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Secondary structure analysis of penicillin G acylase covalently immobilized onto nanobeads with circular dichroism

Problem and subject:

The covalent immobilization of enzymes (see Fig. 1) has two main advantages:
- Possibility of enzyme separation (centrifugation)
- Increased enzyme stability against high temperature, ionic strengths and extreme pH values

→But the immobilization leads in many cases to a substantial loss of activity

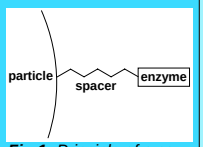

Fig.1: *Principle of a co-valent immobilized enzyme*

Main reasons for the activity losses:
- Sterical hindrance (partial blocking-out of active sites)
- Diffusion limitations (reduced convection near the beads)
- Structural changes (often caused by the immobilization process)

➔ Circular dichroism (CD) measurements were used to determine the secondary structure of immobilized enzyme compared to free enzyme

Sample preparation:

Silica – nanobeads (Ludox®-40, Grace Davison) were used to covalently immobilize penicillin G acylase (EC 3.5.1.11, *E. coli*, sigma-aldrich) via glutardialdehyde spacer.

Fig. 2: *Silica nanobeads*

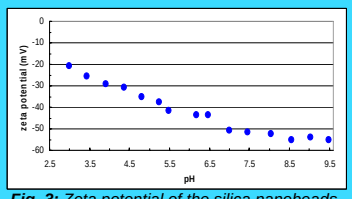

Fig. 3: *Zeta potential of the silica nanobeads*

These nanobeads have a specific surface area of (198-258) m²/g, a specific gravity of 1.292-1.312 (compared with water) and a pH of 9.2-9.9 (25°C). Immersed in water they form a stable and totally transparent suspension (left side of Fig. 2). Dry beads (right side of Fig. 2) are a white powder. The beads have a -52 mV zeta potential at pH 7.5 (working pH of immobilization, see Fig.3). This is important for two reasons: On the one hand this guaranteed a stable colloidal suspension (homogeneous dispersion) but on the other hand the penicillin G acylase is also negatively charged at this pH, which means that the loading is slightly lower than with positively charged beads.

The silicabeads were functionalized with glutardialdehyde solution (0.1M / 1.0 M, 1:10 excess) 1h at 25°C in a thermomixer. After washing with water immobilization of penicillin G acylase was performed for 1 h / 24 h at 25°C in a thermomixer (32.3 mg-penicillin G acylase/g-silicabeads in phosphate buffer [0.2M, pH 7.5]). Afterwards the silicabeads were washed with phosphate buffer (0.2M, pH 7.5).

Characterization of the enzyme via mass spectrometry:

Although the enzyme was bought from sigma-aldrich it was necessary to characterize it because the product information was very poor. Therefore we used mass spectrometry (MALDI-TOF/TOF) and the NCBInr database to identify the enzyme.

Before doing mass spectroscopy the enzyme was separated via 1D-gel electrophoresis and then digested with trypsine.

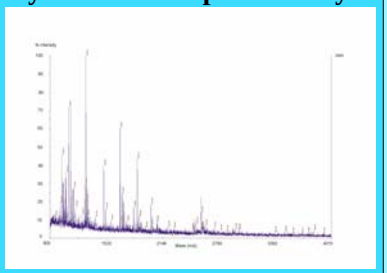

Fig.4: *MS-spectrum of trypsin-digested penicillin G acylase (MALDI-TOF/TOF)*

The masses of the enzyme fragments (see Fig. 4) after digestion found in the MS spectrum could be unambiguously matched with the entries of the precursor enzyme in the data base. In combination with the mass determined in the 1D-gel electrophoresis the maturated enzyme could be clearly identified.

The table on the left side shows the result of the MS-analysis and the table on the bottom the enzyme data.

Accession	Mass (g/mol)	Score	Description
gi 147162	94668	89	penicillin G acylase precursor

Precursor enzyme	AA – Number	1 – 26	27 – 235	236 – 289	290 – 846
	Section	Signal Sequence	A – Subunit	Linker Peptide	B – Subunit
Maturated enzyme	AA – Number	--	A1 – A209	--	B1 – B557
	Section	--	A – Subunit	--	B – Subunit

Circular dichroism analysis:

Circular dichroism (CD) was used to analyze the secondary structure elements of covalently immobilized penicillin G acylase in comparison with the free enzyme (see Fig. 5).

The blue curve indicates the CD spectrum of the free enzyme, the red curve the enzyme immobilized onto silica nanobeads with glutardialdehyde (0.1 M) and the orange curve the same immobilization with the spacer at lower concentration (0.01 M). The enzyme loading was between 0.03 mg/mL and 0.09 mg/mL. All spectra are dominated by the appearance of peaks at 193, 209 and 222 nm, which are typical for a protein that has a significant part of α-helical structural elements.

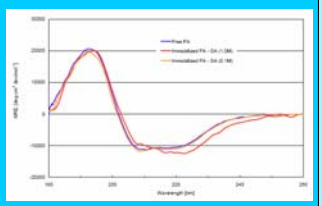

Fig.5: *CD spectra of free and immobilized penicillin G acylase in 10 mM phosphate buffer; JASCO J-810 spectropolarimeter, optical path length: 0.1 cm, T = 20 °C)*

The CD spectrum of the free enzyme (blue curve) is equivalent to the CD spectrum described in [1]. The enzyme immobilized with lower glutardialdehyde concentration shows no substantial change in the CD spectrum, while the enzyme immobilized with higher concentration exhibits a distinct change in the spectrum: The negative band at 209 nm is reduced in intensity and the 222 nm band is slightly increased indicating a minor structural change. Structural changes caused by adsorption and desorption onto nanobeads were investigated earlier [2, 3] but in this work the first time covalent immobilization was investigated.

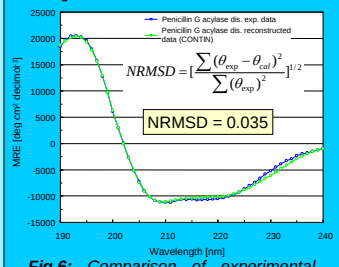

Fig.6: *Comparison of experimental and reconstructed spectrum of the free enzyme (CONTIN algorithm)*

The CD spectrum of free penicillin G acylase was analyzed with the secondary structure estimation algorithms CONTIN, CDSSTR and SELCON3 (see Fig. 6, [4]). The table below gives estimates of secondary structure element fractions of these analyses together with secondary structure data from X-ray diffraction analysis [5,6,7]. The secondary structure element fractions obtained from CD analysis are rather consistent with the data from X-ray crystallography. The normalized root-mean-squared error (NRMSD) is clearly < 0.1, which indicates a good fit between experimental spectrum and the spectrum reconstructed from the secondary structure fractions.

Algorithm	α-helix	β-strand	other	total
CONTIN	0.338	0.192	0.469	0.999
CDSSTR	0.35	0.23	0.42	1.00
SELCON3	0.347	0.177	0.484	1.008
PDB (X-ray Diffraction)	0.348	0.200	0.452	1.00

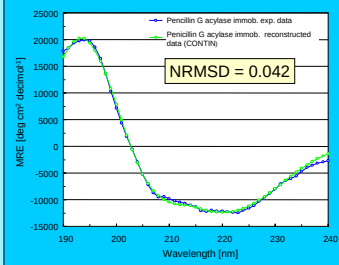

Fig.7: *Comparison of experimental and reconstructed spectrum of the immobilized enzyme (CONTIN algorithm)*

The CD spectrum of immobilized penicillin G acylase was analyzed with the same algorithms (see Fig. 7, [4]). In the table below estimates of secondary structure element fractions of these analyses are presented. Within the error limits of the methods no substantial change for the α-helix content was found. A slight decrease (3 %) in β-strand fraction was observed.

The minor structural change points out that the enzyme specific activity should not be strongly reduced by the immobilization process. This was proven with mass balance measurements using enzyme activity determination.

Algorithm	α-helix	β-strand	other	total
CONTIN	0.34	0.167	0.494	1.001
CDSSTR	0.39	0.17	0.45	1.01
SELCON3	0.364	0.164	0.487	1.015

The activity determinations were done according to the method of [8]. The sample volume was always 1.0 mL containing 25 µL 6-Nitro-3-phenylacetamido benzoic acid (5 mM), 25 µL enzyme solution and 950 µL phosphate buffer (pH 7.5, 0.2 M). The reaction has been carried out by vortexing (50 s) and then centrifuging (10 s) the sample. The optical density was determined at 380 nm.

These measurements show that 97% oft the specific activity is retained,

Conclusions:

- Immobilization of penicillin G acylase onto silica nanobeads was successfully performed using glutardialdehyde spacer
- CD reveals only minimal structural changes of immobilized penicillin G acylase
- Activity measurements show only slight decrease in enzyme specific activity
- Increasing enzyme loading may cause stronger structural changes (data not shown)

Outlook:

- Further experiments have to be done to study the effect of higher enzyme loading
- The investigations will be extended to magnetic nanobeads
- Further characterization of the immobilized enzyme is planned using other physico-chemical methods

References:

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