SUPPORTING INFORMATION

Engineering of *Aerococcus viridans* L-lactate oxidase for site-specific PEGylation: characterization and selective bioorthogonal modification of a S218C mutant

Birgit Unterweger[†], Thomas Stoisser[†], Stefan Leitgeb[†], Ruth Birner-Grünberger[‡], and Bernd Nidetzky[†]*

[†] Research Center Pharmaceutical Engineering, and Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria

[‡] Institute of Pathology and Center for Medical Research, Medical University of Graz, Stiftingtalstraße 24, A-8010 Graz, Austria

Keywords: L-Lactate oxidase; site-specific PEGylation; cysteine-selective bioconjugation; biosensor; cysteine.

* Corresponding author

Telephone: +43-316-873-8400; fax: +43-316-873-8434; e-mail: bernd.nidetzky@tugraz.at

List of contents

Table S1 summarizing kinetic parameters of wild-type enzyme, the S218C mutant, and the PEGylated form of the mutant for α-hydroxyacid substrates other than L-lactate

Figure S1 showing unfolding of PEGylated and unmodified S218C mutant in a thermal-shift differential scanning fluorometry experiment.

Figure S2 showing elution of the S218C mutant in hydrophobic interaction chromatography (panel A) and a SDS polyacrylamide gel in which the different steps of the purification are documented (panel B).

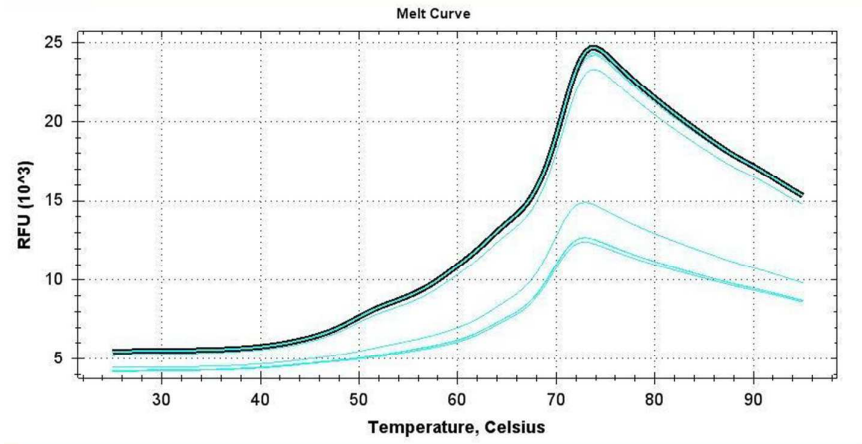
Figure S3 showing Michaelis-Menten plots of data used for determination of kinetic parameters for enzymatic reactions with L-lactate.

Detailed description of methodology used for LC-MS/MS analysis

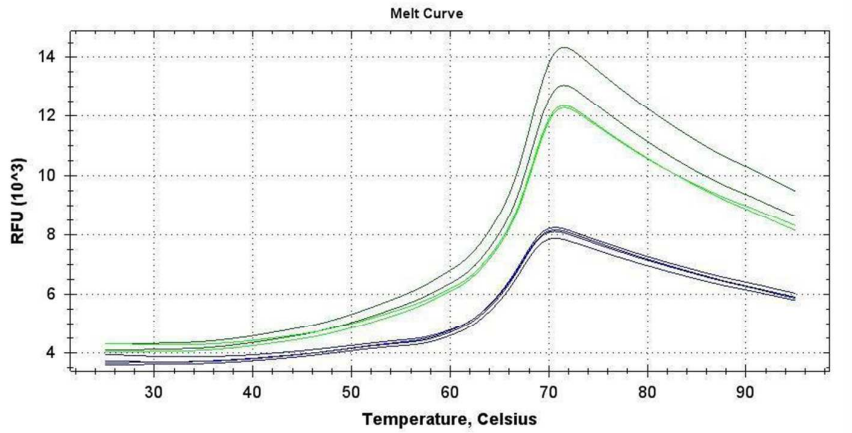
Table S1. Substrate specificity of PEGylated and unmodified S218C mutant as compared to wild-type LOX. Standard deviation on specific activity is from five independent experiments. Standard deviations on kinetic parameters are from non-linear fits of data with the Michaelis-Menten equation.

Enzyme	**Substrate**	**Specific activity [U/mg]**	k_{cat} **[s^{-1}]**	K_M **[mM]**	k_{cat}/K_M **[(s mM)$^{-1}$]**
Wild-type LOX	(*S*)-2-Hydroxybutyric acid	2.0 (± 0.05)	1.40 (± 0.01)	0.43 (± 0.05)	3.2
S218C mutant		2.5 (± 0.07)	1.80 (± 0.05)	4.0 (± 0.41)	0.45
PEGylated S218C		2.4 (± 0.10)	1.60 (± 0.04)	4.1 (± 0.41)	0.41
Wild-type LOX	(*S*)-2-Hydroxyisocaproic acid	1.6 (± 0.08)	1.10 (± 0.01)	0.43 (± 0.05)	2.5
S218C mutant		0.40 (± 0.01)	0.27 (± 0.01)	0.26 (± 0.02)	1.1
PEGylated S218C		0.50 (± 0.03)	0.35 (± 0.02)	0.82 (± 0.16)	0.4

A



B



C

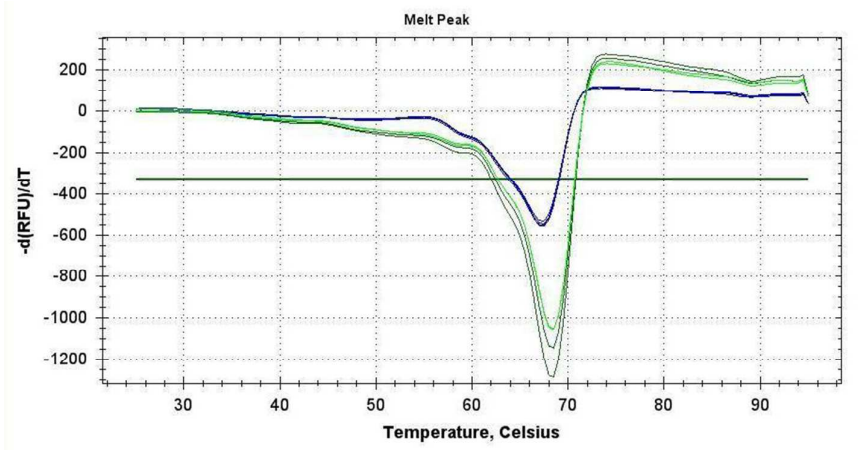


Figure S1. Determination of protein conformational stability in differential scanning fluorimetry experiments. Temperature scans were performed in the presence of the probe SYPRO orange whose fluorescence intensity (relative fluorescence units, RFU) was recorded every 0.5 °C in dependence of the temperature in the range 25 – 95 °C. The scan rate was 0.5°C/min. Panels **A** and **B** show the "melt curves" for unPEGylated and PEGylated S218C mutant, respectively. In panel **A**, measurements were

done at an enzyme concentration of 5 μM and 10 μM (family of curves at higher fluorescence intensity). In panel B, the measurements were done at an enzyme concentration of 3 μM and 5 μM (family of curves at higher fluorescence intensity). Note: the decay of fluorescence intensity in the high-temperature range is due to aggregation of unfolded protein. Panel **C** shows determination of the T_i value (apparent melting point) for PEGylated S218C mutant. The requisite curve fitting was conducted with Bio-Rad CFX Manager software implemented in the Bio-Rad C1000 Thermocycler RT-PCR machine (CFX96 system) that was used for acquisition of the melt curves.

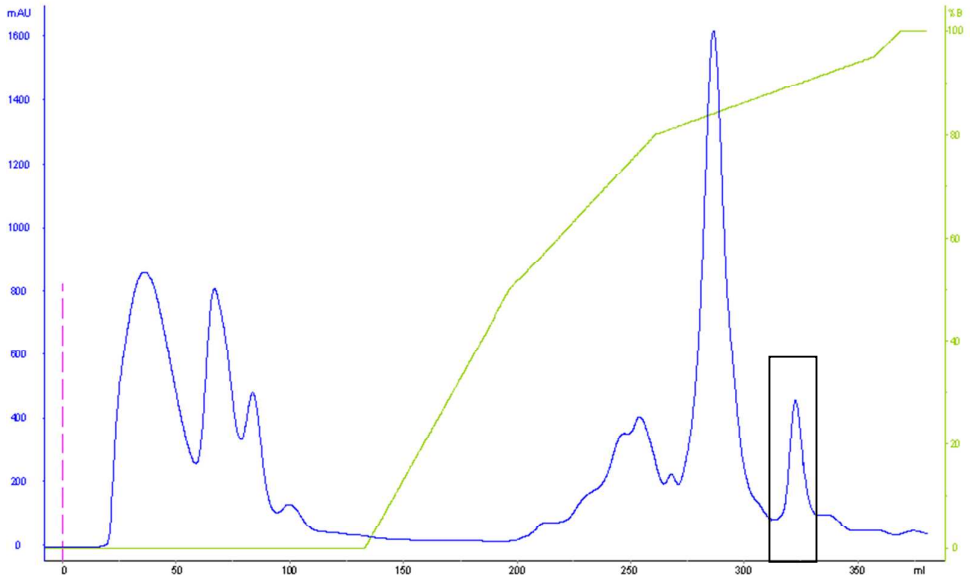


Figure S2A. Purification of the S218C mutant using hydrophobic interaction chromatography. A Phenyl Sepharose 6 Fast Flow (High Sub) column (GE Healthcare Life Sciences; 64 mL) and the Äkta FPLC system (Amersham Biosciences) were used. The protein (about 100 mg in total) was loaded on the column equilibrated with buffer and eluted at 25 °C with a linearly decreasing gradient of $(NH_4)_2SO_4$ (green trace). The flow rate was 5 mL/min, and eluted protein (blue trace) was detected at 280 nm. All steps were performed using 50 mM potassium phosphate buffer, pH 7.0. The original buffer contained 1.5 M $(NH_4)_2SO_4$, the elution buffer (buffer B) contained no $(NH_4)_2SO_4$. The framed absorbance peak shows elution of LOX activity. The peak was divided into two fractions 1and 2, where fraction 2 contained about two-thirds of the total protein eluted in the peak. The specific activity of protein present in fraction 2 was almost twice that contained in fraction 1. Purity determined by SDS PAGE was also higher for fraction 2 than fraction 1(see panel 2B of this figure).

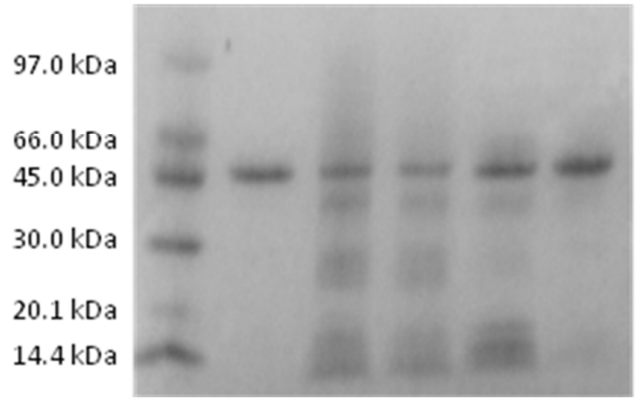


Figure S2B. Purification of S218C mutant documented by SDS PAGE. Lane 1 (from left), molecular mass marker; lane 2, Wild-type enzyme; lane 3, *E. coli* cell extract containing S218C mutant; lane 4, preparation after $(NH_4)_2SO_4$ precipitation; lanes 5 and 6, S218C mutant after HIC purification in fraction 1 (lane 5) and fraction 2 (lane 6).

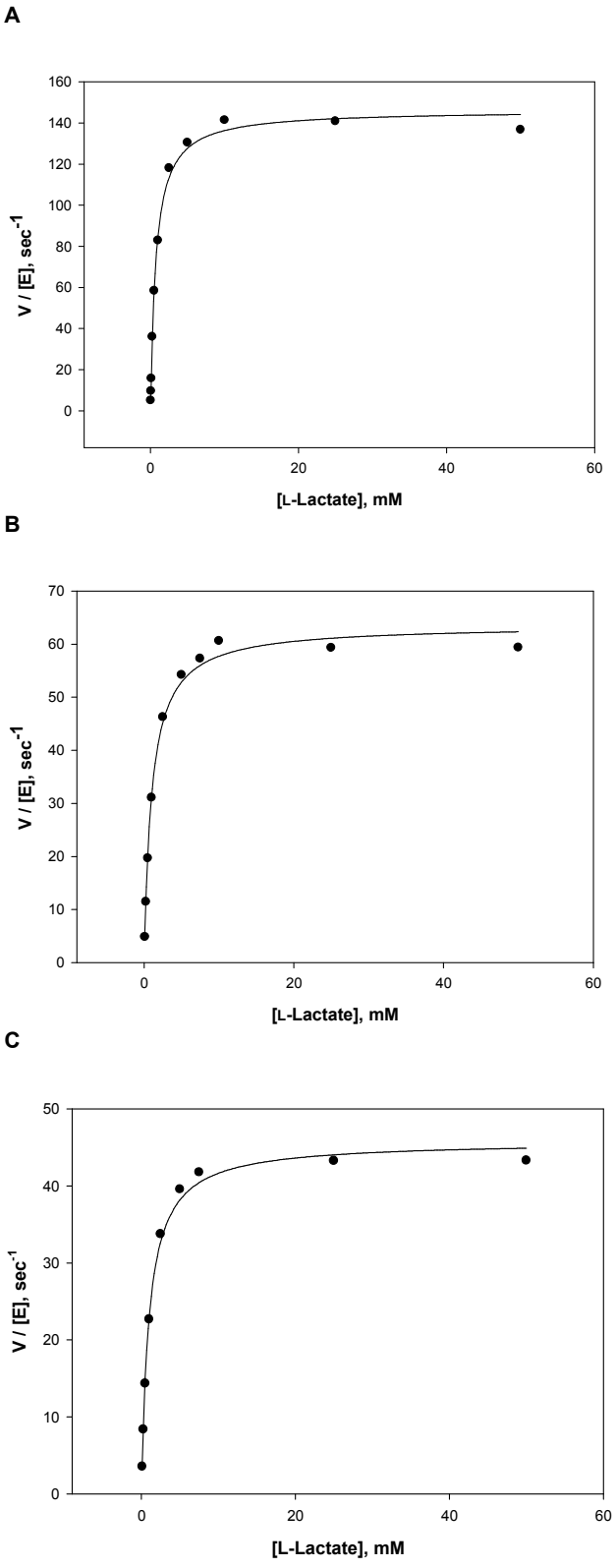


Figure S3. Michaelis-Menten plots of data applied in determination of kinetic parameters for reaction with with L-lactate. Reactions were performed at 37 °C. Panel **A**: wild-type LOX; **B**: S218C mutant; and **C**: PEGylated S218C mutant. Black circles are experimental data, and lines are results of the corresponding non-linear least squares fits.

Protocol of LC-MS/MS analysis

Internal sequence information of the PEGylated LOX, as confirmed by the detected mass shifts by MALDI-TOF analysis, was received from LC-MS/MS. Protein bands were excised from gels and digested with Promega modified trypsin according to the method of Shevchenko and colleagues (see the reference below) and/or with 0.5 µg chymotrypsin (Roche) in 50 mM ammonium bicarbonate containing 10 mM $CaCl_2$ with or without prior reduction and alkylation. Digests were separated by nano-HPLC (Agilent 1200 system; Vienna, Austria) equipped with a Zorbax 300SB-C18 enrichment column (5 µm, 5 × 0.3 mm) and a Zorbax 300SB-C18 nanocolumn (3.5 µm, 150 × 0.075 mm). 40 µL of sample were injected and concentrated on the enrichment column for 6 min using 0.1 % formic acid as isocratic solvent at a flow rate of 20 µL/min. The column was then switched in the nanoflow circuit, and the sample was loaded on the nanocolumn at a flow rate of 300 nL/min. Separation was carried out using the following gradient, where solvent A is 0.3 % formic acid in water and solvent B is a mixture of acetonitrile and water (4 : 1, by vol.) containing 0.3 % formic acid: 0-6min: 13 % B; 6-35 min: 13-28 % B; 35-47 min: 28-50 % B, 47-48 min: 50-100 % B; 48-58 min: 100 % B; 58-58,1 min: 100-13% B; 58,1-70 min: re-equilibration at 13 % B. The sample was ionized in the nanospray source equipped with nanospray tips (PicoTipTM Stock# FS360-75-15-D-20, Coating: 1P-4P, 15±1 µm Emitter, New Objective, Woburn, MA, USA). It was analyzed in a Thermo LTQ-FT mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) operated in positive ion mode, applying alternating full scan MS (m/z 400 to 2000) in the ion cyclotron and MS/MS by collision induced dissociation of the 5 most intense peaks in the ion trap with dynamic exclusion enabled. The LC-MS/MS data were analyzed by searching the protein sequence of LOX of *Aerococcus viridans* (NCBI accession number ZP_06807595) with Mascot 2.2 (MatrixScience, London, UK). The database contained target protein and known contaminants such as the enzymes used for digestion and human keratins. PEGylation with 110 and 111 monomeric units (the two most abundant masses of PEG 5000) was included as variable modification at Cys or the protein N-terminus next to carbamidomethylation on Cys as variable modification. A maximum false discovery rate of 5% using decoy database search, an ion score cut off of 20 and a minimum of 2 identified peptides were chosen as identification criteria.

Shevchenko, A., Wilm, M., Vorm, O., Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem. 68*, 850-858