Calcium Responsive Bioerodible Drug Delivery System

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INTRODUCTION

Polymeric systems that respond to specific molecules are the basis for technological applications such as biosensors, drug delivery devices and actuators (1). In recent years, several research groups have been developing responsive/modulated drug delivery systems that more closely resemble the normal physiological process where the amount of drug released can be affected according to physiological needs.

Modulated drug delivery devices can be broadly classified as externally modulated and self modulated. Externally modulated devices are devices that can alter their drug release in response to an externally generated signal such as an electronic input that alters the rate of delivery from a mechanical pump (2), an oscillating magnetic field (3,4), ultrasound (5,6), temperature (7–10), or an electrical signal (11,12). The self modulated systems utilize several approaches as rate-control mechanisms: pH sensitive polymers (13,14), enzyme-substrate reaction (15–19), pH-sensitive drug solubility (20), competitive binding (21,22), and metal concentration-dependent hydrolysis (23).

Polymeric modulated drug delivery systems can be further segregated into biodegradable and non-biodegradable. The great advantage of using biodegradable polymer is the elimination of the need for removal of the "ghost" drug delivery system after all of the drug is released.

This work presents an enzymatically controlled (self-modulated) biodegradable drug delivery system, that is responsive to calcium, based on a cellulose-starch matrix (biodegradable) and the starch hydrolytic enzyme, α-amylase, in its non active form.

Starch is a polysaccharide consisting of anhydroglucose units linked together primarily through α-D (1 → 4) glucosidic bonds. Most starches contain two types of glucose polymers: (1) amylose—a linear polymer and (2) amylopectin—a branched polymer that contains the α-D (1 → 6) glucosidic bonds that form the branch points.

α-Amylase catalyzes the hydrolysis of α-D (1 → 4) glucosidic linkages of amylose and amylopectin to produce maltose and other lower molecular weight products. All α-amylases so far investigated have been found to contain at least one atom of calcium firmly and specifically bound to the enzyme molecule that is essential for its activity and stability (24–26). Removing the calcium ions causes enzyme instability and loss of its activity. The instability is reversible; with the addition of calcium ions, the enzyme regains its activity. Based on this observation, we propose a novel approach for a calcium responsive delivery system.

One application for our calcium responsive system can be an implantable device for prevention of cardiovascular calcification. Calcification is the formation of calcium phosphate deposits on devices made from synthetic or natural materials. Cardiovascular calcification is a common end-stage phenomenon affecting a wide variety of bioprostheses (27–29). Moreover, the system can be the basis for a novel approach for enzyme-substrate responsive bioerodible drug delivery systems where the non-active enzyme is incorporated in a matrix composed of its substrate and the matrix responds to a trigger molecule that causes the non-active enzyme to become active.

MATERIALS AND METHODS

Enzyme Deactivation by Removal of Calcium Ions

0.15 ml of aqueous solution of α-amylase (EC 3.2.1.1 from *Bacillus species*, Sigma) at different concentrations, and 0.85 ml of 0.5 mg/ml EDTA (ethylenediaminetetraacetic acid, Sigma) in 0.01 M MOPSO buffer (3-[N morpholino]-2 hydroxy propanesulfonic acid, Sigma) pH 6.6, were added to a test-tube with 1.0 ml of 0.01 M MOPSO buffer pH 6.6, and cooled to 4°C. Samples were taken, as a function of time, for enzyme activity determination.

Enzyme Reactivation by Addition of Calcium Ions

1.0 ml of $CaCl_2$ solutions at different concentrations were added to test tubes containing the same amount of non-active enzyme (previously exposed to EDTA). Samples were taken at appropriate times for enzyme activity determination.

Starch Solution

0.5 g corn starch (Sigma) was placed into a volumetric flask (50 ml). Boiling distilled water was added until the volume reached 50 ml, and the solution was rapidly stirred. After cooling to 35–40°C, the solution was immediately used.

Enzyme Activity

α-amylase activity was determined by measuring the concentration of reducing equivalents (maltose as standard), using the "Reducing Sugar" method (30). A 1.0 ml of 10 mg/ml corn starch aqueous solution was added to the sample for 3 min. Enzyme activity was defined as the ratio of amount of maltose (mg) generated during 3 minutes divided by the amount of enzyme (mg) used.

Tablets Without Enzyme

Tablets, 9.5 mm in diameter and with an average weight of 460 mg, were prepared by wet granulation: 15 g corn starch, 15 g cellulose derivatives (methocel E50- 64.65%; metocel E4M- 32.33%; klucel- 3.02%) and 0.48 g myoglobin, as a drug model (from horse heart, Sigma) were ground (IKA-Labontechnik type A10, Janke & Kunkel Gmbh & C0.KG) for 1.0 min

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with 1.0 g of PVP K-30 (povidon) as a binder. 10 g of ethyl alcohol (Frutarom) was added and the mixture was ground for an additional 30 seconds. Then it was distributed on drying trays and placed in an oven at 60°C for 1.0 h. Dried granules were screened through a mesh screen no. 20 and placed in a twin-shell blender with 0.5 g syloid 244 (silica) as a glidant. After 10 min. 0.25 g talc (lubricant) was added, and the granules were blended for an additional 3 minutes. Finally, the granules were compressed on a single-punch press to tablets.

Tablets with Incorporated Enzyme

Tablets, 9.0 mm in diameter and with an average weight of 250 mg, were prepared by wet granulation as described above with the following modifications: 9.4 ml of a non-active enzyme solution (enzyme that undergoes the deactivation process described above; 0.1 mg/ml) was added instead of ethyl alcohol, and the granules were dried in an oven at 45°C for 3.0 h.

Release Experiments

Tablets Without Enzyme

Two tablets were placed in a flask of 100 ml containing 49 ml MOPSO buffer (0.01 M), pH 6.6, with different calcium concentrations and 1.0 ml of non-active α-amylase solution (0.05 mg/ml). The releasing mechanism is shown in Fig. 1a.

Tablets with Enzyme

Four tablets were placed in a flask of 100 ml containing 50 ml MOPSO buffer (0.01 M), pH 6.6, with different calcium concentrations. The releasing scheme is shown in Fig. 1b.

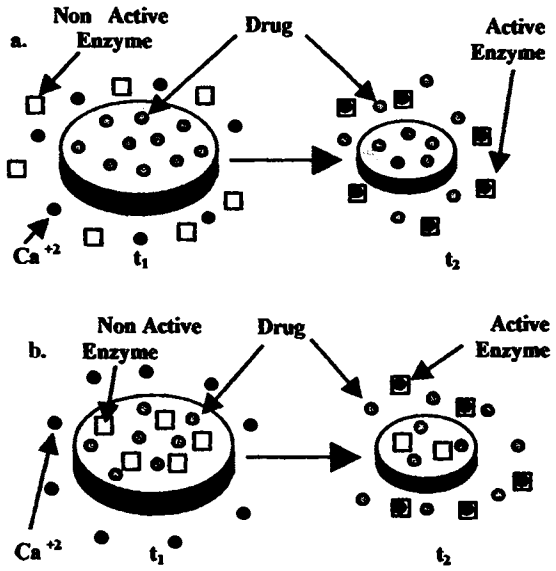

Fig. 1. (a) Schematic presentation of the reactivation of non-active enzyme dissolved in the medium by calcium ions. The active enzyme causes the tablets' degradation followed by myoglobin release. (b) Schematic presentation of the reactivation of non-active enzyme incorporated in cellulose-starch matrices, by calcium ions from the medium. The active enzyme causes the tablets' degradation followed by myoglobin release.

3.0 ml samples were taken from the flasks at the appropriate times, centrifuged at 3500 rpm for 10 minutes for particle removal. Myoglobin release from the tablets was measured with a spectrophotometer at 410 nm. All samples were returned to the flask (because the samples contain the enzyme).

RESULTS AND DISCUSSION

The first step for developing an enzymatically controlled drug delivery system responsive to calcium is to make the enzyme (α-amylase) sensitive to calcium. In order to prevent enzyme denaturation since calcium-free enzyme is not stable, we had to work in a narrow and precise range of pH and temperature. Calcium ions were removed by EDTA, a metal chelating agent, in MOPSO buffer, pH 6.6, at 4°C. MOPSO buffer was chosen because it does not contain phosphate ions that cause calcium ion precipitation.

Figure 2 shows the decrease in α-amylase activity with increased exposure to EDTA; α-amylase exposed to buffer without EDTA shows no loss of activity. The higher the ratio of EDTA to α-amylase, the faster the decrease in enzyme activity. EDTA competes with α-amylase for calcium ions. During EDTA exposure, a calcium-EDTA complex is formed and the enzyme loses its stabilizing calcium ions and therefore its activity.

In order to evaluate the reversibility of the deactivation process, solutions with different concentrations of calcium ions were added to the deactivated α-amylase solution. Enzyme activity values were measured after 10 minutes exposure to different calcium concentrations. The regained activity of the treated α-amylase is dependent upon the calcium concentration in the environment, as can be seen in Fig. 3a. The activity of non-treated enzyme is not affected by calcium. Thus, removal of calcium ions was reversible and enzyme activity could be fully recovered.

In order to verify if 10 minutes exposure to the calcium solution is sufficient for the α-amylase activity recovery, longer exposure times were studied. Figure 3b shows enzyme activity of the non-active α-amylase after 10 minutes and 24 hours

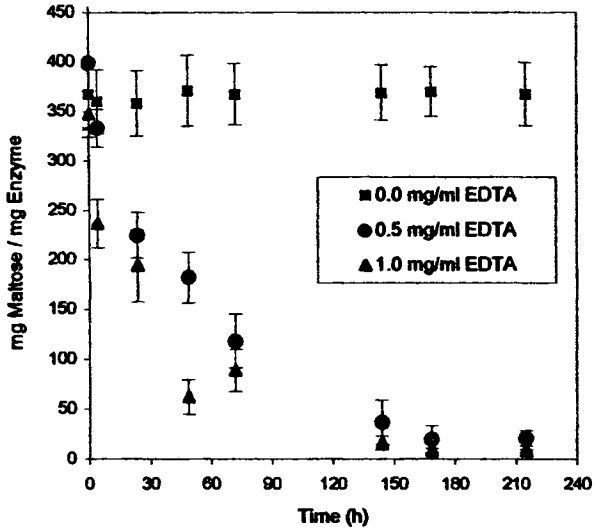

Fig. 2. Effect of exposure time to EDTA on α-amylase activity. EDTA (mg) to enzyme (mg) ratio: ▲ 113.3, ● 56.7, ■ 0.0.

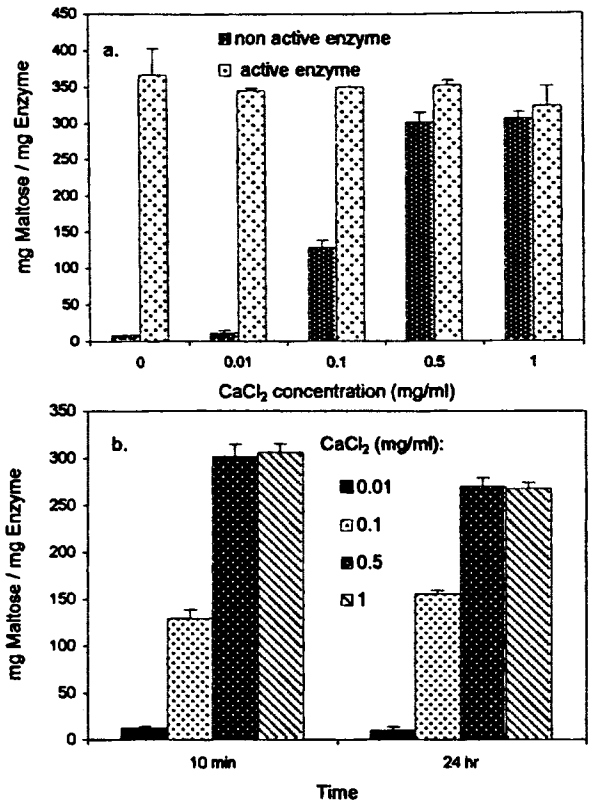

Fig. 3. (a) Enzyme activity regained as a function of 10 minutes exposure to different calcium concentration. (b) Enzyme activity regained as a function of different exposure times to different calcium concentrations.

exposure to different calcium solutions. No significant differences between short and long exposure time can be noted in activation pattern as a function of calcium concentrations. It follows that longer exposure times are not required. This result indicates that the response time of the system to changes in calcium concentration is in order of minutes.

The calcium concentrations that the system responds to are dependent on the amount of EDTA used in the deactivation process. In attempt to shorten the exposure time to EDTA, extent of EDTA was employed in the deactivation process. Enzyme treated with lower EDTA concentrations regained higher activities at the same calcium concentration (results not shown). This can be explained by the presence of free EDTA in the test-tube that competed with the enzyme for the added calcium ions. This suggests that the sensitivity to calcium is higher in a system that uses lower EDTA concentrations. Therefore, in order to increase the sensitivity to calcium, EDTA should be removed from the solution.

One method for increasing sensitivity would be to remove the EDTA at the end of the deactivation process by dialysis in dialysis tubing with molecular weight cut off (MWCO) between 300–50000, against buffer at precise pH and temperature for stabilizing the non-active enzyme. Alternatively, one can perform the deactivation process in dialysis tubing with MWCO < 250 against buffer with EDTA so that the calcium ions can get out but the EDTA can not get in. The product will be a non-active enzyme free from EDTA and therefore sensitive to low concentration of calcium.

In all the experiments that were presented so far, the substrate (starch) was used in its soluble form. Since our objective was to develop a calcium responsive delivery system, we next examined the myoglobin release from starch-cellulose solid tablets, with α-amylase in the releasing medium. Myoglobin release from tablets without incorporated enzyme is shown in Fig. 4a.

Increased calcium concentration in the medium caused an increase in the percentage of myoglobin released. This is consistent with the finding (Fig. 3a) that different calcium concentrations enable the treated enzyme to regain different enzymatic activity levels. Since the activity of the unstabilized α-amylase is calcium dependent, the degradation of the cellulose-starch matrices by α-amylase and therefore the release of the incorporated myoglobin, is dependent upon the calcium concentration in the medium.

After testing the treated α-amylase activity in solution, we then incorporated the treated enzyme into a biodegradable drug delivery system (starch-cellulose matrix). The myoglobin release kinetics from tablets with incorporated enzyme are shown in Fig. 4b. As with the soluble enzyme (Fig. 4a), the higher the calcium concentration in the medium, the higher the percentage of myoglobin released and the faster the release rate (seen as the slope of the data points). It is important to state that EDTA treated, unstabilized α-amylase was reactivated by the calcium ions in the medium after its incorporation in the matrix and that, in fact, these tablets operate as a calcium responsive drug delivery device.

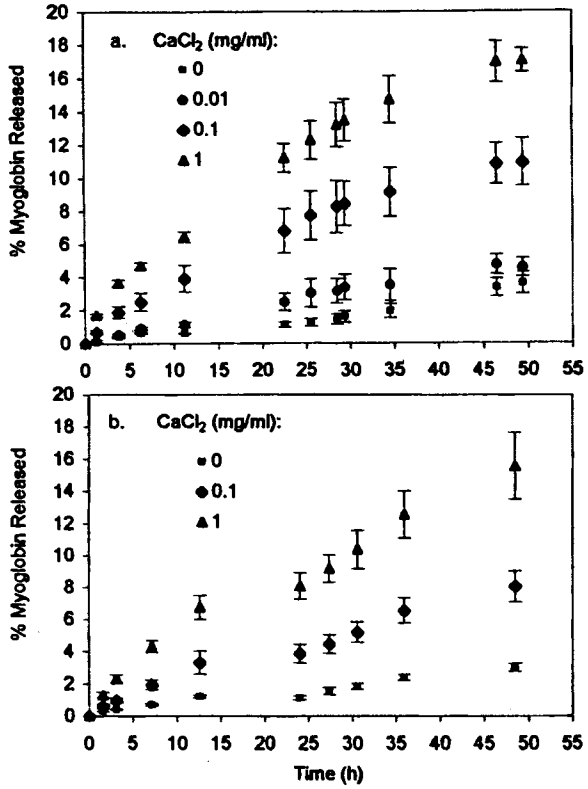

Fig. 4. (a) Kinetics of myoglobin release from tablets exposed to enzyme (0.00 mg/ml) and different calcium concentrations in the releasing medium. (b) Kinetics of myoglobin release from tablets with incorporated enzyme, exposed to different calcium concentrations. Myoglobin percentage of release was defined as the percentage released out of the amount incorporated into the tablets.

Observation of Figs. 4a and 4b seems to indicate that myoglobin is being released by surface erosion mechanism because there is no significant difference between the two experiments (enzyme incorporated in the tablets, or enzyme outside the tablet). However, these two experiments can not be directly compared, since the active enzyme concentration is different in each experiment. The tablets with incorporated enzyme have an active enzyme concentration that increases over the course of the experiment, while the active enzyme concentration in the medium for the tablets without incorporated enzyme remains constant over time. In addition, the ratio of surface area to volume, which known to affect release rates, is different in each experiment.

We have demonstrated that varying the calcium concentration enables us to control the change in the release rate of the model drug, by changing the rate and degree of enzyme reactivation. Other parameters that will influence the drug release rate are matrix composition, change in loading degree of the drug and its size, amount of loaded enzyme, etc. Since all of these parameters are directly measurable and manipulatable, this system lends itself nicely to customizing the drug release rate per application, and is not at all limited to the release rates and quantities presented here.

CONCLUSIONS

This work presents a novel approach for a calcium responsive drug delivery system. These results show that enzymatic activity of α-amylase can be lowered by exposing it to EDTA. The treated α-amylase can be reactivated by addition of calcium ions to the medium and the reactivation degree is calcium concentration dependent. The calcium concentrations that the system responds to are in the range of physiological calcium concentrations. In order to achieve a higher sensitivity to calcium, the EDTA in the system must be removed, at least partially, after treatment. The starch-cellulose tablets with incorporated enzyme can operate as a drug delivery system responsive to calcium, since the release rate of the incorporated drug from these tablets is calcium concentration dependent.

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