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Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 1041–1047
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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In vitro characterization of an in situ microdialysis sampling assay for elastase activity detection

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Received 17 March 2005; received in revised form 6 May 2005; accepted 10 May 2005
Available online 20 October 2005

Abstract

A microdialysis sampling method has been developed to detect the in vitro presence of a proteolytic enzyme, porcine elastase, external to a microdialysis probe. Elastase converts the substrate, succinyl(Ala)$_3$-*p*-nitroanilide (suc(Ala)$_3$-*p*-NA), to *p*-nitroaniline (*p*-NA). The substrate, suc(Ala)$_3$-*p*-NA, was locally delivered through the microdialysis probe to external solutions containing different elastase activities (0.025–0.5 units/mL). The product, *p*-NA, was recovered back into the probe. Dialysates containing both suc(Ala)$_3$-*p*-NA and *p*-NA were quantified using HPLC–UV. Different microdialysis suc(Ala)$_3$-*p*-NA extraction efficiencies (EE) were observed among different elastase-containing solutions (buffer and 0.3% agar solutions). The *p*-NA concentrations recovered back into the microdialysis probe correlated with the elastase activity external to the microdialysis probe. The greatest fraction of *p*-NA recovered as compared to substrate lost occurred with the highest flow rate used (5.0 μL/min). However, the highest concentrations of *p*-NA recovered occurred at the lowest flow rates. This method may allow for microdialysis sampling to be used as a means to study localized enzyme activity.

Keywords: Microdialysis sampling; HPLC–UV; Elastase

1. Introduction

The breakdown of tissue extracellular matrix (ECM) is an important component related to normal biological functions (e.g., angiogenesis, embryonic development, and wound healing) as well as pathological functions (e.g., arthritis, cancer, and cardiovascular disease). A group of proteolytic proteins called matrix metalloproteinases (MMPs) are believed to play vital roles in these processes [1–3]. There are more than 20 different human MMPs that have minor overlap with respect to their substrate selectivity. Many analytical methods have been described for ex vivo assays of the clinically relevant MMPs from biopsies [4]. Immunochemical methods give quantitative information regarding total enzyme concentrations. However, MMPs are released as pro-enzymes into the ECM and are converted in situ into their active forms. Thus, immunochemical methods cannot distinguish between the inactive pro-enzyme form and the activated enzyme. Zymography is a common method to separate and quantify the concentrations between the pro-enzyme and the active enzyme [5]. Zymography is a time-intensive method to quantify MMPs. The presence of active MMP enzymes in tissue extracts can be determined with fluorescence and colorimetric assays [6–10]. Research is ongoing to develop colorimetric and fluorescence assays for all the MMPs [11]. Recently, in vivo optical imaging methods have been described to determine MMP activity [12,13]. However, optical imaging methods can be constrained with respect to depth profiling.

Microdialysis sampling is a well-established in vivo sampling method for use in biologically complex matrices in many different tissues [14,15]. Microdialysis sampling is a diffusion-based separation method that uses a semi-permeable hollow-fiber dialysis membrane that is perfused at μL/min flow rates. Analytes that are smaller than the membrane pores diffuse into the inner fiber lumen and are carried to the outlet by the perfusion fluid. Larger analytes will either be completely rejected by the membrane pores or will diffuse so slowly through these pores that their recovery is negligible. The analyte concentration obtained in the dialysate is a fraction of the external analyte concentration

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doi:10.1016/j.jpba.2005.05.036

in which the probe is immersed. The fraction obtained is related to probe properties such as flow rate, membrane length, membrane material and its molecular weight cutoff (MWCO). Analyte concentrations obtained from the dialysate can be related to the sample concentration by using the membrane extraction efficiency (EE):

$$\mathrm{EE} = \frac{C_{\mathrm{outlet}} - C_{\mathrm{inlet}}}{C_{\mathrm{sample}} - C_{\mathrm{inlet}}}$$

where C_{outlet} and C_{inlet} are the outlet and inlet analyte concentrations of microdialysis probe, respectively, and C_{sample} is the analyte concentration far away from the microdialysis probe in the sample matrix. If C_{inlet} equals zero, EE is also called relative recovery (RR). The recovery describes the overall mass transport of a substance to and from the microdialysis probe and is commonly used as a means to calibrate the device.

The inherent non-selectivity towards molecules that can be recovered across the semi-permeable dialysis membrane makes microdialysis sampling highly useful for a wide variety of biochemical applications. A largely unexplored area of microdialysis sampling is that in which analytes could be included in the perfusion fluid and locally delivered to the probe implant site as a means to study localized biochemistry [16–18]. Microdialysis sampling coupled with appropriate analytical methods such as separations techniques for small molecules or multiplexed flow cytometric immunoassays for larger molecules allows for a nearly universal "biosensor" [19]. Thus, localized biochemistry and physiology could potentially be studied within an intact tissue using microdialysis sampling techniques.

Here we describe well-characterized studies using an in vitro model system of microdialysis sampling as a means to determine the presence of an important serine protease, elastase, external to the microdialysis probe. While microdialysis sampling has been used to assess enzymatic activity in an in vivo context similar to the in vivo imaging methods, there have been no well-controlled studies to determine the needed enzyme concentrations necessary to obtain a measurable signal of the product in the dialysate. The work presented here is to describe some of the challenges unique to using microdialysis sampling for attempting to make quantitative determination of enzymatic activity external to the probe. Porcine pancreatic elastase was chosen as a model enzyme system for MMP-12 (neutrophil elastase) because of its wide availability and ability to cleave the same substrate, suc(Ala)$_3$-*p*-NA [8]. The elastase substrate, suc(Ala)$_3$-*p*-NA, was included in the microdialysis perfusion fluid as shown in Fig. 1. The suc(Ala)$_3$-*p*-NA diffuses out of the microdialysis probe into the surrounding medium and reacts with elastase to form the product *p*-nitroaniline (*p*-NA). The microdialysis probe then collects the *p*-NA produced after the enzymatic reaction.

2. Materials and methods

2.1. Chemicals

Suc(Ala)$_3$-*p*-NA, *p*-NA, porcine pancreatic elastase (lyophilized powder and aqueous suspension), and agar were obtained from Sigma (St. Louis, MO, USA). Elastase and

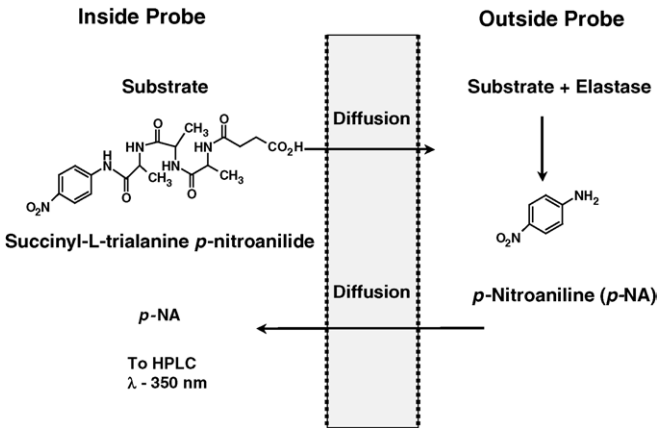

Fig. 1. Schematic of microdialysis method for the indirect detection of elastase. Suc(Ala)$_3$-*p*-NA is perfused through the probe and diffuses into the surrounding solution. Elastase external to the probe converts the substrate to *p*-NA that diffuses into the probe and is collected in the dialysate. The dialysate is protein-free due to the MWCO associated with the microdialysis membrane. The substrate and product in the dialysate are separated and quantified by HPLC–UV (350 nm).

suc(Ala)$_3$-*p*-NA solutions were prepared daily. All other chemicals were reagent grade or better. All solutions were prepared with nanopure water (Barnstead, Dubuque, IA, USA).

2.2. Microdialysis sampling

Microdialysis sampling was performed with a 1 mL Hamilton non-metallic syringe (Hamilton Company, Reno, NV, USA) and a CMA-102 syringe pump (CMA/Microdialysis, North Chelmsford, MA, USA). CMA 20/04 microdialysis probes with a 4 mm 20 kDa molecular weight cutoff (MWCO) polycarbonate/polyether (PC) membrane (CMA/Microdialysis, North Chelmsford, MA, USA) were used. The perfusion fluid consisted of 0.1 M sodium phosphate buffer, pH 7.0, supplemented with 500 μM of the elastase substrate, suc(Ala)$_3$-*p*-NA. The microdialysis probes were perfused at flow rates that ranged between 0.5 and 5.0 μL/min. Elastase was prepared between 0.025 and 0.5 units/mL in either sodium phosphate buffer or 0.3% agar, pH 7.0, in a total volume of 500 μL. Experiments were performed at either room temperature or 37 °C maintained by a dry bath incubator (Fisher Scientific, USA).

2.3. Product concentration as a function of EE

A 0.5 units/mL elastase solution in 0.1 M sodium phosphate buffer, pH 7.0, was prepared and heated to 37 °C in a 750 μL centrifuge vial. The substrate (500 μM) was perfused through the quiescent elastase solution at varying flow rates. A fresh elastase solution was used for each flow rate. Samples were collected at 15 min intervals.

2.4. HPLC

Suc(Ala)$_3$-*p*-NA and *p*-NA were separated and quantified by HPLC–UV. The system consisted of a P1000 pump with a variable wavelength UV 1000 detector set to 350 nm controlled by

PC 1000 software (ThermoSeparations Products, San Jose, CA). A Hypersil 5 micron C18 column (250 mm × 2 mm) with a Phenomenex security guard cartridge was employed. The mobile phase consisted of water:acetonitrile:1.0 M sodium phosphate buffer, pH 7.0 (65:25:10, v:v:v). The flow rate was 0.2 mL/min.

2.5. Elastase activity determination

A Hitachi UV spectrometer was used to determine elastase activity. Elastase solutions (0.01–1 units/mL) were prepared in 0.1 M sodium phosphate buffer, pH 7.0. The elastase solutions were mixed with isotonically matched suc(Ala)$_3$-*p*-NA to achieve a total solution concentration of 500 μM. The change in absorbance for the production of *p*-NA upon mixing the elastase and suc(Ala)$_3$-*p*-NA solutions was measured at 400 nm. The linear relationship between the change in absorbance versus the calculated elastase concentration was used to confirm experimental elastase concentrations.

The elastase solution stability was determined over a 6-h period at room temperature and over a 2-h period at 45 °C (to ensure stability in agar). Standard elastase solutions were prepared and aliquoted. The initial elastase activity was determined by spectrophotometry and the aliquot activity was measured on an hourly basis. Measurements were recorded at room temperature.

2.6. Michaelis–Menten kinetics

Different substrate concentrations (0.5–1 mM) were combined with elastase to an enzyme activity of 0.05 units/mL in 0.1 M sodium phosphate buffer, pH 7.0. The change in absorbance at 400 nm was measured for the production of *p*-NA to determine the initial rate. K_m and V_{max} were determined from a direct linear plot of the data. Measurements were conducted in triplicate at room temperature.

2.7. Limit of detection and quantitation

The signal for seven standard 5 μM *p*-NA samples was measured and the standard deviation calculated. The average signal for seven 0.1 M sodium phosphate buffer solutions was calculated. The LOD and LOQ were then determined at the 98% confidence level by using methods outlined in a standard reference text [20].

3. Results and discussion

3.1. Stability and reproducibility

Standard solutions of *p*-NA were found to be stable for 2 weeks at room temperature. Suc(Ala)$_3$-*p*-NA and elastase solutions were found to be stable for up to 6 h at room temperature. Elastase solutions prepared from lyophilized powder in 0.1 M sodium phosphate buffer, pH 7.0 and stored at 4 °C were found to progressively lose activity over a 48 h period. Porcine pancreatic elastase was determined to be stable at 45 °C as previously reported [21]; thus allowing it to be incorporated into agar gels.

An important aspect to assessment of this microdialysis method for determining enzyme activity external to the probe was the ability to have reproducibility during the run-to-run preparation of the enzyme solutions. Initially a lyophilized powder of porcine pancreatic elastase was used and prepared on a daily basis. Despite care to ensure reproducible weighing and solution transfer, the run-to-run *p*-NA kinetics after inclusion of a fixed amount of suc(Ala)$_3$-*p*-NA was quite variable and had an R.S.D. of 25.4% ($n = 3$, data not shown). This problem with enzymatic activity variability was overcome by switching to a commercially available solution-based elastase preparation, which had a run-to-run kinetic activity R.S.D. of 4.5% ($n = 5$, data not shown).

3.2. Microdialysis sampling

Microdialysis sampling allows for the collection of a wide variety of low molecular weight analytes. Thus, microdialysis sampling coupled with appropriate analytical separations methods to quantify targeted analytes allows it to be a versatile sensor mimic [19,22]. HPLC was used to separate and quantify the suc(Ala)$_3$-*p*-NA from *p*-NA in the dialysate samples. The *p*-NA obtained in the dialysate could be easily quantified via a colorimetric assay because of its high molar absorbtivity at 400 nm. The low dialysate volumes obtained coupled with the need to determine the percentage of the substrate lost necessitates the use of the HPLC method for the analysis. Fig. 2 shows the chromatogram for the isocratic separation of the suc(Ala)$_3$-*p*-NA and *p*-NA demonstrating that these two analytes can be easily separated and quantified. For the product *p*-NA, the LOD for this method was 1.14 ± 0.05 μM ($n = 5$) and the LOQ was 4.00 ± 0.36 μM ($n = 5$).

An important consideration during microdialysis sampling is the microdialysis probe extraction efficiency (EE) at different flow rates. This is important for this work since the product concentrations that are collected in the microdialysis probe are

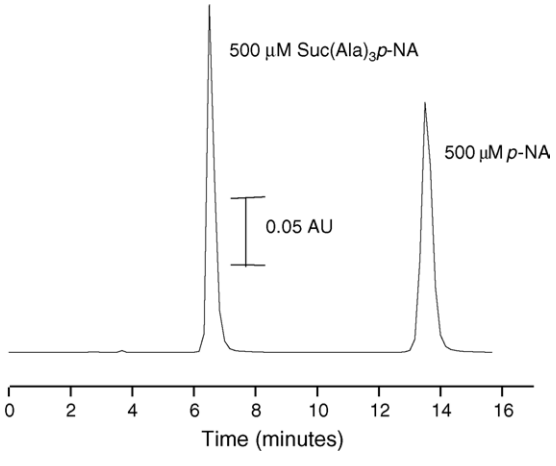

Fig. 2. HPLC separation of 500 μM suc(Ala)$_3$-*p*-NA and 500 μM *p*-NA using isocratic conditions. The detection wavelength was 350 nm.

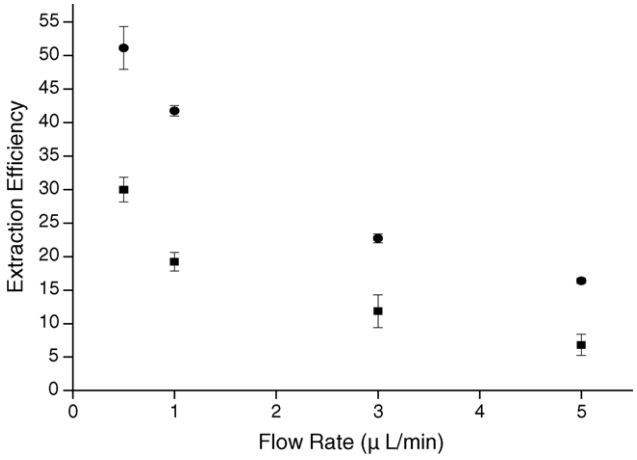

Fig. 3. EE for 500 μM suc(Ala)$_3$-*p*-NA (■) (MW 451.4) and 45 μM *p*-NA (●) (MW 138.1). The dialysate was collected under quiescent conditions at 37 °C in 0.1 M sodium phosphate buffer, pH 7.0. Error bars denote S.D. ($n = 3$) at each flow rate.

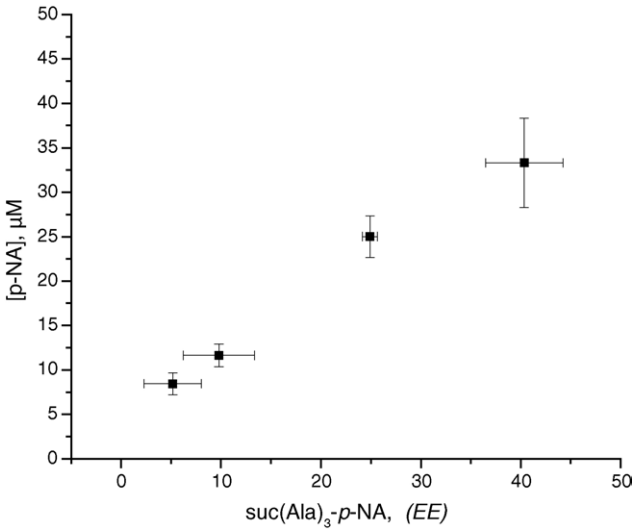

Fig. 4. EE of suc(Ala)$_3$-*p*-NA (■) and subsequent recovery of *p*-NA (●) at different flow rates (0.5–5.0 μL/min) from a 37 °C quiescent solution. A fresh elastase solution containing 0.5 units/mL was used for each flow rate. Error bars denote S.D. ($n = 3$) for each EE value and the *p*-NA concentration.

generally significantly lower than the initial substrate concentrations infused through the probe. For these studies it was important to optimize the microdialysis flow rate conditions so as to obtain the maximum amount of *p*-NA back into the probe after a localized delivery of the substrate.

Fig. 3 shows the microdialysis EE for suc(Ala)$_3$-*p*-NA and *p*-NA in a quiescent buffer solution at 37 °C. Both analytes exhibit typical microdialysis EE behavior with high EE values obtained at low perfusion fluid flow rates and low EE at higher perfusion fluid flow rates. These EE values ranged between 5 and 32% for suc(Ala)$_3$-*p*-NA (MW 451.4) and 20 and 53% for *p*-NA (MW 138.1) using a flow rate range of 0.5–5 μL/min. The difference in EE observed between suc(Ala)$_3$-*p*-NA and *p*-NA is typical for analytes with different molecular weight since microdialysis sampling is a diffusion-based separation process.

3.3. Product recovery in buffer

The *p*-NA concentration recovered as a function of different suc(Ala)$_3$-*p*-NA EE, achieved using different flow rates, is shown in Fig. 4 for a quiescent 0.5 units/mL elastase solution at 37 °C. A fresh elastase solution was used for each flow rate to prevent build up of *p*-NA in the solution. The greatest EE for the substrate and recovery of product is observed at the lowest flow rate. Note that for suc(Ala)$_3$-*p*-NA the EE is higher in this solution than for that shown in Fig. 3 because of the presence of the enzymatic reaction. The coupling of the enzymatic reaction with the diffusion out of the microdialysis probe serves to remove the substrate and thus increase the amount lost across the microdialysis membrane.

Table 1 shows the mean concentrations lost across the dialysate probe for suc(Ala)$_3$-*p*-NA, the mean concentration of *p*-NA recovered, and the fraction of these two values. Contrary to what normally would be expected, the fraction of *p*-NA recovered was actually higher for the higher flow rates and lower for the lower flow rates. However, the highest concentrations of product were obtained at the lowest flow rates. This is consistent with microdialysis sampling because the longest residence times would exist allowing for more diffusion time at these flow rates. When using lower flow rates, much higher molar concentrations of the substrate would exist directly outside of the probe extending into the sample space. This analysis is important as it suggests that in order to obtain measurable concentrations of the product to verify enzymatic presence, it is necessary to use relatively high concentrations of substrates with very low flow rates. This means that high substrate concentrations will exist external to the dialysis probe. However, when compared to imaging approaches, the actual substrate concentrations in the tissue space are not measured and therefore unknown. This could pose an advantage for microdialysis sampling since the overall amounts of material lost are localized unlike a systemic injection for an imaging agent and also quite low in this example (picomole lost per minute).

Table 1
Average suc(Ala)$_3$-*p*-NA loss and *p*-NA recovery

Average (EE)	Flow rate (μL/min)	[Substrate] (μM) lost	Lost substrate (pmol/min)	[*p*-NA] (μM) recovered	Fraction *p*-NA recovered (%)[a]
5.2	5	25.8	129.2	8.4	32.6
9.8	3	48.9	146.7	11.6	23.8
24.9	1	124.6	125.6	25.0	20.1
40.4	0.5	201.8	100.9	33.3	16.5

[a] The fraction of the *p*-NA concentration recovered as compared to the suc(Ala)$_3$-*p*-NA concentration lost.

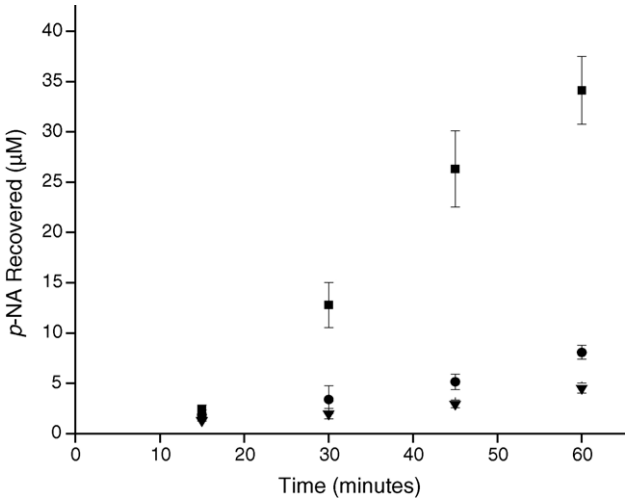

Fig. 5. Recovery of *p*-NA by microdialysis sampling at 1.0 μL/min using various conditions. (■) 0.1 units/mL 0.03% agar/elastase at 37 °C. (▼) 0.1 units/mL elastase in a well stirred solution at 25 °C. (●) 0.1 units/mL elastase in a quiescent solution at 37 °C. All solutions were prepared in 0.1 M sodium phosphate buffer, pH 7.0 with 500 μM substrate and an elastase activity of 0.1 units/mL. Error bars denote S.D. ($n = 3$) for each activity, i.e., three separate solutions were prepared for each enzymatic activity value.

3.4. Comparison of product recovery in quiescent buffer, stirred buffer and agar

The recovery of *p*-NA into the microdialysis probe after the reaction of suc(Ala)$_3$-*p*-NA with elastase was explored using various conditions. Fig. 5 shows the *p*-NA concentrations collected in the microdialysis probe after infusion of 500 μM suc(Ala)$_3$-*p*-NA to a well-stirred elastase solution at room temperature, a quiescent solution at 37 °C and an elastase suspension in 0.3% agar at 37 °C. The 0.3% agar solution provided the greatest concentration recovery of *p*-NA during microdialysis sampling. Performing the microdialysis experiment in a 0.3% agar solution was of interest since it has been commonly used to mimic extracellular matrix conditions [23]. Furthermore, performing the microdialysis sampling experiments in the 0.3% agar provided greater reproducibility as compared experiments performed in quiescent buffer solutions due to decreased possibility for convection at the probe/solution interface in agar. Microdialysis sampling performed in the stirred solution at room temperature shows the least *p*-NA produced. This is likely due to the decreased enzyme activity at 25 °C as well as the external stirring which serves to decrease localized build up of substrate as well as product at the probe/solution interface.

It is interesting to compare the suc(Ala)$_3$-*p*-NA EE values among the different conditions employed shown in Table 2. For the stirred conditions at 25 °C, the loss (EE) of suc(Ala)$_3$-*p*-NA, was not statistically different between solutions with or without enzyme added. The stirred solution creates an external system such that diffusional mass transport resistance is greatly minimized [24]. In other words, the stirring serves to rapidly remove the substrate away from the dialysis probe. Adding an enzyme to the well-stirred system does not change the EE since the stirring is more effective than the enzyme at removing the substrate from the dialysis probe/solution interface. When elastase is added to the solution external to the microdialysis probe, the loss (EE) of suc(Ala)$_3$-*p*-NA is increased as compared to controls that do not contain enzyme. The increased EE is due to the removal of substrate via the enzymatic reaction. The overall extent of measurable differences in suc(Ala)$_3$-*p*-NA microdialysis EE would be expected to be correlated with the enzyme concentration external to the dialysis probe. Comparing the agar solutions, there is a statistically significant difference between the EE in an agar solution containing no enzyme and that containing 0.5 units/mL elastase.

Table 2
Suc(Ala)$_3$-*p*-NA EE between control and elastase-containing solutions

Condition	EE buffer	EE elastase
Stirred	29.4 ± 2.6	28.5 ± 4.9
Agar	22.0 ± 1.8[a]	29.3 ± 2.5[a]
Quiescent	27.1 ± 3.9	33.3 ± 0.8

Suc(Ala)$_3$-*p*-NA (500 μM) was perfused through the microdialysis probe at 1.0 μL/min. The probe was immersed into either a well-stirred solution at room temperature or 0.3% agar and quiescent buffer solution at 37 °C, $n = 3$. The enzyme activity was 0.5 units/mL.

[a] Denotes statistically significant difference as determined by a *t*-test at the 95% confidence level.

3.5. Comparison of product recovery using different elastase concentrations

Fig. 6a shows the concentration recovery of *p*-NA into the dialysis probe during an infusion of 500 μM suc(Ala)$_3$-*p*-NA though a probe immersed in a 0.3% agar/elastase suspension containing a range of elastase concentrations (0.025–0.5 units/mL). As the enzyme activity increased, the resulting *p*-NA concentrations recovered back into the probe also increased. The *p*-NA concentrations collected back into the dialysis probe correlated with the concentration of enzyme external to the probe. It is not clear why the *p*-NA concentrations in Fig. 6a show greater run-to-run variability as denoted by the standard deviation error bars. Perhaps greater error is incurred during the agar preparation at these enzymatic higher concentrations. Fig. 4 depicts the aqueous data using the same enzyme concentration (0.5 units/mL) and the coefficient of variation is actually less when compared to the agar studies. Fig. 6b shows suc(Ala)$_3$-*p*-NA EE obtained for the same experiments shown in Fig. 6a. An important feature to point out for Fig. 6b is that EE is constant and rapidly achieves a steady state that is maintained throughout the duration of the experiment. When the microdialysis EE reaches and maintains a steady-state value, this is indicative of a chemical reaction outside the probe. The EE between the two separate concentrations of elastase concentrations of elastase depicted ranged from 18.1–20.1% for 0.025 units/mL to 30.8–36.1% for 0.5 units/mL. Despite the 20-fold increase in overall enzyme activity external to the dialysis probe, the suc(Ala)$_3$-*p*-NA EE only changes by approximately 50%. This demonstrates how EE is related to the sum of all the mass transport processes in the system including mass transport through the sample, dialysis membrane, and dialysis perfusion

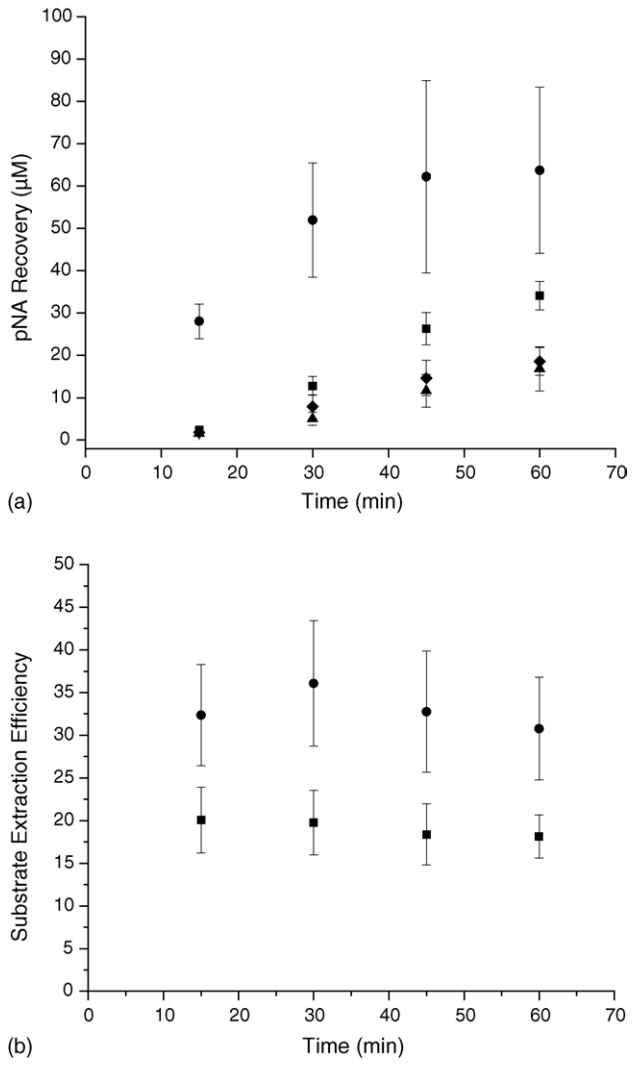

Fig. 6. (a) Recovered *p*-NA into the dialysis probe immersed into a 0.3 wt.% agar solution with varying elastase concentrations. The dialysis probe was perfused with 500 μM substrate and the elastase activities are denoted as ▲ (0.025 units/mL), ♦ (0.05 units/mL), ■ (0.1 units/mL) and ● (0.5 units/mL). Error bars denote S.D. ($n = 3$) for each specified enzymatic activity. A separate solution was prepared for each individual experiment. (b) Substrate EE in for a dialysis probe immersed in 0.025 units/ml (■) and 0.5 units/ml (●) elastase/0.3% agar. The substrate (500 μM) was perfused through elastase/0.3 wt.% agar at a flow rate of 1 μL/min at 37 °C. Error bars denote S.D. ($n = 3$) for each specified enzymatic activity. A separate solution was prepared for each individual experiment.

fluid. Among the lower enzyme activity values studied (0.025, 0.05, and 0.1 units/mL) there was no statistical difference in the suc(Ala)$_3$-*p*-NA EE despite observable differences in the recovered *p*-NA back into the dialysis probe (data not shown).

The overall concentrations of *p*-NA recovered into the microdialysis probe are a small fraction of the amount of substrate that diffuses out of the membrane. For the 0.025 units/mL experiment, the suc(Ala)$_3$-*p*-NA EE is approximately 20% which means from an initial infusion concentration of 500 μM the outlet dialysate concentration is 400 μM. Using a flow rate of 1 μL/min, this means 0.1 nmol/min of suc(Ala)$_3$-*p*-NA was delivered to the enzyme solution. The amount of *p*-NA recovered ranged between 1.5 and 5.3 μM for the 15 and 60 min measurements, respectively. Using this delivery rate, at 60 min, 6 nmol has been delivered to an enzyme solution in 0.5 mL, corresponding to a *p*-NA concentration of 12 μM. The concentrations delivered are well below the overall specified enzymatic activity. For 0.025 units/mL in 500 μL (0.0125 units), the system should be able to convert 12.5 nmol of substrate per minute since 1 unit of elastase converts 1 μmol suc(Ala)$_3$-*p*-NA per minute. Furthermore, the concentration of substrate delivered is much lower than the reported K_m and our experimentally derived K_m for elastase. The Michaelis–Menten constant for the substrate was determined by direct linear plot to be 5.8 mM and the maximum velocity was 3.25 mM/min. This experimentally derived K_m for the substrate was similar to a previously reported value of 6.5 mM [25].

4. Conclusions

Microdialysis sampling allows for simultaneous delivery and recovery of low molecular weight compounds. The presence of porcine elastase which served as a model enzyme external to a microdialysis probe was determined by infusing a known enzymatic substrate through the dialysis probe. This infusion allowed for a localized delivery of substrate to an enzyme solution and the concomitant product recovery was followed for enzyme incorporated into different solutions. The use of 0.3 wt.% agar provided the best overall reproducibility between runs. These results suggest that microdialysis sampling may allow for the active form enzymes to be interrogated at the site of action without the need to remove the exudate ex vivo. Certainly the validity of the microdialysis approach for determining specific enzymatic activity has to be addressed on a case-by-case basis.

Acknowledgements

Funding from the Dreyfus Foundation for Joshua Villeneuve via the Summer Research Program for Minority High School Students at Rensselaer Polytechnic Institute is gratefully acknowledged. Additional supplemental funding from NSF for JV is gratefully acknowledged. Funding from NSF CAREER 99-84150 and NIH EB001441 is also gratefully acknowledged.

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