Stabilization of glucose oxidase within sodium alginate-cellulose sulfate-poly(methylene-co-guanidine) capsules

M. Bucko[1], A. Vikartovska[1], D. Mislovicova[1], V. Patoprsty[1], I. Lacik[2] and P. Gemeiner[1*]
[1]Institute of Chemistry of SAS, Bratislava, Slovakia
e-mail:chembuck@savba.sk
[2]Polymer Institute of SAS, Bratislava, Slovakia

Introduction

Glucose oxidase (GOD, EC 1.1.3.4) was chosen as a model oxidizing enzyme to demonstrate suitability of polyelectrolyte complex (PEC) capsules for enzyme encapsulation and stabilization (Vikartovska 2007). GOD is a flavin containing glycoprotein which catalyses oxidation of β-D-glucose to δ-gluconolactone, followed by spontaneous hydrolysis to gluconic acid (Leskovac 2005). In certain cases, immobilization may produce stabilization, depending on the enzyme nature: multipoint covalent attachment may prevent enzyme unfolding, multisubunit immobilization may prevent enzyme dissociation, generation of microenvironments may improve the enzyme stability (Mateo 2007, Abian 2007). On the other hand an insufficient description of encapsulation protocols and materials together with poorly characterized biooxidation conditions may slow down a more efficient development in the field of GOD encapsulation. Moreover, encapsulation or entrapment of GOD often led to enzyme leakage from polymeric matrix (Blandino 2000). Development of reproducible encapsulation and biooxidation protocols may be achieved by highly controlled and thoroughly described encapsulation, post-encapsulation and biooxidation procedures. Therefore it is desirable to employ PEC capsules made of sodium alginate (SA), cellulose sulfate (CS), poly(methylene-co-guanidine) (PMCG), $CaCl_2$ and NaCl (SA-CS/PMCG capsules) (Lacik 1997, Bucko 2005 and 2006) for encapsulation of GOD.
The main objectives of the present work are as follows: i) encapsulation of GOD into the SA-CS/PMCG capsules; ii) quantification of enzyme loss during each encapsulation step using Electrospray Ionization-Mass Spectrometry (ESI-MS); iii) investigation of the influence of GOD encapsulation into the SA-CS/PMCG capsules on enzyme properties. An influence of co-encapsulated MnO_2 on oxygen availability within the GOD-SA-CS/PMCG capsules is studied. Mechanical resistance of capsules exposed to oxygenation conditions is investigated.

Materials and methods

Materials. GOD from *Aspergillus niger* (specific activity of 199 U/mg) was from Biozyme Laboratories Ltd. (Gwent, UK). High viscosity SA was from ISP Alginates (Girvan, Ayrshire, UK); CS (sodium salt) was from Acros Organics (New Jersey, NJ, USA); PMCG hydrochloride (Scientific Polymer Products Inc.; Ontario, NY, USA) was lyophilized prior use; potassium pectate was prepared from commercial apple pectin (Pectin-Fabrik; Smirice, Czech Rep.) (http://www.chem.sk/products). All other chemicals were of analytical grade.

Encapsulation. A custom-made coaxial air-stripping extrussion device fitted with multiloop reactor (Anilkumar 2001), stroboscopic light and precision air regulators with pressure gauges was used for encapsulation of GOD and co-encapsulation of GOD and MnO_2 (Vikartovska 2007). Briefly, GOD was dissolved in polyanion solution (PA) prepared from 0.9% (w/v) SA and 0.9% (w/v) CS in 0.9% (w/v) NaCl at pH 7.0 to obtain a concentration of 1mg/ml. Prepared solution was air-stripped through concentric nozzle at a flow rate of about 0.5 ml/min into the stream of polycation solution (PC) flowing in the multiloop reactor. The flow rate of PC solution, consisting of 1.8% (w/v) PMCG, 1.0% (w/v) $CaCl_2$ and 0.9% (w/v) NaCl was 50 ml/min providing the reaction time for capsule formation of 70 s. The reaction was quenched by collecting the capsules at the exit of reactor in 150 ml batches of 0.9% (w/v) NaCl solution in the time intervals of 1 min. Capsules were then treated for 5 min with an excess of 50 mM citrate solution containing 0.9% (w/v) NaCl, followed by washing in 0.9% (w/v) NaCl. The second polyanion layer was prepared by subsequent 10 min treatment of the capsules with an excess of 0.1% (w/v) CS solution containing 0.9% (w/v) NaCl. The production of capsules with co-encapsulated MnO_2 involved dispersion of 1% (w/v) MnO_2 in 0.9% (w/v) NaCl solution using ultrasonic cleaner. Obtained dispersion was used for preparation of PA solution with GOD followed by encapsulation procedure described above. Capsules were stored at 4°C in 0.9% (w/v) NaCl.

Entrapment. Entrapment of GOD in calcium pectate gel (CPG) beads was carried out using above described encapsulator without multiloop reactor (Vikartovska 2007). 5.2% (w/v) potassium pectate solution containing GOD (1mg/ml) was air-stripped at a flow rate of 0.5 ml/min into 200 ml of gently stirred solution of $CaCl_2$ (0.1M). Beads were hardened for 1 hour, then washed with distilled water and used immediately in biooxidation experiments.

Determination of GOD activity in soluble and immobilized form. The activity of free and immobilized GOD as well as storage and thermal stability of free GOD was determined spectrophotometrically after horseradish peroxidase catalysed oxidation of *o*-dianisidine with hydrogen peroxide, using D-glucose as a substrate. Flow calorimetry (FC) was used for determination of storage, thermal and operational stabilities of GOD-SA-CS/PMCG capsules as reported previously (Vikartovska 2007).

Quantification of enzyme loss during encapsulation. ESI-MS technique was used for quantification of peptide fragments of GOD to determine overall encapsulation efficiency. The fragments were obtained from trypsin-digested samples, withdrawn after each encapsulation step (Vikartovska 2007).

Mechanical resistance. A Texture Analyser (Model TA-XT2i, Stable Micro Systems, Godalming, UK) equipped with software Texture Expert was used for determination of mechanical resistance of GOD-SA-CS/PMCG capsules exposed to oxygen bubbling during oxidation of D-glucose as reported in detail elsewhere (Vikartovska 2007). The average bursting force and standard deviation were obtained from the individual analysis of at least 30 capsules. Obtained results were compared to those for GOD-SA-CS/PMCG capsules stored in 0.9% (w/v) NaCl at 4°C.

Results and Discussion

Uniform GOD-SA-CS/PMCG capsules with the average size of 0.88±0.02 mm and membrane thickness of 0.11±0.01 mm were produced as depicted on Fig. 1. According to ESI-MS measurement, the overall *encapsulation efficiency* was 43%, thus giwing rise to 0.67 $mg_{GOD}/g_{wet\ capsules}$. The largest portion of GOD of 56.5% was lost either to PC or washing solution during the initial capsule and membrane formation. The loss of GOD during further encapsulation stages was less than 1%.

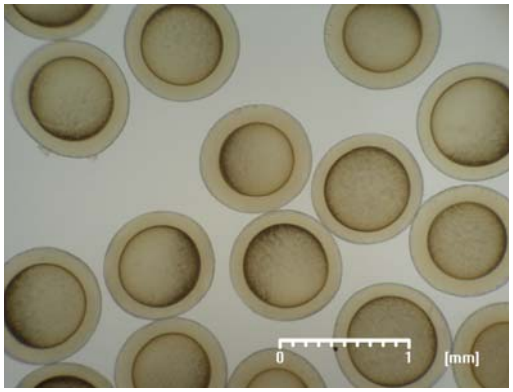

Fig. 1 GOD-SA-CS/PMCG capsules stored in 0.9 % (w/v) NaCl solution.

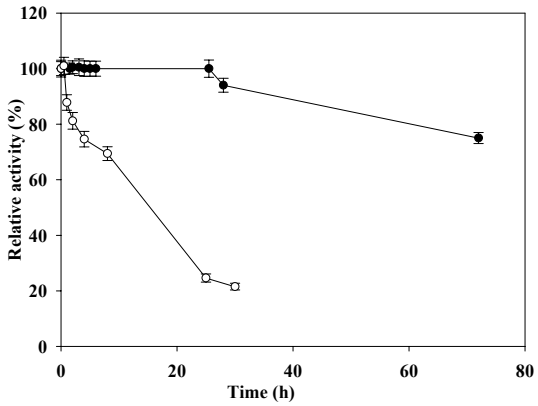

Fig. 2 Thermal stability of free (○) and encapsulated (●) GOD at 45°C, determined by FC

The encapsulated GOD was much more *thermostable* than the free enzyme. After 28-hour incubation at 45°C free and encapsulated GOD retained 21.5% and 94% of activity, respectively. Furthermore, after 75-hour incubation at 45°C the GOD-SA-CS/PMCG capsules retained about 80% of enzyme activity (Fig. 2). The *storage stability* of encapsulated GOD was also higher than that of the free GOD (Fig. 3). While free GOD retained 83% and 59% activity after 100-day storage at 4°C and 25°C, respectively, the encapsulated GOD exhibited 98% and 97% activity at the same conditions.

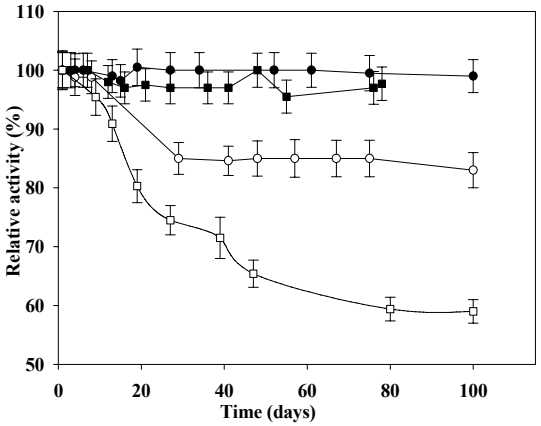

Fig. 3 Storage stabilities of free (empty) and encapsulated GOD (full) by FC. Enzyme or capsules stored at 4˚C (square) or 25˚C (circle)

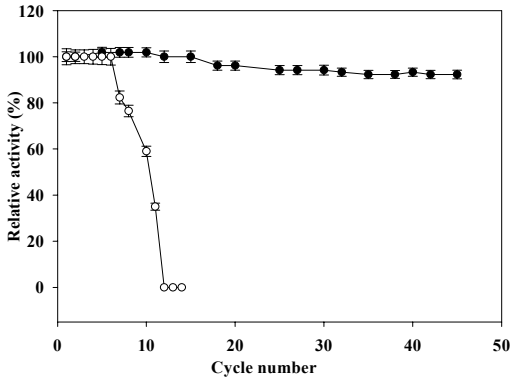

Fig. 4 Operational stability of GOD-SA-CS/ PMCG capsules (●) and GOD-CPG beads (○) determined by FC.

The GOD-SA-CS/PMCG capsules retained 92% of activity after 45 repeated biooxidation cycles during assessment of *operational stability* by flow calorimetry (Fig. 4). The enzyme activity of GOD entrapped in CPG beads, tested as a reference immobilization method, abolished after 12 cycles due to large pores and low resistance of CPG beads towards chelating compounds. A floatation of the GOD-SA-CS/PMCG capsules by oxygen bubbling during biooxidation resulted in a mild drop in their *mechanical resistance*, while the integrity of the capsules was maintained. Texture analyses of the capsules indicated a 25% drop of the average bursting force, from 1.6±0.3 g/capsule (reference sample) to 1.2±0.2 g/capsule for the oxygenated capsules. This effect was probably caused by intensive shear forces imposed to capsules during oxygen bubbling needed for floatation of the capsules. *Co-encapsulation of MnO_2* in GOD-SA-CS/PMCG capsules enhanced enzyme activity. The specific activity of encapsulated GOD with MnO_2 increased by about 30%, from 25 U/mg to 32 U/mg, as compared to encapsulated GOD wihout MnO_2.

Conclusions

Encapsulation of GOD into SA-CS/PMCG capsules with negligible leakage from the formed capsules was accomplished. A novel approach of immobilized protein quantification employing ESI-MS technique was developed to monitor the loss of the GOD during enzyme encapsulation. In comparison with the free enzyme, the encapsulated GOD showed decreased activity probably due to diffusion limitations. This drawback is more than counter-balanced by achieved stabilization of encapsulated GOD in terms of higher thermal, storage and operational stabilities as compared to free GOD. Further enhancement of the enzyme activity was achieved by co-encapsulation of MnO_2 within GOD-SA-CS/PMCG capsules. Presented data suggest that the SA-CS/PMCG capsules may represent a suitable matrix for encapsulation and stabilization of oxidative enzymes (Vikartovska 2007).

Acknowledgement. This work was supported by Science and Technology Assistance Agency under the contract APVV-51-033205, the Slovak Grant Agency for Science VEGA 1/4452/07 and the COST Action 865.

References

Abian et al. (2007) *J. Mol. Catal. B Enzym.* 19-20: 295-303
Anilkumar et al. (2001) *Biotechnol. Bioeng.* 75: 581-589
Blandino et al. (2000) *Enzyme Microb. Technol.* 27: 319-324
Bucko et al. (2005) *Enzyme Microb. Technol.* 36: 118-126
Bucko et al. (2006) *J. Chem. Technol. Biotechnol.* 81: 500-504
Lacik et al. (1997) *J. Biomed. Mater. Res.* 39: 52-56
Leskovac et al. (2005) *Int. J. Biochem. Cell Biol.* 37: 731-750
Mateo et al. (2007) *Enzyme Microb. Technol.* 40: 1451-1463
Vikartovska et al. (2007) *Enzyme Microb. Technol.*, doi:10.1016/j.enzmictec.2007.06.010