酵素電極によるD-およびL-アミノ酸計測のための電圧,電流条件

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SOME VOLTAMMETRIC CONDITIONS FOR THE DETERMINATION OF D- AND L-AMINO ACIDS*

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An electrode for the determination of D- and L-amino acids was prepared by using ferrocene carboxylic acid and D- and L-amino acid oxidases. A linear relationship was obtained between the concentration of D- and L-amino acids and the output current at an operating potential of +360 mV *vs*. an Ag/AgCl reference electrode. The output current of the working electrode consisted of the D-amino acid oxidase immobilized into the gel matrix of hydroxyethyl cellulose was related linearly with the concentration of D-amino acid at an operating potential of +22 mV *vs*. an Ag/AgCl reference electrode. These results enable the development of the practical biosenser for the determination of D- and L-amino acids.

Key words: D-Amino acid, D-Amino acid oxidase, L-Amino acid, L-Amino acid oxidase, Enzyme electrode, Ferrocene monocarboxylic acid, Hydroxyethyl cellulose

Introduction

Recently, the role of D-amino acids on the multiplication and division of cell, an intelligence network, and a symptom of age has been studying[1]. D-Amino acids are determined mainly with HPLC[2]. This method has enabled the exact determination of each amino acid although it requires the time consuming (even if when only one amino acid is analyzed, the assay time may be required that for total amino acid assay) and complicated procedures such as the preparation of L- and D-amino acid derivatives. On the other hand, a biosensor does not require at all the pretreatment as mentioned above on account of using the substrate specificity. It is predictable that one assay of total amino acid is completed within 5 to 10 min as reported in the previous paper on the biosensor[3]. It may be important to clear in what materials D-amino acid exists and under what conditions L- and D-amino acids increase or decrease. The objectives of this study are to obtain the basic data for the development of the biosensor for simple and rapid survey of total L- and D-amino acids, for instance, in fish extract. Therefore, in this paper, some voltammetric conditions of the enzyme electrodes for the determination of L- and D-amino acids were investigated. D-Amino acids were detected with a mediated amino acid oxidase electrode. The main objectives were as follows: (1) to select a mediator which exhibits reversible oxidation and reduction, *i.e.*, which produces a clear, discrete anodic and corresponding cathodic peak current during cyclic voltammetry in aqueous buffer solution, (2) to combine the mediator with amino acid oxidases for catalysis of L- and D-amino acids at a platinum working electrode in a single electrode chamber with Ag/AgCl reference electrode and platinum auxiliary electrode, (3) to identify the operating potential for construction of an amino acid-oxidase electrode for amino acid analysis, and (4) to successfully immobilize the enzyme at the surface of the electrode.

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Materials and Methods

Reagents

D-Amino acid oxidase (EC 1.4.3.3, 89 U/m*l*) Type II from porcine kidney and L-amino acid oxidase (EC 1.4.3.2, 56 U/m*l*) Type IV purified from *Crotalus adainanteus* venom were purchased from Sigma Chemical Co. Both enzymes were stored at 4℃ until use. One unit of D-amino acid oxidase will oxidatively deaminate 1.0 μmol of D-alanine to pyruvate per min at pH 8.3 at 25℃, in the presence of catalase. Similarly, one unit of L-amino acid oxidase will oxidatively deaminate 1.0 μmol of L-phenylalanine per min at pH 6.5 at 37℃. The mediators, ferrocene monocarboxylic acid (FMCA) and 1,1-dimethyl ferrocene, were purchased from Aldrich Chemical Co.

All other reagents, potassium chloride, Tris(hydroxymethyl amino methane), maleic acid, sodium borate, D-alanine, L-alanine, D-leucine, L-leucine, D-phenylalanine, L-phenylalanine and D-proline, were supplied by Wako Pure Chemical Industries Ltd. and were of analytical reagent grade.

Cyclic voltammetry of amino acid oxidase and ferrocene monocarboxylic acid

Electrochemical measurements were performed using a BAS CV-50W Analyzer (BAS, Tokyo), interfaced to a Gateway 2000 computer (Gateway Co., U.S.A.) and a color jet printer. Before each voltammetric experiment the working electrode was cleaned with acetone, polished with an alumina-water slurry (particle size 0.5 mm, Wako Pure Chemical Industries Ltd., Tokyo) and then cleaned electrochemically in 1 M H_2SO_4 by cycling between −0.8 V and +1.4 V in accordance with the

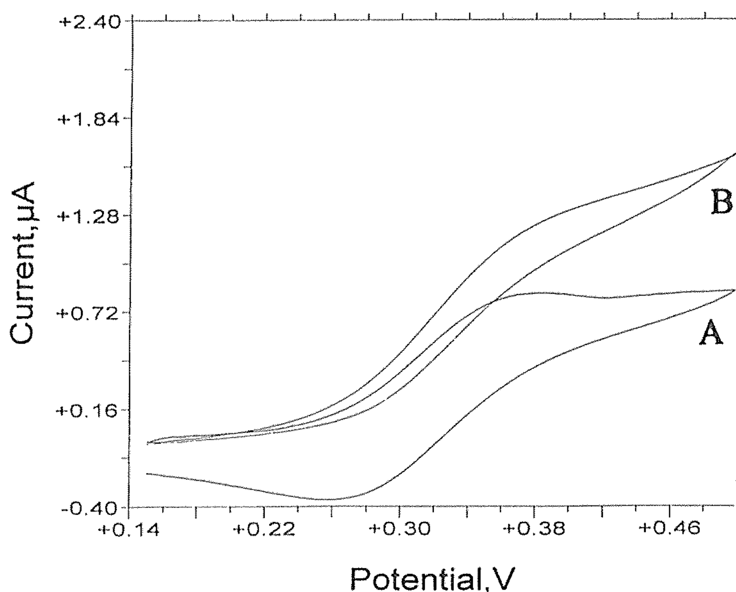

Fig. 1. Cyclic voltammetry of L-amino acid oxidase.
(A) 75 mM ferrocene monocarboxylic acid (FMCA); 125 mM L-alanine, no enzyme. (B) 75 mM FMCA; 125 mM L-alanine (total volume 2 m*l*); addition of 50 μl enzyme (2.80 U).
Assay in 0.1 M Tris–HCI pH 8.3 containing 0.1 M KCI supporting electrolyte; BAS Pt working elctrode (w.e.); BAS Pt counter electrode (c.e.); Ag/AgCl reference electrode (ref.); scan rate 5 mV/s.

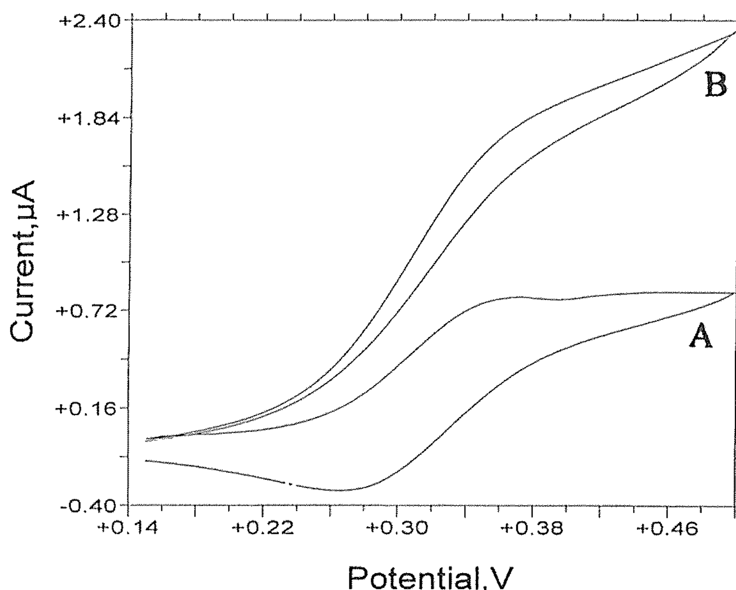

Fig. 2. Cyclic voltammetry of D-amino acid oxidase.
(A) 75 mM FMCA; 125 mM D-alanine, no enzyme, (B) 75 mM FMCA; 125 mM D-alanine (total volume 2 m*l*); addition of 50 μ*l* enzyme (4.45 U).
Assay in 0.1 M Tris–HCl pH 8.3 containing 0.1 M KCl supporting electrolyte; BAS Pt w.e.; BAS Pt c.e.; Ag/AgCl ref.; scan rate 5 mV/s.

method of Sawyer and Roberts[4]. The electrodes were then thoroughly rinsed in distilled water before use. All measurements were carried out at 25℃ in a single compartment electrochemical cell containing a platinum working electrode, a platinum auxiliary electrode and an Ag/AgCl reference electrode all supplied by BAS, Tokyo. The electrode chamber contained 0.1 M Tris–HCl buffer pH 8.3 with 0.1 M KCl supporting electrolyte. Cyclic voltammetry was performed in combination of 75 mM FMCA, L- and D-amino acid oxidases (2.80 and 4.45 U, respectively) and each of the amino acid oxidase substrates (125 mM). Further control experiments involved incubation of enzyme with substrate in the absence of mediator and incubation of substrate with mediators in the absence of enzyme.

Dimethyl ferrocene-modified enzyme sensor based on hydroxyethyl cellulose-glutaraldehyde gel matrix for detection of D-alanine

Twenty μ*l* aliquots of 40 mM dimethyl ferrocene dissolved in acetone were adsorbed on the electrode surface. This procedure was repeated 4 times. The acetone solvent was allowed to evaporate before immobilization of enzyme.

One m*l* of 5% hydroxyethyl cellulose aqueous solution was added to 5 m*l* of solution containing 50 μ*l* ethanol and 5% glutaraldehyde (Tokyo Kasei Chemical Industries). Twenty μ*l* aliquots of D-amino acid oxidase (1.78 U) were then added and the solution was mixed gently in a rotary mixer and incubated at 35℃ for 20 min to form a gel. Small sections of the gel (2×3 mm) were transferred to the electrode surface and incubated at 4℃ for 12 hours.

The enzyme electrode was rinsed in distilled water prior to amperometric detection of D-alanine. Amperometry was performed using a Bio Analytical Systems BAS CV-50 W version 2 Voltammetric

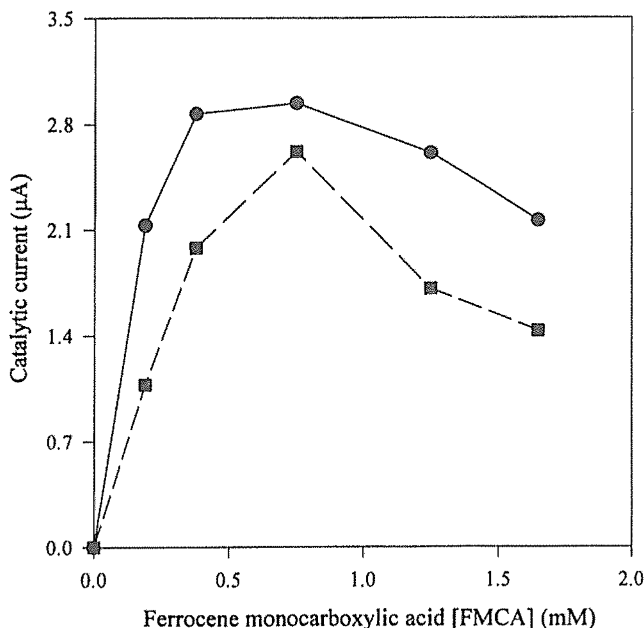

Fig. 3. Optimal mediator concentration for amino acid oxidase catalysis.
●, D-amino acid oxidase; ■, L-amino acid oxidase.
Cyclic voltammetry in 0.1 M Tris–HCl pH 8.3 (total volume 2 m*l*), scan rate 5 mV/s.
BAS Pt w.e.; BAS Pt c.e.; Ag/AgCl ref.; 50 μl enzyme (D- and L-amino acid oxidase; 2.80 and 4.45 U, respectively).

Analyzer interfaced to a Gateway 2000 computer and a color jet printer. The enzyme electrode was immersed in 4.0 m*l* of 0.1 M Tris–HCl buffer, pH 8.3, containing 0.1 M KCl supporting electrolyte thermostated at 25℃ and poised at +225 mV *vs.* an Ag/AgCl reference electrode for amperometric detection of D-alanine in the presence of a platinum auxiliary electrode.

Results and Discussion

Cyclic voltammetry of L- and D-amino acid oxidases

Figure 1 shows the cyclic voltammogram obtained when L-amino acid oxidase was added to a saturated solution of the mediator FMCA in the presence of excess substrate (125 mM L-alanine). A catalytic current of 0.56 mA was recorded at a potential of 360 mV *vs.* an Ag/AgCl reference electrode on addition of 50 μl L-amino acid oxidase to 2 m*l* of 75 mM FMCA.

Similarly, the cyclic voltammogram of D-amino acid oxidase on addition to 75 mM FMCA in the presence of excess D-alanine (125 mM) is presented in Fig. 2. The observed catalytic current for D-amino acid oxidase, 1.12 mA, was two times greater than that observed for L-amino acid oxidase in Fig. 1. This difference can be attributed to the greater activity of D-amino acid oxidase (4.45 U/50 μl) compared with that of L-amino acid oxidase (2.80 U/50 μl).

Effect of mediator concentration

Fifty μl aliquots of L-amino acid oxidase were added to 125 mM L-alanine in the presence of

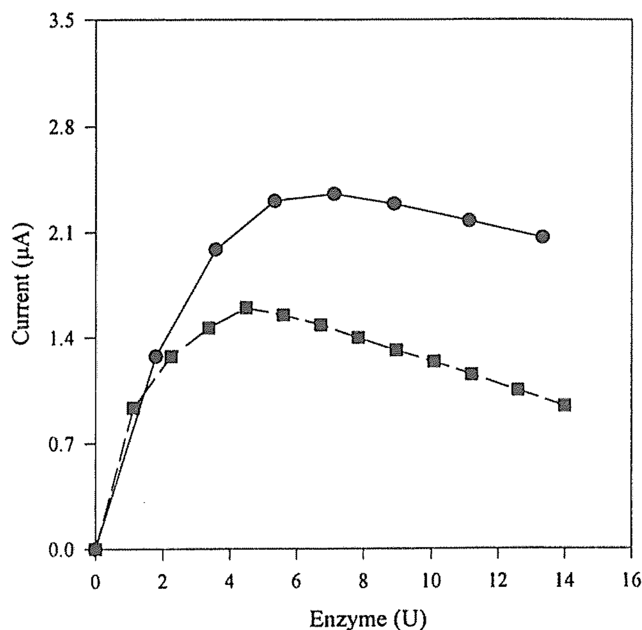

Fig. 4. Optimal enzyme concentration for amino acid oxidase catalysis.
●, D-amino acid oxidase; ■, L-amino acid oxidase.
Cyclic voltammetry in 0.1 M Tris–HCl pH 8.3 (total volume 2 ml), scan rate 5 mV/s.
BAS Pt w.e.; BAS Pt c.e.; Ag/AgCl ref.; substrate concentration; 125 mM.

increasing concentrations of FMCA. Cyclic voltammetry was performed at 5 mV/s as shown in Fig. 1 and the FMCA-amino acid oxidase response was optimized in the presence of 2.5 mM, 1.25 mM, 0.75 mM, 0.375 mM and 0.175 mM FMCA mediator.

The observed catalytic current for each amino acid oxidase at different mediator concentrations was plotted in Fig. 3. The optimal mediator concentration for both D- and L-amino acid oxidases was 0.75 mM FMCA. The decrease in catalytic current at FMCA concentrations of greater than 0.75 mM is assumed to arise from mediator poisoning of the enzymes, *i.e.*, inhibition of enzyme catalysis at high FMCA concentrations.

The control experiments shown in Fig. 3 establish that no oxidation or reduction peaks were observed in the absence of FMCA.

Effect of enzyme concentration

The observed catalytic current for each amino acid oxidase at different enzyme concentrations was plotted in Fig. 4.

The optimal enzyme concentration for amino acid catalysis was 5.34 U for D-amino acid oxidase and 4.48 U for L-amino acid oxidase. The control experiments in D-amino acid oxidase confirmed that its substrate was not oxidized at the electrode surface in the absence of mediator and the control experiments in L-amino acid oxidase established that the amino acid substrate could not be catalyzed by FMCA mediator in the absence of enzyme.

Effect of pH

The catalytic current observed for each amino acid oxidase at different pH was plotted in Fig. 5.

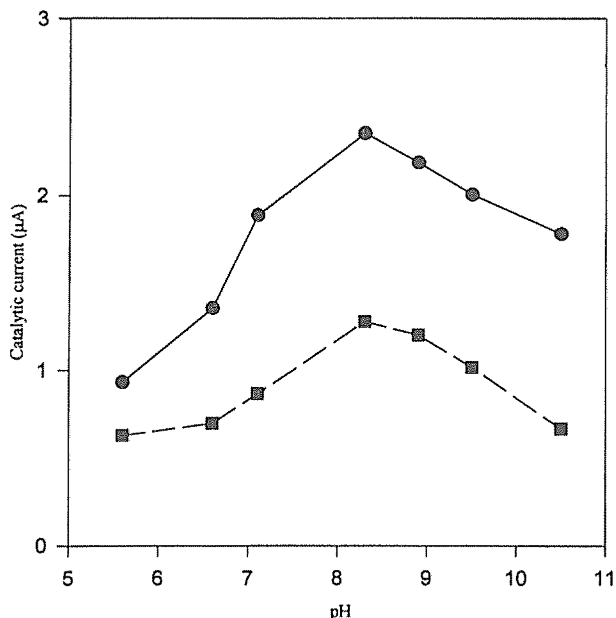

Fig. 5. Optimal pH for amino acid catalysis.
●, D-amino acid oxidase; ■, L-amino acid oxidase.
Cyclic voltammetry with 0.75 mM FMCA (total volume 2 m*l*); 50 μ*l* enzyme (D- and L-aminoacid oxidase; 2.80 and 4.45 U, respectively).
BAS Pt w.e.; BAS Pt. c.e.; Ag/AgCl ref.; substrate concentration; 125 mM; scan rate 5 mV/s.

The optimal pH for catalytic coupling of L- and D-amino acid oxidases with the mediator FMCA was 8.3 which is in good agreement with the optimal pH for the enzyme activities[5]. The responses were observed using 0.2 M Tris–maleate buffer (pH 5.0–7.0), 0.1 M Tris–HCl buffer (pH 7.1–8.9), and 0.1 M borate buffer (pH 9.5–10.5).

Effect of substrate

Cyclic voltammetry of 50 μ*l* L-amino acid oxidase (2.8 U) in the presence of 0.75 mM FMCA was recorded in the presence of increasing concentrations of L-alanine, L-leucine, and L-phenylalanine substrates. Similarly cyclic voltammetry of 50 μ*l* D-amino acid oxidase (4.45 U) and 0.75 mM FMCA was performed in the presence of increasing concentrations of D-alanine, D-leucine, D-phenylalanine and D-proline.

The catalytic response to increasing concentrations of L-amino acid is shown in Fig. 6. The current response to L-alanine increased in the range between 3 and 22 mM before reaching saturation at L-alanine concentrations of greater than 30 mM. The maximum catalytic current of 0.84 μA was observed at 26 mM L-alanine. A similar increase in catalytic current was recorded between 0 and 30 mM L-leucine with a maximum value of 0.93 μA at 26 mM. A smaller catalytic response was observed for increasing concentrations of L-phenylalanine (maximum value 0.49 μA at 20 mM) which showed a significant decrease at phenylalanine concentrations of greater than 30 mM.

Similarly the catalytic response of D-amino acid oxidase in the presence of D-alanine was also optimized with respect to increasing concentrations of D-alanine. The catalytic response of D-amino acid oxidase for different D-amino acids in the presence of FMCA is shown in Fig. 7. The greatest

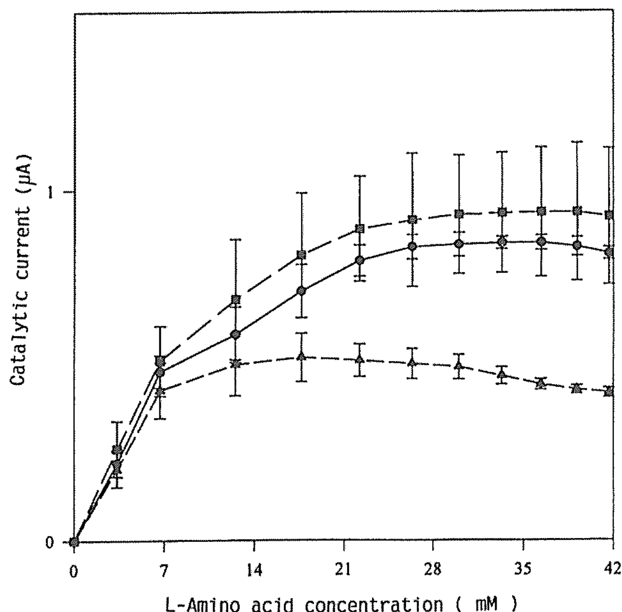

Fig. 6. Cyclic voltammetry with L-amino acid oxidase.
●, L-alanine; ■, L-leucine; ▲, L-phenylalanine.
Cyclic voltammetry in 0.1 M Tris–HCl pH 8.3 (total volume 2 m*l*), scan rate 5 mV/s.
50 μl L-amino acid oxidase (2.8 Units); 0.75 mM FMCA. Catalytic current recorded at 360 mV *vs.* Ag/AgCl ref.; BAS Pt w.e.; BAS Pt c.e.

catalytic current was observed for D-leucine. Although the reason for this is not clear, it should be emphasized that control experiments, in the absence of enzyme, established that amino acids were neither oxidized by the mediator nor by the electrode surface in the absence of the mediator. The differences in the response for each amino acid have to be solved when these electrodes are used practically.

Amperometry of amino acid oxidase

A hydroxyethyl cellulose glutaraldehyde gel matrix was used for immobilization of D-amino acid oxidase. The gel formed a self adhesive layer on the surface of a plane gold electrode. The sensor was capable of determining D-alanine at +225 mV *vs.* an Ag/AgCl reference electrode at pH 8.3 (optimal for enzyme activity). The results presented in Fig. 8 show that the relationship between D-alanine concentration and current output was linear between 1.25 mM and 16 mM ($n=5$). The linear response of this amperometric biosensor correlates well with the total alanine levels of fish and shellfish shown in Standard tables of Japanese food composition[6]. The limit of detection, with respect to a signal-to-noise ratio of 1 : 3, in accordance with the standard definition of clinical chemical analysis[7] was 100 μM D-alanine. The "within batch" precision of the electrode was determined for ten consecutive measurements at 8.75 mM D-alanine (mid-point linear range). The mean current output was $14.53 \pm 0.65\,\mu$A, with a coefficient of variation of 4.5%; $n=10$. These results show that this electrode could be applied for D- and L-amino acids analysis of fish in future.

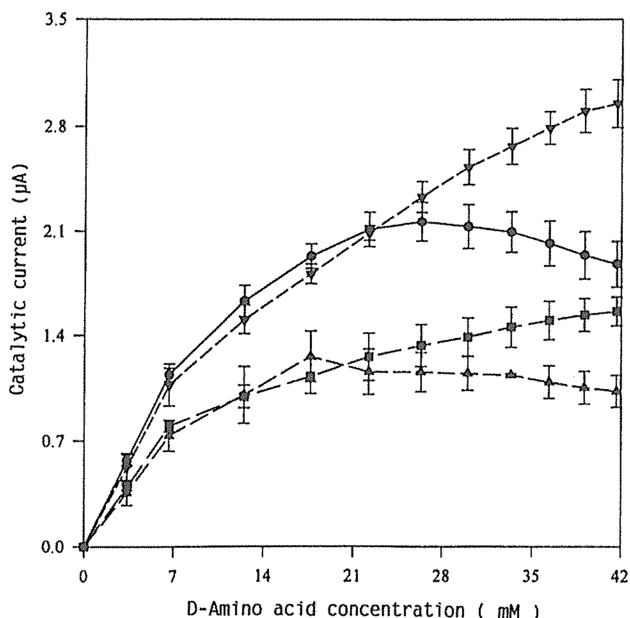

Fig. 7. Cyclic voltammetry with D-amino acid oxidase.
●, D-alanine; ▼, D-leucine; ■, D-phenylalanine; ▲, D-proline.
Cyclic voltammetry in 0.1 M Tris-HCI pH 8.3 (total volume 2 m*l*), scan rate 5 mV/s.
50 *μl* D-amino acid oxidase (4.45 Units); 0.75 mM FMCA.
Catalytic current recorded at 360 mV *vs.* Ag/AgCl ref.; BAS Pt w,e.; BAS Pt c.e.

Conclusion

The results presented here show that the mediator FMCA can be successfully used in conjunction with D-amino acid oxidase and L-amino acid oxidase for rapid measurement of both D- and L-amino acids at a BAS Pt electrode. The mediated catalytic response to amino acid substrate was optimized with respect to mediators, enzymes, and pH. The dynamic range of the voltammetric response to different D- and L-amino acids is in good agreement with total amino acid levels of fish and shellfish listed in Standard tables of Japanese food composition. These results indicate that FMCA could be successfully used to construct an amino acid oxidase electrode for detection of D- and L-amino acids at an operating potential of +360 mV *vs.* an Ag/AgCl reference electrode.

An amperometric biosensor consisted of D- amino acid oxidase immobilized into a hydroxyethyl cellulose-glutaraldehyde gel matrix could also be applied for detection of D-alanine at an operating potential of 22 mV *vs.* an Ag/AgCl reference electrode.

On the basis of the results obtained in this paper and the lack of substrate specificity of the amino acid oxidases (namely, two amino acids can't be readily discriminated from each other by these enzymes), the development of a biosensor for simple and rapid simultaneous determination of total L- and D-amino acids is planning now.

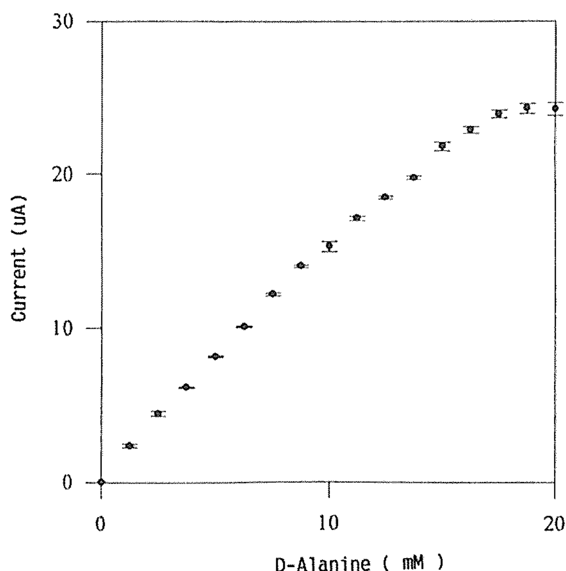

Fig. 8. Standard curve of D-alanine.
Error bars represent standard deviation ($n=5$).

References

1) Yoshimura, T. and Souda, K. 1996. A role of D-amino acids. Protein, Nucleic Acid, and Enzyme, **12**: 14–19.
2) Okuma, E., Fujita, E., Amano, H., Noda, H. and Abe, H. 1995. Distribution of free D-amino acids in tissues of crustaceans. Fisheries Sci., **61**: 157–160.
3) Watanabe, E. and Tanaka, M. 1990. Determination of fish freshness with a biosensor system. In: D. L. Wise (ed.), Bioinstrumentation and Biosensors. New York, Marcel Dekker, Inc., pp. 39–73.
4) Sawyer, D. and Roberts, J. 1974. Experimental Electorchemistry for Chemists. New York, John Wiley, Inc., pp. 67–79.
5) Bergmeyer, U. Hans and Grassl, M. 1983. Handling for reagents for enzymatic analysis. Methods Enzym. Anal., **2**: 102–125.
6) Resources Council, Science and Technology Agency. 1986. Standard tables of Japanese food composition. 2 nd edition. Tokyo, Printing Bureau, Ministry of Finance, pp. 26–40.
7) Buettner, J., Borth, R., Boutwell, H. J., Broughton, G. M. P. and Bowyer, C. R. 1980. Assesment of analytical methods for routine use. J. Clin. Chem. Clin. Biochem., **18**: 78–88.

酵素電極による D- および L-アミノ酸計測のための電圧，電流条件

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電子伝達体（フェロセンモノカルボン酸）と酸化酵素（D- および L-アミノ酸オキシダーゼ）から D- および L-アミノ酸測定用酵素電極を製作したところ，Ag/AgCl 電極に対して還元電位 360 mV における出力電流値と各種 D- および L-アミノ酸の濃度との間に直線的相関が得られた。またヒドロキシエチルセルロースのゲルマトリックス中に D-アミノ酸オキシダーゼを固定化した作用電極は，Ag/AgCl に対する印加電位 22 mV

において，D-アミノ酸と出力電流値との間によい相関が得られた。これら結果は，実用的な D- および L-アミノ酸計測用バイオセンサの開発を可能にする。

キーワード：D-アミノ酸，D-アミノ酸オキシダーゼ，L-アミノ酸，L-アミノ酸オキシダーゼ，酵素電極，フェロセンモノカルボン酸，ヒドロキシエチルセルロース