I&EC research
Industrial & Engineering Chemistry Research

Article

Cite This: Ind. Eng. Chem. Res. 2019, 58, 10692−10697

pubs.acs.org/IECR

Profiling Protease Activity in Laundry Detergents with Peptide Arrays and SAMDI Mass Spectrometry

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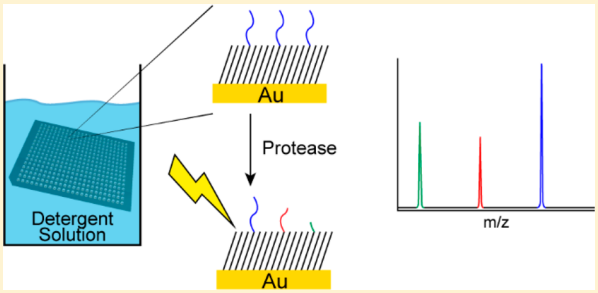
Supporting Information

ABSTRACT: Laundry detergent formulations include proteases to digest and clean protein-based stains. Methods that can profile protease activity and specificity in detergents could be important in guiding the development of engineered proteases for these applications. The work reported here uses peptide arrays with analysis by self-assembled monolayers for matrix-assisted laser desorption/ionization (SAMDI) mass spectrometry to analyze protease activity across a 324-peptide library for five commercial laundry detergents. The results showed differences in cleavage activity across different brands. The combination of peptide arrays and SAMDI mass spectrometry provides a rapid and reliable technique for analyzing protease activities in laundry detergents and has the potential to play an important role in the development of new proteases and detergent formulations.



1. INTRODUCTION

Proteases are important in a wide range of industrial applications—including food manufacturing, leather making, and personal care—and they account for 60% of the market for industrial enzymes.[1,2] The primary application is found in laundry detergents, where the enzymes remove proteinaceous stains and have been engineered to operate efficiently at low temperatures, thereby eliminating the need for energy-consuming high-temperature washes. The use of enzymes has also relieved the inclusion of phosphates, resulting in more environmentally benign detergents.[3] Continued development of proteases for laundry detergents will benefit from technologies that have been developed for protein engineering and characterization in the life sciences. In this paper, we demonstrate the use of peptide arrays to profile the activities and specificities of several commercial detergents, and we suggest this method will be important in comparing and optimizing proteases for detergents.

Proteases are used in laundry detergents to digest protein-based stains including those from blood, grass, sweat, milk, and food soils. Among the important characteristics for selecting proteases are thermal stability, broad alkaline pH stability, and compatibility with detergent ingredients and mild bleaching agents.[4] Serine endoproteases have proven the most effective, as other proteases are incompatible; thiol proteases are oxidized by bleaching agents; metalloproteases lose their metal cofactors due to the binding of water softening agents and hydroxide ions.[5] The subtilisin class of serine proteases from the *Bacillus* species is favored for its high stability and broad substrate specificity and has been used extensively in laundry detergents for decades.[6]

Because of their significance in industry, subtilisin proteases have been the focus of much study, with the goals of improving the production of subtilisin as well as characterizing the activities of different variants on adsorbed substrates.[7,8] Protein engineering efforts have focused on optimizing subtilisin proteases via directed evolution to enhance thermal stability as well as efficiency at low temperatures, alkaline stability, and compatibility with detergent components.[4,9,10] These efforts have revealed a plasticity of protease active sites that allow them to be engineered for a wide range of activities under varying conditions.[1] While assays are used to ensure that proteases remain active at these conditions, experiments are generally not performed to characterize the substrate specificities of the proteases in detergents.[11,12] In principle, it would be useful to engineer proteases that have high activity and little specificity to make them broadly effective at digesting and removing protein stains.

Combinatorial methods have proven important for assessing the specificities of engineered proteases and identifying optimal substrates.[13,14] In this paper, we describe the use of peptide arrays to profile protease activity and specificity in commercial laundry detergents. We separately apply detergent solutions to an array having 324 peptide sequences and allow proteases in the detergent to cleave the peptides. The peptide array is then analyzed with SAMDI (self-assembled monolayers for matrix-assisted laser desorption/ionization) mass spectrometry to

Received: January 4, 2019
Revised: May 22, 2019
Accepted: June 4, 2019
Published: June 4, 2019

DOI: 10.1021/acs.iecr.9b00057
Ind. Eng. Chem. Res. 2019, 58, 10692−10697

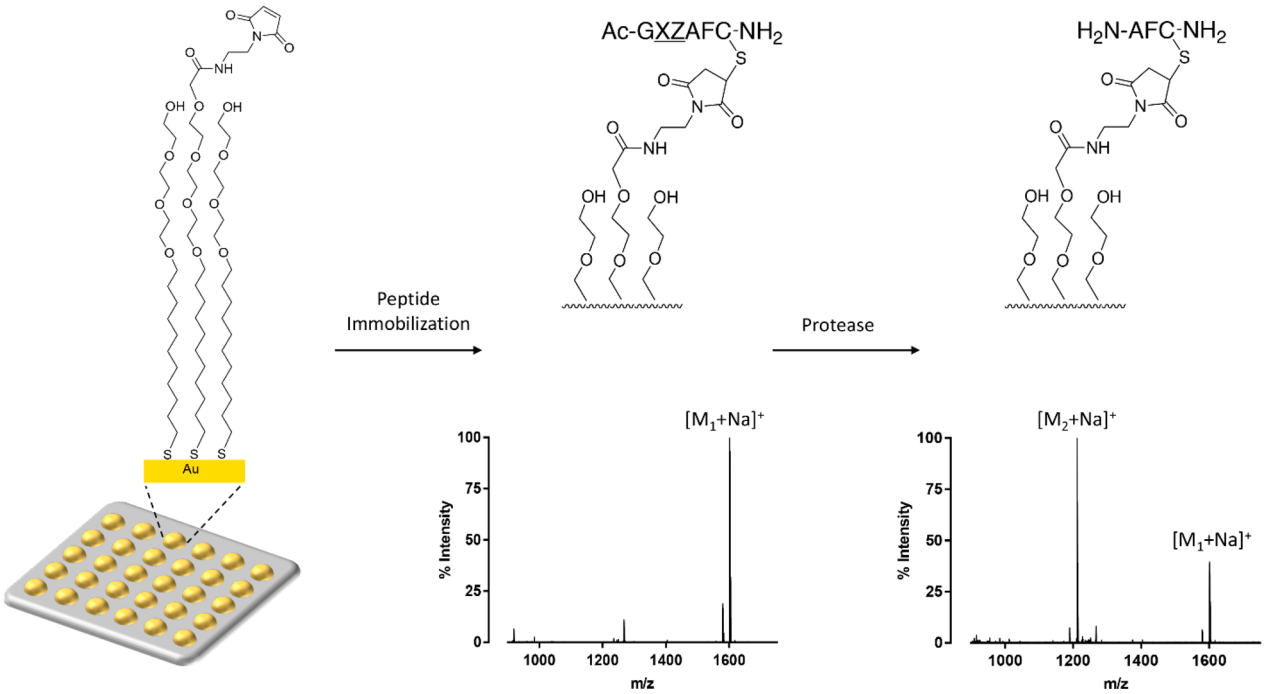


Figure 1. Maleimide-terminated self-assembled monolayers were used to covalently immobilize cysteine-terminated peptide substrates on the surface in an array format. The array was placed in a solution of laundry detergent (using the manufacturer's recommended dilution) and then analyzed by SAMDI MS to quantitate the proteolytic product. Mass spectra corresponding to the substrate and product peptides are shown in the lower left and right, respectively.

identify the cleavage sites in the peptides and to quantitatively measure the extent of cleavage.[15,16] This work provides activity profiles that allow a comparison of the detergents and suggest that this assay may be important in guiding the development of new formulations.

2. EXPERIMENTAL SECTION

2.1. Materials. Metal plates having an array of gold islands were prepared as previously described.[17] Fmoc Rink Amide MBHA resin (0.42 mmol/g) was purchased from AnaSpec Inc. Fmoc amino acids were purchased from AAPPTec and Chem-Impex International Inc. All other solvents and reagents for Fmoc solid-phase peptide synthesis were purchased from Thermo Fisher and Sigma-Aldrich. Arm & Hammer, Persil, Kirkland, Gain, and Tide laundry detergents were purchased from local grocery stores.

2.2. Preparation of Self-Assembled Monolayers on Gold. Steel plates were washed with hexanes, ethanol, water, and ethanol and dried with nitrogen gas. An electron beam evaporator was used to deposit the array of gold spots in a 384 format.[17] The array plates were then soaked in a solution containing a mixture of tri(ethylene glycol)-terminated (EG3) alkane disulfides and a mixed disulfide of EG3-alkanethiol and a maleimide-terminated EG3-alkanethiol for 24 h at room temperature to form self-assembled monolayers on the gold surface.[18] The solution of disulfides (1 mM total concentration) had the two disulfides present in an appropriate ratio to yield a final density of maleimide groups of 10%.

2.3. Synthesis and Immobilization of Peptide Library. A total of 324 peptides having the general sequence Ac-GXZAFC where X and Z represent all 18 natural amino acids except cysteine and methionine were synthesized by using standard Fmoc solid-phase peptide synthesis on Rink Amide resin (0.42 mmol/g) as previously described.[19] The peptides were dissolved in 1% dimethyl sulfoxide (>99.5% pure) in deionized water. The library was then transferred into 384 microtiter well plates and diluted to 100 μM in 200 mM Tris HCl buffer (pH 8.0) by using a Multidrop Combi benchtop robot. A 0.25 mM tris(2-carboxyethyl)phosphine hydrochloride reducing resin in Tris HCl buffer was added to reduce any disulfide bonds from the C-terminal cysteine thiol groups. The peptide library was transferred onto SAMDI array plates (2.5 μL per spot) using the TECAN Freedom EVO automated liquid handling robot with a 384-tip head, and the peptides were allowed to react with the maleimide-terminated monolayers in a humidified chamber for 20 min at 37 °C. The resulting array plate was then rinsed with ethanol, water, and ethanol and dried with nitrogen.

2.4. Application of Proteases and Profiling of Enzyme Activity. A Speed Queen top load washer (model number: AWNE82SP113TW01) was chosen as a representative washing machine, utilizing 19.5 gal (73.8 L) of water for a cold medium wash. Detergent solutions, in water, were prepared as directed by each detergent brand's recommendation for a medium-load cold wash cycle (equivalent of 45−60 mL of each detergent in 73.8 L). The immobilized peptide arrays were then placed in 100 mL of detergent solution in a reaction chamber; the reaction chamber was sealed and covered with aluminum foil and placed on a shaker for agitation for 20 min at room temperature (22−26 °C) to mimic the conditions of the cold wash settings. The array plates were then rinsed with ethanol, water, and ethanol, dried with nitrogen, and treated with 2′,4′,6′-trihydroxyacetophenone matrix solution (THAP, 12 mg/mL in acetone). Matrix-assisted laser desorption/ionization time-of-flight mass spectra were acquired on an AB Sciex TOF-TOF 5800 instrument (~1.5 s per spot) in reflector positive mode to quantitate the cleavage activity on individual peptide substrates

DOI: 10.1021/acs.iecr.9b00057
Ind. Eng. Chem. Res. 2019, 58, 10692−10697

directly on the surface. The data were analyzed by using Data Explorer software to retrieve the integrated areas under the peaks (AUP) of the substrate and product, as follows:

$$\text{activity} = \frac{\text{AUP}_{\text{product}}}{\text{AUP}_{\text{product}} + \text{AUP}_{\text{substrate}}}$$

3. RESULTS AND DISCUSSION

3.1. Preparation of Peptide Arrays. We individually synthesized 324 peptides based on the sequence Ac-GXZAFC, where the variable positions X and Z represent all 18 natural amino acids excluding cysteine and methionine. We also prepared an array of self-assembled monolayers of alkanethiolates on gold terminated in maleimide groups at a density of 10% against a background of tri(ethylene glycol) groups (Figure 1).[18] The maleimide functionality allows for the selective immobilization of cysteine-terminated peptides, while the glycol background prevents nonspecific adsorption of proteins to the surface.[18] The monolayers were prepared on a metal plate having an array of 384 gold islands in the standard format used in the life sciences, as previously described.[17] The use of self-assembled monolayers has the further benefits that the peptides are immobilized in a defined orientation and a uniform density across the array, giving a consistent activity and good reproducibility.[20−22] The 324 peptides were transferred onto the monolayers within the 384-array plates using an automated multichannel liquid transferring robot with a 384-tip head. SAMDI mass spectrometry was then used to confirm the immobilization and purity of each peptide (each of the 324 spectra is presented in Figure S1).[15]

3.2. Characterization of Protease Activity and Selectivity. To measure the proteolytic activity of a laundry detergent, we prepared a working concentration of the detergent by diluting it in water according to the manufacturer's instructions for a medium-load cold wash; note that we are labeling the detergents as A−E and not disclosing the specific detergent brand for each proteolytic profile. We separately immersed immobilized peptide array plates into solutions of detergent in sealed reaction chambers and placed the containers on a shaker to mimic the conditions of a cold laundry wash. After 20 min, we removed the plates from the solutions and rinsed them with water and ethanol, dried under nitrogen, and treated with THAP matrix in acetone. The plates were analyzed by SAMDI mass spectrometry, and protease activity was quantified by quantitating the areas under the peaks of the parent peptide and cleavage products. Each detergent condition was repeated in triplicate and individually analyzed. The standard deviation in these measurements was smaller than 6% for 88% of the peptides.

We first profiled detergent A and observed a wide range of cleavage activity that showed a strong dependence on the peptide sequence. For the peptide substrate GEAAFC, we observed three distinct product peaks resulting from proteolytic activity (Figure 2). First, the peaks at m/z 971.5 (H^+) and m/z 993.5 (Na^+) correspond to the fragment NH_2-Cys-maleimide and therefore represent cleavage of the Phe−Cys bond near the C-terminus of the peptide. Second, the peaks at m/z 1118.6 (H^+) and m/z 1140.6 (Na^+) correspond to the fragment NH_2-Phe-Cys-maleimide and represent cleavage of the Ala−Phe bond. Finally, the peaks at m/z 1189.7 (H^+) and m/z 1211.7 (Na^+) correspond to NH_2-Ala-

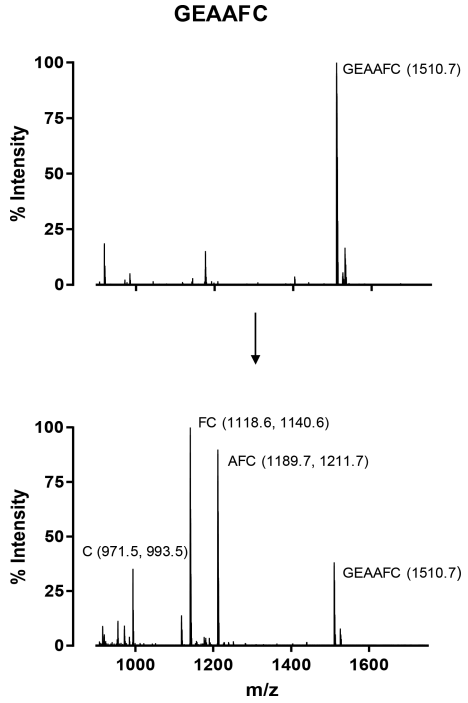


Figure 2. Mass spectra of a monolayer presenting the peptide GEAAFC before (top) and after (bottom) treatment with detergent A. Multiple cleavage sites reveal the enzyme activity on this sequence.

Phe-Cys-maleimide and represent cleavage between the variable position alanine and fixed position alanine in the sequence. Certain peptides in the array showed variations of the three cleavage sites, others were cleaved at only one or two of the positions, and still others were inactive toward any proteolytic activity. Because there existed a possibility for multiple cleavage sites, the total protease activity on each peptide was quantitated using the sum of the area under the peaks (AUP) of the products with the following equation:

$$\text{activity} = \frac{\sum_{i=1}^{n} \text{AUP}_{\text{product},i}}{\sum_{i=1}^{n} \text{AUP}_{\text{product},i} + \text{AUP}_{\text{substrate}}}$$

To compare the total proteolytic activity of detergent A on the peptide array, we generated a heat map that showed the extent of cleavage for each peptide, where each peptide is represented by one square and the degree of purple intensity corresponds to the yield for cleavage products (Figure 3A). The proteases in detergent A were observed to have higher activity toward substrates with hydrophobic amino acids in the variable positions. This trend correlated to previously known selectivities of subtilisin proteases, including a slight preference for hydrophobic residues in the −1 position.[6] Furthermore, the peptide arrays reveal a lower proteolytic activity for substrates having a glycine or histidine in either variable position and almost no activity with a proline in the Z positions, as well as significantly lower activity with asparagine, and arginine in the variable X position. Finally, a control experiment where the array was not treated with detergent showed essentially no proteolysis of the immobilized peptides (Figure S2).

We also analyzed the data to present a cleavage site-specific profile for detergent A (Figure 4A). This detergent's proteases displayed a high relative specificity for the bond between the variable position Z and the fixed position alanine in the GXZAFC array, shown through the orange bars that denote

DOI: 10.1021/acs.iecr.9b00057
Ind. Eng. Chem. Res. 2019, 58, 10692−10697

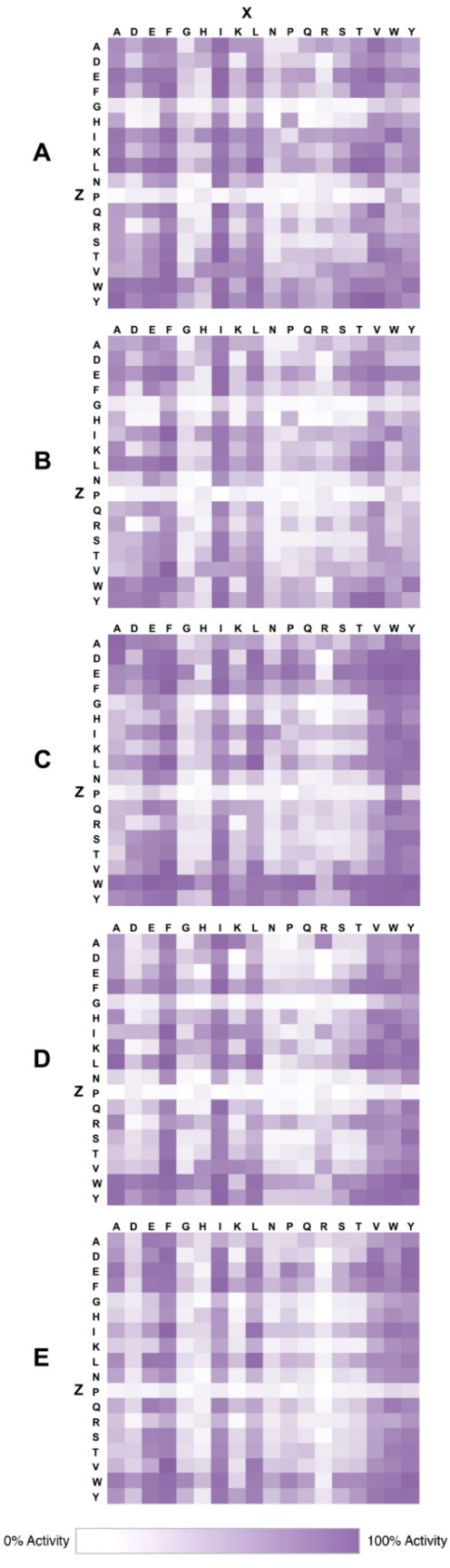


Figure 3. Heat maps represent total proteolytic activity of the peptide library GXZAFC in each of five anonymized detergents, A–E.

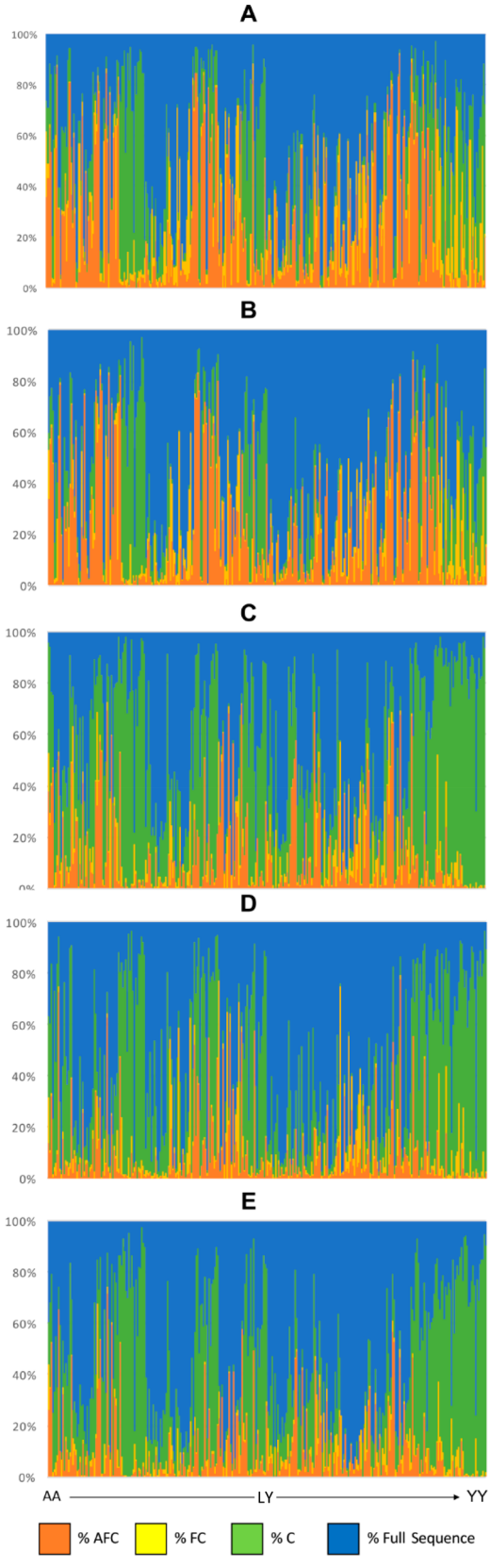


Figure 4. 100% stacked bar graphs display sequence-specific cleavage activity of each detergent protease across the 324-peptide library. The variable dipeptides in GXZAFC on the *X*-axis are arranged in alphabetical order, from AA to YY (left to right).

DOI: 10.1021/acs.iecr.9b00057
Ind. Eng. Chem. Res. 2019, 58, 10692–10697

NH_2-Ala-Phe-Cys as the final product. The second most abundant product was NH_2-Cys shown in green, followed by NH_2-Phe-Cys shown in yellow (the fraction of each cleavage product for each of the detergents is tabulated in Table S1A). These results, together with the heat map, show that both total cleavage activity and specific cleavage activity can be deduced by using SAMDI to analyze peptide arrays.

We also used the peptide arrays to profile the other four commercial laundry detergents (Figures 3B–E and 4B–E, Table S1B–E). We again prepared detergents at the suggested concentrations, treated separate peptide arrays with each solution, and analyzed the arrays with SAMDI to generate heat maps and site-specific profiles as described above to show cleavage activity and specificity for each. A sixth detergent that lacks enzymes was also tested, and no proteolytic activity was observed (data not shown). A brief comparison of the heat maps reveals that detergent C has the highest overall activity of the five detergents. Also, the heat maps showed similar overall activity and specificity for the five detergents, such as the preference for hydrophobic residues in the variable positions and the strong loss of activity for peptides having glycine and proline in positions X and Z, respectively. However, the five detergents can be divided into two groups, based on a slight variation in activity, with detergents A and B in the first group, and C, D, and E in the second. The two groups have a substantially similar specificity, though proteases in the second group show a strong preference for peptides having tryptophan and tyrosine in both variable positions, which is not seen with detergents A and B.

The site-specific cleavage activity depicted in Figure 4 (which quantitates the possible products from proteolysis of the peptide at different amide bonds) gives further information about the relatedness of the detergents. The first group (detergents A and B) has a strong preference for cleaving the amide that is C-terminal to the variable positions (Figure 4, orange bars). The three detergents in the second group are also quite similar (though, as explained further below, there are differences). The differences between the two groups, however, become significantly more apparent when site-specific activities are analyzed. Unlike the proteases in the first group, those in the second show a strong preference for cleavage at the Phe–Cys amide bond near the C-terminus of the peptide (green bars).

Interestingly, although the proteases in the second group (C, D, and E) have similar specificities, careful examination of the activity profiles reveals slight differences. For example, detergent C shows high activity on peptides with Asp and Glu in both variable positions and Pro in position X, while detergent D lacks a preference for those residues and even shows lower activity for Glu and Pro in both variable positions. Likewise, detergent E only shows an increased preference for Glu, but not for Asp, and, unlike C and D, disfavors Arg in the X position.

Finally, we quantitated the total proteolytic activity for each detergent by taking the average total cleavage of each peptide within the array. We observe similar average levels of activity: A, 52.9%; B, 43.2%; C, 56.6%; D, 45.9%; E: 46.3%. This result is not unexpected, as the formulations are developed to have a similar overall activity for removing stains.

Thus, in this study, we showed that the combination of peptide arrays and SAMDI mass spectrometry not only allowed us to profile overall proteolytic activity in commercial detergents but also allowed us to identify differences in the site-specific proteolytic activities of the different compositions. Furthermore, the use of self-assembled monolayers allowed for the selective capture of peptide substrates to the surface in array format while preventing nonspecific adsorption, and the use of MALDI mass spectrometry provided quantitative, label-free detection of proteolytic activity directly on the surface. At ~1.5 s per spot, MALDI mass spectrometry also allowed for fast readouts, with one 324-peptide array plate analysis taking 15 min. This, in addition to the compatibility of this method with standard microtiter plate formats and automated liquid handling robots, it allowed for thousands of individual reactions to be performed in a day.[17]

This study is the first to use peptide arrays to profile laundry detergents and to characterize the protease specificities across a large number of peptide substrates. This work allowed a direct comparison of proteases across five commercial detergent brands. We were able to identify detergents having higher protease activity, which may correspond to better protein stain-removing ability. We were also able to compare the specificities of the protease activities for their peptidic substrates, and this demonstration will be relevant to future work that optimizes protease variants for high activity and low specificity. Furthermore, this approach may be useful for developing detergents that are efficient at specific protein stains based on the sequences of those proteins. This demonstration of screening proteases in commercial formulations may, in addition to laundry detergents, be important for screening products in other areas as well as other enzyme activities. As efforts in protease engineering advance, SAMDI proves a promising method for screening and optimizing new variants for detergents and more.

4. CONCLUSIONS

Peptide arrays and SAMDI mass spectrometry offer a new method for profiling laundry detergent proteases directly in relevant conditions and may guide the development of new detergent formulations with improved abilities in removing protein-based stains.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.iecr.9b00057.

Individual SAMDI mass spectra for each of the 324 peptides used to prepare the array; heat map of negative control (unreacted peptides); site specific cleavage data for 324-peptide array with detergents A–E (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the NTU-NU Institute for NanoMedicine located at the International Institute for Nanotechnology, Northwestern University, and the Nanyang

DOI: 10.1021/acs.iecr.9b00057
Ind. Eng. Chem. Res. 2019, 58, 10692–10697

Technological University, Singapore; Agmt 10/20/14 and the Air Force Research Laboratory (FA8650-15-2-5518). R.D. also acknowledges the Weinberg College Summer Research Grant from Northwestern University for support.

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DOI: 10.1021/acs.iecr.9b00057
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