PROTEASE PRODUCTION DURING GROWTH AND AUTOLYSIS OF SUBMERGED *METARHIZIUM ANISOPLIAE* CULTURES

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ABSTRACT

The growth and autolysis of two strains of the entomopathogenic deuteromycete fungus *Metarhizium anisopliae* var. *anisopliae* were evaluated in medium containing casein or glucose as carbon source. Parameters such as economic coefficient and degree of autolysis were determined for each strain. Protease production was determined throughout the growth and autolysis phases of the cultures on medium under conditions of protease induction (in the presence of casein as sole source of carbon and nitrogen). The fungus was shown to utilize casein as a carbon/energy source in a more efficient manner than glucose. The autolysis shown by the strains was intense under both types of growth conditions, reaching up to 62.7% of the dry mass produced and started soon after the depletion of the exogenous carbon source. The relationship between the proteolytic activities of the two strains evaluated varied significantly (a maximum of 19.78 on the 5th day and a minimum of 2.03 on the 16th day of growth) during the various growth and autolysis phases, clearly showing that the difference between the growth curves and the difference in the kinetics of enzyme production may decisively affect the process of strain selection for protease production.

Key words: *Metarhizium anisopliae*, protease production, fungal growth, fungal autolysis, entomopathogenic fungus

INTRODUCTION

Fungi have been extensively used as producers of different substances of economic interest such as enzymes, antibiotics, vitamins, amino acids and steroids. In many cases, the metabolites produced in culture play an important role in the physiological processes of the microorganism in the environment. This is the case for the hydrolytic enzymes and the toxines produced by the entomopathogenic deuteromycete fungus *M. anisopliae* which, in addition to being of economic interest, are important during the process of host penetration and death. However, the evaluation of the production of these substances and the use of these parameters in programs of selection and genetic breeding of different strains depend on the understanding of the basic aspects related to their development in culture.

Several studies have associated protease production (more specifically chymoelastases acting on insect cuticles or proteases with a toxic action) with the virulence of *M. anisopliae* (4, 6, 7, 8, 23).

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The existence of wide genetic variability in the production of these enzymes has also been observed (2, 13, 19). For practical reasons, most of the studies on enzyme production are limited to a single evaluation performed after a given period of growth. On this basis, we may assume that factors such as the differences between the growth curves of the isolates or the difference in the kinetics of enzyme production by the strains may hamper comparison and selection for the trait of interest.

The objective of the present study was to determine the kinetics of protease production by *M. anisopliae* strains during culture growth and autolysis.

MATERIALS AND METHODS

Origin and maintenance of the strains

Strains CLII and 22 of *M. anisopliae* var. *anisopliae* were obtained from the germplasm bank of the laboratory of entomopathogenic fungi of the State University of Campinas - UNICAMP. Strain CLII was isolated in the state of Alagoas, Brazil, from the sugar cane leaf spittlebug *Mahanarva posticata* (Homoptera: Cercopidae) and strain 22 was isolated in the state of Espírito Santo, Brazil, from pasture spittlebug *Deois flavopicta* (Homoptera: Cercopidae). To obtain spores, the strains were grown on complete solid medium (CSM) (14) at 28°C for 12 days.

Growth conditions

Fifty ml Erlenmeyer flasks containing 30 ml culture medium were inoculated with 3 ml of a suspension containing 3×10^7 conidia ml^{-1} in an aqueous solution containing 0.85% NaCl (w/v) and 0.002% Tween-20 (v/v). The flasks were then incubated at 28°C with shaking at 150 rpm for variable periods of time (1 to 16 days). After each incubation period, the content of each flask was filtered through Inlab type 10 paper, previously washed and tared, in order to retain the mycelial mass produced. The collected filtrate was divided into 1 ml aliquots and stored at -70°C. The presence of possible contaminants was monitored throughout the experiment by microscopic observation of the filtrate and by plating aliquots of the medium onto dishes containing CSM. Controls containing culture medium only were used to determine the changes in medium volume over the 16 days of incubation. All experiments were carried out in triplicate.

The two carbon sources (glucose and casein, Hammarsten, vitamin free) were added individually to basic salt medium (14) at a concentration of 0.5% (w/v). When casein was used, $NaNO_3$ was eliminated. The media were sterilized by autoclaving at 121°C for 20 min.

Determination of proteolytic activity against casein

Caseinolytic activity was determined as shown by Söderhäl and Unestam (21), with the following modifications: test tubes containing 1 ml of the filtrate were incubated in a water bath at 30°C. After 5 min, 1 ml of a 2% (w/v) vitamin-free casein (Hammarsten) solution in 0.05 M Tris-HCl buffer, pH 8.0, of the same temperature was then added to each tube. The mixture was incubated at 30°C for 15 minutes without shaking. The reaction was stopped by the addition of 5 ml of a 10% (w/v) trichloroacetic acid and samples were allowed to stand at 25°C for 1 hour. After centrifugation at 3,000 g (r_{av} = 7.0 cm) for 15 min, the supernatant was filtered through Inlab type 10 paper and its absorbance was determined at 280 nm. Controls in which trichloroacetic acid was added to the filtrate before adding the substrate were prepared in parallel for all treatments. A mixture containing 1 ml buffer was used as blank and treated in the same manner. Enzymatic activity was calculated as the variation in supernatant absorbance during the period of incubation x 10.

Determination of proteolytic activity against elastin

The elastolytic activity of the filtrate was determined by the method used by St. Leger *et al.* (22), with the following modifications. A reaction mixture containing 4 ml of an elastin Congo red (Sigma) suspension, 1.5 mg ml^{-1} in 0.05 M Tris-HCl buffer, pH 8.0, and 1 ml of the filtrate was prepared. The mixture was shaken vigorously for 10 sec, incubated at 30°C for 30 min without shaking, and then centrifuged at 5,000 g (r_{av} = 7.0 cm) for 10 min. The supernatant was filtered through Inlab type 10 paper and absorbance was determined at 450 nm. Controls in which the substrate was excluded from the reaction mixture were carried out in parallel for all treatments. Buffer was used as the blank. Enzymatic activity was determined as the variation in supernatant absorbance during the incubation period x 100.

Determination of residual glucose

Residual glucose present in the medium throughout growth was determined by the method of Nelson (12).

Determination of dry mass and autolysis

The mycelium separated by filtration was dried at 70°C for 72 h. Autolysis was determined by the methods of Reyes and Lahoz (16). The degree of autolysis was defined as percent loss of mycelial dry mass from the day of maximum growth, taken as the initial point, to the day when the sample was evaluated.

Efficiency of growth

The efficiency of growth was estimated by the economic coefficient (EC) according to the following formula:

EC = Mycelial dry weigh (mg) / Quantity of carbon source consumed (mg)

RESULTS AND DISCUSSION

Growth and autolysis

Table 1 shows the production of biomass by strains 22 and CLII growing on medium containing glucose as the only carbon source. Maximum biomass production ocurred on the 5th day of incubation for both strains. The maximum biomass production of strain CLII (71.5 mg/flask) was lower, corresponding to 81.7% of the maximum production reached by strain 22 (87.5 mg/flask). Exogenous glucose depletion ocurred at about the 5th day of growth and on the 7th day both strains presented autolysis process. The beginning of autolysis after depletion of the exogenous carbon source has been observed to a greater or lesser degree in various species of filamentous fungi (1, 9, 10, 11, 17, 18). After 16 days of growth (11 days of autolysis), strain 22 presented 52.4% autolysis and strain CLII presented 61.4% autolysis. These rates of autolysis are observed only when degradation of cytoplasm and cell walls occurs (16). Table 2 shows the efficiency of the utilization of the substrate from the calculation of the economic coefficient. It can be seen that the economic coefficient varied considerably as a function of the time of culture development. The highest economic coefficient was observed on the 5th day for strain 22 when the highest biomass production and total utilization of exogenous substrate occurred. For strain CLII, the highest

Table 1. Variations in pH, glucose availability and degree of autolysis during incubation of strains 22 and CLII in medium containing 0.5% (w/v) glucose as the only carbon source.

Time (days)	Dry mass (mg)	Degree of autolysis (%)[a]	Glucose in the medium (mg/ml)	pH of the medium
Strain 22				
0	-	-	5.19 ± 0.00	6.50 ± 0.00
1	20.5 ± 5.1	-	3.66 ± 0.19	6.63 ± 0.02
2	31.5 ± 6.1	-	3.08 ± 0.15	6.73 ± 0.02
3	45.3 ± 7.4	-	1.72 ± 0.23	6.86 ± 0.07
5	87.5 ± 8.7	-	0.04 ± 0.00	7.82 ± 0.06
7	82.6 ± 4.6	5.6	0.03 ± 0.00	7.94 ± 0.04
9	63.9 ± 0.5	27.0	0.03 ± 0.00	8.14 ± 0.04
13	51.8 ± 4.1	40.8	0.03 ± 0.00	8.27 ± 0.02
16	41.6 ± 2.5	52.4	0.02 ± 0.00	8.40 ± 0.05
Strain CLII				
0	-	-	5.19 ± 0.00	6.50 ± 0.00
1	18.6 ± 3.1	-	3.77 ± 0.19	6.64 ± 0.01
2	36.3 ± 2.0	-	2.75 ± 0.12	6.73 ± 0.02
3	61.5 ± 12.0	-	1.57 ± 0.56	7.82 ± 0.06
5	71.5 ± 8.5	-	0.03 ± 0.00	8.02 ± 0.01
7	66.2 ± 11.8	7.4	0.02 ± 0.00	8.22 ± 0.04
9	37.2 ± 1.0	48.0	0.02 ± 0.00	8.33 ± 0.12
13	26.7 ± 0.6	62.7	0.02 ± 0.00	8.63 ± 0.07
16	27.6 ± 1.7	61.4	0.02 ± 0.00	8.74 ± 0.04

Each result corresponds to the mean of three replicates.

[a] Autolysis was calculated with respect to the highest production of dry mass.

economic coefficient was observerved on the 3rd day, when the biomass production was 86.0% and the utilization of exogenous substrate was 70.0% of the total.

According to Reyes and Lahoz (16), during growth there is a balance between the synthesis and lysis of the polymers that form the walls. The lack of nutrients may cause the cessation of synthesis, provoking a shift of this balance toward lysis of hyphal walls, with the consequent beginning of autolysis. Although glucose, when present in the medium, represses the synthesis of hydrolytic enzymes, it has been shown that, after the beginning of autolysis, the production of hydrolytic enzymes is induced by the presence of soluble oligomers and monomers present in the medium. These substances are derived from the degradation of the cell wall and are found at low concentrations in the culture fluid due to the turnover of substances that occurs during autolysis (15).

Table 2. Glucose utilization by strains 22 and CLII in submerged cultures.

Incubation time (days)	Strain 22			Strain CLII		
	Glucose utilized (mg)	Mycelium formed (mg)	Economic coefficient	Glucose utilized (mg)	Mycelium formed (mg)	Economic coefficient
1	50.5 ± 6.4	20.5 ± 5.1	40.2 ± 6.2	46.8 ± 6.4	18.6 ± 3.1	40.0 ± 2.0
2	69.6 ± 5.0	31.5 ± 6.1	45.0 ± 5.5	80.6 ± 4.0	36.3 ± 2.0	45.5 ± 4.5
3	114.6 ± 7.7	45.3 ± 7.4	39.5 ± 6.3	119.6 ± 18.5	61.5 ± 12.0	51.0 ± 4.9
5	170.0 ± 0.1	87.5 ± 8.7	51.5 ± 5.1	170.5 ± 0.0	71.5 ± 8.5	41.9 ± 5.0
7	170.2 ± 0.0	82.6 ± 4.6	48.5 ± 2.7	170.5 ± 0.1	66.2 ± 11.8	38.8 ± 6.9
9	170.3 ± 0.1	63.9 ± 0.5	37.5 ± 0.3	170.6 ± 0.0	37.2 ± 1.0	21.8 ± 0.6
13	170.5 ± 0.0	51.8 ± 4.1	30.4 ± 2.4	170.6 ± 0.1	26.7 ± 0.6	15.6 ± 0.4
16	170.5 ± 0.1	41.6 ± 2.5	24.4 ± 1.5	170.8 ± 0.1	27.6 ± 1.7	16.1 ± 1.0

Each result represents the mean of three replicates.

Table 3. Variations in pH and in the proteolytic activity of the filtrate and degree of autolysis during incubation of strains 22 and CLII in medium containing 0.5% (w/v) casein as the only carbon and nitrogen source.

Incubation time (days)	Dry mass (mg)	Extent of autolysis (%)[a]	Proteolytic activity vs. casein[b]	Proteolytic activity vs. elastin[c]	pH of the medium
		Strain 22			
1	27.2 ± 0.5	-	0.14 ± 0.08	0.20 ± 0.16	6.45 ± 0.00
3	72.6 ± 0.3	-	0.35 ± 0.15	0.40 ± 0.14	-
5	103.4 ± 0.6	-	0.51 ± 0.13	0.34 ± 0.22	7.78 ± 0.08
7	102.9 ± 7.2	0.4	1.33 ± 0.17	1.03 ± 0.14	8.41 ± 0.03
9	90.5 ± 3.2	12.5	1.50 ± 0.07	1.40 ± 0.18	8.43 ± 0.06
13	70.3 ± 4.4	32.0	1.17 ± 0.14	1.14 ± 0.14	8.53 ± 0.02
16	58.4 ± 1.5	43.5	1.15 ± 0.18	1.22 ± 0.43	8.57 ± 0.09
		Strain CLII			
1	32.0 ± 1.8	-	0.05 ± 0.03	0.19 ± 0.04	6.46 ± 0.02
3	78.9 ± 4.2	-	0.38 ± 0.24	0.22 ± 0.17	-
5	91.7 ± 4.2	-	10.05 ± 0.22	3.93 ± 0.12	8.14 ±0.10
7	58.7 ± 1.5	36.0	7.21 ± 0.92	3.25 ± 0.39	8.32 ± 0.12
9	45.2 ± 4.6	50.7	5.48 ± 0.84	2.79 ± 0.19	8.59 ± 0.05
13	43.7 ± 1.6	52.3	3.22 ± 0.14	2.12 ± 0.25	8.77 ± 0.05
16	39.0 ± 1.7	57.5	2.33 ± 0.48	1.84 ± 0.13	8.67 ± 0.06

Each result represents the mean of three replicates.

[a] Autolysis was calculated with respect to the highest dry mass production.
[b] Activity determined by the variation in absorbance at 280 nm during the period of incubation (5 min.).
[c] Activity determined by the variation in absorbance at 450 nm during the period of incubation (30 min.).

Table 3 shows the production of biomass by strains 22 and CLII growing on medium containing casein as the only source of carbon and nitrogen. Again, the maximum production of strain 22 (103.4 mg/flask) was higher than the production of strain CLII (91.7 mg/ flask). Both presented a production peak on the 5th day of growth. The biomass production data presented in Fig. 1 show that casein is a carbon source that can be used more efficiently by *M. anisopliae* than glucose, favoring a more rapid growth and a greater biomass production. Similar results have been reported for other species of filamentous fungi (5). Using respirometric experiments, we observed that casein and hydrolyzed casein accelerate germination, reduce the duration of the lag phase and increase the growth rate compared to glucose (umpublished data).
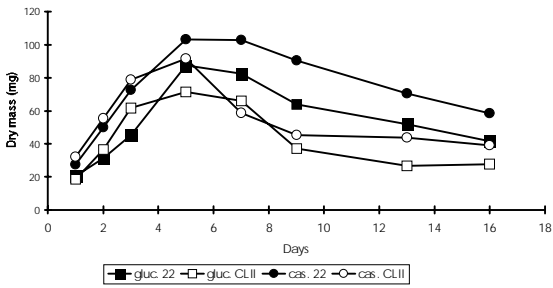
Figure 1. Growth curves for strains 22 and CLII in media containing 0.5% glucose or casein (w/v). Each point represents the mean of three replicates. The standard errors of the means were 3.1 for strain 22 in glicose, 3.9 for strain CLII in glicose, 2.0 for strain 22 in casein and 1.8 for strain CLII in casein.

Tables 1 and 3 show that in the culture media used there was a progressive increase in pH both during the growth phase and the autolysis phase of the two strains. Santamaria and Reyes (20), in a study of protease production during autolysis in different species of filamentous fungi, observed that autolysis occurred at pH values between 6.5 and 8. According to these investigators, the fact that autolysis occurs in these fungi at slightly alkaline pH may be related to the production of neutral and alkaline proteases by fungi from the major taxonomic groups. The main protease produced by *M. anisopliae*, Pr1, has a pH optimum of about 8 (22). The progressive increase in medium pH during autolysis of *M. anisopliae* cultures was previously observed by Campbell *et al.* (3).

Protease production

The caseinolytic activity of the *M. anisopliae* culture filtrates is mainly due to the presence of two proteases, Pr1 and Pr2 (22), both capable of hydrolyzing casein. The elastolytic activity is due to the action of Pr1 which, in addition to having caseinolytic and elastolytic activity, has a strong activity against insect cuticles. Pr1 is being indicated as one of the factors responsible for the virulence of the fungus (4, 23).

In a previous study we determined the proteolytic activity of 16 *M. anisopliae* strains after a fixed time of growth (2). The results permitted us to identify two contrasting strains: strain CLII, with high proteolytic activity, and strain 22, with low proteolytic activity. However, we could not determine whether this difference persisted throughout growth and autolysis or whether there was variation in the kinetics of enzyme production between strains. This information was obtained only by monitoring protease production
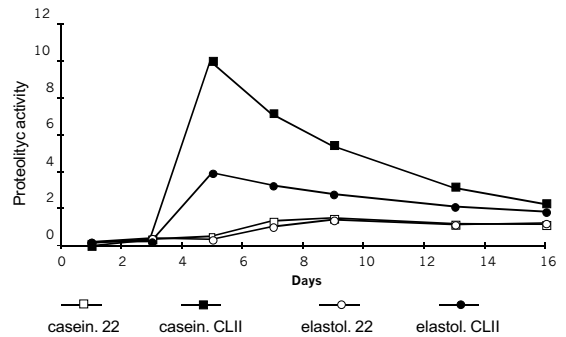

Figure 2. Caseinolytic and elastolytic activities present in the culture filtrates of strains 22 and CLII grown on medium containing 0.5 % casein (w/v) as the single carbon source.Caseinolytic activity was calculated as the variation in absorbance at 280 nm x 10 during the incubation period (15 min). Elastolytic activity was calculated as the variation in absorbance at 450 nm x 100 during the incubation period (30 min). Each point represents the mean of three replicates.

throughout the steps of strain growth and autolysis. Table 3 and Fig. 2 show the variations in the proteolytic activities present in the culture filtrates of the two strains grown on medium containing casein as the only carbon and nitrogen source. Up to the 2nd day the strains presented similar proteolytic activities. Starting at that time, the proteolytic activities of strain CLII were rapidly increased, reaching a maximum at about 5 days, when the highest production of biomass was also observed. A progressive decrease in proteolytic activity occurred thereafter. Protease production by strain 22 increased in a discrete manner, reaching a peak at about 9 days, already during the autolysis phase, and decreasing slowly until the end of the experiment. Studies on other species of fungi have shown that in most cases proteolytic activity increases with the beginning of autolysis; however, it is not uncommon for the maximum proteolytic activity to coincide with the growth phase (5, 20). The highest caseinolytic activity achieved by strain CLII was on average 6.7 times higher than that achieved by strain 22. The highest elastolytic activity observed in strain CLII was on average 2.8 times higher than that observed in strain 22. Fig. 2 shows that the ratios of the proteolytic activities of the two strains varied considerably along the various physiological stages of the cultures. The ratio of the caseinolytic activities of strains CLII and 22 on the 5th day was 19.78 (maximum), and was decreased to 2.03 (minimum) on the 16th day. Although strain 22 never presented a higher proteolytic activity than strain CLII, the variation in the ratio of their activities along

development clearly shows that the difference in the growth curves and in the kinetics of enzyme production may impair the evaluation of the trait if proteolytic activity is determined only once during culture development.

ACKNOWLEDGMENTS

RESUMO

Produção de protease durante o crescimento e análise de culturas submersas de *Metarhizium anisopliae*

O crescimento e a autólise de duas linhagens do deuteromiceto entomopatogênico *Metarhizium anisopliae* var. *anisopliae* foram avaliados em meio contendo caseína ou glicose como fonte de carbono. Foram determinados parâmetros como o coeficiente econômico e o grau de autólise apresentado pelas linhagens. A produção de protease foi determinada durante todas as fases do crescimento e da autólise das culturas, em meio indutor da produção de proteases (meio contendo caseína como única fonte de carbono e de nitrogênio). Pôde-se verificar que o fungo foi capaz de utilizar a caseína como fonte de carbono/energia de maneira mais eficiente do que a glicose. A autólise apresentada pelas linhagens foi intensa em ambas as condições de crescimento, alcançando até 62,7% da massa seca produzida, e se iniciou logo após o esgotamento da fonte exógena de carbono. A relação entre as atividades proteolíticas apresentadas pelas duas linhagens avaliadas variou de maneira significativa (máxima de 19,78 no 5° dia e mínima de 1,55 no 16° dia de incubação) durante as diversas fases do crescimento e da autólise, deixando claro que as diferenças verificadas entre as curvas de crescimento e entre as cinéticas da produção das enzimas podem influenciar decisivamente no processo de seleção de linhagens para a produção de proteases.

Palavras-chave: *Metarhizium anisopliae*, produção de protease, crescimento de fungo, autólise de fungo, fungo entomopatogênico.

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