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ORIGINAL PAPER

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CHARACTERIZATION OF CRUDE 5'-PHOSPHODIESTERASE FROM GERMINATED ADZUKI (*VIGNA ANGULARIS* L.) BEANS

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ABSTRACT

Background. 5'-Phosphodiesterase (5'-PDE) is an enzyme that hydrolyzes RNA to form 5'-inosine monophosphate (5'-IMP) and 5'-guanosine monophosphate (5'-GMP). These 5'-nucleotides can function as flavor enhancers. Adzuki beans (*Vigna angularis* L.) are found to be high in 5'-PDE.

Materials and methods. 5'-phosphodiesterase (5'-PDE) enzyme was characterized from adzuki beans, in which the optimum pH and temperature were determined. In addition, the stability of 5'-PDE was assessed at different pH and temperature. The effects of cations and EDTA were evaluated to characterize the 5'-PDE enzymes further.

Results. The alkaline 5'-phosphodiesterase has an optimum pH of 8.5. This enzyme is also thermostable, with an optimum temperature of 80°C. The stability in terms of temperature and pH was also determined, and was found to be stable in the pH range of 7.0–8.5. This enzyme was found to retain more than 80% of its activity for 4 days at 60 and 65°C. In addition, the effects of 14 different metal ions, 4 types of detergents and ethylenediaminetetraacetic acid (EDTA) on 5'-PDE were studied. Ca^{2+}, K^{+}, Mg^{2+} and Li^{+} activated 5'--PDE while Na^{+}, Zn^{2+}, Ni^{+}, Hg^{+}, Cu^{2+}, Pb^{2+}, Fe^{2+}, Al^{3+}, Ba^{2+} and Co^{2+} were inhibitory. EDTA, Triton X-100 and sodium dodecyl sulfate (SDS) were strong inhibitors of 5'-PDE, while Tween 80 and Tween 20 were slightly inhibitory. The effects of cations and EDTA suggest that 5'-PDE from adzuki beans is a metalloenzyme.

Conclusion. Although 5'-PDE from adzuki beans has a high temperature optimum of 80°C, the enzyme is more stable at 60°C, and different cations affected the activity of the enzyme differently.

Keywords: 5'-phosphodiesterase, thermostability, optimum pH, detergents, cations

INTRODUCTION

5'-Phosphodiesterase (5'-PDE, EC 3.1.4.1) is the enzyme that digests phosphodiester bonds to produce a 5'-hydroxyl group, in which it hydrolyzes RNA to 5'-nucleotides. 5'-PDE is a useful tool in the area of biotechnology, especially in nucleic acid research labeling, characterization of oligonucleotides and also as an aid in sequence analysis (Harvey et al., 1970; Rosemeyer et al., 2002). As well as this, it is utilized in the study of genes related to obesity and insulin resistance and hepatitis C viruma (Santoro et al., 2009; Takahama et al., 2008). 5'-PDE can also be used for the production of mononucleotides, whereby the

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mononucleotides (5'-IMP and 5'-GMP) can function as flavor enhancers in the food industry (Beluhan et al., 2003; Rotola-Pukkila et al., 2019).

Previous studies have been done on germinating beans as sources of 5'-PDE such as barley, mung bean (Khutle et al., 2011), yellow soybean and black soybean (Utami et al., 2011). Also, 5'-PDE with different pH and temperature optima, as well as other characteristics, were reported for microorganisms such as *Xanthomonas axonopodis* (Lassila and Herschlag, 2008), *Rhizobium legumniosarum* (Jones et al., 2008), *Aspergillus niger* (Chohnan et al., 1994), *Aspergillus fumigatus* (Luo et al., 2017) and *Escherichia coli* (Miller et al., 2007); animals such as the cow (Rosemeyer et al., 2002) and plants as well, such as thale cress (Speath et al., 2007) and rose periwinkle flower (Aoyagi et al., 2006). There are even phosphodiesterases being produced artificially (Takebayashi et al., 2004) and obtained from human cells (Maldonado et al., 2008). Among the results (Singh et al., 2006) on 5'-PDE properties, it is interesting to note that 5'-PDE from some sources showed high pH optimum (pH 10). There is also (Lerch and Wolf, 1972) a 5'-PDE (from sugar beet leaves) which showed high-temperature stability (80°C). Germinated adzuki beans (Pui et al., 2012) were found to have the highest amount of 5'-PDE when compared with mung bean, soybean, chickpea, black eye pea and petai (*Parkia speciosa*). Although 5'-PDE has been characterized from different sources, there has been no work on the characterization of 5'--PDE from adzuki beans (*Vigna angularis* L.). Moreover, the effects of metal cations and detergents on the activity of 5'-PDE have been studied by only a few researchers (Henz et al., 2007; Ying et al., 2006). Characterization studies are also important to determine the stability of an enzyme under various conditions and to find ways to minimize denaturation, so that the enzyme may become useful in industry.

This study was conducted to determine the properties of 5'-phosphodiesterase isolated from adzuki (*Vigna angularis* L.) beans that had been germinated for 15 hours. The scope of characterization of 5'-PDE included pH optimum, pH stability, temperature optimum and temperature stability, the stability of 5'-PDE at different storage temperatures and the effects of pH solutions on the storage. This work also describes the effects of metal ions and detergents on the activity of the enzyme.

MATERIALS AND METHODS

Materials

Adzuki (*Vigna angularis* L.) beans imported from Tian Jing, China, were bought at a local dry market in Sri Serdang, Selangor, Malaysia. The substrate for the enzyme assay, sodium thymidine 5'-monophosphate p-nitrophenyl ester (nitrophenyl-pT), was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. All other chemicals in this research were of analytical grade.

Extraction of enzymes from germinated adzuki beans

Adzuki beans (100 g) were germinated by first soaking them in 200 mL of 0.3% (w/v) sodium hypochlorite solution for 5 minutes, after which the beans were rinsed 5 times with distilled water before being placed inside four sterilized beakers which were pre-autoclaved with 15 mL distilled water and cotton wool. Each beaker contained 25 g of seeds. The beakers were covered with aluminum foil, and the beans were allowed to germinate in the dark at room temperature (28 ±2°C) for 15 h. The germinated beans were then pooled and homogenized by grinding them with 400 mL 50 mM acetate buffer (pH 4.5). The homogenate was stirred for 30 minutes before filtering through a piece of muslin cloth. The filtrate was subjected to centrifugation at 10°C for 15 min at 12,5000 × g (Sartorius Model 3-18 k centrifuge, Sartorius AG, Weender Land Strasse, Gottingen, Germany; Dhule et al., 2006).

The resulting enzyme extract obtained above was then subjected to dialysis. Dialysis tubes (15 cm) were boiled in distilled water for half an hour and soaked in 500 mL·1 mM ethylene diamine tetraacetic acid (EDTA) solution for 12 hours before use (Idris, 2000). The enzyme extract (10 mL) was added to individual dialysis tubes, tied and then placed in 1 L·50 mM acetate buffer, pH 4.5 and stirred for 12 hours at 4°C. The buffer solution was replaced every 4 hours. The dialyzed extract was then used to determine the properties of the enzyme.

5'-PDE activity assays

The method of Harvey et al. (1970) was applied with modifications to determine the 5'-PDE activity. The

reaction mixture (consisting of 0.1 mL enzyme extract, 0.2 mL of 200 mM phosphate buffer, pH 7.0, and 0.2 mL·1 mg/mL nitrophenyl-pT as a substrate) was incubated in a water bath (10 min, 55°C), with deactivated enzyme in the same formulation as the control. The reaction was stopped with the addition of 0.5 mL·0.1N NaOH solution, followed by centrifugation (12,5000 × g, 10°C, Sartorius AG, Weender Land Strasse, Germany). After centrifugation, the mixture was measured with a spectrophotometer (405 nm).

Specific activity was expressed as unit activity per mg protein, in which the unit (U) of the enzyme was the amount of enzyme required to form 1 µmol of p--nitrophenol per mL enzyme extract in 1 min, at 55°C and pH 7.0. The protein content was determined according to Pui et al. (2012).

Determination of pH optimum and pH stability

The effect of pH on 5'-PDE was determined by assaying the activity of the enzyme at different pH using buffers (citrate, phosphate, Tris-HCl, and carbonate-bicarbonate) to obtain a buffering capacity between pH 3–10. The concentration of each buffer was 200 mM.

A pH stability study was carried out by pre-incubating the enzyme extract (0.1 mL) in 0.2 mL buffer (at different pH ranging from pH 6 to 10) in a capped Eppendorf tube for 1 h at 80°C in a water bath (Ying et al., 2006). After that, 0.2 mL of 1.0 mg/mL nitrophenyl-pT was added to the test tube, mixed, and incubated at 80°C in a water bath for 10 minutes to allow conversion of the substrate into p-nitrophenol (yellow) and 5'-nucleotides as products. Then, 0.5 mL of 0.1N NaOH solution was added to terminate the reaction. The absorbance was measured at 405 nm.

Determination of temperature optimum and thermostability

Determination of the optimum temperature of 5'-PDE was conducted by measuring the enzyme activity at different temperatures ranging from 20–95°C. Temperature stability was determined by pre-incubating 0.1 mL enzyme plus 0.2 mL·200 mM of Tris-HCl buffer pH 8.5 at different temperatures (20, 30, 40, 50, 60, 70, 80 and 90°C), for 1 hour before assaying the 5'-PDE described earlier at 80°C (Ying et al., 2006).

Determination of stability of 5'-PDE at the optimum temperature and pH, and other temperatures and pH

The pH-temperature stability of the enzyme was studied by incubating the enzyme at 60, 65 and 70°C at pH 7.5 (200 mM phosphate buffer) and pH 8.5 (200 mM Tris-HCl buffer) for 1 hour before measuring the residual activity (Ying et al., 2006). The enzyme activity was determined at different time intervals.

Effects of detergents and EDTA on 5'-phosphodiesterase activity

The effects of detergents (Triton X-100, Tween-20, Tween-80 and Sodium Dodecyl Sulfate, SDS) and ethylenediaminetetraacetic acid (EDTA) on the enzyme activity were determined by pre-incubating the enzyme extract (0.1 mL) with 0.1 mL of detergent or EDTA (all at a final concentration of 10 mM), in 0.1 mL 200 mM Tris-HCl buffer, for 1 h at 80°C (Ying et al., 2006). 0.2 mL 1.0 mg/mL nitrophenyl-pT (substrate) was then added to the enzyme-detergent mixture and enzyme-EDTA mixture and further incubated for 10 min at 80°C. NaOH solution (0.5 mL, 0.1N) was added to stop the reaction. The control reaction mixture which did not contain detergent/EDTA was considered as having 100% activity, and the activity of the enzyme exposed to detergents and EDTA was expressed as a relative activity to that of the control.

Effects of metal cations on 5'-phosphodiesterase activity

The effect of metal ions on the activity of 5'-PDE was determined using various metal ions (Li^{+}, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Al^{3+}, Pb^{2+}) all at a final concentration of 10 mM. Enzyme-metal ion mixtures (0.1 mL enzyme, 0.1 mL cation solution and 0.1 mL·200 mM Tris-HCl buffer) were pre-incubated at 80°C for 1 hour before adding 0.2 mL·1 mg/mL nitrophenyl-pT for further incubation at 80°C for 10 min. The reaction was terminated by adding 0.5 mL·0.1N NaOH. The activity of the enzyme without added cation served as the control, where 0.1 mL enzyme and 0.2 mL·200 mM Tris-HCl buffer were pre-incubated at 80°C for 1 hour before adding 0.2 mL of 1.0 mg/mL nitrophenyl-pT for further incubation at 80°C for 10 min. Enzyme activity is expressed as relative activity based on the activity of the control.

The effect of different concentrations of selected metal ions (Ca^{2+}, K^{+}, Mg^{2+}, Li^{2+}, Na^{+}, Al^{3+}) on the activity of 5'-PDE was determined by pre-incubating 0.1 mL enzyme and 0.1 mL cation solution at 80°C for 1 hour before adding 0.2 mL·1.0 mg/mL nitrophenyl-pT for further incubation at 80°C for 10 min. The concentrations of metal cations were 0, 2, 4, 6, 8 and 10 mM. The reaction was stopped by adding 0.5 mL·0.1N NaOH. The activity of enzyme without any addition of cation functioned and treated as above served as the control. Enzyme activity is expressed as relative activity based on the activity of the control.

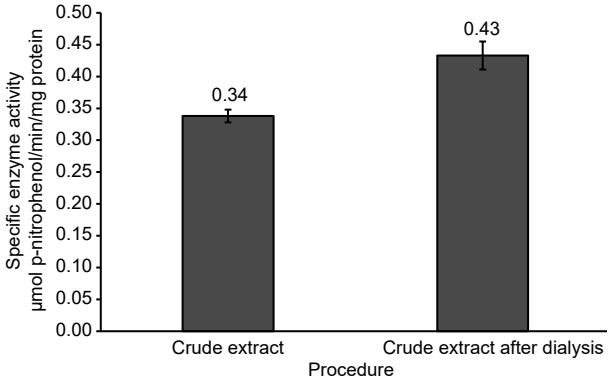

Fig. 1. Specific activity of 5'-PDE after extraction and dialysis. The error bars represent the mean ±standard deviation of three replicates

RESULTS AND DISCUSSIONS

Enzyme extraction and dialysis

The results of the extraction are shown in Figure 1 where dialysis was found to increase 5'-PDE specific enzyme activity from 0.34 µmol p-nitrophenol/min/mg protein (from extraction) to 0.43 µmol p-nitrophenol/min/mg protein. The increase was due to the reduction in protein content during dialysis.

pH optimum and pH stability

The obtained results show that the optimum pH of 5'-PDE was pH 8.5 (Fig. 2), where 5'-PDE activity increased with an increasing pH until pH 8.5. The activity of the enzyme was minimal at acidic pH and increased from pH 6.0 until pH 8.5. After that, the activity dropped significantly as the pH was further increased. This suggests that the enzyme requires a relatively alkaline pH to function effectively. Changes in the pH of the medium resulted in changes in the ionic statues at the active sites and changes in the activity of the enzymes and, hence, the reaction rate. Changes in

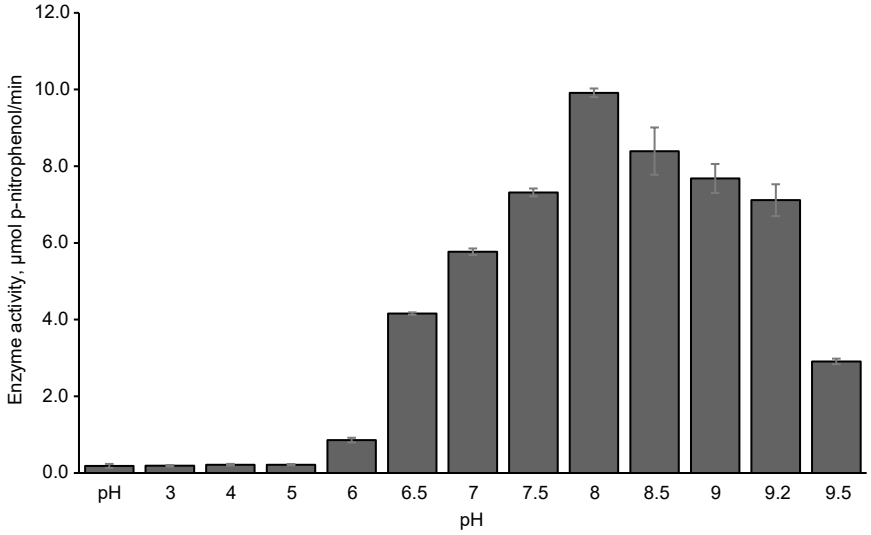

Fig. 2. 5'-PDE activity at different pH. The error bars represent the mean ±standard deviation of three replicates

pH may also alter the three-dimensional shape of the enzyme (Pelczar et al., 2004).

There are several 5'-PDEs that are active in alkaline pH. 5'-PDE from the barley malt rootlet has shown a pH optimum of 8.9 (Beluhan et al., 2003). Higher pH optima have also been reported. Sugar beet leaf 5'-PDE has an optimum pH of 9.2, and the enzyme activity was measured using nitrophenyl p-uridine as the substrate (Lerch and Wolf, 1972). Bowles (1991) found that 5'-PDE from malted barley rootles have a pH optimum ranging from pH 8.5–9.5.

On the other hand, the pH optimum of 5'-PDE from other sources can also vary substantially. Ying et al. (2006) reported that 5'-PDE from *Penicillium citrinum* has a pH optimum of pH 5.4, where the enzyme was shown to have no activity at pH > 8.0. 5'-PDE malt rootlets showed that its pH optimum was between pH 5.5–6.0 (Zou et al., 2008), while 5'-PDE from *Aspergillus fumigatus* has a pH optimum of 5.0 (Luo et al., 2017). From this study, it can be concluded that the pH optimum of 5'-PDE from adzuki bean is in the alkaline region, and the pH optimum is organism-specific.

Table 1 shows that 5'-PDE is stable between pH 7.0 and pH 8.5, where the relative enzyme activity ranges from 100–132.6%. The activity dropped dramatically as the pH was increased or decreased beyond this range. It was also demonstrated that it was most active and stable at pH 7.5 but unstable over pH 9.0, as well as at pH 6.0 and below. The enzyme catalysis activity was at pH 8.5 for 10 minutes, but the enzyme was stable in the range pH 7.0–8.0.

Table 1. The relative stability of 5'-phosphodiesterase at different pH

pH	Relative enzyme activity, %
6.0	6.1 ±1.1
6.5	95.5 ±4.9
7.0	132.6 ±5.2
7.5	384.3 ±5.3
8.0	305.5 ±10.4
8.5	100 ±5.3
9.0	8.9 ±4.0
9.5	7.8 ±1.7

5'-PDE at pH 8.5 (optimum pH) was taken as control (100% relative enzyme activity).

At pH 8.5, the alkaline condition causes slight alteration in 5'-PDE enzyme conformation, which leads to the exposure of more active sites of the enzyme. However, the prolonged exposure of high pH may lead to the changing of conformation or denaturation of the enzyme. There are some 5'-PDE with similar pH stability properties as the enzyme from adzuki beans. Harvey et al. (1967) also found that 5'-PDE from carrots was stable between pH 8.5 to pH 9.0. Overall, the enzyme was stable in slightly alkaline solutions.

Temperature optimum and stability

The activity-temperature curve for 5'-PDE is shown in Figure 3, with the optimum temperature for 5'-PDE fixed at 80°C, and nitrophenyl-pT as the substrate. The activity increased when the temperature was increased from 20 to 80°C. At 95°C, the remaining enzyme activity was only 12% of the activity at the optimum temperature. No enzyme activity was detected at 100°C.

The choice of temperature is critical for the determination of enzyme activity and purification of enzymes. This information will be extremely useful, especially to give a clear picture of the working temperature of the enzyme to avoid denaturation.

According to Sowdhamini and Balaram (1993), an increase in temperature generally increases enzyme activity. As the temperature increases, molecular motion increases resulting in more molecular collisions.

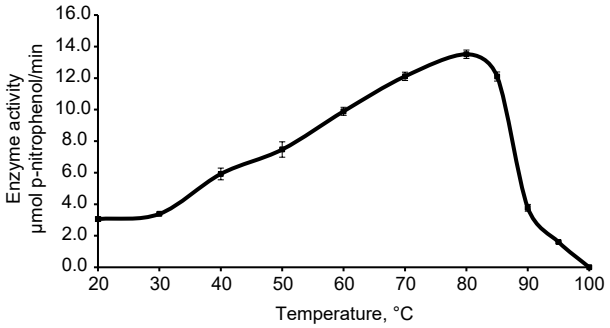

Fig. 3. 5'-PDE activity at the different reaction temperatures. The error bars represent the mean ±standard deviation of three replicates

If, however, the temperature rises above a certain point, that heat will denature the enzyme, causing loss of its three-dimensional functional shape by denaturing its hydrogen bonds. Thus, the protein will denature as the temperature increases beyond the optimum temperature. A slow reaction rate was observed at a low temperature for 5'-PDE activity. The cold temperature slows down enzyme activity by decreasing molecular motion (Sowdhamini and Balaram, 1993). The information obtained from this experiment has also provided a better understanding of the working environment of this crude 5'-PDE preparation that would help to avoid denaturation of the enzyme during purification or application.

The data obtained in this study coincide with the findings of research by Lerch and Wolf (1972), where high-temperature stability (80°C) was displayed by 5'-PDE purified from sugar beet leaves (Lerch and Wolf, 1972). However, in general, the temperature optimum for 5'-PDE was found to be 70°C and below. This can be seen in 5'-PDE from germinated barley (Dhule et al., 2006), *Penicillium citrinum* (Ying et al., 2006), *Aspergillus fumigatus* (Luo et al., 2017) and malt rootlet (Zou et al., 2008) which had temperature optimums of 60°C, 65°C, 60°C and 70°C, respectively.

When compared to 5'-PDE from other sources, the enzyme from adzuki beans has excellent thermostability properties. This is considered an important and useful criterion for industrial applications. The enzyme was stable up to 60°C (Fig. 4) even after exposure for 1 hour at that temperature. After 60°C, the enzyme activity started to decrease drastically, where a complete loss of activity was observed at 90°C. Thus, applications of the enzyme in an industrial setting should be made at temperatures below 60°C. Hua and Huang (2010), in their recent study, found 5'-PDE from barley rootlets to be stable at 70°C for 420 minutes. On the other hand, Beluhan and Maric (2011), in their work on 5'-PDE from barley rootles, observed retention of 70% relative activity after 120 min of incubation at 70°C. Besides that, 5'-PDE from yeast extract, which has an optimum temperature of 65°C, showed decreasing stability with an increase in temperature (Sombutyanuchi et al., 2001). The yeast 5'-PDE was stable at 60°C for 200 minutes. However, at 65, 70 and 75°C, the enzyme was stable for 120, 60 and 40 minutes, respectively, showing a decreasing trend in thermostability. At 80°C, the enzyme was only stable for 20 minutes.

Stability of 5'-PDE at optimum temperature and pH, and other temperatures and pH

5'-PDE had a reduction of 48.5% enzyme activity after 30 min pre-incubation. The activity further reduced with 93% loss of activity when pre-incubated at 120 min. This is in agreement with the work of Lerch and Wolf (1972), where 5'-PDE from sugar beet leaves experienced 50% activity lost after 10 minutes (at 80°C), although its optimum temperature was 80°C.

Figure 6 shows that on the first day of incubation, 5'-PDE was stable (relative enzyme activity more than 80%) under all conditions tested. At 60 and 65°C,

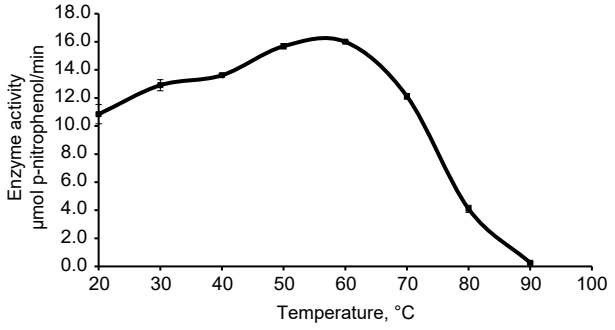

Fig. 4. Stability of 5'-PDE pre-incubated at different temperatures for 1 hour. The pH was maintained at pH 8.5. The error bars represent the mean ±standard deviation of three replicates

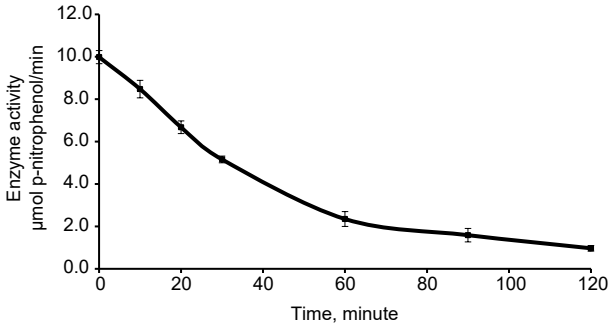

Fig. 5. Stability of 5'-PDE at optimum pH (pH 8.5) and optimum temperature (80°C) with time. The error bars represent the mean ±standard deviation of three replicates

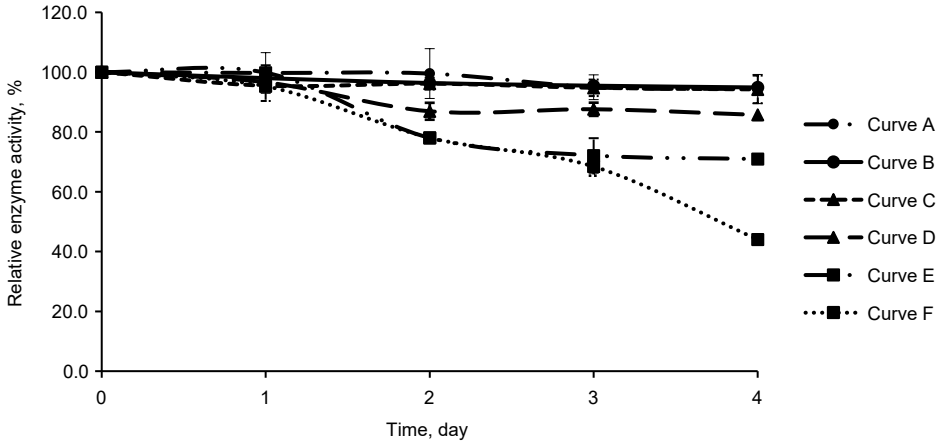

Fig. 6. Stability of 5'-PDE at pH 7.5 and pH 8.5 and 60°C, 65°C and 70°C: condition A – pH 7.5 and 60°C, condition B – pH 8.5 and 60°C, condition C – pH 7.5 and 65°C, condition D – pH 8.5 and 65°C, condition E – pH 7.5, and 70°C, condition F – pH 8.5 and 70°C. The error bars represent the mean ±standard deviation of three replicates

regardless of the pH of the reaction (conditions A, B, C and D), 5'-PDE retained more than 80% of its relative enzyme activity on the 4th-day of incubation. However, at 70°C, the relative activity of 5'-PDE incubated in pH 7.5, and pH 8.5 dropped to 71% (condition E) and 44% (condition F), respectively. Moreover, it was observed that 5'-PDE lost more activity at pH 8.5 compared to pH 7.5, especially on day 4 of incubation, where the activity at pH 7.5 was 1.61 times greater than the activity at pH 8.5. 5'-PDE from barley was found to be stable at 70°C for 2 hours (Beluhan and Maric, 2003). However, there was no report on the stability of 5'-PDE at 70°C for beyond a few hours.

Effects of detergents and EDTA on 5'-phosphodiesterase activity

Figure 7 shows that Tween 80 had a slight inhibitory effect on 5'-PDE activity as it decreased the activity by 23.1% relative to the activity of the control. A greater degree of inhibition was obtained in the presence of Tween 20, where the relative activity of the enzyme was 69.4%. The loss in enzyme activity was 75.2% and 96.8%, respectively, in the presence of Triton X-100 and SDS. From the experiment, it can be concluded that 5'-PDE from adzuki beans is inhibited by detergents.

However, each of the detergents has a different degree of inhibition towards the enzyme. Ying et al. (2006), who studied the effects of detergents on 5'-PDE from *Penicillium citrinum*, reported the presence of 0.1% (v/v) and 0.2% (v/v) as compared to 12% (v/v) Tween 80 (10 mM) used in this experiment. Tween 80 in the reaction medium did not have a profound or high inhibitory effect. When the researchers used Triton X-100 at 0.2% (v/v), a strong inhibitory effect was obtained where the relative activity was 23%. On the other hand, 0.1% (w/v) and 0.2% (w/v) SDS reduced 5'-PDE activity to 55 % and 42%, respectively.

Although the presence of a high concentration of detergents often resulted in protein denaturation, non-ionic detergent concentrations below 0.1% (w/v) are usually not harmful to proteins (Scopes, 1994). The detergents used in this experiment (Tween 80, Tween 20 and Triton X 100) are examples of non-ionic detergents. The most common chelating agent is ethylene diamine tetraacetic acid (EDTA). If the enzyme is stable or its activity increases after pre-incubation with EDTA, it means the enzyme is affected by metals. On the other hand, if the activity of the enzyme drops after the addition of EDTA, the enzyme contains metal as a cofactor that is removed by EDTA. Thus, how EDTA inhibits an enzyme would indicate whether it is a metalloenzyme or a metal-activated enzyme.

The results shown in Figure 7 clearly show that the presence of EDTA greatly affected the activity of the enzyme such that only 0.6% of the control activity remained. This indicates that 5'-PDE from adzuki

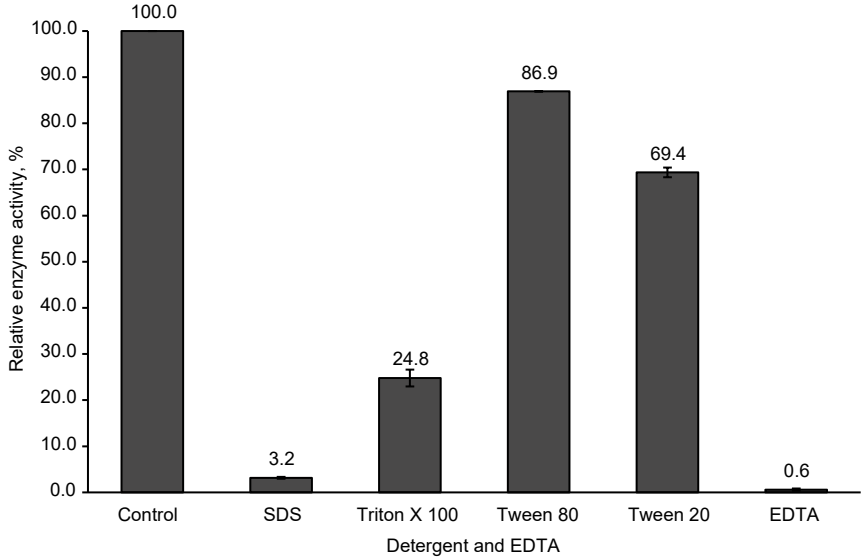

Fig. 7. Effects of detergents and EDTA on the activity of 5'-PDE. Error bars: standard deviations; results are mean of three determinations

beans is probably a metalloenzyme, meaning that the enzyme requires metal as a cofactor. EDTA has been reported as an inhibitor of 5'-PDE from different kinds of sources, such as beet (Lerch and Wolf, 1972) and rat submandibular salivary glands (Henz et al., 2007). A similar effect was also reported by Beluhan and Maric (2003) on 5'-PDE from barley, where 1 mM EDTA and 10 mM decreased relative enzyme activity to 77% and 10% of the control activity, respectively. This indicates that 5'-PDE from adzuki beans is probably a metalloenzyme, meaning that the enzyme requires metal as a cofactor.

Effects of metal cations on 5'-phosphodiesterase activity

Metal ions play important roles in the biological functions of many enzymes, mainly as cofactors. Various metal ions were investigated for their effects on 5'-PDE activity. The effects of different metal ions on 5'-PDE activity are shown in Figure 8. Out of 14 metal cations tested, only four were found to enhance the activity of the enzyme as they increased the activity to more than 100% of the control. The other metal ions caused either a slight or significant inhibition of the enzyme.

From Figure 8, most cations inhibited the enzyme significantly. However, the enzyme activity was strongly activated by Ca^{2+}, K^{+} and Mg^{2+} (at 10 mM concentration) with the relative activity of 249%, 214% and 179%, respectively, compared to the activity of the control. However, the enzyme activity was increased only slightly (120% relative activity) by Li^{+}. These results suggest that the 5'-PDE from adzuki beans is also a metalloenzyme. Figure 8 also shows that Na^{+}, Zn^{2+}, Ni^{2+} and Hg^{2+} (all 10 mM final concentration) acted as an inhibitor of 5'-PDE activity where the activity of the enzyme decreased to 77.3%, 49.0%, 44.4% and 39.8%, respectively. The other metal cations were found to decrease the activity of the enzyme even more. The activity of the enzyme was almost completely removed by Co^{2+}, to 8% of the relative activity compared to initial activity.

The present study also showed that there are metal cations that are capable of inhibiting 5'-PDE from adzuki beans. Zn^{2+} was also found to be inhibitory to 5'-PDE from adzuki beans (Fig. 8). According to Beluhan and Maric (2003), as the concentration of Zn^{2+} increased (from 1 mM to 10 mM), the relative activity of 5'-PDE from barley malt sprouts decreased. It has been reported by Flaganan and Zbarsky (1976) that

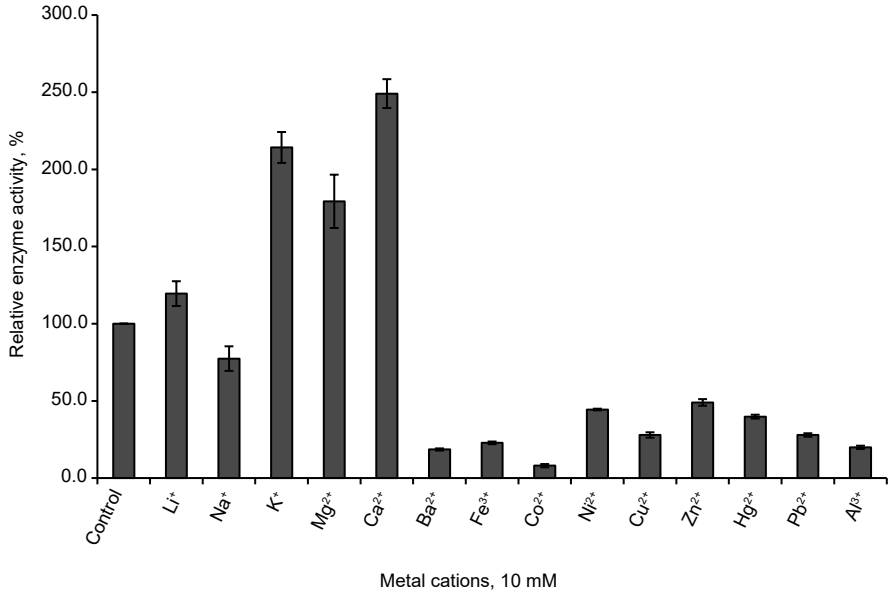

Fig. 8. Effects of metal ions on the activity of 5'-PDE. Error bars: standard deviations; results are mean of three determinations

10 mM Ni^{2+} was only slightly inhibitory (91%) to the enzyme from rat intestine.

Inhibition of 5'-PDE by Hg^{2+}, Cu^{2+}, Pb^{2+}, Al^{3+}, Fe^{2+} and Fe^{3+} has been reported by Chohnan et al. (1994), Lerch and Wolf (1972) and Futai and Mizuno (1967). Ying et al. (2006) reported on the strong inhibitory effect of Cu^{2+} on 5'-PDE from *Penicillium citrinum* where at 1 mM, and 2 mM, the activity of the enzyme declined to 1.6% and 4%, respectively. Flaganan and Zbarsky (1976) also showed that as the concentration of Cu^{2+} in the reaction mixture increased from 1 mM to 10 mM, the relative activity of 5'-PDE from rat intestine decreased from 74% to 34%. On the other hand, Beluhan et al. (2003) reported a mild decrease in 5'-PDE activity from 93% to 69% when 1 mM and 10 mM Cu^{2+} were present in the reaction mixture; while Luo et al. (2017) also reported that 5'-PDE from *Aspergillus fumigatus* were partially inhibited by Cu^{2+}. There is a report on the role of Pb^{2+} as an inhibitor of 5'-PDE.

Chohnan et al. (1994) found that Fe^{2+} and Fe^{3+} inhibited 5'-PDE from *Aspergillus niger*, while Luo et al. (2017) reported on inhibition of the 5'-PDE from *Aspergillus fumigatus* by Fe^{3+}. Inhibition of 5'-PDE from *Aspergillus niger* by Co^{2+} has been reported by Chohnan et al. (1994), although Lerch and Wolf (1972) reported that the cation activated the enzyme from sugar beet. According to Ying et al. (2006), 1 mM of Co^{2+} had a slight inhibitory effect, where it reduced the activity of 5'-PDE from *Penicillium citrinum* to 75%. This is in contrast with other reports which stated that 1 mM Co^{2+} increased the relative activity to 114% (Futai and Mizuno, 1967), and 2 mM increased the relative activity to 116% (Harvey et al., 1967). There has been no report on the effects of Ba^{2+} on 5'-PDE from any sources.

The results of the effects for different concentrations of selected cations on 5'-PDE activity are shown in Table 2. Li^{+} showed no significant effects on 5'-PDE at concentrations less than 4 mM. At 6 mM concentration, Li^{+} caused the activity of the enzyme to increase by 1.48 times that of the control. However, the relative activity of the enzyme decreased (but did not inhibit) as the concentration of Li^{+} was further increased. So far, there has been no report on the effects of Li^{+} on 5'-PDE activity.

When the effect of Na^{+} was investigated further, it was found that different concentrations of Na^{+} affected the enzymatic activity of 5'-PDE differently. Thus, the effect of Na^{+} on 5'-PDE is rather interesting, where

at final concentrations of up to 8 mM, the cation was found to stimulate the activity of 5'-PDE. However, at 8 mM, the relative activity was lower than at 6 mM (Table 2). An inhibitory effect was observed when the concentration of Na^+ was 10 mM. The results obtained are consistent with those from a few studies that reported Na^+ as a slight activator of the enzyme (Futai and Mizuno, 1967; Wang et al., 1993).

However, K^+ has a different effect on the catalytic reaction on the enzyme compared to Ca^{2+} (Table 2). It was observed that the relative activity of 5'-PDE exhibit a proportional increase with an increasing K^+ concentration. At 10 mM K^+ concentration, 5'-PDE activity was 2.15 times greater than that of the control. Ying et al. (2006), who studied the effects of K^+ on 5'-PDE from *Penicillium citritum* found that the activity of the enzyme increased from 150% to 172% as the concentration of K^+ added increased from 1 mM to 2 mM, respectively. They also reported on the activation of enzyme activity by 10 mM of Mg^{2+}. The activity of 5'-PDE increased as the concentration of Mg^{2+} increased from 1 mM to 6 mM. The maximum stimulatory effect of Mg^{2+} on 5'-PDE was observed at 6 mM with a 231% increase in activity. However, above this concentration, the strong stimulatory effect by Mg^{2+} was slightly reduced.

Activation of 5'-PDE from adzuki beans by Mg^{2+} is also consistent with research on the effects of Mg^{2+} on 5'-PDE from different sources, where studies revealed that Mg^{2+} activated the enzyme regardless of the type of sources of the enzyme itself. Harvey et al. (1967) found that 2 mM of Mg^{2+} increased the relative activity to 223%, whereas for 5'-PDE from sugar beet (Lerch and Wolf, 1972), the relative activity increased to 123% (at 2 mM Mg^{2+}) and 127% (at 10 mM Mg^{2+}), respectively. Beluhan et al. (2003) studied the effects of different concentrations of Mg^{2+} on 5'-PDE from barley malt sprouts and found that the relative activity increased as the concentration of Mg^{2+} increased. 5'--PDE appeared to be more sensitive to Ca^{2+} activation compared to the other cations tested, where a significant activation could be seen at 2 mM Ca^{2+}. The highest relative activity was 296.0% at 4 mM Ca^{2+}. After that, the activity of 5'-PDE remained relatively stable.

From Table 2, the relative activity of 5'-PDE increased, with an increase in Ca^{2+} concentration. Harvey et al. (1967) reported a high relative activity of

Table 2. Effects of metal cation concentration on the activity of 5'-phosphodiesterase

Metal cations	Concentration, mM	Relative enzyme activity, %
Li^+	0	100.0 ±0.0^a
	2	95.5 ±6.6^a
	4	102.6 ±15.7^{ac}
	6	148.4 ±8.4^b
	8	132.8 ±5.2^{bc}
	10	121.4 ±8.2^c
Na^+	0	100.0 ±0.0^a
	2	111.1 ±7.1^a
	4	179.4 ±14.4^b
	6	203.6 ±6.4^c
	8	146.6 ±12.9^c
	10	77.8 ±8.1^e
K^+	0	100.0 ±0.0^a
	2	104.4 ±1.2^a
	4	134.8 ±6.7^b
	6	169.6 ±6.3^c
	8	188.8 ±8.4^d
	10	215.4 ±4.6^e
Mg^{2+}	0	100.0 ±0.0^a
	2	142.3 ±7.6^b
	4	191.4 ±27.6^c
	6	231.5 ±15.3^d
	8	207.4 ±6.7^{cd}
	10	193.9 ±16.2^c
Ca^{2+}	0	100.0 ±0.0^a
	2	261.0 ±10.8^b
	4	296.0 ±6.2^c
	6	289.0 ±1.9^c
	8	274.0 ±6.3^b
	10	268.9 ±9.3^b
Al^{3+}	0	100.0 ±0.0^a
	2	71.2 ±1.8^b
	4	66.7 ±2.9^{bc}
	6	56.3 ±3.7^c
	8	50.6 ±0.6^c
	10	25.9 ±5.2^d

Mean residual activity ±standard deviation, %. Residual activity, which has a different alphabet, indicates significant differences ($P < 0.001$) between concentrations, %.

218% among the other metal cations when 2 mM Ca^{2+} is added to the 5'-PDE from carrots. Henz et al. (2007) reported that 0.5 mM and 1 mM of Ca^{2+} had no significant effect on 5'-PDE, which had been inhibited by EDTA from a rat mammary gland.

5'-PDE activity was negatively affected by the presence of Al^{3+} in the reaction mixture. As the concentration of Al^{3+} increased, the relative activity of enzyme activity decreased as well. At 10 mM concentration, the relative activity of the enzyme was only 25.9% of the untreated enzyme. Ying et al. (2006) also reported that the enzyme activity decreased from 65% when 1 mM Al^{3+} was added to the reaction mixture to 54% when 2 mM was incorporated. The results strongly suggest that 5'-PDE from adzuki beans is a metalloenzyme that is also sensitive to the presence of cations, causing the activity to either increase or decrease compared to the control without the addition of cations.

CONCLUSIONS

This study has shown that crude 5'-phosphodiesterase with a high-temperature optimum (80°C) and alkaline pH optimum (pH 8.5) has been extracted from germinated adzuki beans. The enzyme is relatively stable between pH 7.0 and 8.5. Higher stability was observed at pH 7.5 compared to pH 8.5, and at 60°C and 65°C the enzyme activity still possesses good efficiency even after 4 days of incubation. Increasing the temperature to more than 70°C affected the stability of the enzyme. This study has also shown that the activity of 5'-PDE extracted from germinated adzuki beans is affected by exogenously added metal ions in different ways. 5'-PDE was slightly inhibited by detergents such as Tween 80 and Tween 20, while Triton X 100, SDS and EDTA were strong inhibitors. Cations such as Ca^{2+}, K^{+}, Mg^{2+} and Li^{2+} were found to enhance enzyme activities. The results further showed that the activity of the enzyme was at its highest in the presence of Ca^{2+}, followed by K^{+} and Mg^{2+}, while Li^{+} and Na^{+} showed a slight increase up to a certain concentration only. When selected metal ions at different concentrations were tested against the enzyme, Mg^{2+}, Li^{+} and Na^{+} showed a similar pattern of enzyme activation, whereby increasing of metal ion concentration increased 5'-PDE activity until it reached its maximum activity at 6 mM. In all cases, the activity of the enzyme remained greater than the control. In the case of Ca^{2+}, increasing the concentration beyond 4 mM resulted in inhibition of activity. 5'-PDE activity increased as K^{+} concentration increased. On the other hand, 5'-PDE activity decreased as Al^{3+} concentration decreased. In conclusion, the metal cations that inhibited the activity of 5'-PDE were Co^{+}, Cu^{2+}, Zn^{2+}, Hg^{+}, Ba^{2+}, Al^{3+}, Pb^{2+}, Ni^{2+} and Fe^{3+} at 10 mM. Thus, it is concluded that 5'--PDE from adzuki beans is a metalloenzyme. As well as this, activation of the enzyme with the addition of Na^{+} (6 mM) suggests its possible incorporation into the extract to enhance enzyme catalysis.

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REFERENCES

Aoyagi, H., Akimoto-Tomiyama, C., Tanaka, H. (2006). Preparation of mixed alginate elicitors with high activity for the efficient production of 5-phosphodiesterase by *Catharanthus roseus* cells. Biotechnol. Lett., 28, 1567–1571. https://doi.org/10.1007/s10529-006-9124-5

Beluhan, S., Karmelic, I., Novak, S., Maric, V. (2003). Partial purification and biochemical characterization of alkaline 5'-phosphodiesterase from barley malt sprouts. Biotechnol. Lett., 25, 1099–1103. https://doi.org/10.1023/A:1024144215414

Beluhan, S., Maric, V. (2011). Comparative biochemical characterization of 5'-phosphodiesterase and phosphomonoesterase from barley malt sprouts. Nat. Prod. Commun., 6, 73–78. https://doi.org/10.1177/1934578X1100600118

Bowles, L. K. (1991). 5'-phosphodiesterase enzyme preparation and methods for its production. US Pat., 5034325.

Chohnan, S., Nagata, T., Midorikawa, Y. (1994). Purification and characterization of a novel bifunctional enzyme, cyclic-ribonucleotide phosphomutase-5'-phosphodiesterase, from *Aspergillus niger*. Biosci. Biotech. Bioch., 58, 250–255. https://doi.org/10.1271/bbb.58.250

Dhule, S. S., Shetty, P. R., Iyer, J., Singhal, R. S. (2006). Purification and characterization of 5'-phosphodiesterase from germinated barley. Process Biochem., 41, 1899–1902. https://doi.org/10.1016/j.procbio.2006.03.041

Flaganan, P. R., Zbarsky, S. H. (1976). Purification of phosphodiesterase II from rat and guinea-pig intestinal mucosa. Biochem. J., 155, 607–613. https://doi.org/10.1042/bj1550607

Futai, M., Mizuno, D. (1967). A new phosphodiesterase forming nucleotide 5'-monophosphate from rat liver. J. Biol. Chem., 242, 5301–5307.

Harvey, C., Malsman, L., Nussbaum, A. L. (1967). A phosphodiesterase from carrot. Biochemistry, 6, 3689–3694. https://doi.org/10.1021/bi00864a011

Harvey, C., Olson, K. C., Wright, R. (1970). Further purification and properties of phosphodiesterase from the carrot. Biochemistry, 4, 921–925. https://doi.org/10.1021/bi00806a030

Henz, S. L., Furstenau, C. R., Chiarelli, R. A., Sarkis, J. J. F. (2007). Kinetic and biochemical characterization of an ecto-nucleotide pyrophosphate/phosphodiesterase (EC 3.1.4.1) in cells cultured from submandibular salivary glands of rats. Arch. Oral Biol., 1906, 1–8. https://doi.org/10.1016/j.archoralbio.2007.03.006

Hua, J., Huang, K. L. (2010). Preparation and characterization of 5'-phosphodiesterase from barley malt rootlets. Nat. Prod. Commun., 5, 265–268. https://doi.org/10.1177/1934578X1000500220

Idris, Y. M. A. (2000). Kesinai (*Streblus asper*) protease as a potential milk coagulation enzyme. PhD Thesis. University Putra Malaysia.

Jones, A. M. (2003). Practical skills in biology (3rd ed.). Harlow: Prentice Hall.

Khutle, N. M., Vijaya, D. C., Pawar, H., Jodhe, P., Gaikar, A. (2011). Extraction, partial purification and characterization of 5'-phosphodiesterase from germinated *Phaseolus mungo* (mung bean). Int. J. Pharm. Phytopharm. Res., 1, 55–56.

Lassila, J. K., Herschlag, D. (2008). Promiscuous sulfatase activity and thio-effects in a phosphodiesterase of the alkaline phosphatase superfamily. Biochemistry, 47, 12853–12859. https://doi.org/10.1021/bi801488c

Lerch, B., Wolf, G. (1972). Isolation of phosphodiesterase from sugar beet leaves. Biochim. Biophys. Acta, 258, 206–218. https://doi.org/10.1016/0005-2744(72)90979-5

Luo, Z., Fan, Y., Li, Q., Han, B., Liu, Y., Li, S., ..., Pang, Z. (2017). Isolation, purification and characterization of 5'-phosphodiesterase from *Aspergillus fumigatus*. PloS One, 12(10), e0186011. https://doi.org/10.1371/journal.pone.0186011

Maldonado, P. A., de Carvalho Corrêa, M., Becker, L. V., Flores, C., Moretto, M. B., Morsch, V., Schetinger, M. R. C. (2008). Ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) and adenosine deaminase (ADA) activities in patients with uterine cervix neoplasia. Clin. Biochem., 41(6), 400–406. https://doi.org/10.1016/j.clinbiochem.2007.12.019

Miller, D. J., Shuvalova, L., Evdokimova, E., Savchenko, A., Yakunin, A. F., Anderson, W. F. (2007). Structural and biochemical characterization of a novel Mn^{2+}-dependent phosphodiesterase encoded by the *yfcE* gene. Protein Sci., 16, 1338–1348. https://doi.org/10.1110/ps.072764907

Olczak, M., Kobialka, M., Watorek, W. (2000). Characterization of diphosphonucleotide phosphatase/phosphodiesterase from yellow lupin (*Lupinus luteus*) seeds. Biochim. Biophys. Acta, 1478, 239–247. https://doi.org/10.1016/S0167-4838(00)00024-8

Pelczar, M. J., Chan, E. C. S., Kring, N. R. (2004). Microbiology (5th ed.). New Delhi: Tata McGraw-Hill.

Pui, L. P., Abdulkarim, S. M., Ghazali, H. M. (2012). Effects of germination conditions on 5'-phosphodiesterase activity of selected seeds. Int. Food Res. J., 19(4), 1641–1648.

Rosemeyer, H., Ramzaeva, N., Becker, E. M., Feiling, E., Seela, F. (2002). Oligonucleotides incorporating 7-(aminoalkyl)-7-deaza-2'-deoxyguanosines: duplex stability and phosphodiester hydrolysis by exonucleases. Bioconjugate Chem., 13, 1274–1285. https://doi.org/10.1021/bc020024q

Rotola-Pukkila, M., Yang, B., Hopia, A. (2019). The effect of cooking on umami compounds in wild and cultivated mushrooms. Food Chem., 278, 56–66. https://doi.org/10.1016/j.foodchem.2018.11.044

Santoro, N., Cirillo, G., Lepore, M. G., Palma, A., Amato, A., Savarese, P., ..., del Giudice, E. M. (2009). Effect of the rs997509 polymorphism on the association between ectonucleotide pyrophosphatase phosphodiesterase 1 and metabolic syndrome and impaired glucose tolerance in childhood obesity. J. Clin. Endocrinol. Metab., 94, 300–305. https://doi.org/10.1210/jc.2008-1659

Scopes, R. K. (2013). Protein purification: principles and practice. Springer Science & Business Media.

Singh, S. K., Singh, S. S., Pandey, V. D., Mishra, A. K. (2006). Factors modulating alkaline phosphatase activity in the diazotrophic rice-field cyanobacterium, *Anabaena oryzae*. World J. Microb. Biot., 22, 927–935. https://doi.org/10.1007/s11274-006-9137-1

Sombutyanuchit, P., Suphantharika, M., Verduyn, C. (2001). Preparation of 5′-GMP-rich yeast extracts from spent brewer's yeast. World J. Microb. Biot., 17(2), 163–168.

Sowdhamini, R., Balaam, P. (1993). Protein structure and stability. In M. N. Gupta (Ed.), Thermostability of enzymes. Delhi: Narosa Publishing House.

Speath, B., Settele, F., Schilling, O., Dangelo, I., Vogel, A., Feldmann, I., ..., Marchfelder, A. (2007). Metal requirements and phosphodiesterase activity of tRNAase Z enzymes. Biochemistry, 46, 14742–14750. https://doi.org/10.1021/bi7010459

Takahama, Y., Uto, H., Kanmura, S., Oketani, M., Ido, A., Kusumoto, K., ..., Tsubouchi, H. (2008). Association of a genetic polymorphism in ectonucleotide pyrophosphatase/phosphodiesterase 1 with hepatitis C virus infection and hepatitis C virus core antigen levels in subjects in a hyperendemic area of Japan. J. Gastroenterol., 43, 942–950. https://doi.org/10.1007/s00535-008-2256-8

Takebayashi, S., Ikeda, M., Takeuchi, M., Shinkai, S. (2004). Metal ion induced allosteric transition in the catalytic activity of an artificial phosphodiesterase. Org. Biomol. Chem., 6, 493–499.

Utami, T., Maharani, S., Nur Chahyanto, M. (2011). Activity of 5'-phosphodiesterase isolated from various germinated beans. The 12th ASEAN Food Conference, Bangkok, Thailand.

Wang, A. Y., Juang, R. H., Chang, C. T., Sung, H. Y. (1993). Purification and characterization of 5'-phosphodiesterase from barley rootlets. Biochem. Mol. Biol. Int., 6, 1095–1102.

Ying, G. Q., Shi, L. E., Yu, Y., Tang, Z. X., Chen, J. S. (2006). Production, purification and characterization of nuclease p1 from *Penicillium citrinum*. Process Biochem., 41, 1276–1281. https://doi.org/10.1016/j.procbio.2005.12.028

Zou, H. C., Cai, G. Y., Cai, W., Li, H. L., Gu, Y., Park, Y. D., Meng, F. G. (2008). Extraction and DNA digestion of 5'-phosphodiesterase from malt root. Tsinghua Sci. Technol., 13, 480–484.