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Contributed Paper

Partial Purification and Comparison of Precipitation Techniques of Pyruvate Decarboxylase Enzyme

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ABSTRACT

The intracellular pyruvate decarboxylase enzyme (PDC, EC 4.1.1.1) extract from *Candida tropicalis* TISTR 5350 was compared by two different purification methods using ammonium sulphate and acetone precipitation. The total volumetric PDC activity and percentage recovery (yield) of precipitated PDC based on 50% (v/v) cold acetone were significantly higher (1.13 ± 0.02 U/ml and 98.27 ± 2.98 %, respectively) than any other concentration levels of acetone used. Furthermore, all concentration levels of cold acetone also yielded a much higher specific PDC activity than the precipitate obtained using the 40 to 60% (w/v) ammonium sulphate saturation (0.75 ± 0.08 U/mg protein). The precipitated enzyme in buffer solutions from the 50% (v/v) acetone was subsequently freeze dried. Freeze drying of the precipitated PDC by cold acetone resulted in the specific PDC activity of 1.57 ± 0.02 U/mg protein and differed statistically ($p \leq 0.05$) from the crude enzyme extract (control).

Keywords: Pyruvate decarboxylase, Partial purification, Acetone, Ammonium sulphate, Freeze drying

1. INTRODUCTION

Pyruvate decarboxylase enzyme (PDC, EC 4.1.1.1) was the first active enzyme of the glycolytic pathway in many fermentative microorganisms and its role was generally recognized for ethanol production [1].

Moreover, PDC was subsequently utilized for the biotransformation of pyruvate and benzaldehyde to produce (*R*)-phenylacetylcarbinol (PAC). PAC was the precursor for the commercial production of

ephedrine and pseudoephedrine which were used primarily as bronchial dilators and nasal decongestants [2].

The strategy of protein purification was used following cells disruption to remove all contaminants while retaining the amount of desired protein as much as possible. Most protein molecules carried no electrical charge. However, some molecules possessed an electric dipole or multipole due to unequal sharing of electric charge between atoms within a molecule. The addition of water-miscible solvents such as ethanol or acetone to a protein solution would result in the precipitation of proteins. This was due to the decrease of dielectric constant which would make interactions between charged groups on the surface of proteins stronger [3]. Salting out or ammonium sulphate precipitation was useful for concentrating dilute solutions of proteins above 0.5 mg/ml. Ammonium sulphate competed with the protein for water molecules, allowing charged groups to interact. In addition, the precipitation was also due to removal of 'bound' water molecules from hydrophobic surfaces of the protein. The hydrophobic interaction would be stronger at high salt concentration in this case. Finally, protein started to precipitate when there were insufficient molecular interaction between water and protein [4].

Enhanced PAC production with partially purified PDC was observed by Shin and Rogers [5] who prepared purified PDC from *C. utilis* cells growing under fermentative conditions in a 100 l fermenter at a constant temperature of 25 °C and a pH of 6.0. The highest PAC concentration of 190.6 mM was achieved at 7 U/ml PDC activity with 2.0 molar ratio of pyruvate to benzaldehyde. As indicated in the previous study by Tangtua *et al.* [6], *C. tropicalis* TISTR 5350 was the best microbial strain with the highest PDC level of activities (0.39±0.06 U/ml) and PAC production (19.83±3.36 mM).

The aim of this research was to compare two different PDC purification methods using ammonium sulphate and acetone precipitation. The best precipitation method was selected for subsequent freeze dried enzyme preparation and subsequent monitoring of a specific enzyme activity.

2. MATERIALS AND METHODS

2.1 Microorganism

Candida tropicalis TISTR 5350 was purchased from Thailand Institute of Scientific and Technological Research (TISTR). The batch cultivation of *C. tropicalis* TISTR 5350 was initiated from preseed cultivation by transferring 2 × 1.0 ml frozen culture stock to 100 ml inoculum media (composed in one litre of 10.0 g glucose, 3.0 g yeast, 5.0 g malt extract and 5.0 g peptone) in 250 ml Erlenmeyer flasks. All cultivations were done at 30 °C on a rotary shaker at 200 rpm for 24 h. The seed inocula was achieved by diluting 100 ml preseed inocula with 0.9 l medium (composed in one litre of 200 g glucose, 20.0 g yeast extract, 3.0 g KH_2PO_4, 2.0 g $Na_2HPO_4.12H_2O$, 1.0 g $MgSO_4.7H_2O$, 10.0 g $(NH_4)_2SO_4$, and 0.05 g $CaCl_2.2H_2O$) which were used for 10 l scale cultivation in a 16 l stainless steel bioreactor. Air flow rate of 10 l/min was transferred to the bioreactor for 36 h. The microbial growth in the sample was stopped by immersion of the collected sample in liquid nitrogen and kept frozen at -20 °C pending subsequent preparation of crude enzyme extract.

2.2 Preparation of Crude Enzyme Extract

The supernatant and cells pellet in 10 ml culture broth were separated by centrifugation

at 2,822 × g for 15 min using a centrifuge machine. The cells pellet obtained was washed twice with 10 ml distilled water. This was performed prior to resuspension in 200 mM citrate buffer (pH 6.0/ 4.5 M KOH) and subsequent addition with glass beads (0.55 mm diameter) at volume ratio of 1:1. The mixture was vortexed at a maximum speed for 1 min with the similar cooling time in an ice/water mixture for three cycles. Supernatant attained from centrifugation (2,822 × g for 15 min) was collected for subsequent assessment of enzyme activity and protein concentration levels.

2.3 Acetone Precipitation

Acetone precipitation of the crude enzyme extract in the range of 30 - 60% (v/v) at 4 °C was employed to obtain partially purified PDC [7, 8]. Precooled acetone at -20 °C, was slowly added to the crude PDC extract until 30% (v/v) acetone concentration was reached. After the mixture was stood overnight at 4 °C, the precipitate was removed by centrifugation at 12,100×g for 15 min at 4 °C. Further addition of acetone was followed to bring the acetone concentration up to 40% (v/v). The mixture was centrifuged to remove the precipitate while the resulting supernatant was further subjected to acetone precipitation (50 and 60% v/v) in a sequential manner as previously described. The precipitate from each concentration level was dissolved in 0.2 M citrate buffer (pH 6.0/KOH). Acetone was allowed to evaporate from the uncapped tubes after standing at 4 °C for 4 h.

2.4 Ammonium Sulphate Precipitation

Enzyme extract was subjected to fractionation in four stages, namely, 0 to 20%, 20 to 40%, 40 to 60%, and 60 to 80% (w/v) ammonium sulphate saturation. Ammonium sulphate was transferred to the crude PDC extract until the concentration of 20% (w/v) saturation was reached. After the mixture was stood overnight at 4 °C, the precipitate was removed by centrifugation at 12,100 × g for 15 min at 4 °C. Further addition of ammonium sulphate was followed to bring the concentration up to 40% (w/v). The mixture was centrifuged to remove the precipitate while the resulting supernatant was further subjected to ammonium sulphate precipitation (60 and 80% w/v) in a sequential manner as previously described. These stepwise precipitations were referred to as ammonium sulphate "cuts". The amounts of solid ammonium sulphate were added to a known volume in order to obtain the desired percentage saturation. Solid ammonium sulphate should be added slowly while the solution was kept stirring to allow for a uniform increase in the concentration and in powdered form, to ensure rapid equilibration. The precipitate from each concentration was dissolved in 0.2 M citrate buffer (pH 6.0/KOH). The solution was dialyzed against 10 times diluted citrate buffer at 4 °C for 24 h using a dialysis bag [9].

2.5 Preparation of Freeze Dried Enzyme

To freeze dry PDC, the enzyme solution was transferred to aluminium round case (height 10 mm, external diameter 40 mm, fill depth 10 mm, 12 cm3 per vial) and frozen overnight at -20 °C. The frozen solids were placed on a circular aluminium tray before loading in the shelves of a freeze dryer. After completion of the freeze drying process, the enzyme paste was ground to a powder and stored at -20 °C. The PDC activity measurement was performed immediately at the end of the process.

2.6 Analytical Methods

PDC carboligase activity was measured as a formation of PAC in 20 min at 25 °C from 80 mM benzaldehyde and 200 mM pyruvate in carboligase buffer. One unit (U) carboligase activity was defined as the amount of enzyme that produced 1 µmol PAC from pyruvate and benzaldehyde per min at pH 6.4 and 25 °C in a carboligase assay as specified by Rosche *et al.* [2]. The detection of protein concentration was performed according to Bradford assay, using bovine serum albumin for construction of a standard curve. Specific carboligase activity was determined based on protein concentration level in the sample and expressed in unit of enzyme per milligram protein (U/mg).

3. RESULT AND DISCUSSION

3.1 Acetone Precipitation

The comparison of five different concentration levels of acetone at 0, 30, 40, 50, and 60% (v/v) were examined to find out the optimum concentration for partial purification of PDC. From the tabulated results in Table 1, the data revealed that 50% (v/v) acetone was the best solution to achieve the optimum level of total volumetric PDC activity (1.13 ± 0.02 U/ml), specific PDC activity (1.62 ± 0.11 U/mg protein), and enzyme recovery (98.27 ± 2.98 %). This was compared to the 30 and 40% (v/v) acetone in which the corresponding total volumetric PDC activity of 0.96 ± 0.03 and 0.89 ± 0.02 U/ml, specific PDC activity of 1.07 ± 0.06 and 1.55 ± 0.07 U/mg protein, as well as enzyme recovery of 83.75 ± 4.06 and 77.48 ± 2.13%. Such result was similar to Mahdavi *et al.* [10] who precipitated α-amylase from *Bacillus cereus*. The fraction obtained at 50% (v/v) acetone showed the highest specific enzymatic activity of 50 U/mg protein. The miscible acetone solvents by addition of acetone to the solution would decrease the dielectric constant of water. This effect allowed proteins to come close together. The solvation layer around the protein decreased as the organic solvent progressively displaced water from the protein surface. The protein molecules aggregated by attractive electrostatic and dipole forces due to smaller hydration layers. The other important parameters for protein precipitation were temperature, pH, and protein concentration levels in the solution. In addition, the efficiency of this method depended on the protein molecules attaching to the other proteins in solution to form aggregates.

The elevation of 60% (v/v) acetone concentration resulted in the higher specific PDC activity of 2.00 ± 0.10 U/mg protein which was not significantly different ($p > 0.05$) from that of 50% (v/v) acetone. The precipitated fractions of protease from *Bacillus sphaericus* with acetone during the concentration ranges between 50 to 75% (v/v) saturation levels exhibited similar result with the highest volumetric enzyme activity of 1,071 U/ml [1]. Furthermore, Vidyasagar *et al.* [11] observed the gradual loss of protease activity with the increase in acetone concentration levels.

Previous report described the purification of lipase from *Fusarium oxysporum* by acetone in the range of 20 - 90% (v/v). The effectiveness of cold acetone as a purification agent for lipase enzymes revealed that the lowest concentration level of 20% (v/v) was optimal for preserving total volumetric PDC activity, specific PDC activity, and yield (8.1 U/ml, 6.4 U/mg protein, and 46%, respectively). An increased concentration level of acetone caused an 80% decrease in total PDC activity which eventually extinguished the lipase activity [12]. Meanwhile, the best condition for precipitation of xylanase from *Streptomyces* spp. was 70%

(v/v) acetone. The total volumetric PDC activity and specific PDC activity were 0.23 U/ml and 4.76 U/mg protein, respectively [13].

The optimum concentration level of acetone at 50% (v/v) for purified PDC was lower than purified xylanase at 70% (v/v). This might be due to PDC had a higher molecular weight of 60 kD [14] than xylanase which was determined to be 42.4 kDa. The efficiency of enzyme precipitation was practically independent of the molecular weight of proteins [13].

Table 1. Partial purification of PDC from *C. tropicalis* TISTR 5350 using various acetone concentration levels (% (v/v))

Purification steps	Total volumetric PDC activity (U/ml)	Total protein (mg/ml)	Specific PDC activity (U/mg)	Purification (folds)		Recovery (%)
Crude	1.15±0.03 A	1.19±0.05 A	0.98±0.04 C	1.00	BC	100.00
Precipitation						
30%	0.96±0.03 B	0.91±0.04 B	1.07±0.06 C	1.11±0.05	B	83.75±4.06
40%	0.89±0.02 B	0.58±0.03 CD	1.55±0.07 B	1.60±0.14	AB	77.48±2.13
50%	1.13±0.02 A	0.72±0.06 C	1.62±0.11 AB	1.67±0.11	A	98.27±2.98
60%	0.89±0.01 B	0.31±0.04 D	2.00±0.10 A	2.07±0.05	A	77.29±2.19
Supernatant	0.32±0.07 C	0.48±0.02 D	0.66±0.02 C	0.68±0.10	C	27.57±1.99

The number with the same alphabet indicated no significant different ($p > 0.05$) for comparison between different rows of the same columns.

3.2 Ammonium Sulphate Precipitation

Partial purification of crude enzyme sample was achieved by fractionation using ammonium sulphate followed by dialysis. The crude samples were fractionation into four stages, namely, 0 to 20%, 20 to 40%, 40 to 60%, and 60 to 80% (w/v) based on the saturation of ammonium sulphate. As can be seen from Table 2, the precipitation of PDC by using ammonium sulphate at 40 - 60% (w/v) achieved a higher specific enzyme activity of 0.75 ± 0.08 U/mg protein than any other fractionation which corresponded to 1.21 ± 0.11 purification and 97.4 ± 2.7 % recovery.

At this fraction of sufficiently high ionic strength, the protein of interest was almost completely precipitated with the maximum level of protein contaminants in the soluble forms. This technique could remove unwanted proteins by interfering with the sulphate ion interactions between amino acid side-chains, thereby water limiting the available water and protein solubility. As a result, the aggregation of hydrophobic residues on a protein molecule led to the formation of precipitate. A similar study reported a recovery rate of 78.9% xylanase from a fungus *Paecilomyces themophila* upon partial purification using 20 - 50% (w/v) ammonium sulphate saturation [15].

Moreover, it was evident that the last purification steps of 60 - 80% (w/v) supernatant ammonium sulphate resulted in the lower specific enzyme activity (0.52 ± 0.05 U/mg protein) and percentage recovery (63.16 ± 2.06 %) than the concentration range of 40 to 60% (w/v). Soares *et al.* [16] observed that the highest specific enzyme activity (359.28 U/mg protein) of bromelain

was collected in the 20 - 40% (v/v) saturation fraction of ammonium sulphate. Further comparison with higher purification step, the specific enzyme activity of bromelain was significantly lower ($p \leq 0.05$) than 60 U/mg protein. Ammonium sulphate at high concentration levels could influence a drop of pH level and consequently a loss of enzyme activity [12]. Narayan *et al.* [17] also reported that increasing ammonium sulphate concentration resulted in the significant decrease of purification degree. As in conventional salting out, the extent of protein precipitation was a function of ammonium sulphate concentration [18].

Table 2. Partial purification of PDC from *C. tropicalis* TISTR 5350 using various ammonium sulphate concentrations (% (w/v))

Purification steps	Total volumetric activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification (folds)	Recovery (%)
Crude	0.76±0.01 A	1.24±0.01 A	0.62±0.03 A	1.00 A	100.00
Precipitation					
Fraction I (0-20%)	0.44±0.02 C	0.69±0.03 C	0.65±0.02 A	1.05±0.05 A	57.89±2.69
Fraction II (20-40%)	0.58±0.08 BC	0.84±0.07 BC	0.68±0.06 A	1.10±0.14 A	76.32±2.98
Fraction III (40-60%)	0.74±0.02 AB	1.02±0.06 AB	0.75±0.08 A	1.21±0.11 A	97.37±2.66
Fraction IV (60-80%)	0.48±0.03 C	0.94±0.04 B	0.52±0.05 AB	0.84±0.05 AB	63.16±2.06
Supernatant	0.24±0.04 D	0.70±0.05 C	0.34±0.03 B	0.55±0.10 B	31.58±2.42

The number with the same alphabet indicated no significant different ($p > 0.05$) for comparison between different rows of the same columns

3.3 Comparison of Precipitation Techniques

From Table 1 and 2, fractions precipitated with the acetone concentration level of 50% (v/v) exhibited the maximum enzyme activities whereas ammonium sulphate saturation levels were within the ranges of 40 - 60% (w/v). The specific enzyme activity, purification fold, and recovery percentage from acetone precipitation were not only higher than ammonium sulphate precipitation, but this method also had the advantage of being simple and convenient which was appropriated for commercially process. This was compared to the ammonium sulphate procedure which had numerous steps, cumbersome, and time consuming. The dialysis bag must be used to remove the salt which was relatively expensive and unsuitable for use in routine process. The effectiveness of cold acetone as a purification agent for proteolytic enzymes was reported by Popova and Pishtiyski [19] whereas insufficient partial purification by ammonium sulphate was observed by Wang *et al.* [20]. Maehashi *et al.* [21] also reported that cold acetone was a much better purification agent.

In term of commercial PDC purification, the precipitation of proteins by 50% (v/v) acetone was accounted for the cost of 1.05 Baht/sample (0.21 Baht/ml). The economical total enzyme activity from acetone method (1.08 U/Baht/ml) was higher than 40 - 60% (w/v) ammonium sulphate precipitation method (0.005 U/Baht/ml) with the corresponding cost of 0.12 Baht/ sample (0.18 Baht/g). The additional cost relevant to ammonium sulphate method involved the dialysis step to remove the salt from the precipitate with the relatively high price of dialysis tube at 1,460 Baht/meter.

In addition, the salting out method was a much more time consuming process (48 h, in comparison to 28 h of acetone method). A technique of ammonium sulphate precipitation was generally used in a small scale (lower than 500 ml) and was considered unsuitable for a very large scale (up to 500 ml) preparation [22]. These results suggested that cold acetone was a more effective agent than ammonium sulphate for PDC purification.

3.4 Freeze Dried Enzyme

The enzyme solution from 50% (v/v) acetone and 40 - 60% (w/v) ammonium sulphate precipitation were further prepared with freeze drying method. Freeze drying of the PDC by cold acetone precipitation resulted in the highest specific enzyme activity (1.57 ± 0.02 U/mg protein) and residual activity (98.58 ± 2.00 %) which was found to be significantly different ($p \leq 0.05$) from that of crude enzyme extract (control) preparations with the corresponding PDC activity of 0.59 ± 0.01 U/mg protein and residual activity of 94.49 ± 1.67 %. The remaining specific activity of PDC was observed after freeze drying process as shown in Table 3. Devakate *et al.* [23] reported similar result that the residual activity retention of bromelain in freeze drying was found to be 95% as against 78% in spray drying. The reason was the desired low temperature of the material during freeze drying which maintained its structure and the morphology during the process of dewatering. According to Ciurzynska and Lenart [24], freeze-drying had become one of the most important processes for the preservation of heat-sensitive biological substance. The key benefits of freeze-drying included the retention of biochemical, high yield, long shelf life, and reduced weight for storage [25].

This was subsequently compared to ammonium sulphate precipitation with the enzyme activity of 0.72 ± 0.01 U/mg protein (Table 3). The result indicated that the precipitated protein from ammonium sulphate methods had similar PDC activity before freeze drying procedure which was accounted for recovery percentage of 97.70 ± 1.59 % in comparison to total crude enzyme extract. Kawai and Suzuki [26] stated that freeze drying was an important drying process for products requiring bioactivities. However, this method of drying generated low temperature stress, stress of dehydration and ice crystal formation, which might deactivate and destabilize enzymes.

β-galactosidase from *Aspergillus oryzae* had been also reported to lose most of its activity after freeze-drying in the absence of protective solutes [27]. Jiang and Nail [28] explained the loss of protein activity due to freeze drying that the effect of water replacement with the protein molecules which acted as a substitute for water in forming hydrogen bonds with the surface of a protein as water was removed.

Table 3. Freeze dried PDC from acetone and ammonium sulphate precipitation.

Precipitation technique	Total volumetric activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Recovery (%)
Crude extract	0.72±0.01 B	1.23±0.01 A	0.59±0.01 BC	94.49±1.67
Acetone 50% (v/v)	1.11±0.02 A	0.71±0.07 C	1.57±0.02 A	98.58±2.00
Ammonium sulphate 40-60% (w/v)	0.73±0.01 B	1.00±0.01 B	0.72±0.01 B	97.70±1.59

The number with the same alphabet indicated no significant difference ($p > 0.05$) for comparison between different rows of the same columns

4. CONCLUSION

In conclusion, the precipitation of PDC enzyme by 50% (v/v) acetone was selected over 40 - 60% (w/v) ammonium sulphate because of high specific enzyme activity (1.62 ± 0.11 U/mg protein), purification factor (1.67 ± 0.11), and better recovery of activity (98.27 ± 2.98 %). Acetone method was also suitable for application to commercial process which had low cost and uncomplicated process. In case of freeze drying, the maximum specific activity (1.57 ± 0.02 U/mg pretein) of cold acetone precipitation was achieved. To improve the stability of PDC, further investigation such as stabilizer could be done for the preservation during freeze drying and long-term storage.

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