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Immobilisation of active enzymes on novel GFP protein particles

A thesis submitted in complete fulfilment of the requirements of the degree of
Master of Science
in
Microbiology
at Massey University, Palmerston North,
New Zealand.

Mark Piers Venning-Slater
2013
ABSTRACT

Inclusion bodies were previously thought to be aggregations of inactive, mis-folded proteins. However, there is a growing body of evidence that suggests otherwise. In 2011 Jahns et al demonstrated a self-assembling GFP protein particle (GFP particle) that not only exhibited fluorescence, but was also able to display functional antibody and ligand binding sites. These functional GFP particles exhibited reasonable activity, and in many cases outperformed commercially available particles. The GFP particles consisted of an aggregation of fusion proteins. These fusion proteins in turn consisted of an N-terminally extended enhanced GFP protein which was fused at its C-terminus to an inactive polyester synthase (PhaC(C319A)) from Ralstonia eutropha, and a functionality, e.g. antibody/ligand binding site. In this study, GFP particles were investigated to ascertain whether they could serve as a support for the immobilization and display of active enzymes; and provide a technology that is potentially more efficient and cost-effective than other enzyme immobilization methods. Furthermore, their inherent fluorescence would provide an additional advantage. The enzymes used for functionality tests were: a thermostable α-amylase from Bacillus licheniformis that lacked its signal sequence (Bla(-ss)); N-acetyl-D-neuraminic acid aldolase (NanA) from Escherichia coli; and organophosphohydrolase (OpdA) from Agrobacterium radiobacter. These enzymes were chosen for their differing quaternary structure—monomer, tetramer, and dimer, respectively- and were fused to the C-termini of GFP fusion proteins. The results of this investigation showed that it is possible to generate fluorescent GFP particles inside recombinant E. coli BL21(DE3) cells which are also able to display active enzyme. These enzyme-bearing GFP particles exhibited considerable stability across a range of temperature, pH, and storage conditions, and could also be reused. The activity of the particles was also compared to a similar technology—functionalized PHA beads; however, the PHA beads consistently exhibited stronger enzyme activity under all conditions tested. GFP protein particles represent a novel method for the immobilization and display of enzymes. Their ability to immobilise and display active enzymes of different quaternary structure under a range of conditions makes GFP particles particularly attractive to industrial biocatalysis processes. Potential applications include diagnostic assays, food production, pharmaceutical production, and bioremediation.
ACKNOWLEDGEMENTS

Where do I start? As I’m sure every post-grad can appreciate, undertaking a Master’s degree is not to be considered lightly. All those who have gone before can tell you how much work is required: the long days; the lack of sleep; the repetition of assays that fail for unforeseen reasons (or foreseen reasons). My project was no exception, and there are many people I’d like to thank for their words of encouragement and advice for which I’m deeply grateful. First, I’d like to thank my supervisor Professor Bernd Rehm for giving me the opportunity of studying under him. Considering that he did not know me beforehand, he took a chance on an eccentric guy from Wellington, and gave me advice and direction that not only helped me with this study, but will also be of great use in my future career. Next, I’d like to thank David Hooks for his endless support and technical knowhow. I was very much a novice in molecular biology when I started, but thanks to David, and his patience, I finish my Master’s with a wealth of knowledge- and some fancy squash techniques. A thank you must also go out to Jason Lee for making the weekend work and breaks a lot more enjoyable, and Iain Hay for having the answer I need no matter how obscure the question. A special thanks to the whole Rehm lab and Polybatics for being a great bunch of people to work beside; Trevor Loo for his HPLC expertise; and Rosie Bradshaw’s lab for letting me steal their equipment. A big thanks to my flatties Bex Smith for being a whirlwind of energy and laughs; Bob Stewart for helping me get in touch with my Upper Hutt gangsta roots; and Kim Green for the fly-by hugs. As always I’m indebted to my family for their support and encouragement, and financial assistance. I don’t want to think how much money they’ve given me over the years, but I promise it’s been worth it.
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<td>AGE</td>
<td>Agarose Gel Electrophoresis</td>
</tr>
<tr>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Ampicillin resistance gene</td>
</tr>
<tr>
<td>AVTS</td>
<td>Amino acids Alanine-Valine-Threonine-Serine that constitute a short N-terminal extension of GFP in GFP fusion proteins</td>
</tr>
<tr>
<td>BLA</td>
<td>α-amylase from <em>Bacillus licheniformis</em></td>
</tr>
<tr>
<td>Bla(-ss)</td>
<td>α-amylase from <em>Bacillus licheniformis</em> that lacks the signal sequence required for extra-cellular export</td>
</tr>
<tr>
<td>Bla(-ss)-PhaC</td>
<td>α-amylase/ PHA synthase fusion protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CFP</td>
<td>Enhanced cyan-fluorescent protein</td>
</tr>
<tr>
<td>CLEA</td>
<td>Cross-linked enzyme aggregate</td>
</tr>
<tr>
<td>CLEC</td>
<td>Cross-linked enzyme crystal</td>
</tr>
<tr>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Chloramphenicol resistance gene</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoabsorbant assay</td>
</tr>
<tr>
<td>extGFP</td>
<td>N-terminally extended green fluorescent protein</td>
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<td>FHKP</td>
<td>Amino acids Phenylalanine-Histidine-Lysine-Proline that constitute a short N-terminal extension of GFP in GFP fusion proteins</td>
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<td>GB1</td>
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<td>[GB1]&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Triple-repeat of GB1</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GFP particle</td>
<td>GFP fusion protein particle</td>
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GiCL Fusion protein consisting of ext(AVTS)GFP-inactive PHA synthase-pentaglycine linker.

GiCLB Fusion protein consisting of ext(AVTS)GFP-inactive PHA synthase-pentaglycine linker-α-amylase. Also denotes resultant GFP particle.

GiCLN Fusion protein consisting of ext(AVTS)GFP-inactive PHA synthase-pentaglycine linker-[N-acetyl-D-neuraminic acid aldolase]. Also denotes resultant GFP particle.

GiCLO Fusion protein consisting of ext(AVTS)GFP-inactive PHA synthase-pentaglycine linker-organophosphohydrolase. Also denotes resultant GFP particle.

GiCLZ Fusion protein consisting of ext(AVTS)GFP-inactive PHA synthase-pentaglycine linker-ZZ domain. Also denotes resultant GFP particle.

GNL Fusion protein consisting of ext(AVTS)GFP-[N-acetyl-D-neuraminic acid aldolase]-pentaglycine linker.

GNLN Fusion protein consisting of ext(AVTS)GFP-[N-acetyl-D-neuraminic acid aldolase]-pentaglycine linker-[N-acetyl-D-neuraminic acid aldolase]. Also denotes resultant GFP particle.

GNLZ Fusion protein consisting of ext(AVTS)GFP-[N-acetyl-D-neuraminic acid aldolase]-pentaglycine linker-ZZ domain. Also denotes resultant GFP particle.

h Hour

HcR Far red protein HcRed

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC High Performance Liquid Chromatography

IB Inclusion body

IgG Immunoglobulin G

IPTG Isopropyl β-D-1-thiogalactopyranoside

kDa Kilodalton

LAVG Amino acids Leucine-Alanine-Valine-Glycine that constitute a short N-terminal extension of GFP in GFP fusion proteins

LB Luria-Bertani

M mol per litre
μg  Microgram
μl  Microliter
mA  Milliampere
ml  Millilitre
mM  Millimol per litre
MALDI-TOF/MS  Matrix-assisted Laser Desorption/ Ionisation Time-of-Flight Mass Spectrometry
MalE  Maltose binding protein
ManNAc  N-acetyl-D-mannosamine
min  Minute
MOPS  3-Morpholinopropanesulfonic acid
MW  Molecular weight
NanA  N-acetyl-D-neuraminic acid aldolase from E. coli
NanA-PhaC  N-acetyl-D-neuraminic acid aldolase/ PHA synthase fusion protein
Neu5Ac  N-acetyl-D-neuraminic acid
ng  Nanogram
OpdA  Organophosphohydrolase from Agrobacterium radiobacter
PCR  Polymerase Chain Reaction
pET14b-GiCL  pET14b plasmid that encodes the fusion protein GiCL.
pET14b-GiCLB  pET14b plasmid that encodes the fusion protein GiCLB.
pET14b-GiCLN  pET14b plasmid that encodes the fusion protein GiCLN.
pET14b-GiCLO  pET14b plasmid that encodes the fusion protein GiCLO.
pET14b-GiCLZ  pET14b plasmid that encodes the fusion protein GiCLZ.
pET14b-GNL  pET14b plasmid that encodes the fusion protein GNL.
pET14b-GNLN  pET14b plasmid that encodes the fusion protein GNLN.
pET14b-GNLZ  pET14b plasmid that encodes the fusion protein GNLZ.
PHA  Polyhydroxyalkanoate
PhaC  PHA synthase
PhaC (C319A)  Inactive PHA synthase
PhaC-OpdA  PHA synthase/ Organophosphohydrolase fusion protein
PHB  Polyhydroxybutyrate
rpm  Revolutions per minute
R.T.  Room temperature (22 °C - 25 °C)
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>U</td>
<td>Enzyme units</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>x g</td>
<td>Unit denoting centrifugal force as a multiple of standard gravity on Earth</td>
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<tr>
<td>YFP</td>
<td>Enhanced yellow fluorescent protein</td>
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<td>ZZ</td>
<td>IgG binding domain of Protein A from <em>Staphylococcus aureus</em></td>
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