Potentiometric Study of α-Amylase Kinetics Using a Platinum Redox Sensor

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A potentiometric study of α-amylase kinetics using a platinum redox sensor is described in this paper. The sensor measured the amount of triiodide ion released from a starch-triiodide complex after biocatalytic starch degradation, which was correlated with the α-amylase activity. The response characteristics of the platinum redox sensor to the triiodide ion and the working parameters for the starch hydrolysis have been optimized. The kinetics of starch degradation using α-amylase have been measured by direct potentiometry. The two theoretical models for α-amylase kinetics, Michaelis-Menten and Lineweaver-Burk, were calculated from the experimental data and optimized using *Solver*. The calculated parameter values for α-amylase were V_{max} = 2.99E-5 and K_m = 0.4862 for the Michaelis-Menten model, and V_{max} = 3.31E-05 and K_m = 0.5771, Slope = 17449, for the Lineweaver-Burk model.

1. INTRODUCTION

α-Amylases (endo-1,4-α-d-glucan glucanohydrolase, EC 3.2.1.1) are enzymes that hydrolyze starch molecules to produce diverse products including dextrins and progressively smaller polymers composed of glucose units, such as glucose, maltose, or dextrins [1]. α-Amylases are a part of a larger group of amylolytic enzymes.

Today, amylases are of great significance in biotechnology, with applications ranging from food, fermentation, detergent, and textiles to paper industries [2,3] and in synthetic chemistry, with the production of oligosaccharides by transglycosylation [4] accounting approximately 25% of the enzyme market [5].

α-Amylases They play an important role in the carbohydrate metabolism of microorganisms, plants and animals. α-Amylases are also found in human saliva, and as a disease indicator in blood and urine [6].

The difficulties in determining amylolytic activity are reflected by the variety of analytical procedures that have been developed. Two of the most frequently used procedures for the determination of α-amylase activity are the amyloclastic [7] and saccharogenic [8,9] methods. The first method is based on a starch-iodine reaction [10,11] and the second on the formation of reducing sugars, measured as maltose or glucose equivalents. The disadvantages of these methods include long incubation periods, endogenous glucose interference and unstable reaction colors, resulting in poor reproducibility and reliability. Some published methods have been applied to determine the catalytic activity of α-amylase, including spectrophotometry [12], fluorometry [13], amperometry [14], electrophoresis [15], isoelectric focusing [16], chromatography [17] and immunological methods [18].

Recently, we reported a novel sensor for α-amylase determination [19]. A theoretical approach to measure α-amylase activity accurately and rapidly, based on potentiometric principles, was proposed and used to design a sensing methodology involving a platinum redox electrode. The sensor functioning was based on the measurement of the triiodide released from a starch-triiodide complex after biocatalytic starch degradation by α-amylase. Using this method, many of the disadvantages of other methods have been overcome, resulting in a significant reduction of measuring time and costs of analysis.

The aim of this paper was to develop a kinetic model for α-amylase activity determination, based on the experimental and theoretical kinetic model response of direct potentiometric determination of triiodide released from a starch-triiodide complex after biocatalytic starch degradation by α-amylase.

2. EXPERIMENTAL

2.1. Reagents and Solutions

α-Amylase, 0.36 U/mL, was purchased from Fluka (Switzerland) and soluble starch was purchased from Kemika (Croatia). Iodine (I_2), purchased from Kemika (Croatia), and potassium iodide (KI) purchased from Sigma-Aldrich (Germany), were used to prepare the potassium triiodide inhibition solution. Sodium chloride (NaCl) and calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich (Germany). Glacial acetic acid (CH_3COOH) was purchased from Panreac (Spain), and sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$) was purchased from J.T. Baker (Holland).

2.1.1. α-Amylase solution preparation

An α-amylase solution was prepared by dissolving 5 mg of α-amylase (Fluka, Switzerland) in 500 mL (0.36 U/mL) of a 0.1 M acetate buffer (pH 5.5) solution containing 6 mM $CaCl_2$ and 20 mM NaCl. The addition of $CaCl_2$ and NaCl to the amylase solution stabilized enzyme activity. It was

essential that the amylase should be buffered before coming into contact with NaCl because its activity rapidly decreases in the presence of NaCl at pH values below 4.0. The decrease in amylase activity by self-decomposition was less than 3% after 3 days, when stored in a refrigerator.

2.1.2. Starch solution preparation

Determination of starch dry weight was performed by drying soluble starch at 130 °C until a constant mass was achieved. The 5.0 g/L starch stock solution was prepared by taking the equivalent of 2.5 g of anhydrous starch and dissolving it in a previously prepared acetate buffer (pH 5.5) in a 500 mL volumetric flask. The suspension was swirled and boiled gently for 3 minutes. When the starch suspension was completely dissolved, the solution was rapidly cooled under running water to prepare it for use. Furthermore, the starch stock solution was used to prepare a range of concentration dilutions: 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0, and 5.0 mg/mL. The solutions must be prepared on the day of use to avoid microbial degradation and retrogradation.

2.1.3. Inhibition solution preparation

The potassium triiodide solution (100 µM) was prepared by dissolving solid iodine in a 0.05 M potassium iodide solution with a small volume of water. When solid iodine was completely dissolved, acetic acid was added until a final concentration of 1 M was reached. The acetic acid-triiodide solution was used as an α-amylase inhibition solution (IS) and as the reagent for producing colored starch-triiodide complexes.

2.2. Apparatus

A Metrohm 780 pH meter, a 728 Stirrer, a Metrohm 765 Dosimat (all from Metrohm, Switzerland), homemade software and a platinum redox electrode IJ64 (Ionode, Australia) were all used for the triiodide response measurements. A silver/silver (I) chloride reference electrode (Metrohm, Switzerland) was used as a reference.

Direct potentiometric measurements were performed using an eDAQ 186 Quad Amp pH/mV, eDAQ e-corder 821 8-channel data acquisition system, eDAQ EChem 1.5 software (all from eDAQ, Australia), IJ64 platinum redox electrode and a silver/silver (I) chloride reference electrode. A thermostat (PolyScience, USA) was used for the amylase incubation.

2.3. Procedure

2.3.1. Response characteristics of the platinum redox sensor to the triiodide ion

Potassium triiodide solutions containing a fixed iodine concentration (c = 5 mM) and potassium iodide concentration (0.05 M) were prepared with ultrapure deionized water. The potassium iodide

solution was prepared separately. The responses of the platinum redox sensor were measured by accurate incremental addition of the triiodide solutions to 20 mL of potassium iodide solution of an equal iodide concentration. In this way, the triiodide response was always measured at constant iodide concentrations, resulting in a pure triiodide response of the redox sensor. The solution was continuously stirred during the triiodide addition and measurement.

2.3.2. Response parameters optimization for α-amylase measurement

2.3.2.1. pH optimization

Starch solution was inserted into a series of tubes with a pH range of 4-8.5. The tubes were incubated for 10 minutes at 40 ºC. The α-amylase concentration was 10 mg/L.

2.3.2.2. Time and temperature optimization

The starch solution with a pH of 5.5 was inserted into a series of cuvettes. The solution was incubated with α-amylase solution (10 mg/L), and IS solution was added at 30 seconds intervals for the first 5 minutes, and then every minute up to 19 minutes. The same procedure was tested at 35, 40 and 45 ºC.

2.3.3. Potentiometric measurement of α-amylase activity

The α-amylase solution (30 mL) was pipetted into a 100 mL flask and placed in a 40 °C water bath with a second flask containing 30 mL of starch solution. After 15 minutes, 20 mL of the α-amylase solution was added into the starch solution and stirred. At regular time intervals, 3 mL aliquots were taken from the flask and added to 6 mL of the IS solution. The solution was mixed thoroughly, and the potential was immediately measured using the platinum redox sensor. Blank or zero point potential was measured separately using the same procedure as described above, except that the α-amylase solution was replaced with the same volume of deionized water.

3. RESULTS AND DISCUSSION

3.1. Response characteristics of the platinum redox sensor to the triiodide ion

The response characteristics of the platinum redox sensor to the triiodide ion at a constant iodide concentration (0.05 M) are shown in Figure 1. The sensor exhibited a stable, reproducible and linear Nernstian response (slope 29.5 ± 0.2 mV/concentration decade, correl. coefficient 0.9996). The triiodide concentrations, released during α-amylase starch degradation, were always within the linear response range of the sensor.
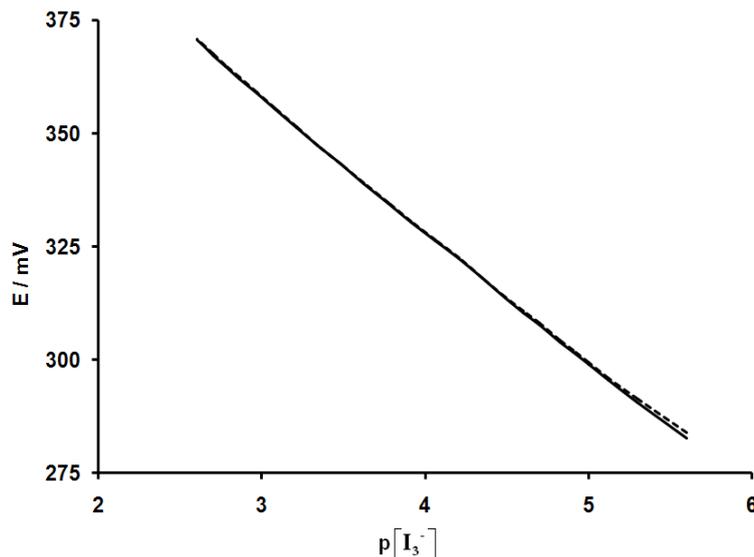
Figure 1. The reproducibility of the two response characteristics of the platinum redox sensor to triiodide ion at a constant iodide concentration (0.05 M).

3.2. Optimization of the working parameters for starch hydrolysis

The working parameters (time, temperature and pH) for amylolytic starch hydrolysis have been optimized. The influence of time and temperature on amylolytic starch hydrolysis in terms platinum redox sensor response is shown in Figure 2a. During the first ten minutes, all of the starch was hydrolyzed. The redox sensor showed the lowest response at 35 °C and increased at temperatures of 40 °C and 45 °C. A temperature of 40 °C was used in further investigations because it is more convenient and easier to use than 45 °C, and the sensor response is similar for both temperatures. Furthermore, the influence of pH values, ranging from 4-8, on α-amylase activity was tested (Figure 2b). The platinum redox sensor showed the highest response at pH 5.5. α-Amylase also demonstrated the highest activity at this pH; thus, further investigations were carried out at pH 5.5.
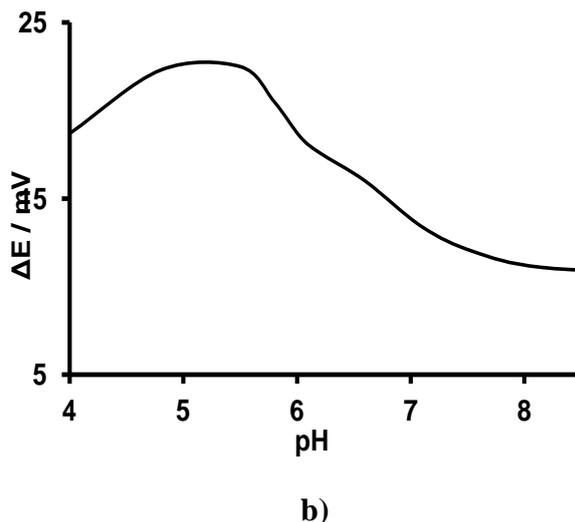

a)

b)
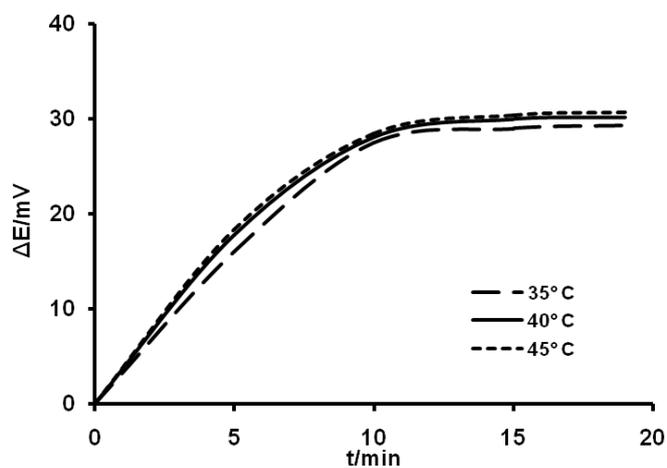
Figure 2. Working parameters optimization. a) Influence of temperature and hydrolysis time on platinum redox sensor response (α-amylase concentration was 10 μg/mL, starch concentration was 5 mg/mL), b) Influence of pH on the platinum redox sensor response (α-amylase concentration was 10 μg/mL, incubation time of 10 minutes at 40 °C, starch concentration was 5 mg/mL).

3.3. Kinetics of starch degradation using α-amylase

After the addition of a known amount of the starch solution to the solution of iodide-triiodide, with a fixed concentration of triiodide ion and an excess of iodide ion, a starch-triiodide complex was formed and the following equilibrium will be established:

$$\left[KI_3\right]_0 + \left[KI\right]_0 + S \rightleftharpoons \left[KI_3 \cdot S\right] + \left[KI_3\right]_f + \left[KI\right]_0 ,$$ (1)
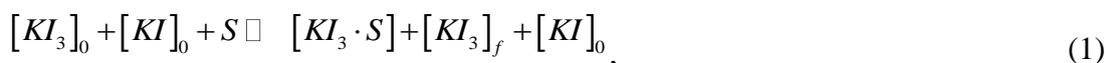
where $\left[KI_3\right]_0$ and $\left[KI\right]_0$ are the initial triiodide and iodide concentrations, respectively, and $\left[KI_3\right]_f$ is the free triodide concentration.

During the starch hydrolysis caused by α-amylase, the initial starch amount decreases at a fixed triiodide concentration. This has the consequence of a decreased starch-triiodide complex concentration, resulting in the continuous increase of free triiodide concentration in the solution. The triiodide equilibrium can be described by Eq. 2:

$$\left[I_3^-\right]_f = \left[I_3^-\right]_0 - \left[I_3^-\right]_b ,$$ (2)

where $\left[I_3^-\right]_f$ is the free triiodide concentration, $\left[I_3^-\right]_0$ is the initial triiodide concentration and $\left[I_3^-\right]_b$ is the concentration of triiodide bound to starch.

The Nernstian response equation [19] for the observed system can be simplified to:

$$E = C + S \log\left[I_3^-\right] \tag{3}$$

where $C = E^0 - S \log\left[I^-\right]^3 = E^0 - 3S \log\left[I^-\right]$ (4)

The iodide concentration must be in great excess and can be considered constant.

α-Amylase starch hydrolysis has been investigated at a fixed α-amylase concentration (c = 10 μg/mL) and various starch solution concentrations (c = 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0 and 5.0 mg/mL). The corresponding responses of the platinum redox sensor to the above solutions are shown in Figure 3.
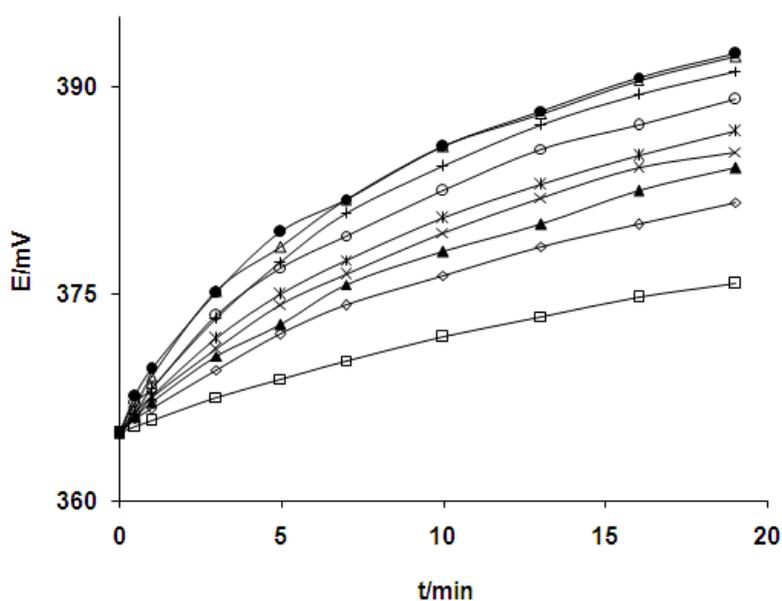

Figure 3. The responses of the platinum redox sensor to the starch solutions of different concentrations (c = 0.1 (□), 0.2 (◇), 0.3 (▲), 0.4 (×), 0.5 (✳), 1.0 (○), 2.0 (+), 3.0 (△), 5.0 (●) mg/mL) during α-amylase starch hydrolysis.

The free triiodide concentration can be calculated from the potentiometric data by using Eq. (5), derived from Eq. (4):

$$\left[I_3^-\right]_f = 10^{\left(\frac{E - Const}{S}\right)} \tag{5}$$

The values of *S* and *Const* were calculated from the initial reaction conditions.

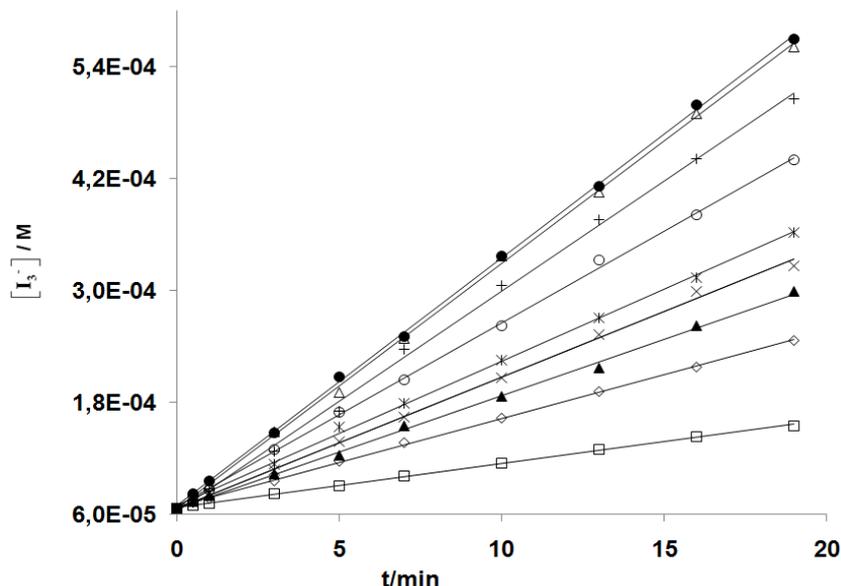The related progress curves displaying $\left[I_3^-\right]$ vs. time are shown in Figure 4.

Figure 4. Progress curves displaying $\left[I_3^-\right]$ vs. time for the starch solutions of different concentrations (c = 0.1 ($\square$), 0.2 ($\diamond$), 0.3 ($\blacktriangle$), 0.4 ($\times$), 0.5 ($*$), 1.0 ($\bigcirc$), 2.0 ($+$), 3.0 ($\triangle$), 5.0 ($\bullet$) mg/mL) during α-amylase starch hydrolysis.

The corresponding experimental and modeled Michaelis-Menten curves, reaction rate vs. substrate concentration, presented in Figure 5, were obtained by fitting the experimental V - [S] data to the Michaelis-Menten equation [20] by nonlinear regression using *Solver*, a spreadsheet optimization modeling system incorporated into Microsoft Excel for Windows:

$$V = \frac{V_{max}\left[S\right]}{\left[S\right]+K_M} , \tag{6}$$

where V is the reaction rate, V_{max} is the maximum rate, K_M is the Michaelis-Menten constant, and [S] is the substrate concentration. The calculated parameter values for α-amylase were $V_{max} =$ 2.99E-5 and $K_m = 0.4862$.

Equation (6) can be linearized by taking reciprocals of each side and rearranging them, resulting in the linear Lineweaver-Burk plot:

$$\frac{1}{V} = \left(\frac{K_M}{V_{max}}\right)\frac{1}{\left[S\right]}+\frac{1}{V_{max}} . \tag{7}$$

The corresponding Lineweaver-Burk plot, reciprocal reaction rate $\left(\dfrac{1}{V}\right)$ vs. reciprocal substrate concentration $\left(\dfrac{1}{\left[S\right]}\right)$, presented in Figure 6, was obtained by fitting the experimental $\left(\dfrac{1}{V}\right)-\left(\dfrac{1}{\left[S\right]}\right)$

data to the Lineweaver-Burk equation (7) using linear regression. The calculated parameter values for α-amylase were $V_{max} = 3.31E-05$ and $K_m = 0.5771$, Slope $= 17449$.
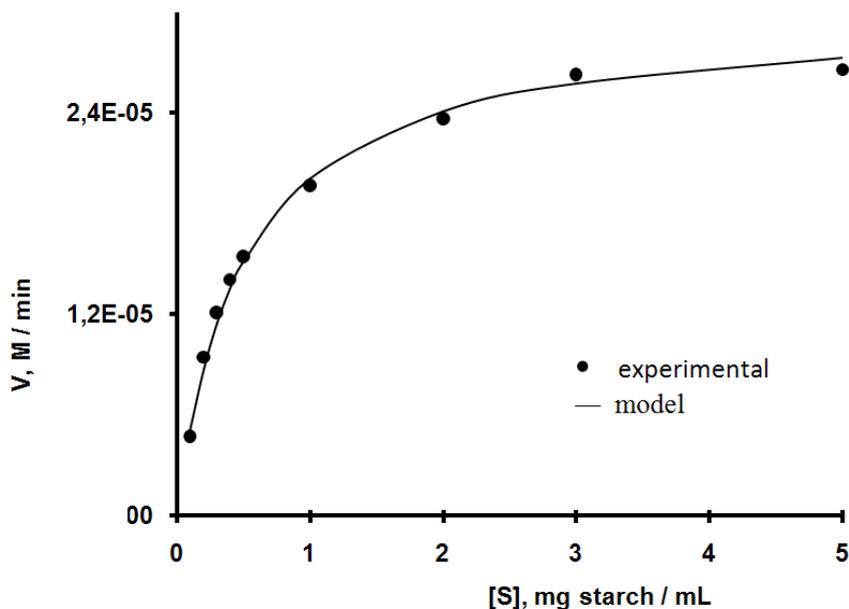

Figure 5. The Michaelis-Menten progress curves of α-amylase-catalyzed hydrolysis of starch.
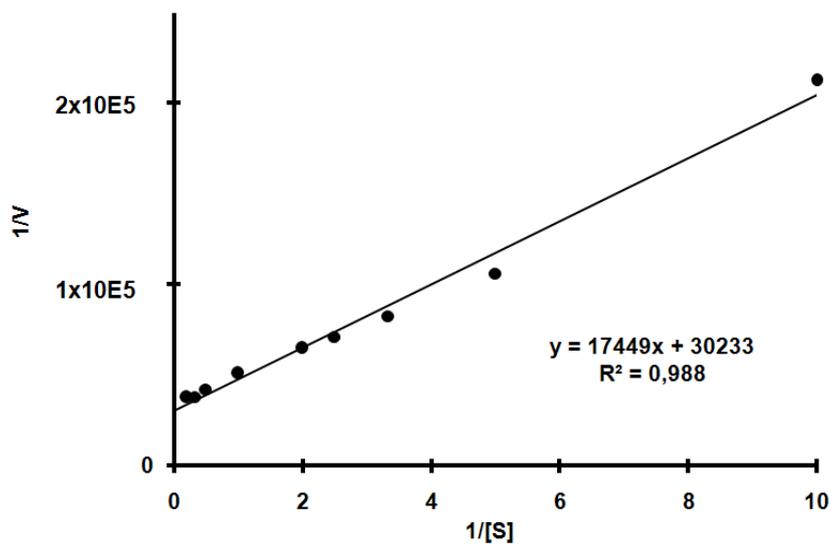

Figure 6. The Lineweaver-Burk progress curve of α-amylase-catalyzed hydrolysis of starch.

4. CONCLUSIONS

An α-amylase kinetics study using direct potentiometry and a platinum redox sensor has been proposed. The sensor measured the amount of triiodide ion released from a starch-triiodide complex,

which was correlated with the α-amylase activity after biocatalytic starch degradation. Experimental data were optimized using nonlinear and linear regression using *Solver*. The α-amylase kinetics parameters were obtained using the two enzyme kinetics models (Michaelis-Menten and Lineweaver-Burk) and revealed a satisfactory mutual agreement.

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