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PURIFICAÇÃO E IMOBILIZAÇÃO DE UMA PROTEASE QUERATINOLÍTICA PRODUZIDA POR *Chryseobacterium* sp. linhagem kr6

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Com o texto a seguir, dedico este trabalho aos meus avós.

Era uma vez, no antigo país das fábulas, uma família em que havia um pai, uma mãe, um avô que era o pai do pai e aquela já mencionada criança de oito anos, um rapazinho. Ora sucedia que o avô já tinha muita idade, por isso tremiam-lhe as mãos e deixava cair a comida da boca quando estavam à mesa, o que causava grande irritação ao filho e à nora, sempre a dizerem-lhe que tivesse cuidado com o que fazia, mas o pobre velho, por mais que quisesse, não conseguia conter as tremuras, pior ainda se lhe ralhavam, e o resultado era estar sempre a sujar a toalha ou a deixar cair comida ao chão, para já não falar do guardanapo que lhe atavam ao pescoço e que era preciso mudar-lhe três vezes ao dia, ao almoço, ao jantar e à ceia. Estavam as cousas neste pé e sem nenhuma expectativa de melhora quando o filho resolveu acabar com a desagradável situação. Apareceu em casa com uma tigela de madeira e disse ao pai, A partir de hoje passará a comer aqui, senta-se na soleira da porta porque é mais fácil de limpar e assim já a sua nora não terá de preocupar-se com tantas toalhas e tantos guardanapos sujos. E assim foi. Almoço, jantar e ceia, o velho sentado sozinho na soleira da porta, levando a comida à boca conforme lhe era possível, metade perdia-se no caminho, uma parte da outra metade escorria-lhe pelo queixo abaixo, não era muito o que lhe descia finalmente pelo que o vulgo chama o canal da sopa. Ao neto parecia não lhe importar o feio tratamento que estavam a dar ao avô. Até que uma tarde, ao regressar do trabalho, o pai viu o filho a trabalhar com uma navalha um pedaço de madeira e julgou que, como era normal e corrente nessas épocas remotas, estivesse a construir um brinquedo por suas próprias mãos. No dia seguinte, porém, deu-se conta de que não se tratava de um carrinho, pelo menos não se via sítio onde se lhe pudessem encaixar umas rodas, e então perguntou, Que estás a fazer. O rapaz fingiu que não tinha ouvido e continuou a escavar na madeira com a ponta da navalha. Não ouviste, que estás a fazer com esse pau, tornou o pai a perguntar, e o filho, sem levantar a vista da operação, respondeu, Estou a fazer uma tigela para quando o pai for velho e lhe tremerem as mãos, para quando o mandarem comer na soleira da porta como fizeram ao avô. Foram palavras santas. Caíram as escamas dos olhos do pai, viu a verdade e a sua luz, e no mesmo instante foi pedir perdão ao progenitor e quando chegou a hora da ceia por suas próprias mãos o ajudou a sentar-se na cadeira, por suas próprias mãos lhe levou a colher à boca, por suas próprias mãos lhe limpou suavemente o queixo, porque ainda o podia fazer e o seu querido pai já não.

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PURIFICAÇÃO E IMOBILIZAÇÃO DE UMA PROTEASE

QUERATINOLÍTICA PRODUZIDA POR *Chryseobacterium* sp. linhagem kr6

RESUMO

Queratinases (E.C. 3.4.21/24/99.11) são um grupo de enzimas proteolíticas que catalisam a hidrólise da queratina. A linhagem queratinolítica *Chryseobacterium* sp. kr6 apresenta elevada produção de queratinases extracelulares, demonstrando potencial para bioconversão de substratos constituídos por queratina. O presente trabalho teve como principal objetivo purificar e imobilizar uma queratinase produzida pelo *Chryseobacterium* sp. kr6. As condições ótimas para a atividade proteolítica da queratinase foram estabelecidas com o auxílo das ferramentas estatísticas de planejamento experimental e superfície de resposta. Os resultados demonstraram que tais condições foram atingidas ao utilizar pH na faixa de 7,4 a 9,2, 35°C a 50°C e concentração de NaCl de 50 a 340 mmol L^{-1}. A especificidade da queratinase frente a diferentes substratos também foi investigada, indicando a preferência da enzima por resíduos hidrofóbicos ou positivamente carregados. A completa purificação da queratinase envolveu a precipitação com sulfato de amônio, cromatografias de permeação em gel e troca aniônica. A amostra obtida após as referidas etapas apresentou um fator de purificação de 40,2 vezes e atividade específica de 21.466 U mg^{-1} de proteína. A massa molecular da enzima, determinada por SDS-PAGE, foi, de aproximadamente, 20 kDa. Os parâmetros cinéticos e termodinâmicos para a inativação térmica da queratinase, sob diferentes condições, foram estimados. A partir dos resultados obtidos, observou-se que a presença de cálcio aumenta significativamente a estabilidade térmica da enzima. Comparando com as amostras controles, o tempo de meia vida da enzima purificada na presença de Ca^{2+} aumentou 7,3, 20,2 e 9,8 vezes, a 50°C, 55°C e 60ºC, respectivamente. A atividade enzimática foi significativamente inibida na presença de EDTA e 1,10-fenantrolina, sugerindo se tratar de uma metaloprotease. O desenvolvimento de um suporte a base de quitosana para a imobilização covalente da queratinase purificada foi investigado. Para isso, foram avaliados os efeitos de diferentes concentrações de glutaraldeído e tempo de contato deste com as esferas de quitosana, sobre o percentual de imobilização da enzima. Os melhores resultados obtidos para a imobilização da queratinase ao suporte foram ao utilizar concentração de glutaraldeído na faixa de 34 a 56 g L^{-1} e um período de ativação das esferas na faixa de 6 a 10 h. Sob tais condições, obteve-se 80% de imobilização da enzima. A partir das condições ótimas para a imobilização da enzima ao suporte, apontadas pela metodologia de superfície de resposta, estimou-se a capacidade de carga máxima do suporte, sendo esta de 58,8 U g^{-1}. A enzima imobilizada apresentou maior estabilidade térmica quando comparada com a forma livre, além de reter 63,4% da atividade enzimática inicial após cinco reutilizações.

PURIFICATION AND IMMOBILIZATION OF A PROTEASE KERATINOLYTIC FROM *Chryseobacterium* sp. strain kr6

ABSTRACT

Keratinases (E.C. 3.4.21/24/99.11) are a group of proteolytic enzymes that are able to catalize the keratin hydrolysis. The keratinolytic *Chryseobacterium* sp. kr6 strain shows high extracellular keratinases production, suggesting potential for bioconversion of keratinous substrates. The present work had as main objective to purify and immobilize a keratinase from *Chryseobacterium* sp. kr6. The optimal conditions for proteolytic activity were established with aid of experimental design and response surface methodology. The results demonstrated that the best conditions were at pH range of 7.4 to 9.2, 35°C to 50°C and NaCl concentration from 50 to 340 mmol L^{-1}. Keratinase specificity for various substrates also was investigated, suggesting that the enzyme had preference for hydrophobic and positively charged residues. The keratinase purification involved precipitation with ammonium sulphate and chromatographic techniques of gel permeation and anionic exchange. The final sample obtained after the purification steps presents a purification factor of 40.2-fold and specific activity of 21,466 U mg^{-1} of protein. The molecular weight of the enzyme, determined by SDS-PAGE, was around 20 kDa. The kinetics and thermodynamics parameters for thermal keratinase inactivation, under different conditions, were estimated. From results, it was possible to observe that the calcium affect significantly the thermal stability of the enzyme. Comparing with the control samples, the half-life time of the purified enzyme with calcium increased about 7.3, 20.2 and 9.8-fold, at 50°C, 55°C and 60°C, respectively. The enzyme activity was significantly inhibited in the presence of EDTA and 1,10-phenanthroline, suggesting that the enzyme belongs to metalloprotease group. The developing of a chitosan support for covalent immobilization of the purified keratinase was investigated. The effects of different glutaraldehyde concentrations, as well as, the activation time required for the chitosan beads on the enzyme immobilization were investigated. The optimal conditions for enzyme immobilization were at glutaraldehyde concentration ranging from 34 to 56 g L^{-1} and activation time of 6 to 10 h. Under these conditions, above 80% of added enzyme was covalently immobilized on the support. From the best conditions, indicated by response surface methodology, the load capacity of the macrospheres was estimated, being of 58.8 U g^{-1}. The immobilized enzyme presented higher thermal stability when compared with free one, besides it retained 63,4% of the initial enzyme activity after five cicles of reuse.

1. INTRODUÇÃO

A produção de diversos setores agroindustriais é responsável pela geração de uma variada gama de subprodutos, sendo que muitos destes possuem elevado conteúdo de biopolímeros, como celulose, lignina, quitina e queratina. Tais resíduos apresentam, atualmente, reduzido valor agregado, em virtude da inexistência de tecnologias que visem seu aproveitamento. A degradação dessas macromoléculas depende de enzimas extracelulares que possuem habilidade de atuar na superfície desses substratos geralmente recalcitrantes.

As indústrias de processamento de aves geram resíduos com elevado conteúdo protéico, principalmente penas, que são constituídas de queratina. Esta, em sua forma nativa, apresenta reduzido valor nutricional, em função da composição e configuração molecular dos seus aminoácidos constituintes, que proporcionam uma rigidez estrutural característica. Modificações na estrutura da queratina através de processos físico-químicos, como tratamento térmico ou alcalino, possuem desvantagens relativas ao custo elevado, além de promoverem a destruição de alguns aminoácidos essenciais.

A aplicação de técnicas de bioconversão, enzimática ou microbiana, é uma alternativa atraente para o tratamento e, consequentemente, melhor aproveitamento dos resíduos queratinosos. Tais técnicas são, em geral, operadas em condições moderadas, requerendo reduzido aporte energético e, ainda, são ambientalmente amigáveis. O mercado industrial de enzimas está em constante crescimento em função da melhoria das tecnologias de produção, avanços na área de engenharia de proteínas, bem como a diversificação do campo de aplicação.

As queratinases microbianas despertam a atenção dos pesquisadores, particularmente em função das suas múltiplas possibilidades de aplicações industriais, em especial, nas indústrias de fertilizantes, farmacêutica, de detergentes, coureiras,

ração animal e alimentos. No entanto, a aplicação de enzimas em escala industrial é, muitas vezes, dificultada em função do elevado custo de produção e purificação, bem como inconvenientes relacionados a sua instabilidade em determinadas condições operacionais e sua solubilidade no meio reacional. A utilização de enzimas em processos industriais é viável apenas se estas permanecerem estáveis frente a variações de temperatura, pH e presença de substâncias químicas.

A imobilização de enzimas em suportes sólidos é, provavelmente, uma das estratégias que apresenta maior potencial para incrementar a estabilidade operacional desses biocatalisadores. As principais vantagens do uso de enzimas imobilizadas estão relacionadas com o fato da enzima tornar-se um catalisador heterogêneo, possibilitando a sua separação e reutilização. A possibilidade de desenvolvimento de sistemas contínuos, maior estabilidade, bem como uma diversidade de desenho dos biorreatores são outras características interessantes atribuídas às enzimas imobilizadas.

Dentre as principais aplicações para proteases imobilizadas, destacam-se a hidrólise de proteínas em aminoácidos, investigação da estrutura de proteínas através da hidrólise controlada, avaliação da digestibilidade de proteínas.

Nesse contexto, o presente trabalho visa fornecer informações relacionadas a purificação e imobilização de uma protease queratinolítica produzida por *Chryseobacterium* sp. kr6, visando contribuir para a sua aplicação em processos de bioconversão.

2. REVISÃO BIBLIOGRÁFICA

2.1 Proteases

A clivagem proteolítica das ligações peptídicas é uma das mais frequentes e importantes modificações enzimáticas das proteínas (BEYNON & BOND, 1996). As proteases são uma classe única de enzimas, haja vista sua imensa importância fisiológica e comercial (RAO et al., 1998). Elas possuem propriedades tanto degradativas quanto sintéticas, pois catalisam a hidrólise das ligações peptídicas em ambiente aquoso e sintetizam-as em ambiente não aquoso (AFRIN et al., 2000; GUPTA & KHARE, 2006). Por serem fisiologicamente necessárias, estão ubiquamente distribuídas em animais, plantas e microrganismos. Estes últimos são a fonte preferida para produção comercial de enzimas, em função do seu rápido crescimento, reduzido espaço requerido para os cultivos, bem como a facilidade com que podem ser manipulados geneticamente para produzir enzimas com propriedades diferenciadas. As proteases microbianas possuem longo histórico de aplicação na indústria de alimentos e de detergentes (RAO et al., 1998). Representam um dos três grandes grupos de enzimas industriais, contribuindo com 60% do total das enzimas comercializadas no mundo (MUKHERJEE et al., 2008).

2.1.1 Classificação

Segundo o Comitê de Nomenclatura Enzimática (EC) da União Internacional de Bioquímica e Biologia Molecular (NC-IUBMB; www.chem.qmul.ac.uk/iubmb/enzyme/), as peptidases são enzimas que pertencem à classe 3 (hidrolases) e a subclasse 3.4 (peptídeo hidrolases ou peptidases. As peptidases que clivam ligações no interior da cadeia polipeptídica são classificadas como

endopeptidases e as que atuam nas extremidades da cadeia, de exopeptidases. As exopeptidases que atuam na região N-terminal da proteína são classificadas como aminopeptidases e as que atuam na região C-terminal são denominadas carboxipeptidases.

Entretanto, a especificidade das proteases não se relaciona apenas à posição da ligação peptídica ou ao tamanho na cadeia de resíduos de aminoácidos, existindo também seletividade em relação a sequência de aminoácidos vizinhos à ligação. Comumente, utiliza-se uma classificação baseada na estrutura química do sítio ativo. Nesse sistema, as carboxipeptidases são subdivididas em serina-, metalo- e cisteína-carboxipeptidases. As endopeptidases são subdivididas em serina-, cisteína-, aspártico-, metalo e treonina-endopeptidases. Existe, atualmente, um sexto tipo catalítico, as glutâmico-peptidases (BON et al., 2008). De acordo com o pH ótimo para a atividade enzimática, as proteases também são referenciadas como ácidas, neutras ou alcalinas (RAO et al., 1998).

As serina endo- e exo-peptidases estão amplamente distribuídas, apresentando funções diversas. Existem muitas famílias distintas, sendo estas agrupadas em seis clãs, dos quais os dois maiores são os clãs tipo-(quimo)tripsina e tipo-subtilisina (SIEZEN & LEUNISSEN, 1997). As serina-proteases são reconhecidas por serem irreversivelmente inibidas por 3,4-dicloroisocumarina (3,4-DSI), L-3-carboxitrans-2,3-epoxipropil-leucilamido (4-guanidina) butano, diisopropilfluorofosfato (DFP), fluoreto de fenilmetilsulfonil (PMSF) (BARRET, 1994; RAO et al., 1998). Segundo LIN et al. (1995), muitas proteases serínicas, produzidas por *Bacillus*, têm sido sequenciadas, clonadas e caracterizadas.

As proteases aspárticas, comumente conhecidas como proteases ácidas, são endopeptidases que dependem de resíduos de ácido aspártico para sua atividade

catalítica, exercem atividade catalítica máxima em valores de pH na faixa de 3,0 a 4,0. Um potente inibidor é a pepstatina, um hexapeptídeo que no estado de transição se parece com o substrato (BEYNON & BOND, 1996).

A atividade de todas as cisteíno proteases depende de uma díade catalítica, cisteína e histidina, sendo que a ordem desses resíduos difere dentro das famílias. Em geral, as cisteína-proteases são ativas apenas na presença de agentes redutores, tais como HCN, ou cisteína, são suscetíveis a agentes sulfidrílicos, porém, não são afetadas por DFP, nem por agentes quelantes de metais (RAO et al., 1998).

As metaloproteases são o grupo que apresenta maior diversidade de sítios catalíticos, caracterizadas por requererem íons metálicos divalentes para sua atividade. Cerca de 30 famílias de metaloproteases são reconhecidas, das quais 17 contêm somente endopeptidases, 12 contêm somente exopeptidases e 1 (M3) contêm tanto endo- quanto exopeptidase. São inibidas por agentes quelantes, tais como EDTA, mas não por agentes sulfidrílicos ou DFP (RAO et al., 1998).

2.1.2 Queratinases microbianas

As queratinases (E.C. 3.4.21/24/99.11) são um importante grupo de enzimas proteolíticas com capacidade para catalisar a degradação de substratos queratinosos (GUPTA & RAMNANI, 2006). Estão amplamente distribuídas na natureza, sendo produzidas por uma variedade de microrganismos, isoladas, principalmente, em efluentes da indústria avícola (RIFFEL & BRANDELLI, 2002).

Dentre os fungos queratinolíticos, destacam-se os gêneros *Chrysosporium* (EL-NAGHY et al., 1998), *Acremonium*, *Alternaria*, *Aspergillus*, *Curvularia*, *Paecilomyces*, *Penicillium* (FARAG & HASSAN, 2004; MARCONDES et al., 2008; VESELÁ & FRIEDRICH, 2009), *Scopulariopsis* (KAUL & SUMBALI, 1997; ANBU et al., 2005),

Cladosporium, *Doratomyces* (VIGNARDET et al., 2001; FRIEDRICH et al., 2005), *Trichophyton* (SINGH, 1997; ANBU et al., 2008).

Na ordem Actinomycetales, mais conhecida por actinomicetos, o gênero *Streptomyces*, como o *S. fradiae* (YOUNG & SMITH, 1975; KUNERT, 1989), *Streptomyces* sp. (MUKHOPADHYAY & CHANDRA, 1990; LETOURNEAU et al., 1998; CHAO et al., 2007; TATINENI et al., 2007; MABROUK, 2008), *S. pactum* (BÖCKLE et al., 1995), *S. albidoflavus* (BRESSOLLIER et al., 1999), *S. gulbargensis* (DASTAGER et al., 2009) e o gênero *Thermoactinomyces*, como o *T. candidus* (IGNATOVA et al., 1999), são correntemente descritos como degradadores de queratina, demonstrando habilidade para atuar em uma ampla variedade de substratos queratinosos, incluindo cabelos, pelos e penas.

Bactérias gram-positivas do gênero *Bacillus* (LEE et al., 2002; DAROIT et al., 2009), em especial, *B. subtilis* (KIM et al., 2001; SUH & LEE, 2001; MACEDO et al., 2005), e *B. licheniformis* (WILLIAMS et al., 1990; ROZS et al., 2001; KORKMAZ et al., 2004), *B. cereus* (NILEGAONKAR et al., 2007) e *B. megaterium* (PARK & SON, 2007), *Lysobacter* (ALLPRESS et al., 2002), *Nesterenkonia* sp. (GESSESSE et al., 2003; BAKHTIAR et al., 2005), *Kocuria* (BERNAL et al., 2006) e *Microbacterium* sp. (THYS & BRANDELLI, 2006) são frequentemente reportadas como produtoras de queratinases. Todavia, poucas linhagens de bactérias gram-negativas, entre elas, *Vibrio* (SANGALI & BRANDELLI, 2000), *Xanthomonas* (DE TONI et al., 2002), *Stenotrophomonas* (LUCAS et al., 2003; CAO et al., 2009) e *Chryseobacterium* (SANGALI & BRANDELLI, 2000, RIFFEL et al., 2003; WANG et al., 2008) são relacionadas à produção de proteases queratinolíticas.

Apesar da produção de proteases com capacidade queratinolítica estar amplamente distribuída em uma diversidade de microrganismos, poucos são aqueles

que atingiram o estágio de exploração comercial. Uma exceção é a queratinase produzida por *Bacillus licheniformis* PWD1 – fonte da Versazyme – a primeira queratinase comercial desenvolvida por WILLIAMS e colaboradores (1990) e LIN e colaboradores (1992).

2.1.2.1 Aplicações

Queratinases microbianas têm atraído uma grande parcela de atenção, particularmente em função de suas múltiplas possibilidades de aplicações, dentre elas destacam-se as indústrias de ração animal, fertilizantes, detergentes, coureira e farmacêutica (GUPTA & RAMNANI, 2006). Em especial, as enzimas queratinolíticas bacterianas possuem potencial para aplicação em processos biotecnológicos não poluentes que visem a bioconversão de efluentes ricos em queratina (BRANDELLI, 2008).

O incremento nutricional da farinha de penas através da aplicação de queratinases têm sido descrito em alguns trabalhos (ONIFADE et al., 1998; GRAZZIOTIN et al., 2006). Conforme relato de LEE et al. (1991) e ODETALLAH et al. (2003) o uso da queratinase bruta aumentou significativamente a digestibilidade aminoacídica de penas não tratatas e de farinha de penas comercial.

O processamento biotecnológico do couro envolve a utilização de uma mistura de enzimas, dentre as quais estão as proteases, lipases e carboidrases (GUPTA & RAMNANI, 2006). A aplicação de proteases queratinolíticas sem atividade colagenolítica e com moderada atividade elastinolítica possibilita a exploração destas na etapa depilatória, em substituição ao sulfeto de sódio (MACEDO et al., 2005). Há, ainda, uma considerável quantidade de efluente orgânico gerado durante o processamento do couro. A biodegradação desse efluente tem sido investigada, um exemplo é a habilidade demonstrada por *Streptomyces* e *Bacillus* para hidrolisar

queratina oriunda de cabelos e pelos (HOOD & HEALY, 1994). Outras características que merecem destaque são a completa remoção dos pelos, elevada eficiência, redução do conteúdo de enxofre no efluente e catálise seletiva (BRANDELLI, 2008). Linhagens proteolíticas de *Bacillus subtilis* e *B. amyloliquefaciens*, anteriormente caracterizadas, apresentaram propriedades adequadas para o processamento do couro (GEORGE et al., 1995; VARELA et al., 1997; GIONGO et al., 2007).

Efluentes ricos em queratina também são considerados fertilizantes de solo. As penas possuem cerca de 15% de nitrogênio, apresentando um grande potencial para serem utilizadas como fertilizantes com liberação lenta de nitrogênio. Caso a estrutura da queratina seja modificada enzimaticamente, a taxa de mineralização aumenta (WILLIANS et al., 1990). VESELÁ & FRIEDRICH (2009) produziram uma mistura contendo proteína solúvel, peptídeos e aminoácidos, a partir da hidrólise de um efluente rico em queratina por uma queratinase produzida por *Paecilomyces marquandii*, sugerindo potencial para produção de fertilizante foliar.

As queratinases que não hidrolisam substratos como o colágeno possuem características adequadas para propósitos cosméticos e farmacêuticos, visto que o colágeno não deve ser danificado. Dentre as aplicações potenciais estão: como ingrediente em produtos para depilação ou em formulações para uso tópico, auxiliando a remoção de calos (VIGNARDET et al., 2001).

Observa-se o aumento no interesse pelo desenvolvimento tecnologias envolvendo a bioconversão de queratinas em filmes e coberturas biodegradáveis, tanto para aplicações na agricultura quanto biomédicas (SCHROOYEN et al., 2001).

As enzimas proteolíticas dominam o mercado de detergentes, sendo que as proteases alcalinas respondem por aproximadamente 89% da comercialização desse grupo de enzimas (GUPTA et al., 2002). Não obstante, há uma incessante busca por

novas enzimas com propriedades que permitam ampliar a utilização de detergentes a base desses biocatalisadores. Nesse sentido, as queratinases são atrativas, pois possuem a habilidade de se ligar e hidrolisar substratos sólidos, característica interessante para formulações de detergentes, que requerem enzimas capazes de atuar sobre substratos protéicos ligados a superfícies sólidas (GUPTA & RAMNANI, 2006).

2.1.2.2 Purificação de queratinases

Muitas vezes, os avanços em biotecnologia dependem do desenvolvimento de técnicas eficazes para a purificação de biomoléculas. O sucesso da purificação de proteínas está na seleção da técnica mais apropriada, combinando-a de forma lógica para maximizar o rendimento e minimizar o número de etapas requeridas. A maioria dos protocolos de purificação utiliza como etapa inicial a concentração da amostra através de agentes precipitantes, em geral, sulfato de amônio, e subsequentemente, técnicas cromatográficas (SAXENA et al., 2003).

WANG et al. (2008) purificaram três metaloproteases queratinolíticas produzidas por *Chryseobacterium indologenes* TKU014. Para tal, utilizaram precipitação do caldo bruto com sulfato de amônio, seguida por cromatografia de troca iônica e interação hidrofóbica. Esta última etapa cromatográfica rendeu três picos com atividade queratinolítica, denominados P1, P2 e P3, resultando em fatores de purificação de 16, 22 e 11, respectivamente, e rendimento global de 9%. A obtenção de mais de um pico com atividade queratinolítica, durante a purificação de queratinases já havia sido reportada por RIFFEL et al. (2007), ao desenvolver um protocolo para purificação de uma queratinase produzida por *Chryseobacterium* sp. kr6, onde foram detectados três picos com atividade queratinolítica. O processo desenvolvido pelos referidos autores envolveu cromatografia de interação hidrofóbica e permeação em gel, sendo que ao final das etapas de purificação obteve-se um fator de purificação de 14,26 vezes e um

rendimento global de 1,18%. Fatores de purificação elevados são, muitas vezes, atingidos ao se utilizar uma extensa sequência de etapas cromatográficas. DE TONI et al. (2002), ao purificar uma queratinase produzida por *Xanthomonas maltophilia*, utilizando quatro passos cromatográficos, obtiveram um fator de purificação de 177 vezes, no entanto o rendimento final foi de apenas 0,9%. Rendimentos na faixa de 20 a 45% são normalmente obtidos para purificação de queratinases, utilizando duas a três etapas cromatográficas (LEE et al., 2002; GESSESSE, et al., 2003; FARAG & HASSAN, 2004; ANBU et al., 2005; THYS & BRANDELLI, 2006).

2.2 Queratinas

A queratina é uma proteína mecanicamente durável e quimicamente não reativa que ocorre em todos os vertebrados superiores. É o principal componente da rígida camada externa da epiderme e de seus apêndices relacionados, como cabelos, chifres, unhas e penas. As queratinas têm sido classificadas em α-queratinas, onde predominam segmentos de polipeptídeos em α-hélice, que se enrolam e formam uma estrutura bastante rígida; e β-queratinas, cujas folhas β formam filamentos flexíveis e macios. Cada mamífero possui cerca de 30 variantes de queratina, cuja expressão varia de tecido para tecido (BON et al., 2008; VOET et al., 2008).

A α-queratina é rica em resíduos de cisteína, que formam ligações dissulfeto responsáveis pela ligação cruzada entre cadeias polipeptídicas adjacentes. As α-queratinas são classificadas como “duras” ou “moles” se possuírem alto ou baixo teor de enxofre, respectivamente. As queratinas “duras”, como as constituintes do cabelo, chifres e unhas, são menos flexíveis do que as queratinas “moles”, como da pele e de calos, resistindo à deformação devido às ligações dissulfeto (VOET et al., 2008).

2.3 Estabilidade das enzimas

As enzimas sofrem uma variedade de reações desnaturantes durante sua produção, armazenagem e utilização na indústria. A desnaturação é o desdobramento da estrutura terciária da enzima gerando um polipeptídeo desordenado, no qual resíduos chave não estão mais alinhados próximos o suficiente para continuar participando das interações de estabilização estrutural ou funcional (IYER & ANANTHANARAYAN, 2008). A estabilidade *in vitro* de enzimas e proteínas permanece um assunto crítico na biotecnologia, visto que tanto a estabilidade operacional quanto a de armazenagem influenciam consideravelmente a aplicação de produtos e processos enzimáticos (FAGÁIN, 2003).

A baixa estabilidade conformacional das enzimas as tornam muito suscetíveis à desnaturação por alteração do balanço das forças fracas (que não as de ligação) que mantêm a conformação nativa. A desnaturação pode ocorrer por uma variedade de condições: aquecimento, variações de pH, presença de detergentes e agentes caotrópicos (VOET et al., 2008). A desnaturação pode ser revertida por meio da retirada do agente desnaturante, no entanto, a proteína pode vir a sofrer mudanças químicas que induzam a uma perda irreversível da atividade (IYER & ANANTHANARAYAN, 2008).

De acordo com o modelo amplamente aceito de Lumry-Eyring, a inativação enzimática é um fenômeno de, pelo menos, duas etapas: o desdobramento reversível da enzima nativa, seguida pela etapa cineticamente irreversível, a qual induz a agregação ou mudanças covalentes na enzima. Visando incrementar a estabilidade das enzimas, três principais rotas de pesquisa são focadas:

a) Isolamento de enzimas produzidas por extremófilos;

b) Produção de enzimas estáveis via manipulação genética de organismos mesófilos;

c) Estabilização de enzimas instáveis por métodos como: engenharia de proteínas, modificações químicas, uso de aditivos e imobilização (FAGÁIN, 2003; IYER & ANANTHANARAYAN, 2008).

2.4 Imobilização de enzimas

A imobilização de enzimas, técnica especificamente desenvolvida para restringir a liberdade de movimento destas, é obtida através da fixação da enzima “em” ou “no interior” de suportes sólidos, resultando em um sistema heterogêneo (BICKERSTAFF, 1997; KRAJEWSKA, 2004). Quando comparada com as enzimas livres, as imobilizadas são mais robustas, além disso, a heterogeneidade do sistema imobilizado possibilita maior facilidade de recuperação tanto do catalisador quanto do produto, múltiplas reutilizações, operações em processos contínuos, bem como uma maior flexibilidade na escolha dos diversos modelos de biorreatores (KRAJEWSKA, 2004; CAO, 2005).

O termo “enzima imobilizada” inclui:

* A modificação das enzimas de forma as torná-las insolúveis em água.

* A utilização de enzimas na forma solúvel em reatores equipados com membranas de ultrafiltração, que permitem o escoamento dos produtos da reação, mas retém a enzima no interior do reator.

* A restrição da mobilidade da enzima pela ligação a outra molécula, que torna o sistema insolúvel no meio de reação. O sistema imobilizado permite a condução de reações em reatores contínuos, com fácil separação de catalisador-produto, aumento da produtividade do processo (BON et al., 2008).

As enzimas podem ser imobilizadas por uma variedade de técnicas, sendo quatro destas consideradas as principais: a) adsorção; b) reticulação; c) encapsulação; d) ligação covalente (BICKERSTAFF, 1997). Numerosos outros protocolos reportados na

literatura são combinações dos supracitados (KRAJEWSKA, 2009). No entanto, não há um único método ou suporte ideal para todas as enzimas, haja vista a diversidade de características e composição desses biocatalisadores, bem como as diferenças nas propriedades dos substratos e produtos e, ainda, a diversidade de aplicação dos produtos (D'SOUZA, 1999; CAO, 2005).

A imobilização por adsorção é a técnica considerada mais simples, envolve interações de superfície reversíveis entre a enzima e o suporte (KRAJEWSKA, 2009). As forças envolvidas são, principalmente, eletrostáticas, como van der Waals, interações de hidrogênio e iônicas, embora interações hidrofóbicas possam ser consideráveis. Tais forças são muito fracas, no entanto, em quantidade suficiente são capazes de proporcionar ligação razoável (BICKERSTAFF, 1997; KRAJEWSKA, 2004). O procedimento consiste em misturar os componentes biológicos e o suporte com propriedades de adsorção, sob condições propícias de pH e força iônica, por um dado período, seguida da coleta do material imobilizado e extensivas lavagens para retirada do material biológico não adsorvido. Quitosana, quitina, alumina e resinas de troca iônica são suportes frequentemente utilizados em imobilização por adsorção (ABDEL-NABY et al., 1998; ROY et al., 2000).

A imobilização por reticulação difere da adsorção e da ligação covalente em função de que as moléculas da enzima estão livres em solução, mas seu movimento é restrito pela estrutura entrelaçada do gel. A porosidade do entrelaçamento do gel é controlada para assegurar que a estrutura seja ajustada o suficiente para prevenir o escape da enzima, contudo, permitindo, ao mesmo tempo, o movimento do substrato e produto. Inevitavelmente, o suporte atuará como uma barreira à transferência de massa, provocando sérias consequências para a cinética da reação (BICKERSTAFF, 1997). Tem sido extensivamente utilizada para imobilização de células e não de enzimas

(D'SOUZA, 1999). Dentre os suportes mais utilizados estão a poliacrilacrilamida, alginatos, politetrafluoroetileno, carragenas, gel de álcool polivinílico (ABDEL-NABY et al., 1998; ABDEL-NABY et al., 1999a; AFRIN et al., 2000; BELYAEVA et al., 2004). ALSARRA et al. (2002) obtiveram sucesso ao utilizar hidrogel de quitosana para imobilizar lipase utilizando a técnica de reticulação.

A encapsulação de enzimas pode ser obtida pela captura dos componentes biológicos no interior de uma membrana semipermeável. É similar a técnica de reticulação, pois a enzima está livre em solução, no entanto em um espaço restrito. Dessa forma, as moléculas de enzima não podem atravessar a membrana, enquanto que o substrato e os produtos atravessam livremente a membrana (BICKERSTAFF, 1997). Consequentemente, somente substratos de baixa massa molecular podem ser empregados com esse tipo de enzima imobilizada (BON et al., 2008).

A imobilização através de ligação covalente envolve a formação de uma ligação forte entre grupos funcionais presentes na superfície do suporte e grupos funcionais pertencentes aos resíduos dos aminoácidos na superfície da enzima. Apesar desse método de imobilização ser um dos mais estudados e difundidos, a seleção das condições de imobilização é mais difícil do que nas outras técnicas (BON et al., 2008). Alguns grupos funcionais dos aminoácidos são adequados para participação na formação da ligação, dentre os quais destacam-se o grupo amino (NH_2) da lisina ou arginina, o grupo carboxila (CO_2H) do ácido aspártico ou glutâmico, o grupo hidroxila (OH) da serina ou treonina, e o grupo sulfidrila (SH) da cisteína. Muitos fatores podem influenciar a seleção de um suporte em particular, as pesquisas têm demonstrado que a hidrofilicidade é o fator mais importante para manutenção da atividade enzimática no ambiente do suporte (BICKERSTAFF, 1997). Na técnica de ligação covalente é crucial escolher um método que não inative a enzima em função de reações com resíduos dos

aminoácidos do sítio ativo. Uma enorme gama de suportes para imobilização covalente é reportada na literatura (CHAE et al., 2000; RAGNITZ et al., 2001; KARIN & HASHINAGA, 2002; FERNANDES et al., 2003; KANNAN & JASRA, 2009). Compósitos formados pela associação de materiais também têm demostrado potencial para aplicação na imobilização de enzimas via ligação covalente (CHANG & JUANG, 2005). ABDEL-NABY et al. (1999b) demonstraram que, dentre as várias técnicas e suportes utilizados para imobilização da tanase produzida por *Aspergillus oryzae*, a ligação covalente em quitosana apresentou os melhores resultados em termos de rendimento da imobilização e atividade específica da enzima imobilizada.

A ativação do grupo ligante é frequentemente realizada no suporte a fim de reduzir o risco de diminuição da atividade catalítica (BON et al., 2008). Um fator relevante para o sucesso da técnica de imobilização por ligação covalente é a escolha do agente bifuncional, também denominado espaçador. Dentre os mais utilizados estão a benzoquinona, carbodiimidas e o glutaraldeído, que apresentam grupos os funcionais similares aos presentes na superfície da enzima (COCHRANE et al., 1996). NOUAIMI et al. (2001) estudaram diferentes estratégias para a imobilização de tripsina em escamas de poliéster no que diz respeito ao comprimento da cadeia do espaçador. Eles observaram que a fixação direta da enzima na superfície do suporte resultou em uma baixa atividade desta na forma imobilizada, bem como reduzida estabilidade. Dentre os espaçadores avaliados, albumina de soro bovino (BSA) se mostrou o mais eficiente.

A principal tarefa para desenvolver um biocatalisador imobilizado está na seleção de um suporte adequado, condições (pH, temperatura, natureza do meio) e a enzima (fonte, natureza e grau de pureza). A natureza do método deve satisfazer tanto os critérios catalíticos (expressos como produtividade, rendimento, estabilidade e seletividade) quanto os critérios não catalíticos (separação, processos de *downstream*,

controle) que são requeridos para uma dada aplicação. Dessa forma, quando ambos requisitos são preenchidos, pode-se considerar que a enzima imobilizada é robusta (BORNSCHEUER, 2003).

2.4.1. Suporte de quitosana

A quitina é o principal componente estrutural do exoesqueleto de invertebrados como crustáceos, insetos e aranhas, estando também presente na parede celular da maioria dos fungos e de muitas algas. É um homopolímero de resíduos de N-acetil-D-glicosamina, unidos por ligações $\beta(1\rightarrow4)$ (VOET et al., 2008). A quitosana ocorre na natureza em alguns fungos, como *A. niger* e *Mucor rouxii*, em quantidades pouco expressivas quando comparada com a quitina. Industrialmente, a quitosana é obtida a partir da desacetilação da quitina (CASTRO, 2008).

Quitina e quitosana podem ser classificadas como copolímeros constituídos por diferentes conteúdos de unidades 2-amino-2-desoxi-D-glicopiranose e 2-acetamido-2-desoxi-D-glicopiranose. Os copolímeros com predominância de unidades acetiladas, solúveis apenas em solventes específicos (como N,N-dimetilacetamida contendo 5% de cloreto de lítio) são denominados quitina, enquanto que os copolímeros com maior abundância de unidades desacetiladas, solúveis em soluções de ácidos diluídos são denominados quitosanas (CARDOSO, 2008). A Figura 1 representa as estruturas da quitina (esquerda) e da quitosana completamente desacetilada.

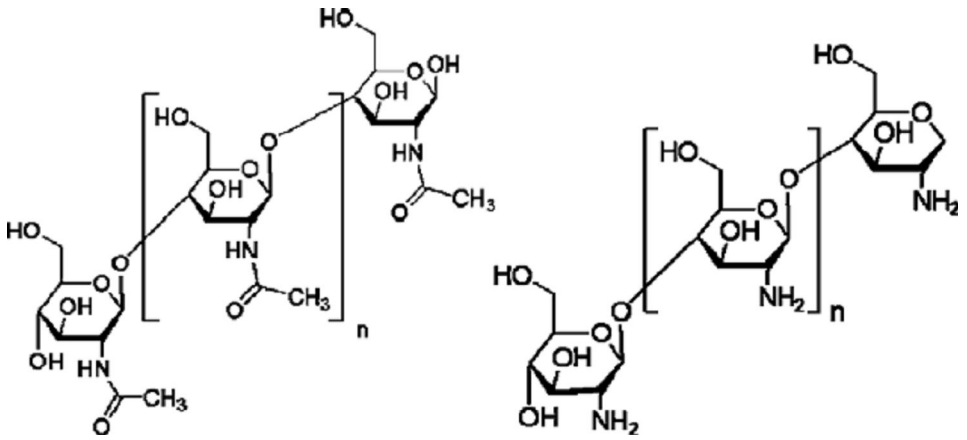

Figura 1: Estruturas da quitina (esquerda) e da quitosana completamente desacetilada (SJOHOLM et al. 2009).

A quitosana possui diversas aplicações tecnológicas, dentre elas destacam-se: utilização no tratamento de efluentes industriais, carreador de drogas na área farmacêutica, material biocompatível na área médica e, ainda, como suporte para imobilização de enzimas (ALSARRA et al., 2002; JAGTAP et al., 2009).

O desenvolvimento de suportes para imobilização de enzimas a base de quitosana é interessante em virtude da sua boa biocompatibilidade, reduzida adsorção não específica, facilidade de produção em várias formas, bem como baixo custo de obtenção (HSIEH et al., 2000, CHANG & JUANG, 2005). Em sua cadeia linear de poliglicosamina de alta massa molecular, a quitosana possui os grupos reativos amino e hidroxila, suscetíveis a modificações químicas (CHANG & JUANG, 2005). A basicidade proporciona a quitosana algumas propriedades singulares, tais como a solubilidade em soluções ácidas (pH < 6,5), quando dissolvida possui elevada quantidade de cargas positivas nos grupos -NH_3 (PILLAI et al., 2009).

A imobilização de enzimas em suporte de quitosana é realizada, principalmente, através da reação do agente bifuncional glutaraldeído entre o grupo amino livre da

quitosana e o grupo amino da enzima (CHIOU & WU, 2004), conforme ilustra a Figura 2.

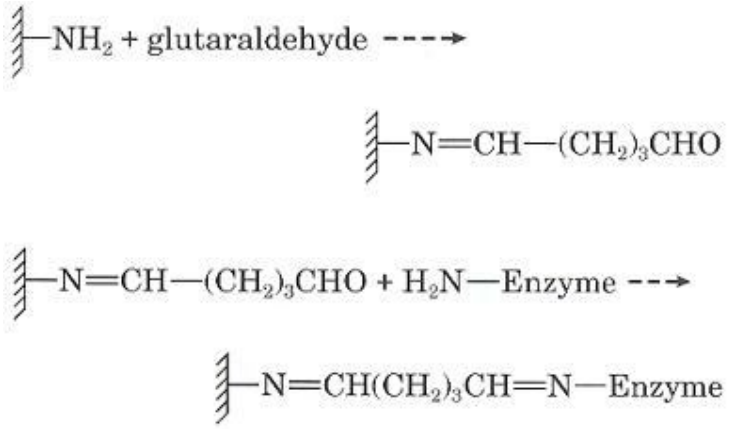

Figura 2. Etapas envolvidas na ativação do leito de quitosana com glutaraldeído e posterior ligação da enzima ao suporte (REJIKUMAR & DEVI, 1995).

A introdução de um espaçador também contribui para a hidrofilização do microambiente da enzima, proporcionando um aumento na estabilidade desse catalisador (CAO, 2005). Sabendo que a atividade enzimática é dependente da manutenção da conformação flexível da molécula, a introdução de um espaçador entre a enzima e a superfície do carregador é utilizada para reduzir a perda na atividade em função da imobilização (YODOYA et al., 2002).

Dentre os diversos formatos de suporte a base de quitosana comumente desenvolvidos, estão os filmes, as partículas esféricas e as escamas (YANG et al., 2004). As partículas esféricas são interessantes no sentido de possibilitarem sua utilização tanto em tanques agitados (HAYASHI & IKADA, 1991; KUMAR et al., 2008) quanto em colunas (KIBA et al., 1993; FIGUEROA et al., 1997; SHIN et al., 1998; TANIAI et al., 2001) e, ainda, proporcionam uma ótima superfície de contato.

O estudo das condições envolvidas na produção e ativação do leito, bem como a subsequente ligação da enzima ao suporte é crucial para o sucesso da imobilização (BIRÓ et al., 2009).

A Tabela 1 apresenta as principais abordagens envolvendo o desenvolvimento e a aplicação do suporte de quitosana para imobilização de enzimas através das diversas técnicas existentes.

Tabela 1: Diferentes abordagens envolvendo a imobilização enzimas em suportes a base de quitosana

Enzima	Abordagem	Referência
Alanina e leucina desidrogenase	Desenvolvimento de um reator de leito fixo contendo as enzimas covalentemente imobilizadas em esferas de quitosana.	KIBA et al., 1993
Álcool desidrogenase	[a]Avaliação da adequação do $P(CH_2OH)_3$ como agente bifuncional durante a imobilização da enzima em filme de quitosana. [b]Caracterização da enzima imobilizada, através de ligação covalente, em esferas de quitosana.	[a]COCHRANE et al., 1996 [b]SONI et al., 2001
Álcool oxidase	Investigação das condições ideais para a imobilização da enzima em esferas quitosana empacotadas em reator do tipo coluna.	TANIAI et al., 2001
α-Amilase	[a]Avaliação dos fatores que influenciam a atividade da enzima imobilizada em microesferas de quitosana, pela técnica de entrecruzamento. [b]Desenvolvimento de esferas produzidas a partir de um compósito de quitosana-carvão ativado para imobilização da enzima.	[a]SISO et al., 1997 [b]CHANG & JUANG, 2005

β-Amilase	[a]Desenvolvimento de um reator contendo a enzima imobilizada em esferas de quitosana pela técnica de adsorção, para produção semi-contínua de maltose. [b]Desenvolvimento de esferas produzidas a partir de um compósito de quitosana-carvão ativado para imobilização da enzima.	[a]NODA et al., 2001 [b]CHANG & JUANG, 2005
Catalase	[a]Determinação das condições operacionais da enzima imobilizada em filmes de quitosana ativados com glutaraldeído. [b]Caracterização da enzima imobilizada em esferas de quitosana, via entrecruzamento.	[a]ÇETINUS & ÖZTOP, 2000 [b]ÇETINUS & ÖZTOP, 2003
Celulase	Determinação dos parâmetros de adsorção da enzima em esferas de quitosana para aplicação em processos de purificação em leito fluidizado.	ROY et al., 2000
Ciclodextrina glicosiltransferase	Avaliação de três diferentes métodos para imobilização da enzima em suporte de quitosana.	SOBRAL et al., 2002
Fosfatase	Investigação de diferentes parâmetros que afetam a produção de esferas de quitosana na imobilização da enzima.	JUANG et al., 2001

β-Galactosidase	[a]Investigação dos principais fatores que afetam a imobilização da enzima em esferas de quitosana através de ligação covalente. [b]Determinação das condições ótimas para a imobilização da enzima em esferas de quitosana. [c]Avaliação da performance de um reator vertical do tipo "plug flow" empacotado com esferas de quitosana contendo a enzima imobilizada. [d]Desenvolvimento de um processo contínuo para hidrólise de lactose utilizando a enzima imobilizada por adsorção em esferas de quitosana. [e]Avaliação das condições para obtenção de oligossacarídeos pela hidrólise da lactose utilizando a enzima imobilizada em esferas de quitosana comercial. [f]Otimização das condições para imobilização da enzima em esferas de quitosana, utilizando a metodologia de superfície de resposta.	[a]CARRARA & RUBIOLO, 1994 [b]REJIKUMAR & SUREKHA, 1995 [c]FIGUEROA et al., 1997 [d]SHEU et al., 1998 [e]SHIN et al., 1998 [f]DWEVEDI & KAYASTHA, 2009
Glicose oxidase	Desenvolvimento de um leito híbrido a base de quitosana e óxido de silício (SiO_2) para imobilização da enzima por entrecruzamento.	YANG et al., 2004

Glicosidase	[a]Avaliação de dois métodos para imobilização de β-glicosidase em quitosana: covalente e entrecruzamento. [b]Determinação das condições para imobilização de duas glicosidases em quitosana através das técnicas de entrecruzamento e adsorção. [c]Investigação dos principais fatores que afetam a imobilização de β-glicosidase em esferas de quitosana por adsorção ou entrecruzamento.	[a]MARTINO et al., 1996 [b]SPAGNA et al., 1998 [c]GALLIFUOCO et al., 1998
Invertase	[a]Avaliação de fatores que influenciam a atividade da enzima imobilizada por entrecruzamento em microesferas de quitosana. [b]Investigação da estabilidade térmica da enzima imobilizada covalentemente em quitosana. [c]Caracterização da enzima covalentemente imobilizada em quitosana.	[a]SISO et al., 1997 [b]GÓMEZ et al., 2000 [c]HSIEH et al., 2000
Lacase	[a]Caracterização da enzima imobilizada por entrecruzamento em quitosana. [b]Trabalho de revisão envolvendo os principais protocolos para imobilização de lacases.	[a]D'ANNIBALE et al., 1999 [b]DURAN et al., 2002

Lipase	[a]Avaliação da influência da massa molecular e grau de desacetilação sobre a eficiência da imobilização da enzima em quitosana, via reticulação. [b]Determinação da atividade da enzima imobilizada covalentemente em esferas de quitosana utilizando espaçadores com diferentes comprimentos. [c]Desenvolvimento de um filme de quitosana para imobilização covalente da enzima. [d]Imobilização covalente da enzima em dois tipos de esferas de quitosana ativadas com carbodiimida.	[a]ITOYAMA et al., 1994 [b]ALSARRA et al., 2002 [c]AMORIM et al., 2003 [d]CHIOU & WU, 2004
Pectinase	Hidrólise de pectato utilizando a enzima imobilizada em reator contínuo do tipo CSTR.	IWASAKI et al., 1998
Protease	[a]Estabelecimento das melhores condições para a atividade de diferentes proteases imobilizadas por adsorção em esferas de quitosana. [b]Imobilização covalente de uma protease alcalina em quitosana. [c]Avaliação da eficiência da hidrólise da caseína pela enzima covalentemente imobilizada em esferas de quitosana, em reator agitado.	[a]KISE e HAYAKAWA, 1991 [b]ABDEL-NABY et al., 1998 [c]BENKHELIFA et al., 2005
Queratinase	Comparação da enzima imobilizada em diferentes suportes, dentre eles a quitosana.	FARAG & HASSAN, 2004

Tirosinase	[a]Otimização das condições de imobilização da enzima via ligação covalente em escamas de quitosana, através de planejamento fatorial completo. [b]Trabalho de revisão envolvendo os principais protocolos para imobilização da enzima.	[a]CARVALHO et al., 2000 [b]DURÁN et al., 2002
Tanase	[a]Caracterização da enzima imobilizada covalentemente em quitosana. [b]Hidrólise de taninos em chás pela enzima encapsulada em quitosana.	[a]ABDEL-NABY et al., 1999b [b]BOADI & NEUFELD, 2001
Transferase	Investigação de diferentes propriedades da enzima imobilizada via ligação covalente em quitosana.	KARIN & HASHINAGA, 2002
Transglutaminase	Desaminação de caseína utilizando a enzima imobilizada em esferas de quitosana.	NONAKA et al., 1996
Urease	[a]Caracterização da enzima imobililzada covalentemente em suporte a base de quitosana e glicidil metacrilato. [b]Otimização das condições para imobilização da enzima via ligação covalente em esferas de quitosana. [c]Avaliação do efeito de diferentes fatores sobre a imobilização da enzima em esferas de quitosana.	[a]CHELLAPANDIAN & KRISHNAN, 1998 [b]KAYASTHA et al., 2001 [c]KUMAR et al., 2008

Xilanase	[a]Caracterização de duas xilanases produzidas por *Aspergillus niger* NRC 106 imobilizadas em quitosana. [b]Desenvolvimento de um hidrogel, preparado pela complexação de quitosana e xantana para imobilização da enzima.	[a]ABDEL-NABY, 1993 [b]DUMITRIU & CHORNET, 1997

Conforme o exposto na Tabela 1, é possível observar a diversidade de técnicas nas quais diferentes enzimas podem ser imobilizadas em suportes a base de quitosana, bem como a variedade de condições e substratos utilizados. Todavia, há, ainda uma lacuna considerável no que concerne ao estudo de queratinases imobilizadas, especialmente em suporte produzido com quitosana.

3. OBJETIVOS

3.1 Objetivo geral

Desenvolver protocolos para a purificação e imobilização de uma protease queratinolítica produzida por *Chryseobacterium* sp. kr6.

3.2 Objetivos específicos

- Estabelecer as condições ótimas para a atividade da queratinase produzida por *Chryseobacterium* sp. kr6, utilizando as ferramentas estatísticas de planejamento experimental e metodologia de superfície de resposta.
- Avaliar a especificidade da enzima parcialmente purificada frente a diferentes substratos.
- Desenvolver um protocolo para a purificação completa da queratinase.
- Determinar os parâmetros cinéticos e termodinâmicos para a inativação térmica da enzima sob diferentes condições.
- Desenvolver um suporte para imobilização da queratinase purificada.
- Avaliar as condições ótimas para imobilização da queratinase em esferas de quitosana.
- Comparar algumas propriedades da enzima livre e imobilizada.

4. RESULTADOS E DISCUSSÃO

Os resultados deste trabalho serão apresentados na forma de artigos científicos, sendo a elaboração neste formato incentivada pelo PPGBCM, para organização da tese. Os referidos artigos estão apresentados nos itens Resultados I, II e III.

No primeiro artigo (Resultados I - "Kinetic data and substrate specificity of a keratinase from *Chryseobacterium* sp. strain kr6") foram determinadas as condições ótimas para a atividade da queratinase produzida por *Chryseobacterium* sp. kr6 com o auxílio das técnicas de planejamento experimental e supefície de resposta. A especificidade da enzima frente a diferentes substratos também foi investigada. Este artigo está publicado no periódico: Journal of Chemical Technology and Biotechnology, v. 84: 361-366, 2008.

O segundo artigo (Resultados II - "Thermodynamics and kinetics of heat inactivation of a novel keratinase from *Chryseobacterium* sp. strain kr6") envolve a purificação da queratinase e disponibiliza os parâmetros cinéticos e termodinâmicos relativos a inativação térmica da enzima sob diferentes condições. Este artigo foi aceito para publicação no periódico: Applied Biochemistry and Biotechnology, em setembro de 2009.

O terceiro artigo (Resultados III – "Immobilization of a keratinolytic protease from *Chryseobacterium* sp. strain kr6 on chitosan beads"), envolve o desenvolvimento de macroesferas de quitosana para a imobilização da queratinase produzida por *Chryseobacterium* sp. kr6. Este artigo foi submetido para publicação no periódico: Food Bioprocess Engineering, em setembro de 2009.

4.1 RESULTADOS I. “Kinetic data and substrate specificity of a keratinase from *Chryseobacterium* sp. strain kr6” - Journal of Chemical Technology and Biotechnology, v. 84: 361-366, 2008.

Research Article

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Kinetic data and substrate specificity of a keratinase from *Chryseobacterium* sp. strain kr6

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Abstract

BACKGROUND: Keratinases are important enzymes for biotechnological processes involving keratin hydrolysis. In this work substrate specificity and kinetic properties of a keratinase from *Chryseobaterium* sp. were investigated.

RESULTS: The optimal conditions for activity of purified keratinase with respect to pH, temperature and sodium chloride concentration were established using factorial design and surface response techniques. The optimum conditions for keratinase activity were pH from 7.4 to 9.2, temperature from 35 °C to 50 °C and NaCl concentration from 50 to 340 mmol L^{-1}, having azocasein as substrate. Subsequently, the kinetic parameters for this substrate were determined to be K_m = 0.75 mg mL^{-1} and V_{max} = 59.5 U min^{-1}. The K_i value for 1,10-phenanthroline was estimated at 0.78 mmol L^{-1}. The enzyme specificity was evaluated over different synthetic and insoluble substrates. The protease exhibited specificity with selectivity for hydrophobic and positively charged residues. In relation to the insoluble substrates, the enzyme hydrolyzed preferably chicken nails.

CONCLUSIONS: This enzyme effectively hydrolyzes insoluble keratin substrates. The knowledge of keratinase properties is an essential step in the development of biotechnological processes involving keratin hydrolysis.

Keywords: enzyme kinetics; factorial design; keratinase; synthetic substrate

INTRODUCTION

Keratins are the insoluble structural proteins of feathers, wool, hooves, scales, hair, nails and *stratum corneum*. They have been subdivided into 'hard' and 'soft' classes according to their chemical and physical properties.[1,2] Feathers constitute up to 10% of total chicken weight, reaching more than 7.7×10^8 kg $year^{-1}$ as a by-product of the poultry industry. Since feathers are almost pure keratin, feather waste represents a potential alternative to produce feedstuff ingredients with high nutritional value. Feather meal is currently produced using physicochemical processes, and is used on a limited basis as an ingredient in animal feed, since it is deficient in some essential amino acids.[3,4] Feathers have about 15% nitrogen, and thus have great potential for use as soil fertilizers. The slow release of nitrogen from raw feathers indicates that soil microorganisms could not easily digest the keratin structure. In the case of feather meal, which undergoes an extensive thermal treatment, total nitrogen release also occurs at 6–7 weeks.[5] Modification of keratin structure can be achieved by thermal treatments and enzymatic hydrolysis, with cleavage of disulfide bridges and peptide bonds.[6,7] In this regard, the use of keratinolytic microorganisms may represent an alternative for development of nitrogen sources for fertilizer utilization.

Although relatively little attention has been given to alternative uses of feather waste, the increasing growth of the poultry industry has stimulated research on keratinolytic enzymes.[8,9] Bioconversion techniques as an alternative process for structural modification of keratins using enzymatic and microbial conversion should receive consideration by scientists, because they play an important part in the biotechnological valorization of keratin-containing wastes.[10,11]

Although the capacity for keratinolysis is widespread among microorganisms only a few have reached commercial exploitation. Keratinases from *Bacillus* spp., in special *B. licheniformis* and *B. subtilis*, and *Streptomyces* have been extensively studied due their effectiveness in terms of feather degradation.[12–14] Most keratinases described to date are reported to belong to the serine-type protease family showing extensive similarity with subtilisins.[15–17] Keratinases generally have broad substrate specificity and are active against both soluble and insoluble protein substrates. However, relatively few studies using synthetic substrates have been reported to date, most of them on keratinolytic serine-type proteases.[18–21]

Factorial design of a limited set of variables has advantages over the conventional method of manipulating a single parameter per trial, as the latter approach frequently fails to locate optimal process conditions due to its failure to consider the effect of possible interactions between factors. Moreover, the factorial design makes it possible to take advantage of practical knowledge about the process during the final response surface analysis. Optimization through factorial design and response surface methodology is a current practice in biotechnology.[22]

We have previously isolated and characterized a feather-degrading *Chryseobacterium* sp. kr6 strain.[23] This strain produces

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a keratinolytic metalloprotease that belongs to the M14 family of peptidases, also known as the carboxypeptidase A family, being the first enzyme of this family associated with keratinolytic activity and to the genus *Chryseobacterium*.[24] In this paper, the effects of pH, temperature and sodium chloride concentration on enzyme activity were evaluated using factorial design and response surface methodology, and then the kinetic characteristics and substrate specificity of the keratinase were determined.

MATERIALS AND METHODS

Microorganism, culture media and conditions

A keratinolytic *Chryseobacterium* sp. strain kr6, isolated from waste feathers produced by poultry processing, was plated on feather meal agar.[23] The basal medium used for growth of the feather-degrading microorganism was feather meal broth containing the following (g L^{-1}): NaCl (0.5), KH_2PO_4 (0.4), $CaCl_2$ (0.015) and feather meal (10.0). The pH was adjusted to 8.0. Cultures were carried out in a 250 mL Erlenmeyer flask (50 mL working volume), at 30 °C, at 100 rpm in an incubator shaker, for 48 h. The culture was centrifuged for 20 min at 10 000 *g* and 4 °C to harvest the keratinase-containing supernatant.

Assay of enzymatic activity

Proteolytic activity was assayed with azocasein as substrate using the following method.[25] The reaction mixture contained 120 μL of enzyme preparation and 480 μL of azocasein 10 g L^{-1} in 50 mmol L^{-1} Tris-HCl buffer (pH 8.0). The mixture was incubated for 40 min at 45 °C; the reaction was stopped by addition of 600 μL of trichloroacetic acid to a final concentration of 100 g L^{-1}. After separation of the un-reacted azocasein by centrifugation, 800 μL of clear supernatant was mixed with 200 μL of 1.8 mol L^{-1} NaOH, resulting in a yellow colored complex that was measured spectrophotometrically at 420 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at the specified conditions. Controls were prepared by adding trichloroacetic acid to the reaction mixture before adding the enzyme preparation.

Protein assay

Protein concentration was measured using the Folin phenol reagent method,[26] using bovine serum albumin as a standard. In steps of column chromatography elution, the amount of protein was estimated in terms of the absorbance at 280 nm.

Enzyme purification

The supernatant containing the enzyme was collected after centrifugation at 10 000 *g* for 20 min, at 4 °C. The enzyme was precipitated from the supernatant by the gradual addition of solid ammonium sulphate, with gentle stirring, to 50% saturation, allowed to stand for 3 h and centrifuged at 10 000 *g* for 20 min, at 4 °C. The pellet was dissolved in Tris-HCl buffer 50 mmol L^{-1} (pH 8.0). The concentrated sample was applied to a Sephadex G-100 gel filtration (0.8 × 30 cm), equilibrated and eluted with Tris-HCl buffer 50 mmol L^{-1} (pH 8.0). Fractions representing the main peak of enzyme activity were pooled. The proteolytic enzyme appeared as a single sharp peak. The overall purification factor was about 16.2-fold, and the final yield was 12.7%. The final product had a specific activity of about 16 800 U mg^{-1}.

Table 1. Coded levels and real values (in parentheses) for a full factorial design, and enzyme activity (U mL^{-1})

Run	X_1	X_2	X_3	Enzyme activity (U mL^{-1})
1	−1 (6.8)	−1 (36.7)	−1 (140)	723
2	+1 (9.2)	−1 (36.7)	−1 (140)	1360
3	−1 (6.8)	+1 (53.3)	−1 (140)	633
4	+1 (9.2)	+1 (53.3)	−1 (140)	583
5	−1 (6.8)	−1 (36.7)	+1 (410)	523
6	+1 (9.2)	−1 (36.7)	+1 (410)	1040
7	−1 (6.8)	+1 (53.3)	+1 (410)	430
8	+1 (9.2)	+1 (53.3)	+1 (410)	523
9	−1.68 (6.0)	0 (45)	0 (275)	567
10	+1.68 (10)	0 (45)	0 (275)	1167
11	0 (8.0)	−1.68 (30)	0 (275)	1597
12	0 (8.0)	+1.68 (60)	0 (275)	313
13	0 (8.0)	0 (45)	−1.68 (50)	2123
14	0 (8.0)	0 (45)	+1.68 (500)	1570
15	0 (8.0)	0 (45)	0 (275)	1680
16	0 (8.0)	0 (45)	0 (275)	1763
17	0 (8.0)	0 (45)	0 (275)	1763
18	0 (8.0)	0 (45)	0 (275)	1910

X_1: pH; X_2: temperature (°C); X_3 sodium chloride concentration (mmol L^{-1}).

Second-order factorial design

The influence of pH, temperature and sodium chloride concentration on enzyme activity was evaluated using a full factorial design (2^3 plus star configuration) with four replicates in the central point, which was a total of 18 runs.[27] Five levels of each independent variable were chosen, the upper and lower limits of these being set in the range described in the literature.[10,11] In the statistical model, Y predicted response; b_0, constant; X_1, pH; X_2, temperature (°C); X_3, salt concentration (mmol l^{-1}); b_1, b_2 and b_3, linear coefficients; b_{11}, b_{22} and b_{33}, quadratic coefficients; b_{12}, b_{13} and b_{23}, interaction coefficients. Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and response. The run numbers 1 to 8 correspond to the linear points of the factorial design, which allow one to investigate the main effect of each independent variable on the evaluated response. The run numbers 9 to 14 correspond to the axial points and the run numbers 15 to 18 are the central points.

This design is represented by a second-order polynomial regression model (Equation (1)) to generate contour plots:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}{X_1}^2 + b_{22}{X_2}^2 + b_{33}{X_3}^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

Statistica 7.0 software (Statsoft, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's *t*-test, the second-order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination (r^2). The effect of pH on enzyme activity was investigated by measurements in buffers of various pH (phosphate for pH 6.0–6.8; Tris-HCl for pH 8.0–9.2; glicine-NaOH for pH 10.0).

Substrate specificity

The substrate specificity was investigated with the following amino-4-methyl coumarin (AMC) substrates (N-α-Benzoyl-L-Arg-

MCA, Ala-Ala-Phe-MCA, L-Leu-MCA, N-t-Boc-Gln-Ala-Arg-MCA, N-t-Boc-Ile-Glu-Gly-Arg-MCA, L-Val-MCA, Gly-MCA, L-Aspartic acid-MCA, Ala-Phe-Lys-MCA and L-Phe-MCA). Substrates were dissolved in dimethyl sulphoxide and added to 50 mmol L^{-1} Tris-HCl buffer (pH 8.0) to a final concentration of 50 μmol L^{-1}. The mixture contained 180 μL of substrate and 20 μL of enzyme, was incubated for 40 min at 45 °C, the reaction was stopped by addition of 2.8 mL of Na_2CO_3 0.2 mol L^{-1}. Hydrolysis leads to an increase of fluorescence, which was measured with fluorometer DyNA Quant™ (Hoefer Scientific, Cortland, USA) (excitation wavelength of 365 nm and an emission wavelength of 460 nm). A standard solution of MCA was used to convert the fluorescence values to product concentration. All assays were done in triplicate.

The hydrolysis of different insoluble substrates (feather, wool and chicken horns) was also evaluated. The reaction mixture consisted of 4 mL of enzyme solution and 4 mL of Tris-HCl pH 8.0 containing 20 g L^{-1} of each substrate. These suspensions were incubated for 6 h at 45 °C and 150 rpm. At each interval (0, 1, 2, 4 and 6 h), 250 μL of sample were withdraw and added 250 μL of 10% (w/v) trichloroacetic acid (TCA). This mixture was centrifuged and the released amino acids measured as tyrosine using the method described by Lowry *et al.*[26] A blank was developed containing distilled water instead enzyme. All assays were done in triplicates.

Keratinolytic activity was also performed with keratin azure as substrate. The reaction mixture contained 0.2 mL of enzyme preparation and 0.8 ml of 0.5% (w/v) keratin azure in 50 mmol L^{-1} Tris-HCl buffer (pH 8.0). The mixture was shaken (200 rpm) for 1 h at 45 °C prior to centrifugation (10 000 *g* for 10 min) and measurement of the supernatant fluid's absorbance at 595 nm. One unit of keratinolytic activity was the amount of enzyme that caused a change of absorbance of 0.01 in the specified conditions.[19]

Kinetic data

The kinetics parameters, both azocasein and fluorogenic substrates N-t-Boc-Ile-Glu-Gly-Arg-MCA and N-t-Boc-Gln-Ala-Arg-MCA, were estimated from product accumulation curves. At least six different concentrations were used, and the steady-state kinetic parameters were calculated using the Michaelis–Menten equation. Kinetic data were calculated by Statistica 7.0 (Statsoft Inc., USA) using nonlinear regression analysis with a quasi-Newton method, for Michaelis–Menten kinetics. The initial concentrations for fluorogenic substrates ranged from 1 μmol L^{-1} to 100 μmol L^{-1}, while azocasein concentration varied from 0.30 mg mL^{-1} to 30 mg mL^{-1}.

Earlier work reported that crude keratinase of strain kr6 is a metallo-type protease.[23] For measurement of the inhibitory (K_i) effects of a typical metalloprotease inhibitor on the keratinase of strain kr6, the partially purified enzyme was preincubated at room temperature for 15 min with 1,10-*o*-phenanthroline at different concentrations prior to measurement of enzyme activity, according reference 25. The kinetic parameters also were estimated using nonlinear regression.

RESULTS

The establishment of optimal conditions for enzyme activity as a function of pH, temperature and sodium chloride concentration was carried out using the statistical tools of experimental design and response surface techniques. The experimental conditions and the results of the enzyme activity evaluated in the experimental design are shown in Table 1. Maximum enzyme activity was 2123 U mL^{-1} (run 13). This value was similar to those obtained at the central points (run numbers 15 to 18). Lowest activity was observed in the run 12 (313 U mL^{-1}).

Table 2. Effects and interactions analysis for enzyme activity

Factor	Effect (U mL^{-1})	Std.Err.	*t*-value	*P*-value
Mean	1802	47.8	37.7	<0.000[a]
pH (L)	323	51.8	6.2	0.008[a]
pH (Q)	−847	53.9	−15.7	0.0005[a]
Temperature (L)	−532	51.8	−10.3	0.002[a]
Temperature (Q)	−784	53.9	−14.5	0.0007[a]
NaCl concentration (L)	−251	51.8	−4.8	0.017[a]
NaCl concentration (Q)	−152	53.9	−2.8	0.066[b]
1 × 2	−277	67.7	−4.1	0.026[a]
1 × 3	5.8	67.7	0.08	0.937
2 × 3	64	67.7	0.9	0.413

[a] = significant factors $P < 0.05$
[b] = significant factors $P < 0.07$

An estimate of a main effect is obtained by evaluating the difference in process performance caused by a change from low (−1) to high (+1) levels of the corresponding factor.[28] The performance of the process was measured by enzyme activity response. Both *t*-test and *P*-value were the statistical parameters used to confirm the significance of the factors studied. Table 2 presents the effects of each independent variable and its interaction on the enzyme activity. The change of pH from 6.8 (−1) to 9.2 (+1) produced an increase on enzyme activity, about 323 U mL^{-1}. The increase of temperature from 36.7 °C to 53.3 °C reduced the enzyme activity, on average, 532 U mL^{-1}, also the variation of sodium chloride concentration from 140 mmol L^{-1} to 410 mmol L^{-1} caused a reduction on related response in 251 U mL^{-1}. The combining effect of pH and temperature resulted in a reduction in the enzyme activity, on average, 277 U mL^{-1}. All effects mentioned were significantly at a 95% confidence level.

To construct a second-order model that can predict the enzyme activity (dependent variable) as a function of pH, temperature and ionic strength (independent variables), the analysis of variance (ANOVA) was used to evaluated the adequacy of the fit. On the basis of the ANOVA ($F_{calc} = 9.84$; $F_{tab} = 3.14$, at 95% confidence level), a second-order model was established (Equation (2)), describing the enzyme activity as a function of independent variables.

$$Y = 1802 + 161.6X_1 - 266.3X_2 - 125.5X_3 - 423.4X_1^2 - 392.2X_2^2 - 76.3X_3^2 - 138.9X_1X_2 \quad (2)$$

Based on an *F*-test, the model is predictive, since its calculated *F*-value is higher than the critical *F*-value. The coded model was used to generate the response surfaces, illustrated in Fig. 1. The response surfaces generated as a function of the evaluated variables indicate that maximum activity was around the central point (pH 8.0, 45 °C, 275 mmol L^{-1} NaCl). These graphics allowed establish the region where the maximum enzyme activity was reached. This region corresponds to a pH range from 7.4 to 9.2, temperature 35 °C to 50 °C and NaCl concentration from 50 to 340 mmol L^{-1}, having azocasein as substrate. Under these conditions an enzyme activity of about 1500 U mL^{-1} was obtained.

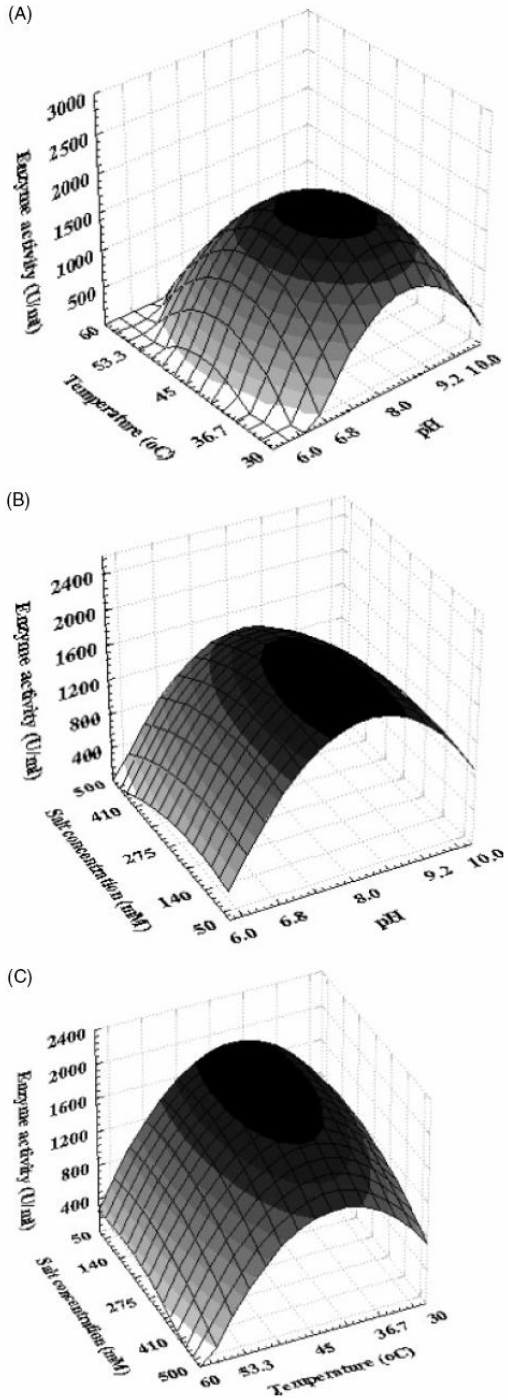

Figure 1. Response surface for keratinase activity as a function of temperature and pH (A), pH and salt concentration (B) and salt concentration and temperature (C).

Table 3. Kinetic parameters for some synthetic substrates and azocasein

Substrate	V_{max}	K_m (μmol L^{-1})	V_{max}/K_m
N-t-Boc-Gln-Ala-Arg-MCA	4.3 nmol L^{-1} min^{-1}	0.61	7.0
N-t-Boc-Ile-Glu-Gly-Arg-MCA	5.4 nmol L^{-1} min^{-1}	0.58	9.5
Azocasein	59.5 U min^{-1}	0.75 mg mL^{-1}	79.3

Table 4. Hydrolysis of synthetic substrates with purified enzyme

Substrate	Velocity (nmol L^{-1} min^{-1})
N-α-Benzoyl-L-Arg-MCA	0
Ala-Ala-Phe-MCA	0
L-Leu-MCA	4.82 ± 0.41
N-t-Boc-Gln-Ala-Arg-MCA	3.48 ± 0.12
N-t-Boc-Ile-Glu-Gly-Arg-MCA	4.58 ± 0.42
L-Val-MCA	0.22 ± 0.01
Gly-MCA	0
L-Asp-MCA	0
Ala-Phe-Lys-MCA	2.18 ± 0.19
L-Phe-MCA	0.44 ± 0.15

The kinetics parameters were estimated with azocasein as substrate (Table 3) at K_m = 0.75 mg mL^{-1} and V_{max} 59.5 U min^{-1}. Protease inhibition studies have indicated that the predominant proteolytic enzymes able to degrade feathers are serine proteases. However, earlier work reported that keratinase of strain kr6 is a metallo-type protease.[23] In the presence of different concentrations of 1–10 phenanthroline, the inhibition constant (K_i) was 0.78 mmol L^{-1}. A high keratinolytic activity was achieved using keratin azure, on average 2320 U mL^{-1} and a specific activity of 15 040 U mg^{-1}.

The enzyme is active on some fluorogenic peptide substrates (Table 4). The protease exhibited preference for positive and hydrophobic amino acids, such as arginine and leucine, respectively. No hydrolysis was detected with L-Asp-MCA, Gly-MCA, Ala-Ala-Phe-MCA and N-α-Benzoyl-L-Arg-MCA. However, some hydrolysis was detected with L-Phe-MCA. Another substrate with hydrophobic amino acid at P1, to which the enzyme presented some affinity, was L-Val-MCA.

The lowest K_m and a higher ratio K_m/V_{max} was observed for the substrate N-t-Boc-Ile-Glu-Gly-Arg-MCA (Table 3).

The keratinase was also examined to determine its ability to hydrolyze some insoluble substrates, such as chicken nails, wool and feathers. Figure 2 shows the data obtained. The largest hydrolysis rate was observed for chicken nails, and was about 120 μg mL^{-1} of tyrosine, after 4 h reaction. Both wool and feathers presented similar hydrolysis rate, between 55 and 60 μg mL^{-1} of tyrosine.

DISCUSSION

Keratinases play an important role in the metabolism of the *Chryseobacterium* sp. kr6, because it can grow in a medium containing feather meal or raw feathers as the sole source of both carbon and nitrogen. Purification with gel filtration

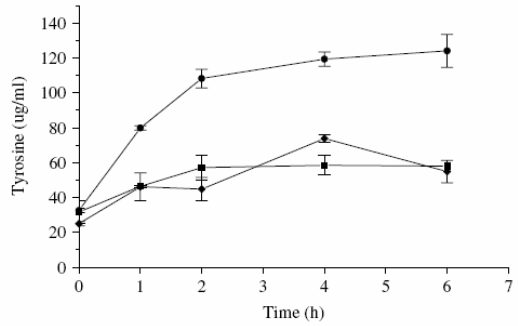

Figure 2. Hydrolysis of insoluble substrates by purified keratinase for nails (circles), feather (squares) and wool (diamonds). Results are the means of three independent experiments.

chromatography separated two peaks, where only one presented proteolytic activity against azocasein. The purification factor is estimated to be at least 16.2-fold and specific activity about 16 800 U mg^{-1}. When keratin azure is used as substrate, the pool obtained after gel permeation shows an enzyme activity of 2,320 U mL^{-1} and a specific activity of 15 040 U mg^{-1}. Bressollier *et al.*[19] achieved a specific activity of 17 593 U mg^{-1}, using the same substrate and conditions, after four purification steps of a keratinolytic serine proteinase from *Streptomyces albidoflavus*.

Figure 1(A), 1(B) and 1(C) illustrate the enzyme activity as a function of pH, temperature and sodium chloride concentration. Besides the optimal points, the analysis of such graphics is quite useful to visualize the boundaries. The contour region for the evaluated response as a function of variables allows one to estimate the range of working conditions for this enzyme. The optimal temperature and pH obtained here are similar to those observed for other keratinases.[29,30] Keratinases from *Bacillus* spp. have an optimum temperature in the range 50–60 °C,[2,31] whereas *Streptomyces* spp. and *Doratomyces microsporus* keratinases have an optimum between 55 and 65 °C.[18,19,32] Rozs *et al.*[33] characterized a new keratinase from *B. licheniformis* strain with respect to temperature and pH, the referred enzyme is a thiol protease and has an optimal activity at pH 8.5 and 52 °C. From the results for experimental design for sodium chloride concentration, illustrated in Fig. 1(B) and 1(C), it is noted that a NaCl concentration higher than 300 mmol L^{-1} promoted the inhibition of the enzyme activity. Korkmaz *et al.*[2] reported that a keratinase from *Bacillus licheniformis* strain HK-1 was inhibited by 50 mmol L^{-1} NaCl.

The enzyme was inhibited by 1,10-phenanthroline, with an inhibition constant of 0.78 mmol L^{-1}.The keratinolytic serine proteinase of *Streptomyces albidoflavus* was slightly affected by 1-10-phenanthroline at a concentration of 10 mmol L^{-1}.[19] A similar effect was reported on the keratinolytic protease of *Microsporum canis* for this same inhibitor.[34] An extracellular alkaline keratinase from *B. licheniformis* strain HK-1 was characterized as a metalloprotease type,[2] an unusual property among keratinases from *Bacillus*. According to Allpress *et al.*,[35] inhibition of the metalloprotease by chelating agents provides a potential method for temporary inactivation during storage, reducing autolysis associated with proteolytic enzymes. The keratinolytic *B. licheniformis* K-508 secreted an unusual trypsin-like thiol protease that is strongly active towards benzoyl-Phe-Val-Arg-*p*-NA and is not inhibited by PMSF.[33]

In order to evaluate if keratinolytic enzyme shows characteristic substrate specificities, the *Chryseobaterium* sp. kr6 protease was tested with different synthetic substrates. The P1 specificity of keratinase was measured with 10 synthetic MCA substrates (Table 4). The results indicate that the keratinase has a preference for hydrophobic and positively charged amino acids at P1 position. However, the affinity for Phe at P1 position was much lower than Leu. Since, no or very low hydrolysis was detected with N-α-Benzoyl-L-Arg-MCA, the presence of an amino acid in P2 appears to be necessary for effective catalysis. The keratinase was less efficient with Lys-MCA, followed by Phe-MCA and Val-MCA, suggesting the preference for hydrophobic residues.

The P1 specificity of the keratinase NAPase of *Nocardiopsis* sp. was tested with 11 synthetic *p*-nitroanilide (*p*NA) substrates. The enzyme also had a preference for aromatic and hydrophobic residues at P1 position.[36] The feather-degrading *Nesterenkonia* sp. AL20 produces an alkaline protease that also exhibited higher activity with tetrapeptides with hydrophobic residues located at P1 site in the order Tyr > Phe > Leu.[37] A keratinolytic metalloprotease from *Lysobacter* was strongly active towards carboxybenzoyl-Phe-*p*NA.[35] *S. albidoflavus* produces a chymotrypsin-like keratinase that exhibited specificity for aromatic and hydrophobic amino acid residues, as demonstrated by using synthetic peptides.[19] The keratinolytic serine protease of *S. pactum* DSM 40530 showed substrate specificity and stereospecificity to *p*NA derivatives of basic amino acids lysine and arginine (L-enantiomers), but hydrolysis of benzoyl-D-Arg-*p*-NA was not detected.[18] De Toni *et al.*[21] characterized an alkaline serine endopeptidase and observed that among all peptide bonds of the fluorogenic substrates tested, the only hydrolyzed bond was that with a modified cysteine at subsite P1.

CONCLUSION

The response surface methodology was successfully used for optimization of enzyme activity for *Chryseobacterium* sp. kr6 protease. The optimum parameters for the improved level of keratinase production were at pH from 7.4 to 9.2, temperature from 35 °C to 50 °C and sodium chloride concentration from 50 to 340 mmol L^{-1}, having azocasein as substrate. Among the synthetic substrates tested, the enzyme exhibited activity mainly with hydrophobic and positive residues. The enzyme also demonstrated the ability to hydrolyze insoluble protein substrates. Whatever hydrolysis was achieved could be attributed to the specificity of the enzyme for hydrophobic amino acids that constituted about 50% of the residues in keratin protein.

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4.2 RESULTADOS II – “Thermodynamics and kinetics of heat inactivation of a novel keratinase from *Chryseobacterium* sp. strain kr6” - Applied Biochemistry and Biotechnology, aceito para publicação (prelo).

Thermodynamics and kinetics of heat inactivation of a novel keratinase from *Chryseobacterium* sp. strain kr6

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Abstract

A novel keratinase from *Chryseobacterium* sp. strain kr6 was purified to homogeneity by $(NH_4)_2SO_4$ precipitation, gel permeation on Sephadex G-100 and Q-Sepharose Fast Flow ion-exchange chromatography. The molecular weight of the purified enzyme was around 20 kDa. Kinetic and thermodynamic parameters for thermal inactivation were determined. The influence of Ca^{2+} and Mg^{2+} ions, and purification degree on the enzyme stability was evaluated in the range of 50 to 60ºC. The results showed that first-order kinetics explained well the thermal denaturation of the keratinase in this temperature interval. The presence of Ca^{2+} increases significantly the enzyme stability. Compared with the controls, the half-life time of the purified enzyme after two purification steps in the presence of Ca^{2+} increased 7.3, 20.2 and 9.8-fold at 50, 55 and 60ºC, respectively. Thermodynamics parameters for thermal inactivation were also determined.

Keywords: biocatalysis; enzyme; kinetics; microbial; protease; thermal inactivation

Nomenclature

C_0	Activity at time $t = 0$
C_t	Activity at time t
k_d	First-order rate constant (min^{-1})
$E_{a,d}$	Activation energy for denaturation (kJ mol^{-1})
$\Delta G^{\#}_d$	Free energy (kJ mol^{-1})
$\Delta H^{\#}_d$	Activation enthalpy (kJ mol^{-1})
$\Delta S^{\#}_d$	Activation entropy (J mol^{-1} K^{-1})
h	Planck constant (J s)
K_B	Boltzmann constant (J K^{-1})
R	Universal gas constant (J mol^{-1} K^{-1})
r^2	Determination coefficient
t	Time (min)
$t_{1/2}$	Half-life time (min)
T	Temperature (K)

1. Introduction

Keratinases (E.C. 3.4.99.11) are proteases able to degrade the scleroprotein keratin. Huge amounts of this protein are available as feathers, a waste by-product generated by the poultry production. Keratinases are very promising from a biotechnological point of view, since they could be applied to process keratin-containing wastes from leather and poultry industries and to improve the nutritional value of feather meal [*1,2*].

Although keratinases from different microorganisms have been purified and characterized to date [*3-6*], thermodynamic data and kinetic studies about thermal inactivation of keratinases are not available. Nonetheless, data about the activity and stability of enzymes can be useful for enhancing biotechnological processes, providing information on the structure of this catalyst and help to optimize the economics feasibility of the industrial process.

Several techniques based on protein engineering [*7*], isolation of thermophilic organisms [*8*], chemically or enzymatically-modified enzymes [*9,10*] and use of additives [*11*] have been reported to successfully increasing enzyme thermostability. It had been reported that the addition of Ca^{2+}, Mg^{2+} or polyhydric alcohols caused an increase in thermal stability of some proteases [*12,13*].

Recently, we isolated and characterized a new keratinolytic bacterium presenting a remarkable feather-degrading activity. This microorganism was identified as a *Chryseobacterium* sp. kr6 and its keratinase showed great potential for biotechnological applications [*14*]. A keratinolytic metalloprotease was recently purified from culture supernatants of strain kr6, being the first keratinase associated with the M14 family of peptidases [*15*]. In this paper, a novel keratinase from *Chryseobacterium* sp. strain kr6 was purified with only two chromatography steps. The aim of this work was to investigate the

thermal stability of this keratinase. On this basis, an Arrhenius plot was derived and the thermodynamic parameters for thermal inactivation were investigated by kinetic method.

2. Materials and methods

2.1. Microorganism, culture media and conditions

The keratinolytic *Chryseobacterium* sp. strain kr6 was isolated from feather waste produced by commercial poultry processing [*14*]. The culture was propagated and maintained on feather meal agar plates at 30 ºC and subcultured at weekly intervals. The basal medium used for growth of the feather-degrading microorganism was feather meal broth: NaCl (0.5 g L^{-1}), KH_2PO_4 (0.4 g L^{-1}), $CaCl_2$ (0.015 g L^{-1}) and feather meal (10 g L^{-1}), pH was adjusted to 8.0. The medium was sterilized by autoclaving at 121 ºC for 15 min. Cultures were incubated in an orbital shaker at 30ºC and 100 rpm, for 48 h. The culture was centrifuged at 4ºC and 10,000 x *g* for 20 min to harvest the keratinase-containing supernatant.

2.2. Assay of enzyme activity

Proteolytic activity was assayed with azocasein as substrate by the following method [*16*]. The reaction mixture contained 120 µL of enzyme preparation and 480 µL of 10 g L^{-1} azocasein (Sigma, St. Louis, MO, USA) in 50 mM Tris-HCl buffer pH 8.0. The mixture was incubated for 40 min at 45ºC; the reaction was stopped by addition of 600 µL of 100 g L^{-1} trichloroacetic acid. After separation of the un-reacted azocasein by centrifugation, 800 µL of clear supernatant was mixed with 200 µL of 1.8 mol L^{-1} NaOH, resulting in a yellow colored complex that was measured spectrophotometrically at 420 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01, under these conditions. Keratinolytic activity was also determined. The reaction mixture, containing

100 µL of enzyme sample and 500 µL of 20 g L^{-1} azokeratin suspension in 50 mmol L^{-1} Tris-HCl buffer pH 8.0, was incubated for 30 min, at 45°C. The reaction was terminated by addition of 600 µL of 100 g L^{-1} trichloroacetic acid, centrifuged and the supernatant measured spectrophotometrically at 450 nm. One unit of keratinolytic activity was defined as an increase in the A_{450} of 0.01, under the specified conditions [*17*].

2.3. Enzyme purification

The supernatant containing the enzyme was collected after centrifugation at 10,000 x *g* for 20 min. The enzyme was precipitated from the supernatant by gradual addition of solid ammonium sulphate, with gentle stirring, to 50% saturation, allowed to stand for 3 h and centrifuged at 10,000 x *g* for 20 min. The pellet was dissolved in 50 mmol L^{-1} Tris-HCl buffer pH 8.0. The concentrated sample was applied to a Sephadex G-100 gel filtration column (Pharmacia, Uppsala, Sweden), equilibrated and eluted with 50 mmol L^{-1} Tris-HCl buffer pH 8.0, at a linear rate of 1 cm min^{-1} (flow rate 0.5 cm^3 min^{-1}). The fractions were monitored by measuring the absorbance at 280 nm and enzyme activity. The fractions with high enzyme activity from gel permeation were pooled and dialyzed overnight against 20 mmol L^{-1} Tris-HCl buffer pH 8.5. The dialyzed enzyme solution was applied to Q-Sepharose Fast Flow column (3.5 x 15 cm) equilibrated with Tris-HCl 20 mmol L^{-1} buffer pH 8.5. The unbound proteins were washed with 20 mmol L^{-1} Tris-HCl buffer pH 8.5. The bound proteins were eluted at a linear rate of 0.074 cm min^{-1} (flow rate 0.712 cm^3 min^{-1}), with a linear gradient of NaCl (0-1 mol L^{-1}) in 20 mmol L^{-1} Tris-HCl buffer pH 7.0. The fractions were monitored by measuring the absorbance at 280 nm and enzyme activity. The concentration of soluble protein was determined by the Folin phenol reagent method [*18*], using bovine serum albumin as standard.

2.4. Electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed with 12% polyacrylamide gels essentially as described elsewhere [*19*]. After electrophoresis the protein was detected with silver staining [*20*].

2.5. Thermal inactivation

Kinetics of thermal inactivation of *Chryseobacterium* kr6 keratinase was determined by incubating the enzyme with different purifications grades, in the absence (control) or presence of Ca^{2+} or Mg^{2+} ions (5 mmol L^{-1} final concentration) at 50, 55 or 60°C. Enzyme solutions (1.0 mL) were heated in sealed tubes, which were incubated in a thermostatically controlled thermal block (Labnet International, Woodbrige, NJ, USA). Tubes were withdrawn at each time intervals and the residual activity was determined as describe earlier, with azocasein as substrate. The activity after 1 min of heating-up time ($t = 0$) was considered to be the initial activity, thereby eliminating the effects of heating-up. Assays were done in triplicate.

2.6. Kinetic model and statistical analysis

The first-order rate constants for denaturation (k_d) of the enzyme at different temperatures were determined from the slopes of semi-logarithmic plots according to Eq. (1).

$$\ln (C_t/C_0) = -k_d t \quad (1)$$

where C_t is the enzyme activity at the time t, C_0 the initial enzyme activity, k_d is the first-order rate constant for denaturation, and t is the time.

The activation energy for denaturation ($E_{a,d}$) was obtained from the slope ($-E_{a,d}/R$) of Arrhenius plot of $ln\ k_d$ verus $1/T$. Free energy ($\Delta G^{\#}_d$), enthalpy ($\Delta H^{\#}_d$) and entropy ($\Delta S^{\#}_d$) of activation for denaturation of the enzyme were calculated from Eq. (2)–(4), respectively.

$$\Delta G^{\#}_{d} = -RT \ln \frac{k_d h}{K_B T} \quad (2)$$

where h (Planck constant) = 6.626×10^{-34} J s, and K_B (Boltzman constant) = 1.381×10^{-23} J K^{-1}.

$$\Delta H^{\#}_{d} = E_{a,d} - RT \quad (3)$$

where R (gas constant) = 8.314 J.mol^{-1} K^{-1}

$$\Delta S^{\#}_{d} = \frac{\Delta H^{\#}_{d} - \Delta G^{\#}_{d}}{T} \quad (4)$$

The half-life time ($t_{½}$) of the enzyme was obtained from Eq. (5).

$$t_{(1/2)} = \ln(2)/k_d \quad (5)$$

The values of k_d and $E_{a,d}$ were estimated by regression analysis using the statistical package of Microsoft Excel® (Microsoft, Seattle, WA). Data were compared by the Tukey's test using the Statistica 5.0 software (Statsoft, Tulsa, OK) and values were considered different each other when $p < 0.05$.

3. Results and discussion

3.1. Keratinase purification

The culture supernatant was submitted to precipitation with ammonium sulphate, gel filtration and ion exchange chromatography. Purification with gel filtration chromatography separated two peaks, where only one presented proteolytic activity against azocasein. While in the Q-Sepharose anionic exchange chromatography it was observed two peaks with enzymatic activity. These peaks were eluted through the NaCl gradient at

approximately 0.27 and 0.61 mol L^{-1}, respectively (Fig. 1). Fractions of the first peak, showing prominent activity, were pooled and used to thermal inactivation studies. This fraction was subjected to SDS-PAGE and a unique band was observed, corresponding to a molecular mass of approximately 20 kDa (Fig. 1, inset). The overall purification factor was about 40.2-fold, and the final yield was 7.1%. The final product had a specific activity of about 21 466 U mg^{-1} (Table 1).

These results indicate that a novel keratinolytic enzyme was purified, since the earlier purified keratinase Q1 from *Chryseobacterium* sp. kr6 showed a molecular mass of 64 kDa [*15*]. The molecular mass of this novel enzyme is in agreement with most keratinases of mesophilic microorganisms, which range from 20 to 50 kDa [*1,4*].

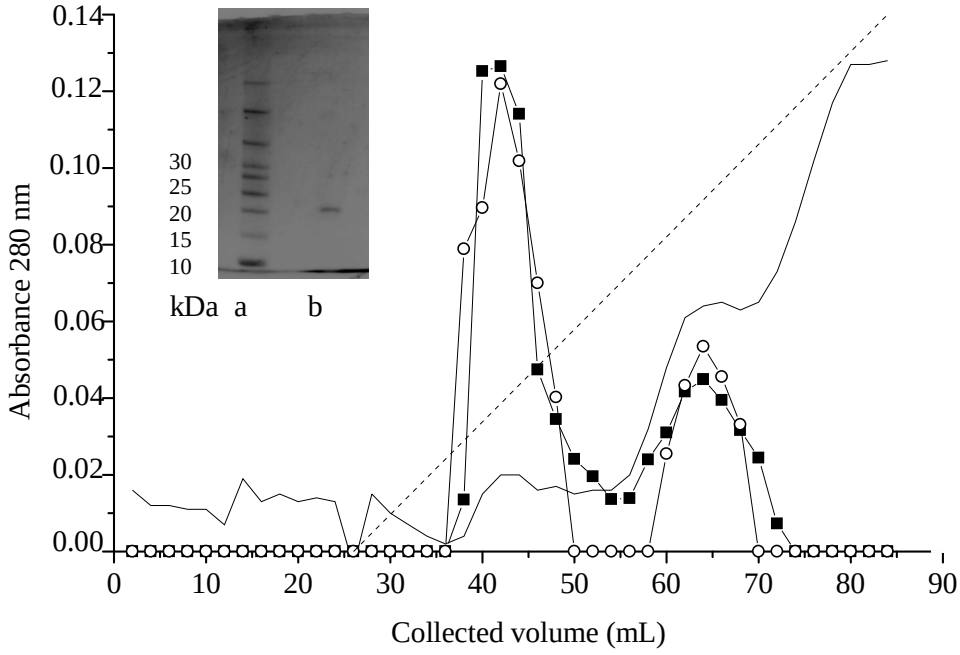


Fig. 1. Elution profile of keratinase from *Chryseobacterium* sp. kr6 on Q-Sepharose Fast Flow equilibrated with 20 mmol L^{-1} Tris-HCl buffer pH 8.5 and eluted with a NaCl gradient (0-1 mol L^{-1}). Proteolytic activity (-■-), keratinolytic activity (-○-), absorbance at

280 nm (—) and NaCl concentration (---). Inset: Silver stained gel showing (a) molecular mass standards and (b) purified enzyme.

Table 1

Purification of the keratinase from *Chryseobacterium* sp. strain kr6 culture

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude enzyme	89,995	168.3	534	1	100
$(NH_4)_2SO_4$ precipitation	68,501	7.9	8,671	16.2	76.1
Sephadex G-100	18,280	1.3	14,061	26.3	20.3
Q-Sepharose Fast Flow	6,440	0.3	21,466	40.2	7.1

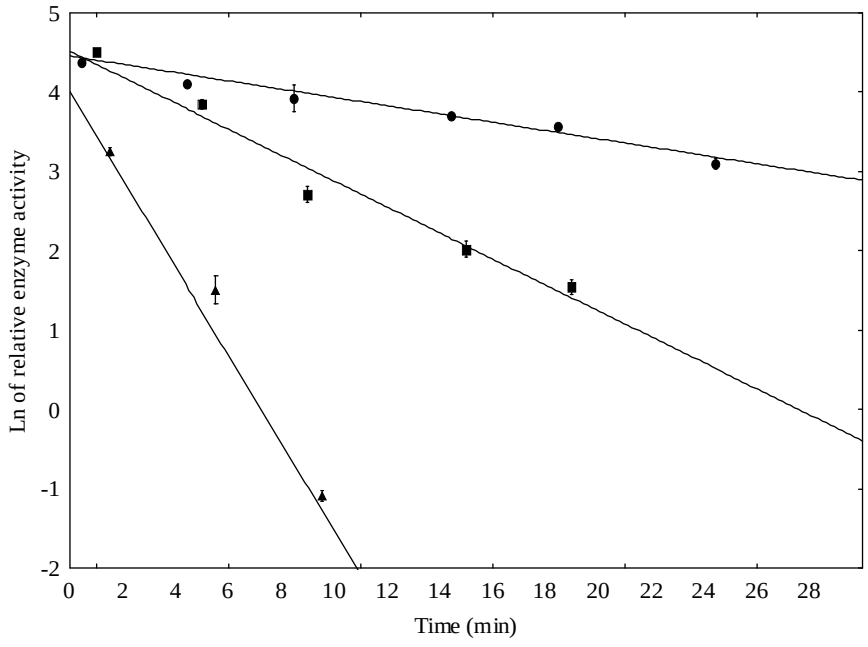
3.2. Kinetics of thermal inactivation

The extent of inactivation after heating the enzyme solution for 10 min at various temperatures was measured. Initially, residual activity was compared after 10 min of thermal treatment in the range from 50 to 80°C, using crude and purified enzyme samples. Both samples retained about 40% its initial activity after 10 min at 50°C, but they were completely inactivated at 65°C or more (results not shown). Residual activity of the different enzyme preparations was then compared over the temperature range from 50 to 60ºC. Kinetic studies about thermal inactivation of proteases are often more complex due to the autolysis effect [*21*]. However, the first-order kinetic model has been used, mainly at low enzyme concentration, to describe the thermal inactivation of proteases [*21,22*].

The influence of temperature on inactivation of the crude enzyme is shown in the Fig. 2 (a, b, c), being the straight lines in these figures the linear fit for each condition, which

were well fixed by the Eq. (1). The slopes in these figures are the first-order rate constants for enzyme denaturation (k_d), estimated by linear regression of the experimental data. The k_d values and the half life time ($t_{½}$) are given in Table 2. Comparing the rate constants (k_d) for enzyme inactivation, smaller values were observed with addition of Ca^{2+} at the different temperatures tested. In addition, excepting for the enzyme with Ca^{2+}, the rate constants increased considerably with the increase of temperature. Compared with control, the $t_{1/2}$ was increased around 2.5-fold at different temperatures in the enzyme samples with calcium.

After 25 min at 50°C the crude enzyme maintained about 22.1% its initial activity, in the same conditions in the presence of calcium the residual activity was 55.8%. When magnesium was added, 36.5% residual activity was observed.



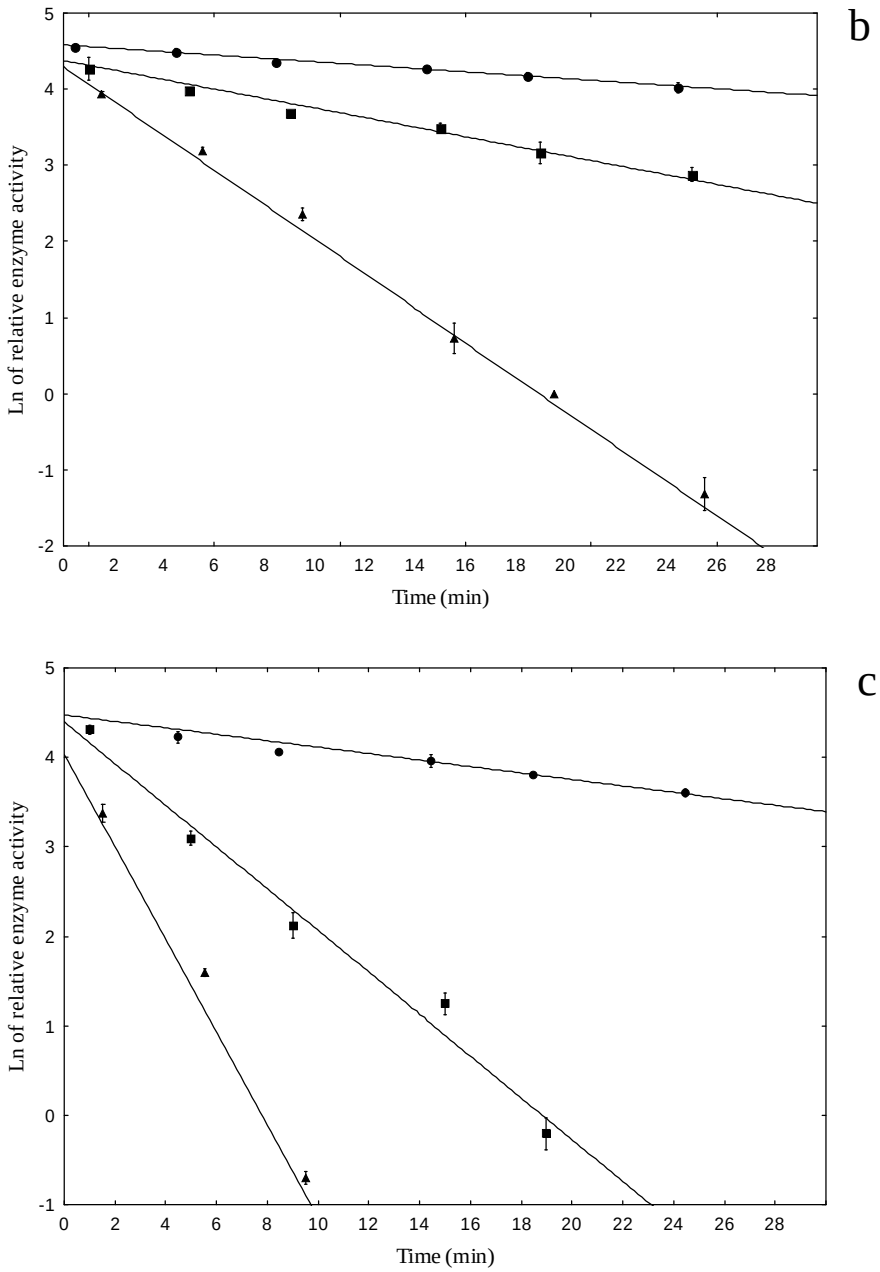


Fig. 2. First-order plots for thermal inactivation of crude enzyme. (a) Control; (b) enzyme with Ca^{+2}; (c) enzyme with Mg^{+2}. The r^2 values for 50°C (•), 55°C (■) and 60°C (▲) were respectively: (a) 0.95, 0.97, 0.99; (b) 0.99, 0.97, 0.99; (c) 0.95, 0.98, 0.99.

Table 2

Kinetics and thermodynamics parameters for thermal denaturation of crude enzyme

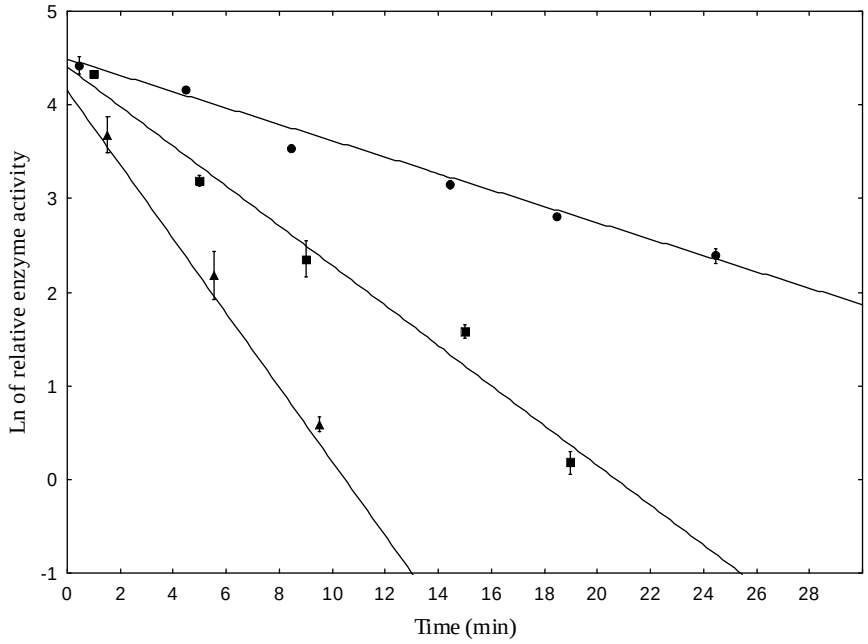
T (K)	k_d (min^{-1})	$t_{1/2}$ (min)	$\Delta G^{\#}_d$ (kJ mol^{-1})	$\Delta H^{\#}_d$ (kJ mol^{-1})	$\Delta S^{\#}_d$ (J mol^{-1} K^{-}
			Control [a]		
323 (50ºC)	0.053±1.41E^{-3}	13.0	87.2	208.9	376.7
328 (55ºC)	0.164±7.07E^{-4}	4.2	85.5	208.8	376.0
333 (60ºC)	0.56±7.07E^{-3}	1.2	83.4	208.8	376.5
			Ca^{2+} [b]		
323 (50ºC)	0.022±3.54E^{-3}	31.2	89.5	206.0	360.6
328 (55ºC)	0.063±2.12E^{-3}	10.9	88.1	205.9	359.3
333 (60ºC)	0.231±2.83E^{-3}	3.0	85.9	205.9	360.3
			Mg^{2+} [c]		
323 (50ºC)	0.036±7.07E^{-4}	19.0	88.2	236.8	460.0
328 (55ºC)	0.235±7.78E^{-3}	2.9	84.5	236.7	464.0
333 (60ºC)	0.528±1.20E^{-2}	1.3	83.6	236.7	459.7

[a] $E_{a,d}$ = 211.6 ± 3.5 kJ mol^{-1}

[b] $E_{a,d}$ = 208.7 ± 15.3 kJ mol^{-1}

[c] $E_{a,d}$ = 239.5 ± 3.7 kJ mol^{-1}

The effect of temperature on inactivation of the partially purified enzyme (after one chromatography step) is shown in the Fig. 3 (a, b, c), the calculated k_d values and the half life are listed in Table 3. Control enzyme maintained 10.9% its initial activity after 25 min, while in the presence of calcium and magnesium it retained 66.5% and 60.8% initial activity, respectively. In the samples with this purification grade it was observed a remarkable effect of calcium on the enzyme stability, the increase on $t_{1/2}$ ratio was 5.6, 7.6 and 8.4-fold for 50, 55 and 60°C, respectively.


Ln of relative enzyme activity
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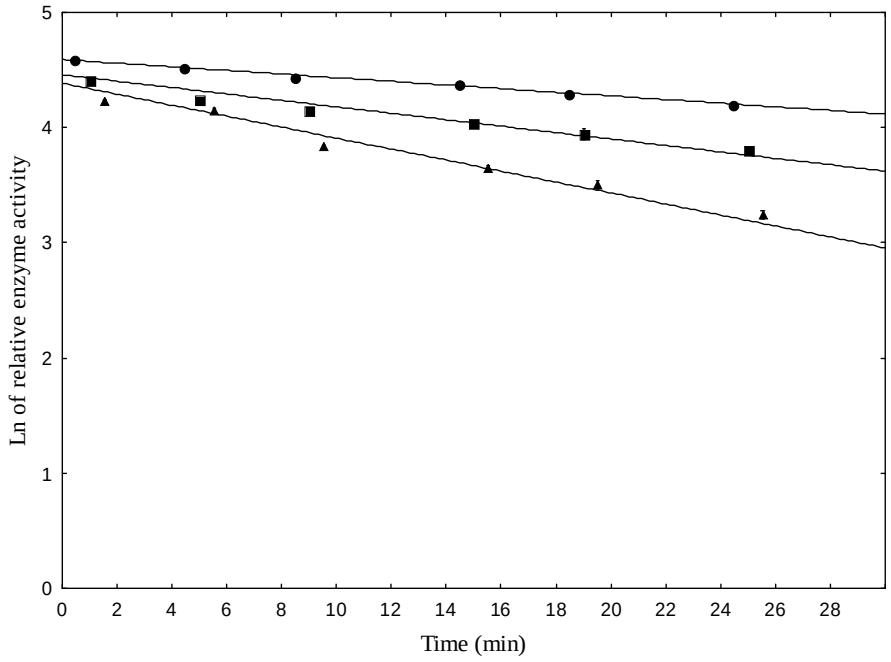
a


Ln of relative enzyme activity
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b

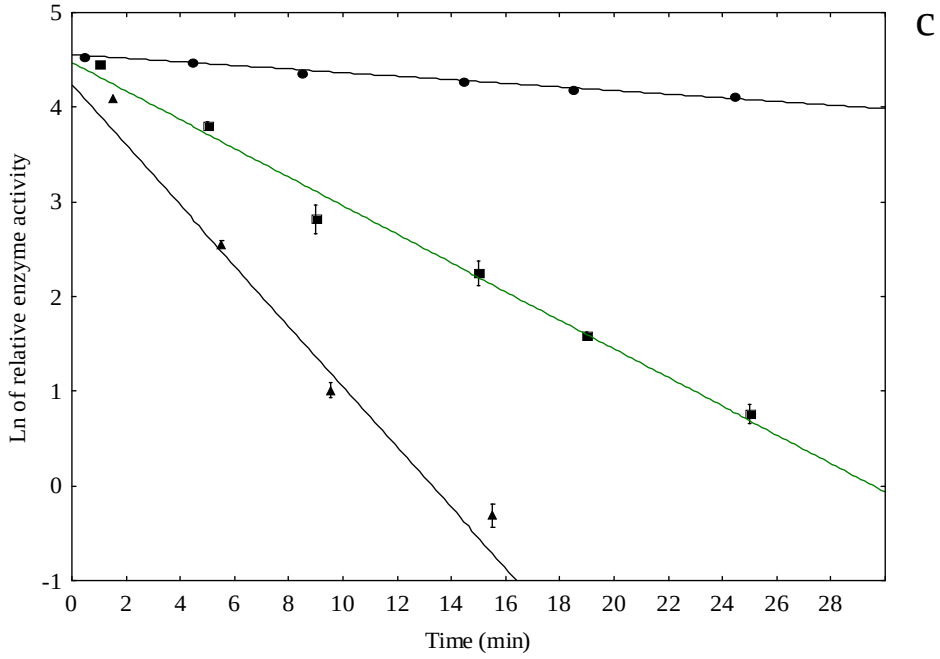


Fig. 3. First-order plots for thermal inactivation of partially purified enzyme. (a) Control; (b) enzyme with Ca^{+2}; (c) enzyme with Mg^{+2}. The r^2 values for 50°C (•), 55°C (■) and 60°C (▲) were respectively: (a) 0.99, 0.98, 0.98; (b) 0.99, 0.97, 0.99; (c) 0.98, 0.99, 0.98.

Table 3

Kinetics and thermodynamics parameters for thermal denaturation of partially purified enzyme

T (K)	k_d (min^{-1})	$t_{1/2}$ (min)	ΔG_d (kJ mol^{-1})	ΔH_d (kJ mol^{-1})	ΔS_d (J mol^{-1} K^{-1})
			Control [a]		
323 (50ºC)	0.089±7.07E^{-4}	7.7	85.8	131.9	142.6
328 (55ºC)	0.214±4.95E^{-3}	3.2	84.8	131.8	143.4
333 (60ºC)	0.402±3.54E^{-3}	1.7	84.4	131.8	142.3
			Ca^{2+} [b]		
323 (50ºC)	0.016±7.07E^{-4}	43.3	90.4	96.4	18.7
328 (55ºC)	0.028±7.07E^{-4}	24.3	90.3	96.4	18.6
333 (60ºC)	0.048±2.12E^{-3}	14.3	90.2	96.4	18.4
			Mg^{2+} [c]		
323 (50ºC)	0.019±7.07E^{-4}	35.5	89.9	252.3	502.9
328 (55ºC)	0.155±2.83E^{-3}	4.5	85.7	252.3	508.0
333 (60ºC)	0.335±9.19E^{-3}	2.0	84.9	252.2	502.6

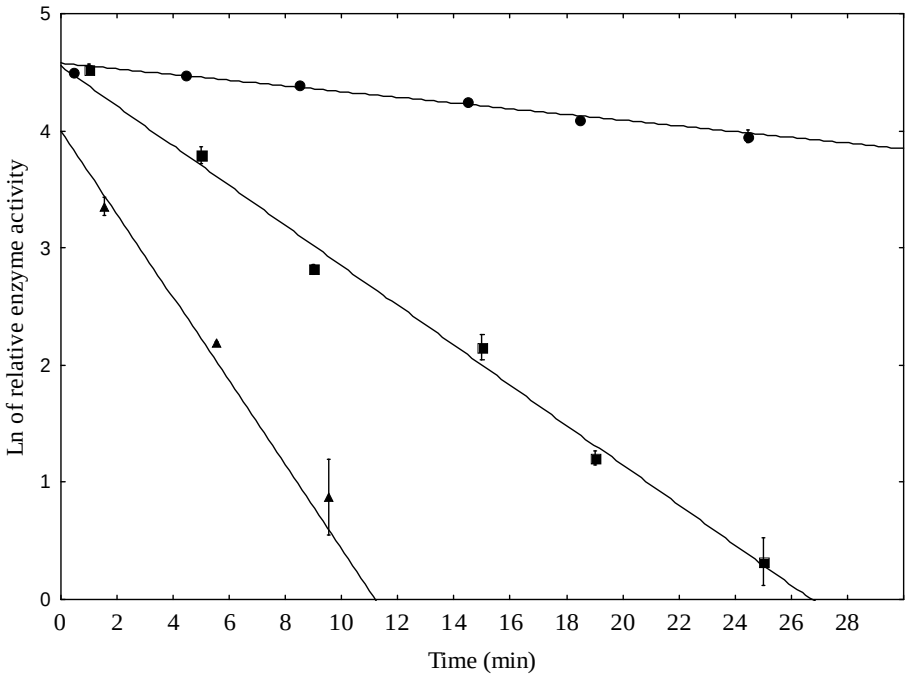
[a] $E_{a,d}$ = 135.4 ± 1.5 kJ mol^{-1}

[b] $E_{a,d}$ = 99.1 ± 3.8 kJ mol^{-1}

[c] $E_{a,d}$ = 255.0 ± 5.7 kJ mol^{-1}

Compared with control, the $t_{1/2}$ of the purified enzyme (after two chromatography steps) in the presence of Ca^{2+} was increased by 7.3, 20.2 and 9.8-fold at 50, 55 and 60ºC, respectively (Table 4). The Fig. 4 (a, b) illustrates the denaturation kinetics of the purified enzyme. After the second chromatographic step, the control enzyme maintained 52% its initial activity, while in the presence of calcium the sample maintained 90.3% residual activity, during 25 min at 50°C. At 55°C for 25 min, only 1.4% residual activity is

observed, while 80.5% is maintained in the presence of calcium. The control enzyme was totally inactivated at 60°C for 10 min, but a residual activity of 39% was observed after 25 min in the presence of calcium. The protective effect caused by the presence of calcium became more pronounced as the purification degree increases.



a

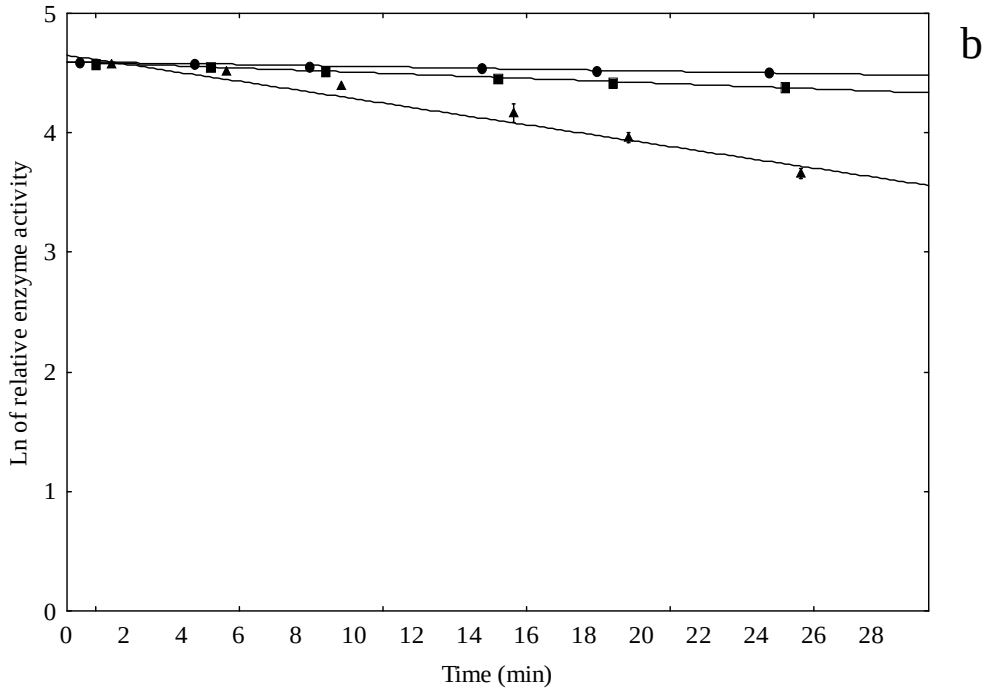


Fig. 4. First-order plots for thermal inactivation of purified enzyme. (a) Control; (b) enzyme with Ca^{+2}. The r^2 values for 50°C (•), 55°C (■) and 60°C (▲) were respectively: (a) 0.98, 0.99, 0.96; (b) 0.98, 0.99, 0.96.

Table 4

Kinetic and thermodynamic parameters for thermal denaturation of purified enzyme.

T (K)	k_d (min^{-1})	$t_{1/2}$ (min)	$\Delta G^{\#}_d$ (kJ mol^{-1})	$\Delta H^{\#}_d$ (kJ mol^{-1})	$\Delta S^{\#}_d$ (J mol^{-1} K^{-1})
		Control [a]			
323 (50ºC)	0.025±1.41E^{-3}	27.7	89.2	236.3	455.4
328 (55ºC)	0.174±2.83E^{-3}	4.0	85.3	236.3	460.1
333 (60ºC)	0.360±3.61E^{-2}	1.9	84.7	236.2	455.1
		Ca^{2+} [b]			
323 (50ºC)	0.003±7.07E^{-4}	202.1	94.5	200.0	326.5
328 (55ºC)	0.008±7.07E^{-4}	81.8	93.6	199.9	324.3
333 (60ºC)	0.037±1.41E^{-3}	18.7	91.0	199.9	327.1

[a] $E_{a,d}$ = 239.0 ± 3.8 kJ mol^{-1}

[b] $E_{a,d}$ = 202.7 ± 8.5 kJ mol^{-1}

The presence of Ca^{2+} and Mg^{2+} ions have been associated with increase of thermal stability of some bacterial proteases. The extracellular protease of *Pseudomonas fluorescens* T20 contained Mg^{2+}, which plays an important role on enzyme stability [*23*]. The presence of calcium on thermolysin, a typical bacterial metalloprotease, contributes to heat stability, prevention of autolysis, and maintenance of structural integrity [*24*]. In this work, only Ca^{2+} appeared to increase the thermal stability of keratinase.

It has been reported that the addition of Ca^{2+} caused an increase in the thermal stability of alkaline proteases. For example, the half-life time was increased 2.5-fold at 50ºC, and 10-fold at 60ºC by adding Ca^{2+} to the alkaline proteases from *Bacillus* sp. GX6638 and *B. sphaericus*, respectively [*25*,*26*]. The addition of $CaCl_2$ increases the half-life of an alkaline protease of *Bacillus mojavensis* from 15 min to 57 min, showing the importance of this ion to improve the thermo resistance [*11*]. In agreement, the half-life

time at 50°C of an alkaline protease from *Conidiobolus coronatus* increased from 17 to 47 min by adding Ca^{2+} [*12*]. The effect of calcium on the improvement of thermal stability against heat inactivation may be explained by the strengthening of interactions inside the protease molecules and by the binding of Ca^{2+} to the autolysis site [*12,13*].

3.3. Thermodynamic parameters

From the values obtained for the denaturation rate constants, it was possible to establish a relationship between the value of k_d and temperature by fitting the data to the Arrhenius equation, which relates rate constants to temperature.

The activation energy ($E_{a,d}$), free energy ($\Delta G^{\#}_{d}$), enthalpy ($\Delta H^{\#}_{d}$) and entropy ($\Delta S^{\#}_{d}$) for enzyme inactivation at different temperatures are listed in Tables 2 to 4. The thermal denaturation of enzyme is accompanied by weakening or disruption of non-covalent linkages with simultaneous increase in the enthalpy of activation, and opening of the enzyme structure is accompanied by an increase in the disorder or entropy [*27,28*].

For crude enzyme, the variation of the $\Delta H^{\#}_{d}$ was observed to be insignificant for the different conditions evaluated (Table 2). However, the presence of calcium reduced the $\Delta H^{\#}_{d}$ values from 131 kJ mol^{-1} to 96.4 kJ mol^{-1} for partially purified enzyme (Table 3) and from 236 kJ mol^{-1} to 200 kJ mol^{-1} for purified keratinase (Table 4).

The smaller activation energy for denaturation of the enzyme was obtained with the partially purified enzyme with Ca^{2+}, and this result differs significantly of the other conditions (p<0.05).

The values of entropy ($\Delta S^{\#}_{d}$) of activation for denaturation of the purified enzyme with Ca^{2+} were remarkably reduced, around 7-fold, changing from 142.6 J $mol^{-1}K^{-1}$ to 18.7 J $mol^{-1}K^{-1}$. Viewed from thermodynamics, decrease in entropy means decrease in disorder degree of molecules [*28*]. The $\Delta G^{\#}_{d}$ value is directly related with the protein stability: the higher $\Delta G^{\#}_{d}$ is higher will be the enzyme stability [*29*]. Results for $\Delta H^{\#}_{d}$ show that the

enthalpy is practically independent of temperature, thus, there is no change in enzyme heat capacity [*30*].

The purity of the enzyme is reported to have a strong effect on the inactivation of pseudomonal proteases [*31*]. The addition of sodium caseinate to an extracellular proteinase from *Pseudomonas fluorescens* caused an increase in inactivation rate, probably due the aggregation of the enzyme molecules with caseinate [*22*]. Though, when the authors compared the kinetic parameters of purified proteinase with the unpurified sample they did not detect statistically significant difference.

Some proteases are protected against thermal inactivation by companion proteins [*28*]. Possibly, the thermostability of purified enzymes may be reduced due to partial denaturation during the purification process, and the effect of autoproteolysis has been also associated with inactivation at moderate temperatures [*22*].

There is no specific information available on kinetics and thermodynamic parameters for keratinase heat-inactivation. The thermodynamic parameters estimated here were similar to those reported by Vicente et al. [*32*] for thermal inactivation of an extracellular aspartic protease from *Phycomyces blakesleeanus.* The fact that no specific studies were found to report kinetics and thermodynamic parameters for heat-inactivation of keratinase gives the present investigation an innovatory character in the analysis of these important proteolytic enzymes.

3.4 Effects of various chemicals

To further characterize the *Chryseobacterium* sp. kr6 keratinase, we examined the effects of some chemicals on the enzyme activity. Protease activity was investigated after preincubation of the enzyme with several chemicals for 30 min, at room temperature. The effects of various inhibitors, metal ions and solvents on the enzyme activity are summarized in Fig. 5. The protease activity of the sample without any reagent (control)

was taken as 100%. Tukey's test was carried out to verify if there was significant difference between the conditions, at 95% of confidence level.

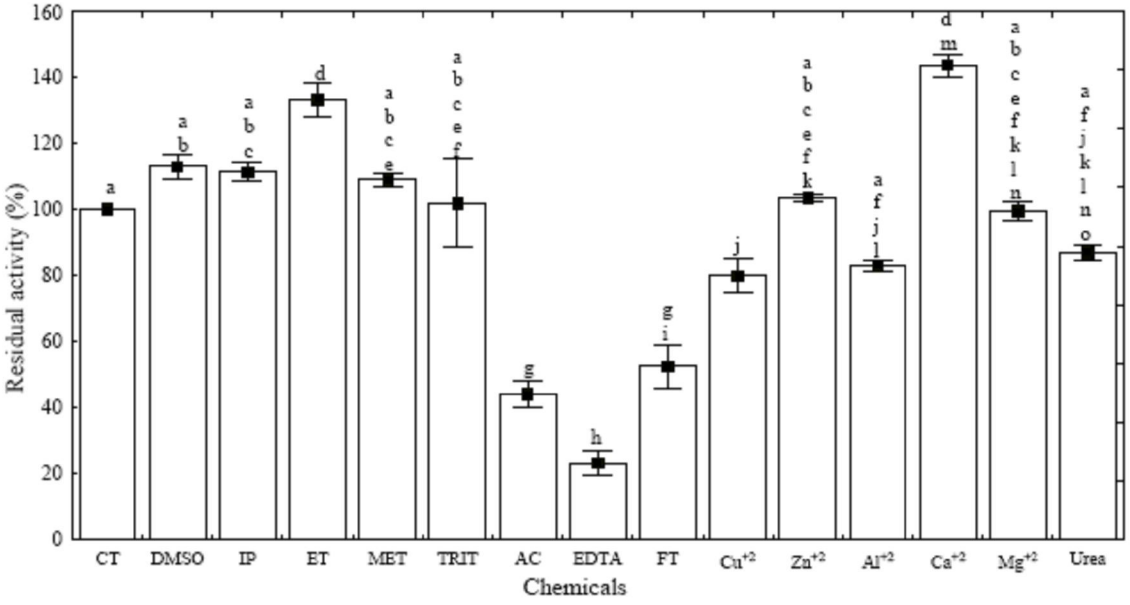


Fig. 5: Effects of various chemicals on enzyme activity. The enzyme was first incubated for 30 min at room temperature. Residual activity was measured according described elsewhere, with azocasein as substrate. CT (control), DMSO, IP (1% v/v isopropyl alcohol), ET (1% v/v ethanol), MET (1% v/v methanol), TRIT (1% v/v Triton X-100), AC (1% v/v acetonitrile), EDTA (5 mmol L^{-1}), FT (5 mmol L^{-1} 1,10 phenanthroline), Cu^{+2}, Zn^{+2}, Al^{+2}, Ca^{+2}, Mg^{+2}, urea: 5 mmol L^{-1}.

Among the solvents, ethanol was only that caused a significantly increase on the enzyme activity, while that the acetonitrile inhibited the proteolytic activity.

The enzyme was significantly inactivated by the presence of 1,10-phenanthroline and EDTA. The inhibitory effect of metal chelator EDTA and Zn-specific chelator 1,10-phenanthroline characterized the enzyme as a metalloprotease. In the presence of some divalent ions Zn^{+2}, Al^{+2} e Mg^{+2} did not detect significantly effects on the enzyme activity. Calcium promoted an increase on proteolytic activity, while copper provide a reduction,

when compared with the control. Finally, the urea also did not exert significantly inhibition, at the same significance interval.

4. Conclusions

We reported here, for the first time, the thermodynamics and kinetics data for thermal denaturation of a keratinase from *Chryseobacterium* sp. strain kr6. An increase in the enzyme stability with the purification degree, and an increase in stability in the presence of calcium were observed. Calcium seems to play an important role in the maintenance of thermal stability of this keratinase.

Acknowledgements

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4.3 RESULTADOS III – "Immobilization of a keratinolytic protease from *Chryseobacterium* sp. strain kr6 on chitosan beads", Food and Bioprocess Technology, submetido em setembro de 2009.

Immobilization of a keratinolytic protease from *Chryseobacterium* sp. strain kr6 on chitosan beads

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Running head: Keratinase immobilization on chitosan

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Abstract

Some process parameters for immobilization of a purified keratinase from *Chryseobacterium* sp. kr6 on chitosan beads were investigated. Production of chitosan beads was optimized using factorial design and surface response techniques. Optimum chitosan beads production for protease immobilization were using 20 g L^{-1} chitosan solution in acetic acid (1.5% *v/v*), glutaraldehyde ranging from 34 g L^{-1} to 56 g L^{-1} and activation time between 6 to 10 h. Under these conditions, above 80% of the enzyme was immobilized on the support. The behavior of the protease loading on chitosan beads surface was well described by Langmuir model. The maximum capacity of the support (q_m) and the constant rate (K_d), were estimated as 58.8 U g^{-1} and 0.245 U mL^{-1}, respectively. The thermal stability of immobilized enzyme was improved around two fold, if compared with the free enzyme, after 30 min at 65°C. The activity of the immobilized enzyme remained 63.4% after it was reused five times. The immobilized enzyme has improved thermal stability and remained active after several uses.

Key words: casein hydrolysis; chitosan; enzyme; immobilized; kinetics; protease

Introduction

An effective use of enzymes may be hampered by some peculiar properties of the enzymatic proteins such as their non-reusability and high sensitivity to several denaturating agents. Many of these undesirable constraints may be removed by the use of immobilized enzymes. This approach has proven to be more advantageous for catalysis than the use of free enzymes (Durán et al. 2002; Saleem et al. 2005). There are several benefits of use immobilized enzymes rather than their soluble counterparts: the reusability of the heterogeneous biocatalysts with the aim of reducing the production cost by efficient recycling and control of the process, as stable and reusable analytic devices for analytic and medical applications, as fundamental tools for solid-phase protein chemistry and as effective microdevices for controlled release of protein drugs (Cao 2005).

Immobilizing enzymes into insoluble supports has been the subject of considerable research for over 30 years and, consequently, many different methodologies and a wide range of applications have been suggested (D´Souza 1999, Isgrove et al. 2001). The various methods devised for enzyme immobilization may be subdivided into two general classes: chemical methods, where covalent bonds are formed with the enzyme, and physical methods, where weak interactions between support and enzyme exist (Çetinus & Öztop 2000). In spite of several techniques for enzyme immobilization that have been reported (Yang et al. 2004; Kannan & Jasra 2009; Ma et al. 2009), many of them are extremely complex to reproduce, expensive and time-consuming. During the immobilization process, the main task is to select a suitable carrier, conditions (pH, temperature and nature of medium) and enzyme itself (source, nature and purity) to design an immobilized biocatalyst (Cao et al. 2005).

Chitosan, a natural polymer, is a good support for enzyme immobilization, since it is non-toxic, user-friendly, and available in different forms: powder, gel, fibers and membranes. This linear copolymer polysaccharide consists of β(1→4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) units (Krajewska 2004; Tang et al. 2006). Chitosan can provide many advantages, such as an excellent hydrophilicity, high porosity, large adhesion area, and thus, small mass transfer resistance to enzymes. In addition, it possesses high protein affinity, allows easy derivatization, and is easily available and inexpensive, since derived from chitin, a by-product of the fishing industry (Krajewska 2004). This polymer has been shown to be an efficient support for immobilization of enzymes like glycosidases, proteases, glucose oxidase, and other (Chang & Juang 2005; Tang et al. 2006).

Factorial design and response surface techniques are important tools to determine the optimal process conditions. This methodology has been successfully used in many areas of biotechnology, particularly to optimize the production of bioactive molecules (Cladera-Olivera et al. 2004; Thys et al. 2006; Casarin et al. 2008). One of the main advantages of experimental design is the reduction of the required number of experiments without remarkable loss of useful information (Kalil et al. 2000).

Keratinases constitute an exciting group of proteases that hydrolyses hard-to-degrade keratin substrates. Bacterial keratinases are mostly related to serine proteases from *Bacillus* and *Streptomyces*, but interesting keratinases from other bacteria has been described (Brandelli 2008). Indeed, *Chryseobacterium* sp. kr6 produces an unusual keratinolytic metalloprotease that belongs to the M14 family of peptidases (Riffel et al. 2007; Silveira et al. 2008). Despite the growing interest on keratinases, relatively few works on their immobilization have been described (Wang et al. 2003; Farag & Hassan

2004; Konwarh et al. 2009). In the present work, some process parameters that affect the immobilization of a purified keratinolytic protease from *Chryseobacterium* sp. kr6 on chitosan beads were investigated. Glutaraldehyde concentration and time required for chitosan beads activation were evaluated. Then, using the optimal conditions established for protease immobilization on chitosan beads, the maximum support capacity (q_m) and the equilibrium constant (K_d) for the enzyme immobilization on chitosan beads were estimated. Also, some properties of free and immobilized enzyme were compared.

Materials and Methods

General

Chitosan flakes were from Sigma Chemical Co. (St. Louis, MO, USA), with a 85% degree of deacetylation. Glutaraldehyde (25% aqueous solution) was from Nuclear (São Paulo, Brazil). A keratinolytic *Chryseobacterium* sp. strain kr6, previously isolated from waste feathers, was plated on feather meal agar (Riffel et al. 2003). Protease production was carried out by submerged culture, using feather meal as only carbon source, as described previously (Silveira et al. 2008). The crude enzyme was collected after 48 h of cultivation at 30°C, by centrifugation at 10,000 x *g*, during 20 min, at 4°C. The enzyme purification involved precipitation with solid ammonium sulphate $(NH_4)SO_4$ at a final concentration of 50% saturation at 0°C. After stand for 3 h, the sample was centrifuged at 10,000 x *g* for 20 min, at 4°C. The pellet was dissolved in a minimum volume of 50 mmol L^{-1} Tris-HCl buffer pH 8.0, centrifuged as above, and the clear supernatant collected. The concentrated sample was applied on a Sephadex G-100 gel filtration column (0.8 x 30 cm) (Pharmacia, Uppsala, Sweden), equilibrated and eluted with the same initial buffer. The fractions with enzyme activity from gel permeation

were pooled and dialyzed overnight against 20 mmol L^{-1} Tris-HCl buffer pH 8.5. The dialyzed enzyme solution was applied on a Q-Sepharose Fast Flow column (3.5 x 15 cm) pre-equilibrated with 20 mmol L^{-1} Tris-HCl buffer pH 8.5. The unbound proteins were washed with 20 mmol L^{-1} Tris-HCl buffer pH 8.5. The bound proteins were eluted at a linear rate of 0.074 cm min^{-1} (flow rate 0.712 cm^3 min^{-1}) NaCl (0-1 mol L^{-1}) in 20 mmol L^{-1} Tris-HCl buffer pH 7.0. The fractions were monitored by measuring the absorbance at 280 nm, enzyme activity having azocasein and azokeratin as substrate, and soluble protein.

Determination of enzyme activity

During the purification steps, the enzymatic activity was carried out with azokeratin as substrate, as follows. The keratinolytic activity of free enzyme was determined by using azokeratin as the insoluble substrate (Riffel et al. 2003). The enzyme sample (100 μL) was mixed with 500 μL of 20 g L^{-1} in 50 mmol L^{-1} Tris-HCl buffer pH 8.0, for 30 min, at 45°. The reaction was stopped by the addition of equal volume of 100 g L^{-1} trichloroacetic acid, the precipitate was removed by centrifugation at 10.000 x *g* during 10 min and the absorbance of the supernatant was measured at 450 nm. Control samples were prepared in a similar manner, except that 100 g L^{-1} trichloroacetic acid was added prior to the enzyme. One unit of keratinolytic activity was defined as an increase in the A_{450} of 0.01, under specified conditions.

While during the optimization of enzyme immobilization on chitosan beads, the enzyme activity was measured using casein as substrate (Böckle et al. 1995). The caseinolytic determinations were done essentially by incubating 100 μL of the enzyme with 500 μL of casein solution (5 g L^{-1}) in 100 mmol L^{-1} Tris-HCl buffer pH 8.0, for 1 h, at 45°C, with a stirring speed of 150 rpm. The reaction was stopped by the addition of

an equal volume of 100 g L^{-1} trichloroacetic acid, the precipitate was removed by centrifugation at 14,000 x *g* during 20 min. The absorbance was measured at 280 nm. One unit of enzyme activity is defined as the amount of enzyme required to increase one absorbance unit at 280 nm due to 1 μmol of tyrosine produced per min during casein hydrolysis, under the described conditions (Böckle et al. 1995). The activity of immobilized enzyme was analyzed following the procedure for corresponding free enzyme with minor modification. In this case, 1 g of enzyme-immobilized beads and 5 mL of casein solution in 100 mmol L^{-1} Tris-HCl buffer pH 8.0. Blanks were prepared under the same conditions, but 100 g L^{-1} trichloroacetic acid was added before the addition of the enzyme.

The protein content of soluble protease was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard. The amount of protein immobilized on chitosan beads was estimated by subtracting the residual protein (protein left in protease solution after incubation plus the protein in washings) from the total protein (enzyme solution used for incubation of chitosan beads). The assays were conducted in triplicate.

Preparation of chitosan beads

Chitosan (2 g) was dissolved in 100 mL of 1.5% (v/v) acetic acid, and heated at 60ºC for 1 h at 150 rpm. The yielded viscous solution was sonicated for 30 min, to remove air bubbles, and then drop-wise sprayed through a syringe, at constant rate, into a neutralization solution containing 1 mol L^{-1} KOH. The beads formed (2-3 mm diameter) were washed with Milli Q water until the solution became neutral and stored at 4ºC in 100 mmol L^{-1} Tris-HCl buffer pH 8.0 until glutaraldehyde activation.

Activation of chitosan beads - second-order factorial design

Chitosan beads were incubated at 30°C and 100 rpm with 50 mL of different glutaraldehyde concentrations (ranging from 10 to 80 g L^{-1}) by different time intervals, (from 0.5 h to 10 h). After activation time of each run, the beads were washed with 100 mmol L^{-1} Tris-HCl buffer pH 8.0 to remove the excess of glutaraldehyde, and stored at 4ºC in the same buffer until further use. The influence of glutaraldehyde concentration (GA) and activation time on enzyme immobilization was evaluated through a full factorial design (2^2 plus star configuration) with four replicates in the central point, a total of 12 runs (Box et al. 1978). Five levels of each independent variable were chosen, the applied ranges of these variables were previously determined by preliminary experiments. In the statistical model, *Y* predicted response: enzyme immobilization (EI); b_0, constant; X_1, glutaraldehyde concentration (GA); X_2, activation time; b_1 and b_2 linear coefficients; b_{11} and b_{22} quadratic coefficients; b_{12}, interaction coefficient.

Table 1 shows the actual levels corresponding to the coded settings, the treatment combinations and the response. The runs 1 to 4 correspond to the linear points of the factorial design, which allow investigating the main effect of each independent variable on the evaluated response. The experiments 5 to 8 correspond to the axial points, and runs 9 to 12 are the central points. This design is represented by a second order polynomial regression model (Eq.(1)), to generate contour plots:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \qquad (1)$$

Statistica 7.0 software (Statsoft, Tulsa, OK, USA) was used for regression and graphical analysis of the data. The significance of the regression coefficients was determined by Student's *t*-test, the second order model equation was determined by Fisher's test. The variance explained by the model is given by the multiple coefficient of determination (r^2).

Enzyme immobilization

An amount of activated wet beads (1 g), prepared according the conditions described above, was incubated with 1 mL of enzyme (specific activity 31 U mg^{-1}) and 3 mL of 100 mmol L^{-1} Tris-HCl buffer pH 8.0, for 12 h. After incubation, the beads were washed with 100 mmol L^{-1} Tris-HCl buffer pH 8.0 to remove unbound protease, and stored at 4°C until further use. Blank was prepared under the same conditions, however without solid support, to quantify the enzyme denaturation during the reaction. The enzyme activity of the supernatant and the chitosan beads was estimated according described above, with casein as substrate. The enzyme loading on chitosan beads achieved during the immobilization process was calculated according Equation 2, in terms of enzyme immobilization (EI):

$$EI=\frac{U_0-U_{remain}}{U_0}*100 \quad (2)$$

where U_0 (U mL^{-1}) is the enzyme activity present in the solution used for immobilization, U_{remain} (U mL^{-1}) is the activity remaining in the supernatant at the end of the immobilization procedure.

Loading capacity of chitosan beads

Experiments to investigate the loading capacity of chitosan beads for protease immobilization consisted of batch reactors with 1 g of previously activated beads (40 g L^{-1} glutaraldehyde and 10 h activation time) and 3 mL of different activities of purified protease (ranging from 1.9 U mL^{-1} to 30 U mL^{-1}). After 12 h of incubation at 20°C and 100 rpm, the enzyme activity in both liquid and solid phases was determined as described above, with casein as substrate. In order to detect any enzyme denaturation during the immobilization procedure, we made up blanks at the same conditions,

excepting by the chitosan beads absence. The experimental data of enzyme activity obtained after the immobilization equilibrium were used to check if the Langmuir model can be able to fit it, allowing to estimate the parameters q_m and K_d. The kinetic equation of protease immobilization can be expressed by Eq. (3), which is transformed in Eq. (4) at equilibrium:

$$\frac{dq}{dt} = k_1 C(q_m - q) - k_2 q \quad (3)$$

$$q^* = \frac{q_m c^*}{c^* + K_d} \quad (4)$$

Equation 4 was used to determine the maximum amount of protease loaded on support (q_m) and the equilibrium constant (K_d), being q* and c* the enzyme activity on solid and liquid phase, at equilibrium, respectively. The parameters were calculated using Statistica 7.0 (Statsoft Inc., USA) by nonlinear estimation analysis with Hook-Jeeves and Quasi-Newton method.

Thermal stability and reuse of immobilized enzyme

Thermal stability of free and immobilized enzyme was tested by measuring the activities after the samples were incubated for 30 min at different temperatures (50, 55, 60 and 65ºC). Free and immobilized enzymes were assayed as described above, with casein as substrate.

The initial activity of the immobilized enzyme was measured and then compared with the activity of the used enzyme obtained after its repeated use for five cycles. After each cycle, the immobilized enzyme was immediately filtered, washed with 100 mmol L^{-1} Tris-HCl buffer pH 8.0, to remove any residual substrate and reintroduced into fresh reaction medium. The remaining of enzyme activity was tested as described above, using casein as substrate. The results of thermal stability and reusability were presented

in a normalized form, with the highest value of each set being assigned the value of 100 % activity.

Results and Discussion

Optimization of chitosan beads production

Beads of chitosan were successfully produced and activated, the particles obtained were often nearly spherical, with diameter ranging from 2.0 to 3.0 mm. Amylases and commercial protease XIX were also successfully immobilized on chitosan beads with this diameter range (Chang & Juang 2005; Benkhelifa et al. 2005), while similar chitosan beads, with 5 mm of diameter, were developed to immobilize β-galactosidase (Dwevedi & Kayastha 2009). Spacers are required for efficient immobilization of biospecific molecules to solid support materials, because it minimizes the hindrance and environmental effects imposed by the surface properties of the matrices (Nouaimi et al. 2001). The modification of chitosan beads with glutaraldehyde had three objectives: (1) the introduction of a spacer arm in order to improve the mobility of the immobilized enzyme; (2) to move the enzyme away from the polymer surface so as to reduce interactions between the protein and the support; and (3) to introduce onto the surface of the polymer carbonyl groups to react of the amino groups of the protease.

The experimental conditions and the response obtained for protease immobilization on chitosan beads are shown in Table 1.

Table 1

Coded levels and real values (in parentheses) for a full factorial design, and enzyme immobilization (EI)

Run	X_1	X_2	EI (%)
1	-1 (12)	-1 (2.2)	46.8
2	+1 (68)	-1 (2.2)	68.7
3	-1 (12)	+1 (8.6)	64.9
4	+1 (68)	+1 (8.6)	78.2
5	0 (40)	-1.41 (0.5)	39.2
6	0 (40)	+1.41 (10)	93.6
7	-1.41 (10)	0 (5.2)	27.7
8	+1.41 (80)	0 (5.2)	66.6
9	0 (40)	0 (5.2)	84.6
10	0 (40)	0 (5.2)	85.4
11	0 (40)	0 (5.2)	84.3
12	0 (40)	0 (5.2)	83.9

X_1: glutaraldehyde concentration (g L^{-1}); X_2: activation time (h)

The higher EI was achieved in experiment 6 (93.6%), which was carried out with a glutaraldehyde concentration of 40 g L^{-1} and 10 h of activation time (Table 1). The smallest EI (27.7%) was observed when glutaraldehyde was used at 10 g L^{-1} and activation time was 5.2 h (run 7). The effects of independent variables on response were presented in Table 2. The results obtained in this experimental design demonstrated that the percent of enzyme immobilization (EI) was significantly influenced by both factors, at 95% of confidence level. Change of glutaraldehyde concentration from inferior level (12 g L^{-1}) to superior level (68 g L^{-1}) promoted an increase of EI, on average, of 22.6%.

Change the activation time from 2.2 h to 8.6 h caused a rise of EI, on average, of 26.1%. Glutaraldehyde concentrations above of 40 g L^{-1} did not favored the protease immobilization. When the effects of variables are interactive, this effect can be evaluated (Box et al. 1978). In this study, the combining effect of glutaraldehyde concentration and activation time resulted in a decrease of EI, on average, of 4.3%. Çetinus and Öztop (2000) evaluated the effect of glutaraldehyde concentration on catalase immobilization in chitosan film. According those authors, when the amount of glutaraldehyde was increased, the enzyme activity increased, however the films became fragile, so, they chose to work with glutaraldehyde at 0.02% (w/v). Optimized immobilization of soybean urease on chitosan beads reached 77% when working at 20 g L^{-1} chitosan beads, 10 g L^{-1} glutaraldehyde and 12 h of coupling time (Kumar et al. 2008). Those authors observed that higher concentrations of glutaraldehyde affected the efficiency of immobilization. The contact time of glutaraldehyde with chitosan to crosslink a β-glucosidase was 30 min only, since additional incubation caused enzyme deactivation due to conformational and surface modifications induced by the glutaraldehyde (Martino et al. 1996). Response surface methodology and central composite design (CCD) were also used to optimize the β-galactosidase immobilization onto chitosan beads (Dwevedi & Kayastha 2009). Factors including the glutaraldehyde concentration, amount of enzyme, and number of beads affected significantly the enzyme immobilization, while pH did not show a significant effect on the response. After the optimal conditions were established, maximum enzyme immobilization was 75%.

Table 2

Main effects and interactions analysis for glutaraldehyde concentration (GA) and activation time (AT) for enzyme immobilization (EI) during protease immobilization on chitosan beads

Factor	Effect	Std.Err.	*t*-value	*p*-value
Mean	84.5	0.32	266.2	<0.000[a]
GA(L)	22.6	0.45	50.2	<0.000[a]
AT (L)	26.1	0.45	58.1	<0.000[a]
1x2	-4.3	0.63	-6.7	<0.00[a]

a = significant factors $p<0.05$

Unless a few mentioned works, most of reports involving the optimization of conditions for support production to enzyme immobilization did not applied statistical tools; they only evaluate a single parameter per trial. In this work, experimental design and surface response techniques were employed to determine the best conditions for chitosan beads production. To construct a second order model that can predict the percent of enzyme immobilization (EI) (dependent variable) as a function of glutaraldehyde concentration and activation time (independent variables), the analysis of variance (ANOVA) was used to evaluate the adequacy of the fit. The second-order model adjusts well to the experimental data, 10.2% of the total variation was not explained by the model (r^2 = 0.898). The error factor was very low, indicating optimal accuracy for the experimental data. Based on the *F*-test, the model is predictive, since its calculated *F*-value ($F_{0.95;5;6}$ = 4.39) was higher than the critical *F*-value (11.1).

On the basis of the ANOVA, a second order model was established (Eq. (6)), describing the EI as a function of the independent variables:

$$EI = 84.5 + 11.3X_1 + 13.0X_2 - 2.1X_1X_2 - 16.7X_1^2 - 7.0X_2^2 \qquad (6)$$

The coded model was used to generate the response surface (Fig. 1). The highest percent of immobilization was achieved at glutaraldehyde concentration ranging from 34 g L^{-1} to 56 g L^{-1} and activation time between 6 to 10 h. Under conditions, it is possible to achieve a percent of enzyme immobilization above of 80%. This good loading efficiency for the immobilization by covalent binding might be due to the formation of stable binding between the carrier and the enzyme through a spacer group (glutaraldehyde).

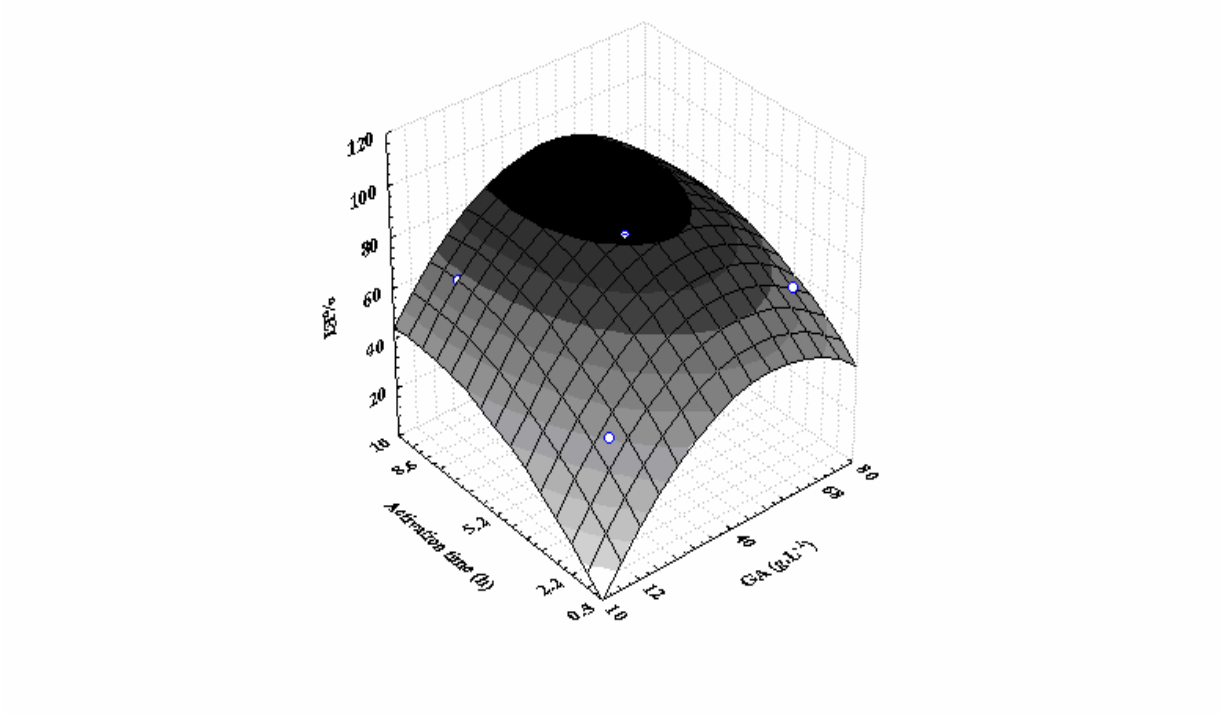

Fig. 1 Response surface for enzyme immobilization (EI) as a function of the glutaraldehyde concentration (GA) and activation time (AT) during protease immobilization on chitosan beads.

Loading capacity of chitosan beads

To investigate the behavior of chitosan-enzyme system and to estimate the maximum load capacity of chitosan beads to the purified protease from *Chryseobacterium* sp. kr6,

experiments using fixed amount of chitosan beads and variable enzyme concentrations were carried out. The results for proteolytic activity in both liquid and solid phases after the immobilization process reached the equilibrium are shown in Fig. 2.

Some parameters of covalent immobilization of lipoprotein lipase onto chitosan beads were investigated (Itoyama et al. 1994). Those authors demonstrated that the amount of immobilized lipoprotein increased as increases the initial enzyme concentration, at low concentrations levels below 4.0 mg mL^{-1}, and a plateau was obtained at concentration higher than 5.0 mg mL^{-1}. The plot presented in the referred work is very closer to that obtained in the present work (Fig. 2).

To fit the experimental data, the Langmuir equation was used, resulting in a good agreement (r^2 =0.976). Thus, it was possible to use this model to estimate the parameters q_m and K_d as 58.8 U g^{-1} and 0.254 U mL^{-1}, respectively. The dissociation coefficient (K_d) is a measure of the affinity of the protein to a support (Monzo et al. 2007).

Langmuir model is often used to investigate the adsorption behavior of molecules to solid supports, especially to evaluate the protein adsorption to affinity and ion exchange resins, aiming to optimize downstream steps (Cano et al. 2005). This model assumes the existence of finite sites and according the sites in the support are filled it becomes increasingly difficult for a solute molecule to find an available vacant site. Recently, the Langmuir model was used to evaluate the adsorption mechanism of keratinase from *Nocardiopsis* sp. TOA-1 on keratin powder (Mitsuiki et al. 2004). Those authors mentioned that the keratinase shows a high adsorbability on keratin, and, in general, insoluble substrate-hydrolyzing enzymes posses a high adsorption capability for insoluble substrates.

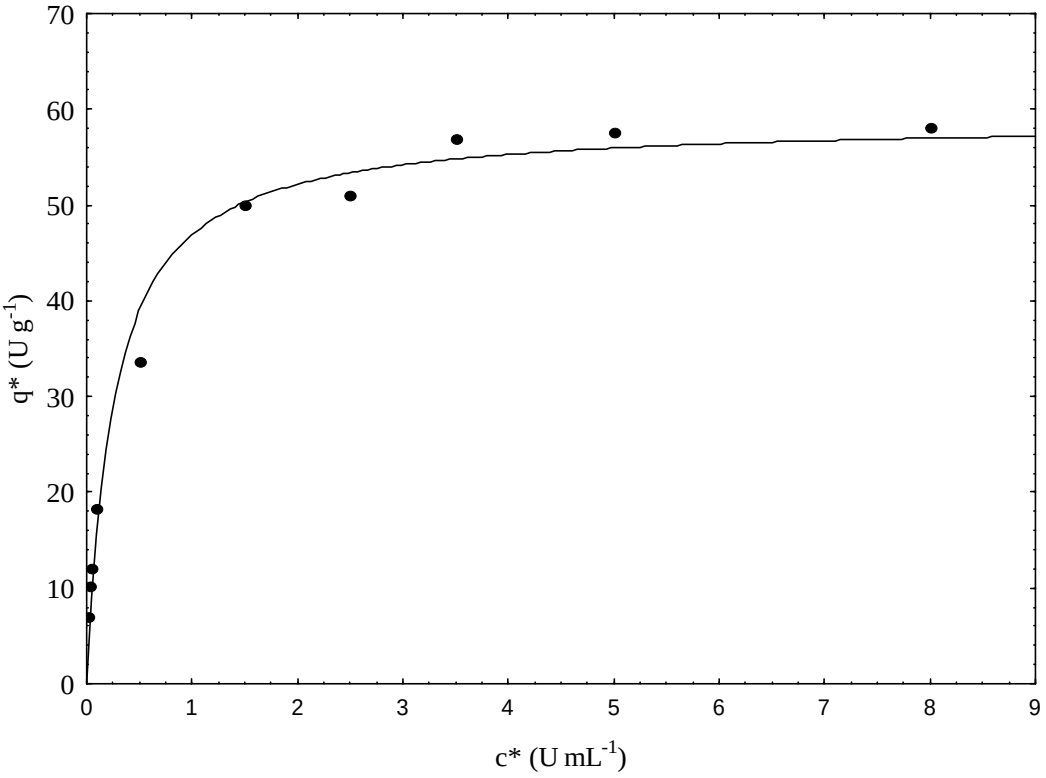

Fig. 2. Binding isotherm of chitosan/protease system. Immobilization studies were performed at 20°C, at 100 rpm, during 12 h.

Thermal stability and recycles

Immobilized and free enzyme was incubated at 50, 55, 60 and 65°C in water bath for 30 min. Fig. 3 illustrates the residual activity for the different temperatures evaluated. The activity loss of immobilized enzyme was lesser than the free enzyme. After 30 min at 65°C, only 13% of residual enzymatic activity of free enzyme was remain, while around 34% residual activity of immobilized enzyme was detected. It seems that the immobilization on chitosan beads caused an increase in the enzyme rigidity and protected it from unfolding, thus immobilized enzyme showed higher thermal stability than that of the free enzyme. The conformational flexibility of the enzyme was affected by immobilization, causing an increase of enzyme rigidity and protected it from

unfolding (Tang et al. 2006). Consequently, the immobilized enzyme showed higher thermal stability than the free enzyme.

Another important factor for application of immobilized enzymes is the evaluation of their capability to reutilization. The results obtained after five cycles of catalysis indicated that it was possible to reutilize the beads containing the immobilized protease from *Chryseobacterium* sp. kr6 for at least five times, since the residual activity after the last cycle was 63.4%. Kannan & Jasra (2009) reported some properties of immobilized alkaline serine endopeptidase from *Bacillus licheniformis* on SBA-15 and MCF by surface covalent binding. The reusability of immobilized enzyme showed 80% of the activity retained even after 15 cycles, the thermal stability decrease gradually after 60°C. The immobilization of a neutral proteinase on chitosan nano-particles resulted in a remaining activity of 88% after nine times of reuse (Tang et al. 2006).

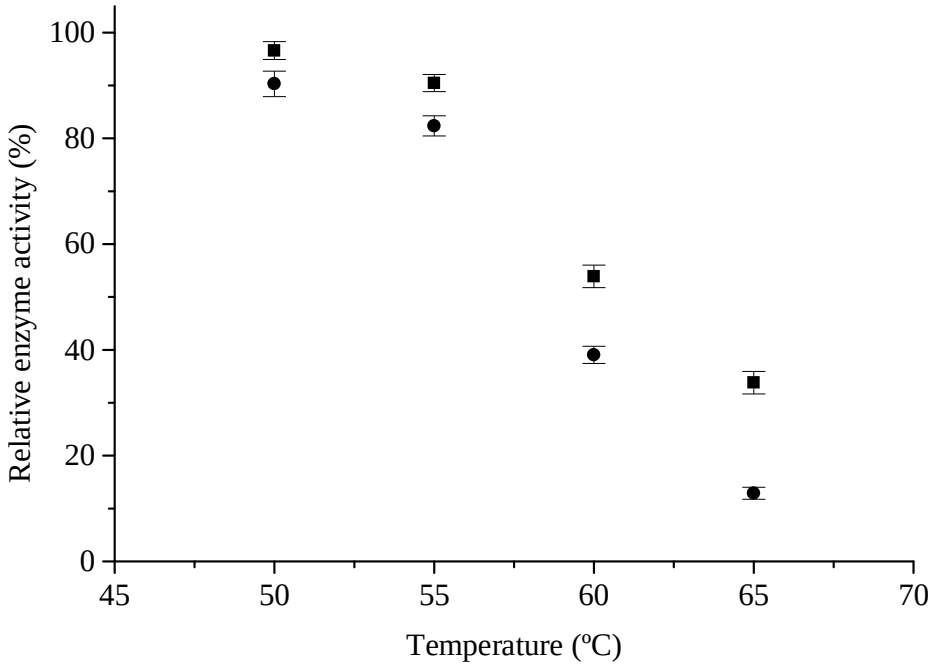

Fig. 3. Effect of temperature on free (•) and immobilized (■) protease.

Conclusions

This paper shows that chitosan macrospheres are suitable for immobilization of a purified protease from *Chryseobacterium* sp. kr6. The optimal conditions for chitosan beads production were 20 g L^{-1} of chitosan concentration, glutaraldehyde ranging from 34 g L^{-1} to 56 g L^{-1} and activation time between 6 to 10 h. Under these conditions, above 80% of the enzyme was immobilized on the support. The behavior of the immobilization protease on chitosan beads was fitted well using Langmuir model, the estimated parameters were q_m of 58.8 U g^{-1} and K_d of 0.254 U mL^{-1}. The thermal stability of immobilized enzyme was improved around two fold, if compared with the free enzyme, after 30 min at 65°C. Also, the activity of the immobilized enzyme remained 63.4% after it was reused five times.

Nomenclature

c^*	Equilibrium concentration of enzyme on liquid phase (U mL^{-1})
EI	Enzyme Immobilization (%)
K_d	Dissociation constant for enzyme/chitosan complex (U mL^{-1})
q_m	Maximum loaded capacity (U g^{-1}solid support)
q^*	Equilibrium concentration of immobilized enzyme on solid support (U g^{-1})
r^2	Determination coefficient
YI	Yield of immobilization

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5. CONCLUSÕES GERAIS

O interesse no desenvolvimento de técnicas para bioconversão dos diversos resíduos constituídos por queratina tem impulsionado a busca por microrganismos produtores de queratinases. A linhagem *Chryseobacterium* sp. kr6 produz queratinases com considerável potencial para aplicação na hidrólise de farinha de penas, bem como outros substratos protéicos como a caseína.

Este trabalho visou disponibilizar informações relacionadas a uma nova queratinase produzida por *Chryseobacterium* sp. kr6, além de desenvolver técnicas relacionadas a sua purificação e imobilização.

Inicialmente, foram estabelecidas as condições ótimas para a atividade proteolítica da queratinase, através da utilização das técnicas de metodologia de superfície de resposta e planejamento experimental. A estratégia escolhida foi construir um delineamento composto central rotacional (DCCR) em função do reduzido número de fatores de estudo. A partir dos resultados obtidos foi possível desenvolver um modelo que prediz a atividade enzimática em função das variáveis independentes (pH, temperatura e concentração de NaCl). As superfícies de resposta, construídas com base no modelo gerado pelo DCCR, possibilitaram a determinação da região ótima para a atividade proteolítica. As condições que promoveram a maior atividade enzimática foram ao utilizar pH na faixa de 7,4 a 9,2, 35ºC a 50°C e concentração de NaCl de 50 a 340 mmol L^{-1}.

A especificidade da queratinase frente a diversos peptídeos sintéticos foi investigada, indicando a preferência da enzima por aminoácidos com cadeias laterais hidrofóbicas ou carregadas positivamente. A enzima também demonstrou habilidade para hidrolisar substratos insolúveis, especialmente farelo de unhas.

Em um segundo momento, buscou-se desenvolver um protocolo visando a purificação completa da queratinase. Para isso, combinaram-se duas técnicas cromatográficas: permeação em gel e troca aniônica. O processo de purificação utilizando as referidas etapas cromatográficas possibilitou a obtenção de uma fração com elevada atividade queratinolítica, que apresentou uma massa molecular de 20 kDa.

De posse da queratinase purificada, avaliou-se a estabilidade térmica da enzima em diferentes estágios de purificação, bem como na presença de íons divalentes. Observou-se que a estabilidade térmica da enzima é dependente da presença de cálcio nas diferentes condições avaliadas.

O desenvolvimento de um suporte a base de quitosana para a imobilização da queratinase purificada foi investigado. Dentre os fatores envolvidos no estudo, destacam-se a concentração de quitosana para a produção das esferas, concentração de glutaraldeído e tempo requerido para ativação das esferas. Nas condições otimizadas, acima de 80% da enzima adicionada foi imobilizada ao suporte.

A enzima imobilizada apresentou um aumento na termoestabilidade em torno de 2 vezes a 65°C, durante 30 min. A reutilização do sistema catalítico heterogêneo foi testado por cinco ciclos repetidos, sendo que no último ciclo foi detectado 63,4% da atividade enzimática.

6. PERSPECTIVAS

Este trabalho demonstrou que a linhagem kr6 do microrganismo *Chryseobacterium* sp. possui capacidade de produzir mais de uma queratinase com elevado potencial para aplicação em processos biotecnológicos. Dentre os assuntos que podem ser melhor explorados estão:

- Determinar a sequência completa de aminoácidos que constituem a queratinase.
- Com a enzima imobilizada em esferas de quitosana:

Determinar os parâmetros cinéticos para a inativação térmica.

Avaliar a atividade da enzima em diferentes valores de pH e temperatura.

Investigar a capacidade de hidrólise de outros substratos.

Estudar a possibilidade de utilização das esferas de quitosana em outros modelos de reatores.

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ANEXOS

ANEXO A.

RESULTADOS NÃO APRESENTADOS NOS MANUSCRITOS

1. Hidrólise de diferentes substratos protéicos

A atividade da queratinase purificada frente a diversos substratos foi investigada. A mistura, consistindo de 800 μL da suspensão do substrato (albumina, caseína, colágeno, farinha de penas, gelatina, hemoglobina, queratina) e 200 μL da enzima, foi incubada a 45°C, sob agitação, por 1 h. Após o período de incubação, a reação foi parada pela adição de igual volume de ácido tricloroacético (100 g L^{-1}), centrifugando-se por 20 min a 14.000 x *g*, para coleta do sobrenadante, o qual foi utilizado para medida de absorbância a 280 nm. Uma unidade de atividade enzimática foi definida como a quantidade de enzima requerida para causar um aumento na A_{280} em uma unidade, nas condições especificadas (BÖCKLE et al., 1995). A atividade enzimática frente a caseína foi considerada 100%, sendo os demais resultados relativizados ao referido substrato.

Dentre os substratos investigados, a caseína, farinha de penas e hemoglobina foram hidrolisados pela enzima, apresentando 100%, 34,5% e 18% de atividade relativa, respectivamente. Não foi detectada hidrólise frente ao colágeno. As metaloproteases produzidas por *Chryseobacterium indologenes* TKU14 também não demonstraram capacidade para hidrolisar a albumina, apresentando uma atividade relativa na faixa de 20 a 30% sobre a hemoglobina (WANG et al., 2008).

Tabela 1

Hidrólise de diferentes substratos pela protease queratinolítica purificada de *Chryseobacterium* sp. kr6

Substrato	**Atividade relativa (%)**
Albumina	0
Caseína	100
Colágeno	0
Farinha de penas	34,5
Gelatina	0
Hemoglobina	18,0

2. Identificação parcial da proteína

A queratinase purificada foi submetida a migração em gel de poliacrilamida (SDS-PAGE) e a coloração da banda foi realizada através de impregnação com Comassie Azul Brilhante R-250. A banda de interesse foi recortada de forma manual, minimizando a quantidade de gel, digerida *in situ* com tripsina, conforme protoloco descrito por SHEVCHENKO et al. (1996). Uma alíquota de 0,5 μL do sobrenadante da digestão foi analisada diretamente em um espectrômetro de massas Q-TOF Micro da Micromass, acoplado a um UPLC nano Acquity da Waters. A identificação dos peptídeos da queratinase foi realizada no Uniprote-MS do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul.

Até o momento a sequência de dois peptídeos foi elucidada:

1) Ser-Pro-Arg-Val-Gly-Leu-Ala-Gly-Leu-Ala-Ala-Gly-Val-Pro-Leu-Thr-Asp-Lys

2) Ala-Ala-Pro-Glu-Lys-Leu-Ala-Ala-Leu-Val-Gly-Glu-Tyr-Arg

Os quadros a seguir apresentam as sequências de peptídeos resultantes da digestão tríptica da queratinase purificada produzida por *Chryseobacterium* sp. linhagem kr6.

Peptídeo 1

Sequência	Organismo
SPRVGLAGLAAGVPLTDK	*Chryseobacterium* sp. kr6
VGLAGLAAGVPLTDK	Putative quitinase *Metarhizium anisopliae*
VGL**V**GLA**S**GVP**F**TD	Putative endoquitinase CHI2 *M. anisopliae*
LA**A**LAAGVPLT	Putative tiolase *Erwinia tasmaniensis*
RV – LAGL**S**AGVPL	Hypothetical protein *Synechococcus* sp.

Peptídeo 2

Sequência	Organismo
AAPEKLAALVGEYR	*Chryseobacterium* sp. kr6
AAPE**Q**LAA**I**VGEYR	Putative quitinase *Metarhizium anisopliae*
AP**K**KLAA**V**VGE**F**R	Putative ATP-dependent helicase *Roseobacter denitrificans*
APE**QV**AALVG**S**YR	Proteína da família AcrB/AcrD/AcrF *Pseudomonas syringae*

O sequenciamento de outros peptídeos está em andamento.

8. CURRICULUM VITAE RESUMIDO

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1. DADOS PESSOAIS

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2. FORMAÇÃO

Engenharia de Alimentos (Fundação Universidade Federal do Rio Grande – FURG, 1997-2003).

Mestrado em Engenharia e Ciência de Alimentos (Fundação Universidade Federal do Rio Grande – FURG, 2003 – 2005).

3. ESTÁGIOS

Estágio curricular obrigatório na empresa FURTADO S/A (Rio Grande, no período de agosto a dezembro de 2001).

Estágio curricular não obrigatório na empresa EFEGÊ (Pelotas, no período de setembro de 2002 a fevereiro de 2003).

Estágio curricular não obrigatório na empresa SALSUL LTDA (Rio Grande RS, no período de fevereiro a agosto de 2003).

4. EXPERIÊNCIA PROFISSIONAL OU DIDÁTICA

Professora (servidora pública federal dedicação exclusiva), do Instituto Federal de Ciência e Tecnologia da Bahia, curso Ténico em Alimentos.

Disciplinas:

Química Orgânica - fevereiro a julho de 2008

Estatística Básica - agosto a dezembro de 2008

Bioquímica de Alimentos – fevereiro de 2009 até o presente

Análise de Alimentos - fevereiro de 2009 até o presente

Introdução à Tecnologia de Alimentos - fevereiro de 2008 até o presente

Métodos de Conservação de Alimentos - fevereiro a agosto de 2009

Atividades administrativas:

Coordenação do curso Técnico em Alimentos do IFBA-Campus Porto Seguro - agosto de 2008 a abril de 2009

Coordenação de Estágios do IFBA-Campus Porto Seguro - maio de 2009 até o presente

5. ARTIGOS COMPLETOS PUBLICADOS

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8. PARTICIPAÇÃO EM BANCAS E COMISSÃO JULGADORAS

SILVEIRA, S. T., SOUZA, M. A., CARVALHO FILHO, E. V. Parcipação em banca de concurso público para professor substituto de Microbiologia de Alimentos do Instituto Federal de Ciência e Tecnologia da Bahia, julho de 2008.

SILVEIRA, SOUZA, M. A. Participação em banca de concurso público para professor efetivo de ensino básico, técnico e tecnológico, na área do conhecimento de ciência e tecnologia de alimentos do Instituto Federal de Ciência e Tecnologia da Bahia, junho de 2009.

9. SUPERVISÕES E ORIENTAÇÕES CONCLUÍDAS

PEÑA, M. G. N. Desenvolvimento de convênio entre CEFET-BA e empresas do setor produtivo de alimentos. Orientação de bolsista do Programa de Apoio ao Educando – PAE, período de junho a dezembro de 2008.

10. SUPERVISÕES E ORIENTAÇÕES EM ANDAMENTO

REIS, G. J. Desenvolvimento de um programa para treinamento dos manipuladores da merenda escolar da Escola Paulo Souto, no Município de Porto Seguro. Trabalho de Conclusão do Curso Técnico em Alimentos.