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Enzymatic degradation of starch thermoplastic blends using samples of different thickness

M. Alberta Araújo · António M. Cunha · Manuel Mota

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Abstract The material studied was a thermoplastic blend of corn starch with a poly(ethylene-vinyl alcohol) copolymer, SEVA-C. The influence of both the material's exposed surface and enzyme concentration on degradation kinetics was studied. As α-amylase is present in the blood plasma, experiments were performed, varying the material thickness and the α-amylase between 50 and 100 units/l, at 37°C, lasting up to 90 days. Four different batches using SEVA-C and starch samples of different thickness were performed. The positive correlation between degradation rate and the exposed material surface was confirmed, since thin films with larger exposed surfaces were degraded faster than thick square plates having the same total mass. The degradation extent depends on the total amount of amorphous starch present in the formulation rather than on the amount of enzyme used and the minimum thickness to ensure maximum degradation was estimated to be close to 0.25 mm.

M. A. Araújo
Escola Superior de Tecnologia e Gestão, IPVC,
Avenida Atlântico, 4900-348 Viana do Castelo, Portugal
e-mail: alberta@estg.ipvc.pt

M. A. Araújo · M. Mota (✉)
IBB-Institute for Biotechnology and Bioengineering,
Centre for Biological Engineering, University of Minho,
Campus Gualtar, 4710-057 Braga, Portugal
e-mail: mmota@deb.uminho.pt

A. M. Cunha
Institute for Polymers and Composites - Department of Polymer Engineering, Universidade do Minho, Campus de Azurém,
4800-058 Guimarães, Portugal
e-mail: amcunha@dep.uminho.pt

1 Introduction

Natural, synthetic polymers and their composites are widely used as biomaterials for biodegradable applications. Biodegradable surgical implants are ideal for the repair and regeneration of healing tissues, since the surgical procedure for implant removal is avoided [1–4].

At an early stage of healing, the biodegradable implant preserves the structure of the tissue and its function. In time, the implant gradually decomposes and their function is progressively transferred to the healed tissue. The time necessary for this process depends on the type of polymer, on its molecular weight and on the specific biochemical and mechanical environment inside the body [3, 4].

Apart from favourable physico-chemical and mechanical properties, a biodegradable polymer to be used in medical applications needs to be biocompatible in a specific environment and its degradation products should not be cytotoxic [5]. The use of synthetic degradable polymers as biomaterials implies they are biocompatible by themselves and the use of particular additives and/or processing technologies should not interfere with the biocompatible behaviour [5].

The development of degradable materials with adequate mechanical properties may lead to a new range of small load bearing applications for these systems. Combination of polymers with bioactive ceramics is expected to minimize the mismatch of mechanical properties of bioceramics and natural tissue. Several polymer matrix and bioactive ceramic reinforcements have been used to develop composites intended to simulate bone properties. Both bioinert and biodegradable polymers have been used as matrices, aiming at both permanent and temporary applications [6].

1.1 Starch biomaterials

Starch blends have shown great processing versatility being proposed for applications such as scaffolds for tissue engineering [7, 8], drug delivery carrier systems [9–12], hydrogels [9, 13], bone replacement/regeneration applications [14–17] and, more recently, tissue engineering scaffolding [18–20]. Starch materials have adequate biocompatibility, non-cytotoxicity and suitable mechanical properties [21–23].

The most studied systems are blends of starch with ethylene-vinyl alcohol copolymer (SEVA-C), cellulose acetate (SCA), poly(ε-caprolactone) (SPCL) and poly(lactic acid) (SPA) [6, 15, 16, 24].

Starch has poor mechanical properties and poor stability caused by water absorption and processing. Some of these problems can be overcome by physical or chemical modifications, including blending with the above mentioned polymers [25].

1.2 Degradation of starch biomaterials

Studies using starch compounds showed that the starch phase in the compound, being more hydrophilic, enhances water infiltration [16, 26–28]. Starch blends degrade when formulated with α-amylase, which catalyses the degradation of starch [24, 29, 30]. The enzyme catalyses the hydrolysis of α-1,4-glycosidic linkages of starch to maltose and dextrins. Typical starch degradation products are lower molecular weight starch chains, fructose and maltose. It is also worth noting that α-amylase is present not only in saliva but also in human blood [31].

Previous results [29, 30] showed that the total mass loss fraction of starch blends was around 35%, implying that only part of the amorphous phase was released and degraded to the solution, the remainder staying embedded in the network structure. This structure hinders the enzyme access to the starch molecules interspersed in the synthetic insoluble component and EVA chains are likely to be responsible for the non-degraded residual starch. The high amount of non-degraded residual starch suggests the possibility of optimising the material degradability by deliberately increasing material porosity.

In this work, starch biomaterials were analysed in terms of physical changes, degradation products and loss of properties with time, when they were immersed in simulated physiological solutions containing α-amylase for periods of up to 90 days. These studies were carried out to investigate the influence of thickness, exposed surface area and enzyme concentration on the enzymatic degradation mechanisms of starch blends. The investigation was also performed to gain insight into how these materials will behave as biodegradable thermoplastic materials.

2 Materials and methods

2.1 Materials

The material used was a thermoplastic blend of corn starch with a poly(ethylene-vinyl alcohol) copolymer (60/40 mol/mol), SEVA-C, supplied by Novamont, Italy (Mater-Bi 1128RR). Typically, the starch in this commercial blend is 50–60% (wt%) with 70% of amylopectin and 30% of amylose. Four different batches were tested, using SEVA-C and starch samples of different thickness (0.15 and 0.5 mm). Two batches consist of films of SEVA-C of 0.15 and 0.5 mm thickness, using always the same weight of 1.6 g. To perform the same fixed weight of 1.6 g, 2 films of 0.5 mm were used in one batch and 4 films of 0.15 mm were used in another batch, according with Fig. 1.

Comparing with the other batches where square plates of 30 × 30 × 2 mm were used, the surface area corresponded to 10 and 20 times (0.5 and 0.15 films with 95 cm^2 and 190 cm^2, respectively) the surface area of the two other batches (square plates of 30 × 30 × 2 mm of starch and SEVA-C with 10 cm^2) (Fig. 2).

SEVA-C samples in individual containers (volume approximately 50 cm^3) were immersed for 90 days at pH 7.4 and placed in a temperature-controlled immersion circulator (37°C ± 1°C), under continuous stirring (150 r.p.m.). A Hank′s balanced salt solution (HBSS) without phenol red

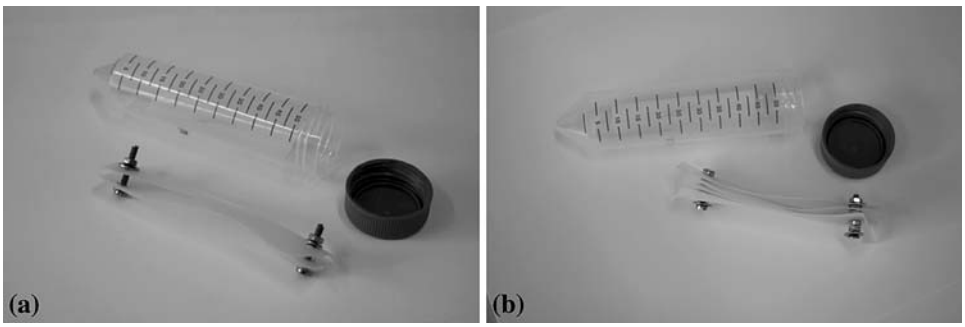

Fig. 1 Batches using films of (**a**) 0.5 mm (2 films) and (**b**) 0.15 mm (4 films) with 1.6 g weight

Fig. 2 Setup of the samples in each assay. (**a**) Squared plate; (**b**) films of 0.5 mm and (**c**) films of 0.15 mm

(HBSS Sigma reference H8264), with α-amylase (from human saliva, Sigma reference A0521) was used to immerse the samples. HBSS ionic composition is similar to the one of human blood plasma. Control samples contained HBSS without enzyme. The weight of samples was recorded and used to determine the mass loss after the enzymatic degradation. The concentration of α-amylase usually found in human blood plasma is 50 U/l [14, 31]. To perform the assays two concentrations of α-amylase were selected, 50 units/l and 100 units/l, in order to simulate the natural conditions and duplicate the enzyme concentration.

The enzyme solution had an activity of 0.35 mg/unit/min at pH 6.9 and 20°C per gram of soluble starch (1% w/v). To stabilize α-amylase, 1 mM calcium chloride was employed.

Square plates and films were used in *in-vitro* degradation experiments under strictly sterile conditions in a laminar flux chamber. Samples were sterilised in a 10/90 gaseous mixture of ethylene oxide (EtO) and carbon dioxide (CO_2), with a cycle time of 20–22 h at 45°C and a chamber pressure of 180 kPa.

2.2 Analytical methods

2.2.1 Detection of starch and polysaccharides

The starch amount in the degradation solutions was determined by a colorimetric method: 100 µl of sulphuric acid 2 M and 0.5 ml of KI-I_2 were added to a 5 ml of sample. The absorbance of the solution was determined in a ELISA reader (microprocessor controlled readers in 96 well microplates) at 580 nm, being the respective concentration obtained from a standard curve, with the same corn starch used in the samples. The total amount of polysaccharides in the degradation solutions was quantified using the Dubois method [32] which is based on the addition of 1 ml of phenol (5% w/v) and 5 ml sulphuric acid (95–97%) to 1 ml of sample of the degradation solution. The absorbance of the resulting mixture was determined with an ELISA reader at 490 nm, using the same control as above.

2.2.2 Detection of reducing sugars

Enzyme activity was determined measuring reducing sugars concentration in the degradation solutions by the dinitrosalicylic acid method (DNS). DNS procedure is based on the reduction of the 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid under alkaline conditions, after what the colour intensity is measured at 540 nm [33] in the ELISA reader. The control sample was the same as above. The respective carbohydrate concentration was obtained by comparison with a standard curve.

2.2.3 Oligosaccharides detection by high performance liquid chromatography

High performance liquid chromatography (HPLC) with 830-RI (Jasco, Japan) refraction index detection and a 880-PU pump (Jasco) was used to separate sugar derivatives from the starch hydrolysates of the degradation solutions.

Commercial standards were used for the calibration of the Chrompack carbohydrates Ca column. A Chrompack guard column at 90°C with ultra-pure water as eluent (0.5 ml/min), was kept at a 6500–7000 kPa pressure. Eluent was filtered through a 0.22 µm sterilized membrane previously degassed with helium, and kept in a container to preclude contamination by airborne bacteria or fungi. Sorbitol (1 g/l) was used as internal standard. Samples were also filtered through 0.22 µm filters. Three replica of each sample were analyzed. A standard curve was obtained using different standard concentrations.

2.2.4 Scanning electron microscopy (SEM)

Modifications on the surface morphology and interconnectivity of SEVA-C specimens and films with enzymatic physiological solution, as a function of immersion time, were characterized by SEM in a Leica Cambridge S360 microscope. Before analysis, plates and films were cleaned and dried at 50°C for 1 week and kept in a desiccator. A non-immersed specimen, stored at controlled environment conditions was used as control.

2.2.5 Statistical analysis

Student's *t*-test for independent samples was performed to test differences among samples. Results were expressed as mean ± standard deviation. Differences between experimental results were analyzed according to Student *t*-test,

with $p < 0.05$ considered statistically significant. Student's *t*-test was plotted along with mean values based on 95% confidence intervals.

3 Results

3.1 Starch amount in the degradation solution

The amount of starch released to the solution was measured in all batches. Figure 3 shows the results obtained using a starch square plate in solution.

Starch was also available on degradation solution after immersion with α-amylase using 0.5 mm films and square plates of SEVA-C.

Figures 4 and 5 show the results for SEVA-C square plate and 0.5 mm films with 50 and 100 units/l α-amylase.

3.2 Reducing sugars in the degradation solution

The amount of reducing sugars in the degradation solutions was measured by DNS for batches with 50 units/l and 100 units/l α-amylase. Figures 6 and 7 shows the results obtained with 50 units/l and 100 unit/l α-amylase, respectively.

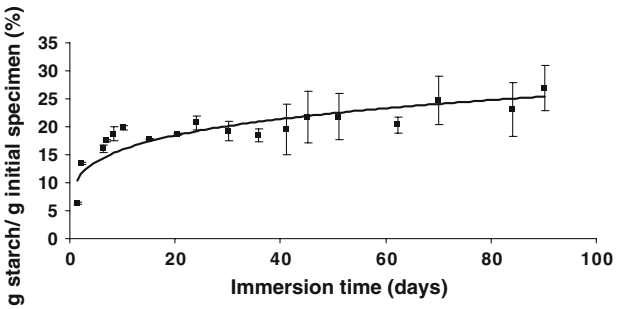

Fig. 3 Mass of starch (square plate) released to the solution with 50 units/l α-amylase, per initial specimen mass (1.6 g) in 50 ml of solution, as a function of immersion time. Each point is the mean of 2 duplicates (4 values in all); error bars are 95% confidence intervals of each mean

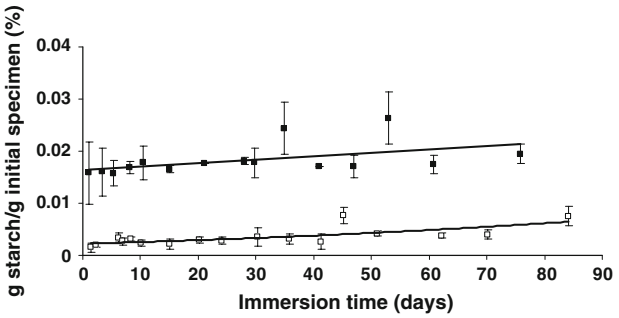

Fig. 4 Mass of starch released to the solution with 100 (■) and 50 (□) units/l α-amylase, per initial specimen (SEVA-C squared plate) mass (1.6 g) in 50 ml of solution, as a function of immersion time. Each point is the mean of 2 duplicates (4 values in all); error bars are 95% confidence intervals of each mean

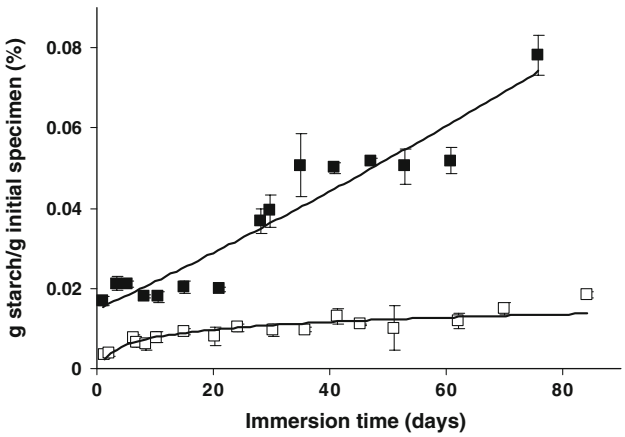

Fig. 5 Mass of starch released to solution with 100 (■) and 50 (□) units/l α-amylase, per initial specimen (SEVA-C 0.5 mm films) mass (1.6 g) in 50 ml of solution, as a function of immersion time. Each point is the mean of 2 duplicates (4 values in all); error bars are 95% confidence intervals of each mean

3.3 Polysaccharides in the degradation solution

The amount of polysaccharides released to the solution was quantified by the Dubois method, as previously described. Figures 8 and 9 show the polysaccharides released to solution for 50 and 100 units/l α-amylase.

Qualitative changes on SEVA-C surface morphology in contact with the enzymatically active solution were also followed by SEM. Figure 10 shows films micrographs before and after 90 immersion days. It was also notice that starch squared plates was completed fragmented after a few immersion days, so it was not possible to observe the structure.

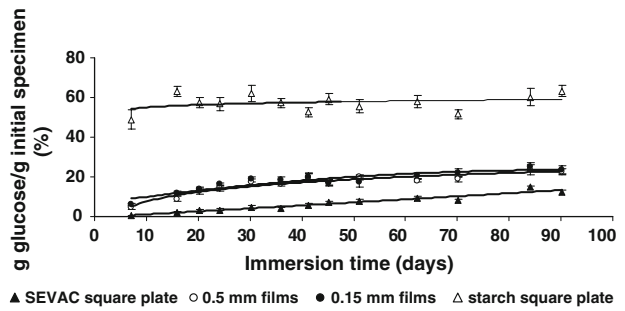

Fig. 6 Mass of glucose released to the solution with 50 units/l α-amylase, per initial specimen (squared plates of SEVA-C and starch and films of SEVA-C) mass (1.6 g) in 50 ml of solution, as a function of immersion time. Each point is the mean of 2 duplicates (4 values in all); error bars are 95% confidence intervals of each mean

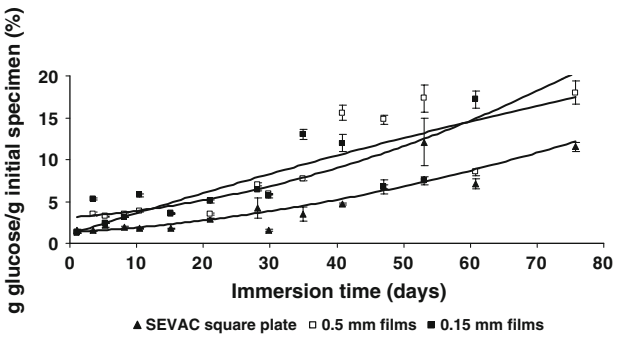

Fig. 7 Mass of glucose released to solution with 100 units/l α-amylase, per initial specimen (squared plates and films) mass (1.6 g) in 50 ml of solution, as a function of immersion time. Each point is the mean of 2 duplicates (4 values in all); error bars are 95% confidence intervals of each mean

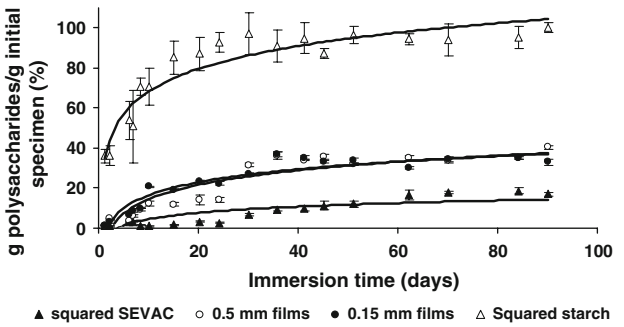

Fig. 8 Mass of total polysaccharides released to solution with 50 units/l α-amylase, per initial specimen (squared plates and films of SEVA-C and starch) mass (1.6 g) in 50 ml of solution, as a function of immersion time. Each point is the mean of 2 duplicates (4 values in all); error bars are 95% confidence intervals of each mean

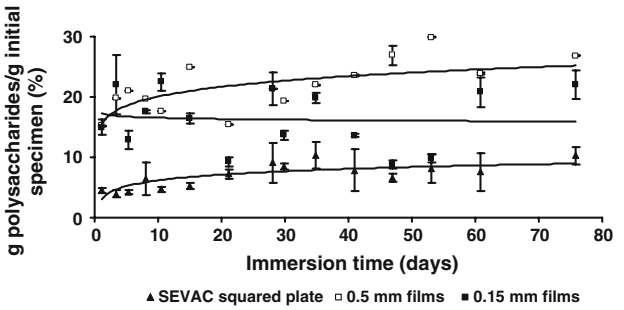

Fig. 9 Mass of total polysaccharides released to solution with 100 units/l α-amylase, per initial specimen (squared plates and films) mass (1.6 g) in 50 ml of solution, as a function of immersion time. Each point is the mean of 2 duplicates (4 values in all); error bars are 95% confidence intervals of each mean

4 Discussion

Analysing Fig. 3, starch released to the solution after 10-day immersion. Starch was completely fragmented, due to the polymer swelling and water lubrication, confirming at the same time the enzymatic action on the starch molecule. The release of starch from the surface of the blends to the bulk solution is also limited by the porosity of the material. Rupture of polymeric chains exposes subsequently amylose and amylopectin, allowing thereby enzyme to proceed its action directly on these molecules.

The amount of starch released to the solution with 100 units/l α-amylase is higher than the one obtained for all the experiments using 50 units/l, even though the quantity released, 0.08%, is still rather small (Fig. 4).

Diffusion of water and access of water-soluble enzymes into the blend is expected to be less for miscible SEVA region than for a pure starch domain (Figs. 3 and 4).

During processing, thermal-oxidative degradation enhances the release of non-polymerized monomers or oligomers soluble in water, as quantified by the method. Starch destruction depends also on the material microstructure, since the starch surrounded by EVA chains is less susceptible to degradation, rendering more difficult the external and internal starch mass diffusion.

The release of starch from the surface to the bulk is also limited by the porosity of the material (Figure 10) and by transport limitation from inside to the surface and from this to the bulk zone (inner and outer diffusion). Global degradation rate is controlled by the slowest step. In fact, the enzyme molecule is too large to allow its diffusion through the polymeric matrix; therefore, only starch at or close to the surface would be available for enzymatic attack. To increase the accessibility of inner starch particles, the percentage of starch in the material should be higher. Degradation of starch would then create a connected network of pores and voids that would enable the transport of enzyme to the inner region of the material.

Figure 6 compares the results of glucose release in all the batches using 50 unit/l of alpha-amylase. The difference between SEVA-C material and square plates is significant, 100% percentage meaning total enzymatic of the specimen to glucose. The difference between SEVA-C square plates and films is also evident, confirming the positive effect of the exposed surface, since the total mass is fixed (1.6 g). The initial linear part of the degradation curve corresponds to the fast hydrolysis phase, and tended to stabilise reaching a constant 20% (w/w) of glucose, after a 60-day immersion. This was expected, since α-amylase randomly hydrolyses α-1,4-glucosidic bonds of starch into dextrins.

Only small molecular weight fragments were released at early stages of degradation, and therefore no significant changes in the total mass of the specimen were observed. It is noticeable the difference between films and square plates, as happened also with 100 units/l α-amylase (Fig. 7). As all the binding and active sites α-amylase are saturated, since the enzyme concentration is much lower than the starch concentration, there was no difference

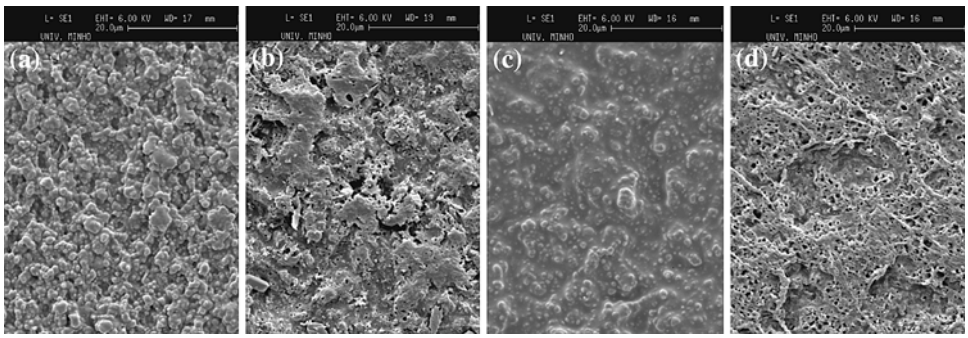

Fig. 10 SEM micrographs of SEVA-C surfaces of control (before degradation) and after 90 immersion days, respectively (x 1500). (**a**), (**b**) 0.5 mm films and (**c**), (**d**) 0.15 mm films. The images represent dried samples after immersion periods

between 0.5 and 0.15 mm films on the amount of released products, despite the variation on the exposed surface between them (90 and 190 cm^2). When the enzyme concentration rises, SEVA-C surface becomes completely coated with enzyme molecules and the reaction rate becomes proportional to the concentration of reactive sites (effective concentration). Enzyme kinetics depends directly on the exposed surface but not on the total mass of degradable material, since the amount of released products on materials with the same mass and different surface is distinct.

There is no significant difference between square plates and films on the final glucose amount as shown in Figs. 6 and 7. The initial hydrolysis phase is faster with 100 units/l α-amylase but tends to the same limit, which is probably related with the limiting number of active and binding sites of the enzyme.

Total amount of polysaccharides was also quantified for 50 and 100 unit/l alpha-amylase as demonstrated in Figs. 8 and 9. The amount of polysaccharides released to solution increased in both assays, evidencing the enzyme action on degradation rate. Stabilization of weight mass loss as a function of ageing is associated to internal diffusion limitation of monosaccharides coming from the inner part of the material.

The large difference between square plates and films of SEVA-C and starch (Fig. 8) is also noticeable. When in solution, enzymatic reaction converts starch completely to short chain monosaccharides. By comparing Figs. 8 and 9, it is clear that increasing enzyme concentration to 100 units/l did not lead to more released products, owing to the unfavourable starch structural arrangement inside the material. No significant differences were seen between 50 and 100 units/l α-amylase for plates or films. The low mass percentage released after 90 days may also be attributed to the nature of the blend, as the enzyme molecules can not reach the starch molecules strongly interpenetrated in the synthetic insoluble component.

The presence of non-reactive ethylene also reduced mass loss, as less accessible hydroxyl groups are present in the blend. Moreover, the low porosity and the lack of connectivity among starch sites avoid complete degradation of this material. Other malto-oligosaccharides might be expected to be present in the solution, as α-amylase cleaves starch glycosidic linkages randomly.

As to compare changes on surface morphology before and after degradation, Fig. 10 shows the differences between the film control (before degradation) and after degradation. Porosity, roughness and heterogeneity surfaces increased as a function of immersion time, as detected on surface microstructure between control (smooth) and more degraded samples (90 days) being more rough.

5 Conclusions

The effect of SEVA-C thickness (different exposed surface) and enzyme concentrations on starch blends biodegradation mechanisms was studied and led to the following conclusions:

- Higher enzyme concentration (100 units units/l α-amylase) increases only the initial degradation rate of amorphous phase, such as starch and other sugar chains like maltotriose, maltose and glucose to solution. Nevertheless, for longer immersion times the same equilibrium value is attained for all batches, which is related with the limited reaction sites and starch structural organization inside the material.
- Square starch plate was completed fragmented by enzymatic action. The amount of accessible starch in the composite proved to be a more important factor than the quantity of enzyme used to promote degradation.
- No significant differences between SEVA-C 0.5 and 0.15 mm films were observed in the amount of saccharides released to solution, although they present different exposed surfaces. There seems to be enzyme saturation at the reaction sites, the same final amount of degraded products was obtained.
- The minimum thickness to ensure maximum degradation may be estimated to be close to 0.25 mm (half of

the 0.5 mm film thickness), as no differences were obtained between SEVA-C 0.5 and 0.15 mm films. The degradation rate depends directly on the exposed surface and not on the total mass.

– The release of starch from the inner structure is also limited by the way it is organised inside the thermoplastic blend, limiting thus the inner and external diffusion and mass transfer of starch, precluding the enzyme access to the innermost starch molecules.
– Chemical composition, processing conditions and the structure of the starch polymeric blend influence the enzymatic hydrolysis rate by limiting the accessibility of enzymes to the starch substrate influencing, therefore, their degradation profiles.

Further optimization and control of the main physical material properties might improve enzymatic degradation processes for specific applications. The proper choice of material thickness (surface structure), surface porosity and roughness and enzyme concentration may elicit the control of the degradation rate.

Nevertheless, the released products should be metabolized and excreted by normal human mechanisms, and *in vivo* assays are necessary to confirm particle resorption and their behaviour inside the body in temporary tissue replacement.

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