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A bienzyme electrochemical biosensor for the detection of collagen L-hydroxyproline

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ABSTRACT

L-hydroxyproline (L-Hyp: *trans*-4-hydroxy-L-proline) is commonly found at high concentrations in connective tissue proteins such as collagen. It is a remarkably useful molecular marker because variation in the level of L-Hyp is associated with various diseases. Recently, the novel enzymes L-hydroxyproline epimerase and D-hydroxyproline dehydrogenase were isolated from bacteria. In this study, a novel electrochemical biosensor for L-Hyp was constructed using these two enzymes. L-hydroxyproline epimerase epimerized L-Hyp to D-hydroxyproline (D-Hyp: *cis*-4-hydroxy-D-proline), and D-Hyp was oxidized with the reaction catalyzed by D-hydroxyproline dehydrogenase and mediated by ferrocene. We found that the sensor could determine L-Hyp concentrations of 10–100 μM with high-selectivity.

1. Introduction

Collagen is the most abundant protein in the extracellular matrix of living organisms. The major amino acid components of collagen are glycine (~33%), L-proline (L-Pro), L-hydroxyproline (L-Hyp) (21%), and alanine (11%). L-Hyp is a collagen-specific amino acid [1]. By measuring L-Hyp in urine, it is possible to determine collagen metabolism in the body, and in particular the degree of collagen degradation. Indeed, because L-Hyp is rarely present without collagen, it has become the specific biomarker for collagen degradation and is used to investigate collagen-related disease [2–4]. For example, collagen is an abundant protein in the bones and skin; therefore, abnormal collagen metabolism can occur with bone [5,6] and skin diseases [7], as well as fibrosis [8]. Additionally, collagen degradation is necessary in the cancer metastatic process [9,10]; thus, L-Hyp is also a useful marker for studying cancer metastasis.

Various analytical techniques can be employed to measure L-Hyp in serum (for determination of bone composition) and urine, and to study rates of bone resorption and collagen metabolism. Colorimetric methods for measuring L-Hyp involve the oxidation of hydrolyzed L-Hyp; however, this process is time consuming, and controlling oxidation and color formation reactions can be difficult [11–13]. L-Hyp can also be analyzed by high-performance liquid chromatography [14–17] and gas chromatography [18,19], but these techniques require expensive equipment that is costly to maintain. An enzymatic method, in which amino acid dehydrogenase is used, could be a rapid and simple method of amino acid analysis. However, it is difficult to detect L-Hyp selectively using such a method because amino acid dehydrogenase has relatively broad substrate specificity, for example proline dehydrogenase *Pyrobaculum calidifontis* catalyzes the dehydrogenation of both L-proline and L-hydroxyproline (relative activity, 72% as compared to L-proline) [20].

Recently, we identified the pathway of L-Hyp metabolism in bacteria, and characterized D-hydroxyproline dehydrogenase. Two types of D-hydroxyproline dehydrogenase were characterized and showed similar high specificity for D-hydroxyproline, one from *Pseudomonas putida* and one from *Pseudomonas aeruginosa* [21]. Although the former is a simple homomeric enzyme containing only FAD as a prosthetic group, the purification of the protein

Abbreviations: L-Hyp, L-hydroxyproline; D-Hyp, D-hydroxyproline; Pyr4H2C, Δ1-pyrroline-4-hydroxy-2-carboxylate; L-Gln, L-glutamine; Gly, glycine; L-Ala, L-alanine; L-Val, L-valine; CV, cyclic voltammetry; SPE, screen-printed electrode.

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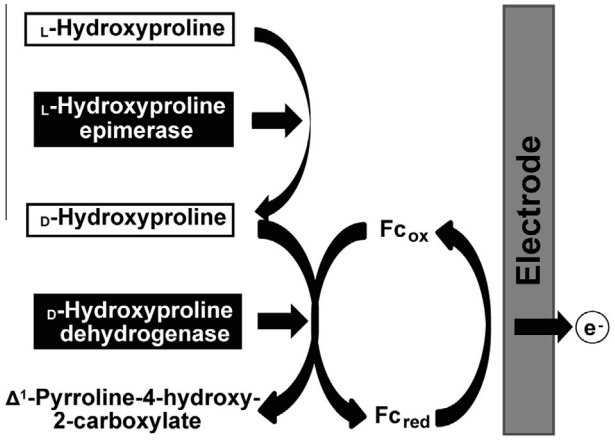

Fig. 1. Schematic diagram of the electrochemical detection of L-hydroxyproline (L-Hyp). Epimerization of L-Hyp to D-hydroxyproline (D-Hyp) was performed by L-hydroxyproline epimerase, and D-Hyp was oxidized on the electrode surface in a reaction catalyzed by D-hydroxyproline dehydrogenase and mediated by ferrocene.

rapidly eliminates its activity. In contrast, the latter has a heterododecameric structure consisting of three different subunits ($\alpha_4\beta_4\gamma_4$), with two FADs, a FMN, and a [2Fe-2S]-iron sulfur cluster contained in $\alpha\beta\gamma$ of the heterotrimeric unit. Additionally, the latter D-hydroxyproline dehydrogenase, in contrast to the former, can be stored for a few months at least, suggesting the possibility that it could have bioindustrial applications. Although an efficient system for preparation of the recombinant enzyme has not been reported, in a recent study we successfully functionally expressed heteromeric D-hydroxyproline dehydrogenase from *Azospirillum brasilense* instead of *P. aeruginosa* in *Escherichia coli* cells [22].

Here, we constructed a novel electrochemical biosensor for L-Hyp using D-hydroxyproline dehydrogenase and another enzyme, L-hydroxyproline epimerase. Electrochemical biosensors offer several advantages; for example, they are readily obtained, easy to use, and involve compact instruments. In this study, we constructed a novel biosensor by using a two-step reaction. First, L-Hyp was epimerized to D-hydroxyproline (D-Hyp) by L-hydroxyproline epimerase; second, D-Hyp was oxidized to Δ1-pyrroline-4-hydroxy-2-carboxylate (Pyr4H2C) by D-hydroxyproline dehydrogenase. Additionally, we obtained the oxidation current of ferrocenecarboxylic acid by D-hydroxyproline dehydrogenase (Fig. 1).

2. Materials and methods

2.1. Reagents

Amino acids (L-Hyp, L-Pro, L-glutamine [L-Gln], glycine [Gly], L-alanine [L-Ala], and L-valine [L-Val]) and ferrocenecarboxylic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of analytical grade. Deionized water, which had been filtered through a Milli-Q water purification system (Millipore Co., Bedford, MA, USA), was used in all experiments.

2.2. Preparation of L-hydroxyproline epimerase and D-hydroxyproline dehydrogenase

L-hydroxyproline epimerase and (heteromeric) D-hydroxyproline dehydrogenase from *A. brasilense* were overexpressed in *E. coli* cells, and then purified homogeneously with a nickel-chelating affinity column, as previously described [22]. Using a spectrophotometric assay, the specific activities of the purified L-hydroxyproline epimerase and D-hydroxyproline dehydrogenase for L-Hyp and D-Hyp were 58.6 and 6.12 units/mg protein, respectively.

2.3. Electrochemical measurement

Cyclic voltammetry (CV) and chronoamperometry experiments were performed using a Model 800B Electrochemical Analyzer (BAS Inc., Tokyo, Japan) and a screen-printed electrode (SPE) (SP-P DEP Chip; Bio Device Technology, Ishikawa, Japan). The SPE consisted of carbon electrodes as the working and counter electrodes and an Ag/AgCl electrode as the reference electrode. All potentials were presented in terms of Ag/AgCl electrode potentials. CV was conducted with a potential range from −0.1 to +0.5 V and with a scan rate of 10 mV s^{-1}. The electrolyte solution was Tris–HCl (15 μL, 50 mM, pH 8.0), and the final concentrations of L-hydroxyproline epimerase, D-hydroxyproline dehydrogenase, and L-Pro were 2.5, 2.5, and 250 μg/mL, respectively.

The electrochemical measurement of L-Hyp was carried out using chronoamperometry with the SPE. Tris–HCl (15 μL, 50 mM, pH 8.0) was dropped onto the SPE, and a constant potential of 250 mV was applied to the working electrode, because ferrocene are oxidized selectively at this potential. When the current had stabilized, 1 μL of each enzyme solution, 0.2 mg/ml L-hydroxyproline epimerase and 0.2 mg/ml D-hydroxyproline dehydrogenase were added. Current responses were defined as the difference between before the addition of a drop of L-Pro, and again 100 s after the addition of the droplets.

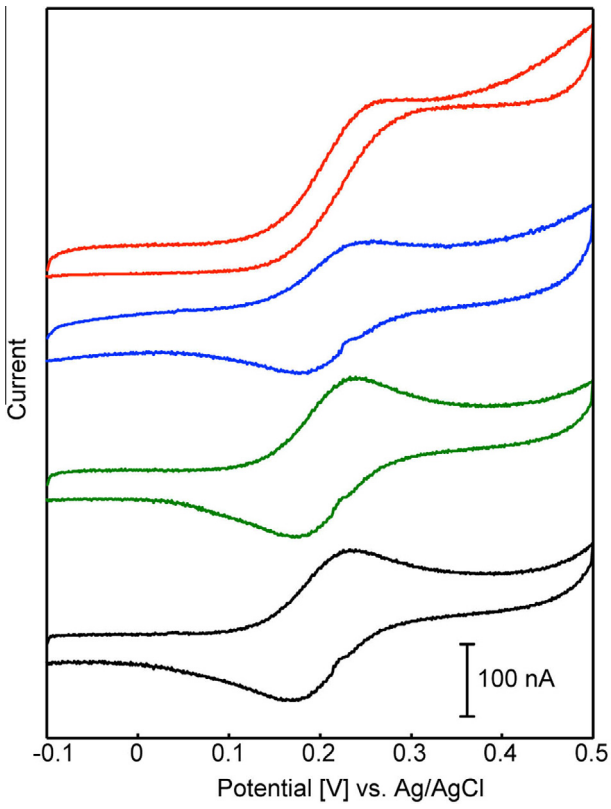

Fig. 2A. Cyclic voltammetry (CV) measurements for each condition. The black lines correspond to ferrocenecarboxylic acid; the green lines correspond to ferrocenecarboxylic acid and L-hydroxyproline (L-Hyp); the blue lines correspond to ferrocenecarboxylic acid, L-hydroxyproline epimerase, and D-hydroxyproline dehydrogenase; and the red lines correspond to ferrocenecarboxylic acid, L-hydroxyproline epimerase, D-hydroxyproline dehydrogenase, and L-Hyp. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

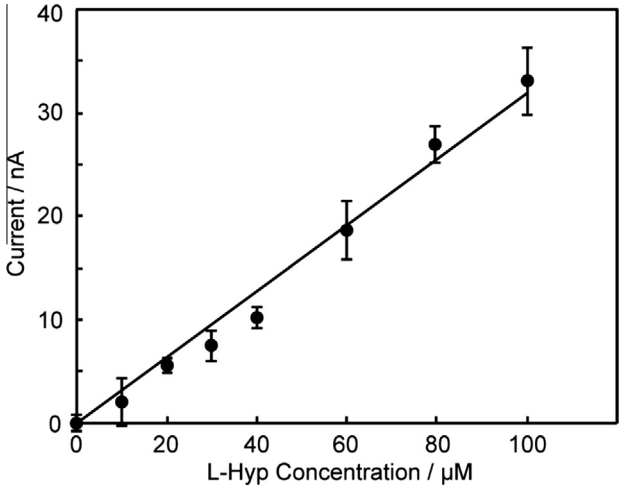

Fig. 2B. Quantification of L-hydroxyproline (L-Hyp) based on current response. The error bars show the standard deviations of triplicate experiments (i.e., $n = 3$).

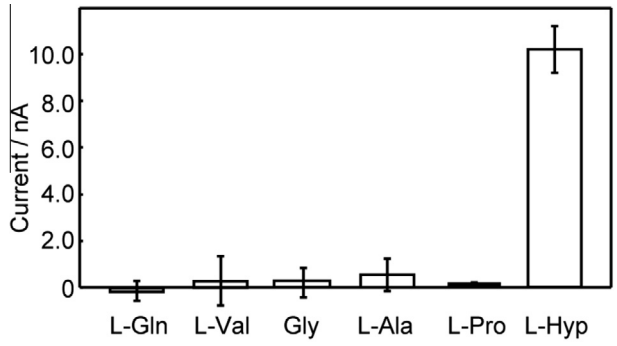

Fig. 2C. Comparison of the current response generated by L-hydroxyproline (L-Hyp) and other amino acids. Additional amino acids were as follows: L-Gln, L-glutamine; L-Val, L-valine; Gly, glycine; L-Ala, L-alanine; L-Pro, L-proline.

3. Results and discussion

We investigated the electrochemical response from the two-step enzymatic reaction using CV (Fig. 2A). We observed a typical pair of redox peaks derived from the redox couple of Fe (III)/Fe (II) ions in ferrocenecarboxylic acid at 0.2 V (Fig. 2A) For L-Hyp, the redox pair of ferrocenecarboxylic acid did not change because L-Hyp is a non-redox molecule (Fig. 2A). For L-hydroxyproline epimerase and D-hydroxyproline dehydrogenase, the redox pair of ferrocenecarboxylic acid also did not change because of the lack of substrate (Fig. 2A). However, for L-hydroxyproline epimerase, D-hydroxyproline dehydrogenase, and L-Hyp, the observed peak indicated that the oxidation current increased and appeared at 0.25 V, while the reduction current decreased (Fig. 2A). These results provided a strong indication that L-Hyp was epimerized by L-hydroxyproline epimerase and dehydrogenized by D-hydroxyproline dehydrogenase, and that the electrons generated from the catalytic reaction between D-hydroxyproline dehydrogenase and D-Hyp were detected through ferrocenecarboxylic acid.

The quantitative capabilities of our system were evaluated by plotting the current response against the amount of L-Hyp in the buffer solution (Fig. 2B). The current intensity value increased in a static dose-dependent manner as the amount of L-Hyp increased. We found that our L-Hyp biosensing system was capable of detecting 10 μM L-Hyp electrochemically. The resultant calibration curve showed linearity, with $R^2 = 0.984$. The detection limit and quantification limit were 2.4 μM and 8.4 μM, respectively. Signaling was linearly correlated to the amount of L-Hyp tested in the range 10–100 μM in a buffer solution.

Serum and urine contain other amino acids at levels of 1–100 μM [23]. In order to evaluate the selectivity of our sensor system, we investigated the current response of L-Gln, Gly, L-Ala, L-Val, and L-Hyp (Fig. 2C), which are all abundant in serum and urine. Results showed that 50 μM of any interferent provided a much weaker response than that of 40 μM of L-Hyp. Therefore, our sensing system distinguished L-Hyp from other amino acids with high-selectivity.

4. Conclusion

Here, we designed and implemented a biosensing system that could detect 10 M L-Hyp in buffer solution, with linear detection for concentrations in the range 10–100 μM. We confirmed that the system distinguishes L-Hyp from other amino acids, including L-Pro. To our knowledge, this is the first reported enzymatic electrochemical biosensor for L-Hyp. The concentration of collagen in urine is in the μM to mM range, and we suggest that our novel biosensing system would be useful for detecting collagen via L-Hyp. Therefore, it could be used in the diagnosis of various diseases, specifically for obtaining information regarding collagen degradation.

Conflict of interest

The authors have declared no conflicts of interest.

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