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CONDUCTOMETRIC MONITORING OF THE AMINO ACID OXIDASE REACTION AS A DETECTOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The deamination of L-amino acids by L-amino acid oxidase, which creates a change in the ionic strength of an HPLC eluent, is used to allow conductometric detection of L-amino acids. An example separation is given, and the conductometric method is compared to other methods of amino acid detection in the area of detection limits. Suggestions are also given for using this principle to create detectors based on other class selective enzymes.

INTRODUCTION

Analysis of amino acids by high-performance liquid chromatography (HPLC) is becoming increasingly widespread. Occasionally UV detection at short wavelengths is used[1], but usually some kind of derivatization step is necessary before detection. Derivatization is used because the amino acids do not have structures which easily lend themselves to the traditional detection methods such as spectroscopy or electrochemistry. These derivatizations can be carried out either before or after the separation of the amino acids. Detection of the derivatized products is by either spectroscopy[2–5], electrochemistry[6] or chemiluminescence[7,8]. The most common methods involve derivatization with either dansyl chloride[5] or with *o*-phthalaldehyde[2,4]. Recently, detection schemes were developed which monitor the decrease in metal ion catalyzed chemiluminescence due to metal ion–amino acid complexes[9,10]. Postcolumn reaction detectors for HPLC have been recently reviewed[11].

The reaction between an amino acid and oxygen in the presence of amino acid oxidase (AAO) is shown below:

$$\text{L-amino acid} + H_2O + O_2 \xrightarrow[\text{pH 7}]{\text{L-AAO}} \text{2-oxo acid} + NH_3 + H_2O_2 \qquad (1)$$

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Amino acid oxidases are specific for either the L or D enantiomer; L-AAO does not act on D-amio acids. If the substrate and enzyme have the right chirality then the reaction is as in eqn. 1. AAO is a flavoprotein with two prosthetic groups[12], and has been isolated from rat liver[13] and snake venom[14]. The rat liver extract also acts on α-hydroxy acids, such as lactic acid. The enzyme has highest activity for amino acids with non-polar groups on the alpha carbon. Non-chiral and charged amino acids, such as alanine and aspartic acid, show little or no reaction[3,13,15]. The enzyme is inhibited by high concentrations of substrate (*e.g.* > 6.7 m*M* leucine)[12]. This inhibition seems to be related to the amount of oxygen present and may be due simply to oxygen depletion. The pH maximum for the enzyme is around 7.2–7.5, although significant activity is retained even at pH 9. Molecular weights of 130 000 and 88 900 have been reported[12–14].

Recent work in our group has demonstrated the feasability of conductometric monitoring of enzyme–substrate reactions in flow injection analysis[16]. The amino acid oxidase reaction was chosen for use as an extension of this technique. The NH_3 and the oxo acid formed in the enzymatic reaction then react to yield NH_4^+ and the oxo acid anion which will increase the conductance of the solution. Since amino acid oxidase responds to several amino acids, some method of separation is needed before the enzyme substrate reaction can take place. In this manner an HPLC detector for amino acids could be created. This paper reports the results of experiments employing conductometric monitoring of the amino acid oxidase catalyzed deamination of amino acids as a detector for liquid chromatography.

EXPERIMENTAL

Instrumentation

The instrumental set-up used can be seen in Fig. 1. The computer controlled bipolar pulse conductance (BICON) instrument used has been described in detail elsewhere[17]. There are a few features of note relative to this work. The instrument has a multiplexed input to make measurements on multiple cells. There are also on-board timing capabilities which were used to control the time between data points on each cell. Extensive use was made of the instrument's floating point calculation capabilities. The instrument was used in the voltage pulse mode with 1 V pulse height and 10 μs pulse width. The conductance reading was output to a chart recorder.

The conductance cells were made from two pieces of plexiglas with platinum rods (1.0 mm dia.) for electrodes. The design was based on previous work in our

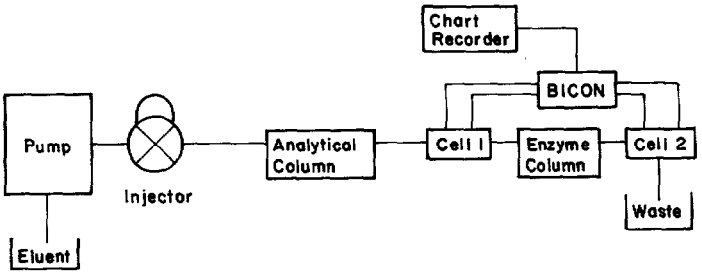

Fig. 1. Block diagram of system.

group on conductance flow cell design[18]. The electrodes were polished flush with the plexiglas surface. The two plexiglas halves were separated by a PTFE spacer 0.38 mm thick. The electrode faces were directly opposite each other when the cell was assembled. The total cell volume was approximately 10 μl for cell 2 and 30 μl for cell 1. The larger volume for cell 1 was needed to reduce pump noise.

Amino acid oxidase was covalently bound to controlled-pore glass (CPG) and contained in an immobilized enzyme reactor (IMER) made of a plexiglas column (57 mm × 3.2 mm) capped with nylon end fitting with 1/4"–28 couplings. The caps had 20-μm stainless-steel frits to contain the CPG.

The HPLC pump was a single piston altex Model 110A The flow-rate was 0.7 ml min^{-1}. The injector was a Rheodyne Model 7120 with a 100-μl injection loop. A Rainin Microsorb Short-one C_{18} column (10.0 cm × 4.6 mm, 3 μm packing) was used as the analytical column. A short pre-column and an in-line filter were used to protect the analytical column. The analytical and enzyme columns were enclosed in urethane foam to regulate the temperature.

Reagents

The amino acids (Sigma), glutaraldehyde (Kodak), $NaH_2PO_4 \cdot H_2O$ (Mallinckrodt), and methanol (Fisher) were used as received. The amino acid oxidase (Sigma, E.C. 1.4.3.2 Type VI) was from *Crotalus Adamanteus*. Millipore/Milli-Q water was used to prepare solutions and mobile phase.

Enzyme immobilization scheme

The enzyme was immobilized on the CPG using a modification of a procedure developed by Klopf and Nieman[19]. A 0.5-g quantity of CPG (Electronucleonics, 250 Å pore diameter, 125–177 μm particle diameter) was washed with concentrated nitric acid, filtered, washed with water, and then silanized by reacting at 90°C for 3 h with (3-aminopropyl)triethoxysilane in 0.05 *M* acetate buffer at pH 5.0. Approximately 0.5 ml of the silane was added to 30 ml of the buffer which had reached the reaction temperature. The CPG was then filtered and washed in a sintered glass filter. To the silanized CPG, 1 ml of glutaraldehyde (25%) and 1 ml of 0.1 *M* pyrophosphate buffer, pH 7.0, were added. The mixture was kept at room temperature for 2.5 h. The first 30 min of the reaction were done under vacuum to reduce the partial pressure of oxygen. The CPG was again filtered and washed. Amino acid oxidase was attached by placing the CPG in a filter flask and adding 10 ml of enzyme solution which contained 10 mg/ml of amino acid oxidase in a 0.1 *M* pyrophosphate buffer at pH 7.0. The reaction was carried out at 4°C. Again the first 30 min of the reaction were done under vacuum. The CPG was left in contact with the enzyme solution for approximately 24 h in a refrigerator. The last step was to filter and wash the CPG and store it in phosphate buffer (0.1 *M*, pH 7).

Procedure

Samples of amino acids were prepared in the mobile phase. The mobile phase for most of the work reported here was 2.5 m*M* phosphate buffer at pH 7.3. A mobile phase of methanol–water (4:96, 2.5 m*M* final phosphate buffer concentration pH 7.3) was also tried. The samples were injected and the conductance monitored on either cell 1 or cell 2 using the chart recorder output. Peak heights were recorded manually

from the chart recorder output. The offset and gain options in the program were used to increase the sensitivity. The analytical column and pump were stored in methanol when not in use. The IMER was stored in phosphate buffer. The IMER was disconnected from the LC set-up until the methanol had been flushed out with water. If this was not done the methanol would cause the enzyme column to lose its activity. The small amount of methanol used in the mobile phase mentioned above did not degrade the IMER.

RESULTS AND DISCUSSION

Initial IMER testing

After immobilization, the column was checked to see if the enzyme was active and to get an estimate of the conversion efficiency. To see if the IMER was active, solutions of phenylalanine (Phe) were injected and the conductance monitored before and after the IMER. No peaks above baseline were seen before the IMER, as expected. After the IMER, injections of varying concentrations of Phe gave peaks of different heights. The peak heights increased monotonically as solutions of 0.1–1 m*M* were injected. When 10 m*M* Phe was injected the peak height was the same as for 1 m*M* Phe but the peak was quite broad. This peak shape could be due to adsorption of the amino acid onto the CPG or interaction of the enzyme and the substrate. For future work the amino acid concentrations were kept at or below 1 m*M*. Further testing of the IMER was done by placing it in a flow injection set-up which monitored the concentration of hydrogen peroxide using electrogenerated luminol chemiluminescence[20]. By comparing the peak from an injection of hydrogen peroxide with one from an injection of Phe, it was possible to estimate the conversion efficiency of the IMER. This method indicated the IMER was 90–100% efficient. No peak was obtained if the IMER was not in the flow system; this indicated that nothing else in the sample was giving false chemiluminescence signals.

Separation of amino acids

Once it had been determined that the IMER would work, the next step was to use it as a detector for the HPLC set-up. Kiba and Kaneko[3] had used AAO in a fluorescence detection mode so initially their separation procedure was used with slight modifications. The mobile phase was methanol–water (4:96) which contained 2.5 m*M* phosphate buffer, pH 7.3. A mixture of Phe, arginine (Arg) and methionine (Met) each at 1m*M* was injected. The elution order was Arg (3 min), Met (6 min), Phe (17 min), just as in the literature[3]. The Arg peak was not retained, but eluted at the same time as the void peak. This was expected since at the pH of the analysis Arg has a net +1 charge and this was a reversed-phase separation. Also, little reaction, based on peak heights before and after the IMER, was seen for Arg. Before the IMER, only the arginine peak was seen. A solution containing Phe, Met, leucine (Leu) and tyrosine (Tyr) was then used. Enough solid Tyr was added to make a 1 m*M* solution, but due to solubility problems the Tyr concentration was approximately 0.7 m*M*. The elution order was Met, Leu, Tyr, Phe. The Tyr and Leu peaks were not well resolved. At this point the mobile phase was changed to 100% water with 2.5 m*M* phosphate buffer. The Tyr and Leu peaks were much better resolved with this mobile phase although still not completely.

Fig. 2 shows the responses for cell 1 and cell 2 following separation of a mixture of amino acids. With the exception of a small void peak there is nothing of interest on cell 1. This void volume peak is proportional to the amount of unretained ions, including background ions. Since the background ions have been separated from the analyte there is no need to monitor the reaction before the IMER. Occasionally a small peak, or dip, may be seen on cell 1 and on cell 2 approx. 5–6 min after injection. This seems to be related to air, or more likely carbon dioxide, in the sample. An injection of deaerated mobile phase gave no peak. An injection of mobile phase after aeration did give a peak. On cell 2, after reaction in the IMER, the eluting amino acids could be seen. The elution order was Met, Tyr, Phe, Trp.

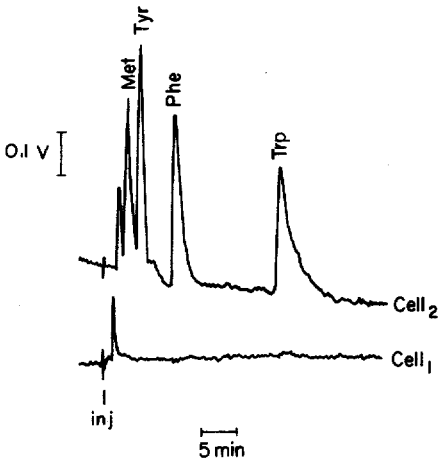

Fig. 2. Chart recorder tracings from a separation of amino acids. Cell 1 is before and cell 2 is after the IMER.

The broadness of the peaks was originally thought to be due to the large size of the CPG in the IMER and the presence of the conductance cell before the column. In an effort to improve the quality of the chromatogram, the enzyme was immobilized on smaller CPG particles (Electronucleonics, 37–74 μm, 500 Å pore size), cell 1 was removed, a smaller (50 μl) sample loop was added, and a Dionex conductance detector (1.5 μl cell volume) was used. Removal of cell 1 and use of the smaller CPG particles were tried to reduce the system dead volume. The smaller sample loop was tried in case too much sample was being placed on the column. None of the modifications significantly reduced the width of the peaks.

Working curves

After establishing that the column would work as a detector for HPLC, working curves with various amino acids were created. Fig. 3 shows the data from injections of mixtures of Phe and Tyr. The upper limit on the Tyr curve is the solubility limit for Tyr. The difference in slopes between the two curves is due to the Phe peak being broader since it is retained longer. Working curves of peak area *vs.* concentration are co-linear. Differences in the relative activity of the enzyme towards the sub-

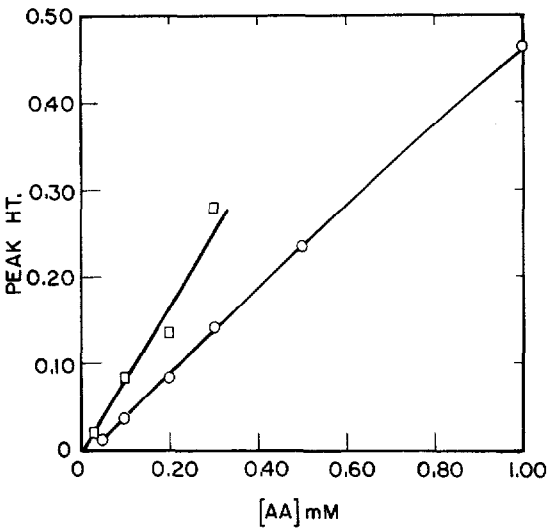

Fig. 3. Working curve for phenylalanine (○) and tyrosine (□) using HPLC with conductometric detection.

strates could also cause slope differences, although this is not the case for Phe and Tyr. The Phe curve is basically linear from 0.05 to 0.5 m*M*. The slight downward trend at 1 m*M* is probably due depletion of oxygen or perhaps peak broadening. The oxygen depletion could be compensated by co-immobilization of catalase with the amino acid oxidase. Catalase catalyzes the conversion of hydrogen peroxide to water and oxygen. The regenerated oxygen could be reacted with more amino acid. The detection limit with this present set-up is approx. 0.03 m*M*. With the 100-μl injector used this corresponds to 3 nmol of injected material. For a comparison with other methods see Table I. The detection limit could be improved by better temperature regulation and better instrumentation.

TABLE I

COMPARISON OF AMINO ACID LC DETECTORS

Detection method	*Detector*	*Limit (pmol)*	*Ref.*
o-Phthalaldehyde derivatization	UV–VIS	500	2
	Fluorescence	50	4
	Voltammetry	0.015	6
Dns-Cl derivatization	Fluorescence	100	5
	Chemiluminescence	0.005	8
Isoluminol	Chemiluminescence	0.025	7
Metal ion complexation	Chemiluminescence	1000	10
	Amperometry	100	21
Reaction with amino acid oxidase	Fluorescence	250–5000	3
	Conductance	3000	This work

CONCLUSIONS

The data show the usefulness of conductiometric monitoring of the amino acid oxidase reaction as a detection method for HPLC. With better instrumentation the detection limits could be made lower, although they would not approach some of the more sensitive detection mechanisms. The ability to find a separation scheme which is compatible with the IMER is critical. A little methanol in the mobile phase does not hurt the IMER. Also using a surfactant in the mobile phase does not hurt enzymatic activity, although no long term data is available; this would allow the use of ion-pair chromatography. It may be necessary to use multiple separation schemes to adequately separate and identify the various components in a mixture. One of the main deficiencies of the method is the enzyme's inactivity to some amino acids (non-chiral and those with charged groups). In those cases where the unreactive amino acid is a charged species, such as aspartic acid, it may stil be possible to use conductance in a manner similar to ion chromatography. Another limitation is the pH range of the enzyme in the IMER. The mobile phase would need to be between pH 6.5 and 9 for the enzyme to operate correctly.

Although in some cases the conductance method may not be sensitive enough for trace analysis, because of the simplicity of the method it certainly presents a viable alternative for routine analyses. Except for this method and that of Kok *et al.*[21], all of the other approaches outlined in Table I require either off-line derivatization (a time disadvantage), or post-column derivitization (a disadvantage due to resolution degradation).

An advantage of an amino acid detector based on post-column reaction with amino acid oxidase is the resulting stereospecific detection. With an L-AAO column (as demonstrated here) only L-amino acids are detected. Similarly, a D-AAO column would result in detection of only D-amino acids. With first an L-AAO column and then a D-AAO column in sequence, one could place conductance cell 1 after the L-AAO and conductance cell 2 after the D-AAO. Then cell 1 would monitor L-amino acids, cell 2 would monitor the sum of L- and D-amino acids, and the difference between the two cells would yield the D-amino acids. All this information would result from a single injection.

In a broader sense, this work with amino acid oxidase indicates the possibility for using other class selective enzymes to create conductometric HPLC detectors. Using class selective enzyme systems with conductometric monitoring for HPLC detection can be extended in two ways. One way is to use another class selective enzyme. Examples include aldehyde oxidase (E.C. 1.2.3.1), amine oxidase (E.C. 1.4.3.4), or lipase (E.C. 3.1.1.3). The second way is to precede the ion-producing IMER with an IMER containing an enzyme which produces compounds which are substrates for the second IMER. For example, amino peptidases (E.C. 3.4.11.X) and carboxypeptidases (E.C. 3.4.17.X) release amino acids from the N and C terminals, respectively, of peptides. The amino acids released could be separated and detected using the amino acid oxidase reactor just described. In these ways, conductometric monitoring of enzymatic reactions as a detector for HPLC could be extended to several areas.

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