Immobilization of ascorbic acid oxidase

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1. INTRODUCTION

Ascorbic acid has long been recognized as an important vitamin [1], and has been shown to exist in high concentrations in the central nervous system of small animals and man. The regional distribution of AA in rat [2] and human [3] brain has been reported. There appear to be homeostatic mechanisms for controlling AA levels, as well as an active uptake mechanism [4]. This, in addition to the many newly reported effects of AA on neuronal tissue, of which just a few are listed, suggests an important role(s) for this molecule in the functioning of mammalian brain [5–7].

Ascorbic acid oxidase (EC 1.10.3.3., AAO) has become a useful tool in the manipulation of [AA] in neuronal tissue preparations [8,9] for the purpose of gaining information on AA directly, as well as removing it as an interference in the electrochemical determination of catecholamine neurotransmitters, serotonin, and their key metabolites. With the intention of obtaining a form of this extremely useful enzyme suitable for use in flowing stream analyses (for example, direct electrochemical analysis of neuronal tissue perfusates), we have immobilized AAO onto Sepharose 4B activated by cyanogen bromide (CNBr). In [10] the enzyme was immobilized in an enzyme electrode configuration; however, this method is unsuitable for the type of flowing stream applications intended. Using the method herein, the enzyme retains a high degree of catalytic activity upon immobilization and appears to be quite stable in the immobilized form. The following is an initial characterization of the immobilized enzyme and a comparison with the soluble form.

Abbreviations: AA, ascorbic acid; AAO, ascorbic acid oxidase; CNBr, cyanogen bromide; DA, dopamine; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid; NE, norepinephrine

2. MATERIALS AND METHODS

AAO was obtained from Boehringer-Mannheim and used without further purification. Other chemicals were obtained from the following commercial sources: Sepharose 4B, CNBr, bovine serum albumin and HEPES (Sigma Chemicals); EDTA and AA (Fisher Chemicals). Buffers and standard stock solutions were prepared as follows:

Physiological buffer:

20 mM HEPES, 5 mM KCl, 120 mM NaCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM glucose, pH adjusted to 7.4 with NaOH.

Assay buffer:

1 mg/ml bovine serum albumin (crystallized and lyophilized), 5×10^{-4} M EDTA, 0.1 M (NaH_2PO_4, Na_2HPO_4) (pH 7.1)

AA stock solution:

10 or 20 mM AA, 0.1 M $HClO_4$, 5×10^{-4} M EDTA (EDTA was present in order to reduce background oxidation of AA).

2.1. *Preparation of immobilized AAO*

The procedure used for CNBr-activation of Sepharose was essentially that in [11]. After CNBr activation, 2–3 ml of settled gel was placed in a small culture tube (~1 cm × 6 cm). To this, 2–3 ml 0.1 M $NaHCO_3$ containing 2–3 mg AAO (weight of lyophilized powder) was added. The

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tube was rotated slowly overnight at 4°C, then the AAO–Sepharose was rinsed with 200 ml H_2O on a glass frit over vacuum. Finally, the resin was reacted with 2 ml 1 M glycine in 0.1 M $NaHCO_3$ (pH8) for 2 h at room temperature with turning as before. After rinsing with 250 ml H_2O, the AAO–Sepharose was ready for use. A control preparation of glycine–Sepharose was prepared by reacting CNBr-activated Sepharose with 1 M glycine.

To determine the extent of protein immobilization, A_{280} of the AAO solution prior to addition to the Sepharose was noted, as was A_{280} for the first 7.5 ml of rinse following incubation. The difference in absorbance (after dilution corrections) was asumed to be due to protein which was immobilized.

2.2. *Assay of AAO and immobilized AAO*

To assay for AAO activity, the change in [AA] was followed by chronoamperometry [12]. This method involves applying a fixed potential (in this case, 0.6 V *vs* and Ag/AgCl reference electrode [Bioanalytical Systems]) to an inert solid electrode (carbon paste or glassy carbon [Bioanalytical Systems]) and measuring the resultant anodic current due to the oxidation of AA after a fixed time (1 s). A PAR model 174 polarographic analyzer and Houston 2000 $X-Y$ recorder with time base were used to generate and record the current-time curves.

All assays were done in 10 ml physiological or assay buffer at ambient temperature. Chronoamperometric measurements were generally taken every 30 s with ~ 5 s of stirring immediately following each measurement. This stirring was necessary to disrupt the diffusion layer at the electrode surface in which AA had been depleted, and to keep the immobilized enzyme in suspension. Kinetic parameters were determined by taking the slope of the [AA] *vs* time curve at various points, thus giving $\Delta\mu M/\Delta T$, or reaction velocity at varying [AA].

3. RESULTS AND DISCUSSION

3.1. *Comparison of the catalytic properties of AAO and AAO–Sepharose*

Fig.1 shows a comparison between the catalytic activity of soluble AAO, AAO–Sepharose, and glycine–Sepharose. Clearly there is a catalytic

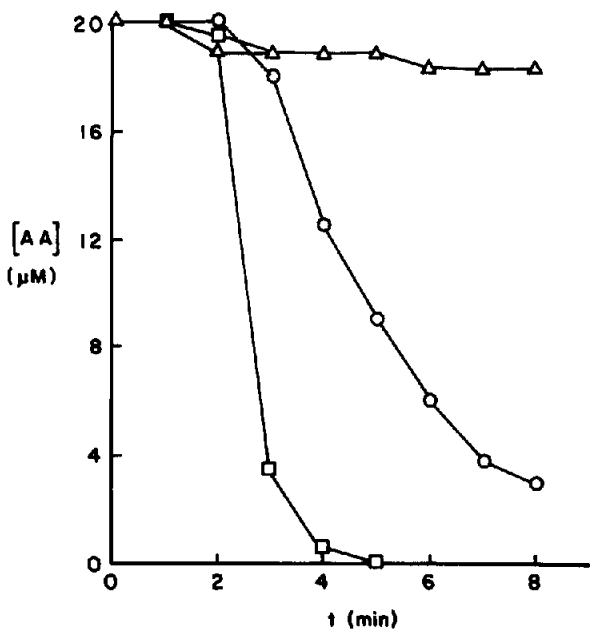


Fig.1. Comparison of the catalytic activity of ~50 μg soluble AAO (lyophilized powder,□), 0.05–0.1 ml AAO–Sepharose. (○) and 0.05–0.1 ml glycine–Sepharose (△). (→) Additions to 20 μM AA in 10 ml physiological buffer. Changes in [AA] were measured as in section 2.

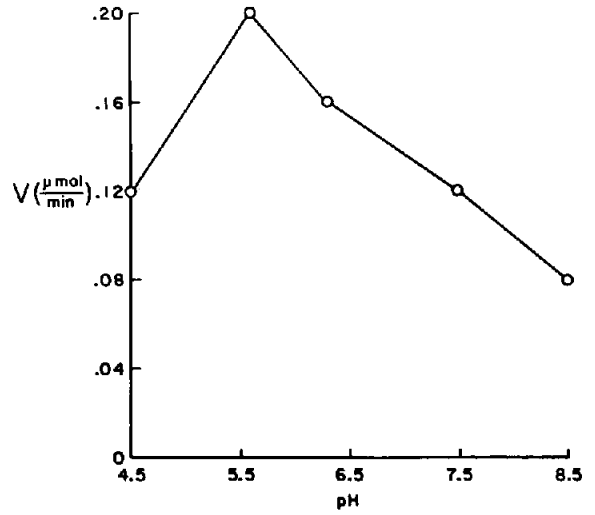
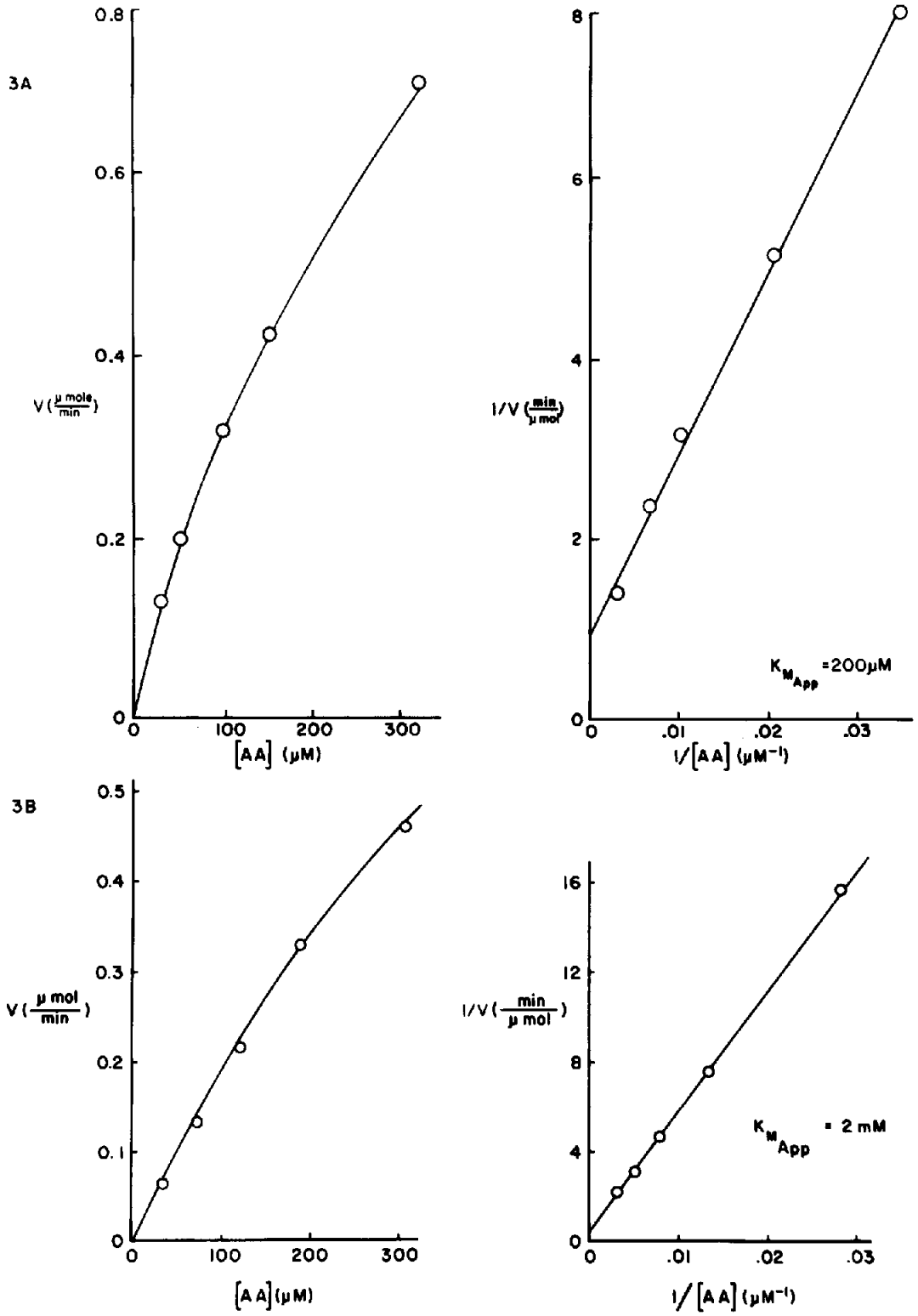


Fig.2. pH profile of AAO–Sepharose activity. Aliquots of AA were added to 54 mg AAO–Sepharose, and activity was determined. Between the addition of each aliquot, sufficient time was allowed for complete oxidation of AA, and the pH was adjusted with NaOH. A control run showed the catalytic activity of AAO–Sepharose was undiminished by repeated additions of AA.


3A
0.8
0.6
0.4
0.2
0
V (μ mole / min)
0
100
200
300
[AA] (μM)
8
6
4
2
0
1/V (min / μ mol)
0
.01
.02
.03
1/[AA] (μM⁻¹)
K_M App = 200μM
3B
0.5
0.4
0.3
0.2
0.1
0
V (μ mol / min)
0
100
200
300
[AA] (μM)
16
12
8
4
0
1/V (min / μ mol)
0
.01
.02
.03
1/[AA] (μM⁻¹)
K_M App = 2 mM

activity which is not an artifactual property of AAO–Sepharose. To insure that AAO was indeed covalently bound to Sepharose, not just strongly adsorbed to the Sepharose, 2 additional control experiments were conducted. The first control experiment was to incubate the AAO overnight with unactivated Sepharose, and after rinsing as with the activated form, the gel was tested for catalytic activity; none was found. In the second control experiment, AAO–Sepharose was assayed for catalytic activity following repetitive rinsings on a glass frit over a vacuum. A sample of AAO–Sepharose was washed 4 times with 250 ml water. Between washes, an aliquot was removed and assayed for AAO activity. No decrease in activity resulted during this exhaustive washing process.

3.2. *Catalytic properties of AAO and AAO–Sepharose*

The pH profile of AAO–Sepharose activity was determined and the results are shown in fig.2. The pH optimum of 5.6 for AAO–Sepharose is the same as that reported for soluble enzyme [13]. K_m-values for AA as a substrate for the soluble and immobilized forms of AAO were also compared. There was a marked increase in the K_m of AA for the immobilized enzyme as compared to that for the soluble enzyme (fig.3). This is consistent with a diffusional process being involved in the catalytic activity of the immobilized AAO [14]. We encountered difficulty in obtaining reproducable K_m-values for the immobilized enzyme, probably because the suspension of AAO–Sepharose and AA was stirred by hand following each chronoamperometric measurement. A lack of a consistent stirring rate would have a marked effect on the diffusion of AA, presumably into recessed areas of the Sepharose beads which contain active enzyme. The K_m-values determined for AA with the soluble AAO ranged from 100 – 350 μM, which is in fairly good agreement with apparent values calculated from the literature of 250 μM [15] and 330 μM [16].

At 50 μM AA, the specific activity of soluble AAO was determined to be 0.016 μmol AA oxidized $\cdot$ min^{-1} $\cdot$ μg lyophilized AAO^{-1}. On two consecutive runs, the specific activity of AAO–Sepharose was found to be 0.0038 and 0.0031 μmol AA oxidized $\cdot$ min^{-1} $\cdot$ μg AAO–Sepharose^{-1}. Based on the amount of protein immobilized, which was determined to be 60%, these specific activities indicate that the enzyme was immobilized with 24% and 19% apparent retention of activity, respectively. The immobilized enzyme when stored at 12°C retained catalytic activity for at least 30 days.

3.3. *Catalytic specificity of AAO–Sepharose*

Soluble AAO will not oxidize dopamine (DA) or dihydroxyphenylacetate, a key metabolite of DA [9]. Because these compounds, in addition to similar catechols, will be present when the AAO–Sepharose is applied to brain tissue perfusates, the specificity of AAO for AA over catechols was reaffirmed. Physiological concentrations of DA and norepinephrine (NE) were not oxidized by the AAO–Sepharose (not shown), conforming the immobilized enzymes' high specificity for AA.

3.4. *Conclusion*

We demonstrate that AAO can be immobilized with reasonable retention of activity onto Sepharose using CNBr activation. The pH optimum agrees with that for soluble AAO. The app. K_m for AA is increased upon immobilization, consistent with a diffusional process being involved in the catalytic activity. The immobilized enzyme retains the specificity necessary for use in neurochemical investigations, and promises to be a useful tool in the study of neuronal tissue perfusates.

ACKNOWLEDGEMENTS

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Fig.3. Comparison of kinetics of soluble AAO (3A), and AAO–Sepharose (3B). Reaction velocities at varying [AA] in assay buffer were determined as in section 2. Apparent K_m-values were calculated using linear regression analysis of Lineweaver–Burke plots.

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