Activity-based probes for monitoring post-proline protease activity

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Abstract

Post-proline proteases constitute a subset of serine proteases involved in the regulation of many signaling events and are emerging as promising therapeutic targets for prevalent diseases such as diabetes and cancer. Therefore, monitoring their activity in different tissues and diverse physiological states would certainly facilitate elucidating their physiological role and the establishment of new therapeutic targets. Here, we have synthesized a dipeptidyl phosphonate activity-based probe that has proved to be highly selective for a specific post-proline protease, prolyl oligopeptidase (POP). Its high sensitivity allows detecting the endogenous activity of POP both by in-gel analysis and mass spectrometry. The evidence provided by mass spectrometry for the high selectivity of the synthesized probe opens the possibility of using dipeptidyl phosphonates not only for activity-based profiling but also for other ABP applications like substrate-based protease identification.

Keywords

activity-based probes; prolyl oligopeptidase; proteomics; phosphorus; peptidomimetics

Introduction

Proline is a DNA-encoded amino acid with unique structural properties. Unlike other amino acids, proline has a side chain that is bound to the α-nitrogen atom forming a pyrrolidine ring. This rigid cyclic structure and the ability of proline to adopt both *cis* and *trans* conformations impose strong conformational restrains on peptide chains with proline tending to introduce kinks into the major secondary structures. Due to its unique properties, proline is a key structural amino acid in many proteins and can function as a molecular switch controlling the timing of several biological processes.[1–3] Moreover, proline is also an important amino acid in many other biologically relevant polypeptide sequences. Indeed, some proline-rich peptides isolated from mammals exhibit antimicrobial activity based on a cell-penetrating mechanism, [4] which has also been recently used in some drug delivery applications.[5,6] Other proline-

rich peptides show neuroprotective and neuroregulatory properties, and have been envisaged as promising drugs for the treatment of Alzheimer's disease and other degenerative disorders.[7] The presence of proline in a peptide affects its interaction with other proteins, which prevents their degradation by most common proteases. This resistance to enzymatic hydrolysis can be especially important for the processing and degradation of many peptide hormones and neuropeptides such as neurotensin, substance P and bradykinin.[8] Indeed, the length of mature neuropeptides can be conditioned by the presence of proline in the vicinity of standard cleavage sites whereas their inactivation might be controlled by specific proteases that cleave neuropeptide sequences after proline residues.[9,10] Post-proline proteases constitute a subset of serine proteases involved in the regulation of many signaling events and are emerging as promising therapeutic targets for prevalent diseases such as diabetes and cancer. This protease subset includes such diverse and important enzymes as prolyl oligopeptidase, dipeptidyl peptidase IV, fibroblast activation protein alpha, acylaminoacyl peptidase and others.[11] Among post-proline proteases, it is worth highlighting prolyl oligopeptidase (POP; EC 3.4.21.26), which is involved in the *in vivo* regulation of many bioactive peptides like substance P and thyrotropin-releasing hormone,[12–15] and has been associated to several neuropsychiatric disorders like schizophrenia and bipolar affective disorder.[16] Several studies suggested that the action mechanism of POP could be mediated by the metabolism of inositol-1,4,5-triphosphate, a key molecule in the transduction cascade of neuropeptide signaling.[17,18]

Monitoring post-proline protease activity to compare the effects of these enzymes in different tissues and diverse physiological states would certainly facilitate the establishment of new therapeutic targets. Activity-based protein profiling (ABPP), which is a chemical strategy that uses probes that covalently bind an enzyme active site, has been recently shown to be a powerful approach for enzyme activity profiling. One of the most effective classes of activity-based probes currently available for monitoring serine proteases is the reporter-tagged fluorophosphonate (FP) probe.[19] These probes contain an FP group coupled to a reporter tag (e.g., rhodamine, biotin) by an alkyl or poly(ethylene glycol) linker and serve as broad spectrum profiling tools for serine proteases in biological samples. FP-peg-rhodamine (FP-peg-Rh), in particular, has proven of particular value for rapid protease activity profiling in a range of previous studies.[20–22] However, when interested in monitoring the activity of specific subset of serine proteases, a more selective probe is preferable.[23] Such target-restricted activity-based probes could be used to both monitor and test the function of individual protease in complex biological samples.

Here, we show the feasibility of using a highly selective activity-based probe to monitor the endogenous activity of a specific post-proline protease, prolyl oligopeptidase, both by in-gel analysis and mass spectrometry.

Results and Discussion

Three peptidyl phosphonate probes were designed and subsequently synthesized to selectively monitor POP post-proline proteolytic activity: Aha-Pro-Pro^{P}(OPh)$_2$ (1), Aha-Pro-Pro^{P}(OEt)$_2$ (2) and Aha-Pro-Pro^{P}(OPh)OH (3) (Figure 1A). The phosphonate reactive group present in probes 1–3 enables the detection of serine proteases by establishing covalent irreversible bonds with their active site. However, to enhance probe selectivity, a dipeptidyl moiety based on the well-known covalent POP inhibitor, Z-prolyl-prolinal (ZPP),[24] was incorporated as a linker between the phosphonate and the alkyne group. The presence of this inhibitor-based dipeptidyl linker should restrict probe reactivity toward the selected post-proline protease and enable selective activity monitoring. Finally, the synthesized peptidyl phosphonate probes did not directly contain a reporter tag but an alkyne functional group at

the N-terminus that allows reporter tag conjugation[25] by Cu(I)-catalyzed Huisgen [3+2] cycloaddition.[26, 27]

Proline-derived phosphonate probes 1–3 were synthesized using the synthetic route outlined in Scheme 1.[28] Briefly, a 1-pyrrolidine trimer was freshly prepared as described previously [29] and used to obtain H-Pro^{P}(OPh)$_2$. Boc-Pro-OH and 5-hexinoic acid were then consecutively coupled to H-Pro^{P}(OPh)$_2$ with *N*,*N*'-dicyclohexylcarbodiimide in solution, which resulted in probe **1**. Finally, probe **2** was synthesized from **1** through a transesterification reaction whereas probe **3** was obtained by hydrolysis of **1** in basic conditions.

Competitive inhibition assays with FP-peg-Rh and recombinant POP allowed the discrimination of phosphonate probes **1**–**3** according to their inhibitory effect towards POP. In these assays POP was incubated for 15 min with either ZPP or increasing amounts of probe and subsequently incubated with FP-peg-Rh for an additional 60 min. After resolving the reaction on an SDS–PAGE, the FP-peg-Rh-labeled POP was visualized using a fluorescence scanner. These assays demonstrated that probes **1** and **3** are unable to recognize the active site of POP (Figure 1B), probably due to steric impediments arising from the presence of bulky phenyl substituents. In contrast, probe **2**, which has smaller ethyl substituents, showed a mild inhibitory effect that has nevertheless proved to be sufficient to use this probe in further activity-based assays to detect POP in mouse brain homogenates (see below). An IC_{50} value of 133 μM was established for probe **2** using a dose-response assay (Figure S1, Supporting Information).

Once phosphonate probe **2** was confirmed as the only probe interacting with the active site of POP, the next assays used probe **2** to selectively detect POP post-proline proteolytic activity by in-gel analysis. First, active and heat-denatured brain soluble proteomes were labeled with probe **2** (25 μM, 1 h at 25 °C), tagged with the fluorescent TriN_3-tag and analyzed with an SDS-PAGE and visualized with a fluorescence scanner (Figure 2A). The functional characterization of mouse brain soluble proteome with probe **2** allowed the activity-dependent detection of both endogenous (lane 1) and recombinant POP (lanes 2 and 3). This detection was activity-dependent as POP recognition was impaired by the presence of a highly selective inhibitor of POP, ZPP (lanes 4–6), or by sample heat-denaturation (lanes 7–9). As expected from previous results, the presence of ZPP completely blocked the labeling of pure POP with probe **2** (lane 6). However, this amount of ZPP seemed not to be sufficient to completely impair the detection of the same quantity of POP when mixed with brain homogenates (lane 5). This effect does not alter the significance of the results and it is probably due to the presence of reactive species in brain homogenates (e.g. primary amines) that interact with ZPP thus reducing the ZPP/Probe ratio. The identity of POP was confirmed by band scission and mass spectrometry (Q-TOF) analysis as described previously.[30] Moreover, these results showed that probe **2** was highly selective as, apart from POP and a 65 kDa-protein (*), no other proteins were detected in an activity-dependent manner (i.e., as defined by labeling in native, but not heat-denatured proteomes). We further evaluated the selectivity of probe **2** by comparing its labeling profiles to those obtained using the broad-spectrum probe FP-peg-Rh (Figure 2B). In this case, active and heat-denatured brain soluble proteomes were labeled with FP-peg-Rh (1 μM, 1 h at 25 °C), analyzed with an SDS-PAGE and visualized with a fluorescence scanner. The activity-based assays performed with the FP-peg-Rh probe identified multiple activity-dependent labeled proteins (*), which did not appear in the previous assays performed with probe **2**. These results confirmed probe **2** not only as an effective activity-based probe, but also as a highly selective probe towards the desired post-proline protease.

The dipeptidyl moiety introduced in the synthesized activity-based probes seems to play a key role in the high selectivity of phosphonate probe **2**. In order to establish the importance of the dipeptidyl moiety in improving probe selectivity towards certain proteases, a new probe, Aha-

Ala-Leu^{P}(OEt)$_2$ (**4**), was synthesized with prolines being replaced by an alanine and a leucine. When using probe **4**, POP activity could not be detected (Figure 3A) confirming the role of the peptidyl region in limiting probe reactivity to certain target proteases.

Shotgun liquid chromatography-mass spectrometry (MudPIT) was used to further confirm the high selectivity of probe **2** in monitoring endogenous POP proteolytic activity, and therefore our success in synthesizing a highly selective probe. After incubating (1 h at 25 °C) brain homogenates with probe **2** (25 μM), probe-protein complexes were labeled with the TriN$_3$-tag, and enriched from the sample with avidin beads. The pulled-down proteins from each of three replicates were analyzed by reverse-phase chromatography and subsequently identified by MS/MS. MS/MS data were analyzed with the SEQUEST software[31] using the mouse IPI database.[32] Protein abundance was estimated from spectral counts[33] and the maximum false positive rate was set to 1% using a reverse-sequence database and the DTASelect software.[34] A heat-denatured sample was used as a control to identify non activity-dependent probe-protein complexes. A second control consisting in samples incubated without any probe was also performed. In these assays, POP and aldehyde dehydrogenase were the only proteins detected with an over 10-fold sample/control ratio and present in all three sample replicates (Table 1). All other proteins were either present in the sample and at least one control or had less than 10 spectral counts (Supporting Information). The evidence provided by mass spectrometry, one of the most sensitive techniques, for the high selectivity of probe **2** opens the possibility of using dipeptidyl phosphonates not only for activity-based profiling but also for other ABP applications like substrate-based protease identification.

Conclusion

Our results have established the viability of synthesizing a dipeptidyl phosphonate activity-based probe to selectively monitor the endogenous activity of a specific post-proline protease (POP) both by in-gel analysis and mass spectrometry. The use of this type of directed probes to monitor post-proline proteolytic activity in different tissues and diverse health/disease states can certainly facilitate the elucidation of the physiological role of POP and other post-proline proteases. Moreover, our results confirm the importance of the peptidyl moiety for modulating probe selectivity, which allows foreseeing the potential of these probes not only to monitor the activity of certain protein subsets, but also for other ABPP applications like substrate-based protease identification. In this case, substrate-based probes would be used to identify unknown proteases once the processed peptides and their sequence were known (e.g. as in the case of many neuropeptides).

Experimental Section

Synthesis

Synthesis of the diphenyl phosphonate 1: Prolyl-derived phosphonate probe 1 was synthesized from H-Pro^{P}(OPh)$_2$ by peptide synthesis in solution. H-Pro^{P}(OPh)$_2$ was synthesized as described previously. [28] Briefly, diphenyl phosphite (150 mmol) was added to freshly prepared 1-pyrroline trimer (150 mmol)[29] and the mixture was stirred for 1.5 h at 85 °C to obtain a yellow pale oil that was dissolved in chloroform, washed with a saturated NaCl aqueous solution and dried over anhydrous $MgSO_4$. The crude product was further purified in a silica column with a $CHCl_3$:MeOH gradient 1:0 to 8:2. Pure H-Pro^{P}(OPh)$_2$ (5 mmol) was then used to synthesize the final phosphonate probes. Boc-Pro-OH (5 mmol) and Aha-OH (5 mmol) were sequentially coupled to H-Pro^{P}(OPh)$_2$ with DCC (6 mmol) in anhydrous DCM through overnight reactions at room temperature. In both couplings, the crude product was filtered and successively washed with H_2O, 1 M HCl (aq) and saturated $NaHCO_3$ (aq), and a further product purification was performed in a silica column with a EtOAc:EtOH gradient 1:0 to 8:2. The Boc protecting group of compound Boc-Pro-Pro^{P}(OPh)$_2$ was removed with HCl

(4 M in 1,4-dioxane) in a 30-min reaction with a temperature gradient from 0 to 25 °C to obtain Cl^{-} NH_3 $^{+}$-Pro-Pro$^{P}(OPh)_2$.

Synthesis of the diethyl phosphonate **2**: Diethyl phosphonate **2** was synthesized by transesterification of diphenyl phosphonate **1**. Compound **1** (0.044 mmol), KF (0.440 mmol) and 18-crown-6 ether (0.003 mmol) in ethanol were bolied for 10 min boiling and stirred overnight at room temperature. Crude product was purified in a silica column with a EtOAc:EtOH gradient 1:0 to 8:2 to obtain pure Aha-Pro-Pro$^{P}(OEt)_2$.

Synthesis of the monophenyl phosphonate **3**: Monophenyl phosphonate **3** was synthesized by partial hydrolysis of diphenyl phosphonate **1.** Compound **1** (0.044 mmol), KF (0.440 mmol) and 18-crown-6 ether (0.003 mmol) in 1 M NaOH aqueous solution were stirred overnight at room temperature. Crude product was purified in a semipreparative HPLC with a 30 to 70 % B gradient in 40 min. Solution A: H_2O + 0.1 % TFA. Solution B: MeCN + 0.05 % TFA.

Synthesis of the diethyl phosphonate **4**: Diethyl phosphonate **4** was obtained by transesterification of Aha-Ala-Leu$^{P}(OPh)_2$ with KF (1 equiv) and 18-crown-6 ether (0.1 equiv) in ethanol as described for probe **2**. Aha-Ala-Leu$^{P}(OPh)_2$ was synthesized by sequential coupling of Boc-Ala-OH (1 mmol) and hexinoic acid (1 mmol) to H-Leu$^{P}(OPh)_2$ using HATU (1 mmol) and DIEA (2 mmol) in anhydrous DMF (16 h at 25 °C). H-Leu$^{P}(OPh)_2$ was obtained by deprotection of Cbz- Leu$^{P}(OPh)_2$ with HBr (33% in acetic acid, 1h at 25 °C) that had previously been synthesized from benzylcarbamate (10 mmol) in acetic acid (7.5 ml), triphenyl phosphite (10 mmol) and 3-methylbutanal (10 mmol) (1 h at 25 °C and 16 h at 50 °C).

For detailed probe characterization, as well as data acquisition and analysis, the reader is referred to the Supporting Information.

In-gel analysis

Recombinant prolyl oligopeptidase—Prolyl oligopeptidase (POP, EC 3.4.21.26) was obtained by expression in *Escherichia coli* of the recombinant plasmid POP-pETM10 and subsequent affinity purification using a His tail fusion as previously reported[35] except for the final desalting step. Here a disposable PD-10 column from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) and a saline phosphate buffer (PBS) at pH 7.4 replaced the HiPrep 26/10 Desalting column and the Tris-HCl (50 mM, pH 8) buffer to enhance Cu(I)-catalyzed Huisgen [3+2] cycloaddition.

Preparation of brain soluble proteome—Brain homogenates were obtained from adult male mice (BALB/c, 8 weeks old). Brains were frozen after extraction and were homogenized using 8 ml of PBS (pH 7.4) and a tight douncer homogenizer from Wheaton Science Products (Millville, NJ USA). Homogenates were centrifuged on a bench centrifuge (5 min, 4 °C, 2000 rpm) and on a ultracentrifuge (1 h, 4 °C, 100,000 *g*). Pellets were discarded and the supernatant was collected to obtain the soluble proteome. The total protein content was quantified with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA USA) using bovine serum albumin as standard. Aliquots of the brain homogenates were immediately prepared and stored at −80 °C.

Competitive inhibition assays with FP-peg-Rh—POP (20 ng) was preincubated for 15 min at 25 °C with either ZPP (25 μM) or synthesized phosphonate probes (5 μM, 25 μM and 100 μM). FP-peg-Rh (1 μM) was then added to the samples, which were vortex-mixed and incubated for one additional hour at 25 °C. Reaction products were analyzed by SDS-PAGE and visualized on a Hitachi FMBIO II fluorescence scanner.

Activity-dependent labeling experiments—Probe **2** (25 μM final) was added to either pure POP (20 ng), brain homogenates (1 mg·ml^{-1}) or a mixture of both, and samples were

gently mixed during 1 h at room temperature (total volume = 50 µl). 1 µl of the $TriN_3$-tag (5 mM in DMSO) was then added, followed by 1 µl of freshly prepared tris(2-carboxyethyl)-phosphine (50 mM in H_2O), 3 µl of tris(triazolyl)amine (1.7 mM in DMSO:t-butanol (1:4)) and 1 µl of $CuSO_4$ (50 mM in H_2O). Samples were incubated at 25 °C during 1 h and were intermittently vortex-mixed. Reaction products were analyzed by SDS-PAGE (20 × 20 cm, 1.5 mm, Dual Gel P10DS-1 Emperor Penguin from Thermo Fisher Scientific Inc., Waltham, MA USA) and visualized on a fluorescence scanner. Heat-denatured samples (Δ) were inactivated (5 min at 95 °C) before adding the phosphonate probe whereas ZPP (25 µM final) was added in some active samples (ZPP +) to be used as a negative control.

Activity-dependent labeling of brain homogenates (1 $mg \cdot ml^{-1}$) and recombinant POP (20 ng) with FP-peg-Rh (1 µM) were performed similarly except for the $TriN_3$-tag labeling. In this case samples and controls were incubated (1 h at 25 °C) with FP-peg-Rh (1 µM) and with no further manipulation were analyzed by SDS-PAGE and visualized on a fluorescence scanner.

Activity-dependent labeling of recombinant POP with probes 2 and 4 was performed incubating (1 h at 25 °C) POP (20 ng) either with probe 2 or probe 4 at different concentrations (25 µM and 100 µM). Samples were labeled with the $TriN_3$-tag as previously described, analyzed by an SDS-PAGE and visualized on a fluorescence scanner.

Mass spectrometry assays

Sample preparation—Brain homogenates (1 $mg \cdot ml^{-1}$) were incubated (1 h at 25 °C) with peptidyl phosphonate probe **2** (25 µM) (total volume = 500 µl) and. $TriN_3$-tag (11.6 µl, 5 mM in DMSO) was subsequently added, followed by freshly prepared tris(2-carboxyethyl)-phosphine (11.6 µl, 50 mM in H_2O), tris(triazolyl)amine (35 µl, 1.7 mM in DMSO:t-butanol (1:4)) and $CuSO_4$ (11.6 µl, 50 mM in H_2O). After another 1h-incubation at 25 °C, samples were centrifuged (4 min, 25 °C, 2000 *g*) and the supernatant was removed. Cold methanol (0.5 ml) was added to the pellet and the samples were sonicated (0.5 Hz, 50 %), shaked (10 min, 4 °C) and centrifuged (4 min, 4 °C, 2000 g). This washing procedure with methanol was repeated twice. 1.2 % SDS in PBS (1 ml) was added to the pellet and samples were sonicated briefly prior to sample heating (5 min at 95 °C) and dilution with 0.2 % SDS in PBS (5 ml). Previously (PBS) washed avidin beads (50 µl, Avidin-Agarose from egg-white from Aldrich, Milwaukee, WI, USA) were added to the samples that were then incubated for 1 h at 25 °C. The supernatant was subsequently removed by centrifugation (3 min, 25 °C, 1400 *g*) and the avidin beads were successively washed with 0.2 % SDS in PBS (1 × 10 ml, 3 min), PBS (3 × 10 ml, 1 min) and H_2O (3 × 10 ml, 1 min). Samples were denatured and reduced (30 min, 25 °C) with 6 M urea in PBS and 10 mM tris(2-carboxyethyl)-phosphine (final volume = 500 µl) and alkylated in the darkness (30 min) with iodoacetamide (25 µl, 400 mM). Urea (200 µl, 2 M) in PBS and trypsin (4 µl, 0.5 mg/ml) were then added and samples were digested overnight at 37 °C. Finally, avidin beads were removed by sample centrifugation (3 min, 25 °C, 1400 g) and samples were used in massspectrometry analysis with no further manipulation. Heat-denatured brain homogenates (5 min, 95 °C) and brain homogenates incubated without any probe were used as controls. All assays were done by triplicate.

Multidimensional protein identification technology (MudPIT)—Each sample was subjected to MudPIT analysis in a Thermo Finnigan LTQ mass spectrometer equipped with a nano-LC electrospray ionization source. Fused-silica microcapillary columns were pulled and packed with C18 reverse-phase material (5 µm) and strong cation exchange material (5 µm) as described previously.[36] Samples were load to a 250 µm precolumn packed with C18 reverse-phase material (5 µm) and an automated 5-step chromatography was performed for each sample. The first step of each run consisted of a 55 min gradient from 0 to 45 % buffer B, 10 min gradient from 45 to 100 % buffer B and a 20 min hold at 100% buffer B. The next

following steps were 112 min each with the following profile: 3 min of 100 % buffer A, 2 min of *X* % buffer C, 1 min of 95 % buffer A, a 10 min gradient from 5 to 15 % buffer B, a 45 min gradient from 15 to 25 % buffer B, and a 52 min gradient from 25 to 55% buffer B. The 2 min buffer C percentages (*X*) in steps 2–5 were: 25, 50, 80 and 100 %. Buffer A: 95 % H_2O, 5 % MeCN and 0.1 % formic acid. Buffer B: 20 % H_2O, 80 % MeCN and 0.1 % formic acid. Buffer C: 500 mM ammonium acetate, 95 % H_2O, 5 % MeCN and 0.1 % formic acid.

MS^2 spectra data were extracted from the raw files using the publicly available RAW Xtractor software (v1.9.1, http://fields.scripps.edu/?q=content/download). Extracted data were then searched using the SEQUEST algorithm (v3.0) against a custom-made database that contained the longest entry of the mouse IPI database (v3.26) associated with each Ensembl gene identifier, resulting in a total of 22833 unique entries. Additionally, each of these entries was reversed and appended to the original database for the assessment of false-positive rates. In total the search database contained 45666 protein sequence entries (22833 real sequences and 22833 decoy sequences). SEQUEST searches allowed for oxidation of methionine residues (16 Da), static modification of cysteine residues (57 Da-due to alkylation), no enzyme specificity and a mass tolerance set to ±1.5 Da for precursor mass and ±0.5 Da for product ion masses. The resulting MS^2 spectra matches were assembled and filtered using the DTASelect software (v2.0.27). For this analysis, non-trypic, half-tryptic and fully-tryptic peptides were individually evaluated and the distribution of Xcorr and DeltaCN values for each direct (to the direct database) and decoy (reversed database) match were separated by quadratic discriminant analysis. Outlier hits in the two distributions were removed and only those spectral matches with Xcorr and DeltaCN values that produced a maximum peptide false positive rate of 1% were retained. In addition, a minimum peptide length of seven amino acids residues was imposed and protein identification required the matching of at least two peptides per protein. Such criteria resulted in the elimination of most decoy database hits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

A) Structure of phosphonate probes Aha-Pro-Pro^{P}(OPh)$_2$ (**1**), Aha-Pro-Pro^{P}(OEt)$_2$ (2), Aha-Pro-Pro^{P}(OPh)OH (3). B) Competitive inhibitory assays to evaluate the recognition of POP active site by the new synthesized probes. In these assays POP (20 ng) was incubated for 15 min at 25 °C with either ZPP (25 μM) or increasing amounts (5 μM, 25 μM and 100 μM) of probe. Afterwards, FP-peg-Rh (1 μM) was added and the incubation was maintained for an additional 60 min at 25 °C. The assay was resolved on an SDS–PAGE and the FP-peg-Rh-labeled POP was visualized using a fluorescence scanner.

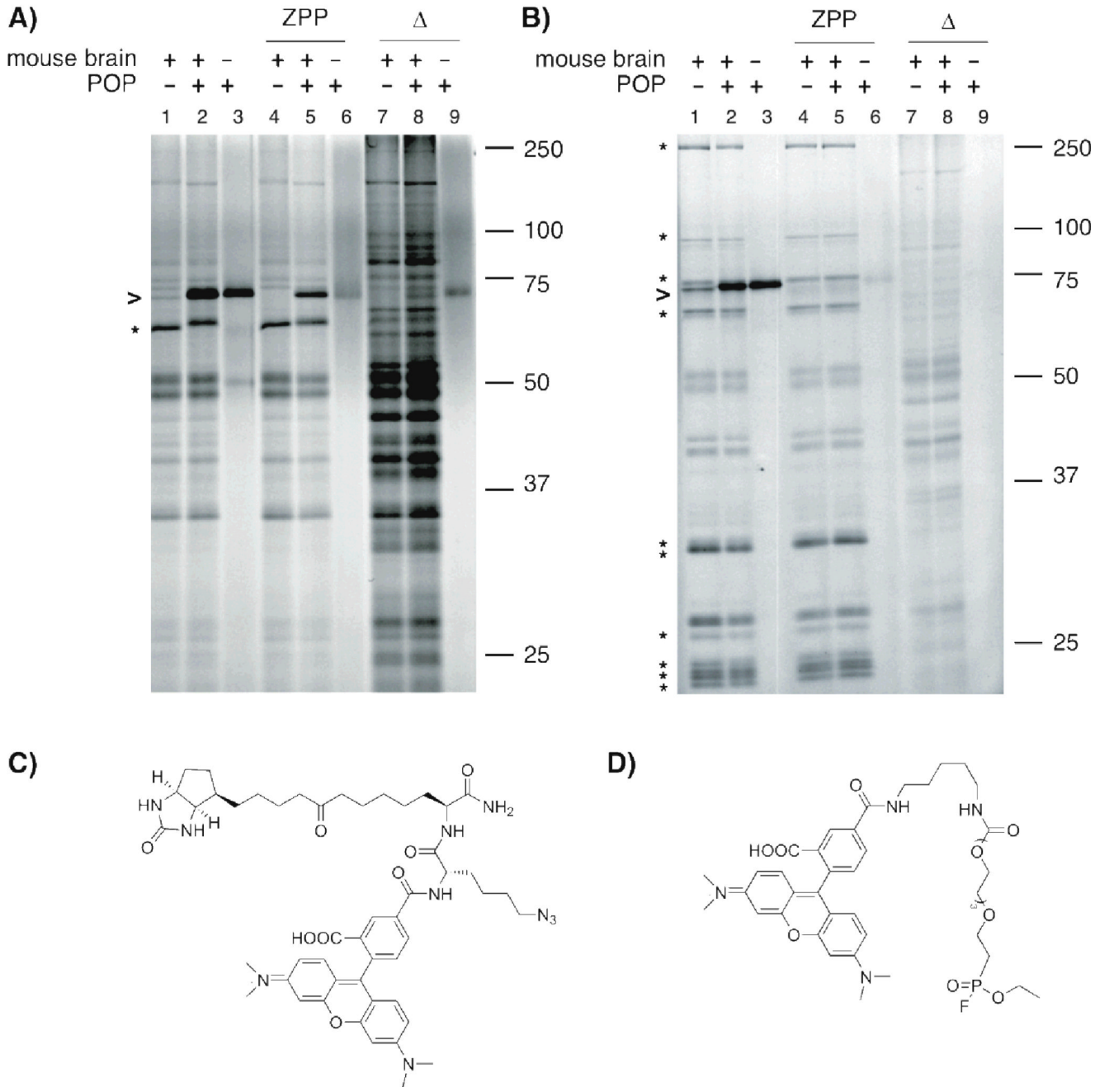


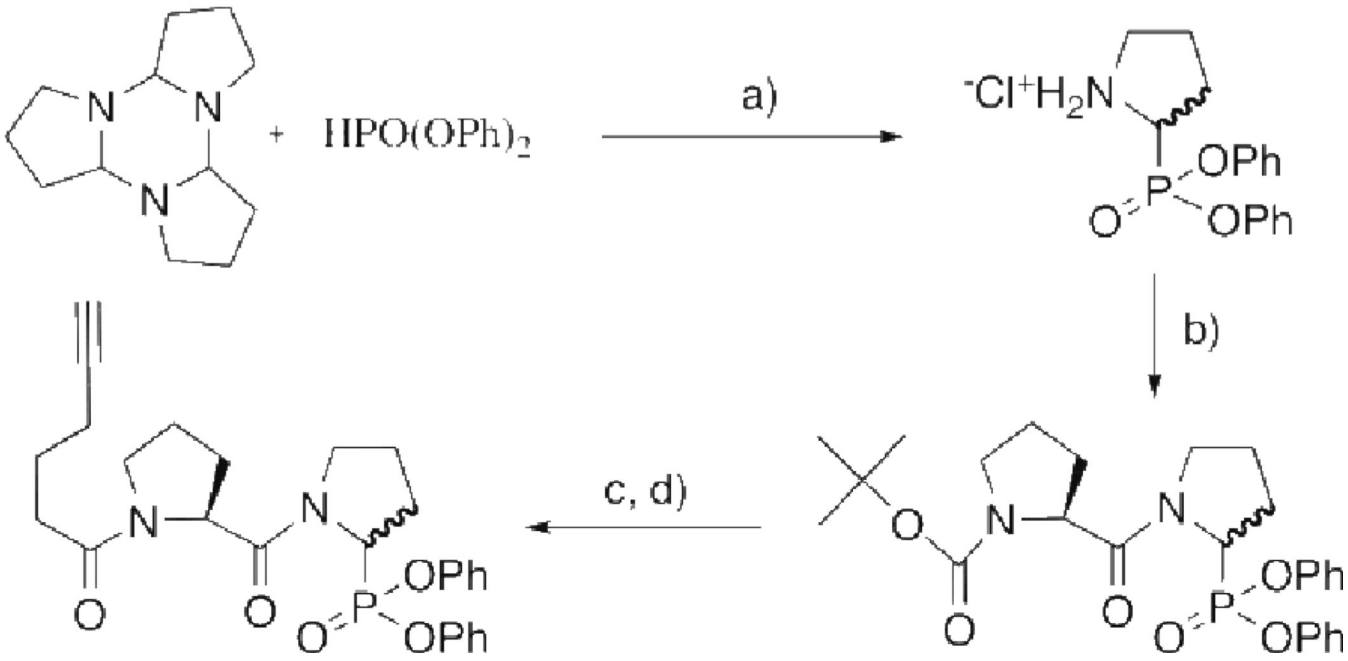
Figure 2.

A) Activity-dependent labeling, using probe **2**, of recombinant and endogenous POP. Active and heat-denatured (Δ) recombinant POP (20 ng) and brain soluble fractions (1 mg/ml) were incubated (1 h at 25 °C) with activity-based probe **2** (25 μM) and ZPP (25 μM). After incubation, samples were labeled with the $TriN_3$-tag, analyzed by SDS-PAGE and visualized with a fluorescence scanner. Asterisks (*) highlight those brain proteins, in addition to POP, that were clearly detected in an activity-dependent manner. B) Activity-dependent labeling of recombinant and endogenous POP using the FP-peg-Rh probe. Active and heat-denatured (Δ) recombinant POP (20 ng) and brain soluble fractions (1 mg/ml) were incubated (1 h at 25 °C) with activity-based FP-peg-Rh (1 μM) and ZPP (25 μM), analyzed by SDS-PAGE and

visualized with a fluorescence scanner. Asterisks (*) highlight those brain proteins, in addition to POP, that were clearly detected in an activity-dependent manner. C) Structure of $TriN_3$-tag. D) Structure of FP-peg-Rh.

Figure 3.

A) Activity-based detection of recombinant POP using probes **2** and **4**. Pure recombinant POP (20 ng) were incubated (1 h at 25 °C) either with probe **2** or probe **4** at different concentrations (25 μM and 100 μM). Samples were labeled with the $TriN_3$-tag, analyzed by an SDS-PAGE and visualized using a fluorescence scanner. B) Structure of probe **4**, Aha-Ala-$Leu^P(OEt)_2$.



Scheme 1.
Reagents and conditions: a) 1h at 85 °C b) Boc-Pro-OH, *N*,*N*'-dicyclohexylcarbodiimide in DCM, 16 h at 25 °C c) 4 M HCl in dioxane, 30 min 0 to 25 °C d) 5-hexinoic acid, *N*,*N*'-dicyclohexylcarbodiimide in DCM, 16 h at 25 °C.

Table 1

Spectral counts of proteins identified by MudPIT with an over 10-fold sample/control ratio.

IPI Number[a] (Mouse)	Sample	Control A (Boiled Sample)	Control B (No probe)	Sample/Controls Ratio
IPI00132089	139±27	0.0±0.0	6.3±2.0	43.9
IPI00111218	16.0±1.5	0.0±0.0	0.0±0.0	n.a.

[a] IPI00132089: Prolyl endopeptidase. IPI00111218: Aldehyde dehydrogenase.