# Application of a glutamate microsensor to brain tissue

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# Chapter 4

## *Improving the performance of glutamate microsensors by purification of ascorbate oxidase.*

*This chapter is based on the following paper:*

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### *Abstract*

*Enzyme-based biosensors have the potential to detect extracellular concentrations of glutamate directly in brain tissue with a high spatial and temporal resolution. To optimize their analytical performance, much attention has been paid to the architectural construction of such biosensors. In particular, the coupling of enzymes to the electrode surface has received much interest, which has resulted in many (derivatives of) 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ generation type of biosensors. However, it is remarkable that in literature little attention, if any, has been paid to the influence of the quality of the enzyme itself on the analytical performance of a biosensor. In the previous chapter (chapter 3) we have reported that different batches of ascorbate oxidase significantly altered the performance of the hydrogel-coated glutamate microsensor. In this chapter it is shown that a simple enzyme purification procedure as buffer exchange leads to a more uniform enzyme quality, which in turn significantly improves the reproducibility and performance of the microsensor. In our opinion, this is an important observation, and of general interest for the construction of enzyme-based biosensors.*

## 4.1 Introduction

To monitor neuronally derived glutamate directly within brain tissue, a detection technique with a high spatial- and temporal resolution is required (Timmerman and Westerink, 1997; Westerink and Timmerman, 1999; Baker et al., 2002). Biosensors or microsensors (determined by the dimensions of the electrode) fulfill these criteria, as they combine the selectivity of a biological recognition element (e.g. an enzyme) with the spatial and temporal resolution of voltammetry (Hu et al., 1994; Kulagina et al., 1999; Burmeister at al., 2002; Rahmann et al., 2005; Day et al., 2006). Many studies have dealt with the construction and evaluation of different types of glutamate biosensors, but the number of biosensors being operated successfully *in vivo* or *in vitro* is rather limited. A promising biosensor concept is the hydrogel-coated glutamate microsensor. Although this microsensor holds promise for *in vivo* applications, the interference by ascorbic acid (AA) is a serious challenge, as discussed previously. AA-ox is incorporated into the hydrogel to limit this electrochemical interference.

Compared to the concentrations of the other enzymes, a rather high AA-ox content in the hydrogel is required. In the previous chapter (chapter 3) we have observed that the performance of the glutamate microsensor was significantly influenced by different batches of AA-ox. Although similar concentrations of the different batches of AA-ox were used (given in units/µl), the microsensors “behaved” as if the concentration of AA-ox in the hydrogel was different. This variation was experienced as a serious limitation in the practical use of the microsensor.

In literature much attention is paid to optimization of biosensor concepts through new developments in their architectural construction. From these developments many (derivatives of) 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ generation of biosensors have emerged. It is remarkable that, in contrast, hardly any attention is paid to the influence of the enzyme itself on the analytical performance of a biosensor. As far as we know only one other report has mentioned alterations in biosensor performance due to differences in enzyme batches (Gregg and Heller, 1991b). In the present chapter the influence of different batches AA-ox and the impact of AA-ox purification on the performance of the microsensor was investigated.

## 4.2 Materials and Methods

### Reagents

All chemicals were of analytical grade unless stated otherwise. Ascorbate oxidase (AA-ox) was obtained from 4 different suppliers. AA-ox from *cucurbita sp* (E.C. 1.10.3.3) with activities of 133.5, 310, 310 and 244.3 units/mg were obtained from respectively Sigma (St. Louise, MO, USA; Product nr. A 0157), Biozyme (Blaenavon, UK; Product nr. AO2), Calbiochem (Darmstadt, Germany; Product nr. 189724) and Seravac (Cape Town, South Africa; Product nr. 011530). Glutamate oxidase (Glu-ox; G-0400; 6.5 units/mg) was obtained from USBiological (Swampscott, MA, USA). Horseradish Peroxidase type II (HRP; P-8250; 158 units/mg), [4-(2-hydroxyethyl)-1-piprazineethane-sulfonic acid] (HEPES), HEPES sodium salt, l-glutamate, l-ascorbic acid, l-glutamine, sucrose, SDS (sodium dodecyl sulfate), DTT (dithiothreitol) and all other chemicals used were obtained from Sigma (St. Louis, MO, USA). Poly(ethylene glycol 400 diglycidyl ether) (PEGDGE), silver chloride and Nafion (5 % Nafion solution,1100 equivalent weight) were obtained from Aldrich, (Milwaukee, WI, USA). Artificial Cerebrospinal Fluid (aCSF), used for the calibration procedures, had the following composition: 145 mM $Na^+$, 1.2 mM $Ca^{2+}$, 2.7 mM $K^+$, 1.0 mM $Mg^{2+}$, 152 mM $Cl^-$ and 2.0 mM phosphate; adjusted to pH 7.4 with sodium hydroxide. Salts were obtained from Merck (Darmstadt, Germany). Solutions were made in ultra-purified water (U.P.; Elgastat maxima, Salm en Kipp). Enzymes solutions were made in HEPES buffer; the salt form of HEPES was added to a 10 mM solution of the acid form, until pH 8.

### Enzyme assay

Different batches of AA-ox were further analyzed within this study. A stock solution of AA-ox (10-20 mg/ml) was purified by buffer exchange using a centrifugal membrane filter unit (Amicon, 10 kDa cut-off). After centrifugation at 13,000 rpm for 30-45 min (dependent on the volume) at 4°C, the filtrate was discarded and resuspended in the same volume of ice-cold HEPES (10 mM; pH 8.0 with NaOH) supplemented with 60 % sucrose (60 % of the stock solution of AA-ox). This procedure was repeated twice. The purified AA-ox solutions were stored at -20°C before future experiments.

The protein content was determined using the modified Lowry method based on the elimination of interfering materials (Lowry et al., 1951; Bensadoun and Weinstein, 1975). Bovine Serum Albumin (BSA) was used as a referential standard.

The sucrose content was determined using an anthrone assay, described by Scott et al. (1953). Briefly, different solutions of sucrose were prepared in a range from 0 – 120 mg/l. Next, the calibration samples and AA-ox samples (100 µl) were incubated for 45 min at room temperature with 200 µl 0.1% anthrone in 97% sulfuric acid. The absorbance was measured at a wavelength of 600 nm using a FLUOstar OPTIMA microtiter plate reader (BMG, Labtech, Offenberg, Germany).

The activity of AA-ox was assessed by incubating 0.02 units of AA-ox with 0.5 µmol AA for exactly 5 min. The reaction was stopped with hydrochloric acid (Bergmeyer et al. (1983): "Enzymatic Assay of Ascorbate Oxidase (E.C. 1.10.3.3.)", Sigma Aldrich). The AA-ox activity was determined by calculating the difference between the absorbance of AA at 245 nm before and after incubation with AA-ox. In the Results section the AA-ox activity is expressed as the ratio: enzyme activity determined ($[A]_{det}$), divided by the specified activity ($[A]_{spec}$). The specified activity is the activity as reported in the certificates of analyses.

To obtain an indication of the salt content of the different batches of AA-ox, the (metal) cations were determined with elemental analysis (ICP-AES; Vista AX CCD). Screening was performed on the following metals: Ag, Al, As, Au, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Hg, I, K, La, Li, Mg, Mn, Mo, Na, Ni, Os, Pb, Pt, Se, Si, Sn, Ti, Tl, U, V, Y and Zn.

In order to obtain an indication of the protein purity of the different batches of AA-ox, an SDS-polyacrylamide gel electrophoresis (PAGE) using Coomassie staining was performed. In short, 10 µl samples containing approximately 5 µg protein were suspended in 20 µl Laemmli buffer (2 % SDS, 350 mM DTT, 25 % (v/v) glycerol, 0.01 % Bromphenol blue in 62.4 mM Tris-HCl, pH 6.8) and boiled for 5 min. Proteins were separated on a 7.5 % SDS-polyacrylamide gel. At these conditions, AA-ox is dissociated into its two identical subunits and gives rise to a single band at approximately 70 kDa (Avigliano et al., 1983).

### Microsensor construction and evaluation

Microsensors were constructed and evaluated according to previously published procedures (chapters 2, 3). Briefly, glutamate microsensors were constructed by

automatic dipcoating of a carbon fiber electrode (CFE), trimmed to a length of 300 - 500 µm, at 37°C in an aqueous mixture containing 20 µl POs-EA (1 mg/ml), 4 µl PEDGE (3 mg/ml), 10 µl HRP ( 711 units/ml), 10 µl AA-ox (1500 units/ml) and 10 µl Glu-ox (6.5 units/ml). The impact of AA-ox on the performance of the microsensor was investigated using 4 different batches of AA-ox, both in a non-purified and purified fashion. The microsensor was evaluated by amperometric calibration at -150 mV versus an Ag/AgCl reference electrode in a flow-injection analysis system. The potential between the microsensor and the reference electrode was applied by a home-made potentiostat. The amplifier was connected to a computer (Intel, Pentium II MMX, 384 MB RAM), where incoming data were processed with home-written software. The calibration of the microsensors was divided into a few steps and was performed as previously described.[1] The following microsensor properties were determined: shape and height of the CV (nA; determined as the absolute value of the anodic ($I_{pa}$) minus the cathodic peak current ($I_{pc}$)), sensitivity (pA/µM), current density (mA $M^{-1}$ $cm^{-2}$), interference (%), linearity ($R^2$), detection limit (µM; defined as 3 times signal to noise ratio) and response time (sec; time required for the signal to increase from 10 to 90%). The present study focuses primarily on the sensitivity and interference by AA, because these are the most critical properties for the practical use of the microsensor. The interference by AA, which was investigated by coinjecting 200 µM AA to 100 µM glutamate, is defined as the percentage of suppression of the original glutamate signal by AA.

### Expression of results and statistics

Experiments are presented as mean ± SEM. Sigmastat 3.0 was used to calculate statistics. Data were analyzed with a Mann-Whitney Rank Sum test. Significance was set at $p < 0.05$.

## 4.3 Results and Discussion

The glutamate microsensor is constructed by dipcoating a CFE into the five component coating solution. The incorporation of an individual hydrogel component, such as AA-ox, into the hydrogel layer is determined by its concentration within the coating solution and its physical chemical properties. In order to investigate why the use of different batches of AA-ox gave rise to a varying microsensor performance, four types of AA-ox were further specified (Table 1A). AA-ox batches are characterized by three major components: protein, (carbohydrate) stabilizers and salts. It was observed that the different batches of AA-ox displayed significant differences in these three components (Table 1B). Salts are known to influence the conformation and precipitation of enzymes and it was shown that the different batches displayed a large variation in cation concentrations. Stabilizers, like sucrose, glycerol and related compounds, stabilize enzymes and offer protection against denaturation by altering the water surface tension around the enzyme (Timasheff, 1993; Ruan et al., 2003). The various batches of AA-ox contained different types and concentrations of stabilizer. In addition, the sucrose content was determined and the values corresponded fairly well with the specified values. The influence of the percentage sucrose present in the AA-ox batch was also investigated and appeared to influence the final microsensor performance only slightly. However, absence of sucrose in the AA-ox batch considerably suppressed the final microsensor performance (results not shown). It was remarkable that two out of four enzymes (Biozyme and Callbiochem) displayed an activity consistent with their specifications, while the other two (Sigma and Seravac) were much more active.

Since the majority of an AA-ox batch consists of stabilizer and salts, the influence of AA-ox purification was investigated. Previous attempts to isolate the protein of interest using affinity chromatography based on capturing copper atoms within AA-ox were unsuccessful (results not shown) and were likely out of focus, as the different batches of AA-ox already displayed a high degree of purity (see Fig. 1). Therefore, the different batches of AA-ox were purified using buffer exchange. Briefly, AA-ox was centrifuged using a membrane-filter (10 kDa cut-off) in order to remove low molecular weight compounds and to standardize all the batches at the same sucrose concentration (60 %). The characteristics of the purified AA-ox batches were presented in Table 1C. In most cases the protein content corresponded nicely with

the specified values (Table 1A), but not the AA-ox supplied by Calbiochem. The sucrose values agreed fairly well with the supplemented 60 %. It was also shown that purification effectively removed salts and promoted uniformity in the salt content. The relatively high sodium concentration originated from the pH adjustment of the HEPES buffer. The Cu values originated from the AA-ox itself, as AA-ox contains approximately 8 atoms of copper per molecule (Dawson et al., 1975; Casella et al., 1999).

***A) Specifications***

| Supplier | Form | Activity (u/mg solid) | Activity (u/mg protein) | Protein (%) | Other compounds |
|---|---|---|---|---|---|
| Sigma | Lyophilized, blue | 133,4 | 1466 | 9,1 | 88.8 % sucrose<br>2,14 % sodium phosphate |
| Seravac | Blue, freeze dried | 244,3 | 814 | ≈ 30 % | 60,8 % sucrose<br>< 1 % of $NH_3SO_4$ and NaCl |
| Biozyme | Blue, freeze dried | 310 | 1265 | 24,5 | 29 % sucrose<br>22.6 % lactilose<br>25.5 % sodium phosphate |
| Calbiochem | Lyophilized, yellow | 310 | 1550 | 20 | 2 % sodium benzoate in<br>0.1 M sodium phosphate |

***B) Non-Purified***

| Supplier | Protein (%) | sucrose (%) | AA-ox activity $[A]_{Det}$ / $[A]_{cal}$ | Cations (mg/l) Cu | K | Na | Ca | Mg | Fe |
|---|---|---|---|---|---|---|---|---|---|
| Sigma | n.d. | 86,1 | 2,8 | 2,2 | 0,4 | ~ 70 | 0 | 0 | 0 |
| Seravac | n.d. | 58 | 2,9 | 5,7 | 0,4 | ~ 80 | 1,6 | 0,3 | 0,1 |
| Biozyme | n.d. | 24 | 1,2 | 20,1 | 1,6 | ~ 400 | 0,5 | 0,03 | 0,1 |
| Calbiochem | n.d. | 0 | 1,1 | 2,2 | ~ 750 | ~ 100 | 0,3 | 0 | 0,5 |

***C) Purified***

| Supplier | Protein (%) | sucrose (%) | AA-ox activity $[A]_{Det}$ / $[A]_{cal}$ | Cations (mg/l) Cu | K | Na | Ca | Mg | Fe |
|---|---|---|---|---|---|---|---|---|---|
| Sigma | 9,1 | 70,6 | 3 | 2,0 | 2,3 | ~ 230 | 0 | 0,04 | 0 |
| Seravac | 23,7 | 59,1 | 2,7 | 6,5 | 2,6 | ~ 220 | 0,3 | 0,04 | 0,1 |
| Biozyme | 24,7 | 55,1 | 1 | 9,7 | 2,3 | ~ 220 | 0,2 | 0,02 | 0 |
| Calbiochem | 58,0 | 64,1 | 0,9 | 1,4 | 1,2 | ~ 130 | 0,2 | 0,02 | 0,3 |

*Table 1: Characterization of AA-ox. **A**) Specifications according the certificates of analysis and by additional information of the supplier. **B**) Analysis of the non-purified AA-ox. **C**) Analysis of the AA-ox after buffer exchange purification. n.d. = not determined.*

To obtain a qualitative indication of the protein purity of the enzyme, an SDS-Page was performed on both the non-purified and purified AA-ox (Fig. 1). It was observed that all batches displayed a large band at approximately 70 kDa, which corresponded to the molecular weight of a single subunit of AA-ox (Avigliano et al., 1983). The AA-ox from Calbiochem seemed to have the lowest purity as indicated by relatively large bands at respectively 37.5 and 52.8 kDa. When the Cu values of Table 1C were used to calculate the AA-ox content, similar results were obtained. Differences in the SDS-PAGE were not observed between the non-purified and purified AA-ox, which was consistent with a buffer exchange permeability < 10 kDa.

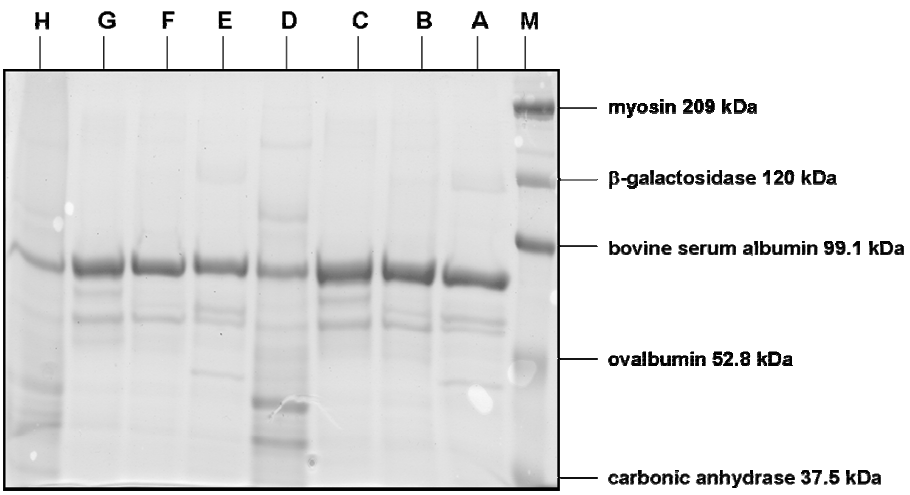


*Figure 1: SDS-PAGE of the different batches of AA-ox. AA-ox from: Sigma: before (A) and after (E) purification; Seravac: before (B) and after (F) purification; Biozyme: before (C) and after (G) purification; Calbiochem: before (D) and after (H) purification. M represents a reference standard.*

In Table 2 the properties of glutamate microsensors constructed with non-purified and purified AA-ox were compared. As shown before, significant differences in sensitivity and interference by AA were observed when the microsensors were constructed with the non-purified enzyme. In contrast, microsensors prepared with the purified enzyme did not show this variability. Moreover, the interference by AA was

significantly lower. In addition, microscopic inspection revealed that the microsensors constructed with the purified AA-ox displayed reproducible hydrogels (smooth, uniformly architected), with a low variation in layer thickness, in contrast to the hydrogels constructed with non-purified AA-ox.

It is likely that standardization of the sucrose and salt content closely controls the physical chemical properties of AA-ox and, in turn, mediates a uniform precipitation into the hydrogel. Whether other factors also contributed is not known. For example, it is possible that deglycation and reglycation during buffer exchange might have contributed, as sugar removal leads to an improved exposure of the catalytic sites of AA-ox (D'Andrea et al., 1989).

The reason that differences in AA-ox incorporation can have such a large effect is because the performance of the microsensor relies heavily on the AA-ox content (see chapter 3). In contrast, differences in microsensor performance were not observed due to different batches of either Glu-ox or HRP. This was explained by the fact that the used concentration of Glu-ox is about 200 times lower than the applied concentration of AA-ox, which automatically makes the performance of the sensor less susceptible to differences in Glu-ox incorporation. The concentration of HRP is in the same range as AA-ox. However, HRP is present in excess in the hydrogel and its function is limited by the activity of Glu-ox.

| | ***A) Non-Purified*** | | | ***B) Purified*** | | |
|---|---|---|---|---|---|---|
| **Supplier** | **n** | **Sensitivity (pA/µM)** | **Interference (%)** | **n** | **Sensitivity (pA/µM)** | **Interference (%)** |
| Sigma | 12 | 8.4 ± 1.2 | 41.5 ± 4.6 | 9 | 3.8 ± 0.5 | 19.9 ± 6.6 |
| Seravac | 10 | 4.0 ± 0.8 | 37.0 ± 4.0 | 9 | 3.8 ± 0.5 | 22.5 ± 6.6 |
| Biozyme | 9 | 1.7 ± 0.5 | 34.0 ± 5.5 | 8 | 4.3 ± 0.5 | 25.1 ± 3.3 |
| Calbiochem | 11 | 3.0 ± 0.7 | 56.5 ± 4.8 | 11 | 3.6 ± 0.7 | 20.9 ± 5.3 |

*Table 2: Performance of microsensors constructed with the non-purified (**A**) and purified (**B**) AA-ox. The sensitivity for glutamate and the interference by AA were investigated. Significant differences in sensitivity and interference were observed in (A), but not in (B) (not marked; $p < 0.05$; Mann-Whitney Rank Sum test).*

Finally, the stability of AA-ox was investigated, as the practical use of the purification procedure would be limited if the purified AA-ox had a restricted life time when stored in solution. Previously, it was reported that AA-ox solutions were stable for several days (stored at room temperature) up to several weeks (storage at 4 °C), but not when stored at -20 °C (decay within 1 day) (Reinhammer et al., 1997; Hazzard et al., 1997). The purified AA-ox was stored at -20°C and to investigate its stability, the AA-ox was freeze-thawed at different time intervals and the resulting activity was compared with the initial activity (Table 3). The purified AA-ox solution was stable for at least 6 months, indicating a great advantage to its practical use. It seems likely that the HEPES buffer contributes to this stability, probably due to its zwitterionic character, which protects the physical chemical state of the copper sites of the AA-ox (Reinhammer et al., 1997).

| Time | Activity $[A]_t$ / $[A]_0$ |
|---|---|
| day 0 | 1,0 |
| day 2 | 1,0 |
| day 5 | 0,9 |
| day 7 | 1,0 |
| day 8 | 0,9 |
| day 12 | 0,9 |
| 2 weeks | 1,0 |
| 3 weeks | 1,2 |
| 5 weeks | 1,0 |
| 6 months | 1,1 |

*Table 3: Activity of AA-ox in time. The purified AA-ox was stored at -20°C and at different time intervals the activity was determined. The AA-ox used was initially obtained from Sigma. The enzyme activity was determined as the activity at different time points ($[A]_t$), divided by the activity at t = 0 ($[A]_0$).*

## 4.4 Conclusion

The performance of the hydrogel-coated glutamate microsensor critically depends on the quality of the enzyme AA-ox. It was shown that the properties of AA-ox could be optimized via a simple purification procedure such as buffer exchange. This resulted in a significant improvement in the reproducibility and performance of the microsensor. This observation might be of general interest for the construction of future enzyme-based biosensors.