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Continuous assay of protein tyrosine phosphatases based on fluorescence resonance energy transfer

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An assay method that continuously measures the protein tyrosine phosphatase (PTP)-catalyzed dephosphorylation reaction based on fluorescence resonance energy transfer (FRET) was developed as an improvement of our previously reported discontinuous version [M. Nishikata, K. Suzuki, Y. Yoshimura, Y. Deyama, A. Matsumoto, *Biochem. J.* 343 (1999) 385-391]. The assay uses oligopeptide substrates that contain Mca [(7-methoxycoumarin-4-yl)acetyl] group as a fluorescence donor and DNP (2,4-dinitrophenyl) group as a fluorescence acceptor, in addition to a phosphotyrosine residue located between these two groups. In the assay, a PTP solution is added to a buffer solution containing a FRET substrate and chymotrypsin. The PTP-catalyzed dephosphorylation of the substrate and subsequent chymotryptic cleavage of the dephosphorylated substrate results in a disruption of FRET, thereby increasing Mca fluorescence. In this study, we used FRET substrates that are much more susceptible to chymotryptic cleavage after dephosphorylation than the substrate used in our discontinuous assay, thus enabling the continuous assay without significant PTP inactivation by chymotrypsin. The rate of fluorescence increase strictly reflected the rate of dephosphorylation at appropriate chymotrypsin concentrations. Since the continuous assay allows the measurement of initial rate of dephosphorylation reaction, kinetic parameters for the dephosphorylation reactions of a FRET substrate by *Yersinia*, T-cell and LAR PTPs were determined. The continuous assay was compatible with the measurement of very low PTP activity in a crude enzyme preparation and was comparable in sensitivity to assays that use radiolabeled substrates.

1. Introduction

Phosphorylation of tyrosine residues of cellular proteins is a major mechanism of signal transduction. The phosphorylation status of these proteins is regulated by the coordinated activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) [1-3]. PTP activity is usually measured using phosphotyrosine-containing peptides or proteins as specific substrates. The assay that measures radioactivity liberated from ^{32}P - or ^{33}P -labeled substrates is probably the most commonly used. This assay is highly sensitive, but it requires separation steps and the use of radioisotopes is problematic. Another commonly used assay relies on the formation of a colored complex between malachite green and phosphomolybdate [4]. This assay is non-radioisotopic, but low in sensitivity and distorted by endogenous phosphate in crude samples. Furthermore, the radioisotopic and colorimetric assays only allow discontinuous (end-point or fixed time) assay format. Although the discontinuous assay may be the easiest way to perform many assays at one time, the observed dephosphorylation rate does not necessarily reflect true initial rate unless the dephosphorylation reaction occurs at a constant rate. These assays are therefore especially disadvantageous to kinetic studies where precise initial rate measurement is essential. Further, very low enzyme activities cannot be measured reliably due to relatively high background radioactivity or absorbance. Continuous PTP assays, on the other hand, allow real-time observation of dephosphorylation reaction, making it easy to determine initial rates rapidly. Dephosphorylation reaction by PTPs can be monitored continuously with small chromogenic or fluorogenic substrates such as *p*-nitrophenylphosphate; however, this type of substrate is artificial and lack specificity for PTPs. In an assay using phosphotyrosine-containing peptides as specific substrates for PTPs, dephosphorylation reaction can be monitored continuously in terms of an increase in absorbance or fluorescence [5,6]. However, a major limitation of this assay results from the fact that proteins in crude enzyme preparations or proteins added as enzyme stabilizers interfere with the measurements, since the assay is conducted at a wavelength where proteins absorb and fluoresce. Therefore, it is strongly desired to develop a new continuous PTP assay that is non-radioisotopic, highly sensitive, and compatible with the presence of inorganic phosphate and proteins.

Previously, we have developed a new fluorimetric assay system for PTPs [7]. This assay takes advantage of the principles of fluorescence resonance energy transfer (FRET) and a coupled enzyme reaction. The substrate used in this assay is a phosphotyrosine-containing oligopeptide, Mca-Gly-Asp-Ala-Glu-Tyr(PO_3H_2)-Ala-Ala-Lys(DNP)-Arg-NH₂ (**I**), where the Mca

[(7-methoxycoumarin-4-yl)acetyl] group serves as a fluorescence donor and the DNP (2,4-dinitrophenyl) group serves as a fluorescence acceptor. Thus, the fluorescence of the Mca group is efficiently quenched by the DNP group by FRET. Although PTP-catalyzed dephosphorylation of the substrate does not change the fluorescence practically, subsequent treatment of the dephosphorylated form of the substrate with chymotrypsin results in a considerable increase in fluorescence as a result of disruption of FRET. Chymotrypsin was chosen as a coupling enzyme because this protease does not cleave the phosphotyrosyl bond but cleaves the tyrosyl bond. Using this assay system, Haase and Maret [8] clearly demonstrated using glial cells that the insulinomimetic effect of zinc is due to its capacity to inhibit PTPs, and Zhao and Bokoch [9] showed the ability of TNF- α to suppress PTP activity in neutrophils. Although this assay is specific, non-radioisotopic, homogeneous, as sensitive as radioisotopic assay methods, and compatible with inorganic phosphate and proteins, it suffers from the fact that the assay is performed in a discontinuous format. It is expected that addition of a PTP solution to the buffer solution containing both chymotrypsin and a phosphorylated substrate will enable continuous measurement of dephosphorylation. We examined this possibility in a preliminary experiment using a *Yersinia* PTP preparation and substrate I [7]. However, the PTP was found to be considerably unstable at chymotrypsin concentrations appropriate for the continuous assay (conditions where chymotryptic cleavage reaction is not rate-limiting). Since we noticed that the chymotrypsin concentration in the assay mixture must be reduced considerably for the continuous assay, we made an attempt to synthesize new substrates suitable for continuous assays, i.e., those with much higher susceptibility to chymotrypsin after dephosphorylation. We show in this paper a continuous PTP assay procedure as an improvement of our discontinuous version [7] using newly synthesized FRET substrates.

2. Materials and methods

2.1. Materials

The following materials were obtained from indicated sources: chymotrypsin (from bovine pancreas, three-times crystallized) (Worthington); *Yersinia* PTP (recombinant Yop51*), T-cell PTP (recombinant TC Δ C11) and LAR PTP (recombinant leukocyte-antigen-related D1) (New England Biolabs); Me-3,4-dephostatin (Sigma); okadaic acid, microcystin LR and calyculin A (Wako); ion-exchange chromatography media (POROS CM/M and POROS 50D) (PerSeptive Biosystems). MT3T3-E1 cell homogenate was prepared as described previously [10]. MT3T3-E1 cell cytosol fraction

was prepared from the cell homogenate by removing insoluble materials by centrifugation ($30,000 \times g$, 50 min). ^{32}P -Myelin basic protein was prepared according to the instruction manual for Protein Tyrosine Phosphatase (PTP) Assay System (New England Biolabs). FRET substrates were synthesized as described previously [7] except for the use of Fmoc-Tyr(PO(OBzl)OH) instead of Fmoc-Tyr(PO₃H₂) as a building block. Amino acid and mass spectral analyses of the substrates gave expected results after purification by HPLC. The substrates were dissolved in dimethylsulfoxide/dimethylformamide (1:1, v/v) and stored in a refrigerator as a stock solution (usually 100 μ M).

2.2. Methods

The amount of PTP was expressed by unit, not by weight or the number of molecule of active enzyme, since no information was available on the amount of active enzyme in commercial preparations of PTP. Phosphatase unit was determined as follows. To 20 mM 3,3-dimethylglutarate buffer (pH 6.6) containing 10 mM *p*-nitrophenylphosphate and 0.01% BSA, a negligible volume of enzyme solution was added. The reaction was allowed to proceed at 25°C and the release of *p*-nitrophenol was monitored continuously at 405 nm. One unit of the enzyme activity was defined as the amount of enzyme required to release 1 nmol of *p*-nitrophenol per min under the above conditions. The molar absorption coefficient of 4,900 was used to calculate the concentration of *p*-nitrophenol at pH 6.6.

Dephosphorylation reaction of FRET substrates was carried out in a 5-mm \times 5-mm square quartz microcuvette. To 196 μ l of buffer solution containing a FRET substrate and 0.01% BSA, 2 μ l of chymotrypsin in 1 mM HCl was added. A PTP solution (2 μ l) was then added and fluorescence intensity was recorded continuously with a Hitachi F-3000 fluorescence spectrophotometer at excitation and emission wavelengths of 328 and 395 nm, respectively. Bandwidths were set at 1.5 nm (ex.) and 40 nm (em.). The reaction was carried out in 20 mM 3,3-dimethylglutarate (pH 6.6) at 25°C unless otherwise stated.

Kinetic parameters were determined from the direct linear plot of Eisenthal and Cornish-Bowden [11]. The best estimates of V_{\max} and K_m were taken as the medians of the two sets of estimates of V_{\max} and K_m , respectively. The mean and standard deviation of each set of estimates were also evaluated in order to estimate the amount of scatter of the data.

3. Results

3.1. Assay principle and optimization

Our continuous PTP assay procedure is based on a general coupled enzyme reaction in which one coupling enzyme is involved [12,13]. The reaction scheme is represented as shown in Scheme 1. A and B represent a phosphorylated substrate and its dephosphorylated form, respectively; C and D represent chymotryptic cleavage products of the second reaction, a fluorescent Mca-peptide and a DNP-peptide, respectively. k_1 and k_2 are rate constants of the primary and the second reactions, respectively. For the rate of fluorescence increase (formation of C) to be a true reflection of the rate of Pi formation, (a) the primary reaction (the reaction of interest) must be irreversible and zero-order with respect to [A], and (b) the following chymotryptic cleavage reaction must be irreversible and first-order with respect to [B]. When these conditions are met, [C] increases at the same rate as Pi formation after a short transient time or lag (τ). Condition a is met since only a small fraction of A is dephosphorylated at least during the early assay period and B is continuously cleaved by chymotrypsin. Condition b is met since the equilibrium lies substantially to the right and the steady state concentration of B ($[B]_{ss}$) can be kept much lower than the K_m value for the second reaction. Practically, this can be done by keeping the transient time negligibly short using sufficient amounts of chymotrypsin since $\tau = 1/k_2$ and $[B]_{ss} = k_1/k_2$.

We synthesized several FRET substrates and examined their susceptibility to chymotrypsin after dephosphorylation (usually with a large amount of *Yersinia* PTP) at a substrate concentration of 1 μ M. We selected three promising substrates based on the results of chymotryptic cleavage experiment. Their structures are as follows; Mca-Gly-Glu-Gly-Thr-pTyr-Gly-Lys(DNP)-Arg-NH₂ (**II**), Mca-Gly-Asp-Gly-Val-pTyr-Ala-Ala-Lys(DNP)-Arg-NH₂ (**III**) and Mca-Gly-Ser-Ala-pTyr-Gly-Lys(DNP)-Arg-NH₂ (**IV**), where pTyr denotes phosphotyrosine residue. The sequences are similar to those around a phosphotyrosine residue in the protein kinases pp34 and p85^{gag-fes}, and lipocortin II, respectively [14]. Arg was introduced to ensure solubility. The dephosphorylated forms of substrates **II**, **III** and **IV** were cleaved by chymotrypsin 18, 13 and 50 times as fast as the dephosphorylated form of substrate **I**, respectively. To optimize assay conditions, we conducted a series of experiments using fixed concentrations of *Yersinia* PTP and substrate **III**, and various concentrations of chymotrypsin. Substrate **III** was chosen because it is, of the three substrates, the least susceptible to chymotrypsin after dephosphorylation and the assays with the other two substrates can be conducted under milder conditions. The PTP was added to the buffer solution containing substrate **III** and various concentrations of chymotrypsin, and the increase in fluorescence intensity

was recorded as shown in Fig. 1A. The rate of fluorescence increase, which corresponds to the chymotryptic cleavage rate of the dephosphorylated form of substrate **III**, was determined from the slope of the linear portion of each line. Very similar values were obtained between chymotrypsin concentrations of 0.005 and 0.05 mg/ml (Fig. 1A, lines a – d). In order to examine whether these values represent the initial rate of substrate dephosphorylation, we measured it by our original discontinuous assay method (30-s incubation of substrate **III** with *Yersinia* PTP). Comparison of the data (not shown) from the two different assay methods (continuous and discontinuous) lead to the conclusion that the value obtained by the continuous assay method is a true reflection of dephosphorylation rate. When the chymotrypsin concentration was raised to 0.2 mg/ml, much lower initial rate was obtained (Fig. 1A, line e). This is probably due to the inactivation of the PTP by chymotrypsin. A short lag period of fluorescence increase was obvious at a low chymotrypsin concentration (Fig. 1, line a). The fact that this lag period did not affect the measurement of initial dephosphorylation rate implies that the steady state concentration of dephosphorylated substrate **III** is much lower than the K_m value for the chymotryptic cleavage reaction. Lines b – e in Fig. 1A have almost no lags, so these lines are real-time traces of the dephosphorylation reaction. The effect of chymotrypsin concentration on the initial rate of fluorescence increase was also examined with T-cell and LAR PTPs. Figure 1B shows the results obtained with the three PTPs. Although the stability to chymotrypsin seems to be different among the three PTPs, they were all stable at chymotrypsin concentrations up to 0.05 mg/ml at least during the initial rate measurement. From a practical point of view, the lag period should be as short as possible. Thus, the chymotrypsin concentration should preferably be in the range of 0.01 – 0.05 mg/ml for substrate **III**. For substrates **II** and **IV**, the lower limits of chymotrypsin concentration may be reduced to 0.007 and 0.003 mg/ml, respectively, judging from their susceptibility to chymotrypsin after dephosphorylation.

3.2. Determination of kinetic parameters

The dependence of the initial dephosphorylation rate on PTP concentration was examined and is shown in Fig. 2. The strict linearity of the plot makes our continuous assay method a reliable tool for kinetic studies. Thus, kinetic parameters for the dephosphorylation of substrates **II**, **III** and **IV** were determined using *Yersinia*, T-cell and LAR PTPs. The continuous assays were performed at several substrate concentrations (0.5 – 20 μ M) at a chymotrypsin concentration of 0.025 mg/ml. Essentially no lag period was observed at any substrate concentrations. The fluorescence measurement is affected by quenching due mainly to an inner filter effect

especially at high substrate concentrations. The increase in fluorescence intensity was therefore corrected for this effect as described previously [7]. A typical direct linear plot [11] for the T-cell PTP-catalyzed dephosphorylation of substrate **III** is shown in Fig. 3. Results of the kinetic experiments are summarized in Table 1.

Figure 4 shows the rates of dephosphorylation of a fixed concentration (1 μ M) of substrates **II**, **III** and **IV** measured by the continuous assay method using the three PTPs. For comparison, results for substrate **I** obtained previously by our original discontinuous assay method [7] are also presented. Each enzyme dephosphorylated the four substrates at quite different rates. Substrate **IV** was the poorest substrate for all the enzymes. The low reactivity of substrate **IV** may be related to the fact that it is the shortest substrate and has no Glu or Asp residues. Preferences for negatively charged residues N-terminal to phosphotyrosine residue have been reported for some PTPs [15-19]. T-cell PTP is seemingly the most active toward all the substrates. However, this does not necessarily mean that this enzyme has the highest catalytic efficiency since the data presented in Fig. 4 are dephosphorylation rates per unit of enzyme and not per molecule of enzyme. Therefore, it is almost meaningless to compare dephosphorylation rates between the PTPs.

3.3. Measurement of low activity in a crude enzyme preparation

A great advantage of the continuous assay method over the discontinuous one reported previously [7] is that even a small increase in fluorescence intensity can be measured accurately. For example, when a homogenate of MC3T3-E1 osteoblastic cells (corresponding to 100 cells) was allowed to react with substrate **III**, for which the homogenate showed the highest activity, a very small but apparently measurable increase in fluorescence intensity was observed (Fig. 5). This increase corresponded to the dephosphorylation of 150 pM per min, which translates into 30 fmol in the assay mixture (200 μ l). This sensitivity compares favorably with that of commonly used radioisotopic assay methods. To examine the specificity of the method, dephosphorylation of substrate **III** by a much larger amount of the homogenate was measured in the presence of various phosphatase inhibitors (Fig. 6). The osteoblastic dephosphorylation activity was not inhibited by 0.1 mM levamisole, an inhibitor of alkaline phosphatase from the osteoblastic cells [20], but almost completely inhibited by 0.1 mM vanadate, a PTP inhibitor [21], or 0.5 mM Me-3,4-dephostatin, a more specific PTP inhibitor [22]. Okadaic acid, a strong inhibitor of protein phosphatase 2A [23], calyculin A and microcystin LR, both strong inhibitors of protein phosphatases 1 and 2A [23], had no inhibitory activity at 1 μ M. Our FRET substrates are peptides, so there is

always a fear of degradation by endopeptidases in crude samples, which results in an increase in fluorescence. However, incubation of substrate **III** with the cell homogenate did not increase fluorescence intensity (data not shown). These findings demonstrate that our assay is insensitive to alkaline phosphatase activity and nonspecific activities, if any, of protein phosphatases 1 and 2A, and hence can be used as a highly specific method for the measurement of PTP activity in crude enzyme preparations.

3.4. Investigation of pH dependence

Our continuous assay method can be used to investigate the pH dependence of PTP activity. The result obtained with MC3T3-E1 osteoblastic cell homogenate and substrate **III** is shown in Fig. 7. The concentration of chymotrypsin employed at each pH was determined so that it could cleave dephosphorylated substrate **III** at the same rate as 0.025 mg/ml chymotrypsin did at pH 6.6. The fluorescence intensity increased linearly at each pH, indicating that almost no inactivation of PTP activity occurred at least during the early assay period. To see if the Mca fluorescence is dependent on pH, we synthesized Mca-Gly-Asp-Gly-Val-Tyr, one of the two chymolytic cleavage products derived from substrate **III**, and measured its fluorescence intensity at various pHs. Practically the same fluorescence intensity was observed at least between pH 5.8 and 8.6 (data not shown), and hence no correction for the observed fluorescence intensity was necessary at this pH range. Previously, we obtained similar pH-activity curves using ^{32}P -Raytide and ^{32}P -RCM (reduced, carboxamidomethylated and maleylated) lysozyme as substrates [10].

3.5. Monitoring of purification

Another example of the application possibility of our continuous assay method is the use in monitoring PTP purification. We partially purified some PTPs from MC3T3-E1 osteoblastic cell cytosol fraction by a combination of cation-exchange (POROS CM/M) and anion-exchange (POROS 50D) column chromatography steps. At each step, PTP activity in the column effluents was measured with substrate **III**. For comparison, we also used a radioisotopic assay with ^{32}P -myelin basic protein as a substrate. The two assay methods gave comparable results at each step. A POROS 50D column chromatographic profile is shown as a representative in Fig. 8. Our method could measure about 20 samples in 1 h. The radioisotopic assay, on the other hand, which required incubation with substrate, stopping the reaction by acid, centrifugation and counting the radioactivity in the supernatant took about 90 min to treat similar number of samples.

4. Discussion

In this paper, we presented a FRET-based assay method that measures PTP-catalyzed dephosphorylation reaction continuously. The method uses a peptide substrate that contains a fluorescent donor (Mca group) and a fluorescent acceptor (DNP group), in addition to a phosphotyrosine residue located between these groups. The continuous assay method was developed by using peptide substrates that are much more susceptible to chymotryptic cleavage after dephosphorylation than the substrate (substrate I) used in our original discontinuous method [7]. The continuous method was found to be especially useful in the determination of kinetic parameters and in the determination of very low PTP activity in crude enzyme preparations.

After we presented our discontinuous assay method for PTPs [7], similar assay methods that use phosphotyrosine-containing peptide substrates were reported. Kupcho et al. [24] presented a fluorogenic assay method that uses a rhodamine 110 derivative in which two phosphotyrosine-containing peptides are conjugated to the rhodamine 110 molecule via its amino groups. When the substrate is dephosphorylated, it becomes sensitive to cleavage by aminopeptidase, releasing free rhodamine 110, which is highly fluorescent. On the other hand, when the substrate is in the phosphorylated form, it is resistant to aminopeptidase digestion and remains nonfluorescent. Since aminopeptidase is an exopeptidase that degrades a peptide stepwise from the N-terminus, it takes time to completely remove the peptide portion from the substrate. In fact, they only presented a discontinuous assay method and the rhodamine 110 fluorescence was read 90 min after the addition of aminopeptidase. To our surprise, Rodems et al. [25], without citing our work [7], presented an assay method which is based on a principle almost identical to that of our original discontinuous assay method [7]. Their peptide substrates contained coumarin as a fluorescence donor and fluorescein as an acceptor instead of Mca and DNP in our peptide substrates. They also used chymotrypsin to cleave the dephosphorylated peptide. However, they only presented a discontinuous assay method. To date, our assay method presented in this paper is therefore the only method that continuously measures PTP activity both in purified and in crude enzyme preparations.

In this paper, we have described a method to measure PTP activity in a single cuvette set in a fluorescence spectrophotometer. In this format, many samples such as column effluents could be measured in relatively short time (Fig. 8). However, the cuvette format is not suitable for high-throughput measurement of a large number of samples because it takes several hours to complete 100 measurements. We therefore examined, in a preliminary experiment, the possibility that our assay principle can be

applied to high-throughput measurement in a microplate format. We measured the initial rates of dephosphorylation of substrate **III** at various concentrations of *Yersinia* PTP in a 96-well microplate. The reaction was started by the addition of 2 μ l of PTP solution to 198 μ l of buffer containing substrate (1 μ M) and chymotrypsin (0.02 mg/ml). The increase in fluorescence intensity was measured using a fluorescence plate reader in a kinetic mode. The initial rate was strictly proportional to the PTP concentration with a high reproducibility and no special difficulties were encountered in performing the assay (not shown). Thus, although more data are required, it seems likely that our assay principle can easily be applied to a microplate assay format, enabling high-throughput screening of PTPs and their inhibitors.

In general, researchers rebel at addition of a protease to an enzyme assay mixture since this may lead to inactivation of the enzyme of interest. However, we demonstrated that our continuous assay could be performed without significant inactivation of PTPs even in the presence of chymotrypsin if the protease concentration was optimized carefully. This is probably because the FRET substrates used in this study are extended in conformation and their primary structures are preferred by chymotrypsin as well. On the other hand, substrate **I**, which was used in our discontinuous assay [7], was not suitable as a substrate for continuous assay since its dephosphorylated form was a much poorer substrate of chymotrypsin. The sequence of substrate **I** is similar to that of substrate **III** except for the large difference in the nature of P₂ (nomenclature of Schechter and Berger [26]) amino acid (Glu vs. Val). Therefore, substrates with acidic amino acid in this position do not seem to be preferred by chymotrypsin. Likewise, according to extensive subsite mapping studies, P₃ Pro, P'₁ Asp, Glu and Pro, and P'₂ Pro are not preferred [27-29]. In addition to the three substrates used in this study, we synthesized Mca-Gly-Glu-Pro-Gln-pTyr-Gln-Pro-Lys(DNP)-Arg-NH₂. However, it was cleaved by chymotrypsin only very slowly after dephosphorylation (30 times more slowly than substrate **III**), and hence it was not employed as a substrate in this study. The low reactivity is probably due to the fact that it contains two Pro residues in the P₃ and P'₂ positions. Our discontinuous version [7], on the other hand, is compatible with any substrate sequence though it has critical drawbacks as described in Introduction.

It is generally accepted that PTP specificity is determined, at least in part, by the substrate's primary structure [30]. This was confirmed in this study by the finding that the reactivity of PTP was sensitive to the sequence of substrate (Table 1 and Fig. 4). Our continuous assay would therefore serve as a valuable tool not only for the fundamental and functional studies of PTPs but also for the development of highly specific substrates for a special PTP if FRET substrates with various primary structures were examined.

Although the above mentioned negative preferences of chymotrypsin for certain amino acids seem to limit the use of the assay, this may be tolerated since such amino acids are few in number and most amino acids can be incorporated at any position of the substrate. For the continuous assay to be compatible with any substrate primary structure, some modification of the assay protocol will be necessary. For example, use of a chymotrypsin derivative conjugated with polyethylene glycol instead of native chymotrypsin may be ideal for such a purpose, since, compared with native enzyme, the modified enzyme shows much reduced hydrolytic activity toward protein substrates while preserving the activity toward small peptide substrates [31]. The possibility of introducing a modified chymotrypsin for use as a coupling enzyme in the assay system is under investigation in our laboratory.

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Figure Legend

Scheme 1. Reaction scheme for the continuous PTP assay.

Fig. 1. Time course of fluorescence increase (A) and initial rate of fluorescence increase (B) in the presence of various concentrations of chymotrypsin. (A) Substrate **III** concentration was 1 μM . Final chymotrypsin concentrations were (a) 0.005 mg/ml, (b) 0.01 mg/ml, (c) 0.02 mg/ml, (d) 0.05 mg/ml and (e) 0.2 mg/ml. *Yersinia* PTP (2 μl , 0.043 unit) was added at the arrows. The fluorescence intensity when the substrate was completely dephosphorylated was determined by adding a large amount (~ 20 units) of *Yersinia* PTP. (B) Plots of initial rate of fluorescence increase vs. chymotrypsin concentration obtained with *Yersinia*, T-cell and LAR PTPs.

Fig. 2. Dependence of initial dephosphorylation rate on PTP concentration. Substrate **III** (1 μM) was allowed to react with various concentrations of *Yersinia* PTP in the presence of 0.025 mg/ml chymotrypsin.

Fig. 3. Direct linear plot for the PTP-catalyzed dephosphorylation reaction. The assay was performed with 0.026 unit/ml T-cell PTP in the presence of 0.025 mg/ml chymotrypsin and various concentrations (0.5 – 3 μM) of substrate **III**.

Fig. 4. Comparison of the dephosphorylation rates of various substrates by different PTPs. Dephosphorylation of substrates **II**, **III**, **IV** was measured by the continuous assay method in the presence of 0.025 mg/ml chymotrypsin. Values are means and the standard deviations of four determinations. Results for substrate **I** are reported values obtained by the discontinuous assay method [7] and presented here for comparison.

Fig. 5. Dephosphorylation by osteoblastic cell homogenate. Substrate **III** (1 μM) was allowed to react with osteoblastic cell homogenate in the presence of 0.025 mg/ml chymotrypsin. The cell homogenate was added at the arrowhead. A small but steady increase in the fluorescence intensity before the addition of the cell homogenate is probably due to continuous UV irradiation [7].

Fig. 6. Effect of phosphatase inhibitors on the dephosphorylation activity of osteoblastic cell homogenate. Osteoblastic cell homogenate (corresponding to 20,000 cells) and a phosphatase inhibitor were preincubated for 3 min at 25°C in a total volume of 196 μl of

20 mM 3,3-dimethylglutarate (pH 6.6) containing 0.01% BSA in a quartz microcuvette. Then, 2 μ l of 2.5 mg/ml chymotrypsin and 2 μ l of 100 μ M substrate **III** were added in this order before measurement of fluorescence intensity. Values are means and the standard deviations of three determinations. The final concentrations of inhibitors are 0.1 mM (levamisole and vanadate), 0.5 mM (Me-3,4-dephostatin) and 1 μ M (okadaic acid, calyculin A and microcystin LR).

Fig. 7. pH dependence of osteoblastic PTP activity. Substrate **III** (0.5 μ M) was allowed to react with osteoblastic cell homogenate in the presence of 0.14 mg/ml (pH 5.8), 0.09 mg/ml (pH 6.2), 0.02 mg/ml (pH 7.0), 0.014 mg/ml (pH 7.5), 0.02 mg/ml (pH 7.9) and 0.02 mg/ml chymotrypsin (pH 8.6). Buffers used were 50 mM citrate (pH 5.8 and 6.2) and 50 mM Tris-HCl (pH 7.0-8.6).

Fig. 8. Monitoring of PTP activity in column effluents. An osteoblastic cytosol fraction in 10 ml of 10 mM Tris-acetate buffer (pH 6.0) containing 10% glycerol (about 1 mg/ml protein) was applied to a POROS CM/M column (4.6 \times 100 mm) equilibrated with the same buffer. PTP activity-containing fractions that had passed through the column were desalted by Hi Prep 26/20 column (Pharmacia). The PTP activity recovered in 10 mM Tris-acetate buffer (pH 8.0) was applied to a POROS 50D column (4.6 \times 100 mm) equilibrated with the same buffer and 2-ml fractions were collected. An elution pattern from the POROS 50D column is shown here. For the assay with substrate **III**, the concentrations of the substrate and chymotrypsin were 1 μ M and 0.02 mg/ml, respectively. Assay with ³²P-myelin basic protein was carried out according to the instruction manual for Protein Tyrosine Phosphatase (PTP) Assay System (New England Biolabs). Both assays were done in 50 mM Tris-HCl (pH 7.0) at 30°C.

Scheme 1

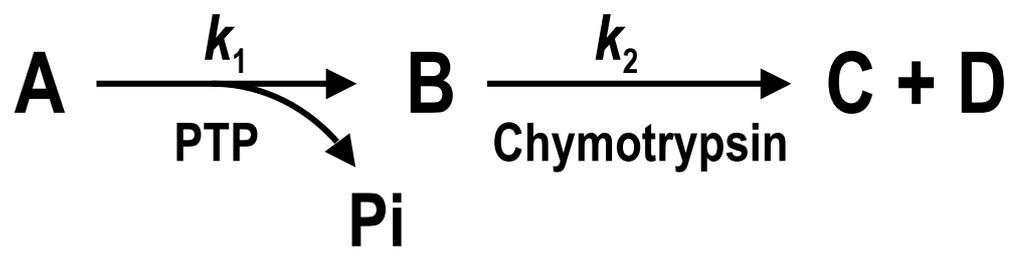


Fig. 1

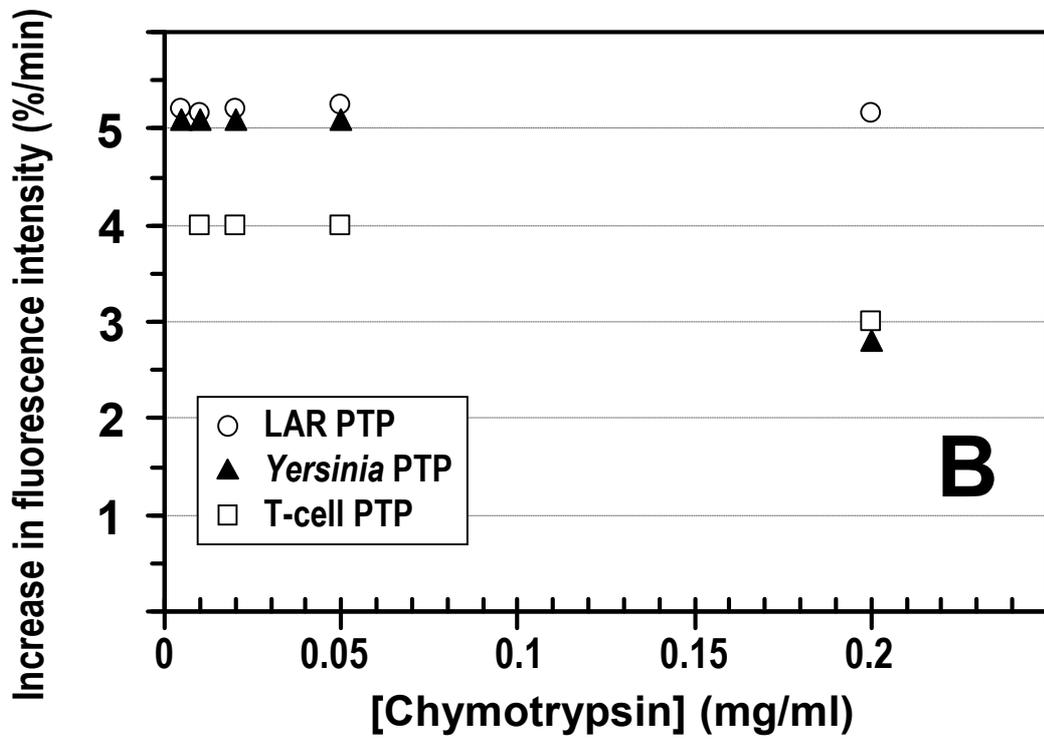
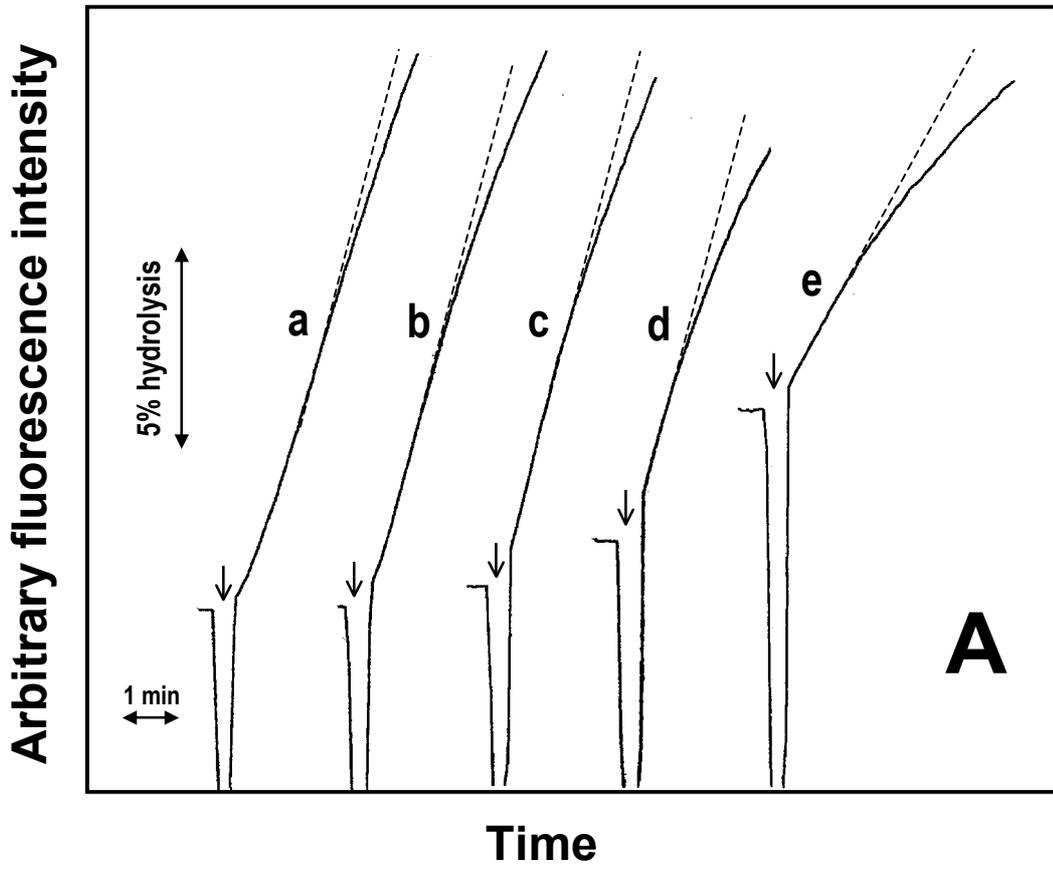


Fig. 2

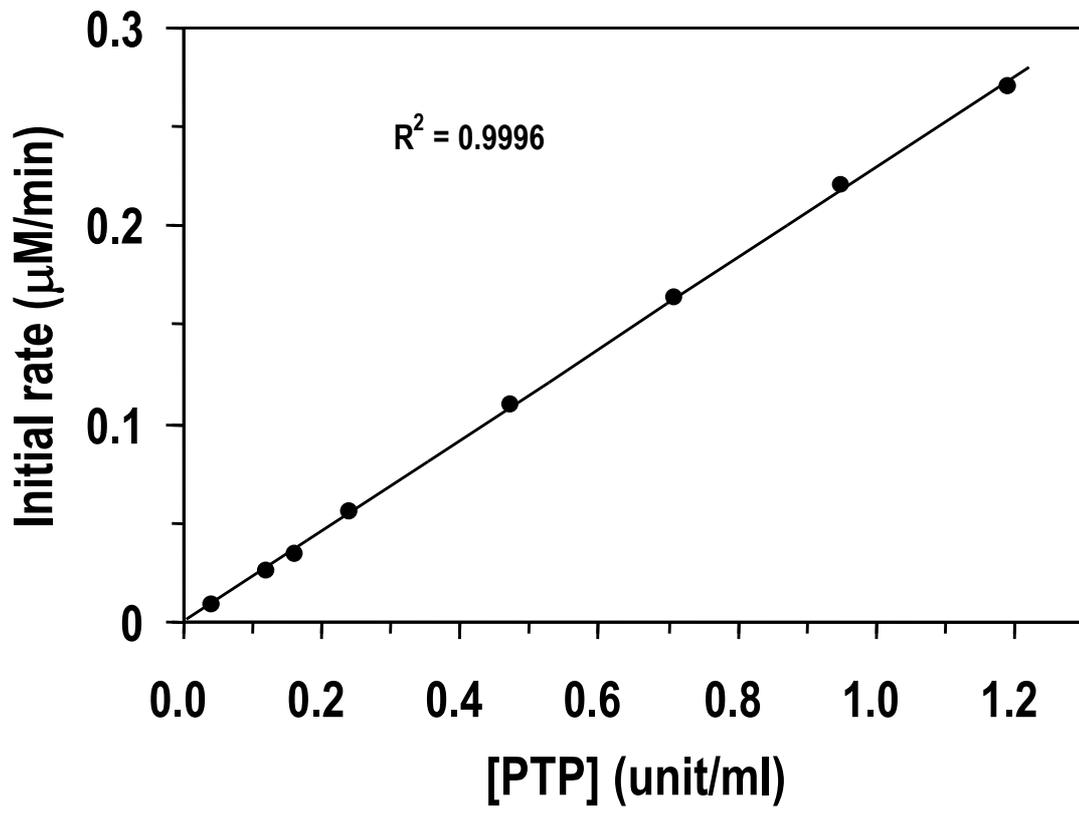


Fig. 3

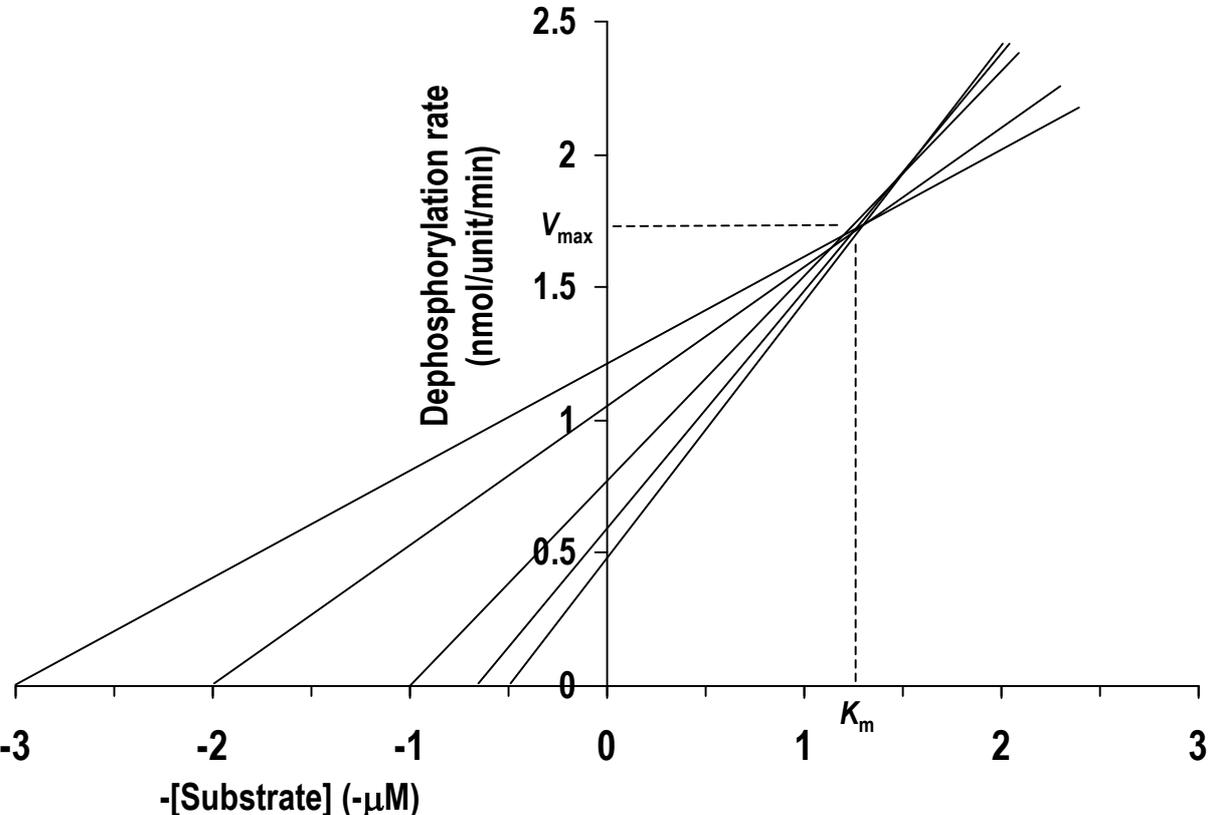


Fig.4

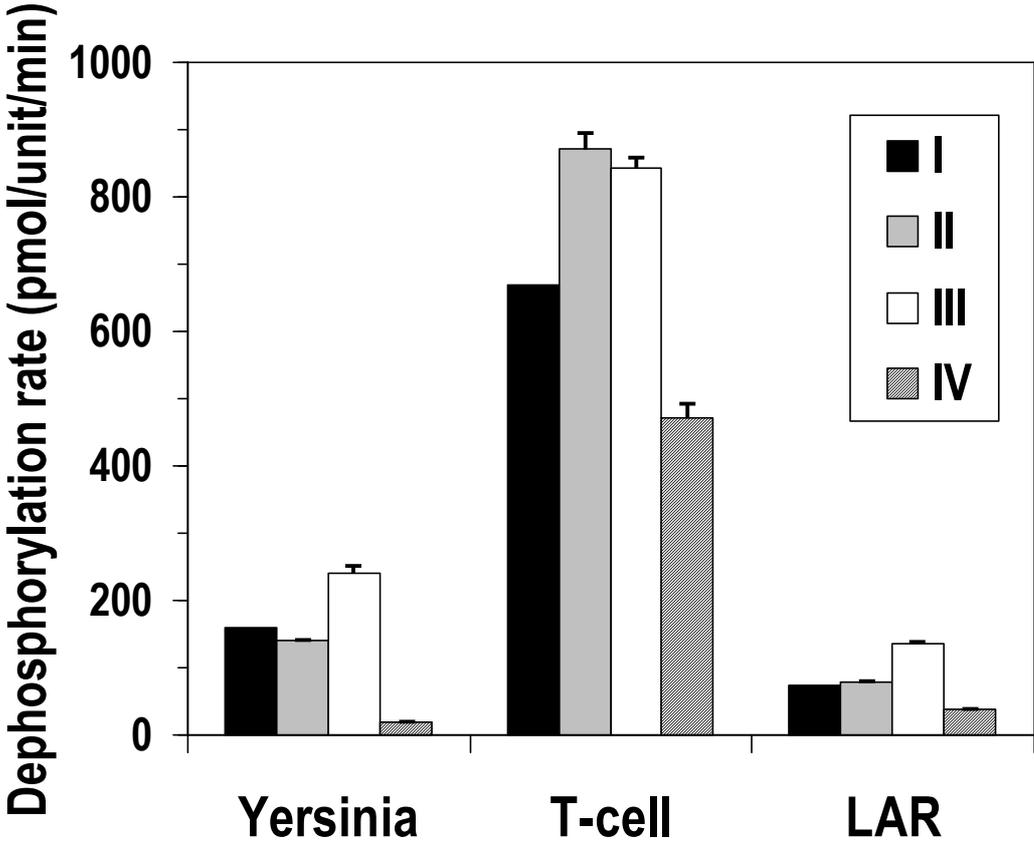


Fig. 5

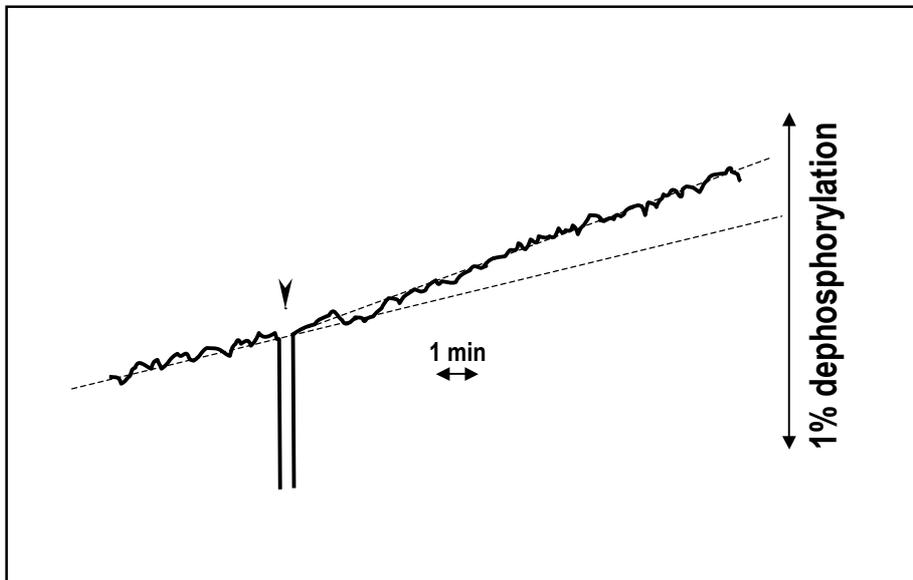


Fig. 6

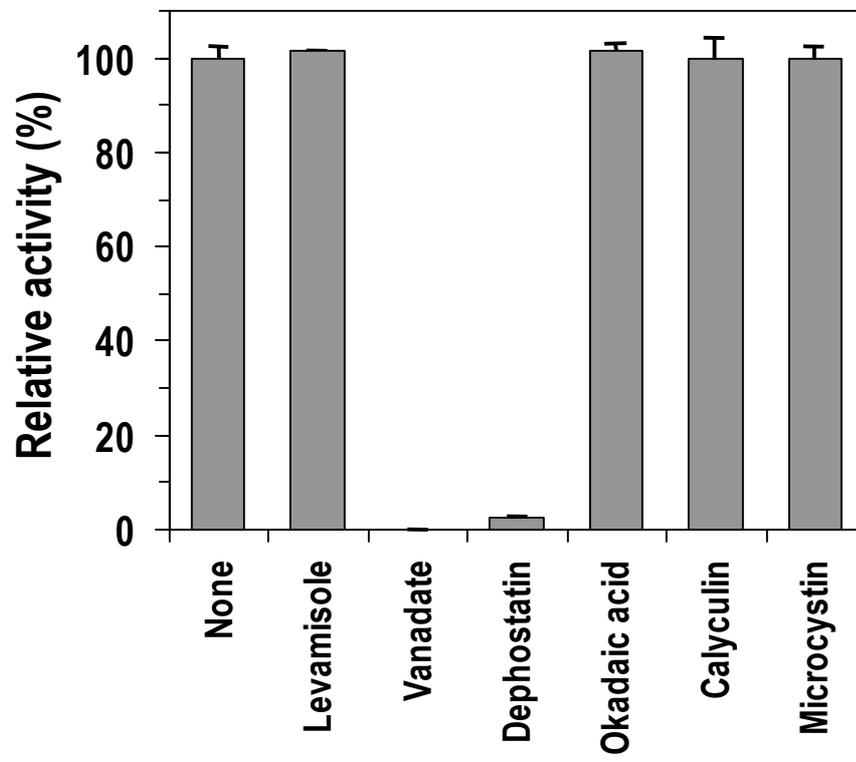


Fig. 7

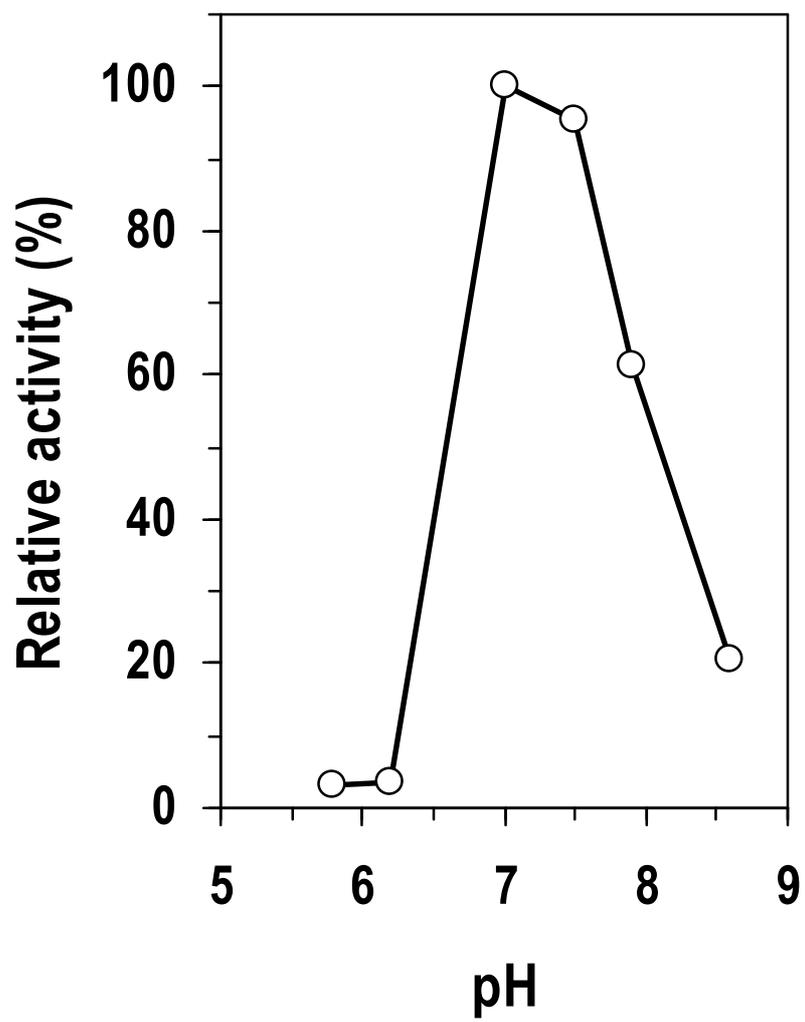


Fig. 8

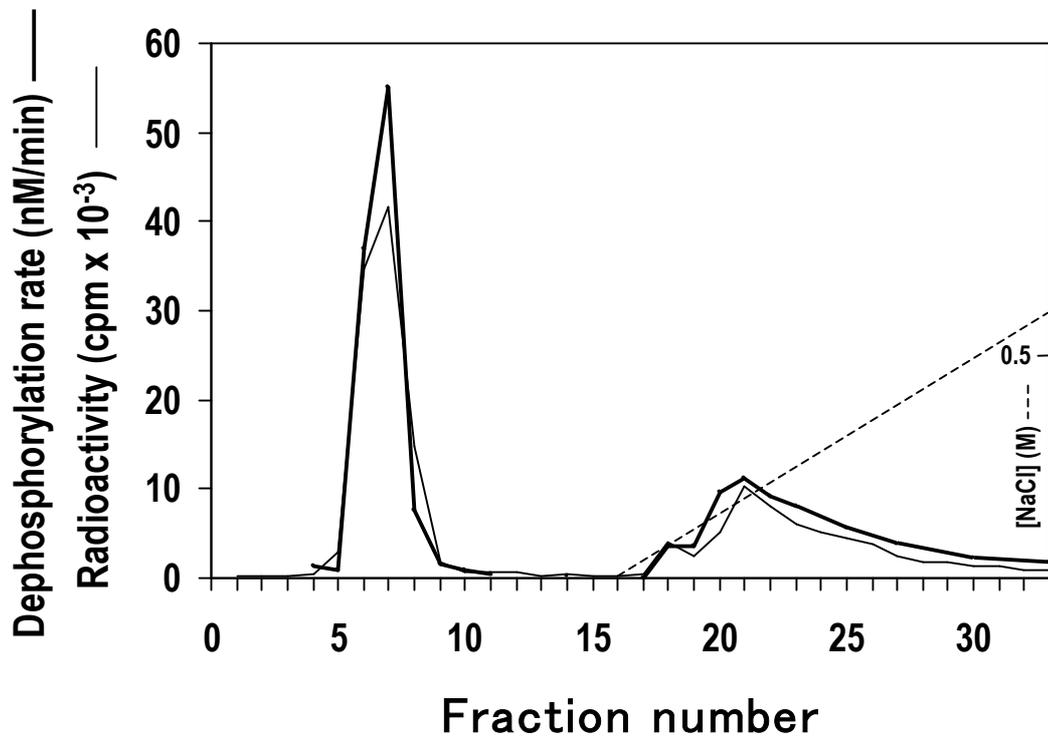


Table 1

Kinetic constants for the PTP-catalyzed dephosphorylation of FRET substrates. The units of K_m and V_{max} are μM and $\text{nmol}\cdot\text{unit}^{-1}\cdot\text{min}^{-1}$, respectively. The values of K_m and V_{max} represent the medians, and those in parentheses represent the mean and the standard deviation (see Methods).

Substrate	Kinetic constants	<i>Yersinia</i> PTP	T-cell PTP	LAR PTP
Substrate II	K_m	17.8 (17.3 \pm 2.7)	0.741 (0.740 \pm 0.091)	2.4 (51.0 \pm 3.7)
	V_{max}	2.25 (2.22 \pm 0.23)	1.28 (1.29 \pm 0.04)	3.66 (3.56 \pm 0.23)
Substrate III	K_m	11.5 (11.5 \pm 1.5)	1.27 (1.29 \pm 0.11)	4.39 (4.45 \pm 0.19)
	V_{max}	3.10 (3.12 \pm 0.30)	1.73 (1.76 \pm 0.09)	1.07 (1.08 \pm 0.03)
Substrate IV	K_m	10.9 (11.1 \pm 2.3)	2.16 (2.24 \pm 0.36)	8.32 (8.38 \pm 1.17)
	V_{max}	0.228 (0.233 \pm 0.038)	1.26 (1.30 \pm 0.14)	0.425 (0.434 \pm 0.040)