

Contents

	Preface	<i>XIII</i>
	List of Contributors	<i>XV</i>
	Introduction	<i>1</i>
	<i>Renaud Sicard and Jean-Louis Reymond</i>	
	Enzyme Assays	<i>1</i>
	Part I: The Chemistry of Enzyme Assays	<i>7</i>
	Part II: Enzyme Assays and Genetic Selection	<i>7</i>
	Part III: Enzyme Profiling	<i>9</i>
	Enzyme Assays in Other Areas	<i>11</i>
	How to Use this Book	<i>11</i>
Part I	High-throughput Screening	<i>15</i>
1	Quantitative Assay of Hydrolases for Activity and Selectivity Using Color Changes	<i>17</i>
	<i>Romas J. Kazlauskas</i>	
1.1	Overview	<i>17</i>
1.2	Direct Assays Using Chromogenic Substrates	<i>18</i>
1.3	Indirect Assays Using Coupled Reactions – pH Indicators	<i>19</i>
1.3.1	Overview of Quantitative Use of pH Indicator Assay	<i>21</i>
1.3.2	Applications	<i>24</i>
1.3.2.1	Searching for an Active Hydrolase (Testing Many Hydrolases Toward One Substrate)	<i>24</i>
1.3.2.2	Substrate Mapping of New Hydrolases (Testing Many Substrates Toward Hydrolase)	<i>25</i>
1.3.3	Comparison with Other Methods	<i>26</i>
1.4	Estimating and Measuring Selectivity	<i>27</i>
1.4.1	Estimating Selectivity without a Reference Compound	<i>28</i>
1.4.2	Quantitative Measure of Selectivity Using a Reference Compound (Quick <i>E</i> and Related Methods)	<i>30</i>

- 1.4.2.1 Chromogenic Substrate 32
- 1.4.2.2 pH Indicators 33
- 1.4.3 Application 33
- 1.4.3.1 Substrate Mapping of Hydrolases 33
- 1.4.3.2 Screening of Mutants in Directed Evolution 33
- 1.4.4 Advantages and Disadvantages 36
- References* 38

2 High-throughput Screening Systems for Assaying the Enantioselectivity of Enzymes 41

Manfred T. Reetz

- 2.1 Introduction 41
- 2.2 UV/Vis Spectroscopy-based Assays 42
- 2.2.1 Assay for Screening Lipases or Esterases in the Kinetic Resolution of Chiral *p*-Nitrophenyl Esters 43
- 2.2.2 Enzyme-coupled UV/Vis-based Assay for Lipases and Esterases 45
- 2.2.3 Enzymatic Method for Determining Enantiomeric Excess (EMDee) 46
- 2.2.4 UV/Vis-based Enzyme Immunoassay as a Means to Measure Enantiomeric Excess 47
- 2.2.5 Other UV/Vis-based *ee*-Assays 48
- 2.3 Assays Using Fluorescence 48
- 2.3.1 Umbelliferone-based Systems 48
- 2.3.2 Fluorescence-based Assay Using DNA Microarrays 51
- 2.3.3 Other Fluorescence-based *ee*-Assays 53
- 2.4 Assays Based on Mass Spectrometry (MS) 53
- 2.4.1 MS-based Assay Using Isotope Labeling 53
- 2.5 Assays Based on Nuclear Magnetic Resonance Spectroscopy 58
- 2.6 Assay Based on Fourier Transform Infrared Spectroscopy for Assaying Lipases or Esterases 62
- 2.7 Assays Based on Gas Chromatography 65
- 2.8 Assays Based on HPLC 68
- 2.9 Assays Based on Capillary Array Electrophoresis 69
- 2.10 Assays Based on Circular Dichroism (CD) 71
- 2.11 Assay Based on Surface-enhanced Resonance Raman Scattering 73
- 2.12 Conclusions 73
- References* 74

3 High-throughput Screening Methods Developed for Oxidoreductases 77

Tyler W. Johannes, Ryan D. Woodyer, and Huimin Zhao

- 3.1 Introduction 77
- 3.2 High-throughput Methods for Various Oxidoreductases 78
- 3.2.1 Dehydrogenases 78

3.2.1.1	Colorimetric Screen Based on NAD(P)H Generation	78
3.2.1.2	Screens Based on NAD(P)H Depletion	79
3.2.2	Oxidases	80
3.2.2.1	Galactose Oxidase	80
3.2.2.2	D-Amino Acid Oxidase	82
3.2.2.3	Peroxidases	82
3.2.3	Oxygenases	85
3.2.3.1	Assays Based on Optical Properties of Substrates and Products	85
3.2.3.2	Assays Based on Gibbs' Reagent and 4-Aminoantipyrine	86
3.2.3.3	<i>para</i> -Nitrophenoxy Analog (pNA) Assay	87
3.2.3.4	Horseradish Peroxidase-coupled Assay	88
3.2.3.5	Indole Assay	89
3.2.4	Laccases	89
3.2.4.1	ABTS Assay	90
3.2.4.2	Poly R-478 Assay	90
3.2.4.3	Other Assays	90
3.3	Conclusions	91
	<i>References</i>	92
4	Industrial Perspectives on Assays	95
	<i>Theo Sonke, Lucien Duchateau, Dick Schipper, Gert-Jan Euverink, Joerd van der Wal, Huub Henderickx, Roland Bezemer, and Aad Vollebregt</i>	
4.1	Introduction	95
4.2	Prerequisites for an Effective Biocatalyst Screening in Chemical Custom Manufacturing	97
4.3	CCM Compliant Screening Methods Based on Optical Spectroscopy (UV/Vis and Fluorescence)	101
4.3.1	Optical Spectroscopic Methods Based on the Spectral Properties of the Product Itself	101
4.3.1.1	Example: Isolation of the D- <i>p</i> -Hydroxyphenylglycine Aminotransferase Gene	102
4.3.2	Optical Spectroscopic Methods Based on Follow-up Conversion of Product	104
4.3.2.1	Example: Fluorometric Detection of Amidase Activity by <i>o</i> -Phthaldehyde/Sulfite Derivatization of Ammonia	106
4.3.2.2	Example: Colorimetric Detection of Amidase Activity by Detection of Ammonia via Glutamate Dehydrogenase-coupled Assay	108
4.3.2.3	Example: Colorimetric Detection of Amino Amidase Activity Using Cu ²⁺ as Sensor for Amino Acids	112
4.4	CCM Compliant Screening Methods Based on Generic Instrumental Assays	114
4.4.1	Flow-injection NMR as Analytical Tool in High-throughput Screening for Enzymatic Activity	115

4.4.1.1	History	115
4.4.1.2	Current Practice	117
4.4.1.3	Practical Aspects	119
4.4.1.4	Example: Screening of a Bacterial Expression Library for Amidase-containing Clones	122
4.4.1.5	Example: Identification of a Phenylpyruvate Decarboxylase Clone	124
4.4.1.6	Example: Identification of Amidase Mutants with Improved Activity towards α -Methylphenylglycine Amide	125
4.4.2	Fast LC/MS for High-throughput Screening of Enzymatic Activity	126
4.4.2.1	Example: Screening of a Bacterial Expression Library for Amidase-containing Clones	127
4.4.2.2	Example: Screening of Enzymatic Racemase Activity	129
4.5	Conclusions	132
	<i>References</i>	133

Part II Genetic Selection 137

5 Agar Plate-based Assays 139

Nicholas J. Turner

5.1	Introduction	139
5.1.1	Directed Evolution of Enzymes: Screening or Selection?	139
5.1.2	General Features of Agar Plate-based Screens	141
5.2	Facilitated Screening-based Methods	143
5.2.1	Amidase	143
5.2.2	Esterase	144
5.2.3	Glycosynthase	144
5.2.4	Galactose Oxidase	146
5.2.5	Monoamine Oxidase	147
5.2.6	P450 Monooxygenases	150
5.2.7	Carotenoid Biosynthesis	151
5.2.8	Biotin Ligase	153
5.3	<i>In vivo</i> Selection-based Methods	154
5.3.1	Glycosynthase	154
5.3.2	Prephenate Dehydratase/Chorismate Mutase	155
5.3.3	Terpene Cyclase	157
5.3.4	Tryptophan Biosynthesis	157
5.3.5	Ribitol Dehydrogenase	157
5.3.6	Inteins	158
5.3.7	Aminoacyl-tRNA Synthetase	159
5.4	Conclusions and Future Prospects	159
	<i>References</i>	160

6	High-throughput Screens and Selections of Enzyme-encoding Genes	163
	<i>Amir Aharoni, Cintia Roodveldt, Andrew D. Griffiths, and Dan S. Tawfik</i>	
6.1	Introduction	163
6.2	The Basics of High-throughput Screens and Selections	164
6.3	High-throughput Selection of Enzymes Using Phage Display	165
6.4	High-throughput Selection of Enzymes Using Cell Display	168
6.5	<i>In vivo</i> Genetic Screens and Selections	169
6.6	Screens for Heterologous Protein Expression and Stability	169
6.6.1	Introduction	169
6.6.2	Screening Methodologies for Heterologous Expression	171
6.6.3	Directed Evolution for Heterologous Expression – Recent Examples	173
6.7	<i>In vitro</i> Compartmentalization	174
6.8	IVC in Double Emulsions	177
6.9	Concluding Remarks	179
	<i>References</i>	179
7	Chemical Complementation	
	<i>Scott Lefurgy and Virginia Cornish</i>	183
7.1	Introduction	183
7.2	Complementation Assays	184
7.2.1	Introduction	184
7.2.2	Early Complementation Assays	184
7.2.3	Enzymology by Complementation	186
7.2.4	Directed Evolution by Complementation	188
7.3	Development of Chemical Complementation	191
7.3.1	Introduction	191
7.3.2	Three-hybrid Assay	192
7.3.2.1	Original Yeast Three-hybrid System	192
7.3.2.2	Dexamethasone–Methotrexate Yeast Three-hybrid System	194
7.3.2.3	Technical Considerations	196
7.3.2.4	Other Three-hybrid Systems	198
7.3.3	Chemical Complementation	198
7.3.3.1	Selection Scheme and Model Reaction	199
7.3.3.2	Results	202
7.3.3.3	General Considerations	203
7.3.3.4	Related Methods	203
7.4	Applications of Chemical Complementation	204
7.4.1	Introduction	204
7.4.2	Enzyme–Inhibitor Interactions	204
7.4.2.1	Rationale	205
7.4.2.2	Screen Strategy	205

7.4.2.3	Enzyme Library Screen	208
7.4.2.4	General Considerations	210
7.4.3	Glycosynthase Evolution	210
7.4.3.1	Rationale	211
7.4.3.2	Selection Scheme	212
7.4.3.3	Glycosynthase Assay	213
7.4.3.4	Directed Evolution	215
7.4.3.5	General Considerations	216
7.5	Conclusion	216
	<i>References</i>	217
8	Molecular Approaches for the Screening of Novel Enzymes	
	<i>Valéria Maia de Oliveira and Gilson Paulo Marfio</i>	221
8.1	Introduction	221
8.2	Use of Nucleic Acid Probes to Detect Enzyme-coding Genes in Cultivated Microorganisms	222
8.2.1	Current Knowledge and Applications	223
8.2.2	Limitations of Probe Technology and the Need for Innovative Approaches	224
8.3	The Microbial Metagenome: a Resource of Novel Natural Products and Enzymes	226
8.3.1	Accessing the Uncultivated Biodiversity: the Community DNA Concept	226
8.3.2	Unravelling Metabolic Function: the BAC Strategy	227
8.3.3	Analysis of Metagenomic Libraries: Activity versus Sequence-driven Strategy, Enrichment for Specific Genomes and Application of High-throughput Screening Methods	230
8.3.4	Follow-up of the Metagenome Harvest	233
8.4	Concluding Remarks	235
	<i>References</i>	236
Part III	Enzyme Fingerprinting	239
9	Fluorescent Probes for Lipolytic Enzymes	241
	<i>Ruth Birner-Grünberger, Hannes Schmidinger, Alice Loidl, Hubert Scholze, and Albin Hermetter</i>	
9.1	Introduction	241
9.2	Fluorogenic and Fluorescent Substrates for Enzyme Activity	242
9.2.1	Triacylglycerol Lipase Activity Assay	245
9.2.2	Diacylglycerol Lipase Activity Assay	247
9.2.3	Cholesteryl Esterase Activity Assay	249
9.2.4	Phospholipase Activity Assay	250
9.2.5	Sphingomyelinase Activity Assay	252
9.3	Fluorescent Inhibitors for Quantitative Analysis of Active Enzymes and Functional Enzyme Fingerprinting	254

- 9.3.1 Lipase and Esterase Profiling 254
- 9.3.1.1 Microbial Lipases and Esterases 255
- 9.3.1.2 Porcine Pancreatic Lipase 257
- 9.3.1.3 Hormone-sensitive Lipase 257
- 9.3.2 Probing Biophysical Enzyme Properties 259
- 9.3.3 Affinity-based Proteome Profiling (ABPP) 262
- 9.3.3.1 Functionality-based Serine Hydrolase Profiling in Tissue Preparations and Cell Lines 263
- References* 267

10 Fingerprinting Methods for Hydrolases

Johann Grognux and Jean-Louis Reymond 271

- 10.1 Introduction 271
- 10.1.1 One Enzyme – One Substrate 273
- 10.1.2 Enzyme Activity Profiles 275
- 10.1.3 The APIZYM System for Microbial Strain Identification 276
- 10.2 Hydrolase Fingerprinting 278
- 10.2.1 Fingerprinting with Fluorogenic and Chromogenic Substrates 279
- 10.2.2 Fingerprinting with Indirect Chromogenic Assays 284
- 10.2.3 Cocktail Fingerprinting 287
- 10.3 Classification from Fingerprinting Data 289
- 10.3.1 Fingerprint Representation 290
- 10.3.2 Data Normalization 293
- 10.3.3 Hierarchical Clustering of Enzyme Fingerprints 295
- 10.3.4 Analysis of Substrate Similarities 297
- 10.4 Outlook 299
- References* 300

11 Protease Substrate Profiling

Jennifer L. Harris 303

- 11.1 Introduction 303
- 11.2 Functional Protease Profiling – Peptide Substrate Libraries 304
- 11.2.1 Solution-based Peptide Substrate Libraries 306
- 11.2.2 Solid Support-based Synthesis and Screening of Peptide Libraries 314
- 11.2.3 Genetic Approaches to Identifying Protease Substrate Specificity 320
- 11.3 Identification of Macromolecular Substrates 322
- 11.3.1 Genetic Approach to the Identification of Macromolecular Substrates 323
- 11.3.2 Proteomic Approaches to Identifying Protease Substrates 326
- 11.4 Conclusions 327
- References* 328

12	Enzyme Assays on Chips	333
	<i>Souvik Chattopadhyaya and Shao Q. Yao</i>	
12.1	Introduction	333
12.2	Immobilization Strategies	335
12.2.1	Covalent versus Noncovalent Immobilization	335
12.2.2	Site-specific versus Nonspecific Immobilization	336
12.2.3	Site-specific Immobilization of Peptides/Small Molecules	336
12.2.4	Site-specific Immobilization of Proteins	337
12.2.4.1	Intein-mediated Protein Biotinylation Strategies	338
12.2.4.2	Puromycin-mediated Protein Biotinylation	343
12.2.4.3	Immobilization of N-terminal Cysteine-containing Proteins	343
12.3	Microarray-based Methods for Detection of Enzymatic Activity	344
12.3.1	Enzyme Assays Using Protein Arrays	345
12.3.2	Enzyme Assays Using Peptide/Small Molecule Substrate Arrays	348
12.3.2.1	Proteases and Other Hydrolytic Enzymes	348
12.3.2.2	Kinases	351
12.3.2.3	Carbohydrate-modifying Enzymes	355
12.3.2.4	Other Enzymes	356
12.3.3	Enzyme Assays Using Other Types of Arrays	356
12.4	Conclusions	357
	<i>References</i>	359
	Subject Index	363