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Development of an Amperometric L-Alanine Sensor Using L-Amino Acid Oxidase from *Neurospora crassa*

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An L-alanine (Ala) sensor was developed using partially purified L-amino acid oxidase (EC 1.4.3.2, L-AAOx) from *Neurospora crassa* (*N. crassa*). The amount of L-Ala was amperometrically determined by flow injection analysis (FIA) involving an L-AAOx reactor, the pyruvate oxidase (EC 1.2.3.3, PyOx) electrode and the contrast electrode. Pyruvic acid, formed from L-Ala through the action of L-AAOx, was further oxidized by PyOx via the L-AAOx reaction. The amount of oxygen consumed in the PyOx reaction, proportional to the amount of L-Ala present, was monitored by the oxygen electrode. The L-Ala concentration was calculated from the difference (C. D.) between the PyOx and contrast electrode output. Optimum assay conditions consisted of 50 mM Tris-HCl (pH 7.4) and a transported buffer flow rate of 0.18 ml min^{-1}. Moreover, TPP and FAD at final concentrations of 1 mM and 10 μM, respectively, were added to the buffer as activators of PyOx. The sample injection volume was fixed at 50 μl. A single assay could be completed in approximately 10 min and the assays were stable for up to 50 repetitions. A linear relationship was obtained between C. D. and the L-Ala concentration with an L-Ala concentration range of 0.05 to 0.7 mM (correlation coefficient of 0.994). The relative standard deviation (R.S.D.) was 4.42% (n = 10) at 0.4 mM L-Ala. The L-Ala content of four beverages was also determined using the proposed sensor system. The results obtained indicated a linear relationship between the amount of L-Ala determined by the proposed sensor and that determined by the conventional method. Thus it was possible to develop a biosensor for the determination of L-Ala from its oxidative product, pyruvic acid. The system utilized L-AAOx, an enzyme with a low substrate specificity and isolated from *N. crassa*.

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1. Introduction

L-alanine (L-Ala) is one of the nonessential amino acids and is closely linked to the carbohydrate metabolism in the liver. This amino acid is also found in extracts from several types of seafood, particularly scallop *Patinopecten yessoensis* and the Sakhalin surf clam *Pseudocardium sachalinensis*. The nutritional functions of several amino acids, including L-Ala, have received considerable interest in recent years. Supplements consisting of major proportions of several amino acids have been developed and are currently being sold in the health foods market in Japan.

The method generally employed to determine levels of L-Ala utilizes high-performance liquid chromatography (HPLC). This method requires that the amino acids separated by HPLC be derivatized to *o*-phthaldialdehyde (OPA)-amino acid derivatives (Ruhemann's purple) using OPA or ninhydrin. Although HPLC is very sensitive, reliable and reproducible in so far as L-Ala measurements are concerned, it requires long measurement times, elaborate techniques and expensive equipment. The enzyme method[1] using glutamic-pyruvic transaminase (EC 2.6.1.2, GPT) and L-lactate dehydrogenase (EC 1.1.1.27, LDH) is based on the principle that the NADH determined from the decrease in absorbance at 340 nm is proportional to the amount of L-Ala present. This method only detects L-Ala but requires 60 min for one assay.

The biosensor is a simple method that can be easily applied. The biosensor has been developed and used in the areas of food science, environmental science and medical technologies. Among the numerous biosensors that have been developed, the enzyme sensor that utilizes an enzyme substrate specificity and an electrochemical transducer selects and measures a specific target molecule in a mixture. Compared with sensors utilizing enzymes with high substrate specificity, those using enzymes with low substrate specificity are not suitable for quantitative determinations of a target molecule in a mixture. Recently, we have developed both a D- and L-Ala assay[2] and a D-Ala sensor[3] utilizing D-amino acid oxidase (EC 1.4.3.3, D-AAOx) and L-amino acid oxidase (EC 1.4.3.2, L-AAOx) with low substrate specificity and PyOx. These methods are based on the amount of pyruvic acid derived through the action of D-AAOx and L-AAOx. The amount of pyruvic acid produced was proportional to the original D- and L-Ala content of a sample. These methods suggested that even a biosensor utilizing an enzyme with low substrate specificity could successfully be employed to quantitatively determine the level of target molecules in a mixture.

The L-Ala sensor system was developed with an amperometric FIA system. Figure 1 shows the enzyme reaction process for the determination of L-Ala. In the first step, L-amino acids are oxidized by L-AAOx with L-Ala in particular being oxidized to pyruvic acid. In the second step, the pyruvic acid formed by way of the first step is further oxidized by PyOx.

Several L-amino acid sensors[4–13] have been constructed that utilize immobilized L-AAOx purified from *Crotalus adamanteus,* that is, snake venom. When an aliquot of L-Ala was injected into these sensors,[13] the responses were slight compared to those of other L-amino acids. The L-AAOx activity was very low and required a long time for the completion of the reaction.[2] The effective properties of L-amino acid sensors are dependent on the substrate specificity of the L-AAOx being utilized.

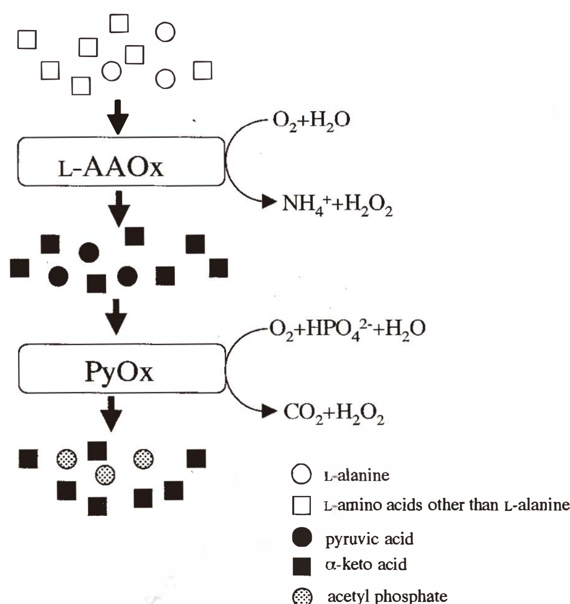

Fig. 1. Enzymatic reaction processes for the determination of L-alanine.

L-AAOxs have been purified from *Neurospora crassa*,[14] *Crotalus adamanteus*,[15] *Corynebacterium*,[16] *Cyanobacterium*,[17] alga,[18,19] *Proteus* sp.[20] and rat kidney.[21,22] The substrate specificities of these L-AAOxs were different. Amongst them, the activity of L-AAOx purified from *N. crassa* in the mycelia and the growth medium after 14 to 18 days growth was very high.[23] This enzyme efficiently converted L-Ala to pyruvic acid.[24,25] The pyruvic acid synthesized was proportional to the amount of L- and D-amino acids added as the sole source of nitrogen.[26] Moreover, the L-AAOx enzyme was induced by the addition of protein synthesis inhibitors or ATP,[27] and by a biotin-limited medium.[23]

In the present study, we investigated the growth conditions of *N. crassa*, the partial purification of L-AAOx from *N. crassa* and the activity of this enzyme in the presence of certain amino acids acting as sources of nitrogen. The L-Ala sensor consisted of a PyOx electrode, an oxygen electrode and the reactor packed with the immobilized L-AAOx by which L-Ala was converted to pyruvic acid. The L-Ala sensor was used to continuously and amperometrically determine the amount of L-Ala in certain beverages.

2. Material and Methods

2.1 *Reagents*

The enzymes used were L-AAOx (EC 1.4.3.2 from *Crotalus adamanteus*, 4 U mg^{-1}; Sigma, St Louis, MO), PyOx (EC 1.2.3.3 from *microorganism*, 6.78 U/mg; Toyobo,

Osaka, Japan) and catalase (EC 1.11.1.6 from *bovine liver*, 16,700 U/mg; Sigma, St Louis, MO). Albumin from bovine serum (BSA) was obtained from Sigma. Long-chain alkylamine controlled pore glass (CPG) was obtained from CPG Inc (New Jersey, USA). The particle size and pore size of the CPG are 74–125 μm and 500 Å, respectively. Photocrosslinkable poly (vinyl alcohol) bearing stilbazolium groups (PVA-SbQ) was obtained from Toyo Gosei Kogyo (Chiba, Japan). L-Ala, L-phenylalanine (L-Phe) and thiamine pyrophosphate (TPP) were obtained from Wako (Tokyo, Japan). Pyruvic acid and flavin adenine dinucleotide (FAD) were obtained from Sigma. Glutaraldehyde solution (50%) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). DEAE-sepharose fast flow and sephacryl S-200 (high resolution) were purchased from Amersham Bioscience (Uppsala, Sweden). Other reagents used were of guaranteed reagent grade.

2.2 *Strain and culture condition*

N. crassa (ATCC 12756) was used in this study. The strain was grown in potato dextrose broth at 27°C for 84 h with shaking. The mycelia were washed with sterilized water and then grown in Vogel's minimal medium[28] (containing 2% sucrose and an amino acid nitrogen source as shown in Table 1) at 27°C for 72 h with shaking. Preparations used for the isolation of active L-AAOx extracts were grown in 3 mM D-Ala. After incubation (39 L), the induced mycelia were washed with distilled water and lyophilized. The lyophilized mycelia were stored at –20°C.

2.3 *Partial purification of L-AAOx from N. crassa*

All of the enzyme preparative steps were carried out in a cold room. The lyophilized mycelia were homogenized in 6 L of 50 mM Tris-HCl (pH 7.4) and 1 mM EDTA with a mortar and then disrupted by ultrasonic treatment. After centrifugation at 8,000 rpm for 10 min, the supernatant was recovered and to this pulverized ammonium sulfate was added to yield a 70% saturated solution. The solution was stored overnight. After centrifugation at 12,000 rpm for 30 min, the supernatant was recovered, brought to 90% saturation with ammonium sulfate and then stored overnight. After centrifugation at 12,000 rpm for 30 min, the supernatant was discarded and the precipitate was dissolved in 100 ml of the above buffer before being dialyzed against the same buffer. The dialyzed protein solution was applied to a 1.6 × 38 cm column of DEAE-sepharose equilibrated in the same buffer. Protein was eluted with a 0 to 0.5 M linear NaCl gradient. Fractions (5.85 ml) were

Table 1
L-amino acid oxidase activity in extracts from *N. crassa* against the different amino acids as nitrogen source.

Nitrogen	Concentration (mM)	Growth (g wet wt%)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
D-alanine	3	2.75	91	3.63	0.0398
L-alanine	3	2.67	75.5	2.55	0.0337
D-methionine	3	2.86	81	2.95	0.0365
D-phenylalanine	3	3.16	86	3.08	0.0359
NH_4NO_3	50	2.71	62.2	1.04	0.0167

collected at a flow rate of 33 ml h^{-1}. The active fractions (total volume 200 ml) were concentrated by filtration using an ultrafiltration membrane (Millipore PM 10). The concentrated fraction sample was applied to a 1.6 × 95 cm column of sephacryl S-200 equilibrated in the same buffer. The active fractions were collected and concentrated by filtration using an ultrafiltration membrane.

2.4 *Enzyme assays*

L-AAOx activity was assayed by measuring the rate of formation of pyruvic acid from L-Ala according to the method of Sikora and Marzluf[26] with modifications. Reagents used for the determination of pyruvic acid were based on Friedemann and Haugen[29] and Friedemann.[30] The cell extract (200 μl) was added to 200 μl of 50 mM phosphate buffer (pH 7.4) containing L-Ala at a final concentration of 3 mM. This reaction mixture (400 μl) was incubated at 37°C for 20 min. Two hundred milliliters of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl was then added and the mixture was incubated for a further 5 min. To this, 400 μl ethanol was added with mixing followed by 1000 μl of 2.5 N NaOH. After 5 min, the absorbance was determined at 550 nm. One unit of L-AAOx activity is defined as that amount of enzyme needed to form 1 μmol of pyruvic acid per min under the above conditions. Protein concentrations were determined according to the micro-biuret method[31] using BSA as standard and the absorbance measured at 280 nm.

2.5 *Construction of L-Ala sensor*

CPG (dried, 1 ml) was activated according to Inaba's method.[3] L-AAOx and catalase were co-immobilized on the activated CPG prepared as previously described. Activated CPG (340 mg) was reacted with 1.25 ml of 10 mg phosphate buffer (pH 7.8) containing 2.43 mg L-AAOx prepared from *N. crassa* and 4 mg catalase at 30°C for 2 h. After washing the immobilized CPG with distilled water, the immobilized CPG was then reacted with 2 ml of the buffer containing 30 mg D-Ala at room temperature for 1.5 h (L-AAOx beads). Alternatively, 220 mg of activated CPG was reacted with 1 ml of the buffer containing 15 mg catalase at 30°C for 2 h. After washing the immobilized CPG with distilled water, the CPG was then reacted with 2 ml of the buffer containing 30 mg D-Ala at room temperature for 1.5 h (catalase beads). The L-AAOx beads and the catalase beads were packed into a polypropylene reactor tube (50 mm × 2 mm) at a ratio of 9 to 1 without mixing and then stored at 4°C. The L-AAOx beads were placed in the lower layer.

An immobilized PyOx membrane was prepared from a mixture of distilled water (300 μl), polymer (100 μl) and PyOx (40 U). The mixture was left at rest on a PS plate and dried at 4°C for 24 h in the dark. Both sides of the dried membrane were irradiated for 10 min with a fluorescent lamp (30 W) to facilitate cross-linking of the polymer and removal from the PS plate. The distance between the light source and the membrane was 10 cm. The membrane was stored at 4°C. The oxygen electrode used (diameter, 3 mm; DG-5G, ABLE, Tokyo) was a Clark-type electrode consisting of a platinum cathode, a lead anode, an alkaline electrolyte (KOH) and an oxygen-permeable teflon membrane (thickness ca. 12.5 μm). The PyOx membrane (5 × 5 mm) was placed on the teflon membrane and covered with the rinsed dialysis membrane (molecular weight cut off, 14000; thickness, 20 μm) (PyOx electrode).

A BSA membrane, prepared by using 2 mg BSA instead of PyOx, was used for the preparation of a contrast electrode in a similar manner to that described above.

As shown in Fig. 2, the FIA system used consisted of the L-AAOx reactor, the PyOx electrode and the contrast electrode which are set to a flow cell, two zero-shunt ammeters (HM-103 Hokuto Denko, Tokyo) two recorders (R-01A Rikadenki, Tokyo), an air pump, a HPLC pump (LD-10AD, Shimadzu, Kyoto) and a buffer tank. The sample was injected from the injection port. Firstly, several L-amino acids in the sample were oxidized by the immobilized L-AAOx in the reactor, and any hydrogen peroxide produced was acted upon by the catalase in the reactor. Second, another L-AAOx reaction product, pyruvic acid, was oxidized by the PyOx electrode. The decrease in the amount of oxygen resulting from the activity of L-AAOx was monitored by the PyOx and contrast electrodes in the flow cell. The decrease in the amount of oxygen resulting from the activity of PyOx was measured by the PyOx electrode. Therefore, the amount of L-Ala was calculated from the difference between the output currents of the PyOx and the contrast electrodes connected directly to the L-AAOx reactor.

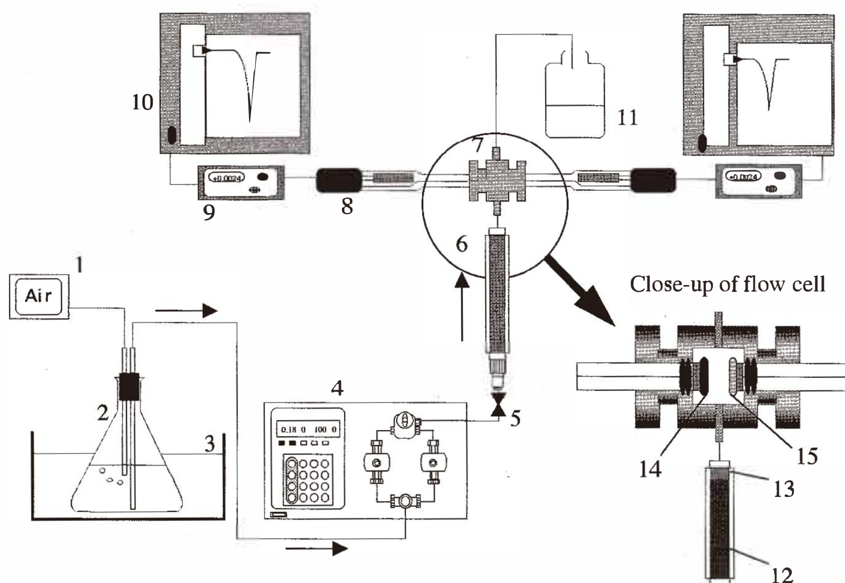

Fig. 2. Schematic diagram of the L-alanine sensor. 1. Air, 2. buffer tank, 3. thermostatically controlled bath, 4. HPLC pump, 5. injection port, 6. L-AAOx reactor, 7. flow cell, 8-1. PyOx electrode, 8-2. contrast electrode, 9. zero shunt ammeter, 10. recorder, 11. waste tank, 12. co-immobilized L-AAOx and catalase beads, 13. immobilized catalase beads, 14. immobilized PyOx membrane, 15. immobilized BSA membrane. The arrowheads show the flow path.

3. Results and Discussion

3.1 *Partial purification and substrate specificity of L-AAOx from N. crassa*

3.1.1 *Effect of addition of amino acids as nitrogen sources on L-AAOx synthesis by N. crassa*

The synthesis of L-AAOx activity was increased by the addition of D-Ala, L-Ala, D-Met and D-Phe, each at a final concentration of 3 mM, and NH_4NO_3 at a final concentration of 50 mM, as nitrogen sources as shown in Table 1. Although L-Leu and L-Phe were usually used as the substrates in L-AAOx activity assays, only L-Ala was used as the substrate in this study. When amino acids constituted the nitrogen source of *N. crassa*, the total activity of L-AAOx (average 3.05 U) formed from *N. crassa* was higher than that formed when NH_4NO_3 (1.04 U) was used as the nitrogen source. The addition of D-Ala induced the highest L-AAOx activity (3.63 U). The results suggested that the synthesis of L-AAOx by *N. crassa* required amino acids as the nitrogen source, consistent with the findings of Sikora and Marzluf.(26) Therefore, D-Ala at a final concentration of 3 mM was added to a large-scale culture medium (39 L) for the preparation of L-AAOx.

3.1.2 *Partial purification of L-AAOx from N. crassa*

The results of the purification of the L-AAOx from *N. crassa* are summarized in Table 2. The L-AAOx obtained represented a partially purified form of the enzyme. Following gel filtration chromatography, 37.2 mg of partially purified L-AAOx was obtained. The specific activity was 0.92 U mg^{-1}. It was shown that the partially purified L-AAOx extract included four proteins as determined by gel electrophoresis (data not shown). In the subsequent experiments, the partially purified L-AAOx extract was used.

3.1.3 *Substrate specificities of immobilized L-AAOx's*

Two amperometric L-amino acid sensors were constructed using the reactor packed with L-AAOx from *N. crassa* or *Crotalus adamanteus*, respectively. These L-amino acid sensors used a FIA system which consisted of an L-AAOx reactor, an oxygen electrode that is set to a flow cell, a zero-shunt ammeter, recorders, an air pump, a HPLC pump and a buffer tank. The L-Ala sensor is essential for L-AAOx activity that quickly converts L-Ala to pyruvic acid. The responses of each L-amino acid sensor to several amino acids were examined. The results indicating that substrate specificities for immobilized L-AAOx from

Table 2
Summary of each purification process of the L-amino acid oxidase.

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Ammonium sulfate (70–90%)	298.6	48.4	0.16	100.0	1.0
DEAE-sepharose FF (NaCl-gradient)	46.3	38.1	0.82	78.7	5.1
Sephacryl S-200HR	40.3	37.2	0.92	76.9	5.7

Crotalus adamanteus and *N. crassa* were obtained are shown in Table 3. The catalysis of L-amino acids observed for immobilized L-AAOx from *N· crassa* was higher than that observed with the enzyme derived from *Crotalus adamanteus*. The response of the sensor using the immobilized L-AAOx from *N. Crassa* was consistent with a report from Bender and Krebs.[24] Moreover, this response changed with differing buffer concentrations. The substrate specificity of L-AAOx from *N. crassa* is that the relative amounts of some neutral amino acids (L-Ala, -Val, -Leu, -Ile and -Ser) were increased and that of the acidic amino acids (L-Glu and -Asp) were decreased when buffer concentration was increased. In the case of L-Ala, the relative activity was 0% of the immobilized L-AAOx from *Crotalus adamanteus*. On the contrary, the immobilized L-AAOx from *N. crassa* produced pyruvic acid only from L-Ala and 118% of the relative activity was obtained in 50 mM Tris-HCl (pH 7.4). These observations suggest that the response of the sensor was affected by the buffer pH, the nature of the buffer solution and the injected substrate concentration. The results suggest that L-AAOx from *N. crassa* optimally converts L-Ala to pyruvic acid in 50 mM Tris-HCl (pH 7.4). Moreover, the purification of L-AAOx from *N· crassa* is more simple and safe than that of L-AAOx from *Cratalaus adamanteus*.

The low substrate specificity and the high activity of L-AAOx from *N· crassa* suggested that it might be possible to construct a more sensitive protein sensor as a substitute for other

Table 3
Effects of some kinds of amino acids on the output of L-amino acid sensors using L-AAOx's from *N. crassa* and *Crotalus adamanteus*.

		Realative amounts (%)			
		Crotalus adamanteus venon		*Neurospora crassa*	
		Buffer concentration (mM)		Buffer concentration (mM)	
L-amino acid		10	50	10	50
aliphatic amino acid	glycine	0	0	0	3
	alanine	0	0	54.3	118
branched chain amino acid	valine	0	0	10.9	19
	leucine	49.7	96.0	57.8	99.2
	isoleucine	3.4	43.7	27.7	67.9
hydroxy amino acid	serine	0	0	10	51
sulfur amino acid	methionine	46.4	92.5	106.5	99.5
	cysteine	6.0	60.7	101.0	92.9
acidic amino acid	aspartic acid	0	0	15	9
	glutamic acid	0	0	92	31
basic amino acid	lysine	0	0	105	104
	ornithine	0	0	102	93
	arginine	0	6.6	81	83.9
	histidine	0	22.0	102	90.1
aromatic amino acid	tyrosine	95.6	95.3	115.1	101.8
	phenylalanine	100.4	100	111.7	100
heterocyclic amino acid	tryptophan	57.0	85.8	104.6	103.0
imino acid	proline	0	0	0	2

*Realative activity is expressed as % of that for L-phenylalanine.
Condition: Tris-HCl buffer (pH 7.4), flow rate 0.18 ml/min, injection volume 50 μl.

methods such as the microbiuret method. The protein sensor was based on a combination of protease, L-AAOx and the oxygen electrode. L-amino acids were oxidized by L-AAOx and the extent of the oxidation was monitored by the oxygen electrode.

3.2 *Construction of L-Ala sensor*

3.2.1 *Construction of L-Ala sensor and procedure for the determination of L-Ala*

Figure 2 depicts the L-Ala sensor system, an amperometric FIA system utilizing dual oxygen electrodes. In the flow cell, the PyOx electrode monitored the amount of oxygen consumed during the PyOx and L-AAOx reactions, and the contrast electrode monitored the amount of the oxygen consumed in the L-AAOx reaction. The output of each of the two electrodes was monitored and recorded. The amount of L-Ala was determined from the amount of pyruvic acid produced from L-Ala through the action of L-AAOx. The amount of pyruvic acid formed was calculated from the difference between the output of the PyOx and contrast electrodes.

A time lag between the start times of the L-AAOx and PyOx reactions was incorporated into the FIA system to negate the oxygen consumed during the L-AAOx reaction. The L-Ala sensor was specifically capable of determining the level of L-Ala, even though other L-amino acids were present in the sample. However, when the total amount of other L-amino acids in the sample was 10 times greater than the amount of L-Ala , the system could not detect L-Ala.

L-Ala was determined according to the report of Inaba.[(3)] Namely, from the correlation curve derived using L-Phe, the output of the contrast electrode corresponding to the measured output of the PyOx electrode was calculated. The amount of L-Ala was calculated from the difference (C.D.) between the measured and the calculated outputs of the contrast electrodes.

3.2.2 *Response curve of the L-Ala sensor*

When 50 μl of L-Ala (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.7 mM) was injected into the sensor system, some of the response curves obtained were as shown in Fig. 3. The assay conditions were as follows: 50 mM Tris-HCl (pH 7.4) containing a final concentration of 1 mM TPP and 10 μM FAD was used as the transport solution at a flow rate of 0.19 ml min^{-1}. The injection volume was 50 μl. One assay was completed within 10 min.

3.2.3 *Effect of assay conditions on the output of the L-Ala sensor system*

Figure 4 shows the effect of pH, buffer concentration and flow rate on the C.D. and the output of the L-Ala sensor. The pH stability of native PyOx was in the 5.7 to 6.5 range. The outputs of the contrast and PyOx electrodes were stable and more sensitive at pH 7.4 – 8.2 and pH 7.4 – 7.8, respectively, whereas that of the PyOx electrode decreased in alkali conditions above pH 7.8. Therefore, the pH of the proposed sensor system was set at 7.4, at which the output was stably maintained and the sharpest response was obtained. Comparing 50 mM Tris-HCl, TES, MOPS, HEPES and phosphate buffers at pH 7.4, it was found that the response curve of L-Ala was the sharpest using Tris-HCl in the 0.2 to 0.7 mM range (data not shown).

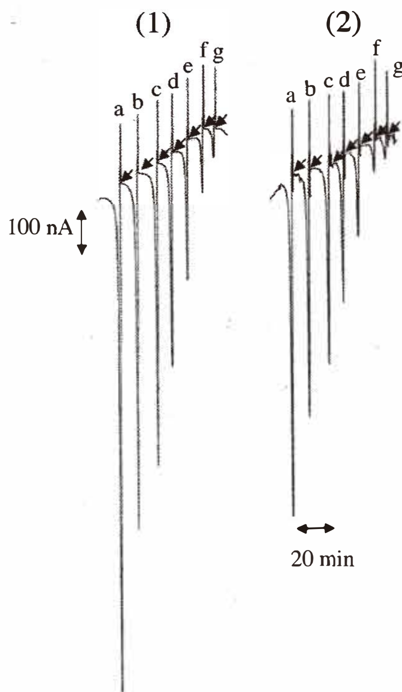

Fig. 3. Response curve of L-alanine sensor. (1) Responses of the PyOx electrode. (2) Responses of the contrast electrode. The signs from a. to g. were 0.7, 0.5, 0.4, 0.35, 0.2, 0.1 and 0.05 mM L-Ala, respectively. Sensor conditions: 50 mM Tris-HCl (pH 7.4); TPP concentration, 1 mM; FAD concentration, 10 μM; flow rate, 0.2 ml min^{-1}; injection volume, 50 μl. Some arrowheads show injection points.

As shown in Fig. 4 (b), the outputs of both the PyOx and contrast electrodes increased with increasing buffer concentration up to 40 mM, and were stable in the 40 to 85 mM range. The results were sensitive to ionic strength. The optimum buffer concentration was established as 50 mM.

As shown in Fig. 4 (c), the outputs of both the PyOx and contrast electrodes decreased monotonically with an increasing in flow rate up to 0.4 ml min^{-1}. The results suggest that catalytic activity in the sensor was inadequate. The reasons are likely to be the low immobilization yield and the relative activity of immobilized L-AAOx. The optimum flow rate of the sensor system was established as 0.18 ml min^{-1} to ensure an adequate reaction time in the L-AAOx reactor and to finish measurement as soon as possible. Moreover, final concentrations of 1 mM TPP and 10 μM FAD were added to the transport solution as activators of PyOx. The activity of PyOx used in this study was proportional to the TPP concentration. The effect of the addition of TPP on the activity of the PyOxs was weakest compared to those from *Pediococcus* sp. and *Aerococcus* sp. The injection volume of the samples was fixed at 50 μl.

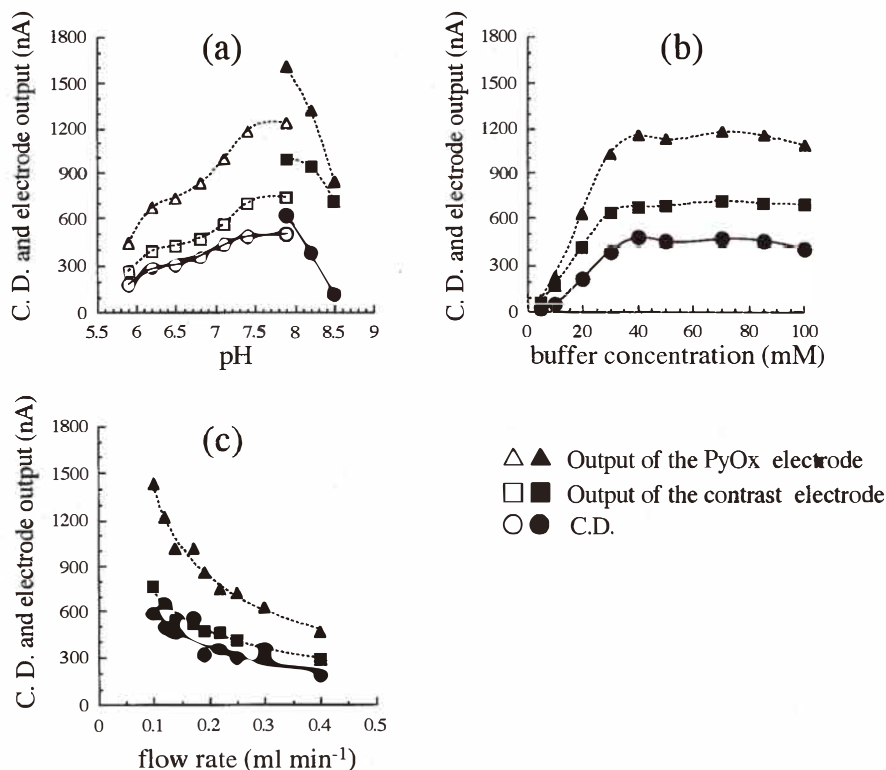

Fig. 4. Effect of conditions on the output of the L-alanine sensor. (a) pH; pH 5.8–7.8 phosphate buffer, pH 7.8–8.9 Tris-HCl, (b) buffer concentration, (c) flow rate. Sensor conditions: phosphate buffer and Tris-HCl; TPP concentration, 1 mM; FAD concentration, 10 μM; injection volume, 50 μl of 0.5 mM L-Ala. The C.D. was the difference between the measured output of the contrast electrode and the calculated output of the contrast electrode, corresponding to the measured output of the PyOx electrode.

3.2.4 *Calibration curve and stability*

The calibration curve for L-Ala using L-AAOx from *N. crassa* is shown in Fig. 5. A linear relationship was observed between the amount of L-Ala and the C.D. when the L-Ala concentration was in the 0.05 to 0.7 mM range (from the results of five successive measurements). The correlation coefficient was 0.994 and the R.S.D. was 4.42% (n=10) against 0.4 mM L-Ala. The sensor system was stable for up to 50 repetitions within a day.

On the other hand, in the case of the L-Ala sensor utilizing L-AAOx from *Crotalus adamanteus*, a linear relationship was observed between the amount of L-Ala and the C. D. when the L-Ala concentration was in the 2 to 5 mM range ($y = 6.786x + 15.714$). The correlation coefficient was 0.992 (data not shown). The assay conditions were as follows: the 30 mM phosphate buffer (pH 6.8) contained final concentrations of 1 mM TPP and 10

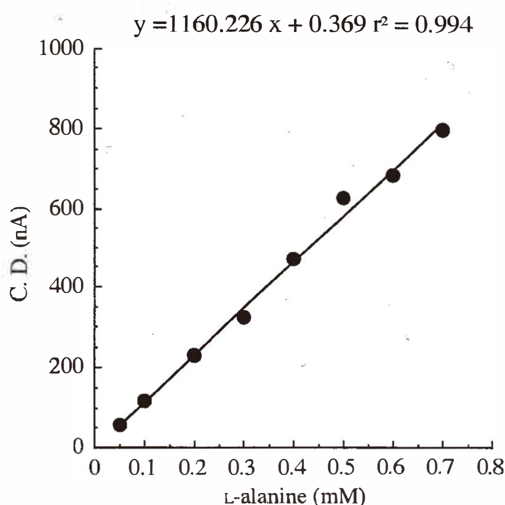

Fig. 5. Calibration curve of the L-alanine sensor. Sensor conditions: 50 mM Tris-HCl (pH 7.4); TPP concentration, 1 mM; FAD concentration, 10 μM; flow rate, 0.18 ml min^{-1}; injection volume, 50 μl.

μM FAD was used as the transport solution at a flow rate of 0.15 ml min^{-1}. The injection volume was 50 μl. One assay was completed within 15 min.

L-Ala sensors using L-AAOx from *N. crassa* were more sensitive and rapid than those using L-AAOx from *Crotalus adamanteus.*

3.2.5 *Recovery of L-Ala from the sample and accuracy of the L-Ala sensor system*

The recovery test was performed using three beverages to which known amounts of L-Ala had been added. The method involved the preparation of a sample of beverage containing an unknown amount of L-Ala and a sample of beverage to which a known amount of L-Ala was added, and the L-Ala content in each sample was determined by the proposed sensor system. The results are shown in Table 4. An average recovery of 101.8% was achieved with a coefficient variation (CV) of 15.6%. This suggested that the determination of L-Ala in the samples was accurately performed by the L-Ala sensor system.

In the case of sample 3, the recovery was 75%. This suggested that the total amount of L-amino acids significantly exceeded the amount of L-Ala present, thus thwarting the sensitivity of the immobilized L-AAOx. The linear relationship between the output of the contrast electrode as a function of the concentration of the sample and the dilution ratio of the sample has been confirmed.

Some beverages were tested by the sensor system and the conventional method.[1] As shown in Table 5, both methods yielded similar results. The correlation equation was $y = 0.942x + 0.111$ ($r = 0.997$). Given the results obtained, it is suggested that the proposed sensor system would be a highly appropriate tool in the area of food analysis.

Table 4
Recovery of L-alanine added to beverage samples.

Sample	(B: mM)	L-alanine added (A: mM)	Sample+A (C: mM)	(C–B: mM)	Recovery (C–B)/A×100
1	0.09	0.05	0.14	0.05	93.0
		0.10	0.22	0.13	126.5
		0.20	0.29	0.20	98.3
2	0.14	0.05	0.19	0.05	102.0
		0.10	0.24	0.10	100.0
3	0.16	0.05	0.22	0.06	120.0
		0.10	0.26	0.10	100.0
		0.20	0.31	0.15	75.0
				Mean	101.8
				CV* (%)	15.6

*Coefficient of variation

Table 5
L-alanine contents in some beverages.

Sample	L-alanine concentration (mM) Sensor method	Conventional method
A	1.87	2.20
B	0.27	0.33
C	1.66	1.40
D	8.27	7.88

4. Conclusion

An L-Ala sensor system was developed using partially purified L-AAOx from *N. crassa*. Activity of the partially purified L-AAOx was higher than that of the commercially available L-AAOx from *Crotalus adamanteus*. The amount of L-Ala was determined by the amperometric FIA system. The FIA system consisted of a L-AAOx reactor, a PyOx electrode and a contrast electrode in the flow cell. Among the oxidative products from the many L-amino acids acted upon by the L-AAOx, pyruvic acid was formed only from L-Ala. The pyruvic acid formed was further oxidized by PyOx via the L-AAOx reaction. The amount of oxygen consumed in the PyOx reaction was proportional to the amount of L-Ala present and was monitored by the oxygen electrode. A time lag between the start times of the L-AAOx and PyOx reactions was incorporated into the FIA system to negate the oxygen consumed during the L-AAOx reaction. The L-Ala concentration was calculated from the difference (C.D.) between the outputs of the PyOx and the contrast electrodes and was measured only for the PyOx reaction.

Optimal reaction conditions consisted of 50 mM Tris-HCl (pH 7.4) and a transported buffer solution flow rate of 0.18 ml min^{-1}. Moreover, TPP and FAD were added to the buffer at final concentrations of 1 mM and 10 μM, respectively, as activators of PyOx. The

injection volume of the sample was fixed at 50 μl. One assay required about 10 min for completion and the assay was stable up to 50 repetitions. A linear relationship between the C.D. and the concentration of L-Ala was obtained when the concentration of L-Ala was in the 0.05 to 0.7 mM range (correlation coefficient of 0.994). The R.S.D. was 4.42% (n = 10) for 0.4 mM L-Ala. The sensitivity of the proposed sensor system was higher than that of the L-Ala sensor system that was constructed using L-AAOx from *Crotalus adamanteus*.

The L-Ala content of four beverages was also determined using the proposed sensor system. The results obtained indicated a linear relationship between the amounts of L-Ala determined by the proposed sensor system and the conventional method. Thus, the possibility of the development of a biosensor utilizing the enzyme with low substrate specificity has been suggested.

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