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.
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, leukocyte 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 a 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):

\[
\text{Total activity} = \frac{\text{Abs.} \times 10^6}{\text{Sample volume (ml)} \times \text{No. of } \varepsilon \times E_o}
\]

Where: Sample volume = 0.1 ml
No. of \(\varepsilon\) (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 °C 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 °C. 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 Nomoto, 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) °C. 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)°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-henylenediamine) 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    | 67000                     | 322.5               |
| (BSA)                   |                           |                     |
| 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.

\[ y = -0.2458x + 6.5822 \]

\[ R^2 = 0.894 \]
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 (Km) 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 (15.38 mmol/liter) and $V_{max}$ (0.833 µmol/min.).

![Fig. 10: Lineweaver-Burk plot of partially purified ceruloplasmin from serum control.](image)

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.](image)

In this study, maximum inhibition was obtained when sodium chloride 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.

![Graph showing the effect of potassium cyanide on ceruloplasmin activity.]

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

![Graph showing the Lineweaver-Burk plot with and without sodium chloride.]

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