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

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18

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

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')$_2$ 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 phophoacceptor [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**

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90

2.1. Materials

91

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, 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).

99

2.2. Hydrolysis of pNPP

100

101

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

114   determined in this study. The kinetic parameters, the molecular activity (k_{cat}) and Michaelis

115   constant (K_m), were calculated from Hanes-Woolf equation (Eq. 1) by

116   least-squares-regression.

117

$$\frac{1}{v_o} = \frac{K_m}{V_{max}\,[S]_o} + \frac{1}{V_{max}} \tag{1}$$

118

119   In this equation, v_o, V_{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_{cat} was calculated

121   from V_{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 20ºC on the substrate concentration are

130   shown in Fig. 2. All plots showed saturated profiles, and the k_{cat} and K_m values of BIALP

131   were determined separately (Table 1). The k_{cat} and K_m values markedly increased with

132   increasing concentrations of diethanolamine. The k_{cat} value at 3.0 M diethanolamine was

133   2330 ± 30 s^{-1}, which was 12-fold higher than that at 0.05 M (190 ± 10 s^{-1}), and the K_m value

134   at 3.0 M diethanolamine was 1.75 ± 0.05 mM, which was 25-fold higher than that at 0.05 M

135   (0.07 ± 0.01 mM). Consequently, the k_{cat}/K_m values were relatively constant in the range

136   0.05-3.0 M.

137

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 ± 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.

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 s^{-1}, which was 180% of that at 0.1 M (840 $\pm$ 30 s^{-1}), and the value at 1.0 M

175   *N*-methylethanolamine was 2250 $\pm$ 80 s^{-1}, which was 450% of that at 0.2 M (500 $\pm$ 10 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 s^{-1}, which was 50% of that at 0.05 M (610 $\pm$ 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 $\pm$ 10 s^{-1}, which was 90% of that at 0.05 M (130 $\pm$ 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 $\pm$ 0.01 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:

8

185   the value at 1.0 M triethanolamine was 0.31 ± 0.01 mM, which was 130% of that at 0.1 M

186   (0.24 ± 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 ± 0.02 mM), and the value at 0.5 M

189   borate was 10.30 ± 0.41 mM, which was 2100% of that at 0.05 M (0.49 ± 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 $Na^+ > K^+ > Li^+$, which was different

215   from Hofmeister's series corresponding to the degree of hydration of ions: $Li^+ > Na^+ > K^+$

216   [22-24].

217

218   *4.2. Effects of aminoalcohols on BIALP activity*

219

220   The k_{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 Zn^{2+} ions and one 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   Zn^{2+} ion (Zn2). The Michaelis complex is formed when the ester oxygen atom of the

230   substrate coordinates another 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-dioxetanephenyl 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

284 **References**

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286 [1] T.W. Reid, I.B. Wilson, *E. coli* alkaline phosphatase, in: P.D. Boyer (Eds.), 3rd ed., The

287 Enzymes, Vol. 4, Academic Press, New York, 1971, pp. 373-415.

288 [2] H.N. Fernley, Mammalian alkaline phosphatases, in: P.D. Boyer (Eds.), 3rd ed., The

289 Enzymes, Vol. 4, Academic Press, New York, 1971, pp. 417-447.

290 [3] E.E. Kim, H.W. Wyckoff, Reaction mechanism of alkaline phosphatase based on crystal

291 structures, J. Mol. Biol. 218 (1991) 449-464.

292 [4] T. Harada, I. Koyama, T. Matsunaga, A. Kikuno, T. Kasahara, M. Hassimoto, D.H.

293 Alpers, T. Komoda, Characterization of structural and catalytic differences in rat intestinal

294 alkaline phosphatase isozymes, FEBS J. 272 (2005) 2477-2486.

295 [5] B. Stec, K.M. Holtz, E.R. Kantrowitz, A revised mechanism for the alkaline phosphatase

296 reaction involving three metal ions, J. Mol. Biol. 299 (2000) 1303-1311.

297 [6] M.M.E. de Backer, S. McSweeney, P.F. Lindley, E. Hough, Ligand-binding and

298 metal–exchange crystallographic studies on shrimp alkaline phosphatase, Acta Cryst. D60

299 (2004) 1555-1561.

300 [7] P. Gettins, J.E. Coleman, ^{31}P nuclear magnetic resonance of phosphoenzyme

301 intermediates of alkaline phosphatase, J. Biol. Chem. 258 (1983) 408-416.

302 [8] J.E Murphy, T.T. Tibbitts, E.R. Kantrowitz, Mutations at positions 153 and 328 in

303 *Escherichia coli* alkaline phosphatase provide insight towards the structure and function of

304 mammalian and yeast alkaline phosphatase, J. Mol. Biol. 253 (1995) 604-617.

305 [9] M.S. Blake, K.H. Johnston, G.R. Russell-Jones, E.C. Gotschlich, A rapid, sensitive

306 methods for detection of alkaline phosphatase-conjugated anti-antibody on Western blots,

307 Anal. Biochem. 136 (1984) 175-179.

308 [10] E. Jablonski, E.W. Moomaw, R.H. Tullis, J.L. Ruth, Preparation of

309 oligodeoxynucleotide-alkaline phosphatase conjugated and their use as hybridization probes,

310 Nucleic Acids Res. 14 (1986) 6115-6128.

311 [11] D.W. Chan, Automation of immunoassays, In: Immunoassay, E.P. Diamandis, T.K.

312 Christopoulos (Eds), Academic Press, New York, 1996, pp. 483-504.

313 [12] J.P. Gosling, Immunoassay, in: E.P. Diamandis, T.K. Christopoulos (Eds), Academic

314 Press, New York, 1996, pp. 287-308.

315 [13] K. Morimoto, K. Inouye, Single-step purification of $F(ab')_2$ fragments of mouse

316 monoclonal antibodies (immunoglobulins G1) by hydrophobic interaction high performance

317 liquid chromatography using TSKgel Phenyl-5PW, J. Biochem. Biophys. Methods 24

318 (1992) 107-117.

319 [14] K. Morimoto, K. Inouye, Single-step purification of $F(ab')_{2\mu}$ fragments of mouse

320 monoclonal antibodies (immunoglobulins M) by hydrophobic interaction high-performance

321 liquid chromatography using TSKgel ether-5PW, J. Biochem. Biophys. Methods 26 (1993)

322 27-39.

323 [15] K. Morimoto, K. Inouye, A sensitive enzyme immunoassay of human

324 thyroid-stimulating hormone (TSH) using bispecific $F(ab')_2$ fragments recognizing

325 polymerized alkaline phosphatase and TSH, J. Immunol. Methods 205 (1997) 81-90.

326 [16] K. Morimoto, K. Inouye, Method for the preparation of bispecific $F(ab')_{2\mu}$ fragments

327 from mouse monoclonal antibodies of the immunoglobulin M class and characterization of

328 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.

353 [26] T.K. Christopoulos, E.P. Diamandis, in: Immunoassay, E.P. Diamandis, T.K.

354 Christopoulos (Eds), Academic Press, New York, 1996, pp. 309-335.

355 [27] L.J. Kricka, in: Immunoassay, Edited by E. P. Diamandis and T. K. Christopoulos (Eds),

356 Academic Press, New York, 1996, pp. 337-353.

357 [28] S. Sekiguchi, H. Kohno, K. Yasukawa, K, Inouye, Study on chemiluminescent enzyme

358 immunoassay for the measurement of leptin, Biosci. Biotechnol. Biochem. in press.

359 [29] R.K. Morton, Some properties of alkaline phosphatase of cow's milk and calf intestinal

360 mucosa, Biochem. J. 60 (1955) 573-582.

361 [30] D. Chappelet-Tordo, M. Fosset, M. Iwatsubo, C. Gache, M. Lazdunski, (1974)

362 Intestinal alkaline phosphatase. Catalytic properties and half of the sites reactivity,

363 Biochemistry 13 (1974) 1788-1795.

364 [31] H.N. Fernley, P.G. Waker, Kinetic behaviour of calf-intestinal alkaline phosphatase with

365 4-methylumbelliferyl phosphate, Biochem. J. 97 (1965) 95-103.

366 [32] D.J. Plocke, B.L. Vallee, Interaction of alkaline phosphatase of *E. coli* with metal ions

367 and chelating agents, Biochemistry 1 (1962) 1039-1043

368 [33] M.L. Applebury, B.P. Johnson, J.E. Coleman, Phosphate binding to alkaline

369 phosphatase. Metal ion dependence, J. Biol. Chem. 245 (1970) 4968-4976.

370 [34] S. Yan, L. Liu, X. Tian, Y. Zhang, H. Zhou, Effect of extraneous zinc on calf intestinal

371 alkaline phosphatase, J. Protein Chem. 22 (2003) 371-375.

372 [35] T.U. Hausamen, R. Helgar, W. Rick, W. Gross, Optimal conditions for the

373 determination of serum alkaline phosphatase by a new kinetic method, Clin. Chim. Acta 15

374 (1967) 241-245.

375 [36] R.B. McComb, G.N. Bowers Jr, Study of optimum buffer conditions for measuring

376 alkaline phosphatase activity in human serum, Clin. Chem. 18 (1972) 97-104.

377   [37] R. Han, J.E. Colman, Dependence of the phosphorylation of alkaline phosphatase by

378   phosphate monoesters on the pK_a of the leaving group, Biochemistry 34 (1995) 4238-4245.

379

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 ($\bigcirc$), 0.25 ($\triangle$), 1.0 ($\square$), and 3.0 ($\diamondsuit$) 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.
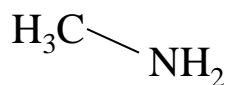
Fig. 3. Dependence of v_o on the pNPP concentration in the BIALP-catalyzed hydrolysis of

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, $\bigcirc$; 1.0 M ethylamine, $\triangle$; and 0.5 M ethanol, $\bullet$; 1.0 M

methylamine, $\square$; and 1.0 M dimethylamine, $\diamondsuit$.

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, $\bigcirc$; 1.0 M ethanolamine, $\triangle$; 1.0 M triethanolamine, $\square$; 1.0 M N-methylethanolamine, $\diamondsuit$; and 0.5 M borate-NaOH. $\bullet$.

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, $\bigcirc$; 0.4, $\triangle$; 0.6, $\square$; 0.8, $\diamondsuit$; 1.0, $\triangledown$; 2.0, $\bullet$; 3.0, $\blacktriangle$; 4.0, $\blacksquare$; and 5.0, $\blacklozenge$. (B) 0.1, $\bigcirc$; 0.2, $\triangle$; 0.4, $\square$; 0.6, $\diamondsuit$; 0.8, $\triangledown$; 1.0, $\bullet$; 1.5, $\blacktriangle$; 2.0, $\blacksquare$; and 3.0, $\blacklozenge$. (C) 0.2, $\bigcirc$; 0.4, $\triangle$; 0.6, $\square$; 1.0, $\diamondsuit$; 1.5, $\triangledown$; 2.0, $\bullet$; 3.0, $\blacktriangle$; 4.0, $\blacksquare$; and 5.0, $\blacklozenge$.
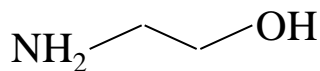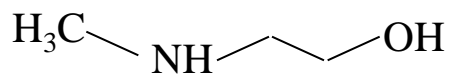
methylamine

ethanolamine

dimethylamine

diethanolamine
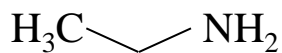
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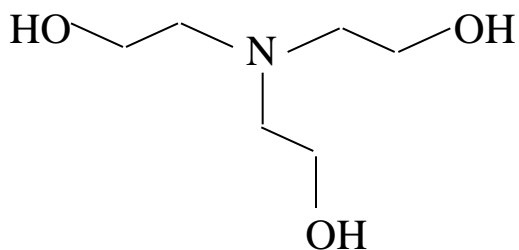
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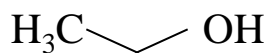
ethanol

N–methylethanolamine

Fig. 1. Sekiguchi *et al.*
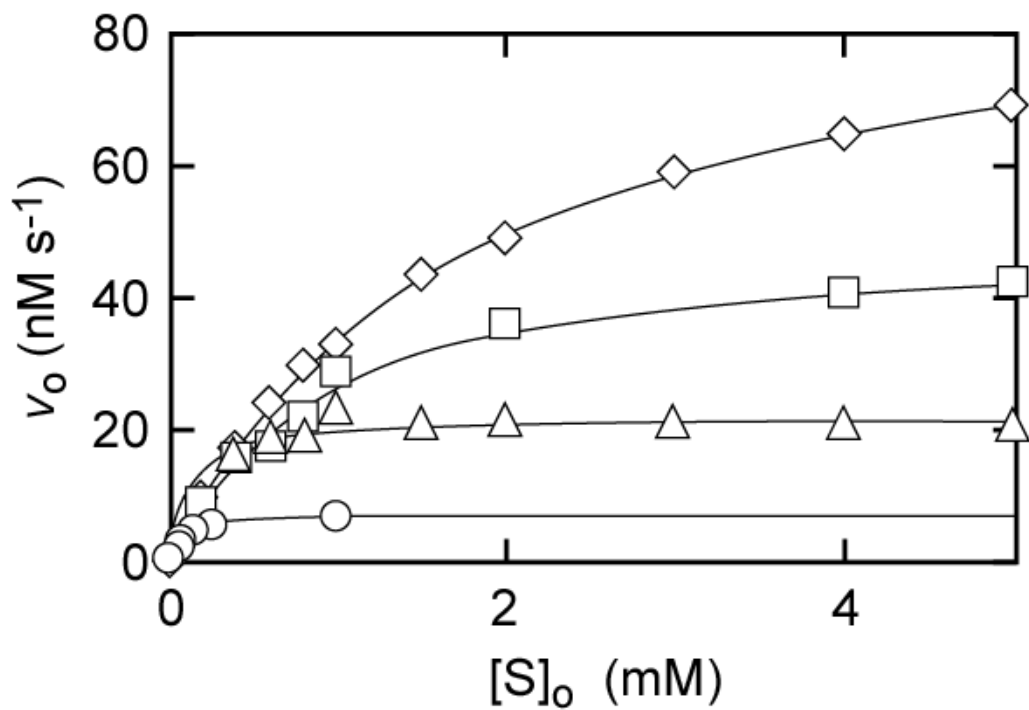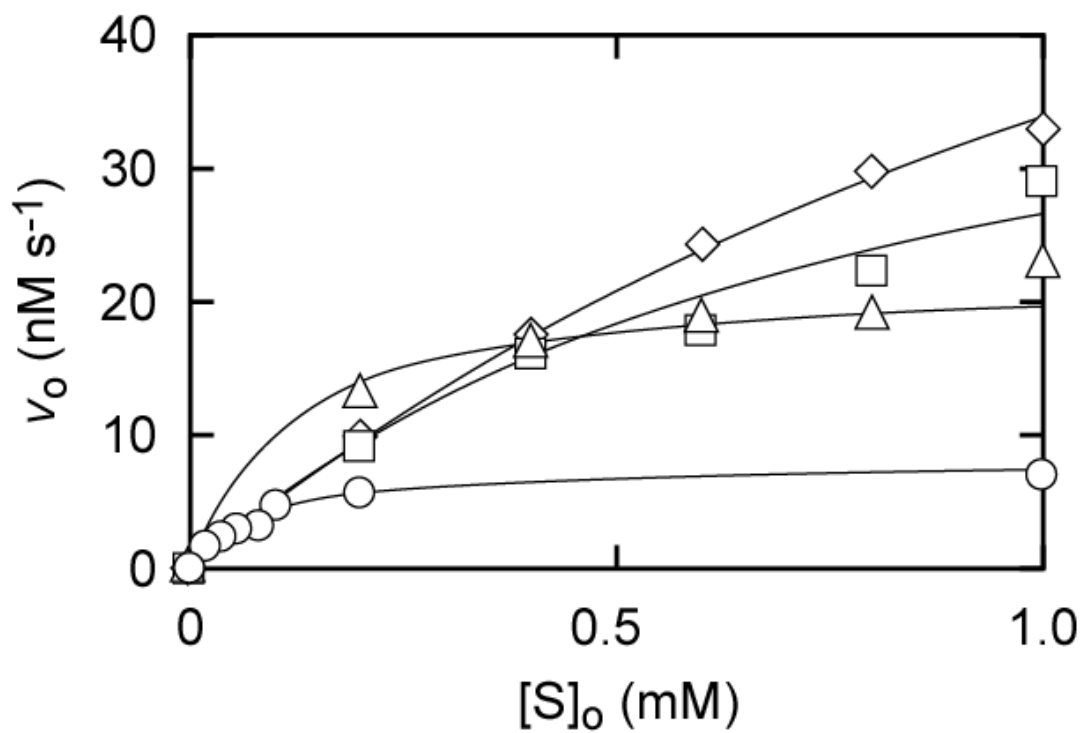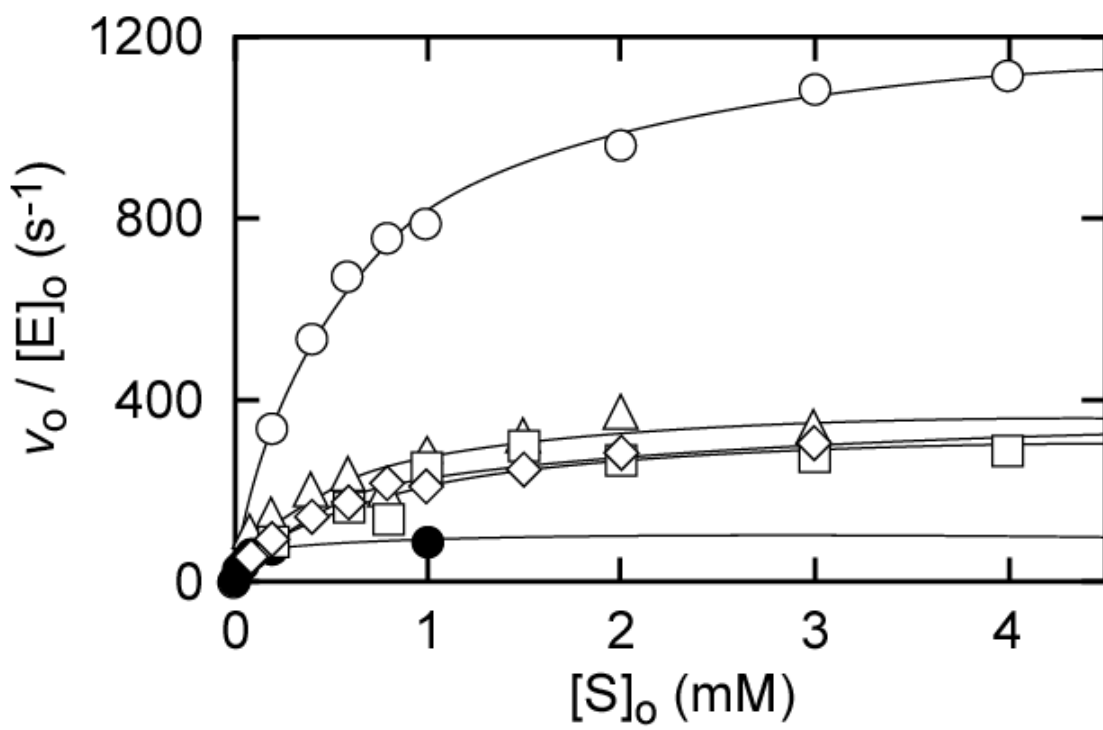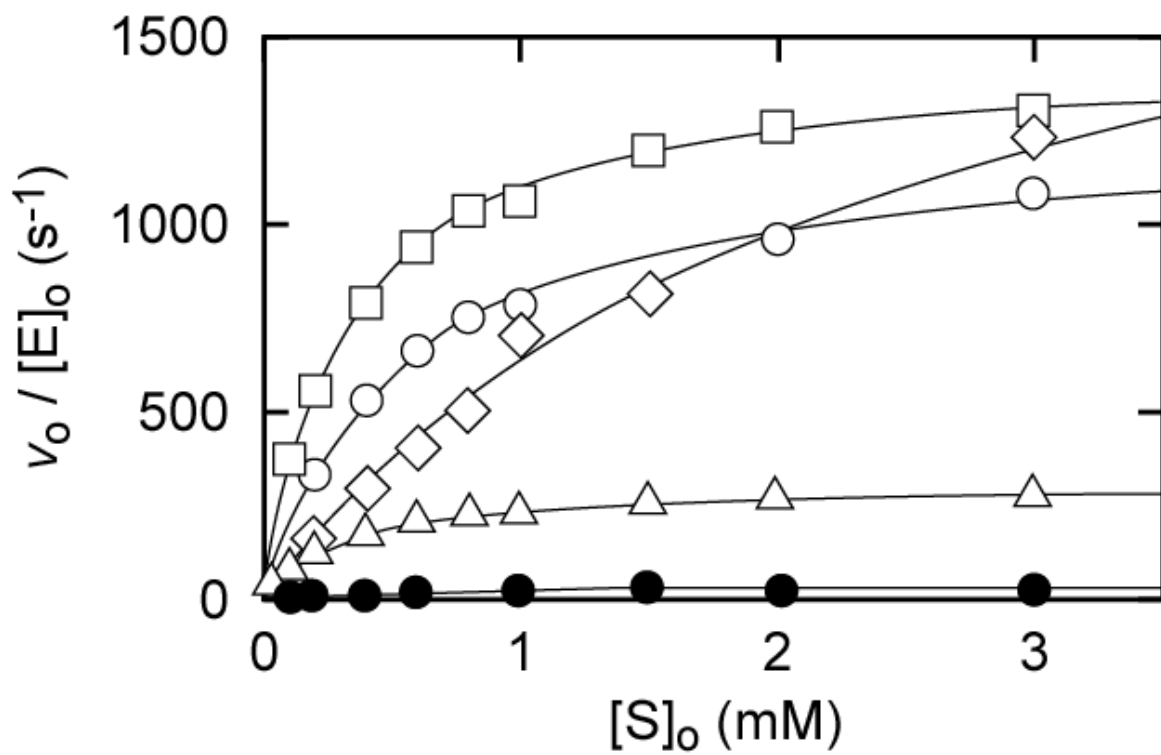
Fig. 2. Sekiguchi *et al.*
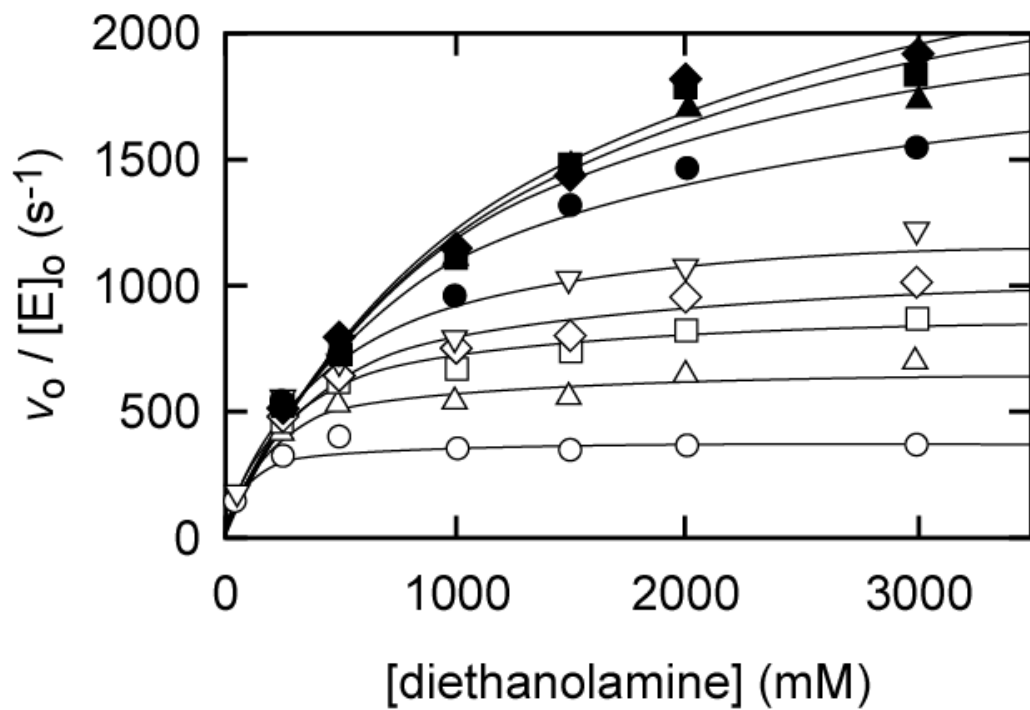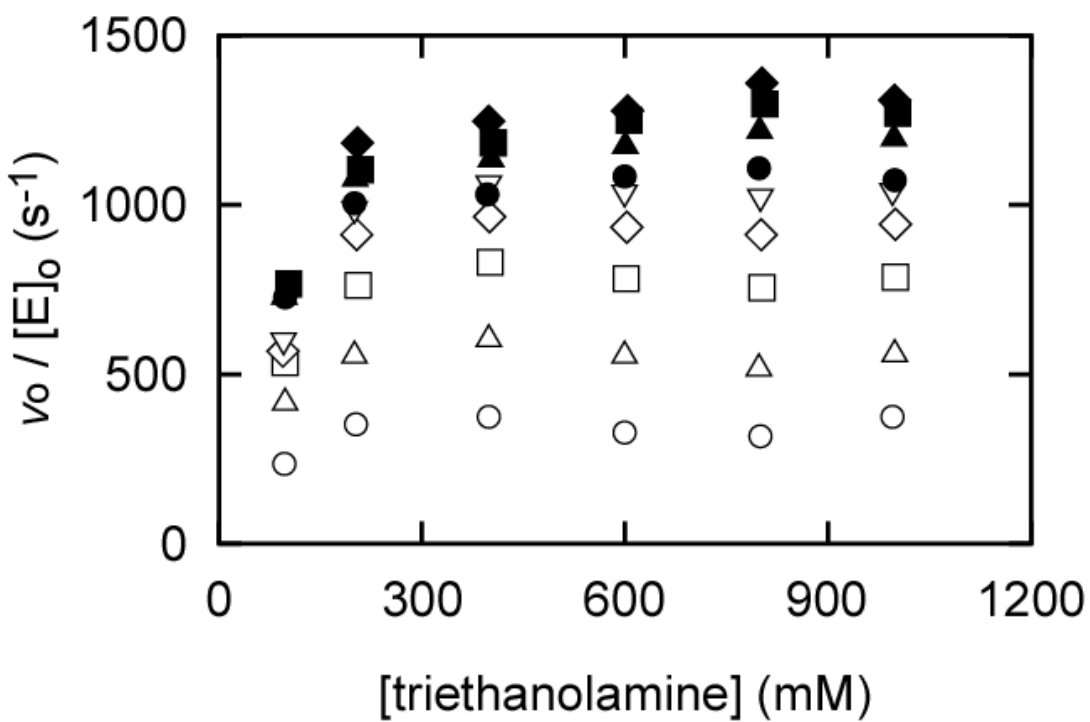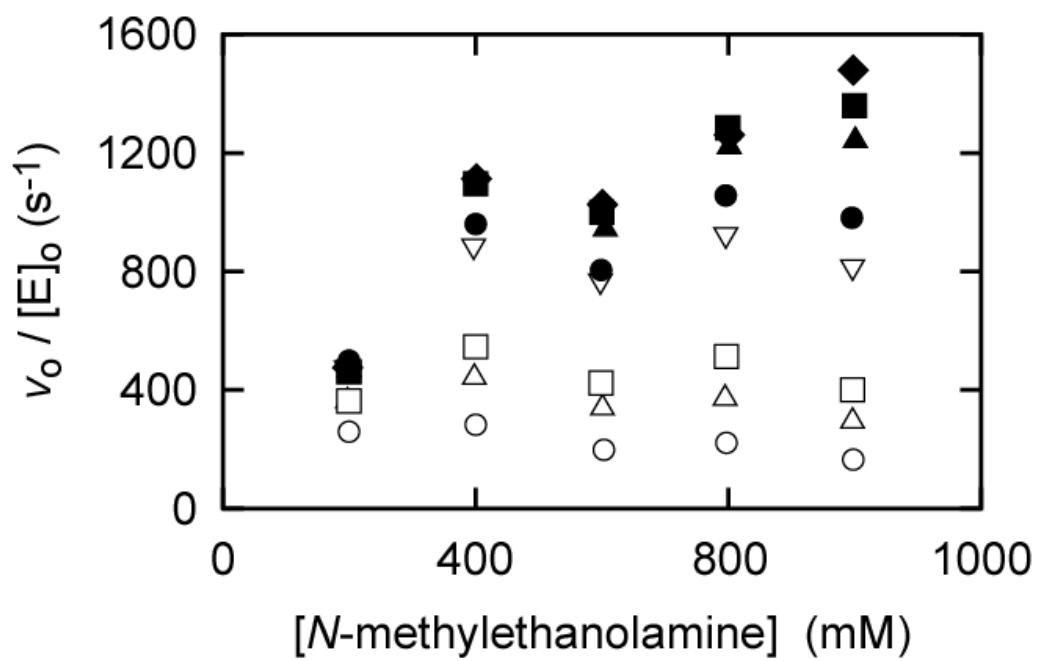
Fig. 3. Sekiguchi *et al.*

Fig. 4. Sekiguchi *et al.*

A

B

Fig. 5. Sekiguchi *et al.*

C

Fig. 5. Sekiguchi *et al.*