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PROPERTIES OF CHITIN SYNTHETASE FROM YEAST AND MYCELIAL PHASES OF BLASTOMYCES DERMATITIDIS

The University of Oklahoma PH.D. 1983

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GRADUATE COLLEGE

PROPERTIES OF CHITIN SYNTHETASE FROM YEAST AND MYCELIAL PHASES OF BLASTOMYCES DERMATITIDIS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirement for the

degree of

DOCTOR OF PHILOSOPHY

By

GLENMORE SHEARER, JR.

Norman, Oklahoma

1983

PROPERTIES OF CHITIN SYNTHETASE FROM YEAST AND MYCELIAL PHASES OF BLASTOMYCES DERMATITIDIS

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ACKNOWLEDGEMENTS

As with any work of this magnitude, the author is responsible for the rough hewn copy and relies on the skills of friends and colleagues to smooth the rasp marks and apply the varnish of proper grammer and logic until the grain of truth if smoothly evident.

I would like to humbly thank the following individuals for their laborious sanding:

Thank McCarthy

Thank Lancaster

Thank McInerney

Thank Johnson

Thank Larsh

Thank Bear

Thank God!

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CHAPTER I

INTRODUCTION

Fungal dimorphism is defined as the reversible conversion of a yeast-like growth phase to a mycelial phase (52). This phenomenon is exhibited by only a few of thousands of known fungi. The etiologic agent of Dutch Elm disease, Ceratocystis ulmi exhibits dimorphism (46) as do several species of the saprophytic genera Mucor (61) and Mycotypha (49). Most dimorphic fungi, however, are human pathogens, e.g., the genera Blastomyces, Histoplasma, Coccidioides, Paracoccidioides, Sporothrix, and Candida.

Many factors influence the growth phase of dimorphic fungi. One factor of particular importance in the human pathogenic fungi is temperature. At body temperature (37°C) growth is yeast-like. At "room temperature" (actually below about 35°C) growth is mycelial This dimorphism plays an important role in the epidemiology of the the pathogenic fungi. In nature, the infective units are spores produced by the mycelium. Spores are carried great distances by the wind and produce disease when inhaled by a susceptible individual. Phytopathogenic and saprophytic fungi do not seem to exhibit thermal dimorphism.

Media composition and cultural conditions control the growth phase of pathogenic and non-pathogenic fungi. The genera Mucor (61) and Mycotypha (19) grow as a yeast in media with high hexose concentration. In Mucor atmosphere

also plays a role in dimorphism. Anaerobic conditions favor the yeast phase while aerobic growth is mycelial (61). Ceratocystis ulmi dimorphism is nitrogen source dependent. Proline favors the yeast phase while arginine or ammonia compounds favor the mycelial phase (46). Zoopathogenic fungi may be grown as a yeast even at room temperatures if a low redox potential is maintained in the medium (50). Cyclic AMP has been implicated in morphogenesis in several fungi. Agents that increase cAMP level favor the yeast phase in Mucor (61). In Histoplasma, however, these same agents induce the mycelial phase (44).

In practically every physiological aspect tested there are marked differences in the yeast and mycelial phase. There are qualitative and quantitative differences in enzymes, e.g., uridine diphosphoglucose dehydrogenase (24), sulfite reductase (5), cystine reductase (40,41) and RNA polymerase (4,37). Sensitivity to antimicrobial agents also varies with growth phase (16,17).

The ultimate physical determinant of morphology is the cell wall. The cell wall of fungi is a rigid structure composed mostly (80-90% by dry weight) of polysaccharides. In most fungi these polysaccharides are in the form of chitin and glucan (2). Chitin, a beta 1-4 homopolymer of N-acetylglucosamine, is considered to be the single most important wall polymer. Chitin is found in the wall as helical molecules with two residues per turn called alpha chitin. Several alpha chitin molecules arranged in an antiparallel fashion make up microfibrils which form the principle structural network of the cell (28).

The second polysaccharide component, glucan, is composed of glucose residues linked mostly by beta and alpha 1-3 links although 1-4 and 1-6 links are occasionally found (2).

Chitin microfibrils are laid down first during de novo wall synthesis and thus form the innermost layer. The wall of the growing tip of hyphae is composed primarily of chitin. A short distance back from the growing tip glucans are deposited to make the wall much thicker and more rigid. In this older portion glucan makes up the majority of the wall (29).

In dimorphic fungi the chemical composition of the cell wall is markedly different in the yeast versus the mycelial phase. In Candida albicans (15), Histoplasma capsulatum (35) and Histoplasma farciminosum (58) chitin makes up a much greater portion of the cell wall in the mycelial phase than in the yeast. In Paracoccidioides brasiliensis, however, the yeast phase has nearly four times as much chitin as the mycelium (36). The three genera listed above show a distinct change in the arrangement of the microfibrils in the yeast versus the mycelial phase. Yeast phase microfibrils are arranged in a random fashion while mycelial phase fibrils are mostly longitudnal (32).

Glucan composition also differs with the phase of growth. Blastomyces dermatitidis yeast glucan is almost all alpha 1-3 linked but in the mycelium beta 1-3 glucan makes up nearly half the total glucan (33). In Paracoccidioides brasiliensis (34) and Histoplasma capsulatum (35) alpha 1-3 glucan predominates in the yeast phase while the mycelial phase contains almost exclusively beta 1-3 glucan.

One method used to study dimorphism is to examine the cell wall biosynthetic system. In yeast-like organisms cell wall material is deposited in an isodiametric manner. In mycelial organisms, however, the deposition of wall material is highly polarized -- wall synthesis occurs only in the first 100 μm of the hyphal tip (28). Most extant wall biosynthesis data concerns the chitin synthesizing system. Chitin synthetase (C-syn, EC 2.4.1.16) was first described

in fungi by Glaser and Brown in 1957 (27). The chitin polymer is formed by incorporation of N-acetylglucosamine (GlcNAc) from the substrate UDP-N-acetylglucosamine with release of free UDP. UDP is inhibitory while free N-acetylglucosamine stimulates enzyme activity. In most preparations tested a divalent cation (usually Mg^{2+}) is needed for maximum activity.

Chitin synthetase has been shown to exist in a latent form in Saccharomyces cerevisiae (11), Mortierella vinacea (47), Aspergillus flavus (38), Phycomyces blakesleeanus (63), Neurospora crassa (1), Blastocladiella emersonii (45), and in the dimorphic fungi Mucor rouxii (53) and Candida albicans (8). Latent chitin synthetase is activated by several proteases in vitro and probably in vivo (9,12,14,54). In Aspergillus nidulans (14) a neutral protease was isolated that increased the activity of chitin synthetase 14 fold over that obtainable by trypsin treatment. Such results have suggestaed that endogenous proteases may play an important regulatory role in vivo.

There are two general theories of regulation of dimorphism. Data from the laboratory of Cabib (10, 11) indicate that chitin synthetase zymogen is uniformly distributed on the inside of the plasma membrane. Zymogen is activated by fusion of vesicles containing activating factor (protease) with the plasma membrane. Excess activating factor is controlled by soluble protease inhibitor in the cytoplasm. Data from the laboratory of Bartnicki-Garcia (39) indicate that chitin synthetase zymogen is contained in small vesicles called chitosomes. Chitosomes migrate to the area where cell wall formation is needed and fuse with the plasma membrane. Zymogen is activated by a protease either just before or just after membrane fusion. A protein inhibitor that acts on the active enzyme itself controls free active chitin synthetase.

Among the fungi that demonstrate thermal dimorphism only Candida albicans chitin synthetase has been examined in the yeast and mycelial phase (8,9). Candida albicans, however, only exhibits a pseudohyphal form rather than eumycelium like that of the other dimorphic zoopathogenic fungi. The objective of this research is to compare the chitin synthetase system of the yeast phase versus the mycelial phase of Blastomyces dermatitidis, a zoopathogenic fungus which exhibits a yeast phase at 37°C and extensive eumycelium at room temperature.

CHAPTER II

MATERIALS AND METHODS

ORGANISM

Blastomyces dermatitidis strain SCB-2 (ATCC 26199) was used throughout this study. The organism was originally isolated from a case of human blastomycosis and was maintained on brain heart infusion (BHI) agar slants at 37°C. Stock cultures were transferred to fresh medium twice weekly.

CULTURAL CONDITIONS

Auto Pow autoclavable tissue culture medium (Flow Laboratories) was used for both growth phases. Auto Pow (10 g/l) was supplemented with 9 g/l glucose and the pH adjusted to 6.0 before autoclaving. Yeast phase organisms from BHI slants were used to inoculate 500 ml of Auto Pow in 100 ml nephlo flasks (Belco Glass). Flasks were incubated at 37°C on an orbital shaker at 160 rpm until mid log phase as determined by direct counts of colony forming units. This mid log culture was used to inoculate fresh Auto Pow medium to grow organisms for enzyme studies.

Cultures for yeast phase enzyme preparation were grown by inoculating 1 l fresh Auto Pow medium in 2 l Erlenmeyer flasks with 5% v/v mid log phase yeast and incubating at 37°C, 160 rpm. The second culture was incubated until mid log phase and was harvested. Cultures for mycelial phase enzyme were similarly

inoculated but incubated at 25°C at 160 rpm. After five days at 25°C the yeast began to form short hyphae. Fresh medium was inoculated (5% v/v) with the five day mycelial culture and incubated for five days at 25°C at 160 rpm. After this second transfer at 25°C the organisms were over 90% mycelial and were harvested.

ENZYME PREPARATION

Cultures were allowed to settle for 60 minutes and the clear media decanted. Organisms were washed twice with 50 mM Tris-HCl buffer pH 7.5 containing 10 mM $MgCl_2$ (buffer A) at 0°C. Organisms were resuspended in cold buffer A, mixed with an equal volume of 0.5 mm glass beads and disrupted for 30 seconds in a Bronwill MSK homogenizer cooled with liquid carbon dioxide. During all subsequent steps the temperature was maintained at 0 to 4°C. The cell lysate was fractionated by successive centrifugations at 1000 x g for 10 minutes, 10,000 x g for 20 minutes and 100,000 x g for 45 minutes to yield 1K, 10K, 100K pellets and 100K supernatant fractions respectively. Each pellet was washed twice with cold buffer A and resuspended in the same buffer to a concentration of approximately 3 mg/ml protein. Each fraction was frozen at -70°C in 0.5 ml aliquots. Unless noted otherwise all experiments were performed with 100K pellet fractions.

PROTEOLYTIC ACTIVATION

Chitin synthetase (C-syn) was activated with trypsin in the following manner. Crystallized bovine pancreas trypsin (type III Sigma, 2 mg/ml in buffer A) was added to C-syn preparations to a final concentration of 200 µg/ml. After 20 minutes incubation at 25°C soybean trypsin inhibitor (3 mg/ml in buffer A) was added to a final concentration of 300 µg/ml.

ENZYME ASSAY

In the standard assay 10 µl of enzyme extract was added to 40 µl of assay mixture containing final concentrations of 50 mM tris pH 7.5, 10 mM $MgCl_2$, 30 mM glucosamine (GlcNAc) and 1.0 mM UDP-(U-^{14}C)-GlcNAc (specific activity 0.2 Ci/mM). Incubation was carried out at 25°C for 30 minutes and terminated by adding 3 ml of 5% w/v trichloroacteic acid (TCA). Samples were filtered through 24mm Whatman GF-C glass fiber filters and washed 5x with 3 ml of 5% TCA and 5x with 3 ml of 95% ethanol. Filters were placed in 3 ml Nalgene Filmware bags and allowed to dry overnight at 37°C. Each bag was filled with 2.5 ml of toluene fluor containing 42 ml/l Spectrafluor (Amersham) and heat sealed. Sealed Filmware bags were placed in 10 ml Nalgene Carrier Vials and counted in a Beckman LS-100C scintillation counter. Variations in the standard assay for kinetic data are noted in individual figures.

IDENTIFICATION OF REACTION PRODUCT

The standard reaction assay was scaled upto 0.5 ml. After 60 minutes incubation at 25°C triplicate 25 µl samples were incubated at 97°C for 10 minutes and processed in the following manner: (1) filtered immediately as in the standard assay; (2) 1 ml of chitinase (10 mg/ml in 50mM succinate buffer pH 6.0 wih 0.01% azide) added and incubated at 30°C for 48 hours; (3) 1 ml of chitinase buffer added and incubated as in #2; (4) 1 ml distilled water added and incubated at 97°C for 60 minutes; (5) 1 ml 1 M HCl added and incubated at 97°C for 60 minutes; (6) 1 ml 1 M NaOH added and incubated at 97°C for 60 minutes. After the indicated incubation period the samples were filtered as in the standard assay.

INHIBITOR AND ACTIVATOR ASSAY

To investigate the presence of a C-syn inhibitor or activator the 100K pellet fraction from yeast or mycelium was resuspended in 100K supernatant or 100K sonicate and incubated at 25°C. At various time intervals triplicate 10 µl samples were removed from the incubation mixture and assayed by the standard method. Sonicate was prepared by resuspending 100K pellets in buffer A and sonicating for 3 five second periods with a Branson Sonifer 200 at a power output of 60. During sonication the enzyme suspension was cooled in ice. Each of the 3 sonication periods was separated by a 10 second pause. The sonicated enzyme was centrifuged at 100,000 x g for 60 minutes. The supernatant from this centrifugation is referred to as 100K sonicate.

MISCELLANEOUS

Biochemicals were obtained from Sigma Chemical Co. UDP-(U-^{14}C)-GlcNAc (282 Ci/M) was from Amersham Inc. Protein was determined by the method of Bradford (6). Determination of Km and Vmax from Lineweaver Burke plots was by weighted linear regression (51).

CHAPTER III

RESULTS

DISTRIBUTION OF ENZYME

The distribution of C-syn in yeast and mycelial fractions is shown in Figure 1. Yeast and mycelial enzyme were similar in that the 1K and 100K pellets had the greatest amount of enzyme activity and no activity was seen in the 100K supernatant. The mycelial extracts had approximately 5 fold greater activity than the yeast extract without trypsin activation.

IDENTIFICATION OF REACTION PRODUCT

The identity of the reaction product as chitin is supported by the data shown in Table 1. Incorporated radioactivity from UDP (^{14}C)-GlcNAc was insoluble in hot waer, hot 1M acid and hot 1M base. Chitinase, however completely digested the reaction product.

PROTEOLYTIC ACTIVATION

The ratios of latent to active enzyme were markedly different in the yeast and mycelial extracts as shown in Figure 2 Trypsin activation of yeast enzyme resulted in a 3 fold increase in activity. Mycelial enzyme, however, showed maximal activity without proteolytic activation. Incubation with trypsin drastically reduced mycelial activity.

Figure 1. Distribution of chitin synthetase in 1000 x g, 10,000 x g, 100,000 x g and 100,000 x g supernatant. Assays were performed without proteolytic activation

Yeast: 

Mycelium: 

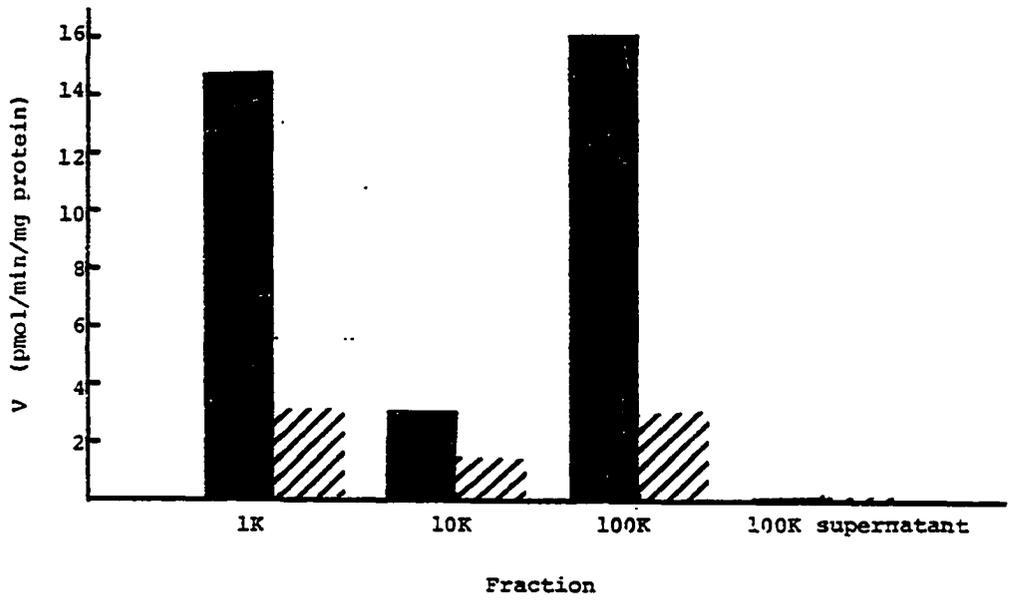

V (pmol/min/mg protein)
16
14
12
10
8
6
4
2
1K
10K
100K
100K supernatant
Fraction

TABLE I

Stability of Reaction Product

treatment [1]	cpm ± std. dev.
none	2461 ± 114
hot H_2O	2515 ± 40
hot 1M NaOH	2682 ± 215
hot 1M HCl	2253 ± 40
chitinase 10mg/ml	12 ± 1
chitinase buffer	2478 ± 145
background	10 ± 1

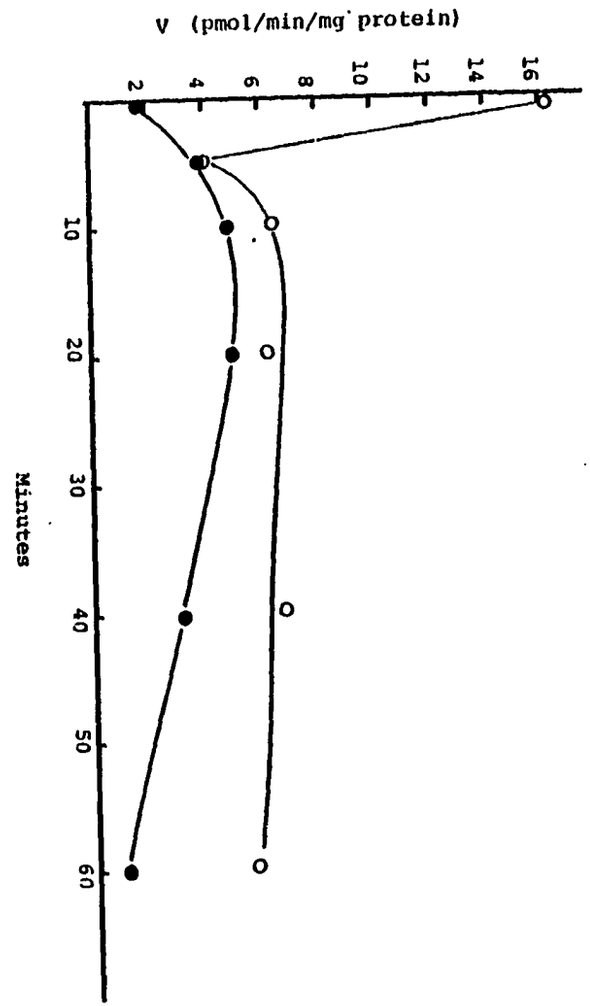
[1] treatment prior to standard assay

[2] hot indicates 97°C for 60 minutes

Figure 2. Proteolytic activation of chitin synthetase with 200 µg/ml trypsin. Enzyme fraction used was 100K pellet.

Yeast (●)

Mycelium (o)


V (pmol/min/mg protein)
2
4
6
8
10
12
14
16
10
20
30
40
50
60
Minutes

STIMULATION BY GLUCOSAMINE

Chitin synthetase from yeast and mycelium was greatly stimulated by free N-acetyl-glucosamine as shown in Figures 3-6. Double reciprocal plots of velocity versus then concentration of (UDP-GlcNAc constant at 1 mM) indicate half maximal activation with 8.5 mM for yeast enzyme and 3.9 mM for mycelial enzyme.

EFFECT OF TEMPERATURE

Yeast and mycelial enzyme showed essentially the same pattern of activity versus temperature as shown in Figure 7. The optimum temperature for both phases was 28°C.

EFFECT OF PH ON ACTIVITY

The effect of pH on activity is shown in Figure 8. The optimal pH was in the 7.0 to 7.5 range for enzyme from both phases. The response to the buffers used differed in that mycelial activity was greatest (by two fold) with imidazole versus tris. Yeast extracts showed greatest activity with tris.

EFFECT OF MAGNESIUM CONCENTRATION

The effect of magnesium concentration on enzyme activity is shown in Figure 9. Yeast enzyme had maximal activity at 10 mM Mg. Activity decreased sharply below 10 mM. Mycelial activity was greatest with 5 mM Mg. Enzyme activity from both phases decreased gradually to approximately 1/2 maximal at 60 mM Mg^{+2}.

EFFECT OF UDP-GLUCOSAMINE

Yeast and mycelial enzyme exhibited sigmoidal kinetics when UDP-GlcNAc concentrations were varied at fixed glucosamine levels as shown in Figures 10-16. The effect of fixed low (5mM), intermediate (30 mM) and high (100 mM) GlcNAc

Figure 3. Effect of GlcNAc on activity of yeast chitin synthetase: Michaelis-Menten plot. UDP-GlcNAc held constant at 1mM.

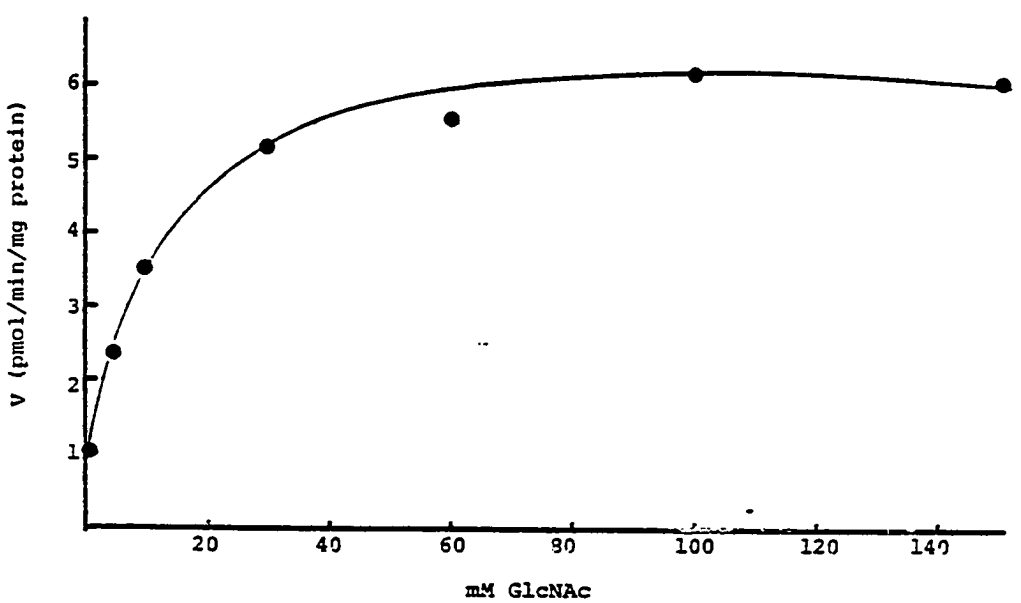

v (pmol/min/mg protein)
1
2
3
4
5
6
20
40
60
80
100
120
140
mM GlcNAc

Figure 4. Effect of GlcNAc on yeast chitin synthetase: Lineweaver-Burke plot. UDP-GlcNAc held constant at 1mM.

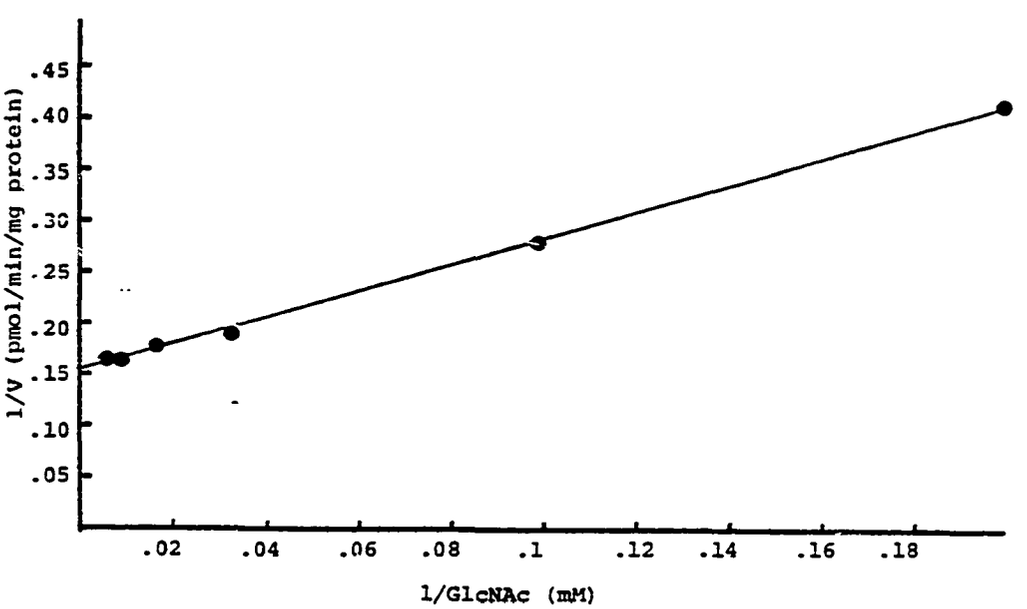

1/V (pmol/min/mg protein)
.45
.40
.35
.30
.25
.20
.15
.10
.05
.02
.04
.06
.08
.1
.12
.14
.16
.18
1/GlcNAc (mM)

Figure 5. Effect of GlcNAc on mycelial chitin synthetase: Michaelis-Menten plot. UDP-GlcNAc held constant at 1mM.

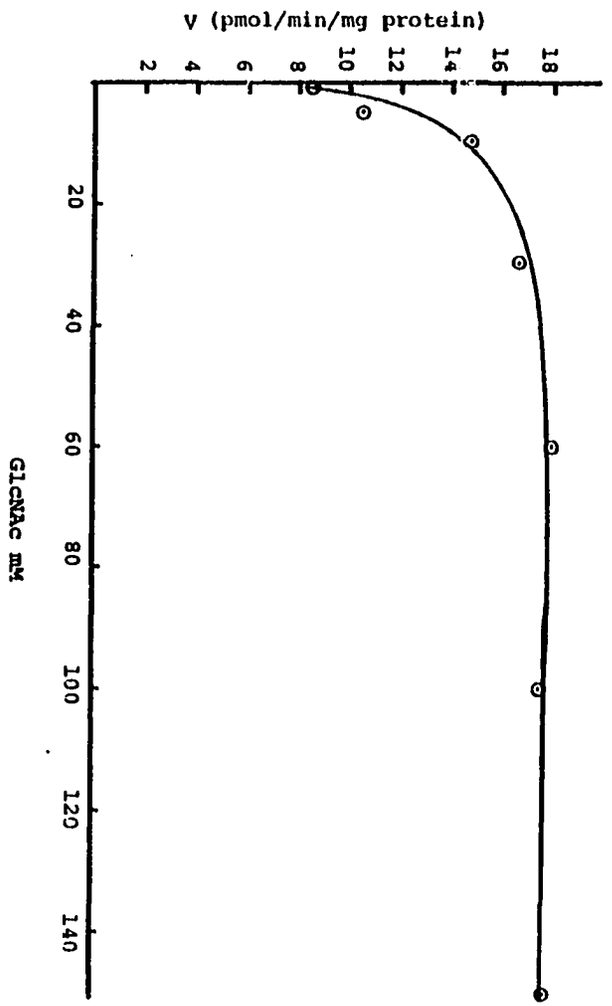

V (pmol/min/mg protein)
2
4
6
8
10
12
14
16
18
20
40
60
80
100
120
140
GlcNAc mM

Figure 6. Effect of GlcNAc on mycelial chitin synthetase: Lineweaver-Burke plot. UDP-GlcNAc held constant at 1mM.

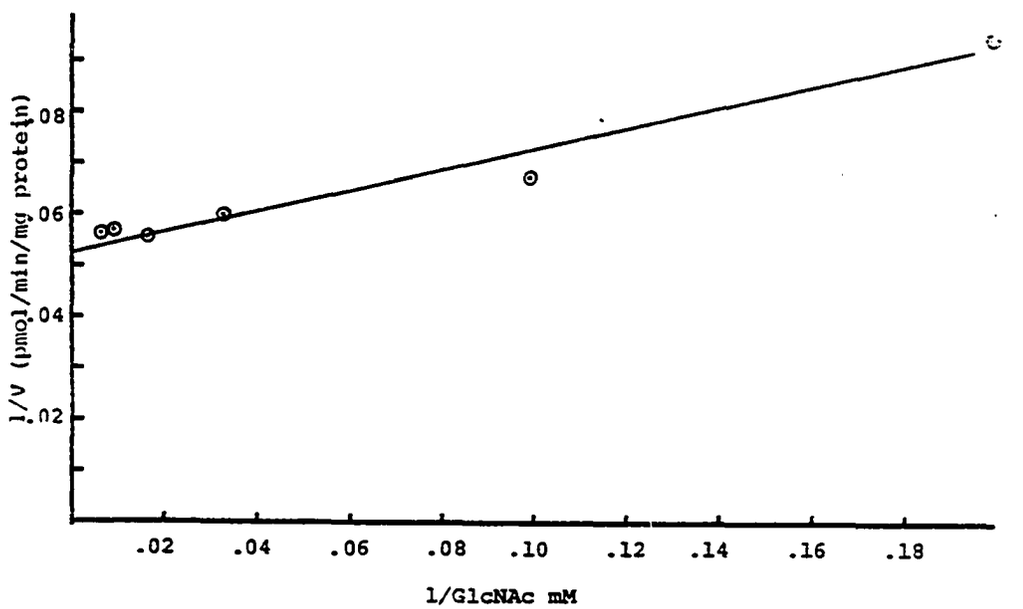

1/V (μmol/min/mg protein)
.08
.06
.04
.02
.02
.04
.06
.08
.10
.12
.14
.16
.18
1/GlcNAc mM

Figure 7. Effect of temperature on chitin synthetase. Standard assay mixture with pH adjusted to 7.5 at each temperature. Yeast activities are multiplied by 3.

Yeast (●)

Mycelium (○)

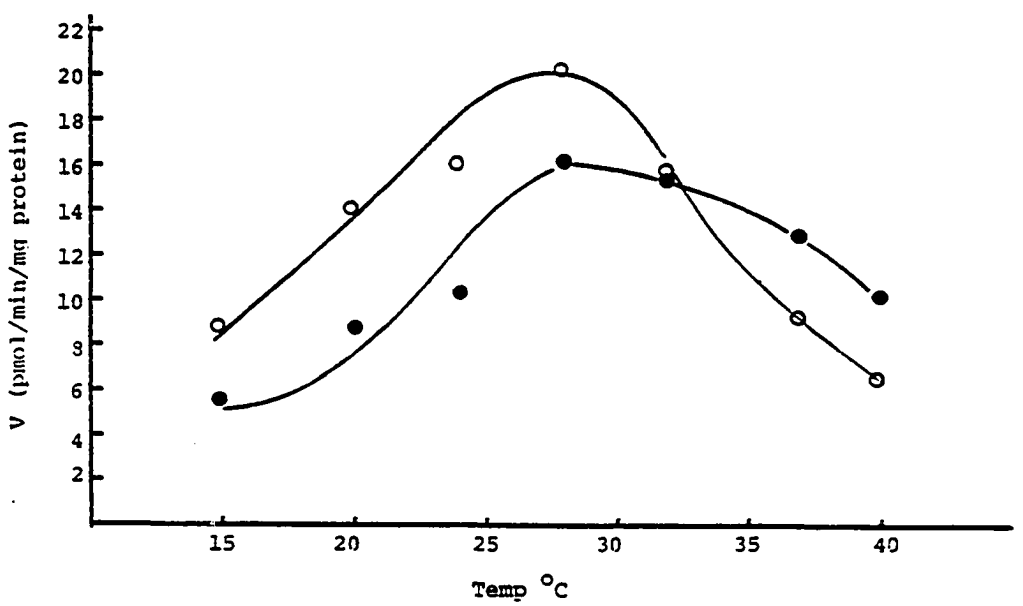

V (μmol/min/mg protein)
22
20
18
16
14
12
10
8
6
4
2
15
20
25
30
35
40
Temp °C

Figure 8. Effect of pH on chitin synthetase. Buffers were 50mM with 10mM $MgCl_2$: Sodium succinate (pH 5.0-6.5), imidazole (pH 6.5-7.5) and tris (pH 7.5-9.0). Yeast activities have been multiplied by 10.

Yeast (●)

Mycelium (o)

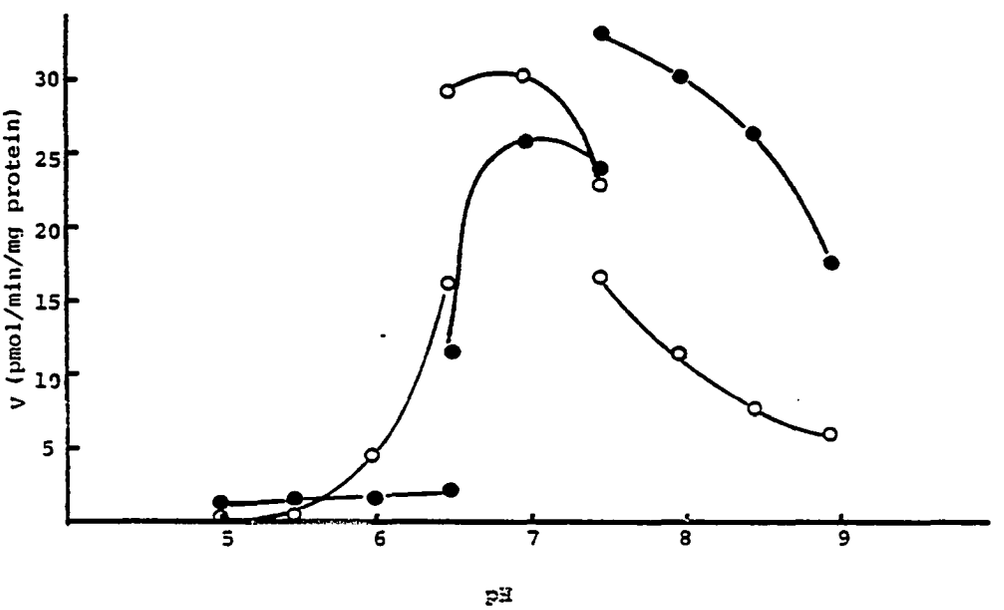

v (µmol/min/mg protein)
30
25
20
15
10
5
5
6
7
8
9
pH

Figure 9. Effect of magnesium on chitin synthetase. Standard assay mixture with only Mg altered. Yeast activities have been multiplied by 3.

Yeast (●)

Mycelium (○)

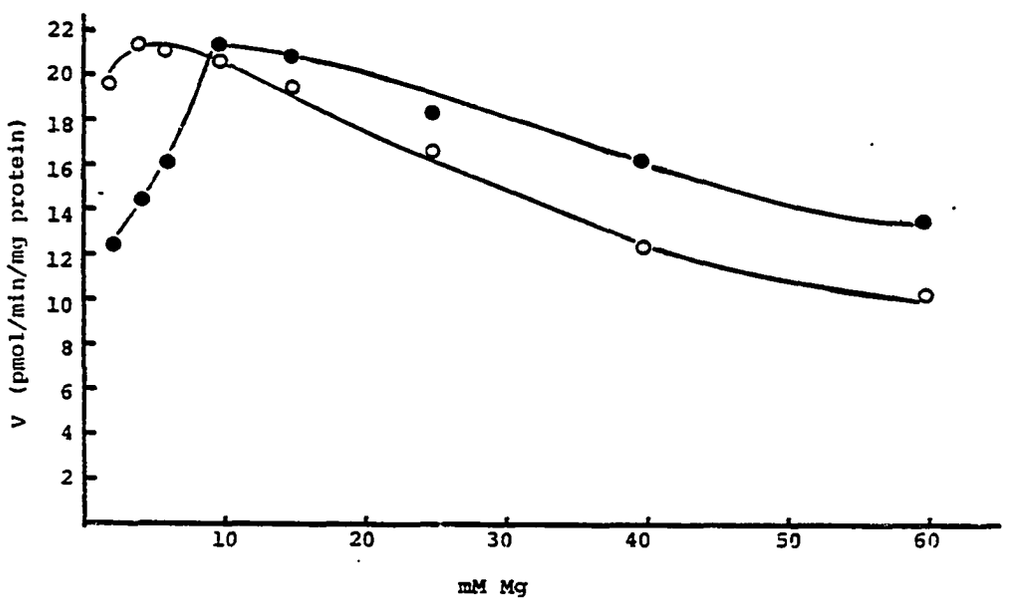

V (pmol/min/mg protein)
22
20
18
16
14
12
10
8
6
4
2
10
20
30
40
50
60
mM Mg

Figure 10. Effect of UDP-GlcNAc on chitin synthetase from yeast at low, intermediate and high GlcNAc concentrations: Michaelis-Menten plot.

GlcNAc = 5mM (○)

GlcNAc = 30mM (△)

GlcNAc = 100mM (□)

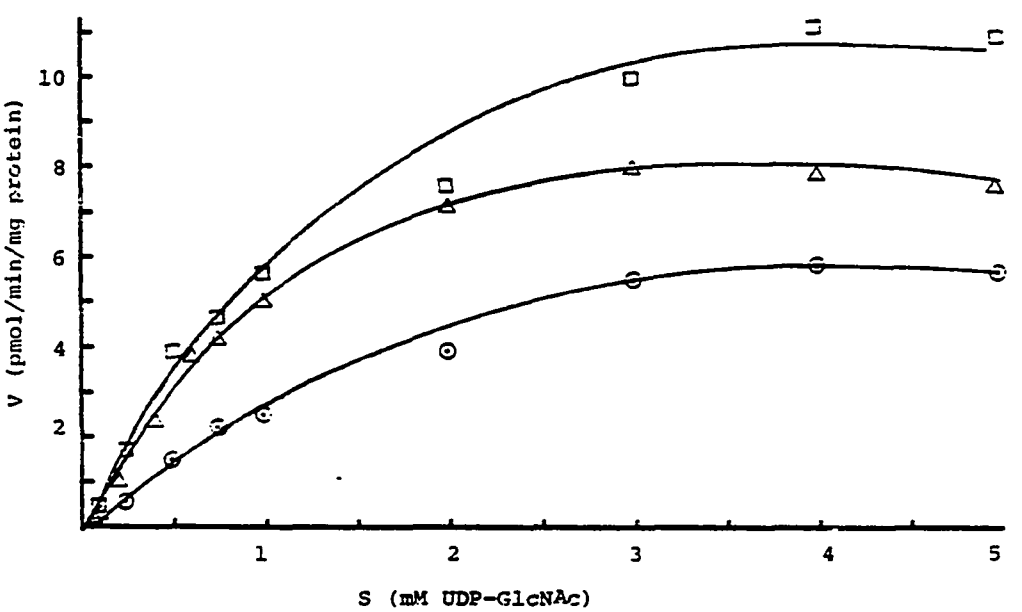

V (pmol/min/mg protein)
10
8
6
4
2
1
2
3
4
5
S (mM UDP-GlcNAc)

Figure 11. Effect of UDP-GlcNAc on chitin synthetase from yeast at low, intermediate and high GlcNAc concentrations: Lineweaver-Burke plot.

GlcNAc = 5mM (○)

GlcNAc = 30mM (△)

GlcNAc = 100mM (□)

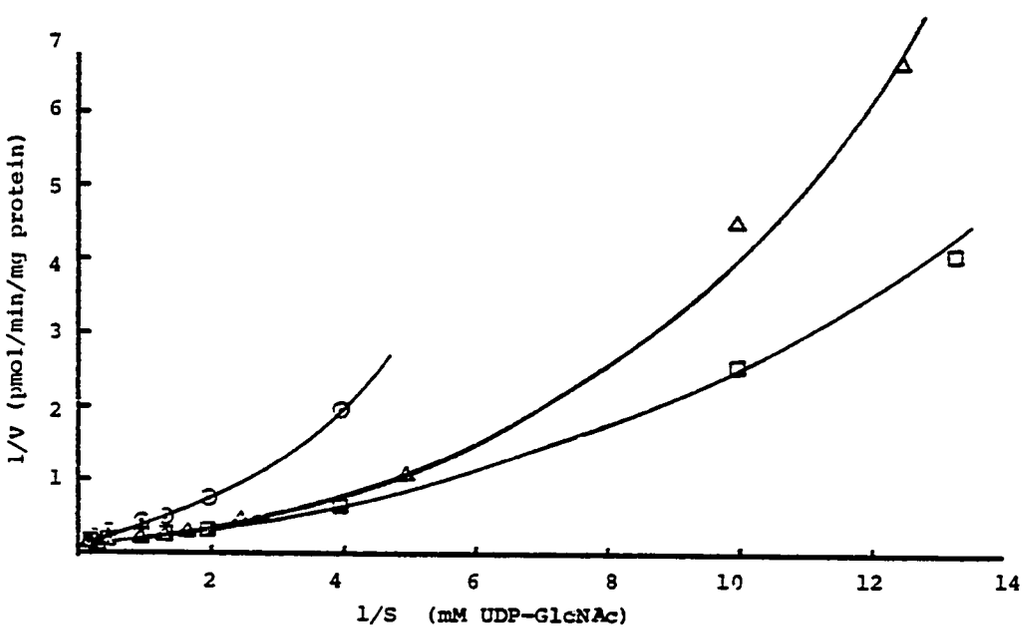

1/V (pmol/min/mg protein)
1
2
3
4
5
6
7
2
4
6
8
10
12
14
1/S (mM UDP-GlcNAc)

Figure 12. Effect of UDP-GlcNAc on chitin synthetase from yeast at low GlcNAc concentration (5mM) expanded scale Lineweaver-Burke plot.

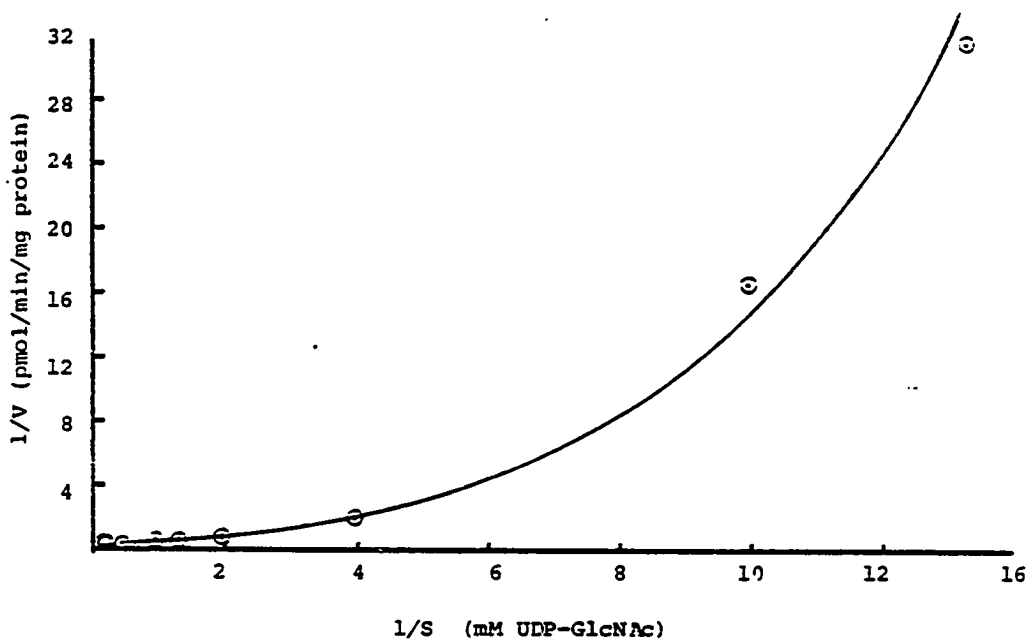

32
28
24
20
16
12
8
4
1/V (pmol/min/mg protein)
2
4
6
8
10
12
16
1/S (mM UDP-GlcNAc)

Figure 13. Effect of UDP-GlcNAc on chitin synthetase from yeast at low, intermediate and high GlcNAc concentrations. UDP-GlcNAc concentrations below 0.5mM have been deleted.

GlcNAc = 5mM (○)

GlcNAc = 30mM (△)

GlcNAc = 100mM (□)

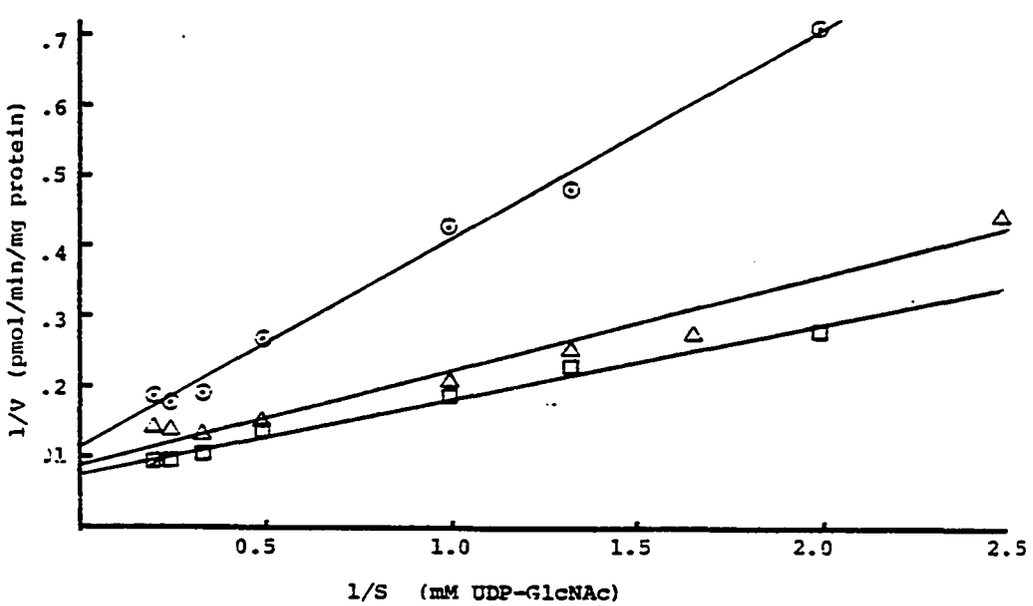

.7
.6
.5
.4
.3
.2
1/V (pmol/min/mg protein)
0.5
1.0
1.5
2.0
2.5
1/S (mM UDP-GlcNAc)

Figure 14. Effect of UDP-GlcNAc on chitin synthetase from mycelium at low, intermediate and high GlcNAc concentrations: Michaelis-Menten plot.

GlcNAc = 5mM (○)

GlcNAc = 30mM (△)

GlcNAc = 100mM (□)

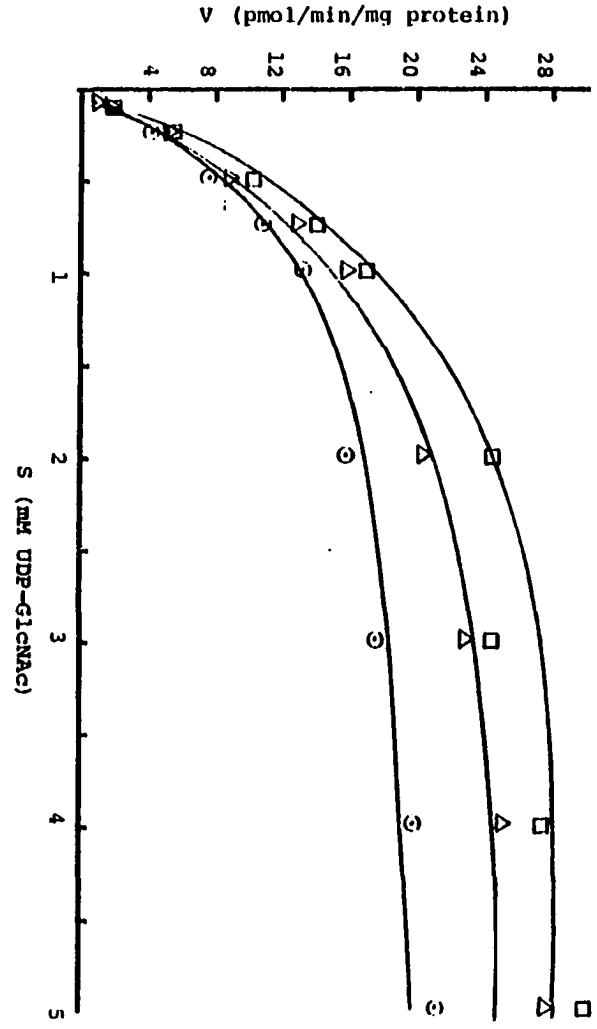

V (pmol/min/mg protein)
4
8
12
16
20
24
28
1
2
3
4
5
S (mM UDP-GlcNAc)

Figure 15. Effect of UDP-GlcNAc on chitin synthetase from mycelium at low, intermediate and high GlcNAc concentrations: Lineweaver-Burke plot.

GlcNAc = 5mM (○)

GlcNAc = 30mM (△)

GlcNAc = 100mM (□)

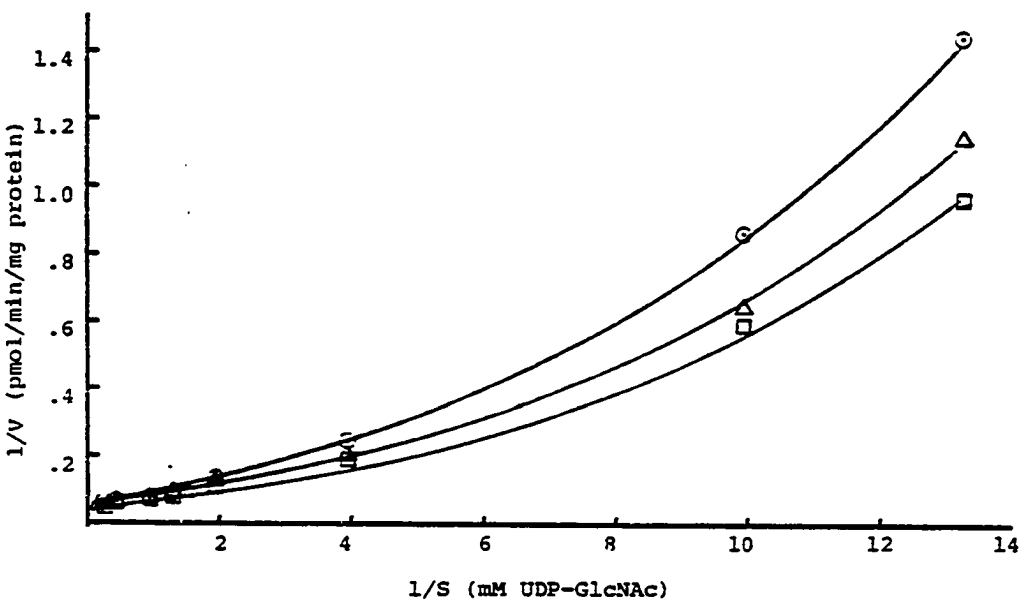
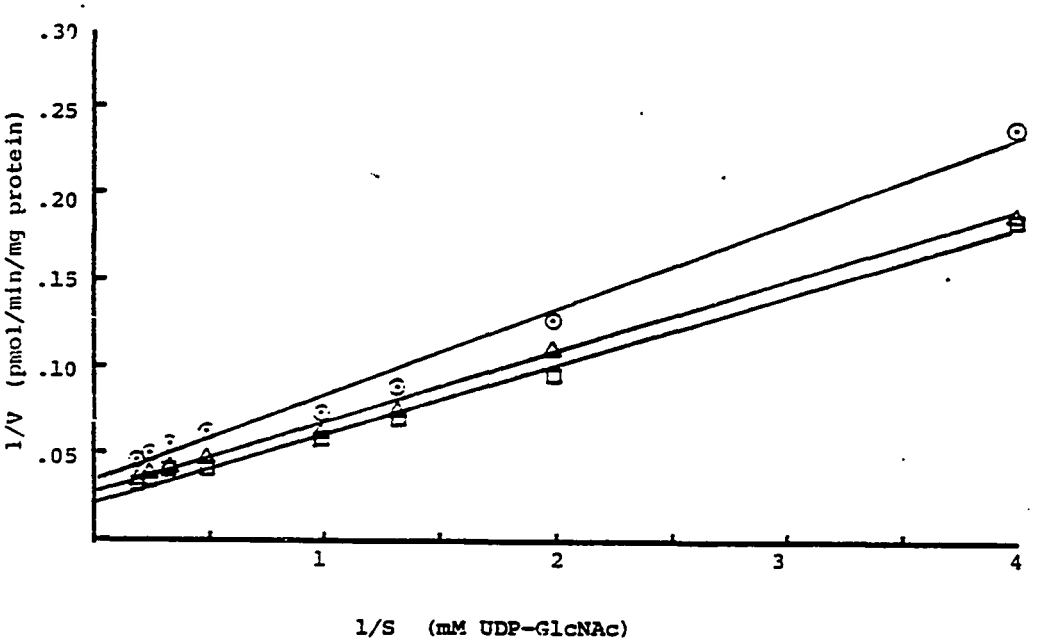

1.4
1.2
1.0
.8
.6
.4
.2
1/V (pmol/min/mg protein)
2
4
6
8
10
12
14
1/S (mM UDP-GlcNAc)

Figure 16. Effect of UDP-GlcNAc on chitin synthetase from mycelium at low, intermediate and high GlcNAc concentrations. UDP-GlcNAc concentrations below 0.25mM have been deleted.

GlcNAc = 5mM (○)

GlcNAc = 30mM (△)

GlcNAc = 100mM (□)


.30
.25
.20
.15
.10
.05
1/V (pmol/min/mg protein)
1
2
3
4
1/S (mM UDP-GlcNAc)

was also investigated. Double reciprocal plots were sharply curved upward. By ignoring UDP GlcNAc concentrations below 0.5 mM for yeast enzyme and below 0.25 mM for mycelial enzyme double reciprocal plots were linear (Figures 13 and 16). Values calculated for Km and V max from linear double reciprocal plots are summarized in Table 2. Maximal velocity inreased with GlcNAc levels in both enzyme extracts. Mycelial Km values were essentially unchanged with increasing GlcNAc levels. Yeast enzyme Km, however, changed with glucosamine levels. Km was greatest at low GlcNAc concentrations and decreased to the same value as with mycelial enzyme at high GlcNAc levels.

Hill plots for high GlcNAc concentration are shown in Figures 17 and 18. Yeast and mycelial enzyme exhibited values for n of approximately 1.3. The slopes for other concentrations of GlcNAc were essentially the same.

INHIBITOR AND ACTIVATOR

No evidence for the presence of an inhibitor of activator was found. Enzyme resuspended in 100K supernatant or 100K sonicate was neither stimulated nor inhibited. Typical results for yeast enzyme are illustrated in Figure 19. Results with mycelial enzyme were similar.

TABLE II

Kinetic Values for Yeast and Mycelial Chitin Synthetase

	yeast	mycelium
substrate Km (G=5)[1]	3.0	1.5
substrate Km (G=30)	1.5	1.4
substrate Km (G=100)	1.4	1.4
activator Km (U=1) [2]	8.5	3.9
Hill number (G=100)	1.3	1.3

[1] Km_{app} for UDP-GlcNAc. G=5 etc., indicates mM concentration of GlcNAC.

[2] Km_{app} for GlcNAc at UDP-GlcNAc = 1mM

Figure 17. Hill plot for yeast chitin synthetase at high GlcNAc concentration (100mM).

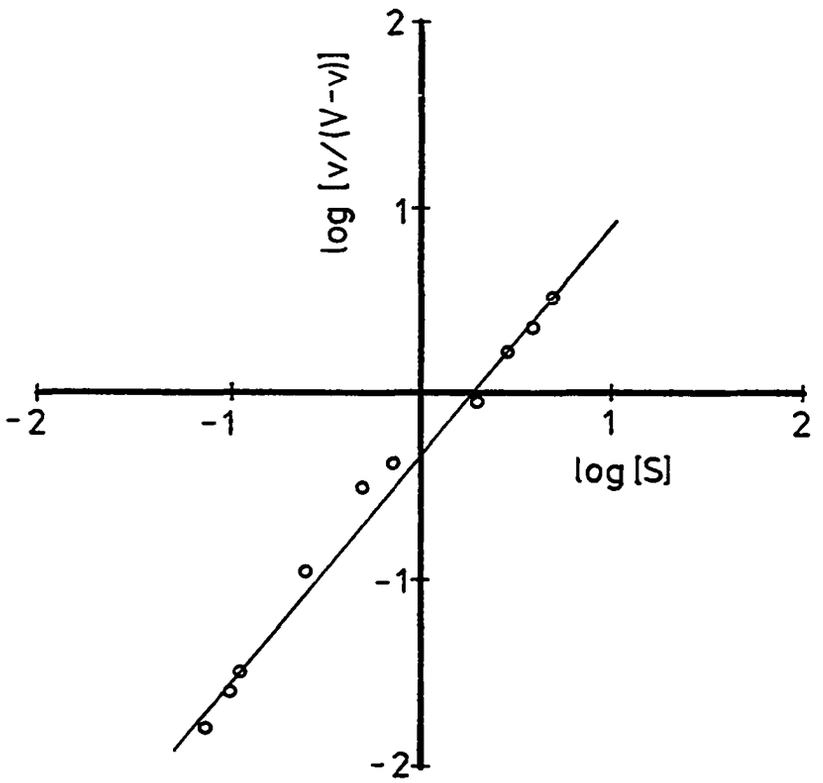

2
1
-1
-2
-2
-1
1
2
log [v/(V-v)]
log [S]

Figure 18. Hill plot for mycelial chitin synthetase at high GlcNAc concentration (100mM)

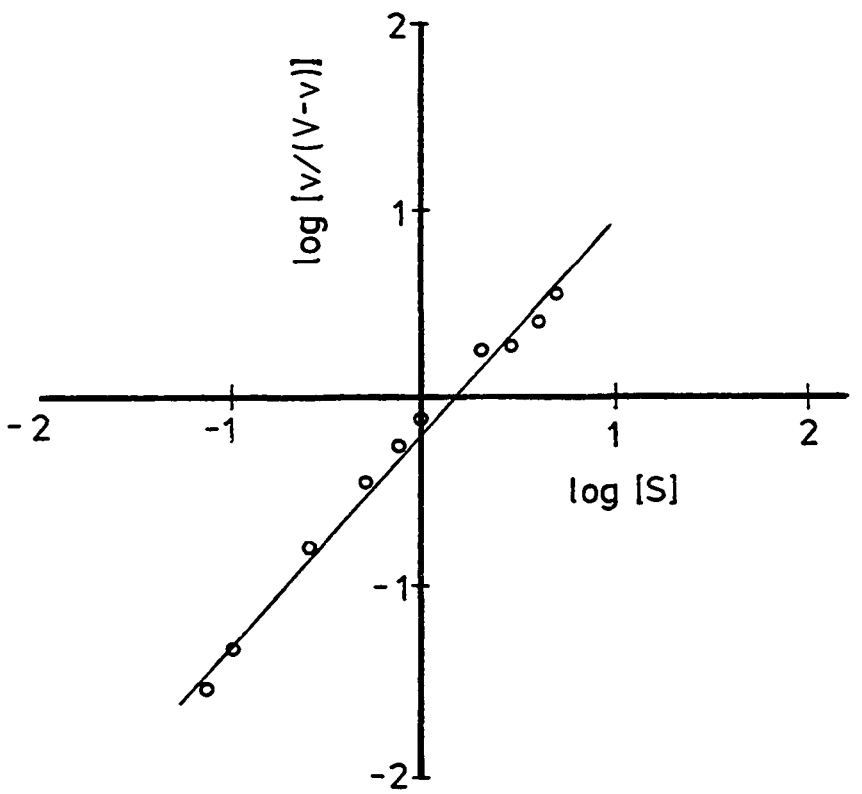
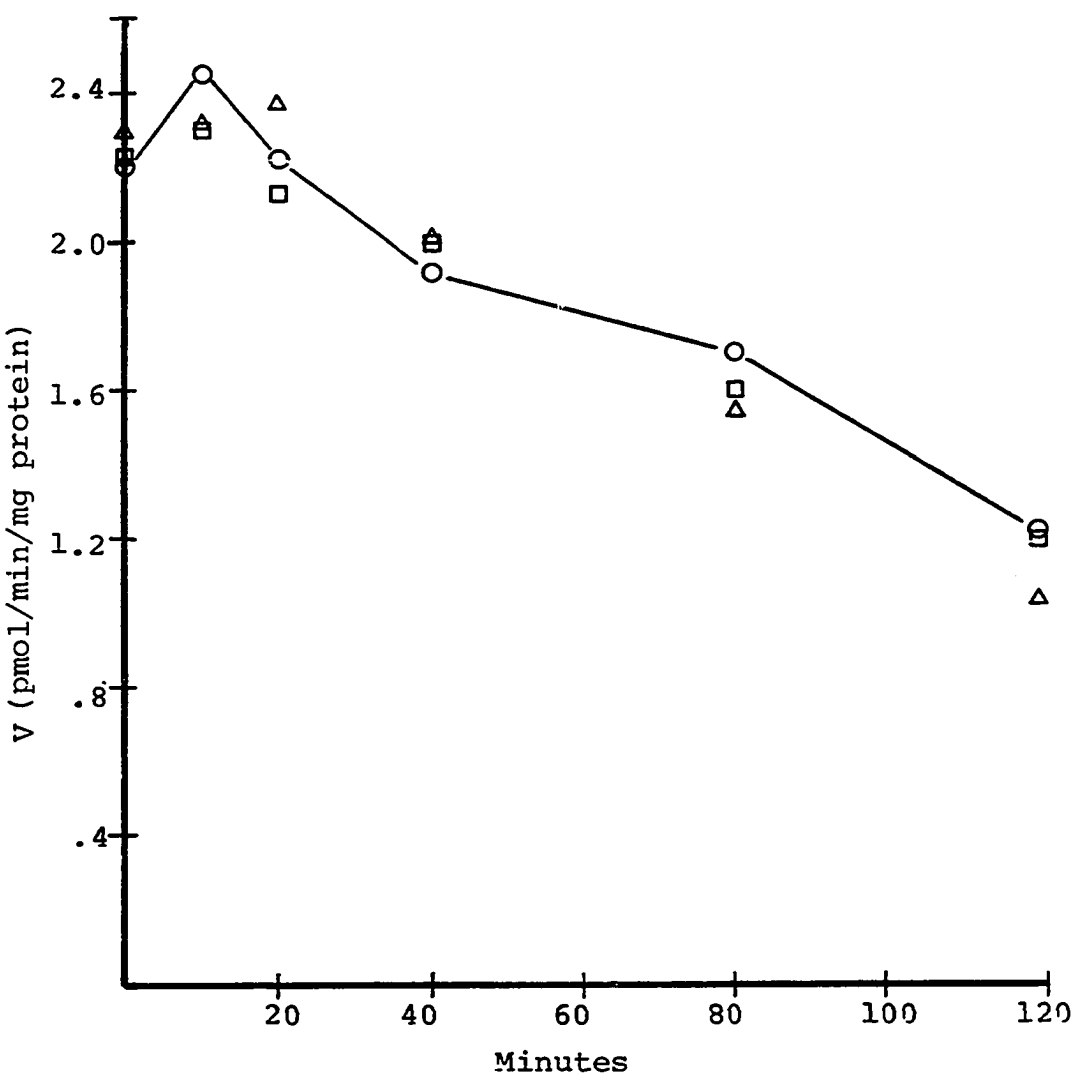

2
1
-1
-2
-2
-1
1
2
log [v/(V-v)]
log [S]

Figure 19. Effect of 100K supernatant on yeast phase chitin synthetase.

Buffer control (○)

Yeast supernatant (□)

Mycelium supernatant (△)


2.4
2.0
1.6
1.2
.8
.4
V (pmol/min/mg protein)
20
40
60
80
100
120
Minutes

CHAPTER IV

DISCUSSION

There are two important cautions that must be considered when interpreting enzyme data based on crude preparations such as those used in this study. First, the assay measures the conversion of soluble substrate to an insoluble product. This product may or may not be formed by one enzyme. Thus the assay could be measuring several isoenzymes which would mean all kinetic data are averages. Second, the contaminating molecules in the preparation may have significant influence on activity by binding substrate, activator, ions, etc. or by masking the enzyme. For example, the increase in activity of the yeast enzyme by protease treatment could be caused by removal of proteins which cover the active sites. The use of a pure enzyme preparation would eliminate these ambiguities. However, since attempts to solubilize Blastomyces chitin synthetase with 1% triton x-100, 1% digitonin and butanol (data not shown) were unsuccessful, the only recourse is to use a crude preparation and keep in mind the limits of such experimentation.

The distribution of chitin synthetase in Blastomyces is similar to other systems in that activity is associated with easily sedimented particles (7,8,18,21, 42,47,63). Usually the low speed (cell wall) and high speed (e.g., 100,000 x g)

fractions exhibit the greatest activity (63) as did Blastomyces. One exception to this is Aspergillus flavus which shows no activity associated with cell walls (38). The enzyme fractions used in the above sudies are derived from cells broken by harsh methods such as ballistic disruption with glass beads. When milder lytic conditions are employed chitin synthetase is usually found only in high speed mixed membrane fractions. Enzyme from Candida albicans protoplasts extracted by metabolic lysis is found only in a membrane fractions sedimenting at 80,000 x g (8). This and other data indicate an intimate association of the enzyme with membranes. In Blastocladiella (21), Candida (8), and Saccharomyces (25,26) there is good evidence that chitin synthetase is attached to the inside of the plasma membrane. Data in many other organisms, however, indicate that chitin synthetase occurs in discrete vesicle-like structures 40 to 70 nm in diameter termed chitosomes by investigators from the laboratory of Bartnicki-Garcia (7). The most accurate theory is still debatable. Perhaps the enzyme is found on the plasma membrane and also in particles. A very likely explanation is that enzyme is located on the plasma membrane which when disrupted by ballistic methods forms small membrane associated particles.

The stimulation of C-syn activity by GlcNAc is apparently universal (see ref # 28 for brief review). Yeast and mycelial C-syn from Blastomyces were stimulated to different degrees. Yeast enzyme activity increased 6 fold with a Km of 8.5 mM and a plateau at approximately 60 mM. The mycelial enzyme was only stimulated 2 fold with a Km of 4 mM and a plateau at approximately 30 mM. These Km values are within the range of other systems, e.g., 3 mM for Blastocladiella emersonii (13) to 12.5 mM for Mucor rouxii (42). The activation by free glucosamine may serve an important function in vivo by stimulating

C-syn in regions of wall autolysis which must occur during yeast budding in particular.

Yeast and mycelial enzyme were very similar as regards temperature optima although one might expect a higher value for yeast or at least a tolerance of higher temperatures. The only significant differences was that the yeast enzyme showed a slower decline in activity at temperatures above the optimum of 28°C. This optimum value is within the range of other systems, e.g., 24°C for Mucor rouxii (55) to 33°C in Mortierella vinacea (47).

As with temperature, the pH optima for yeast and mycelial enzyme were nearly identical at 7.0 to 7.5. Differences consistently noted were the sharp decline in activity in the yeast phase below pH 7 as opposed to very gradual changes for the mycelial enzyme. The yeast enzyme was more tolerant for pH values above 7 however. The most common pH optima for other systems seems to be 6.5 or slightly below (43,47) although the optimum for the slime variant of Neurospora crassa is over 8 (59).

Chitin synthetase from most organisms also requires a divalent cation usually served best by Mg^{+2}. The most common optimum is approximately 10 mM although the published range is from 1 mM in the slime variant of Neurospora crassa (59) to 30 mM in Mucor rouxii (42). Yeast and mycelial enzyme from Blastomyces were stimulated by Mg but as with GlcNAc stimulation the yeast enzyme was more sensitive. The activity dropped quickly below the optimum of 10 mM for yeast but only slightly decreased at Mg concentrations below the 5 mM optimum for mycelial enzyme.

The sigmoidal nature of Michaelis-Menten and Lineweaver-Burke plots with Blastomyces enzyme is similar to most other C-syn systems. In practically every other organism studied double reciprocal plots are curved at low GlcNAc levels

and become linear, indicating normal Michaelis-Menten kinetics are operative, at higher activator concentrations. This linearization usually occurs at GlcNAc levels at or below 50 mM (56,59,63). In some organisms, however, the sigmoidal nature of the curve is lessened as GlcNAc levels increase but is not linearized (22,42). As GlcNAc concentrations increase the apparent Km for UDP-GlcNAc changes from 3 to 4 mM to between 1 and 2 mM (47, 63). Blastomyces enzyme was similar in that sigmoidal kinetics were noted. The yeast enzyme was again most sensitive to activator changes and indicated by the extreme curvature of Lineweaver-Burke plots and apparent Km changing from 3 mM at 5 mM GlcNAc to approximately 1.5 at 30 and 100 mM activator. In contrast to the yeast, the apparent Km for UDP-GlcNAc with the mycelial enzyme remained essentially unchanged at approximately 1.5 mM at low, intermediate and high activator concentrations. In both phases the sigmoidal nature of the data was lessened but not abolished at high GlcNAc levels. Only by disregarding UDP-GlcNAc concentrations below 0.5 mM for yeast enzyme and below 0.25 mM for mycelial enzyme was a linear double reciprocal plot obtained.

Although estimates of binding sites by Hill plots are subject to much interpretation it is interesting to compare apparent n values from Blastomyces enzymes to other systems. Estimates of Hill coefficients for chitin synthetase usually fall below 2 (18) but have been calculated at 4 at low substrate levels with enzyme from the fungus Coprinus cinereus (56). The slope obtained by Hill plots with yeast and mycelial enzyme was approximately 1.3 regardless of activator concentration indicating weak interaction of binding sites.

It is somewhat surprising not to find evidence of an activating protease in Blastomyces since trypsin activation of yeast enzyme resulted in much greater activity. Activating proteases have been noted in several other organisms using similar sonication methods, e.g., Candida albicans (8), Saccharomyces cerevisiae (62) and Aspergillus nidulans (14). In Candida, however, activating protease was only found in the yeast phase (8) and no activating factor was found in Mortierella vinacea (47).

It is more surprising to find no evidence of a soluble inhibitor since such inhibitors are fairly common in yeast and mycelial organisms (11,20,39). In fact even organisms which have no known activating protease do have inhibitor, e.g., Candida albicans (8) in both yeast and mycelium and in Mortierella vinacea (47).

It is possible that activating protease and inhibotor are in such small quantities that the crude sonication method results in a concentration too low to detect. This might especially be true for very slow growing fungi such as Blastomyces. Literature concerning other fungi show several hundred fold more chitin synthetase activity than Blastomyces thus small amounts of activating protease and inhibitor would be expected. Another explanation may be that protease and inhibitor are present but bound to each other and thus inactive and undetectable. Inhibitor and protease from Saccharomyces bind in a equimolar ratio but are segregated since the protease is bound by vesicles and liberated by sonication (62).

The most striking difference in chitin synthetase from Blastomyces yeast and mycelial phase was the response to trypsin activation (Fig. 2). Yeast C-syn apparently exists mianly in a latent form (perhaps a true zymogen or simply masked by protein) that must be activated by protease to achieve maximal

activity. Mycelial C-syn, however, exists almost completely in an active form since trypsin incubation drastically reduced chitin formation. There may be some latent enzyme in the mycelium since after the initial sharp decline of activity upon proteolytic activation a small increase was noted. This difference is very similar to that noted in the yeast and mycelial phase of Mucor rouxii (54). In contrast to Mucor and Blastomyces, Candida albicans C-syn from both yeast and mycelial phase requires proteolytic activation (8). An important point in interpreting this difference in C. albicans may be that Candida sp. form a limited pseudo-hyphal mycelium rather than the extensive eumycelium of Mucor and Blastomyces. The interpretation is further clouded, however, by data showing that chitin synthetase extracted from the filamentous fungus Neurospora crassa by ballistic disintegration is found primarily in an latent form (1).

Based on the similarity of pH, temperature, and magnesium optima and kinetic data, it appears that the chitin synthesizing enzymes from the yeast and mycelial phase of Blastomyces dermatitidis are essentially the same (c.f., Figures 7-9 and Table II). The difference in C-syn from the two growth phases is one of the state of activation rather than presence of different enzymes. The important question is does this difference in the state of activation have a role in dimorphism. Similar results in Mucor (54) have been postulated to play a role in dimorphism in the following manner: if C-syn is transported to areas of wall synthesis by chitosomes which then fuse with the plasma membrane the fully active enzyme would operate for only a brief time before being inactivated by endogenous protease. This would result in tubular growth if chitosomes were transported primarily to the hyphal apex as some data indicate (21,43). Inactive enzyme inserted into the plasma membane would gradually be activated and then

slowly degraded by protease and thus would result in a more spherical or ellipsoidal wall growth. If the chitosome theory is incorrect, however, this active versus inactive insertion idea would be invalid.

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3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

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