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Growth and production of extracellular proteases by the fungus Aspergillus fumigatus on various media I. Media without proteins

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A strain of Aspergillus fumigatus was grown on ten nutrient media containing glucose and various (organic and inorganic) sources of nitrogen. Growth of the mycelia, pH of the cultivation fluid, uptake of nutrients and proteolytic activity on haemoglobin at pH 3 and on azocasein at pH 7.5 were assessed. The aim of the study was to find the physiological conditions for the production of extracellular proteases. High activities of alkaline protease(s) were found only when the following three conditions were fulfilled: (a) exhaustion of glucose from the medium, (b) reaching of the maximum mycelium dry weight, (c) rise of the medium pH to at least 7. These three factors are causally related and coincide in time so that they can hardly be evaluated individually. A neutral to alkaline pH is certainly a necessary condition and a steep rise in pH may itself be a sufficient triggering factor for the synthesis of protease(s). The presence of proteins and/or peptides as inducers is probably not quite necessary but is nevertheless strongly stimulating. The activity of acid (aspartic) protease was small to negligible on all media and its production clearly depended only on the pH of the medium (optimum pH 4 to 6).

Key words: Aspergillus fumigatus, virulence, extracellular proteases, enzyme induction

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Kmen Aspergillus fumigatus byl pěstován na deseti živných půdách s glukózou a různými (organickými i anorganickými) zdroji dusíku a sledován růst mycelia, pH kultivační tekutiny, využívání živin a proteolytická aktivita v pH 3 na hemoglobinu a pH 7,5 na azokaseinu. Hlavním cílem bylo nalézt fyziologické podmínky pro sekreci extracelulárních proteáz. Větší aktivity alkalické proteázy (proteáz) byly nalezeny jen při splnění tří podmínek: a) vyčerpání glukózy z média b) dosažení maximální sušiny mycelia c) vzestup pH média nad cca 7. Zmíněné faktory jsou při vývoji kultur příčinně spojeny a časově korelovány, takže je lze těžko posuzovat jednotlivě. Neutrální až alkalické pH je s určitostí podmínkou nutnou a sám prudký vzestup pH v médiu může být někdy pro tvorbu proteázy postačujícím signálem. Přítomnost peptidů a/nebo bílkovin jako induktorů není zřejmě zcela nutná, ale produkci proteázy silně stimuluje. Aktivita kyselé (aspartátové) proteázy byla u všech půd jen malá až nepatrná a její sekrece závisela zřetelně pouze na pH média (optimální pH 4 až 6).

Aspergillus fumigatus is an important opportunistic fungus and the main causative agent of aspergillosis in humans. Inhalation of airborne conidia by immunocompromised individuals may lead to invasive aspergillosis which is fatal in up to 90 % of the cases.

Among the putative factors of *Aspergillus* virulence an important role is ascribed to proteolytic enzymes ("elastases"). The latter have been studied intensively in the past few years, since advanced methods of biochemistry and molecular genetics have become available. The main alkaline serine protease of the subtilisine type was purified from various strains (Reichard et al. 1990, Monod et al. 1991, Frosco et al. 1992, Larcher et al. 1992, Kolattukudy et al. 1993), followed by a neutral metalloprotease (Monod et al. 1993, Markaryan et al. 1994) and an "acid" (aspartic) protease (Reichard et al. 1994, Lee and Kolattukudy 1995). Although the investigation of these three enzymes had already reached the molecular level of study, little attention was paid to physiology and regulation of their production under different circumstances.

In the seventies, these questions were addressed by Cohen (see e.g. Cohen 1973) in *A. nidulans*. He concluded on the basis of experiments with the transfer of growing mycelium into fresh media lacking some essential nutrients ("shift-down") that the presence of proteins was not a pre-requisite for the synthesis of extracellular proteases. Decisive was derepression caused by the absence of a suitable source of carbon, nitrogen or sulphur in the medium. Good sources of C, N and S (e.g. glucose, ammonium ions or methionine) repressed the synthesis; the repression by ammonium ions was especially strong. The same regulatory system was later found in 25 strains of 21 species of the genus *Aspergillus* (Cohen 1981), and some recent results (Katz et al. 1994) confirm its existence in *A. nidulans*. However, Srinivasan and Dhar (1990) could not achieve the production of proteases by simple derepression without the presence of proteins in their strain of *A. flavus*. The role of proteins as inducers of the synthesis of proteases has been shown in many fungi, e.g. in *Neurospora crassa* (Cohen and Drucker (1977) and *Candida albicans* (Lerner and Goldman 1993).

Because the data on the regulation of protease production in *A. fumigatus* are scattered and hard to generalize we thought it useful to study these questions systematically.

MATERIAL AND METHODS

The *Aspergillus fumigatus* strain Afu-1 isolated from the lung of a chicken with invasive aspergillosis was used throughout. It was kept on Sabouraud glucose (4 %) — peptone (1 %) agar at 26 °C. The conidia from 7 days old cultures on this medium were suspended in sterile distilled water by shaking with glass beads. The suspension was filtered through cotton wool, counted in a haemocytometer and diluted to about 10^6 spores per ml.

Ten media of the following composition (g/l) were used:
(1) glucose 9, bacto-peptone 1; (2) glucose 8, peptone 2; (3) glucose 6, peptone 4; (4) glucose 9, L-glutamine 1; (5) glucose 8, L-glutamine 2; (6) glucose 8, L-serine 2;

(7) glucose 6, L-glutamic acid, L-arginine, L-proline, L-serine and L-tyrosine 0.75 each; (8) glucose 9, ammonium tartrate 1; (9) glucose 8, ammonium tartrate 2; (10) glucose 8, sodium nitrate 2. The main nutrients were dissolved in a mineral solution containing 400 mg KH_2PO_4, 50 mg $MgSO_4.7\ H_2O$, 10 mg $CaCl_2.2\ H_2O$, 5 mg $FeCl_3$, 5 mg $ZnSO_4.7H_2O$ and 5 mg $MnCl_2.4\ H_2O$ per litre. The media were adjusted to pH 6.5 + 0.1 and sterilized by autoclaving. In media nrs. 5 and 6 glutamine was added as a concentrated solution sterilized by filtration.

Fifteen ml of the medium in 100 ml cotton wool-stoppered conical flasks were inoculated with 0.1 ml of spore suspension and the cultures incubated at 28 + 0.5 °C without shaking. At various time intervals three cultures were taken, filtered through pre-weighed paper filters and the dry weight of the mycelia measured after drying at 90 °C. In the cultivation fluid pH, concentration of the remaining nutrients and proteolytic activity at pH 3 and pH 7.5 were determined. The values presented are arithmetic means of three measurements in parallel cultures.

Glucose was assayed with glucose oxidase using a commercial set (Bio-La-Test, Lachema, Brno, Czech Republic) and peptide substances by the Lowry method, calibrated with bacto-peptone. The concentration of ammonium ions was determined colorimetrically by the Berthelot reaction (Bio-La-Test Oxochrom Urea) and that of nitrate by capillary isotachophoresis on the Agrophor apparatus (Development Laboratories of Palacký University, Olomouc, Czech Republic). As leading electrolyte 0.006 M HCl was used and 0.01 M n-caproic acid was the terminating electrolyte. The uptake of amino acids from the medium was followed semiquantitatively by thin layer chromatography on silica gel (Silufol^R plates, Cavalier Glassworks, Sázava, Czech Republic). For elution 70 % n-propanol was used and the substances detected with ninhydrin reagent containing cupric ions (Brenner and Niederwieser 1960).

For the assay of acid protease activity 2 ml 2 % acid –denatured bovine haemoglobin (Sigma, St. Louis, USA) in a 0.2 M citrate buffer (pH 3.0) and 1 ml cultivation fluid were mixed. After 1 hour at 40 °C 0.3 ml 40 % trichloracetic acid was added, left at 40 °C for 30 min., the precipitate centrifuged off and the concentration of peptides in the supernate determined by the Lowry method. To the blank trichloracetic acid was added before the sample. One arbitrary unit of enzyme activity corresponded to an increase in absorbance of 0.1 at 500 nm.

The activity of alkaline protease was assayed on azocasein synthesized according to Langner et al. (1973). To 2 ml 1 % azocasein in a 0.1 M tris-HCl buffer (pH 7.5) 0.2 to 1 ml of cultivation fluid was added and filled up to the volume of 3 ml with water. Incubation and substrate precipitation were as in the previous procedure. In the supernate, absorbance at 440 nm was measured against the blank. One arbitrary unit of activity corresponded to an increase in absorbance of 0.1. Because the dependence of final absorbance on the activity (estimated by serial dilution

of a highly active sample) was not sufficiently linear, the results were read from a calibration graph.

In the text the terms "acid protease" and "alkaline protease" are used. In fact, the activity measured was very likely the sum of the activities of several enzymes active at the respective pHs.

RESULTS

On medium nr. 1, glucose was present in excess so that the growth was terminated by the exhaustion of the nitrogen source (peptone) on about the 5th day of cultivation (Fig. 1B, 2B). Fast uptake of glucose was, however, terminated earlier (Fig. 2A). A long stationary phase was accompanied by a further — albeit much slower — utilization of glucose. Lowry-positive substances were present in the medium throughout the stationary phase (Fig. 2B). The dynamics of medium pH is shown in Fig. 1A. The activity of acid protease peaked in the mid-exponential phase and increased again in the stationary phase (Fig. 3B). The activity of alkaline protease was low (less than 3 U, Fig. 3A).

On medium nr. 2 the C/N ratio was better balanced, both glucose and peptone were exhausted at the same time (Fig. 2A,B) and the growth was faster. The autolytic phase began soon after cessation of the growth (Fig. 1B). The pH of the medium decreased to 4.2 in the exponential phase but later increased quickly to values above 6 (Fig. 1A). Lowry-positive substances were again present in the medium even after cessation of the growth (Fig. 2B). The activity of acid protease was the highest when the pH of the medium was the lowest and disappeared in the autolytic phase (Fig. 3B). The activity of alkaline protease did not exceed 3.5 U (Fig. 3A).

The relative content of the nitrogen source was further increased in medium nr. 3. This made the growth in the exponential phase faster but did not significantly increase the maximum dry weight (Fig. 1B). By the time the growth stopped (3rd day) glucose was nearly exhausted. The content of peptide substances, however, continued to decrease until the 5th day of cultivation (Fig. 2A,B). An intensive autolysis was accompanied by a steep increase in pH from about 5 up to 8.4 (Fig. 1A) and by an increase in the amount of Lowry-positive substances in the medium (Fig. 2B). Acid protease appeared by the time of minimal medium pH on the 3rd day but disappeared one day later (Fig. 3B). Simultaneously with the rapid increase in pH great amounts of alkaline protease were released (Fig. 2A). Later this activity decreased again.

On media nrs. 4 and 5 the source of nitrogen was 0.1 % and 0.2 % glutamine, respectively. The development of the cultures on both media was similar with respect to growth rate, maximum dry weight, medium acidification in the exponential phase (Fig. 4A,B) and glucose utilization (Fig. 5). Moreover, according to

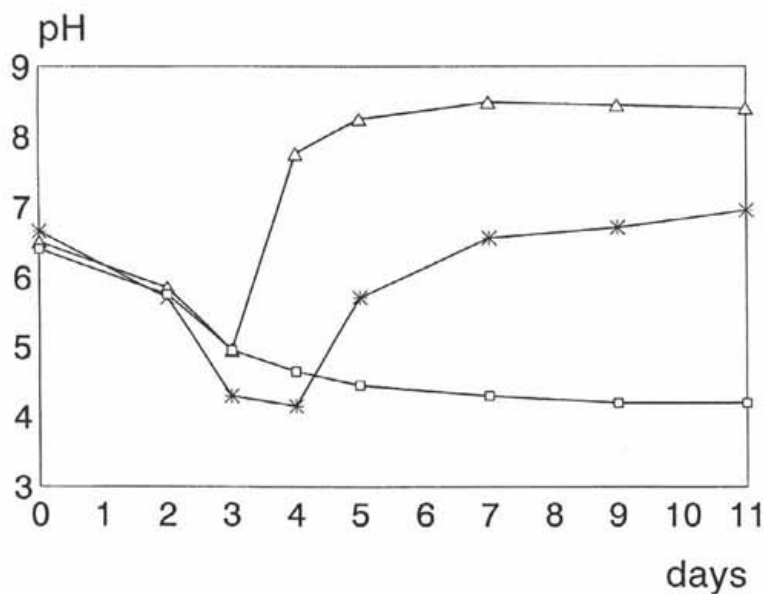

Fig. 1A. pH of the cultivation fluid during growth on glucose-peptone media.
X-axis: time, Y-axis: pH of the cultivation fluid. Squares, asterisks and triangles: medium nrs.1, 2 and 3, respectively.

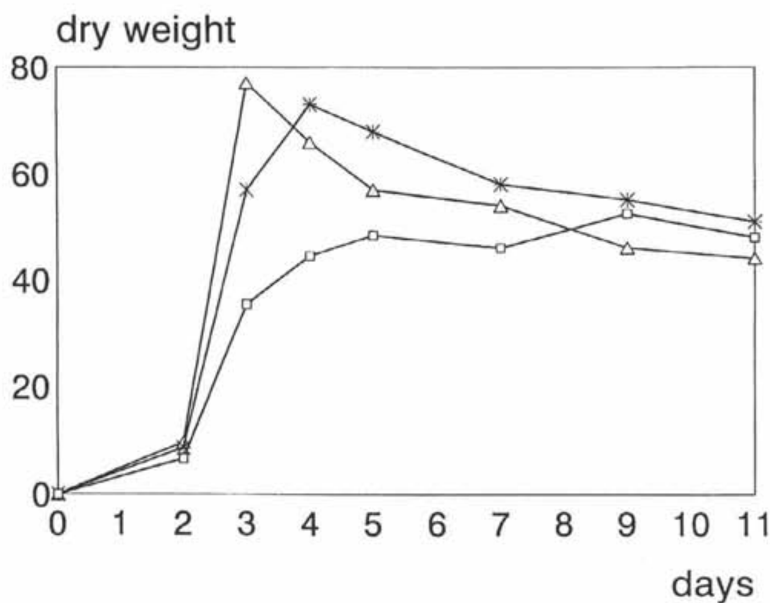

Fig. 1B. Growth of *Aspergillus fumigatus* on glucose-peptone media.
Y-axis: dry weight of the mycelium in milligrams per culture. For legend see Fig. 1A.

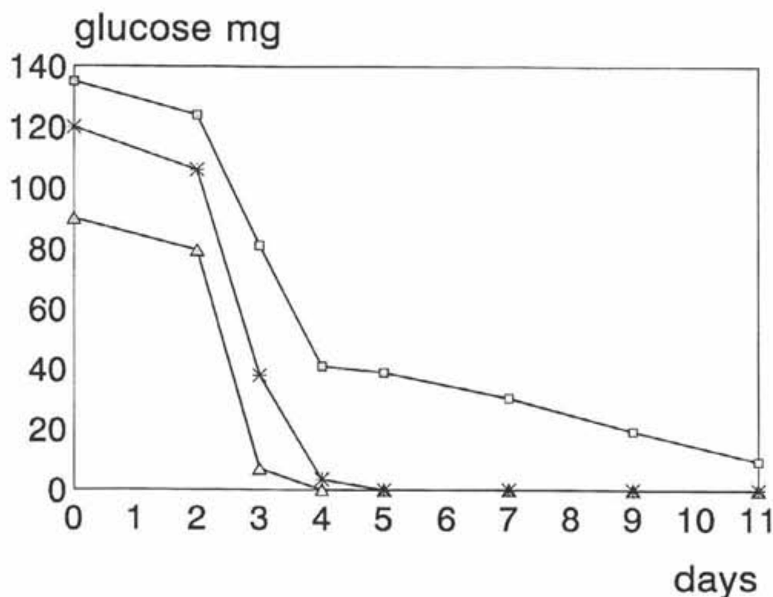

Fig. 2A. Utilization of glucose during growth on glucose-peptone media.
Y-axis: content of glucose in milligrams per culture. For legend see Fig. 1A.

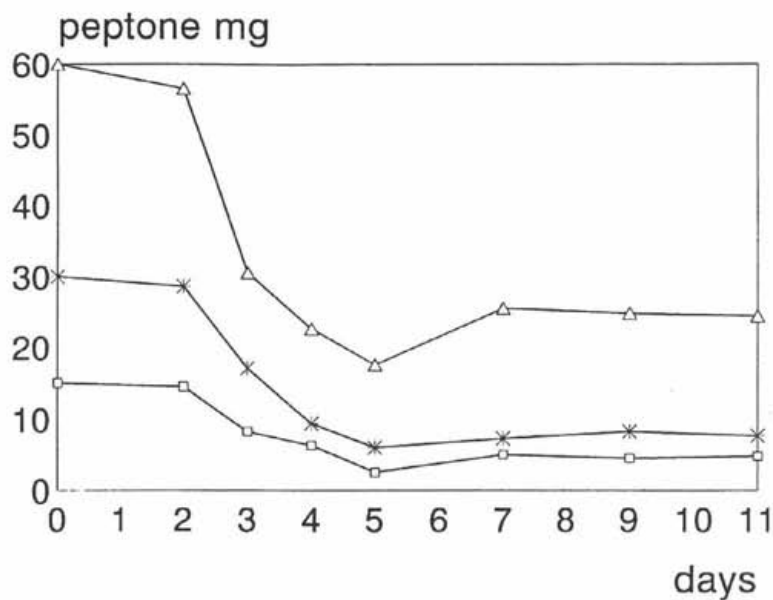

Fig. 2B. Utilization of peptone during growth on glucose-peptone media.
Y-axis: content of peptone (Lowry-positive substances) in milligrams per culture. For legend see Fig. 1A.

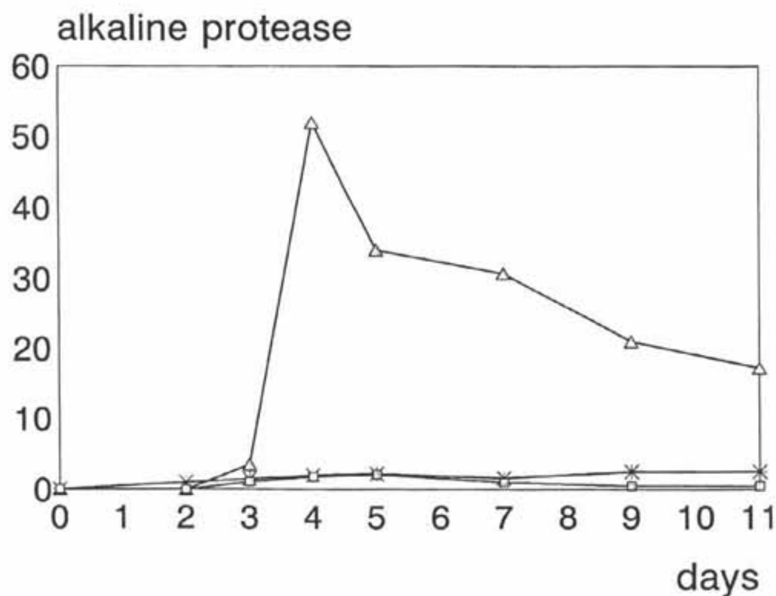

Fig. 3A. Activity of alkaline protease in the cultivation fluid.
Y-axis: activity of alkaline protease in arbitrary units. For legend see Fig. 1A.

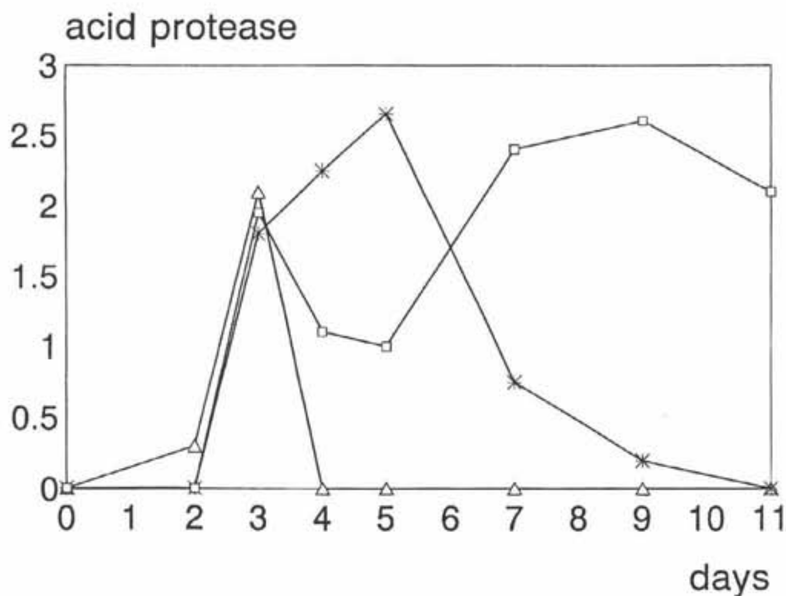

Fig. 3B. Activity of acid protease in the cultivation fluid.
Y-axis: activity of acid proteinase in arbitrary units. For legend see Fig. 1A.

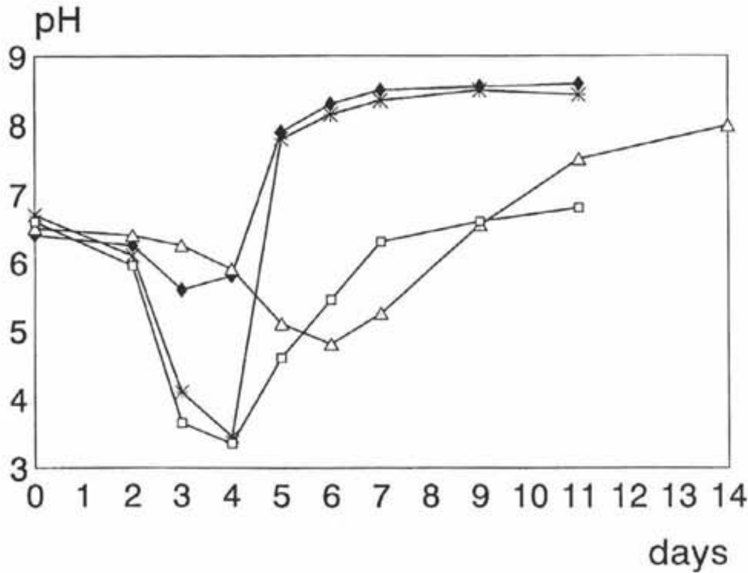

Fig. 4A. pH of the cultivation fluid during growth on media containing glucose and amino acids. X-axis: time, Y-axis: pH of the cultivation fluid. Squares, asterisks, triangles and diamonds: medium nrs. 4, 5, 6 and 7, respectively.

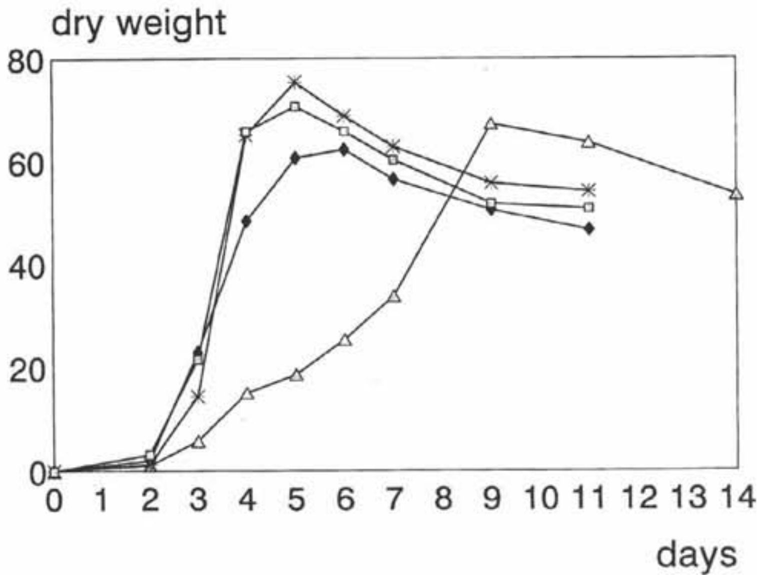

Fig. 4B. Growth of *Aspergillus fumigatus* on media containing glucose and amino acids. Y-axis: dry weight of the mycelium in milligrams per culture. For legend see Fig. 4A.

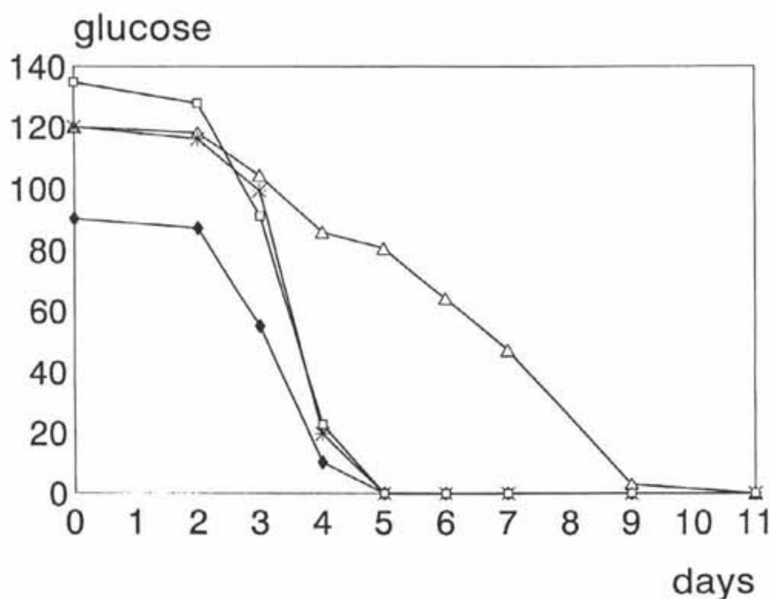

Fig. 5A. Utilization of glucose during growth on media containing glucose and amino acids. Y-axis: content of glucose in milligrams per culture. For legend see Fig. 4A.

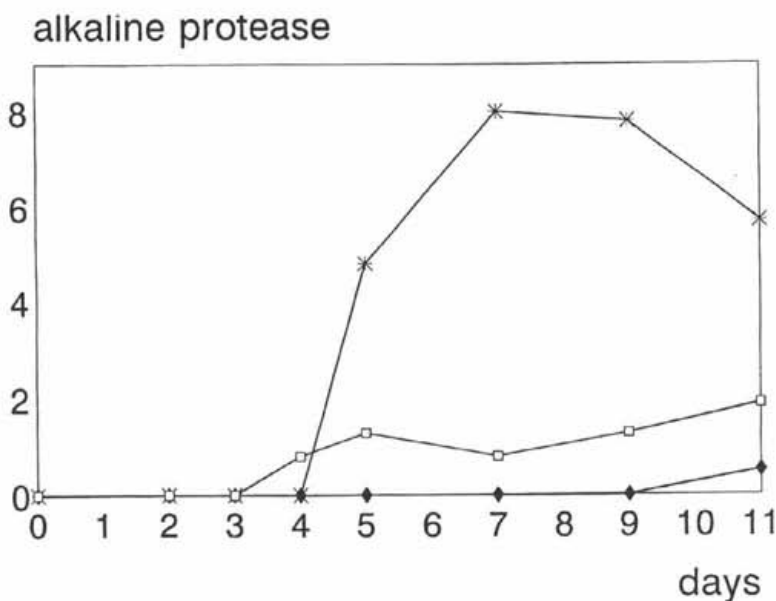

Fig. 6A. Activity of alkaline protease in the cultivation fluid.
Y-axis: activity of alkaline protease in arbitrary units. For legend see Fig. 4A.

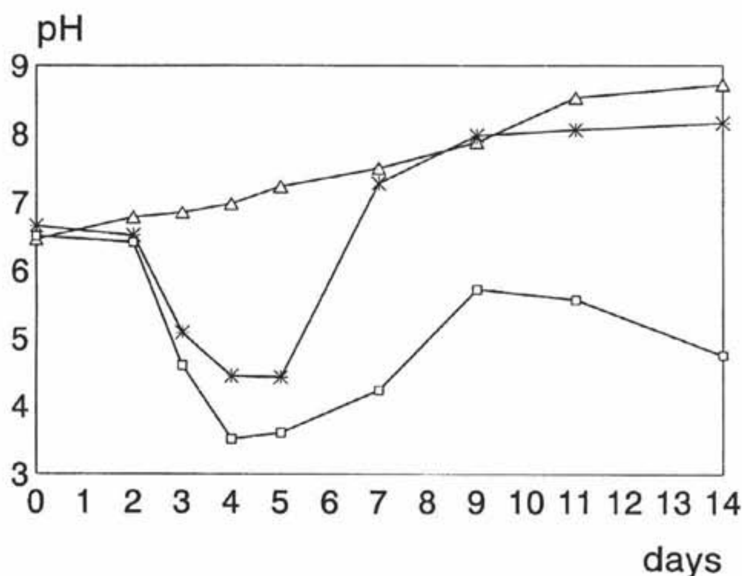

Fig. 7A. pH of the cultivation fluid during growth on media containing inorganic sources of nitrogen.
X-axis: time, Y-axis: pH of the cultivation fluid. Squares, asterisks and triangles: medium nrs. 8, 9 and 10, respectively.

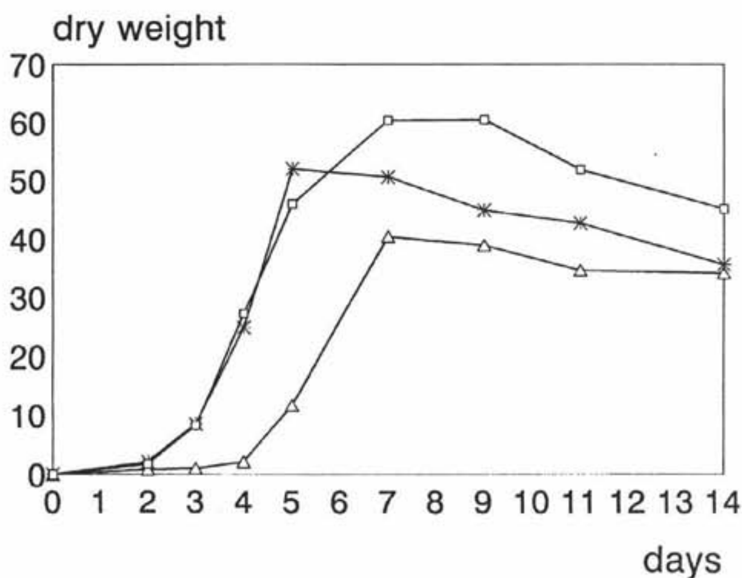

Fig. 7B. Growth of *Aspergillus fumigatus* on media containing inorganic sources of nitrogen.
Y-axis: dry weight of the mycelium in milligrams per culture. For legend see Fig. 7A.

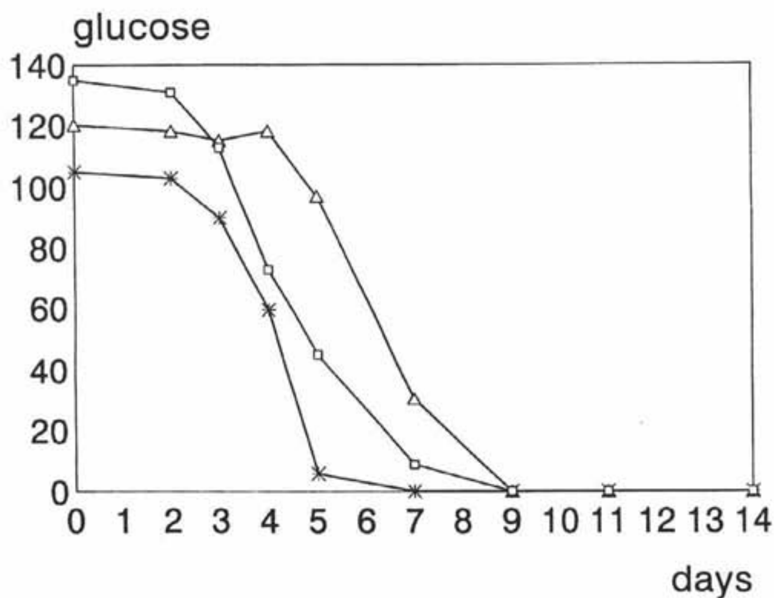

Fig. 8A. Utilization of glucose during growth on media containing inorganic sources of nitrogen. Y-axis: content of glucose in milligrams per culture. For legend see Fig. 7A.

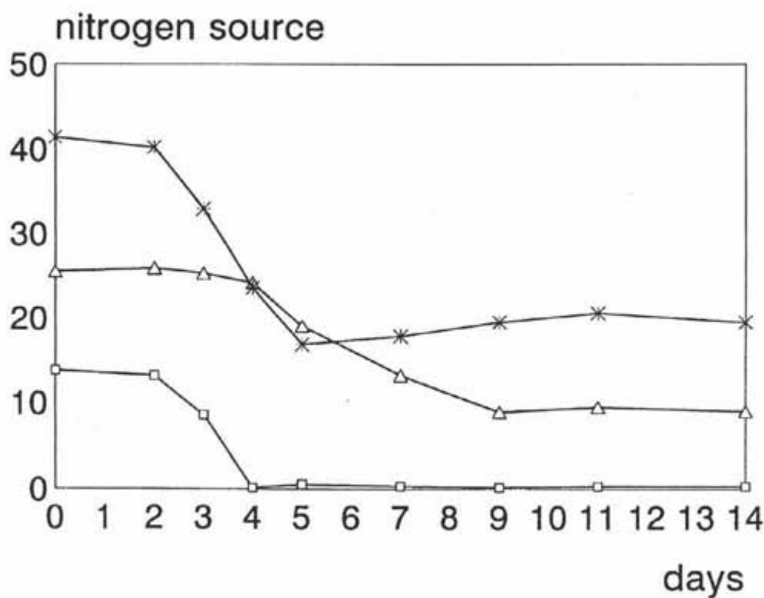

Fig. 8B. Utilization of the inorganic sources of nitrogen.
Y-axis: content of N-source in milligrams per culture. For legend see Fig. 7A.

the results of thin-layer chromatography, glutamine was consumed at the same time (4th to 5th day) from both media. The increase in pH in the autolytic phase was, however, much faster and higher in medium nr. 5 containing more glutamine. A well measurable activity of alkaline protease (up to 8 U, Fig. 6) also appeared on the latter medium only. The activity of acid protease was negligible.

On medium nr. 6 serine was used as the source of nitrogen. This amino acid supported only slow growth (Fig. 4B). Maximum dry weight was reached on the 9th day and by the same time glucose (Fig. 5) and also serine (according to the results of chromatography) were exhausted. The pH of the cultivation fluid decreased to below 5 during active growth but later increased to up to 8 (Fig. 4A). The proteolytic activities were mostly non-detectable, although a small activity of alkaline protease was present as late as in the autolytic phase (Fig. 6).

On medium nr. 7 a mixture of five amino acids (with a total nitrogen content comparable to 0.4 % peptone, cf. medium nr. 3) was used as nitrogen source. Compared to the glucose-peptone medium nr. 3 the growth was slower and the maximum dry weight about 16 % lower (Fig. 4B). Glucose and all amino acids were exhausted by the end of the exponential phase (5th day, Fig. 5). The dynamics of medium pH is shown in Fig. 4A. Proteolytic activities in the cultivation fluid were mostly non-measurable; a small activity of alkaline protease appeared at the end of the experiment (Fig. 6).

Ammonium tartrate (in concentrations of 0.1 and 0.2 %) was used as an inorganic source of nitrogen in media nrs. 8 and 9. In the presence of glucose it enabled medium growth with a maximum dry weight smaller than that on similar media with peptone (Fig. 7B). The cultures on medium nr. 9 with a higher ammonium tartrate content showed faster glucose utilization (Fig. 8A), a rather lower maximum dry weight and a smaller decrease in pH of the medium (Fig. 7A). Ammonium ions were not exhausted at the time of glucose consumption and their concentration increased again in the autolytic phase (Fig. 8B), simultaneously with the fast alkalinization of the cultivation fluid (Fig. 7A). On medium nr. 8 with only 0.1 % ammonium tartrate the development of the cultures was slower (Fig. 7B). Ammonium ions were completely consumed during the exponential phase of the growth and did not appear again in the autolysis (Fig. 8B). The increase in pH of the medium after the cessation of the growth was less pronounced (Fig. 7 A). A small activity (1.8 U at the maximum) of alkaline protease appeared as late as in the autolytic phase on medium nr. 9. There was no detectable activity of acid protease.

The other inorganic N source, sodium nitrate, proved to be a poor nutrient. The growth on medium nr. 10 was rather poor, with a long lag phase and a low maximum dry weight. The stationary phase was long and autolysis slow (Fig. 7B). Glucose was exhausted between the 7th and 9th day of growth, nitrate ions were taken up until the 9th day. About 40 % of nitrate remained in the medium until

the end of the experiment (Fig. 8A,B). The growth was in all phases accompanied by an alkalinization of the medium (Fig. 7A). No activity of the acid protease could be measured, a low (1-2 U) activity of alkaline protease was found in old cultures only.

DISCUSSION

The media used differed in their ability to support growth of the fungus. Best growth was attained on glucose-peptone media (nrs. 2,3) an on medium nr. 5 with glucose and glutamine. Poor media were those with an inorganic source of nitrogen and medium nr. 1 with a lowered content of peptone. The complex nitrogen source (peptone) was better than pure substances including amino acids used individually or in combination.

On five media the growth was terminated by simultaneous exhaustion of both the C and N sources, on media nrs. 3,9 and 10 by exhaustion of the C source (glucose) only. On media nrs. 1 and 8 the N source was consumed first. When glucose was still present in the stationary phase, it continued to be slowly utilized, probably for the growth of the surviving part of the mycelium. Here, substances released from the autolyzing part could have been used as the source of nitrogen. In this way all glucose was consumed during culture development on all media except nr. 1. When peptone was present in excess, it similarly continued to be taken up in the stationary phase after the exhaustion of glucose (medium nr. 3). It might have been utilized as a source of both carbon and nitrogen. A complete exhaustion of peptone from glucose-peptone media could not be determined with certainty because of the release of interfering Lowry-positive substances by the mycelium in the stationary and autolytic phases.

Active growth in the exponential phase was accompanied by an acidification of the medium. Its intensity depended on the C/N ratio, being highest when glucose was present in excess (media nrs. 1,2 and 8, pH as low as 3.5). Thus, acidification of the medium was probably due to the production of organic acids from the catabolism of glucose. Deamination of amino acids and peptides, leading to ammonia production, had an opposite effect. Exceptional was the course of pH on medium nr. 10, where uptake of nitrate ions from sodium nitrate led to the production of sodium hydroxide. The pH values always (with the exception of medium nr. 1 with excess glucose) rose during the stationary and autolytic phases. This was probably due to the release of ammonia during autolysis (cf. concentrations of ammonium ions measured in medium nr. 9, Fig. 8 B). An excess of nitrogen sources stimulated the pH increase. On glucose-peptone media nrs. 2 and 3 the start of medium alkalinization, glucose exhaustion and reaching the maximum dry weight coincided, on the remaining media the rise in pH had already begun in the late exponential phase.

The goal of the present study was to investigate the conditions for the production of extracellular proteolytic enzymes. In experiments with the transfer of growing mycelium to a fresh medium lacking C, N or S sources, this production in aspergilli is triggered by simple derepression (see Introduction). The situation during continuous growth on one medium is obviously different. In our experiments high activities (up to 50 U) of alkaline protease(s) were found only on medium nr. 3 containing substantial amounts of peptone. Medium activities (8 U at maximum) were present on medium nr. 5 containing 0.8 % glucose and 0.2 % glutamine. On both media the protease was induced in the late exponential phase and peaked early in the stationary phase. On the remaining media the activities were low (less than 3 U) and appeared later, often as late as in the autolytic phase.

In general it can be summarized that a high activity of alkaline protease in the media appeared only when (a) glucose had been consumed from the medium, (b) the maximum dry weight of mycelium had been reached, and (c) the pH of the cultivation fluid had risen to about 7 or higher. As stated above, these three conditions are causally related and usually coincide in time. It is thus difficult to analyze them separately. An exhaustion of glucose (the C source) was a necessary condition for the production of alkaline protease(s), in accordance with literature data on the absence of proteolytic activity on media rich in glucose (Klapper et al. 1973, Srinivasan and Dhar 1990, Monod et al. 1991, Jaton-Ogay et al. 1992, Bouchara et al. 1993, Katz et al. 1994, Tomee et al. 1994). In contrast, some authors found the production of proteases on media containing glucose (Reichard et al. 1990, Frosco et al. 1992, Larcher et al. 1992, Moutaouakil et al. 1993, Tronchin et al. 1993). However, these authors did not measure the concentration of glucose in the medium at the moment of protease induction. Consumption of glucose before or simultaneously with the N source did not always lead to the production of protease so that glucose exhaustion is not the only decisive factor.

Similarly, an exhaustion of the source of nitrogen before that of C source (on media nrs. 1 and 8) itself was not sufficient for the induction of protease. The role of peptone in the synthesis of extracellular proteases of *A. fumigatus* may be dual: it is stimulating in lower amounts and inhibiting in higher (0.5 to 1 %) concentrations (Monod et al. 1991, Jaton-Ogay et al. 1992, Larcher et al. 1992, Bouchara et al. 1993, Moutaouakil et al. 1993, Tronchin et al. 1993). The (poly)peptides present act probably as inducers, while ammonium ions are strong repressors (Bouchara et al. 1993).

The production of proteases in the stationary phase after cessation of the growth is a typical phenomenon in fungi. It is, however, hard to say if the main decisive factor is the derepression by the exhaustion of C and/or N sources or if other factors are involved. Increase in pH of the medium to 7 and more (where the alkaline protease of *A. fumigatus* has its optimum) is probably a necessary condition. According to our results, an early and sharp increase in pH is highly

inducing, whereas alkalinization that is slow or shifted to the autolytic phase is usually ineffective. A neutral to alkaline pH was found to be a condition for the production of alkaline proteases by Moutaouakil et al. (1993), too. The results published by Reichard et al. (1990) also point to the importance of the medium pH for the production of proteases.

Some of our results indicate that for the secretion of protease(s) the presence of at least some proteins and/or peptides in the medium is needed in addition to the above mentioned three factors. High protease activities were found only on medium nr. 3 containing well measurable amounts of peptide (Lowry-positive) compounds in the stationary phase while on the similar medium nr. 2 where these compounds were consumed the protease was not produced. In media nr. 7 (with amino acids) and 10 (with sodium nitrate) the three mentioned conditions were fulfilled but in the absence of proteins no proteases were secreted. However, the proteolytic activity was present on glucose-glutamine medium nr. 5 which contained no proteins either. An unusually fast increase in pH (from 3.5 to 7.8 in 24 hours) could be the triggering factor here. Concluding, the presence of proteins and peptides is at least stimulating for the production of proteases in our strain of *A. fumigatus*. The proteolytic activity on media containing proteins is the subject of a following paper (Kunert, in preparation).

The acid (aspartic) protease is an important enzyme in *A. fumigatus*, as shown by the experiments of Reichard et al. (1990, 1994) and Lee and Kolattukudy (1995). These experiments were, however, done on media containing proteins. In the present study acid protease could be found only on glucose-peptone media and its activity was in itself small. Its production was obviously pH-dependent: the activity was present in media with a pH in the acid range (4 to 6) and disappeared in neutral to alkaline cultivation fluid. Acidity of the medium was also postulated by Jarai and Buxton (1994) as the decisive factor. The production of acid protease only on media containing peptone may indicate the role of proteins and/or peptides as inducers.

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