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Modification of Tyrosine Residues in lysozyme by Electrochemical, Sonochemical and Sonoelectrochemicald Nitration

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INTRODUCTION AND OBJECTIVES

The electrochemical and sonochemical modification, separately or combined in sonoelectrochemistry, of residues in proteins and other bioactive molecules offers the production of novel proteins, enzymes and other bioactive species, in comparison with traditional methodologies such as protein engineering and the use of chemical reagents. The aim of this presentation is to investigate the production of electrosynthetically nitrated tyrosine residues in proteins, and to manipulate the selectivity and effectiveness of the modification either by irradiation with ultrasound during the electrolysis or by exposure of the aqueous protein solution to the ultrasonic field without electrolysis. The results have important consequences for the labelling of proteins, specific immobilisation, production of novel modified proteins for pathophysiology in diseases involving oxidative dysfunction, use in biosensors and microencapsulation of proteins in polymeric microspheres.

CONVENTIONAL PROCEDURES FOR PROTEIN MODIFICATION

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DRAWBACKS

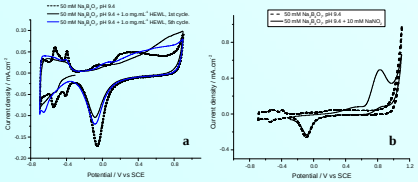
Chemical reaction

- Lack of specificity.
- Side reactions.
- Chemically heating conditions.
- Toxicity of reagents.
- Product separation and purification can be complex.
- requires removal of excess reagent.

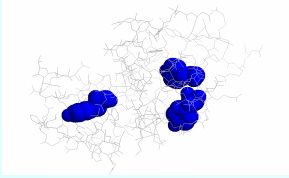
Protein Engineering

*Requires expensive facilities
*Sophisticated methodology.
*Restricted to naturally accuring amino acids.
*Could produce hazardous modified species in the environment

ELECTROCHEMIC AL MODIFICATION OF LYSOZYME HEWL

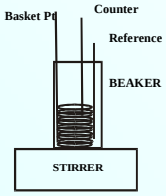


Cyclic voltammogram for the electrochemical behaviour of 50 mM di-sodium tetraborate, pH 9.4. (a) in the presence of lysozyme (1 mg mL^{-1}). (b) in the presence of 10 mM sodium nitrite. Onset of scan at –0.3 V vs SCE. Potential limits between –0.7 and 0.9 V. Scan rate: 0.050 V.s^{-1}.

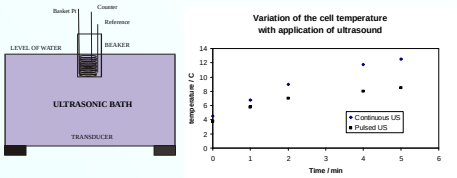


Y23: Most exposed Tyr residue.
Y20 : Partially hidden.
Y53: Buried inside the structure.

EXPERIMENTAL SET-UP FOR ELECTROCHEMICAL, SONOELECTROCHEMICAL AND SONOCHEMICAL PREPARATIVE REACTIONS



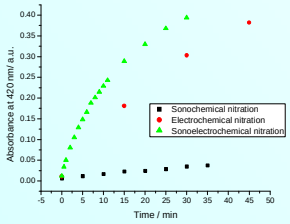
Working: Basket Pt electrode (20 cm^2). Counter: Pt flag electrode. Reference: SCE electrode.
50 mM di-sodium tetraborate + 20 mM sodium nitrite, pH 9.0. Volume: 20 mL. HEWL concentration 1 mg/mL. T= 4 ºC, ice cooled. Controlled potential electrolysis: 0.85 V vs SCE.



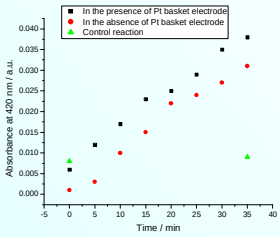
Sonochemical and sonoelectrochemical modification of lysozyme were performed using an ultrasonic cleaning bath (40 kHz, 180 w power output). Prior to the insonation of lysozyme, the cleaning bath is kept at 5 ºC and protein solution equilibrated for 2 min at the same temperature. Ultrasound field is applied through the glass cell for intervals of 5 min each in order to keep the temperature bellow 12 ºC. Tetraborate buffer solutions were de-oxygenated by bubbling argon before starting experiments.

POSSIBLE MECHANISMFOR THE ELECTROCHEMICAL NITRATION

COMPARISON BETWEEN SONOCHEMICAL, ELECTROCHEMICAL AND SONOELECTROCHEMICAL NITRATION



SONOCHEMICAL MODIFICATION OF LYSOZYME



Possible mechanism for the sonochemical nitration of lysozyme

FORMATION OF H AND OH
$H_2O \rightarrow H + OH$
$H + H \rightarrow H_2$
$OH + OH \rightarrow H_2O_2$

FORMATION OF TYROSINILATE RADICALS
$2Tyr + H_2O_2 \rightarrow Tyr + 2H_2O$

FORMATION OF NO_2
$NO_2^- + \cdot OH \rightarrow NO_2 + \cdot OH$

OTHER REACTIONS
$NO_2^- + H \rightarrow NO + OH$
$NO_2 + NO_2 + H_2O \rightarrow NO_2^- + NO_2^- + 2H^+$
$2NO + O_2 \rightarrow 2\ NO_2$

FORMATION OF NITRATED LYSOZYME
$Tyr + NO_2 \rightarrow Tyr\text{-}NO_2$

MASS SPECTROMETRY

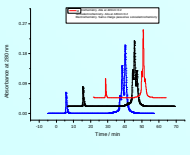
Electrochemical and sonoelectrochemical nitration of lysozyme: MALDI-TOF mass spectrum of digested nitrated lysozyme.

Nozzle-Skimmer: Insufficient
↓
Tryptic digestion and fragments study
↓
Nitration tyrosine 23
↓
Nitration tyrosine 20 and 23

Sonochemical nitration of lysozyme: Applied Biosystems Qstar Pulsar I Electrospry Ionization

Mononitration of Lysozyme
Mass increase of 45 Da

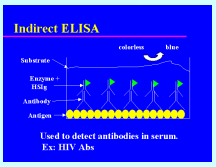
HPLC CHROMATOGRAPHY

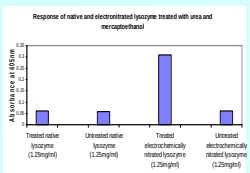


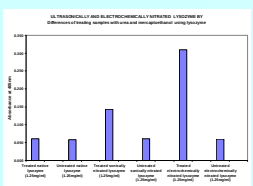
Mono to bis nitrated lysozyme

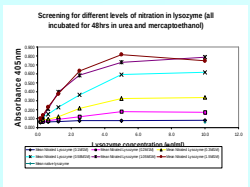
	Electrochemical nitration of lysozyme	Sonoelectrochemical nitration of lysozyme
Same absorbance at 280 nm Abs=0.200	1.797	2.553
Same charge passed for both electrochemical and sonoelectrochemical nitration	1.532	2.553

IDENTIFICATION OF NITRATED LYSOZYME BY ELISA

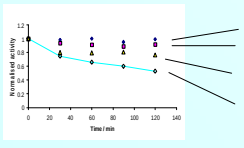






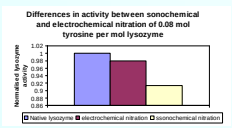


ENZYME ACTIVITY
Cell wall turbidimetric assay for lysozyme activity



50 mM disodium tetraborate pH 6.2, open circuit
50 mM disodium tetraborate pH 9.0, open circuit
50 mM disodium tetraborate pH 9.0, 0.85 V vs SCE
50 mM disodium tetraborate 20 mM nitrite pH 9.0, 0.85 V vs SCE

Basket platinum electrode
Lysozyme conc: 1 mg mL^{-1}



Native lysozyme retains its activity during exposure to ultrasound, while mononitrated lysozyme, purified by HPLC, undergoes a reduction of activity of ca. 60 %.

CONCLUSIONS

(a) An effective procedure has been developed for selective electrochemical nitration of tyrosine in proteins such as lysozyme and myoglobin.
(b) Appropriate electrochemical parameters and selectivity allow retention of activity of the enzyme with consequences in labelling, immobilisation and biosensors.
(c) Nitration at low levels can be achieved sonochemically in lysozyme but with consequent loss of enzymic activity reflecting a change in conformation although this does not lead to availability of nitrotyrosine for antibody binding in an ELISA assay.

Financial Support
EPSRC, Coventry University Funding and European Community Marie Curie Fellowship
J. Iniesta thanks the Spanish Programme Ramón y Cajal