Journal of Industrial Microbiology & Biotechnology (2000) 24, 369–373

www.nature.com/jim

ORIGINAL PAPERS

Nutritional regulation of proteinase production in the fungus, *Tritirachium album*

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The fungus, *Tritirachium album*, produces a number of proteinases under proper conditions. We have studied the nutritional regulation mechanisms for proteinase production in the mold, i.e. the effects of carbon and nitrogen sources, and the influence of starvation. Proteinase production was induced when the nitrogen source was an exogenous protein or peptide, such as peptones or yeast extract. The production rate was affected by the amount of available inducing substrate. Inorganic nitrogen compounds, i.e., ammonium or nitrate salts, had a repressing effect on the production. Production was not induced if a detectable concentration of glucose or sucrose was present in the medium. Starvation did not trigger proteinase production. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 369–373.

Keywords: proteinase; regulation; fungus

Introduction

Regulation of enzyme production in fungi can be studied from different points of view; i.e., genetic regulation, biochemical regulation or regulation by medium composition. The amounts of any enzyme produced by a fungus are generally very closely controlled by its own regulatory mechanisms, by derepression [7] or induction [8,11], and by catabolite repression [14], all operating against a background of breakdown or turnover mechanisms. In particular, the proteolytic enzymes are produced as a response to depletion of more readily utilized nutrients in the growth environment. The limitation of essential nutrients can promote the release of catabolite repression and lead to relevant enzyme synthesis. This mechanism may be combined with a substrate-specific induction mechanism. The production of fungal proteinases has been reported in several cases to vary with the nitrogen source supplied [2,5,13,17].

Tritirachium album Limber, a fungus belonging to the family Moniliaceae, produces at least three different kinds of proteinases and secretes them into the growth medium under proper conditions [9,15,16]. Proteinase K is one of the proteinases produced by *T. album*. It is a very active and stable endopeptidase with a broad activity spectrum. It can hydrolyze even native keratin and remains active in the presence of sodium dodecyl sulfate and urea [9]. It is used for the isolation of native high molecular DNA and RNA, because DNases and RNases from most microorganisms and mammalian cells are rapidly inactivated by proteinase K [12]. Samal et al. [16] isolated two other proteinases, proteinase R and T, from the extramycelial cultivation broth of *T. album*. These proteinases are induced by bovine serum albumin or skim milk and they have similarities with proteinase K with respect to chemical and thermal stability.

We have studied the inducing and repressing effects of different nitrogen sources on proteinase production in *T. album*. The effect of glucose on proteinase production during different cultivation phases was also investigated. Finally, we studied the influence of starvation conditions on production.

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Received 20 January 1999; accepted 6 March 2000

Materials and methods

Microorganism

T. album (Evitech 90.1) is a filamentous fungus, which belongs to the family Moniliaceae. It has been isolated from horn grind and can use keratin as sole carbon and nitrogen source. The fungus was maintained on 3% malt extract agar slants stored at 4°C and subcultured monthly.

Media and cultivation

Media: The propagation medium for inoculum preparation consisted of (per liter of tap water): malt extract, 30 g; peptone, 3 g; tetracycline, 0.8 μg; and penicillin G, 50 μg; pH 5.9. The Ebeling production medium [9] for proteinase production consisted of (per liter of tap water): glucose, 10 g; peptone, 10 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; KH_2PO_4, 2.8 g; $Na_2HPO_4 \cdot 12H_2O$, 0.7 g; pH 5.9. For fed-batch cultivations, the same media were employed and the feeding media consisted of peptone, 150 g l^{-1}; or yeast extract, 150 g l^{-1}; or $NaNO_3$ 150 g l^{-1}; or combined (yeast extract, 150 g+$NaNO_3$, 150 g) l^{-1}; or $(NH_4)_2SO_4$, 75 g l^{-1}; or combined (yeast extract, 75 g+$(NH_4)_2SO_4$, 75 g) l^{-1}, or glucose, 75 g l^{-1}; or combined (yeast extract, 75 g+glucose, 75 g) l^{-1}.

The medium for nutrient requirement tests consisted of (per liter of MilliQ purified water): KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5

Table 1 Effect of nitrogen source on cell mass concentration and proteinase production after 2 and 5 days of cultivation

Nitrogen source (1%)	DW (2 days) (g l^{-1})	DW (5 days) (g l^{-1})	Activity (5 days) (mAnson units)
No nitrogen source	3.5	2.9	0.3
$(NH_4)_2SO_4$	2.8	3.3	0
$(NH_4)H_2PO_4$	2.7	3.1	0
$NaNO_3$	7.7	9.1	0.4
NH_4NO_3	6.0	8.4	0.1
Glutamate	7.9	15.0	0
Urea	1.0	1.0	0
Yeast extract	8.0	13.2	5.3
Meat extract	9.8	13.8	3.2
Tryptone	6.3	14.6	0.7
Bacterial peptone	6.5	15.1	3.4
Mycological peptone	6.0	13.0	5.9
Casamino acids	6.1	13.8	0.1

The experiments were done as shake flask cultivations (28°C, 200 rpm) with the medium for nutrient requirement tests and with varying nitrogen sources. Each value was determined as an average value from duplicate cultivations.

g; $CaCl_2 \cdot H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $CuSO_4 \cdot 5H_2O$, 0.005 g; carbon source (glucose, if not otherwise mentioned), 20 g; nitrogen source (peptone, if not otherwise mentioned), 10 g. In starvation experiments, no nitrogen source was supplied.

Culture conditions: The preculture was prepared in two stages; 50 ml in 250 ml shake flasks (200 rpm, 96 h) and 400 ml in 1 l shake flasks (200 rpm, 96 h) of propagation medium at 28°C. Shake flask experiments were performed under the same conditions, except that medium components were varied as described in the text.

Cultivations for production optimization were performed in bioreactors of 3 l working volume (Marubishi MDL-500/5 L, Bioprocess controller MDIAC S3). The inoculum was 15% of the working volume in all cultures. The aeration was 1 vvm, agitation 200 rpm and temperature 28°C, pH 5.9, if not otherwise stated. The flow rates for nitrogen source feeds during fed-batch culture were 0.3, 0.6 or 0.9 g l^{-1} h^{-1}.

Analytical methods

Growth was measured as dry weight (DW). A constant volume of cultivation broth (50 ml) was filtered through a glass microfibre filter (Whatman GF/A). The cell mass was washed twice with phosphate buffer (pH 6.0) and dried to a constant weight at 105°C.

Enzyme activity was measured in the mycelium-free solution which was obtained when samples were filtered (Whatman GF/A). Proteinase activity was assayed according to Anson [1] with denatured hemoglobin as substrate and tyrosine as the standard compound. One unit of proteinase activity (mAnson unit) is defined as the amount which will digest hemoglobin under standard conditions at an initial rate such that there is liberated per minute an amount of split products, not precipitated by TCA, which gives the same color as with the Folin–Ciocalteu phenol reagent with 1 mEq of tyrosine [1].

Nitrate was quantified enzymatically with Boehringer Mannheim test kit no 905 658 [3]. *Ammonium* was measured spectrophotometrically according to the Finnish standard method SFS 3032 [10]. *Glucose* was assayed enzymatically with Sigma test kit no. 315 [19]. *Protein* was quantified according to the method of Bradford [4].

Results

Growth and induction of proteinase production by different nitrogen sources

Different inorganic nitrogen salts were compared to more complex nitrogen sources with respect to growth and enzyme production (Table 1).

Among the nitrogen sources tested, three groups of compounds were seen. The first consisted of complex nitrogen mixtures containing either peptides or amino acids. All compounds in this group (yeast and meat extract, tryptone, two different peptones and casamino acids) were clearly metabolized and cell mass increased to 13–15 g DW l^{-1}. The second group consisted of two nitrate salts and sodium glutamate. Growth on these compounds stayed under 10 g l^{-1}, but they were also metabolized. The third group was composed of those not metabolized. This group consisted of ammonium salts and urea. Growth was seen with ammonium nitrate but only nitrate was consumed, not ammonium.

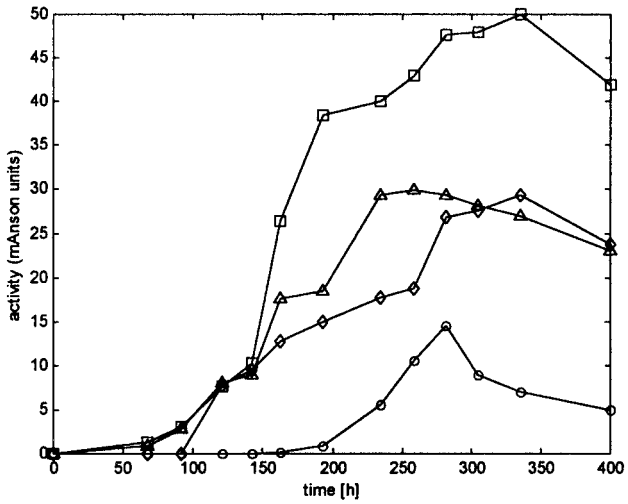

Figure 1 Fed-batch cultures with identical initial medium composition (Ebeling production medium) but with different feeding rates of peptone (-△-, 0.9 l^{-1} h^{-1}; -□-, 0.6 l^{-1} h^{-1}; -◇-, 0.3 l^{-1} h^{-1}; -○- no peptone fed). The peptone feeds were started when all glucose had been consumed, i.e. after about 65 h cultivation.

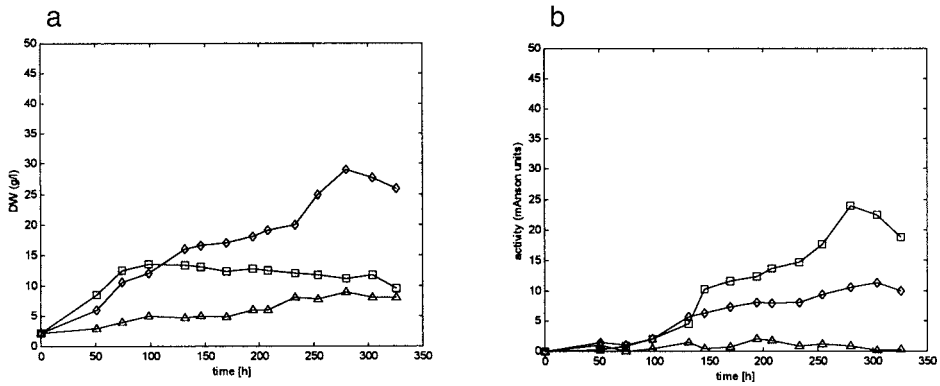

Figure 2 Growth (a) and proteinase activity (b) in fed-batch cultures with sodium nitrate (-△-), yeast extract (-□-) or both sodium nitrate and yeast extract (-◇-) as nitrogen sources. The feeds started after 65 h and consisted of 0.6 g of the nitrogen source per liter of medium per hour.

A low level of extracellular proteinase production could be detected in the shake flasks with nitrogen compounds belonging to the first group. Also, growth on nitrate resulted in a detectable production level, comparable to the level produced in a nitrogen-free culture. Glutamate was utilized, but the growth rate was slower than for growth on compounds from the first peptide-containing group, and no proteinase activity was seen.

When the fungus was cultivated in shake flasks or as batch cultivations in a bioreactor with a peptone-type nitrogen source, enzyme production level stayed under 10 mAnson units, although the cell mass increased up to 20 g l^{-1}. On the other hand, proteinase production was activated as soon as the cultivation was done as a fed-batch cultivation. Feeding of either peptone or yeast extract was started as soon as the sugar had been consumed from the culture medium. Feeding rate and concentration of peptone, or a similar nitrogen source, significantly affected the concentration of produced enzymes (Figure 1). In most cultures, proteinase activity stayed below 30 mAnson units, but with a feeding rate of 0.6 g l^{-1} h^{-1}, it increased to about 50 mAnson units.

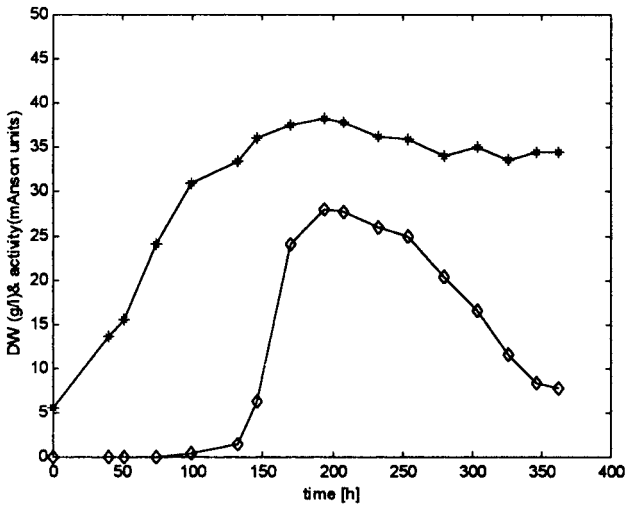

Figure 3 Fed-batch culture with peptone feeding to induce proteinase production during period I (65–180 h), and ammonium sulfate feeding during period II (180–360 h). Both compounds were fed in turn at a rate of 0.3 l^{-1} h^{-1} (-◇-, activity; -*-, growth as DW).

Nitrogen repression

The regulatory effect of nitrate on proteinase production was studied in parallel fed-batch cultures with yeast extract or sodium nitrate or both as the fed nitrogen source. Both compounds were consumed, but growth on nitrate resulted in a lower cell mass (Figure 2a) and also in a lower activity level (Figure 2b). The enzyme productivity coefficient, i.e. enzyme produced per cell mass unit, was 2.1 for peptone, 0.14 for nitrate and 0.35 for the combination of both nitrogen sources. The influence of nitrate can be seen as a damping of the production level.

Figure 3 shows a fed-batch cultivation where the nitrogen solution fed was changed from peptone to a combined ammonium sulfate and peptone feed as soon as proteinase production had clearly been induced by peptone. After the change, the amount of proteinases in the cultivation medium decreased rapidly. Cell mass concentration stayed more or less constant during this second period. To confirm that it was not a question of molecular inactivation, the influence of ammonium on the activity of pure proteinase K was checked (Table 3). No such influence could be seen with ammonium concentrations between 1% and 10%.

Table 2 Effect of carbon source on cell mass concentration and proteinase production after 2 days of cultivation

Carbon source (2%)	DW (g l^{-1})	Activity (mAnson units)
No sugar added	3.4	0
Glucose	8.9	0.3
Lactose	7.8	0.1
Sucrose	8.8	0.1
Xylose	7.4	0.3
Galactose	8.1	1.0
Maltose	8.2	0.8
Starch	n.d.*	0.6
Raffinose	7.3	0.7
Molasses	7.1	0.5
Skimmed milk	7.4	0.8
Whey	6.2	0.2

*Not determined.
The experiments were done as shake flask cultures (28°C, 200 rpm) with Ebeling production medium except that the carbon source was varied. Each value was determined as an average value from duplicate cultivations.

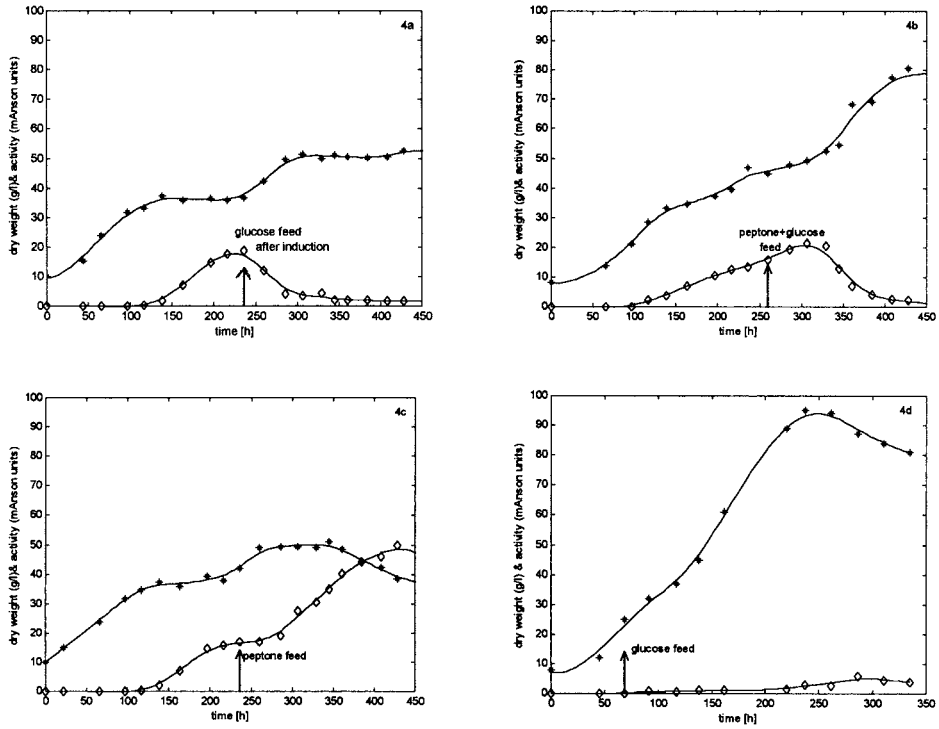

Figure 4 Fed-batch cultures with peptone and/or glucose feeds. The effects on cell growth (-*-) in terms of DW, and proteinase activity (-◇-) measured as mAnson units, were investigated. The arrow indicates the moment for changing from feeding peptone to glucose feed, or to both glucose and peptone feed at the same time. Both compounds were fed at a rate of 0.3 l^{-1} h^{-1}. (a) Glucose was fed to the culture when proteinase production had first been induced by peptone. (b) Glucose was fed at the same time as peptone when proteinase production had first been induced by peptone. (c) No glucose feed, only peptone was fed throughout the cultivation. (d) Only glucose was fed during the cultivation.

Effect of nitrogen starvation on proteinase production

The possibility that nitrogen limitation may trigger protease synthesis was investigated by washing the growing mycelium and then resuspending it in the Ebeling production medium lacking the nitrogen source. The concentration of mycelial mass remained constant, i.e. 10 g l^{-1}, during the starvation period because the starvation time was short enough to avoid breakdown of the mycelia. After a period of 6 h, the feeding of yeast extract, or alternatively albumin, was initiated. In both cases, the shape of the growth curve after a starvation period and when the feeding had started was similar to the curve of unstarved mycelium at the beginning of a cultivation. The only exception was that the lag period after starvation was 3–4 h longer than for a young culture. The same phenomenon was observed in the course of proteinase production: only a low level of extracellular proteinase activity could be detected during the starvation period, but when the feeding had been initiated, the proteinase production started to increase after a lag period. In the prestarved cultures a feeding concentration of 0.6 g l^{-1} resulted in a final proteinase activity of 47 mAnson units. This result corresponded well to the results of between 45 and 50 mAnson units from the unstarved cultures.

Carbohydrate limitation and repression caused by glucose

Eleven different carbon sources (glucose, lactose, sucrose, xylose, galactose, maltose, raffinose, starch, molasses, skimmed milk, and whey) were tested with respect to growth and enzyme production (Table 2). All carbon sources tested were utilized. Glucose, sucrose and starch were tested further in bioreactor cultures. In all three cases, there was no detectable production of proteinases until the carbohydrate was consumed. Even then, the production rate was very low if there was no inducing substrate fed to the culture.

Fed-batch cultures with glucose feed rates of 25 and 50 g l^{-1} h^{-1} gave higher cell mass concentrations (maximum DWs of about 90 g l^{-1}), but had no activating effect on enzyme production (Figure 4d). The repressing effect of glucose can be seen if the glucose supply is started when proteinase production had already been induced by peptone (Figure 4a). The production rate

Table 3 Effect of three nitrogen compounds on proteinase activity

Compound	Concentration (%)	Activity of proteinase K (mAnson units)	
		A	B
$CaCl_2$ (control)	0.02 M	5.7	38
$(NH_4)_2SO_4$	1	5.3	33
$(NH_4)_2SO_4$	2	5.1	34
$(NH_4)_2SO_4$	5	5.4	34
$(NH_4)_2SO_4$	10	5.3	33
$NaNO_3$	1	5.4	32
$NaNO_3$	2	5.4	32
$NaNO_3$	5	5.4	35
$NaNO_3$	10	5.5	34
Peptone	2	5.4	32
Peptone	5	5.5	35
Peptone	10	5.3	37

The tests were done with pure proteinase K enzyme (Merck 1.24568). A 0.02 M $CaCl_2$ solution was used as reference. The enzyme was diluted 1:2000 (A) and 6:2000 (B). Each value was determined as an average value from three replicate determinations.

decreased radically compared to the production rate when no glucose was fed (Figure 4c). This was also the case, even if the inducing substrate, peptone, was fed at the same time (Figure 4b). Still, it is possible that a low concentration of glucose in the feeding medium, i.e. 0.1 g l^{-1} h^{-1}, would not inhibit production as the higher concentrations do.

Discussion

Nutritional regulation of proteinase production in *T. album* was studied in a set of cultures and the results show that the main regulatory mechanism is induction caused by proteins or peptides. Peptones and yeast extract have a clear inducing effect on extracellular proteinase production unlike urea, glutamate or inorganic nitrogen salts. The influence of nitrate on proteinase production was first assumed to be a kind of catabolite repression as found with some other proteinase-producing fungi [18]. A similar influence of ammonium, ''ammonium repression'', has been found in several cases [7,14]. Ebeling et al. [9] also suggested that this is the case with *T. album*, but if nitrate and peptone were fed at the same time to culture of *T. album*, both compounds were consumed simultaneously, a fact that refutes this hypothesis. Since the nitrate supplied decreased the amount of proteinase produced, but did not affect proteinase activity as such (Table 3), the function of nitrate repression seems to be a fine regulation rather than an ''on–off'' regulation, as is the case with ammonium.

The production levels of proteinases in fed-batch cultures responded well to the amounts of peptone fed up to a limiting concentration of about 0.6 g l^{-1} h^{-1}. When the feeding rate was increased over this limiting concentration, substrate inhibition occurred, and the production rate did not rise any further. Peptone did not inhibit the action of the pure proteinase K enzyme (Table 3).

The effect of glucose on proteinase activity in the growth medium could be connected with the fact that phosphorylated sugars reversibly inhibit the proteolytic activity of proteinase K, as reported by Örstan and Gafni [21]. They proposed that it may be a mechanism for keeping the enzyme inactive until it leaves the cell. Glucose does not inhibit proteinase K but when phosphorylated as glucose-6-phosphate, it can prevent proteolytic activity against proteins inside the cell. Glucose also increases cell growth, and proteinase production mainly takes place during the stationary phase. This might be related to morphological changes not investigated yet.

Tomonaga et al. [20] showed in an early work that acid proteinase production in *Aspergillus niger* was depressed by sulfur starvation. Cohen [6] demonstrated that the synthesis of extracellular neutral and alkaline proteinases was repressed in the presence of low molecular weight sources of carbon, nitrogen or sulfur, but if the medium was lacking these compounds, repression did not take place. In the case of *T. album*, neither limitation of nitrogen nor carbon caused derepression. Only a very low level of proteinase production could be detected if the medium was not supplied with a protein-based nitrogen source and, on the other hand, production started as soon as peptone or yeast extract was fed. Our conclusion is that the supplied protein or peptide acts as both inducer and substrate. This assumption is also supported by the fact that if peptone was fed after a starvation period, proteinase production started when peptone was available and resulted in a similar production level as in the case of unstarved cultures.

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