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IMOBILIZAÇÃO DE ENZIMAS EM COMPÓSITOS À BASE DE POLIANILINA

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"Imobilização de enzimas em compósitos à base de polianilina"

TITULARES

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SUMÁRIO

RESUMO

Neste trabalho são relatados os resultados obtidos na síntese, caracterização e aplicação de discos de álcool polivinílico-glutaraldeído-polianilina-glutaraldeído (PVAG-PANIG) como suporte para imobilização das enzimas peroxidase (EC 1.11.1.7) e tripsina (EC 3.4.21.4). A produção dos discos do compósito foi obtida pelo gotejamento de uma solução de álcool polivinílico 2% (p/v) e glutaraldeído 3,6 % (v/v) sobre poços de uma microplaca contendo 120 μL de HCl 3,0 mol L^{-1}, posteriormente, seguido pela síntese química de polianilina sobre a superfície dos discos, utilizando persulfato de amônio como agente oxidante, e pela ativação do compósito com glutaraldeído 2,5% (v/v). A composição do suporte PVAG-PANIG foi investigada mediante análises por espectros de infravermelho de transmitância e absorção por UV-visível e análise termogravimétrica. A superfície do material foi observada em microscopia eletrônica de varredura e em estudos de porosidade pela técnica de adsorção/desorção de nitrogênio e as propriedades condutimétricas foram obtidas utilizando o método padrão de quatro pontas. O compósito apresentou alta hidrofilicidade, baixa porosidade e baixa condutividade elétrica a temperatura ambiente. A imobilização de peroxidase resultou num derivado capaz de remover compostos fenólicos semelhantemente à ação executada pela enzima nativa, com a vantagem da sua reutilização por sete vezes consecutivas. O disco de PVAG-PANIG-tripsina foi capaz de hidrolisar caseína continuamente, produzindo peptídeos de tamanhos diversos.

Palavras-chave: imobilização, álcool polivinílico, polianilina, peroxidase, tripsina.

ABSTRACT

This work presents the results from the synthesis, characterization and application of polyvinyl alcohol-glutaraldehyde-polyaniline-glutaraldehyde discs (PVAG-PANIG) as a support for peroxidase (EC 1.11.1.7) and trypsin (EC 3.4.21.4) immobilization. The synthesis of the composite was obtained by dripping of 2% (w/v) polyvinyl alcohol and 3.6% (v/v) glutaraldehyde mixture into microplate wells containing 3 M HCl (120 μL). On the surface of the discs the polyaniline was chemically synthesized via ammonium persulphate oxidation and the glutaraldehyde (2.5%, v/v) was added again for support activation. The chemical composition of PVAG-PANIG was investigated by infrared and UV-visible spectra and termogravimetric analysis. The both area and porosity of the surface were observed by scanning electron microscopy and Accelerated Surface Area and Porosimetry (ASAP) techniques. Conductivity studies were made using the fourpoint probe method. The composite showed high hydrophilicity, low porosity and low electrical conductivity at the room temperature. The derivative from the peroxidase immobilization was able to remove phenolic compounds in a similar way to the performance of the native enzyme. However, the immobilized form of peroxidase could be reused for seven times consecutively. The PVAG-PANIG-trypsin system could recognize and hydrolyze casein substrate continuously, producing many different sizes of peptides. For the both studied systems PVAG-PANIG discs permitted the immediate removal of enzymes from their catalysis environment.

Key-words: immobilization, polyvinyl alcohol, polyaniline, peroxidase, trypsin.

INTRODUÇÃO

Breve histórico sobre a Biotecnologia

O Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) definiu a Biotecnologia como “a utilização de sistemas celulares para a obtenção de produtos ou desenvolvimento de processos industriais.” Neste contexto, entram as áreas de Bioquímica, Biologia Molecular, Microbiologia e Engenharia Química, de forma que um profissional que trabalhe numa destas esferas deve também interagir com as demais áreas relacionadas a ela.

A Biotecnologia é uma ciência de nome relativamente recente, mas que já existia nos primeiros experimentos que levaram ao uso de materiais biológicos para a produção de diversas técnicas, processos, equipamentos e também à descoberta de novos conhecimentos, desde os tempos mais remotos. As primeiras aplicações de microrganismos para a produção de alimentos e bebidas fermentadas datam de alguns milhares de anos, primeiramente na Babilônia e no Egito e posteriormente na Grécia (Lima et al., 2001).

Antonius van Leeuwenhoeck (1623-1723), um comerciante e técnico especialista em óculos e lupas do século XVII, pesquisou a capacidade de aumentar os objetos, fazendo experimentos com suas lentes. Dessa forma, inventou o primeiro microscópio. Era um equipamento extremamente simples, formado apenas por um tubo com uma lente, mas que tornou possível a visualização de diversas amostras de microrganismos. Leeuwenhoeck foi o

primeiro pesquisador a observar amostras de cerveja em fermentação com seu microscópio.

Já no século XIX as pesquisas realizadas por Louis Pasteur demonstraram que os microrganismos responsáveis pelos processos de fermentação agiam em ambiente de anaerobiose, o que possibilitou o aperfeiçoamento das técnicas tradicionais. Em 1833 os franceses Anselme Payen e Jean-Franois Persoz publicaram no periódico Annales de Chemie et de Physique a descrição de um processo de isolamento de um complexo de amilase de cevada germinante e denominaram-no de diastase. Este complexo era capaz de converter amido gelatinizado em açúcares, como a maltose. Posteriormente, em 1876, William Kuhne propôs que o nome enzima fosse utilizado como o novo termo para denotar fenômenos anteriormente conhecidos como fermentos não organizados, isolados dos organismos vivos dos quais foram formados. A palavra em si significa 'na levedura', sendo derivada do grego *en* que significa em, e *zyme*, que significa levedura ou fermento. No ano de 1897, os irmãos Hans (1850-1902) e Edward Büchner (1860-1917) demonstraram que o processo de fermentação alcoólica ocorria em amostras de leveduras livres de células. Esta descoberta revolucionou o conhecimento da época e auxiliou na derrubada da teoria do vitalismo (Stryer, 1994).

Paralelamente a estes acontecimentos, um pesquisador japonês, o Dr. Jokichi Takamine (1854-1922), desenvolveu a partir de 1894 um método para produção industrial de um complexo enzimático patenteado sob o nome de takadiastase, com base em enzimas amilolíticas produzidas pelo fungo *Aspergillus oryzae*.

Em 1909, Svante Arrhenius (1859-1927) e Soren Sorensen mostraram que a concentração de íon hidrogênio em solução pode ser experimentalmente determinada. Sorensen destacou o efeito do pH na atividade enzimática.

Em 1913, Leonor Michaelis e Maud L. Menten postularam a existência de um complexo enzima-substrato intermediário para explicar o mecanismo de ação enzimática. No ano seguinte o alemão Otto Rohm desenvolve um biodetergente consistindo de uma pastilha de extrato de pâncreas para a remoção de manchas de tecidos. Este foi o primeiro uso comercial da enzima tripsina (van Tilburg, 1984).

Tripsina e Peroxidase: características e aplicações

A enzima tripsina (EC 3.4.21.4) do pâncreas de mamíferos catalisa a hidrólise de proteínas que contenham os resíduos de lisina ou arginina em sua constituição. A tripsina é uma proteína globular, de 24KDa, caracterizada pela predominância de estrutura β e uma pequena parcela de α-hélice. A estrutura terciária da proteína encontra-se organizada em dois barris β antiparalelos, sendo que em um deles encontra-se a extremidade aminoterminal e no segundo a carboxiterminal (Huber & Bode, 1978; Clare et al., 2001).

A tripsina é classificada como uma endopeptidase, enzima que cliva ligações peptídicas distantes dos resíduos N e C terminal. Seu sítio catalítico é constituído de três resíduos de aminoácidos (serina 195, histidina 571 e aspartato 189) (figura 1), dos quais o resíduo de serina, presente em todas as proteínas dessa família, caracteriza a tripsina como uma Serino-protease (Díaz & Balkus Jr., 1996). A clivagem da ligação amida na proteína-substrato inicia-

se pelo resíduo de Ser 195 que se situa na cadeia lateral da tripsina, com a atuação da His 571, também localizada na superfície da proteína. O resíduo de Asp 189, que se localiza na fenda de ligação com o substrato, é o responsável pela seletividade da tripsina por substratos que contenham resíduos de cadeia lateral carregados positivamente, como arginina e lisina (Huber & Bode, 1978).

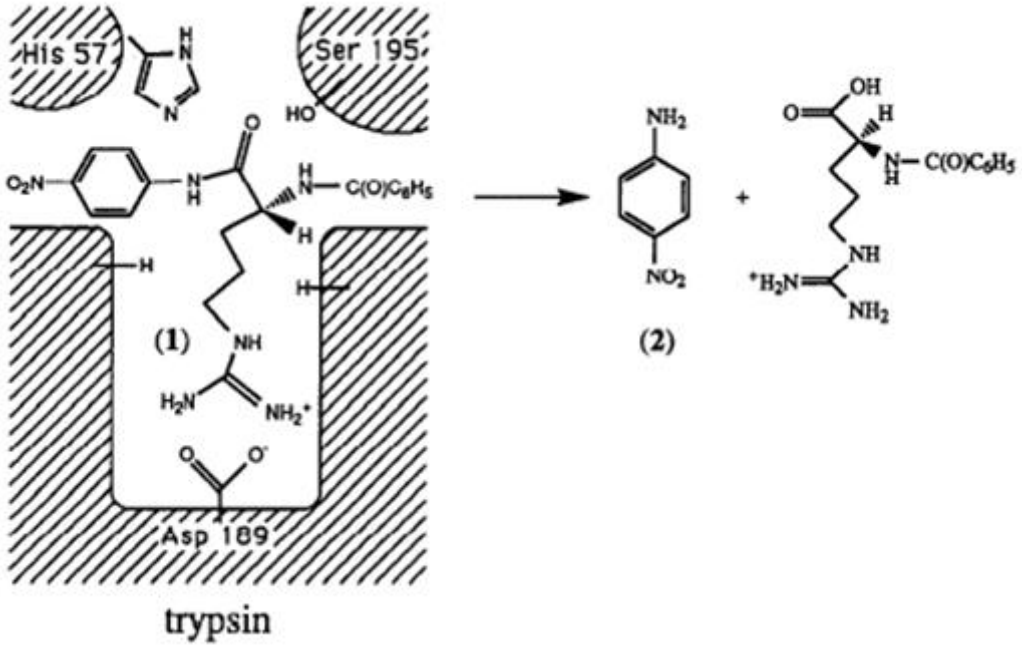

Figura 1. Mecanismo de catálise da enzima tripsina com o substrato sintético N-α-benzoil-DL-arginina-*p*-nitroanilida (BAPNA). Destaque para o sítio ativo (1) e os produtos formados (2) (Díaz & Balkus Jr., 1996).

Na década de 1980, as proteases representavam 60% da produção industrial (Kumar et al., 1998), e são aplicadas em inúmeros processos na indústria de alimentos: da clarificação de cerveja e amaciamento de carne à síntese de aspartame e obtenção de flavorizantes e emulsificantes (hidrolisados protéicos) (Wiselmann, 1975). Além da indústria alimentícia, enzimas proteolíticas, como a tripsina, têm sido utilizadas nos processos de isolamento e purificação de peptídeos, para o seqüenciamento de proteínas (Kornberg, 1990; Clare et al., 2001). Um fator limitante para o uso de proteases

na forma pura é a estabilidade da preparação e o custo de obtenção dessas enzimas em larga escala.

Outra classe de enzima bastante utilizada para os processos industriais é a das oxidorredutases, em que a enzima peroxidase (EC 1.11.1.7) é um dos membros mais proeminentes. As peroxidases são largamente distribuídas no reino vegetal (Halpin & Lee, 1987; Yemenicioglu et al., 1998) e estão fisiologicamente relacionadas à resposta ao estresse salino e hídrico (Lima et al., 1999) e à injúria (Souza et al., 1999).

As peroxidases utilizam peróxido de hidrogênio ou outros peróxidos para a oxidação de uma grande variedade de substratos (Padiglia et al., 1995). Neste tipo de reação o peróxido de hidrogênio é convertido a água e oxigênio e os elétrons transferidos para o íon peróxido são advindos de derivados fenólicos, aminas aromáticas e alguns compostos metoxilados (Figura 2) (Miland et al., 1996).

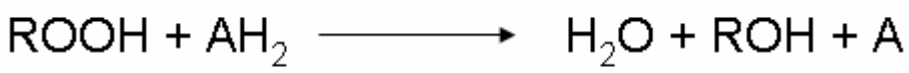

Figura 2. Mecanismo de ação catalítica de peroxidases segundo Chance & Maehly (1955).

A peroxidase de *Amoracia rusticana* (horseradish peroxidase) é uma proteína monomérica de 44 KDa, composta de uma cadeia polipeptídica dividida em dois domínios, que estão glicosilados em oito sítios específicos (Zhao et al., 1996; Azevedo et al., 2001). Esta proteína contém um grupo heme prostético e sítios para ligação aos íons de cálcio (Ryan et al., 1994). A HRP apresenta várias isoformas, das quais a HRP C, a mais estudada, representa 50% da atividade presente na raiz de *A. rusticana* (Nielsen et al., 2001).

Segundo estes autores, a isoforma C possui ponto isoelétrico de 8,8 e foi a primeira peroxidase a ser seqüenciada e ter a sua estrutura determinada.

São muitas as aplicações para peroxidases, em que a HRP é uma das mais utilizadas (Dunford, 1991; Fatima et al., 2007). A utilização dessa enzima vai desde a detecção da concentração de substâncias de interesse clínico, em diagnósticos de teores de glicose (Barham & Trinder, 1972; Pandey & Weetal, 1995), colesterol (Charpentier 1995; Torabi et al., 2007), uréia (Tsai & Doong, 2004), até a detecção e remoção de fenol (Akhtar & Husain, 2006), hidroquinona (Oliveira & Vieira, 2006), e outros compostos presentes em efluentes industriais (Akhtar et al., 2005; Ferreira et al., 2007).

Embora as aplicações para o uso de peroxidases sejam vastas e tragam inúmeros benefícios, existe um inconveniente para a utilização desta enzima em sua forma livre, que é o custo para o seu isolamento e purificação (Fatima et al., 2007).

A imobilização de enzimas

A possibilidade de uso contínuo e repetido das enzimas a partir de técnicas de imobilização proporcionou um grande avanço dos processos industriais, no sentido de diminuir os custos e aumentar a produção. Foi a partir dos anos 1960 que houve um grande avanço das técnicas de imobilização (Vitolo, 2001).

Segundo Chibata et al. (1978), enzimas imobilizadas são aquelas “(...) que estão fisicamente confinadas, ou localizadas em certa região definida do

espaço, com retenção de suas atividades catalíticas, e que podem ser usadas repetidamente e continuamente".

Alguns pesquisadores reuniram as técnicas de imobilização num sistema de classificação que considera a natureza da ligação entre a enzima e a matriz de imobilização. Basicamente, as enzimas ligam-se aos suportes por interações físicas (confinamento, aprisionamento) ou por interações químicas (adsorção, interação iônica, pontes de hidrogênio, forças de Van der Waals, ligação covalente, interação hidrofóbica). O tipo de método empregado depende do tipo de enzima a ser imobilizada ou do material que se deseja utilizar como matriz, além do tipo de produto que se deseja obter (Kennedy & White, 1985).

O álcool polivinílico (PVA) é uma matriz bastante utilizada nos processos de imobilização. Sua estrutura consiste de uma longa cadeia de átomos de carbono ligados entre si por meio de ligações simples e que apresentam grupos laterais hidroxilas que se repetem ao longo da cadeia.

O PVA é um álcool polivalente no qual os grupos hidroxila são todos secundários (figura 3). Eles são facilmente esterificados ou eterificados, levando à formação de álcoois de baixa massa molecular (Sakurada, 1985). O PVA é também um polímero biocompatível (Li et al., 2004), permite a mistura de água ou de solventes orgânicos e pode formar filmes e fibras de propriedades mecânicas excelentes (Hirai et al., 1992; Azevedo et al., 1999). Ele é hidrossolúvel e apresenta um alto grau de cristalinidade (Paradossi et al., 1996; Kim et al., 1992).

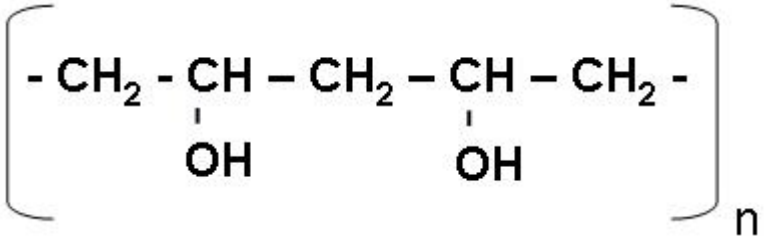

Figura 3. Estrutura química do álcool polivinílico.

A polianilina (PANI) representa uma classe de polímeros altamente promissores, em função de seu baixo custo de síntese, baixo grau de dificuldade de manuseio e ainda por apresentar a propriedade de conduzir elétrons (Gangopadhyay et al., 2001). Dependendo das condições de síntese, o polímero pode atuar como semicondutor (Asturias et al., 1989).

As propriedades condutimétricas da polianilina dependem essencialmente do grau de oxidação do polímero (figura 4). No estado parcialmente oxidado, a polianilina apresenta coloração esverdeada e é conhecida como esmeraldina, o estado que apresenta maior condutividade (Mac Diarmid et al., 1985).

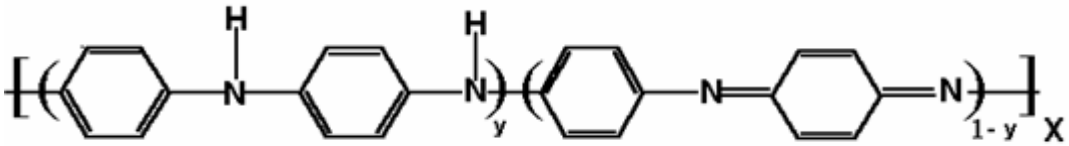

Figura 4. Estrutura química da polianilina segundo Asturias et al., 1989.

A síntese da PANI pode ser realizada quimicamente, através do uso de agentes oxidantes, ou eletroquimicamente, utilizando eletrodos para aplicação de uma corrente elétrica, que ativará a reação de síntese (Gospodinova & Terlemezyan, 1998; Mac Diarmid & Epstein, 1989). Segundo Gospodinova e Terlemezyan (1998), a formação da polianilina se dá inicialmente pela oxidação dos monômeros de anilina, que leva à formação de dímeros. Posteriormente, as espécies diméricas reagem com outros monômeros para formarem

oligômeros, até que o polímero se complete. Durante todo o processo ocorre variação do potencial eletroquímico nas reações, fator limitante para a formação do polímero.

Devido à versatilidade desse polímero, a PANI tem sido proposta para as mais diversas aplicações: construção de supercapacitores (Dong et al., 2007), sensores para detecção de amônia (Xu et al., 2007), despoluição por redução de metais pesados (Olad & Nabavi, 2007), e como matriz para imobilização de enzimas (Singh et al., 2006; Ngamna et al, 2005; Fernandes et al., 2003).

A síntese química de polianilina resulta num pó fino de difícil sedimentação. A dificuldade de paralisação de ensaios enzimáticos em que a polianilina atua como suporte da enzima imobilizada resulta em perda de parte do sistema suporte-enzima e diminuição da precisão na medida de atividade. Por esta razão, alguns autores têm utilizado esse polímero como um compósito com outra matriz (Simon et al., 2002; Caramori & Fernandes, 2004; Luo & Duo, 2004).

O Grupo Proteínas Imobilizadas: interação UFG-UFPE

Em 1996 foi publicado o primeiro trabalho do grupo de Bioquímica da Universidade Federal de Pernambuco, liderado pelo professor Dr. Luiz Bezerra de Carvalho Júnior, que propôs o uso de álcool polivinílico-glutaraldeído para imobilização de proteínas (Carvalho Jr. et al., 1996; Araújo et al., 1997a). Este trabalho trouxe um novo material, capaz de ser moldado de diferentes formas e que apresentou capacidade satisfatória para imobilização das enzimas xantina

oxidase, alfa-amilase e amiloglicosidase. Em função dos bons resultados, a matriz de PVA-Glutaraldeído foi também utilizada para a imobilização de antígenos para diagnósticos de doenças tropicais (Carvalho Jr et al., 1996, Araújo et al., 1996; Araújo et al., 1997b; Araújo et al., 1998). A plasticidade do PVA permitiu a produção de suportes na forma de pérolas (Araújo et al., 1997a), discos (Carvalho Jr. et al., 1996; Araújo et al., 1997a) e ainda uma membrana para o revestimento de papel de filtro (Barbosa et al., 2000).

Foi também durante o ano de 1996 a publicação do primeiro trabalho que utilizava polianilina como suporte para imobilização da enzima xantina oxidase (Nadruz Jr. et al., 1996). A polianilina mostrou-se também promissora, e rendeu ao grupo de pesquisa uma dissertação de mestrado (Coêlho, 1998) e um outro artigo (Coêlho et al., 2001), com a utilização desse polímero como suporte para imobilização de antígenos para imunoensaios.

Paralelamente aos estudos realizados na UFPE, a professora Dra. Kátia Fernandes (Universidade Federal de Goiás - ainda cursando seu doutorado) iniciou seus estudos em imobilização de enzimas, trabalhando com a síntese de diferentes polianilinas em diferentes estados de oxidação para a imobilização de peroxidase, e sua utilização como biossensor. O professor Luiz Carvalho foi convidado a participar da orientação, atuou como membro da banca examinadora de doutorado e, a partir daquele momento, surgiu uma necessidade de estabelecer uma colaboração entre os dois pesquisadores.

O trabalho de tese da Professora Dra. Kátia gerou três artigos: o primeiro, que tratou da síntese e caracterização de cinco tipos de polianilina e sua aplicação para a imobilização de peroxidase (Fernandes et al., 2003), o segundo, que trouxe os parâmetros cinéticos e de estabilidade do sistema

Polianilina-peroxidase (Fernandes et al., 2004) e o terceiro trabalho, com os dados de comportamento de um biossensor para a detecção de peróxido de hidrogênio (Fernandes et al., 2005). Instalada no Laboratório de Química de Proteínas da Universidade Federal de Goiás, a professora Kátia abriu uma nova linha de pesquisa com imobilização de proteínas em suportes à base de polianilina.

A síntese química de polianilina, apesar de seu baixo custo, apresentava um inconveniente na paralisação da reação: o pó fino que constituía o polímero precipitava com grande lentidão. Por causa disto, o grupo de pesquisa da UFG utilizou um material bastante estudado pelo grupo da UFPE: o polietileno-tereftalato (PET, dacron) para sustentar a polianilina e aumentar a precisão dos ensaios enzimáticos contendo enzimas imobilizadas nestes materiais.

A experiência de trabalhos do professor Luiz Carvalho com dacron como matriz para imobilização de proteínas possui mais de 22 anos (Melo, 1984; Amaral et al., 2006). Durante este período, dacron já foi utilizado para imobilização de enzimas (Melo, 1984; Carvalho Jr. et al., 1986; Oliveira et al., 1989; Carneiro-Leão et al., 1991; Duarte, 2002; Amaral et al., 2006) e para ensaios imunológicos (Montenegro, 1991; Montenegro et al., 1991; Carneiro-Leão et al, 1994; Pinheiro et al., 1999), sob a forma de azida, através do tratamento com hidrazina, magnetizado, ou ainda na forma de compósito com polianilina (Coêlho et al., 2001).

Foi a partir desta última experiência que o grupo de pesquisa do Laboratório de Química de Proteínas utilizou o PET de garrafas de refrigerante para construir um compósito com a polianilina, estudo que resultou em uma dissertação de mestrado (Caramori, 2003). O PET-PANI, sob a forma de tiras,

serviu como suporte para a imobilização de peroxidase (Caramori & Fernandes, 2004) e de tripsina (Caramori & Fernandes, 2007), permitindo a estes sistemas um controle refinado do início e término da catálise, através da adição ou remoção da tira de suporte do meio de reação.

Os resultados com PET-Polianilina abriram uma nova perspectiva de trabalho com os dois grupos de pesquisa: a formação de redes interpenetradas (ou semi-interpenetradas) de polissiloxano, PVA e polianilina, o objeto de estudo inicial desta tese de doutorado. Após um ano de trabalho, os compósitos de Polissiloxano-Polianilina e Polissiloxano-PVA-Polianilina não apresentaram reatividade para imobilização das enzimas testadas, mas foram capazes de imobilizar uma lectina de *Brosimum gaudichaudii* (Machado, 2007). Por outro lado, o suporte PVA-Polianilina mostrou-se promissor para a imobilização de peroxidase e tripsina.

Este trabalho apresenta a síntese de uma matriz para imobilização de enzimas, consistido de discos de álcool polivinílico-glutaraldeído-polianilina-glutaraldeído, que permitiu a vantagem de remoção imediata da enzima, separando-a do meio de reação. O primeiro capítulo apresenta todos os testes realizados durante o processo de síntese e padronização do suporte, bem como das análises realizadas para a caracterização do material. No segundo capítulo constam os procedimentos realizados e os resultados obtidos para a imobilização covalente da enzima tripsina na superfície do suporte PVAG-PANIG. Finalmente, no terceiro capítulo estão apresentados todos os dados obtidos nos procedimentos de imobilização da enzima *horseradish peroxidase* no suporte PVAG-PANIG.

OBJETIVOS

Geral

Sintetizar compósitos à base de álcool polivinílico e polianilina e estudar suas propriedades como suporte para imobilização de enzimas.

Específicos

- Sintetizar discos de PVA-Glutaraldeído-Polianilina-Glutaraldeído e estudar suas propriedades físico-químicas e ultra-estruturais;

- Imobilizar covalentemente as enzimas peroxidase e tripsina e comparar as características do sistema suporte-enzima imobilizada com aquelas das enzimas nativas;

- Propor diferentes aplicações para os sistemas PVAG-PANIG-peroxidase e PVAG-PANIG-tripsina.

RESULTADOS

CAPÍTULO I - SYNTHESIS AND CHARACTERIZATION OF COMPOSITES OF POLYVINYL ALCOHOL-GLUTARALDEHYDE AND POLYANILINE

A ser submetido após o aceite para publicação dos artigos descritos nos capítulos II e III.

CAPÍTULO I - SYNTHESIS AND CHARACTERIZATION OF COMPOSITES OF POLYVINYL ALCOHOL-GLUTARALDEHYDE AND POLYANILINE

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Abstract

This work presents the results from the synthesis and characterization of a composite from polyvinyl alcohol-glutaraldehyde-polyaniline-glutaraldehyde discs (PVAG-PANIG). Infrared spectra, UV-visible absorption and Themogravimetric Analysis revealed that the composite conserved similar aspects from the PVA (hydrophilicity, hand molding) and PANI (electrocromical properties). Scanning electron microscopy and surface porosity analysis showed PVAG-PANIG is a macroporous material. Electron conductivity was detected at low temperatures. These results, adding of other studies, suggested PVAG-PANIG disc as a support for enzyme-immobilization applications.

Introduction

Polyvinyl alcohol (PVA) is a largely used polymer because its flexibility, hydrophilicity, transparency and cristalinity properties. Despite of these advantages, PVA was employed as polymers stabilizer (Jayasekara et al., 2004; Gangopadhyay et al., 2001), releaser of Paraquat – a pesticide (Alemzadeh & Vossoughi, 2002), model of mass transfer (Han et al., 2008), a chromatographic matrix gel (Sawatsubashi et al., 2007) and as a matrix for protein immobilization (Araújo et al., 1996; Barbosa et al., 2000).

Polyaniline (PANI) is a vast studied polymer and received much attention of the researchers by its electrochemical behavior (MacDiarmid et al., 1985; Asturias et al., 1989), thermal stability (Hagiwara et al., 1988; Wei & Hsueh, 1989), interesting optical properties (Dutta & De, 2007) and low cost of its synthesis (Azevedo et al., 1999). The property of electrons conduction and the capacity to immobilize biomolecules have made polyaniline a component for biosensors (Arora et al., 2007; Singh et al., 2006; Fernandes et al., 2005).

In our laboratory, PVA and PANI were extensively used as a matrix for protein immobilization. Amylase, xantine oxidase (Nadruz Jr. et al., 1996), peroxidase (Fernandes et al., 2003, 2004) and some antigens of tropical diseases were covalently immobilized in these matrices (Carvalho Jr. et al., 1996; Araujo et al., 1996, 1997). Recently, PVA-PANI-Glutaraldehyde discs were used for immobilization of peroxidase (Caramori et al., 2007a) and trypsin (Caramori et al., 2007b). The first system permitted to release of phenolic compounds in aqueous solution, with additional stability by enzyme storage. PVAG-PANIG-trypsin was applied in the production of peptides of various sizes.

In this work we present a synthesis and characterization of PVA-glutaraldehyde discs coated with polyaniline.

Methods

1. Support synthesis

PVA-Polyaniline discs were synthesized according to Carvalho et al. (1996) and Fernandes et al. (2003). Briefly: PVA (Vetec Química Fina, Brazil; 200 mg) was dissolved in distilled water (10 mL) by heating at 65^{o}C and 25% (v/v) of glutaraldehyde (Vetec Química Fina, Brazil; 1.5 mL) was added. This mixture was vigorously stirred during 50 min after which aliquots (20 µL) were introduced into wells of a microplate (Mumc, Denmark) containing 3 M HCl (120 µL). The network of PVA cross-linked with glutaraldehyde (PVAG) was allowed to be formed for 24 h. Afterwards, the discs were treated with 0.61 M ammonium persulphate (Carlo Erba, Italy) per gram of the discs prepared in 2 M HCl for 30 min. Then the treated discs were incubated with 0.44 M aniline (Merck, Germany) per gram of the discs for 60 min to allow the PANI to be formed. The PVAG-PANI discs (1.0 g) were exhaustively washed with 2 M HCl, incubated with 2.5 % (v/v) glutaraldehyde at 60º C and finally washed five times susceptive with 1 M phosphate buffer, pH 7.6 (Fernandes et al., 2003). These discs (PVAG-PANIG) were kept at 4º C in the phosphate buffer.

2. PVAG-PANIG characterization

Spectrophotometry

PVA, PVAG and PVAG-PANIG discs were analyzed by infrared (FT-IR; KBr method) in the spectral range 400-4000 cm^{-1} (Hartman & Braun MB series -

Michelson). Visible-ultraviolet spectrophotometry analysis of the PVAG and PVAG-PANIG was carried out in the range of 250-900 nm with 2 nm intervals (Beckmann DU-70, Beckmann Instruments GmbH, Germany).

Thermal gravimetry (TGA)

Discs of PVAG-PANIG (15 mg) were heated from 23º C and 1,800º C using the following heating rates: 5º C/min in the 23º C-250º C range; 2º C/min in 251º C-700º C and 5º C/min in the 701-1800º C.

Surface Area Analysis

The surface area measurements of the PVAG-PANIG disc were carried out by using accelerated surface area and porosimetry analyzer (ASAP 2010, Micromeritics Corporate Headquarters, USA). The initial mass of the discs was 0.072 g with P/Po equal to 0.20014508 and the pore measurement capacity was of 1.7 at 35 nm.

Scanning electronic microscopy

The discs were firstly dehydrated in acetone solutions from 30% (v/v) to 100% (v/v) and dried at the critical point (Balzers, model CPD 030, England). Samples were fixed on stubs followed by metallization with gold in argon atmosphere. Finally, they were analyzed by scanning electronic microscopy (JEOL JSM-840A, Japan).

Electrical conductivity

PVAG-PANIG discs were submitted to electrical conductivity variation using Keithley 602 electrometer at 40 V and -146^{o}C to 18.7^{o}C (126.6 K to 291.67 K).

Results and Discussion

About 240 discs of 15 mg (PVAG-PANIG) were synthesized using the procedure here described (11.5 mL of the mixture and three microplates) and typical beads are shown in Figure 1, namely, PVAG bead (yellow) and PVAG-PANIG bead (dark green).

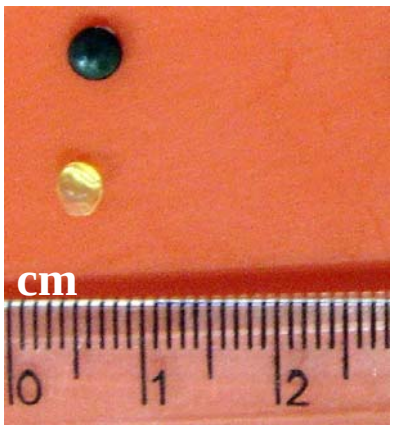

Figure 1 - Beads of PVAG (yellow) and PVAG-PANIG (dark green).

The synthesis of PVAG-PANIG was monitored by infrared spectra in the three stages: PVA, PVAG and PVAG-PANIG (figure 2) when it was observed the pattern of the bands from polyvinyl alcohol, glutaraldehyde and polyaniline. It is possible to observe peaks at the region of the 3400 cm^{-1} in all the stages, from the stretching of -OH from the PVA (Zhang et al., 2006; Bhat et al., 2005). Figure 2C shows a band at 2942 cm^{-1}, related to the presence of CH_2 groups (Kenney & Willcokson, 1966; Bhat et al., 2005). Another band from PVA was found at 1429 cm^{-1} from -OH deformation (figures 2A-C). Additionally, the spectrum of PVA (figure 2A) indicates elevated isotacticity degree on the sample used in this work (1140 cm^{-1}).

Carbonyl bands from glutaraldehyde were observed at the 1700 cm^{-1} region in the figure 2B and 2C (Melo et al., 1999).

The bands from polyaniline in the figure 2C revealed a predominance of 1577 cm^{-1} under 1500 cm^{-1}. This finding, according Melo et al. (1999), indicates that the polyaniline content in the PVAG-PANIG disc appears in the reduced form, named leucoemeraldine. These authors explain these feature based on the hypothesis of redox interactions occurring between glutaraldehyde and polyaniline, yielding the aldehyde groups from the first compound to carboxyl radicals and the quinoid structures from polyaniline to benzoid groups (leucoemeraldine). The HCl doping at 1103 cm^{-1} was also observed in the figure 2C (Kenney & Willcokson, 1966; Melo et al., 1999).

Typical visible-ultraviolet absorbancies for PVA (316 nm, 364 nm, 450 nm e 714 nm), PANI (442 nm) and glutaraldehyde (360 nm) can be observed in the spectrophotometry analysis shown in Figure 3. Some of the PVA peaks were also found by Bhat et al. (2005) and similar PANI absorbance has been reported by Li et al. (2007).

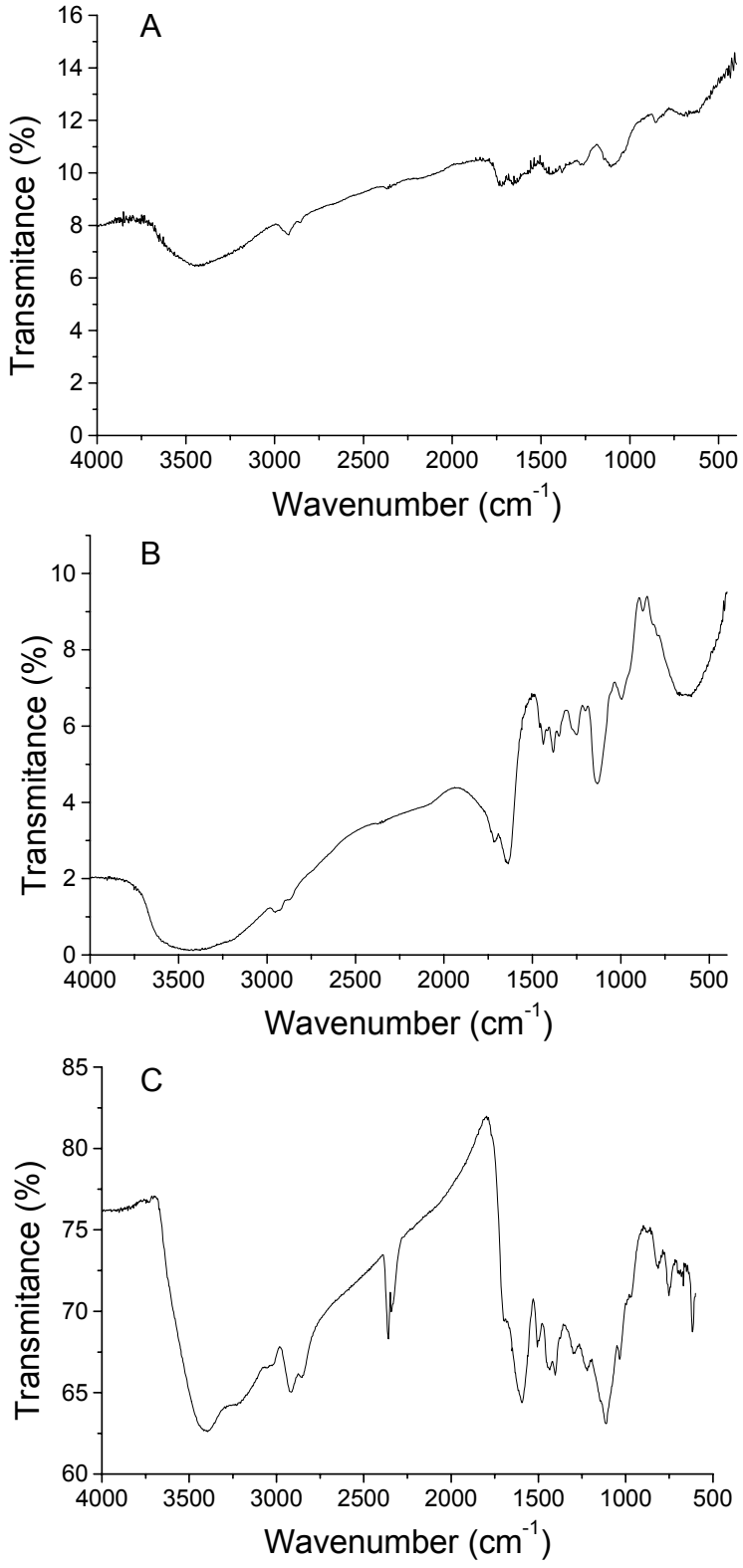

Figure 2 - Infrared spectra of the PVA (A), PVAG (B) and PVAG-PANIG.

Samples were prepared using KBr pellet technique.

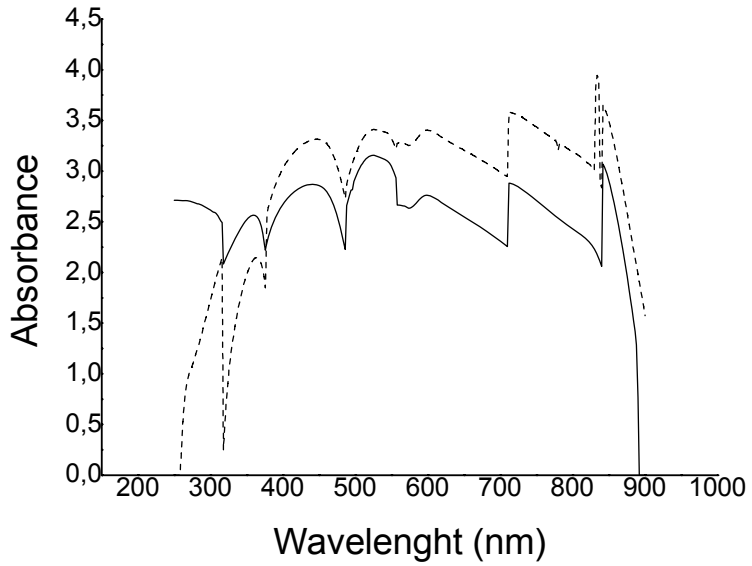

Figure 3 – Visible-ultraviolet spectrophotometry analysis of the PVAG (dash line) and PVAG-PANIG (straight line).

Thermal gravimetry analysis of the PVAG-PANIG is presented in Figure 4. The weight loss was observed in the ranges 50-100ºC and 400-450ºC. The first one can be attributed to the water evaporation from the composite since PVA is a hydrophilic polymer offering to PANI hydroxyl groups able to interact to water molecules. The second loss above 350ºC can be ascribed to the C-C degradation of the PVA and PANI according to Gangopadhyay et al., 2001; Helen et al., 2006; Wei and Hsueh, 1989).

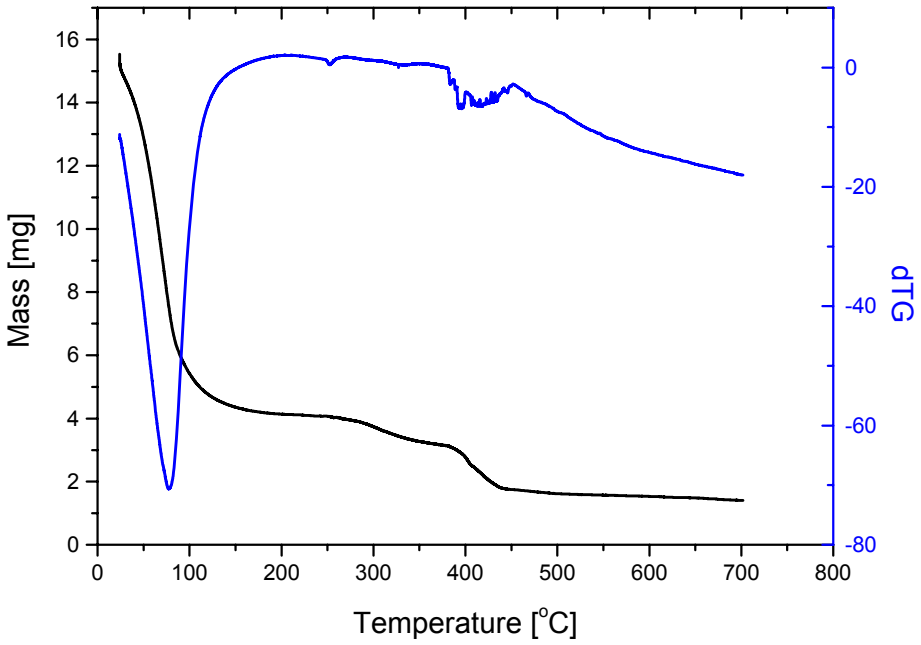

Figure 4 – Thermal gravimetry analysis of the PVAG-PANIG.

Figure 5 presents the results from the porosity analysis of the PVAG-PANIG. Support presented a minor content of pores sizing from 5 to 35 nm diameter (mesopores range) under the analysis condition (0-40 nm). The major adsorved volume content was attributed to the pores with more than 35 nm. Pore volume capacity varied from 0.9 x 10^{-4} cm^3 g^{-1} to 3.58 x 10^{-4} cm^3 g^{-1}.

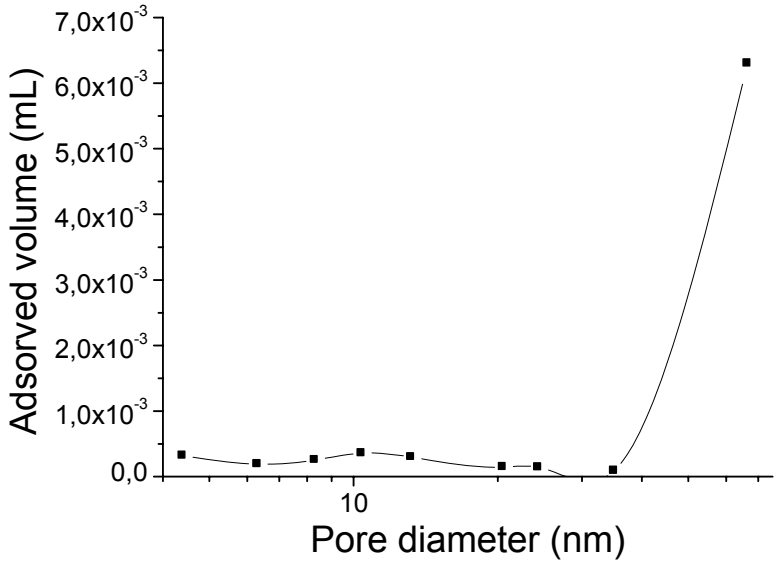

Figure 5 – Pore distribution of the PVAG-PANIG.

PVAG-PANIG adsorption analysis (Figure 6) displayed a behavior comparable to the isotherms of type IV (pores higher than 50 nm) proposed by Brunauer in 1938 (Gregg & Sing, 1982; Teixeira et al., 2001). On the other hand, the pore area measurement of the PVA has been reported to be type I (microporous materials) according to Zhang et al. (2006). Probably, PANI during the synthesis procedure has covered these micropores of the PVA.

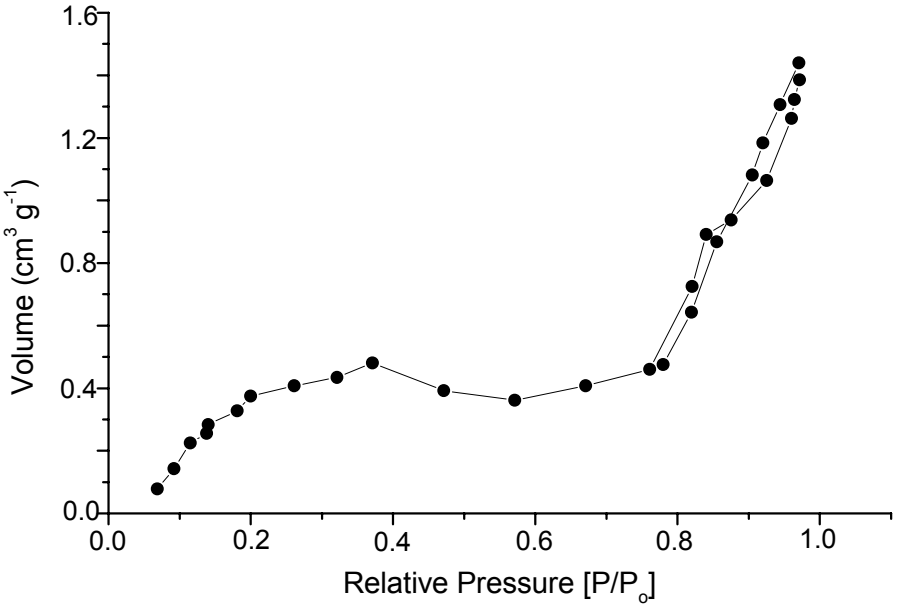

Figure 6 – Isotherm of PVAG-PANIG adsorption.

The absence of micropore in the PVAG-PANIG can also be confirmed by the scanning electronic microscopy (Figure 7) that shows instead of micropores the presence of depressions and protuberances 500 nm apart from each other. According to Gregg and Sing (1982) this is a characteristic of macroporous structure (pores sizing higher than 50 nm). This feature can be an advantage when an enzyme which recognizes voluminous substrate/product is immobilized at the surface of the disc of PVAG-PANIG. Macroporous structure permits the

transit of substrate from the bulk of reaction to the microenvironment of immobilized enzyme, with less mass transfer limitations.

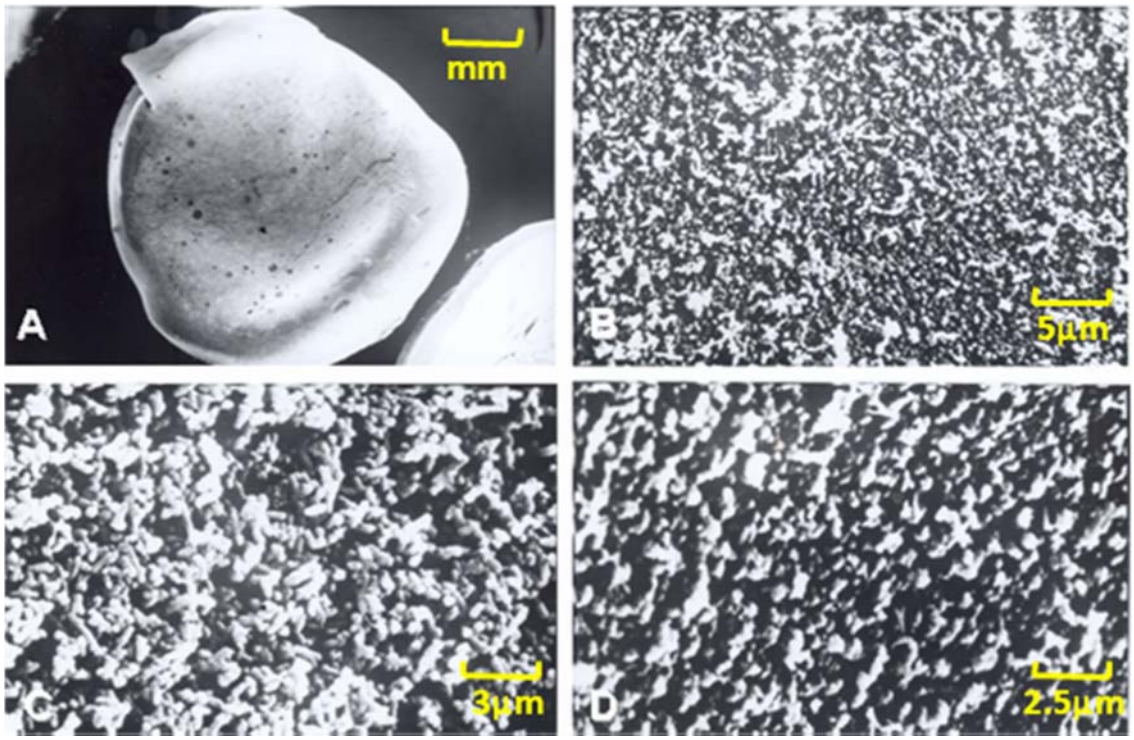

Figure 7. Scanning electronic microscopy of PVAG-PANIG. Magnifications: A – 20x; B – 2000x; C – 6000x; D – 8000x.

Figure 8 presents data related to the influence of the temperature on the electrical resistance of the PVAG-PANIG. No change on the electrical conductivity/resistance was observed in the 120-240 K range (-153 to -73º C). However, after that a dramatic decrease occurred yielding a resistance equal to about 10^6 Ω. Similar conductivity variation influenced by the temperature has been reported for PVA and PANI (Gangopadhyay et al., 2001; Yang et al., 2003; Helen et al., 2006). This phenomenon observed for PANI is explained the HCl evaporation responsible for the conductivity properties of the polymer (Hagiwara et al., 1988; Wei & Hsueh, 1989; Gangopadhyay et al., 2001). In the

case of the PVAG-PANIG, the incubation of the discs in alkaline buffer solution could be responsible for the low conductivity found in this work.

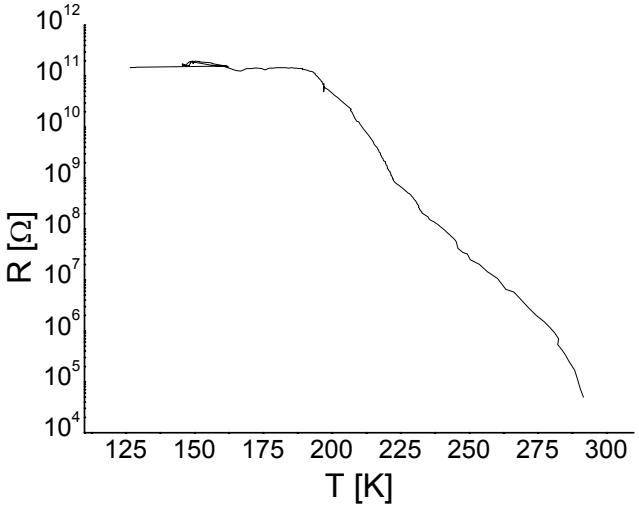

Figure 8 – The influence of the temperature on the electrical resistance of the PVAG-PANIG.

Conclusions

PVAG-PANIG composite discs were synthesized via glutaraldehyde network. Properties from PVA and PANI could be identified by infrared and UV-Vis spectra, and by TG analysis. At room temperature, PVAG-PANIG appears to be a hydrophilic and non-conductive composite. However, surface studies indicated the pattern of recover of PANI on the PVA and appoint to be a promising support for protein immobilization with voluminous/non-voluminous substrate.

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References

ALEMZADEH, I.; VOSSOUGHI, M. Controlled release of paraquat from poly vinyl alcohol hydrogel. *Chemical Engineering and Processing* 41: 707-710, 2002.

AMARAL, T.P**.,** BARRA, G.M.O., BARCIA, F.L., SOARES, B.G., Propriedades de Compósitos Condutivos de Polianilina e Resina Epoxílica. *Polímeros: Ciência e Tecnologia* 11(3): 149-157, 2001.

ARAUJO, A. M.; NEVES JR, M. T.; AZEVEDO, W. M.; OLIVEIRA, G. G.; FERREIRA JR, D. L.; COELHO, R. A. L.; FIGUEIREDO, E. A. P.; CARVALHO JUNIOR, L. B. Polyvinil alcohol-glutaraldehyde network as a support for protein immobilisation. *Biotechnology Techniques* 112: 67-72, 1996.

ARAUJO, A. M.; BARBOSA, G. H. T. S.; DINIZ, J. R. P.; MALAGUENO, E.; AZEVEDO, W. M.; CARVALHO JUNIOR, L. B. Polyvinil alcohol-glutaraldehyde as solid-phase in ELISA for Schistosomiasis. *Revista do Instituto de Medicina Tropical de São Paulo*, 39: 155-158, 1997.

ARORA, K.; PRABHAKAR, N.; CHAND, S.; MALHOTRA, B. D. Ultrasensitive DNA hybridization biosensor based on polyaniline. *Biosensors and Bioelectronics* 23(5): 613-620, 2007.

ASTURIAS, G. E.; MACDIARMID, A. G.; MACCALL, R. P.; EPSTEIN, A. J. The oxidation state of emeraldine base. *Synthetic Metals* 29: E157-E162, 1989.

AZEVEDO, W. M., SOUZA, J. M., MELO, J. V., Semi-interpenetrating polymer networks based on polyaniline and polyvinyl alcohol–glutaraldehyde. *Synthetic Metals* 100(3): 241-248, 1999.

BAHRAMI, S. B.; KORDESTANI, S. S.; MIRZADEH, H.; MANSOORI, P. Poly (vinyl alcohol) – Chitosan Blends: Preparation, Mechanical and Physical Properties. *Iranian Polymer Journal* 12(2): 139-146, 2003.

BARBOSA, G. H. T. S.; SANTANA, E. M.; ALMEIDA, A. M. P.; ARAUJO, A. M.; FATIBELLO, O.; CARVALHO JUNIOR, L. B. The use of filter paper plasticized with polyvinvyl alcohol glutaraldehyde in ELISA. *Brazilian Journal of Medical and Biological Research* 33: 823-827, 2000.

BHAT, N. V., NATE, M. M., KURUP, M. B., BAMBOLE, V. A., SABHARWAL, S., Effect of γ-radiation on the structure and morphology of polyvinyl alcohol films. *Nuclear Instruments and Methods in Physics Research B* 237: 585-592, 2005.

BORA, U., SHARMA, P., KANNAN, K., NAHAR, P., Photoreactive cellulose membrane—A novel matrix for covalent immobilization of biomolecules**.** *Journal of Biotechnology* 126(2):220-229*,* 2006*.*

CAVALCANTE, A. H. M., CARVALHO JR., L. B., CARNEIRO-DA-CUNHA, M. G. Cellulosic exopolysaccharide produced by *Zoogloea* sp. as a film support for trypsin immobilization. *Biochemical Engineering Journal* 29(3): 258-261, 2006.

DUTTA, K.; DE, S. K. Optical and nonlinear electrical properties of SnO_2–polyaniline nanocomposites. *Materials Letters* 61(27): 4967-4971, 2007.

FERNANDES, K. F.; LIMA, C. S.; PINHO, H.; COLLINS, C. H. Immobilization of Horseradish Peroxidase onto Polyaniline Polymers. *Process Biochemistry* 38(9): 1379-1384, 2003.

FERNANDES, K. F.; LIMA, C. S.; LOPES, F. M.; COLLINS, C. H. Properties of horseradish peroxidase immobilised onto polyaniline. *Process Biochemistry* 39, 957-962, 2004.

FERNANDES, K. F.; LIMA, C. S.; LOPES, F. M.; COLLINS, C. H. Hydrogen peroxide detection system consisting of chemically immobilised peroxidase and spectrometer. *Process Biochemistry* 40(11): 3441-3445, 2005.

GANGOPADHYAY, R.; DE, A.; GHOSH, G. Polyaniline-poly(vinyl alcohol) conducting composite: material with easy processability and novel application potential. *Synthetic Metals* 123: 21-31, 2001.

GREGG, S. J., SING, K. S. W., Adsorption, Surface Area and Porosity. Academic Press: London, 1982, p. 41.

HAGIWARA, T.; YAMAURA, M.; IWATA, K. Thermal stability of polyaniline. *Synthetic Metals* 25: 243-252, 1988.

HAN, M.; ZHAO, B.; ZHANG, X. M.; ZHANG, W. J. Model of mass transfer in polyvinyl alcohol membrane for isopropanol/water mixture *Chemical Engineering and Processing: Process Intensification* 47(2): 245-250, 2008.

HELEN, M.; VISWANATHAN, B.; MURTHY, S. S. Fabrication and properties of hybrid membranes based on salts of heteropolyacid, zirconium phosphate and polyvinyl alcohol. *Journal of Power Sources* 163: 433-439, 2006.

HO, W. O., ATHEY, D. MCNEIL, C. J. Amperometric detection of alkaline phosphatase activity at a horseradish peroxidase enzyme electrode based on activated carbon: potential application to electrochemical immunoassay. *Biosensors and Bioelectronics*, 10(8): 683-691, 1995.

HONG, J., GONG, P., XU, D., DONG, L., YAO, S., Stabilization of α-chymotrypsin by covalent immobilization on amine-functionalized superparamagnetic nanogel. *Journal of Biotechnology*, 2007, 128(3): 597-605.

JAYASEKARA, R.; HARDING, I.; BOWATER, I.; CHRISTIE, G.B.Y.; LONERGAN, G.T. Preparation, surface modification and characterization of solution cast starch PVA blended films. *Polymer testing* 23: 17-27, 2004.

KENNEY, J.F., WILLCOKSON, G.W., *Journal of Polymer Science*, 1966, A-I 4: 679.

KIM, D.H., NA, S.K., PARK, J.S., YOON, K.J., lHM, D.W., Studies on the preparation of hydrolyzed starch-g-PAN (HSPAN)/PVA blend films – Effect of the reaction with epichlorohydrin. *European Polymer Journal*, 2002, 38: 1199–1204.

LI, C., YOSHIMOTO, M., FUKUNAGA, K. NAKAO, K. Characterization and immobilization of liposome-bound cellulase for hydrolysis of insoluble cellulose. *Bioresource Technology*, 2007, 98(7): 1366-1372.

MACDIARMID, A. G.; CHIANG, J. C.; HALPERN, M.; HUANG, W. S.; UM, S. L.; SOMASIRI, N. L. D.; WU, W.; YANIGER, S. Polyaniline: Interconversion of metallic and insulating forms. *Molecular Crystals & Liquid Crystals* 121: 173-180, 1985.

MELO, J.V.; BELLO, M.E.; AZEVÊDO, W.M.; SOUZA, J.M.; DINIZ, F.B. The effect of glutaraldehyde on the electrochemical behavior of polyaniline. *Electrochimica acta* 44: 2405-2412, 1999.

NADRUZ JR, W.; MARQUES, E. T. A. ; AZEVEDO, W. M. ; LIMA FILHO, J. L. ; CARVALHO JUNIOR, L. B. Immobilized xanthine oxidase on polyaniline silicone composite. *Brazilian Journal of Medical and Biological Research* 29: 347-350, 1996.

ROMANO, R.C.O.; PANDOLFELLI, V.C. Obtenção e propriedades de cerâmicas porosas pela técnica de incorporação de espuma. *Cerâmica* 52: 213-219, 2006.

SALVINI, V.R.; GARCIA, J.R.; OLIVEIRA, I.R.; PANDOLFELLI, V.C. Agente espumante e seus efeitos nas propriedades físicas de alumina porosa. *Cerâmica* 52: 57-62, 2006.

SAWATSUBASHI, T.; TSUKAHARA, C.; BABA, K.; OHI, E.; SHINODA, A.; MIURA, N. Development of new-type rapid analysis technology of polychlorinated biphenyls by using liquid chromatographic clean-up material (polyvinyl alcohol gel). *Journal of Chromatography* A 1177(1): 138-149, 2008.

SINGH, S.; SOLANKI, P. R.; PANDEY, M. K.; MALHOTRA, B. D. Cholesterol biosensor based on cholesterol esterase, cholesterol oxidase and peroxidase

immobilized onto conducting polyaniline films. *Sensors and Actuators B: Chemical* 115(1): 534-541, 2006.

TEIXEIRA, V.G., COUTINHO, F.M.B., GOMES, A.S., Principais Métodos de Caracterização de Porosidade de Resinas à Base de Divinilbenzeno. *Química Nova*, 2001, 24(6): 808-818.

WEI, Y.; HSUEH, K.F. Thermal analysis of chemically synthesized polyaniline and effects of thermal aging on conductivity. *Journal of Polymer Science: Part A: Polymer Chemistry* 27: 4351-4363, 1989.

YANG, C.C.; LIN, S.J.; HSU, S.T. Synthesis and characterization of alkaline polyvinyl alcohol and poly(epichlorohydrin) blend polymer electrolytes and performance in electrochemical cells. *Journal of Power Sources* 122: 210-218, 2003.

ZHANG, S.J., YU, H.Q., FENG, H.M., PVA-based activated carbon fibers with lotus root-like axially porous structure. *Carbon*, 2006, 44: 2059-2068.

CAPÍTULO II - IMMOBILIZED HORSERADISH PEROXIDASE ON DISCS OF POLYVINYL ALCOHOL GLUTARALDEHYDE COATED WITH POLYANILINE

Data de submissão: 29 de maio de 2007

Chemosphere (Switzerland)

Impact factor (2006): 2.442

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Immobilized horseradish peroxidase on discs of polyvinyl alcohol glutaraldehyde coated with polyaniline

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Abstract

Here it is described an immobilized horseradish peroxidase (HRP) capable to oxidize phenolic compounds that are usually present in the water effluents from textile industry. Discs of the network polyvinyl alcohol-glutaraldehyde (PVAG) were synthesized and coated with polyaniline (PANI) by a simple and inexpensive procedure. HRP was then covalently fixed onto these discs using glutaraldehyde as a chemical arm (HRP-PVAG-PANIG disc). The best conditions for the immobilization were: 1.0 mg ml^{-1} of protein, 60 min and pH 5.5. The optima pH and temperature values for the soluble and HRP-PVAG-PANIG disc were 7.0; 4.5; 40^{o}C and 45^{o}C, respectively. The soluble enzyme lost all activity after incubation at 70^{o}C for 15 min whereas the HRP-PVAG-PANIG disc retained about half of the initial activity. Apparent Michaelis constants for the soluble and HRP-PVAG-PANIG disc were estimated in 5.47 ± 0.71 mM and 12.07 ± 1.35 mM, respectively. The same HRP-PVAG-PANIG disc was used three times without any activity lost and retained 25% of the initial activity at the 7th use despite the presence of H_2O_2, its substrate and well known protein denaturing agent. HRP-PVAG-PANIG disc retained approximately 80% and 60% of its initial activity after 60 and 80 days of storage, respectively. Resorcinol, m-cresol, catechol, pyrogallol, α-naphthol, β-naphthol and 4, 4´-diaminodiphenyl benzidine were efficiently oxidized by the HRP-PVAG-PANIG disc (from about 70% to 90%) and it was less efficient towards aniline, phenol and 2-nitrosonaphthol.

Keywords: horseradish peroxidase; polyvinyl alcohol; polyaniline; phenolic compounds; immobilization.

1. Introduction

Peroxidases (Enzyme Commission number 1.11.1.7) are a huge family of heme-containing enzymes that catalyze oxidation and reduction reactions of large families of substrates. Their broad substrate specificity, polyfunctionality and availability from different sources allow applying them to various biotechnological processes. Oxidative destruction of colored compounds is significantly stimulated by peroxidase and is of practical interest for decoloration processes used in food, textile and paper industry as well as in washing powders. For instance, they are capable to oxidize many compounds bearing the functional group R-N=N-R' (azo-dyings), in which R and R" can be either aryl (aromatic) or alkyl (containing only carbon and hydrogen atoms arranged in a chain) groups. These compounds are widely used in the textile industry and discarded in water collections. They are recognized as pollutants because they have mutagenic and carcinogenic effects and can also alter biological cycles (Houk, 1992; Chung and Cerniglia, 1992). Peroxidases are also extensively used in clinical and immunological analysis. For instance, they are used in glucose (Sanz et al., 2007), cholesterol (Yu et al., 1997) and urea (Mizutani et al., 2000) determinations, procedures involving H_2O_2 releasing. They are also used to label antigen/antibody in enzyme linked immunosorbent assays, abbreviated as ELISA. Horseradish peroxidase (HRP) is the most popular among the sources of peroxidase and can use a variety of organic compounds as electron donors and acceptors.

Immobilized peroxidase obtained from bitter gourd (*Momordica charantia*) has been reported to present several advantages than the free

enzyme in the treatment of wastewater contaminated with phenols (Akhtar and Husain, 2006). Immobilized *Saccharum spontaneum* peroxidase (Shaffiqu et al., 2002) and potato polyphenol oxidase (Khan and Husain, 2007) have also been proposed to decompose textile dyes.

Here it is described the immobilization of HRP on discs of a polyvinyl alcohol glutaraldehyde network (PVAG) coated with polyaniline (PANI), using glutaraldehyde to link the enzyme to the composite (PVAG-PANIG). Previously, in our laboratories HRP was successfully immobilized on powder of PANIG (Fernandes et al., 2003; Fernandes et al., 2004) and discs of PVAG were used for protein immobilization (Araújo et al, 1996; Carvalho et al., 1996; Barbosa et al., 2000). The combined use of both procedures was investigated to propose an alternative immobilized HRP preparation to be used in the decolorizing and detoxification of textile synthetic dyes. Some features affecting the immobilization procedure and properties of this derivative were then investigated.

2. Material and Methods

2.1. Materials

All reagents were purchased from Vetec Química Fina Ltda. (São Paulo, Brazil), except the horseradish peroxidase (HRP) which was kindly provided by Dr. Elba Bon (Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil).

2.2. PVAG-PANIG discs synthesis

The PVAG-PANIG disc synthesis was based on procedures described by Carvalho et al. (1996) and Fernandes et al. (2003). Briefly: 2% (w/v) polyvinyl alcohol (10 mL) was dissolved at 65^{o}C and 2.5% (v/v) glutaraldehyde (1.5 mL) was added. Aliquots (20 µL) of this mixture were transferred to the 96 wells of a microplate containing 3 M HCl (120 µL) and kept at 25^{o}C for 24h, after which the discs were formed (PVAG synthesis). Afterwards, the discs were removed and incubated with 0.61 M ammonium persulphate, prepared in 2 M HCl, for 30 min. Then they were further incubated with 0.44 M aniline for 60 min (PVAG coating with polyaniline: PVAG-PANI). Finally, the PVAG-PANI discs were incubated with 2.5% (v/v) glutaraldehyde for 60 min and exhaustively washed with 0.1 M phosphate buffer, pH 6.0 (PVAG-PANIG synthesis). This process above described yielded 240 discs (wet) weighting 15 about mg each.

2.3. Horseradish peroxidase immobilization

One disc of PVAG-PANIG was incubated with HRP (1.0 mL containing 12,500 U mL^{-1}; 7 µg mL^{-1} prepared in the appropriate buffer) for 60 min under orbital shaker (80 rpm) at 4^{o}C. Afterwards, the immobilized enzyme on the HRP-PVAG disc, from now on abbreviated as HRP-PVAG-PANIG, was washed 3-folds with 0.1 M sodium acetate buffer, pH 5.0, and twice 1 M NaCl and twice with the same buffer. No HRP activity was detected in the final washing buffer, which demonstrated that no physically adsorbed enzyme remained in the derivative.

2.4. Enzyme determinations

The soluble and immobilized HRP were established according to Halpin et al (1989) using pyrogallol and H_2O_2 as substrates. Briefly: either one disc of HRP-PVAG-PANIG or 0.1 mL of soluble HRP (874 U mL^{-1}) was incubated at room temperature (28°C) with 0.5 mL of 12.69 mM pyrogallol (0.5 mL), 5 mM H_2O_2 (0.25 mL) prepared in 0.1 sodium acetate buffer, pH 4.5, or 0.1 M sodium phosphate buffer, pH 7.0 (1.4 mL) and after one min (soluble HRP) or 10 min (HRP-PVAG-PANIG) the formed product (purpurogallin) was spectrophotometrically established at 420 nm (Ultrospec 2000, GE Health Care). A unit of enzyme activity (U) was defined as the amount of enzyme capable to oxidize one micromole of pyrogallol per minute under experimental conditions (ε = 961 M cm^{-1}).

2.5. Immobilization optimization conditions

The HRP-PVAG-PANIG disc was synthesized as above described varying the enzyme concentration (0.502-0.912 µg mL^{-1}), incubation time (30-210 min) and pH (4.0-5.5 using 0.1 M sodium acetate buffer and 6.0-7.5 using 0.1 M sodium phosphate buffer), under orbital shaker (80 rpm) at 4°C. Afterwards, the derivative was washed 3-folds with 0.1 M sodium acetate buffer, pH 5.0, and twice 1 M NaCl and twice with the same buffer. Finally, the activity of the disc was determined as described above. All these experiments were performed in triplicates and the results expressed as mean ± standard deviation.

2.6. Immobilized horseradish peroxidase properties

The following properties of the soluble and immobilized HRP (preparation synthesized under the best conditions) were investigated: the time course of the pyrogallol oxidation, the activity of the preparations as above described at pH varying from 3.5 to 7.0 (3.5-5.5 using 0.1 M acetate buffer and 6.0-7.0 using 0.1 M sodium phosphate buffer) and at temperatures varying from 30º C to 60º C. The thermal stability of both enzymes was established by incubating the preparations at 50º C and 70º C. Samples were then withdrawn at time intervals (15-45 min) and after 37º C equilibration their activities were determined as described. Kinetics was evaluated by assaying the preparation activities at pyrogallol concentrations varying from 4 to 48.7 mM. The reuse of the immobilized enzyme was evaluated by assaying the same preparation seven times and washing it with buffer between each procedure. Finally, the shelf life was analyzed by keeping several HRP-PVAG-PANIG discs at 4º C and assaying the stored disc activity every seven days up to 80 days. The 100% value was established as the activity at the initial incubation time. All these experiments were performed in triplicates and the results expressed as mean ± standard deviation.

2.7. Treatment of phenolic compounds by the soluble and HRP-PVAG_PANIG disc

The soluble enzyme (0.1 mL) and HRP-PVAG-PANIG (01 disc) containing both similar activity (about 90 U) were incubated at 37º C for 1.5 h with 0.1 mM sodium acetate buffer (2.0 mL), pH 4.5, containing 0.75 mmol H_2O_2 and 1 mM of the following phenolic compounds: resorcinol; m-cresol; catechol;

pyrogallol; aniline; phenol; α-naphthol; β-naphthol; 4,4´-diaminodiphenyl benzidine and 2-nitrosonaphthol. Afterwards, the remaining phenolic compounds were spectrophotometrically (750 nm) established according to Lowry et al. (1951) and compared with the amount found in the untreated phenolic solutions (100%). All these experiments were performed in triplicates and the results expressed as mean ± standard deviation.

3. Results and discussion

The PVAG-PANIG disc synthesis is carried out in two steps: firstly, a network of polyvinyl alcohol molecules is formed using glutaraldehyde as an arm under acid catalysis (Araújo et al., 1996) yielding the disc, which is coated with polyaniline chemically synthesized from aniline (Fernandes et al., 2003). The PVAG and PVAG-PANIG forms of the disc are shown in Figure 1.

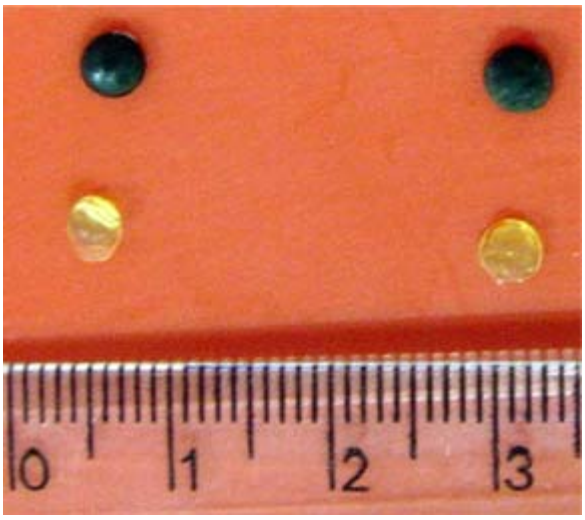

Figure 1 – DISC OF POLYVINYL ALCOHOL-GLUTARALDEHYDE – PVAG (yellow) and coated with polyaniline – PVAG-PANIG (dark-green).

Thus the HRP is covalently fixed onto the PVAG-PANIG disc via glutaraldehyde. It is important to notice that the physical shape of the PVAG

occurs depending on the template used during the synthesis. Here, the wells of a microplate were used and therefore discs were obtained. However, PVAG was already used for plasticizing filter paper which was used as a matrix for protein immobilization (Barbosa et al., 2000).

Figure 2 shows the relationship between the offered HRP and the immobilized enzyme expressed in terms of retained activity and protein per disc, and activity per mg of retained protein (specific activity). It is worthwhile to notice that the PVAG disc itself has carbonyl groups available (Carvalho et al, 1996; Araújo et al, 1996) for the HRP immobilization but the glutaraldehyde treated PANI coat would increase these groups as demonstrated by the increase of the HRP-PVAG-PANIG activity/disc compared to that found for the HRP-PVAG preparation (Figure 2A). This is also reflected by the difference on the specific activity between them (Figure 2B). Intriguingly, the difference in terms of retained of protein is negligible (Figure 2B). Therefore, one can admit that the PANI coating provides some advantage to the PVAG disc as far as the HRP immobilization is concerned. According to this same result there is a linear relationship between the offered HRP and the catalytically active immobilized HRP per disc (Figure 2A). However, this correlation is not followed by the specific activity (Figure 2B) which showed a hyperbolic curve suggesting that there is a limit after which inactive enzyme molecules are immobilized. Steric hindrance due to overloading could be probably attributed to this effect. To avoid this phenomenon (steric hindrance) all experiments to characterize the immobilized enzyme were carried out using a preparation synthesized with 7 μg mL^{-1} of the soluble HRP instead 1 mg mL^{-1}.

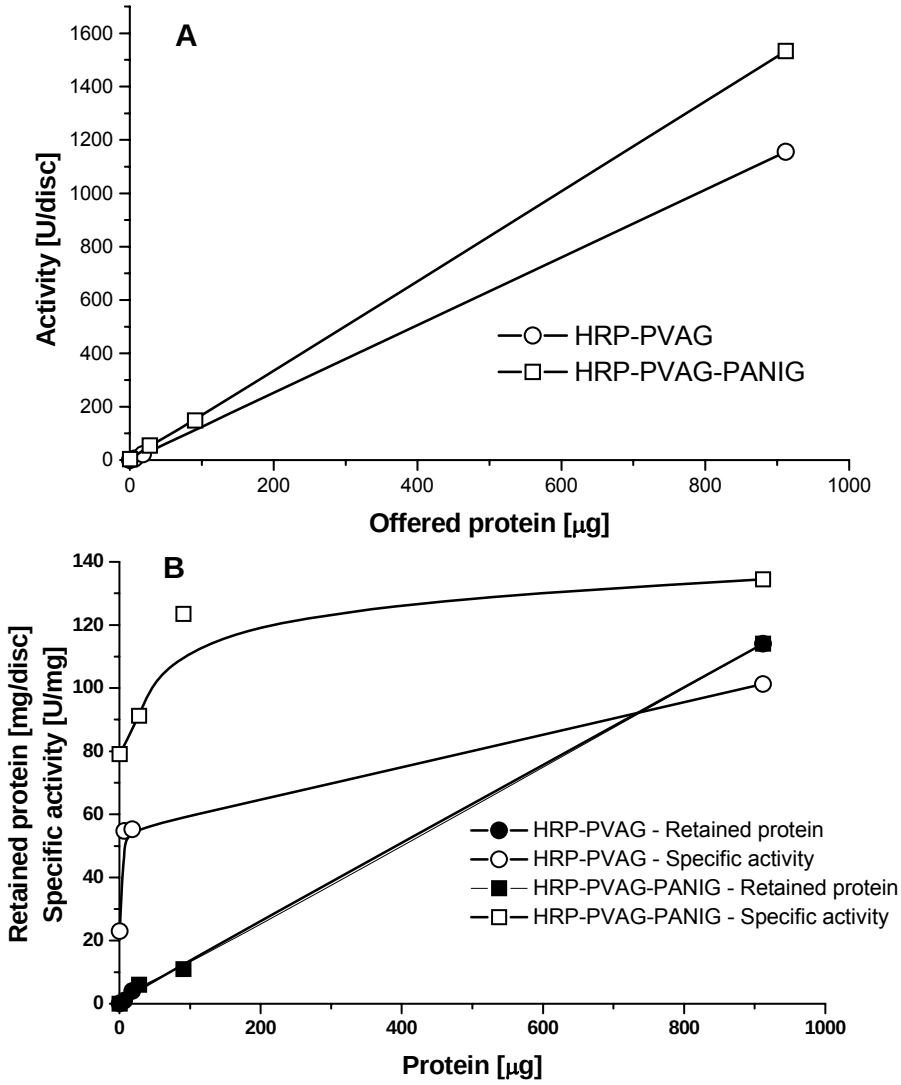

Figure 2 – RELATIONSHIP BETWEEN OFFERED AND RETAINED ACTIVITY (A), PROTEIN (B) AND SPECIFIC ACTIVITY (B) OF THE IMMOBILIZED HRP ON THE PAVG-PANIG DISC. Increasing amounts of HRP were incubated with the PVAG-PANIG disc for 60 min at 4ºC, in 0.1 M sodium phosphate buffer, pH 7.0. After washing the HRP-PVAG-PANIG disc its activity was established and retained protein estimated by the difference between offered protein and that found in the washing solutions. Data are expressed as mean ± standard deviation of triplicates.

Figure 3A shows that 60 min is the best incubation time for the HRP-PVAG-PANIG synthesis, after which longer incubation does not increase the catalytically active immobilized enzyme. This time depends on the support and/or chemical groups involved such as those reported by Rojas-Melgarejo et

al. (2004a) for HRP immobilization on several esters of carbohydrate (2h-25h) and by Liu et al. (2006) on polypropylene membranes (25h).

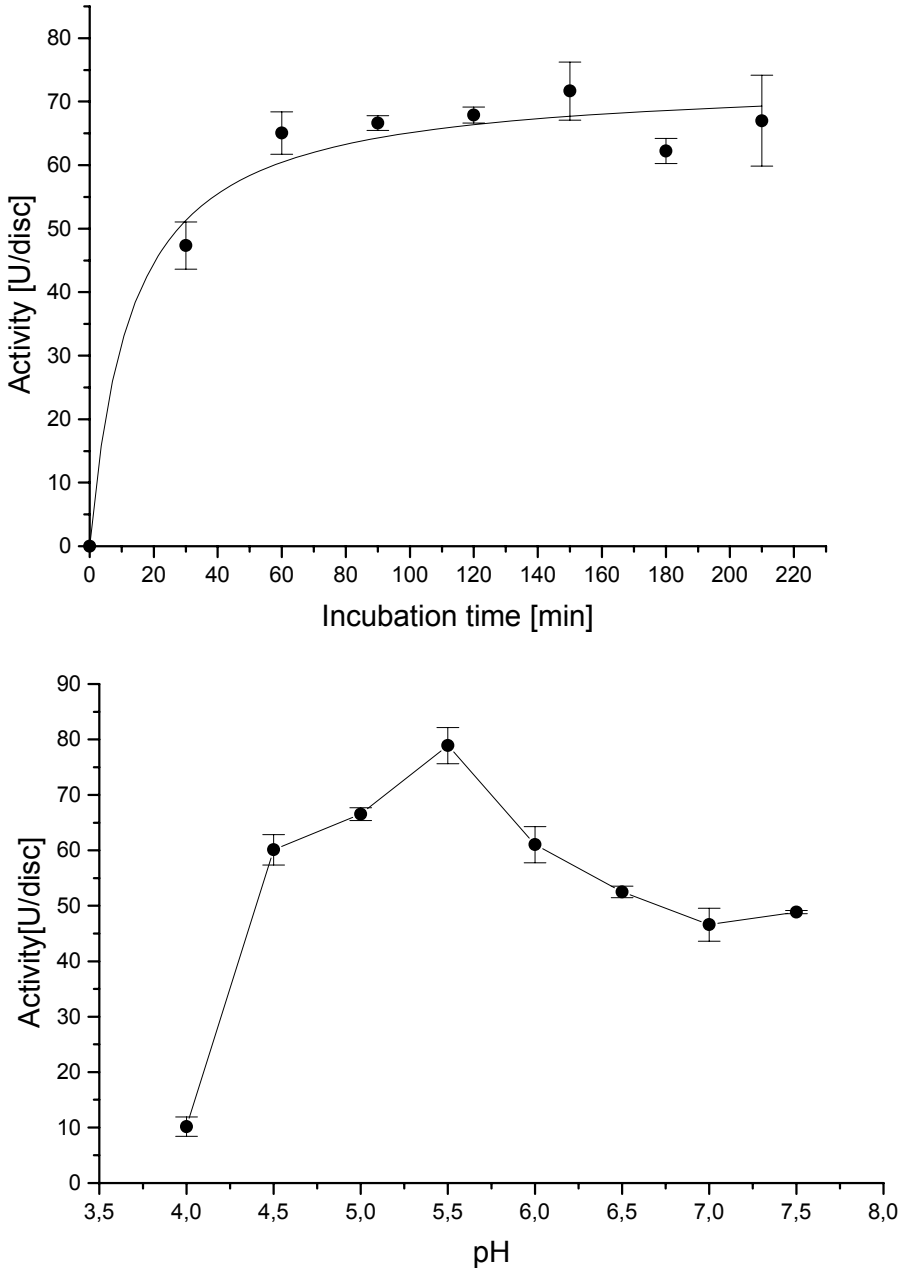

Figure 3 – OPTIMA TIME (A) AND pH (B) FOR THE IMMOBILIZATION OF THE SOLUBLE HRP ON THE PVAG-PANIG. Samples of the soluble enzyme were incubated with the discs at 4ºC, either in 0.1 M sodium phosphate buffer, pH 7.0 during the indicated times or at the indicated pH (0.1 M sodium acetate buffer: 4.0-5.5; 0.1 M sodium phosphate buffer: 6.0-7.0) for 1 h at 4ºC. After washing the HRP-PVAG-PANIG discs their activities were established as describe in Methods. Data are expressed as mean ± standard deviation of triplicates.

The best pH immobilization value of 5.5 for the HRP immobilization on PVAG-PANIG is shown in Figure 3B. This pH value also depends on the support and/or chemical groups involved. Fernandes et al. (2003) immobilizing HRP on PANIG reported a different optimum immobilization pH (6.0). This discrepancy can be probably attributed to the PVAG influence.

The time course of the pyrogallol oxidation catalyzed by the soluble and HRP-PVAG-PANIG disc showed that under the experimental conditions established (item 2.4) a linear curve (first order kinetics) was attained at the 1st and 10th minute, respectively (data not shown). Furthermore, all substrate was oxidized after 30 min of incubation by using both preparations.

The pH profiles for the soluble and HRP-PVAG-PANIG activities are displayed in Figure 4A. Higher pH values than 7.0 were not studied because of the pyrogallol “autoxidation”. There is a markedly difference of the pH influence on the enzyme activities. The immobilized enzyme presents optimum pH value at 4.5 whereas the soluble form at approximately 7.0 as already reported in the literature (Chance and Maehly, 1955). Fernandes et al. (2004) reported a value of 7.0 for the HRP immobilized on PANI. Negatively and positively charged matrices are known to displace the pH profile toward alkaline and acid pH values, respectively, as compared to the native enzymes, at low ionic strength. This left shift of optimum pH for the HRP-PVAG-PANIG suggests that the PVAG-PANIG presents positive charges on its surface. Li and Townshend (1998) also reported different optimum pH for the HRP activity before (7.0) and after immobilization (5.8) on polytetrafluoretilene tubing.

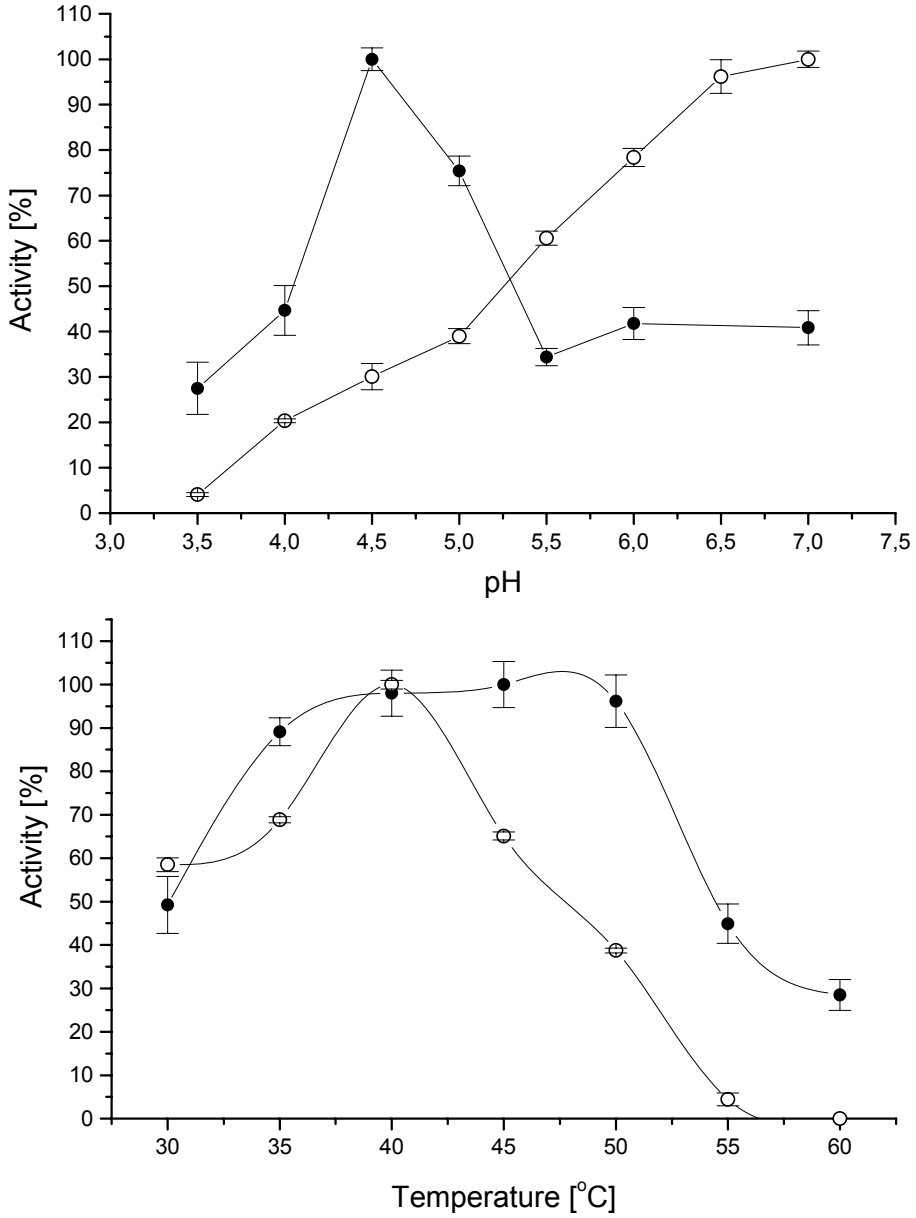

Figure 4. INFLUENCE OF THE pH (A) and TEMPERATURE (B) ON THE SOLUBLE (○) AND HRP-PVAG-PANIG (●). Soluble HRP (0.1 mL) or one disc of HRP-PVAG-PANIG was incubated with 12.69 mM pyrogallol (0.5 mL), 5 mM H_2O_2 (0.25 mL) either at the indicated pH (0.1M acetate buffer: 3.5-5.5; 0.1M sodium phosphate buffer: 6.0-7.0), 28º C, or at the indicated temperatures, pH 4.5. Then after 01 min (soluble) or 10 min (immobilized) aliquots were withdrawn and the formed product (purpurogallin) spectrophotometrically established at 420 nm. Data are expressed as mean ± standard deviation of triplicates.

The effect of the temperature on the soluble and HRP-PVAG-PANIG is shown in Figure 4B and optimal temperatures were found to be 40°C and 40-50°C, respectively. The decays of the descending arms of the curves denote

different effect of the temperature on the soluble and immobilized enzyme. No activity was detected for the soluble enzyme assay at 60^{o}C whereas the immobilized enzyme assay still presented some activity (third of that at 50^{o}C). Similar results were reported by El-Essi et al. (1997) and Lai and Lin (2005) for HRP immobilized on sol-gel matrices and porous glass, respectively. Further evidences for the higher thermal stability for the HRP-PVAG-PANIG can be seen in Figure 5 which showed that the soluble enzyme activity decreased faster than the immobilized preparation when both were incubated at 50^{o}C and 70^{o}C. The soluble enzyme lost all activity after 15 min at 70^{o}C whereas the immobilized one retained about 20% of the initial activity. Increase of the thermal resistance after HRP immobilization has already been reported in the literature (Miland et al., 1996; Bora et al., 2006; Rojas-Melgarejo et al., 2004a; Musthapa et al. 2004).

The action of the soluble and HRP-PVAG-PANIG on pyrogallol followed the Michaelis-Menten kinetics. Values of apparent Michaelis constants were estimated in (mean ± standard deviation) 5.47 ± 0.71 mM and 12.07.± 1.35 mM for the soluble and immobilized HRP (apparent value), respectively. This difference showed to be statistically different ($t = 1.92$ and $p=0.03$). Conformational/steric, partitioning, diffusional/mass-transfer and microenvironmental effects are well known to influence on the immobilized enzyme kinetics. Kinetics parameters, particularly, the apparent Km, can provide the degree of these interferences when compared to that calculated for the soluble enzyme. This apparent constant usually increases as the result of impairment of the enzyme action under those effects.

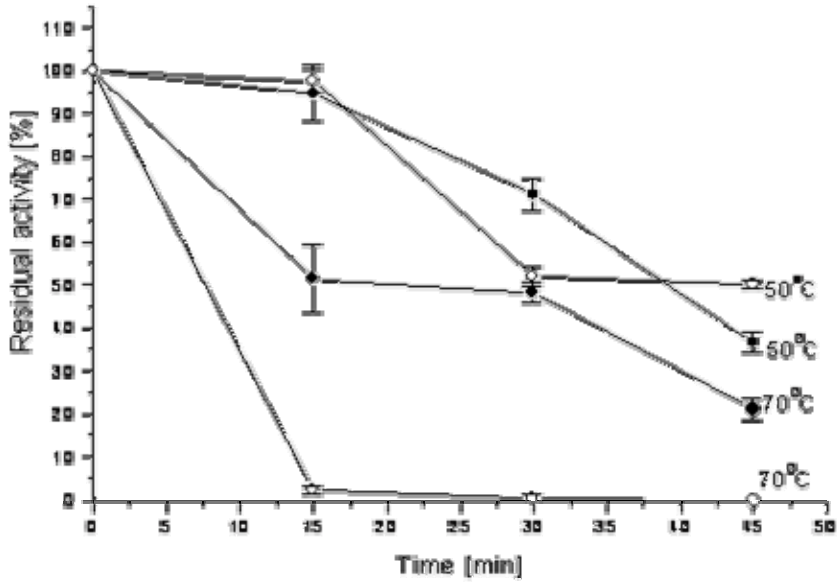

Figure 5. THERMAL STABILITY AT 50ºC AND 70ºC FOR THE SOLUBLE (○) AND HRP-PVAG-PANIG (●). Soluble or discs of HRP-PVAG-PANIG were incubated at 50º C and 70º C. Samples of soluble (0.1 mL) or disc were withdrawn at the indicated times, equilibrated at 37º C and incubated with 12.69 mM pyrogallol (0.5 mL), 5 mM H_2O_2 (0.25 mL) at pH 4.5, at 37º C. Then after 01 min (soluble) or 10 min (immobilized) aliquots were withdrawn for the formed product (purpurogallin) spectrophotometric determination at 420 nm. Data are expressed as mean ± standard deviation of triplicates.

The reuse of the HRP-PVAG-PANIG is presented in Figure 6A. The same disc was used three times without activity lost and retained 25% of the initial activity at the 7th use. It is worthwhile to draw attention to the fact that this preparation was incubated with H_2O_2 one of its substrate which is well known protein denaturing agent. The HRP derivatives (aluminum-pillared interlayered clay) synthesized by Cheng et al. (2006) showed to be not reusable and Akhtar et al (2005) reported poorer performance reusing immobilized bitter gourd peroxidase on Sephadex G-50. The storage stability (shelf life) of the HRP-PVAG-PANIG was investigated for 80 days at 4^{o}C in 0.1 M sodium acetate buffer and Figure 6B resumes the results. The immobilized enzyme derivative retained approximately 80% and 60% of its initial activity after 60 and 80 days of

storage, respectively. This behavior is superior than those reported by Cheng et al. (2006) and Rojas-Melgarejo et al. (2004b).

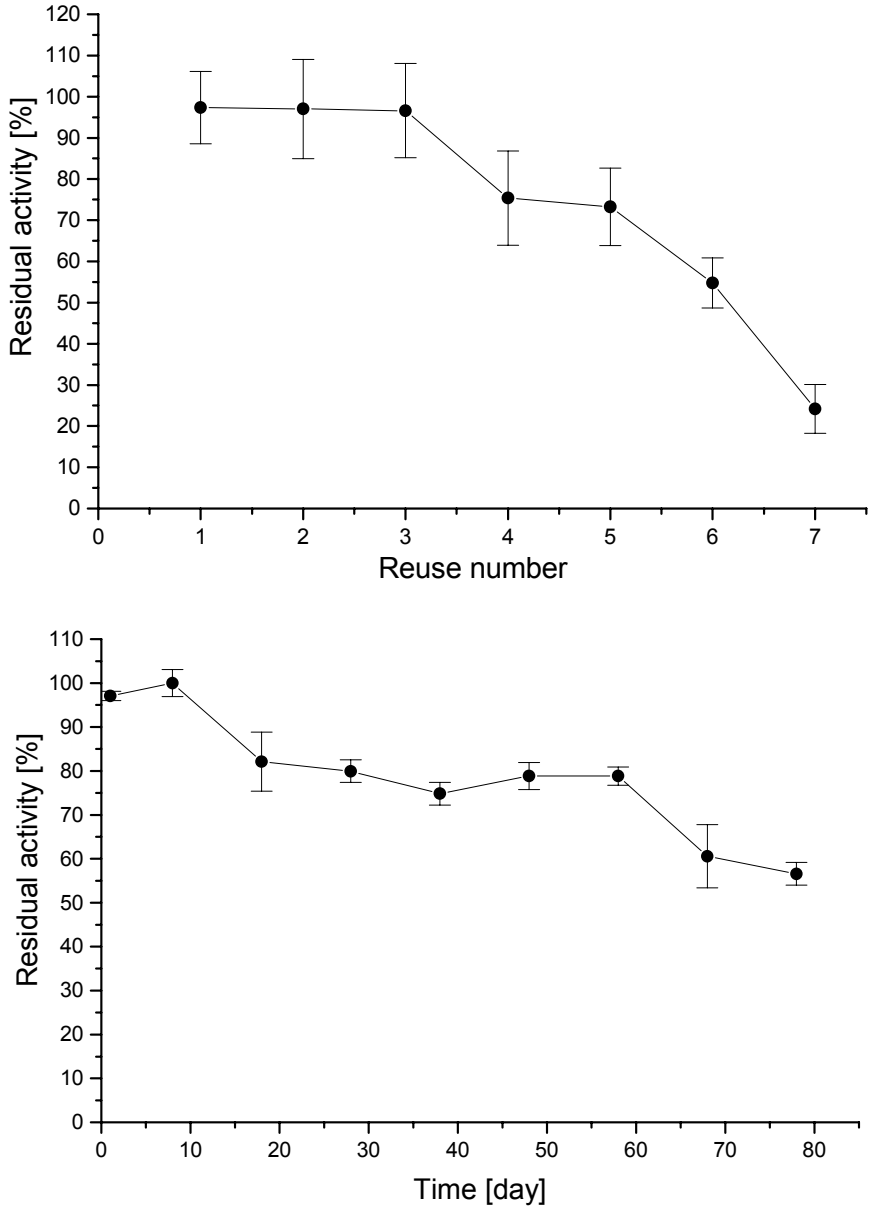

Figure 6. REUSE (A) AND SHELF LIFE (B) OF HRP-PVAG-PANIG. The reuse of HRP-PVAG-PANIG was established by incubating the disc with 12.69 mM pyrogallol (0.5 mL), 5 mM H_2O_2 (0.25 mL) at 40º C and pH 4.5. Then after 10 min aliquot was withdrawn for the formed product (purpurogallin) spectrophotometric determination at 420 nm, the disc washed and its activity determined again (seven times). For the shelf life HRP-PVAG-PANIG discs were stored in 0.1M acetate buffer, pH 4.5, at 4º C and a disc was removed at the indicated times for enzyme activity determination. Data are expressed as mean ± standard deviation of triplicates.

Recently, the enzymatic approach has attracted much interest in the decolorization/degradation of textile and other industrially important dyes from wastewater as an alternative strategy to conventional chemical, physical and biological treatments (Husain, 2006). The ability of the soluble HRP and HRP-PVAG-PANIG disc to remove phenolic compounds is demonstrated in the Table 1. Most of them were efficiently oxidized by both soluble and immobilized enzyme. Pyrogallol, α-naphthol, catechol, β-naphthol, 4, 4´-diaminodiphenyl benzidine, m-cresol and resorcinol were oxidized by the HRP-PVAG-PANIG disc in percents varying from 70% to 90%. As mentioned above pyrogallol was completely oxidized after 30 min of incubation by both enzymatic preparations. The reuse and higher thermal stability of the HRP-PVAG-PANIG disc would offer advantage compared to the soluble enzyme. However, both enzymatic preparations showed to be less efficient towards aniline, phenol and 2-nitrosonaphthol. The HRP-PVAG-PANIG disc showed to be more efficient than the immobilized bitter gourd (*Momordica charantia*) peroxidase on Con A adsorbed-Sephadex G 50 (Akhtar and Husain, 2006). According to these authors resorcinol, m-cresol, catechol, pyrogallol, under similar experimental conditions, were only removed 18%, 11%, 45% and 0%, respectively. On the other hand, the immobilized bitter gourd peroxidase was more efficient to remove phenol (about 90%) than HRP-PVAG-PANIG disc (about 35%).

Table 1 – PHENOLIC COMPOUNDS OXIDATION BY THE SOLUBLE AND HRP-PVG-PANIG.

Phenolic compounds	**Degradation (%)**	
	Soluble HRP	HRP-PVAG-PANIG
Pyrogallol	92.1 ± 0.3	92.1 ± 1.1
α-Naphthol	87.6 ± 0.1	90.0 ± 1.2
Catechol	81.3 ± 0.4	86.5 ± 0.2
β-Naphthol	78.0 ± 0.2	83.2 ± 0.8
4,4´-Diaminodiphenyl benzidine	72.4 ± 1.8	75.1 ± 0.5
m-Cresol	62.0 ± 0.9	71.0 ± 0.4
Resorcinol	75.0 ± 0.3	70.8 ± 0.1
Aniline	28.4 ± 1.0	45.0 ± 3.7
Phenol	15.5 ± 0.9	35.8 ± 1.3
2-Nitrosonaphthol	18.8 ± 2.5	19.7 ± 2.4

Soluble enzyme (0.1 mL) and HRP-PVAG-PANIG (01 disc), both containing about 90 U, were incubated at 37°C, pH 4.5, for 1.5 h with H_2O_2 and the phenolic compounds. These compounds were spectrophotometrically (750 nm) established according to Lowry et al. (1951) and the remaining amounts expressed as mean ± standard deviation (triplicates) and compared to the untreated phenolic solutions (100%).

4. Conclusions

More than 200 discs of the network polyvinyl alcohol-glutaraldehyde (PVAG) coated with polyaniline (PANIG) can be synthesized by a simple and inexpensive procedure. Horseradish peroxidase (HRP) can also be covalently fixed onto these discs via glutaraldehyde (HRP-PVAG-PANIG disc). The best conditions for the immobilization are: 1 mg protein mL^{-1}, 60 min and pH 5.5. The optimum pH of the HRP-PVAG-PANIG disc is lower than that reported for the soluble enzyme, whereas the optimum temperature is higher. The HRP-PVAG-PANIG disc is more thermal resistant than the soluble enzyme. The same HRP-PVAG-PANIG disc can be reused and stored for months without significant activity loss. The apparent Michaelis for HRP-PVAG-PANIG disc is higher than that calculated for the native enzyme. Resorcinol, m-cresol, catechol, pyrogallol, α-naphthol, β-naphthol and 4, 4´-diaminodiphenyl benzidine are efficiently

oxidized by the HRP-PVAG-PANIG disc (approximately about 60% to 90%), while aniline, phenol and 2-nitrosonaphthol are less efficiently oxidized.

Acknowledgments

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References

Akhtar, S., Husain, Q., 2006. Potential applications of immobilized bitter gourd (Momordica charantia) peroxidase in the removal of phenols from polluted water. Chemosphere 65, 1228-1235.

Akhtar, S., Khan, A. A., Husain, Q., 2005. Potential of immobilized bitter gourd (Momordica charantia) peroxidases in the decolorization and removal of textile dyes from polluted wastewater and dyeing effluent. Chemosphere 60, 291-301.

Araújo, A. M., Neves Jr., M. T., Azevedo, W. M., Oliveira, G. G., Ferreira Jr., D. L., Coelho, R. A. L., Figueiredo, E. A. P., Carvalho Jr., L. B., 1996. Polyvinyl alcohol-glutaraldehyde network as a support for protein immobilisation. Biotechnol. Techn. 112, 67-72.

Barbosa, G. H. T. S., Santana, E. M., Almeida, A. M. P., Araújo, A. M., Fatibello, O., Carvalho Jr., L. B., 2000. The use of filter paper plasticized with polyvinyl alcohol glutaraldehyde in ELISA. Braz. J. Med. Biol. Res. 33, 823-827.

Bora, U., Sharma, P., Kumar, S., Kannan, K., Nahar, P., 2006. Photochemical activation of a polycarbonate surface for covalent immobilization of a protein ligand. Talanta 70, 624-629.

Carvalho Jr., L. B., Araújo, A. M., Almeida, A.M.P., Azevedo, W.M., 1996. The use of polyvinyl alcohol glutaraldehyde antigen coated discs for laser induced fluorescence detection of plague. Sensor. Actuat. B-Chem. 36, 427-430.

Chance, B., Maehly, A.C., 1955. Assay of catalase and peroxidase. Method. Enzymol. 2, 764-775.

Cheng, J., Yu, S.M., Zuo, P., 2006. Horseradish peroxidase immobilized on aluminum-pillared interlayered clay for the catalytic oxidation of phenolic wastewater. Water Res. 40, 283-290.

Chung, K.T., Cerniglia, C.E., 1992. Mutagenicity of azo dyes: structure-activity relationships. Mutation Res. 277, 201-220.

El-Essi, F. A., Zuhri, A. Z. A., Al-Khalil, S. I., Abdel-Latif, M. S., 1997. Spectrophotometric determination of enzymatically generated hydrogen

peroxide using Sol-Gel immobilized horseradish peroxidase. Talanta 44, 2051-2058.

Fernandes, K. F., Lima, C. S., Lopes, F. M., Collins, C. H., 2004. Properties of horseradish peroxidase immobilised onto polyaniline. Process Biochem. 39, 957-962.

Fernandes, K. F., Lima, C. S., Pinho, H., Collins, C. H., 2003. Immobilization of horseradish peroxidase onto polyaniline polymers. Process Biochem. 38, 1379-1384.

Halpin, B., Pressey, R., Jen, J., Mondy, N. J., 1989. Purification and characterization of peroxidase isoenzymes from green peas (*Pisum sativum*). J. Food Sci. 54, 644-669.

HOUK, V. S., 1992. The genotoxicity of industrial wastes and effluents. Mutation Res. 277, 91–138.

Husain, Q., 2006. Potential applications of the oxidoreductive enzymes in the decolorization and detoxification of textile and other synthetic dyes from polluted water. Crit. Rev. Biotechn. 26, 201 – 221.

Khan, A. A., Husain, Q., 2007. Decolorization and removal of textile and non-textile dyes from polluted wastewater and dyeing effluent by using

potato (*Solanum tuberosum*) soluble and immobilized polyphenol oxidase. Bioresour. Technol. 98, 1012-1019.

Lai, Y. C., Lin, S. C., 2005. Application of immobilized horseradish peroxidase for the removal of p-chlorophenol from aqueous solution. Process Biochem. 40, 1167-1174.

Li, Y. Z., Townshend, A., 1998. Evaluation of the adsorptive immobilisation of horseradish peroxidase on PTFE tubing in flow systems for hydrogen peroxide determination using fluorescence detection. Anal. Chim. Acta 359, 149-156.

Liu, Z. M., Tingry, S., Innocent, C., Durand, J., Xu, Z.K., Seta, P., 2006. Modification of microfiltration polypropylene membranes by allylamine plasma treatment: Influence of the attachment route on peroxidase immobilization and enzyme efficiency. Enzyme Microb. Techn. 39, 868-876.

Lowry, O. H., Rosebrough, N. J., Farr, A.L. Randall, R. J., 1951. Protein measurement with the Folin-Phenol reagents. J. Biol. Chem. 193, 265-275.

Miland, E., Smyth, M. R., Ófágáin, C., 1996. Increased thermal and solvent tolerance of acetylated horseradish peroxidase. Enzyme Microb. Techn. 19, 63-67.

Mizutani, F., Yabuki, S., Sato, Y., Sawaguchi, T., Iijima, S., 2000. Amperometric determination of pyruvate, phosphate and urea using enzyme electrodes based

on pyruvate oxidase-containing poly (vinyl alcohol)/polyion complex-bilayer membrane. Electrochim. Acta 45, 2945-2952.

Musthapa, S.M., Akhtar, S., Khan, A.A., Husain, Q., 2004. An economical, simple and high yield procedure for the immobilization/stabilization of peroxidases from turnip roots. J. Sci. Ind. Res. India 63, 540-547.

Rojas-Melgarejo, F., Rodríguez-López, J. N., García-Cánovas, F., García-Ruiz, P. A., 2004a. Cinnamic carbohydrate esters: new polymeric supports for the immobilization of horseradish peroxidase. Carbohyd. Polym. 58, 79-88.

Rojas-Melgarejo, F., Rodríguez-López, J. N., García-Cánovas, F., García-Ruiz, P. A., 2004b. Immobilization of horseradish peroxidase on cinnamic carbohydrate esters. Process Biochem. 39, 1455-1464.

Sanz, V., de Marcos, S., Galban, J., 2007. Direct glucose determination in blood using a reagentless optical biosensor. Biosens Bioelectron. 22, 2876-2883.

Shaffiqu, T. S., Roy, J. J., Nair, R. A., Abraham, T. E., 2002. Degradation of textile dyes mediated by plant peroxidases. Appl. Biochem. Biotechnol. 102-103, 315-326.

Yu, H. H., Ginsbrug, G. S., Harris, N., 1997. Evaluation and clinical application of a direct low density lipoprotein cholesterol assay in normolipemic and hyperlipidemic adults. Am. J. Cardiol. 80, 1295-1299.

CAPÍTULO III - TRYPSIN IMMOBILIZATION ON DISCS OF POLYVINYL ALCOHOL GLUTARALDEHYDE/POLYANILINE COMPOSITE

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TRYPSIN IMMOBILIZATION ON DISCS OF POLYVINYL ALCOHOL GLUTARALDEHYDE/POLYANILINE COMPOSITE

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Abstract

Discs of polyvinyl alcohol cross-linked with glutaraldehyde (PVAG) were synthesized under acid catalysis and covered with polyaniline activated with glutaraldehyde (PANIG). Trypsin (EC 3.4.21.4) was covalently immobilized onto this composite. The FT-IR spectra of the discs showed bands of PVA (3300 cm^{-1}, 2930 cm^{-1} and 1440 cm^{-1}) and PANI (1594 cm^{-1} and 1100 cm^{-1}) and appearance of the green emeraldine color of PANI. The best immobilization conditions were achieved at trypsin concentration of 0.01 mg mL^{-1}, pH 7.6 and 60 min of incubation, resulting in 21.1 units per disc (15.0 mg). The PVAG-PANIG-trypsin derivative showed optimal pH and temperature similar to those reported for the native enzyme, namely, 7.0 and 35º C, respectively. This trypsin derivative also showed to be capable to catalyze the casein hydrolysis yielding smaller peptides (hydrolisate). This proposal presents the following advantages: inexpensive and easy support synthesis and immobilization procedure; to be removed immediately from the bulk reaction stopping the catalysis with high precision; reusability (three times with no loss of activity); storage (at 4º C during 37 days) and thermal stability and to act on large substrate (casein).

Keywords: immobilization, trypsin, hydrolysis of casein, stability.

1. Introduction

The use of immobilized trypsin for cleavage of proteins presents several advantages in comparison with application of its soluble form. For instance, immobilized trypsin reactors have been integrated into separation systems such as reversed-phase liquid chromatography or capillary electrophoresis, prior to mass spectrometry analysis, for proteome studies [1]. Furthermore, immobilized trypsin derivatives were used for continuous hydrolysis of casein [2], purification of peptides and cutaneous dressing [3], making these systems very important tools for industrial areas.

In our laboratory, discs of polyvinyl alcohol (PVA) cross-linked with glutaraldehyde were synthesized under acid catalysis (H_2SO_4). Then, the antigen F1 purified from *Yersinia pestis* and soluble adult *Schistosoma mansoni* antigen preparation (SWAP) were covalently linked onto this modified polymer [4, 5]. An enzyme-linked immunosorbent assay (ELISA) was established for the diagnosis of plague in rabbit and human and human schistosomiasis based on these derivatives. The *Y. pestis* antigen was also covalently linked to 5-mm diameter filter paper discs plasticized with polyvinyl alcohol-glutaraldehyde [6]. These discs were used both for ELISA and dot-ELISA in the detection of anti-F1 IgG in rabbits. On the other hand, polyaniline (PANI), a conductive polymer, has been used as a matrix for protein immobilization: *Yersinia pestis* antigen [7] and peroxidase [8].

In this work, discs of polyvinyl alcohol cross-linked with glutaraldehyde (PVAG discs) were coated with polyaniline and activated with glutaraldehyde (PANIG). This composite was employed as a matrix for trypsin immobilization

and some properties of the water insoluble derivative were investigated such as its ability to catalyze casein hydrolysis.

2. Materials

Trypsin (EC 3.4.21.4) and BApNA (Nα-benzoyl-DL-arginine-*p*-nitroanilide) were purchased from Sigma-Aldrich (St. Louis, EUA). PVA and dimethyl sulphoxide were obtained from Vetec Química Fina Ltda. (Rio de Janeiro, Brazil) and all other chemicals were of analytical grade.

3. Methodology

3.1. Synthesis of PVAG-PANIG

Discs of PVAG were synthesized according to Carvalho Jr. et al. [9] with the following modification: an aqueous solution of 2% (w/v) of PVA (12 mL) was heated to 65º C and 25% (v/v) of glutaraldehyde (2 mL) was added. This mixture was vigorously stirred during 50 min and a volume of 20 µL of the mixture was introduced into each microplate wells containing 3 M HCl (120 µL) and kept for 24 h at 25º C to allow the polymerization reaction to occur (disc synthesis). A number of 240 discs in three microplates were synthesized by using the 14.1 mL of mixture. The discs of PVAG were treated with 0.61 M ammonium persulphate prepared in 2.0 M HCl during 30 min. Then, the discs were immersed in 0.44 M aniline and kept for 60 min (PANI coating). PVAG-PANI discs were exhaustively washed with 2.0 M HCl. Following, the activation step was carried out by incubating 1.0 g of PVAG-PANI discs (68 units, approximately) at 60º C with 2.5% (v/v) glutaraldehyde aqueous solution [8].

Finally, PVAG-PANIG discs were washed five times with 0.1 M sodium phosphate buffer, pH 7.6, and stored in the same buffer at 4 ºC until use.

3.2. PVAG-PANIG Characterization

PVAG-PANIG was analyzed by FT-IR spectra (Hartman & Braun – MB Series – Michelson), as KBr pellets, between 500 and 4000 cm^{-1}.

3.3. Trypsin immobilization on PVAG-PANIG parameters

3.3.1. Enzyme concentration:

One PVAG-PANIG disc was incubated, separately, with 1.0 mL of trypsin solution in concentrations varying from 0.05 to 0.5 mg mL^{-1} during 60 min, under orbital agitation, at 4º C. Afterwards, the PVAG-PANIG-trypsin disc was incubated with 0.1 M glycine to block any remaining reactive groups and finally the derivative was exhaustively washed with 1 M NaCl.

3.3.2. Incubation time:

One PVAG-PANIG disc was incubated, separately, with 1.0 mL of trypsin solution (0.1 mg mL^{-1}) at time interval varying from 30-120 min. Blocking remaining active groups with glycine and washings were carried out as described above.

3.3.3. pH:

One PVAG-PANIG disc was incubated, separatety, with 1.0 mL of trypsin solution (0.1 mg mL^{-1}) prepared in different solutions of 0.1 M sodium phosphate buffer at pH ranging from 6.0 to 8.6 for 60 min. Blocking and washings were proceeded as above.

3.4. Measurement of enzyme activity

Trypsin activity was measured (triplicate) according to Alencar et al. [10]. Briefly: one PVAG-PANIG-trypsin disc or 0.15 mL of native trypsin was added to 0.85 mL of 0.6 mM BApNA and 0.1 M sodium phosphate buffer, pH 7.0, and the reaction product (*p*-nitroanilide) was measured at 405 nm. One enzymatic unit was defined as the amount of trypsin capable to produce 1 μmol of *p*-nitroanilide per minute and calculated by the formula U = [(A × v) × 1000]/9.1, where A and v stand for absorbance and the reaction mixture volume in milliliter, respectively, where E = 9.1 for *p*-nitroaniline under these parameters [11]. After the immobilization parameters optimization the activity assay for the PVAG-PANIGtrypsin disc was changed to 35º C (initially, the assays were done at room temperature).

3.5. PVAG-PANIG-trypsin activity characterization

3.5.1. pH profile:

The effect of the pH on the activity of either native or immobilized trypsin on BApNA was established as above except that in 0.1 M of sodium phosphate buffer varying from 6.0 to 8.6.

3.5.2. Temperature:

The effect of temperature on the activity of native and immobilized trypsin on BApNA was established in 0.1 M sodium phosphate buffer, pH 7.0, at temperatures ranging from 30 to 60º C.

3.5.3. Thermal stability:

Either the native and the PVAG-PANIG immobilized were incubated in a water bath at temperatures 40 and 70º C during time intervals from 25 to 125 min. After the incubation, the preparations were left at 25º C for approximately 30 min, before their activities were determined.

3.6. Stability Parameters:

3.6.1. Shelf life:

PVAG-PANIG-trypsin discs were stored in three stabilizer solutions: 1) 0.1mM glycine pH 3.6; 2) 0.1 mM glycine pH 3.6 containing 0.6 mM $CaCl_2$ and 3) 1% (w/v) poly (ethylene glycol) in 0.1 mM sodium phosphate buffer, pH 7.6.

The remaining activity of immobilized trypsin was evaluated each three days, during 46 days.

3.6.2. Reuse:

PVAG-PANIG-trypsin discs, after optimized immobilization and kinetic parameters, were tested for its capacity to hydrolyze BApNA repeatedly. The assays were occurred at the same day: after immobilization of trypsin under optimized conditions, one disc of PVAG-PANIG-trypsin was submitted to hydrolysis of BApNA, washed with sodium phosphate buffer (five times), and submitted to hydrolyze BApNA again. The first measure was assigned as 100% and the assay was processed with triplicate.

4. Application: production of peptides by hydrolysis of casein

Soluble trypsin and PVAG-PANIG-trypsin discs were compared to the capacity of hydrolyze casein. Both trypsin preparations (soluble and immobilized form containing 0.9 U in 0.1 M sodium phosphate buffer at pH 7.0 with 0.6 mM calcium chloride) were stirred with 1.0 mg of casein at pH 7.0, 37º C. Aliquots of 1.0 mL were colleted at the intervals of 0, 15 min, 30 min, 60 min, 120 min, 180 min, 420 min and 480 min. All aliquots were added, separately, to a 10% (w/v) trichloroacetic acid solution, centrifuged, and the supernatant were measured at 280 nm. The samples of 0 and 480 min of hydrolysis (by soluble trypsin and PVAG-PANIG-trypsin discs) were applied in a Sephadex G-50 chromatographic columm for evaluation of the fractions at 280 nm.

5. Results and Discussion

The disc composite here proposed as a matrix consists of a network of polyvinyl alcohol glutaraldehyde (PVAG) coated with polyaniline (PANI) [5]. Figure 1 shows typical discs of PVAG (yellow) and PVAG-PANI (dark green) and also their probable chemical structures. Trypsin was covalently fixed onto PANI via glutaraldehyde [8] and also onto PVAG through the free carbonyl groups [12]. Both chemical possibilities enhanced the disc capacity to attach enzyme molecules. It is important to notice that the disc shape of the PVAG network was provided by the used of microplate wells as template.

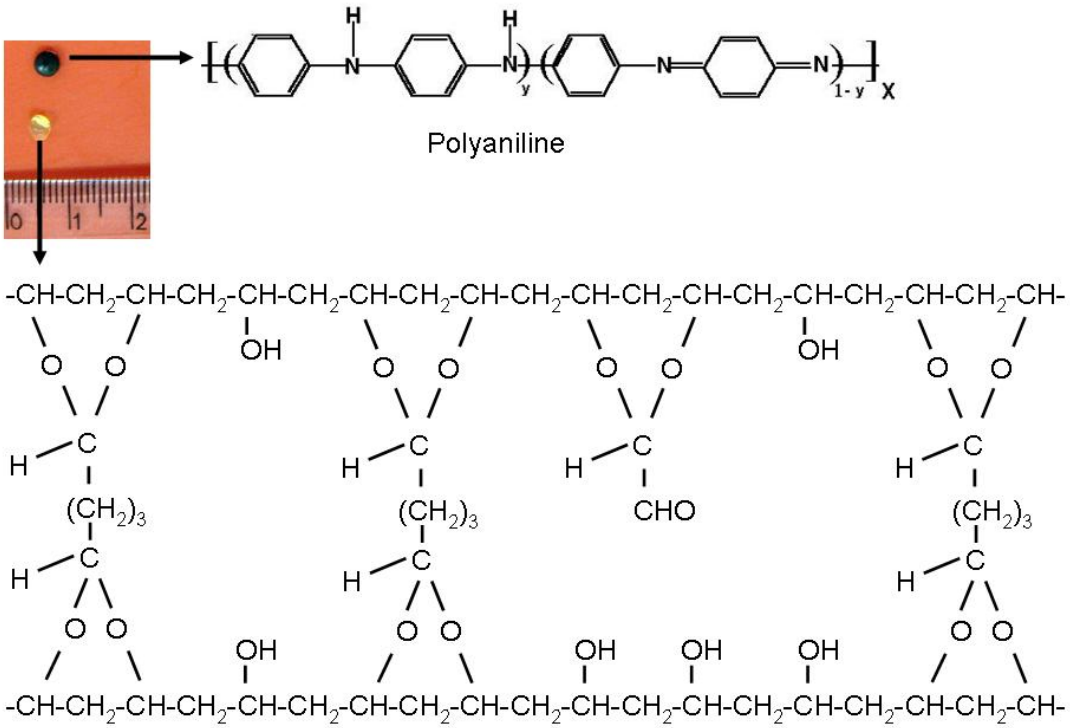
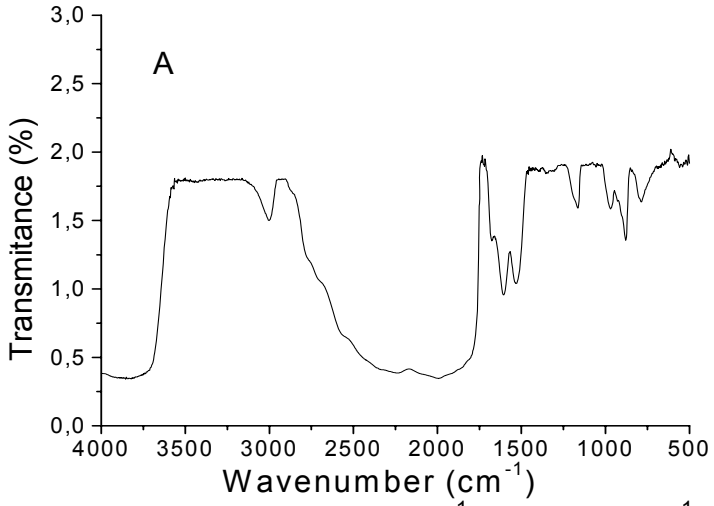


Figure 1 – Discs of PVAG (yellow) and PVAG-PANIG (dark green) and their chemical structures.

Figure 2 displays the infrared spectra of PVA and PVAG-PANIG. The band at 3400cm^{-1} relative to (-OH) stretching, the typical PVA band at 2900 cm^{-1} relative -CH stretching, and the band at 800 cm-1 related to high syndiotacticity were observed in the spectrum of PVA (Figure 2A). The spectrum of PVAG-PANIG composite presented the same characteristic bands of PVA and those



related to PANI, mainly the bands at 1593 cm^{-1} and 1500 cm^{-1} relative to the benzoid/quinoid structures and that at 1107 cm^{-1} relative to doping level of PANI (Figure 2B).

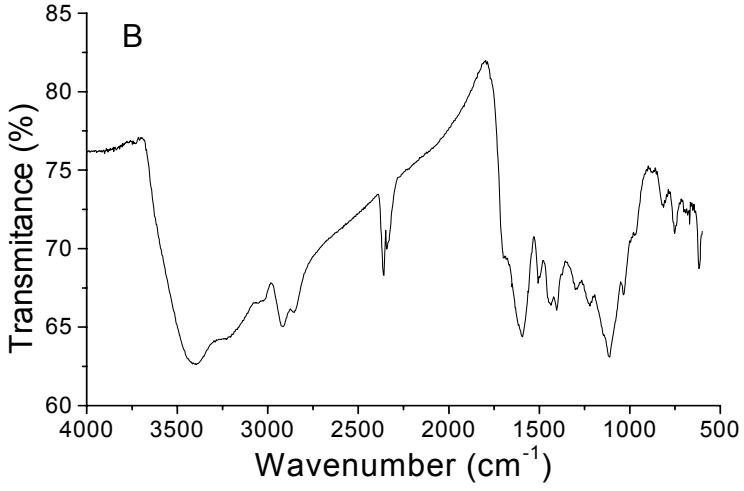


Figure 2 - The infrared spectra of PVA (A) and PVAG-PANIG (B).

The best immobilization parameters are presented in Figure 3. As expected the relationship between the offered trypsin and the fixed protein displayed a hyperbolic curve (Figure 3A). This means that there was a limit of maximum protein load per disc (0.04 mg) which tends to reach at certain amount of offered enzyme (>0.05 mg). Furthermore, similar curve would be achieved relating specific activity *versus* offered enzyme. According to these results a value of 0.2 mg ml^{-1} of trypsin can be established as best enzyme concentration under the experimental conditions. The best incubation time of trypsin on PVAG-PANIG disc was estimated in 60 min as can be depicted from Figure 3B. After this incubation time a decrease in enzyme activity was observed. Similar results were obtained in immobilization of trypsin onto Poly (ethylene terephthalate)-Polyaniline-Glutaraldehyde (PET-PANIG) composites [13]. Probably, longer incubation time results in offered trypsin being hydrolyzed by other soluble trypsin molecules and inactive enzyme (peptides) is fixed onto the composite. Shah et al. [14] working with immobilization of trypsin in Salicylic resorcinol formaldehyde (SRF) resin obtained the same profile of immobilization, with maximum immobilization at 60 min of reaction and reduction in the SRF-trypsin activity after this point. The best pH for the trypsin immobilization on PVAG-PANIG disc was established as 7.6 (Figure 3C). Beyond this value immobilization either in the acidic or in the alkaline range resulted in 30% less efficiency. The same pH profile for trypsin immobilization on poly (ethylene terephthalate) coated with PANIG [13]. Other values have been reported in the literature: values of 6.0 and 7.0 using as supports MCM-41 (molecular sieve) [15] and galleries de γ-zirconium [16], respectively.

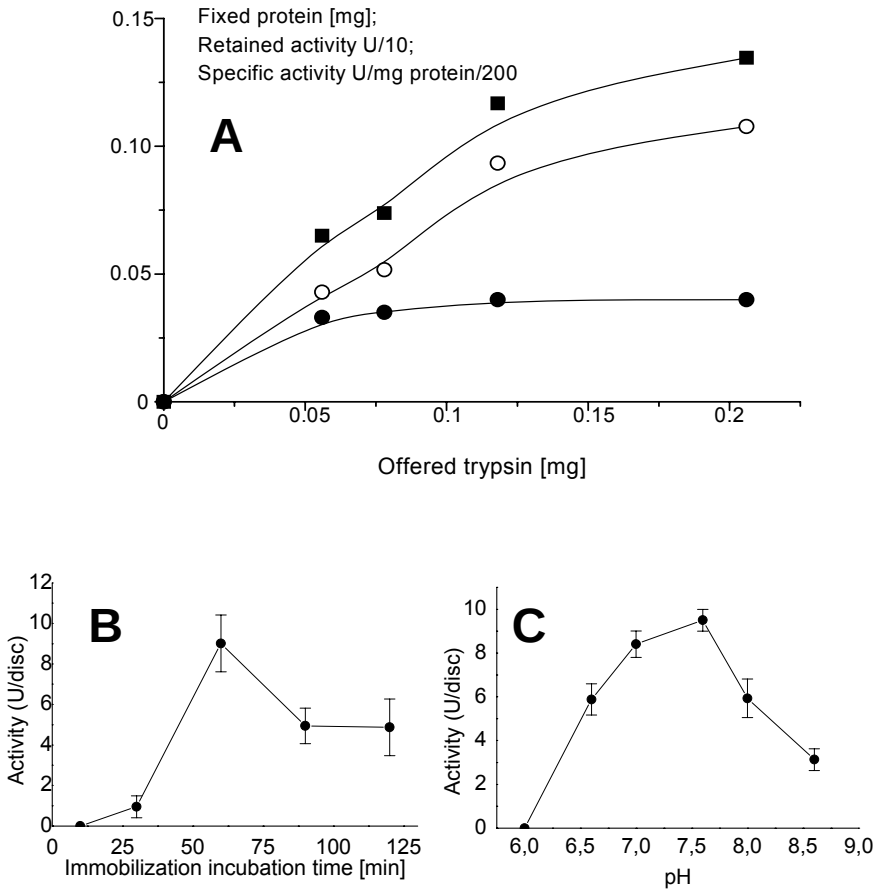


Figure 3 – Best Immobilization conditions: A - Offered trypsin *versus* retained protein (○), activity (●) and specific activity (■); B – immobilization time and C – immobilization pH.

A linear curve (first order kinetics) was observed for the first three hours of both soluble and immobilized trypsin action on BApNA under the experimental conditions established for the enzyme activity determination (data not shown). Figure 4 shows the effect of the pH and temperature on the BApNA hydrolysis catalyzed by trypsin onto PVAG-PANIG discs. The best pH was coincident with that found for the native trypsin, namely, 7.6 (Figure 4A). In spite

of the maintenance of the optimum pH the immobilized trypsin showed higher activity in both acidic and alkaline regions compared with the native trypsin. The increase of activity in acidic or alkaline range observed after immobilization is frequently associated to proton donator or acceptor groups in the support. These groups act adjusting the pH in the surrounding environment of the immobilized enzyme, depending of the support characteristics. In this case, the existence of hydroxyl groups of PVAG and amino groups from PANIG provides an environment able to simultaneously donate and accept protons from bulk solution, resulting in the activity stabilization observed in the Figure 4A. Enhancement of stability of enzymes immobilized in PANI and PANI derivatives, working at pH values up to pH 6.0, has been reported in several works [8, 13, 17]. The optimum temperature (35º C) for the activity of the trypsin onto PVAGPANIG discs was also equal to the native enzyme (Figure 4B).

Figure 5 shows the thermal stability and shelf life of the native and the immobilized enzyme. The PVAG-PANIG-trypsin incubated at 40º C retained the initial activity for 50 min whereas the soluble enzyme lost half of its initial activity (Figure 5A). Furthermore, incubation at 70º C inactivated the soluble enzyme after 50 min whereas the PVAG-PANIG-trypsin retained 20% of its initial (Figure 5A), after the same period. Increase of thermal stability of immobilized trypsin preparations have been reported by several authors using as support acrylic copolymers [18], silica gel chitosan bead [19], galleries of zirconium phosphate [15] and cellulosic exopolysaccharide [11]. Figure 5B shows the residual activity measured when PVAG-PANIG-trypsin was stored in the presence of three stabilizer solutions at 4º C (shelf life). The best result was obtained when 0.1 mM Glycine containing 0.6 mM $CaCl_2$ was used for trypsin storage, resulting in

100% of activity during 37 days. Furthermore, the same preparation was used three times without loss of activity (data not shown). According to Bryjak and Kolarz [18] the storage stability of enzyme-carrier preparations is an important additional factor that determines their usefulness. Kotormán et al. [20], studying the effects of calcium ion on trypsin and α-chymotrypsin stability, reported a change in the trypsin three-dimensional structure in presence of 0.6 mM calcium chloride. The authors pointed an increase of α-helix content when trypsin was stored in the presence of calcium, similar to that changes observed in proteins calcium dependent.

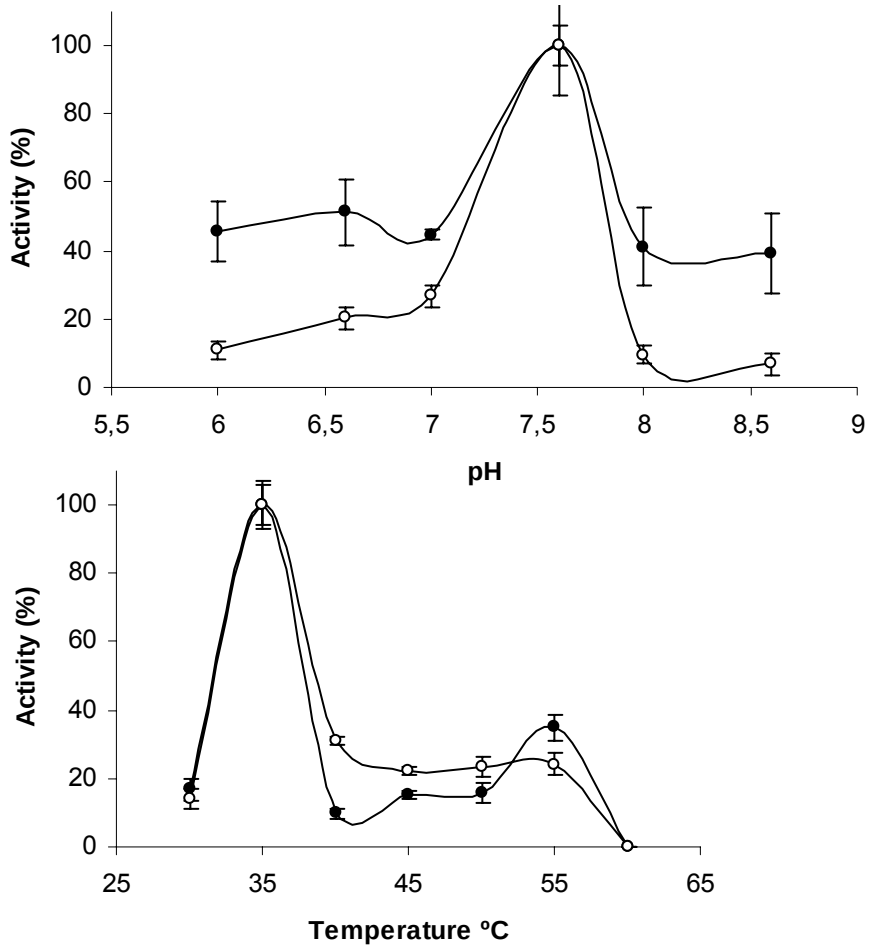


Figure 4 – Influence of pH (A) and temperature (B) on the soluble (○) and PVAGPANIG-Trypsin (●).

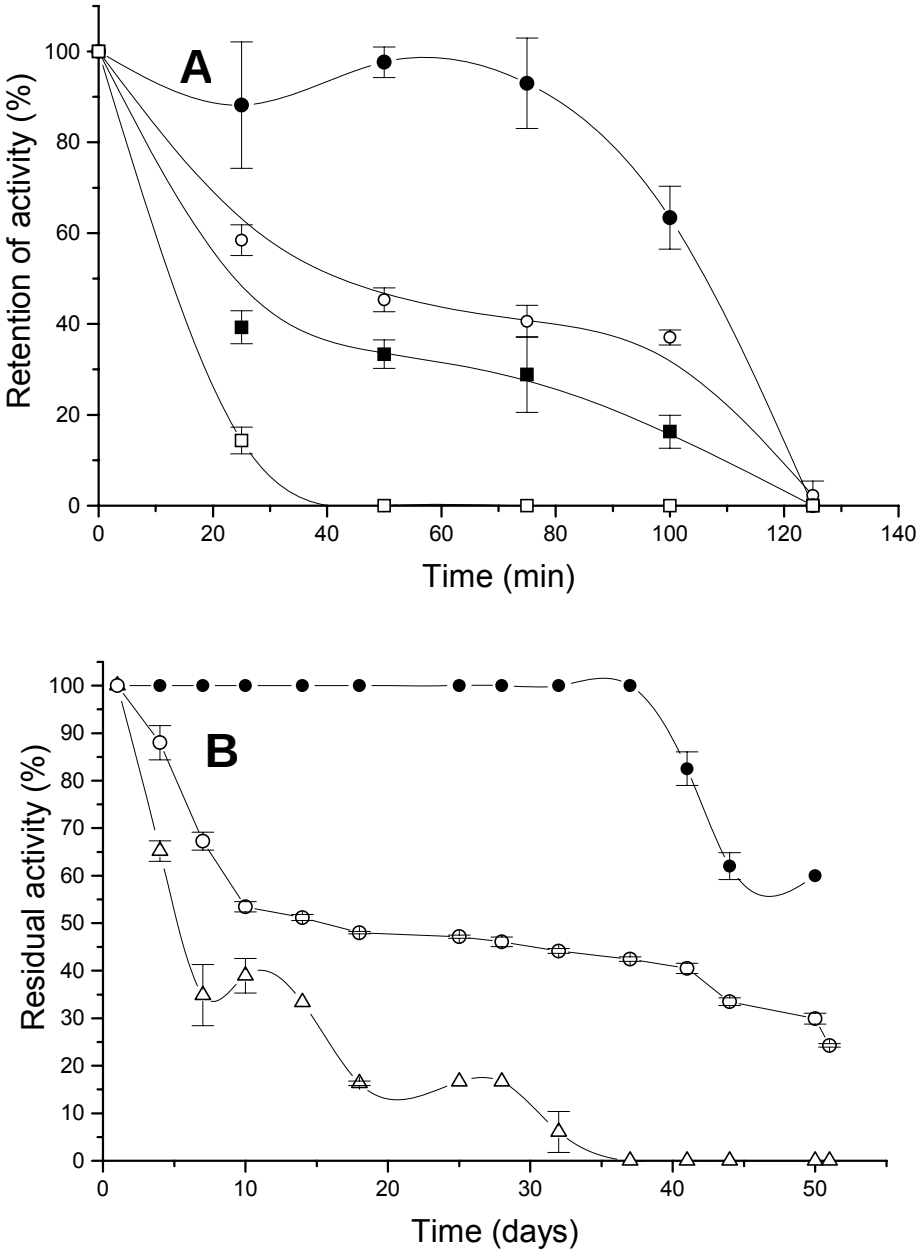


Figure 5 – Thermal stability (A) of the soluble (open symbols) and PVAG-PANIG-Trypsin (closed symbols) at 50ºC (circle) and at 70º C (square). Shelf life (B) of PVAG-PANIG-trypsin at 4ºC stored in glycine (○), polyethyleneglycol (Δ) and glycine + $CaCl_2$ (●).

Finally, Figure 6 presents the action of the soluble and the PVAG-PANIG-trypsin on casein. Full hydrolysis is almost achieved at the 1st hour by the soluble trypsin catalysis (Figure 6A). Casein hydrolysis also occurred by using the PVAGPANIG-trypsin but at a lower rate (Figure 6A). It is worth to note

the fact that in this latter case the trypsin presents smaller degree of freedom and it is acting on a macromolecule substrate (casein). Therefore, a reduction of activity would be expected. However, two third of the maximum hydrolysis promoted by the soluble enzyme was retained by the PVAG-PANIG-trypsin after 8 h of incubation with the substrate casein.

Figure 6B presents the molecular gel filtration (Sephadex G-50) of the hydrolisates obtained from the incubation for 8 h with the soluble and immobilized preparations compared with the casein. Three observations should be stressed: 1) casein showed peptides ranging from the 1st to 43rd fractions; 2) its hydrolisate obtained by the soluble trypsin action presented peptides from the 1st to the 60th fractions and 3) the hydrolisate obtained by the PVAG-PANIG-trypsin action from 10th to 60th fractions. As one can preview smaller peptides were produced by the soluble and immobilized trypsin (peptides from the 44th fraction on). Supporting the evidence of the Figure 6A the PVAG-PANIG-trypsin was also capable to hydrolyze the casein. However, a different pattern was observed because the casein peptides sized between the 1st and 10th fractions disappeared whereas higher amounts of small peptides (45th to 60th fractions) appeared under the PVAG-PANIG-trypsin action (Figure 6B). These findings suggest different mechanism of action of the PVAG-PANIG-trypsin compared to the soluble enzyme.

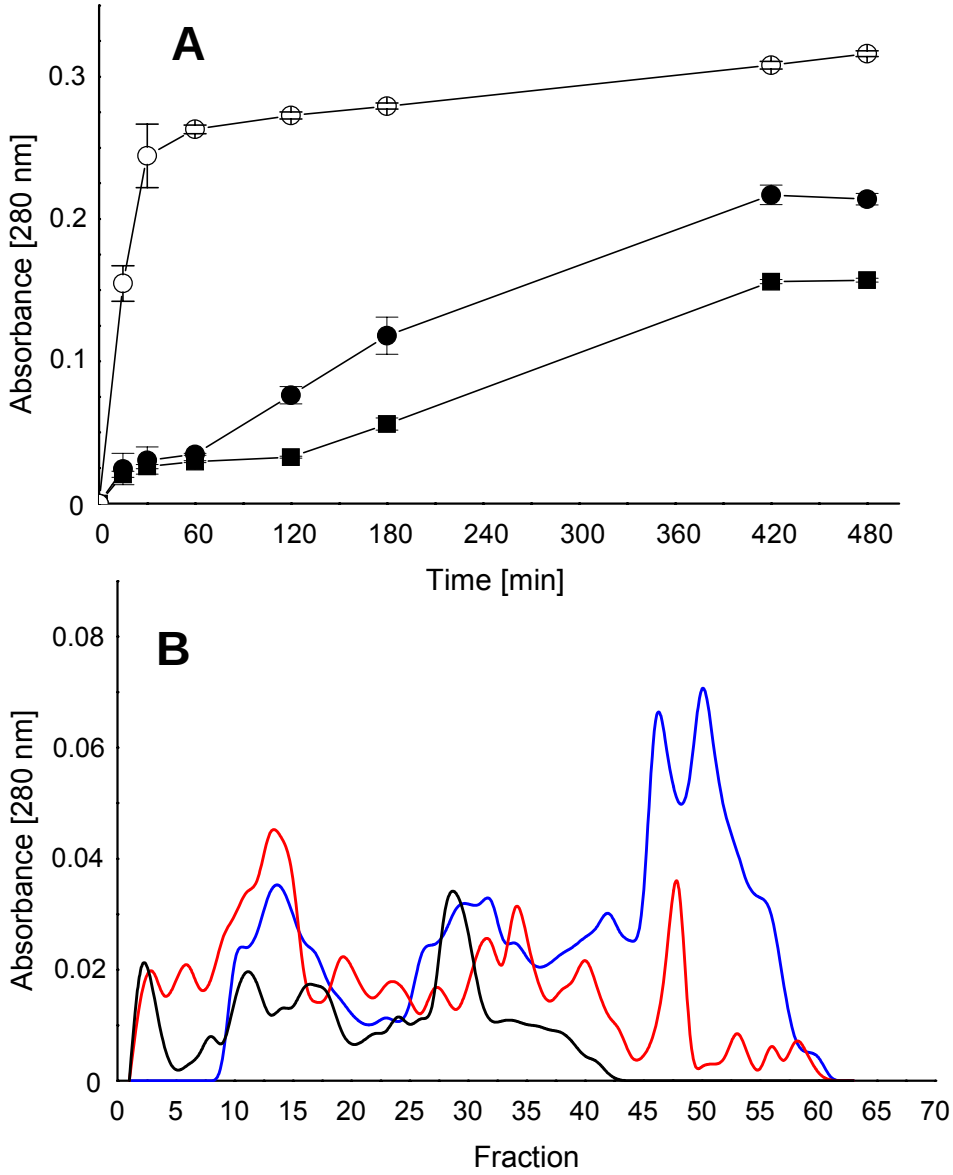


Figure 6 – Action (A) of the soluble (○) and PVAG-PANIG-Trypsin (● first use and ■ for second use) on casein and gel filtration (B) of casein (black line) and the hydrolisates produced by the soluble (red line) and PVAG-PANIG-trypsin (blue line) after 8 h of hydrolysis.

6. Conclusion

From these results one can conclude that discs of polyvinyl alcohol crosslinked with glutaraldehyde, synthesized under acid catalysis, covered with polyaniline, can be used for covalent trypsin immobilization, via glutaraldehyde.

Immobilization conditions were optimized yielding a preparation with 21.1 units per disc (15 mg). This derivative also showed optimal pH and temperature similar to those reported for the native enzyme. The enzyme derivative presents the advantages of reusability; storage and thermal stability and capable to catalyze casein hydrolysis.

6. Acknowledgments

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7. References

[1] G. Massolini, E. Calleri, J. Sep. Sci. 28 (2005): 7-21.

[2] S.J. Ge, H. Bai, L.X. Zhang, Biotechnol. Appl. Biochem. 24 (1996): 1-5 Part 1.

[3] F.M.F. Monteiro, G.M.M. Silva, J.B.R. Silva, C.S. Porto, L.B. Carvalho Jr., J.L. Lima Filho, A.M.A. Carneiro-Leão, M.G. Carneiro-da-Cunha, A.L.F. Porto, Process Biochem. 42 (2007): 884-888.

[4] A.M. Araujo, A.T. Petribu, G.H. Sales-Barbosa, J.R. Diniz, A.M. Almeida, W.M. Azevedo W, E. Malagueno, L.B. Carvalho Jr., Mem. Inst. Oswaldo Cruz 91 (1996): 195-198.

[5] A.M. Araujo, G.H. Barbosa, J.R. Diniz, E. Malagueno, W.M. Azevedo, L.B. Carvalho Jr., Rev. Inst. Med.Trop. São Paulo 39 (1997): 155-158.

[6] G.H. Barbosa, E.M. Santana, A.M. Almeida, A.M. Araujo, O. Fatibello-Filho, L.B. Carvalho Jr., Braz. J. Med. Biol. Res. 33 (2000): 823-827.

[7] R.A.L. Coelho, G.M.P. Santos, P.H.S. Azevedo, G.A. Jaques, W.M. Azevedo, L.B. Carvalho Jr., J. Biomedic. Mat. Res. 56 (2001): 257-260.

[8] K.F. Fernandes, C.S. Lima, H. Pinho, C.H. Collins, Process Biochem. 38 (2003): 1379-1384.

[9] L.B. Carvalho Jr., A.M. Araújo, A.M.P. Almeida, W.M. Azevedo, Sens. Actuators B 36 (1996): 427-430.

[10] R.B. Alencar, M.M. Biondi, P.M.G. Paiva, V.L.A. Vieira, L.B. Carvalho Jr, R.S. Bezerra, Alkaline proteases from digestive tract of four tropical fishes, Braz. J. Food Technol. 6 (2003): 279–284.

[11] A.H.M. Cavalcante, L.B. Carvalho Jr., M.G. Carneiro-da-Cunha, Biochem. Eng. J. 29 (2006): 258-261.

[12] D.R. Walt, V.I. Agayn, Trends Anal. Chem. 13 (1994): 425-430.

[13] S.S. Caramori, K. F. Fernandes, Mater. Sci. Eng. C, in press.

[14] B. Shah, S.R. Kumar, S. Devi, Process Biochem. 30 (1995): 63-68.

[15] J.F. Díaz, K.J. Balkus Jr, J. Mol. Catal. B, 2 (1996): 115-126.

[16] L. Geng, N. Li, M. Xiang, X. Wen, D. Xu, F. Zhao, K. Li, Colloids Surf. B: Biointerf. 30 (2003): 99-109.

[17] R.N. Silva, E.R. Asquieri, K.F. Fernandes, Process Biochem. 40 (2005): 1155-1159.

[19] J. Bryjak, B.N. Kolarz, Process Biochem. 33 (1998): 409-417.

[19] F. Xi, J. Wu, Z. Jia, X. Lin, Process Biochem. 40 (2005): 2833-2840.

[20] M. Kotormán, I. Laczkó, A. Szabó, L.M. Simon, Biochem. Biophys. Res. Commun. 304 (2003): 18-21.

CONCLUSÕES

O compósito de álcool polivinílico-glutaraldeído-polianilina-glutaraldeído pôde ser sintetizado sob a forma de discos, os quais puderam ser caracterizados e utilizados para imobilização de enzimas;

Os estudos de caracterização do compósito de PVAG-PANIG revelaram pequenas alterações nas características originais dos polímeros estudados. Entretanto, para os sistemas utilizados neste trabalho, as mudanças observadas no comportamento dos polímeros aparentemente não exerceram influência sobre a imobilização das enzimas estudadas;

O sistema PVAG-PANIG peroxidase apresentou características bastante similares às da enzima em sua forma nativa, mas com a vantagem de uso contínuo e repetido da enzima imobilizada e a possibilidade de interrupção imediata da catálise pela retirada do sistema suporte-peroxidase do meio de reação;

O sistema PVAG-PANIG-tripsina mostrou-se bastante eficiente na hidrólise do substrato sintético BApNA e na formação de peptídeos a partir de amostras de caseína.

PERSPECTIVAS

Os resultados obtidos ao final desta tese de doutorado apontam para as seguintes perspectivas, que tratam das aplicações do suporte PVAG-PANIG para a imobilização de enzimas:

- Estudo do sistema PVAG-PANIG-HRP:

Tratamento de resíduos industriais que contenham derivados fenólicos, em escala laboratorial e industrial.

- Estudo do sistema PVAG-PANIG-tripsina:

Produção de peptídeos de diversas fontes protéicas. Montagem de reator em batelada e em fluxo contínuo.

Estudo da vibilidade deste sistema na utilização em proteômica.

REFERÊNCIAS BIBLIOGRÁFICAS

AKHTAR, S.; KHAN, A.A.; HUSAIN, Q. Potential applications of immobilized bitter gourd (*Momordica charantia*) peroxidase in the removalof phenols from polluted water. Chemosphere 65: 1228-1235, 2006.

AKHTAR, S.; KHAN, A.A.; HUSAIN, Q. Potential of immobilized bitter gourd (*Momordica charantia*) peroxidases in the decolorization and removal of textile dyes from polluted wastewater and dyeing effluent. Chemosphere 60: 291-301, 2005.

AMARAL, I. P. G.; CARNEIRO-DA-CUNHA, M. G.; CARVALHO JUNIOR, L. B.; BEZERRA, R. S. Fish trypsin immobilized on ferromagnetic Dacron. Process Biochemistry, v. 41, p. 1213-1216, 2006.

ARAUJO, A. M.; PETRIBU, A. T. S.; BARBOSA, G. H. T. S.; DINIZ, J. R. P.; ALMEIDA, A. M. P.; AZEVEDO, W. M.; MALAGUENO, E.; CARVALHO JUNIOR, L. B. The use of polyvinil alcohol glutaraldehyde as solid-phase in ELISA for plague. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, v. 91, p. 195-198, 1996.

a. ARAUJO, A. M.; NEVES Jr, M. T.; AZEVEDO, W. M.; OLIVEIRA, G. G.; FERREIRA Jr, D. L.; COELHO, R. A. L.; FIGUEIREDO, E. A. P.; CARVALHO JUNIOR, L. B. Polyvinil alcohol-glutaraldehyde network as a support for protein immobilisation. Biotechnology Techniques, 11(2): 67-72, 1997.

b. ARAUJO, A. M.; BARBOSA, G. H. T. S.; DINIZ, J. R. P.; MALAGUENO, E.; AZEVEDO, W. M.; CARVALHO JUNIOR, L. B. Polyvinil alcohol-glutaraldehyde as solid-phase in ELISA for Schistosomiasis. Revista do Instituto de Medicina Tropical de São Paulo, Sao Paulo, v. 39, p. 155-158, 1997.

ARAUJO, A. M. ; PETRIBU, A. T. S. ; BARBOSA, G. H. T. S. ; DINIZ, J. R. P. ; ALMEIDA, A. M. P. ; CARVALHO JUNIOR, L. B. . Rapid ELISA for Plague. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, v. 93, n. 1, p. 111-112, 1998.

ASTURIAS, G.E.; MACDIARMID, A.G.; MACCALL, R.P.; EPSTEIN, A.J. The oxidation state of “emeraldine” base. Synthetic Metals 29: E157-E162, 1989.

AZEVEDO, A.M.; PRAZERES, D.M.F.; CABRAL, J.M.S.; FONSECA, L.P. Stability of free and immobilized peroxidase in aqueous-organic solvents mixtures. Journal of Molecular Catalysis B: Enzymatic 15: 147-153, 2001.

AZEVEDO, W. M.; SOUZA, J.M.; MELO, J.V. Semi interpenatrating polymer network based on polyaniline and polyvinyl alcohol glutaraldehyde. Synthetic Metals 100: 241-248, 1999.

BARBOSA, G. H. T. S.; SANTANA, E. M.; ALMEIDA, A. M. P.; ARAUJO, A. M.; FATIBELLO, O.; CARVALHO JUNIOR, L. B. The use of filter paper plasticized

with polyvinvyl alcohol glutaraldehyde in ELISA. Brazilian Journal of Medical and Biological Research, Brasil, v. 33, p. 823-827, 2000.
BARHAM, D.; TRINDER, P. An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst 97: 142-145, 1972.

CARAMORI, S. S. Imobilização de Enzimas em PET-PANIG. 2003. 104 f. Dissertação (Mestrado em Biologia) - Universidade Federal de Goiás. *Orientador:* Kátia Flávia Fernandes Silva.

CARAMORI, S.S.; FERNANDES, K.F. Covalent immobilisation of horseradish peroxidase onto poly(ethylene terephthalate)–poly(aniline) composite. Process Biochemistry 39(7): 883-888, 2004.

CARAMORI, S. S.; FERNANDES, K. F. The Use of Poly(ethylene terephthalate)-Poly(aniline) Composite for Trypsin Immobilisation. Materials Science & Engineering. C, Biomimetic Materials, Sensors and Systems, *in press*, 2007.

CARNEIRO-LEAO, A. M. A. C.; CARVALHO JUNIOR, L. B.; MALAGUENO, E. The use of ferromagnetic dacron as solid-phase in enzyme immunoassays. Memórias do Instituto Oswaldo Cruz, v. 89, p. 189-193, 1994.

CARNEIRO-LEAO, A. M. A.; OLIVEIRA, E. A.; CARVALHO JUNIOR, L. B. Immobilization of protein on ferromagnetic Dacron. Applied Biochemistry and Biotechnology, v. 311, p. 53-58, 1991.

CARVALHO JUNIOR, L. B.; ARAUJO, A. M.; ALMEIDA, A. M. P.; AZEVEDO, W. M. The use of polyvinil alcohol glutaraldehyde antigen coated discs for laser induced fluorescence detection of plague. Sensors and Actuators. B, Chemical, v. 35-36, p. 427-430, 1996.

CARVALHO JUNIOR, L. B.; MELO, E. H. M.; VASCONCELOS, A. R. A.; LIRA, R. R. Glucose oxidase immobilised on gel beads polyacrylamide and polyethyleneterephthalate. Arquivos de Biologia e Tecnologia, v. 291, p. 525-531, 1986.

CHANCE, B.; MAEHLY, A.C. Assay of Catalases and Peroxidases. Methods in Enzymology 2: 764-775, 1955.

CHARPENTIER, L.; EL MURR, N. Amperometric determination of cholesterol in serum with use of a renewable surface peroxidase electrode. Analytica Chimica Acta 318: 89-93, 1995.

CHIBATA, I.; TOSA, T.; SATO, T.; MORY, T. Definition of Immobilized Enzymes. In: CHIBATA, I. (ed), Immobilized Enzymes – Research and Development, cap. 1, New York: John Wiley and Sons, 1978, p. 1-7.

CLARE, D.A.; VALENTINE, V.W.; CATIGNANI, G.I.; SWAISGOOD, H.E. Molecular design, expression and affinity immobilization of a trypsin-stretavidin fusion protein. Enzyme and Microbial Technology 28: 483-491, 2001.

COÊLHO, R. A. L. O Uso da Polianilina em Imunoensaios. 1998. 0 f. Dissertação (Mestrado em Bioquímica) - Universidade Federal de Pernambuco, Conselho Nacional de Desenvolvimento Científico e Tecnológico. *Orientador:* Luiz Bezerra de Carvalho Junior.

COÊLHO, R. A. L.; SANTOS, G. M. P.; AZEVEDO, P. H. S.; JAQUES, G. A.; AZEVEDO, W. M.; CARVALHO JUNIOR, L. B. Polyaniline-Dacron composite as solid phase in ELISA for Yersinia pestis antibody detection. Journal of Biomedical Materials Research. Part A, Estados Unidos, v. 56, n. 2, p. 257-260, 2001.

DÍAZ, J.F.; BALKUS JR, K.J. Enzyme immobilization in MCM-41 molecular sieve. Journal of Molecular Catalysis B: Enzymatic 2(2-3): 115-126, 1996.

DONG, B.; HE, B.L.; XU, C.L.; LI, H.L. Preparation and electrochemical characterization of polyaniline/multi-walled carbon nanotubes composites for supercapacitor. Materials Science and Engineering: B 143(1-3): 7-13, 2007.

DUARTE, D. M. R. Purificação de Tripsina por Cromatografia de Afinidade Empregando o Inibidor de Swartzia pickellii imobilizado em Dacron Ferromagnético. 2002. Dissertação (Mestrado em Bioquímica) - Universidade Federal de Pernambuco. *Orientador:* Luiz Bezerra de Carvalho Junior.

DUNFORD, H.B. In: EVERSE, J.; EVERSE, K.F.; GRISHAM, M.B. (Eds.), Peroxidases in Chemistry and Biology, vol. 2, CRC Press, Boca Raton, FL, 1991, pp. 1–24.

FATIMA, A.; HUSAIN, Q.; KHAN, R.H. A peroxidase from bitter gourd (*Momordica charantia*) with enhanced stability against organic solvent and detergent: A comparison with horseradish peroxidase. Journal of Molecular Catalysis B: Enzymatic 47: 66-71, 2007.

FERNANDES, K.F.; LIMA, C.S.; PINHO, H.; COLLINS, C.H. Immobilisation of horseradish peroxidase onto polynailine polymers. Process Biochemistry 38(9): 1379-1384, 2003.

FERNANDES, K. F.; LIMA, C. S.; LOPES, F. M.; COLLINS, C. H. Properties of horseradish peroxidase immobilised onto polyaniline. Process Biochemistry, v. 39, p. 957-962, 2004.

FERNANDES, K. F.; LIMA, C. S.; LOPES, F. M.; COLLINS, C. H. Hydrogen peroxide detection system consisting of chemically immobilised peroxide and spectrometer. Process Biochemistry, v. 40, p. 3441-3445, 2005.

FERREIRA, O.P.; ALVES, O.L.; MACEDO, J.S.; GIMENEZ, I.F.; BARRETO, L.S. Ecomateriais: desenvolvimento e aplicação de materiais porosos funcionais para proteção ambiental. Química Nova 30(2): 464-467, 2007.

GOSPODINOVA, N.; TERLEMEZYAN, L. Conducting Polymers Prepared by Oxidative Polymerization: Polyaniline. Progams in Polymer Science 23: 1443-1484, 1998.

HIRAI, T.; MARUYAMA, H.; SUZUKI, T.; HAYASHI, S. Shape memorizing properties of a hydrogel of poly(vinyl alcohol). Journal of Applied Polymer Science 45: 1849-1855, 1992.

HUBER, R.; BODE, W. Structural Basis of the Activation and Action of Trypsin. Accounts of Chemical Research 11: 114-122, 1978.

KENNEDY, J.F.; WHITE, C.A. Principles of Immobilization of Enzymes. In: WISELMANN, A. (Ed.) Handbook of Enzyme Biotechnology. Cap 4. New York: Wiley & Sons, 1985, p. 147.

KIM, J.H.; KIM, J.Y.; LEE, Y.M.; KIM, K.Y. Properties and swelling characteristics of cross-linked poly (vinyl alcohol)/chitosan blend membrane, Journal of Applied Polymer Science 45: 1711-1717, 1992.

KORNBERG, A. Why purify enzymes? Methods in Enzymology 182: 1-5, 1990.

KUMAR, C.G.; MALIK, R.K.; TIWARI, M.P. Novel enzyme-based detergents: An Indian perspective. Current Science 75(12): 1312-1218, 1998.

LI, H.; ZHANG, Y.; CHEN, X.; SHI, K.; YUAN, Z.; LIU, B.; SHEN, B.; HE, B. Synthesis and adsorption aspect of crosslinked PVA-based blood compatible adsorbents for LDL apheresis. Reactive & Functional Polymers 58: 53-63.

LIMA, G.P.P.; BRASIL, O.G.; OLIVEIRA, A.M. Poliaminas e atividade da peroxidase em feijão *(Phaseolus vulgaris* l.) cultivado sob estresse salino. Scientia Agricola 56(1): 21-26, 1999.

LIMA, U.A.; BASSO, L.C.; AMORIM, H.V. Produção de Etanol. In: LIMA, U.A.; AQUARONE, E.; BORZANI, W.; SCHMIDELL, W. (coords.) Biotecnologia Industrial: processos fermentativos e enzimáticos, vol. 3, cap. 1, São Paulo: Edgard Blücher, 2001, p. 1-43.

LUO, Y.C.; DO, J.S. Urea biosensor based on PANi(urease)-Nafion®/Au composite electrode. Biosensors and Bioelectronics 20(1): 15-23, 2004.

MACDIARMID, A.G.; CHIANG, J.C.; HALPERN, M.; HUANG, W.S.; MU, S.L.; SOMASIRI, N.L.D.; WU, W.; YANINGER, S.I. Polyaniline: interconvertion of metallic and insulating forms. Molecular Crystals & Liquid Crystals 121: 173-180, 1985.

MACDIARMID, A.G.; EPSTEIN, A.J. Polyanilines: a novel class of conducting polymers. Faraday Discussion Chemical Society 88: 317-332, 1989.

MACHADO, P. M. F. Isolamento, caracterização parcial e imobilização de uma lectina de *Brosimun gaudichaudii* - aplicação para remoção de lactose de leite.

2007. Dissertação (Mestrado em Biologia) - Universidade Federal de Goiás. *Orientador:* Kátia Flávia Fernandes Silva.

MELO, E. H. M. Imobilização covalente de enzimas em polietilenotereftalato sob a forma azida. 1984. 0 f. Dissertação (Mestrado em Bioquímica) - Universidade Federal de Pernambuco. *Orientador:* Luiz Bezerra de Carvalho Junior.

MONTENEGRO, S. M. L. Uso de antigeno imobilizado em Dacron, sob a forma azida, para utilização em ensaio imunoenzimatico. 1991. Dissertação (Mestrado em Bioquímica) - Universidade Federal de Pernambuco. *Orientador:* Luiz Bezerra de Carvalho Junior.

MILAND, E.; SMITH, R.M.; FÁGÁIN, C.O. Increased thermal and solvent tolerance of acetylated horseradish peroxidase. Enzyme and Microbial Technology 19: 63-67, 1996.

MONTENEGRO, S. M. L.; ALMEIDA, A. M. P.; CARVALHO, A. B.; CARVALHO JUNIOR, L. B. The use of Dacron plates for dot enzyme linked immunosorbent assay. Memórias do Instituto Oswaldo Cruz, v. 86, p. 461-465, 1991.

NADRUZ JR, W.; MARQUES, E. T. A.; AZEVEDO, W. M.; LIMA FILHO, J. L.; CARVALHO JUNIOR, L. B. Immobilized xanthine oxidase on polyaniline silicone composite. Brazilian Journal of Medical and Biological Research, v. 29, p. 347-350, 1996.

NGAMNA, O.; MORRIN, A.; MOULTON, S.E.; KILLARD, A.J.; SMYTH , M.R.; WALLACE, G.G. An HRP based biosensor using sulphonated polyaniline. Synthetic Metals 153(1-3): 185-188, 2005.

NIELSEN, K.L.; INDIANI, C.; HENRIKSEN, A.; FEIS, A.; BECUCCI, M.; GAJHEDE, M.; SMULEVICH, G.; WELINDER, K.G. Differential activity and structure of highly similar peroxidases. Spectroscopic, crystallographic, and enzymatic analyses of lignifying *Arabidopsis thaliana* peroxidase A2 and horseradish peroxidase A2. Biochemistry 40: 11013-11021, 2001.

OLAD, A.; NABAVI, R. Application of polyaniline for the reduction of toxic Cr(VI) in water. Journal of Hazardous Materials 147(3): 845-851, 2007.

OLIVEIRA, E. A.; SILVA, M. P. C.; FIGUEIREDO, Z. M. B.; CARVALHO JUNIOR, L. B. Immobilization of proteins on plates of dacron. Applied Biochemistry and Biotechnology, v. 221, p. 109-114, 1989.

OLIVEIRA, I.R.W.Z.; VIEIRA, I.C. Construção e aplicação de biossensores usando diferentes procedimentos deimobilização da peroxidase de vegetal em matriz de quitosana. Química Nova 29(5): 932-939, 2006.

PADIGLIA, A.; CRUCIANI, E.; PAZZAGLIA, G.; MEDDA, R.; FLORIS, G. Purification and Characterization of *Opuntia* Peroxidase. Phytochemistry 38(2): 295-297, 1995.

PANDEY, P.C.; WEETALL, H.H. Peroxidase and tetracyanoquinomethane-modified graphite paste electrode for the measurement of glucose/lactate/glutamate using an enzyme-packed bed reactor. Analytical Biochemistry 224: 428-433, 1995.

PARADOSSI, G.; LISI, R.; PACI, M.; CRESCENZI, V. New hydrogels based on poly (vinyl alcohol). Journal of the Polymer Science Part A: Polymer Chemistry 34: 3417-3495, 1996.

PINHEIRO, S. M. B.; CARVALHO JUNIOR, L. B.; CHAVES, M. E. C. The Use of Feromagnetic Dacron as Solid Phase in Chemiluminescent Assays. Biotechnology Techniques, v. 13, p. 912-922, 1999.

RYAN, O.; SMITH, R.M.; FÁGÁIN, C.O. Thermostabilized chemical derivatives of horseradish peroxidase. Enzyme and Microbial Technology 16: 501-505, 1994.

SAKURADA, I. Polyvinyl alcohol fibers. London: CRC Press, 1985, 476p.

SIMON, E.; HALLIWELL, C.M.; TOH, C.S.; CASS, A.E.; BARTLETT, P.N. Immobilisation of enzymes on poly(aniline)-poly(anion) composite films. Preparation of bioanodes for biofuel cell applications. Bioelectrochemistry 55(1-2): 13-18, 2002.

SINGH, S.; SOLANKI, P.R.; PANDEY, M.K.; MALHOTRA, B.D. Covalent immobilization of cholesterol esterase and cholesterol oxidase on polyaniline films for application to cholesterol biosensor. Analytica Chimica Acta 568(1-2): 126-132, 2006.

SOUZA, A.L.B.; CHITARRA, M.I.F.; CHITARRA, A.B.; MACHADO, J.C. Respostas Bioquímicas em Tecidos de Pêssego Ferido Mecanicamente e Tratado com $CaCl_2$ no Local da Injúria. Ciênc. agrotec. 23(3): 658-666, 1999.

STRYER, L. (2004) Bioquímica. 5 ed. Rio de Janeiro: Guanabara Koogan, 1104 pp.

TORABI, S.F.; KHAJEH, K.; GHASEMPUR, S.; GHAEMI, N.; SIADAT, S.O.R. Covalent attachment of cholesterol oxidase and horseradish peroxidase on perlite through silanization: Activity, stability and co-immobilization. Journal of Biotechnology 131(2): 111-120, 2007.

TSAI, H.C.; DOONG, R. Simultaneous determination of renal clinical analytes in serum using hydrolase – and peroxidase – encapsulated optical array biosensors. Analytical Biochemistry 334(1): 183-192, 2004.

VAN TILBURG, R., Innovations Biotechnol., 1984, 20: 417–422

VITOLO, M. Imobilização de Enzimas. In: LIMA, U.A.; AQUARONE, E.; BORZANI, W.; SCHMIDELL, W. (coords.) Biotecnologia Industrial: processos

fermentativos e enzimáticos, vol. 3, cap. 18, São Paulo: Edgard Blücher, 2001, p. 391-404.

WISELMAN, A. Industrial practice with enzymes: application and sources of industrial enzymes. In: WISEMAN, A. HORWOOD, E. Handbook of Enzyme Biotechnology. New York: Wiley and Sons, 1975. p.252-259.

XU, K.; ZHU, L.; ZHANG, A.; JIANG, G. AND TANG, H. A peculiar cyclic voltammetric behavior of polyaniline in acetonitrile and its application in ammonia vapor sensor. Journal of Electroanalytical Chemistry 608(2): 141-147, 2007.

ZHAO, D.; GILFOYLE, D.J.; SMITH, A.T.; LOEW, G. H. Refinement of 3D models of horseradish peroxidase isoenzyme C: Predictions of 2D NMR assignments and substrate binding sites. Proteins: Structure, Function and Genetics 26(2): 204-216.

ANEXOS

Normas científicas para publicação em periódicos

I - CHEMOSPHERE

Guide for Authors

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Chemosphere accepts Research Papers, Review Papers, Short Communications, Letters to the Editor, Replies and Discussion Papers. Please note that papers with a routine nature and lacking originality, novelty and uniqueness will not be accepted for publication.

A Short Communication should be of significant scientific merit (a novel finding that warrants immediate publication).

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II - JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Guide for Authors

1. Scope

The journal is an international forum devoted to research and developments in the applications of whole-cell and cell-free enzymes as catalysts in organic synthesis. Emphasis is focused on mechanistic and synthetic aspects of the biocatalytic transformation.

Papers deal with the following topics;

• Industrial applications of enzymatic processes, e.g. in fine chemical synthesis;

• Chemo-, regio- and enantioselective transformations;

• Screening for biocatalysts;

• Integration of biocatalytic and chemical steps in organic syntheses;

• Novel biocatalysts, e.g. enzymes from extremophiles and catalytic antibodies;

• Enzyme immobilization and stabilization, particularly in non-conventional media;

• Bioprocess engineering aspects, e.g. membrane bioreactors;

• Improvement of catalytic performance of enzymes, e.g. by protein engineering or chemical modification;

• Structural studies, including computer simulation, relating to substrate specificity and reaction selectivity;

• Biomimetic studies related to enzymatic transformations.

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There are three types:

1. *Full-length*, or regular papers describing original research work not previously published.

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Artigos publicados

CARAMORI, Samantha Salomão; FERNANDES, Kátia Flávia. The Use of Poly(ethylene terephthalate)-Poly(aniline) Composite for Trypsin Immobilisation. Materials Science & Engineering. C, Biomimetic Materials, Sensors and Systems, *in* press, 2007.

CARAMORI, Samantha Salomão; LIMA, C. S.; FERNANDES, K. F. Biochemical Characterization of Selected Plant Species from Brazilian Savannas. Brazilian Archives of Biology and Technology, Curitiba, v. 47, n. 2, p. 253-259, 2004.

CARAMORI, Samantha Salomão; FERNANDES, K. F. Covalent Immobilization of Horseradish Peroxidase onto Poly(ethylene terephthalate)-Poly(aniline) Composite. Process Biochemistry, Bielefeld, v. 39, p. 883-888, 2004.

Trabalhos completos publicados em anais de eventos

FREITAS, Ana Carolyna R Queiroz de; CARAMORI, Samantha Salomão; TEIXEIRA, Juliê Renata Gomes; FERNANDES, K. F. Influência do pH na Extração e Avaliação do Potencial Biotecnológico de Plantas do Cerrado. In: 2º Seminário de Iniciação Científica da UEG, 2004, Anápolis. 2º Seminário de Iniciação Científica da UEG. Anápolis : PRP/UEG, 2004.

Resumos expandidos publicados em anais de eventos

MENEZES, Mara Luanda; CARAMORI, Samantha Salomão; FERNANDES, Kátia Flávia. Verificação de potencialidades bioquímicas visando aplicações biotecnológicas em *Diospyros hispida*. In: XIII Encontro de Biologia da UEG, 2007, Anápolis. Anais do XIII Enbio. Anápolis : UEG, 2007.

ARAÚJO, Amanda; CARAMORI, Samantha Salomão. Caracterização bioquímica de *Inga alba* e *Inga cylindrica*: uma avaliação de potencial biotecnológico. In: V Seminário de Iniciação Científica da UEG, 2007, Anápolis. Anais do V Seminário de Iniciação Científica da UEG. Anápolis : PRP, 2007.

MENEZES, Mara Luanda; CARAMORI, Samantha Salomão. Extração de peroxidase a partir de amostras de frutos de *Campomanesia sp*. e sua utilização para imobilização em suportes à base de polianilina. In: V Seminário de Iniciação Científica da UEG, 2007, Anápolis. Anais do V Seminário de Iniciação Científica da UEG. Anápolis : PRP, 2007.

BORGES, Patrícia Caetano de Medeiros; CARAMORI, Samantha Salomão. Uso de álcool-polivinílico-polianilina para imobilzação de lipase extraída de *Bacillus sp*. In: V Seminário de Iniciação Científica da UEG, 2007, Anápolis. Anais do V Seminário de Iniciação Científica da UEG. Anápolis : PRP, 2007.

Resumos publicados em anais de eventos

CARAMORI, Samantha Salomão; VIANA, Miriam Pereira; FARIA, Flaviana Naves de; FERNANDES, Kátia Flávia; CARVALHO JÚNIOR, Luiz Bezerra de. A practice and stable material for peroxidase assay based in hrp immobilization onto poly(vinyl alcohol)-glutaraldehyde-poly(aniline)-glutaraldehyde. In: XXXVI Reunião Anual da SBBq e 10ª IUBMB, 2007, Salvador. XXXVI SBBq and 10th IUBMB - scientific program. São Paulo: SBBq, 2007.

MENEZES, Mara Luanda; CARAMORI, Samantha Salomão; FERNANDES, Kátia Flávia. Immobilization of peroxidase from *Campomanesia sp*. onto polyaniline supports. In: XXXVI Reunião Anual da SBBq e 10ª IUBMB, 2007, Salvador. XXXVI SBBq and 10th IUBMU - scientific program. São Paulo: SBBq, 2007.

BORGES, Patrícia Caetano de Medeiros; Vieira, Tiago Lima; MITIDIERI, Sydnei; CARAMORI, Samantha Salomão; FERNANDES, Kátia Flávia. Covalent immobilization of lipase from *Bacillus sp*. onto poly(vinyl alcohol)-glutaraldehyde-poly(aniline)-glutaraldehyde discs. In: XXXVI Annual Meeting of the SBBq and 10th IUBMB Conference, 2007, Salvador. XXXVI Annual Meeting of the SBBq. São Paulo: SBBq, 2007.

CARAMORI, Samantha Salomão; FARIA, Flaviana Naves de; VIANA, Miriam Pereira; FERNANDES, Kátia Flávia; CARVALHO JÚNIOR, Luiz Bezerra de. Covalent immobilization of trypsin onto poly(vinyl alcohol)-glutaraldehyde-poly(aniline)-glutaraldehyde composite discs. In: XXXV Reunião Anual da SBBQ, 2006, Águas de Lindóia. XXXV SBBq - Anais e Resumos. São Paulo: SBBq, 2006.

ARAÚJO, Amanda; CARAMORI, Samantha Salomão; FERNANDES, Kátia Flávia. Biochemical characterization of *Inga alba* (sw.) Willd. and *Inga cylindrica* (vell. Conc.) Mart. seeds. In: XXV Reunião da Sociedade Brasileira de

Bioquímica e Biologia Molecular, 2006, Águas de Lindóia. XXXV SBBq - Anais e Resumos. São Paulo: SBBq, 2006.

CARAMORI, Samantha Salomão ; BELTRAO, E. I. C.; FERNANDES, Kátia Flávia; CARVALHO JÚNIOR, Luiz Bezerra de. The use of PVA-PANIG composite for affinity chromatography application. In: XXXIV Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2005, Águas de Lindóia. XXXIV Reunião Anual da SBBq - Programa e Resumos. São Paulo: Sociedade Brasileira de Bioquímica e Biologia Molecular, 2005.

PURCENA, Luiza Luanna Amorim; CARAMORI, Samantha Salomão; FERNANDES, Kátia Flávia. Covalent Immobilization of Trypsin onto Polyaniline. In: 57ª Reunião da Sociedade Brasileira para o Progresso da Ciência, 2005, Fortaleza. 57ª SBPC anais e resumos. São Paulo: SBPC, 2005.

CARAMORI, Samantha Salomão; FREITAS, Ana Carolyna R Queiroz de; TEIXEIRA, Juliê Renata Gomes; FERNANDES, Kátia Flávia Biochemical Characterization of Selected Plant Species from Brazilian Savannas. In: XXXIII Reunião Anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, 2004, Caxambu. XXXIII Reunião Anual SBBQ Programa e Resumos. São Paulo: SBBQ, 2004.