IMMUNOLOGY AND MICROBIOLOGY

Characteristics of Collagenolytic Enzymes Secreted by Deuteromycete Fungi *Aspergillus Flavus*

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Collagen induced the synthesis of collagenolytic enzymes in the culture of *Aspergillus flavus*. Enzyme activity of the culture increased during storage and passage on a medium with this inductor. We developed a method for isolation and purification of collagenolytic enzymes and obtained two electrophoretically homogenous enzyme preparations belonging to neutral thermolabile collagenolytic metalloproteinases.

Key Words: *collagenolytic enzymes of A. flavus; induction; affinity chromatography; EDTA action*

Proteolytic enzymes are widely used in medical practice and scientific studies. For example, some collagenases can be used for removal of scars and keloids and healing of burns and ulcers [1].

Much attention is given to the isolation of collagenolytic enzymes from king crab hepatopancreas [3]. It should be emphasized that enzyme preparations of microbial origin have several advantages: availability and low cost of raw materials, inexhaustible natural sources, and simple procedures of isolation and purification of the final product (compared to animal tissues). Published data suggest that several deuteromycetes produce and secret various proteolytic enzymes. Their synthesis can be inducible [2] or constitutive [4]. The general mechanisms underlying exogenous regulation of enzyme synthesis remain unknown. It is necessary to develop a method for isolation and purification of enzyme preparations with collagenolytic activity from individual microorganisms, bearing in mind that enzymes are usually isolated from multicomponent complexes.

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Here we studied collagenolytic enzymes secreted by deuteromycete fungi *A. flavus*.

MATERIALS AND METHODS

We used a culture of deuteromycetes *A. flavus*. The suspension of spores from 7-day-old cultures (passages 1 and 2) grown on agarized Czapek medium (pH 6.8) with 2% sucrose (control) or 2% collagen (inductor) served as the inoculum. The culture was stored at 4°C under mineral oil.

Submerged culturing was performed at 26°C. The medium contained saline of Czapek medium, 0.5% sucrose, and 1.5% collagen.

For evaluation of collagenolytic activity filtered culture medium obtained at various stages of culturing was added to 1% collagen suspension in 0.01 M phosphate buffer with 0.2 μM $CaCl_2$ (pH 7.4) and incubated at 37°C for 24 h. Centrifugation was performed at 6000 rpm for 60 min. The total amount of free amino acid α-amino groups in the supernatant was determined by the ninhydrin method [6]. Specific collagenolytic activity was estimated by the total amount of free amino acid α-amino groups (μmol) per 1 μg protein.

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Protein content was measured by the method of Lowry. Carbohydrate concentration in the culture medium was evaluated by the anthrone method. After gel filtration on an LKB column (12×150 mm) packed with TOYOPEARL HW-40 gel the material with collagenolytic activity was collected and purified by affinity chromatography. The saline-soluble fraction of collagen was used to obtain the affinity sorbent. The ligand was routinely immobilized on CNBr-activated Sepharose 4B.

The purity of the obtained preparation was determined by disc electrophoresis in polyacrylamide gel. Amino acid assay was performed on an amino acid analyzer (LKB) using a sodium citrate gradient. The amount of amino acid residues was expressed in mole percents.

Enzyme activity was measured using 0.01 M phosphate buffered saline with 0.2 μM $CaCl_2$ at pH 5.8, 6.4, 7.0, 7.4, and 8.0.

Thermostability of the enzyme preparations was estimated by activity of the enzyme after incubation in 0.01 M phosphate buffer with 0.2 μM $CaCl_2$ at 20, 30, 40, 50, and 60°C and pH 7.4 for 60 min.

For evaluation of the effect of metalloproteinases inhibitor EDTA on collagenolytic activity of *A. flavus* surface culturing on agarized Czapek medium with 2% collagen was used. The content of EDTA in the medium varied from 5×10^{-4} to 2×10^{2} M. The control medium contained no EDTA. The culture was inoculated into the agarized medium in three pricks. Activity of secreted proteinases was determined by the index of collagen lysis calculated as the ratio of lysis area to colony area:

$$i_{lys}=R^2_{lys}/R^2_{col},$$

where R_{lys} and R_{col} are radii of the lytic zone and colonies, respectively [5].

The results were analyzed by Student's test for small samples. The data are expressed as $\overline{X}\pm\sigma$.

TABLE 1. Amino Acid Composition of Protein Samples 1 and 2 Isolated from *A. flavus* Culture Medium

Amino acid	Amino acid content, %*	
	enzyme 1	enzyme 2
Aspartic acid	5.91	4.91
Threonine	2.18	2.32
Serine	5.24	4.02
Glutamic acid	6.86	7.79
Proline	2.59	2.67
Glycine	10.6	12.90
Alanine	4.96	4.38
Valine	6.40	5.62
Methionine	1.42	0.24
Isoleucine	4.79	6.03
Leucine	30.9	35.20
Tyrosine	0.8	0.3
Phenylalanine	1.94	0.89
Histidine and lysine	10.8	8.2
Arginine	4.42	4.45

Note. *mole percents.

RESULTS

The composition of the nutrient medium can modulate the synthesis of biologically active substances in micro-

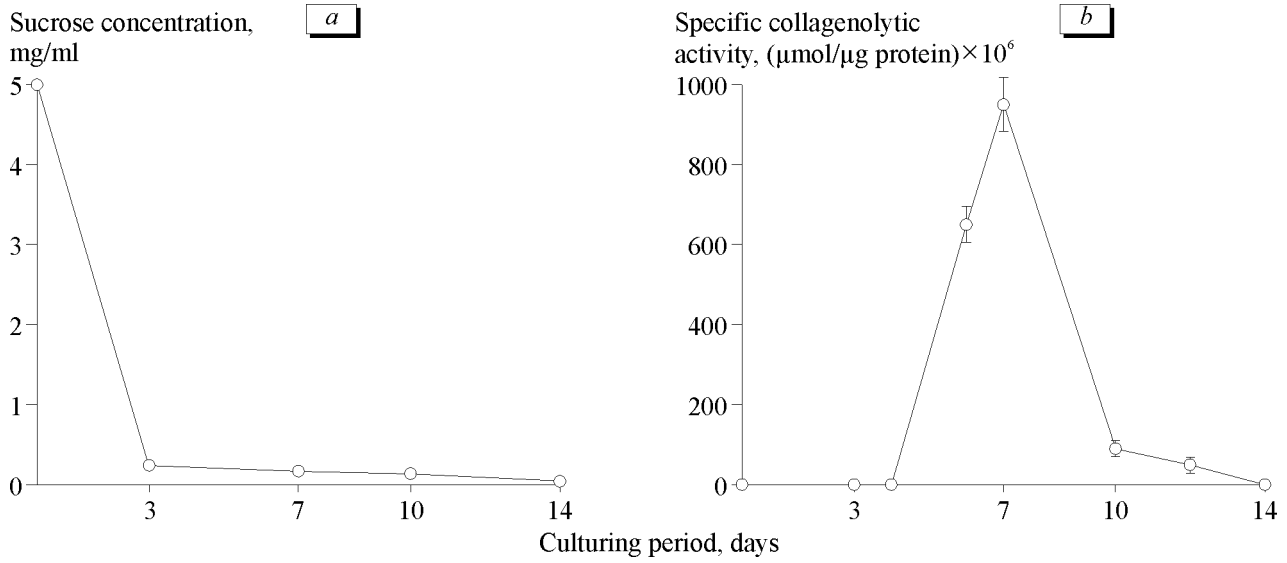

Fig. 1. Sucrose content (*a*) and activity of collagenolytic enzymes (*b*) secreted during culturing of *A. flavus* in Czapek medium with 0.5% sucrose and 1.5 % collagen.

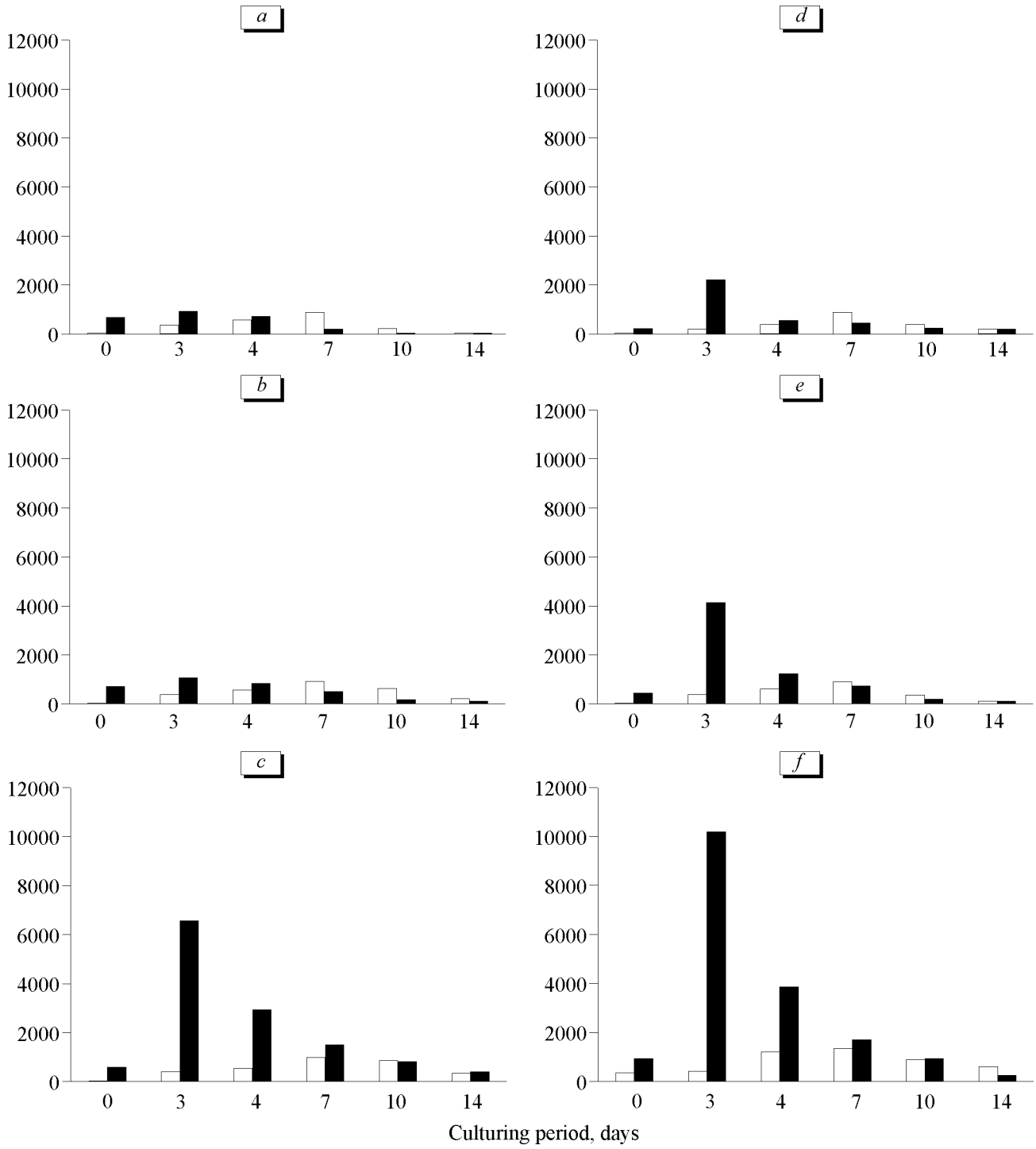

Fig. 2. Collagenolytic activity of enzymes secreted by the culture of *A. flavus* of passages 1 (*a*, *b*, *c*) and 2 (*d*, *e*, *f*) depending on the period of storage in the medium with (dark bars) or without collagen (light bars). Storage of *A. flavus* in the medium for 6 (*a*, *d*), 14 (*b*, *e*), and 21 months (*c*, *f*). Ordinate: specific collagenolytic activity, (μmol/μg)×10^6.

bial cultures. We evaluated the effect of the nutrient medium on the synthesis of collagenolytic enzymes by *A. flavus*. Micromycetes did not secrete collagenolytic enzymes during submerged culturing on Czapek medium. Then we used a nutrient medium containing 0.5% sucrose and 1.5% collagen as the sources of carbon. We assumed that sucrose and collagen should stimulate the growth of deuteromycetes and synthesis of collagenolytic enzymes, respectively.

Sucrose content in the culture medium decreased by 95% after submerged culturing of *A. flavus* for 3 days (Fig. 1, *a*). Then secretion of collagenolytic en-

TABLE 2. Lysis Index of *A. flavus* Culture in Collagen-Containing Czapek Medium with EDTA in Various Concentrations

Culturing period, days	Index of lysis in the presence of EDTA, M					
	0	0.0005	0.001	0.005	0.01	0.02
4	2.51±0.12	2.36±0.11	2.12±0.12*	1.84±0.11*	0	0
7	2.63±0.16	2.44±0.17	2.15±0.10*	1.82±0.09*	0	0

Note. *p<0.05 compared to the control.

zymes increased (Fig. 1, *b*). Our results show that partial substitution of sucrose for collagen in Czapek medium induced the synthesis of collagenolytic enzymes.

The composition of agarized media during storage also can affect biochemical properties of microorganisms. We compared collagenolytic activity of *A. flavus* stored in media with and without collagen (Fig. 2, *a*, *b*, *c*). After storage of *A. flavus* in a medium with collagen for 6, 14, and 21 months the period of fermentation decreased from 7 to 3 days. Under these conditions maximum collagenolytic activity of secreted enzymes increased by 7.5 times (compared to storage in the standard medium).

During storage deuteromycetes were subjected to periodic reinoculations. It was interesting to evaluate whether the inducing effect is preserved after repeated passages. The storage of passage 2 cultures in the medium with collagen increased the maximum level of collagenolytic activity (compared to passage 1 cultures in the same period, Fig. 2, *d*, *e*, *f*).

Collagenolytic enzymes secreted by *A. flavus* into the medium were isolated and purified by gel filtration and affinity chromatography on the synthesized sorbent. The proposed scheme for purification allowed us to obtain two electrophoretically homogenous enzyme preparations with molecular weights of 21.00±1.05 and 24.0±1.2 kDa. These preparations were purified by 34 and 69 times, respectively.

Amino acid analysis of these preparations revealed their high homology (Table 1). However, they differed in the content of some amino acids (phenylalanine, methionine, and tyrosine).

Collagenolytic activity of enzymes was minimum at acid pH (Fig. 3, *a*) and increased with pH increase toward the neutral range. Enzyme activity slightly decreased at pH>7.4. Collagenolytic activity of enzymes remained unchanged at 20-30°C (Fig. 3, *b*). Heating from 40 to 60°C sharply decreased enzyme activity.

The index of collagen lysis decreased with increasing EDTA concentration in the agarized medium. Therefore, EDTA reduced activity of collagenolytic enzymes secreted by *A. flavus* (Table 2).

Our results show that partial substitution of sucrose for collagen during submerged culturing of *A. flavus* in Czapek medium stimulated synthesis of collagenolytic enzymes. Collagen induced the synthesis collagenolytic enzymes by *A. flavus* culture. Enzyme activity of the culture increased during storage and passage in col-

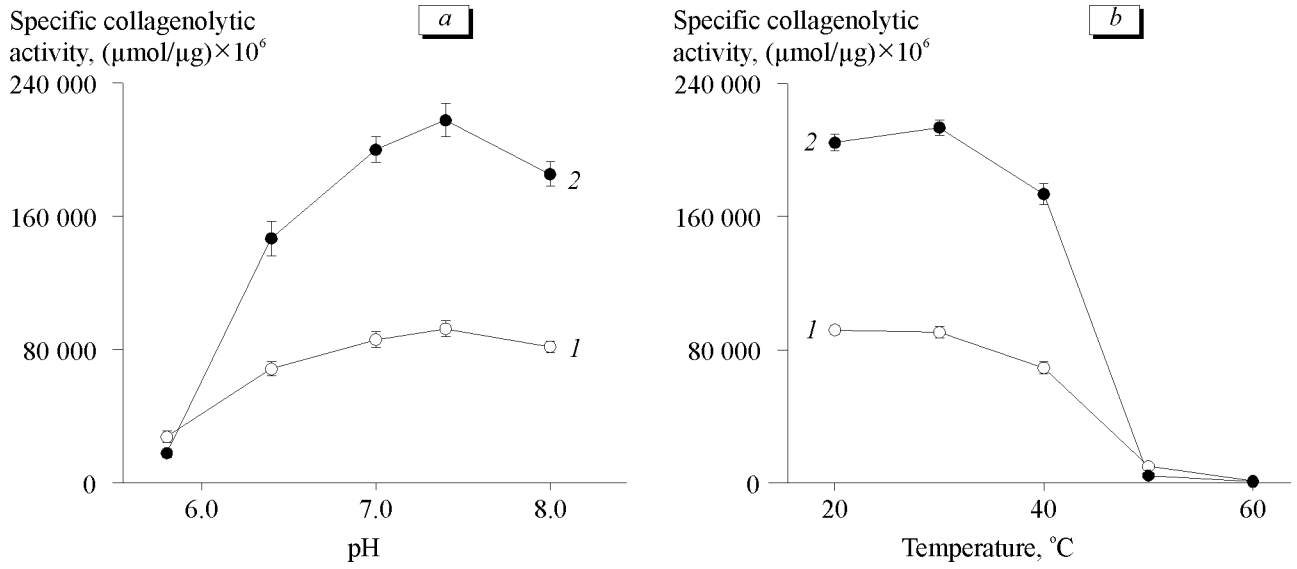

Fig. 3. Collagenolytic activity of enzyme preparations at various pH (*a*) and after incubation under different temperature conditions (*b*): enzymes 1 (*1*) and 2 (*2*).

lagen-containing medium. We developed a method for isolation and purification of enzymes from *A. flavus* culture medium. Studies of physicochemical properties of these enzymes indicate that they belong to neutral thermolabile collagenolytic metalloproteinases.

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