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Improved Activity in Acidic Media of Immobilized Lysozyme

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Abstract

Polyethylene glycols (PEGs) of various chain length were used to crosslink lysozyme onto an insoluble support such as oxirane. A very high degree of modification and no inactivation of lysozyme were obtained with PEG 20000, but enzymatic activity increased up to 20 times at pH 3.0, at which point the activity of the native enzyme was lower when using *Leuconostok oenus* as a macromolecular substrate.

Index Entries: Lysozyme; enzyme immobilization; Eupergit C; polyethylene glycol; covalent attachment.

Introduction

Widely present in nature and well known for its antibacterial activity, lysozyme (E.C. 3.2.1.17), is used in a large number of applications in both the pharmaceutical and the food industries *(1)*.

Immobilization of enzymes on solid supports for technological applications is a highly interesting field because the final products are easily removed from the enzyme by filtration and the enzyme can be used several times, thus making the process more economical. Furthermore, the reaction is more easily controlled, the process can be operated continuously, and both stability and physicochemical behavior are occasionally improved.

Studies on the immobilization of lysozyme through covalent attachment to a solid support *(2,3)* report a significant loss of activity associated

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with the limitation of bacterial diffusion and the steric hindrance of the whole bacterial cells as substrate *(4,5)*.

Our study shows that lysozyme can be successfully immobilized by the method of covalent binding on an acrylic matrix via flexible polyethylene glycol (PEG) spacer with an increase of its enzymatic activity in acidic media. These acrylic polymer beads are considered safe from a toxicological standpoint and are already being used in the pharmaceutical field.

To optimize the lytic activity of immobilized lysozyme toward macromolecular substrates, we investigated spacers of five different lengths to overcome the low diffusion rate and the steric hindrance caused by the enzyme-carrier binding, which is particularly evident with a macrosubstrate such as microorganisms.

Materials and Methods

General

PEG 400, 1000, 3400, 6000, 20,000 (average mol wt = 400, 1000, 3400, 5600–7000, 17,000–20,000, respectively), 4-dimethylaminopyridine (DMAP), and 4-nitrophenyl chloroformate were purchased from Fluka (Buchs, Switzerland). Eupergit C® (oxirane acrylic beads) was obtained from Rohm Pharma, GmbH (Darmstadt, Germany). Hen-egg-white lysozyme was supplied by Fordras SA (Lugano, Switzerland). Lyophilized *Micrococcus lysodeikticus* cell wall substrate was obtained from Sigma (St. Louis, MO). The strain BO of *Leuconostok oenus* was kindly provided by the Institute of Microbiology and Agricultural Industry, University of Torino, and was cultured and prepared for use as substrate as previously described *(6)*. The other reagents were of analytical grade.

Eupergit C-Coupled PEG

To link Eupergit C to PEG at different molecular weights, 1 g of epoxy-activated Eupergit C was mixed by rotation in a roller bottle apparatus, with 10 g of PEG (400, 1000, 3400, 6000, and 20,000, respectively) dissolved in 50 mL of a solution adjusted to pH 12.0 with sodium hydroxide. The reaction mixture was shaken for 16 h at 40°C in a thermostatically controlled environment. The coupled product was washed on a sintered, glass filter funnel until the PEG was no longer detected in the filtrate. To block the residual-free epoxy groups, the product was suspended in 25 mL of 1*M* 2-amino ethanol and then shaken for 12 h at room temperature, subsequently washed thoroughly with water, and dried under vacuum. The amount of PEG bonded to epoxy-activated Eupergit C was determined by using a spectrophotometer to measure the amount of unchanged PEG in the combined solution of the filtrates from the reaction mixture and the washings *(7,8)*. The method utilizing reaction of the oxirane ring with sodium thiosulfate was used to determine the epoxy groups *(9)*.

Eupergit C-PEG 4-Nitrophenyl Carbonate

4-Nitrophenyl chloroformate (5 mmol) dissolved in anhydrous methylene chloride was added dropwise to a suspension of Eupergit C-PEG-OH (1 mmol) and DMAP (5 mmol) in dry methylene chloride at 0°C and shaken. After standing overnight at room temperature, the reaction mixture was filtered and extensively washed with anhydrous methylene chloride, and the activated Eupergit C-PEG 4-nitrophenyl carbonate (Eupergit C-PEG-NPC) was collected. The degree of Eupergit C-PEG activation was 98% (ε of 4-nitrophenol at 400 nm 17,000M^{-1} cm^{-1}) *(10)*, as calculated by spectrophotometer at 400 nm in alkaline media on absorption of 4-nitrophenol released after 15 min.

Coupling of Lysozyme to Eupergit C-PEG Carriers

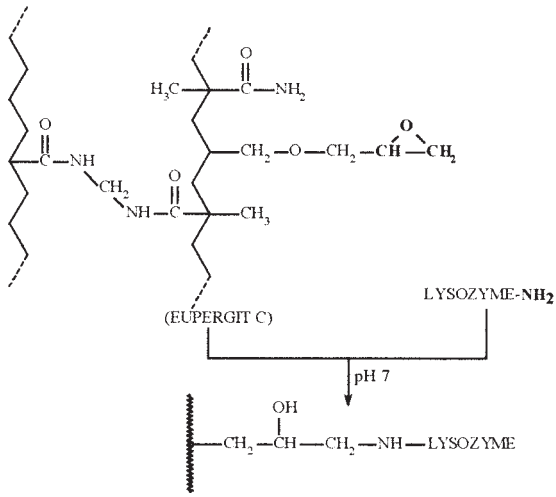
Enzyme solution containing 10 mg/mL of lysozyme ($E_{1\%}$ = 26.1 at 282 nm in water) was prepared in 0.2*M* borate buffer at pH 8.2. The solution was placed in a glass bottle, and activated Eupergit C-PEG beads were added with a 5:1 ratio by mole of polymer/protein-NH_2 (ε-amino group of lysine) residues. The flask was shaken overnight in a roller bottle apparatus and the pH was maintained with pH-stat. The final product, Eupergit C-PEG lysozyme, was filtered and exhaustively washed with distilled water.

The difference in enzyme concentration before and after enzyme coupling was assumed to be the amount of lysozyme bonded to Eupergit C-PEG *(11–13)*. Since the modifying reaction was carried out in 0.2*M* borate buffer at pH 8.2, the unreacted PEG 4-nitrophenyl carbonate was completely hydrolyzed to PEG-OH and 4-nitrophenol.

Enzymatic Assays

The activity of lysozyme was measured using a suspension of *M. lysodeikticus* as substrate *(14)*. Reaction was followed for 3 min with an *M. lysodeikticus* suspension (initial OD_{540} = 0.6 in 100 m*M* phosphate buffer, pH 6.2, and 0.15% NaCl). The lytic activity of lysosyme was also tested on an *L. oenus* suspension *(6)* in phosphate citrate buffer, pH 4.5, 27 m*M* sucrose, and 0.9% NaCl; the decrease in absorbance at 360 nm was recorded for 10 min for soluble enzyme and for 20 min for immobilized lysozyme (initial A_{360} = 1). To finalize optimal enzyme concentration, a curve-plotting lysozyme concentration against activity (Δ Abs/min) was prepared. The specific activity of both the native and the bound enzyme was calculated on the basis of protein content.

To check for the possible influence of PEG and/or Eupergit C-PEG molecules on the lytic activity of lysozyme, assays of enzymatic activity were carried out on both substrates, *M. lysodeikticus* and *L. oenus*, in the presence and absence of PEG or Eupergit C-PEG. The results proved to be completely identical. In other words, the presence of soluble PEG or insoluble support Eupergit C is ineffective on the activity and stability of lysozyme.



Scheme 1. Immobilization of lysozyme onto Eupergit C.

pH-Stability Profiles of Native and Eupergit C-PEG-Modified Lysozyme

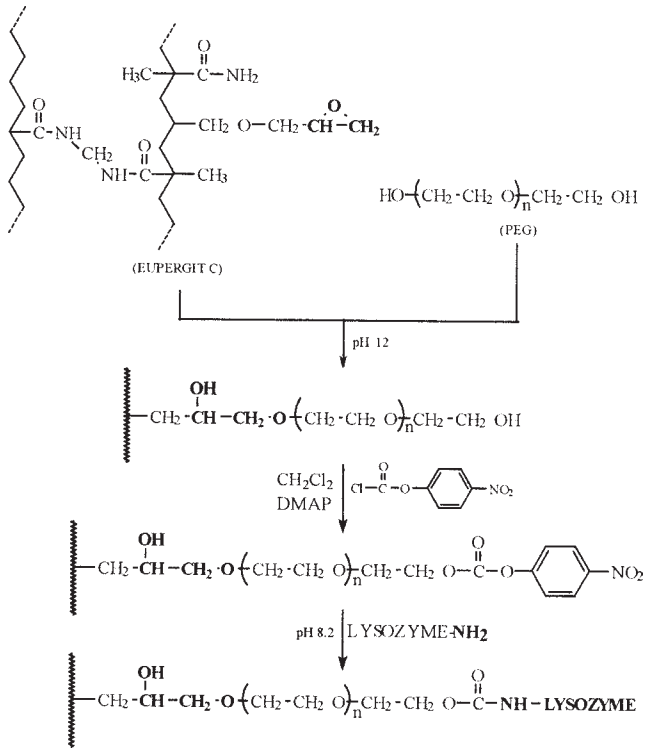
Either native or Eupergit C-PEG-modified lysozyme suspension with the same specific activity in 10 m*M* phosphate buffer at pH 6.2 was added to 50 m*M* buffer at various pH values between 2.0 and 10.0 to make a final volume of 0.5 mL. These enzyme solutions or suspensions at the various pH values were incubated for 2 h at room temperature. Afterward, the lytic activity was tested on *L. oenus* as previously described in enzymatic assays.

Estimation of Optimal pH for Native and Eupergit C-PEG-Modified Lysozyme Activity

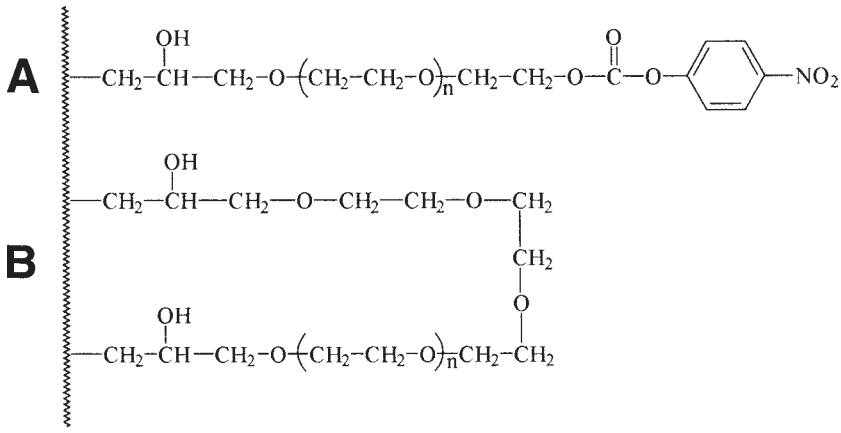
Either 0.1 mL of enzyme solution or Eupergit C-PEG-modified lysozyme suspension with the same specific activity in 10 m*M* phosphate buffer at pH 6.2 was added to 0.4 mL of *L. oenus* suspension in 50 m*M* McIlvaine buffer solution at various pH values between 2.0 and 10.0, 0.9% NaCl, 27 m*M* sucrose. The decrease in optical density at 360 nm was recorded. Immobilization of lysozyme was affected either by direct reaction with the epoxy group in the Eupergit C (Scheme 1) or by reaction with the spacer (Scheme 2) activated with 4-nitrophenyl chloroformate. Furthermore, a good correlation between spacer length and lytic activity of lysozyme was obtained.

Results and Discussion

The use of PEG fulfils the dual function of bridging the gel strands as well as allowing attachment of adsorptive groups or molecules to the matrix via long and flexible amphiphilic spacers. This method has been used successfully for the immobilization of proteins, peptides, and amino acids, and some of these applications have been described earlier *(15,16)*.



Scheme 2. Immobilization of lysozyme via PEG spacers onto Eupergit C.



Scheme 3. Chemical structures of Eupergit C-coupled PEG. **(A)** Active Eupergit C-PEG-NPC; **(B)** intramolecular crosslinks.

Almost all the oxyrane groups on Eupergit C react with the hydroxyl groups on PEG. However, only a small percentage of these groups could be activated as PEG 4-nitrophenyl carbonate (Eupergit C-PEG-NPC) (Scheme 3A) since both the hydroxyl groups on most PEG chains reacted with the oxirane

Table 1
Degree of Lysozyme Immobilization on Eupergit C-PEG

Molecular weight of the PEG	mg PEG/g Eupergit C	Active PEG (%) (Eupergit C-PEG-NPC)	Bound protein (mg/g Eupergit C-PEG)
—	—	—	60
400	225	1.1	51
1000	229	1.9	50
3400	283	4.6	42
6000	327	5.4	38
20,000	340	11.8	32

groups on the acrylic matrix to form intramolecular crosslinks (Scheme 3B). Moreover, the number of intramolecular crosslinks decreases as the molecular weight of the PEG increases (Table 1, column 3).

Both enzymatic activity and amount of bound protein depend on the chain length of the spacer. The amount of bound lysozyme decreases as spacer length increases (Table 1, column 4), whereas specific activity increases and always appears higher as compared to the lysozyme directly bound to Eupergit C (Tables 2 and 3).

The differing sensitivities of *L. oenus* and *M. lysodeikticus* to the enzyme can be attributed to their differing percentage compositions of mucopolysaccharides in the cell wall. The diversity is also emphasized by the shift of the peak of maximum activity with *L. oenus* cells toward acidic pH values, at which values *M. lysodeikticus* cells clump together and precipitate *(6)*.

The high specific activity of the lysozyme bound to Eupergit C-PEG 20000 as compared with the free enzyme is very interesting because it is the first example reported in the literature indicating an increase in the enzymatic activity of lysozyme after immobilization on insoluble supports.

This observation can be explained by the assumption that the macromolecular substrate does not have free access to the active site of the enzyme owing to steric hindrance when this one is bound directly to the carrier. The spacer improves activity by reducing steric hindrance, which is overcome when the spacer reaches sufficient length and mobility. This hypothesis is in agreement with the data obtained with *M. lysodeikticus* substrate, which consists of cell-wall fragments. In this case, the smaller substrate is influenced less by the steric hindrance.

The unexpected enhanced activity of lysozyme on *L. oenus*, when bound with a PEG 20000 spacer, could be related to what is reported by Ibrahim *(17)*, who found higher activity for long-chain fatty acid acylated lysozyme and who attributed this effect to improved interaction of lysozyme with the membrane of the target cells owing to a more lipophilic environment.

As reported in Table 2, the specific activity of Eupergit C–modified lysozyme on *L. oenus* increases with the length of the spacer, but the degree

Table 2
Specific Activity (U/mg protein) at Different pHs of Native and Immobilized Lysozyme on *Leuconostok oenus* as Substrate

pH	Lysozyme (U/mg protein)	Eupergit C-lysozyme (U/mg protein)	Eupergit C-PEG 400-lysozyme (U/mg protein)	Eupergit C-PEG 1000-lysozyme (U/mg protein)	Eupergit C-PEG 3400-lysozyme (U/mg protein)	Eupergit C-PEG 6000-lysozyme (U/mg protein)	Eupergit C-PEG 20000-lysozyme (U/mg protein)
3.0	23.4	5.1	6.3	7.3	14.3	19.8	102.0
5.0	29.7	1.8	2.7	5.9	6.8	12.8	62.4
6.2	14.3	1.2	2.6	3.3	3.7	6.3	35.4
8.0	6.9	1.7	2.4	2.9	3.7	8.3	34.9

Table 3
Specific Activity (U/mg protein) at Different pHs of Native and Immobilized Lysozyme on *Micrococcus lysodeikticus* as Substrate

pH	Lysozyme (U/mg protein)	Eupergit C-lysozyme (U/mg protein)	Eupergit C-PEG 400-lysozyme (U/mg protein)	Eupergit C-PEG 1000-lysozyme (U/mg protein)	Eupergit C-PEG 3400-lysozyme (U/mg protein)	Eupergit C-PEG 6000-lysozyme (U/mg protein)	Eupergit C-PEG 20000-lysozyme (U/mg protein)
6.2	6430	1280	1280	1825	2440	2957	3343
8.0	1110	210	218	330	422	510	573

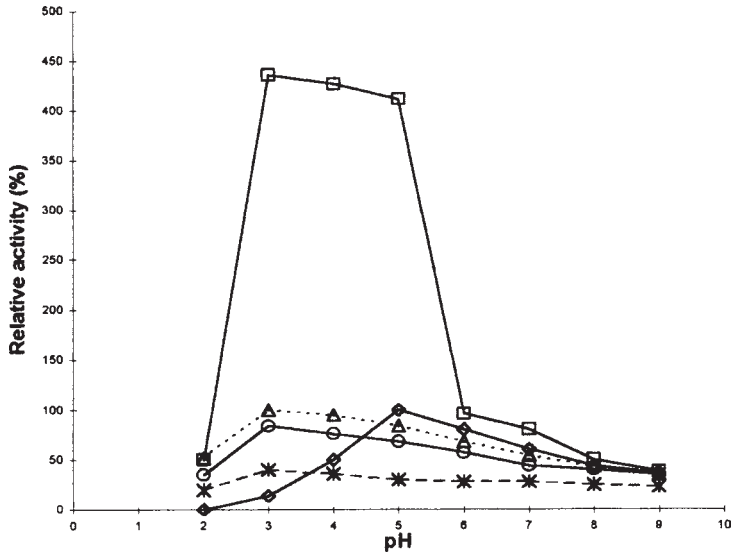


Fig. 1. Profiles of pH activity of the native, Eupergit C, and Eupergit C-PEG-modified lysozyme preparations. Native lysozyme (◆); Eupergit C-PEG 20000-lysozyme (■); Eupergit C-PEG 6000-lysozyme (▲); Eupergit C-PEG 3400-lysozyme (○); Eupergit C-PEG 1000-lysozyme (✳).

of increase is different at the pH tested, with a maximum increase at the extreme pH. In Fig. 1 we can also highlight a shift in optimum pH, for all the modified enzymes, from pH 5.0 to pH 3.0.

For lyophilized *M. lysodeikticus* substrate, the activity is lower than with the native substrate at all the pHs (Table 3).

pH-Stability Profile of Native and Eupergit C-PEG-Modified Lysozyme

The stabilization of enzymes against irreversible pH inactivation is strongly dependent on several factors such as temperature, ionic strength, and chemical nature of the buffer. Increasing the rigidity of the enzyme molecule by chemically linking the ε-amino group of lysine residues of protein molecule is one of the most useful methods of increasing its stability under extreme conditions.

It is well known that ionizable prototrophic groups on the enzyme molecules contribute considerably to the maintenance of the conformational structure of enzyme and that changes in the ionizations of these groups at extreme pH values may partially or completely disrupt both the catalysis mechanism and conformational structure of the enzyme.

The PEG modification of lysine residues of lysozyme occurs at the level of ε group, thus leading to a significant decrease in the number of charged groups present on the protein surface.

These results (Fig. 2) suggest that immobilization of lysozyme on solid support via long and flexible amphiphilic spacers considerably stabilizes

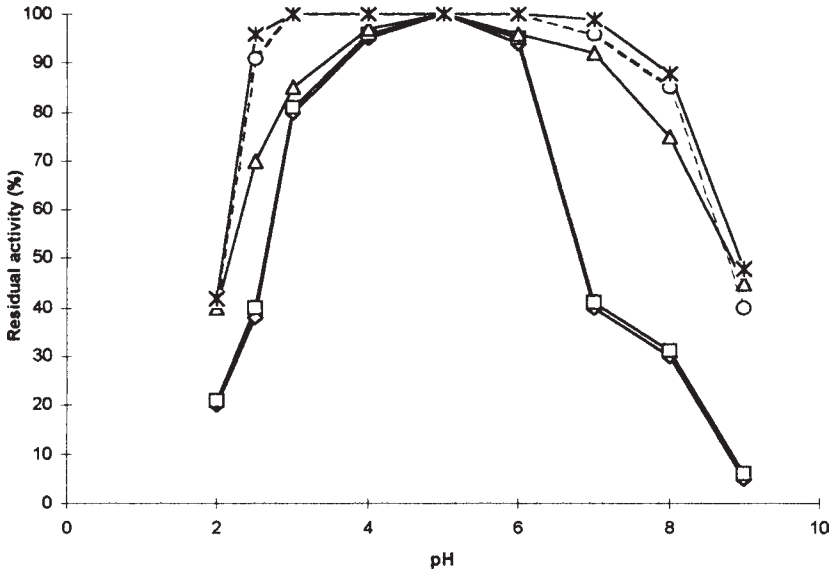


Fig. 2. The effect of pH on the stability of native and Eupergit C-PEG-modified lysozyme. Native lysozyme (◆); Eupergit C-PEG 400-lysozyme (■); Eupergit C-PEG 3400-lysozyme (▲); Eupergit C-PEG 6000-lysozyme (○); Eupergit C-PEG 20000-lysozyme (✳).

the enzyme against pH inactivation, particularly at pH values between 2.5 and 8.0. In this pH range, with PEG 20000, maximum activity is maintained under test conditions.

Conclusion

Lysozyme immobilized on Eupergit C via a flexible PEG spacer arm can enhance the lysis of *L. oenus* and *M. lysodeikticus* cells as compared with lysozyme immobilized directly on various insoluble supports *(2–5)*.

The results of this study indicate that lysozyme coupled to oxirane through a flexible long-chain PEG spacer can increase lytic activity, particularly in extreme acidic media, on complex substrate such as *L. oenus* cells. It is conceivable that this increase of enzymatic activity is determined mainly by the statistical properties of a flexible polymer molecule in solution *(18,19)*.

The data suggest that this technology might be exploited in the beverage industry and in fruit and vegetable food processing, in which such acidic pHs are very common. In particular, lysozyme has been proposed for the inhibition of malo-lactic fermentation and for microbiological stabilization of wine, as a substitute for chemical additives with known or possible toxic effects, such as sulfites. Therefore, the use of immobilized enzymes is very interesting in food processing as well as in many biological and pharmaceutical applications.

This method of immobilization presents the advantages of increased activity and stability. As a result, other support will be studied in order to

optimize all aspects regarding technological applications of an immobilized enzyme.

Acknowledgments

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