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An assay for the enzyme *N*-acetyl-β-D-glucosaminidase (NAGase) based on electrochemical detection using screen-printed carbon electrodes (SPCEs)

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An electrochemical assay for the enzyme *N*-acetyl-β-D-glucosaminidase (NAGase) is described, using bare screen-printed carbon electrodes (SPCEs). The enzyme substrate, 1-naphthyl-*N*-acetyl-β-D-glucosaminide, was added to the NAGase-containing sample under hydrodynamic conditions and was hydrolysed to 1-naphthol, which was monitored amperometrically at an E_{app} of +650 mV *versus* SCE. A pH study revealed the apparent V_{max} for the assay to occur at pH 4.5, corresponding to an apparent substrate K_m of 0.28 mM. In order to be compatible with the analysis of biological fluids, a final operating pH of 5.4 was selected, and, using a data recording time of 100 s post-substrate addition, the assay gave a linear response (r^2 = 0.988) over the range 3.1 to 108 mU ml^{-1} NAGase (RSD = 15.4%). This assay has the potential to monitor NAGase levels in a number of application areas.

Introduction

The enzyme β-*N*-acetylglucosaminidase (EC 3.2.1.52), also known as β-*N*-acetyl-D-hexosaminidase or NAGase, catalyses hydrolytic release of terminal β (1-4)-linked *N*-acetyl-D-hexosamine (-glucosamine or -galactosamine) residues from the non-reducing end of a variety of substrates including glycoproteins, glycolipids and glycosaminoglycans.[1,2] The NAGase enzyme consists of two major isoenzymes, A and B, plus several minor forms and occurs in animals, plants and microorganisms.

The ability to monitor NAGase activity is desirable in a number of potential application areas. For example, in humans the enzyme's function as a lysosomal acid hydrolase is of clinical significance in Tay–Sachs and Sandhoff Diseases, where deficiency in one or more of the A or B isoenzymes leads to imperfect degradation and altered lysosomal storage of glycoconjugates, with resulting pathology.[3] On the other hand, elevated levels of serum NAGase are associated with certain forms of cancer[4] and renal disorder is associated with an increase in total NAGase activity in the urine.[5] NAGase is also a potential marker for autoimmune disease,[6] diabetes[7] and alcoholic liver damage.[8] In animals, bovine mastitis, resulting from colonisation of the udder by pathogenic bacteria, leads to a reduction in milk yield and compositional changes in the milk. As a result of damage to cells and capillaries within the infected udder, elevated levels of NAGase are found in the milk.[9,10] NAGase therefore constitutes a potentially important early marker for the onset of clinical mastitis.

Most existing rate assays for NAGase activity are based upon spectroscopy and involve the incubation of samples with a suitable enzyme substrate in optical tubes or microwells, followed by determination of a product by absorbance[11–13] or fluorimetry.[14] Capillary electrophoresis has been used to separate the reaction product 4-methyl-umbelliferone from the reaction mixture, followed by its detection by UV absorbance or fluorimetry.[15] Enzyme immunoassays for the NAGase isozymes have also been developed using monoclonal antibodies specific for either of the A or B forms.[16]

There appear to be no published reports of NAGase assay methods employing electrochemical detection. Screen-printed carbon electrodes (SPCEs) can be used as transducers for direct electrochemical detection[17–20] and are suitable as base transducers for biosensing applications where inexpensive, disposable devices are required.[21,22] 1-Naphthol has been investigated electrochemically and shown to be capable of sensitive determination at SPCEs.[23] This capability has led to its use as a reaction product, particularly in immunoassays, with a subsequent voltammetric,[23] chronoamperometric,[24] or amperometric[25,26] measurement step. In the present report, an electrochemical assay for NAGase is described, based on the enzymatic generation and subsequent amperometric detection of 1-naphthol at an SPCE. This method has the potential for adaptation into various NAGase sensing application areas.

Experimental

Reagents and buffers

All chemicals were of Analar grade. Water for preparation of solutions was supplied by a Milli-Q Water Purification System (Millipore UK, Watford, UK). A solution of β-*N*-acetylglucosaminidase (EC 3.2.1.52), from bovine kidney, was purchased from Sigma–Aldrich Company Ltd., Poole, Dorset, UK. The enzyme substrate 1-naphthyl *N*-acetyl-β-D-glucosaminide (product number 17007, FW = 347.4) was used for all NAGase assays and was purchased from Glycosynth, Warrington, UK. 1-Naphthol (FW = 144.17) was purchased from Acros Organics, Loughborough, UK.

Phosphate buffer was prepared by mixing 0.5 M stock solutions of Na_2HPO_4, NaH_2PO_4 and H_3PO_4 to achieve the desired pH, followed by a dilution step where appropriate. 2.5

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M Acetate buffer was prepared by combining appropriate ratios of 2.5 M acetic (glacial) acid and 2.5 M $CH_3COONa{\cdot}3H_2O$ to achieve the required pH.

Electrochemical apparatus

Screen-printed carbon electrodes were obtained from Gwent Electronic Materials Ltd. (GEM), Mamhilad, Gwent, Wales. Working electrodes/conducting track were printed from carbon ink, code D14, onto a polyester substrate. For each sensor, a screen-printed layer of dielectric material was then laid down to define the circular working area of 9 mm^2. Individual sensors were cut from the polyester sheet and mounted in the electrochemical cell using a crocodile clip.

All electrochemical operations were performed in a water-jacketed Teflon cell of cylindrical section, diameter 16 mm, at a temperature of 25 °C. A 3-electrode system comprised (1) a screen-printed carbon working electrode; (2) a platinum counter electrode, incorporated into the base of the cell; and (3) a saturated calomel reference electrode. A magnetic stirrer bar, suspended on a small platform above the counter electrode to reduce noise, was incorporated to allow hydrodynamic measurements.

Cyclic voltammetry and hydrodynamic voltammetry of 1-naphthol were performed using a Metrohm E612 VA-Scanner and E611 VA-Detector connected to a JJ-Instruments Chart Recorder. All NAGase assays were performed using a BAS LC-4B Amperometric Detector coupled to a PicoMeter ADC100 converter running PicoScope for data capture.

Electrochemistry of 1-naphthol

Cyclic voltammetry was performed on standard solutions prepared by diluting a stock solution of 2×10^{-3} M 1-naphthol into 5 ml 0.05 M phosphate buffer at the required pH. Solutions were scanned from an initial potential of −100 mV *versus* SCE to a switching potential of +900 mV. Hydrodynamic voltammograms for 1-naphthol were obtained by increasing the applied potential (E_{app}) in +50 mV steps and recording the resulting steady-state currents. After subtraction of values obtained in the absence of 1-naphthol, currents were plotted against E_{app}.

Amperometric determination of NAGase activity

For pH studies of NAGase activity, substrate solutions were prepared at the appropriate concentration, in a 1.92 ml volume of 0.5 M phosphate buffer, containing methanol, at the appropriate pH. Once a steady-state amperometric response had been established, under hydrodynamic conditions, NAGase enzyme was introduced in an 80 μl volume and amperometric responses were recorded as initial rate in pA s^{-1}.

For NAGase assay by substrate addition, steady-state amperometric responses were first obtained for cell solutions comprising 1.8 ml 0.05 M phosphate buffer, pH 6.65, containing NAGase, 0.2 ml ethanediol and 0.2 ml acetate buffer, pH 5.58. Substrate (dissolved in 1 : 1 ethanediol–acetate buffer, pH 5.3) was then added in a 100 μl volume and the subsequent amperometric response was recorded.

Results and discussion

1. Assay scheme

The proposed basis for the electrochemical assay of NAGase is shown in Fig. 1. The enzyme substrate, 1-naphthyl-*N*-acetyl-β-D-glucosaminide, is hydrolysed by NAGase to produce *N*-acetyl-β-D-glucosaminide and 1-naphthol. The production of 1-naphthol is monitored electrochemically by its oxidation at the SPCE surface at the selected applied operating potential. The resulting amperometric response is directly proportional to the concentration of 1-naphthol produced and to the concentration of NAGase present in solution.

2. Electrochemistry of 1-naphthol at SPCE

2.1 Cyclic voltammetry. The electrochemical behaviour of 1-naphthol was examined by performing cyclic voltammetry, at a SPCE in quiescent bulk solution, of a 2×10^{-4} M solution in 0.5 M phosphate buffer, pH 6.65. A single, irreversible oxidation wave was obtained, having a peak potential (E_p) value of +360 mV *versus* SCE at pH 6.65 (Fig. 2A). Other workers have found similar behaviour at carbon paste[27] and screen-printed carbon[23] electrodes.

The diffusional characteristics of the 1-naphthol reaction were examined by performing cyclic voltammetry at various scan rates over the same potential range. At scan rates (v) from 5 to 100 mV s^{-1}, the plot of peak current (i_p) *versus* $v^{1/2}$ (Fig. 3) was linear, obeying the Randles–Sevcik equation for an irreversible reaction ($i_p = 2.99 \times 10^5 n\,[\alpha n_a^{1/2} AD^{1/2} Cv^{1/2}]$),[28] and indicating that oxidation occurred under diffusion-controlled conditions. At higher scan rates, this relationship was no longer obeyed and i_p values decreased. One explanation for this effect is that the active area of the SPCE is reduced at higher scan rates due to an electropolymerised 1-naphthol layer.

From a series of voltammograms, run over the pH range 3.0 to 8.8 (pK_a of 1-naphthol = 9.34), a plot of E_p *versus* pH was constructed (Fig. 4). The slope of this plot was −58 mV per pH unit which, according to the Nernst equation,[28] indicates the removal of one proton per electron during the oxidation process. From the cyclic voltammograms, the value obtained for the electron-transfer coefficient, αn_a,[29] was 0.73, suggesting that a single electron was involved in the rate-limiting step of the oxidation. Together, these data suggest the removal of one electron and one proton from each molecule of 1-naphthol. The magnitude of i_p increased linearly with pH over the range 3.0 to 7.5, with a sensitivity of 0.59 μA per pH unit.

The planar diffusion-controlled cyclic voltammetric peak response obtained for a single cycle under quiescent conditions diminished for repeated cycles at a single SPCE (Fig. 2A) and showed a marked E_p shift, indicating passivation of the

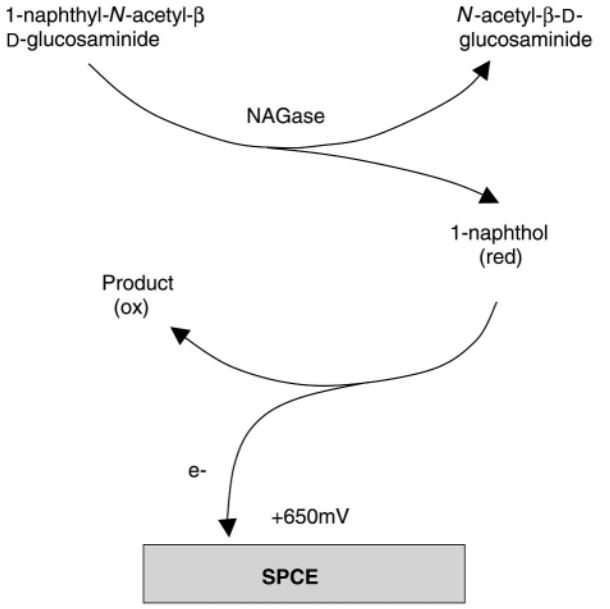

Fig. 1 Schematic diagram for the electrochemical assay of NAGase.

electrode surface, probably as a result of electropolymerisation. When the same experiment was conducted under stirring conditions (Fig. 2B), there was a marked reduction in passivation. There was also a change in the voltammetric profile once the peak current response had been reached, although the stirring rate was not fast enough to maintain a constant diffusion layer thickness and a true steady-state response. This phenomenon was also evident at the lower scan rate of 5 mV s^{-1} (Fig. 2C and D) and indicated that an enzyme assay under hydrodynamic conditions would be favourable for determinations involving 1-naphthol generation.

2.2 Hydrodynamic voltammetry. The proposed NAGase sensor requires amperometric detection of 1-naphthol under hydrodynamic conditions in order to facilitate mixing of sample and reagents, and to avoid potential problems arising from electrode passivation. In order to establish the required applied operating potential (E_{app}), a hydrodynamic voltammogram (HDV) was obtained for a solution of 4×10^{-6} M 1-naphthol in phosphate buffer, pH 6.65. Fig. 5 shows that, at this pH, the oxidation wave for 1-naphthol began at +300 mV *versus* SCE and reached a maximum at around +450 mV. Subsequent experiments at pH 6.65 were therefore performed at an E_{app} of +450 mV, in order to minimise changes in current due to drifts in potential, whilst maximising response sensitivity. The corresponding HDV obtained at a pH of 3.5 (not shown) demonstrated that the oxidation wave plateau occurred at an E_{app} of +600 mV, a positive shift of around 150 mV and in agreement with the effect of pH on E_p (Fig. 4).

2.3 Linear range of 1-naphthol determination. Calibration plots resulting from serial additions of 20 μl volumes of 1-naphthol solution to an SPCE in 0.5 M phosphate buffer, pH 6.65, under hydrodynamic conditions, E_{app} = +450 mV, (not shown) were linear (r^2 = 0.998) over the final concentration range 4×10^{-8} M to 1.5×10^{-6} M, with a sensitivity of 9.07 nA per 10^{-6} M. This result indicated that the transducer would be suitable for determination of enzymatically-generated 1-naphthol over this concentration range.

3. Optimisation of pH for electrochemical determination of NAGase

These experiments were performed by adding NAGase into an amperometric cell containing substrate in buffer solution.

3.1 Determination of apparent V_{max} and K_m values for NAGase. V_{max} and K_m values for NAGase were determined from plots of amperometric response rate (pA s^{-1}) against [S] for stirred phosphate buffer solutions containing 1-naphthyl-*N*-acetyl-β-D-glucosaminide over the range 0.166 mM to 2.66 mM, into which NAGase was introduced, to a final concentra-

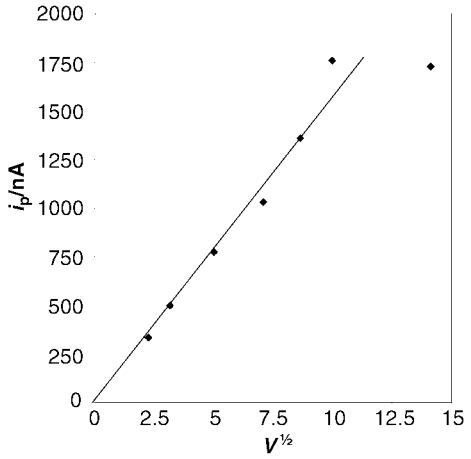

Fig. 3 Plot of i_p *versus* $v^{1/2}$ for cyclic voltammograms of 2×10^{-5} M 1-naphthol in 0.5 M phosphate buffer, pH 6.65 for scan rates (v) from 5 to 200 mV s^{-1}. E_i = 0 V; E_{sw} = +1000 mV.

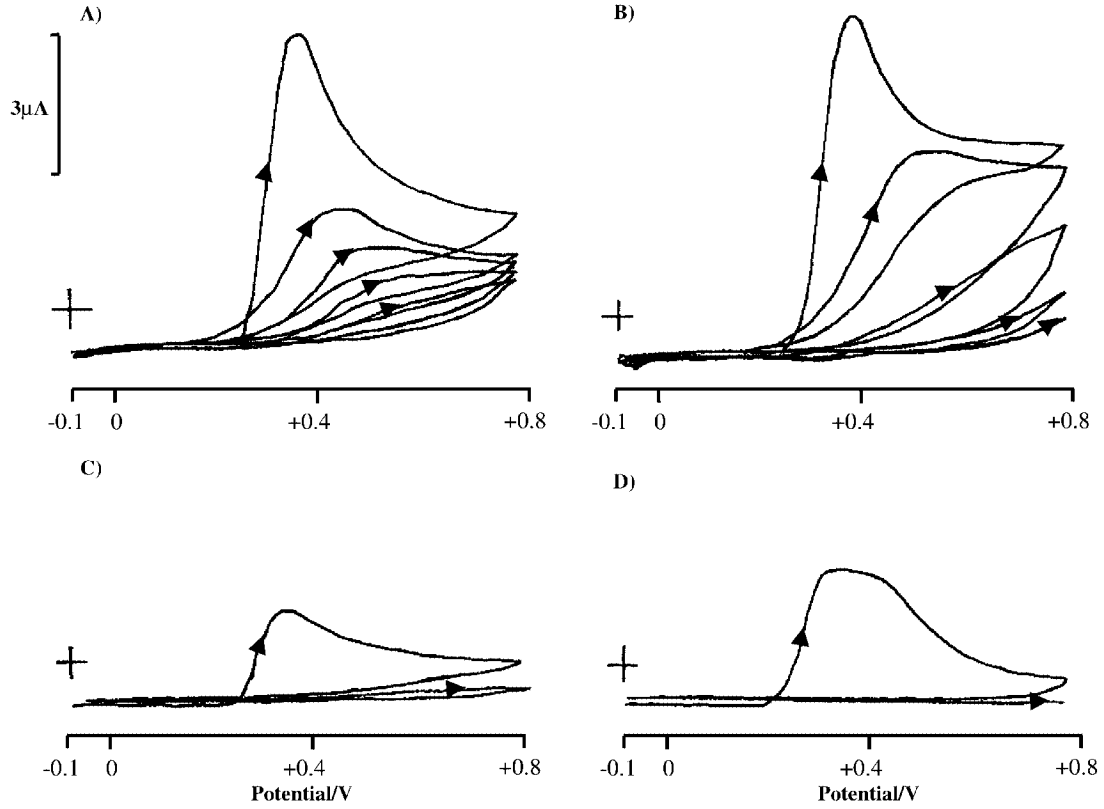

Fig. 2 Cyclic voltammograms of 2×10^{-4} M 1-naphthol in 0.5 M phosphate buffer, pH 6.65 at a SPCE under quiescent (A, C) or hydrodynamic (B, D) conditions, showing five repeat cycles at 50 mV s^{-1} (A, B) or two repeat cycles at 5 mV s^{-1} (C, D).

tion of 51 mU ml^{-1}. The E_{app} used for these experiments was +650 mV. This elevated E_{app} value was chosen to ensure that oxidation of 1-naphthol was still being recorded on the plateau of the electrochemical oxidation wave at all pHs (see above). Plots were obtained for solutions over the pH range 2.5 to 8.5.

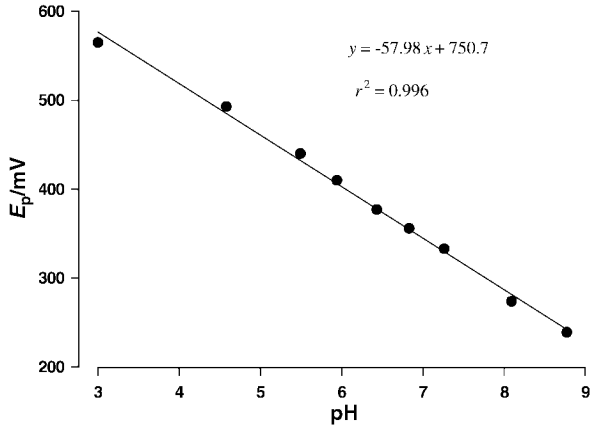

Fig. 4 Plot of E_p *versus* pH obtained from cyclic voltammograms of 2 × 10^{-4} M 1-naphthol in 0.5 M phosphate buffer. E_i = −100 mV; E_{sw} = +800 mV.

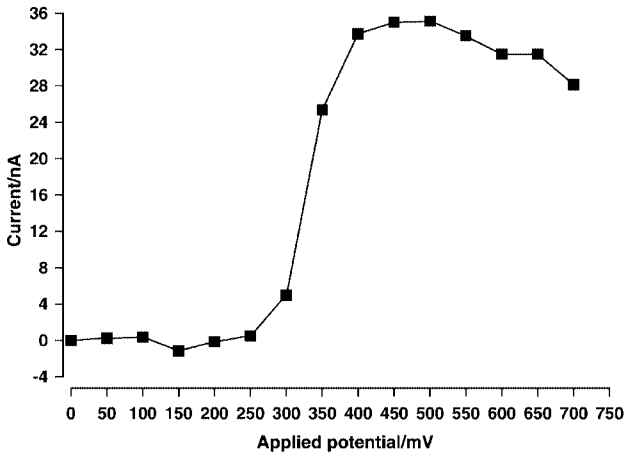

Fig. 5 Hydrodynamic voltammogram for a solution of 4 × 10^{-6} M 1-naphthol in 0.5 M phosphate buffer.

The resulting relationships between pH and V_{max} or K_m are shown in Fig. 6. The maximum rate of reaction (V_{max} = 1641 pA s^{-1}, Fig. 6A) was obtained at pH 4.5, with a sharp decrease in rate being experienced at pHs to either side of this optimum. A pH of 4.5 produced the minimum K_m value for the enzyme (0.28 mM, Fig. 6B), with a sharper increase in K_m value at lower pH than observed at higher pH values. The pH optimum of 4.5 obtained electrochemically agrees well with those quoted using colorimetric methods, where optimum activity is found over the pH range 4.0 to 6.0, depending upon the source of NAGase.

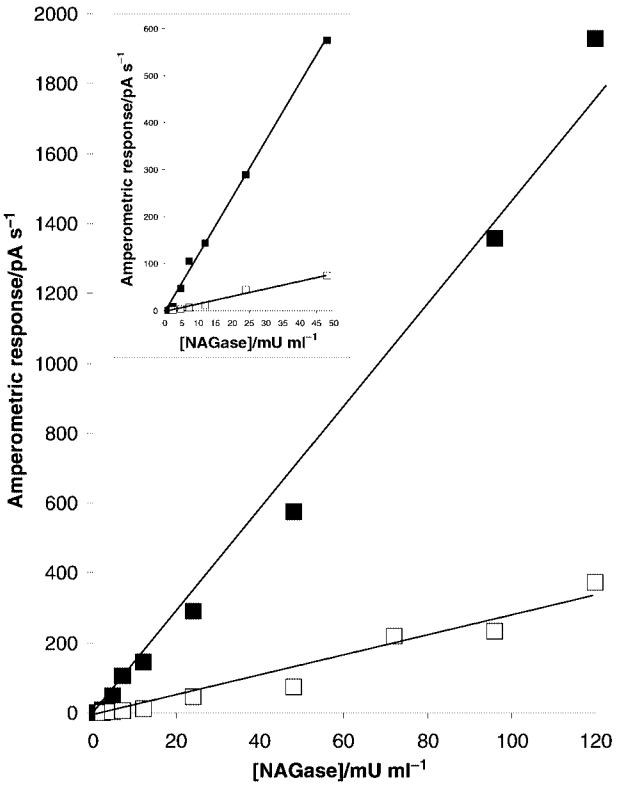

Fig. 7 Calibration plots obtained under hydrodynamic conditions for NAGase added to phosphate buffer solution containing 3.75 mM 1-naphthyl-*N*-acetyl-β-D-glucosaminide substrate at pH 4.5 (solid squares) or pH 6.5 (open squares). Inset shows expanded lower concentration range for clarity.

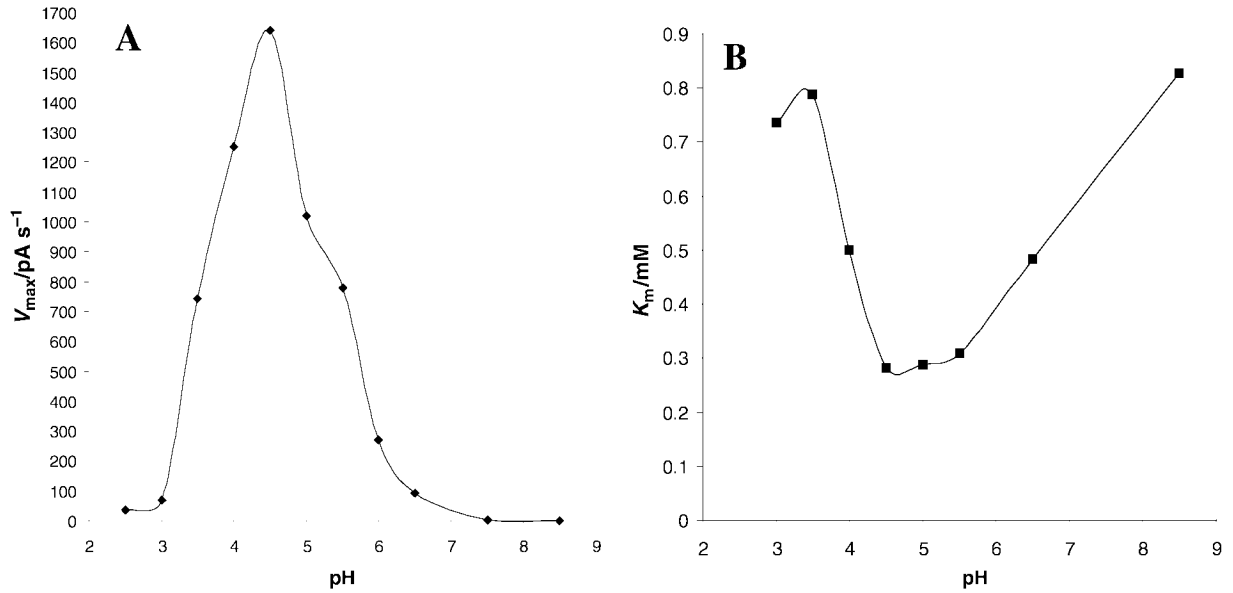

Fig. 6 Plots of (A) V_{max} and (B) K_m *versus* pH for NAGase measured amperometrically at SPCEs in stirred phosphate buffer containing 1-naphthyl-*N*-acetyl-β-D-glucosaminide as substrate.

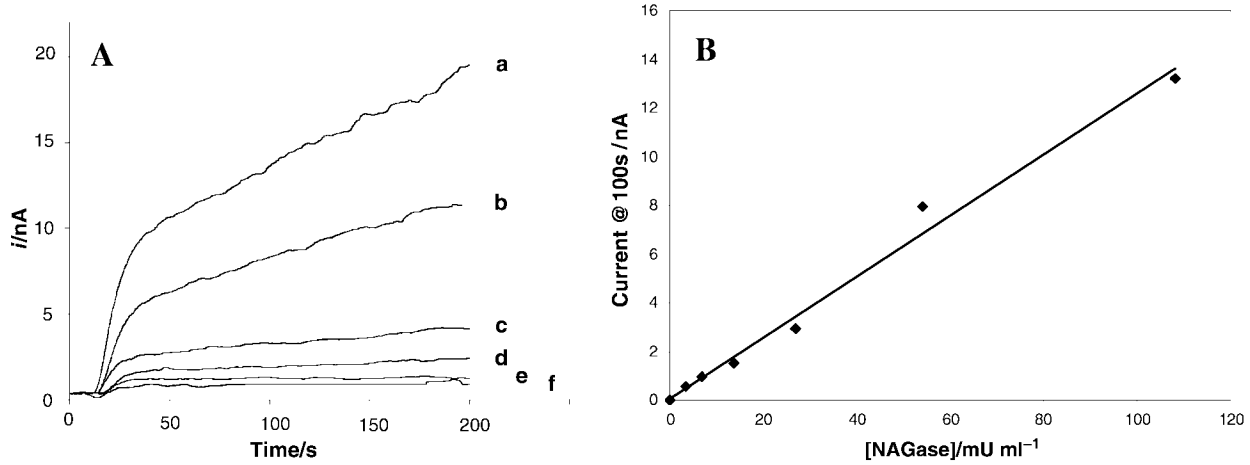

Fig. 8 Electrochemical assay of NAGase in buffer solution. (A) Amperograms obtained for NAGase at: (a) 108; (b) 54; (c) 27; (d) 13.6; (e) 6.8 and; (f) 3.1 mU ml^{-1} final concentration. (B) Calibration plot.

3.2 Effect of pH on calibration response. Although the maximum recorded activity of the NAGase enzyme occurred at pH 4.5, the physiological pH of biological fluids is around 6.5–7.5. In order to decide upon the operational pH for a sensor for NAGase, a comparison was therefore made of the assay performance characteristics at these pHs. Using a separate SPCE for each measurement, amperometric responses were recorded following the addition of NAGase (final concentration range 1.2 to 120 mU ml^{-1}) to phosphate buffer solutions under hydrodynamic conditions, E_{app} = +650 mV. Fig. 7 shows the resulting calibration plots obtained at pHs 4.5 and 6.5. In each case, good linearity was recorded over the range tested. As anticipated from the previous study, sensitivity was enhanced by 5.6-fold at pH 4.5 (14.5 pA s^{-1} per mU ml^{-1}) compared to pH 6.5 (2.6 pA s^{-1} per mU ml^{-1}). Limits of detection were estimated to be 2.4 and 4.8 mU ml^{-1} respectively.

4. Amperometric determination of NAGase activity

These experiments were performed by adding substrate solution into an amperometric cell containing NAGase in solution.

4.1 Selection of pH. The pH of biological fluids is around pH 6.5–7.5. Based upon the results from the previous section, it would be desirable to buffer the sample to obtain a final pH close to pH 4.5, in order to obtain maximum assay sensitivity. However, due to potential problems of protein precipitation in biological fluids (*e.g.*, milk) at such a low pH, it proved necessary to compromise at a slightly higher final pH. This was achieved by introducing acetate buffer into the sample cell. Consequently, all subsequent NAGase determinations were performed at pH 5.4.

4.2 Introduction of substrate solution. 1-Naphthyl-*N*-acetyl-β-D-glucosaminide was initially dissolved in methanol before introduction into the assay cell. However, due to problems of solvent evaporation and therefore volume changes, a less volatile solvent was desirable. Ethanediol proved to be a suitable alternative. Consequently, substrate was mixed with ethanediol, sonicated and then buffered with pH 5.3 acetate buffer, before being introduced to the amperometric cell.

4.3 NAGase assay in buffer solution. For each determination, a steady-state amperometric response was established at E_{app} = +650 mV for an SPCE in a 2.2 ml volume of solution containing ethanediol, acetate buffer and NAGase at pH 5.4. Substrate solution (100 μl) was introduced (to a final concentration of 1.1 mM) and the subsequent increase in amperometric signal was recorded. Fig. 8A shows the amperograms obtained over NAGase concentration range 3.1 to 108 mU ml^{-1}. The corresponding calibration plot (Fig. 8B) was obtained by plotting current values at 100 s post-substrate addition against [NAGase]. Good linearity was obtained over this range (r^2 = 0.988). Based on these data, a RSD of 15.4% was obtained.

Conclusions

This approach has demonstrated the feasibility of determining NAGase amperometrically at concentrations of 10 mU ml^{-1} and above by the simple addition of enzyme substrate under appropriate conditions. Further work is underway to adapt this screen-printed electrochemical NAGase sensor assay for the analysis of biological fluids and to examine the possibility of developing devices for continuous on-line or one-off disposable measurements.

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References

1 Y. Z. Frohwein and S. Gatt, *Biochemistry*, 1967, **6**, 2775.
2 Y. T. Li and S. C. Li, *Methods Enzymol.*, 1972, **28**, 702.
3 E. F. Neufeld, *J. Biol. Chem.*, 1989, **264**, 10927.
4 E. Gil-Martin, F. J. Rodriguez-Berrocal, M. P. de la Cadena and A. Fernandez-Briera, *Oncology*, 1999, **56**, 142.
5 P. Whiting, J. Petersen, D. Power, R. Stewart, G. Catto and N. Edward, *Clin. Chim. Acta*, 1983, **130**, 369.
6 M. P. Iqbal, A. A. Ali, M. A. Waqar and N. Mehboobali, *Exp. Mol. Med.*, 1998, **30**, 165.
7 L. Mandic and D. Filipovic, *Biochem. Mol. Biol. Int.*, 1998, **45**, 545.
8 F. Musshoff and T. Daldrup, *J. Chromatogr. B, Biomed. Appl.*, 1998, **713**, 245.
9 B. J. Kitchen, *J. Dairy Sci.*, 1981, **48**, 167.
10 E. Urech, Z. Puhan and M. Schallibaum, *J. Dairy Sci.*, 1999, **82**, 2402.
11 B. M. Loffler, B. Hesse and H. Kunze, *Anal. Biochem.*, 1984, **142**, 312.
12 G. Humbert, M.-F. Guingamp, A. Choukri and G. Linden, *J. Dairy Res.*, 1995, **62**, 369.

13 H. Shibata and T. Yagi, *Clin. Chim. Acta*, 1996, **251**, 53.
14 S. Linko-Lopponen, *Anal. Biochem.*, 1985, **148**, 50.
15 M. Friedberg and Z. K. Shihabi, *J. Chromatogr. B, Biomed. Appl.*, 1997, **695**, 187.
16 A. Isaksson, B. Hultberg, P. Masson, K. Low, E. Sandgren and A. Lundblad, *Scand. J. Clin. Lab. Invest.*, 1989, **49**, 597.
17 M. A. T. Gilmartin and J. P. Hart, *Analyst*, 1994, **119**, 2431.
18 K. C. Honeychurch, J. P. Hart and D. C. Cowell, *Electroanalysis*, 2000, **12**, 171.
19 R. M. Pemberton and J. P. Hart, *Anal. Chim. Acta*, 1999, **390**, 107.
20 A. Romani, M. Minunni, N. Mulinacci, P. Pinelli and F. F. Vincieri, *J. Agric. Food Chem.*, 2000, **48**, 1197.
21 J. P. Hart and S. Wring, *Trends Anal. Chem.*, 1997, **16**, 89.
22 M. Albareda-Sirvent, A. Merkoci and S. Alegret, *Sens. Actuators B*, 2000, **69**, 153.
23 M. Del Carlo, I. Lionti, M. Taccini, A. Cagnini and M. Mascini, *Anal. Chim. Acta*, 1997, **342**, 189.
24 R. M. Pemberton, J. P. Hart, P. Stoddard and J. A. Foulkes, *Biosens. Bioelectron.*, 1999, **14**, 495.
25 J. Wang, P. V. A. Pamidi and K. R. Rogers, *Anal. Chem.*, 1998, **70**, 1171.
26 R. M. Pemberton, J. P. Hart and T. T. Mottram, *Biosens. Bioelectron.*, 2001, in the press.
27 C. Fernandez-Sanchez and A. Costa-Garcia, *Biosens. Bioelectron.*, 1997, **12**, 403.
28 P. T. Kissinger and W. R. Heinemann, *Laboratory Techniques in Electroanalytical Chemistry*, Marcel Dekker, Inc., New York, 1984, p. 83.
29 Z. Galus, *Fundamentals of Electrochemical Analysis*, Ellis Horwood, Chichester, 1976, p. 237.