

Title	Effects of amines and aminoalcohols on bovine intestine alkaline phosphatase activity
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Citation	Enzyme and Microbial Technology (2011), 49(2): 171-176
Issue Date	2011-07
URL	<a href="http://hdl.handle.net/2433/143675">http://hdl.handle.net/2433/143675</a>
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Type	Journal Article
Textversion	author

1 Enzyme and Microbial Technology

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3 **Effects of amines and aminoalcohols on bovine intestine alkaline phosphatase activity**

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10 *Keywords:* Alkaline phosphatase, Amine, Aminoalcohol, Bovine intestine alkaline  
11 phosphatase, Enzyme immunoassay

12

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

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18

19 **Abstract**

20

21 Bovine intestine alkaline phosphatase (BIALP) is widely used as a signaling enzyme in  
22 sensitive assays such as enzyme immunoassay (EIA). In this study, we evaluated the effects  
23 of various aminoalcohols and amines on the activity of BIALP in the hydrolysis of  
24 *p*-nitrophenyl phosphate (pNPP) at pH 9.8, at 20°C. The  $k_{\text{cat}}$  values at 0.05 M  
25 diethanolamine, 0.1 M triethanolamine, and 0.2 M *N*-methylethanolamine were  $190 \pm 10$ ,  
26  $840 \pm 30$ , and  $500 \pm 10 \text{ s}^{-1}$ , respectively. The  $k_{\text{cat}}$  values increased with increasing  
27 concentrations of diethanolamine, triethanolamine, and *N*-methylethanolamine and reached  
28  $1240 \pm 60$ ,  $1450 \pm 30$ , and  $2250 \pm 80 \text{ s}^{-1}$ , respectively, at 1 M. On the other hand, the  $k_{\text{cat}}$   
29 values at 0.05-1.0 M ethanolamine, ethylamine, methylamine, and dimethylamine were in  
30 the range of 100-600  $\text{s}^{-1}$ . These results indicate that diethanolamine, triethanolamine, and  
31 *N*-methylethanolamine highly activate BIALP and might be suitable as a dilution buffer of  
32 BIALP in EIA. Interestingly, the  $K_{\text{m}}$  values increased with increasing concentrations of  
33 diethanolamine and *N*-methylethanolamine, but not triethanolamine: the  $K_{\text{m}}$  value at 1.0 M  
34 diethanolamine ( $0.83 \pm 0.15 \text{ mM}$ ) was 12-fold higher than that at 0.05 M ( $0.07 \pm 0.01 \text{ mM}$ ),  
35 and that at 1.0 M *N*-methylethanolamine ( $2.53 \pm 0.20 \text{ mM}$ ) was 14-fold higher than that at  
36 0.2 M ( $0.18 \pm 0.02 \text{ mM}$ ), while that at 1.0 M triethanolamine ( $0.31 \pm 0.01 \text{ mM}$ ) was similar  
37 as that at 0.2 M ( $0.25 \pm 0.01 \text{ mM}$ ), suggesting that the mechanisms of BIALP activation are  
38 different between the aminoalcohols.

39

40

41

## 42 1. Introduction

43

44 Phosphatases (EC 3.1.3.1) catalyze the hydrolysis of phosphomonoesters. They are  
45 classified into two groups as alkaline phosphatase (ALP) and acid phosphatase (ACP)  
46 depending on their optimal pH in alkaline and acidic pH regions, respectively. ALPs are  
47 widely distributed in many bacteria and mammals, and play an essential role in biochemical  
48 processes [1-4]. Their structural and functional properties are considered to be commonly  
49 conserved in all ALPs. ALP is a homodimeric metalloenzyme. The subunit has a molecular  
50 mass of about 50 kDa and contains two  $Zn^{2+}$  and one  $Mg^{2+}$  ions [5,6]. The catalytic triad  
51 composed of the two  $Zn^{2+}$  and one  $Mg^{2+}$  ions is conserved in all ALPs from *Escherichia coli*  
52 to mammals [7]. Mammalian ALP is present in the liver, intestine, placenta, kidney, and  
53 other tissues. The molecular activity,  $k_{cat}$ , of mammalian ALP is 10-60 times higher than that  
54 of *E. coli* ALP [8].

55 Bovine intestine ALP (BIALP) has the highest specific activity among mammalian  
56 ALPs. Therefore, it has been applied as a signaling enzyme in sensitive assays such as  
57 enzyme immunoassay (EIA), Western blotting analysis, nucleic acid hybridization assay,  
58 and polymerase chain reaction and has been used in diagnosis, immunology, and molecular  
59 biology [9-11]. We developed a fully automated random-accessible type EIA diagnosis  
60 system, AIA, with BIALP (Tosoh, Tokyo, Japan). In this system, 180 assays could be done  
61 in 1 h with the sensitivity of an attomole level using 0.1 ml sample solution. Generally, in  
62 EIA, the concentration of the analyte is translated to the activity of the signaling enzyme.  
63 The concentration of the enzyme-reaction product is measured using signals such as  
64 absorbance, fluorescence, and luminescence. Various enzymes such as ALP,  $\beta$ -galactosidase,  
65 glucoamylase, and peroxidase have been used for this purpose [12]. Presently, BIALP and

66 horseradish peroxidase (HRP) are the most extensively used due to high activity. According  
67 to the Michaelis-Menten equation, the reaction velocity is proportional to the concentration  
68 and  $k_{cat}$  of the signaling enzyme. Therefore, the sensitivity and rapidness of EIA increase if  
69  $k_{cat}$  of the signaling enzyme increases. This means that activation of the signaling enzyme  
70 makes the EIA system more sensitive and rapid. Regarding this, we developed several  
71 technologies, and demonstrated that they were effective [13-16]. First, to reduce  
72 non-specific binding of the BIALP-labeled antibodies (Abs) to the immobilized Abs, we  
73 produced F(ab')<sub>2</sub> fragments and used them as Abs to be immobilized, instead of intact IgG  
74 or IgM Abs, [13,14]. Secondly, considering that chemical labeling of BIALP to Ab  
75 sometimes makes BIALP and Ab inactivated, we produced bi-specific Abs that bind with  
76 antigen and BIALP simultaneously and used them as the Abs for detection, instead of  
77 covalently BIALP-labeled Abs [15,16]. Thirdly, to increase the reaction velocity, we  
78 produced dimerized and trimerized BIALPs by chemical conjugation with glutaraldehyde  
79 and labeled them to the Abs for detection, instead of monomeric BIALP [15].

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

88

## 89 **2. Materials and methods**

90

## 91 2.1. Materials

92

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,  $\epsilon_{310}$ , of  $10,380 \text{ M}^{-1} \text{ cm}^{-1}$ ,

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).

99

## 100 2.2. Hydrolysis of pNPP

101

102 The BIALP-catalyzed hydrolysis of pNPP was initiated by mixing 2,990  $\mu\text{l}$  of the

103 substrate solution pre-incubated at  $20^\circ\text{C}$  and 10  $\mu\text{l}$  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  $\text{MgCl}_2$

109 and 20  $\mu\text{M}$   $\text{ZnCl}_2$ , 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  $20^\circ\text{C}$  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,  $\Delta\epsilon_{405} = 17,500 \text{ M}^{-1} \text{ cm}^{-1}$ , at  $20^\circ\text{C}$ , which we

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

117

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

118

119 In this equation,  $v_o$ ,  $V_{\text{max}}$ , and  $[S]_o$  are the initial reaction rate, the maximal initial reaction  
120 rate, and the initial substrate concentration, respectively. The value of  $k_{\text{cat}}$  was calculated  
121 from  $V_{\text{max}}$  obtained using a monomer molecular mass of 50 kDa.

122

### 123 **3. Results**

124

#### 125 *3.1. Effects of diethanolamine on the BIALP-catalyzed hydrolysis of pNPP*

126

127 Diethanolamine is commonly used as a dilution buffer of BIALP [19,20]. We first  
128 made kinetic analysis of BIALP in the hydrolysis of pNPP with various concentrations of  
129 diethanolamine. The dependences of  $v_o$  at pH 9.8, at 20°C on the substrate concentration are  
130 shown in Fig. 2. All plots showed saturated profiles, and the  $k_{\text{cat}}$  and  $K_m$  values of BIALP  
131 were determined separately (Table 1). The  $k_{\text{cat}}$  and  $K_m$  values markedly increased with  
132 increasing concentrations of diethanolamine. The  $k_{\text{cat}}$  value at 3.0 M diethanolamine was  
133  $2330 \pm 30 \text{ s}^{-1}$ , which was 12-fold higher than that at 0.05 M ( $190 \pm 10 \text{ s}^{-1}$ ), and the  $K_m$  value  
134 at 3.0 M diethanolamine was  $1.75 \pm 0.05 \text{ mM}$ , which was 25-fold higher than that at 0.05 M  
135 ( $0.07 \pm 0.01 \text{ mM}$ ). Consequently, the  $k_{\text{cat}}/K_m$  values were relatively constant in the range  
136 0.05-3.0 M.

137

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

139

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  $400 \pm 30 \text{ s}^{-1}$ , which was  
152 90% of that at 0.2 M ( $450 \pm 10 \text{ s}^{-1}$ ). The  $k_{cat}$  values decreased with increasing concentrations  
153 of ethanol: the value at 0.5 M ethanol was  $100 \pm 10 \text{ s}^{-1}$ , which was 50% of that at 0.05 M  
154 ( $200 \pm 20 \text{ s}^{-1}$ ). The  $k_{cat}$  values increased with increasing concentrations of methylamine and  
155 dimethylamine: the value at 1.0 M methylamine was  $330 \pm 30 \text{ s}^{-1}$ , which was 240% of that  
156 at 0.05 M ( $140 \pm 20 \text{ s}^{-1}$ ), and that at 1.0 M dimethylamine was  $370 \pm 10 \text{ s}^{-1}$ , which was  
157 250% of that at 0.2 M ( $150 \pm 10 \text{ s}^{-1}$ ). 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.



161

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

163

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  $1450 \pm$   
174  $30 \text{ s}^{-1}$ , which was 180% of that at 0.1 M ( $840 \pm 30 \text{ s}^{-1}$ ), and the value at 1.0 M  
175 *N*-methylethanolamine was  $2250 \pm 80 \text{ s}^{-1}$ , which was 450% of that at 0.2 M ( $500 \pm 10 \text{ s}^{-1}$ ).  
176 The  $k_{cat}$  values decreased with increasing concentrations of ethanolamine: the value at 1.0 M  
177 ethanolamine was  $300 \pm 10 \text{ s}^{-1}$ , which was 50% of that at 0.05 M ( $610 \pm 200 \text{ s}^{-1}$ ). The  $k_{cat}$   
178 values were stable with increasing concentrations of borate: the value at 0.5 M borate was  
179  $120 \pm 10 \text{ s}^{-1}$ , which was 90% of that at 0.05 M ( $130 \pm 10 \text{ s}^{-1}$ ). 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  $0.30 \pm 0.01 \text{ mM}$ , which was identical to that at 0.05 M ( $0.30 \pm 0.02$   
184 mM). The  $K_m$  values slightly increased with increasing concentrations of triethanolamine:

185 the value at 1.0 M triethanolamine was  $0.31 \pm 0.01$  mM, which was 130% of that at 0.1 M  
186 ( $0.24 \pm 0.03$  mM). The  $K_m$  values markedly increased with increasing concentrations of  
187 *N*-methylethanolamine and borate: the value at 1.0 M *N*-methylethanolamine was  $2.53 \pm$   
188  $0.20$  mM, which was 1400% of that at 0.2 M ( $0.18 \pm 0.02$  mM), and the value at 0.5 M  
189 borate was  $10.30 \pm 0.41$  mM, which was 2100% of that at 0.05 M ( $0.49 \pm 0.02$  mM).  
190 Consequently, the  $k_{cat}/K_m$  values increased with increasing concentrations of triethanolamine,  
191 and decreased with increasing concentrations of ethanolamine, *N*-methylethanolamine, and  
192 borate.

193

#### 194 **4. Discussion**

195

##### 196 *4.1. Effects of amines on BIALP activity*

197

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

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

209 ALP activity [21]. They showed that the activating and stabilizing effects of neutral salts  
210 correlated with the Hofmeister series. The effects of neutral salts on BIALP are the next  
211 subject. In regard to this, we reported that neutral salts remarkably activated thermolysin, a  
212 thermostable neutral metalloproteinase produced in the culture broth of *Bacillus*  
213 *thermoproteolyticus* [22]. Importantly, the orders of ions for the efficiency in the activation  
214 and the increase in the solubility of thermolysin were  $\text{Na}^+ > \text{K}^+ > \text{Li}^+$ , which was different  
215 from Hofmeister's series corresponding to the degree of hydration of ions:  $\text{Li}^+ > \text{Na}^+ > \text{K}^+$   
216 [22-24].

217

#### 218 4.2. Effects of aminoalcohols on BIALP activity

219

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

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

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

237       There are two possible mechanisms for the activation of BIALP by aminoalcohols  
238 [17-20]. One is that aminoalcohols are located at the active site of BIALP and receive the  
239 leaving phosphate group more efficiently than water molecule. The  
240 aminoalcohols-phosphate complex is hydrolyzed when the complex releases from the  
241 enzyme. Another possibility is that aminoalcohols bind BIALP out of the active site and  
242 activate it. In both cases, the initial reaction rate could be saturated as the aminoalcohol  
243 concentration increases. To address this issue,  $v_o$  of BIALP in the hydrolysis of pNPP was  
244 plotted against aminoalcohol concentrations for each pNPP concentration (Fig. 5). The  
245 reaction with diethanolamine exhibited Michaelis-Menten profiles (Fig. 5A). The  
246  $K_{m,diethanolamine}$  values at the pNPP concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0, and  
247 5.0 mM were  $64 \pm 23$ ,  $160 \pm 60$ ,  $260 \pm 50$ ,  $360 \pm 80$ ,  $400 \pm 100$ ,  $820 \pm 170$ ,  $1000 \pm 200$ ,  
248  $1300 \pm 300$ , and  $1300 \pm 300$  mM, respectively, indicating the value increased with  
249 increasing concentrations of pNPP. On the other hand, the reaction with triethanolamine and  
250 *N*-methylethanolamine exhibited the saturation curve, but did not exhibit Michaelis-Menten  
251 profiles (Figs. 5B and C). Our results suggest that the mechanisms of BIALP activation are  
252 different between the aminoalcohols although the difference cannot be precisely explained  
253 at this stage.

254

255 4.3. Application of ALP to EIA

256

257 In the application of ALP to EIA, some substrates are used depending on the principle of  
258 the product-detection system. Namely, with the pNPP substrate, the product *p*-nitrophenol  
259 (pNP) gives a strong yellow color at neutral and alkaline pH regions and could be detected  
260 with absorbance at 405 nm; with the 4-methylumbelliferyl phosphate (4MUP) substrate, the  
261 product 4-methylumbelliferone gives a large fluorescent emission intensity at 450 nm with  
262 excitation at 325 nm [26]. BIALP is applied to chemiluminescence immunoassay, too. It  
263 dephosphorylates the substrate, adamantyl 1, 2-dioxetanophenyl phosphate (AMPPD), to  
264 produce a phenoxide intermediate, which decomposes to produce light emission at 470 nm  
265 [27,28]. The detection limit for the enzyme is 1 zmol ( $10^{-21}$  mol) and the light emission is a  
266 long-lived glow ( $> 1$  h). The results presented in this study that diethanolamine,  
267 triethanolamine, and *N*-methylethanolamine activated BIALP with pNPP suggest that they  
268 also activate it with 4MUP or AMPPD although the degree of activation might vary  
269 depending on substrate species.

270 The properties of ALP are described such as pH-activity profile [29-31],  
271 temperature-activity profile [31], thermal stability [31], and metal activation [32] by many  
272 investigations. Substitution of  $Zn^{2+}$  to divalent metals ( $Co^{2+}$ ,  $Mn^{2+}$ ,  $Ni^{2+}$ , and  $Cu^{2+}$ ) at  
273 catalytic site [33], and addition of these metals to reaction buffer [31] were mainstream  
274 investigations in the study of ALP activation. While a buffer constitution in the study of ALP  
275 activation was not enough to know the optimum condition for ALP reaction [17,34,35], it  
276 has suggested that ALP activity depended on reaction medium from kinetic studies of ALP  
277 [19,20,36,37]. Based on these lines of evidence, diethanolamine-HCl buffer is generally  
278 used for measurement of ALP activity [19,20,36].

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

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

283

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285

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379

380 **Figure legends**

381

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

383

384 **Fig. 2.** Dependence of the initial reaction rate ( $v_o$ ) on the pNPP concentration in the  
385 BIALP-catalyzed hydrolysis of pNPP in the presence of diethanolamine. The reaction was  
386 carried out in 0.05 (○), 0.25 (△), 1.0 (□), and 3.0 (◇) M diethanolamine-HCl buffer  
387 containing 1.0 mM MgCl<sub>2</sub>, 20 μM ZnCl<sub>2</sub>, at pH 9.8, at 20°C. The initial enzyme  
388 concentration, [E]<sub>o</sub>, is 40 pM.  $v_o$  is plotted against pNPP concentrations of 0-5.0 mM (A)  
389 and 0-1.0 mM (B). Solid line represents the best fit of the Michaelis-Menten equation using  
390 the nonlinear least-squares methods.

391

392 **Fig. 3.** Dependence of  $v_o$  on the pNPP concentration in the BIALP-catalyzed hydrolysis of  
393 pNPP in the presence of amines and alcohols. The reaction was carried out in the presence  
394 of 1.0 mM MgCl<sub>2</sub> and 20 μM ZnCl<sub>2</sub>, at pH 9.8, at 20°C. [E]<sub>o</sub> is 40-400 pM. The  $v_o/[E]_o$  is  
395 plotted against pNPP concentrations. Solid line represents the best fit of the  
396 Michaelis-Menten equation using the nonlinear least-squares methods. Symbols for the  
397 buffers: 1.0 M diethanolamine, ○; 1.0 M ethylamine, △; and 0.5 M ethanol, ●; 1.0 M  
398 methylamine, □; and 1.0 M dimethylamine, ◇.

399

400 **Fig. 4.** Dependence of  $v_o$  on the pNPP concentration in the BIALP-catalyzed hydrolysis of  
401 pNPP in the presence of aminoalcohols. The reaction was carried out in the presence of 1.0  
402 mM MgCl<sub>2</sub> and 20 μM ZnCl<sub>2</sub>, at pH 9.8, at 20°C. [E]<sub>o</sub> is 40-400 pM. The  $v_o/[E]_o$  is plotted

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

407

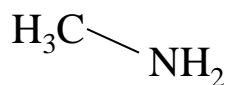
408 **Fig. 5.** Dependence of  $v_o$  on the aminoalcohol concentration in the BIALP-catalyzed  
409 hydrolysis of pNPP in the presence of aminoalcohols. The reaction was carried out in the  
410 presence of 1.0 mM MgCl<sub>2</sub> and 20 μM ZnCl<sub>2</sub>, at pH 9.8, at 20°C. [E]<sub>o</sub> is 40 pM. The  $v_o/[E]_o$   
411 is plotted against the concentrations of diethanolamine (A), triethanolamine (B), and  
412 *N*-methylethanolamine (C). Solid line represents the best fit of the Michaelis-Menten  
413 equation using the nonlinear least-squares methods. Symbols for pNPP concentration (mM):  
414 (A) 0.2, ○; 0.4, △; 0.6, □; 0.8, ◇; 1.0, ▽; 2.0, ●; 3.0, ▲; 4.0, ■; and 5.0, ◆. (B)  
415 0.1, ○; 0.2, △; 0.4, □; 0.6, ◇; 0.8, ▽; 1.0, ●; 1.5, ▲; 2.0, ■; and 3.0, ◆. (C) 0.2, ○;  
416 0.4, △; 0.6, □; 1.0, ◇; 1.5, ▽; 2.0, ●; 3.0, ▲; 4.0, ■; and 5.0, ◆.

417

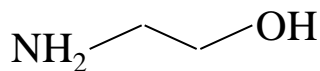
418

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420



methylamine



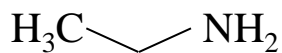
ethanolamine



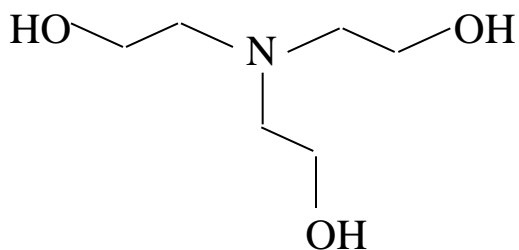
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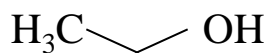
diethanolamine



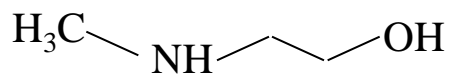
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triethanolamine



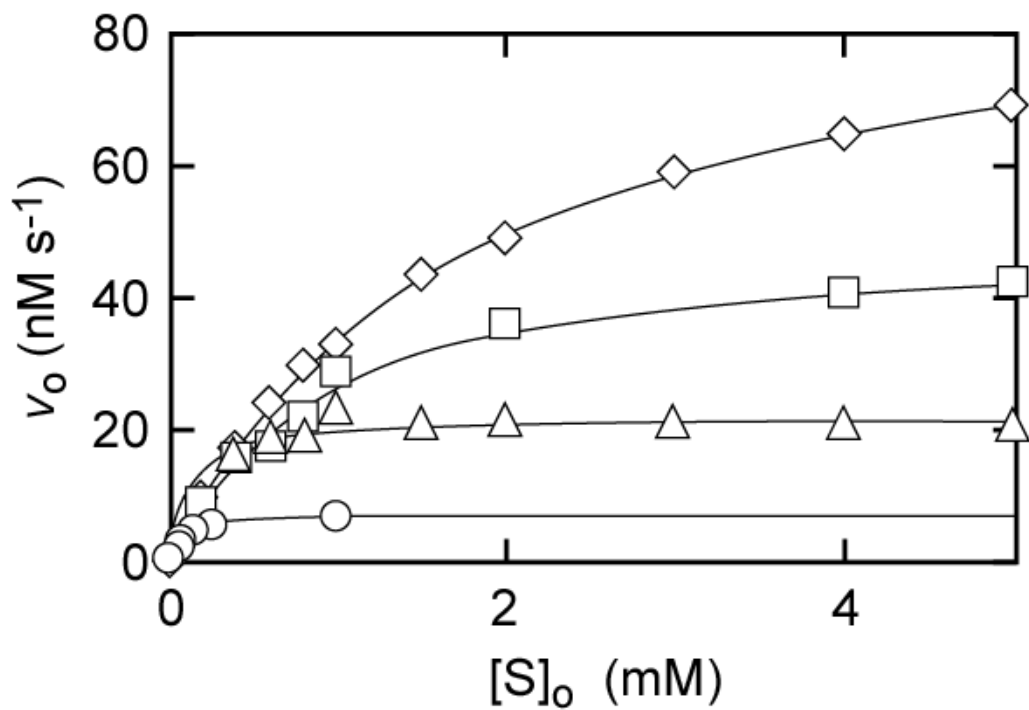
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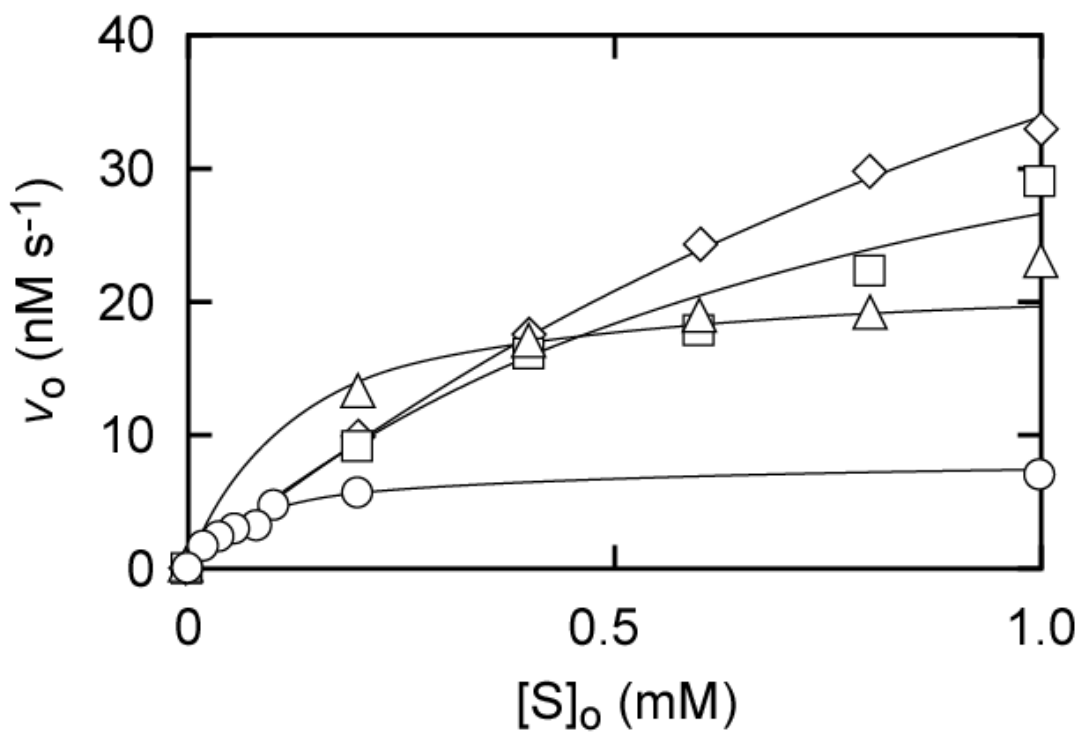
*N*-methylethanolamine

Fig. 1. Sekiguchi *et al.*

A



B

Fig. 2. Sekiguchi *et al.*

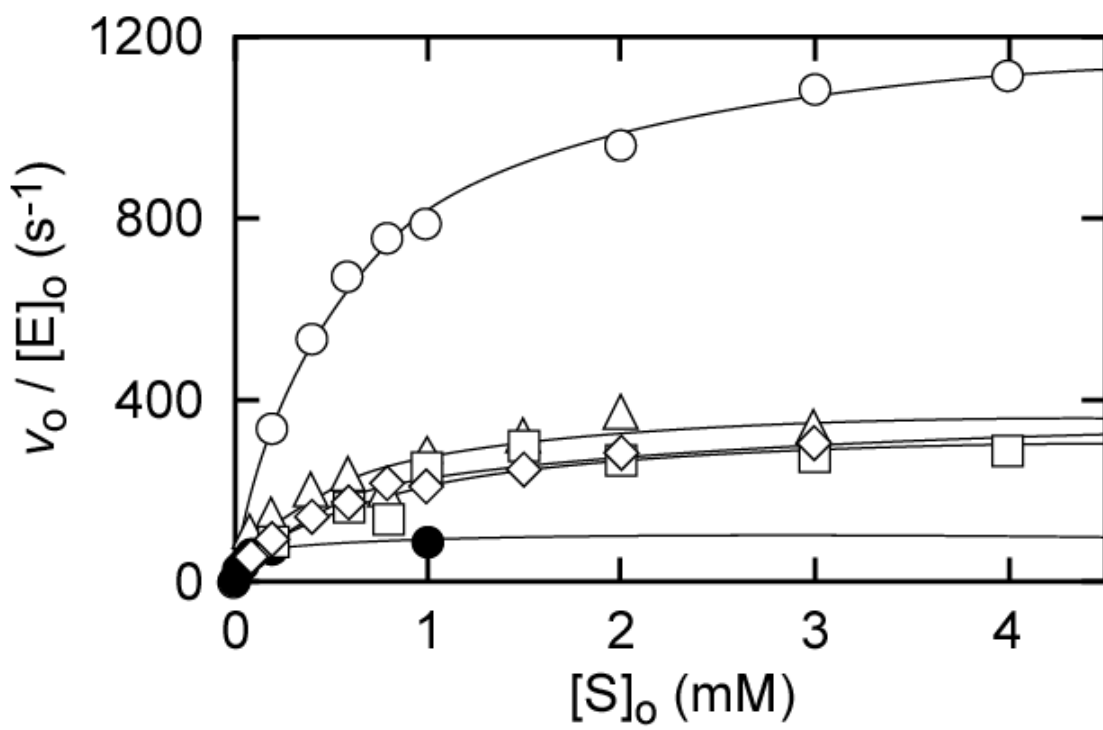


Fig. 3. Sekiguchi *et al.*

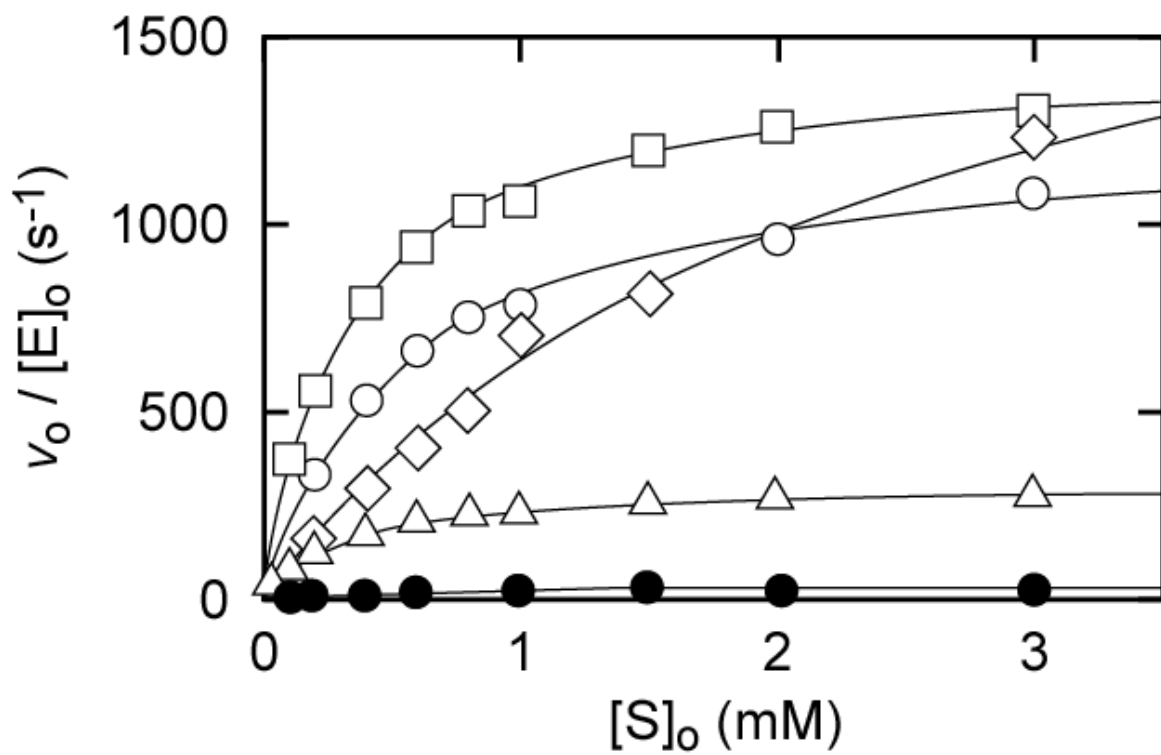
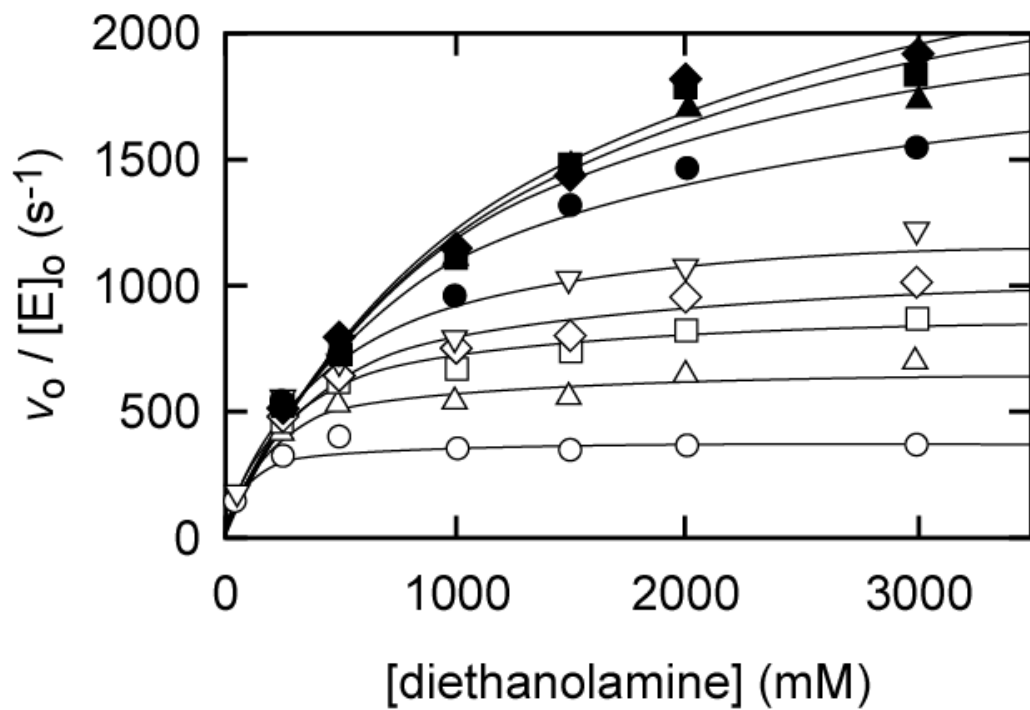


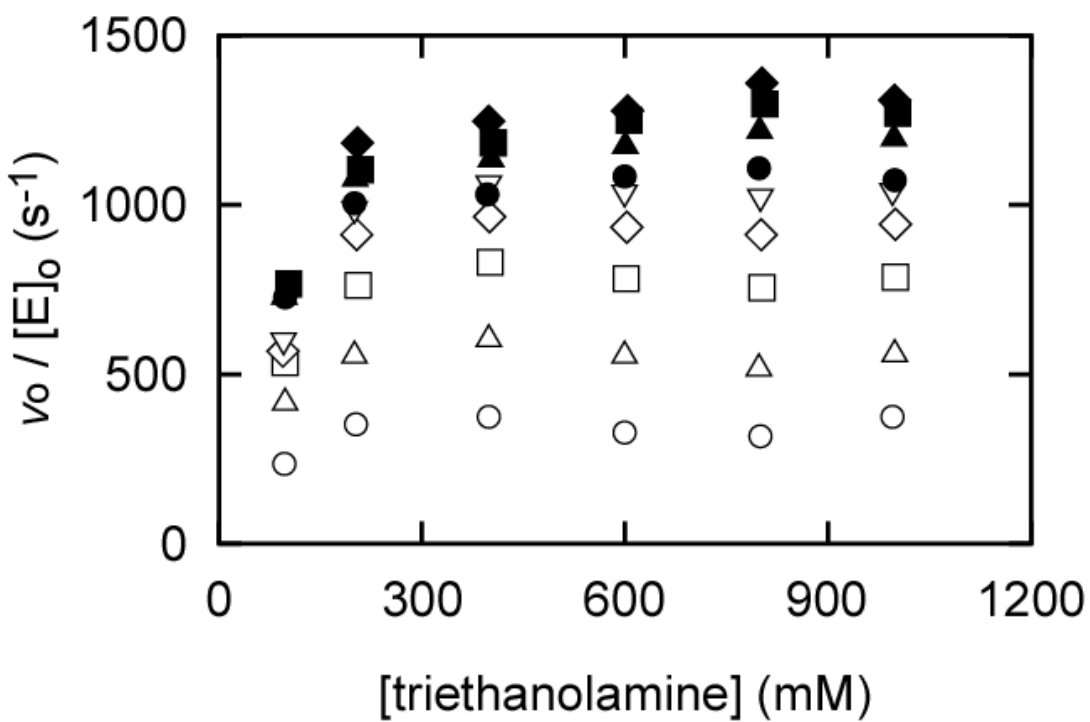
Fig. 4. Sekiguchi *et al.*



A



B

Fig. 5. Sekiguchi *et al.*

C

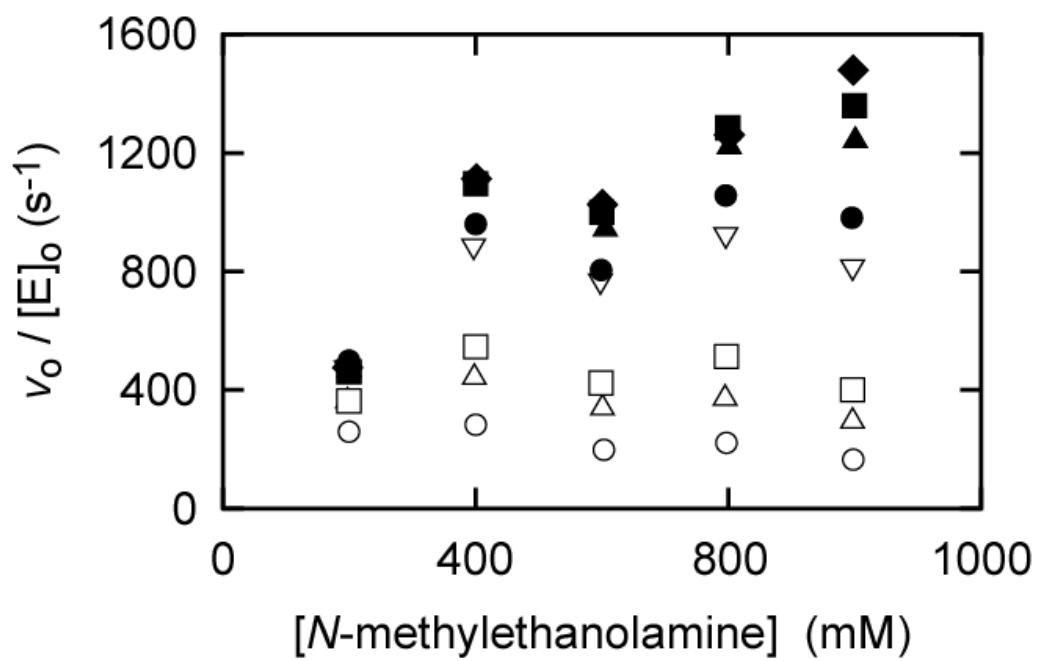


Fig. 5. Sekiguchi *et al.*