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Kinetic studies of serine protease inhibitors in simple and rapid 'active barrier' model systems - Diffusion through an inhibitor barrier

Erika Billinger, Gunnar Johansson*

Department of Chemistry – BMC Uppsala University, Box 576, SE-751 23 Uppsala, Sweden

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ABSTRACT

A model based on gelatin for protease activity studies was designed. The model is also extended to study the efficiency of inhibitors in a separate protective layer covering the layer containing the target substrate. A good correlation between protease concentration and the size of erosion wells formed in a plain gelatin layer was observed. Similarly, increased concentration of inhibitors gave a systematic decrease in well area. Kinetic analyses of the two-layer model in a spectrophotometric plate reader with a fixed concentration of substrate in the bottom layer displayed a strict dependence of both inhibitor concentration and thickness of the top "protective" layer. An apparent, but weaker inhibition effect was also observed without inhibitors due to diffusional and erosion delay of enzyme transport to the substrate-containing layer.

Introduction

The design of models that simulate in-vivo protease action requires a convenient model substrate. In this context, the protease degradation of collagen and collagen derivatives such as commercial gelatin have a long history of discovery and use [1,2]. Gelatin is formed by enzymatic, alkaline or acidic partial hydrolysis of collagen and includes not only the characteristic repeating sequences rich in glycine, alanine, proline and hydroxyproline, but also sections with a more varied amino acid composition that provides the versatility as a substrate. Gelatin can withstand mild heating, is readily available and does easily form a homogeneous gel. Taken together, collagen and its derived product, gelatin, are thus both convenient and versatile as model substrates for proteases [3,4].

The aim of this study is to design a model for a "skin with barrier" based on gelatin. The models are tested for selected proteases in the presence and absence of inhibitors. The target enzymes are the intestinal proteases trypsin and α-chymotrypsin, whereas the inhibitors studied are chymostatin, antipain and leupeptin. In a first stage the inhibitors are included in the substrate layer where the gelatin itself serves as a substrate. The second stage represents a more direct simulation of a barrier system where the enzyme encounters the inhibitor in the top layer, the barrier, before it reaches the target substrate in the bottom layer.

Materials & methods

Chemicals and materials

Commercial household gelatin powder (type A, from porcine source, Bloom number 220-240 g) was of the brand Törsleff's 'favorit gelatin pulver - extra guld'. Trypsin, α-chymotrypsin, N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPA), N-Benzoyl-L-tyrosine p-nitroanilide (BTpNA), antipain, leupeptin, chymostatin, supplement chemicals and buffers were all purchased from Sigma Aldrich. Petri dishes with the diameter of 9.1 cm and 96-well plates were purchased from VWR.

General preparations

The gel used in the experiments contains 0.02 g/ml gelatin powder mixed with deionized water. The powder was dissolved by heating the mixture mildly to approximately 38 °C. The resulting liquid phase was allowed to cool down to 30 °C before mixed with any reagents. The first gel layer was applied to each well/petri dish followed by cooling at 4 °C for 1 h, allowing the gel to solidify before the enzyme solution or the next gel layer was added. For the experiment with the 96-well plates, where enzyme solution was to be added, the gels were allowed to reach room temperature before the addition. Application of enzyme solution to the experiments using petri dishes took place at 4 °C, the experiments in 96-well plates took place at RT (22 °C). All the reagent solutions were initially made in deionized water, except the α-chymotrypsin solutions which, for stability, was dissolved in 80 mM Tris-HCl pH 7.8 containing

* Corresponding author.
E-mail address: gunnar.johansson@kemi.uu.se (G. Johansson).

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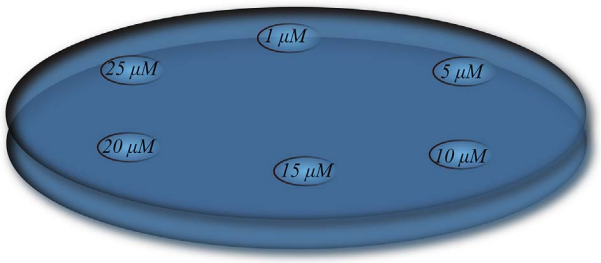

Fig. 1. A schematic picture of the petri dish used in the tests of enzyme activity. The gel layer thickness was 4 mm.

2 mM $CaCl_2$. Stock solutions of protease inhibitors antipain, leupeptin and chymostatin with concentrations of 2.0 mM, 5.4 mM and 4.17 mM respectively, as well as trypsin and α-chymotrypsin stock solutions of the concentration 25 μM were prepared. Inkscape version 0.91 and Word version 15.35 were used for picture drawing.

Kinetic experiments in petri dishes

Protease activity screening: Petri dishes containing 20 ml plain gel solutions were prepared in triplicates. The plates were stored at 4 °C for approximately 90 min until the gel had solidified. 25 μl trypsin or α-chymotrypsin solutions with the concentrations 1, 5, 10, 15, 20 and 25 μM were pipetted onto the plates (see Fig. 1). The chosen volume of 25 μl resulted in a uniform droplet. The plates were left for 24 h at 4 °C after enzyme application. The resulting wells in the gelatin created by the enzyme were dyed red with food coloring solution to simplify observation. The well diameters were measured after 24 h and the corresponding area was plotted against the enzyme concentration by GraphPad Prism 7, as can be seen in Fig. 3 with the corresponding area growth rate in Table 1.

Inhibition studies

Petri dish preparation and experiment procedures were carried out in triplicate as above, with the exception that inhibitors were included at concentrations of 0, 5, 10, 15 and 20 μM. The relative area growth rate (v_i/v_0) was analyzed as a function of the inhibitor concentration by GraphPad Prism 7 using the one-phase decay algorithm (equation (1)) which was most suitable and gave a good empirical fit, as can be seen in Fig. 4. The corresponding plateau values can be found in Table 2. For some points, the error bars would be shorter than the height of the symbol. In these cases, GraphPad Prism 7 simply does not draw the error bars.

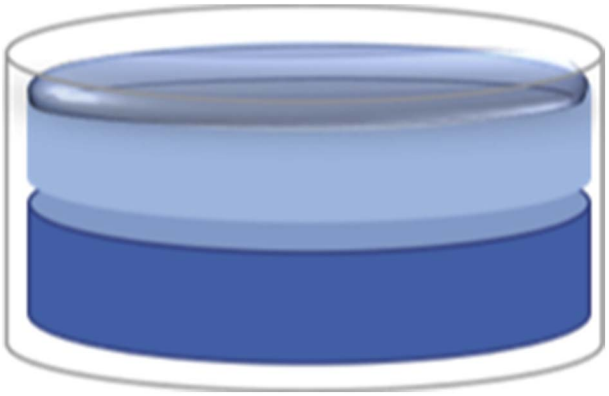

Fig. 2. A model portraying the general composition of a sample well. Note that the barrier layer only consists of gelatin gel in the experiments without inhibitor.

Kinetic experiments in 96-well plates

Three different experiments were performed in 96-well Microtiter plates, namely *Michaelis-Menten kinetics*, *determination of* K_i and *evaluation of different thicknesses of the barrier layer*. Gelatin gel in layers, containing substrate layer and barrier layer, were cast in 96-well Microtiter plates (see Fig. 2). The enzymes examined in this part were trypsin and α-chymotrypsin and the inhibitors examined were leupeptin, antipain and chymostatin. The wells were first cast with a substrate layer which was allowed to solidify before adding the barrier layer with or without inhibitors.

Michaelis-Menten kinetics

Wells were filled in triplicate with 200 μl gel, i. e. thickness of 5.5 mm, containing BAPA (0-15 mM) for trypsin or BTpNA (0.1–3 mM) for α-chymotrypsin. Once the substrate layer had solidified, 100 μl plain gel solution was added for each substrate concentration, corresponding to a 3 mm thick diffusion/erosion layer. Finally, 50 μl 2 μM enzyme was pipetted onto the congealed sample wells of the plates with a ten-tipped multichannel pipette. 50 μl was sufficient enough to cover the whole area in the well. Immediately after enzyme application, the absorbance of the wells was monitored in a SpectraMax Plus 384 Microplate Reader at 410 nm (for trypsin) and 384 nm (for α-chymotrypsin) every 5 min for 3 h. The kinetic parameters V_{max} and K_m were extracted after fitting the Michaelis-Menten equation (equation (2)) by non-linear regression to the initial velocity data using GraphPad Prism 7. For some points, the error bars would be shorter than the height of the symbol. In these cases, GraphPad Prism 7 simply does not draw the error bars.

Inhibition experiment

Determination of K_i

The substrate gel preparation, enzyme application and measurement of increased absorbance was done as mentioned in the *Michaelis-Menten experiment but with* a fixed concentration of enzyme ([Trypsin] = 5.0 μM, [α-chymotrypsin] = 2.0 μM) and substrate ([BAPA] = 2.5 mM for trypsin, [BTpNA] = 0.65 mM for α-chymotrypsin). Once the substrate layers had solidified, 100 μl gel solutions containing inhibitor were added creating the barrier layer. The inhibitor concentration varied from 0 to 25 μM for leupeptin and antipain for the experiment with trypsin and the concentration of leupeptin, antipain and chymostatin varied from 0 to 25 μM for the experiment with α-chymotrypsin. The thickness of the barrier layer containing inhibitor was 3 mm for all the experiments. The kinetic parameter K_i was extracted from GraphPad Prism 7 using the Morrison equation (equations (3) and (4)). For some points, the error bars would be shorter than the height of the symbol. In these cases, GraphPad Prism 7 simply does not draw the error bars.

Evaluation of different thickness of the barrier layer

To test the influence of the barrier layer thickness on the time course of the reaction, three thicknesses (1.5, 3 and 4.5 mm) were investigated in the absence and presence of leupeptin. The substrate layer was set up as mentioned in the *Michaelis-Menten experiment* (but with a final volume of 100 μl due to limitation of the well) for the experiment with different thicknesses of the barrier layer without inhibitor. When adding leupeptin to the barrier layer, a fixed concentration of [BAPA] = 2.5 mM was used. The leupeptin concentration ranged from 1 to 100 μM. Finally, 50 μl 2 μM enzyme was pipetted onto the congealed sample wells of the plates with a ten-tipped multichannel pipette. Immediately after enzyme application, the measurement of increased absorbance was set up as mentioned in the *Michaelis-Menten experiment*. The kinetic parameters V_{max} and K_m were extracted after fitting the Michaelis-Menten equation (equation (2)) by non-linear