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Partial Separation and Some Kinetic Studies of Ceruloplasmin in Human Serum*

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ABSTRACT

The study attempts to isolate the enzyme ceruloplasmin from human serum. Two proteinous components had been isolated by gel filtration chromatography from the precipitate produced by polyethylene glycol (4000). It was found that only the second peak had a high activity for ceruloplasmin. The apparent molecular weight of the isolated ceruloplasmin using gel filtration chromatography and SDS-PAGE was (138111) and (134400) dalton respectively.

Maximum activity for ceruloplasmin was obtained using (35.8) mmol/l of p-phenylenediamine as a substrate for the enzyme, sodium acetate (0.1 mol/l) as a buffer at pH (5.45) for (35) minutes at (56) °C. Using lineweaver–burk plot, it was found that maximum velocity (V_{max}) and Michaelis constant (K_m) had the values of (0.83) µmol/ min and (15.38) mmol/l respectively. The effects of some chemical compounds on the ceruloplasmin activity were investigated. Sodium chloride showed uncompetitive inhibition on the activity of the enzyme at a concentration of (70) mmol/l.

فصل جزئي ودراسة بعض الصفات الحركية لأنزيم السليروبلازمين من مصل الإنسان

الملخص

في هذه الدراسة حاولنا عزل أنزيم سليروبلازمين من المصل، إذ تم فصل حزمتين رئيستين من البروتينات بتقنية الترشيح الهلامي للراسب البروتيني الناتج من الترسيب بـ بولي اثيلين كلايكول (4000)، وأظهرت الحزمة الثانية فعالية عالية لأنزيم السليروبلازمين. بعدها قدر الوزن الجزيئي للأنزيم باستخدام تقنيتي الترشيح الهلامي والهجرة الكهربائية، والتي كانت بحدود (138111) و(134400) دالتون على التوالي.

وتم أيجاد الظروف المثالية لفعالية أنزيم السليروبلازمين وكانت كالاتي: استخدام (35.8) ملي مول/لتر من مادة (p-phenylenediamine) كمادة اساس و(0.1) مول/لتر من المحلول المنظم (Sodium acetate buffer) عند أس هيدروجيني (5.45) ودرجة حرارة (56) م بفترة زمنية مقدارها (35) دقيقة.

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وباستخدام رسم لاين ويفر– برك تم أيجاد قيمة السرعة القصوى (V_{max}) وثابـت مكـياس (K_m) وكانتا مساوية لـ (0.83 مايكرومول/دقيقة) و(15.38) ملي مول/لتر على التوالي. كذلك تمـت دراسـة تأثير المثبطات على فعالية أنزيم السليروبلازمين، وبينت النتائج أن كلوريد الصوديوم مثـبط لاتنافـسي لفعالية الأنزيم بتركيز (70) ملي مول/لتر.

INTRODUCTION

Ceruloplasmin (Cp.) is an α2-globulin that contains approximately 95% of total serum copper. Each molecule of Cp. contains six to eight copper atoms, most of which are tightly bound while others are relatively free (Fleming and Gitlin, 1990; Fox *et al.*, 1995).

It has a single polypeptide chain with 1046 amino acids and three glucosamine-linked oligosaccharide side chains, with a total carbohydrate content of 8 to 9.5%. Cp. in serum shows significant size and charge heterogeneity because of differences in glycosylation. In addition it is very susceptible to proteolysis, both in vivo and in vitro, by many proteases, including trypsin, plasmin, leukuocyte elastase and a plasma metalloproteinase (Burtis and Ashwood, 1999).

It is one of plasma-specific enzymes which are normally present in the plasma at higher levels than in the most tissue cells and perform their primary function in the plasma. It exhibits oxidase activity toward some polyphenols and toward amines, such as epinephrine and serotonin. It can catalyse the oxidation of a variety of polyalcohols, polyphenols and polyamines (such as quinols, catechols and p-phenylenediamine) (Wilkinson, 1962; Halliwell and Gutteridge, 1985).Ceruloplasmin can function either as an prooxidant or an antioxidant depending on other factors, such as the presence of free ferric ions and ferritin binding sites, acting as a ferroxidase. It is vitally important in regulating the ionic state of iron in particular, oxidizing Fe^{+2} to Fe^{+3} (Harris *et al.*, 1997). It thus permits the incorporation of iron into transferrin without the formation of toxic iron products (Henry, 2001). Moreover, it probably transports coppers to tissues, which have separate membrane receptors for Cp. and albumin-bound copper. The importance of Cp. in transport is controversial, however, because turnover of Cp. copper is very slow (Gitlin and Janeway, 1960) and individuals with genetic deficiency of Cp. have no apparent problems related to copper transport (Gillham *et al.*, 2000; Henry, 2001). Cp. does not participate in the transport of copper in the blood. In the blood, copper in Cp. does not exchange with non-Cp. Copper (Bhagaven, 1978).

The epithelial cells are the major source of Cp. in the lung fluid and support ceruloplasmin's critical role in host defense against oxidative damage and infection in the lung. (Yang *et al.*, 1996).

Ceruloplasmin increased in pregnancy, seizure disorders. Decreased in Wilson's disease (Also known as hepatolenticular degeneration) (Bhagaven, 1978).

The aim of the work is to provide a detailed study of ceruloplasmin involving isolation, characterization and purification from human serum in normal (control living in Mosul center) using different biochemical techniques.

MATERIALS AND METHODS

Ceruloplasmin Assay

The results were expressed as gm of ceruloplasmin per liter of serum, and converted to Unit per liter using the following equation (Flayeh, 1988):

$$\text{Total activity} = \frac{\text{Abs. x } 10^6}{\text{Sample volume (ml) x No. of } \bar{e} \text{ x } E_o}$$

Where: Sample volume = 0.1 ml
No. of $\bar{e}$ (electron)= 1
E_o (Excision confession) = 0.68 M^{-1} cm^{-1}

Purification of Ceruloplasmin from Human Serum.

The method given here has yielded an enzyme preparation acceptable for human infusion(Hao and Wickerhauser, 1977). All steps were performed at 4 ^oC unless stated otherwise.

A human fresh serum was obtained from normal person in 21-1-2005: Age (29 year), Body Mass Index (BMI) (25.03 Kg/m^2), Blood group (B^+).

Step I: Polyethylene glycol 4000 (PEG) Fractionation

Solid PEG was added in the amount of 0.2 gm/10ml of serum (Hao and Wickerhauser, 1977). All operations were conducted at 4 ^oC. After stirring for 60 minutes, the suspension was centrifuged at 3850 X g for 30 minutes. The 20% PEG supernatant contained ceruloplasmin and most of the smaller proteins (Noyer *et al.*, 1980).

The protein in precipitate and supernatant are determined using the modified Lowry (Schacterle and Pollak, 1973), and the Cp. is determined in each fraction (Sunderman and Nomato, 1970) .

Step II: Dialysis

The dialysis sac containing the suspension in (Step I) was dialyzed against 0.015M phosphate buffer, pH 6.9, which contained 0.1M sodium chloride. The solution was stirred with a magnetic stirrer overnight at (4) ^oC. The buffer was changed twice during dialysis (Robyt and White, 1987). Then the protein in the supernatant solution containing the enzyme was estimated by modified Lowry (Schacterle and Pollak, 1973) and the Cp. is determined (Sunderman and Nomoto, 1970) then stored for the next step.

Step III: Gel Filtration Chromatography

In the present study, the column has a dimension of 3 × 98 cm which contained a gel Sephadex G-200 to height of (87) cm. The exclusion limit for this type of the gel is (600000) dalton (Robyt and White, 1987) or molecular weight ranges for peptides and globular proteins of (5000 - 800000) dalton. Depending on the volume of this column which is 450 ml, it was packed with a slurry of the gel in water. The slurry was carefully poured down on a glass rod to prevent air bubbles formation.

A concentrated sample (4.5) ml of the proteinaceous material, which was prepared in (Step II), was applied to top of the bed of Sephadex G-200, followed by deionized water.

Elution of the proteinaceous materials was carried out at a flow rate (24) ml / hour with a definite time interval of (10) min, using deionized water, as eluant. The fractions were collected using a fraction collector. The proteinaceous compounds in each fraction collected were detected by following the absorbance at wave length (280) nm using UV/Visible Spectrophotometer. Peak was combined separately from the plot of an absorbance versus elution volumes and Cp was determined in each fraction (Sunderman and Nomato, 1970).

Step IV: Freeze-Dryer (Lyophilization) Technique

The enzyme fraction which was obtained from gel filtration was dried using a freeze-dryer (lyophilization) technique to obtain a powder or a concentrated protein. The enzyme was kept in a deep freeze at (-20)^{o}C in a tight sample tube to be used in further investigations.

Step V: Electrophoresis

Only one sample can be run per gel each tube, (Step IV) which was applied on Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using disc electrophoresis unit quick fit instrumentation (Laemmli, 1970).

RESULTS AND DISCUSSION

Precipitation of the Protein

Ceruloplasmin has been purified, from human plasma (Noyer *et al.*, 1980), camel (Essamadi *et al.*, 2002), sheep (Calabrese *et al.*,1983), dolphin (Bonaccorsi *et al.*, 1992) and others. In this research the ceruloplasmin was isolated from normal human serum.

The PEG method has the advantages that make it particularly suitable for the preparation of a protein that is susceptible to proteolytic degradation such as ceruloplasmin. Some antiproteases are retained in the 20% PEG supernatant together with the ceruloplasmin and thus may inhibit its degradation (Noyer *et al.*, 1980). The conjugation of Cp. with biocompatible polymers is important because the immobilized enzyme conjugates and give higher stability, lower antigenicity and possibility to continuous use in various techniques of biochemistry and other tests (Ganaraja *et al.*, 2004).

Dialysis

As shown in Tables (1), the specific activity was slightly increased after dialysis. This might be due to the removal of the small molecules and increasing the purification of ceruloplasmin.

Gel Filtration Separations

The results (Fig. 1) indicated that there were mainly two peaks in all groups. For example in control, the elution volume of peak (A) was (203) ml, while the elution volume of peak (B) was (304) ml. The specific activity of the enzyme was increased in peak (B) (13.973) folds than the activity in initial extract as shown in Table (1), with total activity (60.044 U). Peak (A) was neglected at the moment, since possessing low activity.

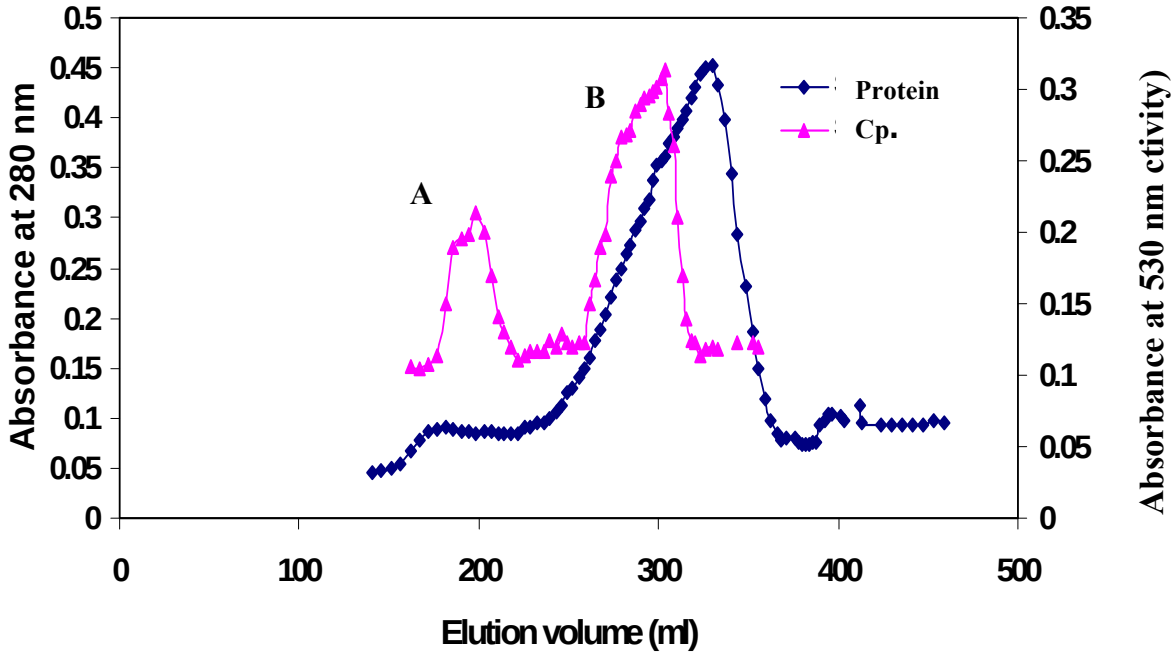


Fig. 1: Elution profile ceruloplasmin for control on Sephadex G- 200.

Table 1: Partial purification steps of ceruloplasmin from control human serum.

Purification stage	Volume taken (ml)	Total protein mg/ml	Activity U*/ml	Total activity (U)	Sp.activity U/min./mg protein	Folds of Purification	Recovery %
Serum	10.0	88.024	16.166	161.666	1.836	1.0	100
Precipitate product from PEG	1.6	12.819	14.366	22.985	1.793	1.0	14
20% PEG supernatant	8.4	59.35	14.966	125.714	2.118	1.2	78
Dialysis	14.0	51.08	9.333	130.662	2.557	1.4	81
Sephadex G-200 (Fraction)							
Peak (A)	53.4	2.119	0.27	14.418	6.804	3.7	9
Peak(B)	55.7	4.297	1.078	60.044	13.973	7.6	37

U*: a mount of ceruloplasmin that oxidize one micromole of substrate (p-henylendiamine) in one min.

Molecular Weight Determination of Cp. by Gel Filtration

The molecular weight of second peak (B) as a source of ceruloplasmin was determined by gel filtration chromatography using sephadex G-200 column (3 x 98) cm calibrated with known molecular weight proteins that were listed in Table (2).

Table 2: Elution volumes of known molecular weight materials on sephadex G-200.

Materials	Molecular weight (Dalton)	Elution volume (ml)
Urease	480000	249.9
Bovine serum albumin (BSA)	67000	322.5
α-Amylase	45000	344.9
Egg albumin	45000	340.2
I	57	44
Pepsin	36,000	442.3
Unknown (peak B)	138111*	304.3

*This value was obtained from Figure (2).

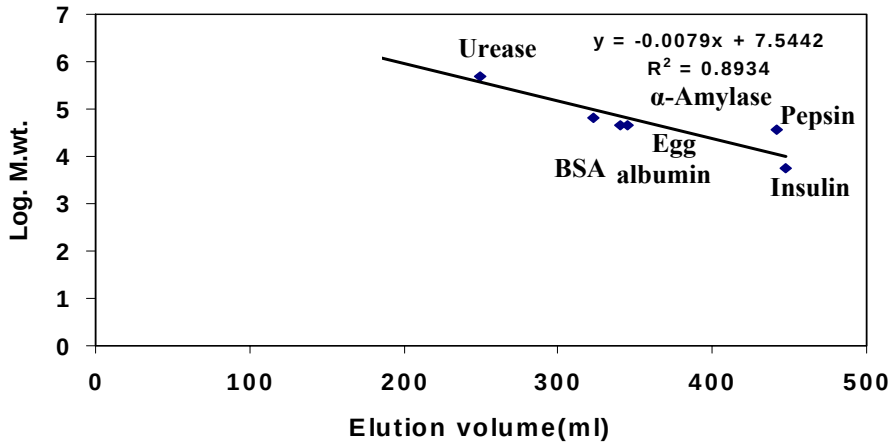


Fig. 2: A plot of the logarithm molecular weights of known proteins versus elution volumes on a sephadex G-200.

A plot of logarithmic molecular weight of each material versus the elution volumes indicated in Table (2) gives a straight line as illustrated in Figure (2).

The molecular weight of the unknown proteinous compound separated by the same column chromatography as shown in (step III) could be determined from the standard curve, which was represented by Figure (2). The comparative molecular weight of peak (B) as a source of ceruloplasmin is approximately equal to (138111) dalton. This finding was in a good agreement with the previous results where it was reported that the molecular weight of ceruloplasmin was (130000-135000) dalton from serum of normal individual (Noyer *et al.*, 1980; Ehrenwald and Fox, 1994; Essamadi *et al.*, 2002).

Molecular Weight Determination by SDS-PAGE

The electrophoretic mobility of ceruloplasmin in SDS gels was determined. The enzyme migrated as a single band in control only as shown in Figure (3) with an apparent molecular weight of (134400) dalton which was determined by using known molecular weight compounds as shown in Figure (4).

Fig. 3: Protein patterns obtained by SDS gel electrophoresis. The tubes from left to right contained (50)µg of standard protein employed to calibrate the columns were:
a. Control. **b**. Urease (M.wt. 480000). **c.** Egg albumin (M.wt. 45000).
d. Pepsin(M.wt. 36000). **e**. Bovin serum albumin (BSA)(M. wt. 67000).

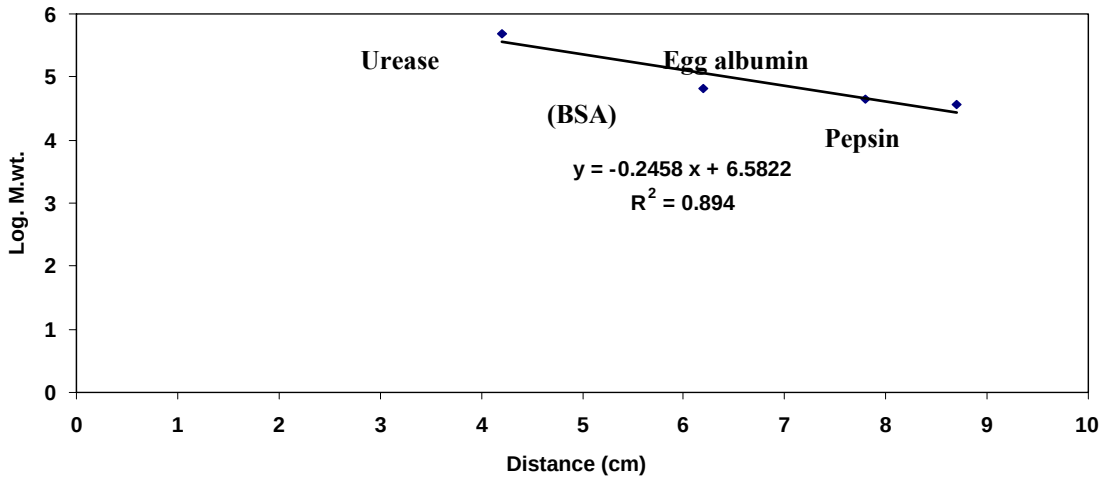


Fig. 4: Calibration plot for molecular weight estimation of ceruloplasmin by (SDS) gel electrophoresis using known molecular weight proteins.

Optimum Conditions for Ceruloplasmin Activity

1.Effect of Enzyme Concentration on Ceruloplasmin Activity:

The activity of enzyme was measured in the presence of different concentrations of partially purified enzyme from serum between (10-80) µg/ml as shown in Figure (5).

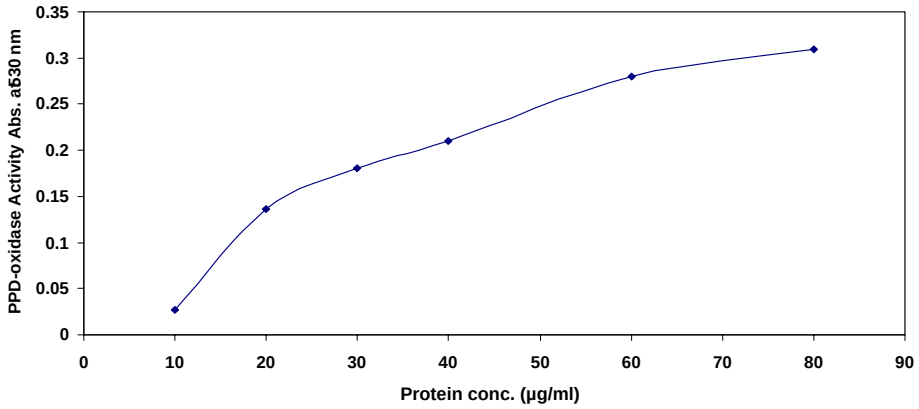


Fig. 5: Effect of different protein concentrations on ceruloplasmin activity

The result indicated that the enzyme activity increased with increasing the concentration of protein as a source of the enzyme. These results were on the direct accord with most enzymes where the activity increases with the increasing the enzyme concentration provided no inhibitors are present. For the next experiment 40µg/ml, as a source of the enzyme was selected for the other optimum conditions.

2-Effect of Buffer Concentration on Ceruloplasmin Activity:

The activity of the enzyme was measured in the presence of different concentrations of buffer solution within the range (0.06-0.16) mol/liter of sodium acetate buffer at pH 5.45. Maximum activity was obtained using (0.1) mol/liter of sodium acetate buffer (Table 3).

Table 3: Effect of buffer concentrations on ceruloplasmin activity.

Acetate buffer pH 5.45 (mol/liter)	PPD-oxidase (ΔA at 530 nm)
0.06	0.21
0.08	0.29
0.1	0.34
0.12	0.31
0.14	0.26
0.16	0.23

3.Effect of pH on the Ceruloplasmin Activity:

The influence of pH upon the activity of ceruloplasmin was investigated using of the (40µg/ml) as a source of the enzyme in (0.1) mol/liter sodium acetate buffer. The assay conditions were conducted in the same manner as described earlier at pH range of (4.2-6.2). Maximum ceruloplasmin activity was obtained at pH (5.55) as indicated in Figure (6).

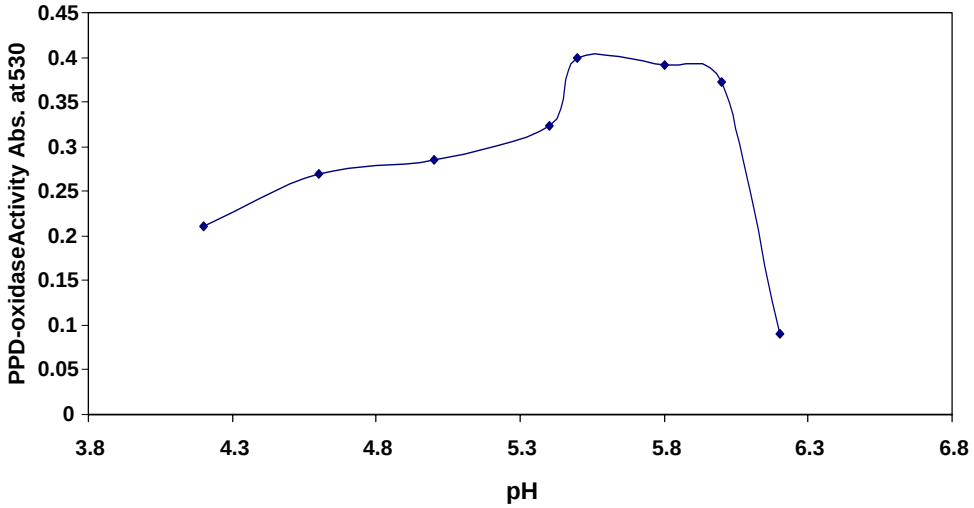


Fig. 6: Effect of pH on ceruloplasmin activity using (0.1) mol/liter sodium acetate buffer and (40µg/ml) as a source for the enzyme.

3. Incubation Time as a Function of Enzyme Activity

To determine the stability of ceruloplasmin activity under assay conditions, a series of experiments were performed at different time intervals. The results indicated that maximum enzyme activity was obtained after (35) mint. (Figure 7).

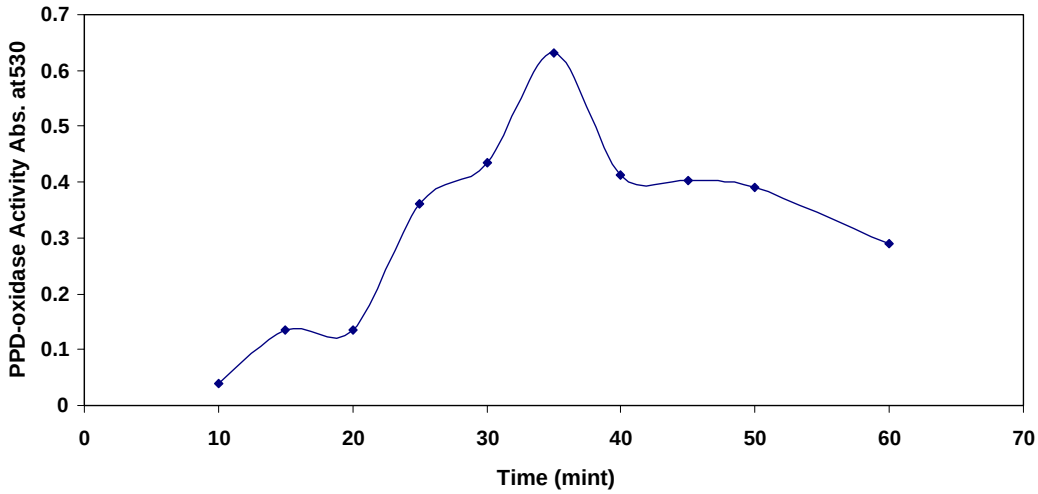


Fig. 7: Effect of incubation time on ceruloplasmin activity.

4.Effect of Temperature on Ceruloplasmin Activity:

The role of enzyme catalyzed reactions, like most chemical reaction, increases with temperature. This means that the initial reaction rate will rise with temperature until it becomes impossible to measure due to almost immediate inactivation. In practice, most enzymes are completely inactivated above (70 °C) (Plummer, 1978).

In this study, it has been found that as the temperature increased, there was a concave up increase in the enzyme activity until it reached a maximum value at a temperature of (56 °C) then dropped gradually after that Figure 8. The most convenient temperature for routine use was judged to be (37°C) (Sunderman and Nomato, 1970).

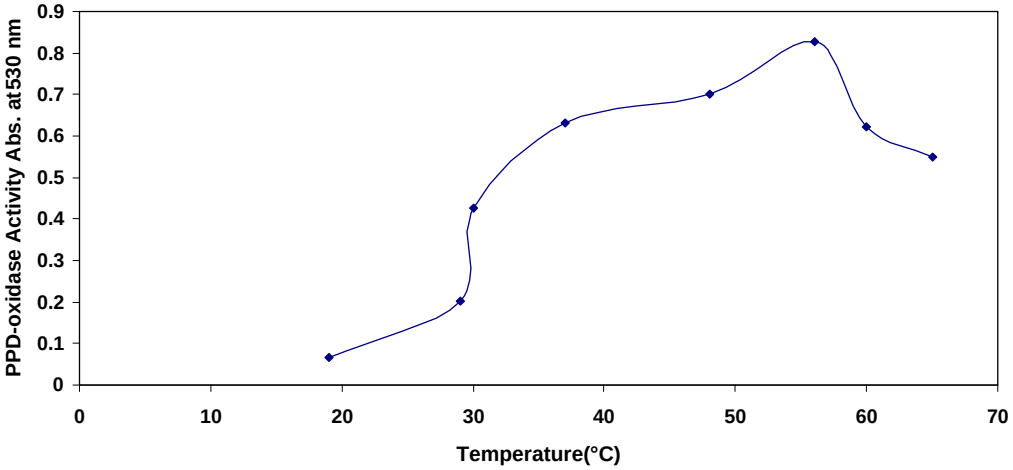


Fig. 8:Effect of temperature (°C) on ceruloplasmin activity.

6.Effect of Substrate Concentration on the Enzyme Activity:

To determine the effect of substrate concentration on the enzyme activity, a series of experiments were performed where the concentration of the substrate was varied Figure (9). Crystalline p-phenylenediamine was preferred for routine use as the substrate, because of its greater stability and commercial availability in satisfactory purity.

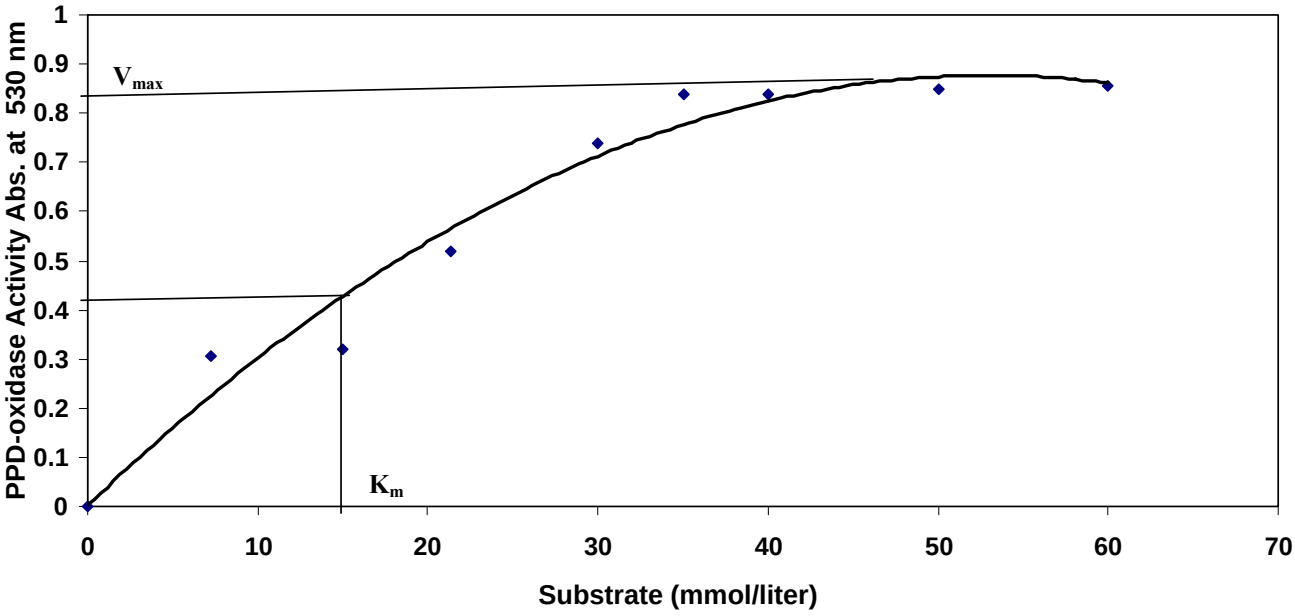


Fig. 9: Effect of substrate concentration [PPD] on the activity of partially purified ceruloplasmin.

The Michaelis-Menten constant (K_m) of the enzyme was determined from Figure (9) and found to be (15.5) mmol/liter. A similar result was obtained using a Lineweaver-

Burk plot by plotting the reciprocal of the initial velocity versus the reciprocal of the substrate concentration. A linear relationship was obtained Figure (10) giving a K_m value of (15.38mmol/liter) and V_{max} (0.833 µmol/min.).

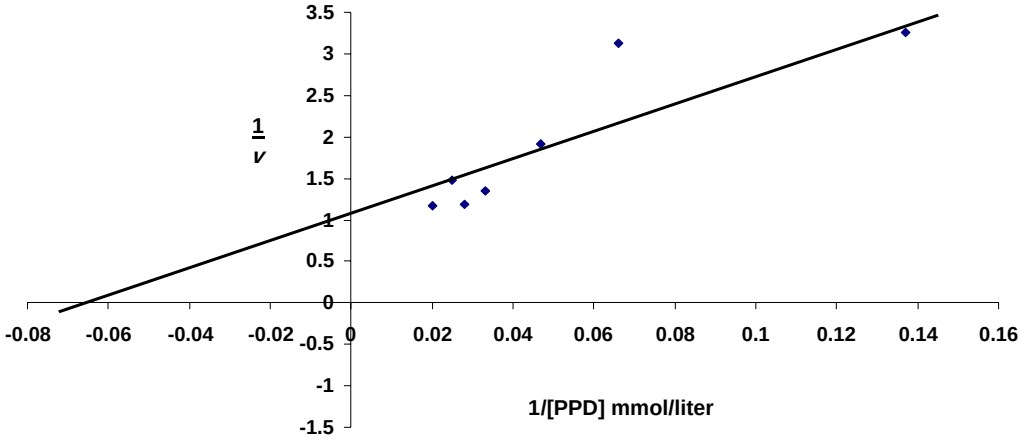


Fig. 10: Lineweaver-Burk plot of partially purified ceruloplasmin from serum control.

7.Inhibition Studies of Ceruloplasmin:

Many investigators observed that some chemical compounds have an inhibitory effect on ceruloplasmin activity.

The results of adding sodium chloride or potassium cyanide on the activity of partially purified ceruloplasmin were shown in Figure (11) and Figure (12).

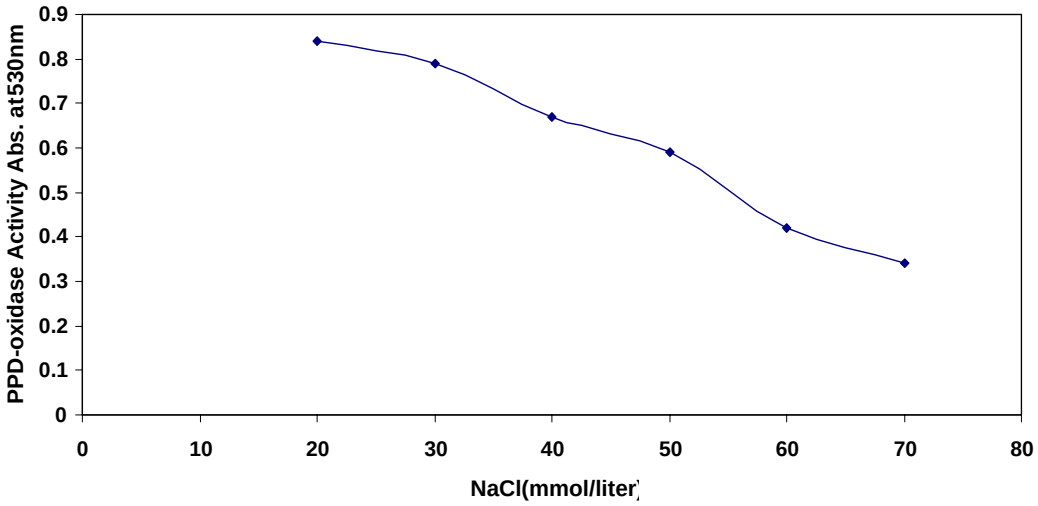
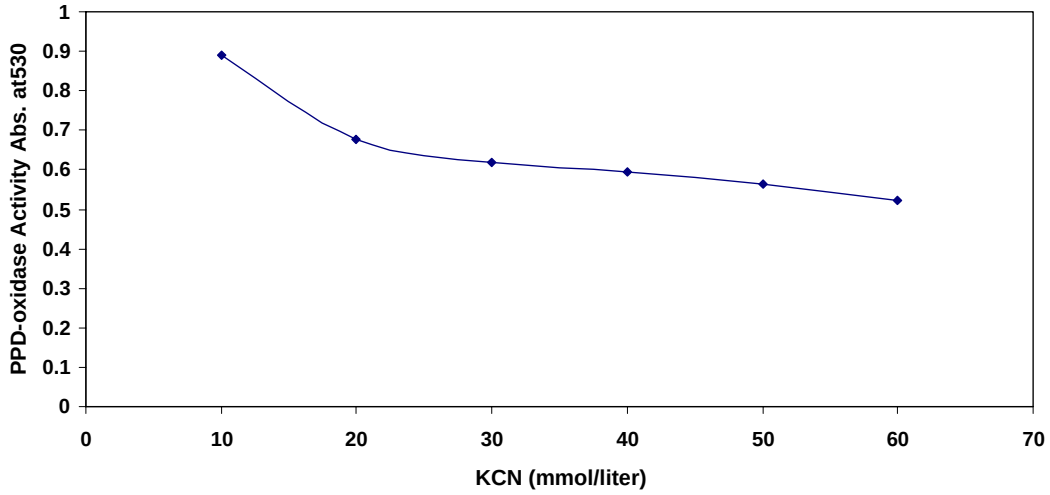


Fig. 11: Effect of sodium chloride on the activity of partially purified ceruloplasmin .

In this study, maximum inhibition was obtained when sodium chlorid was used at a concentration of (70 mmol/liter). A lineweaver-Burk plot was performed Figure (13) where (70 mmol/liter) of sodium chloride as an inhibitor was used. The results showed that a parallel lines were obtained where the slop remains constant, but the intercept on the ordinate was altered by the presence of the inhibitor. This finding predicted that sodium chloride acted as a uncompetitive inhibitor. Uncompetitive-type inhibition is rare

in single-substrate reactions, but is more common in two-substrate reactions (Burits and Ashwood, 1999).

Fig. 12: Effect of potassium cyanide on the activity of partially purified ceruloplasmin.



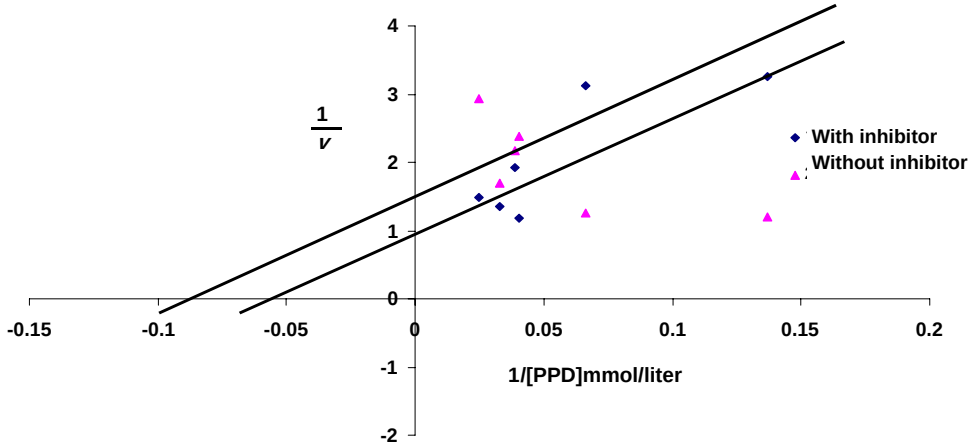


Fig. 13: Lineweaver-Burk plot of ceruloplasmin with and without sodium chloride as an inhibitor.

REFERENCES

Bhagaven, N.V., 1978. Biochemistry. 2nd Edn. J. Blippincott Company, USA. 145p., 1019p., 1020p.

Bonaccorsi di Patti, M.C., Galtieri, A., Giartosio, A., Musci, G., and Calabrese, L., 1992. Dolphin Cerulpolasmin: the First Proteolytically Stable Mammalian Ceruloplasmin. Comp. Biochem. Physiol. 103 B: pp.183–188.

Burtis, C.A., and Ashwood, E.R., 1999. Tietz Textbook of Clinical Chemistry. 3rd Edn.W. B. Saunders Company, USA. 490p., 482p., 1048p., 500p., 1241p.

Calabrese, L.; Cappuzzo, E.; Galtieri, A. and Belloco, E., 1983. Sheep Ceruloplasmin: Isolation and Characterization. Mol. Cell Biochem. 51, pp.129–132.

Essamadi, A.K.; Bengoumi, M.; Zaoui, D., and Faye, B., 2002. Purification and Partial Characterization of Camel (Camelus Dromedarius) Ceruloplasmin. Comp. Bioch. Physiol. Part B .131: pp.509-517.

Flayeh, K.A., 1988. Spermidine Oxidase Activity Serum of Normal and Schizophrenic Subjects. Clin. Chem.34(2): pp.401-403.

Fleming, R.E., and Gitlin, J.D., 1990. Primary Structure of Rat Ceruloplasmin and Analysis of Tissue-Specific Gene Expression During Development. J. Biol. Chem. 265, pp.7701–7707.

Ehrenwald, E., and Fox, P.L., 1994. Isolation of Nonlabile Human Ceruloplasmin by Chromatographic Removal of a Plasma Metalloproteinase. Arch. Biochem. Biophys. 309(2): pp.392-395.

Fox, P.L.; Mukhopadhyay, C., and Ehrenwald, E., 1995. Structure, oxidant activity, and cardiovascular mechanisms of human ceruloplasmin. Life Sci. 56: pp.1749-1758.

Ganaraja, B.; Pavithran, P., and Ghosh, S., 2004. Effect of Estrogen on Plasma Ceruloplasmin Level in Rats Exposed to Acute Stress. Indian J. Med. Scie. 58(4): pp.150-154.

Gillham, B.; Papachristodoulou, D.K., and Thomas, J.H., 2000. Wills` Biochemical Basis of Medicine. 3rd ed. Butterworth-Heinmann.UK.

Gitlin, D., and Janeway, C.A., 1960. Turnover of the Copper and Protein Moieties of Ceruloplasmin. Nature, 185:693p.

Harris, Z.L.; Morita, H., and Gitlin, J.D., 1997. The Biology of Ceruloplasmin. In Multi-Copper Oxidases (Messerschmidt, A., ed.) World Scientific, Singapore., 285p., 305p.

Halliwell, B., and Gutteridge, J.M.C., 1985. Free Radicals in Biology and Medicine. Clarendon Press. Oxford, New York, USA. 101p., 107p., 148p., 185p., 222p., 231p., 33p.

Hao, Y.L., and Wickerhauser, M., 1977. Proc. Of the International Workshop on the Technology for Protein Separation and Improvement of Blood Plasma Fractionation. DHEW Publication No.(NIH). pp.372-379.

Henry, J.B., 2001. Clinical diagnosis and management by laboratory methods. 20th. Edn.W.B.Saunders Company.USA. 258p.,189p.

Laemmli, U.K., 1970. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T_4. Nature, 227: pp.680-85.

Noyer, M.; Dwulet, F.E.; Hao, Y.L., and Putnam, F.W., 1980. Purification and Characterization of Undegraded Human Ceruloplasmin. Anal. Biochem.102: pp.450-458.

Plummer, DT., 1978. An Introduction to Paractical Biochemistry. 2nd Edn. McGraw-Hill Book Company.UK. 142p.

Robyt, J.F., and White, B.J., 1987. Biochemical Techniques, Theory and practice. Wadsworth, Inc., Belmont, California, USA.

Schacterle, G.R., and Pollack, R.L., 1973. A Simplified Method for the Quantitative Assay of Small Amounts of Protein in Biological Material. Anal. Biochem. 51: pp.654-55.

Sunderman, F.W., and Nomato, S., 1970. Measurement of Human Serum Ceruloplasmin by its Para phenylenediamine Oxidase Activity. Clin. Chem. 16(11): pp.903-910.

Wilkinson, J.H., 1962. An Intrudaction to Diagnostic Enzymology. 1st Edn. Spottiswood, Ballantyne and Co.Ltd. UK. 228p.

Yang, F., Friedrichs, W.E., and Cupples, R.L., 1996. Human ceruloplasmin:T specific Expression of Transcripts Produced by Alternative Splicing. J. Biol. Chem. 265: pp.10780-10785.