrolyzed collagen) proved to be a substrate for actinidin at acidic pH. In the present project, collagenase activity of actinidin toward types I and II collagen has been surveyed in different buffer conditions. Furthermore, this protease has been used successfully for isolation and culture of three different cell types: endothelial cells from human umbilical vein (HUVEC), hepatocytes from rat liver, and thymic epithelial cells (TEC) from rat thymus.

Materials and Methods

Purification of actinidin Actinidin was purified from kiwifruit (Hayward cultivar) as described by Boland and Hardman [12]. Briefly, the enzyme fraction was precipitated from kiwifruit extract by 60% saturation of ammonium sulfate. The precipitate was redissolved in 50 mM citrate buffer (pH 5.5) and dialyzed overnight against this buffer. The dialyzate was loaded into a DEAE-Sepharose Fast Flow column (Pharmacia), which pre-equilibrated with the same buffer. The adsorbed fractions were eluted with 0.0–1 M linear gradient of sodium chloride in the buffer.

Protein and protease assay Protein concentration was estimated by the method of Bradford [13] using bovine serum albumin as the standard. Protease activity was determined based on the method of Anson [14] using casein as the substrate.

Collagenase activity of actinidin Stock solutions (3 mg/ml) of collagen type I from rat tail (Roche Applied Sci.) and type II from chicken external cartilage (Sigma Chemical Co.) were prepared in distilled water adjusted to pH 3 with acetic acid. Solutions of collagen type I or II with final concentration of 1 mg/ml were prepared from each of the stock solutions in 20 mM acetate (pH 4), 20 mM citrate (pH 5.5), 20 mM phosphate (pH 7), and 20 mM Tris–HCl (pH 8.5) buffers. About 10 µl of actinidin (1 mg/ml) was added to 990 µl of the collagen substrate solution in each buffer system. The reaction mixtures were incubated for 1 or 2 h at 37 °C. Thereafter, the enzyme activity rapidly arrested by addition of 250 µl of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and the tubes were incubated for 10 min in boiling water. Protein profile of the all samples was analyzed by SDS-PAGE in 10% slab gels according to the method of Laemmli [15]. After electrophoresis, the protein bands were stained with Coomassie brilliant blue R-350 (Pharmacia) and analyzed by densitometry (Helena) in 600 nm to determine the ratios of protein bands.

Isolation of HUVEC HUVEC were isolated and cultured using a modified standard procedure [16]. Briefly, fresh umbilical cords were washed with sterile phosphate-buffered saline (PBS). The umbilical vein was cannulated and thoroughly rinsed with sterile PBS until the vessel is slightly distended to clear excess blood. After clamping the other extremity, the vein was filled with 5–10 ml of 1, 2, 4, 8, or 16 mg/ml of actinidin or similar concentrations of type II or IV collagenase (Sigma Chemical Co.). The cords were incubated at 37 °C for 10, 20, 30, 40, 50, or 60 min to digest selectively the single layer of endothelial cells. Thereafter, the vein was perfused with MCDB131 medium containing 20% fetal calf serum (FCS), and the released cells were collected by centrifugation (150×*g* for 5 min). The pellet was resuspended in MCDB131 containing FCS, penicillin (100 IU/ml), streptomycin (100 μg/ml), and endothelial cell growth factor (20 μg/ml). At confluence, the endothelial cells were detached from the culture flasks using a solution of trypsin-EDTA and passaged. The cells were then plated on culture plates or flasks precoated with gelatin.

Identification of endothelial cells HUVEC were identified by their non-overlapping cobblestone morphology and immunostaining (Immunoperoxidase) with an antibody vs factor-VIII-related antigen (Von Willbrond factor) and a secondary antibody (HRP-conjugated) in representative plates.

Isolation of rat hepatocytes Hepatocytes were isolated from rat liver using the two-step perfusion technique as described by Wang et al. [17] with some modifications. Wistar rats (150–200 g from Razi Institute, Iran) were anesthetized with chlorophorm; then, heparin was injected (200 IU per 100 g of body weight) into the femoral vein. The abdomen was opened up to the sternum, and the vena cava closed with curved tweezers. The small intestine was pushed towards the left side and the liver upward to cannulate the portal vein (with 23-gauge needle). The cannula tightly was fixed with curved tweezers, and liver was perfused with calcium and sulfate-free Krebs–Henseleit buffer at 10 ml/min for 15 min. Few minutes after starting perfusion, one brunch of the portal vein was cut, and then liver was perfused with 0.1, 0.2, 0.4, 0.8, or 1 mg/ml actinidin solution at 7 ml/min for 10, 15, 20, 25, or 30 min. The liver was taken out from the animal and placed within a Petri dish together with the remaining actinidin solution for 20 or 30 min with gentle shaking for further digestion. All the perfusion and digestion steps were kept at 37–38 °C. The digested tissue was filtered through sterilized gauze and centrifuged for 3 min at 150×*g*. The pellet was washed two further times via centrifugation steps. The final cell pellet was resuspended in William's E culture medium containing 10% FCS, 2.5 μl/ml amphotericine B, and 50 μg/ml gentamycine and seeded in culture flasks precoated with collagen type I. The medium was changed daily, and morphology of the cells was observed under microscope. The albumin synthesis of hepatocytes was assessed using SDS-PAGE.

Isolation of rat TEC TEC from rat thymus were isolated as described by Ropke [18] with some modifications. The rats were injected with dexamethasone (1 μl/g weight) and anesthetized with chlorophorm 72 h after injection. The thymus was taken out and washed with PBS buffer. The gland was minced into small pieces and suspended in the PBS containing 1, 2, 4, 8, or 16 mg/ml actinidin for 1, 2, 3, or 4 h at 37 °C with gentle shaking. All released cells were harvested at 150×*g* for 5 min and washed two times with PBS. The cell pellet was resuspended in William's E culture medium containing 20% FCS, 2.5 μl/ml amphotericine B, and 50 μg/ml gentamycine. The cell suspensions were cultured in dishes precoated with collagen type I at 37 °C. After several hours of incubation, the adherent cells were generously washed with the culture medium three times and maintained in the same medium.

Viability Assessment

Isolated cells were washed with PBS and resuspended in this buffer. About 50 µl of trypan blue solution was mixed with 100 µl of the cell suspension and allowed to stand for 2 min at room temperature. The mixture then analyzed for cell viability according to the related formula.

Results

Collagenase activity of actinidin The hydrolysis of types I and II collagen by actinidin under different pH conditions was monitored by SDS-PAGE. Figure 1 shows the SDS-PAGE pattern of collagen type I after incubation for 1 h with actinidin in acetate (pH 4), citrate (pH 5.5), phosphate (pH 7) or Tris–HCl (pH 8.5) buffer system. As the figure indicates, the protein bands of collagen type I completely disappeared after 1 h of hydrolysis in Tris–HCl (pH 8.5) and phosphate (pH 7) buffer systems (lanes 8 and 6 vs 7 and 5 as their controls, respectively). In citrate buffer (pH 5.5), actinidin digested more than half of the collagen type I (lane 4), but in acetate buffer (pH 4), the enzyme did not hydrolyze this substrate (lane 2).

In comparison to collagen type I, actinidin hydrolyzed most of the collagen type II after 2 h incubation at Tris–HCl (pH 8.5) and phosphate (pH 7) buffer systems but did not hydrolyze this protein in acetate (pH 4) or citrate (pH 5.5) buffer systems.

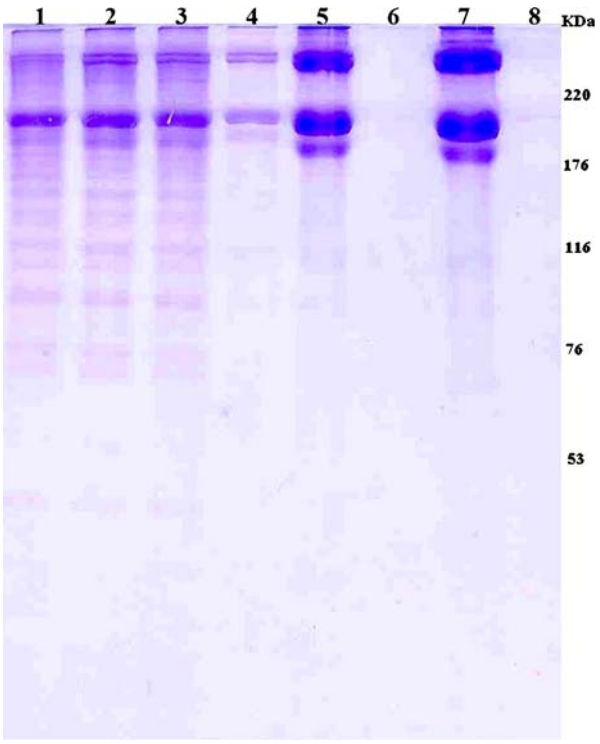

Fig. 1 SDS-PAGE of collagen type I hydrolyzed by actinidin at pH 4 (*lane 2*), pH 5.5 (*lane 4*), pH 7 (*lane 6*), and pH 8.5 (*lane 8*). *Lanes 1*, *3*, *5*, and *7* are controls, respectively. The gel stained with Coomassie brilliant blue

Isolation of HUVEC To find proper concentration of actinidin and necessary time selectively to isolate the single layer of endothelial cells, different doses of actinidin (1 to 16 mg/ml) and different incubation times (from 10 to 60 min) were tested. The results indicated that actinidin in concentration of 2–4 mg/ml for 20–40 min selectively isolates HUVEC with minimal contamination from other cell populations (Fig. 2a). The viability of separated cells was estimated more than 95% in these situations. The separated cells showed morphologic and immunohistologic characters of HUVEC (Fig. 2b).

Isolation of rat hepatocyte Perfusion of 0.4 mg/ml actinidin solution at flow rate of 7 ml/min for 15–20 min and an additional treatment for 20–30 min in Petri dish was isolated $2–3 \times 10^7$ hepatocytes from each liver with viability of 92–95%. Phase-contrast microscopy showed that isolated hepatocytes were translucent and spherical in shape (Fig. 3a). After isolation, the majority of intact hepatocytes adhered to each other, and the cells reconstructed their cellular polarity after 48 h presenting a typical polygonal morphology and many with binuclei (Fig. 3b).

Isolation of rat TEC Rat TEC was properly isolated after digestion of thymus in 4 mg/ml actinidin for 4 h at 37 °C. The isolated cells were adhered to collagen precoated dishes after washing. After 24 h of culture, the adherent cells were flattened and showed polygonal morphology with small nuclei (Fig. 4a,b). The viability of the cells as judged by the trypan blue test was estimated to be 90–95% in all isolations.

Discussion

Actinidin is a thiol protease abundant in kiwifruit [8]. This enzyme shows considerable structural and functional similarities with other plant thiol proteases such as papain [19–21]. Actinidin is known as a meat-tenderizing protease [22]. Therefore, this suggests that it may hydrolyze collagen. Although the effect of actinidin on synthetic or natural substrats has been addressed in several studies [10, 23, 24], there are few reports on the collagenolytic activity of this protease [9–11]. In the most recent study, Morimoto et al. [11] concluded that actinidin has no collagenase activity, but atelocollagen (pepsin-hydrolyzed collagen) proved to be a substrate for this enzyme at acidic pH.

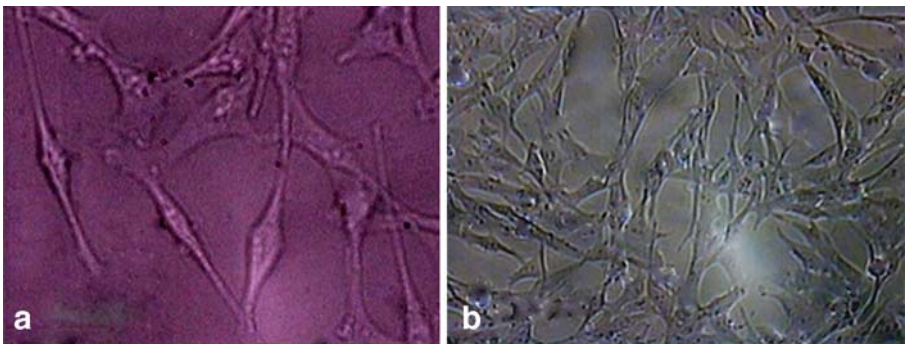

Fig. 2 **a** Morphology of HUVEC 48 h and (**b**) 5 days after isolation (invert microscope ×400)

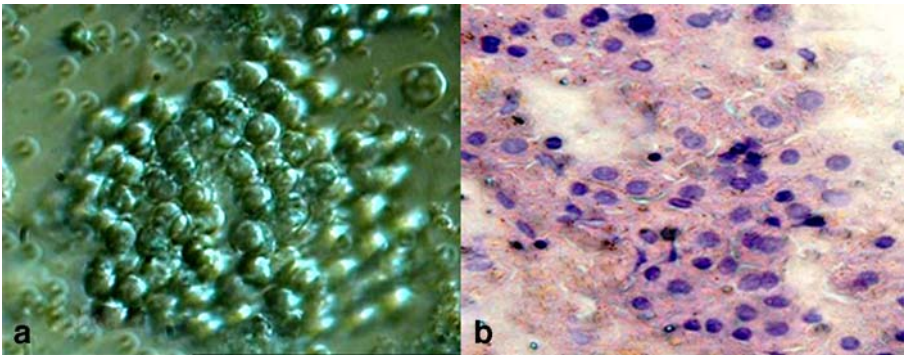

Fig. 3 **a** Morphology of hepatocytes after isolation (invert microscope ×400) and **b** 48 h after isolation (Papanicolaou stained)

In the present project, we examined the hydrolytic effect of actinidin toward types I and II collagens at four different buffers with pH 4, 5.5, 7, and 8.5. Our results revealed that actinidin can digest the two types of collagens at pH 7 and 8.5 but that it had no considerable effect on these substrates at acidic buffers particularly at acetate buffer with pH 4. Furthermore, actinidin digested more efficiently type I collagen than type II, so that the protein bands of collagen type I completely disappeared even after 30 min of hydrolysis in Tris–HCl (pH 8.5) or phosphate (pH 7) buffers as assessed by SDS-PAGE (Fig. 1) and protein assay methods. The pattern of SDS-PAGE indicates that actinidin could cleave collagens at multiple sits. These results suggested that the mode of action of actinidin is similar to bacterial collagenases. Type I collagen is the major structural constituent of most connective tissues, except for cartilage, where homotrimeric type II collagen is prevalent [25, 26]. Furthermore, types I and II collagen fibrils have been shown to have binding sites for other types of collagens and proteoglycans [27, 28]. Therefore, tissue dissociation and cell isolation is achieved by disintegration of these major types of collagens. The interstitial collagenases are a group of endopeptidases having the ability to cleave the helical region of native collagen fibers [29]. The definition excludes proteases capable of hydrolyzing only the non-triple-helical telopeptide portions of collagens and enzymes capable of degrading the triple-helical domains of collagens only in solution or extremes of pH.

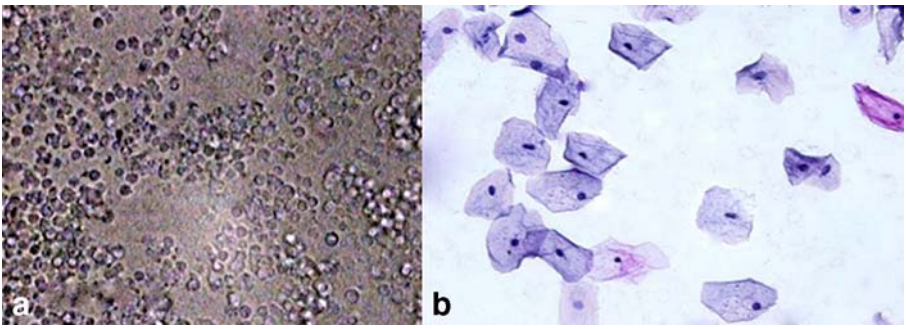

Fig. 4 **a** Morphology of TEC after isolation (invert microscope ×100) and **b** 48 h after isolation (Papanicolaou stained)

According to the meat-tenderizing effect of actinidin and collagenolytic properties of this protease, which has been revealed in this study, we speculated that actinidin can be used for isolation of different cells from various tissues. Hence, in this project, actinidin has been used instead of collagenase to isolate HUVEC, hepatocytes from rat liver, and TEC from rat thymus. The effect of actinidin on umbilical vein in different conditions showed that optimal situation for selective isolation of HUVEC is 4 mg/ml actinidin for 20 min. In this condition, the separated cells had viability of more than 95% and contained minimal contamination from other cell populations as assessed by morphologic and immunohistologic characters. These results were comparable with results of isolation of HUVEC by collagenases in this study and other similar studies that successfully cultured endothelial cells by collagenases treatment of large and small-sized vessels, including human umbilical vein [16], human iliac vessels [30], bovine vena cava [31], rabbit pulmonary artery [32], and heart vessels [33–35]. Endothelial cells form a single-cell layer that lines the inner surface of all blood vessels. Much interest has been generated in isolation and culture of endothelial cells due to their potential involvement in vascular disease, the repair of blood vessels, and angiogenesis in cancer [36, 37].

Extracellular matrix of liver contains collagen types I, III, IV, V, and VI and various glycoproteins [38]. For disintegration of collagenouse fine meshwork and isolation of hepatocytes from rat liver, we used actinidin from 0.1 to 1 mg/ml in different situations. The results indicated that perfusion of 0.4 mg/ml actinidin into the portal vein at 7 ml/min for 15 min and treatment of the taken-out liver for an additional time (15–20 min) in the enzyme solution could isolate $2–3\times10^7$ intact hepatocytes from each liver. The isolated cells had viability of 92–92% in all isolations and survived for at least 3 days in the culture flasks without any growth factor. These results revealed that the use of actinidin in the basic two-step perfusion procedure is a good choice for isolation of hepatocytes from rat and may be other animals. The results were comparable with the results obtained herein and from other studies to used collagenaeses in two-step perfusion method for isolation of hepatocytes from various rodents [17, 39, 40] and human [41, 42] livers. Isolation and primary culture of hepatocytes is an in vitro model widely used to investigate various aspects of liver physiology and pathology [43].

Thymus is a three-dimensional network of distinct cell types and extracellular matrix elements [44]. These elements includes collagen types I and IV, laminin, fibronectin, and various ligands of adhesion molecules. Unlike liver, disintegration of thymus extracellular matrix needs harsh conditions using generally more than one type of hydrolytic enzymes. So far, a mixture of three types of bacterial protease (Liberase) and DNase [45] or collagenase and dispase [46, 47] has been used for disintegration of this tissue to isolate TEC. In this study, TEC sufficiently were isolated after digestion of minced thymus in 4 mg/ml actinidin for 4 h. The isolated TEC had viability of more than 90% in all isolations and showed normal morphology in culture. Isolation and culture of TEC provide a valuable tool to study the role of these critical cells in thymus function [48].

Together, the present study clearly demonstrated collagenolytic activity of actinidin on types I and II collagens, and used this protease for the isolation of HUVEC, hepatocytes, and TEC for the firs time. According to the collagenolytic activity of actinidin, this protease has a potential for isolation of different cell populations from various solid tissues. Furthermore, acinidin can be isolated and purified from kiwifruit in large scale by a simple method, without risk of infections, compared to bacterial or tissue collagenases. These properties collectively address a novel and suitable collagenase for many applications in cell isolation and in medical and biological sciences. However, the precise mode of action of actinidin on collagen types I and II and hydrolytic effect of this protease on the other glycoproteins of extracellular matrix need more quantitative investigations.

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