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Infrared laser ablation and capture of enzymes with conserved activity

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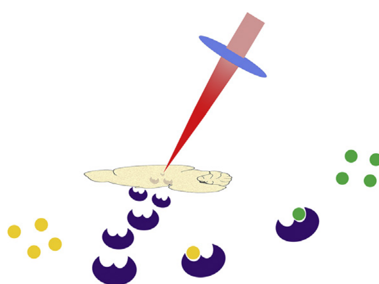
HIGHLIGHTS

- Laser ablation and capture of enzymes from rat brain.
- Enzymes transferred with 75% efficiency with laser fluences in 10–30 kJ/m^2 range.
- 37% of the transferred trypsin was active while 50% of transferred catalase was active.
- Catalase activity measured from ablation transferred rat brain tissue tracks anticipated catalase activity distribution.

GRAPHICAL ABSTRACT



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ABSTRACT

Infrared (IR) laser ablation at 3 μm wavelength was used to extract enzymes from tissue and quantitatively determine their activity. Experiments were conducted with trypsin, which was ablated, captured and then used to digest bovine serum albumin (BSA). BSA digests were evaluated using matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) and sequence coverage of 59% was achieved. Quantification was performed using trypsin and catalase standards and rat brain tissue by fluorescence spectroscopy. Both enzymes were reproducibly transferred with an efficiency of 75 ± 8% at laser fluences between 10 and 30 kJ/m^2. Trypsin retained 37 ± 2% of its activity and catalase retained 50 ± 7%. The activity of catalase from tissue was tested using three consecutive 50 μm thick rat brain sections. Two 4 mm^2 regions were ablated and captured from the cortex and cerebellum regions. The absolute catalase concentration in the two regions was consistent with previously published data, demonstrating transfer of intact enzymes from tissue.

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1. Introduction

Enzyme histochemistry combines the measurement of enzyme activity with localization information and serves as a link between biochemistry and morphology [1]. Enzyme histochemistry has been used in diagnostic pathology and pathobiology, as well as in experimental pathology [2]. The activity of an enzyme is regulated at different levels from mRNA to post translational modifications [3, 4] and from molecular interactions between the cytoplasm and organelles to other regulation mechanisms in the extracellular matrix [5]. Accordingly, the full picture of enzyme activity cannot be determined simply by total protein or mRNA quantification.

Imaging of fresh frozen tissue sections and biopsies using methods such as fluorescent probes, chromogenic probes, and *in situ* zymography [6, 7] allows measurement of enzyme activity with localization information. In the case of fluorescent or chromogenic agents, probes can be sprayed on the tissue section before measuring the localized signal [8, 9]. After enzyme reaction on the surface of the tissue section, the localized indicator is activated

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based on enzyme activity [8-10]. Similarly, *in situ* zymography is an electrophoretic technique that uses fluorescent or chromogenic reactions [11]. There are two general zymography methods [6, 12]: tissue sections can be mounted on a glass slide coated with a fluorescent substrate, or first mounted on a slide, then immersed in a solution containing fluorescent substrate. Unlike fluorescent or chromogenic probes that are able to detect various enzymes [8, 9, 13, 14], the substrates used for zymography are typically protein based [15, 16], such as gelatin or collagen, which make *in situ* zymography well suited for proteases [17]. Imaging based methods require special probes, the design of which can be challenging due to time consuming steps, high costs, [18] and their potential for non-specific binding [19].

Extraction of enzymes from small regions of tissue sections allows the measurement of localized enzyme activity [20, 21]. Regions of interest (ROI) containing enzymes can be isolated via manual microdissection followed by extraction and analysis. Extraction from microdissected tissue allows measurement of isolated cell populations in solution rather than on the tissue section surface. This enables more flexibility in adjustment of reaction conditions such as temperature and pH, which can play an important role on reproducibly of enzyme assay [22]. In addition, extraction of enzymes can facilitate absolute quantification of their activity, whereas imaging techniques are often limited to relative quantification [23-25]. Although ROI can be isolated by manual microdissection, where the material is removed under an optical microscope [21, 26], this technique is somewhat labor intensive and has limited reproducibility [27].

An alternative dissection technique is laser capture microdissection (LCM) [28], which employs a focused laser to cut and isolate regions from a tissue section. There are two general LCM configurations that use either an infrared (IR) or ultraviolet (UV) laser [28, 29]. IR-LCM employs a thin thermoplastic film that covers a tissue section. A near IR laser is used to irradiate and melt part of the film, causing it to fuse with that part of the tissue section. The film is detached together with the tissue material [30]. UV-LCM uses a UV laser to cut the boundary of a ROI on a tissue section and the ROI is then detached using unfocused laser pulses [31]. In both LCM configurations, collected tissue regions require extraction and cell lysis before measuring enzyme activity [20].

An alternative to LCM is laser ablation and capture, where the region of interest is removed with a pulsed infrared laser [32, 33]. The ejected material is collected and biomolecules can be extracted without the need of cell lysis or addition of detergents [32-34]. The main absorber of infrared laser radiation in tissue is water, which as an absorption maximum at 2.94 μm [35]. The optical penetration depth is approximately 1 μm at room temperature, but increases with temperature, facilitating greater material removal at higher pulse energies [36]. Another tissue absorber is protein, which has OH and NH stretch absorbers at 3 μm and CH stretch absorbers at 3.4 μm. Absorption at these wavelengths can produce ablation of proteins even in nominally dry samples [37]. Absorption of pulsed nanosecond mid-IR laser light is sufficiently rapid to produce a volumetric phase change and explosive boiling of the irradiated volume [38, 39]. The recoil stress of the phase explosion leads to the ejection of particulate with size distributions that vary with laser energy and the mechanical strength of the tissue [38, 40]. The removal of material as particulate appears to protect fragile biomolecules from fragmentation allowing the capture of intact peptides, proteins [33], and DNA [34] from tissue using a nanosecond laser. Near-IR and mid-IR picosecond lasers can even more efficiently produce explosive boiling in tissue and have been employed to ablate and capture cells, virus and proteins with conserved function and activity [41-43].

In the work described below, enzymes from thin films as well as from tissue sections were ablated and captured using a nanosecond mid-IR laser, and their activity quantitatively assessed. Trypsin and catalase enzyme standards were laser ablated using a 3 μm wavelength laser and the transfer efficiency was measured using Bradford assay while the activity of trypsin was qualitatively assessed by using it to digest bovine serum albumin (BSA) before analysis by MALDI mass spectrometry. Quantitative assessment of the activity of trypsin and catalase standards after laser ablation was measured using fluorescence assays and the activity of catalase ablated and captured from rat brain tissue sections was determined.

2. Experimental

2.1. Chemicals and materials

Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Reagents DL-dithiothreitol (DDT, 98%), iodoacetamide (IAA, BioUltra, 99%), α-cyano-4-hydroxycinnamic acid (CHCA), and ammonium bicarbonate (ABC, BioUltra, 99.5%) were obtained from Sigma-Aldrich (St Louis, MO, USA). Trifluoroacetic acid (99.5%, LC-MS grade) and acetonitrile (99.9%, LC-MS grade) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Bovine serum albumin (BSA) and glass microscope slides (25 × 75 mm) were obtained from VWR (Radnor, PA). BSA from VWR was used as substrate for trypsin digestion. Ultrapure water (18 MΩ) was produced with a Barnstead Nanopure Diamond Lab Water System (Thermo Fisher Scientific). The ABC buffer was prepared at a concentration of 10 mM and corrected to a pH of 7.4.

A Bradford assay kit (Coomassie Plus, Thermo Fisher Scientific), which included Coomassie dye and BSA protein standard, was used to build calibration curves for protein quantification. A fluorescent protease assay kit (Pierce, Thermo Fisher Scientific) included L-(tosylamido-2-pheyl) ethyl chloromethyl ketone (TPCK) treated trypsin, fluorescein isothiocynante (FTIC) labelled casein, and tris buffered saline (TBS; 25 mM tris; pH 7.2, 150 mM NaCl). An Amplex Red catalase assay kit (Life Technologies, Grand Island, NY, USA) included Amplex Red reagent, dimethylsulfoxide (DMSO), horseradish peroxidase, hydrogen peroxide, reaction buffer, and catalase.

2.2. Sample preparation

Enzymes were reconstituted in TBS or fluorescence reaction buffers and BSA was dissolved in 10 mM ABC buffer (pH 7.4) at a concentration of 0.5 mg/mL. Aliquots of the enzyme solutions were deposited on a plain microscope slide (cleaned with ethanol) and dried for 2 min under vacuum before ablation.

Sprague Dawley rat brain tissue samples were collected from 6 week old rats using procedures approved by the LSU Institutional Animal Care and Use Committee (IACUC) at the LSU School of Veterinary Medicine, Division of Laboratory Animal Medicine (DLAM). The animals were sacrificed by carbon dioxide exposure and tissue samples were collected and snap-frozen within 30 min using liquid nitrogen and stored at −80 °C prior to use. Frozen tissue was sectioned at a thickness of 50 μm and thaw-mounted on the microscope slide at −20 °C using a cryostat (CM 1850, Leica Microsystems, Wetzlar, Germany). Optimal cutting temperature solution (OCT, Sakura Finetek, USA) was used to fix one side of the brain tissue sample to the cryostat support. Slides were stored at −80 °C until further processing and were vacuum dried for 10 min prior to sampling.

2.3. Laser ablation sample transfer

The mid-IR laser ablation system has been described in detail previously [33, 44]. Briefly, a wavelength tunable pulsed IR optical

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