

CHAPTER 34

Thermoacidophilic Extracellular α -Amylase of *Mucor pusillus*

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The thermophilic mold *Mucor pusillus* produces an extracellular thermoacidophilic α -amylase. Maximum enzyme yields were attained after 7 to 8 days of growth in liquid media at 37 C. The amylase was isolated and purified 55-fold by ultrafiltration, gel filtration, and diethylaminoethyl-cellulose ion exchange chromatography and appeared homogeneous on discontinuous polyacrylamide disc gel electrophoresis. Carbohydrate content of the glycoprotein enzyme was 8.7%. The mol wt was estimated to be 48,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimal pH and temp ranges for enzyme activity were 3.5 to 4.0 and 65 to 70 C, respectively. Amylase activity was inhibited by Ca^{2+} and Cu^{2+} . Ethylenediaminetetraacetic acid and thiol reagents had no effect upon enzyme activity. The K_m for starch was 0.54 mg/ml. The amylolytic pattern consisted of a mixture of glucose, maltose, maltotriose, maltotetraose, and maltopentose. The enzyme also hydrolyzed cyclic oligosaccharides.

INTRODUCTION

Thermophilic molds have been reported to synthesize a variety of extracellular enzymes (Barnett and Fergus 1971; Chapman et al. 1975; Cooney and Emerson 1964; Fergus 1969a; Voordouw et al. 1974). *Mucor pusillus*, a thermophilic mold, was found to be the source of several extracellular enzymes, including acid protease (Somkuti and Babel 1968), lipase (Somkuti et al. 1969), and cellulase (Somkuti 1974). Sorensen and Crisan (1974) reported on a thermostable membrane-bound β -galactosidase in *M. pusillus*. The production of extracellular amylase by *M. pusillus* was noted first by Fergus (1969b). Adams and Deploey (1976) studied the effect of different carbon sources on amylase and biomass production by this mold. The persistent presence of amylase in *M. pusillus* milk-clotting protease preparations also was observed by deKoning et al. (1969), who suggested that such content may be useful in differentiating the *Mucor* rennet from other types of microbial rennet.

Since high temp and acidic pH are optimum conditions for the economic hydrolysis of starch, the use of thermostable and acid-stable amylases of microbial origin for industrial purposes has been recommended (Takagi et al. 1971). Thermostable bacterial amylases that have been isolated and extensively studied include enzymes from *Bacillus stearothermophilus* (Manning and Campbell 1961), *B. macerans* (DePinto and Campbell 1968), *B. licheniformis* (Saito 1973), *B. acidocaldarius* (Buonocore et al. 1976), *Thermomonospora vulgaris* (Allam et al. 1975), and *T. curvata* (Glymph and Stutzenberger 1977). However, information on thermostable fungal amylases is limited to one report in the literature (Day 1978).

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This study is concerned with the synthesis, purification, and characterization of a thermoacidophilic amylase from *M. pusillus*.

MATERIALS AND METHODS

Selection and maintenance of microorganisms. Strains of *Mucor pusillus* originated from the following sources: *M. pusillus* 410, Purdue University Culture Collection; *M. pusillus* 6 and 8, D. J. Cooney, University of Nevada; *M. pusillus* 1426, 3638, and 3639, J. J. Ellis, U.S. Department of Agriculture. Stock cultures were incubated on 10% (w/v) potato malt agar (Difco) at 37 C for 72 h, followed by storage at 4 C. Fresh transfers of stock cultures were made weekly.

To isolate the strain producing the highest level of extracellular amylase, cultures were grown in a 4% (w/v) wheat bran infusion medium prepared as described by Somkuti (1974). The volume of medium per 500-ml Erlenmeyer flask was 125 ml. Incubation was at 34 C for 7 days on a Psychrotherm G26 incubator shaker (New Brunswick Scientific Co., Inc.)¹ set at 230 rpm. Aliquots (10 ml) were withdrawn daily, filtered through Whatman No. 2 filter paper, and assayed for amylase activity.

Enzyme production. *M. pusillus* 3638 was selected for further studies. The enzyme production (EP) medium contained (per liter) the following components: starch (J. T. Baker, Phillipsburg, NJ), 15 g; K_2HPO_4 , 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; neopeptone (Difco), 3 g; $FeSO_4 \cdot 7H_2O$, 1.0 mg; $MnCl_2 \cdot 4H_2O$, 1.0 mg; $ZnSO_4 \cdot 7H_2O$, 1.0 mg; and $CaCl_2$, 1.0 mg. The pH before autoclaving was 7.4. The medium (800 ml/2-liter Erlenmeyer flask) was inoculated with a spore suspension of *M. pusillus* 3638 and incubated under conditions described above.

Purification of the enzyme. Mycelial growth was removed by filtration through gauze. The filtrate was clarified by centrifugation at 30,000 g at 4 C for 10 min. The clear liquor (6.5 liters) was concd to 80 ml by ultrafiltration under nitrogen pressure (1.0 kg/cm²) with an Amicon model 401S ultrafiltration cell with a UM-10 membrane. The concd crude enzyme was dialyzed for 16 h at 4 C against 0.02 M ammonium acetate buffer, pH 4.5. A 10-ml portion of the dialyzate (230 mg protein) was applied to a Sephadex G-100 (Pharmacia; superfine grade) gel column (5.0 × 75 cm) equilibrated previously at 4 C with the above buffer and eluted with the same buffer. Fractions (8 ml) were collected at a flow rate of 20 ml/h. Fractions containing amylase activity were combined, adjusted to pH 7.5 with 1 N NaOH and applied to diethylaminoethyl (DEAE)-cellulose (Whatman, DE52), equilibrated with 0.02 M Tris (hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (pH 7.5) in a column (1.0 × 16 cm) at 6 C. Fractions (3 ml) were collected during elution with a linear NaCl gradient (0–0.3 M) in buffer, in a total vol of 250 ml. A 0.05-ml aliquot of each fraction was checked for amylase activity. Active fractions from several trials were pooled, dialyzed against 0.02 M Tris (pH 7.5) and rechromatographed on DEAE-cellulose according to conditions described above. The final preparation was concd in an ultrafiltration unit (Amicon, model 52) with a UM-10 membrane.

Protein and enzyme determinations. Protein concn was determined by the Lowry method (Lowry et al. 1951), with bovine serum albumin as a standard. Amylase activity was assayed by the 2,4-dinitrosalicylic acid (DNS) method of Bernfeld (1955). Enzyme in 1 ml vol was incubated with 5 ml of 0.5% starch in 0.1 M sodium acetate, pH 4.0, for 10 min at 37 C. The enzyme reaction was terminated by mixing 1-ml aliquots with equal volumes of DNS reagent, followed by heating in a boiling water bath for 10 min. The samples were transferred to an ice bath for 10 min and diluted with 5 ml of distilled water. Absorbance at 540 nm was determined in a Zeiss PMQII spectrophotometer. One unit of amylase activity is defined as the amount of enzyme that releases 1 μ g of maltose in 1 min at 37 C under our experimental conditions. Specific activity is expressed as units per milligram of protein.

Disc gel electrophoresis. Polyacrylamide gels prepared according to the procedure of Davis (1964) were used in discontinuous electrophoresis equipment (Buchler Instruments). Standard 7% gels were prepared in tubes (0.57 \times 8 cm) and run at pH 8.3. Samples (0.02 ml) containing approx. 50 μ g of protein were mixed with a 40% sucrose solution. A 1-mA current was applied to each tube until the bromphenol blue tracking dye entered the separating gel. The current was then raised to 4 mA per tube and electrophoresis was continued for 60 min. Gels were stained with Coomassie brilliant blue R250 (Eastman Kodak) and destained according to the procedure of Weber and Osborn (1969). Gels were also stained for the presence of carbohydrate by the procedure of Clarke (1964).

Molecular weight determination and amino acid analysis. The sodium dodecyl sulfate (SDS)-polyacrylamide gel method of Weber and Osborn (1969) was used. Protein samples were prepared in 0.01 M sodium phosphate buffer (pH 7.0), containing 0.3% SDS and 0.1% mercaptoethanol and incubated at 37 C for 16 h. A 1-mA current was applied per tube until the tracking dye entered the gel, at which time the current was raised to 6 mA per tube. Gels were stained with Coomassie brilliant blue R250 and destained by diffusion. Proteins of known mol wt, bovine serum albumin, ovalbumin, pepsin, trypsinogen, β -lactoglobulin, and lysozyme served as reference compounds.

Amino acid composition of the purified amylase was determined according to the procedures of Moore and Stein (1963). Samples were hydrolyzed at 110 C for 24 h with 5.7 N HCl containing phenol (10 μ g/ml). Air-oxidation of hydrolyzed samples was carried out before analysis. Tryptophan was determined on separate samples on a 0.9 \times 14 cm basic column at 50 C with 0.35 M sodium citrate buffer, pH 5.26, following protein hydrolysis with mercaptoethanesulfonic acid (Penke et al. 1974).

Amylolytic action pattern and products of hydrolysis. The action of *M. pusillus* amylase on soluble potato starch (J. T. Baker Chemical Co.), amylose (Type III, Sigma Chemical Co.), amylopectin (ICN Pharmaceuticals), maltotriose, maltose, cyclohexaamylose (*cyclo-G₆*), cycloheptaamylose (*cyclo-G₇*), and cyclooctaamylose (*cyclo-G₈*) was evaluated by thin-layer chromatography. Enzyme (175 μ g) was incubated with 45 mg of each substrate in 0.1 M sodium acetate (pH 4.0) in 5 ml total volume, at 37 C. Samples were removed at 15-min intervals, heated in a boiling water bath for 5 min, cooled, deionized with Amberlite MB-3 resin, and spotted on cellulose thin-layer plates. Chromatograms were developed by ascending chromatography in the solvent system

n-butanol-pyridine-water-benzene (5:3:3:0.5, v/v). After two ascents, the chromatograms were dried at room temp and the products of hydrolysis were detected with triphenyltetrazolium chloride (Stahl and Kaltenbach 1965).

The amount of glucose in enzyme digests of maltotriose, *cyclo-G₆*, *cyclo-G₇*, and *cyclo-G₈* was also measured with a coupled hexokinase-glucose-6-phosphate dehydrogenase assay system, as described in Technical Bulletin No. 15-UV of the Sigma Chemical Co. (1978).

All reagents and carbohydrates were of analytical quality. *Cyclo-G₆*, *cyclo-G₇*, and *cyclo-G₈* were gifts from F. W. Parrish.

RESULTS AND DISCUSSION

Enzyme Production

Mucor pusillus 3638 yielded the highest amount of amylase in a group of six cultures, and all subsequent experiments were carried out with this strain. Table 1 shows the synthesis of amylase in EP medium over a period of 8 days. The largest increase in enzyme titer was measured after 24 to 48 h of incubation, with about 57% of the enzyme produced during the first 72-h period. Maximum enzyme levels were reached after 7 to 8 days. This differed from the findings of Adams and Deploey (1976), who reported a loss of amylase activity in culture filtrates of *M. pusillus* after 3 days of growth in a defined liquid medium. The drop in pH after 24 to 48 h of incubation was a characteristic feature of mold growth and amylase production with both wheat bran infusion and EP media.

The effect of different carbon sources on amylase production was tested by replacement of starch with 1.5% of another carbon source in EP medium (Table 2). After 7 days of incubation, the amylase activity of culture filtrates was determined under standard assay conditions. Starch and amylopectin were the most efficient enzyme inducers. Part of the amylase was synthesized constitutively as suggested by the relatively high level of amylase activity measured in glucose medium.

The effect of different nitrogen sources on amylase synthesis was studied by incorporating each nitrogen source into EP medium at 0.4% (w/v) concn. After 5 days, the amylase activity in EP medium with neopeptone and with NaNO₃ was 540 units/ml and 555 units/ml, respectively. Enzyme production was unsatisfactory with either (NH₄)₂SO₄ (40 units/ml) or NH₄NO₃ (60 units/ml) as the source of nitrogen, since in their presence the pH of the medium dropped below 3.0 after 72 h of incubation.

TABLE 1. *Amylase synthesis by Mucor pusillus in EP medium*

Day	pH	Amylase (units/ml)	Specific Activity ^a
1	7.30	5	2
2	5.62	312	226
3	5.92	405	368
4	6.10	453	429
5	6.89	512	540
6	7.24	631	598
7	7.43	672	700
8	7.68	702	740

^a Units per mg of protein.

TABLE 2. Effect of carbon sources on the production of amylase by *M. pusillus*.

Carbon Source	Amylase Activity (units/ml)
No addition	0
Starch	690
Amylopectin	660
Amylose	378
Maltose	510
Glucose	426

Enzyme Purification

M. pusillus amylase was purified from 7-day-old culture filtrates. A summary of results from the purification procedure is given in Table 3. Overall, a 55-fold purification was achieved, with a yield of 21% of the original activity. Figure 1 shows the elution of concd crude enzyme from Sephadex G-100, and Fig. 2 shows the last step of enzyme purification (DEAE-cellulose chromatography). The final preparation appeared to be homogeneous on discontinuous polyacrylamide disc gel electrophoresis.

The treatment and staining of polyacrylamide gels with periodic acid and Schiff's reagent resulted in the development of a single red band which coincided with the protein band stained with Coomassie brilliant blue. This suggested that the amylase of *M. pusillus* is a glycoprotein, similar to several other fungal amylases produced by *Aspergillus oryzae*, *A. niger*, and *Rhizopus delemar* (Takagi et al. 1971). The purified α -amylase of *M. pusillus* contained 8.7% carbohydrate as determined by the phenol-sulfuric acid method (Dubois et al. 1956). The identities and location of the sugars on the enzyme molecule were not determined.

TABLE 3. Purification of amylase from *M. pusillus*^a

Purification Step	Total Activity ^b (units)	Total Protein (mg)	Sp Act ^c	Purification (fold)	Yield (%)
(1) Crude culture filtrate	4,369,950	6,240	700	1	100
(2) Retentate from UM-10 filter	3,425,600	2,300	1,489	2.1	78
(3) Sephadex G-100 chromatography	2,352,000	210	11,200	16	53
(4) DEAE-cellulose chromatography-I	1,164,800	32	36,400	52	26
(5) DEAE-cellulose chromatography-II	924,000	24	38,500	55	21

^a Purification procedures are described in the text.

^b Micrograms (units) of maltose released per min.

^c Units of enzyme activity per mg of protein.

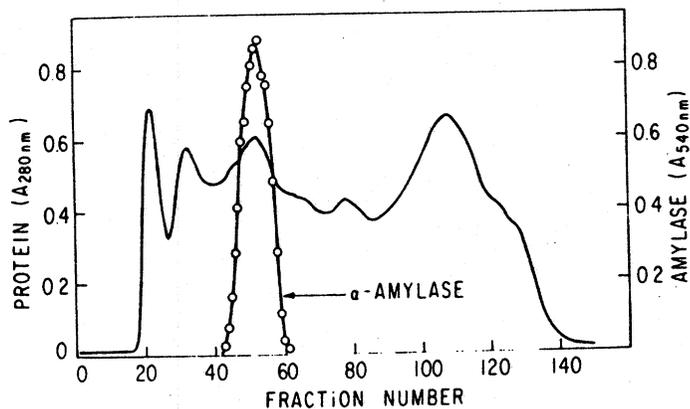


FIG. 1. Elution pattern of crude amylase of *M. pusillus* on Sephadex G-100. A 50- μ l sample of each effluent fraction was assayed for amylase activity as described in the text.

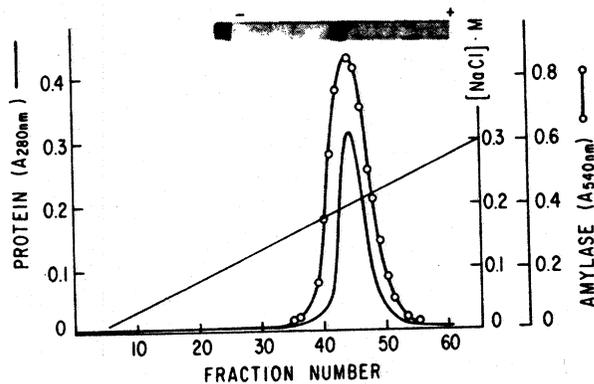


FIG. 2. Chromatography of *M. pusillus* α -amylase on DEAE-cellulose. The inset photo shows the staining of a polyacrylamide disc gel with Coomassie blue.

Molecular Weight Studies

Molecular weight determinations were made in 7.5% and 10% SDS gels. From the results, the apparent mol wt of the amylase was estimated to be 48,000. Although this value is within the range of 40,000 to 60,000 daltons reported for amylases of microbial origin (Takagi et al. 1971), these results are tentative, since glycoproteins have impaired SDS-binding ability and may not behave normally on SDS gels (Bretscher 1971).

The results of amino acid analyses (Table 4) were expressed as minimal no. of residues per amylase molecule. *M. pusillus* amylase lacked half cystine residues and, in this respect, differed from the α -amylases of *A. niger* and *A. oryzae* (Takagi et al. 1971). On the basis of amino acid composition, a minimum mol wt of 9,850 was calculated for the enzyme.

TABLE 4. Amino acid composition of purified amylase from *M. pusillus*

	Molar Ratios for Minimum Mol Wt ^a	Assumed Integer Values
Asp	14.3	14
Thr	5.4 ^b	6
Ser	7.5 ^b	8
Glu	5.8	6
Pro	2.8	3
Gly	7.5	8
Ala	6.4	6
Cys	0	0
Val	6.9	7
Met	2.1	2
Ile	5.8	6
Leu	7.3	7
Tyr	5.7	6
Phe	4.2	4
His	1.8	2
Lys	4.1	4
Try	1.0	1
Arg	1.9	2

^a Based on Try=1, Lys=4, Phe=4.

^b Not corrected for destruction.

Temperature and pH Optima

At pH 4.0, under the conditions of the routine assay system, the enzyme exhibited maximal activity at 65 to 70 C; a sharp decline in activity occurred above 75 C (Fig. 3). Tests on heat stability of the enzyme showed that after a 60-min exposure to 60 C or 65 C, the purified amylase lost 19% and 34% of activity, respectively. In contrast,

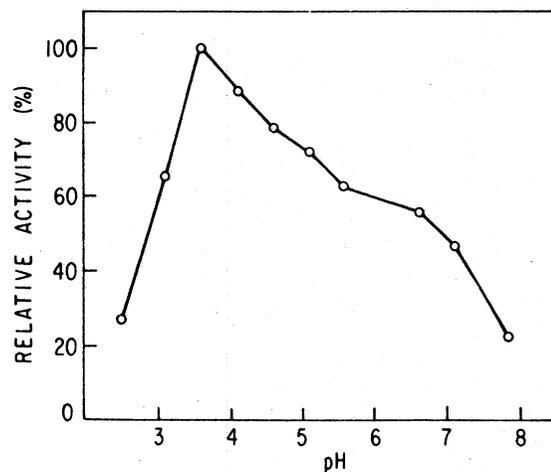


FIG. 3. Effect of temp on amylase activity. Reaction mixtures were buffered with 0.1 M acetate at pH 4.0 and incubated for 10 min at each temp.

an optimal temp of 50 to 55 C was reported for an amylase from *A. oryzae* which lost 75% of activity after being heated at 60 C for 60 min (Kundu and Das 1970).

The pH optimum of the *M. pusillus* amylase was tested at 37 C over the pH range 2.5 to 8.0 in sodium phosphate-citric acid buffer (McIlvaine 1921). The pH optimum for the enzyme was at 3.5 to 4.0 (Fig. 4). This was slightly lower than the optimum pH range (4.5 to 5.0) reported for an α -amylase of *A. oryzae* (Kundu and Das 1970). Incubation of the amylase at 25 C for 6 h at pH 2.0, 2.5, or 3.0, resulted in a 38%, 29%, or 10% loss of activity, respectively. After storage at 25 C for 3 days at pH 2.5, 75% of the enzyme activity was lost.

Effect of Chemicals on Amylase Activity

The effects of several ions and chemicals on *M. pusillus* amylase in 0.1 M Tris-hydrochloride buffer (pH 7.5) are summarized in Table 5. Unlike the α -amylase of *A. oryzae* that requires Ca^{2+} and a free sulfhydryl group for activity and is inhibited by ethylenediaminetetraacetic acid (EDTA) and thiol group reagents (Takagi et al. 1971), the enzyme of *M. pusillus* was inhibited by Ca^{2+} , remained mostly unaffected by EDTA and thiol group reagents, and was stimulated by reducing compounds. With respect to sensitivity to Ca^{2+} and absence of inhibition by thiol reagents, the enzyme was similar to an unusual α -amylase isolated by Kundu and Das (1970) from a strain of *A. oryzae*. However, further studies are required to explain the stimulating effect of mercaptoethanol and dithiothreitol on *M. pusillus* amylase, since amino acid analysis failed to show the presence of cysteine residues in the purified enzyme.

Kinetic Parameters

The effect of substrate concn on purified amylase was determined at 40 C, with soluble starch, amylopectin, amylose, and maltotriose as substrates. As calculated from Line-

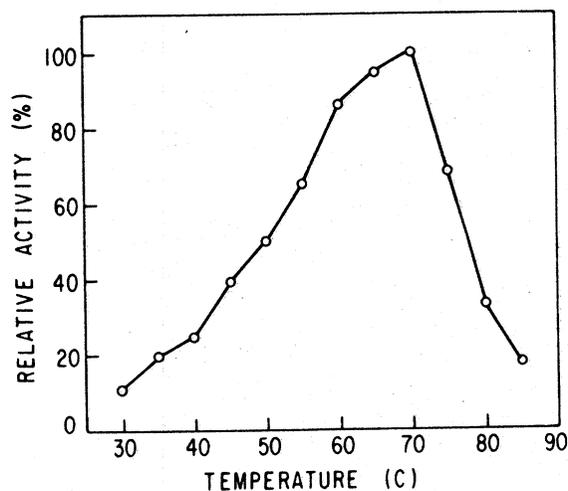


FIG. 4. Effect of pH on amylase activity. Reactions were buffered with Na_2HPO_4 -citric acid and incubated at 37 C for 10 min at each pH value.

TABLE 5. Effect of chemicals on amylase activity

Treatment ^a	Relative Activity (%)
Control	100
EDTA	93
MgCl ₂	99
CaCl ₂	61
CuCl ₂	10
N-ethylmaleimide	110
p-Hydroxymercuribenzoate	101
Iodoacetamide	103
Dithiothreitol	180
Mercaptoethanol	140

^a Each compound was tested at 10 mM final concn. Reaction mixtures were incubated at 40 C for 30 min.

weaver-Burk plots (1/v versus 1/s), the K_m values were 0.54 mg/ml for soluble starch, 0.71 mg/ml for amylopectin, 2.77 mg/ml for amylose, and 7.6 mg/ml for maltotriose. The K_m of 0.54 mg/ml for starch is lower than that reported for thermostable amylases from *Thermomonospora vulgaris*, 1.4 mg/ml (Allam et al. 1975), *Bacillus stearothermophilus*, 1.0 mg/ml (Manning and Campbell 1961), *B. macerans*, 3.3 mg/m; (DePinto and Campbell 1968), and *B. acidocaldarius*, 0.8 mg/ml (Buonocore et al. 1976).

Determination of the Amylolytic Pattern

The purified amylase of *M. pusillus* was able to hydrolyze starch, amylopectin, amylose, *cyclo-G*₆, *cyclo-G*₇, *cyclo-G*₈, and maltotriose, but not maltose. Degradation products in amylase digests were similar to those of other α -amylases previously studied (Robyt and Whelan 1968). Early reaction (15-60 min) products from soluble starch included maltose (G_2), maltotriose (G_3), maltotetraose (G_4), and maltopentaose (G_5). During the late stages of the reaction (3 to 24 h), the intensity of glucose (G_1) and G_2 spots increased on the chromatograms. This was accompanied by the gradual disappearance of the G_3 , G_4 , and G_5 spots. The identification of sugars was based on chromatographic behavior as compared with reference sugars (G_1 , G_2 , and G_3), by the technique of French and Wild (1953), suggested for oligosaccharides.

The enzyme hydrolyzed G_3 to G_2 and G_1 , whereas *cyclo-G*₇, and *cyclo-G*₈ were hydrolyzed to G_3 , G_2 , and G_1 . The amount of G_1 liberated by the enzyme from G_3 , *cyclo-G*₇, and *cyclo-G*₈, after 24 h of incubation at 37 C, was 33%, 19%, and 13%, respectively. The susceptibility of cyclic substrates to the action of *M. pusillus* amylase also supported the classification of the enzyme as an α -amylase, since β -amylases are absolutely inactive against these compounds (Abdullah et al. 1966).

On the basis of the chromatographic data, the *M. pusillus* enzyme apparently has an endoamylolytic nature and yields enzyme digests that are characteristic of alpha-type amylase action patterns. It is apparently the first example of fungal α -amylase exhibiting both acidophily and thermophily simultaneously. Consequently, the α -amylase of *M. pusillus* should be of economic importance.

ACKNOWLEDGMENTS

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