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Three-Phase Partitioning of Peroxidase from Flowers of *Citrus sinensis* for Use in Diagnostic Kit

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This study presents purifying of peroxidase enzyme from flowers of *Citrus sinensis* that used in glucose diagnostic kits by three-phase partitioning technique. Three-phase partitioning technique was chosen due to its simplicity and economical feasibility. An appropriate salt and organic solvent were used to separate the enzyme from aqueous solutions in the system. The homogenate:*t*-butanol ratio and the concentration of ammonium sulphate were determined as 1:1.5 and 40 %, respectively. Optimum pH (9), optimum temperature (40 °C), K_M (9.84 mM) and V_{max} (7.69 U/mL.min) values of the enzyme were calculated using the 4-aminoantipyrine-phenol system. The purity and activity recovery of the enzyme were determined as 9.02-fold and 677.51 %, respectively. The molecular weight (45 kDa) of the enzyme was estimated using SDS-PAGE. The purified enzyme was used in the glucose diagnostic kit. As a result, peroxidase was purified successfully by three-phase partitioning and worked well with the glucose diagnostic kit.

Keywords: Peroxidase, Three-phase partitioning, Purification, *Citrus sinensis*, Glucose diagnostic kit.

INTRODUCTION

Today, the most consumed fruit is orange in the world. The orange flavour is among the luxury flavour due to its rarity for centuries. It has been used in folk medicine and table decoration industry. It was known as the golden apple at the early ages of history. The origin of orange is the North East India and China. The orange is grown widely in many regions due to its adaptation to expanded climatic conditions and endurance to cold weather. Turkey is among a few countries that produce many commercial fruits due to its unique climatological conditions. 1.4 % of the world orange production is cultivated in Turkey that is about 62 million tons. *Citrus* fruits is compatable to the ecological conditions of Mediterranean and Aegean regions. In Turkey, the largest share of the production of orange is from the cities; Antalya (30 %), Içel (27 %), Hatay (15 %) and Adana (31 %). Furthermore, the share of total orange production of East Mediterranean part is 65 % [1].

Peroxidases are widely spread over a large area of nature since they can be found in plant cells, tissues and animal organs. Especially, cell walls, vacuoles and transport organelles have peroxidases in plants. They have a significant role in plant physiological responses including catabolic reaction, lignification, pathogen defense, wound healing and modification of the cell wall. Peroxidases are commonly used in clinical biochemistry, enzyme immuno-assays, wastewater treatment, decolourization, cosmetics and catalytic organic reactions [2,3]. Peroxidases are classified in three superfamilies in terms of their source and form of action. These superfamilies are plant peroxidases, animal peroxidases and catalases [4]. Peroxidase enzyme in plants has vital functions such as hormonal regulation, defense mechanisms, lignin biosynthesis, adjustment of indoleacetic acid of fruit and vegetables. Peroxidase in the industry is used for induction of reactive oxygen species formed during food processing, catalyzing the synthesis of phenolic resins, the elimination of synthetic dye colours and the decomposition of hydrogen peroxide used in bleaching processes and large molecular weight dyes used in the dyeing process. They are also used as catalysts in diagnostic kits which consist of glucose, uric acid, triglycerides and cholesterol diagnostic kits. Furthermore, in immunoassay techniques such as ELISA, labeling of reactants by horseradish peroxidase and applying of its catalytic properties have been reported [5].

Three-phase partitioning (TPP) is an emerging bioseparation method and it has been used for the extraction and purification of enzymes. High concentrations of aqueous ammonium sulphate are added onto crude homogenate and mixed together with a required volume of *t*-butanol as water-miscible aliphatic alcohol. The ethanol, methanol, 1-propanol and *t*-butanol are miscible with pure water but they are insoluble in aqueous solutions of the anti-chaotropic salt, ammonium sulphate. For

this reason, the two phases are in liquid form. The phases are alcohol located on the top and salty water located below. The solubility of lipids, proteins or enzymes will be determined according to their hydrophobicity profile whether it is soluble in polar (lower) phase or apolar (upper) phase. Three-phase partitioning is fundamentally a combination of techniques called salting out, co-solvent precipitation, osmotic and cosmotropic effects. Unique advantages of three-phase partitioning might be specified as simple, inexpensive, versatile, highly efficient technique and it is easily convertible into pilot scale [6].

EXPERIMENTAL

C. sinensis flowers were collected from the rural part of Antalya. The flowers stored at -80 °C in the deep freezer. All the chemicals used for buffers, ammonium sulphate, *t*-butanol, Coomassie Brillant Blue G-250, Bovine Serum Albumin, phenol, 4-aminoantipyrine, hydrogen peroxide glucose diagnostic kit and horseradish peroxidase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of homogenate: About 10 g of fresh flowers of *C. sinensis* were weighed and crushed in a mortar. They were homogenized in 100 mL of 100 mM phosphate buffer of pH 7 using a laboratory blender. The resulting solution which called homogenate was then centrifuged at 20.000×g for 20 min. The supernatant was separated carefully. Peroxidase activity and protein content of the supernatant were measured.

Three-phase partitioning of peroxidase: The slightly modified Roy and Gupta method was used for partitioning of peroxidase [7]. The flowers of *C. sinensis* homogenate was added to *t*-butanol providing the ratios of 1:0.5, 1:1, 1:1.5 and 1:2 (v/v). The mixtures were stirred for 60 min in a 50 mL glass reactor. 4 g of ammonium sulphate was gradually added to 10 mL of homogenate at 25 °C. The ammonium sulphate was completely dissolved in buffer and then 10 mL of *t*-butanol was added to mixture. It was stirred at 200 rpm for 60 min, then centrifuged at 10.000×g for 20 min. The upper phase was discarded. The precipitate and lower phase were separated carefully. The precipitate of protein in intermediate phase was dissolved in 100 mM phosphate buffer of pH 7. The obtained three-phase partitioning phases were dialyzed for 12 h against 100 mM phosphate buffer (pH 7) to remove ammonium sulphate and small salt ions. Then the protein content and peroxidase enzyme activity of obtained phases were determined.

Optimization of the concentration of ammonium sulphate in the partitioning of peroxidase was studied by using the ratio of homogenate:*t*-butanol of 1:1.5. 20, 30, 40 and 50 % (w/v) ammonium sulphate concentrations were studied. The alcoholic phase was discarded. The aqueous phase and the intermediate protein precipitate were separated carefully and dialyzed 12 h against 100 mM pH 7 phosphate buffer at 4 °C. Then the protein content and peroxidase enzyme activity of obtained phases were determined. The results of the phases were compared to determine the optimum conditions for supplying the highest peroxidase recovery and purification fold.

Measurement of protein content: Protein quantification of the obtained homogenate and supernatant was performed according to the slight modification of Bradford method [8]. We also applied the modified method to our previous work. In this study we also used the same modified method.

Peroxidase activity assay: The slightly modified Nicell and Wright method was performed [9]. Peroxidase enzyme activity was measured using a calorimetric method. Phenol, 4-aminoantipyrine (AAP) and hydrogen peroxide (H_2O_2) were the colourization substrates system. Enzyme activity was measured at 510 nm in terms of the consumption rate of H_2O_2.

SDS-PAGE analysis: The Laemmli method was used for SDS-PAGE analysis of peroxidase partitioned from the flowers of *C. sinensis* [10].

Determination of optimum pH, optimum temperature and K_M-V_{max} values: Peroxidase activity studies were performed from pH 3 to 10 to determine the optimum pH of the purified enzyme peroxidase. The different buffer solutions, which were acetate buffer, citrate buffer, phosphate buffer and tris-HCl buffer, were used at appropriate pH ranges to adjust the pH of the medium. Fig. 3A shows the values of enzyme activity values at different pH. Peroxidase activity measurement was performed at 10 °C intervals from 0 to 90 °C to determine the optimum temperature of the purified peroxidase enzyme. Fig. 3B shows the values of enzyme activity values at different temperature. The affinity of the peroxidase enzyme for substrate and the maximum reaction rate were determined using the hydrogen peroxide and 4-aminoantipyrine-phenol substrate system. to calculate. Substrate solutions were prepared at a concentration range of 0.11-1.77 mM. Lineweaver-Burk plot was drawn by performing activity measurement in this range. K_M and V_{max} values were calculated from this graphical equation. Fig. 4 shows the Lineweaver-Burk plot at different substrate concentrations.

Glucose level assay: In order to measure the glucose concentration in human serum, commercial clinical glucose diagnostic kit and 100 Units/100 mL of purified peroxidase from *C. sinensis* were used in the assay. Serum glucose level was measured by enzymatic oxidation of glucose by using glucose oxidase as a catalyst. The H_2O_2 reacts under catalysis of peroxidase with phenol and 4-aminoantipyrine to produce a red-violet quinonimine dye [11]. 10 µL sample was added to 1 mL reagent mixture of 100 mmol/L, pH 7 phosphate buffer, 4 mmol/L phenol, 1 mmol/L 4-aminoantipyrine, > 20 kU/L glucose oxidase, > 2 kU/L peroxidase and 8 mmol/L sodium azide. Glucose standard (std) concentration was 100 mg/dL (5.55 mmol/L). The solutionswere mixed and incubated for 10 min at 37 °C. The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) was measured at 510 nm against reagent blank.

$$\text{Glucose concentration (mg/dL)} = (\text{Standard glucose concentration} \times A_{sample})/A_{standard}$$

RESULTS AND DISCUSSION

Three-phase partitioning of peroxidase: Organic solvent and salt selection are a very important step in three-phase partitioning system. There are various phase forming salts but the ammonium sulphate is generally preferred since it is an effective cosmotropic agent. The different types of C4 alcohol are used for the three-phase partitioning but *t*-butanol is preferred due to its aggregation property. The view of phases

TABLE-1
THREE-PHASE PARTITIONING OF PEROXIDASE FROM *Citrus sinensis* FLOWERS*

Samples	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Activity recovery (%)
Homogenate	205.47	0.158	1300.44	1	100
Three-phase partitioning intermediate phase	1392.1	0.118	11767.46	9.04	677.51
Three-phase partitioning bottom phase	1.22	0.02	61	0.05	0.59

*The experiments were carried out in triplicate for purification steps and the difference in the readings was less than ± 5 %.

and the precipitate observed in the assay are shown in Fig. 1 and overall purification steps are shown in Table-1.

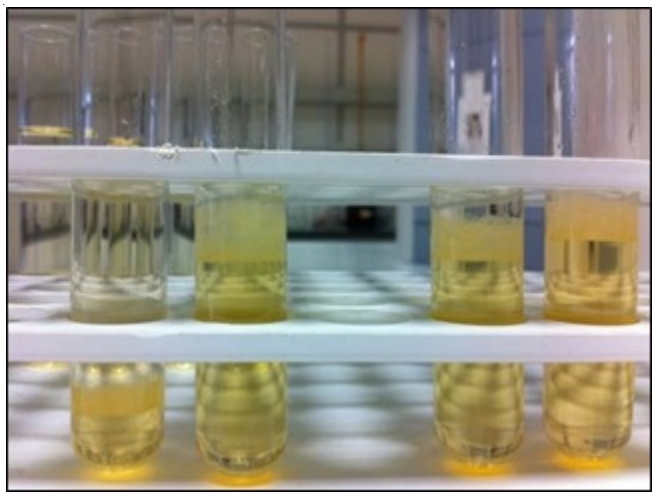

Fig. 1. View of three-phase partitioning peroxidase

Effect of homogenate : *t*-butanol ratio on peroxidase partitioning: *t*-Butanol was chosen due to its particular advantages over other organic solvents. It does not lead denaturation of the partitioned enzymes. Because it has high molecular size that can not penetrate into the three-dimensional structure of protein. It is also an important cosmotropic and aggregation agent at 20-30 °C to enable the partitioning of the enzyme [12]. The homogenate : *t*-butanol ratio of 1:0.5, 1:1, 1:1.5, 1:2 (v/v) were studied with %40 ammonium sulphate. As it can be seen in the Fig. 2A, the best result was obtained with 1:1.5 *t*-butanol.

Effect of ammonium sulphate concentration on peroxidase partitioning: The concentration of ammonium sulphate has a significant parameter in three-phase partitioning system, since it is related to precipitation and interaction of proteins. Salt concentration is effect on the solubility of proteins. Precipitation occurs according to principle of salting out when sufficient salt is added into protein. The salt ions react with the water molecules at higher concentrations. The proteins precipitate by means of hydrophobic interactions [13]. Ammonium sulphate saturations of 20, 30, 40 and 50 % were studied with 1:1.5 homogenate to *t*-butanol ratio. 40 % Solution of ammonium sulphate showed the best activity recovery and the result can be seen in Fig. 2B.

Effect of pH on partitioned peroxidase: The pH is one of the major factors for purification of proteins in three-phase partitioning system.The parameters of temperature, concentration of substrate, ionic strength, buffer type and reaction timehave effect on the optimum pH of the enzymes. The pH change of system influences the partitioning behaviour of the enzyme by changing the net charge of target enzyme. If the pH of buffer is above the isoelectric point of enzyme, it has negative charge and it would be pushed into the lower phase. On the contrary, if the pH of the system is below the isoelectric point of enzyme, it accumulates at the intermediate phase [12]. The pH was studied from 3 to 10 in three-phase partitioning system for 60 min., at the concentration of 40 % ammonium sulphate with homogenate : *t*-butanol ratio of 1:1.5 (v/v) at 25 °C and results are shown in Fig. 3A. It was shown in figure that the negative charge on purifed peroxidase at pH 9 was optimum with an increase in pH of the buffer. It enhanced partitioning of enzyme into the intermediate phase. As maximum purity was obtained at pH 9, it was optimized for future experiments. There are also similar results at different studies [13].

Effect of temperature on partitioned peroxidase: Enzymes are complex protein molecules. The three-dimensional structure of enzymes must be protected in order that their activity remains constant and stable. Temperature is an important parameter for the stability and configuration of enzymes. Analyses for optimizing the temperature have been performed in the range of 0 and 90 °C. The enzyme activity increased while the temperature increased from 0 to 40 °C and then gradually decreased

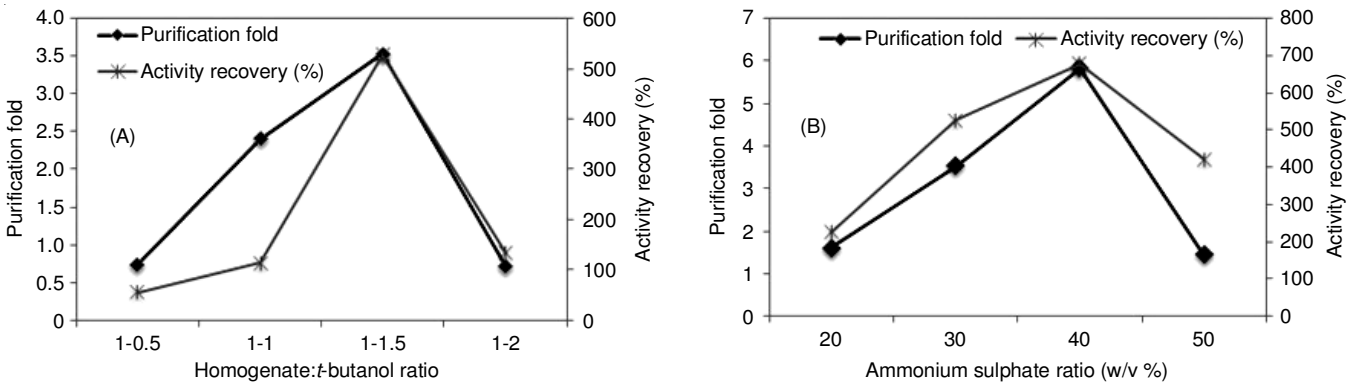

Fig. 2. (A) Effect of homogenate:*t*-butanol ratio, (B) Effect of ammonium sulphate concentration (%, w/v)

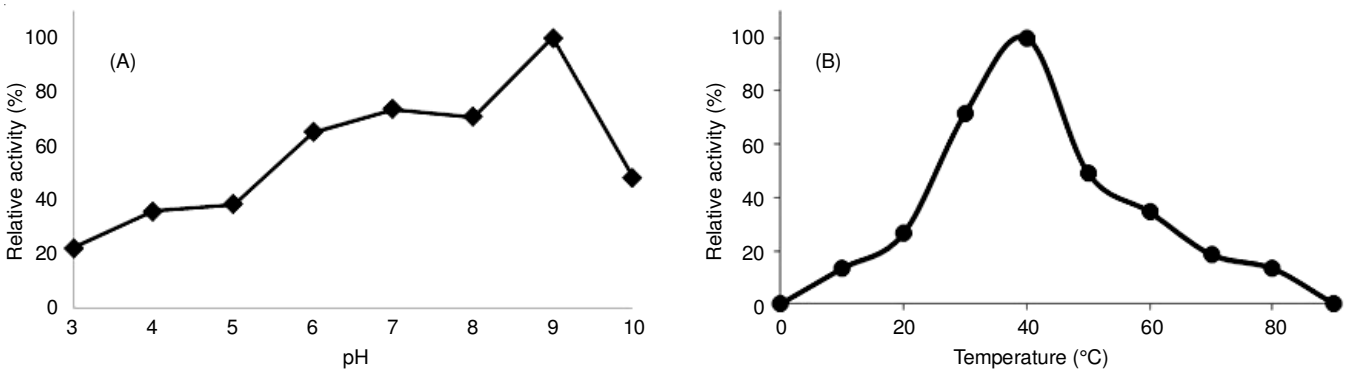

Fig. 3. (A) Effect of pH on the activity of peroxidase, (B) Effect of temperature on the activity of peroxidase

(Fig. 3B). The optimal temperature for purification of peroxidase was found at 40 °C. It was understood that the purified peroxidase was not active above 40 °C. The possible reason behind this may be *t*-butanol works best as a cosmotropic and aggregation agent at 20-30 °C [12]. Narayan *et al.* [13] obtained the similar results in their study.

Kinetics ofpartitioned peroxidase: 4-Aminoantipyrine substrate concentration against reaction rate is shown in Fig. 4. The Michaelis-Menten constant K_M for 4-aminoantipyrine was found as 9.84 mM and value of V_{max} was calculated as 7.69 U/mL min. Triplett and Mellon [14] reported that the K_M value for per-oxidase from cotton was 350 μM in their work.

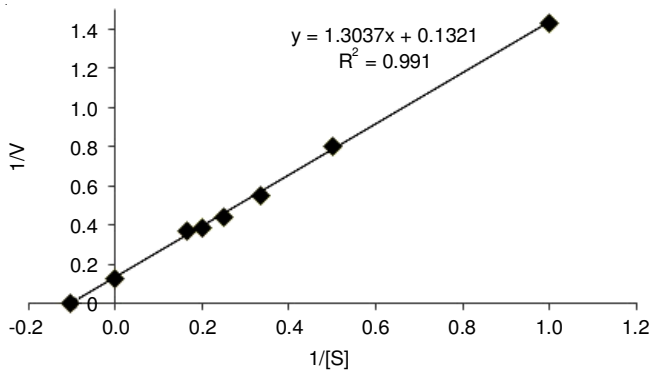

Fig. 4. Lineweaver-Burk plot of the substrate of peroxidase

SDS-PAGE analysis of partitioned peroxidase: Bands of homogenate and purified peroxidase are seen in Fig. 5. After three-phase partitioning, they were found to have similar stains. The other stains on the peroxidase band seem to have disappeared. The molecular weight of peroxidase was estimated about 45 kDa compared the standard protein marker. The similar results about molecular weight have been stated by different researches [15,16].

Glucose level assay of partitioned peroxidase: The main energy source of the body is glucose. It has a significant role in the production of proteins and lipid metabolism. It is stored in the liver as the glycogen molecule. The determination of glucose is significant for diagnosis and controlling of hyperglycemia and hypoglycemia. Hyperglycemia is a condition that an excessive amount of glucose circulates in the blood plasma. It is also known as high blood sugar. In the case of a deficiency in insulin secretion or action, diabetes mellitus can occur and it leads to hyperglycemia. Hypoglycemia is a condition

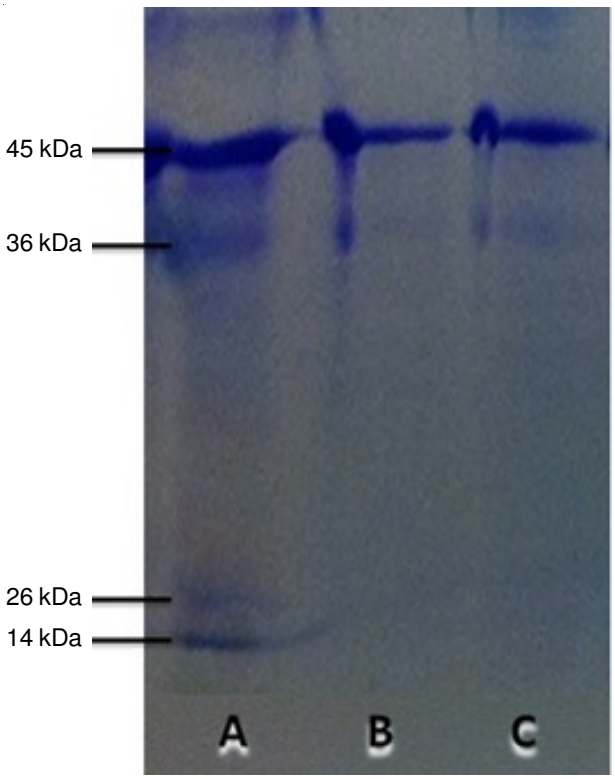

Fig. 5. SDS-PAGE pattern of peroxidases from the flowers of *Citrus sinensis*. Lane A: Molecular weight marker, Lane B: Homogenate, Lane C: three-phase partitioning intermediate phase

characterized by defectively low blood glucose. Insulinoma, insulin administration and fasting cause the hypoglycemia. The glucose level in the blood is regulated by pancreatic hormones. The normal blood glucose level in humans is 4 mM or 72 mg/dL. This value varies according to daily diet [17,18].

In order to control the utility of peroxidase, the enzymatic determination of serum glucose was carried out. The optimal concentration of peroxidase which gave a linear response with time, was 100 Units/100 mL reagents. It was stable at 37 °C upon testing the reference range of glucose; Normal: 74-102 mg/dL and Pathogenic: 235-325 mg/dL. Serum glucose level of human serum sample was calculated as 78 mg/dL in our study.

Conclusion

Three-phase partitioning system is a single step, quick and economic technique has been applied to the flowers of *C. sinensis* for purification of peroxidase. The obtained results

are 40 % (w/v) concentration of ammonium sulphate with 1:1.5 ratio of homogenate : *t*-butanol (v/v) at pH 9 and 40 °C for the best recovery and maximum purification fold of peroxidase. It was proved that molecular weight of peroxidase is near about 45 kDa. In addition to this, purified peroxidase indicated a good activity for determining the serum glucose level. The three-phase partitioning is an efficient and powerful method for purification of peroxidase which has a lot of applications in food, cosmetics and clinical point of care instruments.

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