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On-line quantitation of enzymatic mannan hydrolysates in small-volume bioreactors by microdialysis sampling and column liquid chromatography–integrated pulsed electrochemical detection

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Abstract

Quantitative aspects of on-line microdialysis sampling are investigated with respect to sampling in small-volume bioreactors. Three modes of microdialysis sampling; continuous-flow microdialysis sampling (CFMS), stopped-flow microdialysis sampling with continuous stirring, and stopped-flow microdialysis sampling with stopped stirring were investigated as a possible means for studying bioprocesses. The hydrolytic properties of a well characterised endomannanase from *Aspergillus niger* were studied using 0.01% ivory nut mannan as substrate in a 5-ml reaction vessel. On-line sampling was achieved using a microdialysis probe fitted with an SPS 6005 membrane. Stopped-flow microdialysis sampling was found to give the least analyte depletion and thus used for quantitation of the enzymatic hydrolysates. However, CFMS can be used when analyte depletion is not significant (large-volume reactor). Hydrolysis of ivory nut mannan gave mainly mannobiose and mannotriose in almost equal amounts, which is consistent with an endo-wise hydrolysis. The concentrations of mannose and mannopentaose did not change significantly over the monitoring period, however, that of mannotetraose increased gradually up to 11 h where it starts to decrease.

Keywords: Microdialysis; Sampling methods; Enzymes; β-Mannanase; Polysaccharides; Mannan; Mannoses; Oligosaccharides

1. Introduction

The fast-growing progress within the field of biochemistry, cell- and molecular-biology has made the demand for general assays and analytical methodology obvious for both low and high molecular weight analyte determinations. Target analytes, such as proteins and carbohydrates, are often detected when present in low concentrations. Consequently, there are great demands for analytical methodologies that are able to determine not only the amounts of these target molecules produced, but also yield information from which their characteristics and mechanisms of operation can be deduced. In this regard, there is a requirement for continuous-sampling systems for small bioreactors where such investigations could be carried out on a small scale.

Uncharacterized, biologically active proteins, e.g., enzymes isolated from natural tissues or microorga-

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nisms, are very often only available in pure form in limited quantities. Despite the development of recombinant DNA-techniques it is often necessery to characterize the catalytic properties of an enzyme, before it is available in large quantities. For such experiments, both the enzyme and substrate must be pure. Small-volume bioreactors should be especially useful when there is limited availability of pure substrate. β-mannanases (endo-1,4-β-mannanase, EC 3.2.1.78), is a hemicellulase which endo-wise hydrolyzes galactoglucomannan, the major softwood hemicellulose polysaccharide [1]. β-mannanases are produced by bacteria and fungi. Fungal β-mannanases have been reviewed [2–4]. We have chosen to study the hydrolytic properties of the purified *Aspergillus niger* β-mannanase [5], an enzyme already well characterized [6], using on-line microdialysis sampling coupled to column liquid chromatography and integrated pulsed electrochemical detection.

Microdialysis sampling in combination with column liquid chromatographic (CLC) separation [7–11] has proven to be a successful combination of techniques to evaluate e.g., catalytic properties, mechanisms, stability, activation and inhibition of enzymes. The major advantage of using microdialysis and CLC is that the assay can be separated from the experimental bulk reaction media, and that qualitative [9] and quantitative multianalyte determination can be made. In a homogeneous reaction mixture, products, substrate and enzyme would be sampled, but using this method only the products are sampled leaving the substrate and the enzyme. By choosing a dialysis membrane with an appropriate molecular mass cutoff, a selectivity step is introduced, as the molecular masses of the hydrolysates would affect their recovery. As a constant dialysate flow guarantees reproducible withdrawal from the reaction mixture, the resulting chromatograms reflect the kinetics over this time period. Kinetic data are thereby governed by the sampling cycle time, which, in turn, is determined by the analysis time. This necessitates the need to reduce the analysis time by eliminating sample handling steps, and employing a rapid, sensitive and reproducible detection technique. For applications where the biological effects are very fast, separations by capillary electrophoresis using electrochemical detection has been proven to be highly efficient [11]. As low as nanogram amounts of carbohydrates are sufficient in the reaction vessel to obtain kinetic information [9] which makes this technique highly applicable to the new research fields.

Here we present a CLC system which employs on-line microdialysis sampling and integrated pulsed electrochemical detection (IPED) for the study of the enzymatic hydrolytic properties of an endo-1,4-β-mannanase from *A. niger* in a small-volume reactor. The hydrolysis of a homopolysaccharide, ivory nut mannan, an unbranched β-1,4-mannan, that is commercially available in contrast to the more complex galactoglucomannans, is hydrolysed using the endo-mannanase. Three modes of microdialysis sampling; CFMS, stopped-flow microdialysis sampling with continuous stirring (SFMSCS) and stopped-flow microdialysis sampling with stopped stirring (SFMSSS) are described regarding their suitability for quantitative on-line sampling in the study of bioprocesses. Also, two microdialysis probes, one fitted with a polysulfone membrane (SPS 6005) and the other with a polycarbonate membrane (CMA/10), were investigated regarding their quantitative aspects and the probe of choice was further used to assess different modes of microdialysis sampling.

The microdialysis probe provides a protein free dialysate, which is separated using anion exchange chromatography and detected at high pH (ca.12) using IPED. Mannobiose and mannotriose were produced in equal amounts during the hydrolysis, as is expected in an endo-wise hydrolysis.

2. Experimental

2.1. Chemicals

Mannose, 1,4-β-D-mannobiose, mannotriose, mannotetraose, mannopentaose, mannohexaose and 0.25% ivory nut mannan (insoluble in water) were obtained from Megazyme (Sydney, Australia) and standard solutions were prepared daily. Ivory nut mannan was washed with water, precipitated with ethanol and washed again before being used for hydrolysis experiments.

50% (w/w) NaOH, J.T. Baker (Deventer, Netherlands) was used to prepare the 50 m*M* NaOH mobile phase daily. The mobile phase was sparged with helium and continuously kept under a helium atmosphere regulated to about 2 p.s.i. Water from a Milli-Q system (Millipore, Bedford, MA) was used as a perfusion liquid. 50 m*M* citrate buffer was used to maintain a pH of 5.3 in the reaction vessel. All solutions were prepared using Milli-Q water.

A stock solution of 1,4-β-D-mannanase purified from culture filtrate of the fungi *A. niger* [5] was prepared at 50 nkat/ml. Enzyme activity was determined as in Stålbrand et al. [12] and is expressed in SI units (katals). The endo-1,4-β-mannanase activity was determined by detection of reducing sugars with dinitrosalicylic acid using locust bean gum (galactomannan) as substrate [12]. The enzyme loading for the characterisation was 280 nkat/g of substrate. 2.5 μl of enzyme stock solution were added to 180 μl of substrate (0.25% ivory nut mannan) and the volume was made up to 4.5 ml using citrate buffer, making 0.01% ivory nut mannan and 0.028 nkat/ml.

2.2. Equipment

The experimental set-up is as described in earlier work [9] and consists of a metal-free Dionex 500 Chromatographic system (Dionex, Sunnyvale, USA). The gradient pump (GP 40) delivered the mobile phase at 1.0 ml/min. The dialysate components were separated using a CarboPac PA1 chromatographic column with a CarboPac guard column (both from Dionex) and detected by the integrated pulsed electrochemical detector (ED 40) using the waveform [9]; E_1=0.10 V (t_d=0.20 s, t_1=0.20 s), E_2=0.70 V (t_2=0.19 s) and E_3=−0.75 V (t_3=0.39 s). The GP 40 and ED 40 are linked using DX LAN™ communication and computer-controlled using PeakNet™ software (Dionex). The microdialysis probe was inserted into a 5.0-ml reaction vessel. A syringe pump (CMA/100 Microinjection pump, CMA/Microdialysis, Stockholm, Sweden) delivered the perfusion liquid at a flow rate of 2.0 μl/min, else stated, to the microdialysis probes equipped with a 16 mm SPS 6005 polysulfone membrane with a molecular mass cut-off (MMCO) of 30 kDa (provided by Dr. Thomas Laurell, Department of Electrical Measurements, University of Lund, Lund, Sweden) or CMA/10 polycarbonate membrane, MMCO of 20 kDa (CMA/Microdialysis, Stockholm, Sweden).

3. Quantitative aspects of on-line microdialysis sampling

Static microdialysis sampling was introduced by Bito et al. [13] and further developed by Delgado et al. [14], who introduced the "dialytrode" which was very similar to the present day microdialysis probe.

Use of microdialysis sampling is governed by recoveries during calibration of the microdialysis probe which should equal or closely approximate those achieved in the experiment. This prerequisite is never really fulfilled due to a possible variation in diffusion coefficients in different media, so sometimes recoveries may give an underestimate of the actual concentration.

As described in Bungay et al. [15], the recovery or extraction fraction (E_d) can be expressed as in Eq. 1.

$$E_d = (C_d^{out} - C^{in})/(C_b - C^{in}) = 1 - \exp[-1/Q_d(R_d + R_m + R_{ext})] \quad (1)$$

where C_d^{out} is the concentration of the analyte in the dialysate coming out of the probe, C^{in} is the concentration of the analyte in the perfusion liquid coming in, C_b is the concentration in the bioreactor, Q_d is the perfusion volumetric flow-rate, R_d is the resistance due to the dialysate, R_m is the dialysis membrane resistance and R_{ext} is the solution resistance to diffusion by the analytes. If water is used as the perfusion liquid, C^{in} equals zero and for very soluble low molecular mass analytes in a well-stirred solution as in the hydrolysis experiment, R_{ext} will be equal to the solution resistance (R_s) which also equals zero (R_{ext}=R_s=0) [15]. In such a case Eq. 1 becomes;

$$E_d = (C_d^{out})/(C_b) = 1 - \exp[-1/Q_d(R_d + R_m)] \quad (2)$$

which is more commonly expressed as relative recovery:

$$\text{Relative recovery (RR)} = \text{Recovery} \cdot 100 = C_d^{out}/C_b \cdot 100 \quad (3)$$

The relative recovery will be directly affected by mass transport across the membrane. Mass transport is controlled by the permeability of the analyte through the membrane. If the membrane surface area or length is fixed, selectivity of the analytes will depend upon their effective diffusion coefficients [16,17].

Application of this technique has been concentrated in the area of neuroscience [18] and pharmacopathology *in vivo* and *in vitro* as reflected by the publications in this field [19]. Marko-Varga et al. [8] described the application of on-line microdialysis sampling to monitoring a bioprocess. Also, Marko-Varga et al. [7] and Buttler et al. [10] reported the use of on-line microdialysis sampling in column liquid chromatography with amperometric alcohol biosensors, to follow the fermentation of lignocellulose to ethanol. Torto et al. [9] recently indicated the need for on-line microdialysis sampling to be quantitative, so that the data could be used especially in enzymatic studies. In the case of small-volume reactors, continuous flow microdialysis sampling presents a limitation as the concentration of the monitored analyte is changed significantly. Furthermore, continuous removal of the products of hydrolysis could also affect the reaction equilibrium, thereby distorting the information obtained during the monitoring of a bioprocess. This is more significant in kinetic studies where product formation might be affected by its depletion. The ability of the dialysis process to stop the reaction prior to sampling is very important, thus the need for a better approach, especially for small-volume reactors.

4. Evaluation of a microdialysis experiment

This work is an extension of previously reported, qualitative hydrolysis of ivory nut mannan using the endomannanase from *A. niger* [9]. For microdialysis sampling to be beneficial analytically, the data obtained has to be quantitative to allow interpretations that can be used to elucidate reaction pathways, mechanisms and kinetics in bioprocesses. However, it must be possible to assess the suitability of the microdialysis experiment during hydrolysis compared to the theoretical model. In order to evaluate the quantitative suitability of a microdialysis probe as a sampling device to follow reactions of this kind, two types of probes were tested, as the microdialysis model was developed based on physiology and metabolism [18]. To investigate the validity of the experimental data, it is preferable to examine the relationship of E_d (extraction fraction) and Q_d (volumetric flow-rate). According to Bungay et al. [15], a plot of $-\ln(1-E_d)$ versus $1/Q_d$ should give a straight line if the steady state conditions are met. The gradients for different compounds can be used to evaluate the magnitude of the product of membrane permeability and surface area. Also, this data will show the quantitative range at which the microdialysis probe is useful as a sampling device according to Eq. 1.

Fig. 1a and b show the effect of perfusion rates for two microdialysis probes with the same length, one fitted with a polycarbonate and the other with a polysulfone membrane, respectively, that were characterised under identical conditions. In Fig. 1a for DP 3, 4 and 5 the relationship is linear (r is 0.999) but for DP 2, the last two points deviate from linearity. Extraction fractions for all the points, which satisfy the E_d versus $1/Q_d$ relationship, is less than 0.7. In Fig. 1b, for the same flow-rate range, only the first 5 data points satisfy the steady-state conditions. The correlation coefficient values are between 0.997 and 0.999. For this microdialysis probe, the good data points are also the same points that are in the region with an extraction fraction of 0.7 or less. Although the probe fitted with a polysulfone membrane shows higher extraction fractions, the data points at extraction fractions greater than 0.7 do not satisfy the microdialysis model. However, this membrane was used for the experimental work because relative recovery plots show that the recoveries for the different oligosaccharides are much closer to each other than those found using the probe fitted with a polycarbonate membrane. This is important as the perfusate from the microdialysis experiment is of a very small volume, so taking account of the large volume of eluent required to elute such a sample plug to the detector, higher

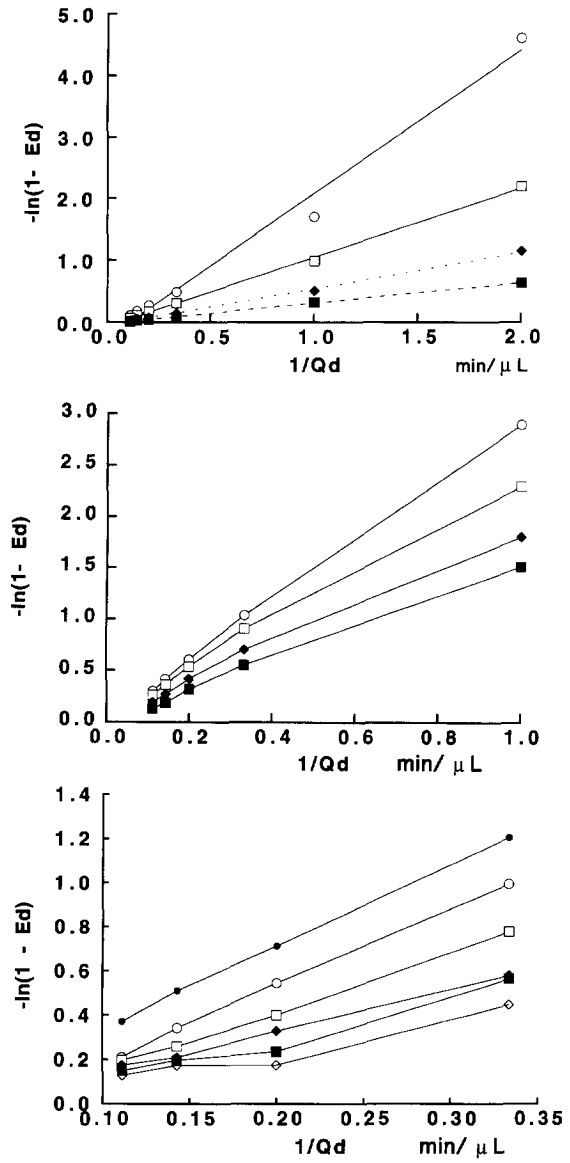

Fig. 1. (a) Effect of perfusion-rate on extraction fraction for the CMA/10 microdialysis probe for different oligomers. (○) represents DP 2, (□) DP 3, (◆) DP 4, (■) DP 5. (b) Effect of perfusion-rate on extraction fraction for the SPS 660 microdialysis probe for different oligomers. (○) represents DP 2, (□) DP 3, (◆) DP 4, (■) DP 5. (c) Effect of perfusion-rate on extraction fraction for the SPS 660 microdialysis probe for different oligomers. (●) represents DP 1, (○) DP 2, (□) DP 3, (◆) DP 4, (■) DP 5, (◇) DP 6.

recoveries are desirable. One disadvantage of microdialysis is that it puts higher sensitivity requirements on the detection system. IPED becomes the method of choice as it allows direct detection of oligosaccharides that are produced without the need for any sample treatment, as this introduces errors considering the small sample volumes. Also, use of on-line microdialysis sampling with IPED after chromatographic separation, when microcolumns are used, allows real time monitoring which facilitates the feedback mechanism.

Fig. 1c shows the effect of perfusion rates on the recovery of a mixture of oligomers of DP 1 to DP 6. This figure has been plotted using points obtained from flow-rates between 3 μl/min to 9 μl/min, as the data points for flow rates less than 3 μl/min do not satisfy the steady-state conditions of the microdialysis model. The trend in Fig. 1c is the same as that seen in Fig. 1b. As shown by the probe fitted with a polycarbonate membrane, if the recoveries of the analytes are not very close [9], and the extraction fraction is less than 0.7, the steady state conditions are met. This implies that for a microdialysis probe membrane of low permeability, use of low flow rates is suitable. At low perfusion rates, $R_d << R_m$ which results in a low extraction fraction (relative recovery). It is important that extraction fractions of less than 0.7 should be used as any higher value will not satisfy the model, hence the data will not be quantitative since such fractions are not well-accounted for by the model [18]. This also emphasises that it is not adequate to evaluate microdialysis probes based on their relative recoveries alone, without actually exploiting the results further using the plot devised by Bungay et al. [15]. Data from such a plot will compliment the information displayed in a percentage relative recovery curve, thus enabling the appropriate choice of the perfusion rate. As the SPS 6005 is home-made, as opposed to the commercially available CMA/10 polycarbonate probe, it would be interesting to investigate further the flow-rates less than 2 μl/min to see if there could be any reason other than the dialysis mechanism that leads to such data, as it is sometimes desirable to have high extraction fractions. Although the SPS 6005 microdialysis probe is checked for leakage before use there could be a possibility that at such low flow-rates, leakage could occur around the glue at the tip of the probe which then becomes significant. For the SPS 6005 microdialysis probe, perfusion rates higher than 2 μl/min are preferable.

Stenken et al. [20] demonstrated the importance of linear velocity with respect to recovery (extraction fraction), which complements the assumption made by Bungay et al. concerning resistances [15]. Although the permeability of the CMA/10 and SPS 6005 microdialysis probe membranes are different, Fig. 1a and b display the same 'mouth of the cone profile' in the region where steady-state is reached. At high perfusion rates (high linear velocity), as demonstrated by the points near the origin, dialysate resistance is dominant. As the perfusion rate is decreased, the extraction factor increases as the diffusion coefficients of the oligomers give them the 'mouth of the cone profile'. Low perfusion rates increase relative recovery as the contact time between the perfusate and the reaction vessel is also increased.

A further decrease in the perfusion rate results in domination by R_m ($R_m >> R_d$). For the SPS 6005 microdialysis probe membrane, the RR is much higher because of a higher permeability as it has a nominal molecular mass cut-off of 30 kDa. compared to 20 kDa. for the CMA/10. In physiological experiments [18] the perfusion rates have to relate to the studied vessels, however, for bioreactor sampling, varying the perfusion rates gives microdialysis sampling an added advantage of on-line controlled dilution.

5. Modes of microdialysis sampling

There are three possible modes of conducting a microdialysis experiment; CFMS, SFMSCS and SFMSSS. In CFMS, the perfusion and stirring are continuous over the monitoring period, for SFMSCS, perfusion is carried out only during sampling but the stirring is maintained continuously and for SFMSSS, the perfusion and stirring are only carried out during sampling. Hydrolysis is thus carried out under vanishing resistance where stirring is continuous and under quiescent conditions for stopped stirring.

5.1. Continuous flow microdialysis sampling

A 5 ml reaction vessel containing mannose was sampled on-line using CFMS. The sampling was monitored by injecting a mannose standard and analysing the dialysate. Fig. 2 shows the concentration change in both the reaction vessel and the collected dialysate (referred to as waste), over a 16 h period.

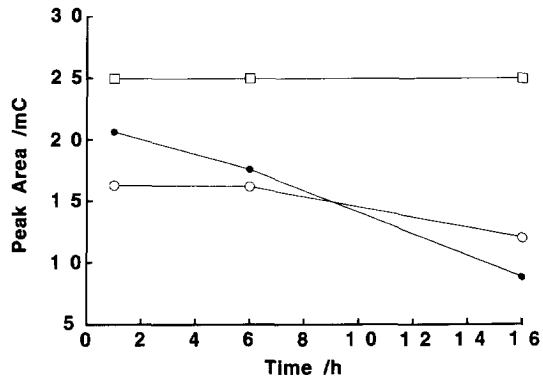

Fig. 2. Analyte depletion during on-line continuous microdialysis sampling, perfusing at 2 μl/min and 20 μl sample volume. (●) represents the reaction vessel, (□) mannose standard and (○) collected dialysate.

After 16 h the concentration of mannose in the reaction vessel has almost decreased by 50%. From the operation mode of microdialysis sampling this is expected as this is part of the driving force of the sampling mechanism. Suffice it to say that, as long as there is mannose in the reactor, there will always be a diffusion gradient between the incoming perfusion liquid (C^{in}) and the reactor (C_b). A decrease in the mannose concentration under experimental conditions would be due to either its consumption during the reaction of interest or the result of microdialysis sampling. Consequently, if the amount of depletion caused by the mode of sampling is unaccounted for, or unknown, then this compromises the quality of data obtained. It is interesting to note that the waste concentration also decreases continuously as it is diluted by an ever decreasing concentration of the analyte from the reaction vessel. This shows that for monitoring of a bioprocess in a small-volume reactor, it is mandatory that the perfusion rate should not lead to depletion of the bioreactor. In 16 h, 1920 μl of perfusion liquid are in contact with the reaction

vessel, resulting in a significant analyte loss. Shorter monitoring times are preferable, despite the limitation imposed by chromatography and the bioprocess. It is sometimes desirable to have enough time to elute analytes from the analytical column, regenerate the analytical column and rinse the sample loop with the perfusate to eliminate memory effects [9].

5.2. Stopped-flow microdialysis sampling with continuous stirring

Investigations with CFMS showed that this mode was not suitable for use with small-volume reactors. Further experiments were carried out with SFMSCS over only 8 h to investigate analyte depletion. SFMSSS was not used as the volume of perfusate in contact with the reaction vessel is equivalent to that in SFMSCS. Fig. 3a-c show the concentration profile for SFMSCS as the sample injection volume is decreased from (a) 20 μl, (b) 10 μl, and (c) 5 μl for a 5-ml sample reactor.

For these investigations, the dead volume between the microdialysis probe and the sample loop was decreased to reduce sample loss. A higher perfusion rate of 3 μl/min was used to encourage sweating of the membrane in order to increase the hydrodynamic flow and to eliminate membrane fouling. However, Fig. 3a shows a high analyte depletion after 8 h. Further reduction of sample loss was carried out by reducing the injection volume to 10 μl (see Fig. 3b). Fig. 3c shows a further reduction in the sample volume, giving a much improved concentration profile. In the case of Fig. 3c, the perfusate volume that comes into contact with the reaction vessel is only 1% of the vessel volume, which reduces analyte depletion significantly. Stopped flow microdialysis sampling modes are much preferable as they yield more quantitative data. Fig. 2, where CFMS was used showed a 50% decrease in analyte concentration compared to about 1% when stopped-flow microdialysis sampling is used. This shows that stopped flow microdialysis sampling is more suited to sampling in small-volume bioreactors. Often a volume change of a bioreactor or reaction vessel of less than 10% during sampling is acceptable for quantitative interpretation of data.

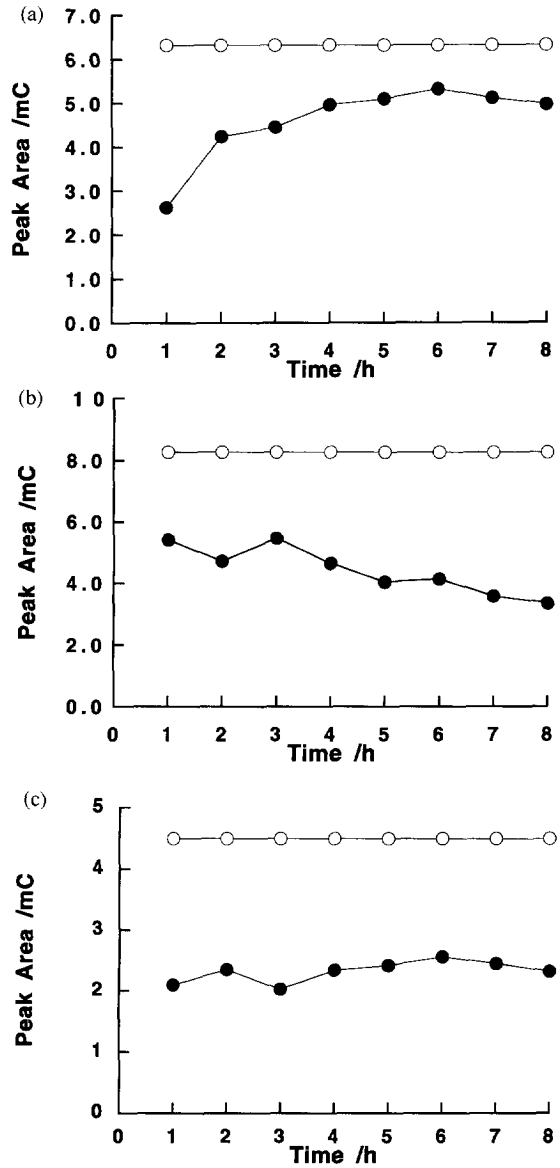

Fig. 3. Analyte depletion during on-line stopped-flow microdialysis sampling, perfusing at 3 μl/min. (●) represents the reaction vessel, (○) mannose standard, where the sample volume is (a) 20 μl, (b) 10 μl, and (c) 5 μl.

6. Quantitation of enzymatic mannan hydrolysis products

Reaction vessel volume investigations illustrated the need to keep sample volumes low in order to

allow on-line monitoring over a long period. Fig. 4a–c show the hydrolysis of ivory nut mannan using the endomannanase from *A. niger*. On-line microdialysis sampling; (a) CFMS, (b) SFMSCS, and (c) SFMSSS were used to sample oligomers of DP 1 to 5 for about 12 h at room temperature.

The data obtained by the three modes of microdialysis sampling demonstrate the reproducibility of the hydrolysis experiment, as they exhibit a similar product formation trend. CFMS has the greatest product formation rate, taking account of the varying sensitivity of IPED for different oligomers. The high product profile contrasts with the observations in Fig. 2 where there is a high analyte depletion. There are two possible reasons: firstly, the continuous removal of oligomers might be affecting the reaction equilibrium and secondly, there could be some product inhibition in the hydrolysis of ivory nut mannan using the endomannanase from *A. niger* as observed for the endomannanase from *Bacillus stearothermophilus* [21]. It has been observed during the use of membrane reactors that the continuous, selective removal of products could affect the chemical equilibrium and thus affect the yield, as a favourable shift towards the products is established [22]. SFMSCS (see Fig. 4b) shows the second highest product formation rate.

Fig. 5 is a cross-sectional view of the microdialysis probe membrane; the dotted inner parallel lines represent the 'blood side' where the dialysis takes place. The diffusion pathway of the analytes from the bioreactor and of those from the 'perfusion plug plug' is shown by arrows. During CFMS, hydrolysis is carried out under vanishing conditions of resistance. There is a steep concentration gradient between the perfusion liquid and the reaction vessel, and analyte diffusion will be at a maximum. For SFMSCS, diffusion of the analyte into the 'perfusion liquid plug' during sampling is limited by a phenomenon called concentration polarisation, which might arise due to non-specific interactions. Hydrophobic, hydrophilic and electrostatic interactions can possibly lead to the formation of a diffusion layer where permeation by the analyte of interest is decreased [23]. Depending on the design of the membrane, clogging can also be a serious problem that results in decreased recoveries. However, SFMSSS is limited

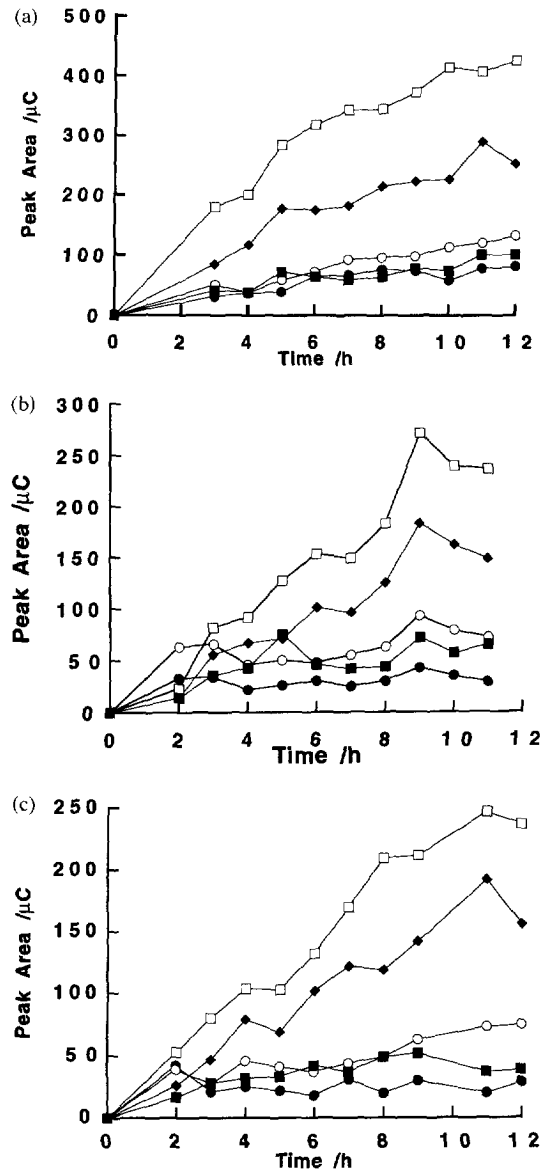

Fig. 4. (a) On-line monitoring of the hydrolysis of ivory nut mannan using continuous flow microdialysis sampling for different oligomers. (●) represents DP 1, (○) DP 2, (□) DP 3, (◆) DP 4, (■) DP 5. (b) On-line monitoring of the hydrolysis of ivory nut mannan using stopped flow microdialysis sampling with continuous stirring for different oligomers. (●) represents DP 1, (○) DP 2, (□) DP 3, (◆) DP 4, (■) DP 5. (c) On-line monitoring of the hydrolysis of ivory nut mannan using stopped-flow microdialysis sampling with stopped stirring for different oligomers. (●) represents DP 1, (○) DP 2, (□) DP 3, (◆) DP 4, (■) DP 5.

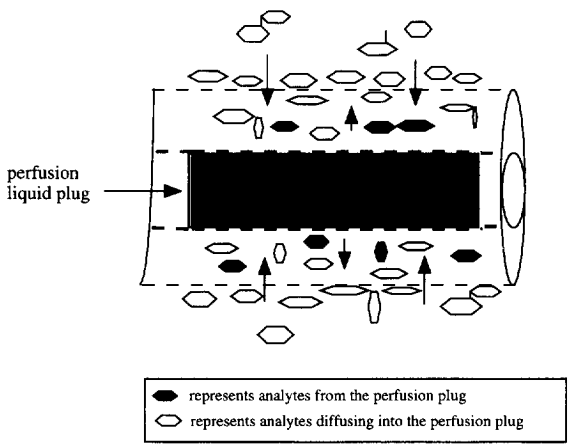

Fig. 5. A schematic cross-sectional view of a microdialysis probe membrane where the arrows show the diffusion pathway for analytes into/from the perfusion liquid plug.

by concentration polarisation in the perfusion liquid and outside the dialysis membrane. As shown by the black analytes (see Fig. 5), in the case of SFMSCS and SFMSSS, there could be some equilibration between the plug and the analytes from the bioreactor. Stirring removes the concentration boundary layer (Prandtl boundary layer [23]) outside the dialysis membrane and thus aids diffusion. Consequently it is expected that SFMSSS will show the least product formation rate. Other factors such as transmembrane pressure or osmolarity due to the presence of large molecules outside the membrane (protein) [24] will also affect the diffusion pathway/and direction. It is therefore important that the choice of a particular membrane which is usually governed by the morphology, porosity, pore size distribution, molecular mass cut-off, chemical resistance, temperature, pH, pressure tolerance and price [23], be complemented by suitable experimental conditions to allow proper functioning of the membrane. This is not only important for reducing operational costs, but also for producing reliable and reproducible samples from the bioprocess of interest.

The difference in hydrolysis products between SFMSCS and SFMSSS is not that significant since the reactions were carried out at room temperature. Hydrolysis data do not show any evidence of fouling of the membrane due to lack of stirring. Therefore,

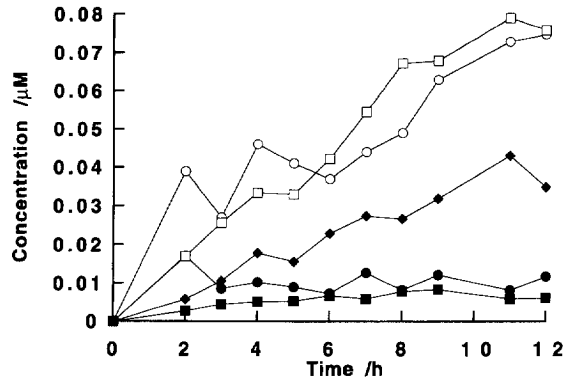

Fig. 6. On-line monitoring of the hydrolysis of ivory nut mannan using the endomannanase from *Aspergillus niger*, where (●) represents DP1, (○) DP2, (□) DP3, (◆) DP4, and [■] DP5.

stopped-flow microdialysis sampling better quantifies the hydrolysis products of the endomannanase from *A. niger*.

The hydrolysis products of ivory nut mannan incubated with *A. niger*-β-mannanase were quantified over a time period of 12 hours (Fig. 6). A typical chromatogram obtained during on-line monitoring is shown in Fig. 7 where the detector maximum output is 2 nC. A build-up of mannotriose, mannobiose and mannotetraose is observed. The concentrations of mannose and mannopentaose are at a considerably lower level and no significant build-up was observed after approx. 3 h. These results indicate that mannopentaose is hydrolysed by the *A.*

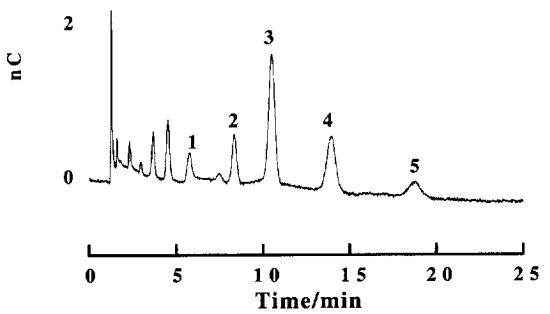

Fig. 7. A typical chromatogram obtained during on-line monitoring of the hydrolysis of 0.01% ivory nut mannan using the endomannanase from *Aspergillus niger*. The numbers represent the following oligosaccharides: (1) mannose, (2) mannobiose, (3) mannotriose, (4) mannotetraose and (5) mannopentaose. For chromatographic conditions, see text.

niger-β-mannanase, that has been observed earlier [25]. The major hydrolysis products of the *A. niger*-β-mannanase have been observed earlier and found to be mannotriose and mannobiose [5,25], typical for endomannanases [26]. At the end of the experiment it can be seen that the concentration of mannotetraose is decreasing. The results presented here are consistent with the observation that mannotetraose is hydrolysed by the β-mannanase, but at a slower rate compared to mannopentaose [25].

7. Conclusion

An on-line analytical system, for characterisation of enzyme hydrolytic properties in small-volume bioreactors, has been described. Three modes of on-line microdialysis sampling have been demonstrated. All three sampling modes are unique as there is no change in bioreactor volume during sampling. Stopped-flow microdialysis sampling was found to provide the most quantitative analyte using an injection volume of 5 μl and a perfusion rate of 3 μl/min, in a 5-ml reaction vessel. Both modes of stopped-flow microdialysis sampling are suitable for quantitation in small-volume bioreactors. However, continuous-flow microdialysis sampling can be used if the depletion of the analyte is not significant.

It is believed that the use of this system could be extended into the areas of biochemistry, biological and biotechnological sciences. The area of genetic engineering could benefit greatly from this technique. Its strength of applicability to small volumes, the ability to eliminate the protein from the detection system and the sensitivity of the IPED makes it a possible diagnostic tool for biochemistry and related fields in the future. As low as picogram quantities of carbohydrates can be detected using IPED especially, with post-column addition of sodium hydroxide which increases the detectibility of carbohydrates.

Also, the system should be useful in the characterization of unknown enzymes by analysis of degradation products, e.g., to discriminate between endo-activity and exo-activity or to observe transferase activity. The technique is limited however, in terms of the smallest possible volume that can be used, by the microdialysis probe design. During the microdialysis experiment, recoveries are increased if the reactor is stirred during sampling, a process which also results in an increase in volume as a stirrer has to be incorporated. However, other agitation methods can be investigated, as can the design of finer probes that will be accommodated in small volumes without necessarily decreasing the extraction fraction. Use of microcolumns is also desirable as this decreases dispersion and analysis time which is highly desirable in the case of on-line monitoring.

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References

[1] E. Sjöström, in Wood Chemistry, Fundamentals and Applications, 2nd Edition, Academic Press, San Diego, 1993.
[2] K.E. Eriksson, R.A. Blanchette and P. Ander, in Microbial Degradation of Wood and Wood Components, Springer-Verlag, Berlin, 1990.
[3] H. Stålbrand, Doctoral Thesis, Lund University, 1995.
[4] L. Viikari, M. Tenkanen, J. Buchert, M. Rättö, M. Bailey, M. Siika-aho and M. Linko, in J.N. Saddler (Editor), Bioconversion of Forest and Agricultural Plant Residues., CAB International, Wallingford, 1993.
[5] H. Stålbrand, P. Jonasson, J. Medve, A. Varga and F. Tjerneld, in preparation.
[6] B.V. McCleary, Meth. Enzymol., 160 (1988) 596.
[7] G. Marko-Varga, T. Buttler, L. Gorton, L. Ohlsson, G. Durand and D. Barceló, J. Chromatogr. A, 665 (1994) 317.
[8] G. Marko-Varga, T. Buttler, L. Gorton and C. Grönsterwall, Chromatographia, 35 (1993) 285.
[9] N. Torto, T. Buttler, L. Gorton, G. Marko-Varga, H. Stålbrand and F. Tjerneld, Anal. Chim. Acta, 313 (1995) 15.

[10] T. Buttler, L. Gorton, H. Jarskog, G. Marko-Varga, B. Hahn-Hägerdal, N. Meinander and L. Ohlsson, Biotechnol. Bioeng., 44 (1994) 322.
[11] B.L. Hogan, S.M. Lunte, J.F. Stobaugh and C.E. Lunte, Anal. Chem., 66 (1994) 596.
[12] H. Stålbrand, M. Siika-aho, M. Tenkanen and L. Viikari, J. Biotechnol., 29 (1993) 229.
[13] L. Bito, H. Davson, M. Murray and N. Snider, J. Neurochem., 13 (1966) 1057.
[14] J.M.R. Delgado, F.V. DeFeudis, R.H. Rooth, D.K. Ryugo and B.M. Mitruka, Arch. Int. Pharmacodyn., 198 (1972) 9.
[15] P.M. Bungay, P.F. Morrison and R.L. Dedrick, Life Sci., 46 (1990) 105.
[16] H. Benveniste, J. Neurochem, 52 (1989) 1667.
[17] K.M. Kendrick, E.B. Keverne, C. Chapman and B.A. Baldwin, Brain Res., 439 (1988) 1.
[18] T.E. Robinson and J.B. Justice Jr, in J.P. Huston (Editor), Microdialysis in the Neurosciences, Elsevier, Amsterdam, 1991.
[19] CMA Bibliography, updated version, Stockholm.
[20] J.A. Stenken, E.M. Topp, M.Z. Southard and C.E. Lunte, Anal. Chem., 65 (1993) 2324.
[21] G. Talbot and J. Sygusch, Appl. Environ. Microbiol., 56 (1990) 3505.
[22] D.M.F. Frazeres and J.M.S. Cabral, Enzyme Microb. Technol., 16 (1994) 738.
[23] P. Nardin, Anal. Biochem., 131 (1983) 492.
[24] M.C. Linhares and P.T. Kissinger., Trends. Anal. Chem., 11 (1992) 171.
[25] B.V. McCleary and N. Mathenson, Carbohydr. Res., 119 (1983) 191.
[26] E.T. Reese and T. Shibata, Can. J. Microb., 11 (1965) 167.