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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2020.561.566

Research Article
Purification and Characterization of D-psicose 3 Epimerase (DPEase) From *Escherichia coli* BL21 (DE3) pET21b *dpe*

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Abstract

Background and Objective: The DPEase enzyme from *Agrobacterium tumefaciens* is more efficient and has a high activity in D-fructose. The *dpe* gene has been successfully cloned to *Escherichia coli* BL21 (DE3) pET-21b dpe but the enzyme has not been purified and its character is unknown. The intent of this study was to purify and assign of DPEase enzyme by recombinant *E. coli*. **Materials and Methods:** The enzyme was clarified by affinity chromatography and then characterized by following pH, temperature, co-factor parameters. Analysis of molecular weight proteins was done by SDS-PAGE. **Results:** Through purification, the purified DPEase activity was increased 1,01 times than crude and with 84.2% of yield. The DPEase had an the maximum temperature is 40°C and pH was 8.5. The presence of Mg^{2+}, Mo^{2+}, Cu^{2+}, Ca^{2+} and Zn^{2+} inhibited the activity of the enzyme while of Co^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+} enhanced the activity. Estimation of molecular weight through SDS-PAGE revealed that weight of DPEase was 32 kDa. **Conclusion:** Purified DPease enzymes shows clear bands that demonstrate successful purification using affinity chromatography. It is expected that after pure enzymes are obtained the character of the enzymes working will be maximized.

Key words: D-psicose 3-epimerase, recombinant *E. coli*, affinity chromatography, D-fructose, ampicillin

Citation: Deby EdyLiani, Yurnaliza Yurnaliza and Budi Saksono, 2020. Purification and characterization of D-psicose 3 epimerase (DPEase) from *Escherichia coli* BL21 (DE3) pET21b *dpe*. Pak. J. Biol. Sci., 23: 561-566.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

D-Psicose (C-3 epimerase of D-Fructose) is a limited amount of monosaccharide with sweetness level of 30% lower than sucrose. D-Psicose is not only synthesized by microorganisms but also present as a free sugar in wheat[1]. A side from being food sweetener, D-psicose also plays a role in increasing insulin levels[2], hepatic lipid accumulation[3], securing pancreatic beta cells[4]. The D-psicose has low calorie[5] and increases blood sugar levels[6]. These characteristics indicate that D-psicose prevent diabetes and other diseases. The D-psicose is used as a sweetener with calories that is safe for consumption by the drug and food regulatory agency in America (In August 2011 GRN No. 400) and has been commonly used as sweetener and food additives[7]. The D-psicose can be applied in the food attentiveness[8].

The D-psicose converted from fructose enzymatically by DPEase encoded by *dpe* gene. This DPEase enzyme is included in the metalloprotein enzyme group because it requires metal ions to increase its enzyme activity[9] especially in Co^{2+} and Mn^{2+} metal ions[10]. This characteristic distinguishes DPEase from other epimerases. The DPEase enzyme was originally announced as D-tagatose 3-epimerase (DTEase), but because the specificity of the product produced was D-psicose, so this enzyme then appeared as the DPEase enzyme[11].

Several studies reported that *dpe* gene were isolated from various microorganisms[8], i.e. *Clostridium cellulolyticum* H10, *C. bolteae* ATCC BAA-613[9], *C. scindens* ATCC 35704[12], *Ruminococcus* sp., 5139 BFAA[13] and *A.tumefaciens*[14]. Among recombinant DPEases, DPEase from *A. tumafaciens* is more effective in the comparison of D-fructose to D-psicose and it is proven that the activity of the enzyme produced is greater with D-fructose than D-tagatose as a substrate[11].

The ability of bacteria in producing D-psicose is low in nature. Genetic engineering is one way to improve bacterial ability to produce D-psicose by isolating *dpe* gene which is responsible for DPEase synthesis. Isolation of *dpe* gene from *A. tumefaciens* and cloning to *E. coli* BL21 (DE3) has been reported from previous study[15]. However, the expression and characterization of the DPEase has not been studied yet. Therefore, the characterization of pure enzyme should be done prior its application.

MATERIALS AND METHODS

This study was conducted in October, 2017 to March, 2018 at the Bioengineering Carbohydrate Research Laboratory (CBRG) LIPI Cibinong Biotechnology Laboratory, Bogor Regency, West Java.

Bacterial strain and culture condition: Different things done in this study were bacterial conditions and culture of *E. coli* BL21 (DE3) pET-21b containing *dpe* gene (recombinant *E. coli* strain) from *A. tumefaciens* local[15]. The recombinant bacteria was cultivated in Luria-Bertani (LB) containing Ampicillin (20 mg mL^{-1}) at 37°C under agitation in rotary shaker (160 rpm) for 24 h.

Production of DPEase: The DPEase enzyme from recombinant *E. coli* strain are made in a specific medium that has been designed by researchers. Overnight raising was vaccinate into 1 L specific medium and incubated at 37°C under agitation. After stretching an OD_{600} of 0.5-1, the inducer, 1 mM IPTG was mixed into culture and maintained at 18°C for 16 h. Induced culture were then centrifuged to obtain biomass. Biomass was redrooping into 10 mM Tris buffer pH 8.0, sonicated and centrifuged to obtain supernatant. The supernatant was applianced as crude enzyme.

Enzyme assay: Determination of the activity of the enzyme DPEase was measured based on the formation of D-psicose with D-fructose as a substrate. The confession mixtures implied 50 µL crude enzyme and 500 µL fructose solution (100 g mL^{-1}) in 50 µL Tris-HCl buffer 1 M pH 8.0 and 400 µL aquadest for 10 min at 40°C stop reaction with heat up at 100°C for 10 min in boiling water. Remaining sucrose was digested by adding culture of *Saccharomyces cerevisiae* into mixture with ratio of 1:1 (v/v) and maintained at 37°C under agitation at 160 rpm for 18 h. To eliminate culture of *S. cerevisiae* using centrifugation at 5.000 rpm for 5 min. The D-psicose is checked by Cysteine-carbazole method[16]. One unit of enzyme activity is an amount enzyme that catalyzes 1 µmol D-psicose per minute. Protein levels were checked by the Lowry analysis[17], with serum albumin protein as standards.

Enzyme purification: The purification of the recombinant DPEase enzyme was carried out by affinity chromatography using the His-Tag resin in HisTrap affinity column (HisTrap HP). The DPEase enzyme was prepared before loading to chromatography column. Crude enzyme was recovered and filtered through 0.22 µm sterile syringe filter. Filtrate enzyme was ready to insert to column after added with 10 mM of $CoCl^{2+}$ solution. The His-Tag resin column was rinsed with distilled water and then equilibrated with 10 mL of buffer A (20 mM imidazole, 50 mM Tris-HCl, 250 mM NaCl, pH 8.0). Two milliliters of crude enzyme was loaded to the list. The list was washed with 20 mL buffer A and with 20 mL buffer B (250 mM imidazole, 50 mM Tris HCl, 250 mM NaCl, pH 8.0), respectively. The fractions were was collected every 1,5 mL.

This study was carried out under temperature 20°C. The purity of the enzyme was checked its activity and the purity. The profile protein of purified DPEase was observed using SDS-PAGE.

Temperature and pH parameters to the DPEase enzyme: To see the effect of temperature on DPEase activity was conducted by reacting enzymes at various temperature of 30, 35, 40, 45 and 50°C. The reaction mixtures containing 0.5 mL of D-fructose (50 g L^{-1}), 0.1 mL of 1 M Tris-HCl buffer pH 8.5 and 0.1 mL were reacted at various temperature parameters for 10 min.

To see the effect of pH on DPEase activity was conducted by reacting enzymes at various pH. Sodium phosphate buffer (1 M) was used pH 6.0-7.0 meanwhile pH 8.0, 8.5, 9.0 was used Tris-HCl buffer (1M) and Glycine buffer (1M) was utilized for pH values 10.0. The enzyme was reacted at 40°C for 10 min.

Metal ions parameters to the DPEase enzyme: To see the effect of metals on the activity of the DPEase enzyme and then reacted by adding nine metal ions i.e., Mn^{2+}, Mg^{2+}, Mo^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+} into the reactions. The crude enzymes was dialyzed prior characterization. The enzyme dialysis was performed at 16°C for 24 h using the 1 M Hepes buffer (pH 8.0). Hepes buffer was replaced to new solution at each 30, 60 and 120 min. After 24 h of dialysis, enzymes are ready to be characterized.

Enzyme assay conducted as above. The reaction mixture contain the metal ions were added each as a solution for 0.1 mL metal solution ($MnCl_2$, $MgCl_2$, $MoCl_2$, $CuSO_4$, $CoCl_2$, $CaCO_3$, $FeSO_4$, $ZnSO_4$, $NiCl_2$), this was followed by incubating an enzyme of 0.1 mL at 40°C for 1 hr. The effect of metal ion as co-factor if increase enzyme activity while inhibitor reduce.

Statistical analysis: Descriptive statistics, data are presented in the mean and standard deviation of the test. Relative activity, the highest value becomes 100% and other values are divided into the highest value.

RESULTS

Enzyme purification and molecular mass determination: Crude DPEase had a specific activity of 5.02 U mg^{-1} and total protein of 9.55 mg from 2 mL of crude enzyme volume. The use of affinity chromatography increased enzyme purity 1.01 times than crude with specific activity of 5.09 U mg^{-1} and yield 84.2% (Table 1).

The purity of pure enzymes is proven through SDS-PAGE, which can be seen at Fig. 1. In addition, to knowing the molecular mass of the protein obtained from the purification results, it was checked using SDS-PAGE. Crude enzyme from supernatant of lysate cell contains many soluble protein including DPEase (Fig. 1, lane 5). In Line 6 was unbound protein fraction which did not interact with His-Tag resins. The washing fraction (lane 7) was the proteins that weak interacting with the resin and will come out. Mean while in lane 8 was eluate, a fraction of target protein which removed from the column (Fig. 1). The existence DPEase protein was shown as a single band at 32 kDa of molecular weight in the eluation fraction. Thus, pure DPEase enzyme was obtained successfully only by single step chromatography using His-Tag affinity chromatography.

Temperature parameters to the DPEase enzyme: Based on the results obtained by testing the various temperatures, the DPEase enzyme activity is obtained in Fig. 2. The DPEase activity increased significantly and reached the optimum activity when the temperature was 40°C. Increasing of temperature more than 40°C will decrease enzyme activity significantly. The DPEase activity was in moderate temperature. The DPEase was categorized in the mesophilic enzyme group. The enzyme belong to this group had maximum activity at the moderate temperatures.

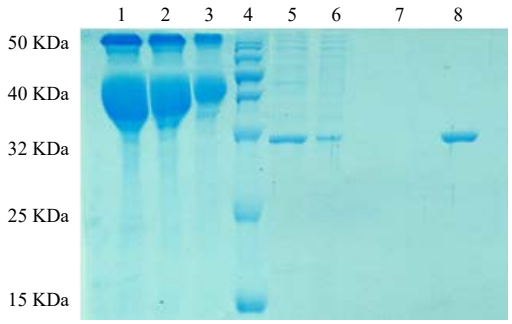

Fig. 1: Molecular mass of purified DPEase from *E. coli* BL21 (DE3) pET-21b contain gene *dpe* by affinity chromatography
Lane 1-3: Bovine serum albumin (BSA) at concentration 2, 1 and 0.5 µg mL^{-1}, Lane 4: Molecular weight marker, Lane 5-8: Crude enzyme, unbound proteins, washing protein, eluent protein of DPEase

Table 1: Profile of crude and purified enzymes of DPEase

Parameters	Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purity	Yield (%)
Crude enzymes	2	9.55	48	5.02	1.00	100
Affinity chromatography	1.5	8.05	41	5.09	1.01	84.2

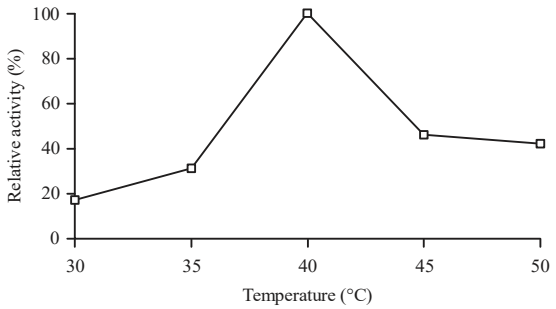

Fig. 2: Testing the temperature parameters for DPEase enzyme activity with temperatures varying at pH 8.5 for 10 min

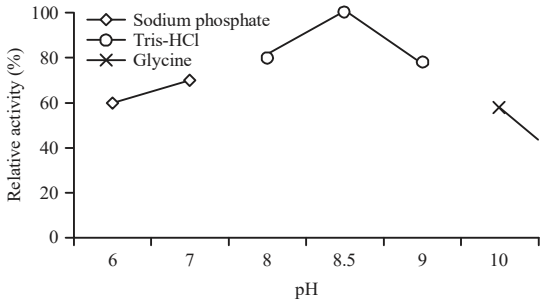

Fig. 3: Testing the pH parameters for DPEase enzyme activity with pH varying at temperature 40°C for 10 min

Table 2: Enzyme activity assay of metal ions

Metal ions	Relative activity (%)
None	100
Mn^{2+}	154
Mg^{2+}	90
Mo^{2+}	79
Cu^{2+}	70
Co^{2+}	184
Ca^{2+}	82
Fe^{2+}	122
Zn^{2+}	83
Ni^{2+}	109

Table 3: Optimum character of various DPEase enzymes produced from strains *A. tumefaciens*

Strains	Temperature (°C)	pH	Metal ion required	References
A. tumefaciens ATCC33970 DPEase	50	8.0	Mn^{2+}	Kim *et al.*[11]
A. tumefaciens CGMCC 1.1488	50	7.5	Co^{2+}, Mn^{2+}	Chen *et al.*[10]
A. tumefaciens ATCC33970 DPEase	50	8.5	-	Lim *et al.*[21]
A. tumefaciens ATCC33970 DPEase	60	8.5	-	Park *et al.*[22]
A. tumefaciens ATCC31749	55	7.5-8.0	Co^{2+}	Tseng *et al.*[23]
A. tumefaciens lokal	40	8.5	Co^{2+}, Mn^{2+}	Present study

pH parameters to the DPEase enzyme: Based on the results obtained by testing the various pH, the DPEase enzyme activity is obtained in Fig. 3. The greatest activity of the DPEase enzyme is pH 8.5. The DPEase activity is at its lowest point at pH 6.0 and increases slowly with increasing pH and optimum at pH 8.5. An increase above of pH 8.5 resulted a significant decrease in activity.

Metal ions parameters to the DPEase enzyme: Based on the results obtained by testing nine type of metal ions, the DPEase enzyme activity was obtained in Table 2. The DPEase activity increased in the presence of co-factors Co^{2+}, Mn^{2+}, Fe^{2+} and Ni^{2+}, but decreased in Mg^{2+}, Mo^{2+}, $Cu^{2+}Ca^{2+}$, Zn^{2+}. The best co-factor to improved the DPEase activity was Co^{2+}, followed by Mn^{2+}, Fe^{2+} and Ni^{2+}. The potential metal inhibitor for decreased of DPEase activity was Cu^{2+}, followed by Mo^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+} (Table 2).

DISCUSSION

The DPEase was successfully purified in active state, by using single step of affinity chromatography with His-Tag resin column and confirmed through SDS-PAGE electrophoresis and enzyme activity of evaluation foil. The DPEase activity in this study was lower than DPEase from Kim *et al.*[11] research that the *dpe* gene also isolated from *A. tumefaciens* by using three purification step with 3 different column (HisTrap HP, HiPrep and Sephacryl S-300 HR). The pure DPEase had lower maximal temperature than crude. Crude DPEase had an optimum temperature at 45°C meanwhile pure DPEase at 40°C. The crude enzyme was more stable at high temperatures than a pure state. Its may be caused by effect of the co-factor. Co-factor will improve enzyme stability to temperature. The pure enzyme was loss its stability because during the purification step, all co-factor was removed from the enzyme[18]. Purified DPEase enzymes have optimum activity at temperature of 40°C and pH 8.5 . This characters similar to DPEase character isolated from *Shinorizobium* sp.[19]. Compared to previous report Kim *et al.*[11], theirs local DPEase possess differences of enzyme activities in pH and temperature. DPEase character[11] have optimum at pH 8.5 and 8.0. The differences amino acids of DPEase constructing may yield a different result of activity of same enzyme[20].

The DPEase activity was increased in the present of co-factors, Co^{2+}, Mn^{2+}, Fe^{2+}, $Ni^{2+}$and inhibited by Mg^{2+}, Mo^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}. Among co-factors, addition of Fe^{2+} was recommended in food industry, considering its benefits to

human bodies and safe for body consumption. Overall, from isolation DPEase enzyme of *Agrobacterium* sp., theirs DPEase has different character has been identified. Low optimum temperature is useful to minimize velocity of miliard reaction on base pH and absolutely useful to minimize dependancy on metal ions of Cu^{2+} and Mn^{2+} danger in body health. And from industry application aspect especially in small industry absolutely has the uniqueness to explore. Fe^{2+} usage as co-factor and very important, because Fe^{2+} is needed to our body. At this time, we are developing functional food based on DPEase enzyme usage with the hope that this research can provide information for readers and other useful research that wants to conduct research on the enzymes DPEase and D-psicose.

The interesting thing is all of the genetic source from *A. tumafaciens* (Table 3). Theirs DPEase has the similarity with DPEase reported by Tseng *et al.*[23] that needs Co^{2+} ion, but it is different from DPEase reported by Kim *et al.*[11] which requires more Mn^{2+} ions. If seen from the character of the optimal temperature of DPEase in this study only about 40°C, this value is relatively lower than the other DPEase 50°C[11,10,21], 55°C[23] and 60°C[22].

What is different in this study is the strain of *Agrobacterium tumafaciens* used is a local strain other than that the media for the production of enzymes uses a specific media that has been designed itself that comes from waste. Means that this research has succeeded in achieving Indonesia's natural wealth and using unused waste is beneficial again.

CONCLUSION

The DPEase enzyme was purified using a single step column His-Tag in the active state. Confirmed through SDS-PAGE electrophoresis and enzyme activity assay. Based on electrophoresis testing, the resulting enzyme appears in a single band with an atomic mass of 32 kDa which shows the success of purification with enzyme specific activity of 5.09 U mg^{-1}. With the acquisition of pure enzymes in this study indicates that the purification conducted by researchers has been successful. Pure enzyme DPEase shows optimum activity at pH 8.5 and temperature of 40°C. the DPEase activity was improved by co-factors, Co^{2+}, Mn^{2+}, Fe^{2+}, $Ni^{2+}$and inhibited by Mg^{2+}, Mo^{2+}, Cu^{2+}, Ca^{2+}, Zn^{2+}. From various metal ion tests, the use of Fe^{2+}metal ions as cofactors in the DPEase enzyme has potential in the food industry.

SIGNIFICANCE STATEMENT

This research succeeded in purifying the cloned DPEase enzyme from a local strain of *Agrobacterium tumafaciens* which is useful in producing D-Psicose as a low-calorie natural sugar, increases insulin levels which is good for diabetes and protects the pancreas.

ACKNOWLEDGEMENT

To the Biotechnology Research Center, Ministry of Research and Higher Education for the 2017 INSINAS Project the authors say many thanks.

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