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Structural analysis of peptide fragments following the hydrolysis of bovine serum albumin by trypsin and chymotrypsin

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Peptide bond hydrolysis of bovine serum albumin (BSA) by chymotrypsin and trypsin was investigated by employing time-resolved fluorescence spectroscopy. As a fluorescent cross-linking reagent, N-(1-pyrenyl) maleimide (PM) was attached to BSA, through all free amine groups of arginine, lysine, and/or single free thiol (Cys34). Time-resolved fluorescence spectroscopy was used to monitor fluorescence decays analyzed by exponential series method to obtain the changes in lifetime distributions. After the exposure of synthesized protein substrate PM-BSA to chymotrypsin and trypsin, it is observed that each protease produced a distinct change in the lifetime distribution profile, which was attributed to distinct chemical environments created by short peptide fragments in each hydrolysate. The persistence of excimer emission at longer lifetime regions for chymotrypsin, as opposed to trypsin, suggested the presence of small-scale hydrophobic clusters that might prevent some excimers from being completely quenched. It is most likely that the formation of these clusters is due to hydrophobic end groups of peptide fragments in chymotrypsin hydrolysate. A similar hydrophobic shield was not suggested for trypsin hydrolysis, as the end groups of peptide fragments would be either arginine or lysine. Overall, in case the target protein's 3D structure is known, the structural analysis of possible excimer formation presented here can be used as a tool to explain the differences in activity between two proteases, i.e. the peak's intensity and location in the profile. Furthermore, this structural evaluation might be helpful in obtaining the optimum experimental conditions in order to generate the highest amount of PM-BSA complexes.

Keywords: excimer lifetime distribution; N-(1-pyrenyl)maleimide; bovine serum albumin; chymotrypsin; trypsin; hydrolysis

Introduction

Spectroscopic techniques have been widely used for studying the proteolytic activities of various enzymes where fluorometric methods were performed by observing the changes in the emission spectra produced directly from the substrates of enzymes or from the fluorescent derivatives of the substrates. (Hermanson, 2008; Lakowicz, 2006; Reymond, 2006; Stubbs & Williams, 2002). Proteolytic enzymes play fundamental roles in metabolic activities of the living organisms by controlling cell cycle, gene expression, enzyme modification, and immune reactions (Rawlings & Salvesen, 2013). Owing to their specificity, they are used as biomarkers in many disease diagnoses. Therefore, monitoring and characterizing the activities of proteolytic enzymes in medicinal, food, and dairy applications are crucial and deserve detailed investigations.

In this work, the effect of proteolytic activities of chymotrypsin and trypsin enzymes on the lifetime distributions of excimer emission originating from the substrate bovine serum albumin (BSA) modified with N-(1-pyrenyl) maleimide (PM) was studied by employing the time-resolved fluorescence spectroscopic technique. Labeling of BSA with PM was achieved by targeting the single free thiol (–SH) in Cys34 (Betcher-Lange & Lehrer, 1978) and/or all free amine (–NH2) groups in the side chains of Arg and Lys residues (Figure 1). The fluorescence decay data of the excimers were analyzed with exponential series method (ESM) to produce lifetime distributions. Ware et al. (James & Ware, 1986; Siemiarczuk, Wagner, & Ware, 1990; Siemiarczuk & Ware, 1987; Wagner & Ware, 1990) have used EMS to obtain fluorescence lifetime distributions from simulated and experimental decay data. It was also shown that ESM was effectively used to determine the dimensionality of restricted geometries in blend like polymeric materials (Pekcan, 1992, 1993a).

A detailed structural analysis was conducted using the three-dimensional crystal structure of the target protein BSA and used to explain the distinct character of

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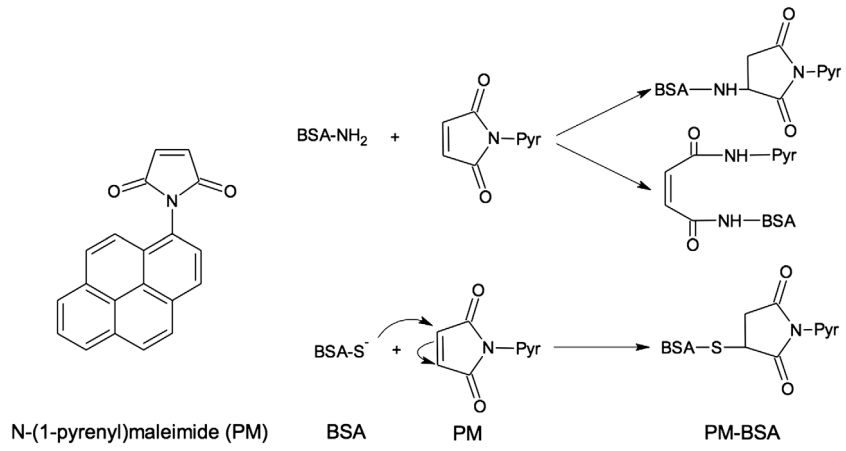

Figure 1. Labeling of bovine serum albumin (BSA) with N-(1-pyrenyl) maleimide by targeting free thiol and free amine groups to produce excimer emission (Wong, 1993).

each protease activity. By simply considering all Cys, Arg, and Lys residues as potential sites for a pyrene molecule, the highest amount of excimers that can be formed by two pyrenes in one BSA molecule was determined. This kind of structural information would be critical when obtaining the optimum experimental conditions to achieve the highest amount of possible excimers. Also, this analysis provided the expected amount of peptide fragments and some unquenched, stable excimers after hydrolysis based on the physicochemical properties of the peptides' end groups (the target site of hydrolysis), which explained the distinctiveness of lifetime distributions for each protease.

Materials and methods

Excimers and ESM

Excimers are short-lived excited state dimers formed by two species, which exist only during the excitation time. Excimer emission spectra appear as a new wide peak at longer wavelengths relative to the monomer peak. Aromatic hydrocarbons such as pyrene, naphthalene, and anthracene can form excimers. Excimer emission generally occurs by either the encounter of the ground-state monomer with an excited neutral monomer, or by the encounter of a monomer radical cation with a monomer radical anion. Monomer and excimer emissions in the solution depend on collisional rate and distance between molecules (Birks, 1975; Panda & Bhattacharyya, 1992). By definition, the excimer is stable only in the excited state and can be observed in the emission but not in the absorption spectrum. The usual model for excimer structure is that of a parallel sandwich model with an interplanar distance of about 0.3–0.5 nm (Bains, Kim, Sorin, & Narayanaswami, 2012; Niwayama, Kassar, Zhao, Sutton, & Altenberg, 2011). Fluorescence decay of excimer can be described as a sum of multi-exponential decays, which is composed of limited number of discrete exponential terms, each of which contains a specific fluorescence lifetime (τ) and a pre-exponential factor as given below (James, Siemiarczuk, & Ware, 1992; James & Ware, 1986; Lakowicz, 2006; Panda & Bhattacharyya, 1992; Pekcan, 1996; Sharma & Schulman, 1999; Siemiarczuk et al., 1990; Stubbs & Williams, 2002; Tkachenko, 2006).

$$F(t) = \sum_{i=1}^{N} a_i \exp(-t/\tau_i) \quad (1)$$

where $F(t)$ is the fluorescence decay intensity, τ_i is the fluorescence lifetime, and a_i is the pre-exponential factor with the normalization $\sum_i a_i = 1, 0$. Each of the terms may correspond to the decays of the local emissions arising from some discrete environments in the sample molecule of interest, which may have different polarities and different accessibilities for solvent molecules. It may be possible to produce the fluorescence lifetimes using distributional approach model, which describes the fluorescence decay as continuous distributions of fluorophore states (Albani, 2004; Alcala, Gratton, & Prendergast, 1987; James & Ware, 1986; James et al., 1992; Lakowicz, 2006; Pekcan, 1992, 1993b, 1996; Sharma & Schulman, 1999; Siemiarczuk & Ware, 1987; Siemiarczuk et al., 1990; Stubbs & Williams, 2002; Valeur, 2001).

In time-dependent fluorescence spectroscopy, because the excitation lamp pulse has finite width, almost in all the time-resolved fluorescence instruments, the experimental fluorescence decay intensity is distorted by the pulse whose decay function is called as "Instrument (or Impulse, Pulse, Lamp) Response Function (IRF), $L(t)$". Therefore, the observed decay is convoluted and its function is known as convolution integral $I(t)$, given in

(Equation (2)) (Albani, 2004; Birks, 1975; James & Ware, 1986; James et al., 1992; Lakowicz, 2006; Panda & Bhattacharyya, 1992; Pekcan, 1996; Sharma & Schulman, 1999; Tkachenko, 2006; Valeur, 2001).

$$I(t) = \int_0^t L(t)F(t-s)\mathrm{d}s \qquad (2)$$

where $I(t)$ is the observed decay of fluorescence intensity, and $L(t)$ is the instrument response function. In exponential series method, a series of exponentials (up to 200 terms) is used as a probe function with fixed, logarithmically spaced lifetimes (τ_i) and variable pre-exponentials (a_i) to analyze the smooth distributions of τ_i and a_i. This is achieved by allowing only a_i amplitudes to vary and by recovering amplitudes a_i with the iterative reconvolution method. The changes of lifetime distributions of protein substrates by the protease activity may give valuable information about the proteolytic characters of the proteases and the process of proteolysis.

Time-resolved fluorescence technique

Time-resolved fluorescence measurements were carried out with "PTI Time Master C-71" spectrofluorometer, including a stroboscobic detector and a nanoflash nitrogen arc lamp. In the stroboscobic pulse sampling technique (Albani, 2004; Lakowicz, 2006; Sharma & Schulman, 1999; Tkachenko, 2006; Valeur, 2001), the sample was excited with a pulsed light source, where the photo-multiplier tube (PMT) is gated or strobed by a voltage pulse that is synchronized with the pulsed light source. The intensity of fluorescence emission was measured in a very narrow time window on each pulse and saved in a computer. The time window was moved after each pulse. The strobe had the effect of turning on the PMT and measuring the emission intensity over a very short time window. When the data had been sampled over the appropriate range of time, a decay curve of fluorescence intensity vs. time can be constructed. Since the strobe technique was intensity dependent, the strobe instrument was much faster than single photon counting (SPC) and even faster than a phase measuring instrument. The strobe instrument was much simpler to use than SPC, and the data were more easily interpreted than in the phase system. Here, the fluorescence decay data were analyzed with the exponential series method embedded in the software PTI FeliX32.

Synthesis of PM-BSA

250 µl of (20 mg BSA/ml D.Water, MWBSA: 66,000; Sigma) stock solution was mixed with 222 µl of (4 mg PM/ml DMSO, MWPM: 297,32; Sigma) stock solution in an eppendorf tube so that the molecular ratio for the conjugation reaction was 100:1(PM/BSA). Total volume was completed with phosphate-buffered solution (pH 9) to 1 ml, and the reaction mixture was incubated in dark at 30 °C for an overnight.

Purification

Gel filtration column filled with Sephadex G-75 from Sigma (the sizes of filling; 1.5 × 20 cm) was used for purification of PM-BSA. Bidistilled water was applied to the column as mobile phase, and the fractions were collected in 2-ml tubes. The fractions including PM-BSA were determined with "Perkin Elmer LAMBDA 25 UV/Vis" spectrophotometer. The selected ones according to the absorption maximum at 344 nm were pooled in "Vivaspin 6 Centrifugal Concentrator (Sartorius Stedim Biotech GmbH, Vivaproducts)," and the pooled samples were condensed by ultrafiltration with SIGMA 3K30 Centrifuge at 10,000 rpm for 2 h. Two samples of 3 ml were prepared from the condensate by dilution with Tris-HCl (pH 7.8) for chymotrypsin and trypsin. Molecular PM/BSA ratio was calculated as ~9 according to the method in reference 18 using molar absorptivities εPM (344 nm) = 16,125 and εBSA(280 nm) = 37,246 and correction factor = 0.9.

For complete hydrolysis, molecular "Protease/PM-BSA" ratio was chosen as 1:1 and the reaction mixture was incubated for an overnight in dark at 30 °C. The hydrolysis was controlled with the gel permeation chromatography (GPC) instrument "Viscotek GPC max VE 2001" using the column "Shimadzu Shim-Pack 300 diol." The retention volumes shifted from about 11 ml to about 21 ml that indicates the completion of the hydrolysis.

Results and discussion

Structural analysis of BSA for possible excimers

In the protein solution under study, BSA exists as a single subunit, which consists of a polypeptide chain of 583 amino acid residues. The three-dimensional structure of BSA previously determined by X-ray crystallography with a resolution of 2.47 Å (Bujacz, 2012) was extracted from Protein Data Bank (PDB id: 4F5S) (Figure 2(a)). The fluorescent probe, PM, has been attached to BSA through the single free thiol (Cys34) and/or all the primary amines of Arg and Lys side chains (Figure 2(b)).

On one BSA molecule, there exist 23 Arg, 59 Lys and one free Cys (with no disulfide bridge), of which 83% were considered as highly exposed to the solvent, as their solvent accessible surface area (SASA) was greater than 35 $Å^2$. All SASA values reported here were calculated with the SASA module of VMD program

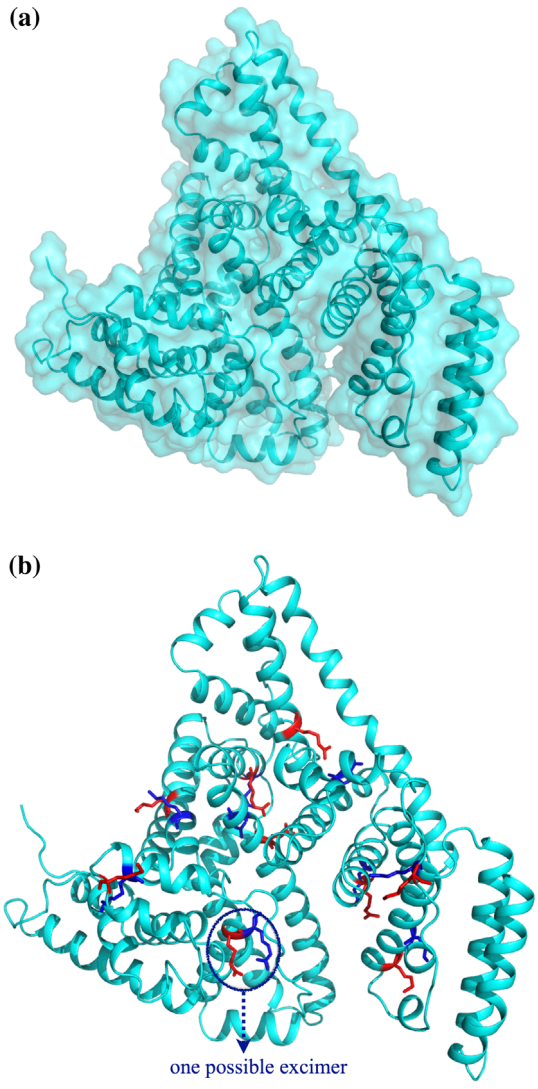

Figure 2. (a) The three-dimensional structure of bovine serum albumin with one subunit extracted from Protein Data Bank (PDB id: 4F5S) in surface representation, (b) 9 possible excimer locations observed in BSA, with two anchor residues represented with red and blue sticks. All molecular graphics were prepared using the PyMOL Molecular Graphics System (Schrödinger) in this article.

(Humphrey, Dalke, & Schulten, 1996) using a probe (solvent) radius of 1.4 Å. Based on the *xyz* coordinates of Cys34, Arg and Lys, the so-called anchor residues that can be labelled with probe molecules, a total of 9 potential excimers have been proposed to exist whether all possible sites were completely labelled with PM. This value represents the maximum amount of excimers that can exist in one BSA molecule, if all the sites were accessible for PM labeling. On the other hand, our experimental measurements indicated a molar ratio of PM/BSA of 9, which indicates a maximum of 4 excimers in one BSA molecule, considering the fact that one excimer consists of two PM groups.

The following distance evaluation was conducted in order to determine all possible excimers; first, we consider all 83 (= 23 Arg + 59 Lys + 1 Cys) residues that possess either amine or free thiol group, which yield 3403 (= 83 × 82/2) possible PM pairs that can be formed. However, for a significant fraction of these pairs, it is not possible to form an excimer due to their relative positions in the enzyme. For each pair, the pairwise distance between the side chain atoms was calculated. Consider the Arg-Lys pair illustrated in Figure 3. With 5 heavy atoms in Lys, and 7 heavy atoms in Arg, (only the heavy atoms which are carbon, nitrogen, and oxygen, were counted), there exist 35 (= 5 × 7) possible pairwise distances to be evaluated. The Arg-Lys pair was then considered as a potential site for an excimer, if it holds at least 4 pairwise distances that are less than 5 Å, which is usually selected as the most favorable distance for observing an excimer in some experimental studies (Bains et al., 2012; Niwayama et al., 2011). After treating all 3403 residue pairs which accounts to 103,579 distance evaluations, only 9 residue (or PM) pairs were found to be in a favorable orientation for generating a potential excimer. Afterward, each excimer has been categorized as *exposed*, *partially buried,* and *buried* based on the SASA values of its two constituent residues to which PM groups were attached. Table 1 lists all 9 excimers, the corresponding constituent residues with their SASA values. Interestingly, none of the 9 excimers had Cys34 as partner (or anchor) residue.

Based on the experimental molar ratio of PM/BSA which was determined as ~9, only 9 of 18 residues listed in Table 1 can be potentially labeled with PM molecules, which can only generate 4 excimers per BSA at maximum. However, in a protein solution, the labelling will not necessarily involve the same 9 residues for every

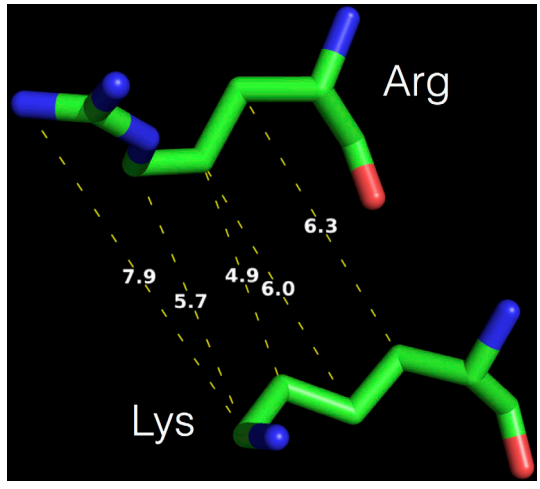

Figure 3. Distance calculations between side chain atoms in an Arg-Lys residue pair, given in Å.

Table 1. List of 9 possible excimers with their corresponding member residue names and ids as found in 4F5S.pdb (Bujacz, 2012). SASA value of each residue in the pair was also listed.

Excimer ID	Residue #1	Residue #2	Number of pairwise distances	SASA for residue #1 (Å^2)	SASA for residue #2 (Å^2)	SASA degree (E, PB, B)
1[a]	LYS239	LYS242	7	76.57	85.25	E[b]
2	ARG194	ARG198	5	95.02	41.56	E[b]
3[a]	LYS93	ARG98	4	97.79	25.97	PB[c]
4	LYS499	LYS533	7	84.32	18.26	PB[c]
5[a]	ARG143	ARG144	10	20.51	52.77	PB[c]
6	ARG217	LYS221	9	95.55	14.85	PB[c]
7	ARG427	LYS431	4	29.71	77.79	PB[c]
8[a]	LYS520	LYS524	6	32.43	57.30	PB[c]
9	ARG347	ARG484	7	34.04	33.00	B[d]

[a]The excimers that would be on the same peptide segment after chymotrypsin hydrolysis.
[b]excimer is exposed: both partner residues have their SASA values greater than 35 Å^2.
[c]excimer is partially buried: one of the partner residues has their SASA value greater than 35 Å^2.
[d]excimer is buried: both partner residues have their SASA values less than 35 Å^2.

BSA molecule, but will be selected from the list of potential residues provided in Table 1. Based on the SASA values, it is highly probable to label the exposed residues more often than the buried ones. Two excimers were categorized as exposed since both residues pairs (Lys239-Lys242 and Arg194-Arg198) have their SASA values greater than 35 Å^2. Furthermore, 1 excimer was completely found buried (Arg347-Arg484), while the remaining 6 excimers were found to be partially buried.

Hydrolysis with trypsin will naturally generate peptides with either Arg, Lys, or Cys34 as the end group, since these residues are also the sites for peptide bond

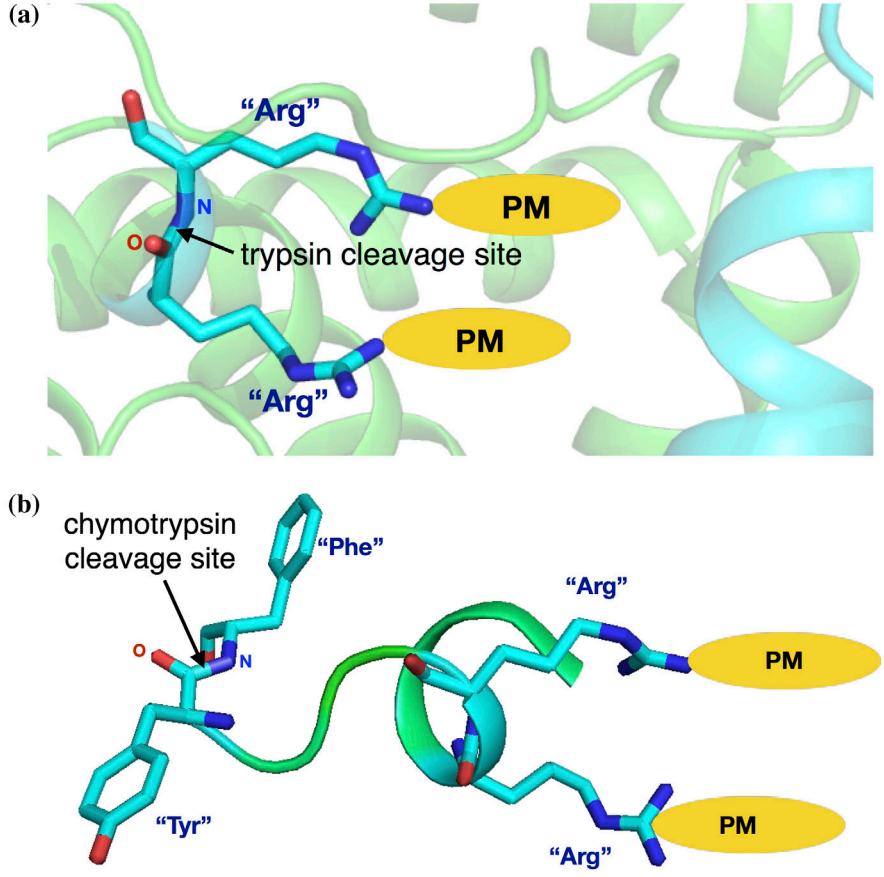

Figure 4. (a) Trypsin cleavage site where each anchor residue (both Arg) holds a PM group. (b) Chymotrypsin cleavage site at the carboxyl site of tyrosine (Tyr) next to phenylalanine (Phe).

cleavage (Figure 4(a)). Thus, any excimer's coherent structure is expected to be disrupted upon hydrolysis with trypsin. On the other hand, hydrolysis with chymotrypsin is expected to create short peptides which ends with either one of the five hydrophobic residues (leucine (Leu), tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), or methionine (Met)) since all five are potential sites for peptide bond cleavage (Figure 4(b)). As a result, some excimers will have its PM units on the same peptide, while others will have its PM on two separate peptides. Moreover, the hydrophobic ends will tend to cluster and form small-scale dense, hydrophobic clusters. Those excimers with PM groups on the same peptide will potentially be a part of those clusters, where they will be shielded from the solvent environment, similar to an inside region of the protein before the hydrolysis. Thus, it becomes crucial to determine the total number of peptide segments with hydrophobic end residues and both PM units of an excimer, in order to explain the persistence of the peak at longer lifetime regions as a result of some unquenched excimers.

Excimer decay analysis

PM-BSA complex presents two peaks in the emission spectra located at 384 nm (monomer) and 462 nm (excimer). It was observed that the excimer emission was dramatically diminished as the monomer emission intensified as a result of proteolytic activities of chymotrypsin and trypsin enzymes on PM-BSA. Fluorescence decay measurements were carried out at emission wavelength of 462 nm, before and after exposure of PM-BSA to chymotrypsin and trypsin. Before the interaction of PM-BSA complex with enzymes, the excimer decay curve was presented as in Figure 5. The produced data from excimer fluorescence decay measurements were used for ESM analysis by minimizing the chi-square function to 1. Here, excitation

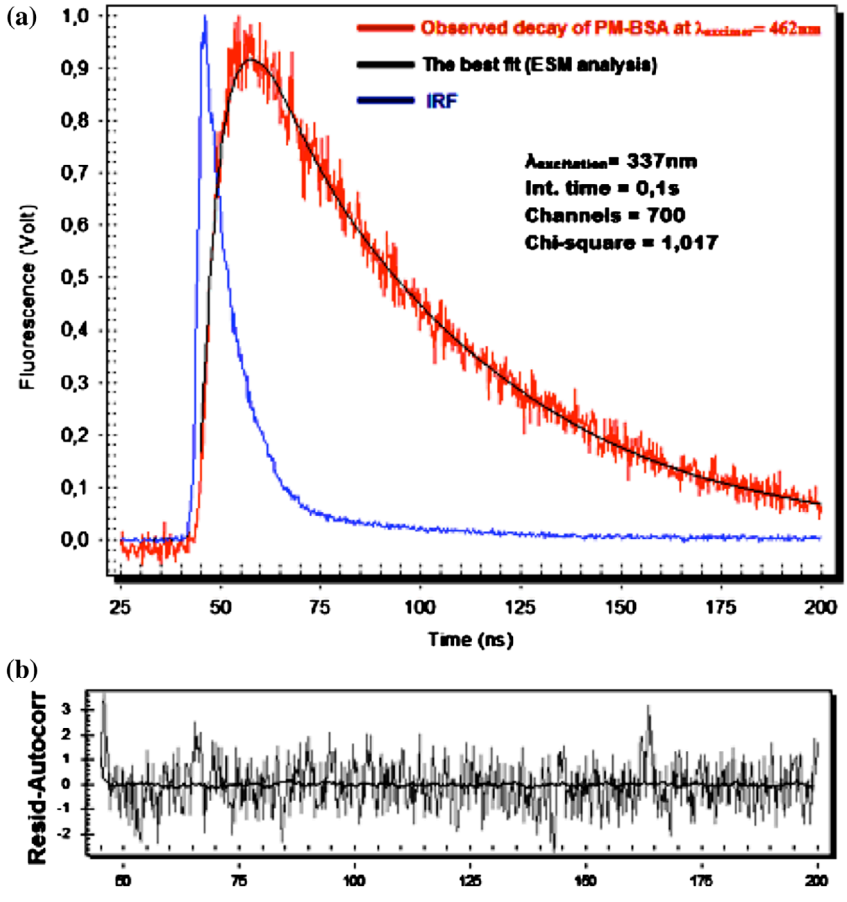

Figure 5. (a) Fluorescence decay curve (red line) of 0.8×10^{-5} M PM-BSA in Tris-HCl, for pH 7.8 at $T = 30$ °C, where excitation and emission wavelengths are 337 and 462 nm, respectively. Decay time was monitored over 700 Channels with integration time of 0.1 s. The solid line represents the best fit of the data. The lower curve is the instrument (or impulse, lamp) Response Function (IRF). The uniqueness of the fit of the data to the model is determined by chi-square, χ^2 ($\chi^2 = 1.017$), (b) the distribution of the weighted residuals and/or the autocorrelation of the residuals.

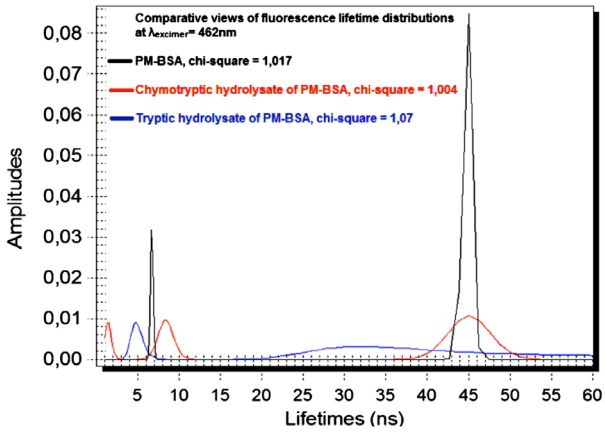

Figure 6. Comparison of fluorescence lifetime distributions, obtained from ESM analysis at $\lambda_{excitation} = 337$ nm and $\lambda_{emission} = 462$ nm wavelengths, for PM-BSA (black line) before and after hydrolysis with trypsin (blue line) and chymotrypsin (red line). Chi-square, χ^2 values for the best fits are presented inset of the figure.

wavelength was chosen as 337 nm. Seven hundred channels were used for each experiment (Özyiğit, Karakuş & Pekcan, in press).

Lifetime distributions obtained from ESM analysis before and after treatments by proteases were illustrated all together in Figure 6 for comparison. The short lifetimes correspond to the mobile region in the receptor where excimers are totally and/or partially exposed to the liquid and collisional quenching can take place. On the other hand, lifetimes at longer time region at around 45 ns belong to those excimers that are buried inside the PM-BSA substrate, so that they avoid from being quenched by the liquid.

In Figure 6, it is observed that the sharp peak at longer lifetimes around 45 ns widened into an area between 35 and 55 ns with a low intensity of 0.01 for chymotrypsin hydrolysates. On the other hand, after treatment with trypsin, the peak at 45 ns presented relatively very low intensity and a widened distribution. The sharp peak at shorter lifetimes around 7 ns widened for both hydrolysis and the distribution shifted to shorter lifetimes around 5 ns for trypsin and slightly longer lifetimes around 9 ns for chymotrypsin.

A hydrophobic environment in chymotrypsin hydrolysate

The areas under the distribution curves at shorter and longer lifetime regions can be associated with the amount of excimers that might exist on the surface and the interior regions of BSA, respectively. Prior to hydrolysis, the areas under the two sharp peaks at 7 and 45 ns were determined as 1.25 and 13.7, respectively (Figure 5(a)). This gives an intensity ratio of approximately 11 (= 13.7/1.25), which indicates that there exist 11 buried excimers as opposed to one exposed excimer in the solution. Based on these experimental findings, we can speculate that only a few BSA molecules would hold the exposed excimer, which is either located on Lys239-Lys242 pair or Arg194-Arg198 pair (Table 1). Averaging over 7.6×10^8 moles of BSA molecules in the solution (4.6×10^{16} protein molecules), the number of buried (or partially buried) excimers was expected to be 11 times more than the exposed ones.

Furthermore, the list of excimers in Table 1 was determined for one single conformation of the protein, which is the snapshot obtained from X-ray crystallography. However, the experiments were conducted for a solution composed of many BSA molecules (4.6×10^{16} protein molecules), which represent different conformations of the same protein. As a result, a partially buried excimer in one conformer may be more exposed in another conformer of the same protein.

The difference between the lifetime distributions of two hydrolysates can be further explained by investigating the possible local arrangements of excimers following the hydrolysis. It is well known that the serine protease trypsin cleaves the peptide chain at the carboxyl side of Arg or Lys. Consequently, this would yield excimers where the peptide bond in between the anchor residues (Arg or Lys) would be broken (Figure 4(a)). This rupture would potentially destabilize several such excimers (buried or exposed) in the hydrolyzed protein. As a result, a significant decrease was expected in the amount of excimers after trypsin hydrolysis, which was well reflected by the obvious disappearance of the two sharp peaks at 7 and 45 ns. Even the excimers with short lifetimes shifted to shorter lifetime regions with smaller intensities. Overall, the lifetime distribution after trypsin hydrolysis shows no sign of stable excimers or any kind of structural rearrangements that might shield a few remaining excimers which became even more solvent exposed after hydrolysis.

On the other hand, the second serine protease chymotrypsin cleaves the peptide chain at the carboxyl side of hydrophobic amino acids such as tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe), leucine (Leu), and methionine (Met). Consequently, all the peptide fragments in the hydrolysate will terminate with an hydrophobic residue (Figure 4(b)). However, different from trypsin hydrolysis, two kinds of peptide fragments will appear, one that hold both anchor residues (PM units) of the excimer, and one that hold only one. The peptides that hold both PM units of the excimer will produce a different environment in the solution. Shortly after hydrolysis, some of the hydrophobic ends will tend to cluster and create a hydrophobic environment. The excimers located on these peptides will naturally become

an integral part of this association and will be shielded from outside in a well protected and buried environment, comparable to an interior region of a protein before hydrolysis. Our structural analysis revealed that there would be 4 such excimers (with ids 1, 3, 5, 8 in Table 1), which can be found on the same peptide and thus have a good chance of remaining buried even after hydrolysis. The distribution profile persistently observed at longer lifetimes of 45 ns even after hydrolysis also supports the existence of these unquenched excimers in the solution (Figure 6). Furthermore, the hydrophobic cluster formation might also favor the association of two PM groups found on two different peptide fragments to form a novel excimer.

In Figure 6, the sharp peak at short lifetimes of around 7 ns became wider and shifted to slightly longer lifetime regions of 9 ns after chymotrypsin hydrolysis. This is in the opposite direction to the one observed in trypsin hydrolysis. Most likely, some of the solvent exposed excimers observed prior to the hydrolysis became slightly less exposed due to the presence of a hydrophobic environment nearby. Furthermore, favorable alignments observed in some of the anchor residues (with 9 interacting distances less than 5 Å) would delay the disruption of the corresponding excimer, as the cleavage site would be at a distant hydrophobic residue. Also, following the chymotrypsin hydrolysis, the ratio of the area at longer lifetimes to the area at shorter lifetimes was experimentally determined as 3.00 (= 6.7/2.23), which indicates a considerable amount of buried excimers that still exist in the solution, most likely in suggested hydrophobic cluster formations.

Conclusion

Following the exposure of the synthesized substrate PM-BSA to the effects of two proteases, trypsin and chymotrypsin, the fluorescence lifetime distributions of the excimer emission have undergone noticeable changes depending on the distinct proteolytic characters of the enzymes. The trypsin hydrolysis caused almost all excimers to become unstable as a result of trypsin cleavage targeting the carboxyl site of both anchor residues. This inevitably disrupted almost all excimer formations that were either buried or exposed before hydrolysis and finally created a solution of short peptides, which all end with either Arg or Lys residues, some attached to one PM group. This kind of structural change further decreased the probability of two PM groups on two different peptides reassembling to form an excimer after hydrolysis, which is well reflected by experimentally measured excimer's lifetime distributions.

On the other hand, the chymotrypsin hydrolysis produced an environment, which was very distinct from trypsin hydrolysis. Since the chymotrypsin cleavage site was on the carboxyl site of a hydrophobic residue, the Arg and Lys residues holding the PM groups were not disrupted. As a result, two kinds of peptide segments were suggested to emerge, one with both PM groups and one with one PM group only. The excimers attached to two PM groups located on the same peptide were expected to survive longer than the ones attached to PM groups located on different peptides. From the structural analysis, it was found that 4 of 9 excimers might exist on the same peptide. Furthermore, it is highly probable that the hydrophobic ends of the peptides would tend to cluster and form small-scale hydrophobic entities, which would further increase the lifetime of excimers that would be shielded in this new structural association. Finally, it is also probable that, the dense, hydrophobic cluster formation might facilitate the two PM groups on different segments to approach to each other to create a novel excimer even after hydrolysis. All this is well reflected by the persistent peak at long lifetime region of 45 ns, and the shift of the peak at the short lifetime region of 7 ns to slightly higher lifetime region of 9 ns.

Moreover, the structural analysis presented in this work provided the highest amount of possible excimers as 9 (corresponding to 18 PM groups), yet the experimental molar ratio of 9 PM/BSA suggested only 4 such excimers to exist (2 PM groups per excimer) in BSA at maximum. It can be suggested that the experimental conditions, such as the pH, temperature, or overnight incubation can be adjusted in order to achieve the maximum amount of labeling which would be 18 residue sites for one BSA molecule.

Overall, in addition to explaining the distinct character of a protease activity, which would be the "fingerprint" of that protease, the structural analysis presented in this work can also be used as a way of tuning the experimental conditions to obtain the highest possible amount of excimer emission.

Disclosure statement

No potential conflict of interest was reported by the authors.

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