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ORIGINAL PAPER

Qualitative analysis of the fluorophosphonate-based chemical probes using the serine hydrolases from mouse liver and poly-3-hydroxybutyrate depolymerase (PhaZ) from *Bacillus thuringiensis*

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Abstract The serine hydrolase family consists of more than 200 members and is one of the largest enzyme families in the human genome. Although up to 50 % of this family remains unannotated, there are increasing evidences that activities of certain serine hydrolases are associated with diseases like cancer neoplasia, invasiveness, etc. By now, several activity-based chemical probes have been developed and are applied to profile the global activity of serine hydrolases in diverse proteomes. In this study, two fluorophosphonate (FP)-based chemical probes were synthesized. Further examination of their abilities to label and pull down serine hydrolases was conducted. In addition, the poly-3-hydroxybutyrate depolymerase (PhaZ) from *Bacillus thuringiensis* was demonstrated as an appropriate standard serine hydrolase, which can be applied to measure the labeling ability and pull-down efficiency of FP-based probes. Furthermore, mass spectrometry (MS) was used to identify the serine residue that covalently bonded to the active probes. Finally, these FP-based probes were shown capable of establishing the serine hydrolase profiles in diverse mouse tissues; the serine hydrolases pulled down from

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mouse liver organ were further identified by MS. In summary, our study provides an adequate method to evaluate the reactivity of FP-based probes targeting serine hydrolases.

Keywords Activity-based probe · Serine hydrolase · PhaZ · Esterase · Fluorophosphonate

Abbreviations

ABP	Activity-based probe
ABPP	Activity-based protein profiling
DPP7	Dipeptidylpeptidase 7
edo	Ethylenedioxy
FP	Fluorophosphonate
PhaZ	Poly-3-hydroxybutyrate depolymerase
peg	Polyethylene glycol

Introduction

Activity-based protein profiling (ABPP) has been widely used as an important tool to detect global enzyme activity in biological samples [1–4]. ABPP is based on the development of activity-based probes (ABPs) capable of labeling a group of activated/active enzymes by stable covalent linkage. Detailed classifications and potential applications of ABPs in drug discovery have been extensively reviewed [1, 5–10]. Among them, members of the serine hydrolase family, including serine proteases, esterases, lipases, and amidases, are specific targets of fluorophosphonate (FP)-based chemical probes [11]. Since the development of the first biotin-tagged FP probe against serine hydrolases in 1999 [12], several structurally modified FP-based probes have been proposed either with [13–17] or without [18–20] a polyethylene glycol linker (Fig. 1a, b). Many targets of FP probes have been identified as serine hydrolases across proteomes of mammalian tissues [21, 22], cancer cells [23], *Arabidopsis thaliana* [24], and *Saccharomyces cerevisiae* [25] (Electronic supplementary material (ESM) Table S1). The resulted global profile identified by FP probes provides an opportunity to investigate the specific or conserved serine hydrolases from prokaryotes to eukaryotes. Up to this date, only a few serine hydrolases have been studied for their in vitro reactivity with FP-based probes [26]. In addition, the kinetic study of FP probes using a single protein was only reported on human sera AChE and BChE [27], but systematic kinetics analysis of serine hydrolase probe using a standard enzyme remains unexplored. In the present study, we reported that a recently identified poly-3-hydroxybutyrate depolymerase (PhaZ) from *Bacillus thuringiensis* [28] reacts well with FP probes in vitro. In addition, we have prepared two hydrophilic FP-based probes, FP-edo-biotin and FP-edo-fluorescein, with an ethylenedioxy (edo) group in the linker region (Fig. 1c). The kinetic studies of

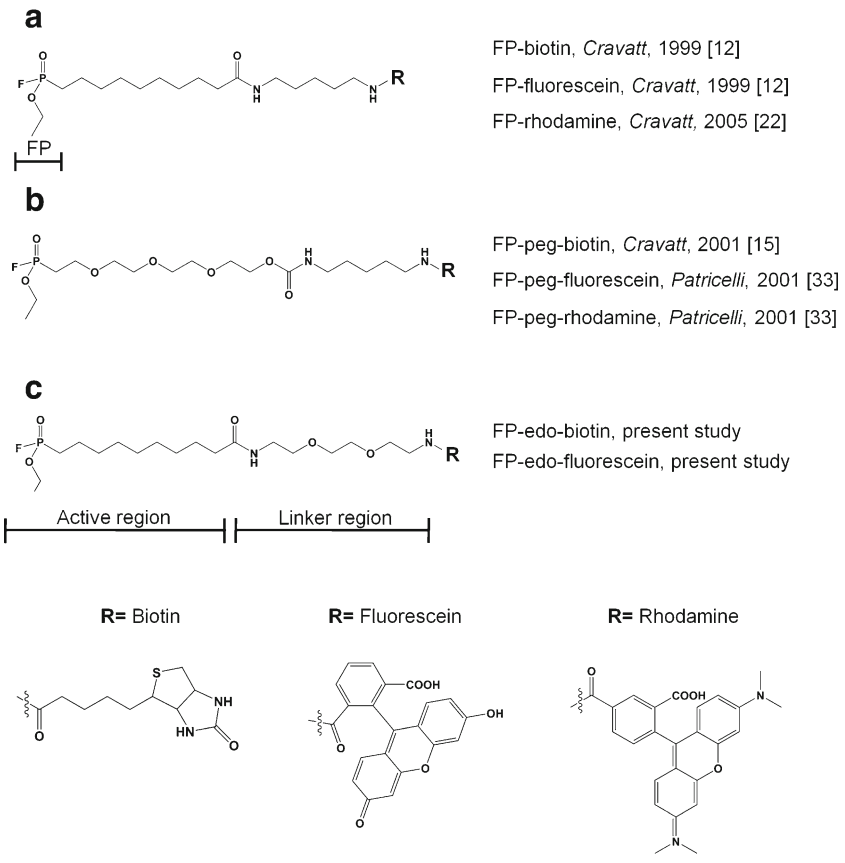

Fig. 1 Chemical structures of FP-based activity probes in the previous and present studies. **a** FP probes with R substituent as biotin [12], fluorescein [12], or rhodamine [22]. **b** FP-peg probes with R substituent as biotin [15], rhodamine [33], or fluorescein [33]. **c** FP-edo probes with R substituent as biotin or fluorescein

these probes using PhaZ were carried out to illustrate their characteristics. Furthermore, 30 serine hydrolases could be identified in mouse liver homogenate by FP-edo-biotin and LC-MS/MS. Most of these proteins were also reported by an original FP–biotin probe [29], indicating that FP-edo-probes are suitable reagents for targeting serine hydrolases.

Materials and methods

Preparation of probes

Syntheses of FP-edo-biotin and FP-edo-fluorescein were performed following a previous procedure [12], with a slight modification, and described in ESM Fig. S1 and ESM Method. FP-edo-biotin and FP-edo-fluorescein were dissolved in chloroform to make a stock solution and then stored at −20 °C. Prior to the reaction, the stock solution was added to a clean Eppendorf tube and chloroform was removed under reduced pressure using a vacuum pump. The Eppendorf tube with the dried probe was ready for the labeling reaction with protein samples.

Recombinant expression and purification of His-tagged PhaZ

Purification of wild-type or mutant PhaZ proteins in *Escherichia coli* JM109 was performed as previously reported [28]. The final eluted fraction from Ni-NTA beads was dialyzed twice against a buffer containing 50 mM Tris (pH 8) and 150 mM NaCl to remove excess imidazole. The resulting protein can be stored at 4 °C for up to 6 months with minimal loss of enzyme activity.

Labeling reaction

The purified wild-type or S102A mutant PhaZ was adjusted to 1 μM (0.034 mg/mL) using a buffer containing 50 mM Tris–Cl (pH 8) and 150 mM NaCl. Proteome samples from mouse tissues were adjusted to 1 mg/mL using 50 mM Tris–Cl (pH 8) buffer. Protein solution was added into the probe-containing Eppendorf tube and allowed to react at 25 °C for a selected period for the concentration- or time-dependent experiments. SDS-PAGE sample buffer was then added to stop the reaction. For pH profiling experiment, each pH buffer (50 mM Tris, 50 mM CAPS, and 50 mM citrate) was adjusted by HCl or NaOH to a different pH value and then mixed with the protein stock solution to a dilution of at least ten fold.

Colloidal Coomassie brilliant blue staining

First, the gel was fixed in buffer containing 50 % methanol and 10 % acetic acid for at least 30 min. Next, the gel was stained with colloidal Coomassie solution (3 % acid phosphoric, 17 % $(NH_4)_2SO_4$, 30 % methanol, and 0.1 % Coomassie brilliant blue G250) for 12–16 h. The resulting gel was immersed in distilled water until the background was clear.

Western blot analysis

Protein samples were separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane, and the membrane blocked in 3 % bovine serum albumin (BSA) in a solution of Tris-buffered saline/Tween 20 (TBST). After incubation with streptavidin–horseradish peroxidase (HRP; BD Science) in 3 % BSA/TBST, the membrane was washed three times with TBST and exposed to an X-ray film. Proteins were visualized by chemiluminescence. For the His-tagged rabbit antibody, 5 % non-fat dry milk is used as the blocking solution instead of BSA in the protocol.

In-gel fluorescence scanning

SDS-PAGE gel was soaked in distilled water and then scanned using a Typhoon Trio imager (GE Healthcare). Excitation was provided by a 30-mW Argon ion laser (488 nm), and a 520DF30 filter for fluorescein was used to detect the fluorescence signal. Voltage was set at 400–500 V.

In-gel digestion

Protein bands of interest on the gel were excised, destained by 50 % acetonitrile/ammonium bicarbonate (25 mM), and dehydrated with 100 % acetonitrile to give a gel pellet. After incubation with ammonium bicarbonate solution containing sequencing grade modified Trypsin (Promega), the gel piece was digested at 37 °C for 12 h. The resulting peptides were extracted by sonication in 50:50 acetonitrile/water (1 % trifluoroacetic acid).

Preparation of mouse tissue proteome

After washing in PBS buffer, mouse tissue samples were subjected to cell disruption using a motor-driven Potter–Elvehjem tissue homogenizer (Wheaton) in buffer containing 50 mM Tris–Cl (pH 8) and sucrose (0.3 M). Next, the homogenate was centrifuged sequentially at 1,100×*g* (10 min), 13,000×*g* (20 min), and 13,000×*g* (30 min). The final supernatant was collected and quantified using a protein assay rapid kit (Wako Chemicals) as the cytosolic fraction to be treated with FP probes.

Neutravidin pull-down for biotinylated proteins

After FP-edo-biotin reacted with the proteome (1 mg/mL) at 25 °C for 1 h, excess free probes were removed using a YM-

10 Microcon centrifugal filter device (Millipore). In addition, proteins were denatured by the addition of SDS to the solution (final concentration, 0.2 %, *w*/*v*) with 10 min heating at 95 °C and cooled to room temperature. Then, 100 μL (50 % slurry) of Neutravidin agarose beads (Thermo Scientific) was added to 1 mg of the labeled proteome and incubated at 4 °C for 12–16 h. The beads were washed sequentially twice with PBS/SDS (0.2 %) and twice with PBS and eluted by heating in 2X SDS-PAGE sample buffers.

Mass spectrometry analysis (MALDI-TOF/MS)

For peptide mass fingerprinting of PhaZ, tryptic digests were spotted on Anchorchip with 2 mg/mL α-cyano-4-hydroxycinnamic acid as the matrix. Ultraflex II matrix-assisted laser desorption/ionization–tandem time-of-flight (MALDI-TOF/TOF) mass spectrometer (Bruker Daltonics) was used to analyze the peptide profile. The *m*/*z* peak list from each spectrum was generated using FlexAnalysis 2.0. Online MASCOT software (Matrixscience) with the NCBInr database was used for protein identification. Data were searched with a peptide mass tolerance of 200 ppm and trypsin with two maximum missed cleavage sites.

Mass spectrometry analysis (LC-MS/MS)

For labeling site verification and pull-down identification, tryptic digest was dried and dissolved in H_2O (0.1 % formic acid) for LTQ-Orbitrap hybrid tandem mass spectrometry (Thermo Fisher). The mass spectrometer was inline-coupled with an Agilent 1200 nanoflow HPLC system equipped with LC Packing C18 PepMap 100 (5-mm length, 300-μm internal diameter, and 5-μm beads) as the trap column and an Agilent ZORBAX XDB-C18 (5-cm length, 75-μm internal diameter, and 3.5-μm beads) as the separation column. An LC gradient elution of peptide separation was set from 5 to 70 % acetonitrile in H_2O (0.1 % formic acid) at a flow rate of 300 nL/min for 30 min. The MS/MS spectra ranging from 200 to 2,000 *m*/*z* were acquired from the five most abundant ions in every MS scan. The mass spectra were processed by the Distiller software. Online MASCOT software (Matrixscience) with mouse-specific NCBInr database was used for protein identification and annotation. Data were searched with a precursor mass tolerance of 20 ppm and a fragment ion mass tolerance of 0.8 Da. The maximum of trypsin missed cleavage sites was set at 1. Variable modification was set to the oxidation of methionine and FP-edo-biotinylation of serine. In addition, the fixed modification was set to the carbamidomethylation of cysteine.

Results and discussion

Characterization of FP probe labeling ability

A previous study showed that FP-peg-rhodamine exhibited better activity than FP-rhodamine in profiling the serine hydrolases of *Arabidopsis* leaf extracts [24], suggesting that the introduction of polyethylene glycol (peg) into the probe structure might increase its solubility and, thus, the contact frequency to targets. The application of FP-peg-rhodamine in profiling the serine hydrolase of proteome has been frequently reported [21, 23, 25]. However, a hydrophobic pocket is usually preferred for the catalytic mechanism of serine hydrolases [30], and the hydrophilicity of polyethylene glycol in the active region of the probe structure may cause high entropy during enzyme/probe interaction. We thus designed and prepared the FP-edo-biotin and FP-edo-fluorescein, which both contain an edo structure in the linker region, to maintain hydrophilicity without affecting the part that interacts with the hydrophobic pocket of the enzyme (Fig. 1 and ESM Fig. S1). The in vitro reaction of these probes with trypsin and other enzymes demonstrated their adequate ability toward serine hydrolases (data not shown). Among the diverse enzymes capable of reacting with FP-edo-probes, the PhaZ of *B. thuringiensis* [28] showed comparable reactivity with trypsin. Notably, trypsin has been known to cause autocleavage of itself during probe labeling reaction and is not suitable to serve as a standard protein to monitor probe activity. Conversely, PhaZ showed great stability during the probe labeling reaction and is further examined for its reaction details with FP-edo-biotin and FP-edo-fluorescein.

Labeling of PhaZ by FP-edo-biotin or FP-edo-fluorescein is dose-dependent

PhaZ was labeled with FP-edo-biotin at diverse ratios, including 1:1, 1:2, 1:4, and 1:8, for 30 min at 25 °C and analyzed in SDS-PAGE, as shown in Fig. 2a. Notably, a higher band shift of PhaZ on PAGE was observed upon its labeling with FP-edo-biotin, which could be used to monitor reaction progress. In addition, probe-induced biotinylation of PhaZ could be detected using streptavidin–HRP blotting. The results showed that the ratio of 1:8 PhaZ/probe reaction gave the strongest signal, indicating that probe labeling is dose-dependent. On the other hand, either using an activity-dead mutant PhaZ (S102A) or a preheating protocol prior to labeling both abolished the band shift and biotin intensity completely, indicating that such probe labeling of PhaZ only occurred on the catalytic serine residue and is activity-dependent. Collectively, we have demonstrated that FP-edo-biotin is able to label PhaZ and induces a band shift of the probe-labeled PhaZ. To our knowledge, this special

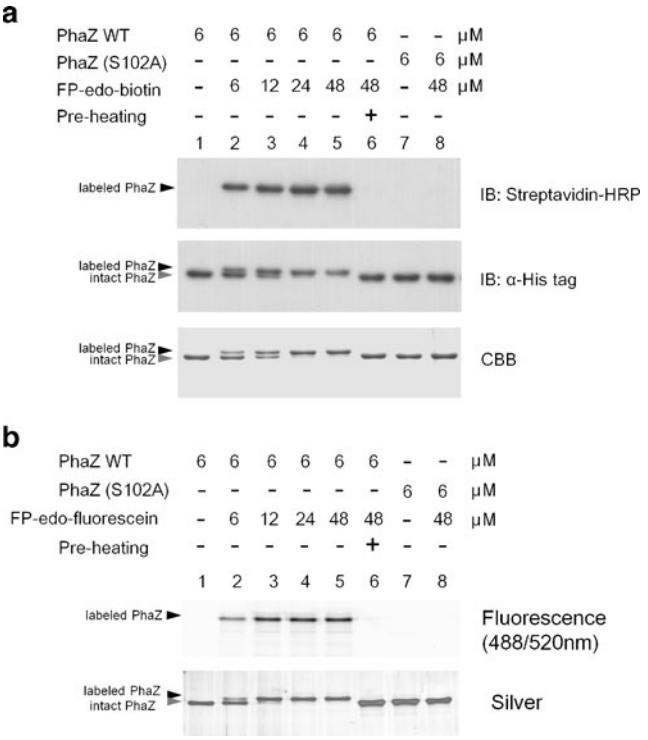

Fig. 2 Concentration-dependent labeling of PhaZ by FP-edo-biotin and FP-edo-fluorescein. **a** Wild-type PhaZ or the S102A mutant (6 μM) was incubated with various amounts of FP-edo-biotin, followed by immunoblotting with anti-His antibody and streptavidin–HRP. Coomassie blue staining was performed in another gel with the same loading. **b** Wild-type or S102A PhaZ (6 μM) was incubated with various amounts of FP-edo-fluorescein, followed by fluorescence scanning. The gel was further stained by silver staining

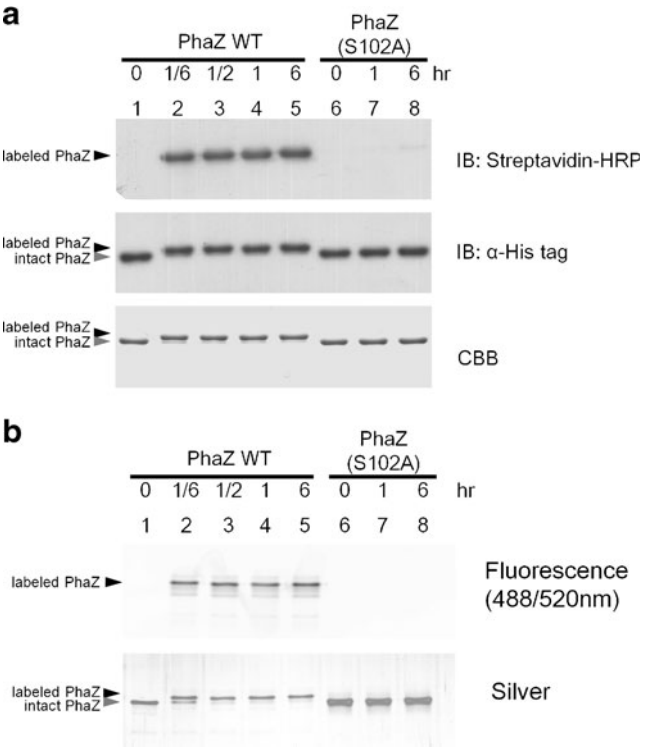

Fig. 3 Time course analysis of PhaZ labeling by FP-edo-biotin and FP-edo-fluorescein. **a** Wild-type PhaZ or the S102A mutant (6 μM) was incubated with FP-edo-biotin (24 μM) for various times, followed by immunoblotting with anti-His antibody and streptavidin–HRP. Coomassie blue staining was performed in another gel with the same loading. **b** Wild-type or S102A PhaZ (6 μM) was incubated with FP-edo-fluorescein (24 μM) for various times, followed by fluorescence scanning. The gel was further stained by silver staining

phenomenon has never been reported in serine hydrolase labeling with any FP-based probes. Similarly, FP-edo-fluorescein could also label PhaZ in the same way, as shown in Fig. 2b.

Labeling of PhaZ by FP-edo-biotin or FP-edo-fluorescein is time-dependent

PhaZ was further labeled with FP-edo-biotin at a 1:4 ratio of protein/probe for 10, 30, 60, and 360 min at 25 °C and analyzed in SDS-PAGE, as shown in Fig. 3a. The labeling started efficiently and gave a product (the shifted band) within 10 min. Conversely, the PhaZ (S102A) mutant exhibited a very weak signal after a 6-h incubation. Time-dependent experiments of FP-edo-fluorescein also showed similar results (Fig. 3b). In addition, the esterase activity of PhaZ upon probe labeling was further determined using *p*-nitrophenylacetate (pNPA), a pan substrate of esterase. As shown in ESM Fig. S2, the esterase activity of PhaZ was indeed blocked by FP-edo-biotin, whereas PhaZ without treatment showed normal esterase activity. In addition, S102A PhaZ, a previously known activity-null mutant, was used as a negative control to represent the auto-hydrolysis of pNPA in the experiment. Since the preparation of PhaZ is relatively simple and easy, its efficient labeling by FP-

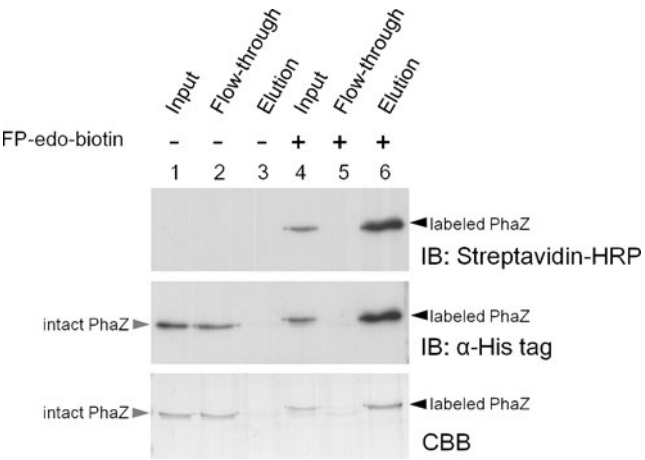

Fig. 4 Pull-down of probe-labeled PhaZ by Neutravidin agarose beads. Wild-type PhaZ (6 μM) was incubated with FP-edo-biotin (24 μM) for 30 min, followed by affinity purification using Neutravidin agarose beads. SDS-PAGE analysis followed by Coomassie blue staining and Western blot showed that probe-labeled PhaZ was present in the eluted fraction, but not in flow-through

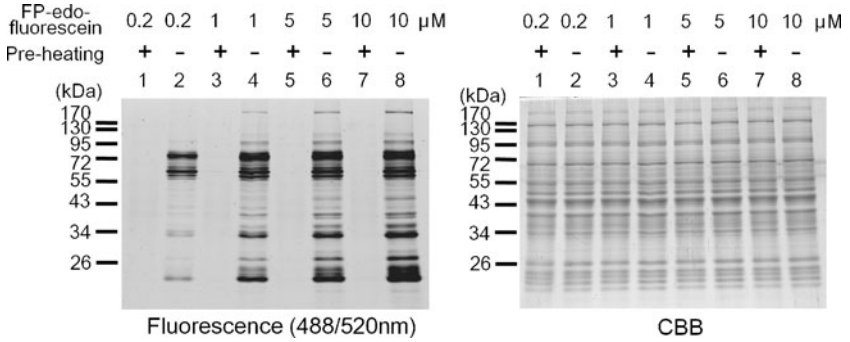

Fig. 5 Concentration-dependent labeling of mouse liver proteome by FP-edo-fluorescein. Cytosolic fraction of the liver tissue lysate was respectively incubated with 0.2, 1, 5, or 10 μM of FP-edo-fluorescein for 60 min and resolved by SDS-PAGE. Fluorescent proteins were detected by laser excitation at 488 nm and emission at 520 nm. The gel was further stained with Coomassie blue to indicate the equal quantity of protein loading in each lane

based probes makes it a suitable standard serine hydrolase for measuring the kinetics of probe activity.

Enrichment of FP-edo-biotinylated proteins using Neutravidin agarose beads

The FP-edo-biotinylated PhaZ was further examined to determine whether it can be pulled down by Neutravidin agarose beads. The results showed that the labeled PhaZ was mostly present in the elution buffer, whereas it was hardly detectable in the flow-through fraction, indicating that the biotin-mediated enrichment of PhaZ was effective (Fig. 4). In addition, FP-edo-biotin was also investigated to determine whether it can enrich PhaZ from a complex protein mixture. Our results showed that probe-labeled PhaZ could be successfully pulled down from the PhaZ-overexpressing *E. coli*, while other *E. coli* serine hydrolases were not significantly enriched (ESM Fig. S3). Although at least 40 serine proteases are encoded in *E. coli* [31], the absence of these enzymes in the elution fraction is probably due to their relatively low abundance compared to the largely induced expression of PhaZ in *E. coli*. Collectively, our data suggested that activity-based biotinylation by FP-edo-biotin provides a basis to further enrich its targeted serine hydrolases.

Establishment and optimization of serine hydrolase profiling by FP-edo-fluorescein

Activity measurement of diverse serine hydrolases in biological samples provides a profile to reflect the enzymatic status. Accordingly, the highly sensitive FP-edo-fluorescein was used to establish the fluorescence-based profiles on various tissue samples. As shown in Fig. 5, the activities of serine hydrolases in mouse liver tissue were indicated by fluorescent signals from the labeling of FP-edo-fluorescein on protein targets within an hour, whereas preheating abolished these signals. In addition, increasing the dose of FP-edo-fluorescein resulted in more and stronger bands on SDS-PAGE, as anticipated. On the other hand, phenylmethanesulfonyl fluoride (PMSF) is a broad serine protease inhibitor. We further determined whether PMSF can deactivate the FP probe labeling of serine hydrolase in liver proteome. Our results showed that PMSF indeed blocked

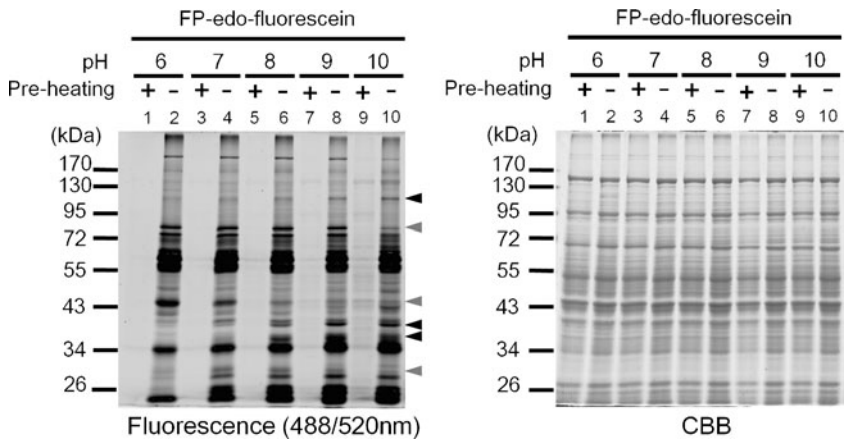

Fig. 6 pH-dependent labeling of mouse liver proteome by FP-edo-fluorescein. Cytosolic fraction of the liver tissue lysate was incubated with 10 μM FP-edo-fluorescein at pH 6, 7, 8, 9, or 10 for 60 min and resolved by SDS-PAGE. Fluorescence signals were detected as previously described. *Black arrowheads* indicate the potential serine hydrolases with optimal activity at high pH, whereas *gray arrowheads* indicate proteins with preference at a low pH

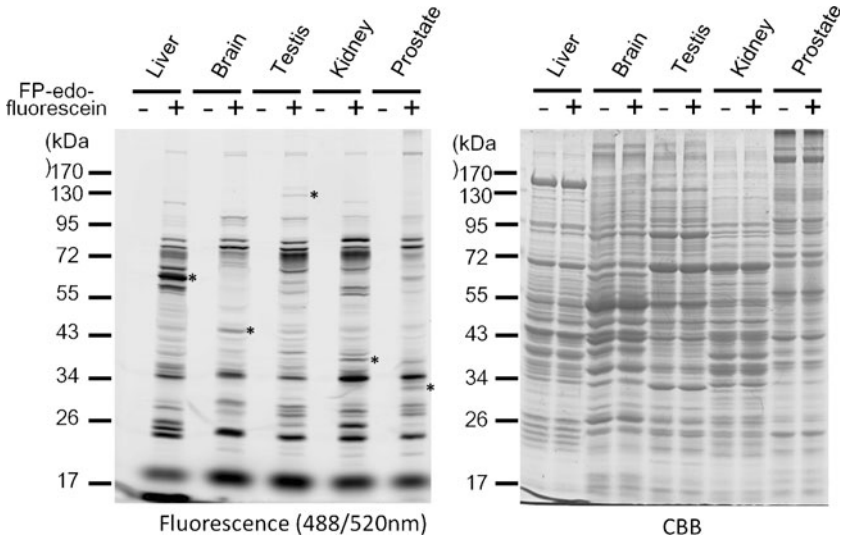

Fig. 7 Tissue profiling of serine hydrolases by FP-edo-fluorescein. Protein samples from diverse mouse tissues (1 mg/mL) were incubated with 10 μM FP-edo-fluorescein for 60 min and resolved by SDS-PAGE. Fluorescent signals were detected as previously described. *Asterisks* indicate the tissue-specific fluorescent signals

the probe labeling of several serine hydrolases in liver proteome, as shown in ESM Fig. S4. The PMSF-resistant activity for the remaining serine hydrolase may be due to the presence of lipases and esterases rather than proteases. Furthermore, protein labeling of FP-edo-fluorescein in mouse liver tissue was performed at diverse pH conditions. As shown in Fig. 6, the activity profiles at diverse pH values were slightly different, given that a few enzymes favored

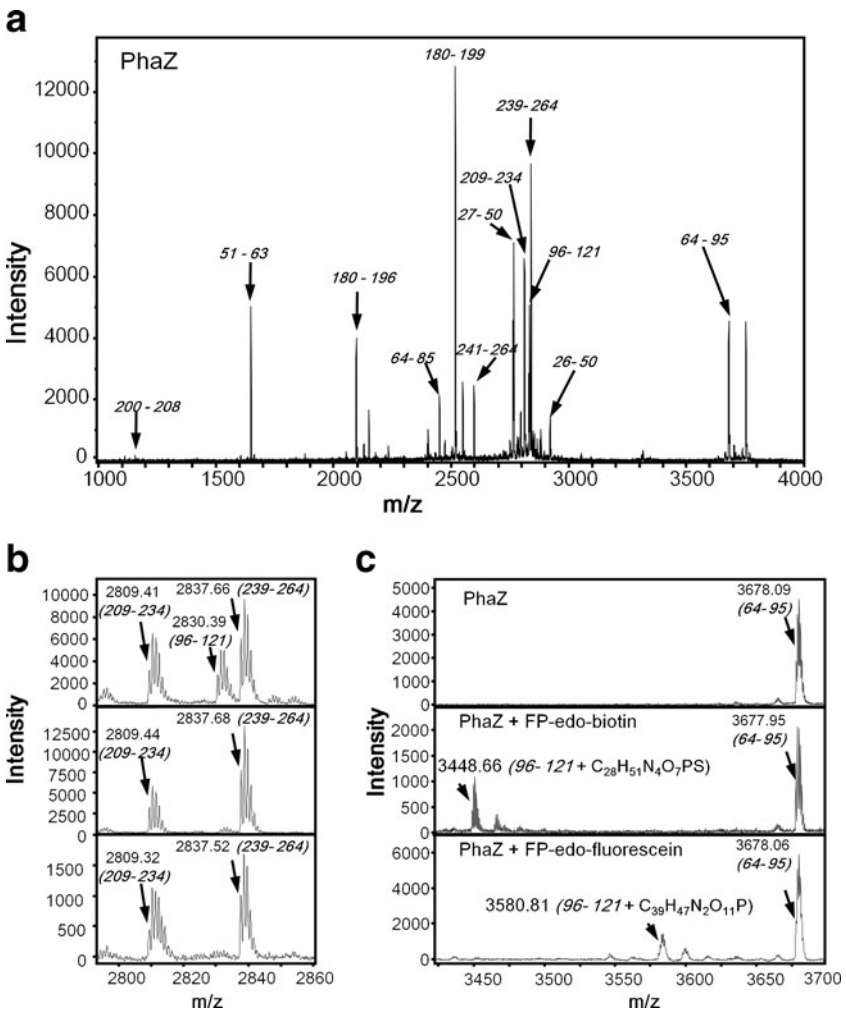

Fig. 8 Identification of FP probe-labeled peptides in PhaZ. **a** Unlabeled PhaZ was digested and analyzed by MALDI-TOF/MS. A total of 12 peptides in the spectrum were matched to the PhaZ of *B. thuringiensis* (58 % coverage). **b** The *upper*, *middle*, and *lower panels* respectively represent the spectrum of PhaZ before and after treatments of FP-edo-biotin or FP-edo-fluorescein. The signal of the active site peptide (residues 96–121) was significantly decreased after both FP probe labeling. **c** Peptide labeled by either FP-edo-biotin or FP-edo-fluorescein was identified as *m*/*z* 3,448.66 and 3,580.81, respectively. The intensity of these peptides was normalized based on another PhaZ peptide (residues 64–95)

Table 1 List of the identified serine hydrolases from the mouse liver proteome by FP-edo-biotin

Description	Accession no.	Mass (Da)	Gene name	Mascot score	Peptide no.
Fatty acid synthase	148702861	276,695	FASN	141	2
Dipeptidyl peptidase 9	26347125	99,013	DPP9	30	1
Prolyl endopeptidase	6755152	81,669	PREP	307	7
Acylpeptide hydrolase	19343726	80,767	APEH	234	4
Butyrylcholineesterase	416798	68,990	BCHE	149	1
Fatty-acid amide hydrolase	13905142	64,206	FAAH	68	1
Liver carboxylesterase 31	29476863	63,790	ES31	1,251	18
Liver Carboxylesterase 1	148679152	63,170	CES1	386	10
Carboxylesterase 2 G	20072612	62,980	CES2G	231	7
Carboxylesterase 2	21704206	62,714	CES2	878	15
Carboxylesterase 5	27370126	62,676	CES5	824	13
Carboxylesterase 6	19527178	62,356	CES6	554	8
Carboxyesterase 2B	37718991	62,172	CES2B	434	9
Carboxylesterase 3	14269427	62,133	CES3	697	12
Carboxylesterase ML1	21450339	61,972	CES1F	1,132	23
Liver carboxylesterase 22	19526804	61,828	ES22	329	8
Sialate O-acetylesterase	1373055	61,522	SIAE	60	1
Liver carboxylesterase N	192854	61,387	ES1	449	9
Liver carboxylesterase 31-like	81915140	58,529	CES3B	656	12
Dipeptidyl-peptidase 2	13626390	56,804	DPP7	87	2
Cathepsin A	12860234	54,423	CTSA	62	1
Lysosomal Pro-X carboxypeptidase	20072291	51,454	PRCP	141	2
Carboxylesterase 2 F	109730703	49,194	CES2F	112	3
Arylacetamide deacetylase	13184050	45,392	AADAC	163	2
S-formylglutathione hydrolase	12846304	35,569	ESD	97	3
Kynurenine formamidase	21746157	34,322	AFMID	90	2
Monoglyceride lipase	6754690	33,708	MGLL	83	2
Isoamyl acetate-hydrolyzing esterase 1	27754071	28,412	IAH1	242	6
Platelet-activating factor acetylhydrolase 1B β subunit	1373363	25,647	PAFAH1B2	60	1
Acyl-protein thioesterase 1	6678760	25,014	LYPLA1	155	3

acidic pH whereas several enzymes exhibited an increased activity upon the elevation of pH. However, the signals observed at a high pH may be due to the increase of nonspecific labeling caused by alkaline conditions as such nonspecific labeling persisted even after the preheating procedure. In summary, our results demonstrated that the use of FP-edo-fluorescein could easily and rapidly establish a fluorescence-based profile of serine hydrolase activities. Moreover, it is anticipated that certain enzymes may not have optimal activity at physiological pH. Activity labeling of FP-edo-fluorescein at diverse conditions helps identify such serine hydrolases for their physiological preferences. Finally, FP-edo-fluorescein was applied to establish the individual serine hydrolase profile of diverse mouse tissues at pH 8. As shown in Fig. 7, such FP probe not only could display cellular serine hydrolases in diverse tissues but also was able to recognize tissue-specific enzymes. Interestingly, a comparison among these profiles indicated that several serine hydrolases are particularly active in certain tissues, which might not be revealed by a traditional expression-based study. Since the physiological functions of diverse tissues are different, the identification of tissue-specific serine hydrolases provides a strategy to illustrate functional regulation in these tissues.

Verification of the probe labeling of protein

It has been reported that S102A mutation of PhaZ lost its enzymatic activity [28], and so was its labeling with FP probes in our experiments (Figs. 2 and 3). However, point mutation of the conserved serine residue other than the catalytic serine can also lead to the abolishment of the enzyme activity as well as the ability to react with the probe [32]. To rule out this possibility and verify whether the FP

probe directly labeled to active site serine, the MALDI-TOF/MS analysis was used to identify and compare the peptide contents of proteins from the shifted and non-shifted PhaZ bands. As shown in Fig. 8a, the tryptic peptides were identified with a 58 % sequence coverage of PhaZ. In Fig. 8b, c, the parental peptide 96–121 (m/z 2,830.39) decreased upon probe labeling, but new peptides whose mass matched the addition of probe to the parental peptide (m/z3,448.66 for FP-edo-biotin and m/z3,580.81 for FP-edo-fluorescein) were identified. Furthermore, labeling of FP-edo-biotin on the Ser102 residue of PhaZ was confirmed from the peptide fragmentation pattern generated by LC-MS/MS analysis (ESM Fig. S5). To determine whether other serine residues in PhaZ were labeled by FP-edo-biotin, a systematic search of serine-containing peptides with a modification of FP-edo-biotin was conducted using LC-MS/MS. A total of 36 peptides covering all 17 serine residues of His-tagged PhaZ upon probe labeling were identified ($p<0.05$), covering 98 % sequence coverage. However, only serine 102-containing peptides were found with the labeling of FP-edo-biotin, as shown in ESM Fig. S6. By far, up to 50 % of the serine hydrolase family are unknown for their substrates and functions [11]. Mass spectrometric identification of the catalytic serine responsible for probe labeling of serine hydrolases in biological samples may increase the understanding of their enzyme chemistry and putative function.

Identification of serine hydrolases in mouse liver

To evaluate the ability of FP-based probes in the identification of endogenous serine hydrolases, mouse liver tissue was used as a model proteome. As shown in Table 1, a total of 30 cytosolic serine hydrolases were identified using LC-MS/MS, most of which were among the previously reported 47 enzymes [29]. Notably, the identified dipeptidylpeptidase 7 (DPP7) in this study has not been reported in liver. However, it is anticipated that a slight change in the chemical structure of FP-based probes might alter their targeting specificity. About half of 30 serine hydrolase targets are carboxylesterases (EC 3.1.1.1), which are involved in xenobiotic detoxification and prodrug activation. The broad reactivity of carboxylesterase has been stated and may account for the abundant presence of these enzymes in the targets of FP-based probes in this study and others [29].

Conclusions

In the present study, we have shown that PhaZ reacted efficiently and effectively with FP-based probes and exhibited a gel shift on SDS-PAGE. These features make this *E. coli*-produced esterase suitable as a standard serine hydrolase for evaluating the activity and kinetics of FP-based chemical probes, which makes it possible to compare the activities among diverse probes for establishing the basis to develop better probes. In addition, two new hydrophilic FP-based probes, FP-edo-fluorescein and FP-edo-biotin, were prepared and demonstrated to be capable of labeling as well as the pull-down of their serine hydrolase targets. The better solubility of these probes is anticipated to provide more identification of serine hydrolase targets. Moreover, the activity profiles of serine hydrolases in diverse mouse tissues were established using FP-edo-fluorescein, which provides a comparative basis for the enzymatic activity among different samples. Finally, the pull-down of FP-edo-biotin-labeled proteins followed by mass spectrometric analysis were shown to be feasible for identifying active serine hydrolases in mouse liver. Since a large portion of serine hydrolases remained uncharacterized, the use of such an identification strategy between normal and diseased samples is expected to discover abnormally regulated serine hydrolases which might be associated with diseases and should be further investigated.

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