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Biomaterials 25 (2004) 2687–2693

Biomaterials

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Enzymatic degradation of starch-based thermoplastic compounds used in protheses: identification of the degradation products in solution

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Received 9 May 2003; accepted 5 September 2003

Abstract

Apart from favourable physico-chemical and mechanical properties, the most important requirement for a biodegradable polymer to be used in medical applications is its biocompatibility and the non-cytotoxicity of its degradation products. Their combined effect should assure the safe material degradation under controlled kinetics. The present work analyses the degradation behaviour of blends of corn starch with poly(ethylene-vinyl alcohol) copolymer (SEVA-C). The characterization included long-term degradation trials on simulated physiological solution with α-amylase up to 200 days. The degradation solutions were analysed by several techniques. High-performance liquid chromatography (HPLC) and colorimetric methods were used to monitor the liberation of carbohydrate as a consequence of starch hydrolysis by α-amylase. The hydration degree was followed by thermogravimetric analysis (TGA). Several degradation products such as carbohydrates ranging from C_6 to C_{18} were identified. After α-amylase action, biodegradation was more pronounced in the first 100 days, after which the biodegradation rate decreased probably due to the structure and porosity of the material. The action of α-amylase solely led to the starch degradation, in contrast with other assays without enzymes where no carbohydrates were found in the degradation solutions.

Keywords: Biodegradable polymer; In vitro test; Starch; Enzyme; Polysaccharide; Polyvinylalcohol

1. Introduction

Starch-based blends present an enormous potential to be widely used in the biomedical and environmental fields, as they are biodegradable, inexpensive and an almost unfailing source of raw material [1–2].

In the orthopaedic field, the aim is to develop systems that will be able to sustain their integrity and mechanical performance in the presence of aqueous media in the first implantation stages starting to degrade thereafter [3–6]. The material must be designed with a degradation rate adequate to retain the scaffold strength until the newly grown tissue takes over the synthetic support [7–10].

Starch is in general totally biodegradable. It consists of two major components: amylose, a mostly linear α-D-(1-4)-glucan and amylopectin, an α-D-(1-4)-glucan, which has α-D-(1-6) linkages at the branch point and contains only glucose as monomer [11]. Both fractions are readily hydrolysed at the acetal link by enzymes. The α-1-4-link in both components of starch is attacked by amylase; the α-1-6-link in amylopectin is attacked by glucosidases.

The endoamylases generally hydrolyse only the main chain acetal bonds in either amylose or amylopectin and are not active on the branch points of the latter, but many exoamylases can cleave either main chain on branch bonds. The exoamylases can generate either glucose or the dimer (maltose) or the trimer (maltotriose) by attacking the non-reducing end of the starch molecules. In many cases, as soon as an enzyme molecule associates with a polymer chain and causes the first chain cleavage, either by an exo- or an

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doi:10.1016/j.biomaterials.2003.09.093

endo-cleavage, it remains associated with the fragmented chain, and can catalyse the hydrolysis of several units before dissociating.

When a sufficient amount of starch is degraded and removed, the sample should lose its strength and/or integrity and disaggregate. The effective connectivity and accessibility of the starch granules, which is required for extensive enzymatic hydrolysis and removal, is achieved only at relatively high starch contents [12].

The addition of poly(ethylene-vinylalcohol), EVOH, to starch blends improves the processability, increases the toughness, and ameliorates the product moisture sensitivity, but will also decrease the overall rate of biodegradability of the system [13–17]. The major concerns associated to the use of synthetic degradable polymers as biomaterials are: (i) to assure they are biocompatible by themselves; and (ii) to assure that the use of particular additives and/or processing technologies required to obtain different properties and/or geometries, will not compromise neither the biocompatible behaviour nor induce cytotoxicity[18].

For example, polymers often have low molecular weight "leachables" (additives, low molecular weight components, initiator fragments) that exhibit varying levels of physiological activity and cell toxicity [19–22]. However, only in very few cases, consideration has been given to these byproducts formed by biodegradation and to their subsequent effects.

The low molecular mass components that are identified in degraded polymers are, in principle, related to synthesis, processability and/or service. Polymers with weak linkages, where specific degradation occurs (like hydrolysis), generally give rise to few and well-defined degradation products [23–24].

The knowledge of the degradation process of a biodegradable polymer and of the effects that their degradation products might have on the body is crucial for long-term success of a biomaterial [25].

Within this frame of interest, a structural investigation of starch/EVOH blends was developed in order to assess the behaviour of these biodegradable thermoplastics.

2. Materials and methods

2.1. Materials

The material studied was a thermoplastic blend of corn starch with a poly(ethylene-vinyl alcohol) copolymer (60/40 mol/mol), SEVA-C, supplied by Novamont, Novara, Italy. The typical amount of starch in this commercially available blend is 50–60% (wt%). Injection moulded square plates 30 mm wide and 2 mm thick were used for the assays. The SEVA-C samples were weighed and immersed for several pre-fixed ageing periods as long as 6 months (200 days) at pH 7.4 and $37^{\circ}C \pm 1^{\circ}C$ in individual containers (volume approximately $50\,cm^3$) with a Hank's balanced salt solution (HBSS) without phenol red (HBSS Sigma reference H8264), with α-amylase (from human saliva, Sigma reference A0521) at a concentration similar to the one usually found in human blood plasma (50 U/l). The enzyme solution had an activity of 0.35 mg/U/min at pH 6.9 and 20°C per gram of soluble starch. To stabilise α-amylase, 1 mM calcium chloride was employed.

The square plates were used in in vitro degradation experiments under strictly controlled conditions in a laminar flux chamber.

The samples were sterilized by autoclave in an atmosphere of 10/90 mixture of ethylene oxide (EtO) and carbon dioxide (CO_2), with a cycle time of 20–22 h at a working temperature of 45°C, and a chamber pressure of 180 kPa.

2.2. Analytical methods

2.2.1. Detection of polysaccharides

The total amount of polysaccharides in the degradation solutions was quantified using the Dubois method [26], 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 (microprocessor controlled reader in 96-well microplates) at 490 nm using the control sample as the reference cell. A solution of HBSS and α-amylase without SEVA-C specimen was used as control.

2.2.2. Acid hydrolysis of dried SEVA-C powder specimens

The amount of starch present in the SEVA-C specimens was obtained by acid hydrolysis and quantified by HPLC and Dubois methods. A sample of about 5 mg of dried SEVA-C powder was dissolved in 250 μl of 78% H_2SO_4 and incubated at 25°C during 30 min in a sealed tube. After that, 1.2 ml of distilled water and 0.5 ml of glucose (0.05 g/l) and sorbitol (1 g/l), used as internal standards for the Dubois method and HPLC detection, respectively, were added to the mixture. The tubes were incubated at 100°C for 120 min. After cooling the samples for 15–30 min, 0.6 ml of 25% ammonia solution (NH_3) was added and cooled again for 10 min.

In each case (HPLC and Dubois) three replica were made. The final percentage of starch present in the material was considered as the average of all the results.

2.2.3. Detection of reducing sugars

The reducing sugars in the degradation solutions were quantified by the dinitrosalicylic acid method: 0.5 ml of reagent DNS was added to 0.5 ml of the sample to be analysed [27]. At the same time, the blank was prepared

using 0.5 ml of control sample. As previously, HBSS and α-amylase without SEVA-C were used as control. The mixture was heated at 100°C for 10 min. After cooling to room temperature, 5 ml of distilled water was added, and the absorbance at 540 nm was measured in the ELISA reader. The respective carbohydrate concentration was obtained by comparison with a standard curve.

2.2.4. Oligosaccharides and glycerol detection by high-performance liquid chromatography (HPLC)

HPLC with 830-RI (Jasco, Japan) refraction index detection and an 880-PU pump (Jasco) was used to separate the sugar derivatives and glycerol 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 maintained at a pressure 6500–7000 kPa. The eluent was filtered through a 0.2 μm sterilized membrane degassed with helium prior to being used, and kept in a container that precludes introduction of airborne bacterial and fungal contamination. Sorbitol (1 g/l) was used as the internal standard. Prior to the injection, the samples were filtered through 0.22-mm filters (Milipore Corporation) to remove the particles present in the degradation solutions. Three replica of each sample were performed. A standard curve was previously built using different standard concentrations.

2.2.5. Glycerol quantification

The glycerol amount in the degradation solutions was quantified by HPLC and HPAE-PAD. HPLC conditions were the same as for the oligosaccharides detection. The glycerol extracted by acid hydrolysis of SEVA-C powder without any treatment was quantified by HPLC.

High-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) equipped with a Carbopac PA1 ($4 \times 250\,mm^2$) column and Carbopac guard column ($4 \times 50\,mm^2$) was used to separate glycerol from the degradation solutions.

Separation was performed with a flow rate of 1 ml/min using an eluent gradient of 50% of 0.2 M NaOH and ultra-pure water (referred to as the 50% gradient). Eluents were flushed with helium to remove dissolved gases and were continuously pressurized.

Lactose (50 mg/l) was used as the internal standard. The glycerol determination in the non-treated degraded SEVA-C specimens was performed in a Soxhlet system during 8 h. SEVA-C square specimens were weighed (≈ 2 g) into thimbles and inserted into the extraction unit. After solvent addition (distilled water was used as the extraction medium), the sample was immersed in boiling water to dissolve most of the soluble material. Finally, the extraction cups were dried and weighed, enabling the calculation of the percentage of glycerol in the specimen.

2.2.6. Thermogravimetric analysis (TGA)

TGA was performed in a TGA-50 Shimadzu-thermogravimetric equipment using aluminium pans, under the following conditions: heating rate of 5°C/min, hold temperature and time of 120°C and 110 min respectively, and a constant helium flow of 30 ml/min.

The pre-condition of the samples was as follows: samples of about 10 mg were removed for testing at a pre-defined immersion time (until 200 days) and kept in a desiccator, with controlled temperature and humidity, until constant weight. Equilibrium hydration degree was considered when no weight change (0.01 g) was observed. The assays were performed in three duplicates, considering the average of all the results as the final result. Two determinations were performed: the SEVA-C water absorption during the TGA analysis and the percentage of water loss during the pre-condition of the samples.

The swelling data percentage of the blends was fitted using the following equation (%SEVA-C water absorption):

$$\frac{W_s - W_f}{W_s} 100\%,$$

where W_s is the weight of the sample after pre-condition at controlled conditions and W_f is the weight of the sample after the TGA analysis.

The water loss percentage of the blends was determined by using the following equation:

$$\frac{W_i - W_s}{W_i} \times 100\%,$$

where W_i is the initial weight of the sample and W_s is the weight of the sample after pre-conditioning, at controlled conditions.

3. Results and discussion

3.1. Reducing sugars in the degradation solution

The amount of reducing sugars in the degradation solutions, reduced by dinitrosalicylic acid, increased since the beginning until the end of the assay (Fig. 1). The initial linear part of the degradation curve corresponds to the fast hydrolysis phase. After 100 days of immersion, the glucose mass (in %w/w) tended to stabilised towards a final value of about 16% (w/w). For longer periods, only small increases in the degradation rate were detected.

The relative amount of reducing sugars in the degradation solutions in similar assays without enzymes was about 100 times lower.

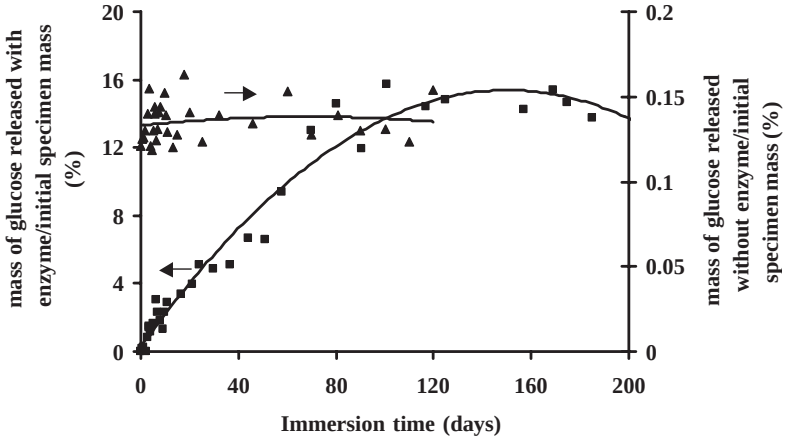

Fig. 1. Mass of glucose released to the solution, with and without enzymes, per initial specimen mass (1.61 g) in 50 ml of solution as a function of immersion time.

3.2. Saccharides in the degradation solution

In order to separate and quantify all the saccharides in solution released by enzymatic action on the starch amount of the SEVA-C blend, liquid chromatography was used with a carbohydrate Ca column.

Analysing Fig. 2, the most abundant compound identified is glucose, followed by maltose and finally maltotriose. This is expected, since α-amylase randomly hydrolyses α-1,4-glucosidic bonds of starch into dextrins. The hydrolysis of the starch bond by α-amylase leads to a significant increase in the mass loss percentage. Values up to 20% were measured, as demonstrated by the polysaccharides curve whose rise is mainly due to the glucose oligomers. The digestion degraded the amylose into maltose and glucose. The enzyme increases the release of long-chain oligosaccharide substrates.

All the oligosaccharides in solution tend to increase with longer immersion times, from monosaccharides to trisaccharides, being an evidence of the degradation of SEVA-C material and favouring the effective area available for degradation.

The change in regime occurred after 100 days was considered to be associated to the specificity of the blend structure. In fact, as the starch is embedded in the matrix, the interior domains are difficult to reach by the enzymes.

The release of starch from the surface to the bulk is also limited by the porosity of the material. Some starch may be dispersed within the chains of the vinyl-alcohol copolymer, increasing the difficulty for enzymes to reach the starch molecules strongly interspersed in the synthetic insoluble component.

The low mass percentage released after 100 days can also be attributed to the nature of the blend, in which starch and ethylene-vinylalcohol are combined as an interpenetrating network. The presence of ethylene also reduced the mass loss, as less accessible hydroxyl groups are present in the blend.

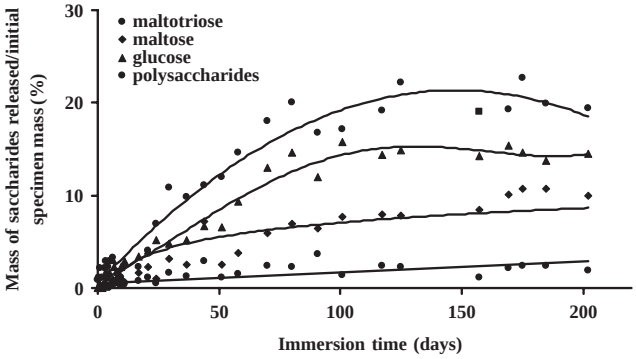

Fig. 2. Mass of saccharides of glucose released to the solution per initial specimen mass (1.61 g) in 50 ml of solution as a function of immersion time.

3.3. Polysaccharides in the degradation solution

Fig. 3 shows the comparison between the sum of all the saccharides in the degradation solutions and the total polysaccharides quantified. The curves presented a similar behaviour along with the immersion time, increasing since the beginning and stabilizing after 100 days of immersion.

The increased enzymatic activity rate may be due to the fact that when the enzyme attacks the reducing groups, the number of domains available for enzymatic degradation increased.

After 75 days of immersion the curves show a small difference. This deviation may be explained considering that in the Dubois method, one molecule of water is released for each molecule of reduced sugar. The saccharides in solution represent almost 25% of the mass weight loss of the material, so only part of the starch present in the material was degraded to

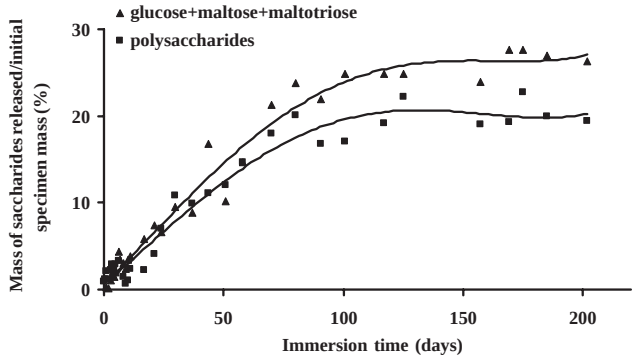

Fig. 3. Mass of all the saccharides and polysaccharides released to the solution, per initial specimen mass (1.61 g) in 50 ml of solution as a function of immersion time.

the solution, the other part remaining inside the structure.

3.4. Total amorphous phase in SEVA-C blend

To quantify the percentage of starch present in the SEVA-C material, an acid hydrolysis with sulphuric acid was performed on a dried SEVA-C powder. The extract was separated and quantified in the solvent phase.

Table 1 shows the results obtained for the two methods used: HPLC and Dubois. From 5 mg of dried powder, it was possible to detect 40% and 43% (w/w) for the Dubois and HPLC methods, respectively, of the starch amount (w/w). An average of the results gave a value of 42%, significantly different from the theoretically expected, 60% (w/w).

The percentage of saccharides found in the degradation solutions (25%) is almost half from the mass of starch in the material structure (42%). This difference may be due to the difficulty of the enzymes to reach the starch molecules strongly interpenetrated with the synthetic insoluble component. Moreover, the low porosity of the material makes the polysaccharides not completely accessible to the enzymatic attack.

3.5. Glycerol in the solution and in the SEVA-C blend

The leaching of glycerol to the solution (Fig. 4) was quantified by two chromatography methods: HPLC and HPAE-PAD. The methods give a difference of about 2% in w/w. The leaching of glycerol to the solution increased in the first days, stabilizing thereafter, until the end of the assay. The mass of glycerol released to the solution per initial specimen mass is around 5% and 3% by HPLC and HPAE-PAD, respectively. In the first days the release of plasticiser is the main effect, after which there is a period without any significant loss of weight (after 20 days). This should correspond to the

Table 1
Mass of saccharides released from acid hydrolysis obtained for HPLC and Dubois methods, from 5 mg of SEVA-C dried power

	HPLC	Dubois
Mass of saccharides released/initial specimen mass (%)	43	40

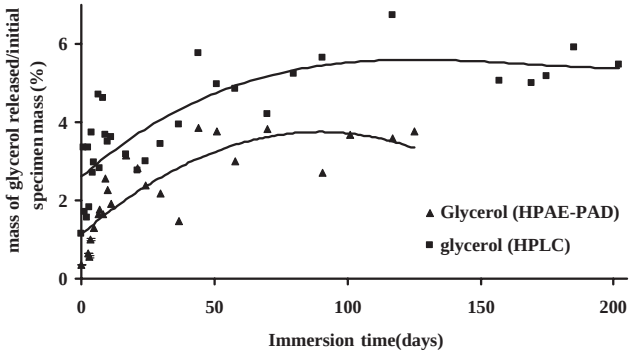

Fig. 4. Mass of glycerol released to the solution per initial specimen mass (1.61 g) in 50 ml of solution as a function of immersion time (obtained from HPLC and HPAE-PAD).

random breakdown of some accessible covalent bondings, as the previous figures of the carbohydrates released demonstrated.

Table 2 shows the results obtained from the Soxhlet extraction to two SEVA-C specimens, one from the enzymatic degradation and another without any treatment. The table also shows the glycerol quantified by HPLC, extracted from the acid hydrolysis of dried SEVA-C powder without degradation.

The difference between them, the specimens subject to degradation and without any treatment, gives approximately the quantity obtained in the degradation solutions (5%) by HPLC. Not all the glycerol was released to the solution, about 2% remaining in the material. When in solution, the glycerol moves away from the material by hydrolysis, being completely soluble as it has no interaction with the copolymer. As expected, this process is followed by the material embrittlement as a result of the glycerol release.

3.6. Swelling behaviour

Swelling behaviour is important when a polymeric system is applied in the biomedical field as its hydration degree influences the surface properties, the mobility and the type of solute transport mechanisms.

The data obtained by TGA are presented in Figs. 5 and 6. The SEVA-C water absorption measured by TGA presented a value of about a 9% for 100 days of immersion. It is important to underline that this percentage was calculated considering the mass of the specimen after the pre-condition process as reference.

Table 2
Mass of glycerol released from Soxhlet extraction per initial specimen mass without any treatment and after degradation, and extracted by acid hydrolysis and quantified by HPLC per initial specimen mass without any treatment

	Sohlet	HPLC
Mass of glycerol released/initial specimen mass without treatment (%)	7.71±0.1	7.35±2.12
Mass of glycerol released/initial specimen mass after degradation (%)	2.20±0.1	

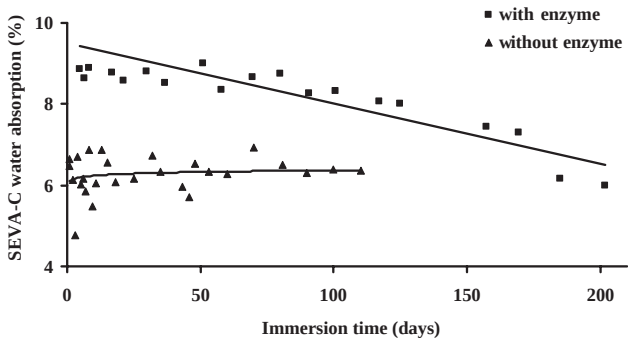

Fig. 5. SEVA-C water absorption with and without enzymes versus immersion time (data from TGA analysis).

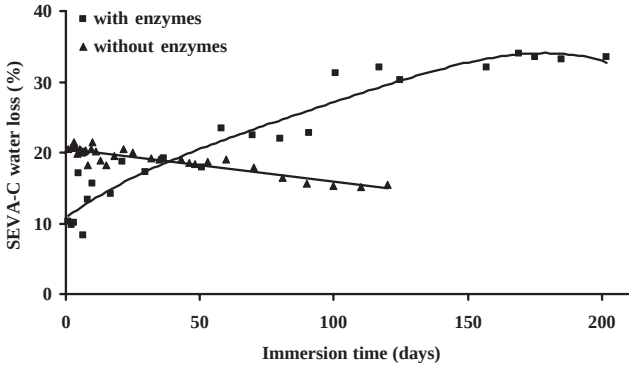

Fig. 6. SEVA-C water loss with and without enzymes versus immersion time (data obtained during the pre-condition of the samples).

The specimens that evidenced lighter water losses in the pre-conditions showed small water release during TGA analysis. Although, the difference between each sample and the reference may be smaller, it should be noted that the reference also lost water during pre-condition stage.

Probably, the presence of the ethylene-vinyl alcohol copolymer, which has hydrophobic ethylene units, is responsible for lowering the hydration degree and for the hindered diffusion of water molecules into the polymer network. The same assays without enzymes present about 3% less in water absorption.

Fig. 6 shows the cumulative water loss during the pre-conditioning process. The absorbed water was lost to the atmosphere. This may be explained by the increased

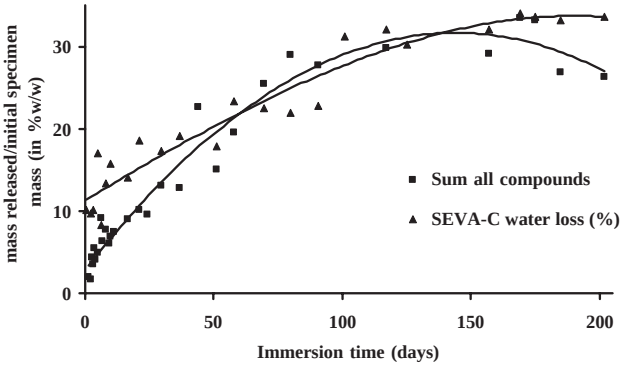

Fig. 7. Comparison between the mass of all the compounds released to the solution per initial specimen mass (1.61 g) in 50 ml of solution, and the SEVA-C water loss as a function of immersion time.

degradation with time and the corresponding increase in surface porosity.

After 100 days, the SEVA-C water loss stabilized at a value of 35%. The enzyme attack to the surface structure enables that the broken low molecular weight chains leach to the solution, enlarging the material porosity that may lead to an increase in water absorption. The water loss in the assays without enzymes was constant and equal to 20%, mainly due to the glycerol released to the solution. Fig. 7 compares the curves of the water loss and the total mass of compounds leached to the solution. The two curves exhibited a similar behaviour. The evaluation of the total degraded mass compounds is reasonably aligned with the obtained through the pre-conditioning analysis, showing a good correlation between the hydration degree and the amount of degradation products.

3.7. Degradation kinetics rate reaction

Considering the concentration of all the compounds leached to the solution, the degradation rate kinetics as a function of the immersion time is depicted in Fig. 8. As expected, the rate follows a logarithmically decay as a function of the immersion time. The decrease of the degradation rate observed in the final stage can be explained to the lower degradability of the amylose-EVOH domains that remain in the material.

After 75–100 days, the variation is almost negligible, nearly zero, as no saccharides and other compounds leached to the solution, as demonstrated before. The rate tends to a zero asymptotic limit. The reduction of the degradation rate is also influenced by the water uptake ability of these polymers.

4. Conclusions

The biodegradability of starch blends with α-amylase increased until nearly 100 days of immersion, being the

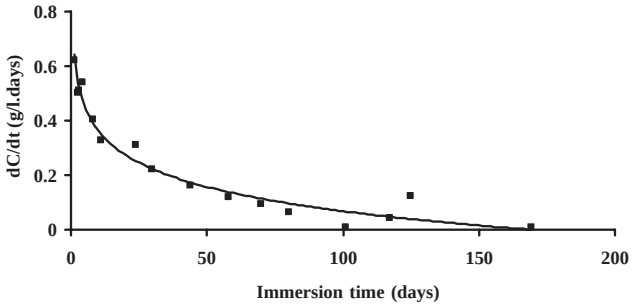

Fig. 8. Degradation rate kinetics of all the compounds released to the solution as a function of immersion time.

total mass loss percentage of 35%. The main saccharides in the degradation solutions were glucose, maltose and maltotriose. The enzyme increases the release of oligosacharide substrates, mainly glucose oligomers. During the experimentation, only part of amorphous of SEVA-C blend was released and degraded to the solution, the remainder stayed embedded in the network structure.

The relatively low levels of starch degradation observed were associated to the specific structure of this thermoplastic interpenetrated blend. This starch phase is embedded in the matrix of vinyl-alcohol copolymer, increasing the difficulty for enzymes to reach the starch molecules strongly interpenetrated with the synthetic insoluble component. The materials exhibited low water uptake ability and slow degradation kinetics.

The present experimentation evidenced the major role of enzymes, always present in the human body on SEVA-C degradation. However, the high amount of residual non-degraded starch, after 100 days degradation, suggests the possibility of optimising the material degradability by means of inducing deliberatety porosity (e.g., foamed structures) in the material.

Furthermore, these systems are expected to be resorbed and degraded faster in "in vivo" experiments, as the enzymatic hydrolysis, expected to be easier with a wide range of available enzymes, will accelerate degradation.

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