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Effects of Polyethylene Glycol on Bovine Intestine Alkaline Phosphatase Activity and Stability

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In this study, we evaluated the effects of polyethylene glycol (PEG) on bovine intestine alkaline phosphatase (BIALP) activity and stability. In the hydrolysis of *p*-nitrophenylphosphate (pNPP) at pH 9.8 at 20 °C, the k_{cat}/K_m values of BIALP plus 5–15% w/v free PEG with molecular masses of 1, 2, 6, and 20 kDa (PEG1000, PEG2000, PEG6000, and PEG20000 respectively) were 120–140%, 180–300%, 130–170%, and 110–140% respectively of that of BIALP without free PEG ($1.8\,\mu M^{-1}\,s^{-1}$), indicating that activation by PEG2000 was the highest. Unmodified BIALP plus 5% PEG2000 and BIALP pegylated with 2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine exhibited 1.3-fold higher activity on average than that of BIALP without free PEG under various conditions, including pH 7.0–10.0 and 20–65 °C. The temperatures reducing initial activity by 50% in 30-min incubation of unmodified BIALP plus 5% PEG2000 and pegylated BIALP were 51 and 47 °C respectively, similar to that of BIALP without free PEG (49 °C). These results indicate that the addition of PEG2000 and pegylation increase BIALP activity without affecting its stability, suggesting that they can be used in enzyme immunoassay with BIALP to increase sensitivity and rapidity.

Key words: alkaline phosphatase; bovine intestine alkaline phosphatase; enzyme immunoassay; pegylation; polyethylene glycol

Phosphatases (EC 3.1.3.1) catalyze the hydrolysis of phosphomonoesters. They are classified into two groups, as alkaline phosphatase (ALP) and acid phosphatase (ACP), depending on their optimal pH in alkaline and acidic pH regions respectively. ALPs are widely distributed in bacteria and mammals, and play essential roles in biochemical processes.[1–4] Their structural and functional properties are considered to be commonly conserved in all ALPs. ALP is a homodimeric metalloenzyme. The subunit has a molecular mass of about 50 kDa and contains two Zn^{2+} ions and one Mg^{2+} ion.[5,6] The catalytic triad composed of the two Zn^{2+} and one Mg^{2+} ions is conserved in all ALPs, from *Escherichia coli* to mammals.[7] Mammalian ALP is present in the liver, intestine, placenta, kidney, and other tissues. The molecular activity (k_{cat}) of mammalian ALP is 10–60 times higher than that of *E. coli* ALP.[8] Bovine intestine ALP (BIALP) has the highest specific activity among mammalian ALPs. It has been applied as a signaling enzyme in sensitive assays such as enzyme immunoassay (EIA), Western blotting analysis, nucleic acid hybridization assay, polymerase chain reaction, *etc.*, and is used in diagnosis, immunology, and molecular biology.[9–12] Therefore, increase in the activity and stability of BIALP is an important subject for practical use. We have found that amines and aminoalcohols activate BIALP.[13]

Polyethylene glycol (PEG) is a hydrophilic, biocompatible polymer. The covalent attachment of PEG to proteins (pegylation) is effective for advanced clinical uses. It drastically increases the serum half lives of therapeutic proteins administered to humans and decreases their immunogenicity. Pegylated adenosine deaminase[14] and asparaginase[15] have been approved for human use by the US Food and Drug Administration, but the effects of pegylation and the external addition of free PEG on the *in vitro* uses of enzymes have not well been described. In this study, we examined the effects of the external addition of free PEG and pegylation on BIALP activity and stability.

Materials and Methods

Materials. BIALP (lot 13609227) was purchased from Roche Diagnostics (Basel, Switzerland). The preparation was used without further purification. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) (lot ALN6692) was from Wako Pure Chemical (Osaka, Japan). *p*-Nitrophenyl phosphate (pNPP) (lot M9T3188) and monomethoxy PEG of 1, 2, 6, and 20 kDa (PEG1000, PEG2000, PEG6000, and PEG20000 respectively) were from Nacalai Tesque (Kyoto, Japan). The concentration of pNPP was determined spectrophotometrically using the molar absorption coefficient, ε_{310}, of $10{,}380\,M^{-1}\,cm^{-1}$.[13] 2,4-Bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (10 kDa) (mPEG) was from Seikagaku (Tokyo). BIALP-conjugated goat anti-rabbit IgG antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of reagent grade and were purchased from Nacalai Tesque or Wako Pure Chemical.

Preparation of pegylated BIALP. Pegylation of BIALP was accomplished by adding solid mPEG (160 mg) to 2.0 mL of 2.0 mg/mL of BIALP in 50 mM borate-NaOH, 1.0 mM $MgCl_2$, and 20 μM $ZnCl_2$ at pH 10.0. The reaction mixture was maintained at 20 °C for 2 h with continuous stirring and subjected to Amicon Ultra YM-30 (Amicon, Bedford, MA) using 30 mM triethanolamine-HCl, 3.0 M NaCl, 1.0 mM $MgCl_2$, and 100 μM $ZnCl_2$ at pH 7.6 at 4 °C to remove unbound mPEG.

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Abbreviations: ALP, alkaline phosphatase; BIALP, bovine intestine alkaline phosphatase; EIA, enzyme immunoassay; PEG, polyethylene glycol; pNPP, *p*-nitrophenyl phosphate; TNBS, 2,4,6-trinitrobenzenesulfonic acid

The degree of pegylation of the amino groups of BIALP was evaluated using TNBS.[16,17] Briefly, solid TNBS (5.5 mg) was dissolved in 1.0 mL of 0.4 mg/mL of unmodified, pegylated BIALPs in 50 mM borate-NaOH, pH 9.0. The reaction mixture was maintained at 25 °C for 2 h with continuous stirring and was dialyzed against the same buffer to remove unbound TNBS. The number of amino groups modified with TNBS was spectrophotometrically determined from the absorbance at 280 nm, A_{280}, and at 344 nm, A_{344}, using the following equations:

$$A_{280} = 50500t + 3300n \quad (1)$$

$$A_{344} = 10900n \quad (2)$$

where t and n represent the molar concentrations of total BIALP and the amino groups modified with TNBS, respectively. An ε_{280} of 50,500 $M^{-1}\,cm^{-1}$ was used for unmodified BIALP according to the numbers of Tyr and Trp residues in one BIALP molecule (19 and 4 respectively),[18] an ε_{280} of 1,490 $M^{-1}\,cm^{-1}$ for Tyr, and an ε_{280} of 5,500 $M^{-1}\,cm^{-1}$ for Trp. An ε_{344} of 10,900 $M^{-1}\,cm^{-1}$ was used for TNBS-modified amino groups.[16,17] The number of pegylated amino groups was calculated by the difference in the numbers of TNBS-modified amino groups of the pegylated and unmodified BIALPs.

SDS–PAGE. SDS–PAGE was performed in a 10% polyacrylamide gel under reducing conditions by the method of Laemmli.[19] Proteins were reduced by treatment with 2.5% of 2-mercaptoethanol at 100 °C for 10 min, and then applied onto the gel. A constant current of 40 mA was applied for 60 min. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250. A molecular mass marker kit consisting of pig myosin (200 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase B (97.2 kDa), bovine serum albumin (66.4 kDa), hen egg-white ovalbumin (44.3 kDa), and bovine carbonic anhydrase (29.0 kDa) was purchased from Takara Bio. (Otsu, Japan).

Hydrolysis of pNPP. BIALP-catalyzed hydrolysis of pNPP was initiated by mixing 2,990 μL of the substrate solution in 1.0 M diethanolamine-HCl, 1.0 mM $MgCl_2$, and 20 μM $ZnCl_2$ at pH 9.8 pre-incubated at 20 °C and 10 μL of 12 nM BIALP solution, unless otherwise specified. The initial enzyme and substrate concentrations were 40 pM and 0.1–4.0 mM respectively. The reaction was carried out at 20 °C and was measured by following the increase in absorbance at 405 nm, A_{405}, for 3 min with a Jasco V-550 spectrophotometer (Jasco, Tokyo). The product, *p*-nitrophenol, was estimated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{405} = 17{,}500\ M^{-1}\,cm^{-1}$, at pH 9.8 at 20 °C.[20] The kinetic parameters, the molecular activity, k_{cat}, and the Michaelis constant, K_m, were calculated from the Hanes-Woolf equation (eq. 3) by least-squares regression.

$$\frac{1}{v_0} = \frac{K_m}{V_{max}[S]_0} + \frac{1}{V_{max}} \quad (3)$$

In this equation, v_0, V_{max}, and $[S]_0$ are the initial reaction rate, the maximal initial reaction rate, and the initial substrate concentration respectively. The value of k_{cat} was calculated from V_{max} using a monomer molecular mass of 50 kDa.

Irreversible thermal inactivation of BIALP. BIALP (1.25 nM) in 1.0 M diethanolamine-HCl, 1.0 mM $MgCl_2$, and 20 μM $ZnCl_2$ at pH 9.8 was incubated at a set temperature for 30 min and subsequently incubated at 20 °C for 3 min. The remaining activity of BIALP in pNPP hydrolysis was determined at 20 °C, as described above.

Results

Effects of the addition of free PEG on BIALP activity

First we examined the effects of the addition of 1–15% w/v free PEG with molecular masses of 1, 2, 6, and 20 kDa (PEG1000, PEG2000, PEG6000, and PEG20000 respectively) on BIALP activity in the hydrolysis of 0.1–4.0 mM pNPP at 20 °C. The pH was set to 9.8 based on previous reports.[21–23] Figure 1 shows the dependences of v_0 of BIALP with 5% free PEG on the pNPP concentration. All the plots showed saturated

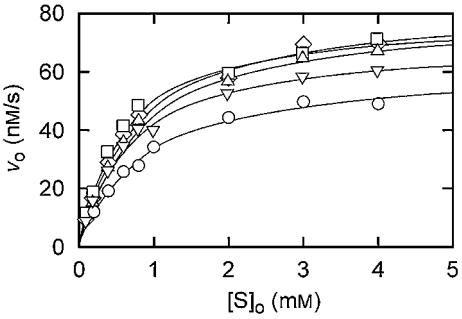

Fig. 1. Dependence of the Initial Reaction Rate on the pNPP Concentration.

The pNPP-hydrolysis reaction was carried out with 40 pM BIALP in 1.0 M diethanolamine-HCl, 1.0 mM $MgCl_2$, 20 μM $ZnCl_2$, and various concentrations of PEGs at pH 9.8 at 20 °C. The initial reaction rates, v_0, are plotted against the pNPP concentration. Solid line represents the best fit of the Michaelis-Menten equation using the nonlinear least-squares methods. Symbols for the enzymes: ○, BIALP without free PEG; △, BIALP plus 5% PEG1000; □, BIALP plus 5% PEG2000; ◇, BIALP plus 5% PEG6000; ▽, BIALP plus 5% PEG20000.

profiles. The same profiles were obtained for 1, 10, and 15% free PEG (data not shown). Table 1 shows the k_{cat}, K_m, and k_{cat}/K_m values of BIALP without and with 1–15% free PEG. The k_{cat} value of BIALP without free PEG was $1{,}440 \pm 30\ s^{-1}$. The k_{cat} values increased with increasing PEG1000, PEG2000, and PEG6000 concentrations, and reached maximum at 5% for PEG1000 ($2{,}020 \pm 60\ s^{-1}$), 15% for PEG2000 ($2{,}020 \pm 90\ s^{-1}$), and 5% for PEG6000 ($2{,}080 \pm 90\ s^{-1}$), while they did not change much with increasing PEG20000 concentrations. The K_m values of BIALP without free PEG decreased with increasing PEG2000 concentrations and reached a minimum (0.19 ± 0.06 mM) at 30%, while they did not with increasing PEG1000, PEG6000, or PEG20000 concentrations. The k_{cat}/K_m values of BIALP plus 5–15% PEG1000, PEG2000, PEG6000, and PEG20000 were 120–140%, 180–300%, 130–170%, and 110–140% respectively of that of BIALP without free PEG. These results indicate that the addition of free PEG activates BIALP, and that the effect of PEG2000 is the highest. We used PEG2000 in subsequent analysis.

Pegylation of BIALP

Next we prepared pegylated BIALP. mPEG (Fig. 2A) is designed to bind to the N-terminal amino group and the ε-amino group of the Lys residue of a protein (Fig. 2B). Pegylation of BIALP was carried out at the [mPEG]/[BIALP] ratio of 400:1 mol/mol. The average number of TNBS-modified amino groups of the unmodified and pegylated BIALPs were 12 and four respectively. Hence it was calculated that eight out of 22 amino groups of monomeric BIALP were pegylated. The pegylated BIALP not subsequently modified with TNBS exhibited a UV absorption spectrum with a peak at 280 nm, while that subsequently modified with TNBS exhibited a spectrum with peaks, at 280 and 344 nm (Fig. 2C). On SDS–PAGE under reducing conditions, unmodified BIALP exhibited a protein band corresponding to 50 kDa, while the pegylated BIALP exhibited a protein band corresponding to 200 kDa at a rough estimate (Fig. 2D), which did not agree with the speculated molecular mass of the pegylated BIALP

Table 1. Kinetic Parameters of BIALP in the Hydrolysis of pNPP

	[PEG] (%, w/v)	Kinetic parameters		
		$k_{cat} \times 10^{-2}$ (s^{-1})	K_m (mM)	k_{cat}/K_m ($\mu M^{-1}\,s^{-1}$)
BIALP		14.4 ± 0.3 (1.0)	0.79 ± 0.03 (1.0)	1.8 ± 0.1 (1.0)
BIALP + PEG1000	1	15.8 ± 0.4 (1.1)	0.62 ± 0.04 (0.8)	2.5 ± 0.1 (1.4)
BIALP + PEG1000	5	20.2 ± 0.6 (1.4)	0.80 ± 0.06 (1.0)	2.5 ± 0.1 (1.4)
BIALP + PEG1000	10	17.9 ± 0.5 (1.2)	0.74 ± 0.04 (0.9)	2.4 ± 0.1 (1.3)
BIALP + PEG1000	15	18.7 ± 0.5 (1.3)	0.88 ± 0.06 (1.1)	2.1 ± 0.1 (1.2)
BIALP + PEG2000	1	16.9 ± 0.4 (1.2)	0.69 ± 0.06 (0.9)	2.4 ± 0.1 (1.2)
BIALP + PEG2000	5	20.0 ± 0.8 (1.4)	0.63 ± 0.06 (0.8)	3.2 ± 0.1 (1.8)
BIALP + PEG2000	10	18.8 ± 0.5 (1.3)	0.43 ± 0.03 (0.5)	4.4 ± 0.1 (2.4)
BIALP + PEG2000	15	20.2 ± 0.9 (1.4)	0.37 ± 0.06 (0.5)	5.4 ± 0.3 (3.0)
BIALP + PEG6000	1	16.7 ± 0.4 (1.2)	0.60 ± 0.04 (0.8)	2.8 ± 0.1 (1.6)
BIALP + PEG6000	5	20.8 ± 0.9 (1.4)	0.75 ± 0.08 (1.0)	2.8 ± 0.1 (1.6)
BIALP + PEG6000	10	21.1 ± 1.3 (1.5)	0.70 ± 0.14 (0.9)	3.0 ± 0.2 (1.7)
BIALP + PEG6000	15	21.3 ± 1.4 (1.5)	0.89 ± 0.14 (1.1)	2.4 ± 0.2 (1.3)
BIALP + PEG20000	1	15.2 ± 0.5 (1.1)	0.61 ± 0.05 (0.8)	2.5 ± 0.1 (1.4)
BIALP + PEG20000	5	17.6 ± 0.4 (1.2)	0.66 ± 0.03 (0.8)	2.6 ± 0.1 (1.4)
BIALP + PEG20000	10	17.2 ± 0.6 (1.2)	0.76 ± 0.06 (1.0)	2.2 ± 0.1 (1.2)
BIALP + PEG20000	15	15.1 ± 0.6 (1.1)	0.81 ± 0.09 (1.0)	1.9 ± 0.1 (1.1)

The reaction was carried out with an initial enzyme concentration of 40 pM in 1.0 M diethanolamine-HCl containing 1.0 mM $MgCl_2$, 20 μM $ZnCl_2$ at pH 9.8 at 20 °C. Numbers in parenthesis indicate values relative to unmodified BIALP without free PEG.

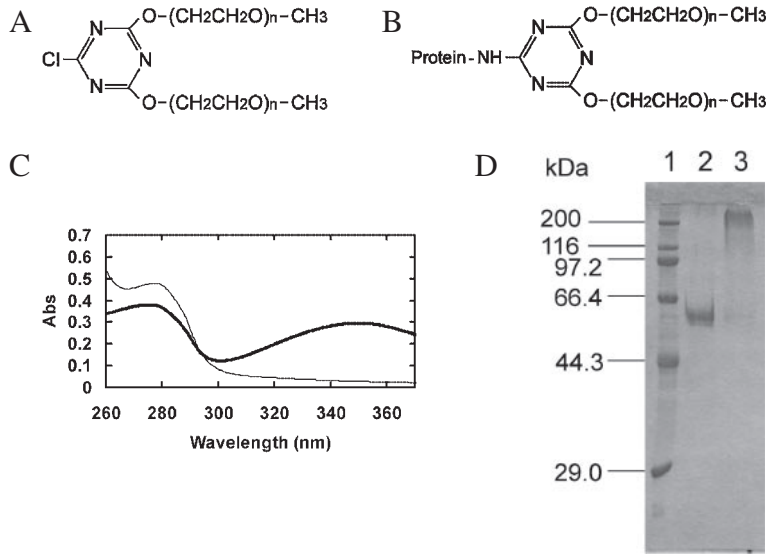

Fig. 2. Pegylation of BIALP with mPEG.

A, Molecular structure of mPEG. B, Molecular structure of the pegylated amino group. C, Absorption spectrum of the pegylated BIALP (thin line) and the TNBS-modified pegylated BIALP (bold line) in 30 mM triethanolamine-HCl, 3.0 M NaCl, 1.0 mM $MgCl_2$, and 100 μM $ZnCl_2$ at pH 7.6. D, SDS–PAGE of BIALP under reducing conditions. Coomassie Brilliant Blue-stained 10% polyacrylamide gel is shown. Lane 1, molecular-mass marker; lane 2, unmodified BIALP; and lane 3, pegylated BIALP.

(130 kDa) comprising monomeric BIALP (50 kDa) and eight mPEG molecules (80 kDa). It is known that on SDS–PAGE, pegylated proteins exhibit a protein band of higher molecular mass than expected.[24] We used this preparation in subsequent analysis.

Effects of the addition of PEG2000 and pegylation on BIALP activity under various conditions

We examined the effects of the addition of PEG2000 and pegylation on BIALP activity under various conditions. Figure 3A shows the pH dependences of v_0 of unmodified BIALP without and with 5% PEG2000 and pegylated BIALP in the hydrolysis of pNPP at 20 °C. The initial pNPP concentration was set at 3 mM, considerably higher than the K_m values of BIALP with 1–15% PEG2000 (0.37–0.69 mM) (Table 1). Because the k_{cat} values of BIALP with 1–15% PEG2000 were almost entirely stable (1,690–2,020 s^{-1}) (Table 1), the PEG2000 concentration was set at 5%. Unmodified BIALP without free PEG showed profiles with an optimal pH of 8.5–9.5, which agreed well with previous reports.[25–27] Unmodified BIALP plus 5% PEG2000 and pegylated BIALP showed the same profiles. The v_0 values at pH 9.0 of unmodified BIALP plus 5% PEG2000 and pegylated BIALP were 86 ± 2 and 83 ± 3 nM/s respectively, higher than of that of unmodified BIALP without free PEG (64 ± 2 nM/s). These results indicate that the addition of PEG2000 and pegylation increased BIALP activity at pH 7.0–10.0.

Figure 3B shows the temperature dependences of v_0 at pH 9.8. Measurements at >65 °C were not done due to the difficulty in preparing buffers at the corresponding temperatures. Unmodified BIALP showed an optimal temperature of 55–60 °C, which agreed well with a

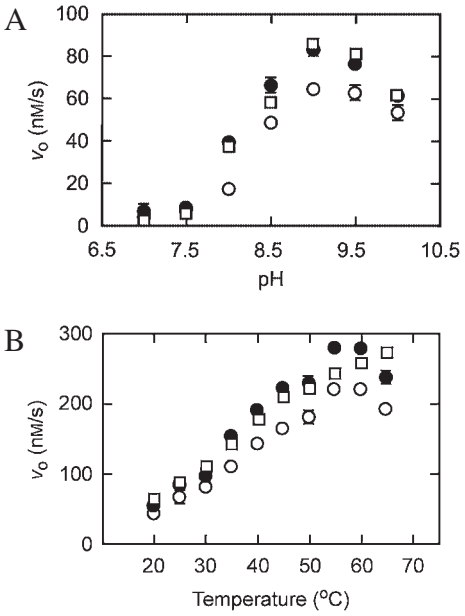

Fig. 3. Effects of Pegylation and the Addition of Free PEG on BIALP Activity.

The pNPP-hydrolysis reaction was carried out with 40 pM BIALP and 3 mM pNPP in 1.0 M diethanolamine-HCl, 1.0 mM $MgCl_2$, 20 μM $ZnCl_2$. A, pH-dependence of v_0. The reaction was carried out at 20 °C. B, Temperature-dependence of v_0. The reaction was carried out at pH 9.8. Error bars indicate SD values of the triplicate determination. Symbols for the enzymes: ○, unmodified BIALP without free PEG; □, unmodified BIALP plus 5% PEG2000; ●, pegylated BIALP.

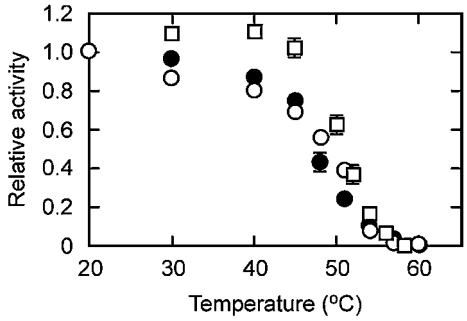

Fig. 4. Effects of Pegylation and the Addition of Free PEG on BIALP Stability.

The relative activity of BIALP for pNPP hydrolysis was defined as the ratio of v_0 with the 30 min of incubation at the temperatures indicated to that at 20 °C (unmodified BIALP, 57 ± 1 nM/s; BIALP plus 5% PEG2000, 64 ± 3 nM/s; pegylated BIALP, 64 ± 2 nM/s). Error bars indicate SD values of the triplicate determination. Symbols for the enzymes: ○, unmodified BIALP without free PEG; □, unmodified BIALP plus 5% PEG2000; ●, pegylated BIALP.

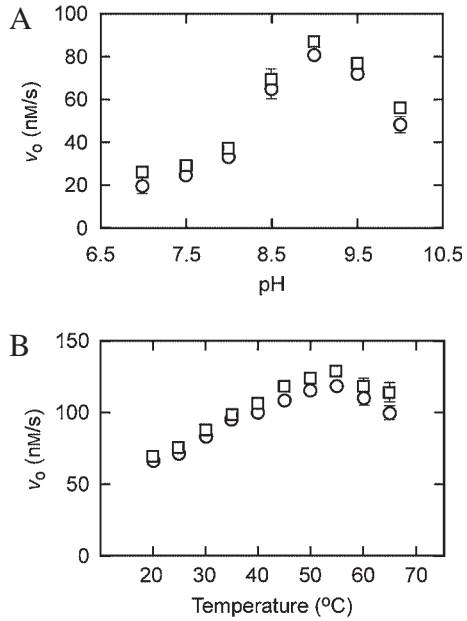

Fig. 5. Effects of the Addition of Free PEG on the Activity of Antibody-Conjugated BIALP.

The pNPP-hydrolysis reaction was carried out with 1 ng/mL of antibody-conjugated BIALP and 3 mM pNPP in 1.0 M diethanolamine-HCl, 1.0 mM $MgCl_2$, 20 μM $ZnCl_2$. A, pH-dependence of v_0. The reaction was carried out at 20 °C. B, Temperature-dependence of v_0. The reaction was carried out at pH 9.8. Error bars indicate SD values of the triplicate determination. Symbols for the enzymes: ○, antibody-conjugated BIALP without free PEG; □, antibody-conjugated BIALP plus 5% PEG2000.

previous report.[23] The optimal temperature of pegylated BIALP was 55–60 °C, and that of unmodified BIALP with 5% PEG2000 was 65 °C or higher. The v_0 values at 55 °C of unmodified BIALP with PEG2000 and of pegylated BIALP were 243 ± 3 and 278 ± 2 nM/s respectively, higher than that of unmodified BIALP without free PEG (219 ± 2 nM/s). These results indicate that the addition of PEG2000 and pegylation increased BIALP activity at 20–65 °C.

Effects of the addition of PEG2000 and pegylation on BIALP stability

We examined the effects of the addition of PEG2000 and pegylation on BIALP stability. BIALP was treated at various temperatures (30–60 °C) for 30 min. Figure 4 shows the relative activity of heat-treated BIALP in the hydrolysis of pNPP. All the plots showed similar sigmoid curves. The temperatures required to reduce initial activity by 50% in 30 min, T_{50}, of unmodified BIALP plus 5% PEG2000 and pegylated BIALP were 51 ± 1 and 47 ± 2 °C respectively, similar to that of unmodified BIALP without free PEG (49 ± 1 °C). This indicates that neither the addition of PEG2000 nor pegylation affects BIALP stability.

Effects of the addition of PEG2000 on the activity of antibody-conjugated BIALP

To explore further the usefulness of the addition of PEG in EIA, we examined the effects of the addition of PEG2000 on the activity of antibody-conjugated BIALP. Figure 5A shows the pH dependences of v_0 without and with 5% PEG2000 in the hydrolysis of pNPP at 20 °C. Antibody-conjugated BIALP plus 5% PEG2000 showed the same profile as that without free PEG. The v_0 value at pH 9.0 of antibody-conjugated BIALP plus 5% PEG2000 was 87 ± 1 nM/s, higher than that of antibody-conjugated BIALP without free PEG (79 ± 1 nM/s). This indicates that the addition of PEG2000 increases the activity of antibody-conjugated BIALP at pH 7.0–10.0. Figure 5B shows the temperature dependences of v_0 at pH 9.8. The optimal temperature of antibody-conjugated BIALP with and without 5% PEG2000 was 55 °C. The v_0 value at 55 °C of antibody-conjugated BIALP plus 5% PEG2000 was 130 ± 1 nM/s, higher than that of antibody-conjugated

BIALP without free PEG (118 ± 1 nM/s). This indicates that the addition of PEG2000 increases the activity of antibody-conjugated BIALP at 20–65 °C.

Discussion

Activation of BIALP by the addition of free PEG and pegylation

The pH and temperatures for the reaction with BIALP in EIA vary depending on individual assay. For example, the pH and temperature were 9.5 and 37 °C for the EIA for leptin,[12] 9.5 and 30 °C for the EIA for 1,2-dioxetane,[28] 9.5 and 25 °C for the EIA for thyroid-stimulating hormone,[29] and 9.5 and 37 °C for the EIA for 17β-oestradiol.[30] In this study, under various conditions including pH 7.0–10.0 and 20–65 °C, the addition of PEG2000 and pegylation increased the activity of BIALP by 30% on average (Fig. 3), and the addition of PEG2000 increased the activity of antibody-conjugated BIALP by 15% on average (Fig. 5). This suggests that the addition of PEG2000 and pegylation can be used in EIA to increase sensitivity and rapidity.

In EIA, the enzyme reaction velocity, v_0, is proportional to the initial enzyme concentration, $[E]_0$, and the k_{cat} of the enzyme according to the Michaelis-Menten equation. Therefore, high enzyme activity is favorable in order to get higher v_0 values. In other words, when the k_{cat} of the signaling enzyme was increased, the sensitivity of EIA was enhanced, and thus the time needed to detect a signal of the product was reduced. In this study, the k_{cat} values of the BIALP with 5–15% PEG1000, PEG2000, and PEG6000 were 120–150% of that of unmodified BIALP without free PEG (Table 1), suggesting that the addition of PEG2000 is most effective in EIA.

In this study, no significant difference was observed between the effects of the addition of PEG2000 and those of pegylation on the activity and stability of BIALP, but the experimental conditions were substantially different. For example, in the hydrolyzing reaction with 40 pM BIALP, the concentration of PEG2000 was 5% w/v, while the concentration of BIALP-bound PEG can be calculated to have been 320 pM because the average number of bound PEG (10 kDa) in one pegylated BIALP molecule was eight. Hence it cannot be concluded that there is no difference between the effects of the addition of PEG2000 and those of pegylation on the activity and stability of BIALP.

The mechanism of activation of BIALP by free PEG is unclear. Activation of BIALP by free PEG was brought about by increases in k_{cat} rather than decreases in K_m (Table 1). Regarding this, we have examined the activation of the activity of thermolysin, a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus*, by neutral salts, and have reported that it is brought about by increases in k_{cat}, but not by decreases in K_m.[31–33] We have also examined the inhibition of the activities of neuraminidase,[34] thermolysin,[35] human matrix metalloproteinase 7 (MMP-7),[36] and *Streptomyces caespitosus* neutral protease (ScNP)[37] by various alcohols. We speculate that not only the dielectric constant of the reaction medium but also the interaction between neutral salts or alcohols and particular surface sites of enzymes are involved in neutral salts-induced activation and alcohol-induced inhibition.[31–37] If this is the case for BIALP and the sites of BIALP to which PEG binds are identified, site-specific pegylation might be effective.[38]

Effects of PEG on enzyme activity

Effects on enzyme activities of the addition of free PEG and pegylation were not predicted. They are particular to the various enzymes and must be investigated on an individual basis. Chiu *et al.* reported that pegylation activates and stabilizes trypsin, although the mechanisms are not known.[39] In non-aqueous solution, pegylation increases the activities of various enzymes such as lipase[40] and subtilisin Carlsberg,[41] but in aqueous solution, it decreases the activities of various enzymes such as α-chymotrypsin[42] and lysozyme.[43] In the case of α-chymotrypsin, pegylation decreased the k_{cat} values without changing the K_m values.[44] This decrease in k_{cat} was explained by the fact that six out of 14 Lys residues of α-chymotrypsin are located near the active site.[39] Gonnelli and Strambini found that pegylation did not affect the internal dynamics of ribonuclease T1, azurin, alcohol dehydrogenase, or *E. coli* ALP, as assessed by fluorescence from internal Trp residues.[45] Hence it can be said that pegylation does not decrease enzyme activities unless the amino acid residues to which PEG is linked are located at the active site.

The effects of pegylation on trypsin activity varied depending on the sizes of the conjugated PEGs.[38,46] In those studies, the *p*-nitroanilide hydrolysis activities of porcine pancreatic trypsin modified with 2, 5, or 10-kDa PEG at 4–5 out of 11 amino groups of one trypsin molecule were about 120% of that of unmodified trypsin, while the activities of trypsin modified with 20-kDa PEG were about 80% of that of unmodified trypsin. This is similar to our results, that the k_{cat}/K_m values of BIALP plus 5–15% PEG1000, PEG2000, PEG6000, and PEG20000 were 120–140%, 180–300%, 130–170%, and 110–140% respectively of that of BIALP without free PEG (Table 1). Thus it can be said that the effects of the addition of free PEG and pegylation on enzyme activities depend on the size of PEG.

In conclusion, it is suggested that the addition of free PEG to and pegylation of BIALP can be used in EIA to increase sensitivity and rapidity. To elucidate the mechanisms, further study is required of the interaction between PEG and BIALP.

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