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High-performance Liquid Chromatographic Analysis of Analogous Amino and Oxo Acids for the Determination of Amino Acid Oxidase and Transaminase Activities

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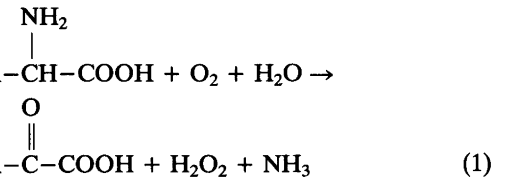
An analytical method, based on reversed-phase high-performance liquid chromatography, for the determination of analogous amino and oxo acids, has been developed. The assay may be employed to monitor amino acid oxidase and transaminase activities. Simultaneous quantitative determination of substrate and product is readily achieved. In particular, the amino acid oxidase activity within whole microbial cells immobilized by entrapment in Ca^{2+}-alginate has been studied. The assay of transaminases has been exemplified with glutamic-pyruvic transaminase.

During recent years we have been involved in the development of a technical process for the production of 2-oxo acids, in particular the analogues of the essential amino acids, which have a potential use in the treatment of acute uremia. To this end we have immobilized microbial cells containing an amino acid oxidase by entrapment in Ca^{2+}-alginate gels.[1,2] The amino acid oxidase within the entrapped cells converts amino acids to the corresponding oxo acids according to eqn. (1).

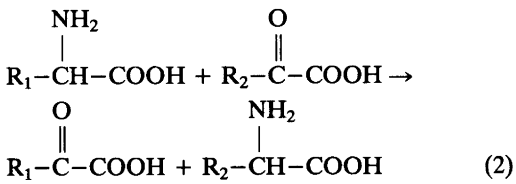


$$R-\underset{\displaystyle NH_2}{\underset{|}{CH}}-COOH + O_2 + H_2O \rightarrow R-\underset{\displaystyle O}{\underset{\|}{C}}-COOH + H_2O_2 + NH_3 \qquad (1)$$

In the initial studies on immobilized amino acid oxidases, standard assays for the determination of amino and oxo acid concentrations were employed, *i.e.* formation of the 2,4,6-trinitrophenyl and 2,4-dinitrophenyl hydrazone derivatives, respectively, with subsequent colorimetric measurements.

A more convenient method of assaying the oxidase activity has, however, been developed. The new method, which is based on reversed-phase HPLC, has some distinct advantages over the colorimetric methods mentioned above, such as simultaneous determination of substrate and product concentrations. Furthermore, when studying crude systems, *e.g.* enzymes within whole cells, the method allows for detection of side reactions.

The HPLC-assay may also be used for the determination of the activity of transaminases, which catalyze the reaction of eqn. (2).



$$R_1-\underset{\displaystyle NH_2}{\underset{|}{CH}}-COOH + R_2-\underset{\displaystyle O}{\underset{\|}{C}}-COOH \rightarrow R_1-\underset{\displaystyle O}{\underset{\|}{C}}-COOH + R_2-\underset{\displaystyle NH_2}{\underset{|}{CH}}-COOH \qquad (2)$$

In principle, the concentration of the four compounds may be determined simultaneously.

RESULTS AND DISCUSSION

The method presented here has been developed in order to determine the activity of

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Table 1. Retention times and capacity factors for various α-amino and α-keto acid pairs.

R–	H_2O:MeOH (9:1) R–$CHNH_2COOH$ t_R (min)	k'	R–COCOOH t_R (min)	k'	H_2O:MeOH (8:2) R–$CHNH_2COOH$ t_R (min)	k'	R–COCOOH t_R (min)	k'
$HOOC-CH_2-CH_2-$	2.10	0.05	2.00	0.00	2.05	0.025	2.00	0.00
H_3C-	2.40	0.20	2.30	0.15	2.25	0.125	2.15	0.075
H_3C-CH_2-	2.50	0.25	2.75	0.375	2.30	0.15	2.40	0.20
$H_3C-CH_2-CH_2-$[b]	2.80	0.40	3.85	0.925	2.60	0.30	3.15	0.575
H_3C / H_3C CH–	2.70	0.35	3.55	0.775	2.55	0.275	2.85	0.425
$H_3C-CH_2-CH_2-CH_2-$[b]	3.90	0.95	7.60	2.80	3.40	0.70	4.90	1.45
H_3C / H_3C $CH-CH_2$	3.70	0.85	7.05	2.525	3.15	0.575	4.55	1.275
CH_3 / H_3C-CH_2-CH-	3.40	0.70	5.80	1.90	3.05	0.525	3.95	0.975
$H_3C-S-CH_2-CH_2-$	3.20	0.60	4.45	1.225	2.80	0.40	3.25	0.625
$H_3C-CH_2-S-CH_2-CH_2-$[b]	4.65	1.325	7.90	2.95	3.80	0.90	4.80	1.40
$Ph-CH_2-$	7.00	2.50	12.00	5.00	4.60	2.30	6.25	2.125
$3,4-OH-Ph-CH_2-$	4.20	1.10	5.80	1.90	3.10	0.55	4.10	1.05
$4-OH-Ph-CH_2-$	3.50	0.75	4.35	1.175	2.70	0.35	3.00	0.50
$3\text{-Indolyl}-CH_2-$	n.d.[a]	n.d.	n.d.	n.d.	6.70	2.35	8.50	3.25

[a] n.d.=not determined. [b] The keto acid was prepared from the amino acid as described in Experimentals.

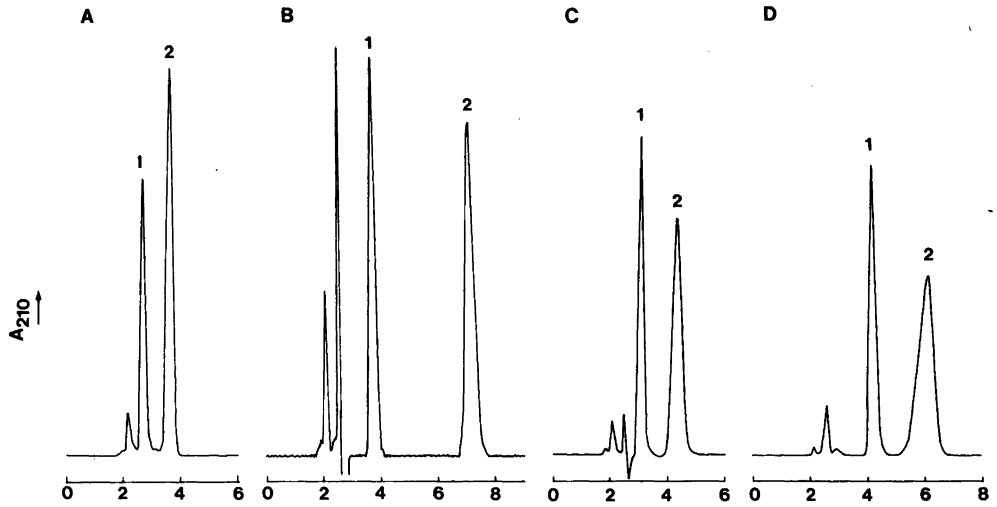

Fig. 1. Isocratic separation of some analogous amino and oxo acids by HPLC. A. 1=methionine, 2=4-methylthio-2-oxobutanoic acid; medium: H_2O:MeOH (8:2). B. 1=leucine, 2=4-methyl-2-oxopentanoic acid; medium: H_2O:MeOH (9:1). C. 1=tyrosine, 2=3-(4-hydroxyphenyl)-2-oxopropionic acid; medium: H_2O:MeOH (8:2). D. 1=phenylalanine, 2=phenylpyruvic acid; medium: H_2O:MeOH (8:2).

amino acid oxidases within immobilized microbial cells.[1,2] The main advantages of a HPLC assay over standard colorimetric methods are the following:

1. Simultaneous determination of substrate and product concentrations is possible.
2. Since whole microbial cells are utilized, side reactions may take place which are readily detected with this assay.
3. The assay is relatively simple, rapid and accurate.

Fig. 1 shows some typical examples of amino and oxo acid separations. Table 1 lists a number of acids with their corresponding retention times (t_R) and capacity factors (k') in two elution systems.

Some of the keto acids listed, a few of which are not commercially available, were prepared from the corresponding amino acids by utilizing immobilized *Trigonopsis variabilis* cells, which contain a D-amino acid oxidase in relatively high amounts.[1,3] The conversion is conveniently followed by application of the appropriate HPLC system. As the amino acid peak decreases in size, a new peak is observed which continuously increases in size until the D-amino acid is consumed. It can be pointed out that catalase was included in the reaction mixture to avoid a secondary spontaneous reaction between the formed oxo acid and hydrogen peroxide, *i.e.* decarboxylation of the oxo acid.[1] It was also shown that a carboxyl compound was formed by formation of the 2,4-dinitrophenylhydrazone derivative.[1] The new peak observed in the elution profile was therefore assigned the oxo analogue of the amino acid used. The oxo analogues of norvaline, norleucine, 4-ethylthio-2-aminobutanoic acid (ethionine) and 3,4-dihydroxyphenylalanine were prepared in this way (Table 1).

As can be seen in Table 1, the most polar acid pairs (*e.g.* alanine–pyruvic acid and glutamic acid–2-oxoglutaric acid) are not separated in the systems investigated and consequently cannot be assayed by this method. Acids with an aliphatic or aromatic side chain are, on the other hand, separated and the concentration of these acids is readily determined with standard curves. It can be pointed out that most of the interesting acids, *i.e.* the acids containing the essential carbon skeleton for potential use in the treatment of acute uremia, can be analyzed. Some standard curves are shown in Fig. 2. Here it can be seen that amino acids with a side chain without substantial absorbance at 210 nm, such as valine, leucine, isoleucine, norvaline and norleucine,

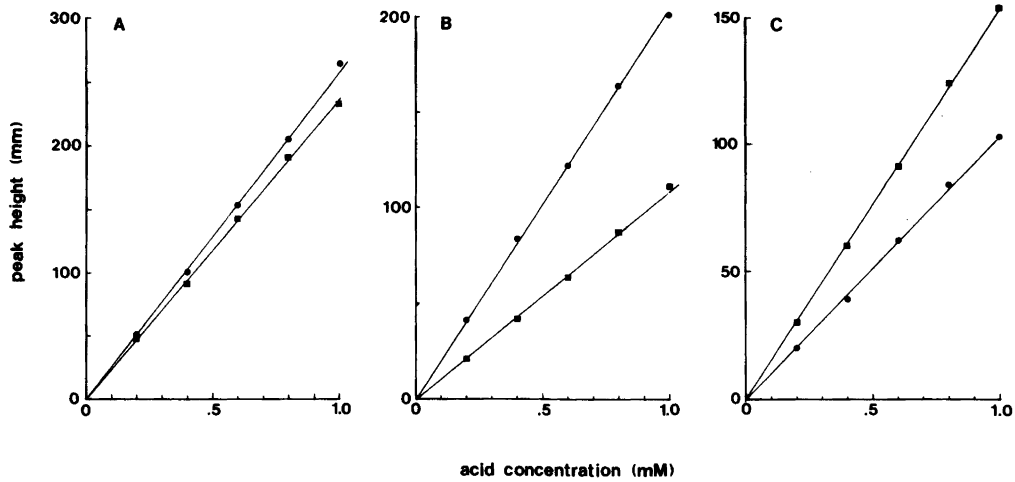

Fig. 2. Calibration curves for some analoguous amino and oxo acids. A. Methionine (■) and 4-methylthio-2-oxobutanoic acid (●); recorder sensitivity 0.1 a.u.f.s. B. leucine (■); recorder sensitivity 0.01 a.u.f.s. 4-methyl-2-oxopentanoic acid (●); recorder sensitivity 0.1 a.u.f.s. C. phenylalanine (■) and phenylpyruvic acid (●): recorder sensitivity 0.1 a.u.f.s.

require a relatively high sensitivity for detection. The minimum concentration of all these amino acids which can be determined (at a peak to noise ratio of 3) is around 50 μM (with a sample size of 10 μl corresponding to 50–100 ng of injected amino acid). The sensivity of the analysis can, of course, be improved by injecting larger samples. For keto acids and amino acids with side chains absorbing at 210 nm (*e.g.* methionine, 4-ethylthio-2-aminobutanoic acid, phenylalanine, tyrosine and tryptophan) the minimum concentration which can be determined is considerably lower. For instance, for phenylalanine and methionine, the minimum concentration is about one tenth of that for aliphatic amino acids, or 5–10 ng of injected acid. As can be seen in Fig. 2B, the observed peak heights of the oxo analogue of leucine is about 20 times higher than those for leucine itself. For simultaneous determination of acid pairs of this kind it is very convenient to use a two channel recorder set at two appropriate sensitivities.

The retention times were, as expected, decreased when the methanol content in the mobile phase was increased (Table 1). The decrease in retention time was accompanied by a decreased separation of the amino and oxo acids. When the methanol content was increased to 30 % (v/v) most of the studied acid pairs were not separated at all. Only tryptophan–3-(3-indolyl) propanoic acid and phenylalanine–phenylpyruvic acid were separated at this high methanol concentration with retention times of 4.50–4.90 and 3.25–3.90 min, respectively. With 10 or 20 % methanol in the mobile phase, most of the acid pairs studied could be separated and their concentrations determined within 5 min.

To illustrate the use of the method described, the D-amino acid oxidase within immobilized *T. variabilis* cells was assayed using various D- and D,L-amino acids as substrates. The continuous conversion of D-methionine to 4-methylthio-2-oxobutanoic acid is illustrated in Fig. 3. It can be seen that the reaction is quantitative. Catalase was added to the reaction mixture to avoid the secondary reaction mentioned above. Similar patterns are observed for a number of amino acids, such as valine, norvaline, leucine, norleucine, isoleucine and phenylalanine. The D-amino acid oxidase in *T. variabilis* has a broad substrate specificity[1] and can therefore be used in a technical process for the production of various oxo acids from the corresponding amino acids.

To demonstrate the possible use of this HPLC assay for the determination of transaminase activity, glutamic-pyruvic transaminase was employed as a model system. This enzyme normally

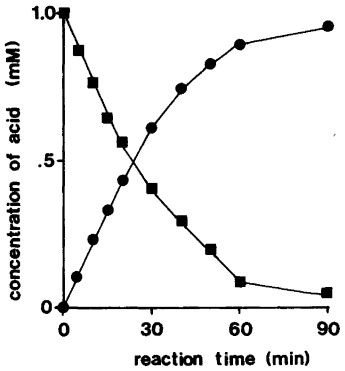

Fig. 3. Stoichiometry of the transformation of methionine to 4-methylthio-2-oxobutanoic acid by immobilized cells of *T. variabilis* as analyzed by HPLC. Methionine (■) and 4-methylthio-2-oxobutanoic acid (●).

catalyzes the interconversion of 2-oxoglutaric acid–alanine and glutamic acid–pyruvic acid. The enzyme shows, however, a very low activity (0.08 %) with norleucine and 2-oxo-glutaric acid as substrates[4] and this could also be verified with the assay method described here. Some 2-oxo-hexanoic acid was formed in the presence of the enzyme after 24 h.

Normally, transaminases are assayed by a coupled enzymatic assay. With such an assay it is, however, impossible to determine the concentration of reactants at equilibrium which may be of interest in some studies, such as the investigation of metabolic concentrations of various amino and oxo acids.

EXPERIMENTALS

A. Chemicals. All 2-amino and 2-oxo acids (used without further purification), glutamic-pyruvic transaminase (E.C. 2.6.1.2) and catalase (E.C. 1.11.1.6) were obtained from Sigma Chemical Co. Methanol (analytical grade) was supplied by BDH and sodium alginate (Manucol DH) by Alginate Industries. Cells of *Trigonopsis variabilis* were grown and immobilized as described elsewhere.[1]

B. Chromatography. Isocratic chromatography was carried out using an Altex Model 1104 pump at a flow rate of 1.2 ml/min; a Valco valve fitted with a 10 μl loop; a 200×4.6 mm stainless steel column packed with Nucleosile-C_{18} (5 μm); a LDC Spectromonitor III variable UV detector operating at a wavelength of 210 nm; and a Cole-Parmer 8373-20 double channel recorder operating at a chart speed of 1.0 cm/min with a full scale sensitivity varying from 0.01 to 2.0 a.u. (depending on the sample analyzed). The mobile phase was mixtures of 10 mM sodium phosphate buffer, pH 6.0, and methanol in various proportions, *i.e.* 9:1, 8:2 or 7:3. Standard solutions (0–1.0 mM) of the appropriate amino or oxo acid were used to obtain a standard curve of peak height versus concentration (see Fig. 2).

C. Preparation of 2-oxo acids. Some 2-oxo acids (see Table 1) were prepared from the corresponding amino acids by utilizing immobilized amino acid oxidase in the following way: to the 2 mM amino acid solution (2.5 ml) in Tris-HCl buffer, pH 8.0, containing catalase (~5 U), alginate beads (0.5 g wet weight) containing *T. variabilis* cells (10 % w/w) were added and the conversion was monitored by removal of samples for analysis in the appropriate HPLC system. The obtained oxo acids were used without purification for determining retention times and capacity factors in the various mobile phases.

D. Assay of enzyme activites. D-amino acid oxidase within immobilized cells of *T. variabilis* was assayed in the following way: to a 1 mM D-amino acid solution (5.0 ml) in Triss-HCl buffer, pH 8.0, containing catalase (~5 U), alginate beads (0.2 g wet weight) containing the microbial cells (10 % w/w) were added and samples were withdrawn every 10 min for analysis in the appropriate HPLC system.

Glutamic-pyruvic transaminase was incubated in the following way: to a substrate solution (2.0 ml) containing 10 mM D,L-norleucine and 5 mM 2-oxo-glutaric acid in 50 mM Tris-HCl buffer, pH 8.0, the enzyme in 1.8 M $(NH_4)_2SO_4$ (5 μl; ~1 U) was added and samples were withdrawn and analyzed in the 8:2-system.

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