UNIVERSIDADE ESTADUAL DE MARINGÁ
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PRODUÇÃO DE ENZIMAS HIDROLÍTICAS PELO FUNGO *Myrothecium verrucaria* COM ÊNFASE EM SUA ATIVIDADE QUERATINOLÍTICA.

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Dedico esta tese a aqueles para os quais sei que esta tem a mesma importância que para mim, meu marido Marcos, meus pais Aloisio e Maria e meus irmãos Veridiana e Aloisio.

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APRESENTAÇÃO

Esta tese de doutoramento está apresentada na forma de três artigos científicos:

Moreira, F.G., Reis, D., Costa, M.A.F., Souza, C.G.M. and Peralta, R.M. (2005). Production of hydrolytic enzymes by the plant pathogenic fungus *Myrothecium verrucaria* in submerged cultures. Brazilian Journal of Microbiology, 36, 7-11

Moreira, F.G., Souza, C.G.M., Costa, M.A., Reis, S. and Peralta, R.M. Degradation of chicken feathers by *Myrothecium verrucaria*. Mycopathologia

Moreira, F.G. and Peralta, R.M. Purification and partial characterization of a keratinolytic protease from the non dermatophytic fungus *Myrothecium verrucaria.* Enzyme and Microbial Technology.

RESUMO

Os fungos são microrganismos heterotróficos que utilizam moléculas orgânicas como fonte de carbono. Em relação ao nutriente nitrogênio, os fungos podem utilizar tanto fontes orgânicas quanto inorgânicas. Sob condições naturais, os fungos necessitam secretar enzimas extracelulares capazes de degradar as moléculas orgânicas complexas. A maioria das enzimas extracelulares dos microrganismos são depolimerases, principalmente hidrolases, que atuam em polissacarídeos, proteínas, lipídeos e ácidos nucléicos. Proteases extracelulares são amplamente encontradas entre as enzimas secretadas pelos microrganismos. Estas enzimas são facilmente detectadas e são freqüentemente sintetizadas em altos títulos, sendo objeto de muitas investigações. As numerosas funções e a variabilidade de condições nas quais as proteases são sintetizadas explicam a multiplicidade destas enzimas. O papel das enzimas proteolíticas extracelulares de fungos não está restrito à sua participação na nutrição destes microrganismos. Enzimas proteolíticas secretadas pelos fungos podem ter papel em sua patogenicidade, provocando severas doenças.

O termo protease tem como sinônimos os termos peptidase, enzima proteolítica, proteinase e peptídeo hidrolase. As proteases incluem todas as enzimas que catalisam a clivagem das ligações peptídicas das proteínas, resultando na produção de peptídeos e aminoácidos livres. Algumas proteínas apresentam certas características estruturais que tornam mais difícil sua hidrólise. As queratinas são encontradas em células epiteliais de vertebrados e representam os maiores constituintes da pele e seus apêndices tais como unhas, cabelos, penas e lã. A queratina do cabelo (queratina dura) é semelhante a encontrada na epiderme (queratina macia), visto possuir estrutura em α-hélice , mas difere desta última por conter menor quantidade de cisteína. Nas penas, a queratina assume conformação β, que é mais facilmente hidrolisada quando comparada com a α-queratina. A resistência à hidrólise da queratina é conferida principalmente pelo seu alto teor em pontes dissulfeto. Entretanto, ela pode ser degradada por algumas espécies de fungos e em menor extensão por bactérias que secretam as enzimas queratinolíticas (queratinases, EC 3.4.99.11), que tem a habilidade degradar queratina nativa em peptideos menores que são subseqüentemente absorvidos pelas células.

Milhões de toneladas de penas de aves são produzidas anualmente como resíduos da criação de aves. O desenvolvimento de métodos enzimáticos e/ou microbiológicos para a hidrólise de queratina a proteínas solúveis e aminoácidos é extremamente atraente, visto que oferece uma condição barata e suave para a obtenção de produtos de alto valor. Este processo pode ser fonte de aminoácidos raros como cisteína, serina e prolina, ou pode ser usado no desenvolvimento de

fertilizantes de lenta liberação de nitrogênio, colas e biofilmes. Diversos microrganismos produtores de queratinases tem sido descritos como hábeis em degradar penas de aves e uma estratégia simples de selecionar microrganismos com potencial queratinolítica é o uso de penas de aves como substrato único nos meios de cultivos.

Myrothecium verrucaria (Albertini and Schwein) Ditmar:Fr, é um fungo fitopatogênico que ataca uma série de plantas, incluindo soja, girassol, arroz e tomate. *M. verrucaria* tem sido também investigado devido ao seu potencial uso no controle biológico de pragas e também pelo fato de ser produtor da enzima bilirrubina oxidase (BOD). Esta enzima é utilizada na determinação da bilirrubina, um produto do catabolismo de heme-proteínas. Outras enzimas envolvidas na degradação de polissacarídeos de parede celular e lignina também tem sido objeto de estudos. *M. verrucaria* é conhecido produtor de endoquitinases, xilanases, pectinases e lacases.

A produção de proteases extracelulares por fungos fitopatogênicos tem sido bem documentada. Tem sido proposto que em algumas interações planta-fungo, essas enzimas tem função patogênica, mas nenhum estudo prévio havia sido realizado em *M. verrucaria.* O objetivo do primeiro trabalho foi, portanto, avaliar as enzimas produzidas por *M. verrucaria* em culturas submersas potencialmente envolvidas em sua fitopatogenicidade utilizando diversos substratos. O fungo produziu diferentes depolimerases e glicosidases, sendo xilanase, pectinase e protease as mais importantes. Atividade lípase foi encontrada nos cultivos em óleo de oliva, enquanto atividade protease foi detectada em todas as culturas. Xilanase e pectinase foram otimamente ativas em pH 4,5-5,5, enquanto protease foi ativa em ampla faixa de pH variando de 3,5 a 11,0. As três enzimas tiveram atividade máxima a 40º C e foram estáveis por várias horas quando armazenadas em temperaturas inferiores a 50º C.

No segundo trabalho, descrevemos a capacidade de *M. verrucaria* crescer em culturas submersas e em estado sólido utilizando penas de aves como únicas fontes de carbono e nitrogênio e eficientemente degradar queratina de penas de aves. Nos dois tipos de culturas, máxima atividade proteolítica foi obtida após quatro dias de cultivo (130 e 250 U/ml, para culturas submersas e em estado sólido, respectivamente). Foi observado que o crescimento *M. verrucaria* em culturas submersas usando penas de aves intactas, resultou em quase completa degradação das mesmas após 48 h. Os filtrados das culturas hidrolisou material queratinoso na seguinte ordem: queratina de penas de aves > queratina de lã de carneiro > queratina de cabelo humano > queratina de unha humana. A melhor condição para os filtrados de cultura degradarem queratina de penas foi pH 9,0 e 40o C. Atividade queratinolítica foi fortemente inibida por PMSF e EDTA, sugerindo a presença de metalo-serina-protease. Análise por SDS-PAGE mostrou que protease foi a principal e menor proteína secretada pelo fungo sob as condições de cultura usada neste trabalho. Os resultados

obtidos neste estudo permitiram concluir que a conversão microbiana de penas de aves por *M. verrucaria* representa uma alternativa atraente para o uso de penas como fonte de proteínas e para reduzir o impacto ambiente do acúmulo de resíduos de penas de aves após o processamento de aves para o consumo humano.

No terceiro estudo, uma protease queratinolítica de *Myrothecium verrucaria* foi purificada até aparente homogeneidade eletroforética usando uma única etapa cromatográfica com um rendimento de 66.7% A enzima é uma proteína monomérica com massa molecular de 22 e 23 kDa determinada por SDS-PAGE e gel filtração, respectivamente. A enzima mostrou-se estável em ampla faixa de pH (5,0-12,0) e a temperaturas de até 45º C. Ela foi severamente inibida por PMSF e parcialmente inibida por EDTA, o que sugere ser uma metalo-serina-protease. A enzima purificada foi capaz de hidrolisar queratina de farinha de penas com a produção de moléculas com grupos amino livres. A eficiência da hidrólise foi aumentada quando a queratina foi previamente tratada com agentes redutores.

Entre os fungos, a maior parte dos estudos de produção, isolamento, purificação e caracterização de queratinases tem sido realizados com fungos dermatofíticos, embora algumas queratinases de fungos não dermatofíticos tenham sido descritas. Os dados apresentados neste trabalho podem ser úteis na compreensão dos mecanismos envolvidos na degradação de queratina

Palavras-chaves: protease, queratinase, *Myrothecium verrucaria*, hidrolases

ABSTRACT

It is well know that fungi are heterotrophic microorganisms using organic substances as a source of carbon. With regard to nitrogen nutrition, fungi can use both inorganic and organic sources. Under natural conditions fungi have to secrete extra cellular enzymes capable of degrading complex natural organic substances. Most of the extracellular enzymes of micro organisms are depolymerises, mostly hydrolases, which act on polysaccharides, proteins, lipids, and nucleic acids. Extracellular proteases, are, apparently, widely found among enzymes secreted by microorganisms. These enzymes can be easily detected, are often synthesized with high yields and have become an object of thorough investigation. Their numerous functions and the variety of conditions in which proteases are synthesized and work explain the multiplicity of these enzymes. The role of extracellular proteolytic enzymes of fungi is not restricted to their participation in nutrition of these micro organisms. Proteolytic enzymes secreted by the fungi can also play a role of factors of pathogenicity, provoking serious diseases.

The term protease is synonymous with peptidase, proteolytic enzyme, proteinase and peptide hydrolase. The proteases include all enzymes that catalyse the cleavage of the peptide bonds of proteins, digesting these proteins into peptides or free amino acids. However, some proteins have certain structural characteristics that make their hydrolysis more difficult. The structural protein keratin is resistant to the activity of a broad range of proteases, mainly due to its high content of disulfide bridges. Keratins are the most abundant proteins in epithelial cells of vertebrates and are the major constituents of skin and its appendages such as nail, hair, feather, and wool. The keratin chain of hair (hard keratin) is similar to that of epidermis (soft keratin), since it is tightly packed in an α-helix, but differs from the latter in that it contains a several fold higher amount of cystein. However, in feathers, the polypeptide assumes a β-conformation, which is more readily hydrolyzed compared with α-keratin. Keratin can be degraded by some species of fungi and to a less extend in bacteria, that secrete keratinolytic enzymes (keratinases, EC 3.4.99.11). These enzymes have the ability to degrade native keratin into smaller peptides entities that can subsequently be absorbed by cells.

Worldwide poultry processing plants produce millions of tons of feathers as a waste product annually. The development of enzymatic and/or microbiological methods for the hydrolysis of feather into soluble proteins and amino acids is extremely attractive, as it offers a cheap and easy method for the production of valuable products. It could be used to produce rare amino acids like serine, cystein and proline, or for the development of slow-release nitrogen fertilizers, glues, and biodegradable films. Keratinase-producing microorganisms have been described as able to degrade

feathers, and one strategy for selecting potential new keratinolytic micro organism is the use of poultry feathers as the only substrate in the cultures.

Myrothecium verrucaria (Albertini and Schwein) Ditmar:Fr, is a ubiquitous phytopathogenic fungus which attacks a wide range of plant crops, including soybean, sunflower, tomato, and rice. *M. verrucaria* has also been investigated due to its potential use in biological weed control and due to its capability to produce the enzyme bilirubin oxidase (BOD). This enzyme is used for the enzymatic determination of bilirubin, a catabolic product of heme proteins. Other enzymes involved in degradation of cell wall polysaccharides and lignin has also been studied, including endochitinases, xylanases, pectinases and laccases.

The production of extracellular proteases by plant pathogenic fungi is well documented, and it has been proposed that in some fungus-plant interactions these enzymes may function as pathogenic factors, but until now, no work has been done on *M. verrucaria.* Considering this, the aim of the first study was to get an overview of the enzymes produced by *M. verrucaria* in submerged cultures that are potentially involved in its phytopathogenicity. The capability of *M. verrucaria* to produce extra cellular hydrolytic enzymes in submerged cultures was studied using several isolated substrates. The fungus was able to produce different depolymerases and glycosidases, with xylanase, pectinase and protease being the most important. Xylanase and pectinase were optimally active at pH 4.5-5.5, while protease was active in a large range of pH 3.5 to 11.0. All three enzymes were maximally active at 40^{o} C and were stable for several hours at temperature up to 50^{o} C.

In the second study, the capability of *M. verrucaria* to grown in submerged (SC) and solid state (SSC) cultures using chicken feathers as the only source of carbon and nitrogen and its ability to efficiently degrade chicken feather keratin is reported. In both types of cultures, maximal protease production was obtained after 4 days of cultivation (130 and 250 U/ml in SC and SSC, respectively). It was observed that the growth of *M. verrucaria* in submerged culture using intact feathers resulted in almost complete degradation of intact feathers within 48 h. The culture filtrates hydrolyzed keratinous substrates in the following order: chicken feather keratin > sheep wool keratin > human nail keratin > human hair keratin. The best conditions for culture filtrates to efficiently degraded feather keratin was pH 9.0 and 40^{o} C. Keratinolytic activity was strongly inhibited by PMSF and EDTA, indicating the presence of serine and metallo protease. SDS-PAGE analysis showed that protease was the main and smaller protein secreted by the fungus under the culture conditions used in this study. The results obtained in this study permit the conclusion that chicken feather microbial conversion by *M. verrucaria* represents an attractive alternative for improving the use of feather as a feed protein and to reduce the impact of poultry feather accumulation as a waste product after the processing of chickens for human consumption.

In the third study, a *Myrothecium verrucaria* keratinolytic protease was purified to apparent electrophoretic homogeneity using one unique chromatographic step with a high yield of 66.7%. The enzyme was a monomeric protein with molecular mass of 22 and 23 kDa by SDS-PAGE and gel filtration, respectively. The enzyme was stable in a broad range of pH (5.0-12.0) and temperature up to 45^{o} C, being optimally active at 40^{o}C and pH 8.0-9.0. It was strongly inhibited by PMSF and partially inhibited by EDTA, which suggests to be a metallo serine protease. The purified enzyme was able to hydrolyze chicken feather meal producing free amino groups. The efficiency of hydrolysis was improved when the keratin was previously treated with reducing agents.

Among fungi, the most part of studies on production, isolation, purification and characterization of keratinase have been carried out with dermatophytic fungi, although some non dermatophytic fungi keratinase has been already described. The data presented in this work may be useful in understanding the mechanism of keratin degradation.

Key-words: protease, keratinase, *Myrothecium verrucaria*, hydrolases

ARTIGO 1

PRODUCTION OF HYDROLYTIC ENZYMES BY THE PLANT PATHOGENIC FUNGUS *Myrothecium verrucaria* IN SUBMERGED CULTURES.

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ABSTRACT

The capability of the plant pathogenic fungus *Myrothecium verrucaria* to produce extracellular hydrolytic enzymes in submerged cultures was studied using several substrates. The fungus was able to produce different depolymerases and glycosidases, being xylanase, pectinase and protease the most important of them. Lipase was found in cultures developed in the presence of olive oil, while protease activity was detected in all cultures. Xylanase and pectinase were optimally active at pH 4.5-5.5, while protease was active in a large range of pH 3.5 to 11.0. All three enzymes were maximally active at 40° C and they were stable for several hours at temperature up to 50° C.

Key words: carbohydrases, hydrolytic enzymes, *Myrothecium verrucaria*, protease.

RESUMO

A capacidade do fungo fitopatogênico *Myrothecium verrucaria* produzir enzimas hidrolíticas extracelulares em culturas submersas foi estudada utilizando diversos substratos. O fungo foi capaz de produzir diferentes depolimerases e glicosidases, sendo xilanases, pectinases e proteases as mais importantes. Atividade lipase foi encontrada nos filtrados das culturas desenvolvidas na presença de óleo de oliva, enquanto atividade proteolítica foi detectada em todas as culturas. Xilanase e pectinase foram otimamente ativas em pH 4,5 a 5,5, enquanto protease foi ativa em ampla faixa de pH (3.5 a 11.0). As três enzimas foram otimamente ativas 40° C e estáveis por várias horas a temperaturas até 50° C.

Palavras-chave: carboidrases, enzimas hidrolíticas, *Myrothecium verrucaria*, protease.

INTRODUCTION

Plant pathogens produce a range of enzymes capable of degrading plant cell wall components (1,23). Among the economically important plant pathogens, *Myrothecium verrucaria* (Albertini and Schwein) Ditmar:Fr, is a ubiquitous phytopathogenic fungus which attacks a wide range of plants, including cucumber (20), soybean (16), upland cotton (11), sunflower (3), birdsfoot trefoil (6), tomato (25), rice (24) and corn (27).

Extracellular proteins secreted by fungus are able to macerate tissues and degrade cell wall components. They must thus contain the enzymes corresponding to the types of glycosidic linkages present in the cell wall polysaccharides. Extracellular enzymes are important to fungi not only for digestion but also in many instances for the pathogenic process: the enzymes may function in overcoming the natural resistance of the host as well as in providing soluble products that can be absorbed and used as food (9). *M. verrucaria* is known to produce endochitinase (8,26), xylanases (7), pectinases (5) and bilirrubin oxidases (10). The production of extracellular proteases by plant pathogenic fungi is also well documented, and it has been proposed that in some fungus-plant interactions these enzymes may function as pathogenic factors (4,17,19,20,23).

Studies of enzyme production by a phytopathogenic fungus are complicated by the presence of plant, particularly by the presence of plant enzymes and microbial enzyme inhibitors that occur in the plants. The most practical way to study the production of enzyme by a fungus is therefore to study the production of its enzymes on artificial growth media that contain no plant or enzyme inhibitors produced by the plant. Considering that, the aim of this study was to get an overview of the enzymes that are produced by *M. verrucaria* in submerged cultures possibly involved with its phytopathogenicity.

MATERIAL AND METHODS

Microorganism *Myrothecium verrucaria* (Albertini and Schwein) Ditmar:Fr CCT 1886 was obtained from the Collection culture of Fundação André Tosello, Campinas, SP, Brazil and it was maintained on potato dextrose agar.

Enzyme production. For enzyme production in submerged cultures, 1 x 10^9 spores were transferred to 50 mL of mineral media (15) containing 0.5% (w/v) of one carbon source, and pH adjusted to 6.0. The cultures were incubated at 28^o C on a rotary shaker at 120 rpm. After 7 days, the mycelia were removed from the culture media by filtration. To determine the dry weight of the

mycelia, they were dried overnight at 60° C. To study the effect of association of different substrates in the production of enzymes, each substrate or mixture of substrates were added to a final concentration of 1% (w/v).

Enzyme determinations. Glycoside hydrolase activities (β-glucosidase and β-xylosidase) were determined by measuring the rate of p-nitrophenol released from the appropriate p-nitrophenyl derivatives (p-nitrophenyl-β-glucopiranoside and p-nitrophenyl-β-xylopyranoside, respectively). The standard reaction mixture contained 20 μl of enzyme solution and 1 mg/mL of substrate in phosphate buffer 0.1 M, pH 6.0. After 15 min of incubation at 50° C, reactions were stopped by the addition of 2 ml of 0.1 M Na_2CO_3 and the p-nitrophenol liberated was determined spectrophotometrically at 410 nm (22). Polysaccharidase activities (as xylanase, pectinase and carboxymethylcellulase, CMCase) were determined by measuring the amount of reducing sugar (xylose, galacturonic acid and glucose) released from various substrates (xylan, polygalacturonic acid and carboxymethylcellulose, respectively). A 20 μl volume of enzyme solution was incubated at 50° C for 30 min. in 1.0 ml of substrate solution polysaccharide (2 mg/ml) dissolved in phosphate buffer, 0.1 M, pH 6.0. Reactions were stopped by the addition of 1 ml of dinitrosalicylic reagent (14). Tubes were placed in a boiled water bath for 5 min. The $A_{540\ nm}$ was read with appropriate single sugars as standards (glucose, xylose and galacturonic acid, respectively to carboxymethylcellulase, xylanase and polygalacturonase activities). Lipase was determined using an olive oil emulsion as substrate. The liberated free fatty acids were titrated with 0.05 M NaOH and phenolphtalein as indicator (18). Protease was determined using casein as substrate. The released tyrosine was estimated by Lowry's method (12).

Effect of pH on the activity of xylanase, polygalacturonase and protease. The effects of pH on the activity of enzymes were determined in a series of McIlvaine's buffers with pH values from 3.5 to 8.3 (13), and 0.1 M glycine-NaOH (pH 8.5 to 10.0).

Effect of temperature on enzyme activity and stability. The effects of temperature on the activity of enzymes were carried out at temperature ranging from 25 to 70° C. Thermal stability was investigated by incubating the enzyme at 30, 40, 50 and 60° C for 1 hour. Immediately afterwards the enzymes were immersed in an ice bath and then the activities were tested under standard conditions.

Experimental design and statistical analysis. To study the effect of each carbon source in the enzyme production, the experimental outline used was a factorial experiment based on 1 x 13 block design (one culture time *versus* 13 different carbon sources) with three repetitions for each one. To study the effect of association of carbon sources in the enzyme production, it was used a 1 x 10 block design (one culture time *versus* 10 different carbon source combination) with three repetitions for each one. The analysis was done using the statistical pack program GraphPad Prism® (3.0). Tukey test ($p<0.05$) was applied for comparison of the averages.

Chemicals. The enzymatic substrates and carbon sources were obtained from Sigma Chemical Corp. (St. Louis, Mo). All other reagents were of analytical grade.

RESULTS

Enzymes capable of degrading a wide range of glucosides and polysaccharides were detected in cell-free culture supernatants (Table 1). Growth of *M. verrucaria* on various polysaccharides used as the sole carbon source demonstrated that the fungus secretes enzymes that convert cellulosic, pectinolytic and hemicellulolytic substrates to simple sugars. Glucose and xylose grown cultures did not exhibit polysaccharidase activities, suggesting the presence of simple sugars repressed the production of such enzymes. Among polysaccharide depolimerases, xylanase and polygalaturonase seemed to be the major enzymes secreted by the fungus.

Lipase was barely produced by the fungus when carbohydrates were offered as carbon source. However, its production was increased ten times when the culture medium was enriched with olive oil at 1% (Table 1). Protease was produced in all cultures and its production was not apparently affected by the carbon source ($P>0.05$)

Figure 1 shows the production of xylanase, pectinase and protease when mixtures of different polymers were used as carbon source. An association of pectin and other carbon source (starch, xylan or casein) increased the production of pectinase from 3.2 U/ml (cultures using orange pectin as the only carbon source) to 8.1, 7.6, 9.9 and 10.1 U/ml when starch, casein, xylan and xylan plus casein were added in the pectin cultures, respectively. In fact, the highest levels of three enzymes were obtained in the cultures where wheat bran, a rich substrate containing several carbohydrates and proteins was the carbon source (12.9, 78.3 and 150 U/ml to pectinase, xylanase and protease, respectively).

Some properties of the main extracellular enzymes (xylanase, pectinase and protease) from *M. verrucaria* were studied. Xylanase and pectinase were more actives in an acidic of pH (4.5-5.5),

while the best pH for protease activity was between pH 8.0 and 9.0 (Fig 2). Figure 2 suggest the existence of more than one group of protease, one with optimum pH between 6.0-7.0, and other with optimum pH between 8.0-9.0. All enzymes were optimally active at 40° C and they retained more than 95% of initial activity after 60 min at 50° C (data not shown).

DISCUSSION

In the present work we have shown that *M. verrucaria* produces polysaccharide depolymerases and glucosidases necessary to degrade important structural cell wall polysaccharides, particularly pectin and hemicellulose (Table 1). The secretion of several enzymes provides this phytopathogenic fungus with the ability to attack hosts which differ in their polysaccharide cell wall compositions (22). The secretion of glycosidases combined with the polysaccharide depolymerases may also remove side groups of heteropolysaccharides, making easier the action of endoenzymes (21).

Our results showed the capability of *M. verrucaria* to produce other hydrolytic enzymes such as proteases and lipases. These enzymes may be involved in the capability of the fungus to invade vegetal tissues. *M. verrucaria* protease was identified as an alkaline protease (Fig. 2). However, differently from xylanase and pectinase that were active in a an acidic range of pH, protease from *M. verrucaria* showed high activity at alkaline pH, although it remained active in a large range of pH, what could indicate the presence of multiple isoenzymes, with different optimum pH.

It has been suggested that the proteases may facilitate located penetration of the plant cell wall by breaking down the fibrous glycoproteins that contribute to cell wall stability (2). Some phytopathogenic fungi such as *Fusarium*, *Alternaria,* and *Rhizoctonia* produced serine alkaline proteases, which are indispensable for their growth (9,19). They are probably nutrient-mobilizing enzymes whose primary function is the support of fungal growth after host cell death has occurred.

The highest levels of enzymic activities have been obtained when wheat bran was the main substrate (Fig. 1). Wheat bran is a very rich substrate consisting of a mixture of proteins, fat, soluble and insoluble carbohydrates. It is probable that the several hydrolytic enzymes secreted by the fungus present a synergy in the degradation of wheat bran components.

In conclusion, in the present study, it was observed the capability of *M. verrucaria* to produce and secrete different hydrolytic enzymes. Studies of production of enzymes using several plant materials is in progress in our laboratory. This study will serve to increase the understanding of factors that control the production, activity, and the role of *M. verrucaria* hydrolytic enzymes in its phyto-pathogenicity.

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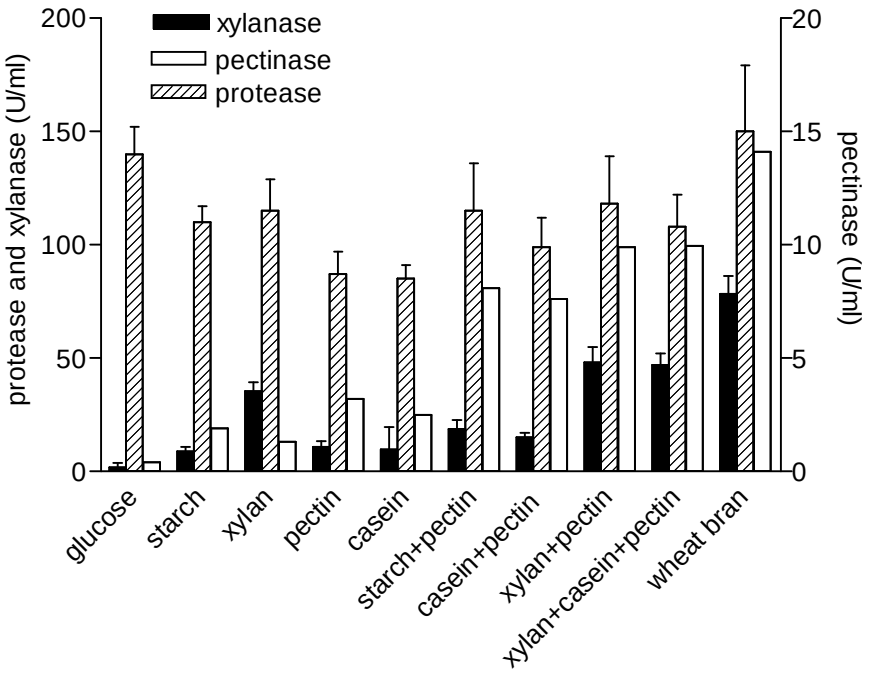

Figure 1. Effect of different mixtures of carbon source in the production of hydrolytic enzymes by *Myrothecium verrucaria*. The carbon source or mixture of carbon source was added to a final concentration of 0.5% (w/v). When more than one carbon source was used, they were used at the same concentration. The cultures were developed at 120 rpm and 28º C for 7 days. The results represent the media ± SD of three independent experiments.

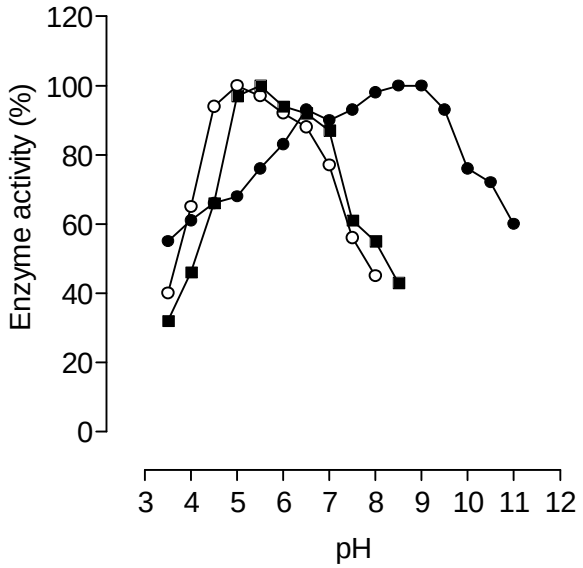

Figure 2. Effect of pH in the activity of *Myrothecium. verrucaria* enzymes. Pectinase (○), xylanase (■) and protease (●) activities. The pH curves were done using the substrate for each enzyme in McIlvaine's buffer (pH 3.5 to 8.3) and 0.1 M glycine buffer (pH 8.5 to 10.0).

Table 1. Production of enzymes by *M. verrucaria* in submerged cultures

Carbon source (0.5%)	Biomass (mg)	β-glu (U/mL)	β-xyl (U/mL)	Xylanase (U/mL)	Pectinase (**) (U/mL)	CMCase (U/mL)	Protease U/mL)	Lipase (U/mL)
Xylose	58±7	4.2±1.3	0.9±0.1	1.4±0.5	Nd	Nd	45.9±4.6	0.11±0.05
Glucose	137±15	8.4±2.4	1.9±0.5	1.9±0.3	Nd	Nd	81.4±11.5	1.23±0.08
Maltose	83±9	9.1±2.6	1.5±0.2	6.1±0.3	1.3±0.2	Nd	58.1±6.8	0.83±0.07
Lactose	125±14	7.8±1.0	1.3±0.3	14.7±1.0	Nd	Nd	89.0±9.0	0.52±0.05
Cellobiose	77±9	8.3±3.8	3.7±0.5	7.9±0.6	1.5±0.6	0.3±0.1	56.8±6.0	0.37±0.04
Sucrose	55±9	5.1±1.1	0.9±0.2	5.4±0.7	1.2±0.4	Nd	35.3±4.4	0.35±0.03
Xylan	56±8	18.7±0.8 (*)	15.8±0.6 (*)	26.8±3.5 (*)	2.7±0.9	0.2±0.1	33.7±3.2	0.50±0.05
CMcellulose	21±3	3.2±0.3	0.1±0.04	1.7±0.2	0.4±0.04	0.2±0.04	10.1±0.9	0.07±0.01
Orange pectin	79±9	18.0±2.2 (*)	15.1±1.1 (*)	7.7±2.2	7.7±0.3 (*)	0.3±0.2	60.2±4.6	0.36±0.05
Starch	85±10	7.8±3.2	3.1±0.3	7.6±1.7	3.6±0.5	Nd	58.4±5.4	0.48±0.05
Casein	57±7	5.2±2.2	1.3±0.2	6.9±1.6	2.4±0.4	Nd	33.4±3.4	0.23±0.02
Ovoalbumin	66±8	4.0±1.8	4.1±0.7	10.4±4.0	1.3±0.1	Nd	43.4±3.7	0.30±0.04
Olive oil	139±19	6.6±2.5	5.0±0.8	14.2±1.9	3.3±0.8	0.5±0.3	69.5±5.8	7.42±0.4 (*)

The cultures were developed at 120 rpm and 28^{o} C for 7 days. The results represent the media±SD of three independent experiments. ND=not detectable activity; (*) significantly different among classes, $p<0.05$ (Tukey's test) ; (**) as polygalacturonase activity.

ARTIGO 2

DEGRADATION OF CHICKEN FEATHERS BY *Myrothecium verrucaria*

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Abstract

Aims: Protease production by *Myrothecium verrucaria* in submerged (SC) and solid state cultures (SSC) using chicken feather meal, a poultry-processing industry waste.

Methods and Results: In both types of cultures, maximal protease production was obtained after 4 days of cultivation (130 and 250 U/ml in SC and SSC, respectively). It was observed that the growth of *M. verrucaria* in submerged culture using intact feathers resulted in almost complete degradation of intact feathers within 48 h. The culture filtrates have hydrolyzed keratinous substrates in the following order: chicken feather keratin > sheep wool keratin > human nail keratin > human hair keratin. The best condition for efficient degradation of feather keratin by *M. verrucaria* culture filtrate was pH 9.0 and 40^{o} C. Keratinolytic activity was strongly inhibited by PMSF and EDTA, indicating the presence of serine and metalo protease. SDS-PAGE analysis has shown that protease was the main and smaller protein secreted by the fungus under the culture conditions used in this work.

Conclusion: The filamentous fungus *Myrothecium verrucaria* was characterized for the first time as a producer of a keratinase with ability to completely hydrolyze chicken feathers with industrial purpose.

Significance and impact of the study: Chicken feather microbial conversion by *M. verrucaria* represents an attractive alternative for improving the use of feather as a feed protein and to reduce the impact of poultry feather accumulation as a waste, after processing the chickens for human consumption.

Key words: chicken feather, poultry waste, protease, *Myrothecium verrucaria*, keratinase

Introduction

Feathers are poultry by-product rich in protein, mainly β-keratin, generated in very large amounts as a waste product from the poultry-processing industry (Onifade *et al*. 1998). Industrially, a great part of the feather waste is cooked under high pressure and temperature, producing a feather meal that can be incorporated into poultry food-stuff as a protein supplement (Bhargava and O'Neil 1975, Papadopoulos *et al.* 1986). However, due to the high degree of cross-linking by disulphide bond, hydrogen bonding and hydrophobic interactions, keratin is insoluble and shows high mechanical stability and resistance to proteolysis. Indeed, keratin is poorly digested by common digestive enzymes, such as trypsin and pepsin (Baker *et al*. 1981).

Development of enzymatic and/or microbiological methods for the hydrolysis of feather releasing soluble proteins and amino acids is extremely attractive, because it offers a cheap and mild reaction condition for the production of valuable products. It could be a source of rare amino acids like serine, cystein and proline, or could be used to the development of slow-release nitrogen fertilizers, glues, and biodegradable films (Dalev and Neitchev 1991; Choi and Nelson 1996). Environmental considerations make attractive the use of keratinolytic enzymes in the production of amino acids and peptides. Keratinase-producing microorganisms have been described as able to degrade feathers, and one strategy to select potential new keratinolytic microorganism is the use of poultry feathers as the only substrate in the cultures (Onifade *et al*. 1998; Sharma and Rajak 2003).

Different keratinases have been isolated and characterized from saprophytic and parasitic fungi (Wawrzkiewcz *et al* 1991; Gradisar *et al* 2000; Santos *et al* 1996; Farag and Hassan 2004; Friedrich *et al.* 1999), and actinomycetes (Bockle *et al.* 1995; Letourneau *et al* 1998; Bernal *et al* 2006; De Azeredo *et al.* 2006). Some of these microorganisms are pathogenic, such as dermatophytes, making them unsuitable for large-scale applications.

The fungus *Myrothecium verrucaria* is a filamentous fungi able to produce several hydrolytic enzymes including endochitinase (Govindsamy *et al.* 1998), xylanases (Filho *et al*. 1994), and pectinases (Faheild and Murad 1992). Recently we described its capability to produce protease when cultivated in submerged cultures using several carbohydrates and proteins as substrate (Moreira *et al*, 2005). In this paper, we describe its capability to grow on chicken feather and efficiently degrade chicken feather keratin.

2. Materials and methods

2.1. Microorganism.

Myrothecium verrucaria (Albertini and Schwein) Ditmar:Fr CCT 1886 was obtained from the Collection culture of Fundação André Tosello, Campinas, SP, Brazil, and it was maintained on potato dextrose agar.

2.2. Pretreatment of the feathers.

White chicken feathers were supplied by a local industry. Freshly plucked wet feathers were washed extensively with water and detergent. Wet feathers were dried in a ventilated oven at 40° C, for 72 h. The feathers were then milled in a ball mill and passed through a small-mesh grid to remove coarse particles. In some experiments, intact feathers were used as substrate.

2.3. Culture conditions

2.3.1. Submerged cultures

One x 10^9 spores of *M. verrucaria* were transferred to 250 ml Erlenmeyer flasks containing 50 ml of mineral media (Montenecourt and Eveileigh 1977) and 1.0% (w/v) of chicken feathers as substrate. The medium was previously sterilized by autoclaving at 121° C for 15 min. The cultures were incubated at 28° C on a rotary shaker, at 120 rpm. At periodic intervals, the cultures were filtered, and the filtrates were used as a source of enzymes. Results were expressed as the mean of at least three different cultures.

2.3.2. Solid state cultures

One x 10^9 spores of *M. verrucaria* were transferred to 250 ml Erlenmeyer flasks containing 5 g of chicken feathers. Mineral medium (Montenecourt and Eveileigh, 1977) was used to adjust the moisture content to 75%. Dry weight of the substrate and moisture content were determined gravimetrically, after drying samples at 60° C. Incubation was carried out at 28° C. At periodic intervals, 25 ml of cold water was added to the cultures and the mixtures were shaken for 1 h, at 4° C. The mixtures were filtered, and the filtrates were used as a source of enzymes. Results were expressed as the mean of at least three different cultures.

2.4. Enzyme assays.

The protease activity of the culture filtrates was assayed using 1.0% (w/v) casein in 0.1 M borate buffer, pH 9.0, as substrate (Boer and Peralta, 2000). One (1.0) ml casein was incubated at 40^{o} C for 1 h with 1.0 ml of a suitable diluted culture filtrate. The reaction was stopped by the addition of 2.0 ml of 0.6 M trichloroacetic acid. After incubation of 15 min in an ice bath, the reactions were mixed on a vortex mixer, and the tubes were centrifuged at 3,000 rpm for 10 min. The tyrosine released was estimated by Lowry's method using a tyrosine standard curve. A unit of enzyme activity was defined as the amount of enzyme producing 1 µg of tyrosine per min. Keratinolytic activity was assayed by the method of Nickerson *et al.* (1963) using feather meal as substrate. A reaction mixture containing 10 mg of substrate, 1.0 ml of borate buffer (0.1 M, pH 9.0), and 1.0 ml of a suitable diluted culture filtrate was incubated in a magnetic stirrer with a stirring bar for 1 h, at 40^{o} C. The reaction was stopped by boiling and the mixture was filtered through filter paper. The production of free amino group was estimated using a reaction with the ninhydrin reagent (Starcher, 2001).

2.5. Partial crude enzyme characterization

2.5.1. Effect of temperature and pH on keratinolytic activity.

The effect of temperature on keratinolytic activity was carried out at temperature ranging from 25 to 60^{o} C. The effect of pH on the activity of the enzyme was determined in a series of McIlvaine's buffers with pH values from 5.0 to 8.3, 0.1 M tris-HCl buffer (pH 8.0-9.5) and 0.1 M borate buffer (pH 8.5-12.0)

2.5.2. Effect of protease inhibitors.

The protease inhibitors phenylmethanesulphonyl fluoride (PMSF, 1 mM), EDTA (5 mM), and pepstatin A (0.7 µg/ml) were added to the enzyme preparations and incubated 15 min at room temperature, before being tested for keratinase activity.

2.5.3. SDS-PAGE and zymogram.

SDS-PAGE was performed on 10% polyacrilamide gel (Laemmli 1970). Protein bands were visualized by silver staining. Proteolytic activity was detected *in situ* with 0.2% (wt/vol) casein as the substrate. After the electrophoresis, the gel was washed once with a mixture of equal parts of isopropanol-borate buffer (0.1 M, pH 9.0), for 30 min, and once with borate buffer to remove the SDS and fix the proteins. The enzymatic reaction was performed incubating the polyacrilamide gel with

the casein gel for 1 h at 30° C. The casein gel was stained with Coomassie brilliant blue R-250 and then bleached with 10% glacial acetic acid/45% water/45% ethanol. Protease bands appeared as clear zones on a blue background.

2.5.4. Degradation of feather meal and production of free amino acids and soluble peptides by crude enzyme.

One ml of culture filtrates conveniently diluted was added to 100 mg of native feather meal in 10 ml of 0.1 M borate buffer, pH 9.0 in a shaking water bath at 130 rpm for up to 48 h. As controls feather meal was incubated in a sterilized buffer under the same conditions, and using the same amount of culture filtrate previously boiled for 5 min. The dry weight of the remaining feather meal substrate was determined on membrane filters (pore size, 0.2 µm) after drying at 105° C for 12 h. The production of free amino acid and soluble peptides from native feather meal was measured using ninhydrin method with alanin as standard (Starcher, 2001).

2.6. Materials

Yeast extract and nutrient agar were from Difco laboratories. Ninhydrin, casein and inhibitors were purchase from Sigma Chemical Corp. (St Louis, Mo). All other reagents were of analytical grade.

3. Results

3.1. Production of protease and keratinous waste degradation in submerged and solid state cultures

Degradation of different source of keratin by *M. verrucaria* was firstly evaluated using a keratin-salt agar media containing white chicken feathers, commercial keratin, human hair or sheep wool. Fungal growth was observed either visually or determined by measuring colony diameters, at periodic intervals during the incubation period. Chicken feather was the most favorable substrate for the fungus growth, and after 30-40 days, the medium was completely colonized by mycelial fungus (data not shown). Similar results were obtained using sheep wool keratin, but hair keratin and nail keratin did not support fungal growth.

In both, submerged and solid state cultures, *M. verrucaria* was able to grow in a mineral medium containing autoclaved chicken feather as the sole carbon and nitrogen sources. Proteolytic activity was maximally produced after 4 days of cultivation (130 and 250 U/ml, respectively to

submerged and solid state cultures (FIG 1A-1B). The alcalinization of the culture media was observed together with an increase in the protease level.

Intact chicken feathers were introduced into 250 ml Erelenmeyer flasks containing 50 ml of mineral medium and inoculated with *M. verrucaria.* It was observed that the growth of *M. verrucaria* on feathers resulted in almost complete degradation of the feather within 48 h. Barbs and barbules were completely hydrolyzed while the rachis was partially degraded (Fig. 2).

3.2. Characterization of crude *M. verrucaria* keratinase

The keratinolytic activity obtained from submerged and solid state cultures was detectable between 25 and 55^{o} C, showing a maximum at 40^{o} C (Figure 3A). Concerning stability, the proteases present in the culture broth remained fully active at 5^{o} C for several weeks and more than 24 h when stored at room temperature (25-28^{o} C).

The keratinolytic activity of the culture filtrate from both types of cultivation was detectable over a wide range of pH values, with an optimum between 9 and 10 (Figure 3B).

At pH 9.0 and 40^{o} C, the enzyme was able to efficiently hydrolyze keratin from chicken feather and from sheep wool while human nail and hair keratins were poorly hydrolyzed (Fig 4). The action of keratinase in chicken feather meal reduced its weight to 40% after 48 h (Fig 5A), while the amount of free amino groups (free aminoacids and peptides) increased in the same period (Fig 5B).

The levels of inhibition on keratinolytic activity by treatment with some proteolytic inhibitors are shown in Figure 6. The keratinolytic activities were inhibited by PMSF and EDTA, indicating the presence of serine and metal requiring proteases in both types of cultures.

Figure 7 shows the electrophoretic patterns of culture filtrates obtained from submerged and solid state conditions. A few different proteins were secreted by the fungus under the conditions used in this study. In both types of cultures, zymogram analysis using casein as substrate identified one protein with protease activity.

4. Discussion

Keratinases which are able to degrade keratins are mostly isolated from pathogenic dermatophytes or bacteria (Gradisar *et al*., 2005, Nickerson *et al.* 1963, Lin *et al.* 1992). The ability of nondermatophytic fungi to degrade native feathers which are composed of 95% protein seems to be very rare. Some examples of this group include *Chrysosporium keratinophilum* (Dozie *et al*. 1994), *Aspergillus oryzae* (Farag and Hassan, 2004), *Doratmyces microsporus* (Gradisar *et al.* 2000),

Scopulariopsis brevicaulis (Malviya *et al*., 1992), and *Aspergillus fumigatus* (Santos *et al.* 1996). This paper describes for the first time the production of keratinase and chicken feather degradation in submerged and solid state cultures by the nondermatophytic fungus *Myrothecium verrucaria,* using chicken feathers as the only carbon and nitrogen sources (Fig 1 and 2). Similar proteic profiles were obtained in the two types of cultivations, and protease activity was related with the main and smaller protein secreted by the fungus under the conditions used in this work (Fig 7).

M. verrucaria has been investigated due to its potential use in biological weed control (Abbas *et al*., 2002). The most studied enzyme produced by this fungus is bilirubin oxidase (BOD). This enzyme is used for the enzymatic determination of bilirubin, a catabolic product of heme proteins formed in the reticuloendothelial system (Kurosaka *et al.* 1998). Other enzymes involved in the degradation of cell wall polysaccharides and lignin has also been studied (Sulistyaningdyah *et al.* 2004). A recent study has described the capability of *M. verrucaria* to produce an alkaline protease in submerged cultures with different carbon sources, including glucose (Moreira *et al*. 2005). The results described in this paper have showed the production of a protease able to hydrolyze keratinous substrates in the following order: chicken feather keratin > sheep wool keratin > human nail keratin > human hair keratin. (Fig 4). The best condition for culture filtrates efficiently degraded feather keratin was pH 9.0 and 40^{o} C (Fig 3 and 5). Considering that the keratinolytic activity was strongly inhibited by PMSF and EDTA (Fig 6), would be reasonable to classify the enzyme as a serine-metalo proteases To support this classification, is the finding in the literature that the activity of keratin degrading protease is normally associated with serine protease, with the exception of keratinases from yeasts, which belong to the aspartic proteases (Monod *et al.* 2002).

The complete mechanism of keratin degradation is not fully understood. Basically, microbial keratinolysis is a proteolytic, protein-degrading process since the substrate (keratin) is essentially a protein. However, not all serine type protease are able to degrade keratin (Eggen *et al.* 1990). The high mechanical stability and resistance to proteolytic degradation of keratins is due to mainly tight packing of the protein chains through intensive interlinkage by cystine bridges (Böckle and Müller 1997). Considering that, it has been proposed that for filamentous fungi, the keratin degradation is result of a combination of mechanical keratinolysis (mycelial pressure and/or penetration of the keratinous substrate), sulphitolysis (reduction of disulphide bonds by sulphite excreted by mycelia), and proteolysis (Onifade *et al.* 1998).

Several investigators agreed that microbial conversion of feather represents a biotechnological process to improve the use of feather as a feed protein. Biodegradation of feather can be achieved by cultivation of keratin-degrading microorganism on feather, and subsequent elaboration of extracellular keratinase, or by the use of culture filtrates containing the keratinase or

crude enzyme without the microorganism. The data presented in this paper showed that *M. verrucaria* is an attractive option for both strategies.

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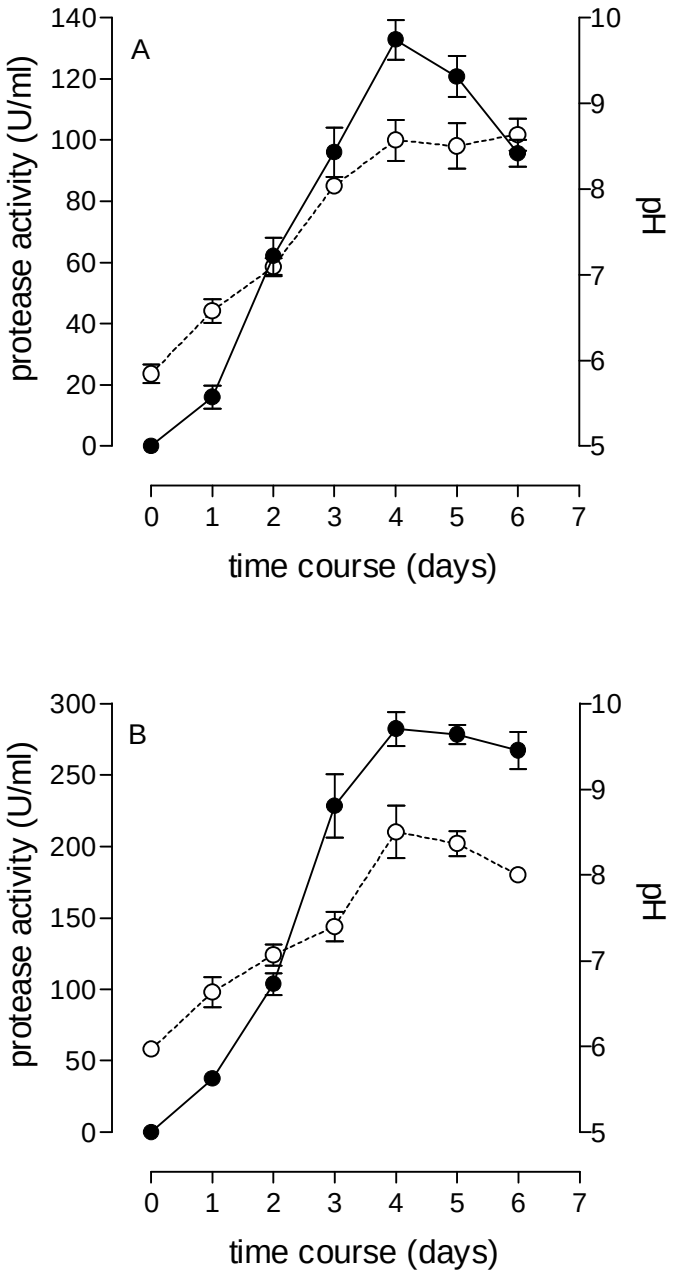

Figure 1. Time course of protease production by *M. verrucaria in* submerged (A) and solid state cultures (B). The submerged cultures were developed at 120 rpm and 28º C for up 6 days in mineral medium containing chicken feather (1.0% w/v) as the only carbon and nitrogen sources. In B: solid state cultures. Protease activity was determined in the culture filtrates using casein as substrate. The results represent the media ± SD of three independent experiments. (●) protease activity; (○) pH

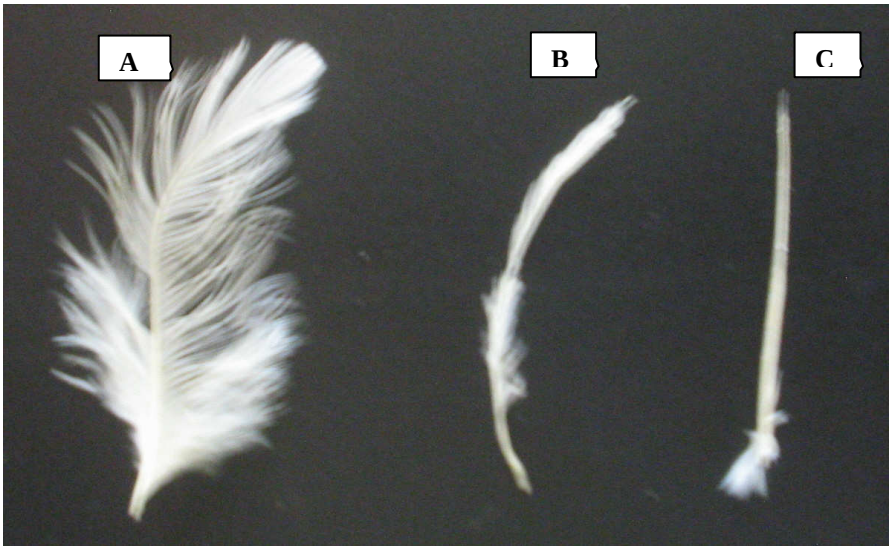

Figure 2. Degradation of chicken feathers by *M. verrucaria* in submerged culture after 0 (A), 48 (B) and 72 h (C) of cultivation.

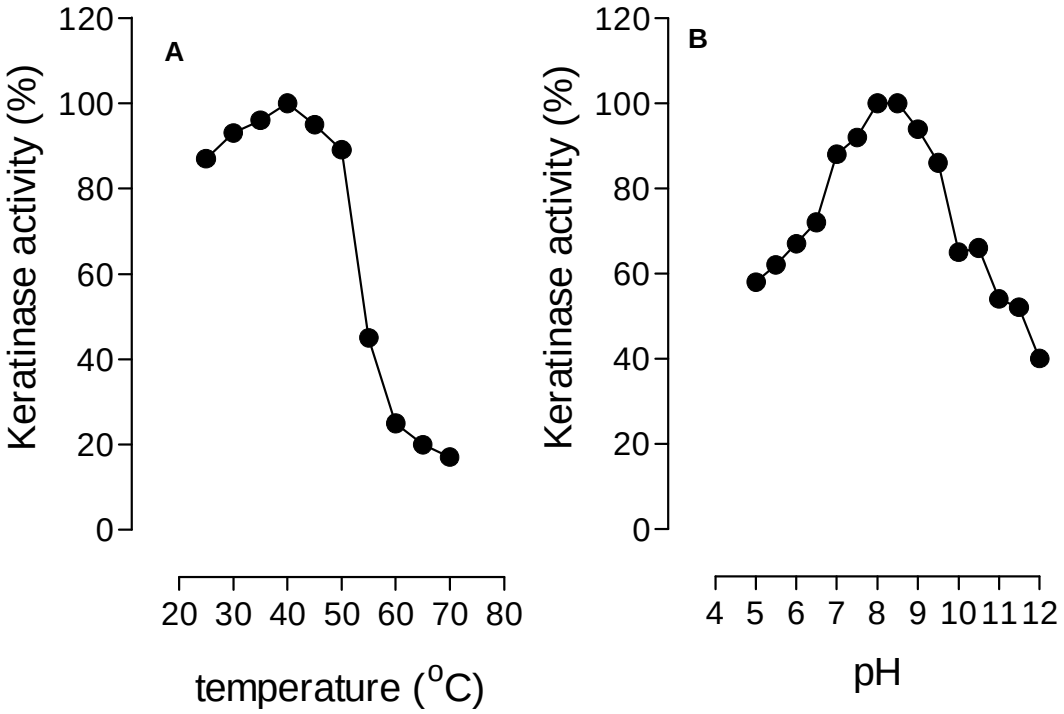

Figure 3. Effect of temperature (A) and pH (B) on crude *M. verrucaria* keratinolytic activity.

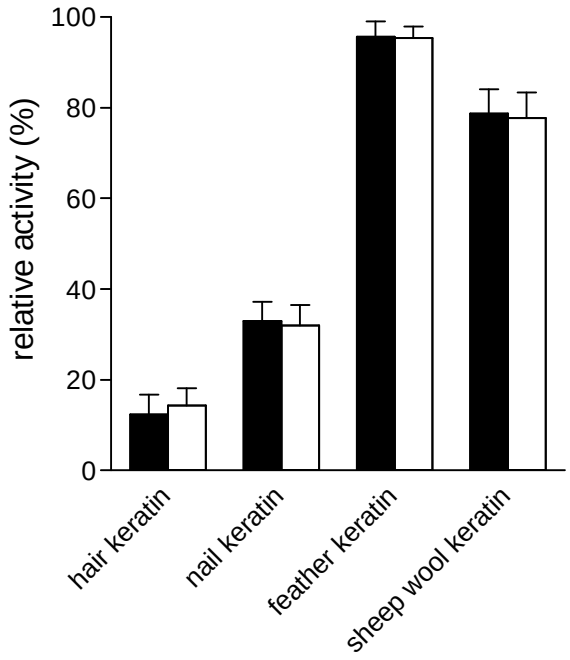

Figure 4. Hydrolysis of several different keratines by crude *M. verrucaria* protease. The keratinase activity was assayed as described in the material and methods section (item 2.4) using the different keratin source. Full bars: crude enzyme from submerged cultures; Clear bars: crude enzyme from solid state cultures.

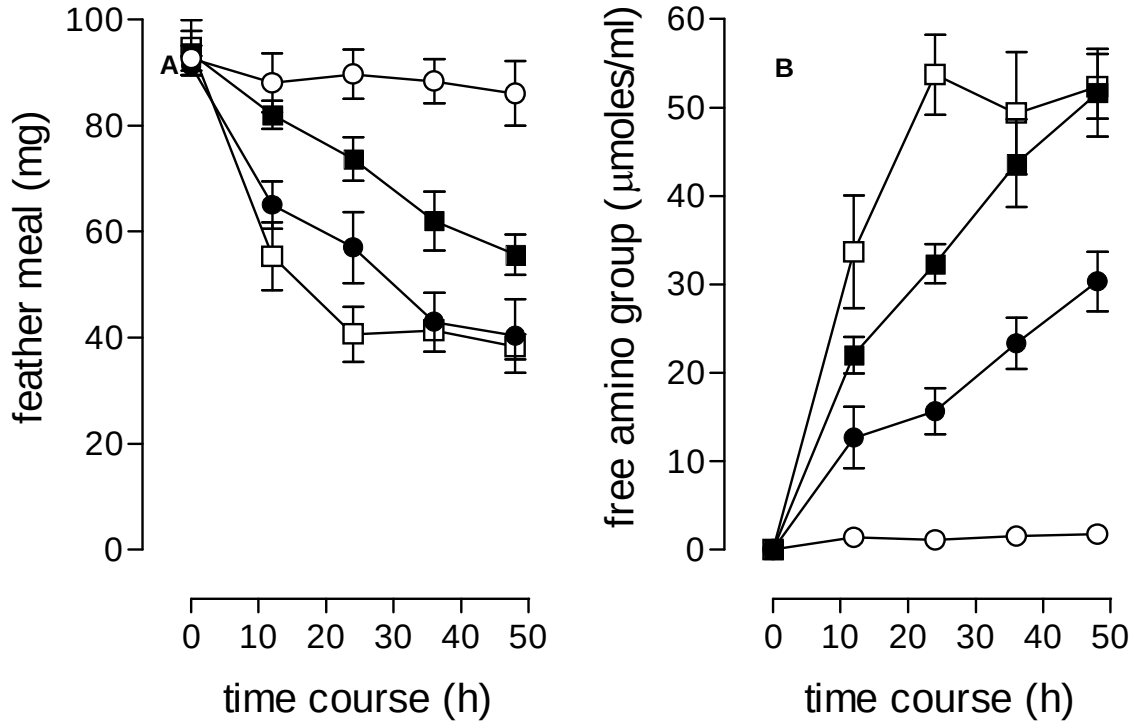

Figure 5. *In vitro* degradation of chicken feather powder by crude *M. verrucaria* protease. One ml of culture filtrates with different keratinolytic activity was added to 100 mg of native feather meal in 10 ml of 0.1 M borate buffer, pH 9.0 in a shaking water bath at 130 rpm for up to 48 h. In A: The dry weight of the remaining feather meal substrate was determined on membrane filters (pore size, 0.2 µm) after drying at 105^{o} C for 12 h. In B: The production of free amino acid and soluble peptides from native feather meal was measured using ninhydrin method with alanin as standard. Final keratinase activity in the assay: (○) 0.0 U/ml; (■) 5 U/ml; (●) 7.5 U/ml); (□) 10.0 U/ml.

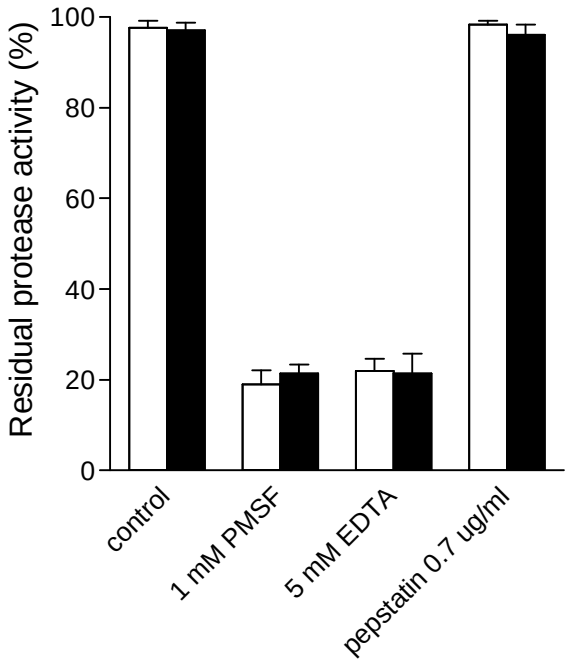

Figure 6. Effects of protease inhibitors on crude *M. verrucaria* enzyme. Full bars: crude enzyme from submerged cultures; Clear bars: crude enzyme from solid state cultures.

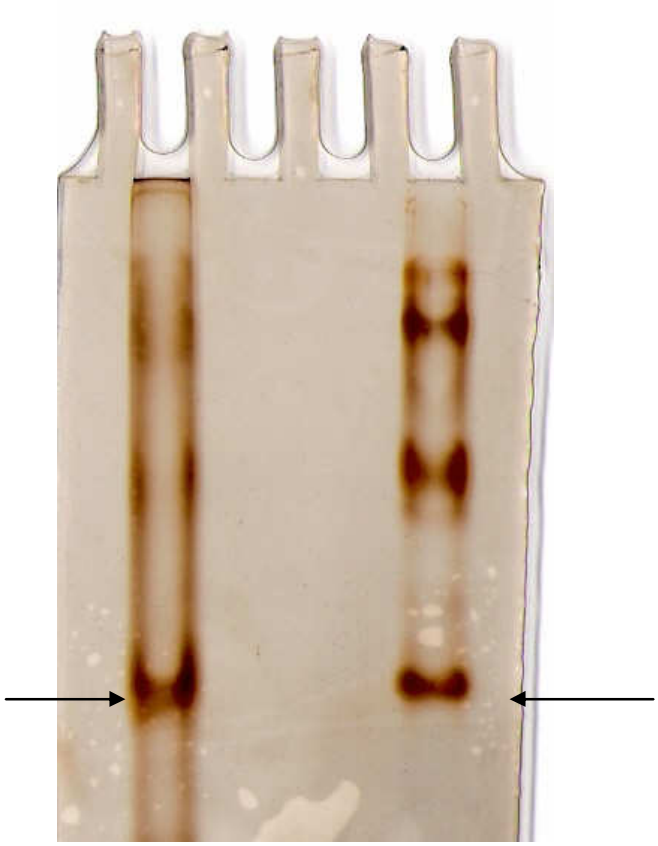

Figure 7. Silver stained SDS-PAGE of protein secreted by *M. verrucaria* grown on 1% chicken feather cultures. Protein bands were visualized by silver staining.The identification of protease was done by using zymogram technique with casein as substrate. The arrows show the proteic band with protease activity. A: submerged culture filtrate proteins; B: solid state culture filtrate proteins.

ARTIGO 3

PURIFICATION AND PARTIAL CHARACTERIZATION OF A KERATINOLYTIC PROTEASE FROM THE NON DERMATOPHYTIC FUNGUS *Myrothecium verrucaria*

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ABSTRACT

A Myrothecium verrucaria keratinolytic protease was purified to apparent electrophoretic homogeneity using an unique chromatographic step with a high yield of 62%. The molecular mass was estimated to be 22 kDa (SDS-PAGE analysis) or 23 kDa (gel chromatography analysis), suggesting a monomeric structure. The enzyme was stable in a broad range of pH (5.0-12.0) and temperature up to 45° C, being optimally active at 40°C and pH 8.0-9.0. It was strongly inhibited by PMSF and partially inhibited by EDTA, which suggests to be a metallo serine protease. The purified enzyme was able to hydrolyze chicken feather meal producing free amino groups. The efficiency of hydrolysis was improved when the keratin was previously treated with reducing agents.

KEY-WORDS: *Myrothecium verrucaria*, protease, keratinase, chicken feather.

INTRODUCTION

Keratins are the most abundant proteins in epithelial cells of vertebrates and represent the major constituents of skin and its appendages such as nail, hair, feather, and wool (Gradisar et al. 2005). Keratins comprise long polypeptide chains stabilized by disulfide bonds, hydrophobic interactions, and hydrogen bonds what result in insolubility and resistance to the activity of non-substrate-specific proteases (Safranek and Goos 1982).

A group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinases and are produced specially by microorganisms (Onifade et al. 1998). Secretion of keratinolytic enzymes is associated with dermatophytic fungi, for which keratin is the major substrate, but there are some reports describing the production, purification and characterization of the keratinases from bacteria and non dermatophytic fungus (Friedrich et al. 1999, Monod et al. 2002, Safranek and Goos 1982, Sharma and Rajac 2003).

The degradation of keratinous material is important medically and agriculturally (Shih 1993, Matsumoto 1996). Feathers are poultry by-product rich in β-keratin (Onifade et al. 1998). World-wide poultry processing plants produce millions of tons of feathers as a waste product annually (Santos et al. 1996) and their content in keratin is largely responsible for their high degree of recalcitrance. However, they also represent a potentially valuable source of protein as animal feedstock if keratinolysis can be achieved (Shih 1993). In addition to this, keratin partial or completely hydrolyzed by keratinases, could be used to the development of slow nitrogen fertilizers, glues and biodegradable films (Dalev and Neitchev 1991, Choi and Nelson 1996).

The fungus *Myrothecium verrucaria* is a filamentous fungi able to produce several hydrolytic enzymes including endochitinase (Govindsamy et al. 1998), xylanases (Filho et al., 1994), and pectinases (Faheild and Murad, 1992). Recently we described its capability to produce a protease with keratinase activity when cultivated in submerged and solid state cultures using chicken feathers as the only carbon and nitrogen source (Moreira et al. submitted). In this paper, we described the purification and partial biochemical characterization of a extracellular keratinase obtained from submerged culture of *M. verrucaria* with chicken feather as the only carbon and nitrogen source.

2. MATERIAL AND METHODS

2. Materials and methods

2.1. Microorganism.

Myrothecium verrucaria (Albertini and Schwein) Ditmar:Fr CCT 1886 was obtained from the Collection Culture of Fundação André Tosello, Campinas, SP, and it was maintained on potato dextrose agar.

2.2. Pretreatment of the feathers.

White chicken feathers were supplied by a local industry. Freshly plucked wet feathers were washed extensively with water and detergent. Wet feathers were dried in a ventilated oven at 40º C for 72 h. The feathers were then milled in a ball mill and passed through a small-mesh grid to remove coarse particles.

2.3. Culture conditions

One x 10^9 *M. verrucaria* spores were transferred to 250 ml Erlenmeyer flasks containing 50 ml of mineral media (Montenecourt and Eveileigh 1977) and 1.0% (w/v) chicken feathers as substrate. The medium was previously sterilized by autoclaving at 121º C for 15 min. The cultures were incubated at 28º C on a rotary shaker at 120 rpm. After 4 days of cultivation, the culture broth was filtered thorough Whatman nº 1 filter paper to retain non-degraded feathers, and centrifuged (5000 x g for 20 min at 4º C). The supernatant was recovered and used for further purification.

2.4. Enzyme assay.

Keratinolytic activity was assayed by the method of Nickerson et al. (1963) using chicken feather meal as substrate. The reaction mixture containing 10 mg of substrate, 1.0 ml of borate buffer (0.1 M, pH 9.0), and 1.0 ml of a suitable diluted culture filtrate was incubated under agitation using a stirring bar for 1 h at 40º C. The reaction was stopped by boiling and the mixture was filtered through filter paper. The production of free amino group was estimated using a reaction with ninhydrin reagent and alanin as the standard (Starcher, 2001). The protease activity of the culture

filtrates was assayed using 1.0% (w/v) casein in 0.1 M borate buffer, pH 9.0, as substrate (Boer and Peralta, 2000). One (1.0) ml casein was incubated at 40^{o} C for 1 h with 1.0 ml of a suitable diluted culture filtrate. The reaction was stopped by the addition of 2.0 ml of 0.6 M trichloroacetic acid. After 15 min in an ice bath, the mixtures were mixing on a vortex mixer, and the tubes were centrifuged at 3,000 rpm for 10 min. The tyrosine released was estimated by Lowry's method using a tyrosine standard curve. A unit of enzyme activity was defined as the amount of enzyme producing 1 µg of tyrosine per min.

2.5. Estimation of protein

Protein was estimated by dye-binding method (Bradford 1982), using bovine serum albumin as standard protein. In the column chromatography elution, the amount of protein was measured in terms of the absorbance at 280 nm. The specific activity was expressed as the enzymatic activity per mg of protein.

2.6. Enzyme purification

All purification steps were carried out below 15^{o} C unless otherwise specified. The material containing crude protease was centrifuged at 15,000 g for 30 min to remove particulate material. The culture filtrate was concentrated about 10-fold by ultra filtration (Amicon YM-10, 10 kDa cut-off). After removal of insoluble substances by centrifugation, the material was dialyzed against water and concentrated by freeze-drying. The material was re suspended in 1.0 ml of 50 mM borate buffer, pH 9.0 and applied to a Sephadex G-100 gel filtration (90 cm x 25 mm) equilibrated and eluted with 50 mM borate buffer (pH 9.0) at a flow rate of 10 ml/h. The protease active fractions were pooled and concentrated by freeze-drying, and stored at -10^{o} C.

2.7. Partial characterization of purified protease

2.7.1. Effect of temperature and pH on keratinolytic activity and stabilityy.

The effect of temperature on keratinolytic activity was carried out at temperature ranging from 25 to 60^{o} C. The effect of pH on the activity of enzyme was determined in a series of McIlvaine's buffers with pH values from 5.0 to 8.3, 0.1 M tris-HCl buffer (pH 8.0-9.5) and 0.1 M borate buffer (pH 8.5-12.0). The thermo stability and pH stability was assessed of *M. verrucaria* keratinase, incubating

aliquots of enzymes at different temperatures (between 5 and 60° C) for different times, and stored at different pH value (between pH 5 and 12) for 12 h. After the treatments, the samples were assayed for residual keratinolytic activity.

2.7.2. Effect of protease inhibitors.

To study the influence of protease inhibitors, purified protease was pre incubated with the following protease inhibitors: phenylmethanesulphonyl fluoride (PMSF, 1 mM), EDTA (5 mM), pepstatin A (0.7 µg/ml), iodoacetamide (0.05 mM), dithiothreitol (DTT, 1 mM), β-mercaptoethanol (5 mM); all of them in 0.1 M borate buffer (pH 9.0) for 15 min at 30° C. Keratinase and protease activities were determined as standard conditions.

2.7.3. Effect of other chemicals on the keratinolytic activity

The effects of solvents and detergents on keratinase and protease activities were tested by incubating purified enzyme in 0.1 M of borate buffer (pH 9.0) containing, isopropanol, methanol, ethanol, buthanol, glycerol, acetonitrile, Triton X-100, SDS, Tween 80 and NaN_3 were incubated for 15 min at 30° C. The enzymatic assay was started by adding the substrate and enzyme activity was measured as described above. To determine the effect of cations on keratinase and protease activities, the purified enzyme was incubated in the presence of each cation (as chloride salt) at 5.0 mM.

2.7.4. Electrophoretic analysis and determination of molecular mass.

The molecular mass of the purified protease was estimated under denaturing conditions electrophoresis. SDS-PAGE was performed on 10% polyacrilamide gel (Laemmli 1970). The following Mr standards (MW-70 kit-Sigma) were used: bovine serum albumin (66 kDa), ovoalbumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), and β-lactoglobulin (18. kDa). Protein bands were visualized by silver staining. The protease molecular mass was also estimated by gel filtration chromatography using Sephadex G-100 equilibrated with 50 mM borate buffer, pH 9.0 plus 0.1 M NaCl. The column was calibrated with standards proteins (MWGF-70 kit-Sigma).

2.8. Materials

Yeast extract and nutrient agar were from Difco laboratories. Ninhydrin, casein and inhibitors were purchase from Sigma Chemical Corp. (St Louis, Mo). All other reagents were of analytical grade).

3. RESULTS

3.1. Purification of M. verrucaria protease.

Protease was the main and smaller protein secreted by *M. verrucaria* under the conditions used in this work (Fig 1, line 1). The culture filtrate concentrated by freeze drying was applied in a gel filtration column. Fractions with high protease activity were pooled, dialyzed and lyophilized without any apparent loss of activity. Table 1 presents a summary of a typical purification of protease. At the end of the process, protease was purified 6.1–fold with a yield of 68% (Table 1) Homogeneity of purified enzyme was revealed by SDS-PAGE, showing a single protein band by silver staining (Fig 1, line 3). The molecular mass of the enzyme was 22 kDa (SDS-PAGE) and 23 kDa (Sephadex G-100 column).

3.2 Enzyme biochemical properties

The purified *M. verrucaria* keratinolytic protease was active at a broad range of temperature (25-55^{o} C) and pH values (5-12). The optimum temperature and pH were 40^{o} C and pH 8.0-9.5, respectively (Fig 2 and 3). The enzyme was stable at pH large range of pH (5.0-12.0) for 1 h (Fig. 2). Thermal inactivation was determined incubating the enzyme for different times in temperatures ranging from 30 to 50^{o} C (Fig. 4). The enzyme was stable at 30 and 35^{o} C for more than 5 hours, and the half-lives at 40, 45 and 50^{o} C were 120, 45 and 25 min, respectively.

Table 2 shows the keratinolytic and proteolytic activities of purified enzyme from *M. verrucaria* when incubated with different inhibitors. The enzyme was strongly inhibited by PMSF and partially inhibited by EDTA. Reducing agents improved the keratinolytic activity but barely affect the proteolytic activity when casein was used as substrate.

The effects of several chemicals in the purified protein were determined using casein and feather meal as substrates. The enzyme was stable in the presence of reducing agent 2-mercaptoethanol, anionic (SDS) and non-ionic (Triton X-100) detergents (Table 3). Different organic solvents such as methanol, ethanol, isopropanol, buthanol, glycerol, and acetonitrile in high

concentrations had a low significant influence on the keratinolytic and proteolytic activities. The most divalente cations tested in concentrations as high as 5 mM, had not influence on the keratinolytic and proteolytic activities. The enzyme was inhibited by Ag^+, Cd^+, Pb^+ and Hg^+. The enzyme was not affected by sodium azide.

At pH 9.0 and 40° C, the purified enzyme was able to hydrolyze efficiently keratin from chicken feather meal, producing soluble free amino group molecules (Fig 5). When the feather meal was previously reduced by 2-mercaptoethanol, the efficiency of hydrolysis was higher.

Figure 6 show the action of purified keratinase on intact feather. *In vitro*, keratinase altered the structure of intact feather, but the degradation of feather was not efficient than that obtained *in vivo*, when the feather was used as substrate for the growth of the fungus.

4. DISCUSSION

In this paper we described the purification and partial biochemical characterization of *M. verrucaria* protease with keratinolytic activity produced in submerged cultures using chicken feather meal as the only substrate. The enzyme was purified 9.29 fold to a specific activity of 12,851.85 U/mg (Table 1). By SDS-PAGE, a single protein band was obtained (Fig 1). The enzyme was a monomeric protein with molecular mass of 22 kDa and optimally active at pH 8.0-9.0 and 40° C (Table 2, Fig 1). Most keratinases have some common characteristics despite their different origins. They belong mainly to the extracellular serine proteases, with the exception of keratinases from yeasts, which belong to the aspartic proteases (Monod et al. 2002). The molecular masses of these type or protease range from 20 kDa to 60 kDa. They are mostly active in alkaline environments, with optimal activity at temperature up to 50° C (Gradisar et al. 2005).

The analysis of the effect of protease inhibitors (Table 3) showed that purified enzyme was totally inhibited by the serine protease inhibitor PMSF and partially by EDTA. The maximum increase of keratinolytic activity was observed in the presence of the reducing agents DTT and βME. Since DTT and βME are know to cleave disulphide bridges, an influence either on enzyme or on substrate was possible. However, the reducing agents improved the enzyme activity only when the substrate was keratin and the activity of enzyme remained unchanged when casein was the substrate. This result permits to conclude that the reducing agents acted on the keratin and not on the enzymes. In fact a more efficient hydrolysis of feather meal could be observed when the substrate was previously reduced by the addition of βME (Fig.5). The reduction of disulfide bonds improved significantly the keratin degradation. However, the results presented in this paper did not support the capability of purified protease from *M. verrucaria* efficiently degrade native feather (Fig

6B). The complete mechanism of keratin degradation was not fully understood. Basically, microbial keratinolysis is a proteolytic, protein-degrading process since the substrate (keratin) is essentially protein. However, not all serine type protease are able to degrade keratin (Eggen *et al.* 1990). The high resistance to proteolytic degradation of keratins is due to principally tight packing of the protein chains through intensive interlinkage by disulphide bridges (Böckle and Müller 1997). Considering that, it has been proposed that to filamentous fungi, the keratin degradation is result of a combination of mechanical keratinolysis (mycelial pressure and/or penetration of the keratinous substrate), sulphitolysis (reduction of disulphide bonds by sulphite excreted by mycelia) and proteolysis (Onifade *et al.* 1998). In fact, our results confirm the fact that the feather degradation was more efficient during the growth of *M. verrucaria* on feather where several feather-degrading factors could be acting cooperatively.

Among fungi, the most part of purified keratinase belongs to dermatophytic fungi (Asahi et al. 1985, Lee et al. 1987, Mushsin et al. 2000, Brouta et al. 2001), although the purification of non dermatophytic fungi keratinase has been already described (Anbu et al. 2005, Farag and Hassan 2004). In this paper we described a very simple process to obtain a homogeneous fraction of a keratinolytic protease from a non dermatophytic fungus which can be useful in future studies to understand the mechanism of keratin degradation.

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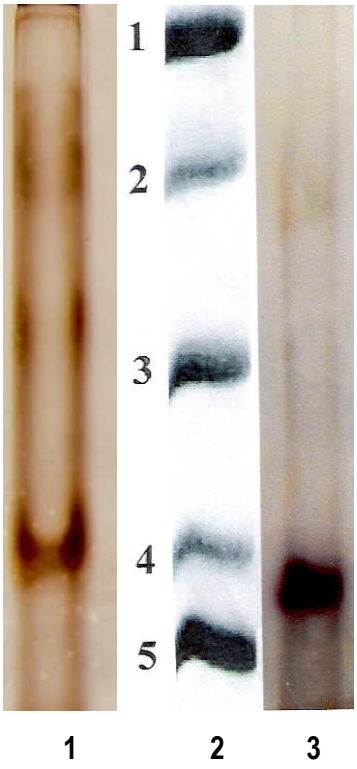

Figure 1. Denaturing SDS-PAGE of *M. verrucaria* keratinase (silver staining). Lane 1: crude culture filtrate proteins; Line 2: Standards proteins, (1) bovine serum albumin (66 kDa), (2) ovoalbumin (45 kDa), (3) pepsin (34.7 kDa), (4) trypsinogen (24 kDa), (5) β-lactoglobulin (18.4 kDa); Lane 3: purified *M. verrucaria* protease.

Table 1. Purification of *M. verrucaria* protease

Purification step	activity (U)	protein (mg)	SA (U/mg)	yield (%)	fold
Culture filtrate (CF)	26,000	18.80	1,383.00	100.0	1.00
Concentrated CF	25,100	12.60	1,992.10	96.5	1.44
Post-Gel filtration	17,680	1.43	12,363.63	68	8.94
Post gel filtration conc.	17,350	1.35	12,851.85	66.7	9.29

SA= specific activity. The protease activity was determined using casein as substrate.

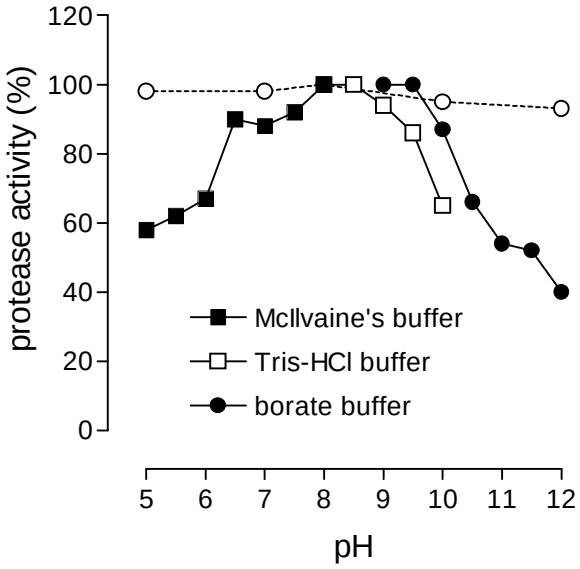

Figure 2. Effect of pH on activity and stability of *M. verrucaria* protease. (-----) stability; (____) activity.

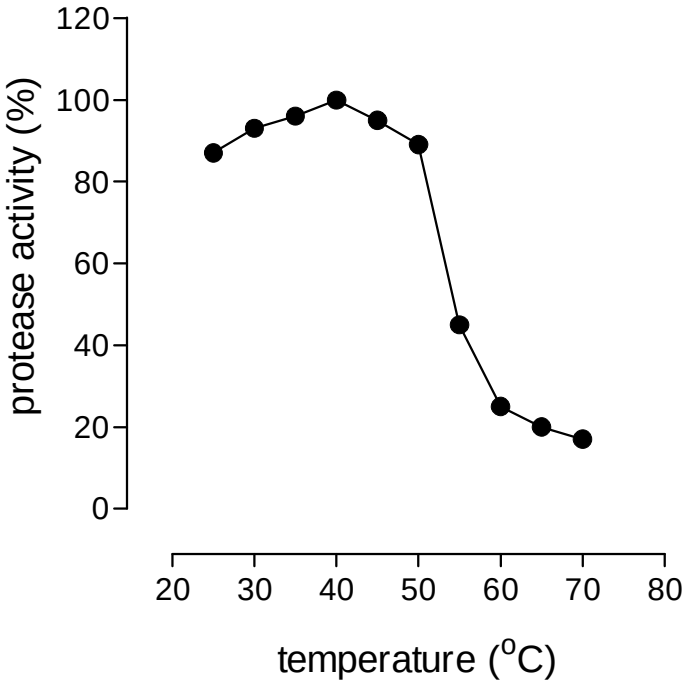

Figure 3. Effect of temperature in the purified *M. verrucaria* protease activity

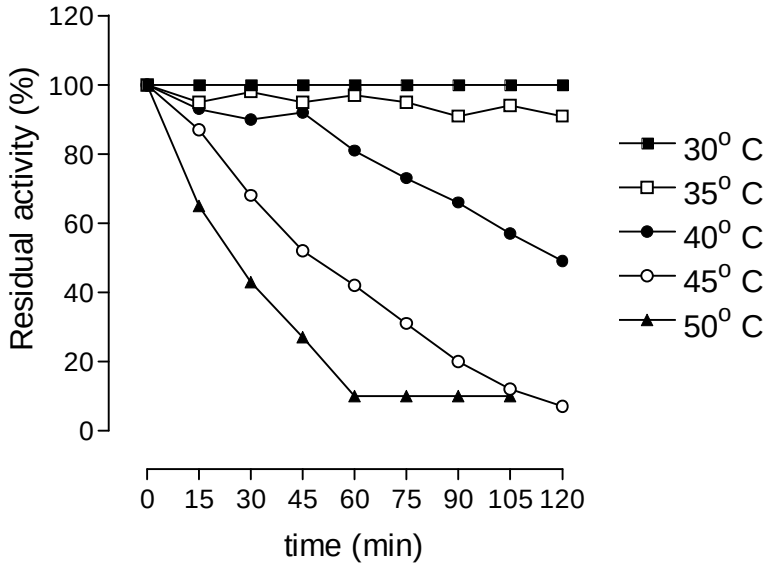

Figure 4. Effect of temperature on stability of *M. verrucaria* protease activity.

Table 2. Effect of chemicals on purified *Myrothecium verrucaria* protease.

Chemical	keratinase activity (%)	protease activity (%)
None	100.0	100.0
1 mM PMSF	7.0	11.8
5.0 mM EDTA	28.6	30.5
0.7 μg/ml Pepstatin A	98.7	95.9
0.05 mM Iodoacetamide	98.4	99.0
1 mM DTT	134.9	98.4
5 mM β-mercaptoethanol	143.5	97.0

Table 3. Effect of several chemicals on purified *M. verrucaria* protease

Chemical	Concentration	Residual activity (%)	
		Proteolytic activity	keratinolytic activity
None		100	100
SDS	5 mM	97.2	98.0
Triton X-100	2.5%	83.8	86.9
Tween 80	2.5%	125.0	139.0
NaN_3	5 mM	103.6	98.9
Methanol	25%	85.8	90.0
Ethanol	25%	96.8	91.9
Isopropanol	25%	81.8	85.4
Buthanol	25%	80.3	76.8
Glycerol	25%	86.8	81.0
Acetonitrile	25%	88.2	86.4
Ca^{2+}	5 mM	100	105
Mg^{2+}	5 mM	100	102
Cu^{2+}	5 mM	100	98.1
Zn^{2+}	5 mM	93.9	89.1
Co^{2+}	5 mM	84.8	89.3
Ba^{2+}	5 mM	80.8	87.6
Al^{3+}	5 mM	80.0	84.9
Mn^{2+}	5 mM	74.4	73.4
Pb^{2+}	5 mM	70.0	77.2
Hg^{2+}	5 mM	29.8	35.3
Ag^{2+}	5 mM	4.0	5.8
Cd^{2+}	5 mM	1.0	2.7
Fe^{3+}	5 mM	1.0	2.6

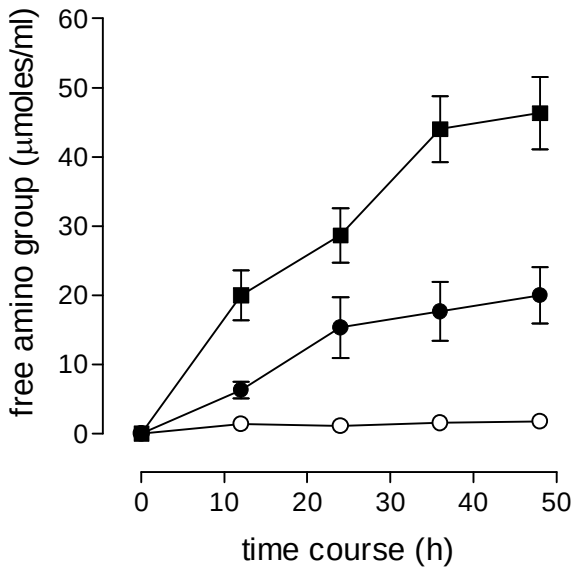

Figure 5. In *vitro* degradation of chicken feather powder by purified *M. verrucaria* protease. Purified *M. verrucaria* protease (as proteolytic activity) was added to 100 mg of feather meal in 10 ml of 0.1 M borate buffer, pH 9.0 in a shaking water bath at 130 rpm for up to 48 h. The production of free amino acid and soluble peptides from feather meal was measured using ninhydrin method with alanin as standard. (○) control without enzyme; (●) enzyme plus feather meal; (■) enzyme plus feather meal pre treated with 5 mM β mercaptoethanol for 1 h.

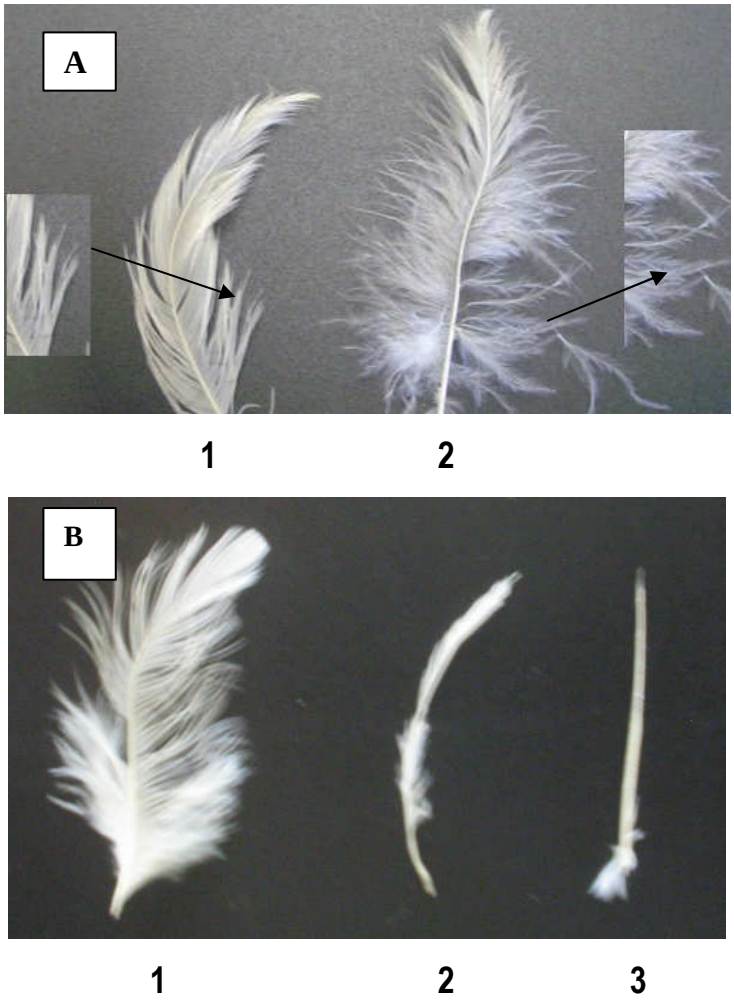

Figure 6. Degradation of native chicken feather *in vivo* (*M. verrucaria* culture) and *in vitro M. verrucaria* purified protease*)*. In A: Native feather was incubated in 50 mM borate buffer (pH 9.0) containing purified *M. verrucaria* enzyme (100 U/ml, as protease activity) and maintained at 30º C for 0 (1) and 48 h (2) under agitation of 130 rpm. In B: Native feather was used as substrate for growth of *M. verrucaria*. The cultures were developed at 30º C at 130 rpm for 0 (1), 48 (2) and 72 h (3).

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