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Increase of Peroxidase Activity of Haptoglobin (2-2)-Hemoglobin at Pathologic Temperature and Presence of Antibiotics

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***Abstract*—**Free Hemoglobin promotes the accumulation of hydroxyl radicals by the heme iron, which can react with endogenous hydrogen peroxide to produce free radicals which may cause severe oxidative cell damage. Haptoglobin binds to Hemoglobin strongly and Haptoglobin-Hemoglobin binding is irreversible. Peroxidase activity of Haptoglobin(2-2)-Hemoglobin complex was assayed by following increase of absorption of produced tetraguaiacol as the second substrate of Haptoglobin-Hemoglobin complex at 470 nm and 42˚C by UV-Vis spectrophotometer. The results have shown that peroxidase activity of Haptoglobin(2-2)-Hemoglobin complex is modulated via homotropic effect of hydrogen peroxide as allostric substrate. On the other hand antioxidant property of Haptoglobin(2-2)-Hemoglobin was increased via heterotropic effect of the two drugs (especially ampicillin) on peroxidase activity of the complex. Both drugs also have mild effect on quality of homotropic property of peroxidase activity of Haptoglobin(2-2)-Hemoglobin complex. Therefore, *in vitro* studies show that the two drugs may help Hp-Hb complex to remove hydrogen peroxide from serum at pathologic temperature (42˚C).

***Keywords*—**Haptoglobin, Hemoglobin, Antioxidant, Antibiotics.

I. Introduction

HAPTOGLOBIN (Hp) is a unique acute phase protein that primarily scavenges hemoglobin (Hb) released into the circulation by hemolysis or normal red blood cell turnover. The Hp genotypes show differences in geographical distribution. In the northwestern part of Europe, the Hp1-1 genotype is carried by approximately 16% of individuals, the Hp2-2 genotype by 36%, and the Hp2-1 genotype by 48% (corresponding to allele frequencies of 0.4 (Hp1) and 0.6 (Hp2). The Hp1 allele frequency is lowest in South-East Asia (0.1%) and highest in South America (0.8%) [1]. Some studies indicate possible association between specific Hp genotypes/phenotypes and particular disorders, including cardiovascular disease, autoimmune disorders, cancerous diseases, and psychiatric disorders. It is shown that the Hp2 allele is associated with a higher level of lipid peroxidation products than Hp1 allele. The Hp2-2 phenotype has consistently been observed to be a risk factor in inflammatory disease [1].

'Free' hemoglobin (Hb) is released into plasma from red blood cells or red blood cell precursors during physiological and pathological hemolysis represent a highly toxic substance, unless rapidly cleared. Free radicals such as $O2^{\circ-}$ and ˚OH are extremely reactive molecules that can cause cell damage by peroxidation of membrane lipids [1]. Free Hb promotes the accumulation of hydroxyl radicals (˚OH) by the heme iron, which can react with endogenous hydrogen peroxide by means of Fenton reaction and Haber-Weiss reaction to produce free radicals that may cause severe oxidative cell damage [2]. Furthermore the auto oxidation of the oxy Hb can produce superoxide ion $O2^{\circ-}$. Hemoglobin has negligible peroxidase activity in the presence of endogenous oxidants such as H_2O_2 and either ascorbate as second substrate [3]. Haptoglobin (Hp) is a serum acute phase protein that forms a soluble complex with Hb. The binding of Hp with Hb is very stable and irreversible [1], [4], [5].It has been reported that peroxidase activity of free hemoglobin is very low and binding haptoglobin to hemoglobin implies a considerable increase of the peroxidase activity of hemoglobin. The binding site of haptoglobin on hemoglobin is located on the globin moiety, although the increased peroxidase activity of hemoglobin is related to the configuration of the heme group [6]. We were used tetraguaiacol as second substrate, in vitro, instead of vitamin C (ascorbic acid) which is a non-enzymatic antioxidant.

When released from red blood cells into plasma, Hb rapidly dissociates into αβ-dimers which bind to Hp in a 1:1 stoichiometry, i.e. one Hb dimer per Hp (αβ)-unit [7], [8]. Both the α- and β-chains of Hb are reported to be active in terms of Hp binding [9- [11] The β globin chain of human Hb contains two specific binding sites for Hp, at amino acid residues β 11-25 and β 131-146, whereas the Hb α globin chain has one Hp-binding region, comprising residues α 121-l27 [12].The plasma concentration of Hp increases several fold in the event of an inflammatory stimulus such as infection, injury or malignancy, whether local (vascular) or systemic (exteravascular). It is hypothesized that neutrophils attracted to sites of infection/injury release stored Hp to facilitate local clearance of 'free' Hb, thereby preventing oxidative tissue damage and irondependent bacterial growth [13].Since ampicillin and coamoxiclav as a common antibiotic are used in a wide variety of infections, in the present study, activity of Hp-Hb complex at pathologic temperature in the presence of therapeutic doses of ampicillin and coamoxiclav have been investigated.

II. Materials and Methods

A. Hydrogen peroxide

A solution of hydrogen peroxide, 0.5M was prepared in phosphate buffer 50 mM and PH 7.5. Peroxide solution is discarded after 30 min and if necessary a fresh dilution is prepared [14].

B. Guaiacol reagent

A buffered solution of guaiacol, 0.03 M, is prepared as follows: 1.86 g of guaiacol and 50 ml of acetic acid are added to 400 ml of

water. The volume is made up to 500 ml with water. The guaiacol reagent is stable for several weeks since stored in the cold [14].

C. Met-hemoglobin solution

Hemoglobin solution was prepared at a concentration of 10 mg/ml in phosphate buffer 50 mM and PH 7.5. An equal volume of 0.4 mM potassium ferricyanide is added to convert completely the oxy-hemoglobin to met-hemoglobin. The met-hemoglobin solution is then carefully diluted using the same buffer to 0.03 mM [14].

D. Haptoglobin 2-2 solution

A solution of Hp 2-2 (0.03 mM) was prepared in phosphate buffer 50 mM, pH 7.5 and the concentration of the solution was determined using reported extinction coefficient of haptoglobin (εmM 58.65 Hp2-2). The mole amount of Hp2-2 was based on its monomer properties because each Hp2-2 monomer is thought to be capable of binding a single hemoglobin molecule [4], [15].

E. Preparation of Hp(2-2)-Hb complex

Hp(2-2)-Hb complexes were formed by mixing 50 ml of each above solution of Hp2-2 and Hb, incubating for 30 min at room temperature with gentle agitation to ensure that all Hp molecules were saturated with Hb.

F. Enzymatic activity assay

Peroxidase activity of Hp(2-2)-Hb complex was assayed by following increase of absorption of produced tetraguaiacol as the second substrate of Hb-Hp complex at 470 nm and 42˚C by UV-Vis spectrophotometer (CICEL Model 1900) [16]. The assay mixture contained Hp(2-2)-Hb complex 64 nM, 0.03 M guaiacol and different concentration of H_2O_2 in phosphate buffer 50 mM, PH 7.5 in 1 ml final volume. All components except for Hp-Hb complex were added directly to a quartz cuvette and mixed by inverting 6 times. Five micro liters Hp-Hb solution was then added to the mixture, and the cuvette was inverted three times to mix the ingredients. The zero time point for following A470 was designated as the time at which Hb-Hp complex was added to the solution. For each concentration of hydrogen peroxide, the reaction was performed at least 3 separate times. To investigate the effect of antibiotic drugs assay mixture of 14 μg/ml ampicillin and coamoxiclav (20 μg/ml Amoxicillin, and 4 μg/ml Clavolonic acid) which corresponded to their maximum concentrations in the plasma after injection.

G. Determination of enzymatic parameters

Maximum initial velocities (V_{max}) were obtained graphically using appropriate saturation curves. The amount of S50 (a concentration of H_2O_2 in which velocity of enzymatic activity is half of V_{max}) were obtained from the H_2O_2 concentration related to cross point of Hill plot to X-axis. The amount of (CL_{max}) (maximum clearance) and S_{max} (a concentration of H_2O_2 in which CL_{max} occurs) were graphically obtained using clearance plots.

H. Calculation of V-cal

Calculated initial velocity (V-cal) was obtained using Hill equation;

$$v = \frac{V_{\max} \times S^m}{S_{50}{}^m + S^m}$$

Where V_{max} is maximum initial velocity, S50 is the substrate concentration corresponding to half of V_{max}, m is Hill coefficient (slope of Hill plot) and S is various substrate concentrations [17].

III. Results and Discussion

Haptoglobin proteins are known as acute phase plasma glycoprotein [18], [19]. After more than 70 years of research, haptoglobin is recognized as a multifunctional protein. they have served physiological roles involved in regulation of a variety of processes, including immune responses, angiogenesis, prostaglandin synthesis and reverse cholesterol transport [20], [21] and reviewed in reference 8. However, the primary function of Hp still appears to be binding of 'free' Hb and peroxidase activity, thereby protecting from heme-catalyzed oxidative stress and facilitating Hb-uptake by macrophages [22]. Initially, methods for determining Hp were based on enhancement of the peroxidase activity of Hb by Hp-Hb binding [4].

It has been reported that peroxidase activity of free Hemoglobin is very low and binding haptoglobin to hemoglobin implies a considerable increase of the peroxidase activity of hemoglobin. The binding site of haptoglobin on hemoglobin is located on the globin moiety, although the increased peroxidase activity of hemoglobin is related to the configuration of the heme group [4], denoting physiological importance of removing hydrogen peroxide molecules produced under acute situations. Because of the lack of kinetic studies at literatures on peroxidase activity of Haptoglobin-Hemoglobin complex, the present study has investigated kinetic properties of the complexe enzymatic activity. The results indicate that antioxidant activity of Hp(2-2)-Hb complex could not be analyzed using Michaelis-Menten model (because of sigmoidal shape of its saturation curve, Figure 1) and requires the adoption of an enzyme model with multiple sites showing cooperative binding for the substrate (H_2O_2). Eadie-Hofstee plot (Figure 2.A) confirms positive cooperativity resulting from right-side curvature of the plot condition. Consideration of Figure 2.B shows that there is a well defined maximum for the clearance of the substrate CL_{max}. CL_{max} (like Km) is determined under in vitro condition but it is appropriate parameter for investigation of the activity under in vivo condition. The concentration of hydrogen peroxide that causes maximum clearance (CL_{max}) is named S_{max}. Both of the parameters were obtained from Clearance plot (Figure 2.B). S_{max} was observed in relatively low hydrogen peroxide concentrations, because of the maximum complex autoactivation. CL_{max} provides an estimate of the highest clearance attained as substrate concentration increases before any saturation of the enzyme sites. Thus if the assumption is made that in vivo activation occurs via endogenous activators, then CL_{max} may be an appropriate parameter for describing the salient feature of the subsystem that can be used for predictive purposes [14].

Hill plot (Figure 2.C) not only confirms positive allostric effect (the slope of the lines are more than 1, m>1), but also shows that there are two sequential positive allostric effects (m1=1.8, m2=3.7) with increasing H_2O_2 concentration (Table1). At Figure 2.D it has been demonstrated that calculated initial velocities (V-cal) obtained from Hill equation (see Materials& Methods), are in good coincidence with experimental initial velocity (V-exp).

As it is seen in Figure 3; both ampicillin and coamoxiclav have activating effect on Hp-Hb peroxidase activity (heterotropic effect or heteroactivation), but they have not changed the sigmoidal shape of saturation curve (homotropic effect or autoactivation). It seems that both drugs induce a new conformation to the complex which is more

active. On the other word, autoactivation and heteroactivation have been occurred simultaneously.

Analysis of the Hp-Hb complex saturation curves in the presence of the two drugs (Figure 3), has shown that Hp(2-2)-Hb complex maintains the homotropic behavior of its peroxidase activity. Moreover the drugs have not changed the first Hill coefficient, while the second Hill coefficient has decreased in the presence of ampicillin and increased in the presence of coamoxiclav (Figure 4.C and 4.D respectively). These results have been summarized in Table1. Comparison of the amounts of CL_{max} shows that the drugs increase CL_{max} of complex to remove H_2O_2 (Figure 4.B, Table1). This effect in the presence of ampicillin is f more than of coamoxiclav (about 29% for ampicillin and 7% for coamoxiclv). S_{max} has been shifted to relatively higher concentrations of H_2O_2 in the presence of coamoxiclav, while ampicillin has not considerable effect on S_{max} (Figure 4.B, Table1). S50 has not been changed in the presence of drugs (Table1).

In conclusion, ampicillin and coamoxiclav can increase antioxidant property of Hp(2-2)-Hb complex via heterotropic effect on peroxidase activity of the complex. It will be meaningful if we remind that the two drugs are usually used under pathologic condition caused by infectious disease, and haptoglobin is known as an acute phase protein [15], [16]. Therefore in vitro studies show that the two drugs (especially ampicillin) may help Hp-Hb complex to remove hydrogen peroxide from serum. We were used tetraguaiacol as second substrate in vitro instead of vitamin C (ascorbic acid) which is a non-enzymatic antioxidant. Our study suggests that antioxidant activity in individual with Hp2-2 that encounters to fever and consume above antibiotics increases if they use more vitamin C.

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TABLE I

UNITS FOR MAGNETIC PROPERTIES ENZYMATIC AND ALLOSTERIC PARAMETERS OF ANTIOXIDANT ACTIVITY OF HP(2-2)-HB COMPLEX IN THE PRESENCE OF AMPICILLIN AND COAMOXICLAV.

Complex	Temperature	CL_{max} ($\times 10^6$)	S_{max} (M)	S_{50} (M)	m_1	m_2
Hp(2-2)-Hb	37	7.2	0.027	0.027	1.8	3.7
	42	42	9.3	0.025	1.4	2.3

The values have been directly obtained from saturation curve (Figures are 1 and 3) Clearance plots (Figures are 2.B, 4.B) and Hill plots (Figures are 2.C, 4.C, 4.D) as it has been mentioned in "Materials and Method

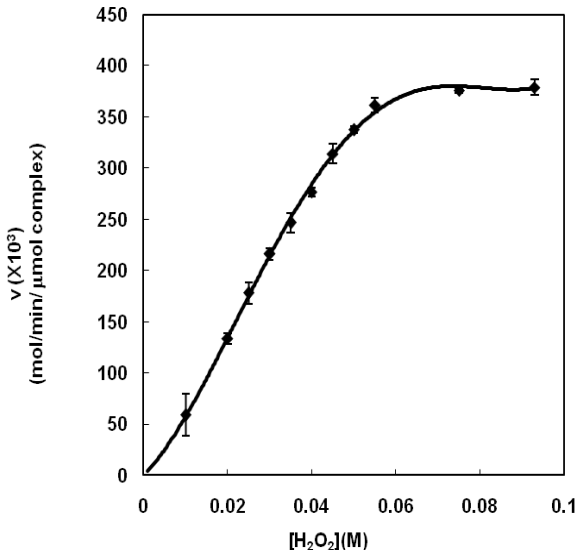

Fig. 1 Saturation curve for antioxidant activity of Hp(2-2)-Hb using hydrogen peroxide as substrate in phosphate buffer 50 Mm, PH 7.5 at 42°C. Each enzymatic assay was done using 0.03 M guaiacol as second substrate and following of A_{470}. Sigmoidal shape of the curvature shows non-michaelis behavior.

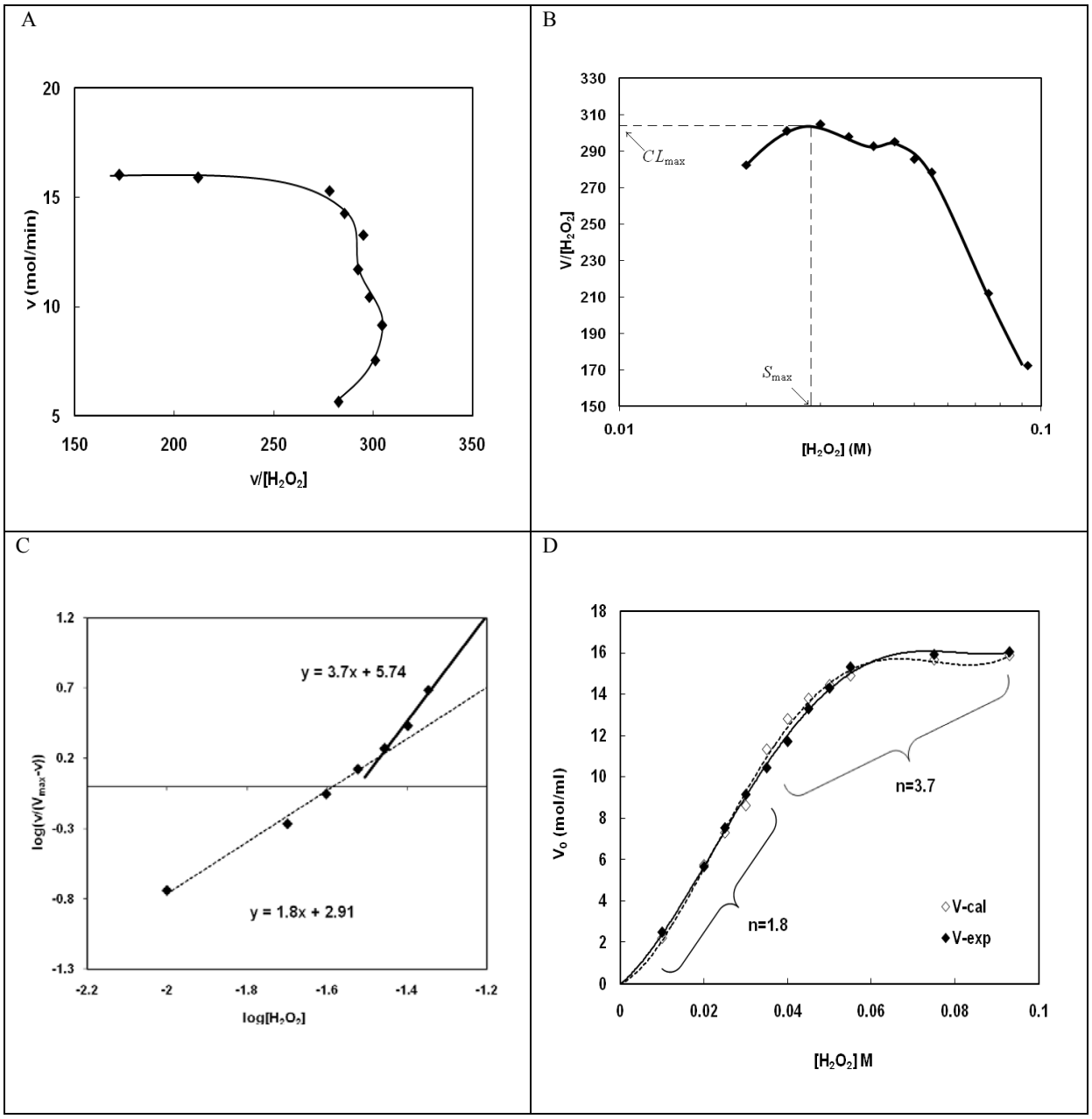

Fig. 2 Non-Michaelis analysis of peroxidase activity of Hp(2-2)-Hb complex. (A) Eadie-Hofstee plot. The nonlinear shape and the curvature toward right show non-Michaelis homotropic allostric effect. B) Clearance plot. As it is observed the upward curvature confirms homotropic property. Maximum clearance (CL_{max}) and S_{max} have been determined graphically as shown. C) Hill plot. It is seen the points of this graphs lay on at least two consecutive linear parts. The slope of each line (m) is more than unit (m>1). This observation not only confirms positive cooperativity and homotropic effect but also demonstrates the behavior is changed with H_2O_2 concentrations so that the two sequential homotropic behaviors lead to increased activity. D) Comparison between experimental and calculated saturation curve. Experimental initial velocities (—) are directly from figure 1. Calculated initial velocities (.....) have been calculated using Hill coefficients (Fig 2.C) and Hill equation as explained in Material and Methods

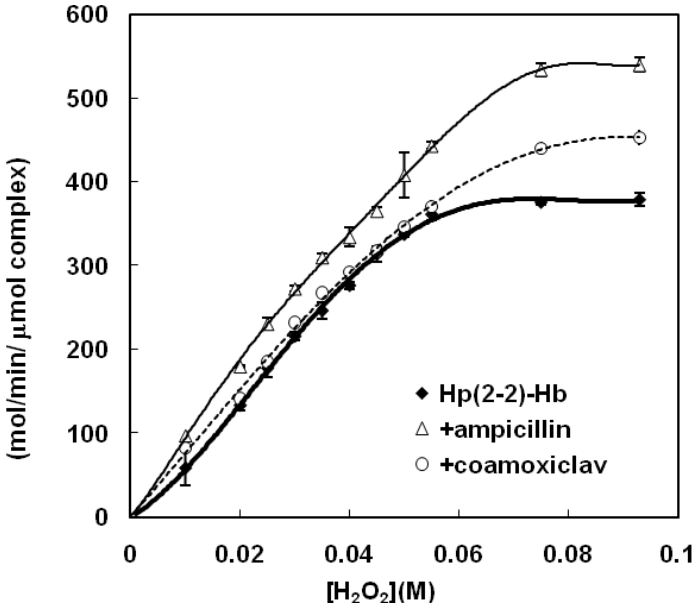

Fig. 3 Saturation curves for peroxidase activity of Hp(2-2)-Hb complex using hydrogen peroxide as substrate in phosphate buffer 50 Mm, PH 7.5 at 42˚C in the presence of therapeutic concentration of ampicillin and coamoxiclav. Saturation curve of Figure 1 has been incorporated here, for comparision

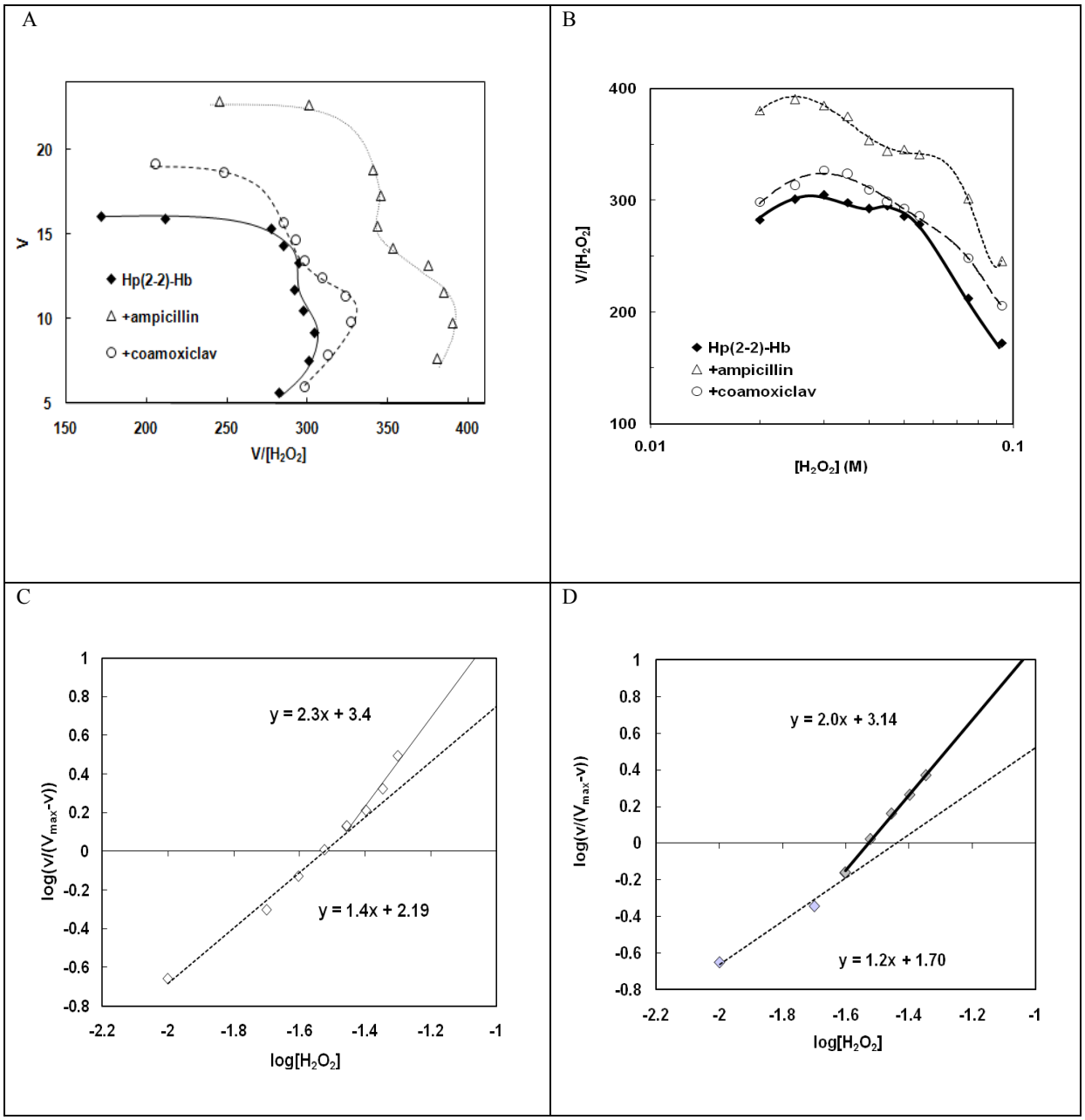

Fig. 4 Non-Michaelis analysis of peroxidase activity of Hp(2-2)-Hb complex. A) Eadie-Hofstee plot. The nonlinear shape and the curvature toward right show non-Michaelis homotropic allostric effect. Eadie-Hofstee plot in the absence of drugs (Figure 2.A) has been incorporated here, for comparison. B) Clearance plot. As it is observed the upward curvature confirms homotropic property. Maximum clearance (CL_{max}) and S_{max} have been determined graphically as shown. Clearance plot in the absence of drugs (Figure 2.B) has been incorporated here, for comparison. C) Hill plot in the presence of ampicillin. It is seen the points of this graphs lay on at least two consecutive linear parts. The slope of each line (m) is more than unit (m>1). This observation not only confirms positive cooperativity and homotropic effect but also demonstrates the behavior is changed with H_2O_2 concentrations so that the two sequential homotropic behaviors lead to increased activity. D) Hill plot in the presence of coamoxiclav. The same effect can be observed here moreover the slope of second line in the presence of coamoxiclav, is more than of ampicillin. The obtained values of CL_{max}, S_{max} and Hill coefficients have been summarized in Table1.