Enhanced expression of active recombinant alginate lyase AlyPEEC cloned from a marine bacterium *Pseudoalteromonas elyakovii* in *Escherichia coli* by calcium compounds

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Abstract

Recombinant protein production in *Escherichia coli* is well suited to applications in the basic and applied sciences due to its associated simplicity, cost-effectiveness and the large number of genetic strategies available. However, the active-form of the marine bacterial enzyme, alginate lyase, was difficult to express in *E. coli* cells under standard culture conditions. In this study, we found various calcium compounds that enhanced the expression of the active enzyme. The *alyPEEC* gene encoding extracellular alginate lyase of *Pseudoalteromonas elyakovii* IAM 14594 was comprised of a 1,197 bp open reading frame encoding 398 amino acid residues with the domain G^{165} to N^{398} functioning as the mature enzyme. Three clones, pTPB24 with a 2.7 kb insert containing *alyPEEC* gene and *paeX* and their respective promoter regions, pTPB31 with a 1.6 kb insert containing only the *alyPEEC* gene its own promoter, and pCD11

containing the truncated domain encoding G^{165} to N^{398} of AlyPEEC inserted into the pTrcHisB expression vector were constructed and their expression was analyzed. Alginate lyase activity for the three clones was detected in cell-free extract cultured in LB broth containing 50% artificial seawater (ASW), but not in media with LB broth alone. Maximum activity was observed in the clones cultured in broth containing 50% to 100% ASW, respectively. Further expression analysis using one-by-one element-deficient ASW showed that calcium sulfate affected active AlyPEEC expression. Furthermore, in contrast to inorganic calcium, calcium lactate, glyceric acid calcium and calcium propionate enhanced active AlyPEEC expression markedly.

Title page

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Keywords: Alginate lyase, *Pseudoalteromonas elyakovii*, AlyPEEC, calcium, expression.

Abbreviations: LB, Luria-Bertani medium; BCIP/NBT, 5-bromo-4-chloroindolyl phosphate/ nitroblue tetrazorium; CBB, Coomassie Brilliant Blue

1. Introduction

Recombinant protein production in *Escherichia coli* is well suited to studies in a variety of fields including protein chemistry, medical, agricultural and environmental applications. This is primarily due to the simplicity, economic viability, fast high-density cultivation, well-characterized genetics, and the large number of compatible expression systems that are available [1]. Approximately 80% of the proteins that have been employed to ascertain the three-dimensional structure of moieties submitted to protein data banks in 2003 were prepared in *E. coli* expression systems [1]. Considerable evidence obtained from genetic studies of protein expression and in the molecular biology of protein folding mechanisms in *E. coli* has facilitated mechanisms for the effective production of biologically active form of proteins [1-3]. However, the active-form of a marine bacterial biopolymer-degrading enzyme has rarely been expressed in *E. coli* under the highly controlled conditions of standard protein expression systems [4].

Since the original description of alginate lyases more than 45 years ago [5-6], more than 50 enzymes have been characterized from a variety of microbial, animal and plant sources [7]. Of these enzymes, unique substrate specificity has been discovered in an alginate lyase produced by *Pseudolateromonas elyakovii* IAM 14596 [8], which is capable of degrading all block structures derived from sodium alginate and produces a series of tri- to octa-oligouronates [9]. This novel alginate lyase with broad substrate specificity is particularly useful in the preparation of specific food products [10-12] and for the preparation of protoplasts in the brown alga *Laminaria japonica* [13]. The gene encoding the alginate lyase, *alyPEEC*, has been cloned and expressed in *E. coli*,

however, the alginate lyase activity of AlyPEEC has only been detected in cultures of LB broth supplemented with seawater [4]. This seawater-dependent production of biologically active proteins, such as AlyPEEC alginate lyase, has never been reported previously. In this study we characterize the expression mechanism of the alginate lyase using *E. coli* system.

2. Materials and Methods

2.1. Bacterial strains

Alginate lyase positive clones pTPB24, pTPB31 and pCD11 [4] were used for the expression of the enzyme in this study. The gene, *alyPEEC*, responsible for encoding the extracellular alginate lyase in the marine bacterium, *Pseudoalteromonas elyakovii* IAM 14594, was found to be comprised of a 1,197 bp open reading frame encoding 398 amino acid residues, and the domain G^{165} to N^{398} is functioned as the mature enzyme [4]. Three clones were designed to carry the *alyPEEC* gene or a part of the gene product. These were pTPB24, designed by inserting *alyPEEC* and *paeX* genes into pUC18, pTPB31 by inserting *alyPEEC* into pUC18, and pCD11 consisting of the PCR amplified mature enzyme domain inserted into pTrcHisB expression vector (Invitrogen, USA) lacking the *P. elyakovii* promoter (Fig. 1). Specifically, pTPB24 was designed by inserting a 2.7 kb fragment containing the *alyPEEC* gene and *paeX* (gene of unknown function) including their own promoter regions, pTPB31 was designed by inserting a 1.6 kb containing only *alyPEEC* gene including the promoter region, and pCD11 was designed by inserting only the domain encoding G^{165} to N^{398} of AlyPEEC into the

pTrcHisB expression vector. The clones were produced and their expression was analyzed. The gene of unknown function, *paeX* (Fig. 1.), had 34% homology to a putative ECF sigma factor gene from *Pseudomonas aeruginosa* (unpublished data). The clones were kept as glycerol stock under -80°C.

2.2 Assay for alginate lyase activity

Alginate lyase activity was assayed using the thiobarbituric acid (TBA) method [5]. The alginate lyase expressing clones were cultured in LB broth or LB broth containing either artificial seawater (ASW; 3% (w/v) NaCl, 0.07% (w/v) KCl, 0.53% (w/v) $MgSO_4$, 1.08% (w/v) $MgCl_2$, and 0.13% (w/v) $CaSO_4$) or calcium compounds with 5 mM IPTG at 30°C with agitation (130 rpm). All media contained 100 μg mL^{-1} ampicillin. After 30 hours incubation, the culture medium was centrifuged (8,000 x g for 10 min at 4°C). Cells were vortexed in the presence of 50 μL chloroform and then suspended in 1.0 mL of 0.1 M Tris-HCl buffer, pH 7.5 [14]. The suspensions were kept at –20°C overnight before being centrifuged (12,000 x g for 5 min at 4°C) to produce cell-free extracts. The centrifuged culture medium (supernatants) and the cell-free extracts were used for enzyme assays. The reaction mixture was composed of 0.1 M Tris-HCl buffer, pH 7.5 and 0.1% (w/v) sodium alginate with reactions performed at 30°C.

2.3. Effect of calcium compounds and concentration on active enzyme production

Seven calcium compounds were selected to screen for the enhancement of active recombinant alginate enzyme production. These compounds were calcium sulfate, calcium chloride, calcium bromide, calcium lactate, calcium gluconate, glyceric acid calcium, and calcium propionate, all of which are soluble in LB broth even after

autoclaving. Clones were cultured in LB broth supplemented with respective calcium compounds with alginate lyase activities in cell-free extracts measured as described above. Calcium compounds exhibiting enhanced active alginate lyase expression were examined further to determine the effect of concentration dependency.

2.4. Electrophoresis, western blotting and antibody staining of the expressed AlyPEEC

pCD11 was constructed by insertion of the mature region of AlyPEEC into the expression vector pTrcHisB [4]. The mature alginate enzyme was expressed as a fusion protein with Xpress protein and hexa-histidine. The Xpress protein is a specific target of the expressed recombinant AlyPEEC by anti-Xpress antibody. Consequently, immunostaining after electroblotting can be used as an assay for the target protein. pCD11 was cultured in LB broth supplemented with ASW (100% v/v), calcium sulfate (0.13% w/v), calcium lactate (1.4% w/v), glyceric acid calcium (0.64% w/v), and propionate calcium (0.7% w/v), at 30°C for 30 h. These media were also supplemented with ampicillin (final concentration of 100 μg ml^{-1}) and IPTG (5 mM). Cell density was recorded spectrophotometrically (Pharmacia, Sweden) at 600 nm. Cell-free extract for determining the enzyme activity and protein concentration were prepared by the chloroform method descried above.

Cell pellets were obtained from the same culture and resuspended in 100 ml distilled water. These were mixed with equal volumes of loading dye and boiled for 5 min. The resulting cell-lysate was subjected to SDS-polyacrylamide gel electrophoresis [4] and western blotting [15]. Electroblotting to nitrocellulose was done using semi-dry system (TE70, Pharmacia, Sweden) according the manufacturer's instructions at 0.8 mA cm^{-2}

for 2 h. The nitrocellulose membrane was subjected to for immunological staining using anti-Xpress antibody (Invitrogen, USA) with the expressed AlyPEEC visualized by Western Blue^{TM} BCIP/NBT substrate (Promega, USA) for the antibody-conjugated alkaline phosphatase. The visualized AlyPEECs on the nitrocellulose membrane were scanned and with concentrations measured using Image Tool software (version 2.0, UTHSCSA Image tool IT, USA). Concentrations of the recombinant AlyPEEC proteins before and after electroblotting in the CBB stained SDS-PAGE gels were estimated by using image processing techniques and blotting efficiency of the AlyPEEC were calculated. Chloramphenicol acetyl transferase gene expression in the pTrcHisB vector was used as a control for immunological staining according to manufacturer's instructions.

2.5. Post-treatment of the recombinant AlyPEEC by calcium compounds

Calcium compounds (calcium chloride, calcium sulfate, calcium lactate, and calcium propionate) were added to the cell-free extract of pCD11 cultured in LB with or without ASW to determine the effect of post-treatment on alginate lyase activity of the cell-free extracts. The concentration of calcium compounds was adjusted to 10 mM in the substrate solution for the enzyme activity assay. Alginate lyase activity was measured by the TBA method described above. Protein concentration was measured by spectrophotometrically at 260 nm with the relative activities of each cell-free extract calculated based on the specific activity of the cell-free extract of pCD11 cultured in LB broth. Magnesium chloride (50 mM), a positive activator of the alginate lyase activity [9], was used as positive control.

3. Results

3.1 Effects of seawater concentration on recombinant alginate lyase production

Alginate lyase activity was detected from cell-free extracts of all clones cultured in LB broth containing ASW, but relatively low or no enzyme activities were observed in cell-free extracts cultured in LB containing 0.5 M NaCl or 50 mM $MgCl_2$ (Fig. 2). Maximum enzyme activity was detected in cell-free extracts cultured with LB broth containing 50% ASW in pTPB24, 75% ASW in pTPB31, and 100% ASW in pCD11 (Fig. 2). The ASW used in this study contained NaCl, KCl, $MgSO_4$, $MgCl_2$ and $CaSO_4$. Therefore, effect of remaining inorganic compounds (KCl, $MgSO_4$, or $CaSO_4$) was evaluated for enhancing expression of the active recombinant AlyPEEC. The enzyme activities were less in culture supernatant than in cell-free extract in almost clones (Fig. 2), only enzyme activity in the cell free extracts was further analyzed.

3.2 Effects of one-by-one element deficient seawater on the expression of AlyPEEC

To confirm which ingredient in ASW had the most marked affect on active recombinant AlyPEEC expression, one-by-one element deficient seawater on the expression of AlyPEEC were investigated. Remarkably low enzyme activity was observed in cultures of $CaSO_4$-deficient ASW on pTPB24 and pTPB31 (Fig. 3. A and B). A significant decrease in enzyme activity was observed in pCD11 (Fig. 3. C). Single supplementation of $CaSO_4$ as the sole calcium source into LB broth stimulated alginate lyase expression in all clones (data not shown). Slight decreases in alginate lyase activity in cultures of NaCl-deficient ASW were also observed (Fig. 3). Conversely, single supplementation of NaCl in LB broth did not affect an increase in alginate lyase production in all clones (Fig. 3).

3.3 Effects of various calcium compounds on the expression of active enzyme

Effects of seven calcium compounds, all of which were easily soluble in LB broth, on the expression of the active form of AlyPEEC from pTPB31 were determined. The molar concentrations of calcium in each compound were adjusted to 1.5×10^{-2} M so that concentrations were similar to those of the calcium concentration in 50% ASW. The activities were higher in cultures supplemented with calcium lactate, glyceric acid calcium, and calcium propionate than they were in 50% ASW or $CaSO_4$ (Fig. 4). Compared to cultures in 50% ASW, the increase in enzyme activity was four fold in the calcium lactate supplemented culture, seven fold in the glyceric acid calcium, and 14 fold in the calcium propionate.

The detected alginate lyase activities of pTPB31 and pCD11 increased in proportion to the concentration of the supplemented calcium compounds (Fig. 5), with the high activities observed in 0.7%, 0.64%, and 0.7% of the cultures supplemented with calcium lactate, glyceric acid calcium, and calcium propionate in pTPB31, respectively (Fig. 5. A). Similarly, high activities were also determined in 1.4%, 0.64%, and 0.7% in cultures supplemented with calcium lactate, glyceric acid calcium, and calcium propionate in pCD11, respectively (Fig. 5. B). High alginate lyase activities were observed in pTPB31 and pCD11 in calcium propionate supplemented cultures, and were two and seven times higher than those of ASW supplemented cultures.

3.4. Detection of expressed AlyPEEC as active and inactive forms

A single sharp signal was detected in cell-lysate from pCD11 in all media with and without calcium compounds tested in this study (Fig. 6). The integrated density of each

immunostained band was corrected by determining blotting efficiency, and expression of recombinant AlyPEEC was estimated (Table 1). The highest level of AlyPEEC production was observed in a culture supplemented with 100% (v/v) ASW, followed by a culture supplemented with 0.64% (w/v) glyceric acid calcium supplemented broth (Table 1). AlyPEEC production was relatively low in LB broth cultures and cultures supplemented with 0.7% (w/v) calcium propionate (Table 1). Specific AlyPEEC production per growth rate was highest in a culture of LB supplemented with 100% (v/v) ASW followed by in a culture with glyceric acid calcium (73%). However, the specific enzyme productions were only 12 and 13% in LB and the broth supplemented with propionate calcium, respectively (Table 1). Conversely, estimated specific enzyme activity per expressed AlyPEEC was remarkably high in LB broth supplemented with propionate calcium. The specific enzyme activity was 30 fold higher than those observed in ASW supplemented culture, followed by those in LB broths supplemented with calcium sulfate and calcium lactate. Therefore, while AlyPEEC production was distinct, protein expression in LB both appeared be less than that in ASW (Table 1).

3.5. Post-treatment of the expressed AlyPEEC

Post-treatment of the inactive recombinant AlyPEEC by calcium compounds increased enzyme activity slightly by 1.5 (calcium propionate) to 5.4 fold (glyceric acid calcium) (Table 2). However, these increases were less than those associated with pre-treated cell-free extract of pCD11 in ASW supplemented broth culture (15.6 fold). Increases in the enzyme activity of the active recombinant AlyPEEC by post-treatment of calcium compounds ranged from 1 to 2 fold (Table 2).

4. Discussion

Single calcium compounds were observed to enhance expression of an active recombinant biopolymer degrading enzyme (AlyPEEC) derived from the marine *Pseudoalteromonas* gene in *E. coli*. (Figs. 2 to 4). Calcium lactate, glyceric acid calcium and calcium propionate greatly enhanced the active AlyPEEC expression, with calcium propionate being the most effective (Figs. 4 and 5). Unit-activity of recombinant AlyPEEC expressed in calcium propionate supplemented LB broth was 30 fold higher than that of LB broth alone (Table 1). The effect of calcium compounds on the activity of inactive AlyPEEC by the “post”-treatment method was relatively smaller than the “pre”-treatment (co-culture) method (Table 2). Use of calcium supplemented media for the cultivation of recombinant *E. coli* could be considered an alternative cost effective method for the expression of active recombinant marine bacterial biopolymer degrading enzymes when active protein production is low under standard culture conditions in *E. coli*.

The enhancement of active AlyPEEC expression by calcium compounds was observed in all three clones examined in this study; pTPB24, pTPB31, and pCD11 (Fig. 3). The primary differences in the gene composition among these clones were related to promoter and secretion signals (pTPB24 and pTPB31), to the function of the unknown *paeX* gene (pTPB24) from *P. elyakovii*, and of truncated minimum catalytic region of AlyPEEC (pCD11) (Fig. 1). Recombinant AlyPEEC expression in pCD11 was driven by the vector-controlled promoter and occurred in the cytoplasm [4]. The recombinant AlyPEEC production was apparent in *E. coli* without calcium but the enzyme was inactive (Fig. 6, and Table 1). The concentrations of the recombinant AlyPEEC proteins

were the same in pCD11 *E. coli* clones, irrespective of whether calcium propionate was supplemented in the LB broth or not (Fig. 6, and Table 1). It therefore appears that, (i) the *paeX* gene was not associated with calcium dependent AlyPEEC expression (Fig. 1), (ii) calcium may not affect transcription of *alyPEEC* gene, and, (iii) calcium may penetrate the plasma membrane and affect the form the active enzymes in the cytoplasm. However, we were unable to determine the presence of the calcium binding sites in AlyPEEC nor how calcium interacts with the inactive recombinant AlyPEEC to form the active enzyme. Future studies will therefore require the use of calcium tracer to understand the manner in which the calcium affects active AlyPEEC expression.

The actual mechanism responsible for increasing AlyPEEC activity by the organic calcium compounds rather than inorganic calcium is unclear at present. The intracellular calcium levels of all bacterial cells are maintained below that contained within the growth medium by cell membrane transporters. This membrane transport functions as either a primary ATPase or as secondary Ca^{2+}/H^{+} chemiosmotic exchanger and ensure that the intracellular calcium concentrations of *E. coli* are maintained at 0.1 μM [16]. To the best of our knowledge, while intracellular calcium content is controlled by this homeostasis-like mechanism, studies of calcium transport, especially on organic calcium compounds, in *E. coli* have not been reported to date. In pCD11, AlyPEEC expression has been reported to occur in the cytoplasm [4]. Therefore, calcium compounds enhanced the AlyPEEC activity must reach the cytosol in pCD11 and affect on the production of the active recombinant AlyPEEC. One possible explanation for calcium enhanced active AlyPEEC production may be related to the findings of Trinidad *et al*. (1999) [17] in their human absorption model. They reported that calcium

absorption by the human distal colon was increased in the presence of organic acid (acetic acid and propionic acid). They suggested that calcium absorption may be promoted by the formation of less-charged organic acid calcium compounds, [Calcium Acetate]$^{+}$ or [Calcium Propionate]$^{+}$, comparing to charged [Ca^{2+}] alone. Since AlyPEEC expression may have occurred in the cytosol (pCD11) before being transported out of plasma membrane (pTPB24 and pTPB31), calcium compounds with lower charges (calcium lactate, glyceric acid calcium, and calcium propionate) could easily reach the cytosol or periplasmic space through outer membrane to interact with the AlyPEEC for folding the mature active enzyme, even in *E. coli*. No remarkable leakage of the expressed recombinant enzyme into culture supernatant was observed in the calcium or seawater supplemented cultures (Fig. 2).

AlyPEEC is effective for producing protoplasts or alginate oligosaccharides, and the finding of stable over-expression of the active recombinant enzyme could thus be an important contribution to marine biotechnology. This is particularly relevant given the recent publication of a global-gene expression study of cellulase-like genes from the western Arctic Ocean and Sargasso Sea metagenomic library by Cottrell et al. (2005) [18]. Enzymes involved in biopolymer degradation are key enzymes in marine mineral cycle. Alginate lyases may also be a suitable candidate for the biopolymer degradation process in oceanic environments. Simple and cost-effective recombinant enzyme expression strategies involving *E. coli* using organic calcium supplemented cultures, especially on genes from marine bacteria such as that demonstrated in this study, may constitute a significant contribution to further investigations involving the degradation by unsealed biopolymer enzymes and their role in the metabolic pathways associated

with oceanic mineral cycles.

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Figure captions

Fig. 1. Gene structure of alginate lyase positive clones for the expression studies. P*aly*: *P. elyakovii* own promoter, *lac*: *lac* promoter, *tac*: *tac* promoter, pre: *alyPEEC* precursor protein region, mature: *alyPEEC* mature enzyme region, *paeX*: function-unkown ECF sigma factor-like protein coding region.

Fig. 2. Effect of seawater concentration on the active AlyPEEC production by the clones. ■: Intracellular enzyme activity, □: Extracellular enzyme activity.

Fig. 3. Effect of on-by-one element deficient seawater on the active AlyPEEC production by the clones. ■: Intracellular enzyme activity, □: Extracellular enzyme activity.

Fig. 4. Effect of various calcium compounds on alginate lyase production in pTPB31. All calcium compounds added in the broth were adjusted to $1.5X10^{-2}$ M.

Fig. 5. Concentration dependency of active AlyPEEC production in calcium supplemented LB broth in *Escherichia coli*. ●: Calcium lactate, ■: glyceric acid calcium, ▲: calcium propionate, ◆: ASW supplemented control.

Fig. 6. Immunological detection of the expressed AlyPEEC. Lane 1: CAT gene control, Lane 2: LB broth, Lane 3:100%ASW supplemented LB, Lane 4: 0.13% calcium sulfate supplemented LB broth, Lane 5: 1.4% calcium lactate supplemented LB, Lane 6: 0.64%

1 Glyceric acid calcium supplemented LB, Lane 7: 0.7% calcium propionate
2 supplemented LB.

Table 1. Estimation of the active recombinat AlyPEEC prodcution in pCD11 on various culture media

Culture medium	Integrated density *1	Blotting efficiency (%)	Corrected amounts of putative enzyme production *2	Growth (OD600)	Activity (U)	Relative value (%)	
						Production /Growth	Activity / Production
LB	7533	100	7533	1.793	7.7×10^{-7}	12	13
100% ASW-LB	52564	99	53095	1.543	4.2×10^{-5}	100	100
0.13% Calcium sulfate-LB	16785	85	19747	1.86	1.2×10^{-4}	31	792
1.4% Calcium lactate-LB	13706	74	18522	2.117	1.0×10^{-4}	25	678
0.64% Glyceric acide calcium-LB	32560	62	52516	2.102	2.0×10^{-4}	73	483
0.7% Calcium propionate-LB	8900	95	9368	2.037	2.2×10^{-4}	13	3017

*1 Integrated dencity of the experssed recombinant AlyPEEC was measured using ImageTool.

*2 Corrected amounts were calculated with integrated density and blotting efficiency.

Table 2. Post-treatment by calcium compounds on the expressed recombinant AlyPEEC

Basal medium	Additives	Relative activity (Times)
LB	Non (inactive-form)	1
LB	50mM Magnesium chloride	1.8
LB	10mM Calcium chloride	4.5
LB	10mM Calcium sulfate	4.0
LB	10mM Calcium lactate	2.1
LB	10mM Glyceric acid calcium	5.4
LB	10mM Calcium propionate	1.5
100%ASW-LB	Non	15.6
100%ASW-LB	50mM Magnesium chloride	26.9
100%ASW-LB	10mM Calcium chloride	14.0
100%ASW-LB	10mM Calcium sulfate	16.6
100%ASW-LB	10mM Calcium lactate	19.6
100%ASW-LB	10mM Glyceric acid calcium	12.2
100%ASW-LB	10mM Calcium propionate	37.5

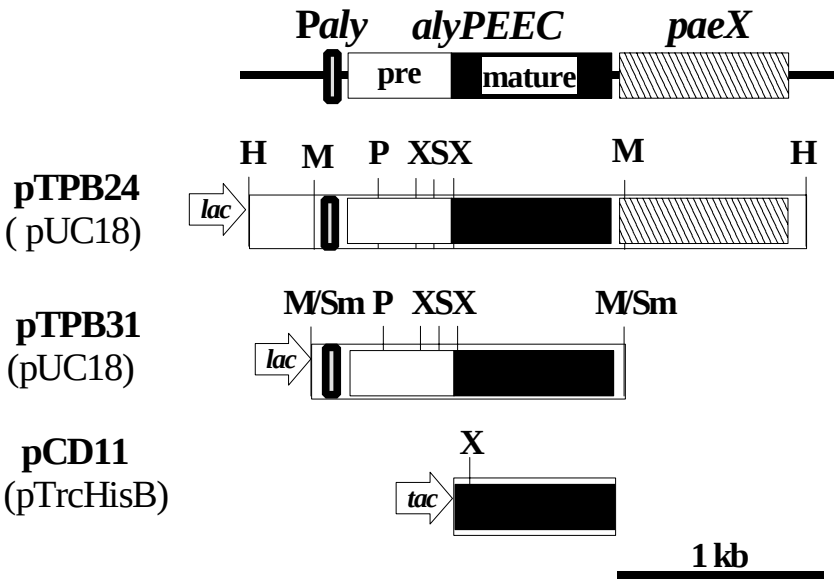
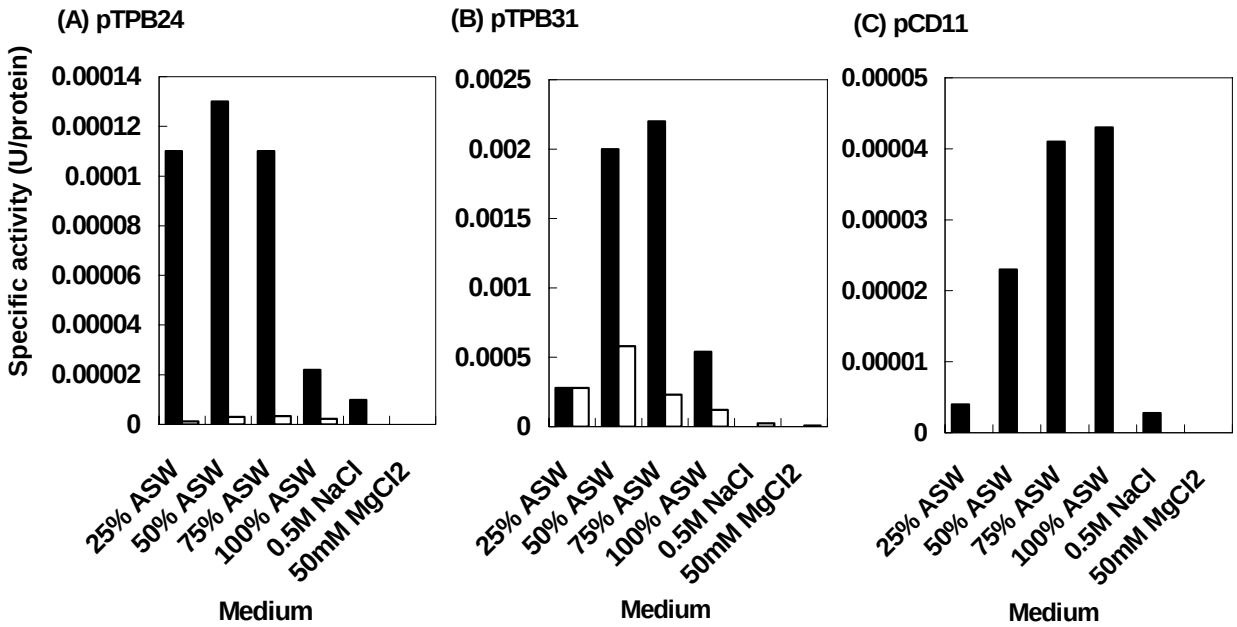


Fig. 1. Sawabe et al.



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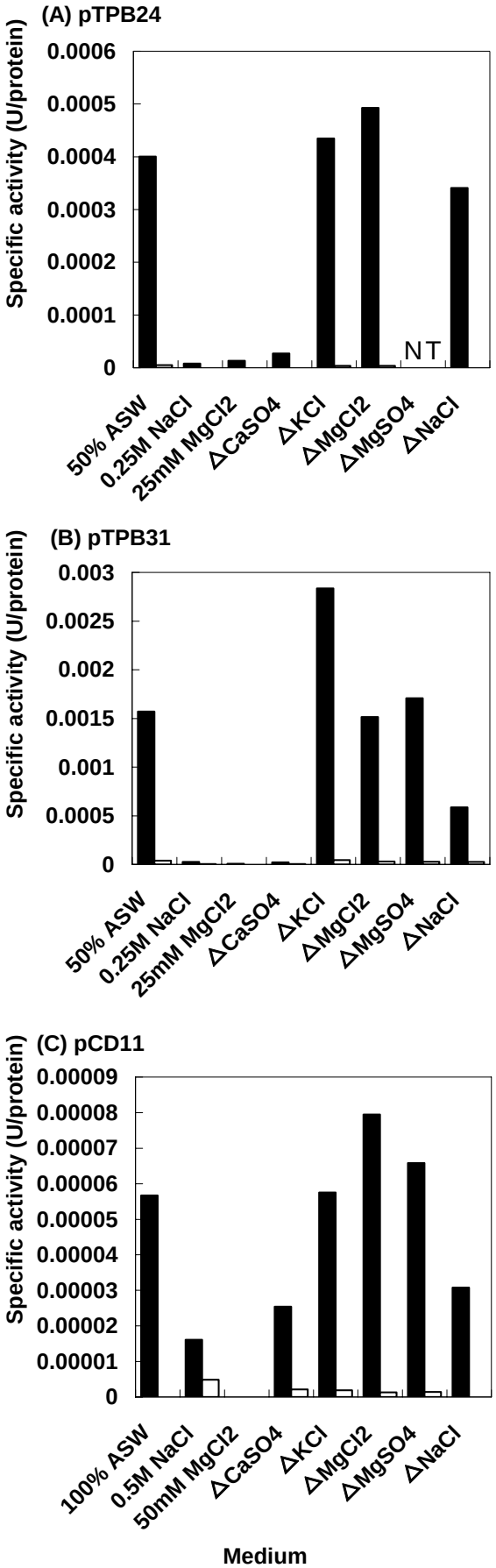

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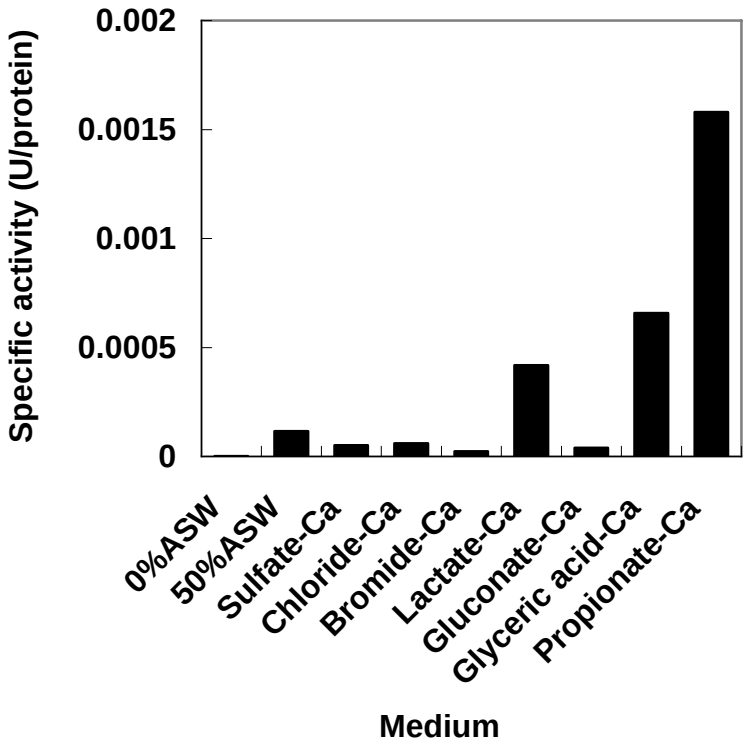

Fig. 4. Sawabe et al.

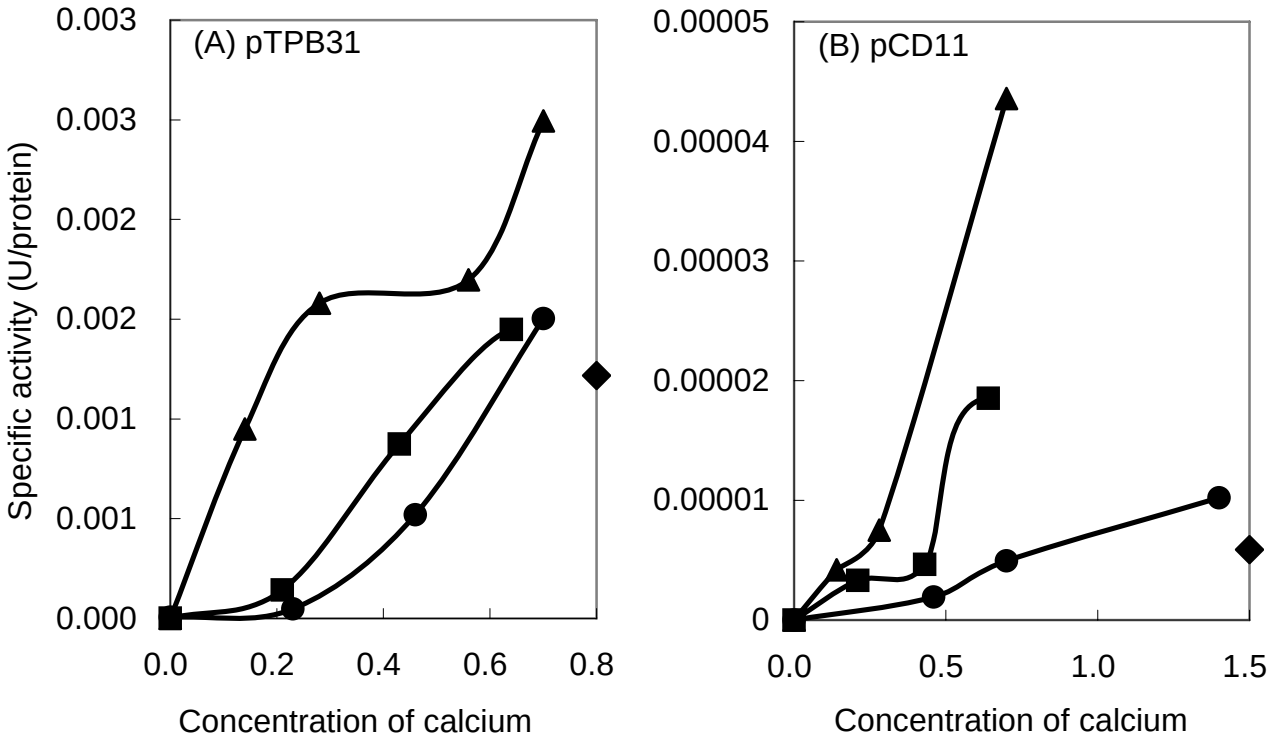

Fig. 5. Sawabe et al.

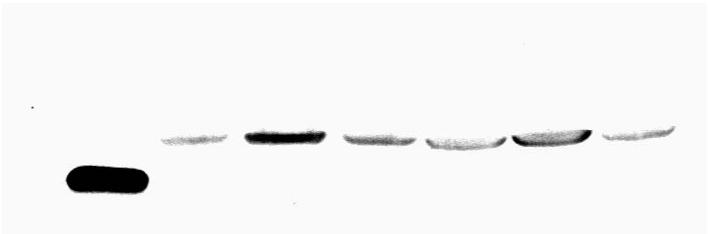

Fig.6. Sawabe et al.