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Bioelectrochemistry 72 (2008) 47–52
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

3,3′–diaminobenzidine (DAB)–H_2O_2–HRP voltammetric enzyme-linked immunoassay for the detection of carcionembryonic antigen

Shusheng Zhang *, Jun Yang, Jiehua Lin

Key Laboratory of Eco-Biochemical Engineering (Education Ministry of China), College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, PR China

Received 16 April 2007; received in revised form 19 September 2007; accepted 11 November 2007
Available online 3 December 2007

Abstract

A new voltammetric enzyme-linked immunoassay system of 3,3′–diaminobenzidine (DAB)–H_2O_2–horseradish peroxidase (HRP) has been presented and used for the sensitive detection of carcinoembryonic antigen (CEA) in human serum. In this proposed procedure, DAB was firstly used as the electroactive substrate in the HRP catalyzed oxidation reaction in the present of H_2O_2. The generated product produced a sensitive second-order derivative linear sweep voltammetric peak at potential of −0.62 V (*vs*. SCE) in Britton-Robinson (BR) buffer solution. The free HRP could be measured in a linear range from $2.5\times10^{-6}-2.5\times10^{-2}$ unit/ml and a detection limit of about 1.5×10^{-6} unit/ml. Under the optimal experiment conditions, CEA could be detected in the linear range from 0.50 to 80 ng/ml with a detection limit of 0.5 ng/ml. The proposed electrochemical enzyme-linked immunosorbent assay method is simple, inexpensive, reproducible and sensitive, which shows promising for detecting CEA in the clinical diagnosis.

Keywords: Carcionembryonic antigen; 3,3′–diaminobenzidine; Electrochemical immunoassay; Horseradish peroxidase; Voltammetric enzyme-linked immunoassay

1. Introduction

Carcinoembryonic antigen (CEA) is one of the most widely used tumor markers. It was first described in 1965 by Gold and Freedman [1] as a tumor-specific antigen in the diagnosis of colonic cancer. Up to now, it has been found that the serum CEA level is related to colon cancer [1–4], lung cancer [5,6], ovarian carcinoma [7], breast cancer [8–10] etc. Also, the determination of CEA is very helpful for evaluating curative effect, judging recrudescence or metastasis [11–15]. Thus, the detection of serum CEA level plays an important role in the initial diagnostic evaluation and the follow-up examination during therapy.

The traditional detection of serum CEA is usually performed by immunological methods, based on the specificity and selectivity of antibody–antigen reaction [16,17]. Owing to the disadvantages of radioimmunoassay, strong efforts have been undertaken to replace the radioactive substances. Enzyme-linked immunosorbent assay (ELISA) is a conventional assay for clinic examination, but its detection limit is relatively poor by spectrophotometry. Recently, electrochemical technique emerges as the very attractive alternative in ELISA due to the prominent characteristics such as the fast detection rate under the enzyme catalysis and the low detection limit [18–24].

Also, the voltammetric enzyme immunoassay has been successfully proposed and used in the detection of tumor markers [25–28]. The obtained linear sweep second-order derivative polarography possesses the advantages such as high sensitivity, low detection limit, short experimental time, fast electrochemical procedure and simple manipulation. Compared with the expensive microtiter plate reader used in the traditional spectrophotometric detection, the microtiter plates used in the voltammetric enzyme immunoassay were low cost, sensitive and free from color and turbid interferences [25–28]. The enzymes used widely as labels in enzyme immunoassay include horseradish peroxidase (HRP) and alkaline phosphatase [29]. HRP or labelled HRP could catalyze the substrate oxidation by

Abbreviations: BR, Britton-Robinson; CEA, Carcinoembryonic antigen; DAB, 3,3′-diaminobenzidine; ELISA, Enzyme-linked immunosorbent assay; HRP, Horseradish peroxidase; PBS, Phosphate buffer solution; TMB, 3,3′,5,5′-Tetramethyl benzidine.

* Corresponding author. Tel.: +86 532 84022750; fax: +86 532 84023927.
E-mail address: shushzhang@126.com (S. Zhang).

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doi:10.1016/j.bioelechem.2007.11.011

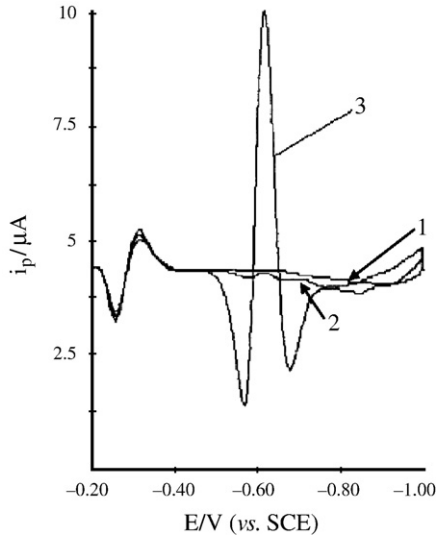


Fig. 1. Second-order derivative linear-sweep voltammogram of BR+H_2O_2 (1), BR+DAB+H_2O_2 (2), BR+DAB+H_2O_2+HRP (3).

H_2O_2, and the obtained product was reduced in the dropping mercury electrode to produce a sensitive voltammetric peak. A series of substrates for HRP-mediated electrochemical assay have been reported such as *o*-dianisidine [25], *m*-aminophenol [26], *o*-phenylenediamine [27] and 3,3′,5,5′-tetramethyl benzidine (TMB) [28].

In current study, a new voltammetric enzyme immunoassay system was proposed for the detection of CEA in human serum. 3,3′-diaminobenzidine (DAB) was firstly used as the substrate in HRP catalysis reaction. The electroactive product 4,4′-diimino-bicyclohexylidene-2,5,2′,5′-tetraene-3,3′-diamine, obtained from the oxidation reaction of DAB in HRP-H_2O_2 system in pH 5.0 BR buffer solution, could be measured by voltammetric method. In comparison with the traditional ELISA method, this proposed method improved the sensitivity of CEA about ten times. And also the method was practical, conventional and reliable, implying a promising alternative approach for detecting serum CEA in the clinical diagnosis.

2. Materials and methods

2.1. Instrumentation

The electrochemical measurement was carried out with a MP-2 voltammetric analyzer (Shandong No. 7 Electric Communication Corp., China). A three-electrode system was employed with a dropping mercury electrode or a hanging mercury drop electrode as working electrode, a platinum electrode as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. The instrumental conditions were as follows: initial potential, −0.20 V; mercury drop standing time, 7 s; potential scanning rate, 300 mV/s. OG3022A enzyme-linked immunity measure implement (Huadong Electronal Group Medical Treatment Instrument Ltd., China) was used for the spectrophotometric ELISA.

2.2. Chemicals

DAB working solution was prepared by dissolving 0.0108 g DAB (Acros Organics, 99%) in doubly distilled water and diluted to 50.0 ml (1.0 mM). The solution of HRP (250 units per mg enzyme, Xueman Biochemical Technique Corp., China) was prepared by dissolving 10.00 mg HRP in 10.0 ml doubly distilled water (250 unit/ml), and then was stored at 4 °C. The CEA ELISA kit was purchased from Shanghai Jiemen Biotechnique Company and stored at 2–8 °C. The kit included microplate coated by anti-CEA serum, HRP labeled anti-CEA, standard sample of CEA, TMB substrate solution, rinsing solution (PBS) and stop liquid (H_2SO_4). Other chemicals were of analytical grade and were prepared by the doubly distilled water.

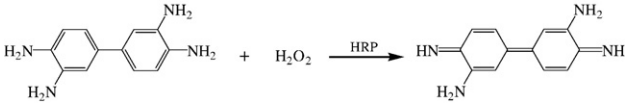
3. Results and discussion

3.1. The second-order derivative linear-sweep voltammograms

Differential pulse voltammetry, modern square wave voltammetry and linear sweep second-order derivative polarography all have excellent voltammetric peaks or polarographic waves in the detection of the product formed by H_2O_2 oxidizing DAB, which is catalyzed by HRP in 0.2 M BR at pH 3.5. Among these methods, linear sweep second-order derivative polarography was the most optimal method with the advantages such as high sensitivity, low detection limit, short experimental time and simple manipulation. The product of the enzyme-catalyzed reaction has a well-defined voltammetric peak. Fig. 1 shows the results of the second-order derivative linear-sweep voltammograms. Curve 1 is the voltammogram of BR buffer solution, which has no voltammetric peak. Curve 2 is that of the BR–DAB–H_2O_2, which has a small voltammetric peak at −0.62 V. The small peak is due to the product of slow oxidation of DAB by H_2O_2. Curve 3 is that of the enzyme-catalyzed reaction solution. Owing to the addition of HRP, which quickens greatly the oxidation of DAB by H_2O_2, the reaction product produces a large and well-defined voltammetric peak at −0.62 V. Although the HRP content is as low as 1.5×10^{-6} unit/ml, a distinctive increase of this voltammetric peak still can be observed. The oxidation of DAB by H_2O_2 yields a stable product, if the enzyme catalyzed reaction happens in 0.2 M BR at pH 5.0.

3.2. Optimal conditions for enzyme-catalyzed reaction

As seen from the voltammograms, HRP intensely catalyzed the oxidation reaction of DAB by H_2O_2. Considering the structure of the product and catalysis cycle of HRP in reaction, the process of HRP-catalyzed oxidation reaction of DAB by H_2O_2 is concluded in Scheme 1. The enzymatic oxidation of DAB yielded a stable



Scheme 1. The process of the HRP-catalyzed oxidation reaction of DAB by H_2O_2.

product, 4,4′–diimino–bicyclohexylidene–2,5,2′,5′–tetraene–3,3′–diamine in BR buffer solution at pH 3.5.

Through measuring the electrochemical response of the enzymatic product, the effect of pH on enzyme-catalyzed reaction was studied between 2.0 to 12.0. The electrochemical peak current reached the maximum value at pH 3.5 BR buffer solution. Additionally, the concentrations of each component of the substrate solution, including BR buffer solution, DAB and H_2O_2, were also optimized. When the final 10 ml substrate solution consisted of 2.0 ml of BR buffer solution (0.2 M, pH 3.5), 2.0 ml DAB solution (1.0 mM) and 1.0 ml H_2O_2 solution (1.0 mM), the electrochemical peak current reached the maximum value. Under the above optimal conditions, the electrochemical peak current of the product changed slightly after 40 min at 37 °C, indicating the reaction reached the equilibrium. Thus, 40 min was selected as the optimal incubation time for the enzyme-catalyzed reaction.

3.3. Optimal electrochemical conditions for the detection

The fine second-order derivative linear-sweep voltammetric peak for the enzyme-catalyzed product was obtained in BR buffer solution. After the enzyme-catalyzed reaction, the effect of pH value on the second-order derivative linear-sweep voltammetric peak of enzymatic product was investigated. Results showed that the peak potential shifted negatively with the increased pH. The peak current reached the maximum value at pH 5.0. Thus pH 5.0 was selected as the optimal pH value of BR for the electrochemical detection.

3.4. The electrode procedure of the enzymatic product

For the enzymatic solution, the linear sweep voltammetric peak was linearly increased with the scanning rate. This means that the product of the enzymatic reaction could be adsorbed on the mercury electrode. Under the optimal conditions, the single sweep cyclic voltammogram (shown in Fig. 2) and the multiple

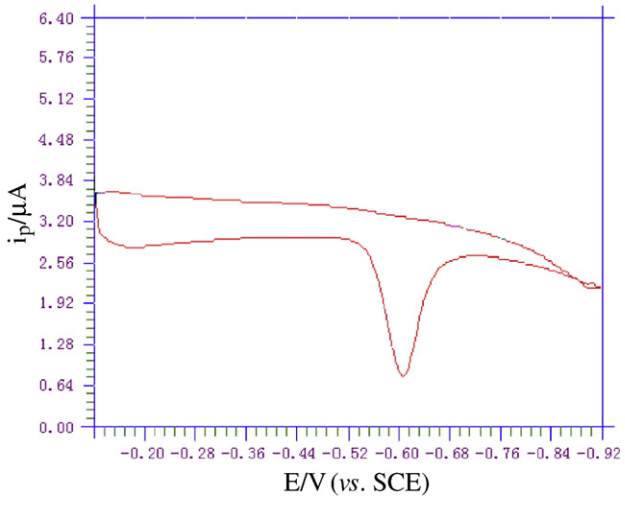


Fig. 2. Cyclic voltammograms of enzymatic product in BR buffer solution at pH 5.0. The enzymatic product was generated by oxidation reaction of DAB in BR buffer solution at pH 3.5. T=1 s, υ=300 mV/s.

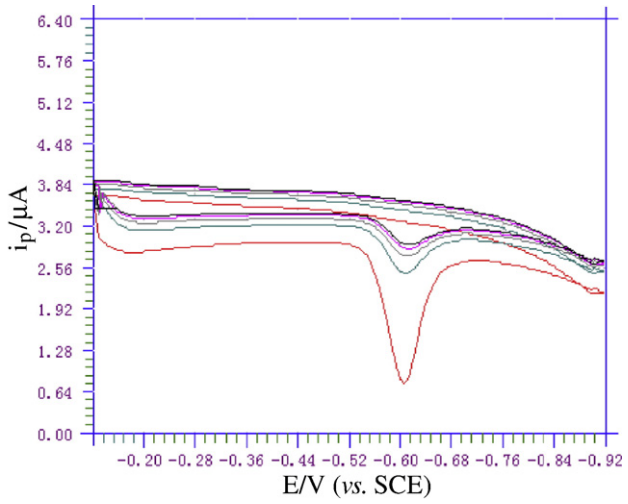


Fig. 3. Multiple-sweep cyclic voltammograms of the BR+DAB+H_2O_2+HRP. T=1 s, υ=300 mV/s.

sweep cyclic voltammogram (shown in Fig. 3) were recorded. The peak current on the multiple sweep cyclic voltammogram decreased with the increasing scanning times. This result indicated that both the electrode reactant and the product could be strongly adsorbed on the mercury electrode, and the reaction rate on the mercury electrode was improved. This is the characteristic of an adsorptive in-reversible wave.

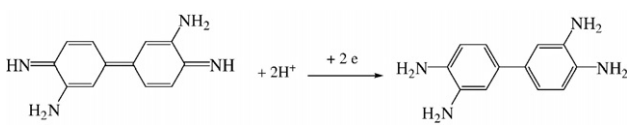
The electrode process was shown in Scheme 2. Based on the theory advanced by Nicholson [30], where $W_{1/2}$ is the width of the peak at half height. The value of

$$W_{1/2} = 3.53RT/nF = 90.6/n(\mathrm{mV}, 25°\mathrm{C})$$

$W_{1/2}$ in this experimental is about 48 mV. So this is a two-electron in-reversible electrode process. The oxidation product of DAB by H_2O_2, 4,4′–diimino–bicyclohexylidene–2,5,2′,5′–tetraene–3,3′–diamine, was produced in BR at pH 3.5. In pH 5.0 BR, such enzyme-oxidized product could be reduced through a two-electron transfer process. Based on such electro-reduction peak, free HRP and labelled HRP can be determined.

3.5. Electrochemical determination of free HRP

Different concentrations of free HRP were used to catalyze the oxidation reaction of DAB by H_2O_2. 2.0 ml of 1.0 mM DAB, 1.0 ml of 1.0 mM H_2O_2 and 2.0 ml of 0.2 M BR at pH 3.5 was mixed with 1.0 ml of HRP solution with different concentrations. The mixture was diluted to 10.0 ml and kept at 37 °C for 40 min in a water bath. Then, 3.0 ml of the above solution and 7.0 ml of 0.2 M BR at pH 5.0 were mixed in a 10.0 ml colorimetric tube, and then was detected by the MP-2



Scheme 2. A two-electron redox process of HRP-catalyzed oxidation reaction product of DAB in BR buffer solution.

voltammetric analyzer in a 10 ml electrolyte cell. The second-order derivative linear-sweep voltammogram was recorded. The peak current in BR exhibits a good linear relation with HRP concentration in the range of $2.5\times10^{-6}-2.5\times10^{-2}$ unit/ml and a detection limit of about 1.5×10^{-6} unit/ml. The relative standard derivative (RSD, $n=11$) of HRP response to 1.25×10^{-5} unit/ml HRP is 2.1%.

3.6. Determination of labelled HRP

Similar with free HRP, labelled HRP could also be determined. We compared the sensitivity for determining free HRP and labelled HRP using five HRP-conjugated antibodies including α-Fetus Proteid (αFPAb–HRP, 1), Ferritin–Ab–HRP (2), Carcinoma Embryo (CEAAb–HRP, 3), PSA (PSAAb–HRP, 4), Full-mouthed Disease (FMDAb–HRP, 5). The dilution curves shown in Fig. 4 demonstrated the proposed sensitive method for the detection of labeled HRP. In comparison with the TMB spectrophotometric ELISA, the newly developed method showed higher sensitivity. The highest dilution ratios for the two methods were shown in Table 1. The highest dilution ratio of electrochemical method was 1:10,000, whereas, that of the TMB spectrophotometric ELISA method was 1:1000. Thus, the proposed electrochemical enzyme-linked immunoassay system could detect 10 times lower concentration of labelled HRP than that of the TMB spectrophotometric ELISA.

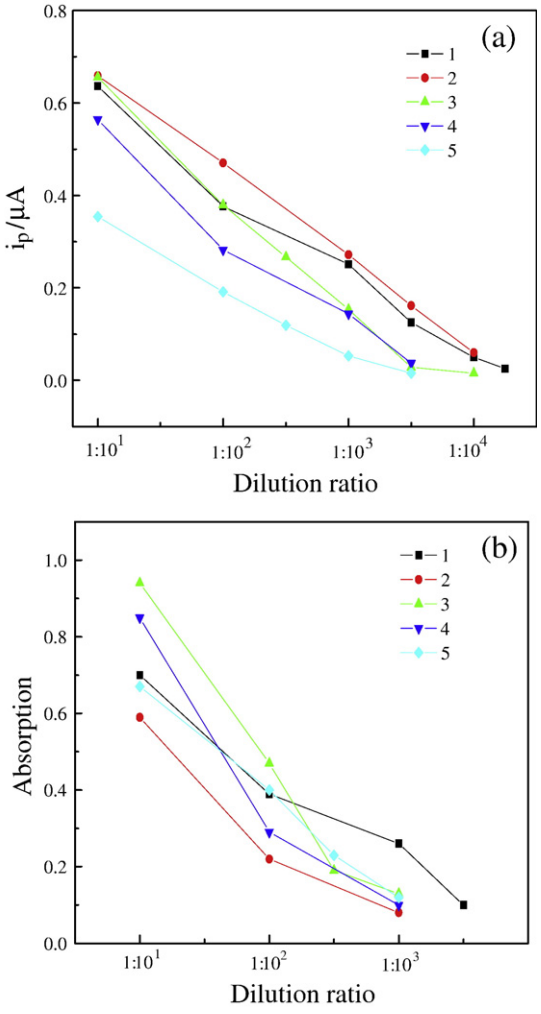


Fig. 4. Dilution curves of detecting the labelled HRP of αFPAb–HRP (1), FerritinAb–HRP (2), CEAAb–HRP (3), PSAAb–HRP (4), and FMDAb–HRP (5) by electrochemical ELISA (a) and spectrophotometric ELISA (b).

Table 1
The comparisons of electrochemical ELISA (i_p) with spectrophotometric ELISA (A_{450}) for the detection of labelled HRP using HRP labeled anti-CEA

Dilution ratio	1:10	1:10^2	1:10^3	1:10^4	1:10^5
i_p (μA)	0.656	0.380	0.154	0.016	0
A_{450}	0.94	0.47	0.10	0.00	0.00

3.7. The linear range, the detection limit and the precision of CEA detection

The detection of CEA was based on sandwich immunoassay format. The immunoassay conditions were controlled according to the procedure recommended by the commercial CEA Kit. 50 μl of CEA solutions with different concentrations were added to the wells coated by CEA monoclonal antibody. The mixture was incubated for 20 min at 37 °C, and then rinsed with PBS (300 μl) and doubly distilled water respectively. After that, 100 μl HRP labeled anti-CEA were added to the wells and incubated for 15 min. After the wells were rinsed, 300 μl of the substrate solutions were added to each well and incubated for 40 min, and then transferred to a 5 ml electrochemical cell for the electrochemical detection. The second-order derivative linear-sweep voltammogram was recorded in the presence of

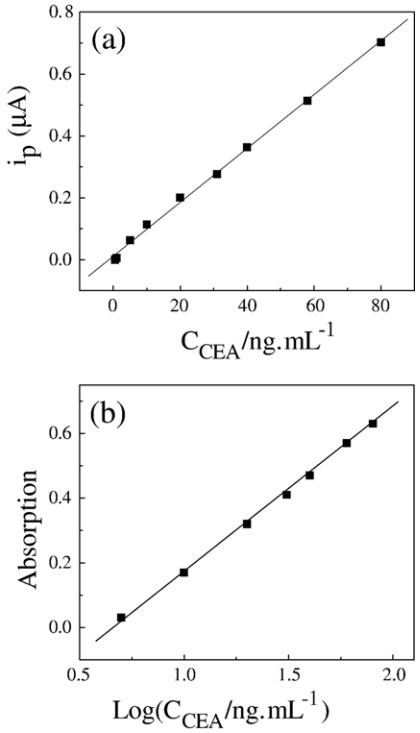


Fig. 5. Calibration plot for detection of CEA by electrochemical ELISA and spectrophotometric ELISA.

Table 2
The comparison of results of this method with the TMB spectrophotometric ELISA method for the detection of CEA in human serum

Sample	This method (ng/ml)	TMB spectrophotometric ELISA method (ng/ml)
1	2.86	–
2	7.99	7.50
3	9.02	8.65
4	15.7	15.9
5	24.0	26.6
6	3.56	–
7	32.0	31.2
8	4.25	–
9	63.9	62.3
10	74.9	75.4

700 μl BR buffer (pH 5.0). For comparison, spectrophotometric detection of ELISA was also preformed in parallel using OG3022A enzyme-linked immunity measure implement.

Under the optimized conditions, the proposed new DAB–H_2O_2–HRP system could detect CEA in a linear range from 0.5 to 80 ng/ml with a detection limit of 0.5 ng/ml. The calibration plot was shown in Fig. 5a. The regression is $y=0.01161+0.0087x$ ($\gamma=0.9989$), where y means Δi_p", i_p" is the peak current (μA), x is the concentration of CEA. For TMB spectrophotometric ELISA method, the linear range of CEA is 5.0–80 ng/ml with a detection limit of 5.0 ng/ml as shown in Fig. 5B. The equation of linear regression is $A=-0.327+0.50\mathrm{Log}C$ ($\gamma=0.9995$), where A is the absorbency, C is the concentration of CEA. Therefore, the detection limit of our electrochemical enzyme-linked immunoassay method was 10 times lower than that of the TMB spectrophotometric ELISA method.

3.8. Determination of CEA in human serum samples

The CEA levels in the human serum samples were detected using both electrochemical and spectrophotometric ELISA methods. The comparison results were listed in Table 2. We could find that the two methods showed good agreement. The results of electrochemical method are linear proportional to that of spectrophotometric method. The regression is $y=0.2587+0.9922x$ ($\gamma=0.9988$), where x is the results of electrochemical method, y is results of spectrophotometric method.

The specificity for detecting CEA was investigated using dilutions of CEA, α-fetoprotein (AFP), prostate specific antigen (PSA), ferritin, and full-mouthed disease (FMD). 0.2 ng/ml CEA could be readily detected. However, there were no significant reactions with the AFP, PSA, ferritin and FMD samples in the concentration of 10.0–1.0 $\mu g\cdot ml^{-1}$. In addition, uninfected human serum and human serum infected with AFP, PSA, ferritin and FMD, respectively, gave no detectable reactions.

4. Conclusions

Based on the new system of DAB–H_2O_2–HRP, the developed electrochemical enzyme-linked immunoassay showed promising performance for detection of CEA in human serum. The processes of the enzyme-catalyzed reaction and electro-reduction of the product on the electrode was investigated. The detection limit for CEA is 10 times lower than that of the traditional spectrophotometric ELISA method. The simple, inexpensive, reproducible and sensitive assay showed a promising alternative approach in the clinical diagnosis.

Acknowledgements

The work was supported by the Natural Science Foundation of Shandong Province (Z2006B01), the Program for New Century Excellent Talents in Universities (No. NCET-04-0649) and the Doctoral Fund of QUST (0022141).

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