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METHODS PAGE

Quantitation and visualization of protease activation in pancreatic acini

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A premature intracellular activation of digestive proteases has long been thought to represent either an initial or an initiating event in the pathogenesis of pancreatitis. The reasons for this assumption are manyfold and include the original observation that the pancreas of patients who do not survive necrotizing pancreatitis appears auto-digested on autopsy. More recently germline mutations associated with an autosomal dominant variety of hereditary pancreatitis have been shown to exclusively affect digestive proteases, primarily trypsinogen. In addition, trypsin activation is a prominent feature of most animal models of pancreatitis.

In order to study the mechanisms of intracellular trypsinogen activation in living pancreatic acinar cells we have developed an assay based on cell permeant fluorogenic substrates specific for trypsin and elastase that permits quantification of the activity of proteases in either single cells or single acini as well as in supensions of acini studied in a cuvette or 96-well plate over a time course of up to 4 hours. More importantly, it allows visual localization of the subcellular compartment where protease activation begins by means of fluorescence microscopy or laser confocal microscopy.

The original description of the method can be found in reference (10), a pdf copy of this paper can be downloaded from your library or obtained from the authors. The method is now widely used by laboratories studying the cell biology of pancreatic acinar cells and the pathophysiology of pancreatitis and allowed us to characterize the changes in intracellular calcium signalling that trigger protease activation and pancreatitis (9), a mechanism that has been confirmed by others (17).

Since the initial description of fluorogenic substrates in 1983 (14, 15) bisamide derivatives of rhodamine 110 (R110) have been widely used as sensitive and selective substrates for non-pancreatic (1-3, 5, 7, 15, 16, 18, 20) and pancreatic (4, 8-12, 17, 19) proteases. Proteolytic selectivity is reached by specific benzyloxycarbonyl (CBZ)- peptides. The tripeptide derivative bis-(CBZ-Ile-Pro-Arg)-R110 (BZiPAR) allows specific detection of trypsinogen activation, whereas the tetrapeptide derivative bis-(CBZ-Ala_4)-R110 (BZAla_4R) specifically detects

activation of proelastase. The peptides are covalently linked to both of rhodamine 110's amino groups and effectively suppress the molecule's fluorescence. Upon enzymatic cleavage, the non-fluorescent bisamide is converted first to the fluorescent monoamide and then to rhodamine 110, with further increase in fluorescence. Once liberated, the green fluorescence of rhodamine 110 can be monitored over time at excitation and emission wavelengths of 490 nm and 520 nm, respectively. The fluorescence intensity is constant from pH 3-9. (**Note 1**)

Here we describe procedures for monitoring intracellular activation of trypsinogen and proelastase in isolated pancreatic acini, quantified by cytofluorometry and visualized by fluorescence microscopy. Protease activation is induced by supramaximal stimulation with the cholecystokinin analogue caerulein.

1. Materials

1.1 Isolation and cultivation of pancreatic acini

1. Secretory units of acinar cells (acini) are freshly prepared from the pancreas of male Wistar rats with body weight between 150 and 200 g, as described by Williams et al. (21), but with slight modifications: Anaesthetic: Rompun (Bayer AG Leverkusen, Germany), Ketamin (CuraMed Karlsruhe, Germany).
2. Digestion medium: 0.01 % (w/v) collagenase NB 8 (1 PZU/mg with lowest tryptic activity, SERVA, Heidelberg, Germany) in HEPES-Eagle medium: 8 % (v/v) 125 mM HEPES buffer (pH 7.6 adjusted with NaOH), 72 % (v/v) Eagle's Minimum Essential Medium (EMEM), 20 % (v/v) of a freshly prepared aqueous solution of 50 mg/ml bovine serum albumin, fraction V (Sigma), $CaCl_2$ (12.5 mM) and aprotinin (15.0 U/ml; Sigma). Adjust the pH of the digestion medium to 7.4.
3. Culture medium contains (in mM): 24.5 HEPES, 96 NaCl, 6 KCl, 1 $MgCl_2$, 2.5 Na_2HPO_4, 0.5 $CaCl_2$, 11.5 glucose, 5 Na pyruvate, 5 Na glutamate, and 5 Na fumarate, containing Eagle's Minimum Essential Medium amino acids (1 % vol/vol) and bovine serum albumin, fraction V (1 % w/v), at pH 7.4 (adjusted with 3N NaOH).
4. Tabletop centrifuge.
5. Oxygen for aeration (bottle with reducing valve).
6. Special filter material (Polymon PES-200/45, Angst+Pfister AG, Zürich, Switzerland).
7. Cell analysis system CASY TTC (Roche Innovatis AG, Germany) for determination of acinar biovolume in normal saline (NaCl 0.9 % w/v) in plastic CASY cups.
8. Cholecystokinin analogue caerulein (Bachem, Switzerland), 500 µM stock solution in PBS stored in aliquots of 15 µl at –20°C.
9. Cell-permeable protease inhibitors, e.g. FUT-175 (Futhan; BD Pharmingen) for tryptic activity and Boc-Ala_3-NHO-Bz (Merck) for elastolytic activity.
10. Speed and temperature controlled water bath shaker (e.g. Lab-Line benchtop shaker).

1.2 Quantitation of protease activation

1. 30 µM bis-(CBZ-Ile-Pro-Arg)-R110 (Molecular Probes/Invitrogen) for monitoring trypsinogen activation, and 30 µM bis-(CBZ-Ala_4)-R110 (Molecular Probes/Invitrogen) for monitoring proelastase activation in substrate medium (in mM): 24.5 HEPES, 11.5 glucose, bovine albumin fraction V (1 % w/v), at pH 7.4 (adjusted with 3N NaOH). Substrate stock solutions: 30 mM in DMSO stored at 4°C. The bisamide substrates are supplied as lyophilised powder and should be stored desiccated at –20°C.

2. Fluorescence microtiter plate reader with probe scan across the bottom of the wells (e.g. Cytofluor 2350 or 4000 fluorescence measurement system for both soluble and cell-associated fluorescence, Perseptive Biosystems).
3. Tabletop or other refrigerated centrifuge.
4. 96-well microtiter plates with flat bottom (e.g. Greiner bio-one, Germany) suitable for fluorescence measurement.

1.3 Visualization of protease activation

1. Inverted fluorescence microscope with high sensitive imaging system. We use an IX70 microscope (Olympus) associated with Polychrome II illumination system, IMAGO high resolution CCD camera, and TILLvision software (TILL Photonics GmbH, Germany) operating on a Vibration Isolation Table System Micro-g (TMC Peabody, MA).
2. Temperature controlled open dish system Delta TC3 (Bioptechs Inc. Butler, PA).
3. Lab-Tek II Chambered Coverglass with 8 wells (NUNC).
4. 100 µM bis-(CBZ-Ile-Pro-Arg)-R110 for monitoring trypsinogen activation, and 100 µM bis-(CBZ-Ala_4)-R110 for monitoring proelastase activation (Molecular Probes / Invitrogen) in substrate medium (in mM): 24.5 HEPES, 11.5 glucose, bovine albumin, faction V (1 % w/v), at pH 7.4 (adjusted with 3N NaOH). They are made from substrate stock solutions (30 mM in DMSO stored at 4°C).

2. Methods

2.1 Isolation and cultivation of pancreatic acini

1. Preparation of pancreatic acini from one Wistar rat is sufficient for a typical experiment. When mouse pancreatic acini are used you may consider using the pancreata of two or three animals. Best experimental results are achieved with acinar units consisting of 3 to 20 (max) acinar cells. The rat is anaesthetized with i.p. Rompun (0.2 ml) plus Ketamin (0.5 ml), sacrificed by heart aspiration, and prepared for injection of 5 ml digestion medium through the pancreatic duct. 10 min later pancreas must be dissected, cleaned from fat and lymph nodes, transferred to 2 ml digestion medium, and incubated under oxygenation and shaking (100 cycles / min) at 37°C for 15 min. Then the pancreas is minced with small scissors, washed twice with 5 ml digestion medium, and incubated with 5 ml fresh digestion medium for another 15 min under the same conditions. Now the suspension is sucked through pipette tips with decreasing apertures (5, 2.5, 1, 0.5 mm), followed by filtering through a Polymon PES-200/45 filter. Filtrate (containing isolated acini) is washed five times with culture medium by centrifugation (500 rpm = 30 g, 1min). The last acinar pellet is re-suspended in culture medium, oxygenated and ready for use. All steps should be performed under cold (4 - 10°C) conditions.
2. For most of our experiments we adjust the biovolume of pancreatic acini to 2 mm^3 per ml medium to get reproducible results. Initial biovolume concentration is measured with the cell analysis system CASY TTC. (**Note 2**). It is estimated in CASY cups diluting 50 µl of the isolated acinar culture in 10 ml physiological saline (1:200). Depending on the initial concentration, biovolume is adjusted with culture medium to a final concentration of 2 mm^3 / ml.
3. Normally, we use a set of four experimental conditions, each with 5 ml of acinar culture in 25 ml nalgene Erlenmeyer flasks: 1. control (acini in culture medium), 2. physiological stimulation (acini in culture medium with 0.1 nM caerulein), 3. supra-physiological stimulation (acini in culture medium with 10 nM caerulein), 4. supra-physiological

stimulation (acini in culture medium with 10 nM caerulein) adding selected protease inhibitors (e.g. 10µM FUT-175 or Boc-Ala_3-NHO-Bz). These cultures are oxygenated and incubated for 60 min at 37°C in a water bath shaker (50 cycles / min). Sampling frequency for quantitation of protease activation: every 20 min.

2.2 Quantitation of protease activation

1. Aliquots (1 ml) of acinar suspension are taken from each of the four flasks at 0, 20, 40 and 60 min of incubation. Samples are washed twice by centrifugation (1,000 rpm = 110g, 1 min) and re-suspended in culture medium containing up to 5 % ethanol (**Note 3**).

2. 3 x 100 µl aliquots of the four different samples are transferred to the wells of a 96 well microtiter plate, in duplicate, first row (12 wells) for trypsin activity, the second row (12 wells) for elastase activity. To each well of the first row 50 µl of BZiPAR (30 µM) is added, to each well of the second row 50 µl of $BZAla_4R$ is added. Immediately, begin fluorescence data collection with the Cytofluor microplate reader using excitation at 490 nm and emission at 520 nm. Data acquisition every 10 min, measuring time 90 min. Protease activation is temperature-dependent, thus temperature control (e.g. at 25°C) during measurements is preferable.

3. Trypsinogen and proelastase activation correspond with the increase of rhodamine 110 fluorescence per time period. Normally, after a short lag phase this fluorescence increase is linear. For this reason, protease activation is expressed as □F/□t, the linear slope of fluorescence increase in the corresponding time interval (6). Summarizing the means of all triplicate slopes allow a time-dependent comparison of the effect of caerulein and other cellular modulators on intracellular protease activation (examples of final graphs are shown in Krüger et al. [9-11]).

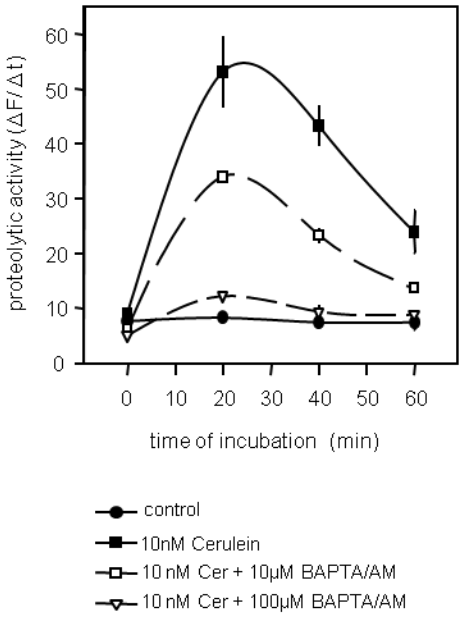

Figure 1. The **graph shows the rise - and subsequent spontaneous disappearance - of intracellular trypsin activity in living pancreatic acinar cells when induced by supramaximal concentrations of caerulein and measured by microfluorometry in a 96 well plate.** When BAPTA/AM, an intracellular calcium chelator, is added at the beginning, no intracellular trypsin activity arises. This shows that premature intracellular protease activation is completely calcium dependent. From reference 9 with permission.

2.3 Visualization of protease activation

1. Freshly prepared acini are suspended at a biovolume of up to 1 mm^3 per ml culture medium. Acini are loaded with the respective rhodamine-110 protease substrate (10 µM) for 30 min at room temperature (**Note 4**). Addition of up to 5% ethanol increases cell permeability for the fluorophore (**Note 3**). Once loaded acini are washed and re-suspended in fresh culture medium. Aliquots of the final suspension are transferred to an 8-chambered coverglass (Lab-Tek, NUNC), actually 500 µl per well.

   Alternatively, if a temperature controlled activation process is required, studies are performed in Delta T dishes (Bioptechs Inc. Butler PA) instead of NUNC chambered coverglasses.

2. Under the light path of an inverse fluorescence microscope (e.g. Olympus IX70 with TILL Photonics imaging system) focus on one exposed acinar cell of a suitable acinus (consisting of no more than 3-5 cells). Use an oil immersion objective with high magnification (x100, n.a. 1.0 – 1.2). Take a brightfield snapshot of the selected acinar cell at the start and end of the experiment, differential interference contrast (DIC) is especially recommended.

3. Carefully add caerulein to the well to get a final concentration of 10 nM (10 µl of a 0.5 µM caerulein stock solution to a well with 500 µl cell suspension). Take care, do not wash away the fixed acinus from the microscopic light path. You can fix the acinus with micromanipulators, if available.

4. From the individual acinus record an image sequence of one fluorescence snapshot per 10 sec for up to 60 min (Ex 485 nm, Em 520 nm for rhodamine-110 detection). Use exposure time as short as possible (10-20 ms) to prevent fading of the fluorophore.

5. TILLVision software produces a proprietary sequence file format, which must be converted to AVI or MPEG video files. At the end of the experiment use one of the brightfield images as sequence overlay to co-localize the activation spots of increasing fluorescence with the respective cell compartments (examples of single overlay images are shown in Krüger et al. (9-11).

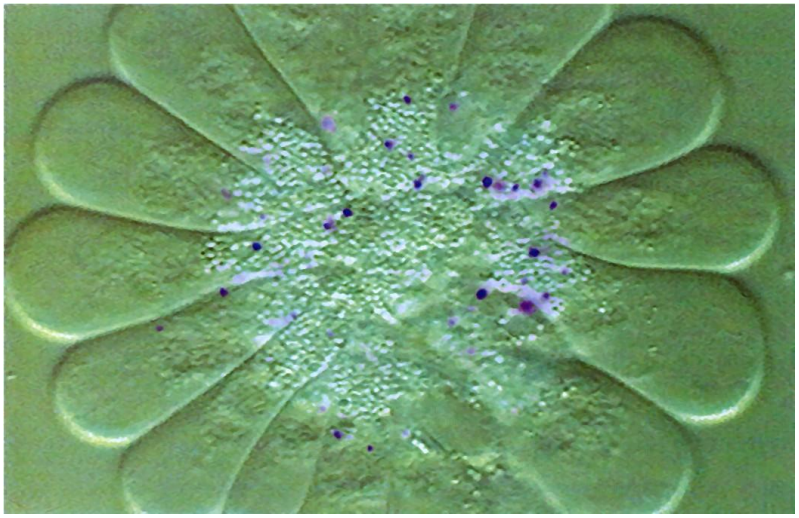

Figure 2. Fluorescence microscopy combined with differential interference contrast microscopy shows an acinus after supramaximal caerulein stimulation. The magenta dots indicate microsites at which the specific trypsin substrate is being cleaved at the time the photo was taken. From reference 10 with permission.

3. Notes

1. As an alternative, 7-amino-4-methylcoumarin-(AMC-) based protease substrates can be used. After cleavage by proteases, AMC produces a blue fluorescence with excitation / emission maxima at 351 nm / 430 nm. However, at these wavelengths cells show a high background fluorescence. On the other hand, published reports indicate a good correlation between AMC- and R110-based substrates.

   Once released, R110 or AMC quickly diffuse out of the cells. Therefore chloromethyl derivatives of R110- and AMC-substrates may exhibit better retention than the unmodified substrates. These thiol-reactive chloromethyl moieties can potentially react with intracellular thiols such as the abundant tripeptide glutathione. Unfortunately, the production of most of these substrate derivatives, sold by Molecular Probes in the past, has been discontinued.

2. Cell density of an acinar culture cannot be determined by cell counting, because acini may contain different numbers of cells. That's why as a standardization parameter for acinar suspensions we use the acinar biovolume concentration, which is fast, reproducible and easy measured with the CASY cell analyser.

3. R110- and AMC-based substrates are cell-permeant. Nevertheless, membrane penetration of these substrates is slow. Addition of up to 5% ethanol to the culture medium accelerates penetration process significantly without affecting intracellular protease activation.

4. To study activation of two different proteases simultaneously, it is necessary to use two substrates with different fluorophores. For example, trypsinogen and proelastase activation have been analysed in acini with CBZ-Ile-Pro-Arg-AMC and $(CBZ\text{-}Ala_4)_2$-R110, each at a concentration of 10 µM in culture medium. Blue fluorescence after excitation at 351 nm revealed trypsin activity, and green fluorescence after excitation at 490 nm shows elastase activity.

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