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HARTIS, EILEEN HARLEY. Comparative Thermal Properties of Aryl-β-Glucosidase Isozymes in Neurospora crassa. (1975) Directed by: Dr. Bruce M. Eberhart Pp. 52

Thin layer gel filtration of crude conidial washes from 26 different strains of Neurospora crassa demonstrated three distinct areas of β-glucosidic activity. The area farthest from the origin was the largest enzyme, aryl-β-glucosidase "Y". The second, smaller isozyme "W" was located closer to the origin, and the third, smallest, newly discovered isozyme, designated "V", was closest to the origin. The new aryl-β-glucosidase "V" was present in extremely low concentration, and required 9-12 hours incubation with substrate (4-methyl-umbelliferyl-β-D-glucopyranoside) in order to note activity. Attempts to purify and isolate "V" were only partially successful.

The largest aryl-β-glucosidase "Y" was the predominant form, although the ratio of "Y" to "W" varied among the exotics tested. The "V" activity was negligible compared to "Y" and "W". Intra-strain activity ratios for "Y" and "W", as determined by thin layer gel filtration, were recorded for fresh preparations and the same preparations after ageing and freeze-thawing. Some strains demonstrated more "W" after ageing than was previously noted in the fresh preparations. A slow association-dissociation process of the type $(4n) \rightleftharpoons 2\ (2n) \rightleftharpoons 4\ (n)$ is proposed, where n is the monomer, and ("Y) $\rightleftharpoons$ 2 ("W") $\rightleftharpoons$ 4 ("V").

The thermal relationship between the isozymes "Y" and "W" was investigated using exotic strains which demonstrated large quantities of "W" in addition to "Y". The exotic P-278 normally produced low levels of "W" when grown on complete medium (GSCP), but produced more "W" when grown on GSCP + 5% ethylene glycol. Preliminary thermal inactivation experiments utilized crude conidial washes which contained both "Y" and "W".

Heating of washes at 55 C produced a two component inactivation curve where the first, more thermolabile component corresponded to the "W" enzyme, and the second, more thermostable component was "Y". The addition of an equal amount of various buffers to crude washes indicated a protective effect, and a higher temperature was required to demonstrate a similar biphasic inactivation curve.

The main thermal inactivation experiments were done with purified "Y" and "W" enzymes from selected exotic strains and a wild-type control. Thermal half-lives were calculated using linear regression analysis. Intra-strain and inter-strain comparisons of independent "Y" and "W" half-lives were utilized as the basis for comparative statements concerning the distinct thermal relationship between the isozymes from any given strain. Strains which produced a relatively stable "Y" also produced a relatively labile "W". The converse relationship was also observed where a labile "Y" was associated with a stable "W". The possible adaptive significance of isozymes is discussed with respect to their relative thermal stabilities.

COMPARATIVE THERMAL PROPERTIES OF

ARYL-β-GLUCOSIDASE ISOZYMES

IN NEUROSPORA CRASSA

by

Eileen Harley Hartis

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Approved by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

The development of the high resolution "zymogram" technique of electrophoresis by Hunter and Markert in 1957 (Shaw, 1969) made possible extensive investigation of numerous enzymes which exist in more than one molecular form. In 1959, the term isozyme was introduced by Markert and Moller (Shaw, 1969) to refer to those enzymes which do occur in multiple molecular forms with similar or identical substrate specificity within the same organism. Since the coining of the term, the occurrence of isozymes has been recognized as a common biological phenomenon, and, in fact, Scandalios (1974) suggests that isozymes are the rule rather than the exception. However, he cautions that his statement must be qualified since negative data are rarely published. Shaw (1969) estimates that approximately one-half of all enzymes occur as isozymes.

During the early years of study after 1957, a large number of enzymes in a variety of organs and tissues, both plant and animal, displayed multiple zones of activity on zymograms. This led many researchers to question the significance of isozymes in relation to the organism and natural selection. One group believed that isozymes were an *in vitro* effect of the electrophoretic technique while another group held that isozymes did exist *in vivo* and presumably were of some biological advantage to the organism (Shaw, 1969). Shaw maintains that the evidence supports both positions, in that some isozymes may be artifacts, but

increasing numbers of studies indicate that isozymes do serve cellular economy since they are not all absolutely biologically identical.

It is highly probable that although isozymes may show many similarities including identical catalytic activity, there are often significant differences between them (Shaw, 1969). Shaw (1969) distinguishes three major types of differences between isozymes as variations in their: (1) role in development and differentiation of tissues, (2) regulation, and (3) enzyme activity.

Many isozymes have been found to differ with respect to the development and differentiation of the organism. Two valuable reviews on this subject have been written by Masters and Holmes (1972) and John Scandalios (1974). Both reviews cite a number of different organisms and tissue specific isozyme systems including the archetypal isozyme system of lactate dehydrogenase (LDH). A notable example of a variation in isozymic pattern during development was reported by Coston and Loomis (1969). They found two different varieties of β-glucosidase which occurred at two different stages in the morphogenesis of *Dictyostelium discoideum*. Both enzymes were electrophoretically distinct from each other.

A second significant difference between isozymes can result when they respond differently to changes in the cellular environment. Such isozymes exhibit a regulatory function which Shaw (1969) believes must be under complex genetic control. Tsao and Madley (1969) reported a definite shift in the electrophoretic pattern of phosphofructokinase isozymes from *Neurospora crassa* in response to depletion of nutrients in the culture medium. *Neurospora* isocitrate lyase enzymes (Sjogren and Romano, 1967)

of strains grown on glucose or acetate differed in pH activity curves, K_m, and sensitivity to inhibition.

The third and most interesting aspect of the significance of isozymes relates to differences in enzymatic activity. Although the zymogram technique has been instrumental in the discovery of many isozyme systems, electrophoretic procedures tend to emphasize catalytic similarities between isozymes and are of little use in detecting kinetic differences. Also, since demonstration of multiple enzyme forms using electrophoresis is based on separation by charge, many isozymes may not be revealed because they do not involve charge variation (Masters and Holmes, 1972).

Shaw (1969) points out that it is tempting to hypothesize, based on kinetic differences between isozymes, that one form of an enzyme provides an adaptive advantage over another form; and, therefore, is maintained in the population through selection. However, one team of researchers claim that the majority of enzyme polymorphisms are "adaptively neutral" and have no significance to survival or reproduction (Johnson, 1974). In his review Johnson (1974) does present some evidence that isozymes and enzyme polymorphisms are not selectively neutral, but are related to metabolic regulatory function. Scandalios (1974) supports the idea that kinetic differences between isozymes may be great enough to "allow for flexibility of the biological role". However, proving the selective advantage of multiple forms of an enzymatic molecule is extremely difficult and presents a real exercise in experimental design (Shaw, 1969).

In order to better understand isozyme systems, it is helpful to consider possible mechanisms of isozyme formation. John Scandalios (1974) suggests two major mechanisms associated with genetic and chemical or

physical alterations. The first mechanism maintains that isozymes may result from "gene duplication and subsequent mutation of daughter and parental loci". Therefore, this method is postulated to contribute to the structure and function of any enzyme composed of more than one kind of subunit. The second means by which isozymes may arise involves chemical or physical modification of a single polypeptide. Such modifications include the binding of coenzyme molecules and prosthetic groups, conjugation or deletion of molecules with reactive groups, and variation of tertiary or quaternary structure of a given primary polypeptide. Scandalios (1974) also recognizes that isozymes may be generated during storage and preparative procedures. Shaw (1969) suggests a similar classification of isozymes based on origin. He denotes "primary" isozymes as those which are distinctly different polypeptides and presumably produced from different genetic loci, while "secondary" isozymes are produced by secondary alterations of a single polypeptide.

One of the most important implications of isozyme systems is related to their genetic potential. Isozymes may differ in primary structure because they are encoded in different genes, either allelic or non-allelic (Scandalios, 1969). Scandalios (1969) describes the genetic control of isozymes in various plants and refers to isozymes as a "natural 'built-in' marker system" which can be an effective tool in the study of inter-allelic complementation, heterosis, and differential gene action in the development of higher organisms. In their review, Isozymes and Ontogeny, Masters and Holmes (1972) suggest that isozyme systems in morphogenesis have the extraordinary potential of "conjuring up relationships with phylogeny". Recently, isozyme electrophoresis has been

used extensively in the taxonomy of the fungus, Neurospora (Reddy and Threlkeld, 1971 a,b, and 1972; Reddy, 1973).

Fungi are well suited for biochemical and genetic analysis due to their brief life cycle, simplified sexual and asexual reproduction, and well-defined nutritional requirements. Fungi can be studied easily as microorganisms and provide a basis of comparison between prokaryotes and eukaryotes. Neurospora crassa is an especially useful research organism because of its carefully defined genetic background and convenient growth characteristics.

Many enzymes in Neurospora have been demonstrated to exist in more than one molecular form. The isozyme systems reported include: β-galactosidase (Bates and Woodward, 1964; Johnson and DeBusk, 1970 a,b), isocitrate lyase (Sjogren and Romano, 1967), glutamate dehydrogenase (Sundarum and Fincham, 1964; Fincham and Garner, 1967), mitochondrial dehydrogenase (Benveniste and Munkres, 1973), esterase (Reddy, 1973), invertase (Metzenburg, 1964; Trevethick and Metzenburg, 1964), trehalase (Hill and Sussman, 1963; Yu, et al., 1971; Hecker and Sussman, 1973 a,b), and β-glucosidase (Eberhart, et al., 1964; Eberhart and Beck, 1970; Madden, 1971).

The β-glucosidases of Neurospora crassa are of particular interest since they are believed to be involved in the carbon cycle via the degradation of cellulose to CO_2 and water. Madden (1971) refers to the following scheme of the degradative process:

$$\text{native cellulose} \xrightarrow{\text{unidentified cellulase}} \text{modified cellulose} \xrightarrow{\text{cellulase}}$$

$$\text{cellobiose} \xrightarrow{\text{cellobiase}} \text{glucose}$$

In 1961, Berger and Eberhart reported p-nitrophenyl-β-D-glucosidase and cellobiase-transglucosidase activity in conidial washes of Neurospora crassa. The test for β-glucosidase activity was based on the solubility of the conidial enzyme in water (Eberhart, 1961). Later, Eberhart, Cross, and Chase (1964) demonstrated two β-glucosidases and two cellulases in mycelial extracts using ammonium sulfate precipitation. The thermostable β-glucosidase was designated aryl-β-glucosidase "Y", indicating the substrate most effectively attacked. The enzyme "Y" was present in the mycelium and in conidia. A second, more thermolabile β-glucosidase, designated "X" or cellobiase, was found only in the mycelium.

The genetic regulation of aryl-β-glucosidase "Y" has been investigated. Mutant strains were isolated in which two genes act as repressors to β-glucosidase production. The gluc-1 allele was dominant and permitted only 10% of the normal production of the enzyme (Eberhart, 1962; Mahadevan and Eberhart, 1962; Eberhart, et al., 1964). Eberhart, Cross and Chase (1964) also reported that Neurospora produced at least two cellulases, neither of which were effected by the gluc-1 gene. Meyers and Eberhart (1966) isolated a mutant strain in which the recessive regulatory gene, designated cell-1, resulted in constitutive production of cellulase and cellobiase. An apparent second allele, gluc-2, was found which decreased production of aryl-β-glucosidase to less than 1% of normal (Mahadevan and Eberhart, 1964 a,b; Eberhart and Beck, 1970). Aryl-β-glucosidase was identified as primarily a mural (associated with the cell wall) enzyme and cellobiase as primarily cryptic (endocellular).

The thermal stability of both aryl-β-glucosidase "Y" and cellobiase "X" has been determined. Only a slight variation in half-lives within

groups of wild-type "Y" and "X" enzymes was noted (Eberhart and Beck, 1970). However, Mahadevan and Eberhart (1964 c) reported striking differences in the "Y" half-lives of exotic strains. In these studies thermal properties were used as a standard method of distinguishing between enzymes when they were both present in the same solution. Differential heat inactivation was often used as a means of physical separation.

During a survey of various exotic strains of Neurospora crassa, Madden (1971) discovered a second aryl-β-glucosidase isozyme, designated "W", in addition to aryl-β-glucosidase "Y". The "new" β-glucosidase was detected in some crude conidial washes using electrophoresis. Purified preparations of the isozyme "W" were obtained from the exotic strain P-212, and an intensive investigation of the physical properties of "W" undertaken. Madden (1971) found that attempts to induce "W" with a variety of substrates were unsuccessful, but "Y" was easily induced by cellobiose. In addition, "W" had an extremely low affinity for cellobiose compared to "Y". In fact, Madden (1971) referred to "W" as a "super" aryl-β-glucosidase since it demonstrated a much greater affinity for aryl compounds than "Y". Thermal inactivation of purified "W" at 60 C indicated that "W" was more thermolabile than "Y".

This report is primarily a more "in-depth" study of the thermal relationship of the isozymes "W" and "Y" from selected exotic strains and a wild-type control. Few investigators of isozyme systems go beyond establishing a basic comparative statement of thermal stability. My goal was to refine the existing statement that "W" is more thermolabile than "Y". Experimentation was directed to determine whether strains which

produced a stable "Y" (relative to those of other strains) also produced a relatively stable "W". By utilizing intra-strain and inter-strain comparisons of independent thermal half-lives of "W" and "Y", this report presents evidence for a definite thermal relationship between aryl-β-glucosidase isozymes. In addition, observations on the effect of "ageing" on frozen isozyme preparations, the effect of growth conditions on isozyme production, and evidence for the existence of another "new" isozyme, designated "V", are discussed.

CHAPTER II

MATERIALS AND METHODS

Chemicals

P-nitrophenyl-β-D-glucopyranoside (PNPG) was obtained from Calbiochem. Nutritional Biochemicals Corporation were suppliers of 4-methyl-umbelliferyl-β-D-glucopyranoside (umbelliferone), and the 12 buffer kit. Bio-Rad Laboratories were suppliers of Bio-Gel P-200 and Bio-Gel P-150. Lyphogel and all electrophoretic products were obtained from Gelman Instrument Company. Carbowax Polyethylene Glycol Compound 20-M and 6000 were purchased from Union Carbide.

Maintenance and Growth Media of Cultures for Crude Conidial Washes

Stocks were maintained on agar slants of modified glycerol complete medium (Eberhart, et al., 1964) containing 1% sucrose, 0.8% glycerol, 1.0% vitamin stock solution, 1.0% Vogel's minimal salts (Vogel, 1956), 0.25% yeast extract, 0.1% Bactocasitone, and 1.5% agar. In some instances, the above medium (GSCP) was supplemented with 5% ethylene glycol and designated GSCP + 5% E.G.

Conidia in quantity were obtained by inoculation of conidial suspensions in glass distilled water into 500 ml Erlenmeyer flasks containing 100 ml of medium. Wide mouth flasks were preferable for growth and conidiation, and were used in most experiments. Occasionally, strains were inoculated directly into flasks from glass distilled water suspensions of silica gel cultures in an effort to minimize subculturing and contamination.

The conidial flasks were incubated at 30 C for three days, then maintained at room temperature (approximately 23 C) for 4 days. The mature cultures were harvested for crude β-glucosidase preparations (containing both "Y" and "W") based on the solubility of the conidial enzymes in water (Eberhart, 1961). One hundred ml of glass distilled water were added to each mature conidial flask and gently shaken with the cotton plug in place. The resulting conidial suspension was filtered through four layers of gauze to remove any mycelium. The filtrate was then centrifuged in a Sorvall SP/X centrifuge at 3,400 X g for 15 minutes or centrifuged at 3,500 X g for 15 minutes at 5 C in a Sorvall Superspeed RC2-B centrifuge.

The supernate was placed in dialysis tubing (Fisher Scientific Company) and concentrated with polyethylene glycol compound (Carbowax) in the refrigerator. Any further concentration was done with lyphogel (Gelman Instrument Company).

Thin Layer Gel Filtration

Thin layer gel filtration was introduced in our laboratory as an additional method of separation of aryl-β-glucosidase isozymes based on their molecular weight and shape. Occasionally, highly concentrated crude conidial washes were diluted with glass distilled water to eliminate viscosity effects that would result in lagging. A control preparation of 740R23-1A was consistantly used in all comparative size evaluations. Each control was carefully diluted to obtain maximum mobility and run on T.L.G.

Thin layer gel filtration was performed with the Pharmacia Fine Chemicals-TLG-apparatus. Initial experiments utilized Bio-Gel P-200

-400 mesh (5.0g/100 ml 0.05 M potassium-phosphate, pH 6.0) spread to 0.6 mm thickness on one 40 x 20 cm glass plate. The eluant was 0.05 M potassium-phosphate, pH 6.0 (60 ml was placed in the top reservoir and 40 ml in the bottom). The gel layer was connected to the reservoirs by Whatmann #3 filter paper bridges. The plate was equilibrated overnight at a 10 degree angle. From 5-6, 10 λ samples were applied, and allowed to run for 6-8 hours. After a separation, the plate was removed from the chamber and covered with filter paper pre-soaked in a saturated solution of 4-methyl-umbelliferyl-β-D-glucopyranoside (umbelliferone). Plastic wrap was applied and the origins carefully marked.

As the β-glucosidic bonds present in the substrate were cleaved, a fluorescence was observed under ultra-violet light. Very small amounts of enzymatic activity could be detected since prolonged incubation with substrate showed a minimum of diffusion effects. Areas of activity were marked on the plastic wrap and later transferred to a permanent record.

Later in the study, a shorter method of T.L.G. filtration was developed. Bio-Gel P-150 -400 mesh was selected for relative ease of swelling with less lumps produced. Two 20 x 20 cm plates were connected by a central plate spacer. For economy, one or both plates could be spread with gel as long as control samples were run on both plates (if comparisons were desired). Sample applications were reduced to 5 λ since resolution was greater, 6-8 samples could be applied easily, and enzyme solutions conserved. Runs were carried out at a 15 degree angle and required 2.0-2.5 hours for efficient separation.

Electrophoresis

Electrophoretic experiments were performed with the Gelman Sepratek System according to the method of Madden (1971).

Ammonium Sulfate Precipitation

Ammonium sulfate precipitation is a method that has been utilized in the separation of the β-glucosidases from Neurospora crassa (Eberhart, et al., 1964; Madden, 1971). My purpose was to investigate the separation of "V" from "W" and "Y" by using ammonium sulfate precipitation.

Ammonium sulfate was added to 30 ml of concentrated crude conidial wash from P-278 to make a 20% saturated solution (Colowick and Kaplan, 1955). The mixture was stirred constantly in an ice bath at 0 C. After equilibration for 5-10 minutes in the bath, the sample was centrifuged at 10,000 X g for 10 minutes at 5 C. The supernate was carefully decanted and replaced in the ice bath. The remaining precipitate was suspended in 0.05 M potassium-phosphate buffer, pH 6.0, and frozen. Ammonium sulfate was added to the supernatant fraction to obtain a 30% saturated solution. The above procedure was repeated. The resulting supernate was treated with ammonium sulfate at 5% increments until an 80% saturation point was reached. The last addition of ammonium sulfate was performed at the 80-90% saturation level. Thin layer gel filtration and electrophoresis of fractions was used to determine whether a separation had been achieved.

PNPG Assay

The substrate, p-nitrophenyl-β-D-glucoside (PNPG), was used for quantitative assays of β-glucosidase activity. The glucoside is attacked by β-glucosidase which releases a chromogenic p-nitrophenyl moiety (at an

alkaline pH) from the ends of the molecule. The discontinuous colorimetric method reported by Eberhart (1961) was modified for this study. A standard 10 ml substrate solution was prepared by addition of 20 mg PNPG to 10 ml 0.05 M citrate-phosphate buffer, pH 6.0 (yielding a final PNPG concentration of 2 mg/ml). The above solution was kept frozen when not in use. The assay was initiated by the addition of 0.2 ml PNPG to a 0.2 ml enzyme sample to give a final substrate concentration of 1 mg/ml. After the appropriate incubation interval (depending on the enzymatic activity of the sample), the reaction was stopped by the addition of 0.2 ml 1M Tris, resulting in the conversion of the released p-nitrophenol to its colored form. Controls consisted of enzyme samples in which the order of addition of reagents was reversed (Tris first, then substrate). The optical density of each sample was read at 410 nm on a Beckman/Spinco 151 Spectro-Colorimeter and the readings were converted to optical density/minute.

Gel Column Filtration

Gel column filtration was used to obtain purified preparations of the extracellular β-glucosidases ("W" and "Y") from exotic strains and the wild-type control. Approximately 10 ml of concentrated crude conidial wash were pipetted onto a Pharmacia Column (50 K, 42 x 5 cm) packed with Bio-Gel P-150 100-200 mesh. The eluant in all cases was 0.05 M potassium phosphate buffer, pH 6.0. Approximately 7 ml fractions were automatically collected and placed in the refrigerator until assay. Later in the study, the procedure for collection of samples was modified to eliminate the need for manually placing fractions in the refrigerator. Fractions were automatically collected in test tubes suspended in a 4.5 C water bath (Buchler Instruments). Collected fractions remained in the bath until assay with

PNPG and then were frozen. Elution profiles were plotted directly from the results of the assay. Later, thawed fractions were run on T.L.G. to determine which were purified enough to be used for further study.

Thermal Inactivation

All thermal inactivation of enzyme solutions was done in glass tubes (7.5 cm x 1.0 cm). Using an Oxford Laboratory Sampler, 0.2 ml of enzyme was pipetted into each tube and placed in a rack. The samples were immediately submersed in an ice bath at 0 C and chilled until ready for use. Individual tubes were transferred to a heated water bath maintained ± 0.5 C, the desired temperature for inactivation. After specified times, the sample was removed and replaced in the ice bath. The rack was immediately swirrled for 10-15 seconds to cool the solution and prevent further thermal inactivation. The control sample and the initial activity (0 time heating) sample were maintained in the ice bath throughout the above procedure.

The rack was transferred to a 25 C water bath for a 10 minute equilibration. The assay was initiated by pipetting 0.2 ml PNPG (2 mg/ml in 0.05 M citrate-phosphate buffer, pH 6.0) into each tube. After various incubation periods (depending on the activity of the sample), the reaction was stopped by the addition of 0.2 ml 1M Tris. The optical density of each sample was read at 410 nm on the Spectro-Colorimeter and the readings were converted to optical density/minute.

Thermal half-lives for purified enzymes were calculated using linear regression analysis based on the $\log_{10}$ of the enzymatic activity. A least squares program was developed by Dr. W.K. Bates for the regression line equation of $\log_{10} Y = a_0 + a_1 t$. A Wang Model 450 programable

calculator was used to determine the thermal half-life where the time value was entered as the X co-ordinate and the optical density/minute as the Y co-ordinate. Each set of data was entered two times to eliminate any mechanical error when entering the numbers. The Y-intercept (a_0) and the slope (a_1) of the regression line were recorded for future reference.

CHAPTER III

RESULTS

This study originally began as a survey of the exogenous aryl-β-glucosidase isozymes ("W" and "Y") produced by exotic strains of Neurospora crassa (obtained from Dr. David Perkins of Stanford University) to determine whether any qualitative or quantitative differences exist. In this laboratory using electrophoresis of crude conidial washes, Madden (1971) not only confirmed the presence of the known aryl-β-glucosidase "Y", but also discovered a new isozyme "W" in some exotic strains. My objective was the further investigation of the occurrence and physical properties of "W" and "Y" in exotic strains utilizing a method new to our laboratory, thin layer gel filtration. This method, with its many useful applications to enzyme and protein research, made possible the detection of very low levels of enzymatic activity and a comparative evaluation of molecular size.

The data presented in this section represents the results of the two broad areas of my research. The first area was concerned with the development, refinement, and application of the T.L.G. filtration technique. During this time intra-strain enzymatic activity ratios for "W" to "Y" were noted, and in subsequent experiments the effect of ageing on these ratios was observed. Also, a third, new aryl-β-glucosidase was discovered and experiments were devoted to partially successful attempts at isolation and purification of the enzyme. The results indicated that the emphasis of this research should be shifted to a more productive area,

focusing on particular exotics which produced larger amounts of "W" in addition to "Y". These exotics could be used to better determine the isozyme relationship of "W" and "Y".

Therefore, the second area and major emphasis of this research was the investigation of the exact thermal relationship (stable or labile) between two aryl-β-glucosidase isozymes "W" and "Y". The experiments in this area will be presented under two headings:

Preliminary experiments using crude conidial washes for the determination of parameters for further thermal experimentation and the comparison of gross thermal characteristics between individual strains.

Main experiments involving the thermal inactivation of purified preparations of "W" and "Y" from selected strains and the subsequent analysis of thermal half-lives.

Thin Layer Gel Filtration of Aryl-β-Glucosidase Isozymes

Thin layer gel filtration of crude conidial washes from 26 different exotic strains of Neurospora crassa demonstrated distinct areas of enzymatic activity indicating the presence of both "W" and "Y" aryl-β-glucosidase isozymes in all strains tested. In addition, a third, new area of β-glucosidase activity, designated "V", was detected in very low concentration after 9-12 hours incubation with substrate.

The area farthest from the origin was the largest enzyme, aryl-β-glucosidase "Y". The smaller isozyme "W" was located closer to the origin than "Y". The smallest enzyme "V" was closest to the origin. The average migration rates on T.L.G. for "V", "W", and "Y" are summarized in Table 1.

TABLE 1

AVERAGE MIGRATION RATES ON THIN LAYER GEL

OF THE ARYL-β-GLUCOSIDASE ISOZYMES

Bio-Gel Type -400 mesh	Angle	Number of Experiments	Migration Rate in cm/hr.		
			"V"	"W"	"Y"
P-200	10	4	0.35	0.72	1.15
P-200	15	3	0.88	1.55	2.27
P-150	15	10	no data	1.94	2.92

Distances in cm were computed from the origin to the center of activity. Each experiment was represented by an average of 6 samples. The average migration rate was calculated from a minimum of 3 individual experiments. The mobility of individual samples within an experiment was very similar and no consistant size differences could be detected. Figure 1 depicts the characteristic mobilities on T.L.G. of Neurospora β-glucosidases.

Most of the enzymatic activity detected was due to "Y", but the ratio of "Y" to "W" varied among the exotics tested. In all strains, the "V" activity was negligible compared to that of "Y" and "W". Also, "V" could not be detected at all after electrophoresis.

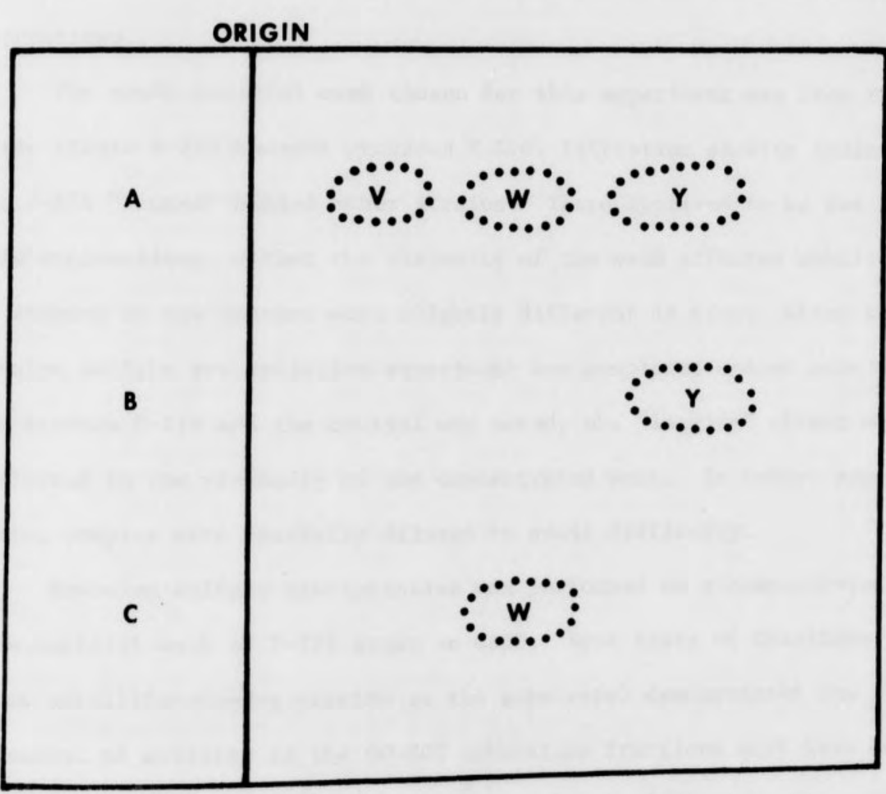
Early T.L.G. filtration experiments utilized fresh crude conidial wash preparations (harvested, concentrated, frozen; and then tested within 1-6 weeks) to evaluate intra-strain activity ratios of "W" to "Y". Later, after 1-1½ years, T.L.G. filtration was again performed on the same frozen preparations to determine the effect of ageing and prolonged frozen

Figure 1. Characteristic Mobilities on Thin Layer Gel of Neurospora β-Glucosidases.

A concentrated crude conidial wash

B purified "Y"

C purified "W"



storage. In several cases, there appeared to be more "W" present after storage than was observed initially in the fresh preparations (Table 2).

Separation of the β-Glucosidases by Ammonium Sulfate Precipitation

Ammonium sulfate precipitation has been utilized as a method of separation of the β-glucosidasesof N. crassa (Eberhart, et al., 1964; Madden, 1971). In this study, the procedure was used to investigate the separation of "V" from "W" and "Y" to obtain partially purified enzyme preparations.

The crude conidial wash chosen for this experiment was from the exotic strain P-278 because previous T.L.G. filtration studies indicated that P-278 "lagged" behind other strains. There appeared to be two plausible explanations; either the viscosity of the wash effected mobility of the enzymes or the enzymes were slightly different in size. After the ammonium sulfate precipitation experiment was completed and no size variation between P-278 and the control was noted, the "lagging" effect was attributed to the viscosity of the concentrated wash. In future experiments, samples were carefully diluted to avoid difficulty.

Ammonium sulfate precipitation was performed on a concentrated crude conidial wash of P-278 grown on GSCP. Spot tests of fractions (using umbelliferone-β-glucoside as the substrate) demonstrated the greatest amount of activity in the 60-80% saturation fractions with less activity in the 45-55% fractions. Separation by T.L.G. filtration was used to determine the enzymatic composition of each fraction (Figure 2) and confirmed using electrophoresis. Pooled fractions (60-80% saturation) contained mostly "Y" with traces of "W". Most of the 55% fraction was "W". The 50% saturation fraction contained an area of limited enzymatic

TABLE 2

OBSERVATIONS OF INTRA-STRAIN ACTIVITY RATIOS OF "W" AND "Y"

AFTER SHORT AND PROLONGED FROZEN STORAGE

		β-Glucosidases			
		Short Storage		Prolonged Storage	
Strain	Origin	"W"	"Y"	"W"	"Y"
P-3	Japan	+	++++	+++	++++
P-30	Philippines	no data	no data	+	++++
P-56	Taiwan	no data	no data	+	++++
P-57	Taiwan	trace	++++	trace	+++
P-58	Taiwan	+	++++	+++	++++
P-61	Taiwan	trace	+	trace	+
P-64	New Guinea	++	+++	+	++++
P-91	Australia	+	+++	++	+++
P-113	Australia	+++	++++	++++	+++
P-142	Indonesia	+	++++	+++	++++
P-153	Indonesia	no data	no data	+++	++++
P-168	Indonesia	+	+++	++	+++
P-190	Indonesia	trace	+	none	+++
P-204	Indonesia	+	+++	+++	++++
P-212	Indonesia	++++	++++	++++	++++
P-249	Malaya	trace	+	trace	+
P-266	Malaya	no data	no data	+	++++
P-271	Malaya	+	trace	++	trace
P-278	Singapore	+	++++	+++	+
P-285	India	trace	+++	++	trace
P-291	India	+	++++	++	+++
P-296	India	trace	++++	+	++++
P-321	India	+++	++++	+	++++
P-343	India	+	+	++++	+++
P-348	India	+	++++	++	++++
P-349	India	+	++++	+	++++

All exotic strains were of the A mating type.

Figure 2. Thin Layer Gel Filtration of Ammonium Sulfate Fractions

% SAT.	ENZYME	ORIGIN
20–45	W, Y	
50	V	
55	W	
control (P–212)	V, W, Y	
60–80	W, Y	

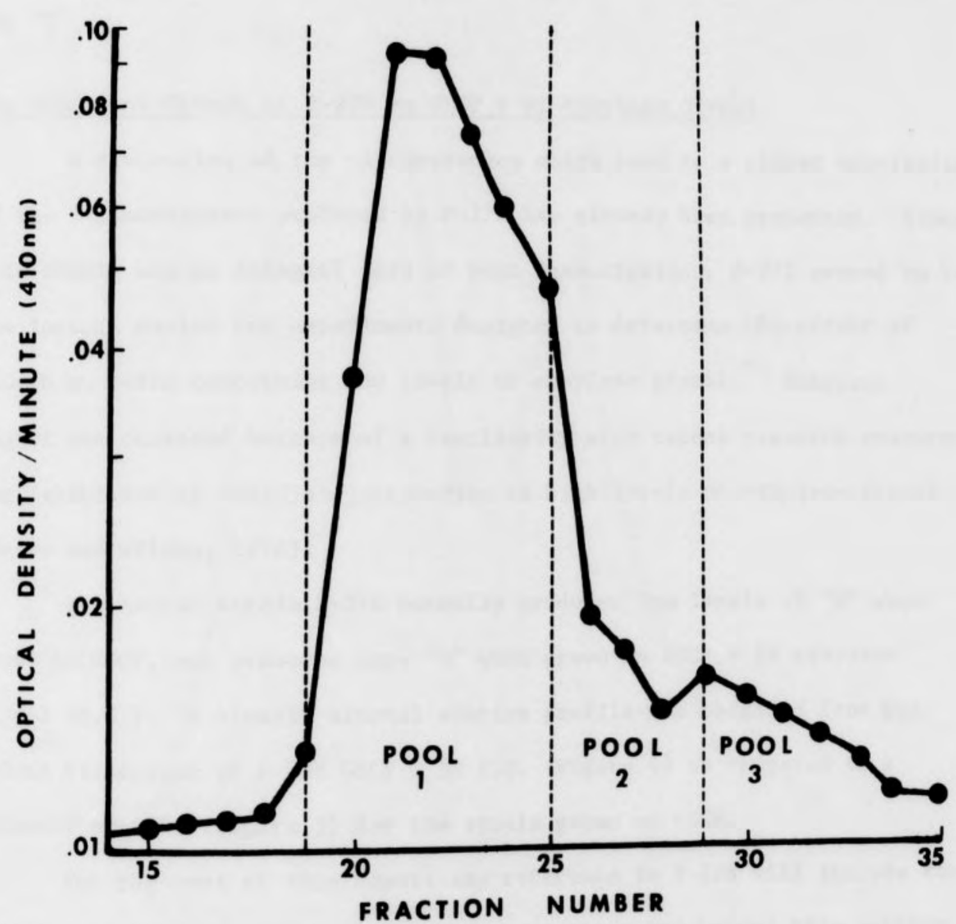
activity which could be attributed to "V". However, attempts to observe any activity in this fraction after electrophoresis were unsuccessful.

Although ammonium sulfate precipitation did achieve a separation of the β-glucosidases, the activity levels were very low. This could be attributed to low initial activity (especially "W" and "V") in the crude conidial wash. However, ammonium sulfate precipitation followed by electrophoresis may have significantly decreased activity, especially in the case of a very labile enzyme. Without an additional means of detection other than T.L.G. filtration, the existence of "V" in the 50% fraction could not be confirmed. Another attempt at purifying "V" will be presented in the next section.

Gel Column Filtration of a Crude Conidial Wash from P-278

Gel column filtration of P-278 was performed in an effort to separate "V" from "W" and "Y" using a simpler, "milder" method of purification than used before. Approximately 7 ml of a concentrated crude conidial wash was pipetted onto a Bio-Gel P-150 column as described in the methods. One peak of activity was plotted after an assay of the fractions using PNPG as the substrate (Figure 3). The fractions were combined (as shown in Figure 3) into 3 pools, concentrated, and frozen. T.L.G. filtration of Pool #1 demonstrated that the larger aryl-β-glucosidase "Y" was eluted first, followed by a mixture of "Y" and "W" in Pool #2. Pool #3 contained enzymatic activity at the time of assay but after concentration and T.L.G. filtration, no activity could be detected. If it were possible to separate "V" and recover activity, one would expect this small enzyme to be located in Pool #3.

Figure 3. Elution Profile of Extracellular β-Glucosidases of P-278 from a Bio-Gel P-150 Column.



The results of this experiment and those using ammonium sulfate did not encourage further attempts at purifying "V" at this time. Therefore, the emphasis of this research was shifted to a more productive area where the larger quantities of "W" produced by some exotic strains could be of advantage in determining more about the isozyme relationship of "W" and "Y".

The Effect of Growth of P-278 on GSCP + 5% Ethylene Glycol

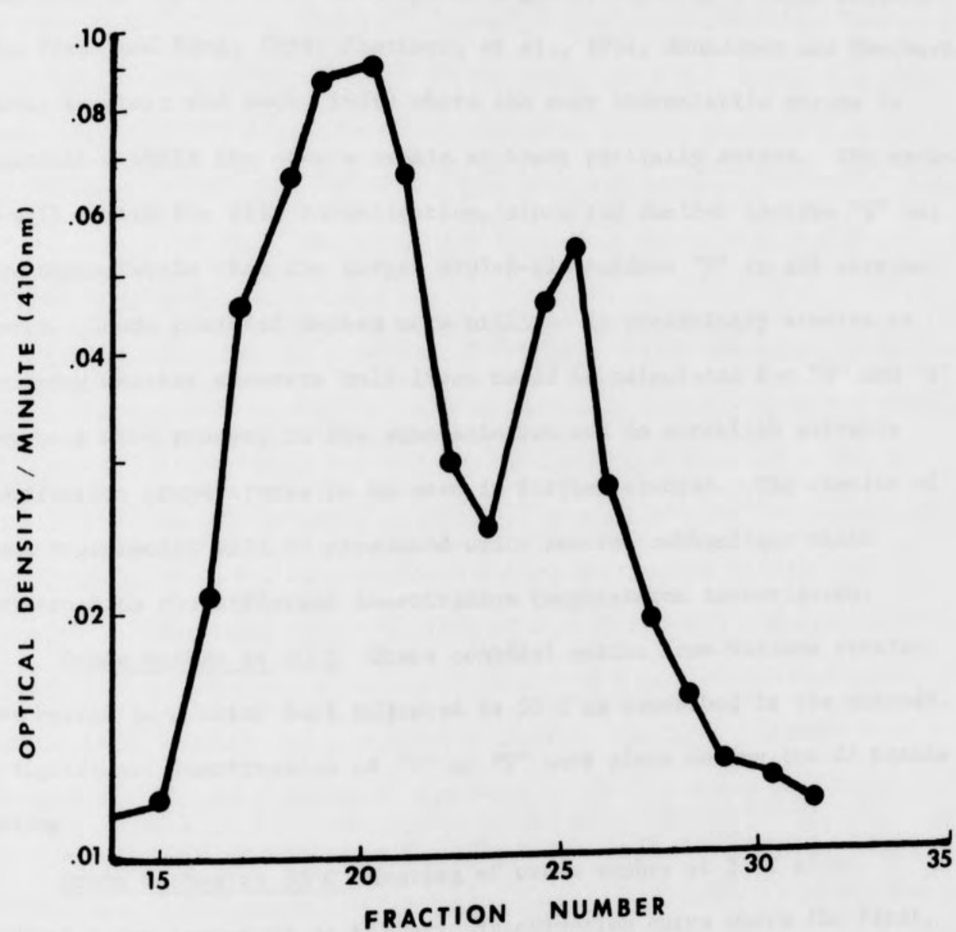
A discussion of the circumstances which lead to a closer examination of the β-glucosidases produced by P-278 has already been presented. Since this strain was an integral part of past investigation, P-278 seemed to be the logical choice for experiments designed to determine the effect of growth on media containing low levels of ethylene glycol.* Ethylene glycol was selected because of a familiarity with recent research concerning inhibition of conidial germination in high levels of ethylene glycol (Bates and Wilson, 1974).

The exotic strain P-278 normally produces low levels of "W" when grown on GSCP, but produces more "W" when grown on GSCP + 5% ethylene glycol (E.G.). A clearly bimodal elution profile was obtained from gel column filtration of P-278 GSCP + 5% E.G. (Figure 4) as compared to a unimodal profile (Figure 3) for the strain grown on GSCP.

For the rest of this report any reference to P-278 will include the notation of GSCP or GSCP + 5% E.G. Experiments cited before this section refer to P-278 grown on GSCP. Thermal inactivation data concerning

*My appreciation to Mr. Doug Gerringer for his collaboration regarding experiments using ethylene glycol.

Figure 4. Elution Profile of Extracellular β-Glucosidases of P-278 GSCP + 5% Ethylene Glycol from a Bio-Gel P-150 Column.



differences in the stability of the isozymes produced under the two growth conditions will be presented at a later time.

Preliminary Thermal Inactivation Studies: Crude Conidial Washes

Differential heat inactivation of enzymes is a method which has been used to separate and distinguish β-glucosidases in a mixed preparation (Hash and King, 1958; Eberhart, et al., 1964; Mahadevan and Eberhart, 1964c; Eberhart and Beck, 1970) where the more thermolabile enzyme is inactivated while the others remain at least partially active. The method is well suited for this investigation, since the smaller isozyme "W" was more thermolabile than the larger aryl-β-glucosidase "Y" in all strains tested. Crude conidial washes were utilized in preliminary studies to determine whether accurate half-lives could be calculated for "W" and "Y" when both were present in the same solution and to establish suitable inactivation temperatures to be used in further studies. The results of these experiments will be presented under several subheadings which correspond to the different inactivation temperatures investigated.

Crude Washes at 50 C Crude conidial washes from various strains were heated in a water bath adjusted to 50 C as described in the methods. No significant inactivation of "W" or "Y" took place during the 25 minute heating.

Crude Washes at 55 C Heating of crude washes at 55 C often produced a two component or biphasic inactivation curve where the first, more thermolabile component corresponded to the "W" enzyme and the second, more thermostable component was "Y". This relationship was further illustrated by applying heated enzyme solutions (0,4,9,25 minutes) directly to T.L.G. plates without the customary quantitative PNPG assay of

β-glucosidase activity. After T.L.G. filtration was performed, the relative enzymatic activity was scored as shown in Figure 5.

Strains were selected for study on the basis of their relative production of "W" and "Y". Thermal inactivation curves were found to be an effective means of screening relative activities and crude calculation of thermal half-lives. Figure 6 depicts 2 distinctly different thermal inactivation curves. One strain (P-278 GSCP + 5% E.G.) produced a significant amount of "W" as evidenced by the sharp initial decline in activity, and the same strain grown on GSCP alone produced no detectable "W". Since "Y" was stable at 55 C, the graph for P-278 GSCP shows practically no decline in activity over time.

In cases where the presence of "W" was evident (as in P-278 GSCP + 5% E.G.), a thermal half-life could be estimated directly from the graph. Calculation of half-lives for "Y" was much more difficult than for "W", since the decline in activity over time was often slight. It is emphasized that thermal half-life data gathered during preliminary studies were intended to illuminate trends in thermal stability. All numerical half-lives for crude conidial washes at 55 C and 60 C were determined graphically from a plot of $\log_{10}$ enzymatic activity vs. time. A summary of thermal half-lives for two exotic strains at 55 C is given in Table 3.

An observation was made that the addition of buffer to a crude conidial wash afforded the "W" enzyme additional protection against heating and subsequent inactivation at 55 C. Equal amounts of various buffers (citrate-phosphate, potassium-phosphate, sodium-phosphate, citrate, bicine, glycyl-glycine, and glycyl-glycine + NaCl) pH 5.7-6.0 were added to crude water washes and then designated buffer-conidial wash preparations. These

Figure 5. Thin Layer Gel Filtration of a Crude Conidial Wash of P-278 After Heating at 55 C.

GROWTH CONDITIONS	MINUTES HEATING	ORIGIN		
GSCP	no heat		trace	++
GSCP + 5% E.G.	no heat		+++	+++
GSCP + 5% E.G.	4 min.		++	+++
GSCP + 5% E.G.	9 min.		+	++
GSCP + 5% E.G.	25 min.		trace	+

Figure 6. Thermal Inactivation of P-278 Crude Conidial Washes at 55 C

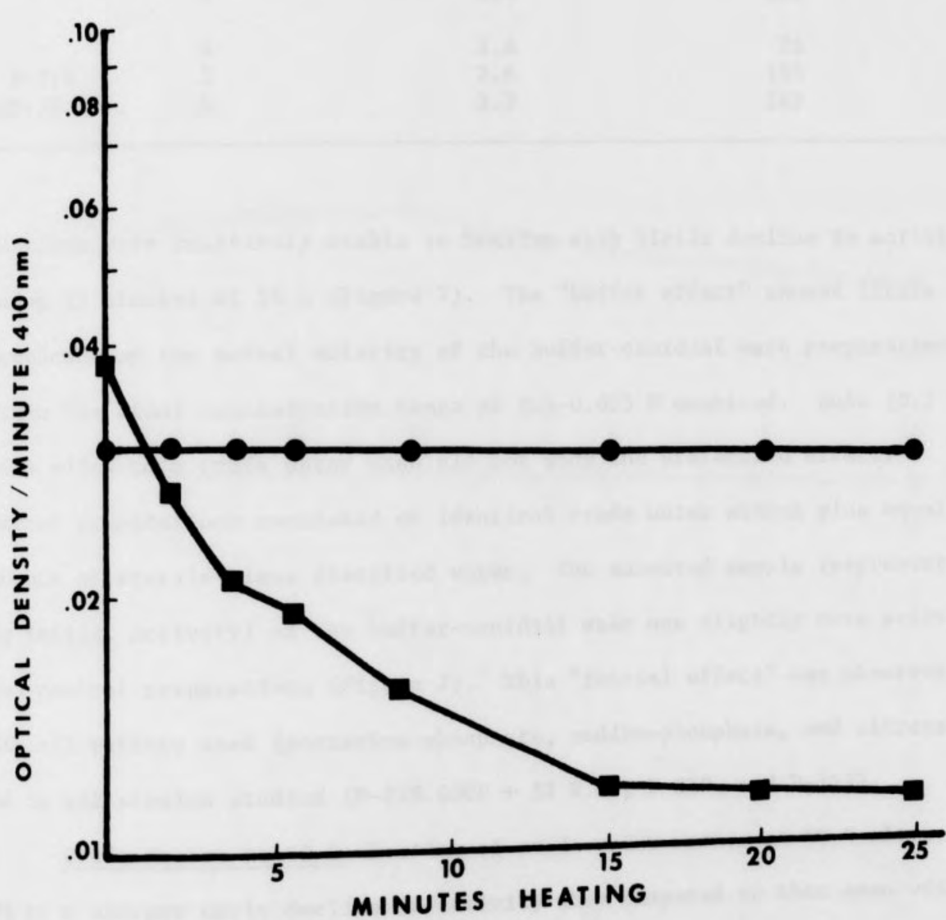


TABLE 3

THERMAL HALF-LIVES OF CRUDE CONIDIAL WASHES AT 55 C

Strain	Expt. No.	Thermal half-lives in minutes "W"	"Y"
P-212	1	3.5	76
	2	3.3	44
	3	2.0	124
P-278 GSCP+5% E.G.	4	3.6	75
	5	2.6	155
	6	3.3	143

solutions were relatively stable to heating with little decline in activity during 25 minutes at 55 C (Figure 7). The "buffer effect" showed little dependence on the actual molarity of the buffer-conidial wash preparation within the final concentration range of 0.1-0.025 M examined. Salt (0.1 M NaCl) added to a crude water wash did not show the protective effect. Control preparations consisted of identical crude water washes plus equal amounts of sterile glass distilled water. The unheated sample (representing initial activity) of the buffer-conidial wash was slightly more active than control preparations (Figure 7). This "initial effect" was observed with all buffers used (potassium-phosphate, sodium-phosphate, and citrate) and in all strains studied (P-278 GSCP + 5% E.G., P-212, and P-113).

Crude Washes at 60 C Heating of crude water washes at 60 C often led to a sharper early decline in activity when compared to that seen with buffer-conidial washes at 60 C (Figure 8). In these cases, it appeared as though "W" was stabilized to a greater extent in buffer solutions than in water solutions. This observation is consistant with the "buffer effect"

Figure 7. Thermal Inactivation of P-278 GSCP + 5% E.G. and P-212 at 55 C Final Molarity 0.05 in Sodium-Phosphate Buffer pH 6.0

● P-278 Buffer-Conidial Wash

ϴ P-278 (Control)

■ P-212 Buffer-Conidial Wash

□ P-212 (Control)

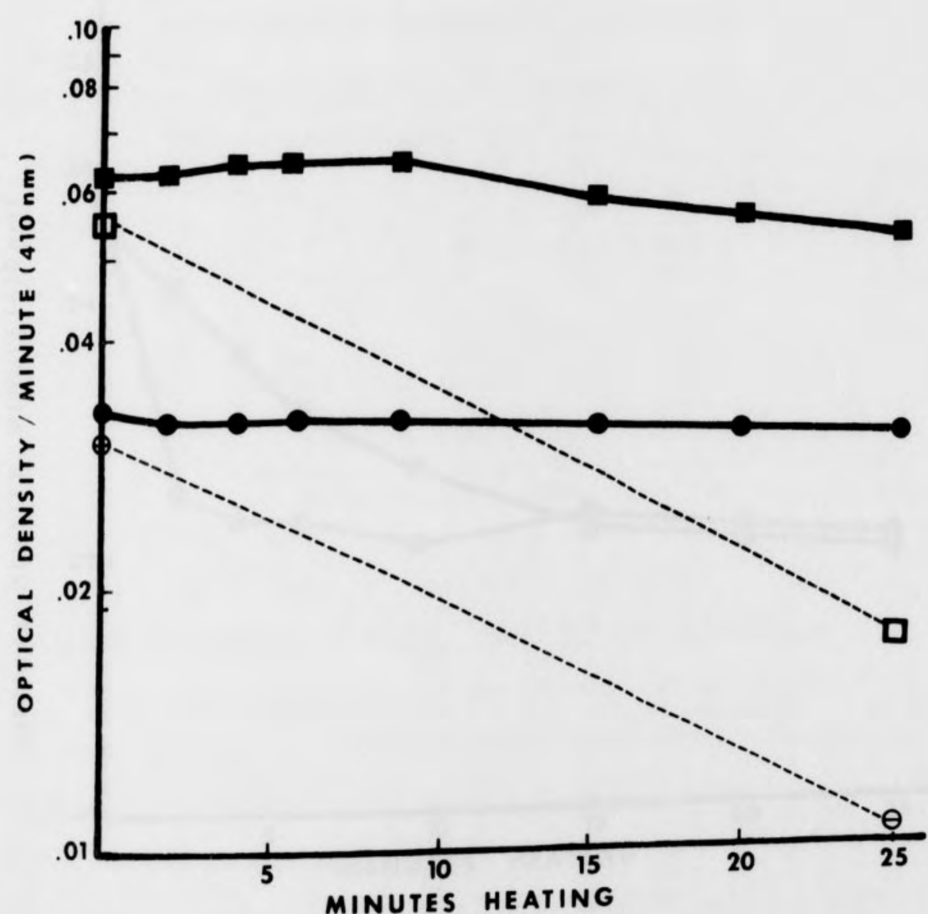
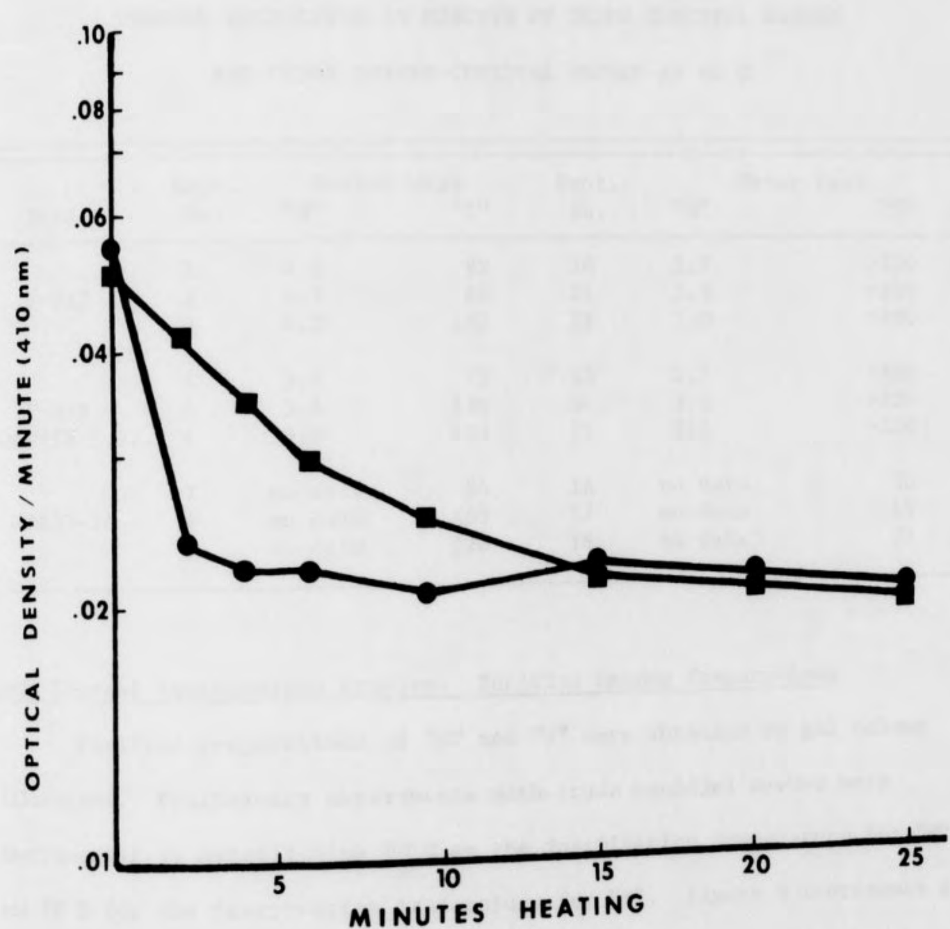


Figure 8. Thermal Inactivation of P-278 GSCP + 5% Ethylene Glycol at 60 C.

● Crude Buffer-Conidial Wash (Sodium-phosphate pH 6.0)

■ Crude Water-Conidial Wash



noted at the lower temperature of 55 C. However, no definitive relationship between the half-lives in buffer and water could be determined except in the case of 740R23-1A where buffer washes were approximately three times more stable than comparable water washes (Table 4).

TABLE 4

THERMAL HALF-LIVES IN MINUTES OF CRUDE CONIDIAL WASHES AND CRUDE BUFFER-CONIDIAL WASHES AT 60 C

Strain	Expt. No.	Buffer Wash "W"	Buffer Wash "Y"	Expt. No.	Water Wash "W"	Water Wash "Y"
	1	4.9	92	10	1.7	>200
P-212	2	4.7	86	11	1.3	>200
	3	4.0	132	12	1.0	>200
	4	3.4	75	13	1.7	>200
P-278	5	3.4	138	14	3.5	>200
GSCP+5% E.G.	6	3.8	163	15	3.5	>200
	7	no data	84	16	no data	30
740R23-1A	8	no data	105	17	no data	19
	9	no data	120	18	no data	21

Main Thermal Inactivation Studies: Purified Enzyme Preparations

Purified preparations of "W" and "Y" were obtained by gel column filtration. Preliminary experiments with crude conidial washes were instrumental in establishing 60 C as the inactivation temperature for "W" and 70 C for the inactivation temperature for "Y". Figure 9 represents a typical heat inactivation experiment for purified "W" and "Y" although actual rates and calculated half-lives varied according to the strain. Thermal half-lives calculated for P-212 from Figure 9 correspond to the first values of "W" and "Y" in Table 5.

Figure 9. Thermal Inactivation of Purified "W" and "Y" from P-212

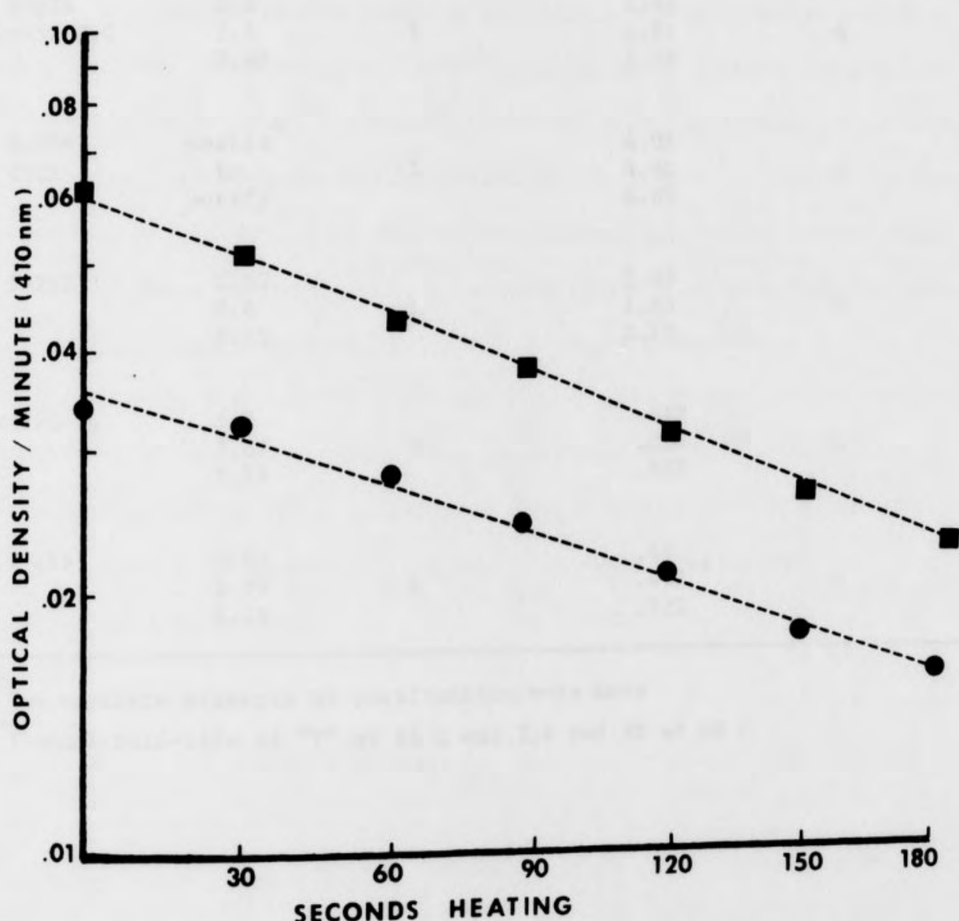


TABLE 5

THERMAL HALF-LIVES IN MINUTES OF PURIFIED "W" AND "Y"

Strain	"W" Half-life at 60 C	"W" Relative Thermal Designation	"Y" Half-life at 70 C	"Y" Relative Thermal Designation
P-278 GSCP+5% E.G.	2.0 2.4 2.48	L	1.96 1.82 1.79	S
P-278 GSCP	unable* to purify	L	4.03 4.82 4.09	S
P-212	2.52 2.8 3.45	L	2.05 1.83 2.11	S
740R23-1A	3.7 3.87 3.79	S	.842 .884 .867	L
P-113	3.83 3.99 4.15	S	.45 .497** .722	L

*Two separate attempts at purification were made

**Thermal half-life of "Y" at 65 C was 2.4 and 30 at 60 C

Thermal half-lives for "W" and "Y" from exotic strains and the wild-type control were calculated using regression analysis based on the $\log_{10}$ of the enzymatic activity as described in the methods. The half-lives were recorded and compiled in Table 5. An inter-strain comparison of each independent column of "W" values and "Y" values was made. The "W" of each strain and the "Y" of each strain was designated either S or L (stable or labile) on the basis of one strain's half-lives in relation to the other's. Each strain's inter-strain relative thermal designation (S or L) was recorded in the column adjacent to the appropriate half-lives. The relative inter-strain thermal stability of "W" and "Y" for each strain was used as the basis for an intra-strain comparison of the thermal relationship of the aryl-β-glucosidase isozymes. One will note from the data presented in Table 5 that strains which produced a relatively stable "Y" (P-278 GSCP + 5% E.G., P-278 GSCP, and P-212) also produce a relatively labile "W". A converse relationship is observed in strains 740R23-1A and P-113 where a labile "Y" is associated with a stable "W". A discussion of the thermal relationship of the aryl-β-glucosidase isozymes will be presented at a later time.

CHAPTER IV

DISCUSSION

The research in this thesis will be discussed under several headings corresponding to the major areas of study.

The Occurrence and Significance of the Isozyme "V"

Thin layer gel filtration of crude conidial washes from 26 different exotic strains demonstrated three distinct areas of β-glucosidic activity corresponding to the enzymes "Y", "W", and "V" (Figure 1). The occurrence of the "new" isozyme "V" was widespread, at least within the population of exotics sampled, but "V" was not observed in conidial washes of the wild-type 74OR23-1A. Since only one standard laboratory wild-type strain was investigated, it is impossible to make a general statement about "V" in other strains. However, it is conceivable that crude washes from wild-type strains do contain "V", but the enzyme could remain undetected if the substrate incubation period is insufficient to note activity. There appears to be an "optimum reading time" before which the enzymatic activity is too slight to notice, but after which the plate becomes one big slur of activity. The total T.L.G. filtration run time including incubation period often exceeds 14-16 hours.

The low intensity of the "V" spot and the extended incubation period required to read activity indicated that "V" was present in very low concentration, and was probably very labile. Also, attempts to purify and isolate "V" were only partially successful, further attesting to the lability and low concentration of the enzyme. What is certain, however,

is that "V" represents only a minor component of the aryl-β-glucosidase isozyme system.

The isozyme "V" was first detected in exotic strains, and interestingly enough, so was the isozyme "W" (Madden, 1971). These two observations probably are related since exotic strains tend to generally produce more "W" than standard wild-type laboratory stocks, and could possibly produce more "V". Morton (1975) has generated "W" from purified "Y" of the exotic P-212 using various concentrations of guanidine hydrochloride (GuHCl), but was unable to generate "W" from wild-type "Y". She proposed a dimeric structure for "Y" in which "Y" was composed of two "W's" associated with a carbohydrate moiety. The tendency for exotic "Y's" to dissociate to "W's" may be a more general phenomenon than previously realized should further experiments prove affirmative. Similarly, exotic "W" could be treated with GuHCl or other effective denaturants to determine if "V" could be generated and stabilized under appropriate conditions.

Some exotic strains of <u>Neurospora crassa</u> may possess a tendency to dissociate into a homologous series of lower molecular weight species of enzymes. If "Y" is visualized to be a tetrameric enzymatic molecule composed of four closely associated monomeric subunits, then "W" and "V" could represent enzymatically active molecules of a series. The dimeric "W" isozyme being composed of two associated monomers, and "V" representing the transient, highly labile, but enzymatically active monomeric species. An association-dissociation relationship similar to this type is possible: $(4n) \rightleftharpoons 2\ (2n) \rightleftharpoons 4\ (n)$ where n is the monomer. High resolution electron microscopy of purified "Y" and "W" could help clarify the spacial and structural relationship of the isozymes. Umezurike (1975) has postulated

a similar association-dissociation process for the β-glucosidases of Botryodiplodia theobromae Pat in which the purified high molecular weight species slowly dissociates to enzymatically active lower molecular weight components during storage. Crude culture filtrates demonstrated multiple molecular weight isozymes (Umezurike, 1971). β-galactosidase from Escherichia coli dissociates and reassociates in commonly used buffer systems during storage to species > (4n) and < (n) (Contaxis, et al., 1973). Erickson and Steers (1970a) reported that the non-tetrameric forms of P-lac β-galactosidase were less stable to heat and storage at 4 C than was the predominant tetramer species. I have observed a dissociation phenomenon during the storage of crude conidial washes from exotic strains of Neurospora crassa. The aryl-β-glucosidase isozyme system probably reflects a slow association-dissociation process which could be accelerated by current preparative and storage procedures.

The Effect of Ageing

The effect of ageing and freeze-thawing of crude conidial washes was observed in fresh preparations and the same preparations after prolonged frozen storage. Intra-strain activity ratios of "W" and "Y" are compared in Table 2. Strains such as P-3, P-58, P-142, and P-278 demonstrated more "W" activity after ageing than was previously noted in the fresh preparations. One possible explanation assumes that "Y" dissociates into two "W's", and in this way, contributes to the previously existing "pool" of "W". Subsequent dissociation of the newly generated "W" and the previously existing "W" could produce additional "V", but detection of increased quantities of the enzyme may be difficult. This possibility has not been adequately investigated since aged washes were not checked for "V" content.

Both the ageing and freeze-thawing of isozyme preparations may influence the dissociation process. In his book, A Physio-chemical Approach to the Denaturation of Proteins, Joly (1965) describes the dissociative effect of ageing in egg-albumin, horse serum albumin, and soybean proteins as typical denaturations. Denaturation being defined as "any modification of the secondary, tertiary, or quaternary structure of the protein molecule, excluding any breaking of covalent bond" (Joly, 1965). The dissociation of β-glucosidase (Umezurike, 1971 and 1975) and β-galactosidase (Contaxis, et al., 1973; Erickson and Steers, 1970) has been attributed to the ageing process during storage. Other enzymes are sensitive to cold inactivation (Graves, et al., 1965). However, in the case of the β-glucosidases of Neurospora crassa, the situation is further complicated by an occasional freeze-thaw cycle during the prolonged frozen storage period. Sometimes these preparations were thawed, an aliquot used in an experiment, and the remainder refrozen. Freeze-thawing has been shown to effect the conformation of proteins in solution (Taborsky, 1970) and could, conceivably, hasten the dissociation process.

Because of current storage procedures used in this laboratory, it would be beneficial to determine whether ageing and freeze-thawing has an independent or additive effect. Experiments directed to determine which is the case could employ purified "Y" under controlled conditions. Ageing and freeze-thawing experiments are a convenient point of departure for further study of the proposed association-dissociation process. The four exotic strains mentioned above, which tend to dissociate, may prove to be valuable sources of purified "W" and "Y" isozymes.

The Effect of Growth of P-278 on GSCP + 5% Ethylene Glycol

The exotic P-278 normally produces low levels of "W" when grown on standard media (GSCP), but produces more "W" when grown on GSCP + 5% ethylene glycol (E.G.). The effect is clearly visible upon examination of elution profiles (Figures 3 and 4), thin layer gel filtration studies (Figure 5), and thermal inactivation curves (Figure 6). The assumption is made that the additional "W" is in response to the added ethylene glycol and not some unrecognized factors. The "W" isozyme of P-278 GSCP comprises approximately 1/6 the total enzymatic activity observed after T.L.G. filtration, as compared to 1/3 of the total activity for P-278 GSCP + 5% E.G.

Ethylene glycol is generally recognized to be an effective denaturing agent (Laidler and Bunting, 1973). Contaxis and Reithel (1971 a,b) investigated the association-dissociation behavior of bacterial β-galactosidase and urease in high concentrations of ethylene glycol and glycerol. From their results they proposed a possible scheme for the formation of isozymes. However, it is highly improbable that a 5% ethylene glycol concentration could effect the process _in vitro_, but it could possibly influence a similar process _in vivo_.

Neurospora aryl-β-glucosidase (Eberhart and Beck, 1970) and invertase (Trevithick and Metzenberg, 1964) have been localized primarily in the intra-mural space between the cell wall and the cell membrane. Invertase exists in two forms, "heavy" and "light", which dissociate to active subunits upon exposure to heat or high salt concentrations (Metzenberg, 1964). Trevithick and Metzenberg (1964) suggest that the "light" subunit may undergo an aggregation or association process to the "heavy" form _in vivo_ within the mural space. Because of aryl-β-glucosidase's similar

location (Chung and Trevithick, 1970), it is tempting to hypothesize that ethylene glycol could enter the cell, and due to its dissociative ability, influence the character of the isozymes in vivo. This mechanism is purely speculative and awaits further investigation. However, some supportive evidence exists in that isozymes of P-278 GSCP have been observed to dissociate upon ageing. Other exotic strains (P-3, P-58, and P-142) and wild-type controls could be grown on GSCP + 5% E.G. for comparative studies.

Preliminary data suggests that growth of P-278 on GSCP + 2% xylose as a carbon source has approximately the same effect on "W" as ethylene glycol. The presence of these two substances in the media could produce less than maximal cell growth rates. This condition does, as in the case of trehalase (Metzenberg, 1972; Hanks and Sussman, 1969) and possibly aryl-β-glucosidase (Eberhart and Beck, 1973), activate enzyme production, and may result in various isozyme patterns (Erickson and Steers, 1970). Since inconclusive results have been obtained in the area of regulation and induction of "W" (Madden, 1971), any further comment should be reserved until more data is collected.

The Thermal Relationship of the Aryl-β-Glucosidase Isozymes

Heat is a classical example of a physical denaturation agent. During the heating process the "native" protein undergoes changes in its secondary and tertiary bonding patterns which ultimately change the general shape of the molecule (Laidler and Bunting, 1973). Since the catalytic effectiveness of an enzyme depends profoundly on the position of the different groups in the polypeptide chains, any change in the overall structure may lead to inactivation (Laidler and Bunting, 1973). The more

gentle the conditions (mild heating or small pH changes), the more likely that the denaturation will be reversible and the protein may regain its original characteristics (Laidler and Bunting, 1973).

The heat stability of an enzyme, as measured by the thermal half-life, provides a convenient criterion for comparative statements concerning the physical properties of isozymes. Generally, higher molecular weight isozymes are more thermostable than the lower molecular weight species. This has been found to be a valid statement in relation to the aryl-β-glucosidases of Neurospora crassa. The larger isozyme "Y" was more thermostable than the smaller "W" in all strains tested. In addition, this study presents evidence for a distinct thermal relationship between the "W" and "Y" produced by any given strain. Inter-strain and intra-strain comparisons of purified "W" and "Y" half-lives were used as the basis for statements concerning the relative stability of the isozymes (see results). Strains which produced a relatively stable "Y" (P-278 GSCP + 5% E.G., P-278 GSCP, P-212) also produced a relatively labile "W". Other strains (P-113 and 74OR23-1A) produced a labile "Y" associated with a stable "W" (Table 5). Although thermal half-lives are routinely determined for each newly discovered isozyme, nowhere, to the knowledge of this investigator, has a similar study been conducted.

Erickson and Steers (1970) published a comparative study of the heat and urea stability of different bacterial (Escherichia coli, Proteus mirabilis, Serratia marcescens and others) β-galactosidases. They used crude cell extracts and did not determine a thermal half-life, as such, but rather proposed generalized statements of relative stabilities based directly on graphical representations. The relative stability of the

bacterial β-galactosidases may be related to the quantity of the heavier isozymes formed (Erickson and Steers, 1970), where the more stable isozymes will be produced in larger quantities. This does appear to be the case in the aryl-β-glucosidase system of Neurospora crassa. The isozyme "Y" is the predominant and the most stable form in all strains investigated. The fundamental question raised by this study is: Why is there a correlation between the thermal stabilities of "W" and "Y" in any given strain? The immediate response, and perhaps the hardest to justify, is that under certain environmental conditions the presence of a certain type of "W" is an adaptive advantage. If a particular substrate or growth condition were limiting, a highly specific enzyme could be selectively advantageous. Madden (1971) reported that "W" has a greater affinity for aryl compounds than "Y". If this were the case, it follows that the stability of "W" may be correlated with the amount of "W" that is normally produced. The more stable "W's" would be observed in larger quantities. The results of this study neither confirm nor disprove this assumption. For instance, P-278 grown on GSCP + 5% E.G. produced more "W" than on standard media, and the thermal stability of the isozymes were altered. The "W" produced by P-278 GSCP + 5% E.G. was more stable than that of P-278 GSCP (Table 5). On the other hand, 740R23-1A produced little "W", and the enzyme was almost twice as stable as the "W" of P-278 GSCP + 5% E.G.

It is difficult to explain the significance of the correlation between thermal stabilities of "W" and "Y" without relying on quantitative comparisons. The possibility exists that the isozymes are structurally related, but independently regulated. Variation in the inter-strain thermal properties of "W" and "Y" could reflect differences in the primary

structure of the enzymes. A single amino acid substitution may profoundly effect the conformation and stability of an enzyme, and still produce no noticeable charge or size variation. Since little is known about the genetics and regulation of the isozyme "W" (Madden, 1971), it would be difficult to interpret these results in this light. However, perhaps further work will determine more about the significance of a refined system of relative thermal stabilities. Some suggestions of what could be done are as follows:

1. Conduct a similar study of the relative thermal stabilities of "W" and "Y" from wild-type strains. Is the wild-type range of thermal half-lives less variable than the exotics?

2. Determine the genetic basis of thermal stability by introducing different exotic genes into a standard genetic background. What is the genetic range of control and how many genes are involved?

3. Investigate the biochemical and physical properties of β-glucosidases from closely related species of Neurospora. Do the enzymes demonstrate thermal relationships which could shed light on evolutionary pathways?

CHAPTER V

SUMMARY

Thin layer gel filtration of crude conidial washes from 26 different strains of Neurospora crassa demonstrated three distinct areas of β-glucosidic activity. The area farthest from the origin was the largest enzyme, aryl-β-glucosidase "Y". The second, smaller isozyme "W" was located closer to the origin, and the third, smallest, newly discovered isozyme, designated "V", was closest to the origin. The new aryl-β-glucosidase "V" was present in extremely low concentration, and required 9-12 hours incubation with substrate (4-methyl-umbelliferyl-β-D-glucopyranoside) in order to note activity. Attempts to purify and isolate "V" were only partially successful.

The largest aryl-β-glucosidase "Y" was the predominant form, although the ratio of "Y" to "W" varied among the exotics tested. The "V" activity was negligible compared to "Y" and "W". Intra-strain activity ratios for "Y" and "W", as determined by thin layer gel filtration, were recorded for fresh preparations and the same preparations after ageing and freeze-thawing. Some strains demonstrated more "W" after ageing than was previously noted in the fresh preparations. A slow association-dissociation process of the type $(4n) \rightleftharpoons 2\ (2n) \rightleftharpoons 4\ (n)$ is proposed, where n is the monomer, and $(\text{"Y"}) \rightleftharpoons 2\ (\text{"W"}) \rightleftharpoons 4\ (\text{"V"})$.

The thermal relationship between the isozymes "Y" and "W" was investigated using exotic strains which demonstrated large quantities of "W" in addition to "Y". The exotic P-278 normally produced low levels of

"W" when grown on complete medium (GSCP), but produced more "W" when grown on GSCP + 5% ethylene glycol. Preliminary thermal inactivation experiments utilized crude conidial washes which contained both "Y" and "W". Heating of washes at 55 C produced a two component inactivation curve where the first more thermolabile component corresponded to the "W" enzyme, and the second, more thermostable component was "Y". The addition of an equal amount of various buffers to crude washes indicated a protective effect, and a higher temperature was required to demonstrate a similar biphasic inactivation curve.

The main thermal inactivation experiments were done with purified "Y" and "W" enzymes from selected exotic strains and a wild-type control. Thermal half-lives were calculated using linear regression analysis. Intra-strain and inter-strain comparisons of independent "Y" and "W" half-lives were utilized as the basis for comparative statements concerning the distinct thermal relationship between the isozymes from any given strain. Strains which produced a relatively stable "Y" also produced a relatively labile "W". The converse relationship was also observed where a labile "Y" was associated with a stable "W". The possible adaptive significance of isozymes is discussed with respect to their relative thermal stabilities.

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