



Immobilization of trypsin onto poly(ethylene terephthalate)/poly(lactic acid) nonwoven nanofiber mats

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ABSTRACT

Mixed solutions of poly(ethylene terephthalate) (PET) and three types of poly(lactic acid) (PLA; a commercial sample and two branched PLAs) were used to prepare nonwoven nanofibrous mats by electrospinning. These mats were used as supports for trypsin immobilization, with the aim of an application in whey protein hydrolysis. Covalent attachment of crosslinked trypsin aggregates to amine-derivatized PET/PLA mats, followed by reduction with sodium cyanoborohydride, gave the best immobilized trypsin activity and showed no enzyme leaching. The activity of immobilized trypsin was higher than that of free trypsin at pH 6, a pH value representative of sweet whey. The PET/PLA mats with immobilized trypsin could be stored at 4 °C in water for at least 30 days, and could be reused 8 times without losing activity and without enzyme leaching.

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1. Introduction

The 2014 cheese production by the major cheese producing countries has been forecasted to 18 million tons [1]. Whey, the liquid phase remaining after precipitation and removal of milk casein during the cheese-making process, represents ca. 85–95% of the milk volume and contains ca. 55% of milk nutrients, in particular, proteins (0.6–0.8% w/v) and lactose (4.5–5% w/v) [2]. Although several possibilities for the exploitation of whey have been developed and already implemented in the food, cosmetics and pharmaceutical industries [3], whey is generally considered a waste product, due to its low percentage of dry matter (ca. 6–7%). As such, about half of the whey produced worldwide is discarded as an effluent, posing environmental problems. This work is concerned with the development of an immobilized enzymatic system aimed at production of bioactive peptides through hydrolysis of whey proteins. As simultaneous hydrolysis and separation of the protein hydrolysate from whey is advantageous and allows the implementation of a continuous process, we have immobilized trypsin, a model proteolytic

enzyme used widely in industrial biotechnological processes and, in particular, in the food industry [4], onto nonwoven nanofiber mats (NNMs) suitable for separation.

Proteases for whey hydrolysis have already been immobilized onto organic and inorganic particulate supports (e.g., [5]) or immobilized through retention in ultrafiltration membranes [6], and bioactive peptides derived from whey have already been exploited in the cosmetics and pharmaceutical areas [3]. Since the first report of trypsin immobilization onto charcoal in the early 1900s [7], trypsin has been immobilized in a variety of organic and inorganic supports [8]. In enzyme immobilization, features of the immobilization support, such as its degree of hydrophilicity, electric charge, morphology, pore size and dimensions, determine the performance of the immobilized enzyme system [9]. Nanostructured supports – nanoparticles, nanofibers, nanotubes and nanoporous materials – are considered a valuable option for enzyme immobilization, since they may ensure high immobilization yields, low mass transfer limitations, high catalytic performance and enzyme stabilization, owing to their very large surface-to-mass ratio, high porosity and high pore interconnectivity [10]. These features can provide not only high biocatalytic activity, but also easy reuse and implementation in continuous processes. Nanofiber assemblies known as nonwoven nanofiber mats (NNMs) can be obtained by electrospinning [11], a process in which a polymer solution or melt is exposed

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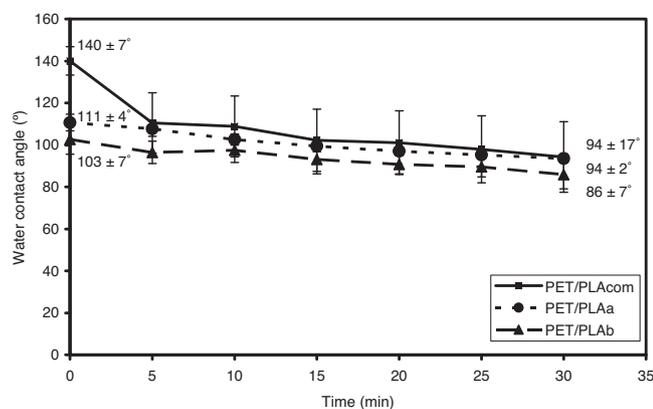


Fig. 1. Variation of the water contact angle (sessile drop) with the time of drop residence on the surface of the PET/PLA mats. Error bars: standard deviation ($n = 10$). The lines shown are only guides to the eye.

to a high electric potential difference while forced to pass through a blunt-tip syringe needle. The nanofibers so formed may be collected onto a rotating drum placed at a specific distance from the needle tip, resulting a self-supporting mat on the drum [12]. Due to their low resistance to the flow of liquids, electrospun NNMs can be used for separation. When used as supports for enzyme immobilization, simultaneous biocatalysis and separation can be attained, allowing the implementation of high throughput, continuous flow processes employing enzymatic membrane bioreactors [13].

Poly(ethylene terephthalate) (PET) is an inexpensive polymer, which is readily electrospun, producing NNMs with good structural, thermal and mechanical properties [14]. However, its hydrophobicity and the reduced number of reactive groups preclude its general use as a support for enzyme immobilization. Nevertheless, a few reports exist on the immobilization of trypsin onto PET-based supports. Among them, trypsin was covalently attached to PET fibers grafted with poly(acrylic acid), employing a carbodiimide [15], and to an hydrazinolyzed PET/polyaniline composite, employing activation with glutaraldehyde (GA) before enzyme addition [16]. With the advent of proteomics in the late 90's, reports concerning immobilized trypsin reactors for protein digestion started to appear [17]. Trypsin was recently immobilized onto electrospun nanofibers and applied in proteomics [18,19]. Reported immobilization strategies include immobilization onto NNMs made from a mixture of polystyrene and a poly[styrene-co-(maleic anhydride)] copolymer (PS/PSMA) and also onto dispersed nanofibers of PS/PSMA, either by direct covalent attachment to the maleic anhydride groups or by direct covalent attachment to the anhydride groups of GA-crosslinked enzyme aggregates [20–23]. The approaches employing crosslinked trypsin aggregates yielded high enzymatic activity and high operational and storage stabilities.

In this work, in order to increase the number of reactive groups and reduce the hydrophobicity of the mats, PET was mixed with three different types of poly(lactic acid) (PLA) – a commercially available PLA (PLAcom) and two branched PLAs (PLAa and PLAb, obtained by esterification of lactic acid with branched polyols [24]). These mixed solutions were electrospun and the resulting PET/PLA mats were evaluated for trypsin immobilization. Three immobilization strategies were assessed: (i) immobilization by direct covalent attachment to the carboxylic groups, employing a carbodiimide; (ii) immobilization by adsorption and crosslinking with GA; and (iii) immobilization by covalent attachment of crosslinked trypsin aggregates to amine-derivatized PET/PLA mats, employing GA for both covalent attachment and crosslinking. The immobilized trypsin activity was quantified and the best NNMs were characterized in terms of chemical composition, thermal properties, surface

Table 1

Activity of trypsin immobilized by direct covalent attachment to carboxylic groups of the different PET/PLA-based mats, employing EDAC.

Mat	Immobilized trypsin activity (\pm SD) ($\mu\text{mol pNA min}^{-1} \text{g}^{-1} \text{mat}$) ^a
PET/PLAcom	0.34 \pm 0.12
PET/PLAa	0.29 \pm 0.13 ^b
PET/PLAb	0.62 \pm 0.14 ^b

EDAC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide pNA – *p*-nitroaniline SD – standard deviation ($n = 3$).

^a Activity of immobilized enzyme expressed in $\mu\text{mol p}$ -nitroaniline (pNA) per min and per g of dry mat.

^b Difference to PET/PLAcom not statistically significant ($p > 0.05$; one-way ANOVA followed by Dunnett's post test, at a confidence level of 95%).

wettability and surface morphology. For the best system, the effect of temperature, pH and substrate solution recirculation speed on the activity of immobilized trypsin, as well as its operational and storage stability, were assessed.

2. Materials and methods

2.1. Preparation of PET/PLA nonwoven nanofiber mats (NNMs)

NNMs were prepared from solutions of poly(ethylene terephthalate) (PET, supplied by Flexitex, Portugal) and three types of poly(lactic acid): (i) PLAcom, a commercial sample (Ingeo 2002D, NatureWorks LLC, USA; $M_w = 187,000$); and (ii) two branched PLAs, obtained by esterification of lactic acid with branched polyols [24] – PLAa, $M_w = 67,000$, polydispersion index (PDI) = 1.4; and PLAb, $M_w = 43,000$, PDI = 1.4). The following mixed PET/PLA solutions were electrospun, at room temperature, employing an electrospinning apparatus already described [14]: 30% (w/v) solutions of PET/PLAcom, PET/PLAa or PET/PLAb, all at a PET/PLA weight ratio of 4:1, in 4:1 TFA/DCM (trifluoroacetic acid/dichloromethane, both from Sigma–Aldrich, USA). Electrospinning was conducted at a voltage of 26 kV and at a flow rate of 0.2 mL/min. Fibers were collected as nonwoven membranes (mats) on an aluminium foil-covered rotating drum (rotation speed: 900 rpm), employing a needle-tip-to-collector distance of 12 cm. The obtained mats were washed with acetone and dried at 35 °C. From the central zone of the mats, several squares of 1.5 × 1.5 cm squares were cut for use in the enzyme immobilization studies.

2.2. Trypsin immobilization onto PET/PLA nanofiber mats

2.2.1. Covalent attachment via EDAC activation

Dry PET/PLA square mats of 1.5 × 1.5 cm were placed in magnetically stirred vials containing 4 mL of a trypsin (EC 3.4.21.4, from bovine pancreas, lyophilized, essentially salt-free, ~9000 BAEE units/mg; Sigma–Aldrich, USA) solution at 1 mg/mL in 0.1 M MES (2-(*N*-morpholino) ethanesulfonic acid) buffer, at pH 5.5. After 5 min under magnetic stirring, at room temperature, 10 mg of *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were added and, after 2 h of reaction, the mats were thoroughly washed with the 0.1 M MES buffer. Unreacted, activated carboxyl groups were neutralized by a 10 min treatment with a 0.1 M TRIS–NaCl buffer (tris(hydroxymethyl) aminomethane buffer solution at pH 7.6, containing 0.5 M NaCl). Finally, the mats were thoroughly washed with the buffer used in the enzyme activity assay (Section 2.3.1) and stored at 4 °C, in the same buffer. This immobilization was carried out in triplicate, using a single mat per vial. The mats were assayed either on the same or on the following day, in which case they were stored at 4 °C in the buffer used in the enzyme activity assay (Section 2.3.1). All reagents and buffer salts were from Sigma–Aldrich, USA.

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