Magnetically modified spent grain as a low-cost, biocompatible and smart carrier for enzyme immobilisation

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Abstract

BACKGROUND: Food and feed technology and biotechnology benefit from the use of immobilised enzymes. New low-cost enzyme carriers exhibiting high biocompatibility and response to external magnetic field can substantially improve the application potential of immobilised enzyme systems.

RESULTS: Ferrofluid-modified spent grain was used as a low-cost, biocompatible and magnetically responsive carrier for the immobilisation of Candida rugosa lipase. Several immobilisation procedures were tested using both native and poly(ethyleneimine)-modified magnetic spent grain. Activity of immobilised lipase per unit mass of carrier, operational stability, time stability and Michaelis constant were compared. In general, magnetic spent grain modified with poly(ethyleneimine) bound a smaller amount of active lipase than unmodified magnetic spent grain, but the operational and storage stabilities of enzyme immobilised on poly(ethyleneimine)-modified carrier were very high.

CONCLUSION: Ferrofluid-modified spent grain can be a promising low-cost magnetic carrier for enzyme immobilisation, applicable e.g. in food and feed technology and biotechnology.

INTRODUCTION

Enzymes are able to catalyse the most complex chemical processes under the most benign experimental and environmental conditions. For many industrial and analytical applications, enzymes have to be immobilised on appropriate carriers via very simple and cost-effective protocols in order to be reused over very long periods of time. From this point of view, immobilisation, simplicity and stabilisation have to be strongly related concepts. A great number of enzyme immobilisation protocols have been described, but very few are simple and useful enough.1

The potential application of immobilised enzymes on a large scale, such as in food technology or biotechnology, requires the use of inexpensive carriers. Recently, several types of low cost material of biological origin, such as bagasse,2 coconut fibre,3 rice husk,4 sawdust5 and spent grain,6 have been used as carriers for enzyme immobilisation. Immobilisation of studied enzymes could be performed in a simple way and their activities were usually high and stable. Since plant-derived carriers are biocompatible, at least some of these materials can find interesting applications in the food industry. Spent grain, which originates as a by-product of the beer industry, is an excellent example of a highly food technology-compatible carrier. The production of 100 L of beer corresponds to the production of approximately 20 kg of spent grain; brewers’ spent grain is available at low or no cost throughout the year and is produced in large quantities in all types of brewery.7

In order to improve manipulation with immobilised enzymes, magnetic carriers have been used for many years.8 Magnetically responsive carriers belong to the group of smart materials and enable simple separation of immobilised enzymes from a reaction mixture that can also contain particulate impurities; alternatively, immobilised enzymes can be targeted to the desired place. In many cases, magnetic carriers consist of small magnetic particles (most often formed by magnetite, maghemite or various ferrites), usually in the nanometre to micrometre range, dispersed in a polymer, biopolymer or inorganic matrix; alternatively, magnetic particles can be adsorbed on the outer surface of diamagnetic particles. Various chemical routes (e.g. classic synthesis by precipitation, high-temperature reactions, reactions in steric environments, sol–gel reactions, decomposition of organometallic precursors, polyol methods, microwave-assisted synthesis, etc.) have been used for the preparation of magnetic iron oxide nano- and microparticles.9,10 Standard covalent conjugation strategies developed in enzymology, immunology and affinity

Keywords: magnetic fluid; spent grain; lipase; magnetic carrier; immobilisation
chromatography, using amine, hydroxyl, carboxyl, aldehyde or thiol groups exposed on the surface of magnetic particles, can be employed for compound immobilisation.1

Recently, a magnetic derivative of spent grain has been developed and used as an adsorbent for organic xenobiotics removal.11 Here we would like to show that magnetic spent grain can also serve as a low-cost, biocompatible and smart carrier for the immobilisation of important enzymes. Owing to the biological origin of the carrier, the whole complex could be efficiently used also in the food and feed industries and biotechnology.

_Candida rugosa_ lipase has been chosen as a model enzyme for the current study. This enzyme is often used in biotechnology applications.12 Recently, _C. rugosa_ lipase has been immobilised on various magnetic carriers such as lauric acid-stabilised magnetic particles,13 magnetic Dacron,14 magnetised polysiloxane polyvinyl alcohol particles15 and magnetic Fe3O4-chitosan nanoparticles.16 Immobilisation of _C. rugosa_ lipase on magnetic spent grain represents a new way to obtain an extremely cheap and smart biocatalyst applicable in food technology and biotechnology.

**MATERIALS AND METHODS**

**Materials**

_Candida rugosa_ lipase (EC 3.1.1.3) was purchased from Sigma, St. Louis, MO, USA. Spent grain obtained from Samson Brewery (Ceske Budejovice, Czech Republic) was repeatedly washed with boiling water to remove extractable components and then air dried. The dry material was homogenised in a knife coffee mill and, after sieving, the fraction below 1 mm was used. Water-based magnetic fluid (ferrofluid) stabilised with perchloric acid was prepared using the standard procedure.17 The ferrofluid was composed of maghemite nanoparticles with diameters ranging between 10 and 20 nm (electron microscopy measurements, not shown). The relative magnetic fluid concentration (25.2 mg mL−1) is given as the maghemite content determined by a colorimetric method.18 Branched poly(ethyleneimine) (PEI; P3143), glutaraldehyde (GA), 1,4-butanediol diglycidyl ether (BDDE), sodium periodate (NaIO4), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and 4-nitrophenyl butyrate were purchased from Sigma. 4-Nitrophenol and common chemicals were obtained from Lach-Ner (Neratovice, Czech Republic).

**Preparation of magnetic spent grain**

Magnetic derivative of spent grain (MSG) was prepared similarly as described previously.11,19 Briefly, washed and sieved spent grain (3 g) was suspended in 40 mL of methanol, and 3 mL of perchloric acid-stabilised ferrofluid was added. The suspension was mixed for 2 h, then, after magnetic separation of the modified spent grain, the material was washed twice with methanol and subsequently air dried at room temperature. Before the immobilisation process, MSG was washed thoroughly with distilled water. The presence of iron in the ferrofluid-modified spent grain was detected using a slightly modified version of Perl’s staining procedure described previously.20

**Immobilisation of lipase on magnetic spent grain**

Immobilisation of lipase on untreated and PEI-treated MSG (PEI modification was performed by suspending 1 g of MSG in 50 mL of PEI (15 g L−1); after 6 h of mixing, the modified material was washed with water and air dried) was performed using several different procedures, as follows.

**Immobilisation by adsorption**

- Adsorption of enzyme on PEI-treated MSG (MSG-PEI-enz).
- Adsorption of enzyme on untreated MSG (MSG-enz).

In both cases, 30 mg of MSG was mixed with 1.5 mL of enzyme solution (1 mg mL−1 in 50 mmol L−1 potassium phosphate buffer, pH 7.5) and shaken for 20 h at 4 °C using an automatic rotator (0.33 Hz). Then the unbound enzyme was removed and the magnetic material was washed with buffer until no enzyme activity was detected.

**Immobilisation by adsorption followed by crosslinking**

- Adsorption of enzyme on PEI-treated MSG followed by GA crosslinking (MSG-PEI-GA-enz).
- Adsorption of enzyme on untreated MSG followed by GA crosslinking (MSG-enz-GA).

In both cases, 30 mg of MSG was mixed with 1.5 mL of enzyme solution (1 mg mL−1 in 50 mmol L−1 potassium phosphate buffer, pH 7.5) and shaken for 20 h at 4 °C. Then the unbound enzyme was removed and 1.5 mL of GA solution (30 mL L−1) in buffer was added. This mixture was shaken for 3 h at 4 °C, then the supernatant was removed and the magnetic material was washed with buffer until no enzyme activity was detected.

**Covalent immobilisation**

- Immobilisation on PEI-treated MSG using the carbodiimide method (MSG-PEI-EDC+NHS-enz).

In this method, 30 mg of MSG was mixed with 1.5 mL of potassium phosphate buffer (50 mmol L−1, pH 7.5) containing enzyme, EDC and NHS in the ratio 5:5:6 (1.5:1:5:1.8 mg) and shaken for 20 h at 4 °C. Then the unbound enzyme was removed and the magnetic material was washed with buffer until no enzyme activity was detected.

- Immobilisation on PEI-treated MSG activated by glutaraldehyde (MSG-PEI-GA-enz).
- Immobilisation on untreated MSG activated by epoxide (MSG-BDDE-enz).
- Immobilisation on untreated MSG activated by periodate (MSG-NaIO4-enz).

In these three methods, 30 mg of MSG was mixed with 1.5 mL of activating agent (50 mL L−1 GA solution in distilled water for glutaraldehyde method, shaking for 3 h at room temperature; 30 mL L−1 BDDE in 0.25 mol L−1 NaOH for epoxide method, shaking for 24 h at room temperature in the dark; 0.047 mmol L−1 NaIO4 in 0.1 mol L−1 acetate buffer (pH 4) for periodate method, shaking for 24 h at room temperature in the dark). Then the supernatant was removed and the magnetic material was washed thoroughly with distilled water. Next, activated MSG was mixed with 1.5 mL of enzyme solution (1 mg mL−1 in 50 mmol L−1 potassium phosphate buffer, pH 7.5) and shaken for 20 h at 4 °C. Then the unbound enzyme was removed and the magnetic material was washed with buffer until no enzyme activity was detected.
Lipase assay and \( K_M \) determination

The activity of lipase was determined spectrophotometrically at 405 nm using the artificial substrate 4-nitrophenyl butyrate. Lipase hydrolysed 0.5 mmol L\(^{-1}\) 4-nitrophenyl butyrate (stock solution dissolved in ethanol) in 50 mmol L\(^{-1}\) potassium phosphate buffer (pH 7.5). Particles of MSG with attached enzyme were stirred in the reaction mixture for a certain time interval, then magnetically separated to the bottom of the cuvette using an NdFeB permanent magnet in order to stop the reaction. The increase in the amount of 4-nitrophenol, the yellow-coloured product of hydrolysis, was measured. The whole process was repeated as necessary. Enzyme activity was determined from the difference between measured absorbance values. The activity of immobilised lipase was expressed in nanokatalis (nkat) mg\(^{-1}\) MSG. Owing to the fact that the extinction coefficient \( (\varepsilon) \) values for 4-nitrophenol found in the literature vary substantially, a value of 13 815 L mol\(^{-1}\) cm\(^{-1}\) was used here, which was determined from spectrophotometric measurement at 405 nm using commercially available 4-nitrophenol in 50 mmol L\(^{-1}\) potassium phosphate buffer (pH 7.5). Values of the Michaelis constant \( (K_M) \) of both the free enzyme and immobilised lipase preparations were determined using 4-nitrophenyl butyrate as substrate in the concentration range 0–2 mmol L\(^{-1}\). The experimental data were fitted to the Michaelis–Menten equation using SigmaPlot software (Systat Software, Inc., USA).

Operational stability of immobilised lipase

The operational stability of lipase immobilised on MSG was determined as its reusability over eight cycles. The activity of lipase was measured as described previously. MSG was washed twice with buffer after each cycle of substrate hydrolysis to remove any substrate or product. The immobilised system was reused eight times. The residual activity of immobilised enzyme (expressed in %) after each cycle was compared with the initial activity in the first cycle (100%).

Time stability of immobilised lipase

MSG with immobilised lipase was stored in buffer at 4 °C for 30 days. The activity of immobilised enzyme was measured every 10 days and the residual activity was determined in comparison with the initial activity (100%) at the beginning of storage. Potential leaching of lipase from the support (MSG) was also tested during this period.

Other procedures

The morphological structure of the original and the magnetically modified spent grain was characterised using scanning electron microscopy (SEM).

![Figure 1. SEM images of native (left) and ferrofluid-modified (right) spent grain.](Image)

RESULTS AND DISCUSSION

Ferrofluid modification of spent grain led to the formation of magnetically responsive material that could be easily separated using simple permanent magnets or magnetic separators.\(^{11}\) Spent grain magnetic modification was caused by the deposition of magnetic iron oxide nanoparticles on the plant material surface. The presence of Fe\(^{3+}\) ions on the surface of ferrofluid-modified spent grain was confirmed by Perl’s Prussian Blue Stain, causing intensive blue colouration of the material. As can be seen from the SEM images in Fig. 1, the spent grain surface was coated by both individual and agglomerated magnetic iron oxide nanoparticles, which is in agreement with other magnetically modified plant materials such as ferrofluid-modified sawdust.\(^{19,21}\)

Spent grain, owing to its origin (a secondary product from beer production), is a highly biocompatible and food technology-compatible material. Magnetic iron oxide particles, used as a magnetic modifier, are an accepted food additive (E172). Magnetically modified spent grain can thus be used as a low-cost, fully biotechnology/food technology-compatible carrier for enzyme immobilisation.

Candida rugosa lipase, an important hydrolase, has been selected as a model enzyme to test the potential of magnetically modified spent grain (MSG) as an enzyme carrier. This lipase is one of the enzymes most frequently used in biotransformations, both in hydrolytic (aqueous media) and in synthetic (organic media) approaches.\(^{12}\) To fully exploit the technical and economic advantages of lipases, it is recommended to use them in an immobilised form to reduce cost and improve enzyme stability.\(^{22}\)

Two types of MSG (unmodified and PEI-modified) and several immobilisation procedures were used to find the optimal combination of parameters for lipase immobilisation. PEI modification has been used to introduce additional amino groups on the surface of lignocellulosic particles.\(^{4}\) Table 1 shows the results based on the amount of immobilised lipase activity per unit mass of MSG (nkat mg\(^{-1}\)). The highest amount of immobilised lipase was observed when unmodified MSG was used for physical adsorption (2.57 nkat mg\(^{-1}\)). Similar results were observed for unmodified MSG with covalently bound lipase using the epoxide (2.54 nkat mg\(^{-1}\)) and periodate (2.35 nkat mg\(^{-1}\)) methods of immobilisation and for physical adsorption of enzyme followed by GA crosslinking (2.19 nkat mg\(^{-1}\)), which helps to stabilise the immobilised enzyme structure. A similar amount of active enzyme was bound by adsorption on PEI-modified MSG (2.15 nkat mg\(^{-1}\)). Smaller amounts of lipase were bound covalently on PEI-modified MSG using the GA (1.35 nkat mg\(^{-1}\)) and carbodiimide (1.33 nkat mg\(^{-1}\)) immobilisation methods. The smallest amount of active enzyme was adsorbed on PEI-modified MSG with subsequent GA crosslinking (1.19 nkat mg\(^{-1}\)). During the immobilisation process, the Michaelis–Menten equation was used here, which was determined from spectrophotometric measurement at 405 nm using 50 mmol L\(^{-1}\) potassium phosphate buffer (pH 7.5). Values of the Michaelis constant \( (K_M) \) of both the free enzyme and immobilised lipase preparations were determined using 4-nitrophenyl butyrate as substrate in the concentration range 0–2 mmol L\(^{-1}\). The experimental data were fitted to the Michaelis–Menten equation using SigmaPlot software (Systat Software, Inc., USA).

**Figure 1.** SEM images of native (left) and ferrofluid-modified (right) spent grain.
processes, 43–67% of active enzyme was immobilised on the carriers, taking the starting lipase activity as 100% (Table 1). These results are difficult to compare with those reported for other immobilised lipases owing to differences in substrate and reaction conditions.

Operational stability is the most important parameter for testing the stability of bonds between immobilised enzyme and magnetic carrier, indicating the possibility of repeated application of the biocatalyst. Figure 2 shows the operational stability of lipase immobilised on MSG using different immobilisation techniques. Although the amounts of active enzyme immobilised on PEI-modified MSG were lower than those on unmodified MSG, PEI-MSG carrier proved to be the best from the point of view of operational stability. Lipase adsorbed on MSG-PEI and crosslinked with GA and lipase covalently immobilised on MSG-PEI using GA or carbodiimide as activating agent were reused eight times without loss of activity. Enzyme adsorbed on unmodified MSG or adsorbed with subsequent GA crosslinking retained nearly 90% of initial activity. Lipase adsorbed on MSG-PEI or covalently bound on unmodified MSG using the periodate method retained about 85% of initial activity, while lipase covalently bound on MSG using the epoxide method retained about 80% of initial activity. In general, these residual activities are much higher than those reported for other immobilised C. rugosa lipase preparations: 11% after three reuses (immobilisation on Celite\textsuperscript{23}), 27% after seven reuses (immobilisation on a macroporous copolymer support\textsuperscript{24}), 78% after ten reuses (immobilisation on dry chitosan beads\textsuperscript{25}) and 74% after ten reuses (immobilisation on chitosan using a binary method\textsuperscript{26}).

Figure 3 shows the results obtained from measurements of the time stability of lipase immobilised on MSG during 30 days of storage at 4 °C. Lipase covalently bound on unmodified MSG using the epoxide method or adsorbed on MSG-PEI followed by GA crosslinking retained full enzyme activity after storage. Lipase adsorbed on MSG or covalently bound on MSG-PEI using the carbodiimide method retained about 95% of initial activity. Lipase covalently bound on MSG-PEI treated with GA or adsorbed on MSG-PEI retained about 90% of initial activity, while lipase immobilised on MSG using the periodate method retained about 85% and lipase adsorbed on MSG with subsequent GA crosslinking more than 80% of initial activity. Again, these residual activities are much higher than those reported for other immobilised C. rugosa lipase preparations: around 55% after 5 days (immobilisation on dry chitosan beads\textsuperscript{26}) and 67% after 7 days (immobilisation on chitosan using a binary method\textsuperscript{27}). The time stability of free lipase was very low: it retained only 8% of initial activity after 30 days of storage. Immobilisation of lipase substantially increases its technology potential.

Both free and immobilised lipase exhibited Michaelis–Menten behaviour using 4-nitrophenyl butyrate as substrate. The higher $K_M$ values observed for the immobilised lipase preparations (Table 1) can be explained by the structural changes in the enzyme induced by immobilisation, which lead to lower accessibility of the substrate to active sites.\textsuperscript{14}

The presented results for immobilised lipase activity per unit mass of MSG have to be correlated with the measured storage and operational stabilities of immobilised lipase. There was a slight difference in immobilisation capacity between unmodified and PEI-modified MSG. In general, PEI-MSG bound a smaller amount of active lipase than unmodified MSG, but the operational and storage stabilities of immobilised enzyme on PEI-modified carrier were very high. Spent grain as a plant-based biomaterial consists of lignocellulose units with a large number of hydroxyl groups

### Table 1. Activity of lipase immobilised on MSG using different methods, retention of lipase activity on MSG, and $K_M$ of immobilised lipase

<table>
<thead>
<tr>
<th>Immobilisation method</th>
<th>Activity of lipase per unit mass of MSG (nkat mg$^{-1}$)</th>
<th>Retention of lipase activity on MSG (%)</th>
<th>$K_M$ of immobilised lipase (mmol L$^{-1}$)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSG-enz</td>
<td>2.57</td>
<td>60</td>
<td>0.360</td>
</tr>
<tr>
<td>MSG-PEI-enz</td>
<td>2.15</td>
<td>67</td>
<td>0.476</td>
</tr>
<tr>
<td>MSG-enz-GA</td>
<td>2.19</td>
<td>57</td>
<td>0.326</td>
</tr>
<tr>
<td>MSG-ENZ-enz-GA</td>
<td>1.19</td>
<td>64</td>
<td>0.329</td>
</tr>
<tr>
<td>MSG-BDDE-enz</td>
<td>2.54</td>
<td>59</td>
<td>0.283</td>
</tr>
<tr>
<td>MSG-NaIO$_4$-enz</td>
<td>2.35</td>
<td>50</td>
<td>0.414</td>
</tr>
<tr>
<td>MSG-PEI-enz-GA</td>
<td>1.35</td>
<td>43</td>
<td>0.422</td>
</tr>
<tr>
<td>MSG-PEI-EDC+NHS+enz</td>
<td>1.33</td>
<td>57</td>
<td>0.626</td>
</tr>
</tbody>
</table>

$^a$ The $K_M$ value of free lipase was 0.270 mmol L$^{-1}$.
in the structure, which can be modified during the periodate or epoxide method of immobilisation. Spent grain modified by poly(ethyleneimine) contains primary, secondary and tertiary amino groups; in particular, primary amino groups are utilised for activation of PEI-MSG by GA or during the carbodiimide method of immobilisation. PEI is a cationic polymer affecting the process of enzyme adsorption on the surface of MSG-PEI. For long-term application of immobilised lipase, poly(ethyleneimine)-modified MSG is preferable for enzyme adsorption followed by GA crosslinking.

CONCLUSIONS
As can be seen from the results, spent grain is a very promising carrier for the immobilisation of target enzymes. Magnetic modification of this carrier enables the use of magnetic separation techniques for its rapid separation from complex samples containing different impurities, including suspended solids. Simple magnetic modification was performed with the perchloric acid-stabilised ferrofluid; magnetic nanoparticles precipitated on the spent grain surface in the form of both individual particles and their aggregates. Various immobilisation procedures can be used for successful enzyme immobilisation; even very simple strategies such as enzyme adsorption (either as such or followed by glutaraldehyde crosslinking) enabled efficient enzyme immobilisation exhibiting high stability (at least 80% during 30 days of storage). This fact is of great importance, because the free enzyme retained just 8% of initial activity after 30 days of storage. Immobilised lipase exhibited slightly higher $K_m$ in comparison with free lipase. Ferrofluid-modified spent grain can thus be a promising low-cost, biocompatible magnetic carrier for enzyme immobilisation, applicable e.g. in food technology and biotechnology.

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