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Affinity purification of recombinant protein-tyrosine phosphatase 1B using a highly selective inhibitor

Anja K. Pedersen[a,1], Sven Branner[b,1], Steen B. Mortensen[d], H. Sune Andersen[e], Kirsten M. Klausen[b], Karin B. Møller[c], Niels Peter H. Møller[c], Lars F. Iversen[b,*]

[a] *Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, DK-2100 Copenhagen, Denmark*
[b] *Protein Science, Novo Nordisk A/S, DK-2880 Bagsvaerd, Denmark*
[c] *Signal Transduction, Novo Nordisk A/S, DK-2880 Bagsvaerd, Denmark*
[d] *Protein Characterization, Novo Nordisk A/S, DK-2820 Gentofte, Denmark*
[e] *Med Chem Research I, Novo Nordisk A/S, DK-2760 Måløv, Denmark*

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Abstract

Our structure-based drug discovery program within the field of protein-tyrosine phosphatases (PTPs) demands delivery of significant amounts of protein with extraordinary purity specifications over prolonged time periods. Hence, replacement of classical, multi-step, low-yield protein purifications with efficient affinity techniques would be desirable. For this purpose, the highly selective PTP1B inhibitor 2-(oxalyl-amino)-4,5,6,7-tetrahydro-thieno[2,3-*c*]pyridine-3-carboxylic acid (OTP) was coupled to epoxy-activated Sepharose 6B (OTP Sepharose) and used for one-step affinity purification of tag-free PTP1B. The elution was performed with a combined pH and salt gradient. Importantly, since OTP Sepharose binds PTP1B with an intact active site only, the method ensures that the purified enzyme is fully active, a feature that might be particularly important in PTP research.

Keywords: Affinity purification; Inhibitor; Crystallization; Protein-tyrosine phosphatases

1. Introduction

Protein-tyrosine phosphatases (PTPs) comprise a group of signal transduction enzymes that control the levels of cellular protein-tyrosine phosphorylation by removal of phosphate from phosphorylated tyrosine residues. It has been shown that PTP1B knock-out mice have increased insulin sensitivity and resistance to diet-induced obesity [1,2]. Since these effects are likely to reflect an important role for PTP1B in negative regulation of insulin and leptin signaling [3,4], PTP1B appears to be an important drug target for the treatment of type 2 diabetes and obesity [5,6]. In the pursuit of developing highly selective and potent PTP1B inhibitors, the need for large quantities of extraordinarily pure preparations of PTP1B has been growing steadily to meet the demands of our iterative structure-based drug design program, which include: (i) high throughput screening of compound libraries; (ii) detailed enzyme kinetic experiments; (iii) mutational studies; (iv) isothermal titration calorimetry; (v) protein NMR studies; and (vi) protein X-ray crystallography. These demands made classical protein purification procedures unsuitable, and we turned our attention towards development of high-speed affinity purification techniques. Already in the landmark publication of the first PTP to be purified and characterized, an affinity method using thiophosphorylated RCM lysozyme coupled to Sepharose was introduced by Tonks et al. [7]. Later, the non-hydrolysable phosphotyrosine analogue, L-histidyl-diazobenzylphosphonic acid agarose, previously used for purification of alkaline phosphatases, was introduced for purification of PTP1B by Pallen et al. [8]. However, although these affinity purification procedures significantly advanced the field, they were not found sufficiently reproducible and specific for a structure-based drug design program.

One of our early, selective PTP1B inhibitors, 2-(oxalyl-amino)-4,5,6,7-tetrahydro-thieno[2,3-*c*]pyridine-3-carboxylic acid (OTP, [9]) (see Fig. 1) seemed particularly useful

* Corresponding author. Tel.: +45-44426120; fax: +45-44427359.
E-mail address: lfiv@novonordisk.com (L.F. Iversen).
[1] Both authors contributed equally to this paper.

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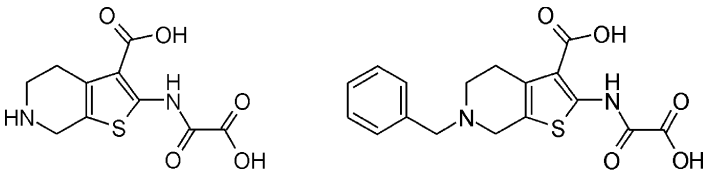
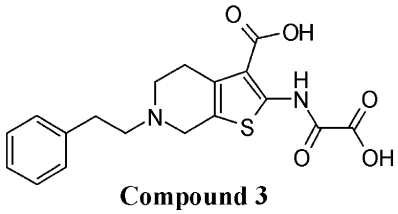


Compound 1 (OTP)

Compound 2



Compound 3

Fig. 1. Compounds used in the study.

for affinity purification since (i) OTP is highly selective for PTP1B (and TC-PTP) versus most other PTPs [9], (ii) OTP has an appropriate affinity for PTP1B in the low micromolar range at neutral pH (i.e. sufficiently high affinity to allow efficient binding, and sufficiently low to allow elution under mild conditions) [9], (iii) OTP has a free secondary amine in the tetrahydropyridine ring suitable for coupling to an appropriate, commercially available matrix, (iv) compounds with an alkyl aryl group extension on the secondary amine group possessed comparable inhibitory profiles to OTP (see Figs. 1 and 2 and [10]), (v) the X-ray structure of PTP1B in complex with OTP showed the secondary amine to be appropriately surface exposed [9], and (vi) molecular modeling showed that a suitable spacer would allow sufficient distance between the matrix and OTP for undisturbed binding to the enzyme active site (see below).

Here we demonstrate the use of OTP to function as an efficient chromatography ligand to purify PTP1B. This method has greatly improved the throughput and the quality of PTP1B used in our structure-based drug design research program [11,12]. Further, TC-PTP, the closest homologue to PTP1B, has been purified by a similar OTP-based method.

2. Experimental

2.1. Chemicals

The inhibitors 2-(oxalyl-amino)-4,5,6,7-tetrahydro-thieno[2,3-*c*]pyridine-3-carboxylic acid (compound 1), 6-benzyl-2-(oxalyl-amino)-4,5,6,7-tetrahydro-thieno[2,3-*c*]pyridine-3-carboxylic acid (compound 2), and 2-(oxalyl-amino)-6-phenethyl-4,5,6,7-tetrahydro-thieno[2,3-*c*]pyridine-3-carboxylic acid (compound 3) were synthesized as described [9,10].

2.2. Cloning and expression

cDNA encoding the catalytic domain of PTP1B was obtained by polymerase chain reaction using primers with convenient cloning sites and placenta cDNA template as described previously [13]. The construct was inserted into the pET11a expression vector, and the coding sequence was confirmed by DNA sequencing. *Escherichia coli* BL21 (DE3) was transformed with the pET11a expression plasmid. An overnight culture was diluted 1:160 into a total volume of 2 l of SOB medium (2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$) and grown at 37 °C until A_{600} was 0.6. Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.1 mM, and the incubation was continued at room temperature for 3 h.

The cells were harvested by centrifugation for 15 min at $6000 \times g$ and broken by cell disruption in a Constant Systems Inc. Cell Disrupter. Remaining cell debris was removed by centrifugation for 30 min at 36,000 × *g*.

2.3. Preparation of the affinity resin

The OTP coupling kinetics to epoxy-activated Sepharose 6B was investigated in a 24 h experiment using three different OTP concentrations (10, 20 and 40 mM). The coupling yield was monitored by HPLC analysis of uncoupled OTP using a Jupiter C18 column equilibrated with buffer A: 20 mM KH_2PO_4 pH 8.0. OTP was eluted in 0–6% buffer B (10 mM KH_2PO_4 pH 8.0 in 70% CH_3CN) over 10 min. A standard curve of OTP was prepared to determine the amount of uncoupled OTP in the coupling experiment.

The final gel was prepared as follows: 3.5 g epoxy-activated Sepharose 6B (Amersham Biosciences) was prepared for coupling according to the manufacturers instructions. An 8 ml gel suspension, corresponding to 4 ml drained gel, was mixed with an equal volume of OTP dissolved to 40 mM in a 0.2 M sodium carbonate coupling buffer pH 9. The gel suspension was agitated gently overnight at room temperature.

Excess ligand was removed by washing with coupling buffer, the remaining active groups were blocked with 1 M ethanolamine and the product was washed extensively using

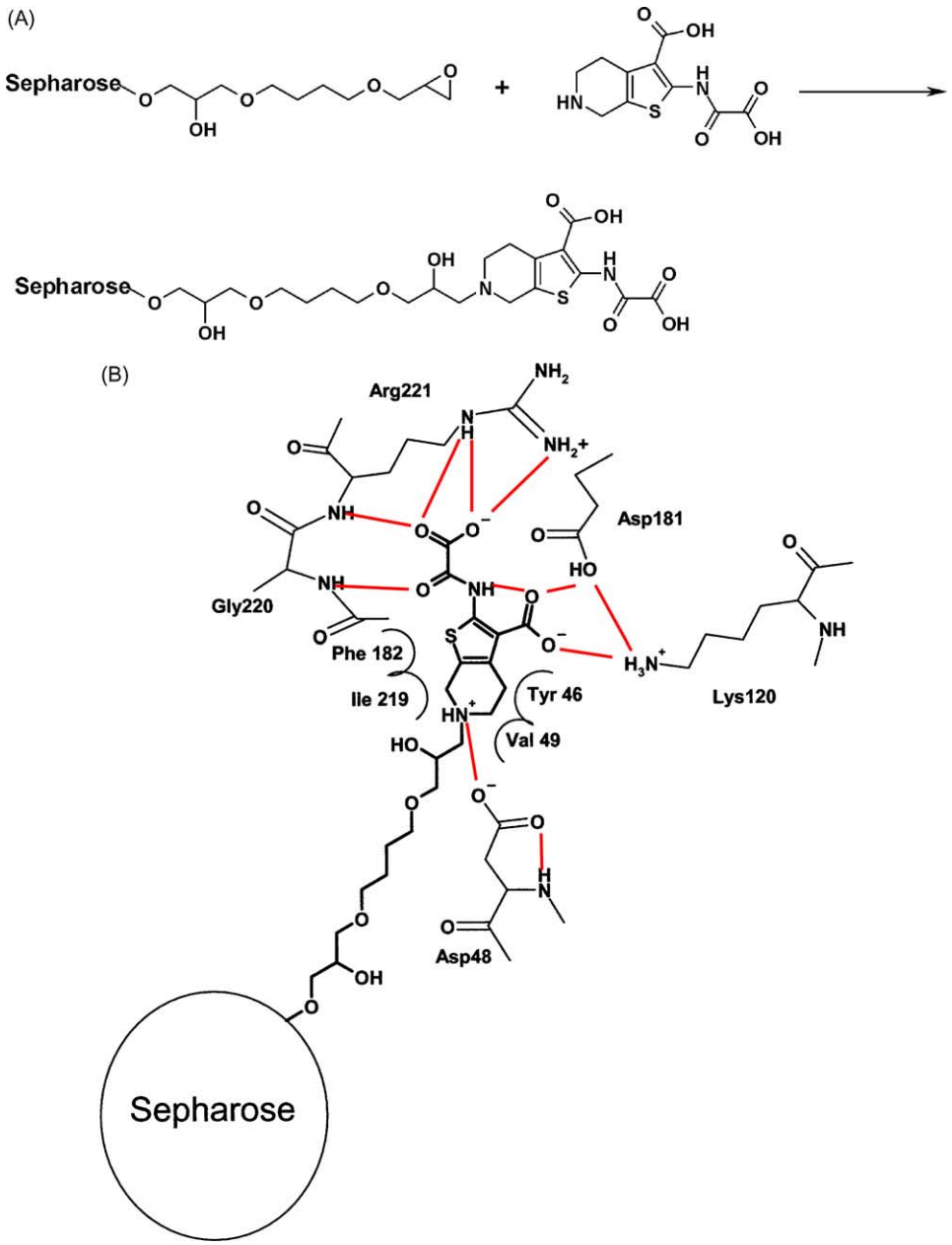


Fig. 2. (A) Coupling of ligand to affinity chromatography material. (B) Schematic representation of 2-(oxalyl-amino)-4,5,6,7-tetrahydro-thieno[2,3-*c*]pyridine-3-carboxylic acid bound to the spacer and Sepharose beads in the active site pocket of PTP1B. Possible hydrogen bonds are shown with red lines.

buffers at alternating pH, all according to the manufacturer's instructions.

The gel was stored refrigerated in 0.1 M acetate pH 4.0 containing 0.5 M sodium chloride. After use the OTP Sepharose was regenerated by washing at alternating pH and stored at 4 °C in 20% ethanol.

2.4. Chromatographic procedure

2.4.1. Affinity chromatography

The OTP Sepharose (1.7 ml) was loaded onto a 1.6 cm diameter column (Amersham Biosciences) and equilibrated with buffer C: 20 mM L-histidine pH 6.2, 100 mM sodium chloride, 1 mM EDTA, and 7 mM 2-mercaptoethanol. Crude cell extract (from ~150 ml of culture) adjusted to pH 6.2 was loaded onto the column at a flow rate of 0.3 ml/min. The column was washed with 17 column volumes of buffer C. PTP1B was eluted by a linear combined salt and pH gradient using buffer D containing 20 mM L-histidine pH 9.0, 1 M sodium chloride, 1 mM EDTA and 7 mM 2-mercaptoethanol over 20 column volumes at a flow rate of 2 ml/min (see Fig. 3).

2.4.2. Superdex 200 size exclusion chromatography

Eluted PTP1B from the OTP Sepharose was loaded onto a Superdex 200 column (2.5 cm × 60 cm) (Amersham

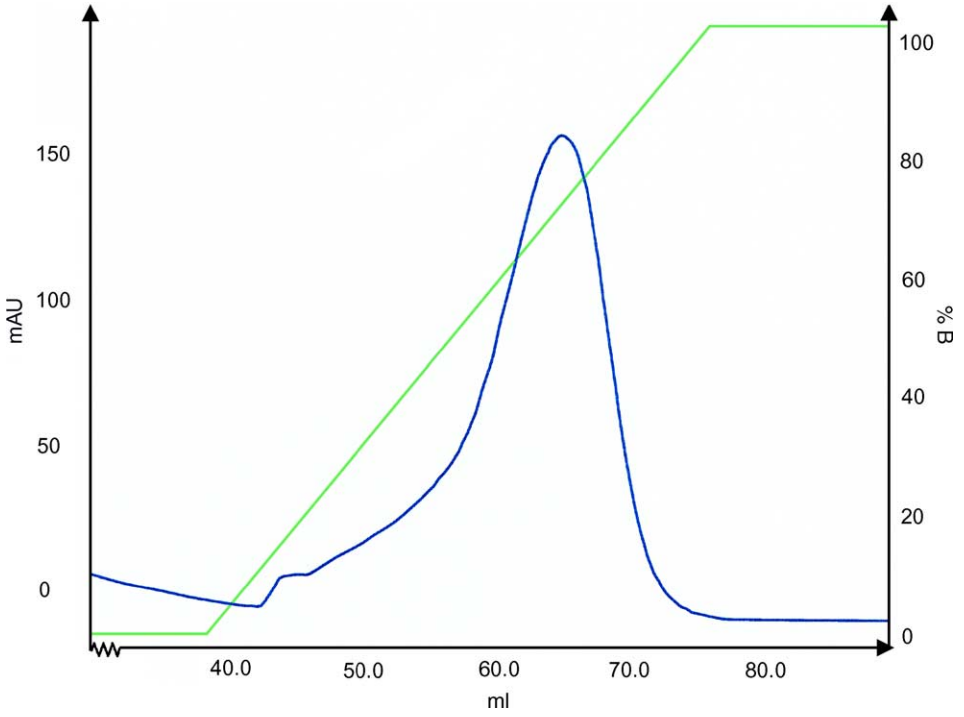


Fig. 3. OTP Sepharose purification of PTP1B. PTP1B enzyme activity was detected in non-bound fractions and in the main eluting peak. The blue curve represents the 280 nm UV trace. The green curve is buffer D (20 mM L-histidine pH 9.0, 1 M sodium chloride, 1 mM EDTA, 7 mM mercaptoethanol). Minimum 0% buffer D. Maximum 100% buffer D. Of note, the UV base line shift reflects the pH dependent UV absorption of the buffer component histidine.

Biosciences) equilibrated in 10 mM Tris, 25 mM sodium chloride, 0.2 mM EDTA and 3 mM dithiothreitol at pH 7.5 at a flow rate of 2 ml/min. Fractions containing PTP1B, measured by enzyme activity assay and SDS-PAGE, were pooled and concentrated to 15 mg/ml using Centriprep and Centricon YM-10 Centrifugal Filter Devices (Millipore) and subsequently stored at −80 °C.

2.5. OTP Sepharose binding capacity determination

The binding capacity of the OTP Sepharose was determined by loading purified PTP1B in buffer C (Section 2.4.1) onto 1.7 ml of OTP Sepharose until OD_{280} absorbing material with PTP activity was detected from the column. The OTP Sepharose was washed with 15 column volumes of buffer C. PTP1B was eluted in 20 column volumes of buffer D and the amount of bound PTP1B was determined by OD_{280} measurement.

2.6. Enzyme assays

2.6.1. Enzyme activity assay

The enzyme activity was assayed at 25 °C and pH 7.2 in a buffer containing 50 mM HEPES, 5 mM EDTA, 5% (v/v) glycerol, 0.03% (v/v) Brij 35, and 0.01% (v/v) 2-mercaptoethanol. The concentration of the substrate *p*-nitrophenylphosphate (*p*NPP) was 5 mM. The release of *p*-nitrophenol (*p*NP) was followed at 405 nm. One unit is defined as the amount of PTP that releases 1 μmol/min at the above specified conditions. The extinction coefficient of a reference sample of *p*NP (Sigma 104-8) was measured to 11,000 M^{-1} cm^{-1} at pH 7.2 and 405 nm.

2.6.2. Determination of kinetic and inhibition constants

The final product, affinity-purified PTP1B, was characterized by detailed enzyme kinetic experiments by determining k_{cat} and K_m as well as K_i values for the compounds shown in Fig. 1. The assay conditions were essentially as described in [11]. The phosphatase activity of PTP1B was determined using the substrate *p*NPP (concentration range 0.26–16.4 mM). For the determination of inhibition constants, K_i, four different inhibitor concentrations (three-fold dilutions) were used. The assays were carried out in microtiter plates at pH 7.0 and 25 °C using a three-component buffer, consisting of 50 mM Tris, 50 mM bis-Tris and 100 mM acetic acid, 0.1% (w/v) human serum albumin, and 5 mM dithiothreitol [14,15]. The assay was carried out in a total reaction volume of 100 μl. The reaction was started by addition of enzyme and stopped after 15 min by adding 20 μl 0.5M NaOH in 50% ethanol. The *p*NP concentration was determined by measuring the absorbance at 405 nm with appropriate corrections. The data were analysed using a non-linear regression hyperbolic fit to the classical Michaelis–Menten enzyme kinetic model. The inhibitors behave as reversible, competitive inhibitors and the K_i values are found by plotting apparent K_m values as a function of the inhibitor concentrations. The presented

values for k_{cat}, K_m, and K_i (±standard deviations) were calculated from at least three independent experiments.

2.6.3. Determination of inhibition with the OTP Sepharose

An assay for determining the inhibition (IC_{50}/K_i) of PTP1B by the OTP Sepharose was established. The reactions were carried out in the three-component buffer as in the above kinetic studies. Suspensions of OTP Sepharose were diluted with suspensions of uncoupled, but ethanolamine-blocked Sepharose gel in order to create a concentration gradient (three-fold dilutions). For each concentration determined, 100 mg of drained gel was used. The gel was incubated with PTP1B for 10 min before 1 mM *p*NPP was added and the reactions were carried out for 15 min at 25 °C under vigorous shaking. The reactions were stopped by addition of 0.5M NaOH in 50% ethanol. OD_{405} of the supernatant was measured after clarifying by centrifugation (to isolate the gel). Three independent experiments were used to calculate the IC_{50} value. The K_i was calculated using the equation: $K_i = IC_{50}/(1 + S/K_m)$.

2.6.4. Specific enzyme activity determination

The activity of PTP1B purified by the classical method (yellow-86 dye adsorption chromatography and subsequent Q-HiLoad ion-chromatography [16]) versus OTP Sepharose purification was determined as described in Section 2.6.1.

2.7. Protein characterization

The purified PTP1B was analyzed by enzyme activity assays as described in Section 2.6.1 and by SDS-PAGE (see Fig. 4), N-terminal sequencing, and MALDI mass spectrometry (MALDI MS).

2.8. PTP1B crystallization

The purified PTP1B was co-crystallized with OTP essentially as previously described [9].

3. Results and discussion

The main aim of the present study was to establish an efficient and easy-to-use method for purification of PTP1B. We hypothesized that OTP could be used for affinity chromatography in the purification of PTP1B since OTP is highly selective for PTP1B, has an appropriate affinity for PTP1B, and can be coupled to a suitable commercially available matrix without affinity loss (as described above). First, we used molecular modeling to analyze the suitability of several resins that are commercially available for coupling to $–NH_2$ groups. This analysis identified epoxy-activated Sepharose 6B as particular useful. Of significance, coupling of OTP to this matrix maintains the basic character and will still allow the basic nitrogen of OTP to interact with Asp48 in PTP1B (see Fig. 2). Thus, this key interaction between ligand and

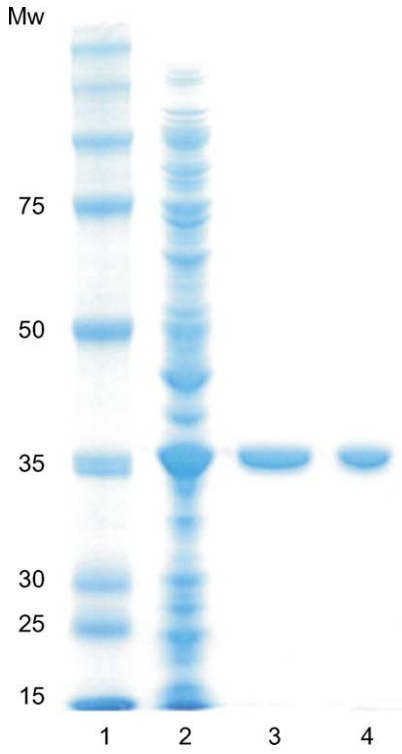


Fig. 4. SDS-PAGE stained by Gelcode Coomassie Blue Stain Reagent of the PTP1B purification process. Lane 1: M_w markers. Lane 2: starting material. Lane 3: eluted PTP1B from the OTP Sepharose column. Lane 4: PTP1B after Superdex 200 size exclusion chromatography.

enzyme will ensure a high degree of selectivity for PTP1B (and the highly homologous TC-PTP) versus other PTPs. Also, and as visualized in Fig. 2, the spacer arm would not be expected to cause significant steric hindrance. The coupling yield of OTP to epoxy-activated Sepharose 6B was monitored by HPLC analysis and found to be 8.5 μmol OTP/ml Sepharose at a concentration of 10 mM OTP, 12.0 μmol OTP/ml Sepharose at 20 mM OTP and 14.8 μmol OTP/ml Sepharose at 40 mM OTP after an overnight incubation at 25 °C. To further evaluate OTP coupling and the binding properties of the resin, the OTP Sepharose was tested in a PTP inhibitor assay. The IC_{50} value was determined to 60 μM, which corresponds to a K_i of ∼23 μM (see Table 4). Comparing the K_i of the OTP Sepharose and the K_i values of compounds 1–3 (see Table 1), an apparent four–five-fold potency loss is found to the parent OTP compound 1 and to the substituted compound 3. The K_i of compound 2 is comparable to the K_i of the OTP Sepharose. Several explanations for the apparent loss of potency can be given. Thus, the coupling density of OTP may be too high, i.e. only a fraction of the coupled OTP molecules may be available for

Table 1
Inhibition constants at pH 7.0 and 25 °C (K_i values in μM)

Enzyme	Compound 1	Compound 2	Compound 3
PTP1B	4.3 ± 0.3	20.4 ± 1.1	6.0 ± 0.4

Table 2
Affinity chromatography of PTP1B. Mass balance scheme

	Volume (ml)	OD_{280}[a] (mg/ml)	Total protein (mg)	Total activity (μmol/min)	Specific activity (nmol/(min mg))	Recovery (%)
Crude extract	9.4	61.0	573.5	231	403	100
OTP affinity column	21	0.39	8.1	165	20263	71
Gel filtration	33	0.18	6.0	122	20467	52

[a] The protein concentrations were calculated using molar absorption coefficients of 1 ml cm^{-1} mg^{-1} for the crude extract and 0.8 ml cm^{-1} mg^{-1} for the OTP affinity column and gel filtration (calculated from the sequence of PTP1B using the program Vector NTI), respectively.

PTP1B binding and diffusion limitations may play a role too. Indeed, the capacity of the OTP Sepharose was determined to be 7 mg PTP1B per ml OTP Sepharose corresponding to 0.2 μmol PTP1B versus 14.8 μmol OTP/ml Sepharose, showing that only a fraction of the coupled OTP molecules bind PTP1B. In addition, a negative entropy contribution for binding of PTP1B can be expected by the imposed restraining of OTP to the Sepharose matrix. Finally, the epoxy spacer arm may negatively influence binding of PTP1B. In agreement with this notion, we have recently shown that most OTP derivatives with substitutions at the secondary amine inhibits PTP1B with lower potency than OTP with several compounds having K_i values above 20 μM [10].

To purify PTP1B using the OTP Sepharose, crude, cleared supernatant from disrupted *E. coli* cells was loaded onto the OTP Sepharose column as described in Section 2. A single peak was eluted from the column (Fig. 3). The purification process was monitored by enzyme activity assays using *p*NPP as substrate and by SDS-PAGE. The enzyme activity profile of the peak fractions (not shown) corresponds to the OD_{280} trace from the OTP Sepharose column, demonstrating PTP1B to be located in these fractions. The SDS-PAGE from the purification of PTP1B is shown in Fig. 4 and PTP1B is eluted from the OTP Sepharose as a single band, thus being 99% pure. Hence, the OTP Sepharose represents a one-step purification method for PTP1B. Importantly, the method is extremely efficient with an overall yield of 71% (Table 2). Previously, Hoppe et al. [16] reported purification of recombinant PTP1B (termed the classical method) using yellow-86 dye adsorption- and Q-HiLoad ion-chromatography with a yield of 14%, proving the OTP Sepharose to be five times as efficient as the classical purification method (with comparable purity between the two methods, not shown). The one-step OTP Sepharose purification compared to the classical purification method represents an obvious move forward in purification technology. Another widely used method for rapid protein purification is to use a tagged version of the protein of interest. His tags and glutathione S-transferase (GST) tags have been used for the PTP purification process for a number of years. These tags often have to be removed, especially when the protein is intended for high resolution X-ray analysis. As an example, PTPα crystals could only be grown after digestion of a GST-PTPα fusion protein with trypsin to liberate the catalytic PTP domain [17,18]. However, the reaction mixture normally has to be purified after the enzymatic digestion to give the final product, thus adding process steps and thereby lowering the overall yield. Further, efficient proteolytic cleavage of the fusion protein is not always possible.

In vitro and in vivo oxidation of the active site cysteine of PTPs can lead to three different inactive enzyme species [19–21]. The active site affinity purification approach described here assures full active site functionality of the purified enzyme since OTP (and OTP Sepharose) only binds to an intact active site pocket of PTP1B. This cannot be achieved using the classical purification method or a tag-based method where the active site pocket of the enzyme is not used for purification at any point during the enzyme isolation. The specific activities of the PTP1B purified by the classical method and OTP Sepharose were measured to be 13815 and 20263 nmol/(min mg), respectively (not shown). The higher specific activity of the OTP Sepharose enzyme clearly underlines the above addressed active site cysteine reactivity and highlights that a significant proportion of PTP1B is without activity (or with lowered activity) when purified by the classical purification method. Thus, the OTP affinity purification ensures a homogeneous enzyme preparation, which is of significant importance in structure-based drug design (or other types of enzyme experiments).

Recombinant purified PTP1B has been used in a wide range of technologies in our structure-based drug design program. Especially, protein crystallization and isothermal titration calorimetry represent a challenge with respect to protein amounts, purity, and functionality. For crystallization purposes, the pooled PTP1B fractions from the OTP Sepharose were subsequently passed onto a Superdex 200 size exclusion chromatography (SEC) column for buffer exchange (crystallization buffer) and thereafter concentrated. Subsequent co-crystallization experiments with OTP showed that large crystals (of data collection quality) could easily be grown (see Fig. 5) demonstrating the high quality of this purification method.

The final product was further characterized by MALDI MS. The M_w was found to be 37.393 Da in agreement with the theoretical mass calculated to be 37.387 Da (corresponding to the mass of PTP1B residues 1–321 with one bound 2-mercaptoethanol molecule (to cysteine 92)). N-terminal sequencing of the first 10 amino acids confirmed the correct N-terminal sequence to be Met-Glu-Met-Glu-Lys-Glu-Phe-Glu-Gln-Ile. The enzyme was further characterized

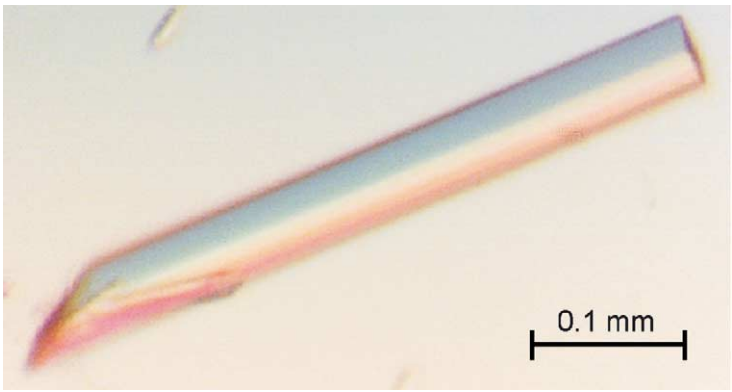


Fig. 5. Crystallization of PTP1B after purification. The crystal has the dimensions 400 μm × 50 μm × 50 μm.

Table 3
Kinetic constants for the hydrolysis of *p*-nitrophenylphosphate at pH 7.0 and 25 °C

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (10^{-3}) ($s^{-1}\,M^{-1}$)
PTP1B	19.7 ± 1.0	0.62 ± 0.03	31.8

Table 4
IC_{50} and K_i determination of OTP Sepharose

Enzyme	IC_{50} (μM)	*S* (mM)	K_i (μM)
PTP1B	60 ± 5	1	23

by detailed enzyme kinetic experiments. Although direct comparison is not possible due to different assay conditions, the measured k_{cat}, K_m and K_i values (see Tables 1 and 3) are in agreement with previously published values [10] and comparable with other data in the literature [22–24].

This OTP Sepharose method has been a valuable tool in our research program on PTP1B. As mentioned, OTP is also a potent TC-PTP inhibitor (similar potency as for PTP1B). We recently published the X-ray structure of TC-PTP, which was purified according to the current OTP procedure [12]. The OTP Sepharose might not be limited to purification of recombinant PTPs. In theory, OTP Sepharose could be useful in other situations where isolation of PTPs, e.g. from tissue samples pull down, is needed.

4. Conclusions

PTP1B (and TC-PTP) can be purified in one step to nearly 99% purity by the use of a highly selective inhibitor (OTP) coupled to Sepharose. The purified enzyme possesses all required properties for use in a structure-based drug design program. In a medium to long term drug discovery program, it can be beneficial to identify and use ligands for active site affinity purification purposes, since the affinity method in most cases will prove to be more efficient than both conventional and tag-based methods.

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