Investigating Alternative Acidic Proteases for H/D Exchange Coupled to Mass Spectrometry: Plasmepsin 2 but not Plasmepsin 4 Is Active Under Quenching Conditions

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Structural studies of proteins by hydrogen/deuterium exchange coupled to mass spectrometry (DXMS) require the use of proteases working at acidic pH and low temperatures. The spatial resolution of this technique can be improved by combining several acidic proteases, each generating a set of different peptides. Three commercial aspartic proteases are used, namely, pepsin, and proteases XIII and XVIII. However, given their low purity, high enzyme/protein ratios have to be used with proteases XIII and XVIII. In the present work, we investigate the activity of two alternative acidic proteases from *Plasmodium falciparum* under different pH and temperature conditions. Peptide mapping of four different proteins after digestion with pepsin, plasmepsin 2 (PSM2), and plasmepsin 4 (PSM4) were compared. PSM4 is inactive at pH 2.2 and 0 °C, making it unusable for DXMS studies. However, PSM2 showed low but reproducible activity under DXMS conditions. It displayed no substrate specificity and, like pepsin, no strict sequence specificity. Altogether, these results show that PSM2 but not PSM4 is a potential new tool for DXMS studies. (J Am Soc Mass Spectrom 2010, 21, 76–79) © 2010 American Society for Mass Spectrometry

DXMS has recently been widely used to improve the structural knowledge of protein complexes that are too large for NMR [1] or too flexible for crystallographic [2] studies, and to obtain complementary information on ligand binding [3] or protein dynamics [4]. One of the main time-consuming steps of this method is the peptide mapping of the protein of interest, after digestion with a nonspecific protease. Acidic proteases are used since their proteolytic activity is maintained under hydrogen/deuterium exchange mass spectrometry (DXMS) experimental conditions, i.e., between pH 2 and 3 and at 0 °C where H/D back-exchange is minimal [5]. To reduce back-exchange, one must also work with proteases able to digest proteins within a few minutes. Recently, new insights in fragmentation techniques, limiting the intramolecular migration of hydrogens, have allowed reaching single-residue resolution. However, so far these techniques have been tested only with synthetic peptides or small proteins such as myoglobin [6]. The combined use of alternative acidic proteases with different specificities is another way to improve the method's resolution. Our laboratory has developed the use of commercial-type XIII and XVIII fungal proteases [7, 8] that are now routinely used to improve the resolution of DXMS studies [9, 10]. However, the presence of impurities requires high enzyme/protein ratios (10.5:1 (wt/wt) for protease type XIII and 17:1 for protease type XVIII) [7]. Recently, we developed recombinant type XVIII protease with higher purity and activity [11].

In the present study, the use of two alternative recombinant aspartic proteases from the parasite *Plasmodium falciparum*, plasmepsins 2 (EC 3.4.23.39) and 4 (EC 3.4.23.B14), is evaluated with the goal of improving protein mapping and spatial resolution in DXMS experiments. Plasmepsins play an important role in the intra-erythrocytic digestion of hemoglobin responsible for the tropical disease malaria. Structural and enzymatic studies were previously reported [12, 13]. However, very little is known about their sequence and substrate specificities. This study shows for the first time that PSM2 can be used to digest nonerythrocytic proteins. We obtained peptide maps and were thus able to compare plasmepsin activity under DXMS conditions with other proteolytic results (pepsin), not only on their natural substrate (hemoglobin), but also on myoglobin

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and the PX domain of $p47^{phox}$ ($p47^{phox}$-PX), a cytosolic factor of the neutrophil NADPH oxydase [4].

Experimental

Materials

All proteins were from Sigma-Aldrich (St. Louis, MO, USA). The reversed-phase C18 column Jupiter (50 × 1.00 mm; 5 μm, 300 Å) was from Phenomenex (Torrance, CA, USA) and the protein MacroTrap C8 was from Michrom Bioresources (Auburn, CA, USA).

Protein Expression and Purification

PSM2 (residues 78–453) and PSM4 (residues 74–448) clones were the kind gifts of Professor Ben M. Dunn (University of Florida). Plasmepsin expression and purification were carried out according to Westling et al. [14]. $p47^{phox}$-PX was expressed in *E. coli* BL21(DE3) and purified according to the protocol used for the entire $p47^{phox}$ described in Durand et al. [15].

Protein Digestion and Protease Cleavage

A total of 338 pmol of protein were digested for 2 min at a 1:1 enzyme/protein ratio (wt/wt). Digestions were carried out in 10 mM glycine pH 2.2 or in 50 mM citrate pH 4.5. PSM4 was activated for 40 min in 50 mM citrate pH 4.5.

LC-MS and LC-MS/MS

Before MS analysis, peptides were desalted for 1 min on a MacroTrap column at a 400-μL/min flow rate with a 0.03% TFA water solution. Peptides were then eluted with an 8-min step gradient (0-10-15-20-25-30-35 and 45% of 95% acetonitrile, 0.03% TFA) for peptide mapping, which was performed on a quadrupole ion trap mass spectrometer (ESQUIRE 3000+; Bruker Daltonics, Billerica, MA, USA) equipped with an electrospray ionization source (ESI). For MS/MS experiments, the three most intense ions were fragmented and then excluded after two spectra had been obtained. Data processing was done using Bruker Data Analysis 3.0 software and Mascot server. MS/MS spectra were checked manually and confirmed by accurate mass measurement on an ESI-time-of-flight (TOF) mass spectrometer (6210; Agilent Technologies, Santa Clara, CA, USA). The Agilent software MassHunter and MagTran were used for data processing. Peptide maps were made with scripts available at http://ms.biomed.cas.cz/MSTools/.

Results and Discussion

PSM2 and PSM4 are generally studied under physiological conditions (37 °C, pH 4.5). Maturation of the 43-kDa PSM2 precursor into the 38-kDa activated form was achieved most often at 37 °C or room temperature for 40 min to 2 h and pH 4.5 to pH 5 [16–18]. Maturation of PSM4 was shown to be efficient after 5 min at pH 4.5 and 37 °C [19]. We determined the optimal time for plasmepsin maturation by following their auto-cleavage on ESI-TOF MS (Figure S1 available as supplementary material, which can be found in the electronic version of this article). PSM2 auto-cleavage after F112 was complete after 20 min in 10 mM citrate pH 4.5 (Figure S1 available as supplementary material, which can be found in the electronic version of this article). After 10 min at room temperature in 10 mM citrate pH 4.5, PSM4 was partly cleaved after Phe109 (38,218 Da), Phe110 (38,071 Da), or Phe81 (41,508 Da); however, cleavage was not complete, even after 7 h (data not shown). We finally used a 40-min maturation time for further experiments with PSM4. PSM2 and PSM4 activity on bovine hemoglobin was then tested before and after maturation at pH 4.5, at two different acidic pHs and two different temperatures (Figure 1a). Table S1 (available as supplementary material, which can be found in the electronic version of this article) lists the identified cleavage sites. Reproducibility of the digestion was confirmed by triplicate ESI-TOF MS accurate mass measurement. PSM2 was active in all tested conditions, but at pH 4.5, the number of cleavage sites was lower at 0 °C than at room temperature (13 versus 19 cleavage sites for the nonmature form). PSM2 produced more cleavage sites at pH 4.5 than at pH 2.2 (13 versus 10 cleavage sites, at 0 °C for the nonmature form). Interestingly, PSM2 was less active after maturation (19 versus 12 cleavage sites at room temperature). At pH 2.2, PSM2 activity was surprisingly observed only without maturation (intact plasmepsin was identified at the end of the LC-MS run). Contrary to PSM2, PSM4 was more active after maturation. It was able to digest bovine hemoglobin in different pH and temperature conditions, but its activity was considerably lowered at pH 2.2 (five cleavage sites at room temperature and only one at 0 °C), making it unusable for DXMS. Proteolytic activity was also tested on human hemoglobin with nonmature PSM2 at pH 2.2 and 0 °C. More than 70% of sequence coverage was obtained with 16 identified cleavage sites (Figure 1b). Similar results were obtained with mature PSM4 but at pH 4.5 and room temperature (11 identified cleavage sites). In conclusion, these first experiments using hemoglobin as substrate showed PSM2 as a candidate for DXMS experiments since it was active at pH 2.2 and 0 °C for 2 min of digestion time, at an 1:1 enzyme/protein (wt/wt) ratio, in contrast with PSM4.

To investigate the substrate specificity of PSM2 and PSM4, we tested their activity on nonerythrocytic proteins. Horse myoglobin was chosen because of its structural similarity with hemoglobin and $p47^{phox}$-PX because of its nonrelated structure. Horse myoglobin and $p47^{phox}$-PX were digested by pepsin and PSM2 (Figure 2) but not by PSM4, even at pH 4.5 and room

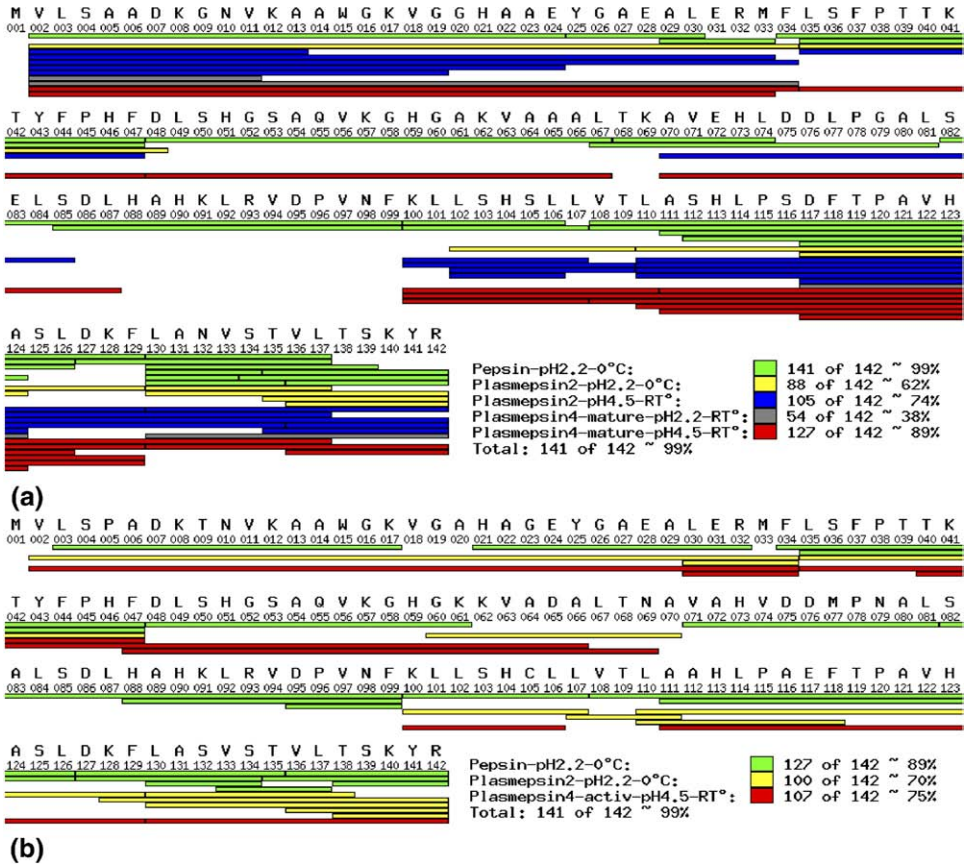

Figure 1. Peptide map of bovine (**a**) and human (**b**) hemoglobin α subunit after digestion with pepsin, non-mature PSM2, and mature PSM4. Different pH and temperature conditions and percentages of coverage are indicated in the insets.

temperature. These results confirm the broad substrate specificity of PSM2, which is able to digest proteins not only from erythrocyte cytoskeleton [18], but also from muscle cells and neutrophils. On the contrary, PSM4 seems to be only able to digest its physiologic substrate (human hemoglobin) or species homologues.

A broad-spectrum substrate does not exclude any sequence motif recognition or at least any preference for certain amino acids. Acidic proteases used for H/D exchange such as pepsin and proteases type XIII and XVIII are not really specific even though pepsin was described to cleave preferentially on the C-terminal end of Met, Phe, and Leu [20]. Protease type XIII was recently shown to favor cleavage after basic amino acids [10]. Specific acidic proteases would considerably reduce the experimental and data analysis time by avoid-

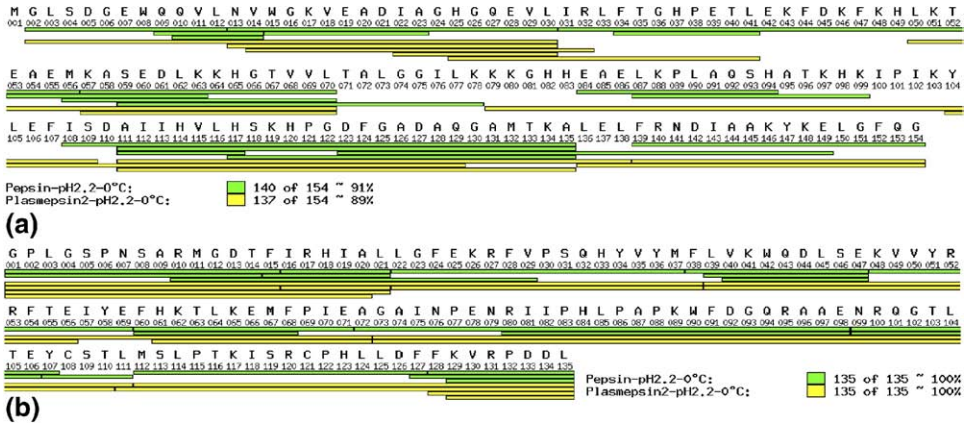

Figure 2. Peptide map of (**a**) horse myoglobin and (**b**) $p47^{phox}$-PX after digestion with pepsin and PSM2 at pH 2.2 and 0 °C. Percentages of coverage are indicated in the insets.

ing the MS/MS peptide identification step. However, low specificity is generally considered to be an advantage for DXMS experiments because it leads to a large number of peptides [20], which increases the spatial resolution of the analysis. We identified here different cleavage sites on various proteins to evaluate plasmepsin specificity. Digestion of bovine hemoglobin α subunit, at pH 2.2 and 0 °C, with either pepsin or PSM2, generated peptides by cleavage after eight different types of residues. This large number of recognized residues seems to indicate low sequence specificity. The proportions of cleavage sites that are obtained with PSM2 but not with pepsin were 30%, 50%, 47% and 45% for bovine and human hemoglobin, myoglobin, and $p47^{phox}$-PX, respectively (Figures 1 and 2), showing the complementarities of both enzymes. Finally, the average size of peptides generated by digestion of the four proteins under DXMS conditions was 12 versus 15.5 for pepsin and PSM2, respectively.

Conclusions

Recombinant PSM2 and PSM4 from *Plasmodium falciparum* were studied for the first time as new tools for structural biology studies. PSM2 activity does not seem to be enhanced upon maturation. However, it was still active at pH 2.2 and 0 °C, contrary to PSM4, which therefore cannot be used for DXMS experiments. Moreover, in these conditions, PSM2 was able to digest not only bovine and human hemoglobin (its physiologic substrate), but also horse myoglobin and $p47^{phox}$-PX, chosen as representatives of nonerythrocytic and structurally different proteins. Despite close sequence specificity of PSM2 with pepsin, both proteases were able to generate unique cleavage sites, thus showing their complementarity. The ability of PM2 to work under quenching conditions and its low substrate and sequence specificities positions it as a potential novel protease to increase DXMS resolution, even though it was less active than pepsin under quenching conditions. Further studies need to be conducted in this field to develop an increasing number of proteolytic tools for DXMS. Comparison of plasmepsin activity and specificity from different *Plasmodium* species could be interesting, since some of them have been described as more efficient in acidic conditions [19].

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Appendix A. Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2009.09.005.

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