Journal of Molecular Catalysis B: Enzymatic 71 (2011) 10–15

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

# β-Galactosidase entrapment in silica gel matrices for a more effective treatment of lactose intolerance

Valentina Nichele, Michela Signoretto*, Elena Ghedini

*Dipartimento di Scienze Molecolari e Nanosistemi, Università Ca' Foscari Venezia, Consorzio INSTM-UR di Venezia, Calle Larga Santa Marta, 2137, 30123 Venezia, Italy*

ARTICLE INFO

*Article history:*
Received 25 October 2010
Received in revised form 10 February 2011
Accepted 10 March 2011
Available online 16 March 2011

*Keywords:*
Enzyme immobilization
Sol–gel
β-Galactosidase
Lactose intolerance

ABSTRACT

In this work we present a preliminary study directed to the realization of a new pharmaceutical formulation for the treatment of lactose intolerance. The main aim of the work is to increase the stability of β-galactosidase in order to prolong its activity in the course of time, thus improving its performance as dietary supplement in the digestion of lactose.

We describe a reliable and effective procedure for the immobilization of β-galactosidase in a three-dimensional silica network. A one-step approach was optimized by using the sol–gel method to obtain homogeneous and stable β-galactosidase/silica gel composites; the textural properties of the porous surface were characterized by $N_2$ physisorption analyses. The activity of β-galactosidase was evaluated *in vitro* in the hydrolysis of o-nitrophenyl-β-D-galactopyranoside (used instead of lactose) at pH 7.4 and 37 °C, thus reproducing the conditions of the human intestine.



## 1. Introduction

Lactose intolerance is a very common disorder due to the inability to digest lactose into its constituents, glucose and galactose, because of low levels of lactase.

Lactase, an enzyme of the β-galactosidase (EC 3.2.1.23) family, is produced on the brush border of the small intestine and is responsible for the hydrolysis of lactose into its constituents [1]. Enzyme levels reach their maximum shortly after birth, but they decline with aging: it is estimated that 75% of adults worldwide shows some decrease in lactase activity during adulthood [2]. Lactase deficiency can result in lactose maldigestion, but only in presence of clinical symptoms such as abdominal bloating and pain, flatulence, diarrhea, nausea and borborygmi lactose intolerance occurs [3]. The diagnosis or even the suggestion of lactose intolerance leads many people to avoid milk and/or to consume food prepared with digestive aids. The treatment of lactose intolerance includes four general principles: (i) reduction or restriction of dietary lactose, (ii) substitution with alternate nutrient sources to avoid reductions in energy and protein intake, (iii) regulation of calcium and vitamin D intake and (iv) use of exogenous β-galactosidase [4–6]. In particular, the intake of digestive supplements is a successful way to alleviate the symptoms of lactose intolerance; actually a number of lactase preparations is commercially available. These supplements are formulated in tablet or capsule form to be taken just before or with meal [7] and the proper dosage should be suited as a function of the seriousness of the symptoms; the final formulation of these preparations consists of a capsule which delivers the enzyme in the small intestine, where it can carry out its therapeutic action.

Enzyme therapy is promising in the treatment of several diseases, in particular of in-born enzyme deficiencies, but many limitations exist for the clinical use of native enzymes because, in addition to the cost, they are unstable and have a short lifetime in the circulation [8]. The polymeric structure of enzymes, in fact, is stabilized by a large number of low-energy bonds, so deactivation is relatively easy. The inactivation phenomena of an enzyme can be of inter- or intramolecular nature: intermolecular phenomena include autolysis and aggregation, while the intramolecular phenomena are due to interactions of the enzyme with poisons such as irreversible inhibitors, or to extremes values of pH or temperature [9].

In the small intestine lactase is exposed to a deactivating environment because of the presence of inhibitors (glucose and galactose) and proteases. Moreover, when taken orally, β-galactosidase has to cross the acid pH of the stomach which can compromise the structural integrity of the enzyme and, as a consequence, its activity in the hydrolysis of lactose: if lactose passes indigested from the small intestine into the colon it can cause physiological effects that result in the clinical manifestations typical of lactose intolerance.

The major lack of the commercial lactase supplements is then the rapid inactivation of the enzyme, which shortens the activity of β-galactosidase thus forcing to several assumptions during the day.

* Corresponding author. Tel.: +39 041 2348650; fax: +39 041 2348517.
*E-mail address:* miky@unive.it (M. Signoretto).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.03.002

Immobilization can make enzymes more stable and impart a longer circulation lifetime in the organism [8]. It is well known that the immobilization of enzymes (physical adsorption, covalent bonding, gel entrapping, etc.) on proper matrices can prevent their chemical and biological degradation and enhance their stability [10–12]; it is then potentially possible to prevent the denaturation of β-galactosidase in the intestine and prolong its therapeutic action in the course of time by its entrapment inside a matrix. The entrapment of β-galactosidase within a porous matrix allows to avoid a direct contact of the enzyme with the surrounding medium, thus increasing its stability, but, at the same time, it enables the reagents to reach the catalytic site.

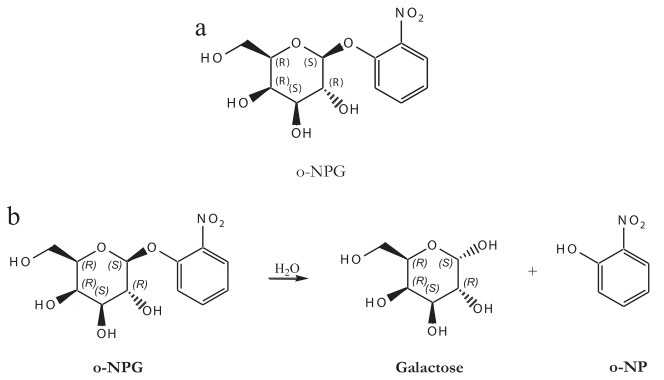
A large number of different supports has been used for lactase immobilization [13–16], but in the case of a pharmaceutical application the support must possess proper features, biocompatibility at first. In the last years, several ceramic and inorganic oxides have attracted the attention of researchers for their potential application in the biomedical field [17]; in this context silica has gained increasing importance and its use for the entrapment of enzymes, antibody, cells and for the design of controlled drug delivery systems has been investigated [18–24]. In fact, silica matrices show high biocompatibility–biodegradability, resistance to microbial attack and exhibit higher mechanical strength, enhanced thermal stability and negligible swelling in organic solvents compared to most organic polymers [25–28]. In addition, silica possesses physico-chemical and textural properties (hydrophilicity/hydrophobicity, surface area, pore volume, etc.) that can be modulated *ad hoc* according to the final application and the nature of the guest molecule.

The choice of the proper immobilization technique is fundamental to preserve to a high degree the structural integrity of the enzyme, which is related to its catalytic activity; an effective technique for this purpose is the sol–gel process [29,30]. The sol–gel method is particularly attractive for the entrapment of biological molecules. It is characterized by a number of unique features including: (i) mild operative conditions (in particular low temperature); (ii) high versatility; (iii) encapsulation of a guest molecule in the inorganic matrix by a one-step approach [28–31]. It is now well established that a wide variety of enzymes and other proteins retains its characteristic reactivity and chemical function when confined within the pores of the sol–gel derived matrix, which isolates the biomolecules protecting them from self-aggregation [32]. It is important to highlight that protein molecules encapsulated in a sol–gel matrix are not covalently bound to the support, but they are physically entrapped in the gel network that has grown around them [33]. This can prevent the enzyme denaturation (chemical modification of the protein) that frequently occurs in the presence of covalent linkages between the matrix and the biological molecule and guarantees a sufficient mobility to the enzyme, which experiences conformational changes when it binds the reagent molecule.

Another important parameter in the design of a pharmaceutical system for the treatment of lactose intolerance is the choice of the proper source of the enzyme.

β-Galactosidase, in fact, can be obtained from a wide variety of sources, such as microorganisms, plants and animals, but its properties differs markedly according to the source. In particular it is well known that the optimum pH of enzymes obtained from fungi is 3.5 to 4.5, while β-galactosidases from yeasts have their optimum pH between 6.5 and 7.0 [34]. The commercially available lactase supplements contain a β-galactosidase obtained from GRAS yeasts or fungi but, considering that in the small intestine the pH is close to neutrality, β-galactosidase from yeasts should be more suitable for this application.

The aim of this work has been the optimization of a reliable procedure for the synthesis of a stable β-galactosidase/silica gel composite by using a sol–gel approach. In particular we have investigated the effect of several operative parameters (pH, aging time, enzyme amount, etc.) on the final features (structural and physico-chemical) of the system and subsequently on its stability. This is a preliminary study directed to the realization of a new pharmaceutical formulation for the treatment of lactose intolerance. The main aim is the design of a stable system able to explain its therapeutic action for a long period of time, thus avoiding the frequent administrations required by the traditional commercial preparations.




**Scheme 1.** Structure formula of o-NPG (a). Hydrolysis of o-NPG to galactose and o-NP (b).

## 2. Materials and methods

### 2.1. Materials

Lactozym 3000L®, a liquid preparation of β-galactosidase from *Kluyveromyces lactis* (specific activity: 4097 U/mL), Tetraethoxysilane (TEOS) (98%, Aldrich), $H_2O$ milliQ, Potassium Phosphate monobasic (99%, Aldrich), Potassium Phosphate dibasic (≥98%, Aldrich), o-nitrophenyl-β-D-galactopyranoside (99%, o-NPG, Aldrich), o-nitrophenol (99%, o-NP, Aldrich). All reagents have been used as received.

### 2.2. Synthesis

Enzyme/silica composites were synthesized by a one-step sol–gel process. In a typical experiment, the silica precursor (TEOS) was combined with ultra pure water (acidified with the addition of a few drops of HCl 0.1 M) in the proper molar ratio (1TEOS:6$H_2O$) and the mixture was homogenised by sonication for 3 h. A solution of Lactozym 3000L® in phosphate buffer (pH 7.4) containing the proper amount of β-galactosidase (in particular, we tested three different enzyme concentrations: 137 U, 273 U, 546 U) was added to the obtained sol, thermostated at 4 °C, under continuous stirring in order to favour the homogeneous dispersion of the enzyme in the final material. 1 mL of the sol was dispensed into polyethylene cylindrical vials (diameter: 1.7 cm, depth: 1.0 cm). The obtained monolithic and transparent gel were washed with a phosphate buffer solution (pH 7.4) and then stored at 4 °C for 21 days covered with the buffer (0.4 mL). The buffer was replaced with a fresh amount every day and analyzed in order to determine the amount of enzyme released from the gel during the aging time.

### 2.3. Catalytic activity

The activity of β-galactosidase was assayed by means of a colorimetric test using o-NPG as substrate; o-NPG is a colourless compound which is hydrolyzed to galactose (colourless) and o-NP (yellow) (Scheme 1). The operational conditions reproduced the characteristic pH and temperature (7.4 and 37 °C, respectively)

of the small intestine, where the enzyme carries out its catalytic action.

A solution of o-NPG in phosphate buffer (4.1 mg/mL) was added to a proper volume of diluted enzyme solution (0.054 U): one unit of β-galactosidase is defined as the amount of enzyme hydrolizing in 1 min 1 μmol of substrate at pH 7.4 and 37 °C.

The rate of formation of free o-NP (the chromophore) was recorded spectrophotometrically ($\lambda$ = 420 nm) using a 1-cm path length cuvette. The activity (a) is then defined as the conversion rate of the substrate (o-NPG) to the products for unitary volume of Lactozym 3000L® (μmol $min^{-1}$ $mL^{-1}$).

The activity of the encapsulated enzyme was measured in the same conditions (o-NPG concentration, substrate/enzyme ratio) used for the free enzyme. The gel was dipped for 10 min in a phosphate buffered solution thermostated at 37 °C, then it was incubated for 20 min in the substrate solution and the o-NP amount in solution was evaluated spectrophotometrically. At the end of the reaction the gel was transferred in the original buffered solution where it was kept until the following test.

### 2.4. Temperature and pH effect

The effect of pH and temperature on the activity of β-galactosidase was evaluated.

The pH effect was determined in the conditions detailed in Section 2.3 at pH in the range 2–8.5, whereas the temperature effect was established in the range 30–60 °C at the constant pH of 7.4.

### 2.5. Characterization

Specific surface area and pores size distribution were obtained from $N_2$ adsorption–desorption isotherms at 77 K (MICROMERITICS ASAP 2000 Analyser). Surface area was calculated by the BET equation [35], whereas the mesopore size distribution was determined by the BJH method [36], applied to the $N_2$ adsorption isotherm branch.

## 3. Results and discussion

### 3.1. Entrapment of β-galactosidase in the silica gel

#### 3.1.1. Synthetic procedure

It is well known that the sol–gel process is a suitable technique for the immobilization of a wide series of molecules in a porous matrix; we have already verified the effectiveness of the process in the encapsulation of enzymes [19] and in the design of controlled drug delivery systems by the entrapment of several drugs in silica gel matrices [23,24,37]. However, in the design of the process parameters it is fundamental to consider the nature of the guest molecule and the final application conceived for the material in question: several issues in fact could compromise the entrapment stage.

First of all, in the immobilization of biomolecules such as enzymes, mild operative conditions are required to prevent their denaturation. For this reason, in the synthesis we optimized for the encapsulation of β-galactosidase we operated at low temperature (4 °C) and at pH near to neutrality: the enzyme preparation (Lactozym 3000L®) was suspended in a proper volume of phosphate buffer (pH 7.4) before its addition to the sol in order to neutralize the original pH of the sol itself (which is weakly acid because of the addition of a few drops of HCl).

Another issue concerns the use of alcohol. In the sol–gel synthesis it is a common practice to add to the reagents (TEOS and water in this case) an alcohol as solvent: it is nevertheless well known that, if present in large amount, it can depress the enzymatic activity [28,33]. Consequently in our synthesis we avoided the use of alcohol.

However one should consider that in the course of the gel reticulation little fractions of alcohol are released, as a consequence of the condensation reaction which occurs even after the gel time; to overcome this problem, the gel was washed and covered with fresh buffered solutions (pH 7.4) every day.

After the addition of β-galactosidase to the sol we obtained, in a few minutes, transparent, monolithic and homogeneous silica gel; moreover the absence of supernatant liquid guarantees that the whole amount of enzyme added to the sol has been entrapped in the gel matrix. The gel exhibits the same appearance at the three different enzyme concentrations we tested and it remains unaltered in the course of time. These features are indicative of a homogeneous distribution of the enzyme in the gel, which is an essential requirement in the design of a pharmaceutical system.

The last problem we considered concerns the possibility of a partial desorption of the enzyme from the matrix forced by the reticulation process.

In order to determine the real enzyme amount present in the silica gel when it has reached its definitive structure, the supernatants were daily analyzed spectrophotometrically by the enzymatic assay (see Section 2.3). We have verified that the enzymatic units desorbed from the gel in 21 days (time required for the complete gel reticulation) are ~0.23% (independently from the enzyme concentration) of the initially loaded units; this is a very satisfactory result as the yield of the encapsulation process is close to 100%.

The results we obtained confirm the effectiveness of the sol–gel process: the gel network grows around the enzyme, independently from its dimension, with the formation of a "cage" that prevents its leaching. Moreover, the β-galactosidase/silica gel composites have the desired features in terms of gelation rate, control of the amount of loaded enzyme and handiness; these results have been achieved through the optimization of the synthesis parameters, in particular the Si precursor:water ratio, the temperature of the enzyme addition and the aging conditions. All these experimental evidences suggest the reliability of the optimized process for the successful encapsulation of β-galactosidase in a silica gel, which is the first aim of our work.

The most important issues in the immobilization of a biomolecule are the retention of its activity upon immobilization and the increase of its stability. Therefore, the following steps of the investigation have been the characterization of the systems and the check of their catalytic activity and stability.

#### 3.1.2. Activity

The activity of the encapsulated enzyme was evaluated *in vitro* in the hydrolysis reaction of o-NPG at 37 °C and pH 7.4 (intestinal conditions) as a function of the aging time of the gel; this test allows to verify if the enzyme kept its bio-functionality within the silica matrix and how its activity varies in the course of the reticulation process.

Fig. 1 shows the profiles of the enzymatic activity as a function of the reaction time and of the gel aging. The bio-functionality of the encapsulated enzyme was experimentally confirmed by the appearance of a yellow colour (o-NP formation) first on the gel surface and gradually in the bulk (see pictures in Fig. 1); this evidence confirms that the reagent diffuses from the bulk solution into the pores of the matrix, reaching the catalytic site of the enzyme.

For what concerns the catalytic kinetic, the first evident result is the decrease of the initial activity with the aging progress; at the same time, a clear improvement of the stability and of the reproducibility (see error bars, standard deviations) of the analyses can be detected. Moreover, after 21 days since the system preparation

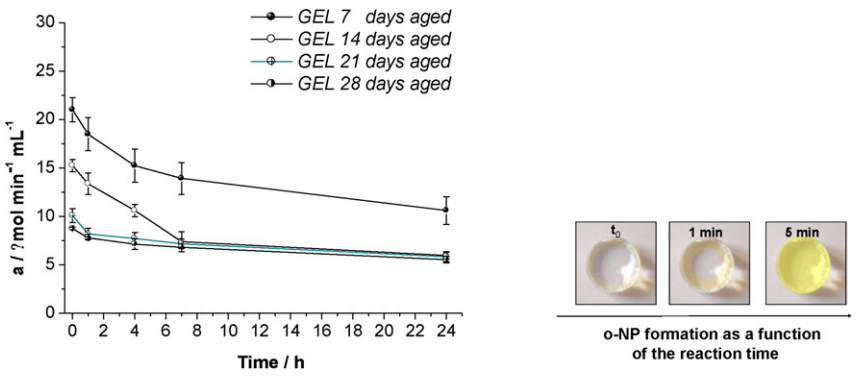



**Fig. 1.** Enzymatic activity vs time profile as a function of the aging time, ($n$ = 3, mean sd) and colour change of the gel tablet following the formation of the yellow o-NP product. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

aging does not affect any longer the reaction kinetic profile, that corresponds to that obtained for the gel 28 days aged.

The activity reduction as a function of the reticulation degree can be plausibly justified considering the evolution of the β-galactosidase/silica gel system during reticulation. The superficial enzyme can be more easily reached by the substrate (o-NPG) and consequently it is more reactive; however, it has been ascertained that the reticulation of the silica gel forces the enzyme molecules inside the matrix. Taking into account that the accessibility of the enzyme to the substrate is the key factor in determining the activity of the final system [30], it is possible to justify the catalytic activity decrease also as a consequence of the onset of diffusion limitations [12].

Moreover, the activity decrease of the less reticulated systems during the catalytic tests can be ascribed to the fact that, at low reticulation degrees, the enzyme is more exposed, so its inactivation (in particular a thermal inactivation) is easier.

In order to validate these hypotheses and obtain more information about the structure evolution of the silica gel (surface area and porosity) with aging progress, physisorption analyses were carried out.

### 3.1.3. Physico-chemical characterization

In Fig. 2 the adsorption–desorption isotherms of the gel loaded with 137 U at different aging times (1, 7, 21 and 40 days since the preparation) are showed.

It is interesting to note that aging and reticulation progress originate an evident evolution of the gel structure from microporous (gel 1 day aged) to mesoporous (gel 21 days aged), as denoted by the clear hysteresis loop in the corresponding adsorption/desorption isotherm. This is a confirmation of the mutual interaction between the silica precursor and the enzyme in the formation of the final enzyme/gel system. In fact, in the as-prepared gel a significant fraction of β-galactosidase molecules are located on the surface of the silica matrix; with the reticulation progress the enzyme moves inside the gel, modulating the gel network conformation to a mesoporous system: it can be said that the protein acts as a structural template [38]. This evidence confirms the supposed correlation between the gel structure evolution and the catalytic activity behaviour. We can observe, moreover, that 21 days are sufficient for the complete reticulation of the silica network that reaches a stable structural conformation. This is confirmed by the isotherm shape of the gel 40 days aged (see Fig. 2b), that agrees with that obtained after 21 days.

In the light of these results, all the following tests were carried out on silica gels 21 days aged.

### 3.2. Temperature and pH effect

In order to evaluate to what extent the silica matrix is capable of preserving the integrity of the enzyme, thus improving its stability, a series of activity runs on the β-galactosidase/silica gel were performed by employing different pH and temperature of work.

The activity trend of the β-galactosidase/silica gel composite as a function of pH (range 2–8.5) is reported in Fig. 3. A strongly acid pH (~2) was selected to check the stability of the entrapped enzyme in extreme pH conditions as those present in the stomach, with a view to the bio-medical application conceived for the synthesized systems; the enzymatic activity is usually inhibited at extreme pH values, as the whole protein structure may suffer of a general unfolding leading to the partial or complete inactivation [39].

First of all it is possible to note that the activity of the encapsulated enzyme reaches its maximum at pH 7.4, i.e. the pH of the small

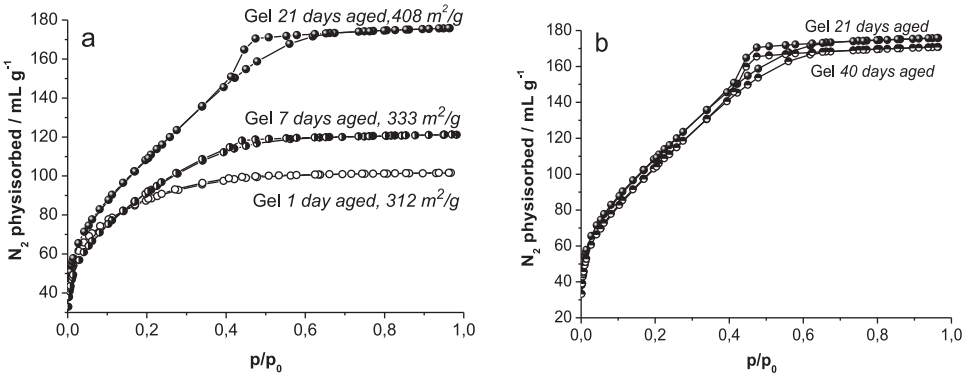



**Fig. 2.** Adsorption–desorption isotherms of the β-galactosidase/silica gel composite containing 137 U at different aging times.

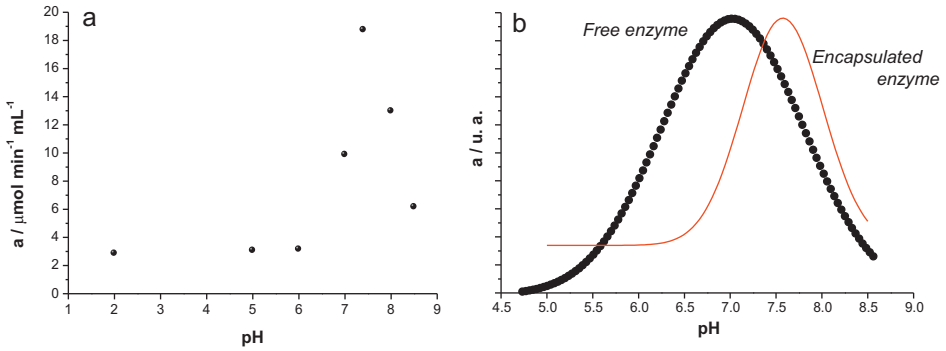



**Fig. 3.** Enzymatic activity trend as a function of the reaction pH for the encapsulated enzyme (a). Comparison of the activity vs pH profiles for the free and the encapsulated enzyme (b).

intestine: β-galactosidase obtained from a yeast is then suitable for this application.

A very interesting result is the preservation of a partial activity (15% of the highest activity) at strongly acid pH (pH 2); in the same conditions the free enzyme (and the silica matrix alone) is totally inactive. This highlights the positive effect that the matrix plays in protecting the enzyme from the external solicitations. Another evidence is the shift of the maximum activity from pH 7.0 for the free enzyme to higher pH values for the encapsulated one (see Fig. 3b). One possible explanation, as reported by Bathia et al. [28], can be that the encapsulated enzyme is experiencing a local pH lower than that of the bulk solution. At neutral pH, silanol groups in a silica matrix are negatively charged and hence the electrical double layer consists of primarily cationic buffer ions and hydrogen ions. An excess of $H^+$ makes the pH in the double layer lower than that of the bulk solution.

In Fig. 4 the temperature effect on the activity of the encapsulated enzyme is showed.

The temperature of maximum activity is 50 °C and it is evident that the encapsulated enzyme retains a partial bio-functionality also at 60 °C and 70 °C, while at these temperatures the free enzyme is completely denatured.

These results confirm once more that the silica matrix can effectively preserve the enzymatic activity, even in extreme operative conditions. This is a significant success with a view to the potential technological applications of these β-galactosidase/silica gel systems.

### 3.3. Stability

The key element of this work was the increase of the stability of β-galactosidase in intestinal conditions to prolong its activity in the digestion of lactose in lactose intolerant people.

The stability of the encapsulated enzyme in the conditions of pH and temperature typical of the small intestine was evaluated *in vitro* and compared with the values obtained for the free enzyme. The immobilized enzymes were incubated in buffered solutions at pH 7.4 at 37 °C for 3 h; the residual enzymatic activity was evaluated by immersion of the gel in a buffered solution (pH 7.4) containing the substrate (o-NPG). The conversion rate of o-NPG to o-NP was determined spectrophotometrically at 420 nm. Fig. 5 shows the test results.

It is evident that the siliceous matrix gives rise to an evident improvement of the enzymatic stability: after 3 h the residual activity of the protein in solution is 60% against 81% of the immobilized β-galactosidase. This is a very important goal because it implies a significant increase of the therapeutic action duration in the case of the pharmaceutical application conceived for these systems.

### 3.4. Enzyme concentration effect

The last parameter we investigated was the correlation between the catalytic activity and the enzyme amount. The kinetic test results are plotted in Fig. 6. It is evident that the catalytic activity of the gel is not proportionally correlated with the enzymatic content: the best catalytic result was achieved, in fact, by the gel containing an intermediate amount of protein (273 U).

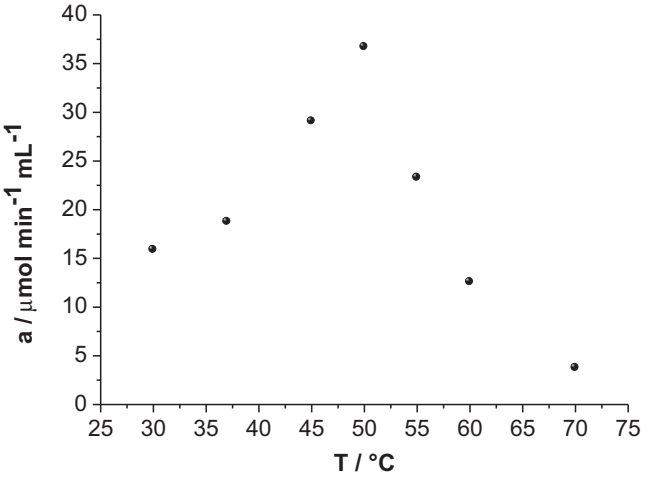



**Fig. 4.** Profile of the enzymatic activity as a function of temperature for the encapsulated enzyme.

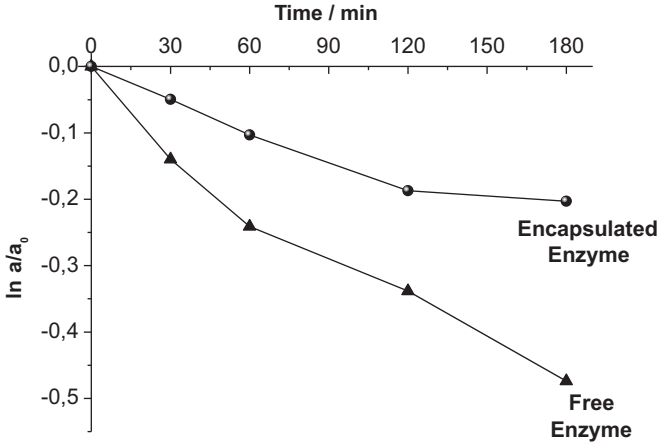



**Fig. 5.** Comparison of the stability of the free and the encapsulated enzyme in intestinal conditions ($a$: activity at the time $t$, $a_0$: initial activity at zero time).

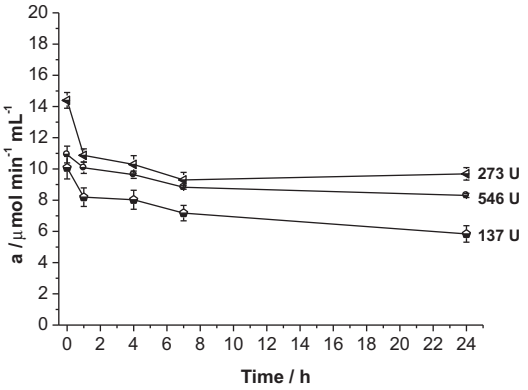



**Fig. 6.** Influence of the enzyme amount on the catalytic activity of the β-galactosidase/silica gel composites.

This result can be explained on the basis of the steric hindrance arising from the increase of the enzyme concentration. As reported by Badjić and Kostić [40], the decrease in activity as the enzyme concentration increases should not be caused by partial loading, aggregation or denaturation of the enzyme, since the sol–gel incapsulation prevents these phenomena, but rather to the fact that the accessibility to the catalytic site decreases, thus reducing the overall activity of the β-galactosidase/silica gel composite. Moreover, the diffusion of the products to the bulk is hampered too, so their detection is delayed.

Therefore these results highlight that an excessive enzyme load is detrimental for the enzymatic functionality as a consequence of the lower accessibility of the catalytic sites by the substrate.

## 4. Conclusions

The obtained results can be summarized in the following points:

1. The optimized sol–gel approach is an effective technique for the immobilization of β-galactosidase in a silica matrix. After 21 days since the gel preparation it is possible to obtain monolithic and homogeneous β-galactosidase/silica gel systems, which are stable in terms of textural properties, enzyme content and catalytic activity.
2. The silica matrix protects the enzyme molecules from the external environment, preserving their bio-functionality even in extreme conditions of pH and temperature.
3. The stability of the systems in the operative conditions of pH 7.4 and 37 °C is clearly improved compared to the free enzyme.

This study, despite its preliminary nature, is very promising and could be useful in the formulation of new pharmaceutical preparations for the treatment of lactose intolerance. The β-galactosidase/silica gel stability could assure, in fact, an adequate enzymatic activity for a prolonged lapse of time, improving the therapeutic efficacy of the actually available commercial formulations.

## Acknowledgement

The authors want to thank Mrs. Tania Fantinel for the excellent technical assistance.

## References

[1] C. Ortolani, E.A. Pastorello, Best Pract. Res. Clin. Gastroenterol. 20 (2006) 467–483.
[2] T.H. Vesa, P. Marteau, R. Korpela, J. Am. Coll. Nutr. 19 (2000) 165–175.
[3] M.C.E. Lomer, G.C. Parkes, J.D. Sanderson, Aliment. Pharmacol. Ther. 27 (2008) 93–103.
[4] M. Montalto, V. Curigliano, L. Santoro, M. Vastola, G. Cammarota, R. Manna, A. Gasbarrini, G. Gasbarrini, World J. Gastroenterol. 12 (2006) 187–191.
[5] M.Y. Lin, J.A. Dipalma, M.C. Martini, C.J. Gross, S.K. Harlander, D.A. Saviano, Dig. Dis. Sci. 38 (1993) 2022–2027.
[6] T.M.M. Coenen, A.M.C. Bertens, S.C.M. De Hoog, C.M. Verspeek-Rip, Food Chem. Toxicol. 38 (2000) 671–677.
[7] R.J. Grand, R.K. Montgomery, Curr. Treat. Options Gastroenterol. 11 (2008) 19–25.
[8] V.P Torchilin, Adv. Drug Deliv. Rev. 1 (1987) 41–86.
[9] M. Ladero, A. Santos, F. Garcia-Ochoa, Enzyme Microb. Technol. 38 (2006) 1–9.
[10] P.S.J. Cheetham, in: A. Wiseman (Ed.), Handbook of Enzyme Biotechnology, Horwood Limited, New York, 1985.
[11] G.F. Bickerstaff, Immobilization of Enzymes and Cells, Humana press, Totowa, 1997.
[12] U. Hanefeld, L. Gardossi, E. Magner, Chem. Soc. Rev. 38 (2009) 453–468.
[13] V. Gekas, M. Lopez-Leiva, Process Biochem. 20 (1985) 2–12.
[14] J.G. Zadow, Whey and Lactose Processing, Elsevier Applied Science, London, 1992.
[15] C. Giacomini, A. Villarino, L. Franco-Fraguas, F. Batista-Viera, J. Mol. Catal. B: Enzym. 4 (1998) 313–327.
[16] M. Di Serio, C. Maturo, E.D. Alteriis, P. Parascandola, R. Tesser, E. Santacesaria, Catal. Today 79–80 (2003) 333–339.
[17] E. Gultepe, D. Nagesha, B.D.F. Casse, R. Banyal, T. Fitchorov, A. Karma, M. Amiji, S. Sridhar, Small 6 (2010) 213–216.
[18] X.S. Zhao, X.Y. Bao, W. Guo, F.Y. Lee, Mater. Today 9 (2006) 32–38.
[19] V. Trevisan, M. Signoretto, S. Colonna, V. Pironti, G. Strukul, Angew. Chem. Int. Ed. 43 (2004) 4097–4099.
[20] R. Wang, U. Narang, P.N. Prasad, F.V. Bright, Anal. Chem. 65 (1993) 2671–2675.
[21] M. Al-Saraj, M.S. Abdel-Latif, I. El-Nahal, R. Baraka, J. Non-Cryst. Solids 248 (1999) 137–140.
[22] Y.F. Zhu, J.L. Shi, Y.S. Li, H.R. Chen, W.H. Shen, X.P. Dong, Micropor. Mesopor. Mater. 85 (2005) 75–81.
[23] L. Contessotto, E. Ghedini, F. Pinna, M. Signoretto, G. Cerrato, V. Crocellà, Chem. Eur. J. 15 (2009) 12043–12049.
[24] E. Ghedini, M. Signoretto, F. Pinna, V. Crocellà, L. Bertinetti, G. Cerrato, Micropor. Mesopor. Mater. 132 (2010) 258–267.
[25] M. Hartmann, D. Jung, J. Mater. Chem. 20 (2010) 844–857.
[26] Z. Wu, H. Joo, T.G. Lee, K. Lee, J. Control. Release 104 (2005) 497–505.
[27] H. Böttcher, P. Slowiik, W. Sÿβ, J. Sol–Gel Sci. Technol. 13 (1998) 277–281.
[28] R.B. Bathia, C.J. Brinker, A.K. Gupta, A.K. Singh, Chem. Mater. 12 (2000) 2434–2441.
[29] K. Smith, N.J. Silvernail, K.R. Rodgers, T.E. Elgren, M. Castro, R.M. Parker, J. Am. Chem. Soc. 124 (2002) 4247–4252.
[30] D. Avnir, S. Braun, O. Lev, M. Ottolenghi, Chem. Mater. 6 (1994) 1605–1614.
[31] S. Braun, S. Rappoport, R. Zusman, D. Avnir, M. Ottolenghi, Mater. Lett. 10 (1990) 1–5.
[32] M.C. Crescimbeni, V. Nolan, P.D. Clop, G.N. Marín, M.A. Perillo, Coll. Surf. B: Biointerface 76 (2010) 387–396.
[33] B.C. Dave, B. Dunn, J.S. Valentine, J.I. Zink, Anal. Chem. 66 (1994) 1120A–1127A.
[34] R. Panesar, P.S. Panesar, R.M. Singh, J.F. Kennedy, M.B. Bera, Food Chem. 101 (2007) 786–790.
[35] S. Brunauer, P.H. Emmett, E. Teller, J. Am. Chem. Soc. 60 (1938) 309–319.
[36] E.P. Barrett, L.G. Joyne, P.P. Halenda, J. Am. Chem. Soc. 73 (1951) 373–380.
[37] M. Signoretto, E. Ghedini, F. Pinna, V. Nichele, G. Cerrato, in: A. Gedeon, P. Massiani, F. Babonneau (Eds.), Studies in Surface Science and Catalysis, vol. 174A, Elsevier, Amsterdam, 2008, pp. 489–492.
[38] B. Dunn, J.M. Miller, B.C. Dave, J.S. Valentine, J.I. Zink, Acta Mater. 46 (1998) 737–741.
[39] K.F. Tipton, H.B.F. Dixon, Methods Enzymol. 63 (1979) 183–234.
[40] J.D. Badjić, N.M. Kostić, Chem. Mater. 11 (1999) 3671–3679.