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Note

Use of immobilized amino acid oxidase as post-column reactor in the high-performance liquid chromatography of amino acids

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Amino acids have been successfully separated using reversed-phase high-performance liquid chromatography (HPLC)[1,2], and post-column derivatization of the amino acids with *o*-phthalaldehyde has been investigated[2,3]. One of problems with the post-column derivatization method is the additional peak broadening caused by the reactor. Packed-bed reactors are preferable to open-tubular reactors with regard to dispersion and pressure drop[4]. Post-column derivatization by using an immobilized enzyme reactor in conjunction with reversed-phase HPLC gives good specificity and has been applied in practical analytical procedures[5–9]. Generally, packed-bed reactors supported enzymes have higher activity than open-tubular reactors[10]. In this work, we used an immobilized L-amino acid oxidase column reactor for the deamination of amino acids.

An L-amino acid oxidase (E.C. 1.4.3.2) (AAO) catalyses the deamination of L-amino acids:

$$\text{L-amino acid} + O_2 + H_2O = \text{2-keto acid} + NH_3 + H_2O_2$$

Several methods have been proposed for the determination of L-amino acids using this enzyme[11]. AAO possesses low specificity and L-amino acids such as leucine, methionine, phenylalanine, norvaline, tyrosine, tryptophan, norleucine, cysteine and citrulline are oxidized[12]. Hence mixtures of amino acids have to be separated by some method prior to the determination of the individual amino acids. AAO has been immobilized by a diazotization procedure by Weetall and Baum[13]. The peroxidase (E.C. 1.11.1.7) (PO)–homovanillic acid system has often been used as an indicator in fluorimetric determinations of oxidative enzymes, their substrates and hydrogen peroxide[14,15]. PO has also been immobilized covalently, coupled with diazotization, and an enzyme column reactor has been applied for the determination of hydrogen peroxide[16].

We prepared co-immobilized AAO–PO by covalent coupling to an aminoaryl derivative of controlled-pore glass (CPG) beads and packed the enzymes into a col-

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umn. The column reactor was placed in a continuous-flow system for HPLC. This paper describes the preparation of the co-immobilized AAO–PO and its adaptation to the post-column system for the simultaneous determination of tyrosine, phenylalanine, tryptophan and methionine.

EXPERIMENTAL

Reagents

L-Amino acids, L-amino acid oxidase (type III) and peroxidase (type VI) were obtained from Sigma (St. Louis, MO, U.S.A.). Flavine adenine dinucleotide (FAD) disodium salt, homovanillic acid (HVA), KH_2PO_4 and tris(hydroxymethyl)aminomethane (Tris) were purchased from Nakarai Chemicals (Kyoto, Japan). An L-amino acid standard solution containing alanine, arginine, asparagine, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine was prepared at a concentration of 1.0 m*M* of each amino acid in 0.1 *M* HCl.

Enzyme immobilization

Aminoaryl-CPG beads (400 mesh) (Electro-Nucleonics, Fairfield, U.S.A.) (4.0 g) were diazotized for 30 min at 0–3°C with 0.5% sodium nitrite in 2 *M* hydrochloric acid; after washing with cold water, they were mixed with 50 ml of an ice-cold solution of AAO (30 mg) and FAD (4 mg) in Tris–HCl buffer solution (I = 0.1, pH 8.5). After the first coupling for 4 h, PO (100 mg) was added to the mixture and additional coupling was allowed to proceed for 12 h. Then, in order to destroy the unreactive diazo group, the immobilized enzymes were allowed to stand for 2 h at 35°C with occasional stirring. The evolution of nitrogen in the column influenced both the reactor efficiency (enzyme activity) and the reproducibility of the peaks. Highly active immobilized AAO could only be obtained in the presence of FAD during the immobilization process. A loose packing was one of causes of the broadening of the peaks. The immobilized enzyme was tightly packed into a column by the use of a packer (Model KSC-20s, Kyowa Seimitsu, Tokyo, Japan) for HPLC.

Procedure

Fig. 1 is a schematic diagram of the flow system. The eluent (methanol–0.1 *M* KH_2PO_4 (4:96%, v/v) (A) and regent solution (1.0 m*M* HVA, 0.4 *M* Tris) (B) are pumped by a piston pump (Model KHU-W-104, Kyowa Seimitsu) (C) through pressure gauges (D). The eluent passes through the injector (E) into a stainless-steel column (250 × 4.6 mm I.D.) (F) packed with Finepak SIL C_{18}-10 (JASCO, Tokyo, Japan). Both streams enter opposing chanels of a tee connector (G), where they are mixed. The solution then passes into the reaction column (stainless steel, 250 × 4.0 mm I.D.) (H), which is packed with the immobilized enzymes. The column is thermostated at 40°C. The stream passes to a fluorescende spectrophotometer (Model 650-10s; Hitachi, Tokyo, Japan) (I) fitted with a flow cell (cell volume 18 μl). The excitation and emission wavelengths were 315 and 425 nm, respectively, and the response was monitored on a Model 056 recorder (Hitachi) (J).

The sample volume was 50 μl. L-Tyrosine was used for calibration. Standard tyrosine solutions were prepared just prior to each calibration.

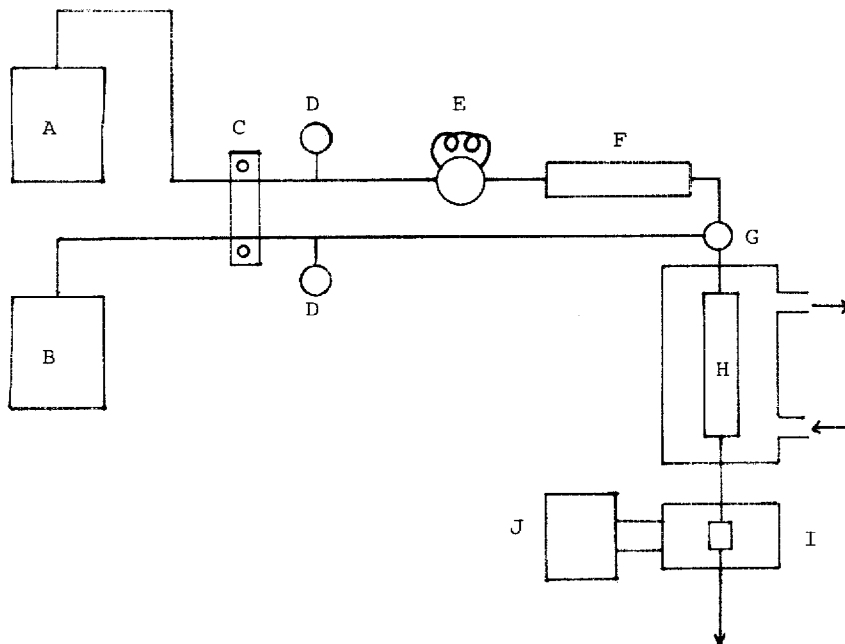

Fig. 1. Schematic diagram of the chromatographic system. A = Eluent; B = reagent solution; C = piston pump; D = pressure gauges; E = injection valve; F = separation column; G = tee connector; H = immobilized enzyme reactor; I = spectrofluorimeter; J = recorder.

RESULTS AND DISCUSSION

Characterization of the immobilized enzyme reactor

AAO is active towards several L-amino acids. As measured with 0.1 m*M* amino acids and 0.5 m*M* HVA in pH 8.0 Tris–HCl buffer and a flow-rate of 0.8 ml/min at 40°C the order of reactivity of L-amino acids with the reactor was tyrosine (Tyr) 100, phenylalanine (Phe) 100, tryptophan (Try) 100, methionine (Met) 100, leucine (Leu) 100, isoleucine (Ileu) 25, arginine (Arg) 12, histidine (His) 8, lysine (Lys) 7, cysteine (CySH) 4. There was no reaction with proline, threonine, asparagine, alanine, serine, valine, glutamine and D-amino acids.

Fig. 2 shows that the peak height of Tyr is almost independent of the pH of the Tris buffer in the range 7.8–8.9. Identical results were observed with Phe, Try, Met and Leu as substrates.

The dependence of peak height on flow rate was studied over the range 0.4–1.8 ml/min (Fig. 3). The peak height was constant at flow-rates between 0.6 and 1.0 ml/min. The peak area did not decrease at lower flow-rates (0.4, 0.5 ml/min) (not shown in Fig. 3). The decrease in peak height at 0.4 ml/min is attributed to the broadening of the peak.

The effect of temperature was examined in the range 25–50°C (Fig. 4). The peak height was constant above 40°C.

Fig. 5 shows the peak height as a function of the amount of methanol in the buffer (pH 8.0). The peak height decreased slowly with methanol content up to 5%.

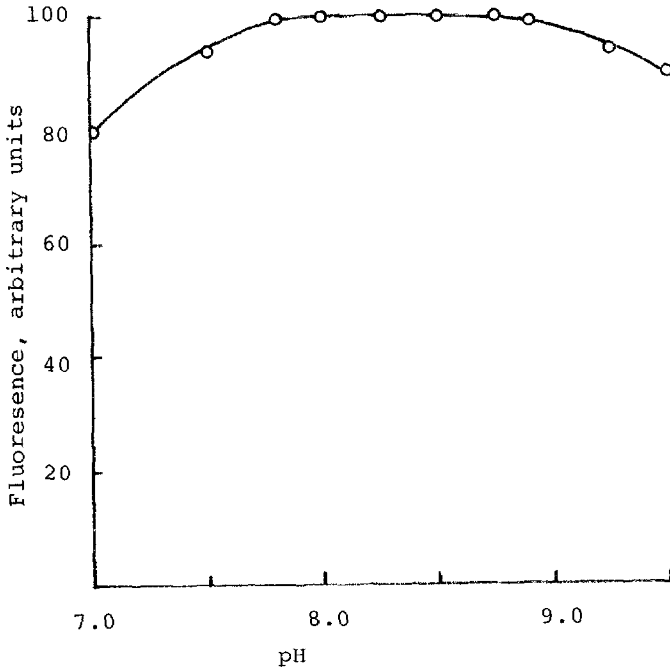

Fig. 2. Effect of pH of the buffer on fluorescence intensity. Tyr, $5 \cdot 10^{-5}$ *M* per 50 μl; flow-rate, 0.8 ml/min; reactor temperature, 40°C.

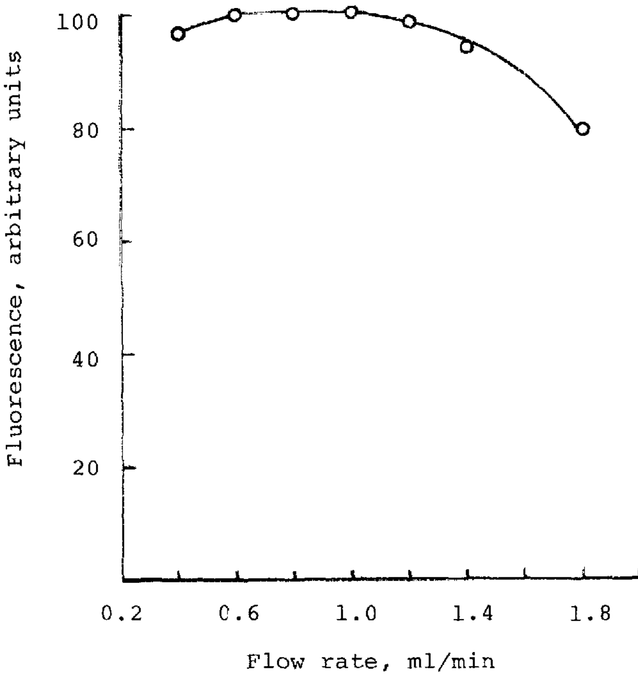

Fig. 3. Effect of flow-rate on fluorescence intensity. Tyr, $5 \cdot 10^{-5}$ *M* per 50 μl; pH 8.0; reactor temperature, 40°C.

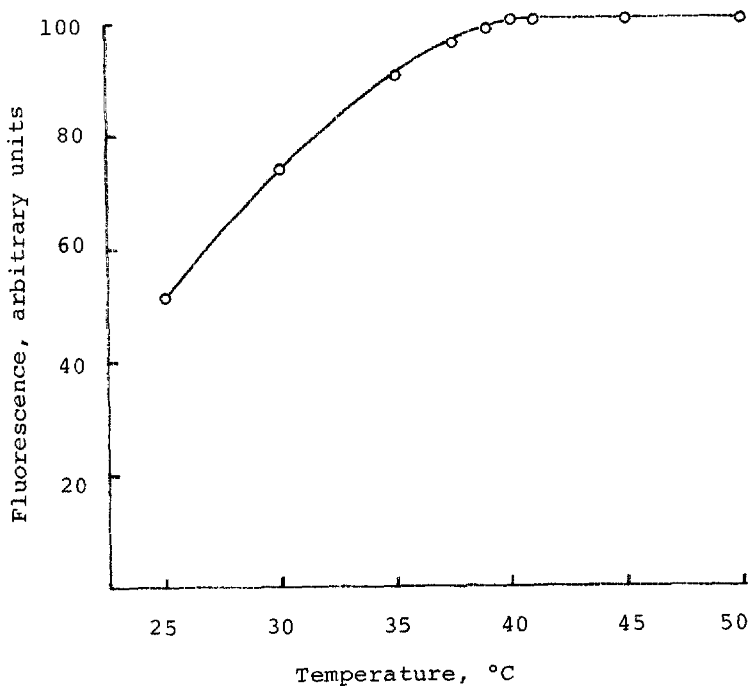

Fig. 4. Effect of reactor temperature on fluorescence intensity. Tyr, $5 \cdot 10^{-5}$ *M* per 50 μl; pH, 8.0; flow-rate, 1.0 ml/min.

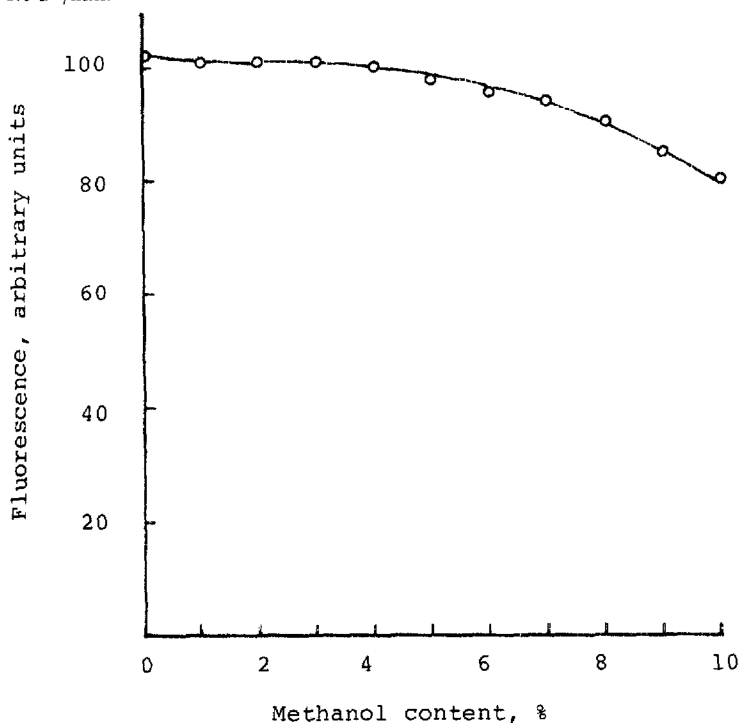

Fig. 5. Effect of methanol content in the buffer on fluorescence intensity. Tyr, $5 \cdot 10^{-5}$ *M* per 50 μl; pH, 8.0; flow-rate, 1.0 ml/min; reactor temperature, 40°C.

The shape of the curve depended only on the activity of the immobilized PO. Addition of small amounts of ethanol to the buffer resulted in a steep decrease in the peak height provided that PO of lower activity was used, even if the content was less than 1%.

When stored at 4°C in the presence of FAD at pH 7.0 in phosphate buffer (0.1 *M*), the immobilized enzyme was stable for at least 2 months. As the inactivation of AAO might be caused by the loss of FAD[17], the stability of the reactor was dependent only on the elution volume under operation. The activity decreased linearly with increasing elution volume. The half-life period at 40°C was about 2 weeks at a flow-rate of 1.0 ml/min (the total effluent volume was about 10 l). At this point the linearity of the calibration was restricted to low substrate concentrations under the conditions described in the procedure. At 50°C the half-life period was about 3 days at a flow-rate of 0.8 ml/min.

Metal ions such as Fe(III), Cu(II) and Co(II) acted as inhibitors. However, the interferences from these ions could be prevented by adding phosphate salt to the stream.

Separation of amino acids

The separation of ten amino acids that are enzymatically oxidized by the immobilized AAO was investigated by using HPLC and the immobilized enzyme reactor. Met, Phe and Try were well resolved on 10 μm ODS-Finepak SIL in a 250 ×

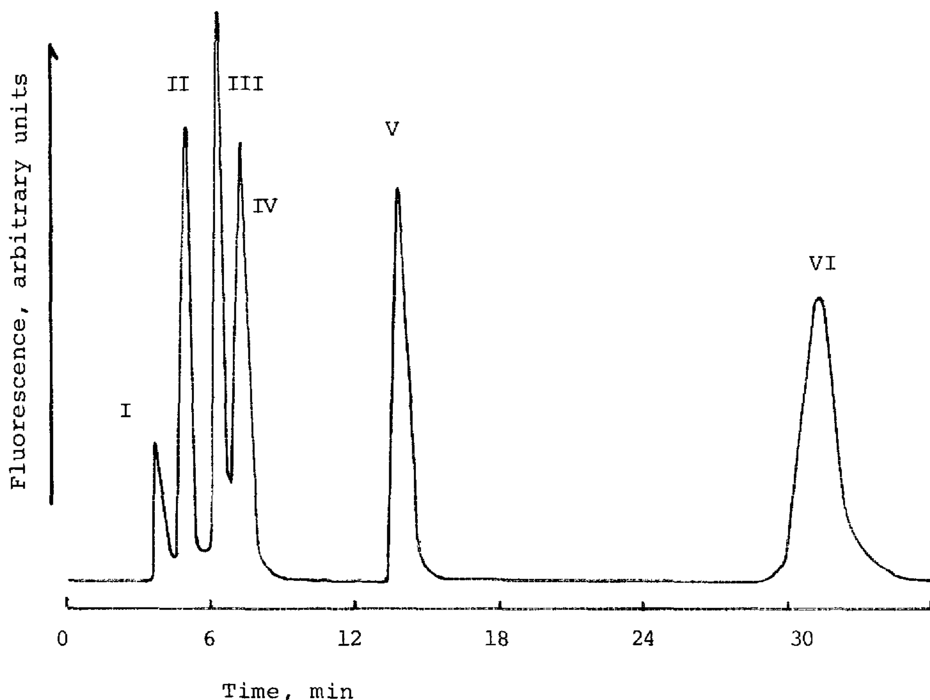

Fig. 6. Separation of a mixture of ten amino acids ($1 \cdot 10^{-4}$ *M* of each). Sample, 50 μl. I = Lys, His, CySH and Arg; II = Met; III = Leu and Ileu; IV = Tyr; V = Phe; VI = Try. Eluent, methanol–0.1 *M* KH_2PO_4 (4:96, v/v); flow-rate, 0.8 ml/min; reagent solution, 1.0 m*M* HVA in 0.4 *M* Tris at 0.2 ml/min; separation column, 250 × 4.6 mm I.D. ODS.

TABLE I

ANALYSIS OF L-AMINO ACID STANDARD SOLUTIONS

L-Amino acid	Taken (nmol)	Found* (nmol)	Recovery (%)	Coefficient of variation (%) (n = 7)
Met	0.267	0.256	96	7.4
Tyr	0.255	0.280	110	8.9
Phe	0.252	0.244	97	9.0
Try	0.251	0.16	–	–
Met	2.67	2.59	97	3.8
Tyr	2.55	2.55	100	4.7
Phe	2.52	2.47	98	4.3
Try	2.51	2.31	92	7.0
Met	5.34	5.29	99	3.8
Tyr	5.10	5.10	100	4.4
Phe	5.04	5.04	100	4.3
Try	5.02	4.97	99	5.4

* Average of seven determinations.

4.6 mm I.D. column, with methanol–0.1 M KH_2PO_4 as the eluent, as shown in Fig. 6. The capacity ratios were Lys, His, CySH and Arg 0.00, Met 0.24, Leu and Ileu 0.52, Tyr 0.76, Phe 2.3 and Try 6.7. To obtain a stream of pH 8.0 and with a flow-rate of 1.0 ml/min at the entrance end of the enzyme column, the flow-rate of the eluent was fixed at 0.8 ml/min.

Calibration

Two calibration graphs were prepared, plotting amino acid concentration in the ranges 5–50 μM and 50 μM–0.1 mM against peak area. There was a downward curvature at concentrations above 0.1 mM because of oxygen deficiency. The calibration graph was found to be suitable for Tyr, Phen, Try and Met. The linearity of the calibration graph for Try was limited to the concentration range 10 μM–0.1 mM. The gradient elution technique was not suitable for this method because a calibration graph must be prepared for each amino acid.

The results for standard solutions showed that Tyr, Phe and Met in the range range 0.25–5 nmol and Try in the range 0.5–5 nmol can be determined with a coefficient of variation not exceeding 10% (Table I).

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