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Enzyme Based Self-Prepared Kit to Measure the Glucose Concentration in Comparison with Standard Kit

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Abstract: In the present study, glucose oxidase was produced in shake culture by the parent *Aspergillus niger* with corn steep liquor as substrate. Glucose oxidase produced was then purified by subjecting to 60-85% saturation of ammonium sulfate precipitation and further purified by ion exchange and gel filtration chromatography respectively. Desalted sample of glucose oxidase showed the activity and specific activity of 39.19 U/ml and 9.07 U/mg. For ion exchange chromatography, 8th fraction of glucose oxidase had the maximum activity of 19.99 U/ml with 17.70 U/mg specific activities. Gel filtration chromatography was applied by sephadex G-200 and glucose oxidase showed a specific activity of 54.243 U/mg. Peroxidase from horseradish obtained activity and specific activity of 21.78 U/ml and 9.64 U/mg, respectively after ammonium sulfate precipitation. DEAE-cellulose chromatography of peroxidase showed activity of 10.36 U/ml and specific activity 57.88 U/mg that was 5.07 U/ml and 66.71 U/mg after gel filtration. These purified enzymes were used to design 3 kits to estimate glucose with varying parameters. The self-prepared kit was compared with standard AMP kit. The self-prepared kit which gave closer result with the standard kit was kit A with enzyme concentrations of 2 ml glucose oxidase and 0.1 ml peroxidase at 546 nm wavelength in serum as well as in plasma.

Key words: Diabetes · *Aspergillus niger* · Purification · Enzyme Based Kit

INTRODUCTION

Diabetes mellitus is a metabolic disorder. Its common features are chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action. The effects of diabetes mellitus include long term damage, dysfunction and failure of different organs [1]. Type 1 diabetes mellitus is categorized by an entire deficiency of insulin. The disease is initiated by an auto immune-induced discerning damage of insulin producing beta-cells in the pancreatic Langerhans islets [2]. The typical signs of Type 1 diabetes mellitus are thirst, polyuria and polydipsia and weight loss. The secretion of insulin may be diminished for years but still sufficient to prevent ketoacidosis [2]. The processes of beta-cell destruction that ultimately lead to diabetes mellitus type 1 in which "insulin is required for survival". Insulin prevents the development of ketoacidosis, coma and death.

Type 2 is the predominant form of diabetes mellitus. The patients are not really dependent on exogenous insulin because production of their own insulin usually is not decreased. The cause of this disease lies in disorder of action of insulin. Decreased cellular effects of insulin on the target organs are mentioned as insulin resistance, due to a disorder at the level of insulin receptor or transduction of signal from the receptor inside the target cell. Unlike the Type 1 the patients are not prone to ketoacidosis. In 60-90% the patients are obese [2]. There are some unusual causes of diabetes which result from genetically determined abnormalities of insulin action. The metabolic abnormalities associated with mutations of the insulin receptor may range from hyperinsulinaemia and modest hyperglycemia to symptomatic diabetes. Some individuals with these mutations have acanthosis nigricans [3].

Glucose level is a key diagnostic parameter for many metabolic disorders. Glucose level is tightly regulated in the human body. Failure to maintain blood glucose in the

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normal range leads to conditions of persistently high or low blood sugar [4]. Diabetes mellitus, characterized by persistent hyperglycemia, is a metabolic problem and is prevalent in many parts of the world. It is the most prominent disease related failure of blood sugar regulation [5]. People with diabetes mellitus need to regularly check their blood glucose levels in order to detect fluctuations in glucose level that could lead to hyperglycemia and hypoglycemia so as to control the disease. This monitoring is done using finger-prick blood samples and a portable meter several times a day [4]. The present study was conducted with an aim to measure the glucose concentration by using indigenously prepared enzymes and to compare the glucose level with standard kit.

MATERIALS AND METHODS

Plan of Research Project: The research work was performed in Enzyme Biotechnology Laboratory, Department of Chemistry and Biochemistry University of Agriculture, Faisalabad. The work was planned to measure the glucose concentration by using self-prepared enzymes.

Isolation of Glucose Oxidase

Microorganism and its Maintenance: A pure culture of fungus *Aspergillus niger* was obtained from EBL, Depart of Chemistry and Biochemistry, University of Agriculture, Faisalabad. The culture was maintained on potato dextrose agar slants [5]. Corn steep liquor was the nutrient source used for glucose oxidase isolation [6]. Inoculum was developed in 250 ml conical flasks with working volume 50 ml per flask. The duplicate media were then autoclaved at 121°C and 15 lbs. After the media was autoclaved and cooled at room temperature, it was inoculated in a sterilized environment with the spores obtained on the PDA slants. The media was then placed in a rotary shaker for 24 hrs at 120 rpm and 30°C [7].

Submerged Fermentation: Fermentation was carried out in 250 ml conical flasks with working volume of 50 ml per flask. The ingredients were dissolved in 130 ml of distilled water in a 250 ml beaker. The pH was corrected to 5 and volume was made up to 150 ml. The 50 ml medium was then transferred separately to three 250 ml flasks to run triplicate. The triplicate media was then autoclaved at 121°C and 15 lbs. After the media was autoclaved and cooled at room temperature, the inoculation was done in a sterilized environment with 2.5 ml of inoculum by a micropipette. The media was then placed in shaker for 36 hours at 120 rpm and 30°C [8].

Preparation of Crude Extract: When the growth was attained after 36 hours, it was filtered. The filtrate was set aside for assay of enzyme activity. The mycelia from the flask having best growth were disrupted in mortar pestle for 10 min. to homogenize by adding potassium phosphate buffer to form a suspension. The suspension was subjected to centrifugation at speed 10,000 rpm for 15 min at 0°C. The suspension was filtered and the filtrate was proceeded to assay of enzyme activity and determination of protein [7].

Assay of Glucose Oxidase Activity: The spectrophotometer was fixed at 460 nm wavelength against blank. In a cuvette, buffer-substrate solution was taken in which 0.2 ml of glucose oxidase enzyme was added. It was placed in the spectrophotometer and after 2-3 minutes of reaction period, the absorbance was checked [9].

Determination of Protein: Protein contents were determined by Biuret method [10].

Purification of Glucose Oxidase: Intracellular glucose oxidase enzyme was purified by ammonium sulfate precipitation technique further proceeded by ion exchange and gel filtration chromatography. Crude enzyme was subjected to ammonium sulfate precipitation by the method of Jiang *et al*. [11 and 7]. The dialyzed sample was preceded to DEAE-cellulose chromatography. A column of DEAE-(Diethyl amino ethyl) cellulose was prepared by the method of Borisy [12]. The buffer from the column was removed up to slightly left on the top of the column. The desalted sample is applied on the surface of the column of about 1 ml with a pipette. The column is left for 5 minutes so that the sample is penetrated in the column. After that sodium phosphate buffer was added in the column. 50 fractions were taken in test tubes on a constant flow rate by adding small amount of buffer all the time as it approaches the column. Each fraction was of 2 ml. All the fractions were preceded for assay of enzyme activity and protein determination. A column of Sephadex G-200 was prepared by the method described by Leach and O'Shea [13]. The outlet tube was opened and the distilled water

present in column was removed until there was a small layer on the top of column. The sample of about 0.5 ml obtained after ion exchange chromatography and having the maximum specific activity were applied on it and outlet was closed. The sample was allowed to penetrate in packed column. Elution was carried out by 0.1 M phosphate buffer (pH 6) at a constant drop rate. A total 30 fractions of 2 ml each were collected which were then subjected to assay of enzyme activity, determination of protein and glucose estimation.

Extraction of Peroxidase from Horseradish: Fresh horseradish was got from the market. Firstly it was thoroughly washed and cut into small pieces. The weighed horseradish (50 g) was put into blender to homogenize by adding 100 ml of distilled water for 10 to 15 minutes [14]. The blended mixture was then centrifuged in the centrifugation machine at 10,000 rpm for 15 minutes. The supernatant obtained was filtered and the sediments were discarded. The crude extract was put in an air tight jar to avoid the contamination [15].

Assay of Peroxidase Activity: The readings were taken on spectrophotometer at 470 nm wavelength against blank. In a cuvette, 3 ml blank solution was inserted and in the other cuvette 3 ml of buffer-substrate solution was taken in which 60 µl of peroxidase enzyme was added. Both the cuvettes were placed in the spectrophotometer and after 2-3 minutes of reaction period the absorbance was checked [16].

Glucose Estimation: We designed three kits having variable concentration of glucose oxidase and peroxidase enzymes.

Kit A

Peroxidase Reagent: 8 ml of phosphate buffer was taken and 3.5 mg of 4-aminoantipyrine (4-aminophenazone) and 0.1 ml of peroxidase (5.07 U/ml) was added to it.

Glucose Oxidase-Peroxidase Chromogen Reagent: 2 ml of purified glucose oxidase (1.1825 U/ml) was added to previously made peroxidase reagent. The final volume was made up to 10 ml.

Kit B

Peroxidase Reagent: 8 ml of phosphate buffer was taken and 3.5 mg of 4-aminoantipyrine (4-aminophenazone) and 0.05 ml of peroxidase (5.07 U/ml) was added to it.

Glucose Oxidase-Peroxidase Chromogen Reagent: 2 ml of purified glucose oxidase (1.1825 U/ml) was added to previously made peroxidase reagent. The final volume was made up to 10 ml.

Kit C

Peroxidase Reagent: 5 ml of phosphate buffer was taken and 3.5 mg of 4-aminoantipyrine (4-aminophenazone) and 0.1 ml of peroxidase (5.07 U/ml) was added to it.

Glucose Oxidase-Peroxidase Chromogen Reagent: 4 ml of glucose oxidase (1.1825 U/ml) was added to previously made peroxidase reagent. The final volume was made up to 10 ml by adding phosphate buffer.

Procedure: Fresh blood was taken from a non-diabetic volunteer. It was left for 2-3 minutes and then it was centrifuged at 3000 rpm for 15 minutes. Urine sample was taken from the same volunteer and filtered for glucose detection. Serum and plasma were separated and were taken immediately for glucose estimation. Three set of 5 test tubes for three kits was designed and labeled as blank, standard and sample 1 (serum), sample 2 (plasma) and sample 3 (urine) for kits A, B and C. Same amount of samples that is 0.5 ml of was poured in its relevant test tube.

The test tubes were placed in an incubator for 10-15 minutes at 37°C. The absorbance of the standard and test were checked at spectrophotometer at different wavelengths (470 nm, 500 nm and 546 nm). These self-prepared kits were then compared with standard AMP kit. The self-prepared kits giving closer results were then led for clinical trials of 20 diabetic patients.

20 diabetic patients from Allied Hospital, Faisalabad were taken as volunteers to analyse the kit for clinical trials. In these patients, 7 were female (32-63 years), 10 male (45-57 years) and 3 children (3-7 years). Fresh blood from the patients was taken separately and centrifuged to separate serum that was used as sample for these patients.

RESULTS AND DISCUSSION

The aim of the research project was to measure the glucose concentration in serum, plasma and urine, by using indigenously prepared and purified enzymes i.e. glucose oxidase and peroxidase. The activity and specific activity of crude glucose oxidase was observed as 20.24 U/ml and 3.641 U/mg respectively having 5.559 mg/ml of

protein contents. The activity and specific activity of desalted sample of glucose oxidase was observed as 39.19 U/ml and 9.07 U/mg respectively with protein contents 4.32 mg/ml. The maximum activity and specific activity of glucose oxidase was possessed by 8th fraction obtained from DEAE-cellulose chromatography and it was found to be 19.99 U/ml and 17.70 U/mg respectively having 0.116 mg/ml of protein contents. The maximum activity and specific activity of glucose oxidase was possessed by 3rd fraction obtained from gel filtration chromatography and it was found to be 1.1825 U/ml and 54.243 U/mg respectively.

The activity and specific activity of crude peroxidase was observed as 19.19 U/ml and 2.081 U/mg, respectively having 9.22 mg/ml of protein contents. The activity and specific activity of desalted sample of peroxidase was observed as 21.78 U/ml and 9.64 U/mg respectively with protein contents 2.26 mg/ml. The maximum activity and specific activity of peroxidase was possessed by 6th fraction of DEAE-cellulose chromatography and it was found to be 10.36 U/ml and 57.887 U/mg, respectively. The maximum activity and specific activity of peroxidase was possessed by 28th fraction of gel filtration chromatography and it was found to be 5.07 U/ml and 66.71 U/mg, respectively. These purified enzymes were used to design 3 kits to estimate glucose with varying parameters. The parameters which were varied are enzyme concentrations and wavelengths (470 nm, 500 nm and 546 nm) and glucose was estimated in serum, plasma and urine. The self-prepared kit was compared with standard AMP kit. The self-prepared kit which gave closer result with the standard kit was kit A with enzyme concentrations of glucose oxidase 2 ml (1.1825 U/ml) and peroxidase 0.1 ml (5.07 U/ml) at the wavelength of 546 nm in serum and plasma. The kit obtained 60 and 63.22 mg/dl glucose values in serum and plasma. This kit A was led for clinical trials for 20 diabetic patients of different age groups.

Purification of Glucose Oxidase: The crude extracellular enzyme possessed enzyme activity 17.325 U/ml and specific activity 3.381 U/mg. The crude intracellular enzyme possessed enzyme activity 20.24 and specific activity 3.641 U/mg. Hatzinikolaou and Macris [17] produced glucose oxidase from *Aspergillus niger* with enzyme activity as high as 5.7 U/ml. Bankar *et al*. [18] produced glucose oxidase from *Aspergillus niger* possessing activities 0.29 to 2.05 U/ml. Zia *et al.* [7] isolated glucose oxidase from mycelium extracts of *A niger* with specific activity of crude extract 2.192 U/mg protein.

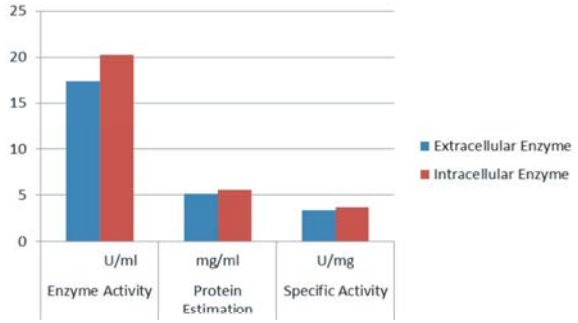

Fig. 1: Analysis of glucose oxidase activity in crude enzyme

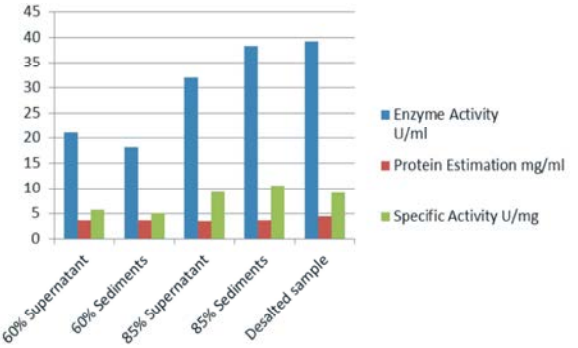

Fig. 2: Analysis of glucose oxidase activity of ammonium sulfate precipitated enzyme

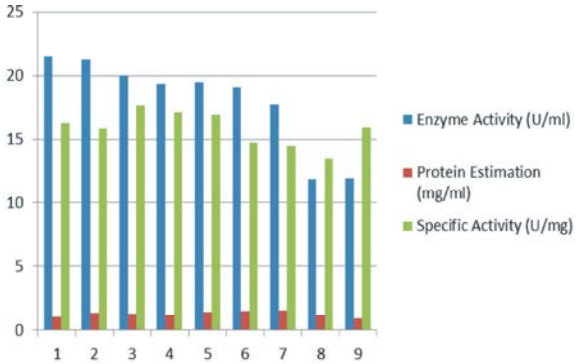

Fig. 3: Graphical sketch of DEAE-cellulose chromatography

Glucose oxidase produced was then partially purified by subjecting the crude enzyme extract to 60-85% ammonium sulfate precipitation.

The fraction of 60% supernatant and sediments of glucose oxidase contained the activity of 21.04 U/ml and 18.18 U/ml respectively. The 85% supernatant and sediments of *Aspergillus niger* showed the activity of 32.04 U/ml and 38.25 U/ml respectively. Desalted sample of glucose oxidase showed the activity and specific activity of 39.19 U/ml and 9.07 U/mg, respectively. Simpson *et al*. [19] partially purified glucose oxidase

isolated from *Penicillium* sp. by 60-70% ammonium sulfate precipitation. The specific activity of the partially purified enzyme was 137.7 U/mg with 4.9-fold purification.

DEAE-Cellulose Chromatography: Desalted sample of glucose oxidase showed the activity and specific activity of 39.19 U/ml and 9.07 U/mg was subjected to DEAE-cellulose chromatography.

It was noted that 8th fraction of glucose oxidase had the maximum activity of 19.99 U/ml with 17.70 U/mg specific activity. Protein contents were somewhat decreased as 1.264 mg/ml that indicated that the technique is reliable. 60% of the enzyme was recovered with 5.4 fold purification. Zia *et al.* [7] purified glucose oxidase isolated from *A. niger* by DEAE-cellulose. The specific activity was 22.53 U/mg proteins. Purification of the enzyme on anion exchange column was 10.27 fold with 97.83 % recovery.

Gel Filtration Chromatography: 8th fraction of glucose oxidase from DEAE-cellulose chromatography having the maximum activity of 19.99 U/ml with 17.70 U/mg specific activities was applied to sephadex G-200. 3rd fraction of glucose oxidase from gel filtration showed 1.1825 U/ml enzyme activities and 54.243 U/mg was its specific activity. Simpson *et al*. [19] purified glucose oxidase from *Penicillium* sp. CBS 120262 to 11.1-fold with 14.1 % yield. Zia *et al*. [7] purified glucose oxidase from *A. niger* by Sephadex G-200.

Extraction of Peroxidase: The enzyme peroxidase was extracted from horseradish by blending it for 15 min in distilled water. The blended mixture was then centrifuged at 10,000 rpm for 15 min. The supernatant obtained was filtered and the sediments were discarded catalase was in activated. The activity and specific activity of crude enzyme were 19.19 U/ml and 2.081 U/mg, respectively. Rehman *et al*. [15] extracted peroxidase from horseradish with activity and specific activity equals to 1.955 U/ml and 1.080 U/mg respectively.

Purification of Peroxidase: The activity and specific activity of crude enzyme were 19.19 U/ml and 2.081 U/mg, respectively reported the degree of purification as 1.93 in horseradish peroxidase.

The fraction of 50% supernatant and sediments of glucose oxidase contained the activity of 22.30 U/ml and 29.67 U/ml respectively. The 85% supernatant and sediments of peroxidase showed the activity of 15.34 U/ml and 13.42 U/ml respectively.

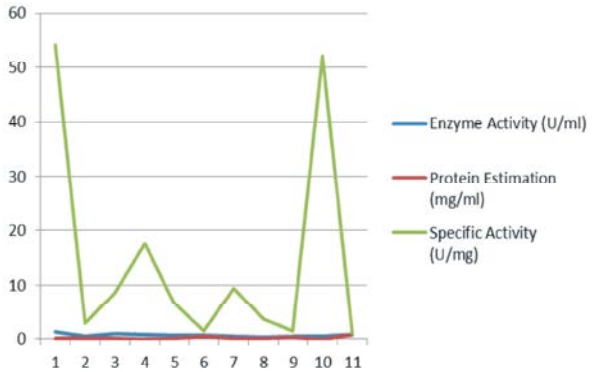

Fig. 4: Graphical sketch of purification of glucose oxidase by gel filtration

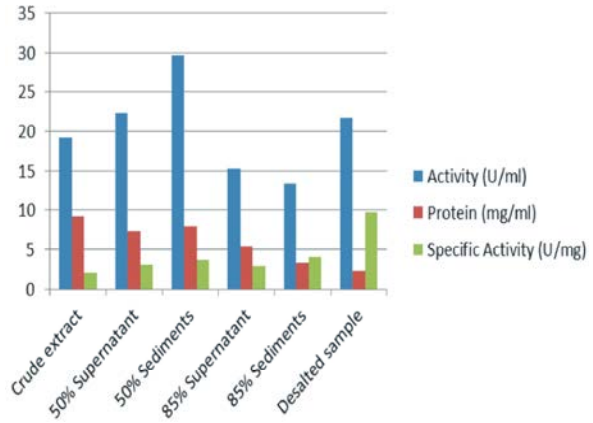

Fig. 5: Graphical sketch of peroxidase activity in crude extract and after $(NH_4)2\ SO_4$ precipitation

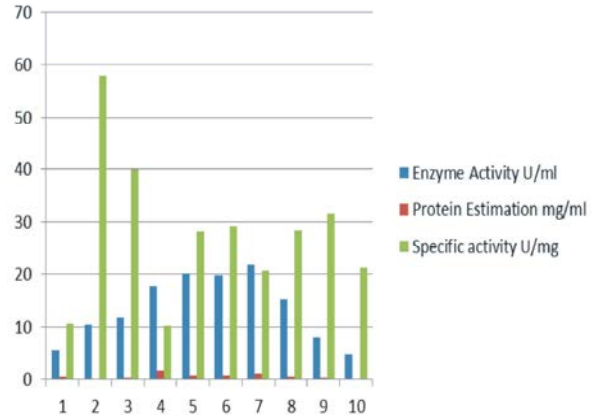

Fig. 6: Graphical sketch of purification of peroxidase by DEAE-cellulose

Purification of Peroxidase by DEAE-Cellulose: Desalted sample of horseradish peroxidase has enzyme activity 21.78 U/ml and specific activity 9.64 U/mg and was subjected to DEAE-cellulose column for ion exchange chromatography.

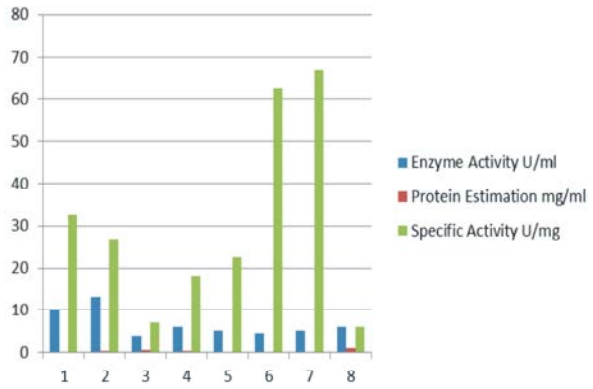

Fig. 7: Graphical sketch of purification of peroxidase by gel filtration

Table 1: Glucose estimation with kits made from self-prepared enzymes

Wavelength (nm)	Kits	Serum (mg/dl)	Plasma (mg/dl)	Urine (mg/dl)
470	Kit A	277.98	233.11	328.29
	Kit B	288.98	280.55	450.32
	Kit C	609.33	577.44	703.11
500	Kit A	307.34	340.55	500.22
	Kit B	337.16	368.03	220.66
	Kit C	219.87	250.14	250.33
546	Kit A	60.45	63.22	390.12
	Kit B	190.33	210.15	110.29
	Kit C	146.86	150.22	430.66
Standard AMP Kit (500 nm)		95	100	46

Table 2: Glucose estimation with standard AMP-kit and self-prepared enzymes kit

Diabetic Patients	Kit A (546 nm)	Standard AMP (500 nm)
1	128.34	328.55
2	190.22	388.66
3	90.21	283.44
4	150.79	301.89
5	155.59	211.43
6	160.31	351.00
7	170.72	275.11
8	165.47	119.63
9	200.07	333.13
10	99.61	204.56
11	94.40	286.71
12	101.51	258.58
13	103.90	334.50
14	71.47	179.93
15	99.64	235.49
16	104.71	190.27
17	220.33	468.53
18	180.41	336.22
19	131.34	239.59
20	229.22	457.25

It was noted that 6th fraction of peroxidase had the maximum activity of 10.36 U/ml and specific activity 57.887 U/mg. 60% of the enzyme was recovered with 5.4 fold purification. Rehman *et al*. [15] purified horseradish peroxidase by DEAE-cellulose column.

Purification of Peroxidase by Gel Filtration: Gel filtration chromatography was applied by Sephadex G-200. Purification of the enzyme by gel filtration technique on sephadex G-200 column at pH 6.0 increase the specific activity and protein contents of the enzyme [20].

The 28th fraction of peroxidase showed a maximum enzyme activity of 5.07 U/ml specific activity of 66.71 U/mg was its specific activity. Zia *et al.* [14] purified horseradish peroxidase to 18.644 fold purification after gel filtration chromatography.

Glucose Estimation: 3 kits were designed to estimate glucose with varying parameters. The parameters which were varied are enzyme concentrations and wavelength. Standard solution was prepared and the absorbance of standard and samples were taken against blank at 470, 500 and 546 nm [4]. The varying concentration of glucose oxidase and peroxidase is given in the Table 1.

The self-prepared kit which gave closer result with the standard kit was kit A with enzyme concentrations: glucose oxidase 2 ml (1.1825 U/ml) and peroxidase 0.1 ml (5.07 U/ml). The kit gave glucose value in serum 60 mg/dl in plasma 63.22 mg/dl. Leary *et al*. [21] determined glucose quantity in human blood with glucose oxidase method. The glucose oxidase-peroxidase give results with concentration from 0 to 50 mmol/L. Relijic *et al*. [22] used glucose oxidase-peroxidase assay to determine glucose quantity in human serum equals to 27.8 mmol/L, with a sample/ reagent volume ratio as low as 0.0025.

Clinical Trials in Terms of Kit Efficiency: When compared with the standard kit, the self-made "Kit A" was found to give nearest results was led for clinical trials. 20 diabetic patients from Allied Hospital, Faisalabad were taken as volunteers to experiment the kit for clinical trials. The estimated glucose values by standard AMP kit and Kit made by self-prepared enzymes are given in Table 2.

The glucose values in twenty diabetic patients ranges from 90-230 mg/dl by self-prepared kit when it was led to clinical trial in Allied Hospital Faisalabad. Kuwa *et al.* [23] used enzymatic method to determine glucose in diabetic patients and the glucose value range was about 0.52 mmol/l. Dungan *et al.* [24] achieved glucose values between 80 and 110 mg/dl in critically ill diabetic patients.

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