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Effects of amines and aminoalcohols on bovine intestine alkaline phosphatase activity

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Abbreviations: ALP, alkaline phosphatase; BIALP, bovine intestine alkaline phosphatase; EIA, enzyme immunoassay; pNPP, *p*-nitrophenyl phosphate

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Abstract

Bovine intestine alkaline phosphatase (BIALP) is widely used as a signaling enzyme in sensitive assays such as enzyme immunoassay (EIA). In this study, we evaluated the effects of various aminoalcohols and amines on the activity of BIALP in the hydrolysis of *p*-nitrophenyl phosphate (pNPP) at pH 9.8, at 20°C. The k_{cat} values at 0.05 M diethanolamine, 0.1 M triethanolamine, and 0.2 M *N*-methylethanolamine were 190 ± 10, 840 ± 30, and 500 ± 10 s^{-1}, respectively. The k_{cat} values increased with increasing concentrations of diethanolamine, triethanolamine, and *N*-methylethanolamine and reached 1240 ± 60, 1450 ± 30, and 2250 ± 80 s^{-1}, respectively, at 1 M. On the other hand, the k_{cat} values at 0.05-1.0 M ethanolamine, ethylamine, methylamine, and dimethylamine were in the range of 100-600 s^{-1}. These results indicate that diethanolamine, triethanolamine, and *N*-methylethanolamine highly activate BIALP and might be suitable as a dilution buffer of BIALP in EIA. Interestingly, the K_m values increased with increasing concentrations of diethanolamine and *N*-methylethanolamine, but not triethanolamine: the K_m value at 1.0 M diethanolamine (0.83 ± 0.15 mM) was 12-fold higher than that at 0.05 M (0.07 ± 0.01 mM), and that at 1.0 M *N*-methylethanolamine (2.53 ± 0.20 mM) was 14-fold higher than that at 0.2 M (0.18 ± 0.02 mM), while that at 1.0 M triethanolamine (0.31 ± 0.01 mM) was similar as that at 0.2 M (0.25 ± 0.01 mM), suggesting that the mechanisms of BIALP activation are different between the aminoalcohols.

1. Introduction

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 many bacteria and mammals, and play an essential role 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+} and one Mg^{2+} ions [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. Therefore, it has been applied as a signaling enzyme in sensitive assays such as enzyme immunoassay (EIA), Western blotting analysis, nucleic acid hybridization assay, and polymerase chain reaction and has been used in diagnosis, immunology, and molecular biology [9-11]. We developed a fully automated random-accessible type EIA diagnosis system, AIA, with BIALP (Tosoh, Tokyo, Japan). In this system, 180 assays could be done in 1 h with the sensitivity of an attomole level using 0.1 ml sample solution. Generally, in EIA, the concentration of the analyte is translated to the activity of the signaling enzyme. The concentration of the enzyme-reaction product is measured using signals such as absorbance, fluorescence, and luminescence. Various enzymes such as ALP, β-galactosidase, glucoamylase, and peroxidase have been used for this purpose [12]. Presently, BIALP and

horseradish peroxidase (HRP) are the most extensively used due to high activity. According to the Michaelis-Menten equation, the reaction velocity is proportional to the concentration and k_{cat} of the signaling enzyme. Therefore, the sensitivity and rapidness of EIA increase if k_{cat} of the signaling enzyme increases. This means that activation of the signaling enzyme makes the EIA system more sensitive and rapid. Regarding this, we developed several technologies, and demonstrated that they were effective [13-16]. First, to reduce non-specific binding of the BIALP-labeled antibodies (Abs) to the immobilized Abs, we produced $F(ab')_2$ fragments and used them as Abs to be immobilized, instead of intact IgG or IgM Abs, [13,14]. Secondly, considering that chemical labeling of BIALP to Ab sometimes makes BIALP and Ab inactivated, we produced bi-specific Abs that bind with antigen and BIALP simultaneously and used them as the Abs for detection, instead of covalently BIALP-labeled Abs [15,16]. Thirdly, to increase the reaction velocity, we produced dimerized and trimerized BIALPs by chemical conjugation with glutaraldehyde and labeled them to the Abs for detection, instead of monomeric BIALP [15].

Enzyme activity depends on buffer species as well as pH. Bannister and Foster reported that tris, imidazole, phosphate ion, and bicarbonate ion increased BIALP activity [17]. Stinson reported that 2-(ethylamino)ethanol increased BIALP probably by acting as a phophoacceptor [18]. Today, diethanolamine is commonly used as a dilution buffer of BIALP [19,20]. However, the activation mechanism of these substances has not been well elucidated. In this study, to address this issue, we examined the effects of various amines and aminoalcohols (Fig. 1) on BIALP activity. We also discuss the importance of BIALP activation from a viewpoint of its diagnostic use.

2. Materials and methods

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91 *2.1. Materials*

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93 BIALP (lot 92958657) was purchased from Roche Diagnostics (Basel, Switzerland).
94 The preparation was used without further purification. *p*-Nitrophenyl phosphate (pNPP) (lot
95 M4R4749) was from Nacalai Tesque (Kyoto, Japan). Its concentration was determined
96 spectrophotometrically using the molar absorption coefficient, ε_{310}, of 10,380 M^{-1} cm^{-1},
97 which we determined in this study. All other chemicals were of reagent grade and purchased
98 from Nacalai Tesque and Wako Pure Chemical (Osaka, Japan).

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100 *2.2. Hydrolysis of pNPP*

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102 The BIALP-catalyzed hydrolysis of pNPP was initiated by mixing 2,990 μl of the
103 substrate solution pre-incubated at 20°C and 10 μl of the BIALP solution (12 nM). The
104 substrate solutions were 0.05-3.0 M diethanolamine-HCl, 0.2-1.0 M ethylamine-HCl,
105 0.05-0.5 M ethanol containing 0.05 M diethanolamine-HCl, 0.05-1.0 M methylamine-HCl
106 containing 0.05 M diethanolamine, 0.2-1.0 M dimethylamine-HCl containing 0.05
107 diethanolamine, 0.05-1.0 M ethanolamine-HCl, 0.1-1.0 M triethanolamine-HCl, 0.2-1.0 M
108 *N*-methylethanolamine-HCl, and 0.05-0.5 M borate-NaOH, each containing 1.0 mM $MgCl_2$
109 and 20 μM $ZnCl_2$, at pH 9.8. The initial enzyme and substrate concentrations were 40-400
110 pM and 0.01-15 mM, respectively. The reaction was carried out at 20°C and measured by
111 following the increase in absorbance at 405 nm, A_{405}, with a JASCO V-550
112 spectrophotometer (Tokyo). The product, *p*-nitrophenol, was estimated using the molar
113 absorption difference due to the hydrolysis, $\Delta\varepsilon_{405}$ = 17,500 M^{-1} cm^{-1}, at 20°C, which we

determined in this study. The kinetic parameters, the molecular activity (k_{cat}) and Michaelis constant (K_m), were calculated from Hanes-Woolf equation (Eq. 1) by least-squares-regression.

$$\frac{1}{v_o} = \frac{K_m}{V_{max}[S]_o} + \frac{1}{V_{max}} \quad (1)$$

In this equation, v_o, V_{max}, and $[S]_o$ 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} obtained using a monomer molecular mass of 50 kDa.

3. Results

3.1. Effects of diethanolamine on the BIALP-catalyzed hydrolysis of pNPP

Diethanolamine is commonly used as a dilution buffer of BIALP [19,20]. We first made kinetic analysis of BIALP in the hydrolysis of pNPP with various concentrations of diethanolamine. The dependences of v_o at pH 9.8, at 20ºC on the substrate concentration are shown in Fig. 2. All plots showed saturated profiles, and the k_{cat} and K_m values of BIALP were determined separately (Table 1). The k_{cat} and K_m values markedly increased with increasing concentrations of diethanolamine. The k_{cat} value at 3.0 M diethanolamine was 2330 ± 30 s^{-1}, which was 12-fold higher than that at 0.05 M (190 ± 10 s^{-1}), and the K_m value at 3.0 M diethanolamine was 1.75 ± 0.05 mM, which was 25-fold higher than that at 0.05 M (0.07 ± 0.01 mM). Consequently, the k_{cat}/K_m values were relatively constant in the range 0.05-3.0 M.

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138 *3.2. Effects of amines on the BIALP-catalyzed hydrolysis of pNPP*

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140 Diethanolamine has amino and hydroxyl groups. Based on the assumption that the
141 hydroxyl group of diethanolamine is involved in the activation of BIALP, we made kinetic
142 analysis of BIALP in the hydrolysis of pNPP with varying concentrations (0.05-1.0 M) of
143 ethylamine, ethanol, methylamine, and dimethylamine. To maintain pH of the solution at
144 9.8, 0.05 M diethanolamine was contained in the solutions with methylamine,
145 dimethylamine, and ethanol. The dependences of $v_o/[E]_o$ on the substrate concentration in
146 the presence of 1.0 M diethanolamine, ethylamine, methylamine, or dimethylamine or 0.5 M
147 ethanol are shown in Fig. 3. The $v_o/[E]_o$ values with ethylamine, methylamine,
148 dimethylamine, and ethanol were considerably lower than those with diethanolamine at all
149 substrate concentrations examined. Because all plots showed saturated profiles, the k_{cat} and
150 K_m values were determined separately (Table 2). The k_{cat} values were stable with increasing
151 concentrations of ethylamine: the value at 1.0 M ethylamine was 400 ± 30 s^{-1}, which was
152 90% of that at 0.2 M (450 ± 10 s^{-1}). The k_{cat} values decreased with increasing concentrations
153 of ethanol: the value at 0.5 M ethanol was 100 ± 10 s^{-1}, which was 50% of that at 0.05 M
154 (200 ± 20 s^{-1}). The k_{cat} values increased with increasing concentrations of methylamine and
155 dimethylamine: the value at 1.0 M methylamine was 330 ± 30 s^{-1}, which was 240% of that
156 at 0.05 M (140 ± 20 s^{-1}), and that at 1.0 M dimethylamine was 370 ± 10 s^{-1}, which was
157 250% of that at 0.2 M (150 ± 10 s^{-1}). These results indicate that ethylamine and ethanol do
158 not activate and methylamine and dimethylamine activate BIALP. However, the magnitudes
159 of the activation by methylamine and dimethylamine were not remarkable compared to that
160 by diethanolamine.

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162 *3.3. Effects of aminoalcohols on the BIALP-catalyzed hydrolysis of pNPP*

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164 To see whether aminoalcohols other than diethanolamine activate BIALP, we made
165 kinetic analysis of BIALP in the hydrolysis of pNPP with varying concentrations (0.05-1.0
166 M) of ethanolamine, triethanolamine, and *N*-methylethanolamine. Borate was also used as a
167 negative control. The dependences of $v_o/[E]_o$ on the substrate concentration at 1.0 M
168 diethanolamine, ethanolamine, triethanolamine, and *N*-methylethanolamine and 0.5 M
169 borate are shown in Fig. 4. The $v_o/[E]_o$ values with *N*-methylethanolamine were the highest
170 for 0.4-1.0 mM pNPP and that with triethanolamine was the highest for 0.2 mM pNPP.
171 Because all plots showed saturated profiles, the k_{cat} and K_m values were determined
172 separately (Table 3). The k_{cat} values increased with increasing concentrations of
173 triethanolamine and *N*-methylethanolamine: the value at 1.0 M triethanolamine was 1450 ±
174 30 s^{-1}, which was 180% of that at 0.1 M (840 ± 30 s^{-1}), and the value at 1.0 M
175 *N*-methylethanolamine was 2250 ± 80 s^{-1}, which was 450% of that at 0.2 M (500 ± 10 s^{-1}).
176 The k_{cat} values decreased with increasing concentrations of ethanolamine: the value at 1.0 M
177 ethanolamine was 300 ± 10 s^{-1}, which was 50% of that at 0.05 M (610 ± 200 s^{-1}). The k_{cat}
178 values were stable with increasing concentrations of borate: the value at 0.5 M borate was
179 120 ± 10 s^{-1}, which was 90% of that at 0.05 M (130 ± 10 s^{-1}). These results indicate that
180 ethanolamine does not activate and triethanolamine and *N*-methylethanolamine activate
181 BIALP.

182 The K_m values were stable with increasing concentrations of ethanolamine: the value at
183 1.0 M ethanolamine was 0.30 ± 0.01 mM, which was identical to that at 0.05 M (0.30 ± 0.02
184 mM). The K_m values slightly increased with increasing concentrations of triethanolamine:

the value at 1.0 M triethanolamine was 0.31 ± 0.01 mM, which was 130% of that at 0.1 M (0.24 ± 0.03 mM). The K_m values markedly increased with increasing concentrations of *N*-methylethanolamine and borate: the value at 1.0 M *N*-methylethanolamine was 2.53 ± 0.20 mM, which was 1400% of that at 0.2 M (0.18 ± 0.02 mM), and the value at 0.5 M borate was 10.30 ± 0.41 mM, which was 2100% of that at 0.05 M (0.49 ± 0.02 mM). Consequently, the k_{cat}/K_m values increased with increasing concentrations of triethanolamine, and decreased with increasing concentrations of ethanolamine, *N*-methylethanolamine, and borate.

4. Discussion

4.1. Effects of amines on BIALP activity

In this study, we determined the k_{cat} and K_m values of BIALP in the hydrolysis of pNPP with varying concentrations of three amines, methylamine, dimethylamine, and ethylamine, one alcohol, ethanol, and four aminoalcohols, ethanolamine, diethanolamine, triethanolamine, and *N*-methylethanolamine (Fig. 1). We demonstrate that diethanolamine, triethanolamine, and *N*-methylethanolamine activate BIALP.

The k_{cat} values increased with increasing concentrations of methylamine and dimethylamine, and were stable with increasing concentrations of ethylamine (Table 2). The magnitudes of the activation by methylamine and dimethylamine are not remarkable compared to that by diethanolamine (Table 1). This suggests that methyl group has activating effects on BIALP although the precise mechanism is not known. Recently, Yang et al. reported the effects of high concentrations (about 1 M) of neutral salts on calf intestine

ALP activity [21]. They showed that the activating and stabilizing effects of neutral salts correlated with the Hofmeister series. The effects of neutral salts on BIALP are the next subject. In regard to this, we reported that neutral salts remarkably activated thermolysin, a thermostable neutral metalloproteinase produced in the culture broth of *Bacillus thermoproteolyticus* [22]. Importantly, the orders of ions for the efficiency in the activation and the increase in the solubility of thermolysin were $Na^+ > K^+ > Li^+$, which was different from Hofmeister's series corresponding to the degree of hydration of ions: $Li^+ > Na^+ > K^+$ [22-24].

4.2. Effects of aminoalcohols on BIALP activity

The k_{cat} values of BIALP in the hydrolysis of pNPP increased with increasing concentrations of diethanolamine, triethanolamine, and *N*-methylethanolamine, indicating that they activate BIALP and might be suitable as a dilution buffer of BIALP in EIA. In contrast, The K_m values increased with increasing concentrations of diethanolamine and *N*-methylethanolamine, but not triethanolamine, suggesting that the mechanisms of BIALP activation are different between the aminoalcohols.

The active site of ALP has two Zn^{2+} ions and one Mg^{2+} ion. According to X-ray structural study of *E. coli* ALP [5,6,25], the catalytic mechanism of ALP has been thought as follows: in free enzyme, the hydroxyl group of active-site Ser residue coordinates one Zn^{2+} ion (Zn2). The Michaelis complex is formed when the ester oxygen atom of the substrate coordinates another Zn^{2+} ion (Zn1) and the non-bridging oxygen atom of the substrate coordinates Zn1. Zn2 polarizes the hydroxyl group the Ser. Ionized Mg-bound water molecule accepts a proton from the Ser. The covalent enzyme-phosphate intermediate

is formed when the ionized hydroxyl group of the Ser attacks the phosphorus atom. Then, the first product is released. Ionized Zn1-bound water molecule attacks the phosphorus atom. The Mg-bound water molecule now gives a proton to the Ser. Finally, the phosphate group is released.

There are two possible mechanisms for the activation of BIALP by aminoalcohols [17-20]. One is that aminoalcohols are located at the active site of BIALP and receive the leaving phosphate group more efficiently than water molecule. The aminoalcohols-phosphate complex is hydrolyzed when the complex releases from the enzyme. Another possibility is that aminoalcohols bind BIALP out of the active site and activate it. In both cases, the initial reaction rate could be saturated as the aminoalcohol concentration increases. To address this issue, v_o of BIALP in the hydrolysis of pNPP was plotted against aminoalcohol concentrations for each pNPP concentration (Fig. 5). The reaction with diethanolamine exhibited Michaelis-Menten profiles (Fig. 5A). The $K_{m,diethanolamine}$ values at the pNPP concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, and 5.0 mM were 64 ± 23, 160 ± 60, 260 ± 50, 360 ± 80, 400 ± 100, 820 ± 170, 1000 ± 200, 1300 ± 300, and 1300 ± 300 mM, respectively, indicating the value increased with increasing concentrations of pNPP. On the other hand, the reaction with triethanolamine and *N*-methylethanolamine exhibited the saturation curve, but did not exhibit Michaelis-Menten profiles (Figs. 5B and C). Our results suggest that the mechanisms of BIALP activation are different between the aminoalcohols although the difference cannot be precisely explained at this stage.

4.3. Application of ALP to EIA

In the application of ALP to EIA, some substrates are used depending on the principle of the product-detection system. Namely, with the pNPP substrate, the product *p*-nitrophenol (pNP) gives a strong yellow color at neutral and alkaline pH regions and could be detected with absorbance at 405 nm; with the 4-methylumbelliferyl phosphate (4MUP) substrate, the product 4-methylumbelliferone gives a large fluorescent emission intensity at 450 nm with excitation at 325 nm [26]. BIALP is applied to chemiluminescence immunoassay, too. It dephosphorylates the substrate, adamantyl 1, 2-dioxetanephenyl phosphate (AMPPD), to produce a phenoxide intermediate, which decomposes to produce light emission at 470 nm [27,28]. The detection limit for the enzyme is 1 zmol (10^{-21} mol) and the light emission is a long-lived glow (> 1 h). The results presented in this study that diethanolamine, triethanolamine, and *N*-methylethanolamine activated BIALP with pNPP suggest that they also activate it with 4MUP or AMPPD although the degree of activation might vary depending on substrate species.

The properties of ALP are described such as pH-activity profile [29-31], temperature-activity profile [31], thermal stability [31], and metal activation [32] by many investigations. Substitution of Zn^{2+} to divalent metals (Co^{2+}, Mn^{2+}, Ni^{2+}, and Cu^{2+}) at catalytic site [33], and addition of these metals to reaction buffer [31] were mainstream investigations in the study of ALP activation. While a buffer constitution in the study of ALP activation was not enough to know the optimum condition for ALP reaction [17,34,35], it has suggested that ALP activity depended on reaction medium from kinetic studies of ALP [19,20,36,37]. Based on these lines of evidence, diethanolamine-HCl buffer is generally used for measurement of ALP activity [19,20,36].

In conclusion, diethanolamine, triethanolamine, and *N*-methylethanolamine highly activate BIALP and might be suitable as a dilution buffer of BIALP in EIA. Our results also

suggest that certain additives might increase ALP activity and stability. The effects of sugars and polyalcohols on BIALP activity and stability are currently underway.

References

[1] T.W. Reid, I.B. Wilson, *E. coli* alkaline phosphatase, in: P.D. Boyer (Eds.), 3rd ed., The Enzymes, Vol. 4, Academic Press, New York, 1971, pp. 373-415.

[2] H.N. Fernley, Mammalian alkaline phosphatases, in: P.D. Boyer (Eds.), 3rd ed., The Enzymes, Vol. 4, Academic Press, New York, 1971, pp. 417-447.

[3] E.E. Kim, H.W. Wyckoff, Reaction mechanism of alkaline phosphatase based on crystal structures, J. Mol. Biol. 218 (1991) 449-464.

[4] T. Harada, I. Koyama, T. Matsunaga, A. Kikuno, T. Kasahara, M. Hassimoto, D.H. Alpers, T. Komoda, Characterization of structural and catalytic differences in rat intestinal alkaline phosphatase isozymes, FEBS J. 272 (2005) 2477-2486.

[5] B. Stec, K.M. Holtz, E.R. Kantrowitz, A revised mechanism for the alkaline phosphatase reaction involving three metal ions, J. Mol. Biol. 299 (2000) 1303-1311.

[6] M.M.E. de Backer, S. McSweeney, P.F. Lindley, E. Hough, Ligand-binding and metal–exchange crystallographic studies on shrimp alkaline phosphatase, Acta Cryst. D60 (2004) 1555-1561.

[7] P. Gettins, J.E. Coleman, ^{31}P nuclear magnetic resonance of phosphoenzyme intermediates of alkaline phosphatase, J. Biol. Chem. 258 (1983) 408-416.

[8] J.E Murphy, T.T. Tibbitts, E.R. Kantrowitz, Mutations at positions 153 and 328 in *Escherichia coli* alkaline phosphatase provide insight towards the structure and function of mammalian and yeast alkaline phosphatase, J. Mol. Biol. 253 (1995) 604-617.

[9] M.S. Blake, K.H. Johnston, G.R. Russell-Jones, E.C. Gotschlich, A rapid, sensitive methods for detection of alkaline phosphatase-conjugated anti-antibody on Western blots, Anal. Biochem. 136 (1984) 175-179.

[10] E. Jablonski, E.W. Moomaw, R.H. Tullis, J.L. Ruth, Preparation of oligodeoxynucleotide-alkaline phosphatase conjugated and their use as hybridization probes, Nucleic Acids Res. 14 (1986) 6115-6128.

[11] D.W. Chan, Automation of immunoassays, In: Immunoassay, E.P. Diamandis, T.K. Christopoulos (Eds), Academic Press, New York, 1996, pp. 483-504.

[12] J.P. Gosling, Immunoassay, in: E.P. Diamandis, T.K. Christopoulos (Eds), Academic Press, New York, 1996, pp. 287-308.

[13] K. Morimoto, K. Inouye, Single-step purification of $F(ab')_2$ fragments of mouse monoclonal antibodies (immunoglobulins G1) by hydrophobic interaction high performance liquid chromatography using TSKgel Phenyl-5PW, J. Biochem. Biophys. Methods 24 (1992) 107-117.

[14] K. Morimoto, K. Inouye, Single-step purification of $F(ab')_{2\mu}$ fragments of mouse monoclonal antibodies (immunoglobulins M) by hydrophobic interaction high-performance liquid chromatography using TSKgel ether-5PW, J. Biochem. Biophys. Methods 26 (1993) 27-39.

[15] K. Morimoto, K. Inouye, A sensitive enzyme immunoassay of human thyroid-stimulating hormone (TSH) using bispecific $F(ab')_2$ fragments recognizing polymerized alkaline phosphatase and TSH, J. Immunol. Methods 205 (1997) 81-90.

[16] K. Morimoto, K. Inouye, Method for the preparation of bispecific $F(ab')_{2\mu}$ fragments from mouse monoclonal antibodies of the immunoglobulin M class and characterization of the fragments, J. Immunol. Methods 224 (1999) 43-50.

329 [17] A. Bannister, R.L. Foster, Buffer-induced activation of calf intestinal alkaline phosphate,
330 Eur. J. Biochem. 113 (1980) 199-203.
331 [18] R.A. Stinson, Kinetic parameters for the cleaved substrate, and enzyme and substrate
332 stability, vary with the phosphoacceptor in alkaline phosphatase catalysis, Clin. Chem. 39
333 (1993) 2293-2297.
334 [19] T. Manes, M.F. Hoylaerts, R. Muller, F. Lottspeich, W. Holke, J.L. Millan, Genetic
335 complexity, Structure, and characterization of highly active bovine intestinal alkaline
336 phosphatases, J. Biol. Chem. 273 (1998) 23353-23360.
337 [20] R.A. Stinson, J.L. McPhee, H.B. Collier, Phosphotransferase activity of human alkaline
338 phosphatase and the role of enzyme Zn^{2+}, Biochim. Biophys. Acta 913 (1987) 272-278.
339 [21] Z. Yang, X.J. Liu, C. Chen, P.J. Halling, Hofmeister effects on activity and stability of
340 alkaline phosphatase, Biochim. Biophys. Acta 1084 (2010) 821-828.
341 [22] K. Inouye, Effects of salts on thermolysin: activation of hydrolysis and synthesis of
342 *N*-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester, and a unique change in the
343 absorption spectrum of thermolysin, J. Biochem. 112 (1992) 335-340.
344 [23] K. Inouye, S.-B. Lee, B. Tonomura, Effect of amino acid residues at the cleavable site
345 of substrates on the remarkable activation of thermolysin by salts, Biochem. J. 315 (1996)
346 133-138.
347 [24] K. Inouye, K. Kuzuya, B. Tonomura, Effect of salts on the solubility of thermolysin: a
348 remarkable increase in the solubility as well as the activity by the addition of salts without
349 aggregation or dispersion of thermolysin, J. Biochem. 123 (1998) 847-852.
350 [25] J.G. Zalatan, T.D. Fenn, D. Herschlag, Comparative enzymology in the alkaline
351 phosphates superfamily to determine the catalytic role of an active-site metal ion, J. Mol.
352 Biol. 384 (2008) 1174-1189.

[26] T.K. Christopoulos, E.P. Diamandis, in: Immunoassay, E.P. Diamandis, T.K. Christopoulos (Eds), Academic Press, New York, 1996, pp. 309-335.

[27] L.J. Kricka, in: Immunoassay, Edited by E. P. Diamandis and T. K. Christopoulos (Eds), Academic Press, New York, 1996, pp. 337-353.

[28] S. Sekiguchi, H. Kohno, K. Yasukawa, K, Inouye, Study on chemiluminescent enzyme immunoassay for the measurement of leptin, Biosci. Biotechnol. Biochem. in press.

[29] R.K. Morton, Some properties of alkaline phosphatase of cow's milk and calf intestinal mucosa, Biochem. J. 60 (1955) 573-582.

[30] D. Chappelet-Tordo, M. Fosset, M. Iwatsubo, C. Gache, M. Lazdunski, (1974) Intestinal alkaline phosphatase. Catalytic properties and half of the sites reactivity, Biochemistry 13 (1974) 1788-1795.

[31] H.N. Fernley, P.G. Waker, Kinetic behaviour of calf-intestinal alkaline phosphatase with 4-methylumbelliferyl phosphate, Biochem. J. 97 (1965) 95-103.

[32] D.J. Plocke, B.L. Vallee, Interaction of alkaline phosphatase of *E. coli* with metal ions and chelating agents, Biochemistry 1 (1962) 1039-1043

[33] M.L. Applebury, B.P. Johnson, J.E. Coleman, Phosphate binding to alkaline phosphatase. Metal ion dependence, J. Biol. Chem. 245 (1970) 4968-4976.

[34] S. Yan, L. Liu, X. Tian, Y. Zhang, H. Zhou, Effect of extraneous zinc on calf intestinal alkaline phosphatase, J. Protein Chem. 22 (2003) 371-375.

[35] T.U. Hausamen, R. Helgar, W. Rick, W. Gross, Optimal conditions for the determination of serum alkaline phosphatase by a new kinetic method, Clin. Chim. Acta 15 (1967) 241-245.

[36] R.B. McComb, G.N. Bowers Jr, Study of optimum buffer conditions for measuring alkaline phosphatase activity in human serum, Clin. Chem. 18 (1972) 97-104.

[37] R. Han, J.E. Colman, Dependence of the phosphorylation of alkaline phosphatase by phosphate monoesters on the pK_a of the leaving group, Biochemistry 34 (1995) 4238-4245.

Figure legends

Fig. 1. Molecular structures of the aminoalcohols and amines examined.

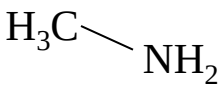
Fig. 2. Dependence of the initial reaction rate (v_o) on the pNPP concentration in the BIALP-catalyzed hydrolysis of pNPP in the presence of diethanolamine. The reaction was carried out in 0.05 (◯), 0.25 (△), 1.0 (□), and 3.0 (◇) M diethanolamine-HCl buffer containing 1.0 mM $MgCl_2$, 20 μM $ZnCl_2$, at pH 9.8, at 20ºC. The initial enzyme concentration, $[E]_o$, is 40 pM. v_o is plotted against pNPP concentrations of 0-5.0 mM (A) and 0-1.0 mM (B). Solid line represents the best fit of the Michaelis-Menten equation using the nonlinear least-squares methods.

Fig. 3. Dependence of v_o on the pNPP concentration in the BIALP-catalyzed hydrolysis of pNPP in the presence of amines and alcohols. The reaction was carried out in the presence of 1.0 mM $MgCl_2$ and 20 μM $ZnCl_2$, at pH 9.8, at 20ºC. $[E]_o$ is 40-400 pM. The $v_o/[E]_o$ is plotted against pNPP concentrations. Solid line represents the best fit of the Michaelis-Menten equation using the nonlinear least-squares methods. Symbols for the buffers: 1.0 M diethanolamine, ◯; 1.0 M ethylamine, △; and 0.5 M ethanol, ●; 1.0 M methylamine, □; and 1.0 M dimethylamine, ◇.

Fig. 4. Dependence of v_o on the pNPP concentration in the BIALP-catalyzed hydrolysis of pNPP in the presence of aminoalcohols. The reaction was carried out in the presence of 1.0 mM $MgCl_2$ and 20 μM $ZnCl_2$, at pH 9.8, at 20ºC. $[E]_o$ is 40-400 pM. The $v_o/[E]_o$ is plotted

against pNPP concentrations. Solid line represents the best fit of the Michaelis-Menten equation using the nonlinear least-squares methods. Symbols for the buffers: 1.0 M diethanolamine, ○; 1.0 M ethanolamine, △; 1.0 M triethanolamine, □; 1.0 M *N*-methylethanolamine, ◇; and 0.5 M borate-NaOH. ●.

Fig. 5. Dependence of v_o on the aminoalcohol concentration in the BIALP-catalyzed hydrolysis of pNPP in the presence of aminoalcohols. The reaction was carried out in the presence of 1.0 mM $MgCl_2$ and 20 μM $ZnCl_2$, at pH 9.8, at 20ºC. $[E]_o$ is 40 pM. The $v_o/[E]_o$ is plotted against the concentrations of diethanolamine (A), triethanolamine (B), and *N*-methylethanolamine (C). Solid line represents the best fit of the Michaelis-Menten equation using the nonlinear least-squares methods. Symbols for pNPP concentration (mM): (A) 0.2, ○; 0.4, △; 0.6, □; 0.8, ◇; 1.0, ▽; 2.0, ●; 3.0, ▲; 4.0, ■; and 5.0, ◆. (B) 0.1, ○; 0.2, △; 0.4, □; 0.6, ◇; 0.8, ▽; 1.0, ●; 1.5, ▲; 2.0,■; and 3.0, ◆. (C) 0.2, ○; 0.4, △; 0.6, □; 1.0, ◇; 1.5, ▽; 2.0, ●; 3.0, ▲; 4.0, ■; and 5.0, ◆.



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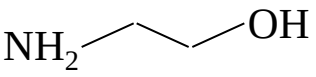
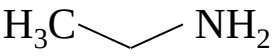


ethanolamine

dimethylamine

diethanolamine



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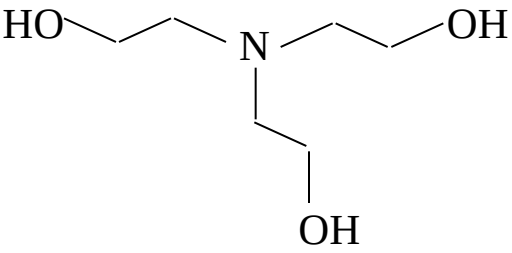

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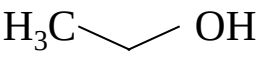
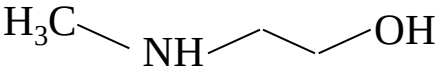


ethanol



N–methylethanolamine

Fig. 1. Sekiguchi *et al.*

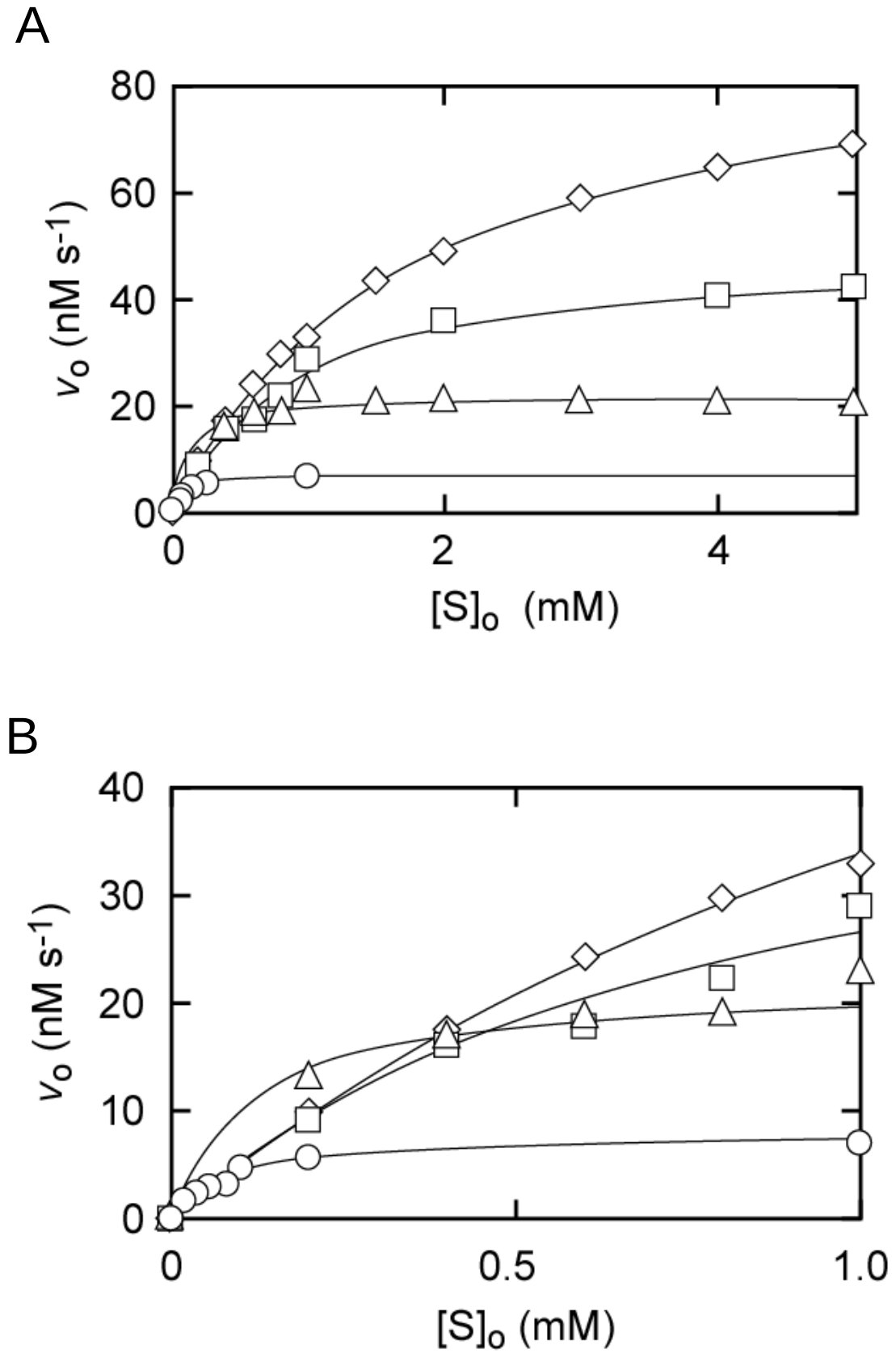
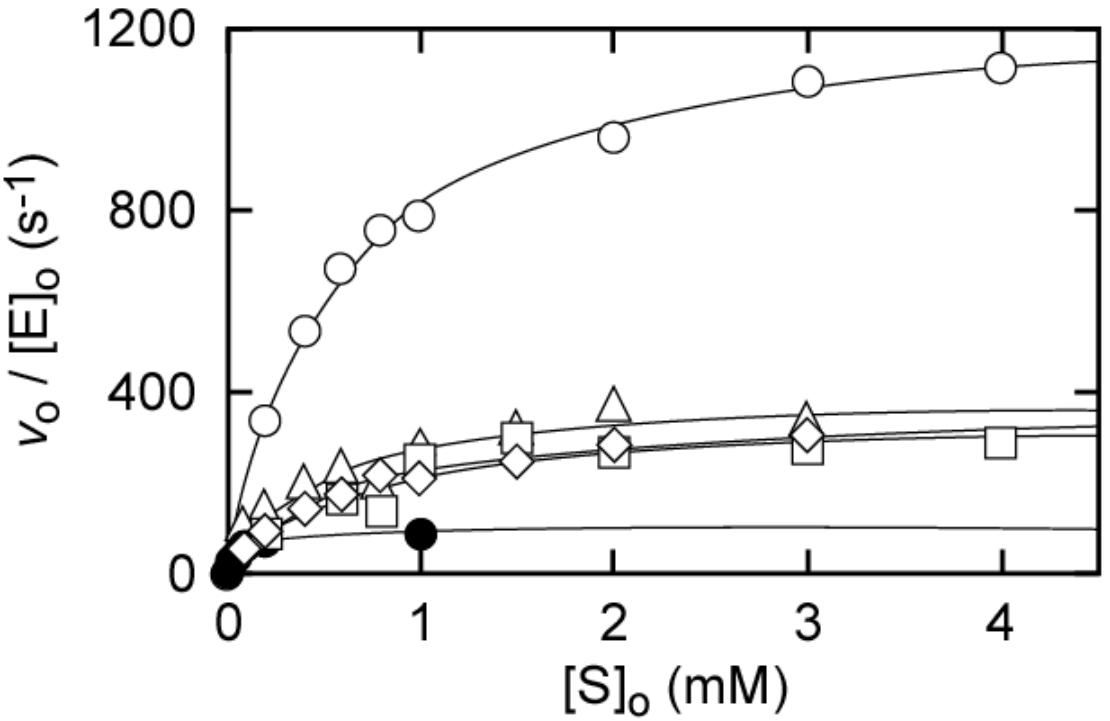


Fig. 2. Sekiguchi *et al.*



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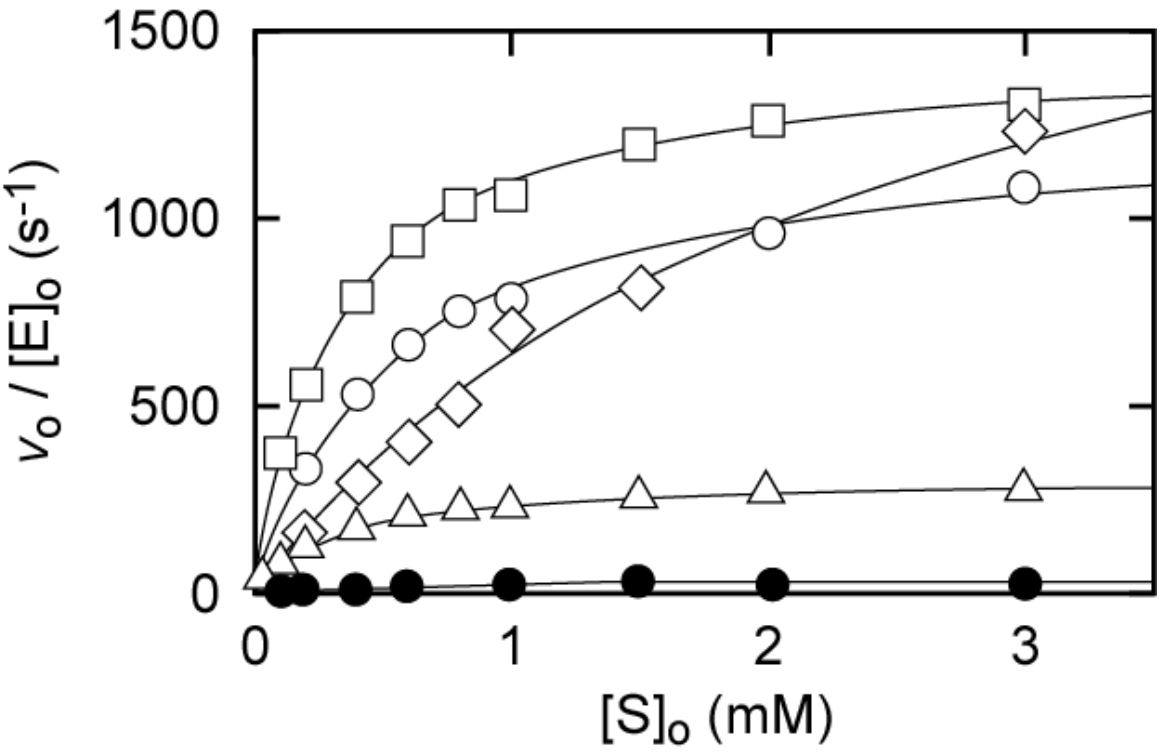
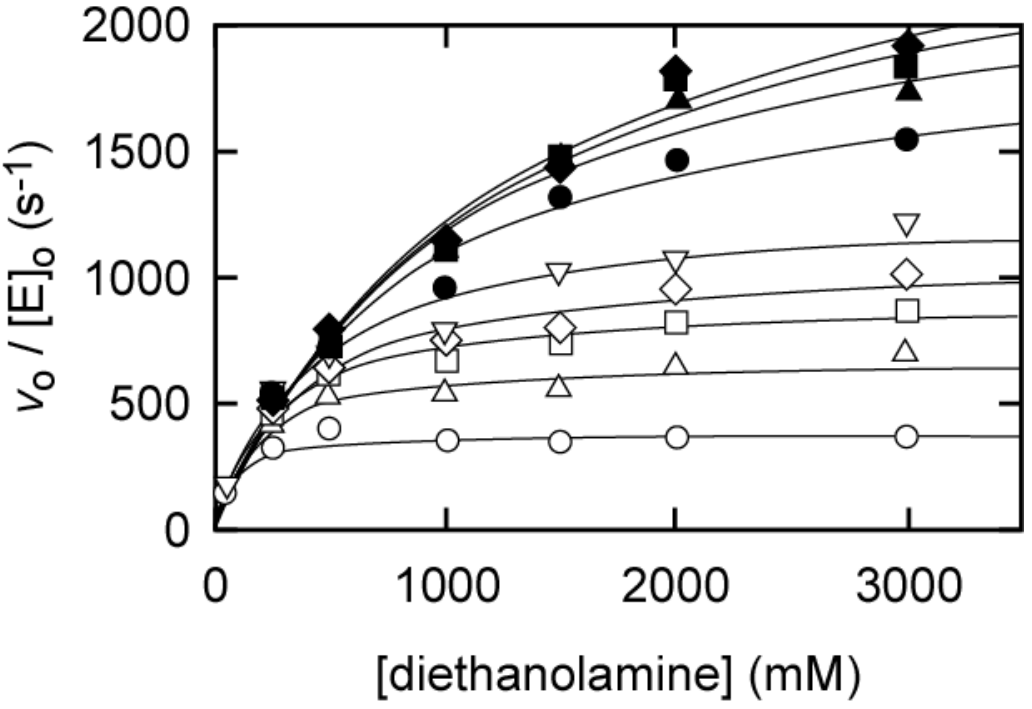


Fig. 4. Sekiguchi *et al.*

A



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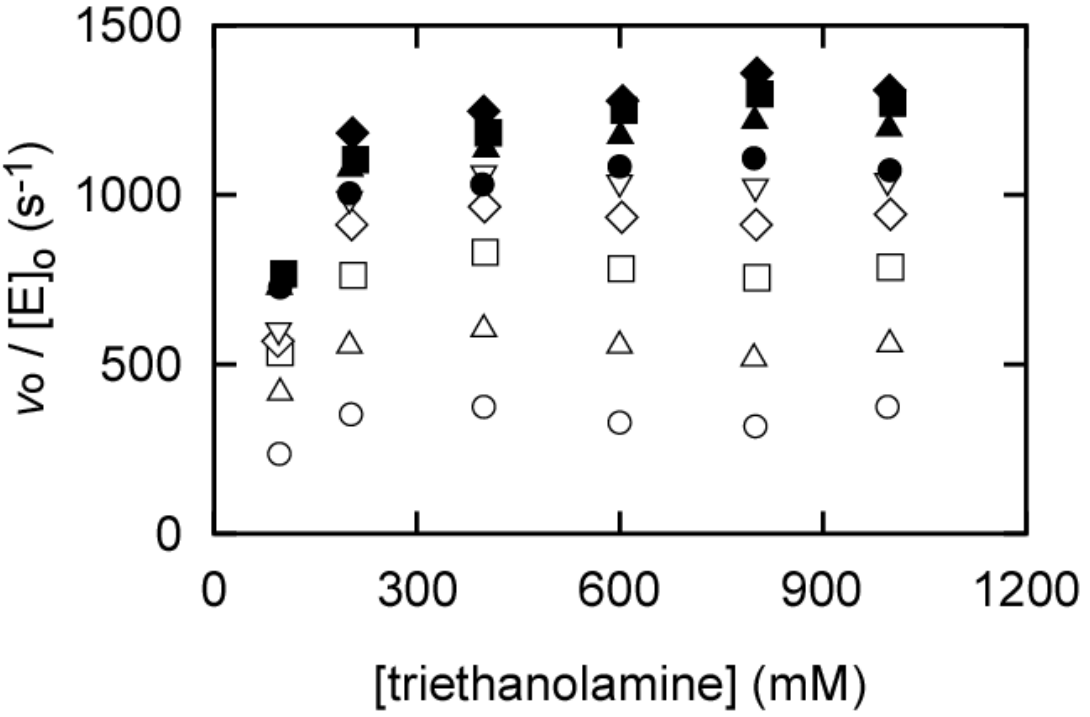

Fig. 5. Sekiguchi *et al.*

C

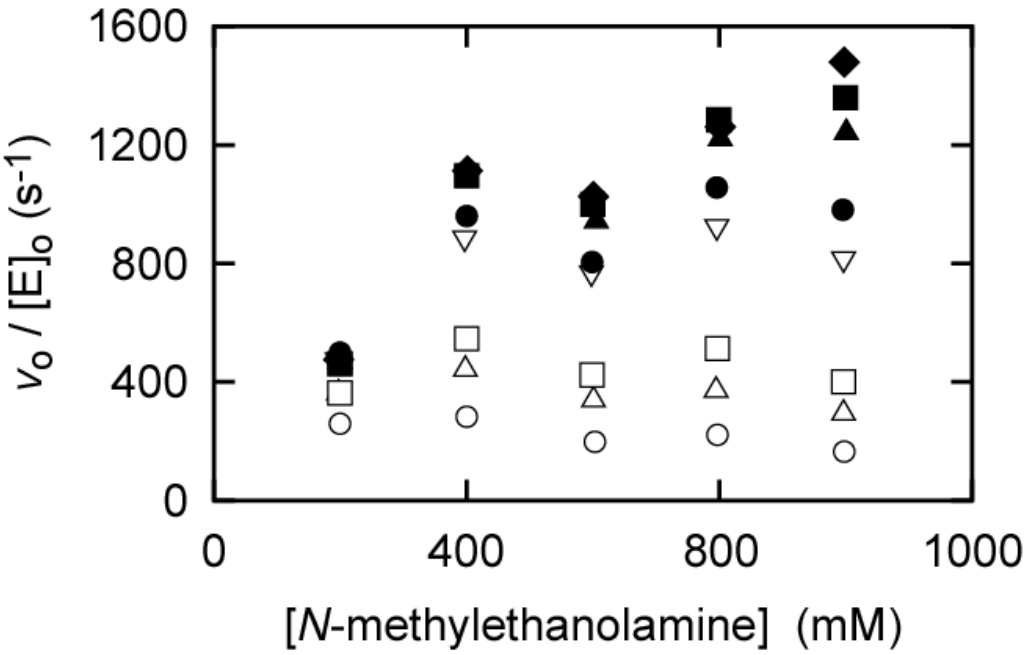

Fig. 5. Sekiguchi *et al.*