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Development of activity-based probes for trypsin-family serine proteases

Zhengying Pan,* Douglas A. Jeffery,† Kareem Chehade, Jerlyn Beltman,‡ James M. Clark, Paul Grothaus, Matthew Bogyo*,§ and Amos Baruch*

Celera Genomics, 180 Kimball Way, South San Francisco, CA 94080, USA

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Abstract—A series of diphenylphosphonate-based probes were developed for the trypsin-like serine proteases. These probes selectively target serine proteases rather than general serine hydrolases that are targets for fluorophosphonate-based probes. This increased selectivity allows detection of low abundance serine proteases in complex proteomes using simple SDS–PAGE methods. We present here the application of multiple probes in enzyme activity profiling of intact mast cells, a type of inflammatory cell implicated in allergy and autoimmune diseases.

The primary goal of proteomics is to assign functions to all proteins in a given cell, tissue or organism.[1] The challenges implicit in this field have generated novel approaches to functionally dissect the proteome. Chemical proteomics or activity-based protein profiling (ABPP) makes use of small molecule probes that form covalent complexes with their targets using an activity-dependent chemical reaction. These activity-based probes (ABPs) can be used to profile enzymatic activities in complex proteomes and provide information on protein targets at the functional, rather than the expression level.[2] Moreover, due to its use of small molecules, this approach naturally focuses on 'druggable' enzymes that show specific ligand binding.

A number of classes of ABPs have successfully been designed and applied to serine hydrolases[3] and cysteine proteases.[4] Recently the scope of this approach has expanded with the development of chemical probes that target additional important enzyme families including phosphatases[5] and kinases.[6] Many approaches have focused on broad-spectrum probes that target multiple related enzyme family members.[7] Thus, there is a great need to develop new chemical probes to selectively target sub-classes of enzymes to study their roles in biological processes.

Serine hydrolases represent a large family of enzymes, members of which participate in many crucial biological processes. One of the most intriguing sub-classes of this family is the trypsin-like serine proteases. This subgroup is comprised of 65 enzymes in humans with unique cellular and physiological regulatory roles in health and disease.[8] Several enzymes within this group, such as thrombin, factor VIIa, factor Xa, and tryptase, are being extensively pursued as drug targets in cardiovascular and inflammatory indications. A fluorophosphonate (FP) probe, similar to probe **1** (Bio-FP), has been previously reported to target the serine hydrolase family (Ref. 3). The broad reactivity of probe **1** enables simultaneous labeling of a large number of enzymes including esterases, proteases, lipases, and amidases. In many cases, this probe generates a highly complex activity profile that prevents its usage for studying low-abundance, specific enzymes using simple analytical methods such as SDS–PAGE. To

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* Corresponding authors; e-mail addresses: zypan@yahoo.com; zhengying.pan@celera.com; mbogyo@stanford.edu; amos.baruch@celera.com
† Present address: Novartis Institute of Biomedical Research, Boston, MA 02139, USA.
‡ Present address: Chiron Corporation, Emeryville, CA 94608, USA.
§ Present address: Department of Pathology, School of Medicine, Stanford University, 300 Pasteur Drive, Stanford, CA 94305, USA.

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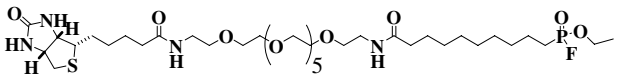


Figure 1. A general serine hydrolase probe 1.

circumvent this issue, we developed a series of selective probes that specifically target the trypsin-like serine proteases (see Fig. 1).

To achieve selectivity toward serine proteases, we focused our efforts on phosphonate-based probes. Initial attempts to use an α-amino fluorophosphonate reactive group failed due to its short half-life in an aqueous environment.[9] The more stable reactive group, diphenylphosphonate, has previously been used to generate potent irreversible serine protease inhibitors[10]. Here, we describe our efforts in developing diphenylphosphonate (DPP)-derived probes for activity profiling of trypsin-like serine proteases[11] (see Scheme 1).

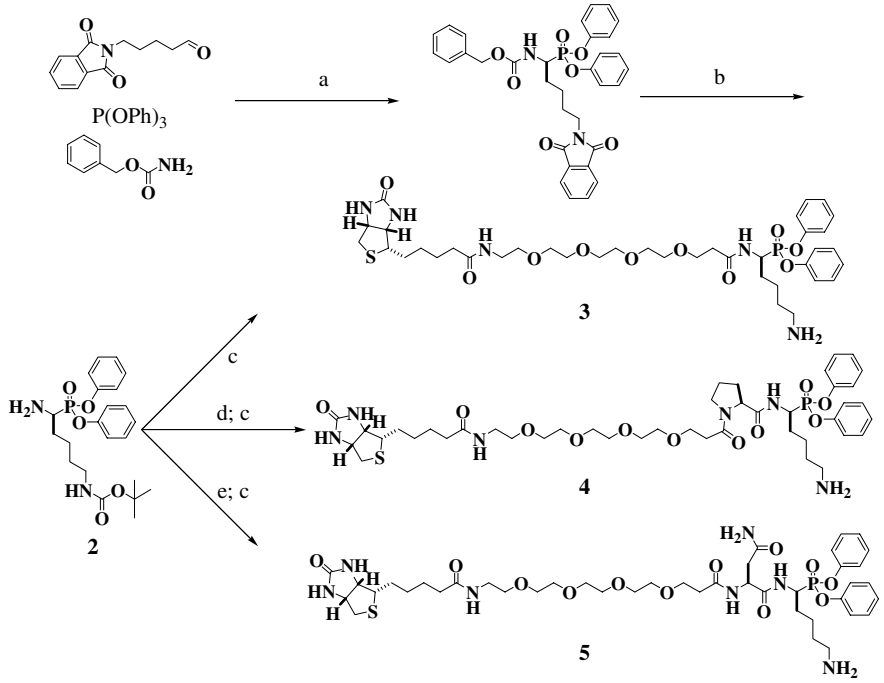
Based on previous studies of substrate specificity,[12] a lysine residue was incorporated at the P_1 site with either a proline or asparagine at the P_2 position to generate a general trypsin-family protease probe **4** (Bio-PK-DPP) and a β-tryptase-selective probe **5** (Bio-NK-DPP), respectively. Lysine-based DPPs can be made through the Oleksyszyn 3-component reaction using either conventional heating or microwave-assisted agitation. Intermediate **2** was obtained through three steps of protecting group manipulations. A biotinylated probe **3** (Bio-K-DPP) was made by coupling **2** with a commercially available biotinylation reagent NHS-$(PEG)_4$-biotin. Following addition of an amino acid residue at the P_2 position, similar procedures produced biotinylated probes **4** and **5**.

Apparent inhibition constants [$K_{i(app)}$] were obtained for these probes against four trypsin-like serine proteases (Table 1). The addition of a P_2 proline residue increased the overall reactivity of probe **4** toward trypsin-like serine proteases. Incorporation of an asparagine residue at P_2 yielded a 23-fold selectivity increase for probe **5** favoring tryptase over its closely related family member trypsin.

To confirm that the potency and selectivity observed in kinetic assays were reflected in enzyme labeling profiles, probes **1**, **4**, and **5** were used for activity-based labeling of a panel of recombinant enzymes from different serine hydrolase families (Fig. 2). The extent of covalent labeling was determined by Western blotting with streptavidin detection of the biotin reporter group. Probe **1** labeled all recombinant enzymes tested including butyrylcholine esterase (BCE), a hydrolase enzyme. In contrast, the lysine-DPP-based probes were completely inactive against both chymotrypsin and BCE. The general trypsin-family probe **4** efficiently labeled all trypsin-

Table 1. Apparent inhibition constants [$K_{i(app)}$ μM] of probes **1**, **4**, and **5** for trypsin-like serine proteases following 30 min of incubation[13]

Enzymes	Bio-FP **1**	Bio-PK-DPP **4**	Bio-NK-DPP **5**
β-Tryptase	30	6.2	2.5
Trypsin	16.5	0.57	57.3
Thrombin	7.9	1.07	>100
Plasmin	>100	2.9	4.03



Scheme 1. Synthesis of ABPs with the diphenylphosphonate reactive group. Reagents and conditions: (a) HOAC as solvent, microwave, 150 °C, 5–10 min or oil bath, 70 °C, 1–3 h; (b) hydrazine; Boc_2O; H_2, Pd–C; (c) NHS-$(PEG)_4$-biotin, triethylamine; then TFA; (d) Cbz-Pro-OH, EDCI, HOBt; H_2, Pd–C; (e) Cbz-Asn(Trt)-OH, EDCI, HOBt; H_2, Pd–C.

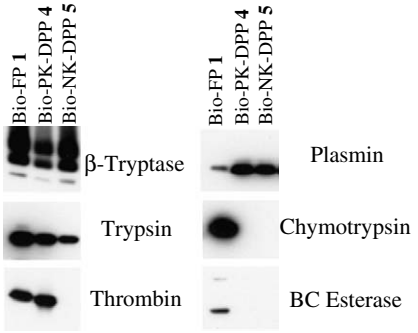


Figure 2. Labeling of recombinant enzymes with probes Bio-FP **1**, Bio-PK-DPP **4**, and Bio-NK-DPP **5**.

like enzymes tested. Interestingly, this probe was even more potent in labeling thrombin than probe **1** even though it has a much less reactive functional group. Probe **5** labeled three of the four trypsin-like serine proteases but not thrombin, with a clear preference for β-tryptase. Together, these results suggest that the incorporation of a P_2 element enhances both the selectivity and potency of the probes for trypsin-like enzymes.

Next we determined whether the probes can selectively label trypsin-like serine proteases in a complex proteome. Initially, the labeling pattern of the serine protease probe **3** was compared to that of the general serine hydrolase probe **1**. Activity-based profiling was performed using the human mast cell line HMC-1. Mast cells are inflammatory cells that provide protection against parasitic infections but are also known to play a major role in allergic responses and autoimmune diseases.[14] Intact HMC-1 cells were incubated for 1 h with probe **1** or probe **3**. Cell lysates were then analyzed by SDS–PAGE followed by Western blotting using streptavidin-HRP detection reagent (Fig. 3). Multiple active mast cell serine hydrolases were labeled by probe **1** (Fig. 3, left panel). Pretreatment with 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a general serine hydrolase inhibitor, blocked the labeling of several enzymes. Affinity purification of probe **1** modified enzymes followed by tryptic digestion and analysis by mass spectrometry, resulted in identification of 15 membrane-bound and secreted enzymes.[15] The majority of these enzymes were serine hydrolases that were not proteases. In addition, several identified serine hydrolases were abundant housekeeping enzymes such as acyl-transferase, lysophospholipase, and acyl-CoA thioester hydrolase. In sharp contrast, the trypsin-family-selective probe **3** selectively labeled β-tryptase (Fig. 3, right panel). Further analysis of the labeling with probe **1** revealed that acyl-transferase and β-tryptase co-migrate on the gel, thus making it difficult to distinguish activity-profiles for these two enzymes in HMC-1 cells. Since no labeling of acyl-transferase was observed with probe **3**, β-tryptase activity could be directly evaluated in the mast cell proteome without a need for any further purification steps. This exemplifies the utility of protease-selective probes for profiling trypsin-like proteases within a complex proteome.

HMC-1 cells are immature cells that do not fully reflect properties of mature mast cells. A prominent feature of mast cell is the presence of granules that store preformed mediators including proteases such as tryptases. Upon activation, the content of the granules is released into the extracellular milieu to promote local wound healing and inflammatory responses. We characterized serine hydrolase and protease activities in more physiologically relevant, mature mast cells that were derived from CD34+ hematopoietic progenitors. These cells were stimulated to degranulate via activation of the high affinity receptor for IgE (FcεR) (Fig. 4). Following activation, intact cells were incubated with probes for 1 h, conditioned medium was collected, and labeled enzymes were purified and identified. Probe **1** yielded a complex labeling pattern regardless of the mast cell activation state (Fig. 4, lanes 1 and 2). The tryptase-selective probe **5** predominantly labeled β-tryptase (Fig. 4, lanes 3 and 4). Notably, when the general trypsin-family probe **4** was used for labeling, both tryptase and cathepsin G were labeled (Fig. 4, lanes 5 and 6). The observed reactivity of probe **4** is consistent with the known substrate specificity of these two enzymes, with tryptase exhibiting trypsin-like activity and cathepsin G having both tryptic and chymotryptic activities. Labeling with probes **4** and **5** showed clear up-regulation in the activity of these enzymes following IgE stimulation (Fig. 4, lane 3 vs lane 4 and lane 5 vs lane 6). This demonstrates that probes **4**

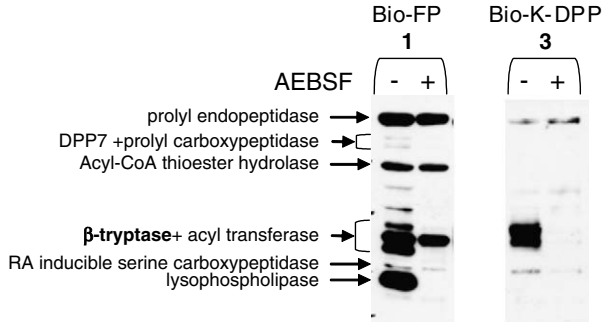


Figure 3. Selective labeling of β-tryptase in HMC-1 cell lysates by Bio-K-DPP probe **3**, with the absence (−) and presence (+) of AEBSF pretreatment prior to probes addition.

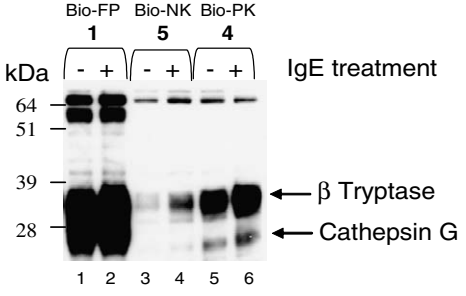


Figure 4. Selective activity-based labeling of mast cell trypsin-like serine proteases by DPP-based ABPs. Mature human mast cells derived from CD34+ hematopoietic stem cells were induced for degranulation with IgE followed by anti-IgE treatment (lanes 2, 4, and 6).

and **5** can be used to study the cellular β-tryptase's activity upon biological stimulation.

In summary, we have demonstrated the design, synthesis, and application of selective trypsin-family protease-selective probes **3**, **4**, and **5**. These probes are capable of specifically labeling trypsin-like serine proteases either in their pure forms or as components of complex proteomes. Thus, these probes represent powerful biochemical tools for monitoring the activity of proteases in their natural environment. Attachment of other reporter groups such as fluorophores may also allow imaging of protease activity in situ as recently described for cysteine proteases.[16] Finally, probes that target other sub-families of serine proteases can be designed using a similar approach. Increasing the number and type of chemical proteomics probes targeting serine proteases will significantly enhance our ability to understand the activity, regulation, and function of these important enzymes.

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