ROLES OF TANNASE AND HYDROLYZABLE TANNINS IN CHESTNUT BLIGHT

by

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(ABSTRACT)

Endothia parasitica (Murr.) P. J. & H. E. Anderson (syn: Cryphonectria parasitica (Murr.) Barr), the causal agent of chestnut blight, was able to grow in total aqueous and tannin extracts from blight-susceptible American chestnut as well as in blight-resistant Chinese chestnut bark extracts, from winter and summer bark. Differences in the amount of conidial germination and growth in extracts of the two species were small. The E. parasitica tannase was more abundant intracellularly than extracellularly. Total tannase activities from cultures in American chestnut aqueous and tannin extracts were greater than in the Chinese chestnut extracts, for both winter and summer bark.

The tannase was isolated from the mycelium of E. parasitica and purified 142-fold with a 10 % yield by anion exchange chromatography and gel filtration. The estimated molecular weight was 240 kD and the molecule may be a tetramer composed of four subunits with a molecular weight of 58 kD. The pH optimum of the purified tannase was 5.5 and the temperature optimum for activity was 30 C. The enzyme was separated into six bands in the pH range of 4.6 to 5.1 which may represent isoenzymes or post-translational modifications.

Based on the Michaelis-Menten constant (K_m) of the tannase for three substrates tested, aleppo tannic acid was the best substrate (K_m = 0.95 mM). The V_{max}/K_m ratio was almost five times higher than that for hamamelitannin and seven times higher than that for methyl gallate. Hamamelitannin, a tannin present in blight-susceptible American chestnut bark but absent in blight-resistant Chinese chestnut bark, was a good substrate for the enzyme (K_m = 5.07 mM). When the relative activities of the enzyme on eight substrate analogues were examined, activity was slightly greater on pentagalloyl glucose, a substrate with five gallic acids linked to the sugar by ester bonds, than on aleppo tannic acid, a substrate with seven gallic acids but two linked in a a depside. Substrates with lower gallic acid content had lower activities. The ellagitannins, vescalagin and castalagin, were not good substrates for the enzyme and no inhibition of E. parasitica tannase by these compounds was found. Gallic acid was an effective competitive inhibitor of the tannase with all substrates and concentrations tested (K_i = 11.0-13.1 mM).

From the results of this dissertation it is apparent that E. parasitica tannase may be an important enzyme during pathogenesis as it enables the fungus to obtain organic carbon for the formation of the high-biomass mycelial fans. Differences in tannase activity on chestnut bark extracts may be used as a tool to identify sources of resistance for Chinese-American chestnut hybrids or American chestnut.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Endothia parasitica (Murr.) A. and A. (syn: *Cryphonectria parasitica* Murr.) Barr) is the causal agent of chestnut blight and the fungus responsible for the devastation of the American chestnut (*Castanea dentata* (Marsh.) Borkh.). In spite of attempts to control the disease, chestnut blight was so destructive that about 3.5 billion trees, greater than 99.99 % of the original population, were killed in the eastern United States in the 40 years following the discovery of chestnut blight in New York (Griffin and Elkins, 1986). It has been replaced mainly by oaks. According to Stephenson (1986) *Quercus rubra* (northern red oak) has become the dominant species in some areas. Chestnut survived mainly by sprouting at the stump of dead trees and eventually, the understory forest trees became blighted and the cycle was repeated, as it is today (Griffin and Elkins, 1986).

Before its devastation, the American chestnut was the predominant species in the eastern hardwood community (Woods and Shanks, 1957; Thor and Summer, 1971). The trees grew to a height of 100 ft with a diameter at breast height (d.b.h.) up to 8 ft. It was a valuable species providing lumber and supporting a large tannin extractive industry. Chestnut produced straight slender trunks with wood highly resistant to decay and highly esteemed for poles, ties, fence rails, and fence posts and bore large quantities of edible nuts, food for man and animals (Beattie and Diller, 1954).

Several attempts to obtain blight-resistant trees have been made. Early work of Clapper and Gravatt (1943) included hybridization with the more resistant Japanese (C. crenata Sieb & Zucc.) and Chinese (C. mollisima Blume) chestnuts (Clapper and Gravatt, 1943; Jaynes, 1964). The goal was to combine the blight resistance of the oriental species with the form and growth rate of the American species. According to Jaynes (1978), the reasons for the poor success of this approach were: 1) lack of good screening techniques for young seedlings; 2) no means to propagate vegetatively and thus test the selections on their own roots and 3) populations of hybrids were too small to obtain desired segregation. If blight resistance is controlled by more than one gene pair, a large population will be required to obtained segregation of resistance and form (Hebard 1982).

A second approach to control blight with resistance has been to use American chestnut. There are two variants to this approach. The first has been irradiation of the American nuts (MacDonald, Thor and Andes, 1962; Dietz, 1978) to induce mutations. The second variant has been to select large surviving American chestnut that may have blight resistance (Diller, 1956; Thor, 1978; Given and Haynes, 1978; Elkins et al., 1980; Griffin et al., 1978; Stambaugh and Nash, 1982). Some of these programs were initiated to improve blight resistance in American chestnut but blight resistance was not tested and no breeding results were published. Evidence for blight resistance in American chestnut was obtained by Griffin et al. (1983) using four simultaneous approaches with both large, surviving trees and blight-susceptible trees: inoculation of virulent E. parasitica 1) on bark-grafted scions, 2) on seedlings, 3) on excised dormant stems in the laboratory, and 4) in situ inoculations of branches or stems of large, surviving trees and susceptible stump sprouts. The inability to clone by rooting mature American chestnut stems and the lack of early selection criteria for seedlings were difficulties confronted by the breeders.

Chestnut tannins are thought to be involved in resistance (Nienstaedt, 1953) and enzymes and metabolites involved in tannin metabolism appear to be very important to both E. parasitica and the chestnut hosts (Hebard et al. 1984, Elkins et al. 1979 and 1982; Griffin and Elkins 1986). Hamamelitannin, present in susceptible American and European chestnut, but not in resistant Chinese and Japanese chestnut, is utilized as a carbon and energy source by E. parasitica

(Elkins et al. 1982) and may be a principal nutrient that supports the formation of mycelial fans by the pathogen. Other hydrolyzable tannin components in chestnut may have qualitatively similar roles in pathogenesis. Quantitative effects of tannins from American, European, and Chinese chestnuts on E. parasitica growth appear to be quite different (Elkins et al. 1978 b).

The objectives of this study are : 1) To determine the relationship between tannase activity and E. parasitica growth on total and tannin bark extracts from susceptible American and resistant Chinese chestnuts; 2) To purify and characterize the tannase from the mycelium of E. parasitica; 3) To determine the tannin substrate and inhibitor relationships of the tannase using purified enzyme preparations.

LITERATURE REVIEW

CHESTNUT BLIGHT

The fungus causing chestnut blight was first isolated by Merkel (1906) from American chestnut (Castanea dentata (Marsh.) trees in the Bronx Zoological Park, New York City. It was first described by Murrill in 1906 as Diaporthe parasitica and in 1912 was renamed Endothia parasitica (Murr.) P.J. and H. W. Anderson (1912). In 1978, Barr altered the taxonomy of the genus Endothia, including the classification of E.

parasitica that was transfered to the genus Cryphonectria and renamed as C. parasitica (Murr.) Barr (Barr, 1978). The fungus was apparently introduced into New York City and Long Island on nursery stock of Oriental chestnuts, possibly from Japan (Griffin and Elkins, 1986). This hypothesis was supported by the discovery of the fungus on Chinese chestnut (C. mollisima Bl.) in 1913 by Meyer in China and in 1915 on Japanese chestnut (C. crenata Sieb. and Zucc.) in Japan (Shear et al., 1917). By 1908, the disease was serious in Long Island, Connecticut, Massachusetts, New Jersey, and Pennsylvania, and it was present in isolated areas of Delaware, Maryland and Virginia (Heald, 1926). After 40 years, the disease had spread to all eastern areas in which the American chestnut was naturally present and to some western states. The fungus caused the almost complete destruction of the American chestnut population, a destruction that is unparalleled in forest history (Griffin and Elkins, 1986).

Intensive research was initiated in 1911 and by 1914, 399 articles were published on the disease or related subjects (Beattie, 1914). All efforts at eradication of advance disease centers, sanitation, and other controls eventually failed (Griffin and Elkins, 1986). In California and other western states, eradication was partially effective; it took 25 years to eradicate the fungus from a western planting (Holdeman, 1984).

In Italy, the fungus was found on European chestnut (C. sativa Mill.) in 1938 by Biraghi (1946) and in 30 years the disease spread throughout the country (Mittempergher, 1978). But in 1950, Biraghi (1953) observed recovery from blight in some areas and this led to the discovery of hypovirulence in E. parasitica. Blight is also present in France, Switzerland, Yugoslavia near the Italian border (Gravatt and Gill, 1930) and in Spain (Griffin and Elkins, 1986). The disease is also found in Turkey, Greece, Hungary and the USSR (Griffin and Elkins, 1986). Blight is also present in China on Chinese chestnut and in Japan on Japanese chestnut, where it is sometimes destructive (Uchida, 1977).

Symptoms and signs of the disease

The most obvious symptoms on American chestnut are yellowing and wilting of the foliage of a branch or main stem, followed by browning and shriveling of the leaves and flowers. A rusty brown, ellipsoid canker, sunken or with exposed wood at the center is present on the stem below the portion that shows foliar symptoms. Abundant orange-brown erumpent stromata are present on smooth bark. Yellow tendrils of conidia may be present in moist weather, and perithecia, with black necks, are apparent on all but young cankers or cankers caused by many hypovirulent strains (Griffin and Elkins, 1986).

Histopathology and histochemistry of lesion formation

In artificially induced cankers, Hebard et al. (1984)

showed that an initial lesion develops in bark tissue of American chestnut inmediately following inoculation of wounds with mycelium. The initial lesion expands for about 9 days and then ceases growth. Enlargement of the lesion begins again at about 26 days. The cessation of the initial lesion expansion is associated with the formation of a zone of lignified cells, 1 to 15 cells wide. This lignified zone is a water-impermeable barrier (Hebard et al. 1984), and also limits the growth of hyphae from the initial lesion into bark tissues.

Wound periderm forms adjacent to the lignified zone, at the deepest part of the initial lesion, and progresses outward to the outer periderm (Hebard et al. 1984). Lignified zones or wound-periderm-induction barriers, and/or wound periderm have been found in C. dentata, C. sativa, C. mollisima and C. crenata, either in association with wounds, initial lesions, mycelial fans or all three (Biraghi, 1953; Bramble, 1936; Bazzigher, 1957; Uchida, 1977; Hebard et al., 1984). A fully formed wound periderm, but not a lignified zone, appears capable of limiting the expansion of mycelia fans in bark tissue. However, thin or incomplete wound periderm or small gaps in the wound periderm may be penetrated by mycelial fans (Hebard et al., 1984). The relationship between the rate of wound periderm formation in the inner and outer bark, and the rate of mycelial fan growth appear to be the key elements that lead to superficial canker development (Hebard et al., 1984).

Superficial cankers are important to blight control as they do not kill trees. A fully formed wound periderm stops mycelial fan growth and eventually leads to sloughing of necrotic bark tissue from the tree in American, European and Japanese chestnuts (Hebard et al., 1984; Uchida, 1977; Bazzigher, 1957).

Hebard et al. (1984) found that the number, location and rate of growth of mycelial fans are the principal factors governing pathogenicity of _E_. _parasitica_ on chestnut species. Fans of virulent _E_. _parasitica_ on resistant chestnut trees grew slowly and only one was formed, usually in the outer bark (Hebard et al. 1984). In contrast, on susceptible chestnut, _C_. _dentata_, fans of _E_. _parasitica_ grow rapidly and are present in several layers of bark (multiple fans) with one often at the vascular cambium. Fans typically are oriented parallel to the bark surface. Based on lesion growth rates and lesion shape (Bazzigher, 1981; Bazzigher and Miller, 1984), the same appears to occur for virulent _E_ _parasitica_ on susceptible _C_. _sativa_, although mycelial fan growth may be considerably greater in the outer bark, versus the inner bark, especially for trees older than 6 years.

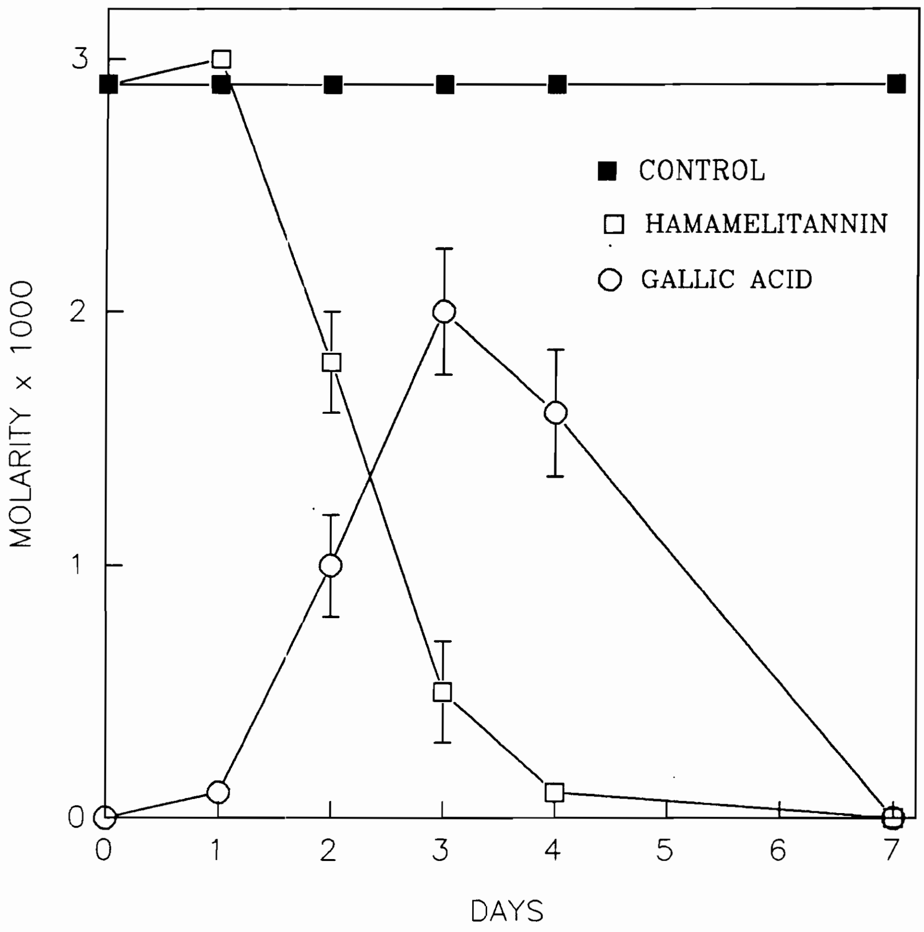
Mycelial fans formed in resistant and susceptible hosts soon after initial lesion formation and, in susceptible _C_. _dentata_, were thicker for a virulent strain than for a hypovirulent strain (Hebard et al., 1984). Aggregation of hyphae was noted previous to fan formation and these aggregates may be precursors of mycelial fans. Physical deformation of bark tissue and splitting of tissue in front of fans were observed by Bramble (1936) and Hebard et al. (1984) on _C_. _dentata_ and by Biraghi (1953) on _C_. _sativa_. In addition, Hebard et al. (1984) noted death of parenchyma cells at least 350 mm in front of the advancing mycelial fans. Activity of peroxidase, ß-glucosidase and esterase were diminished in colonized tissue, but esterase persisted much longer in the resistant Chinese than in the susceptible American chestnut.

Physiology of lesion formation

The rate and extent of mycelial fan formation (Hebard et al., 1984) and acidity of the canker margin (McCarroll and Thor 1978a and 1978b) appear to be the most important factors in pathogenesis of _E_. _parasitica_ on chestnut. The rapid growth of mycelial fans requires a large nutrient base. Cook and Wilson (1915) showed that tannins from American chestnut are utilized by _E_. _parasitica_ as a nutrient source. Bazzigher (1955) confirmed the ability of _E_. _parasitica_ to utilize tannins from chestnut (European and Chinese) as a nutrient source and identified an inducible esterase capable of degrading hydrolyzable tannins from chestnut. Barnett (1973) demonstrated the degradation by _E._ _parasitica_ of components from extracts of American chestnut, and Uchida (1977) showed that _E_. _parasitica_ could grow on extracts from Japanese chestnut. Elkins et al. (1978 b) found that utilization of tannins by E. parasitica from American, European, and Chinese extracts resulted in increased acidity of the media.

Mayer (1959, 1971) has chemically characterized hamamelitannin, castalin, vescalin, castalagin, and vescalagin in the tannin fraction of European chestnut. Elkins et al. (1979, 1981) have shown the presence of hamamelitannin in high concentrations in blight-susceptible American and European chestnuts and the absence of hamamelitannin in blight-resistant Chinese and Japanese chestnuts. They also demonstrated the utilization of hamamelitannin by three virulent isolates and one native hypovirulent American isolate of E. parasitica (1979). Elkins found (Griffin and Elkins, 1986) that hamamelitannin completely disappeared from the culture medium at 4 days and, in the meantime, gallic acid concentration increased to reach a maximum at 3 days and completely disappeared at 7 days (Fig. 1.1). Elkins et al. (1982) reported the utilization of castalagin and vescalagin by one virulent isolate (CR) of E. parasitica. These ellagitannins supported growth of the fungus when they were the only carbon source in a minimal medium. Nienstaedt (1953) observed that extracts from blight-susceptible American chestnut contained condensed and hydrolyzable tannins, whereas extracts from Chinese and Japanese chestnuts contain only

Figure 1.1 Utilization of hamamelitannin by Endothia parasitica. Values are given as average for three American virulent isolates (CR, WK, Ep39) and one American hypovirulent isolate (W2). Initial hamamelitannin concentration calculated to contain the same amount of carbon as 1 % glucose. Concentrations of hamamelitannin and gallic acid were normalized relative to the initial hamamelitannin concentration in controls to compensate for changes resulting from column instability. (From Griffin and Elkins, 1986).


CONTROL
HAMAMELITANNIN
GALLIC ACID
MOLARITY x 1000
DAYS
0
1
2
3
4
5
6
7

hydrolyzable tannins. Hebard and Kaufman (1978) by use of the anthocyanin test, also observed high concentrations of condensed tannins associated only with American callus tissue, and Elkins and Lewis (1978 a), by use of the vanillin-HCl test, obtained similar results for bark. However, there is no known physiological role of condensed tannins in blight susceptibility.

Hebard and Kaufman (1978) associated high concentrations of hydrolyzable ellagitannins and gallotannins with blight-resistant callus tissue. Of the known constituents in chestnut bark, vescalagin and castalagin are ellagitannins which are found in all the chestnut species (Griffin and Elkins, 1986).

Interpretation of the association of resistant callus tissue with high concentration of ellagitannins can be made in terms of the weak acidity of the hydrolysis products of ellagitannins and not in terms of the toxicity of ellagitannins for E. parasitica (Griffin and Elkins, 1986). Hydrolysis of the ellagitannins castalagin and vescalagin yields castalin and vescalin, respectively, and hexahydroxydiphenic acid which esterifies spontaneously to form the very weak and slightly soluble ellagic acid (with phenolic groups and no carboxylic acid groups) (Griffin and Elkins, 1986).

Englander and Corden (1971) observed the formation of oxalate by an isolate of E. parasitica. McCarroll and Thor

(1978b) also observed formation of oxalate by two virulent and to a lesser extent by two hypovirulent isolates of E. parasitica. Havir and Anagnostakis (1983) obtained similar results with three virulent isolates. However, they observed no oxalate formation by their three E. parasitica isolates after conversion with a common hypovirulent factor.

McCarroll and Thor (1978b) observed increased acidity at the canker margins of American and Chinese chestnuts infected with E. parasitica. They attributed this increased acidity to oxalic acid concentration (measured by dissolving insoluble oxalate in hydrochloric acid) at the canker margin of American chestnut (9.28 mg oxalate/g dry bark) as compared with sound bark (3.78 mg/g outer bark and 6.10 mg/g inner bark). However Elkins et al. (1983) were unable to find any free, water-soluble oxalic acid in healthy or diseased American chestnut bark.

Increased acidity is likely to be derived from the free acids released from the tannins upon hydrolysis. Of these, the most important to pathogenesis in American and European chestnut is likely to be the gallic acid released from hamamelitannin (Griffin and Elkins, 1986). Even though the gallic acid may be eventually utilized by E. parasitica, its concentration, in vitro, does build up to a high level soon after inoculation [since some gallic acid is already present (Griffin and Elkins, 1986)]. Gallic acid [pK = 4.42

(Beilstein, 1932)] is a stronger acid than acetic acid [pK = 4.76 (Meites, 1963)] but a weaker acid than citric acid [pk_1 = 3.13 (Meites, 1963)] or oxalic acid (pK_1 = 1.27 and pK_2 = 4.27 [Meites, 1963]). All of these acids can kill chestnut tissues when present at high enough concentrations (McCarroll and Thor, 1978 b); hence, gallic acid would appear to have the potential to kill chestnut tissue. The slightly higher toxicity of oxalic acid to chestnut tissue, as compared to acetic and citric acids, may be attributed to the higher acidity of oxalic acid without having to invoke the hypothesis of McCarroll and Thor (1978b) that oxalic acid helps breaks down pectin (Griffin and Elkins, 1986).

Of other physiological factors involved in lesion development, diaporthin, a toxin produced by E. parasitica in vitro (Hardegger et al., 1966), has been studied most. Crude (Bazzigher, 1953) and purified (Gaufman and Naef-Roth, 1957) diaporthin wilts tomato plants. Although no causal relationship has been established between diaporthin and lesion development, it seems reasonable that a wilt toxin could contribute to tylose formation in the sapwood of blighted chestnut (Bramble,1938). A possible role for diaporthin in pathogenesis is also implied by the presence of a phytotoxin (formed under the same condition and possessing the same characteristics as diaporthin) in culture filtrates of virulent E. parasitica but not in culture filtrates of hypovirulent _E_. _parasitica_ (Bonifacio and Turchetti, 1973).

McCarroll and Thor (1978 b) proposed that _E_. _parasitica_ secretes polygalacturonases and oxalic acid, which together kill host tissue and produce a "gelatinous" zone at the canker margin. They observed that both extracts and cell-wall protein from blight-resistant Chinese chestnut have more, or more effective, inhibitors of polygalacturonase and endopolygalacturonase than similar preparations from blight-susceptible American chestnut. Hebard et al. (1984) suggested, however, that the zone is made up of densely stainable hyphae of _E_. _parasitica_ and not degradation products from chestnut tissue.

Blight control with disease resistance

In breeding programs for blight-resistant hybrid forest chestnut trees, Chinese, Japanese and American chestnuts have been used; one program was initiated by Clapper and Gravatt of the USDA in 1922 (Jaynes, 1978). In 1929, Graves started his chestnut breeding program. He evaluated the resistance of many species of _Castanea_ and their hybrids. His evaluation was based on numerous inoculatons of the trees with the chestnut blight fungus and on his personal observations over 30 years (Graves, 1950). The program initiated by Graves was continued by Jaynes at the Connecticut Agricultural Experimental Station (Jaynes, 1978). Graves (1950) suggested that blight resistance was a character or a combination of characters which followed the Mendelian pattern of inheritance. He also stated that the Chinese, Japanese, and American chestnuts were heterozygous, a condition that explained the lack of uniformity in the F_1 generation and he concluded that blight resistance was not controlled by a single gene and suggested a genetic linkage with other characters (Graves, 1950). Clapper (1952) determined that first generation hybrids had a level of blight resistance that was greater than that of American chestnut. He stated that the level of resistance was considered sufficient for experimental forest planting (Clapper, 1952) but not for field planting, even when the trees had good form and growth rate (Jaynes, 1978). In order to increase the level of resistance, the first generation was backcrossed to Japanese or Chinese chestnuts and although the level of resistant increased (Clapper, 1952), the trees had the apple-tree-like form of the Oriental parents rather than the form of the American tree (Griffin and Elkins, 1986). Based on results of artificial inoculation trials, Clapper (1952) suggested that probably two pairs of genes control Chinese chestnut blight resistance, since the progeny of Chinese x (Chinese x American) [=C x (C x A)] approximated a ratio of 3 resistant : 1 susceptible.

Burnham (1981) and Rutter and Burnham (1982) proposed a different approach to breeding a blight-resistant chestnut. The idea was to transfer blight-resistant genes from Chinese chestnut to American chestnut by backrossing the C x A first generation to American chestnut up to three times and then intercrossing the blight-resistant progeny to obtain the level of blight resistance of Chinese chestnut. These authors supported the hypothesis that two genes are involved in resistance and they concluded that the intermediate resistance indicated incomplete dominance. They discarded the idea of a genetic linkage between resistance and poor form of C x (C x A) progeny since the 75 % Chinese genotype of the trees alone is enough to explain the poor tree form.

Based on canker area, Clapper (1952) and Berry (1960) determined that the F_2 of the Chinese-American cross [(C x A) x (C x A)] had a level of resistance that was closer to the Chinese parents than to the F_1 progeny.

Bingham et al (1971) suggested that three kinds of blight resistance in Castanea are indicated by the data of Berry (1960) and Clapper (1952): retarded canker growth rate, rate of death, and resistance to the establishment of infection. The latter type of resistance may inhibit establishment of mycelial fan formation and colonization, rather than infection, because initial lesions, following artificial inoculation, appear similar in Chinese and American chestnuts (Hebard et al., 1984). Following artificial inoculation, Clapper (1952) and Berry (1960), found high percentages of cankered American trees and very low percentages of cankered

Chinese trees.

Working with Japanese chestnut, Graves (1950) and Clapper (1952) found that they were less blight resistant than Chinese chestnuts and that the resistance in Japanese was variable and influenced by the environment.

Working with European chestnuts, Bazzigher (1975, 1981) and Bazzigher and Miller (1982) characterized the nature of lesions and factors affecting blight resistance in this species. Graves (1950) reported this species to be more resistant than American chestnut and Berry (1960) presented data on canker area following artificial inoculation that support his conclusion. Also the lower mortality of European chestnut in regions of Switzerland (Bazzigher, 1975) compared with the mortality of American chestnut, supports this conclusion.

Dietz (1978), tried to obtain a blight-resistant American chestnut by mutation induced by ionization radiation with gamma rays and thermal neutrons. Mutations for blight resistance are to be selected in progeny of the second and subsequent generations (Dietz, 1978).

Most programs in the United States depend on natural infection in the blight resistance trials (Griffin and Elkins, 1986). A different approach is being used in a cooperative program between Virginia Polytechnic Institute and State University and Concord College, West Virginia (Griffin and

Elkins, 1986). The program involves controlled intraspecific crossing of American chestnut clones with some degree of blight resistance (Griffin et al. 1978 and 1983) and selection among the progeny for a greater level of resistance than found in parents. To make quantitative assessment of blight resistance, canker length or area and degree of canker superficiality, following artificial inoculation with virulent strains, are considered and trees 5 years or older are tested (Griffin and Elkins, 1986). Their results provided evidence that blight resistance exists in the population of large, surviving American chestnut trees and suggested that resistance may be heritable (Griffin et al., 1983). Thor (1978) had a long-term breeding program for selection and breeding of blight resistant American chestnut, but the trees were never tested for blight resistance and were destroyed in the 1980's (Griffin and Elkins, 1986). Given and Haynes (1978) had a similar program. Stambaugh and Nash (1982) and Dierauf (1977) also have been examining surviving American trees for blight resistance. Most of these programs rely on open pollination among chestnut clones and on natural selection among the progeny (Griffin and Elkins, 1986).

TANNINS

The plant tannins are a unique group of water soluble polyphenols of relatively high molecular weight which have the ability to complex strongly with carbohydrates and proteins. (Porter, 1989). For this reason they were among the first natural products to be utilized industrially in the process of tanning. Tanning (waterproofing and preserving) animal hides to make leather has been known for perhaps the last four millenia (Porter, 1989). Tannins are found in a large number of woody and herbaceous plant species. They can accumulate in large amounts, often more than 10 % of dry weight in any plant part (bark, wood, leaves, fruits or roots) (Haslam, 1989).

In higher plants, tannins consist of two major groups of metabolites: the hydrolyzable and the condensed tannins or proanthocyanidins (Haslam, 1975). Hydrolyzable tannins are esters of a phenolic acid and a polyol which is usually glucose. The phenolic acid is either gallic acid in gallotannins or ellagic acid in ellagitannins; ellagic acid is formed by lactonization of hexahydroxydiphenic acid released upon hydrolysis of ellagitannins (Ribereau-Gayon, 1972). Tannin molecular weights vary widely (usually between 500 and 3000) depending on the number of galloyl residues in galloyl esters or the eventual dimerization or polymerization of ellagitannins; for condensed tannins molecular weight depends on the number of flavonol units (Haslam, 1975).

Plants that contain tannins may have a significant evolutionary advantage over their enemies and they are thought to constitute one of the most important groups of higher plant defensive secondary metabolites (Scalbert, 1991). Tannins may deter herbivores from predation (Feeny, 1970, 1975). They may also deter microorganisms, either by increasing resistance against pathogens (Brownlee et al.. 1990), or by protecting wood against decay (Hart and Hillis, 1972). Many of the timber trees selected for their durability are tannin-rich, e.g. oak, chestnut, black locust, redwood and some eucalyptus (Scalbert, 1991).

Toxicity of tannins

Tannins have been shown to inhibit the growth of many filamentous fungi (Scalbert, 1991). The minimum inhibitory concentration (MIC) is usually higher than 0.5 g/l for Fomes annosus, on commercial tannic acid (polygalloylglucose). In liquid medium, Haars et al. (1981) found that a concentration of 0.2 % inhibited growth of Fomes annosus up to 90 %; when tannic acid was applied to agar. A concentration of 0.05 % caused a marked decrease in the radial growth of the mycelium. Yeast seems to be more resistant than fungi (Jacob and Pignal, 1972). Some species of yeasts are inhibited at a tannin level of 25 g/l whereas other require levels as high as 125 g/l (Jacob and Pignal, 1972). The MIC for bacteria is usually lower and can vary between 0.012 and 1 g/l (Nishizawa et al. 1990, Henis et al. 1964).

The effect of tannin molecular structure on toxicity is largely unknown. Some authors have compared the toxicity of tannic acid with other hydrolyzable tannin extracts from myrobalan, oak, chestnut and condensed tannin from wattle, quebracho, sorghum, cocoa or carob pod. Hydrolyzable and condensed tannin have been compared for their toxicity toward filamentous fungi (Sivaswamy and Mahadevan, 1986) and bacteria (Basaraba, 1966; Henis et al. 1964). The authors found no significant differences between the toxicity of hydrolyzable and condensed tannins toward filamentous fungi and bacteria. However, yeast were more sensitive to chestnut tannin than to quebracho tannins and even more sensitive to tannic acid (Jacob and Pignal, 1972).

Toxicity of tannins and lower molecular weight phenols has been related to their oxidation state. Catechin, a condensed tannin monomer, was shown to have no toxicity toward methanogenic bacteria but after autoxidation, strongly reduced methane production (Field et al. 1989). Field concluded that toxicity increased as catechin was polymerized to oligomeric tannins. Similarly, bactericidal activity against *Xanthomonas phaseoli* of catechol, protocatechuic acid or caffeic acid is enhanced upon oxidation with peroxidase (Urs and Dunleavy, 1975). This is possibly related to the effect of phenol oxidation with laccase or alkali on the inhibition of fungal enzymes such as pectinase, cellulase or peroxidase; oxidation increases inhibition of these last enzymes by cathecol, protocatechuic acid or caffeic acid but decreases inhibition by a tannin of unknown origin (Lyr, 1965).

Working with E. parasitica, Nienstaedt (1953) found that tannins from the resistant Chinese chestnut retarded the growth of E. parasitica when present at 1.2 % in potato-dextrose agar cultures. Tannin extract from susceptible American and moderately resistant Japanese chestnut did not have this effect. He concluded that the tannase complex of E. parasitica decomposes pyrogallol tannin and produces a simple phenol that is toxic to the fungus. The tannins in the bark of Chinese chestnut belong to the pyrogallol group, whereas the bark of American and Japanese chestnuts contains a mixture of pyrogallol and catechol tannins (Griffin and Elkins, 1986). In a medium containing bark extract from the latter chestnuts, the concentration of the pyrogallol tannin is reduced and the toxicity similarly reduced. In a recent study S. L. Anagnostakis (unpublished) did not find any inhibitory effect of Chinese tannin. She suggested that other factors may be implicated in the resistance of the Asian chestnut trees to the fungus and concluded that tannin toxicity cannot be the answer.

Mechanisms of tannin toxicity

One of the mechanisms of tannin toxicity can be related to the physico-chemical properties of tannins, and in particular to their astringency. The astringent character of tannins may induce complexing with enzymes or substrates

(Scalbert, 1991). Many fungal enzymes were inhibited when crude extracts or purified enzymes were mixed with tannins: cellulase (Benoit and Starkey, 1968), pectinase, xylanase (Lyr, 1961), peroxidase (Lyr, 1965, 1961), and glucosyltransferase (Kakiuchi et al. 1986). Natural polymers treated with tannins were poorly degraded by microbial enzymes. Decomposition of gelatin (Benoit et al. 1968) or polysaccharides such as pectins or polygalacturonic acid (Benoit and Starkey, 1968), into CO_2 and ammonia by a soil inoculum was strongly reduced by chestnut or wattle tannin when a ratio of tannin/substrate greater than 1 was used. In comparison, decomposition of peptides, amino acids (Basaraba and Starkey, 1966) or lysine and glucuronic acid (Benoit et al., 1968) was less affected. The authors concluded that this was probably due to a lower affinity of these small molecules for tannins, as compared to polymers. The same mechanism based on the astringent character may explain the reduction of virulence in viruses. Tannins have been reported to prevent the infection of plants by tobacco mosaic virus by adsorption on the virus surface (Cadman, 1960). Konishi and Hota (1979) showed that tannic acid inactivates Chikungunya virus _in vitro_. The inactivation was pH dependent and was suppressed by bovine serum albumin (BSA). They concluded that the inactivating capacity of tannins was attributable to its binding to the protein coat of the virus. Takechi et al.

(1985) showed that several tannins have antiherpetic activities and that the activity was dependent on the number of galloyl and hexahydrodiphenic groups for hydrolyzable tannins and for condensed tannins on the degree of condensation. They concluded that active groups interact with protein of virus particles and host surface and reduce viral infectivity.

The astringency of tannins is closely related to their chemical structure (Haslam, 1975; Scalbert, 1991). Dimeric ellagitannins are more astringent than the related monomers (Okuda et al. 1985). Affinity of bovine serum albumin increases with the number of galloyl residues (Okuda et al., 1985; Verzele et al., 1986).

Tannins may also directly affect the metabolism of microorganisms, as in Crinipellis perniciosa in which the morphology of the germ tube is modified at low tannin concentration (0.063 g/l) (Brownlee et al., 1990). Tannins also can interfere with metabolism of microorganisms through action on their membranes. Low concentrations of tannic acid (less than 1 mg/l) can inhibit oxidative phosphorylation by mitochondria of blowfly flight muscle (Duncan et al., 1970); inhibition of electron transport system was also observed on rat liver mitochondria. Similar mechanisms were used to explain antibacterial properties of tannic acid on Photobacterium phosphoreum (Konishi et al. 1987).

Other toxicity mechanisms might involve complexing of metal ions by tannins (Scalbert, 1991). Antimicrobial activity through iron depletion is well documented (Weinberg, 1984). Depletion of metal ions through precipitation might be the mechanism by which tannins exert their antimicrobial action (Scalbert, 1991). Most tannins have o-diphenol groups in their molecules, which can form chelates with many metal ions such as cupric or ferric ions (Harris and Livingstone, 1964).

It is possible that tannins reduce availability of essential metal ions for microorganisms (Scalbert, 1991). Also metal depletion by tannins may inhibit the activity of fungal metalloenzymes such as peroxidase and laccase (Lyr, 1961).

Two reasons may possibly explain the positive effect of phenol oxidation on enzyme inhibition (Scalbert, 1991). First, oxidized phenols may react with sulfhydryl groups of enzymes and form covalent linkages with them (Lyr, 1961; Mason and Wasserman, 1987). Second, an increase in molecular weight through oxidative polymerization (Hathway and Seakins, 1957) could also contribute to increasing the efficiency of binding to protein through non-covalent linkages.

Microbial defenses against tannins

Although tannins have antimicrobial properties, many microorganisms can grow and develop on tannin-rich materials. Many woods such as those from oak or chestnut contain over 10 % of their dry weight as tannins and are among the most durable organic materials that living organisms synthesize (Scalbert, 1991). However some molds develop easily on the surface of tannin-rich woods such as quebracho or european oak. Molds such as *Aspergillus niger* or *Penicillium glaucum* grow on the surface of liquid of tannery pits (Meunier and Vaney, 1903). Several detoxification mechanisms may contribute to the explanation of the growth of microorganisms on tannin rich media (Scalbert, 1991).

Microorganisms may secrete outside the cell polymers with a high affinity for tannins with which they will combine and tannins will not be available for microbial enzymes that are essential for the growth of the microorganism (Scalbert, 1991). Condensed tannins induce the secretion of a water-soluble mucilage by spores of *Colletotrichum graminicola* (Nicholson et al.,1986). The mucilage is made of glycoproteins which have a high affinity for condensed tannins. The same strategy is used by plants and animals to reduce astringency of tannins. The reduction of astringency in ripening fruits seems to depend on the release of tannin-binding molecules (Joslyn and Godstein, 1964).

Enzymes resistant to tannins

It is well known that many enzymes are inhibited by tannins. Some enzymes such as tannases or an invertase (Strumeyer and Malin, 1970) and an amylase (Komninos et al., 1988) are known to maintain full activity in the presence of high concentrations of tannins.

Polyphenoloxidases can oxidize tannins and might cause their detoxification. Tannins induce the secretion of laccase by some fungi such as Trametes hirsuta (Scalbert, 1991). Fomes annosus laccases were induced also by catechin (Haars et al., 1981). However no clear relationship could be established between extracellular laccases and the toxicity of phenols (Haars et al,, 1981). Laccases may oxidize tannins and affect their toxicity (Lyr, 1961) or they may also induce the co-polymerization of soluble protein with tannin or other phenols and thus inactivate fungal enzymes to the detriment of the invader (Leatham et al., 1980).

Tannin degradation

Some soil microorganisms grown in pure culture were shown to develop in media containing tannin as the sole source of carbon (Scalbert, 1991). Several condensed and hydrolyzable tannins were compared (Lewis and Starkey, 1969). Aspergillus spp., Penicillium spp., and other fungal species grew better on gallotannin and catechin than on chestnut tannin or wattle tannin (condensed tannin). The fungus E. parasitica could grow on chestnut tannin extracts from chestnut trees (Cook and Wilson, 1914; Elkins et al., 1978; S. Anagnostakis, unpublished). Among soil bacteria, Achromobacter sp. attacked the hydrolyzable tannins, gallotannin and chestnut tannin, but did not decompose catechin or wattle tannin (Lewis and

Starkey, 1969). Other soil bacteria such as Klebsiella spp. were able to grow on tannic acid as well as on gallic acid (Deschamps et al. 1980).

Not enough information exists on the tannin biodegradation pathways used by microorganisms. Degradation of hydrolyzable tannins, and particularly gallotannins is best understood (Scalbert, 1991). Some fungi produce an enzyme, tannase (tannin acyl hydrolase) which allows them to grow on hydrolyzable tannins. Over twenty fungi were tested for growth on tannic acid . Although most Penicilium spp. and Trichoderma spp. tested were able to grow at a low concentration of tannic acid (2.5 g/l), only a few Penicillium and all the Aspergillus species tested showed good growth under concentration as high as 100 g/l (Knudson, 1913). These last fungi produce a tannase that is induced by methyl ester of gallic acid or tannic acid (Haslam and Stangroom, 1966; Iibuchi et al., 1972). The enzyme has been purified (Iibuchi et al., 1968; Rajakumar and Nandy, 1983) and characterized (Thomas and Murtagh, 1985)

Tannase catalyses the hydrolysis of an ester between a phenolic acid and an alcohol. The nature of the alcohol in the ester bond has little influence on activity. It can be methanol (Haslam, 1966; Haslam and Stangroom, 1966), glucose (Haslam et al., 1961; Iibuchi et al., 1972) or gluconic acid (Tanaka et al., 1986). The requirement on the carboxylic acid is stronger. It must belong to a phenolic acid carrying two hydroxyl groups ortho to one another such as in gallic acid (Iibuchi et al., 1972). Tannase is active on galloyl residues of galloyl esters, as well as on hexahydroxydiphenyl, dehydrogalloyl (Yoshida et al., 1985) and valoneoyl (Hatano et al., 1988) residues of ellagitannins. Galloyl residues are more easily hydrolysed than the other groups (Nonaka et al., 1981, 1982, 1989). Their hydrolysis will depend on the position of the substituent on the glucose. 1-O-galloyl residues will be hydrolyzed first, followed by 2-O-galloyl residues and last 3-O-galloyl residues (Hatano et al., 1989).

4,6-O-Hexahydroxydiphenyl residues are more easily hydrolyzed than the 2,3-O hexahydroxydiphenyl residues (Yoshida et al., 1985). The duration of the hydrolysis of ellagitannins with the tannase can vary from several minutes for stenophyllanin B (Nonaka et al., 1985), to hours for cornusiin E (Hatano et al., 1989), and days for gemini A (Yoshida et al., 1985). The analysis of the products can be used as an aid for structure determination of hydrolyzable tannins (Nonaka et al, 1989; Yoshida et al., 1989).

Several attempts have been made to isolate different tannases from the culture media of <u>Aspergillus</u> <u>oryzae</u>, and in particular to separate the esterase activity catalyzing the hydrolysis of galloyl esters on glucose from the depsidase activity catalyzing the hydrolysis of ester linkages between the two galloyl residues (Haslam et al., 1961; Haslam and

Stangroom, 1966) The authors concluded that two tannase isoenzymes were involved but they were not able to identify fractions with exclusive esterase or depsidase activity. A recent study (Beverini and Metche, 1990) describes evidence for independent esterase and depsidase activity.

Gallic acid produced by microorganisms can be accumulated as shown for E. parasitica (Elkins, 1981) and further degraded. Molds growing on the surface of gallotannin containing tannery pits do not degrade gallic acid if the solution is mixed every day. If the fungi are allowed to grow freely, most of the gallic acid will be degraded (Scalbert, 1991). Pathways of gallic acid degradation have been determined. Aspergillus strain 64 converted gallic acid into pyrogallol (Yoshida et al. 1982). Beveridge and Hugo (1964) isolated ß-ketoglutarate from Pseudomonas convexa grown on tannic acid. Gallic acid was cleaved by A. niger into cis-aconitic, α-ketoglutaric and citric acid (Watanabe 1965). Gurujeyalakshmi and Mahadevan (1987) found that A. flavus produced an enzyme that oxidized gallic acid. They proposed a degradative pathway that went from gallic acid to muconic acid, oxadipidic acid, oxaloacetic acid and finally pyruvic acid. Rajakumar and Nandy (1986) concluded that P. chrysogenum produces an oxidase that decomposed gallic acid into oxalic acid. The degradation of hexahydroxydiphenyl groups has not been studied (Scalbert, 1991). Proanthocyanidin degradation is much less well understood. Depolymerization by yeast was suggested (Vennat et al., 1986). Degradation may be similar to the pathway identified for the degradation of catechin (Chandra et al., 1969).

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CHAPTER 2

TANNASE ACTIVITY ASSOCIATED WITH GROWTH OF ENDOTHIA PARASITICA IN AMERICAN AND CHINESE CHESTNUT EXTRACTS AND PROPERTIES OF THE PARTIALLY PURIFIED ENZYME

INTRODUCTION

The fungus *Endothia parasitica* (Murr.) P. J. & H. E. Anderson (syn: *Cryphonectria parasitica* (Murr.) Barr) is the causal agent of chestnut blight, a devastating disease that destroyed almost all the American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) that were growing in the eastern United States at the beginning of the twentieth century. European chestnut (*C. sativa* Mill.) is similarly blight-susceptible.

Chinese chestnut (*C. mollissima* Bl.) and Japanese chestnut (*C. crenata* Sieb. and Zucc.) are resistant to the disease while American chestnut is susceptible. One difference between susceptible and resistant chestnuts is the type of tannin present in the bark tissues. Elkins et al. (1979, 1981) found high concentrations of hamamelitannin in blight-susceptible American and European chestnuts but none in blight-resistant Chinese and Japanese chestnuts.

The rate and extent of mycelial fan formation appear to be important factors in the pathogenesis of E. parasitica on chestnut (Hebard et al, 1984). The rapid growth of mycelial fans probably requires a large nutrient base. Tannins are abundant in chestnut bark and there is general agreement that E. parasitica can utilize tannins. Cook and Wilson (1915) showed that E. parasitica used tannin from American chestnut as a nutrient source and Elkins et al (1979) reported that three virulent isolates of E. parasitica used hamamelitannin as the sole carbon source. Bazzigher (1955) reported the ability of E. parasitica to utilize tannins from European and Chinese chestnuts and identified an inducible esterase able to degrade hydrolyzable tannins from chestnut.

Tannin acyl hydrolase (tannase) is an enzyme hydrolyzing the ester bonds of tannic acids. In American chestnut, E. parasitica is thought to produce a tannase that hydrolyzes hamamelitannin and releases gallic acid and hamamelose which may be used by the fungus as carbon sources (Elkins et al., 1979; Griffin and Elkins, 1986).

Fungal tannases from Aspergillus spp. and Penicillium spp. have been investigated. Iibuchi et al. (1968, 1972) purified the enzyme from culture broth of A. oryzae and investigated the substrate specificity and inhibition of the enzyme. Rajakumar and Nandy (1983) isolated and purified the tannase from mycelial extract of P. chrysogenum. Studies of tannase from E. parasitica have not been reported.

The present study was undertaken to characterize the properties of the tannase of *E. parasitica* growing on aleppo-tannic acid and to determine the relationship between tannase activity and *E. parasitica* growth on total and tannin extracts from American and Chinese chestnut bark.

MATERIALS AND METHODS

Organisms and cultures

Virulent *E. parasitica* strain WK (Hebard et al., 1984) was obtained from Dr. Gary Griffin (Virginia Polytechnic Institute and State University) and used in all experiments. Strain WK was found to be highly representative of the virulent *E. parasitica* population in Appalachian forests (Griffin et al., 1983) and has been virulent in pathogenicity trials every year since 1976 (G. Griffin, personal communication). The organism was maintained on potato-glucose agar (PDA, Difco Laboratories, Detroit, MI) slants. Ten-day-old cultures were used to prepare aqueous conidial suspensions for inoculation of the different culture media.

Culture media

The four media used for each chestnut species were total aqueous extracts from winter and summer bark and tannin extracts from winter and summer bark plus minimal medium. Our minimal medium consisted of 125 ml/l of inorganic salt solution (Puhalla and Anagnostakis, 1971); 4mg/l of thiamine HCl and 0.1 M phosphate buffer (pH 6.0). Winter bark (November to March) and summer bark (May to September) from thin-barked American and Chinese chestnut stems were used to obtain total aqueous chestnut and tannin extracts. Branches and stems less than 15 mm were collected at random from ten trees. The American chestnut trees used for all experiments were blight susceptible (Grifin et al., 1983) and were located at Glade Road experimental plot (Blacksburg, Va). The Chinese chestnut trees (cv. Nanking) used were located at the former Virginia Tech Horticulture Farm (Blacksburg, Va). After peeling the stems, the bark was dried at room temperature for 48 hr and finely ground. For total aqueous extract, 20 g of ground bark was added to 500 ml of water. After stirring for 10 min, the mixture was centrifuged at 5,000 rpm for 5 min . The supernatant was filter-sterilized through a 0.45-m pore size Millipore membrane and 50 ml of solution were dispensed into 250-ml Erlermeyer flasks. The tannins were extracted by the following procedure: 50 g of ground bark was extracted three times with 500 ml of 1:1 methanol: water. The tannins were completely precipitated with 3% basic lead acetate and reconstituted with 1% sulfuric acid. The resulting concentrated tannin solution was made up to give a growth medium containing minimal medium plus 2 % tannin, equivalent to 2 % (w/v) aleppo tannic acid (Mallinckrodt Chemical Co. St.

Louis, MO.) as determined by precipitation with lead acetate. Unfractionated tannins that include gallic acid and other phenolics are also precipitated by this method. Two percent aleppo tannic acid was previously established as the optimum concentration for the culture medium. The solutions were filter-sterilized through a 0.45-μm pore size Millipore membrane. After the pH of the medium was adjusted to pH 5.5, 50 ml of medium were dispensed into sterile 250 ml Erlenmeyer flasks. For all media, one ml of a mixed suspension of $1x10^6$ spores per ml, determined with an hemacytometer, was placed in each flask and the inoculated flasks were incubated for 10 days at 28 C. Ten flasks per medium were harvested each day starting at day 1. Each experiment was repeated three times.

Conidium germination experiments

Conidium germination experiments were carried out using American and Chinese chestnut tannin and total extracts. A 250-ml Erlenmeyer flask containing 50 ml of extract was inoculated with 1 ml of a washed WK conidial suspension containing $1x10^6$ conidia per ml. The contents of each Erlenmeyer flask were divided among three 9-mm-diameter, flat-bottom, glass petri dishes and incubated at 28 C. Percentage conidium germination and the length of germination tubes were assayed microscopically after 12 and 18 hr. One hundred conidia were measured for each petri dish and the experiment was repeated twice. Statistical differences between American and Chinese extracts were determined by means of Student's t-test.

Estimation of fungal growth, tannin utilization and tannase activity

Mycelium in the flasks was harvested daily and dry weight of mycelium was determined. The mycelium was harvested on a tared Whatman paper filter placed over a Buchner funnel. The filter and mycelium were then dried to constant weight at 75 C. Aliquots of 10 ml of culture filtrate were tested for tannin disappearance by the lead acetate-precipitation method.

Culture filtrate and mycelial extract were tested for tannase activity following growth of E. parasitica in aleppo tannic acid medium. The culture medium was filtered through a 0.45-μm pore size Millipore membrane to separate the mycelial mat from the culture medium. Both the mycelial mat and the filtrate were tested for tannase activity. The mycelial mat was washed with distilled water and homogenized at 0 C in 50 ml of 0.01 M acetate buffer, first with IKA Ultra Turrax T25 homogenizer (IKA Works, Inc., Cincinnati, Ohio) for 5 min and then sonicated for 60 sec (Sonicator^{TM}, Heat System, Ultrasonic, Inc, Model W220F). The mycelial debris was removed by centrifugation at 5,000 rpm and 5 C for 10 min and the supernatant used for enzyme activity determination.

Tannase activity determination

Tannase activity was determined in culture filtrate and mycelial extract by estimating the amount of gallic acid produced during hydrolysis of methyl gallate using a Metrohm autotitrator (Brinkmann Instruments Co., Westbury, NY) in pH stat mode. The procedure used was similar to that used by Iibuchi et al.(1972). Temperature and pH were adjusted to 28 C and 5.5 respectively. The substrate was stirred continuosly and the crude enzyme extract was added with a pipette. Autotitration with 0.01 M NaOH was begun immediatly after enzyme addition and was allowed to continue for 15 min. Except for the pH studies the endpoint setting of the pH stat was 5.5. Reaction rates were calculated from the initial slopes of the pH stat curves (volume of titrant vs. time) and expressed as nmoles/min. Control determinations were carried out with boiled enzyme extract. Enzyme activity (unit) was defined as the amount of gallic acid (nmoles) liberated in 1 min from 1 ml of crude extract.

Total Protein Analysis

Protein in culture filtrate and mycelial extract was determined using the Commassie Blue binding assay (Bradford, 1976). Protein values for crude desalted extracts were obtained from a standard protein curve which was generated based on the dye binding capacity for Bovine Serum Albumin-Standard II (Bio-Rad, Richmond, CA) vs the change in absorbance at 595 nm.

Properties of the tannase

Effect of pH on tannase activity. To find the optimal pH for activity of the enzyme, the crude extract produced in aleppo tannic acid was partially purified by ammonium sulfate precipitation. Solid ammonium sulfate was added to the enzyme extract to 80% saturation for 30 min at 0 C and the mixture was centrifuged at 18,000 x g for 30 min. The precipitate was collected, desalted by the microcentrifuge method (Helmerhorst and Stokes, 1980), and reconstituted in 0.01M solutions of the following buffers: glycine (pH 2.5, 3.0), acetate (pH 4.0, 5.5), citrate-phosphate (pH 6.0, 6.5), and Tris (pH 8.0, 8.5). The pH optimum was determined using timed assays held constant with the pH stat, followed by titration to an endpoint of 5.5 for lower pH values.

Effect of temperature on tannase activity. The crude enzyme extract, prepared in aleppo tannic acid and partially purified as described above, was reconstituted in 0.01 M solution of acetate buffer (pH 5.5) and 3-ml aliquots were kept at 10, 20, 28, 40, 50 and 60 C for 15 minutes. Before and after the treatment, the pH of the solutions at each temperature was determined and activity estimated by the amount of NaOH that was delivered by the autotitrator to bring the pH of the solutions to the values before the temperature treatments.

RESULTS

Enzyme activity of the culture filtrate and mycelial extract.

Mycelial extract had greater specific activities than culture filtrate at 5 and 6 days (Fig. 2.1 A), when aleppo tannic acid was the substrate. The highest specific activity for mycelial extract was recorded at 6 days and for culture filtrate at 7 days. The total activity of the tannase (units/ml) for mycelial extract and culture filtrates increased very rapidly between 3 and 6 days (Fig. 2.1 B). Between days 6 and 8, total activity decreased for the mycelium but remained relatively constant for culture filtrate.

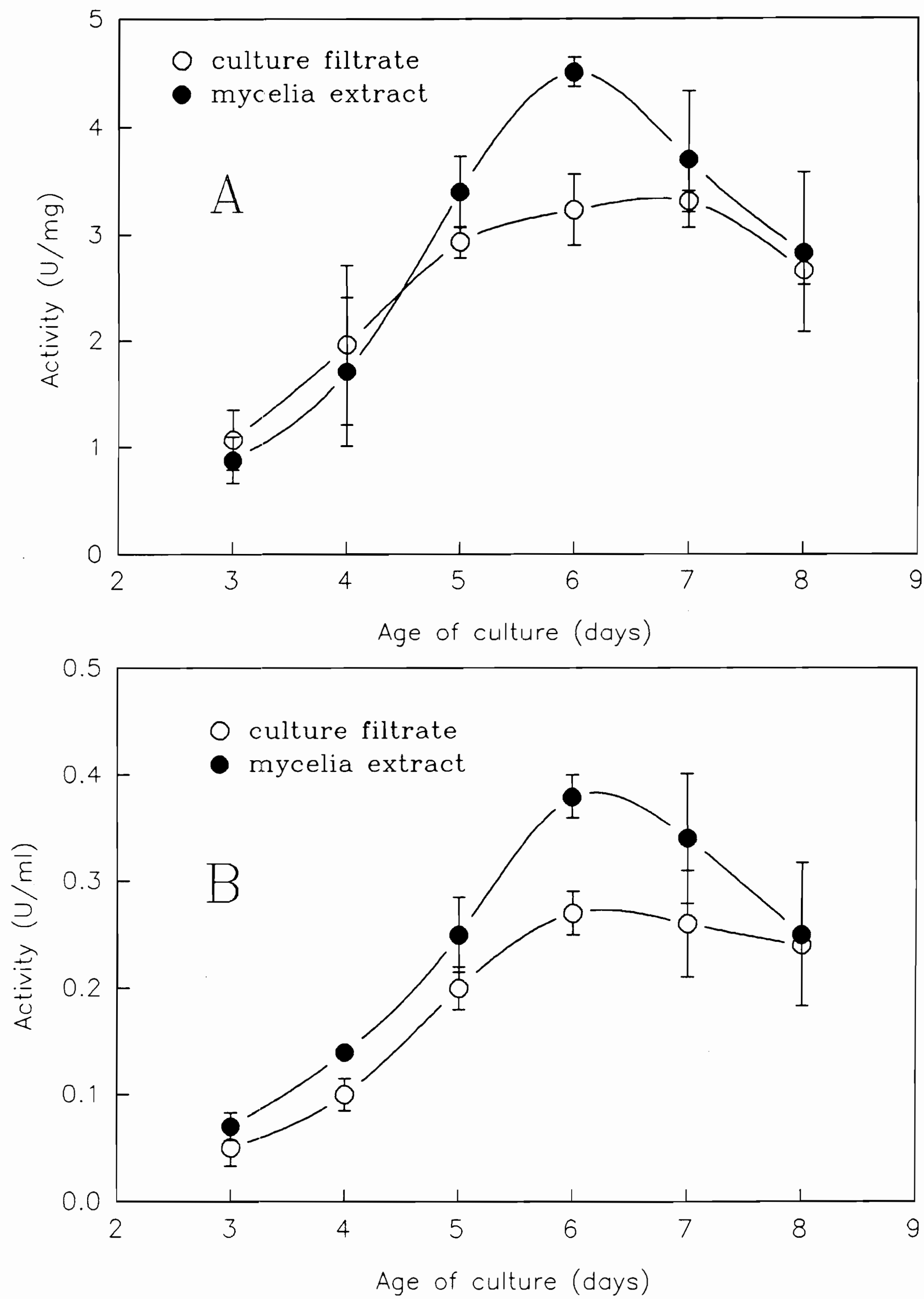
Properties of the tannase

Effect of pH - The optimum pH for enzyme activity was 5.5. Low activity was recorded at pH 2.5 and 8.5 (Fig. 2.2 A).

Effect of temperature - The optimum temperature for the enzyme activity was found to be 30 C (Fig. 2.2 B). Good enzyme activity was observed between 25 and 40 C. The activity was minimal at 10 and 50 C and was lost at 55 C.

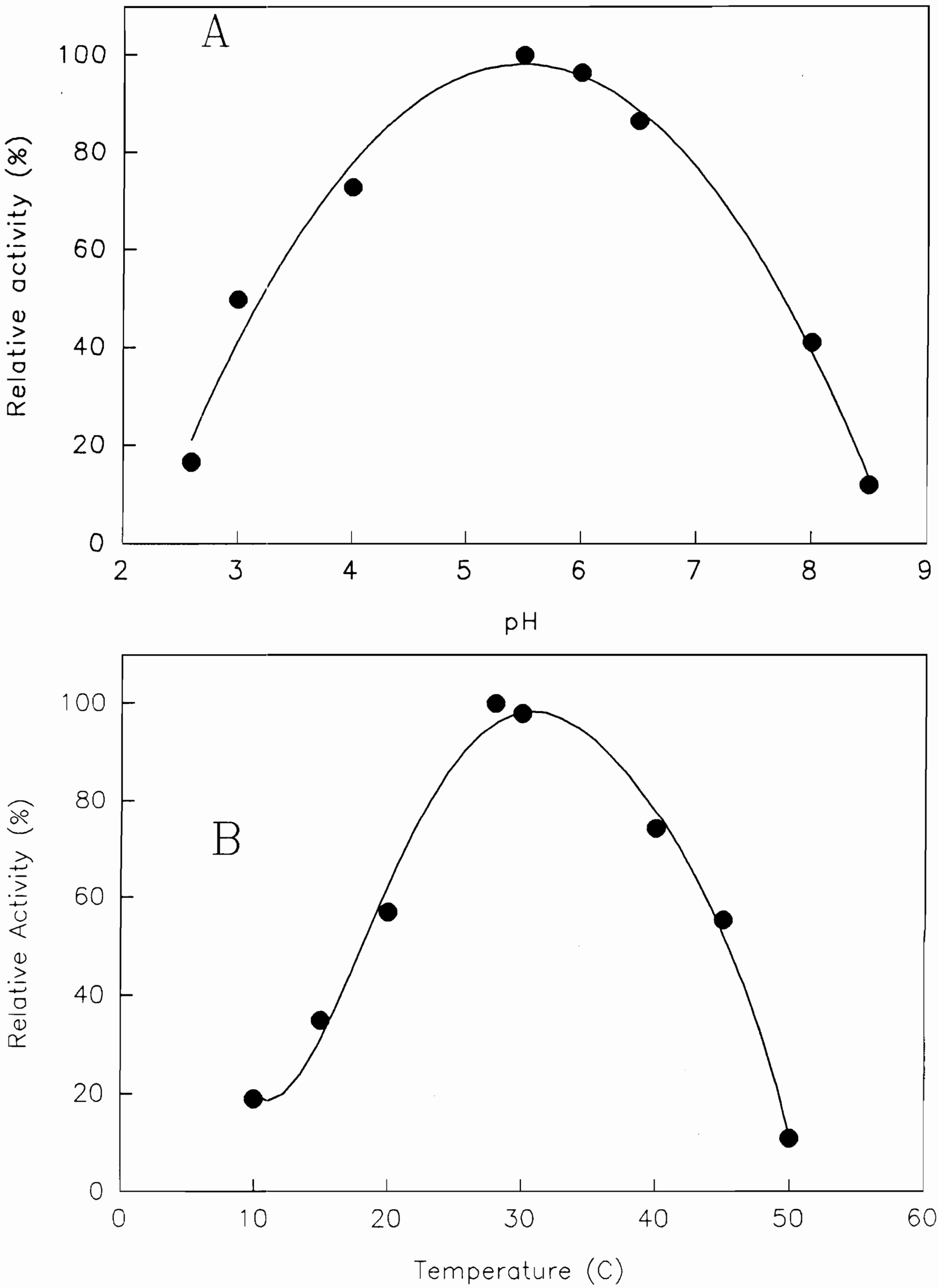
Figure 2.1. Activity of the <u>E</u>. <u>parasitica</u> tannase from culture filtrate and mycelia extract. One unit of activity was defined as nmol of gallic acid released per ml of enzyme extract per minute. A. Specific activity expressed as units per mg of protein. B. Total activity expressed as units per ml of enzyme extract.

Mean of 3 replicates; 95% confidence limits are indicated.



culture filtrate
mycelia extract
A
Activity (U/mg)
Age of culture (days)
culture filtrate
mycelia extract
B
Activity (U/ml)
Age of culture (days)

Figure 2.2. pH and temperature optima of the tannase from *E. parasitica*. A. Optimum pH. The activity of each pH value is expressed as the percentages of the activity at pH 5.5.Buffer solutions were: Glycine (pH 2.5, 3.0), acetate (pH 4.0, 5.5), phosphate (ph 6.0, 6.5) and Tris (pH 8.0, 8.5). B. Optimum Temperature. The activity determined in 15 min reaction using 0.01 M acetate buffer pH 5.5. Relative activity in the figure is the activity at each temperature expressed as percentage of the activity at 28 C.


A
Relative activity (%)
0
20
40
60
80
100
2
3
4
5
6
7
8
9
pH
B
Relative Activity (%)
0
20
40
60
80
100
0
10
20
30
40
50
60
Temperature (C)

Germination experiments

Results of the germination experiments are shown in Table 2.1. The length of germ tubes was significantly greater in American than in Chinese total extracts after 18 hr. No significant differences were detected between American and Chinese tannin extracts. Percentage conidium germination showed no significant differences between American and Chinese total aqueous extracts nor between the respective tannin extracts. Germination was observed in all extracts as early as 12 hr.

Fungal growth and tannase activity.

When winter bark was used to prepare media, growth of E. parasitica was grater in American chestnut total aqueous extract during the first three days than in Chinese chestnut total aqueous extract, but no significant differences between American and Chinese total aqueous extract were detected afterwards (Fig. 2.3 A). When tannin extract from American and Chinese chestnut winter bark was used as the sole carbon source, E. parasitica grew more in Chinese chestnut tannin than in American chestnut tannin but no significant differences were found (Fig. 2.3 A). Growth in tannin extract was slower starting than in total aqueous extracts, but final dry weights were comparable. When summer bark was used, the growth of E. parasitica was the same in American and Chinese chestnut total aqueous extracts during the first

Table 2.1. Conidium germination of E. parasitica in chestnut total aqueous and tannin bark extracts.[1]

Medium	Length germ tube[2] µm	Germination[2] %
EXPERIMENT 1		
American total aqueous bark extract	34.8 A	82 A
Chinese total aqueous bark extract	29.0 B	71 A
EXPERIMENT 2		
American tannin bark extract	22.2 A	95 A
Chinese tannin bark extract	21.4 A	89 A

[1] Observations were made at 18 h. Values are mean of 300 observations.

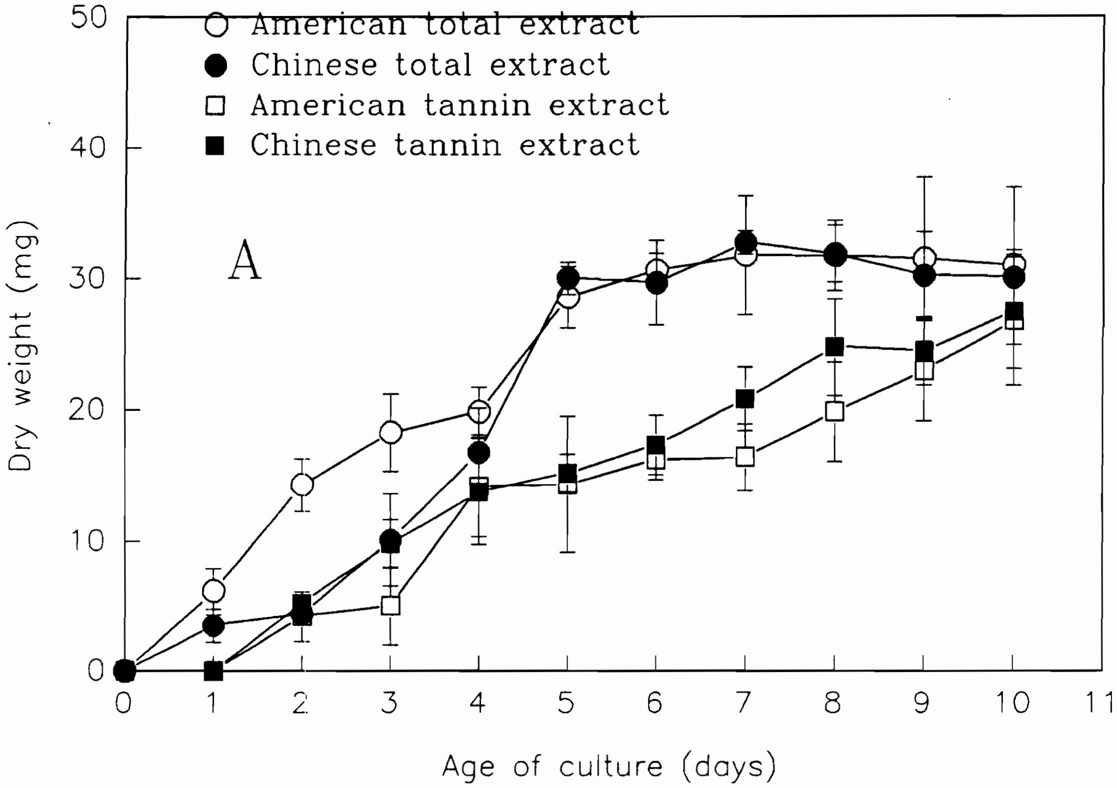
[2] Means within each column and each experiment, followed by same letters are not significantly different according to Student's t-test.

3 days (Fig. 2.3 B). Between days 4 and 6, the growth in Chinese chestnut total aqueous extract was significantly greater than in American chestnut total aqueous extract; no significant differences were detected afterward. When the fungus grew in tannin extracts (Fig. 2.3 B), no significant differences were observed between American and Chinese tannins up to day 4. At 5 and 6 days, growth was significantly greater in Chinese than in American tannin. After day 6, growth in American tannin was higher but no significant differences were detected. In aleppo tannic acid, E. parasitica grew at a nearly constant rate for 10 days (Fig. 2.3 B).

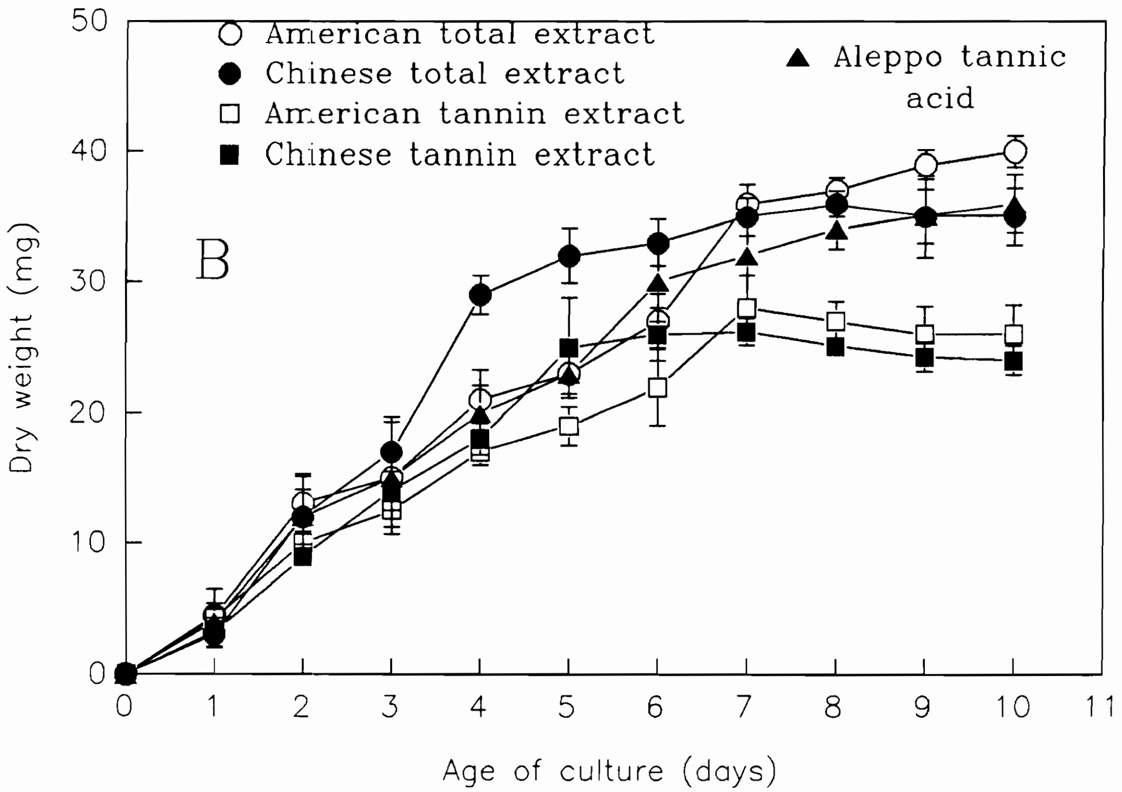
Total tannase activity (units/ml) in American chestnut total extract was greater than in Chinese chestnut total extract (Fig. 2.4 A). Maximum activity for both media was recorded at day 5. When E. parasitica grew in tannin extract, activity in American chestnut tannin was significantly greater than activity in Chinese chestnut tannin. Maximum activity was reached at day 3 for American chestnut tannin extract and day 4 for Chinese chestnut tannin extract.

The results for enzyme activity, following growth in summer bark (Fig. 2.4 B), show that total tannase activity in American chestnut total aqueous extract was greater than in Chinese chestnut total extract. Maximum activity was recorded at 4 days for Chinese chestnut extract and 7 days for American chestnut extract. When the fungus grew in tannin extracts,

Figure 2.3. Growth of E. parasitica in aleppo tannic acid, chestnut total aqueous, and chestnut tannin extracts from bark collected during winter and summer. A. Winter bark collected from November to March. B. Summer bark collected from May to September. Mean of 3 replicates with 10 flasks each; 95% confidence limits are indicated.

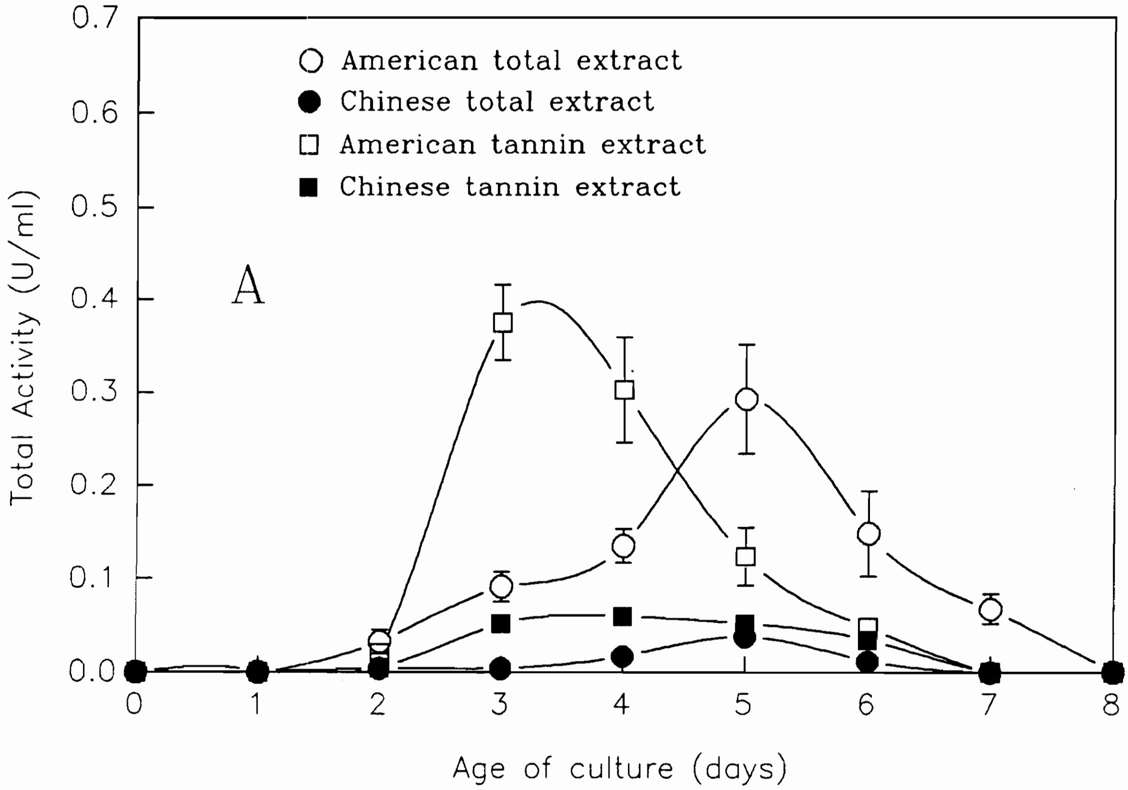


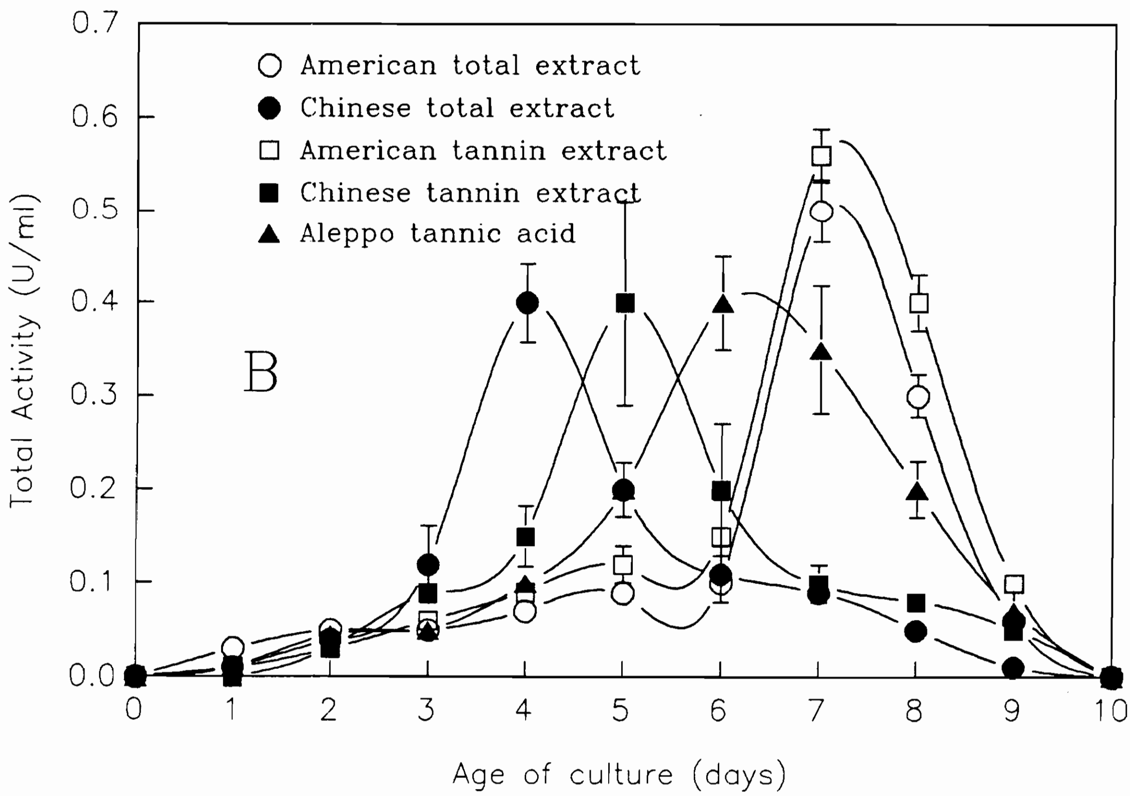
American total extract
Chinese total extract
American tannin extract
Chinese tannin extract
A
Dry weight (mg)
Age of culture (days)
0
10
20
30
40
50
0
1
2
3
4
5
6
7
8
9
10
11



American total extract
Chinese total extract
American tannin extract
Chinese tannin extract
Aleppo tannic acid
B
Dry weight (mg)
Age of culture (days)
0
10
20
30
40
50
0
1
2
3
4
5
6
7
8
9
10
11

Figure 2.4. Total activity of the *E. parasitica* tannase from cultures grown in chestnut total aqueous and tannin extracts. One unit of enzyme activity was defined as nmol of gallic acid released per minute per ml of enzyme extract. A. Activity in winter bark extracts. Bark was collected from November to March. B. Activity in summer bark extracts. Bark collected from May to September. Means of 3 replicates with ten flasks each; 95% confidence limits are indicated.


American total extract
Chinese total extract
American tannin extract
Chinese tannin extract
A
Total Activity (U/ml)
Age of culture (days)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3
4
5
6
7
8


American total extract
Chinese total extract
American tannin extract
Chinese tannin extract
Aleppo tannic acid
B
Total Activity (U/ml)
Age of culture (days)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3
4
5
6
7
8
9
10

tannase activity in American chestnut tannin was significantly greater than in Chinese chestnut tannin. Maximum activity was reached at day 5 for Chinese tannin and day 7 for American tannin. in aleppo tannic acid (Fig. 2.4 B), total tannase activity was comparable to that in Chinese total aqueous and tannin extracts but maximum activity was reached at day 6. The high growth observed in Chinese tannin summer bark extract between days 5 and 6 is coincident with the peak of enzyme activity that occurred at day 5. For American total aqueous and tannin summer extracts, the high growth recorded at day 7 day also is coincident with the activity of the tannase that showed a peak at 7 days.

DISCUSSION

E. parasitica was able to grow in American chestnut total aqueous and tannin bark extracts as well as in Chinese total aqueous and tannin bark extracts. Cook and Wilson (1915) showed that tannins from American chestnut are utilized by E. parasitica as a nutrient source. In growth experiments, Nienstaedt (1953) found that purified tannin extracts from American, Japanese and Chinese chestnuts retarded the growth of E. parasitica when present at 1.2 % in PDA. No differences in growth inhibition were found for American and Japanese purified chestnut tannin extracts but Chinese purified tannin extracts was completely inhibitory. In the present study, 2 % Chinese chestnut tannin extracts were not inhibitory. Elkins et al. (1979) demonstrated that E. parasitica was capable of utilizing tannins from total aqueous extracts of American, Chinese and European chestnut bark, and Elkins (1981) found that hamamelitannin, present in susceptible American and European chestnuts, but not in resistant Chinese and Japanese chestnuts, is utilized as a carbon and energy source by E. parasitica. Total aqueous extracts likely have other utilizable compounds besides tannins, i.e. sugars, amino acids, starch.

Small E. parasitica growth differences were noted between chestnut species. The conidium germination experiments in chestnut summer bark total aqueous extracts showed that germ tubes at 18 h were longer for American extracts than for the Chinese extracts. Differences in dry weight among extracts at 1 day were only minimal, however. The fungus generally grew slower in American total aqueous summer bark extracts between days 4 and 6 than in the Chinese extracts, but the final amount of growth was slightly greater in American total aqueous bark extracts. In contrast, E. parasitica grew more in American chestnut total aqueous winter bark extract during the first 3 days than in Chinese total aqueous winter bark extract. Because the differences in fungal growth in American and Chinese extracts were small, it is unlikely that these differences provide information on the role of tannins in blight susceptibility. S. L. Anagnostakis (unpublished) found slight differences in fungal growth rate on Noble agar containing tannin extracts from Chinese, Japanese, and American chestnut trees.

The pH and temperature optima for activity of the tannase were similar to those found for the tannase of other fungi (Iibuchi et al, 1968; Rajakumar and Nandy, 1983). The activity of tannase produced by E. parasitica was lower in Chinese total aqueous and tannin extracts than in the respective American extracts for both winter and summer bark. A possible explanation, in part, for low tannase activity but high fungal growth in Chinese chestnut tannin extracts from winter bark is that the fungus may have grown on non-tannin phenolics that possibly precipitated along with the tannins during the extraction procedure.

The activity of E. parasitica tannase in American chestnut summer bark tannin extract was grater than in aleppo tannic acid. A prominent tannin in American chestnut bark is hamamelitannin (Elkins et al. 1981), which has one sugar,two ester bonds and a molecular weight of 484. Aleppo tannic acid has one sugar, seven ester bonds and a molecular weight of 1216. Iibuchi et al (1972) found an increase of relative tannase activity with increase in the number of ester bonds for gallic acid esters of glucose. When their tabular data was prepared by us in graph form, the relationship was almost linear. Their results are based on supply of the substrates at equivalent number of ester bonds. These findings suggest that less tannase may be required to liberate the same amount of gallic acid from tannins with a high gallic acid:sugar ratio, such as aleppo tannic acid, than from tannins with a low gallic acid:sugar ratio, such as hamamelitannin. The greater tannase activity recorded following E. parasitica growth in American chestnut tannin extract, than recorded for aleppo tannic acid, may result from high hamamelitannin level in the extract medium. Also if Chinese chestnut bark has gallotannins with a high gallic acid:sugar ratio, a similar relationship may exist. Our future experiments on enzyme kinetics using different tannins as substrate, may help clarify this aspect.

When the fungus grew in total aqueous and tannin extracts from Chinese and American chestnut winter bark, the peak of tannase activity preceded maximum growth. When the fungus grew in total aqueous and tannin extracts from Chinese and American chestnut summer bark, peak of tannase activity either preceded or was coincident with maximum growth. It is not clear why tannase activity peaks during early growth for American winter bark but during late growth for American summer bark. Doi et al. (1973) found a close association between tannase activity and growth of Aspergillus oryzae on tannic acid after an initial lag phase of 30 hr. The tannase of E. parasitica was detected after 1 day for summer total and tannin extracts of both species, but only after 2 days for the corresponding winter bark extracts. In the present study, E. parasitica germ tubes were observed as early as 12 hr in tannin extracts; this observation suggests that tannase was formed within the first 12 hr since E. parasitica conidium germination is exogeneous-carbon dependent (Puhalla and Anagnostakis, 1971; G. J. Griffin, unpublished). If present, tannin may support conidium germination in chestnut bark wounds, the main infectiom court of the fungus.

Nienstadt (1953) found that tannins in American chestnut were mixtures of cathecol and pyrogallol tannins while Chinese tannins contained pyrogallol tannins but only small amounts of catechol tannins. Hebard and Kaufman (1978), presented evidence that hydrolyzable tannin levels in callus tissue cultures of different chestnut species may be an index of the blight resistance. They reported that Chinese chestnut callus tissue has high levels of gallotannins and ellagitannins and low levels of condensed tannins. The levels of gallotannins and ellagitannins were higher in callus tissue of Chinese chestnut than in American chestnut. According to these findings, high tannase activity should be expected in Chinese chestnut since tannase may have specificity for galloyl ester bonds (Iibuchi et al. 1972). Possibly, as indicated above, differences in the type of gallotannins present (gallic acid:sugar ratio) in the bark of Chinese and American chestnut, may explain, in part, the differences in *E. parasitica* tannase activity found in bark extracts of the two species.

Our results suggest there is a relationship between tannase activity and the susceptibility of the two chestnut species to blight. One of the obstacles in breeding for resistance in chestnut is the lack of a chemical test to determine the relative resistance of chestnut progeny at an early stage of growth. Differences in *E. parasitica* tannase activity in chestnut bark extracts, if proved reliable, may be used as a tool to identify sources of resistance for Chinese-American hybrids in early stages of a breeding program. Also, tannase may be a critical enzyme in pathogenesis, supplying essential organic carbon for the high-biomass mycelial fans of *E. parasitica* that are so important in colonization of American chestnut bark tissue (Hebard et al., 1984). Genes in *E. parasitica* responsible for tannase activity may be influenced by dsRNA in *E. parasitica* strains with cytoplasmic hypovirulence.

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CHAPTER 3

PURIFICATION, CHARACTERIZATION, AND SUBSTRATE RELATIONSHIPS OF THE TANNASE FROM *ENDOTHIA PARASITICA*

INTRODUCTION

Tannase (tannin acyl hydrolase EC. 3.1.1.20) was discovered in 1786 by Scheele (Yamada et al. 1968). It is known to hydrolyze the ester linkage as well as the depside linkage of tannic acid to gallic acid and glucose (Haworth et al. 1958). This enzyme has been reported to be present in some plants (Madhavakrishna and Bose, 1961), bacteria (Deschamps et al., 1980, 1983) and fungi. Fungal tannases from *Aspergillus* spp. and *Penicillum* spp. have been well investigated and some of them were reported to be purified to homogeneity (Freudenberg et al., Haslam et al., Dhar and Bose, Iibuchi et al., Rajakumar and Nandy). Freudenberg et al. (1927) prepared the tannase from *A. niger*, Haslam et al. (1961) obtained the partially purified enzyme from this fungus, and Dhar and Bose (1963) reported purification, crystallization, and physicochemical properties of the tannase from *A. niger*. Iibuchi et al. (1968) purified tannase from the culture broth of *A. oryzae* and investigated the hydrolyzing pathway, substrate specificity, and inhibition of the enzyme (Iibuchi et al, 1972). Yamada et al.(1968) purified the enzyme from the mycelial extract of _A_. _flavus_ and Adachi et al. (1968) reported its physicochemical and catalytic properties. Working with _P_. _chrysogenum_, Rajakumar and Nandy (1983) reported the isolation and purification of the tannase from fungal mycelium. The production of tannase was reported also in yeast (Aoki et al., 1970) and in bacteria (Deschamps et al., 1983).

In American chestnut, _E_. _parasitica_ is thought to produce a tannase that hydrolyses hamamelitannin and releases gallic acid and hamamelose, which may be used by the fungus as a carbon source (Elkins et al., 1979; Griffin and Elkins, 1986). Bazzigher (1955) reported the ability of _E_. _parasitica_ to utilize tannins from European and Chinese chestnuts and identified an inducible esterase able to degrade hydrolyzable tannins from chestnuts. Mayer (1959, 1971) chemically characterized hamamelitannin, castalin, vescalin, castalagin and vescalagin in the tannin fraction of European chestnut, and Elkins et al. (1979, 1981) showed the presence of hamamelitannin in high concentration in blight-susceptible American and European chestnuts and its absence in blight-resistant Chinese and Japanese chestnuts.

Preliminary results by have indicated that blight resistance might be related, in part, to the absence of hamamelitannin in these species, and that the greater blight resistance of European chestnut relative to American chestnut might be attributable to the lower ratio of hamamelitannin to vescalagin plus castalagin (Griffin and Elkins, 1986). One explanation is that perhaps the larger fraction of ellagitannins in European chestnut would tie up the E. parasitica tannase and make it less available for the hydrolysis of hamamelitannin to gallic acid (J. Elkins, personal communication). As was indicated in the previous chapter, gallic acid accumulates following hamamelitannin hydrolysis by E. parasitica tannase and has the potential to kill chestnut tissue (Griffin and Elkins, 1986). Also, certain levels of gallic acid accumulation may cause inhibition of the tannase.

The present study includes the purification and characterization of the tannase from the mycelium of E. parasitica growing on aleppo tannic acid. Studies of substrate specificity and evaluation of possible inhibition of the tannase using two ellagitannins, vescalagin and castalagin, and one product, gallic acid, are reported also.

MATERIALS AND METHODS

Growth of the organism - Virulent E. parasitica strain WK was obtained from Dr. Gary Griffin (Virginia Polytechnic Institute and State University). The organism was maintained on PDA (Difco Laboratories, Detroit, MI) slants as described in

Chapter 2. The liquid culture medium used was 2 % aleppo tannic acid (Sigma Chemical Co. St. Louis, MO.) plus minimal medium and was prepared as described in Chapter 2. For enzyme production and purification, 1 l of medium was dispensed into sterile 4-l Erlenmeyer flasks, inoculated as stated in Chapter 2, and incubated for 6 days at room temperature (about 24 C). The mycelium (1-2 g/flask) was harvested by filtration using a Buchner funnel with cheesecloth on top, washed with distilled water, then with 10 mM acetate buffer (pH 5.5), pressed to free moisture and, stored at 0 C until used.

Purification procedures

A 50-60 g batch of mycelium was rinsed with cold 10 mM acetate buffer (pH 5.5), and homogenized in 10 volumes of the same buffer containing 1 mM of phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor. The homogenization was done with a IKA Ultra Turrax T25 homogenizer (IKA WORKS, Inc.,Cincinnati, Ohio) at 0 C for 15 min and then sonicated (Sonicator^{TM}, model W-220F, Heat System-Ultrasonic, Inc.) for 60 sec at 0 C. The mycelial debris was removed by centrifugation at 10,000 g for 15 min at 5 C. The supernatant was fractionated by ammonium sulfate precipitation. The 35-80 % saturated-ammonium sulfate fraction was resuspended in a small volume of 10 mM acetate buffer (pH 5.5), and dialyzed for 24 h against the same buffer at 4 C.

Chromatography on DE52 column - The dialyzed solution (15 ml) was placed on a column of DE52 (Whatman International Ltd., Maidstone, England), which was equilibrated with the buffer (20 mM Tris/HCL). This was done to remove tannins and other phenols and obtain a clear enzyme solution. The elution was carried out using 15 ml of 20 mM Tris/HCl pH 7.5, containing 1 M NaCl.The effluent was collected in one 20-ml fraction and dialyzed against distilled water for 24 h at 4 C.

Anion-exchange chromatography [Fast protein liquid chromatography (FPLC)] on Mono Q HR 5/5 - The sample (20 ml) was filtered through a 22 micron Acrodisk (Gelman Science Inc., Ann Arbor, MI) and applied to a Mono Q HR 5/5 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) column, which was eluted (1.0 ml/min) with 20 ml of the starting buffer (20 mM Tris/HCl) followed by 50 ml of a linear gradient of 0-1.0 M-NaCl in the starting buffer. Fractions containing the highest activity were combined (5-6 ml), dialyzed for 12 h at 4 C, and concentrated to 0.5-1 ml by means of an Amicon Centriprep 10 and then with a Centricon-30 (Amicon, Danvers, MA) microconcentrators.

Gel permeation (FPLC) on Superose 12 - The concentrate was applied to a gel permeation column (Superose 12, Pharmacia) and protein was eluted at 1 ml/min with 20 mM Tris/HCl buffer (pH 7.5) containing 0.1 % NaCl.

Enzyme assay method

During the purification procedure and for determination of the enzyme properties, tannase activity was monitored by the spectrophotometric method. The method was similar to that used by Iibuchi et al. (1967) except that methyl gallate was used as substrate instead of tannic acid. The method was based on estimation of the amount of hydrolyzed substrate by measuring the decrease of absorbance at 310 nm (Yamada et al., 1968) using a Beckman DU 65 spectrophotometer. All the spectrophotometric assays were performed at 30 C in a 1 ml reaction mixture and for enzyme properties determination experiments, the final concentration of the purified enzyme was 5 μg/ml unless otherwise specified. During the purification procedure, total protein was used for calculations. The enzyme solution and 0.5 ml of 10 mM acetate buffer (pH 5.5) to a final volume of 0.5 ml were pre-incubated at 30 C for 5 min, and 0.5 ml of methyl gallate solution was added. Typically, the reaction mixture contained a final substrate concentration of 20 mM methyl gallate. The reaction was carried out for 10 min and stopped by the addition of 99 % ethanol. To a 0.3-ml aliquot of the reaction mixture, 0.7 ml of water was added and the optical density at 310 nm was measured. Control experiments were done under the same conditions using boiled enzyme solution. The activity (units) of the enzyme was expressed in μmoles of methyl gallate that were hydrolyzed in one min at 30 C. The specific extinction coefficient of methyl gallate at 310 nm was determined to be 1,343 M^{-1} cm^{-1} and used for calculations. Specific activity was expressed in units of activity per mg of enzyme protein.

Protein determination - Protein concentrations were determined by the Coomassie Blue method (Bradford, 1976). Protein values were obtained from a standard protein curve which was generated on the dye binding capacity for bovine serum albumin-standard (Bio-Rad Laboratories, Richmond, CA) versus the change in absorbance at 595 nm.

SDS-Polyacrylamide gel electrophoresis - The subunit molecular weight of the enzyme was determined using sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) as described by Laemmli (1970). Enzyme preparations (25 μg) were boiled for 5 min in the presence of 1 % (w/v) SDS and 1 % (v/v) ß-mercaptoethanol, with 0.0013 % bromophenol blue tracking dye. Samples were then subjected to SDS-PAGE in 10 % polyacrylamide gels. Electrophoresis was performed at a constant current of 25 mA/gel, using a vertical system (Mini gel System from Bio-Rad Laboratories, Richmond, CA). Gels were stained overnight with Coomassie Brilliant Blue R-250 (Schleif and Wensink, 1981) and destained with 7 % acetic acid.

Polyacrylamide gel electrophoresis (PAGE) - The protein was located in the gel by preparing two native (no SDS) gels one of which was stained overnight with Coomassie Brilliant Blue.

After destaining the gel, the band with the protein became visible. To determine enzyme activity, the non-stained gel (store overnight ar 4 C) was superimposed with the stained gel and the area corresponding to the band was gently sliced. The gel slice was macerated by chopping finely with a scalpel following the method of Hames (1986). The enzyme was allowed to elute from the gel into 2 ml of 0.1 M acetate buffer by diffusion, for 24 hr at 4 C. Gel fragments were removed by centrifugation and the activity in the supernatant was determined spectrophotometrically as described.

Determination of molecular mass - The native molecular mass of the enzyme was determined by gel filtration chromatography using a 1.5 x 30 cm Superose 6 FPLC column (Pharmacia) The column was precalibrated with gel filtration molecular weight standards (Bio-Rad Laboratories).

Elution volumes (V_e) of standard protein were determined by measuring absorbance at 280 nm. The void volume (V_o) of the column was determined using blue dextran. The value of Kd ($V_e - V_o / V_i - V_o$) and logarithm of the molecular weights were used to determine the molecular mass of the tannase.

Isoelectric focusing in gels - Isoelectric focusing (IEF) was performed using an horizontal isoelectric focusing instrument (Isobox HE 950, Hoefer Scientific Inst., San Francisco, CA) with 1.25 ml of ampholytes 3 to 10 (Pharmacia) and 0.225 ml of ampholytes 4 to 6 (Pharmacia). IEF gels were prepared and run by the method of Ried and Collmer (1985). The gels were stained with Coomasie Brilliant Blue. Purified tannase (20 μg) was applied on the IEF gel.

Effect of pH

pH stability - Purified enzyme was treated for 12 hr with 0.05 ml of 10 mM solutions of the following buffers: glycine (2.5, 3.0), acetate (pH 4.0, 5.5), citrate-phosphate (pH 6.0, 6.5), and Tris (pH 7.5, 8.5). After treatment, the solutions were diluted 10-fold with 10 mM acetate buffer (pH 5.5) and the remaining activity (%) was determined spectrophotometrically using 0.3 ml methyl gallate as the substrate in a 10 min reaction. Concentration of methyl gallate in the reaction mixture (1 ml) was 20 mM with purified enzyme concentration of 5 μg/ml.

Optimum pH - The substrate methyl gallate was dissolved in buffer solutions of varying pH as indicated above. The concentration of methyl gallate in the reaction mixture was 20 mM. The purified enzyme was added to initiate the reaction and tannase activity was determined spectrophotometrically in a 10 min reaction. Final concentration of the enzyme in the reaction mixture was 5 μg/ml.

Effect of temperature

Stability - The tannase, in 0.5 ml of 10 mM acetate buffer (pH 5.5) was incubated at 10, 20, 30, 40, 50, and 60 C. For each temperature treatment, samples were removed after 10 and

20 min and immediately assayed with methyl gallate as substrate. Purified enzyme concentration in the reaction mixture was 5 μg/ml. The activity remaining in the incubation mixture (%) was assayed spectrophotometrically in a 10 min reaction.

Optimum temperature - Methyl gallate in 10 mM acetate buffer pH 5.5 at a final concentration of 20 mM was pre-equilibrated to the temperatures indicated above and then the purified enzyme (5 μg/ml) was added to initiate the reaction. The activity was determined spectrophotometrically in a 10 min reaction.

Substrate relationships of *E. parasitica* tannase - The compounds methyl gallate, hamamelitannin and aleppo-tannic acid were used as substrates for the enzyme. The final concentration of the substrates in the reaction mixtures were for methyl gallate: 5, 10, 20, 30, 50, and 60 mM; for hamamelitannin: 2.5, 5, 15, 25, 35, 45, and 55 mM; for aleppo-tannic acid: 0.5, 1, 2, 3, 6, and 8 mM. In all cases, the reaction mixture contained the substrate at the concentration described above, dissolved in 10 mM acetate buffer (pH 5.5). The purified tannase (10 μg/ml) was added to initiate the reaction and the hydrolysis of the substrates was followed titrimetrically by means of the pH stat technique using the Metrohm (Brinkman Instruments, Co., Westbury, NY) autotitrator with automatic burette. The enzymatically released gallic acid was traced by the titration with 10 mM NaOH and the initial velocity of the enzyme reaction was read from the initial slope of pH stat curve (volume of titrant versus time). The enzyme activity (units) was defined as μmoles of gallic acid released in one min at 30 C. Specific activity was expressed in units of activity per mg of enzyme protein. All the titrimetric assays were performed at 30 C in a 2.5 ml reaction mixture and the final concentration of the purified tannase was 10 μg/ml unless otherwise specified.

To calculate kinetic constants (K_m and V_{max}), data were plotted and fitted directly to the Michaelis-Menten equation using a computer program (Pennzyme, University of Pennsylvania). Calculations were also performed by using the linear transform method of Lineweaver-Burk (1934).

Enzyme activity on substrate analogues - All substrate analogues used were diluted with 10 mM acetate buffer to a final concentration of equivalent of ester bonds so that the reaction mixture contained 50 mM of gallic acid equivalents in the substrates (Iibuchi et al., 1972). Aleppo-tannic acid was obtained from Mallinckrodt Chemical, Co. St. Louis, MO. Hamamelitannin was extracted from witchazel and its purity was spectrophotometrically determined. Castalagin, vescalagin, 3,4,6-trigalloyl, 2,3,4,6-tetragalloyl and 1,2,3,4,6-pentagalloyl glucose were a generous gift from Dr. A. Scalbert (Laboratoire de Chimie Biologique ,INRA, Thiverval-Grignon, France). The enzyme solution was added to initiate the reaction and the hydrolysis of the different substrates was followed titrimetrically as described for substrate specificities.

Relation between the spectrophotometric enzyme activity method and the titration method - To determine the relation between the two methods, an experiment was conducted using methyl gallate as the substrate. For the titration method, the reaction was started by the addition of the purified enzyme and the amount of 10 mM NaOH used was recorded at different time periods. The reaction was followed for 20 min with the autotitrator using the procedure outlined above. For the spectrophotometric method, the procedure used was as previously described. The enzyme reaction was stopped by the addition of 99 % methanol at different time periods (2-20 min) and the absorbance at 310 nm determined with the spectrophotometer.

Enzyme inhibition experiments - The tannase inhibition studies were done using gallic acid, castalagin, and vescalagin as possible inhibitors. Gallic acid was tested at two concentrations: 50 mM and 25 mM. The substrates used and their final concentration in the reaction mixture were as indicated for the substrate relationships experiments. The hydrolysis of the substrates by tannase at pH 5.5 and 30 C was measured titrimetrically in the presence of gallic acid. To determine type of inhibition, the linear transform method of Lineweaver-Burk (1934) was used. Inhibition constants (K_i) were calculated using Pennzyme.

For other tests, castalagin and vescalagin were used as inhibitors and hamamelitannin was used as a substrate. The final concentration of the inhibitors and the substrate in the reaction mixture was 50 mM. The procedure used was similar to that described above.

RESULTS

Purification of tannase

The tannase was isolated from mycelia of the fungus E. parasitica. A typical purification table is shown in Table 3.1. The substrate methyl gallate was used to monitor tannase activity throughout the purification procedure. The enzyme showed two activity peaks when separated by FPLC anion exchange chromatography (Fig. 3.1). The peaks eluted very close together between 340 and 380 mM NaCL and were combined and subsequently purified to homogeneity by FPLC gel permeation (Fig. 3.2). The enzyme was purified 142-fold with a 10 % yield. The final preparation had a specific activity of 170 units/mg protein (Table 3.1).

Table 3.1. Purification of the E. parasitica tannase.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mgP)	Recovery (%)	Purification factor
Crude	1300	1540	1.2	100	1
Ammonium sulfate	45	840	18.6	54	16
Mono Q	3	306	102	20	85
Superose	0.9	153	170	10	142

Tannase was purified as described in Materials and Methods. Activity was measured spectrophotometrically using methyl gallate as substrate at pH 5.5 and 30 C. One unit = 1 μmol methyl gallate hydrolyzed per min. Mono Q = FLPC anion exchange chromatography. Superose = FPLC gel permeation column

Figure 3.1. Mono Q protein and activity profiles of the tannase from *E. parasitica*. After homogenization, proteins from the mycelia were precipitated with 30-80 % ammonium sulfate, dissolved in 10 vol of 10 mM acetate buffer and desalted by dialysis. The desalted preparation was applied to a FPLC Mono Q preparative column and eluted with a NaCl gradient in 20 mM Tris-HCl buffer, pH 7.5, as described under Materials and Methods. Fractions of 1 ml were collected and assayed for methyl gallate-hydrolysing activity. Arrow A indicates the point of washing with the equilibrating buffer. Arrow B indicates the point of initiation of a linear salt gradient of 0 to 1.0 M NaCl in the equilibration buffer. Protein concentration is indicated as absorbance at 280 nm, tannase activity is reported as units per ml. One unit = 1 μmol methyl gallate hydrolyzed per min.

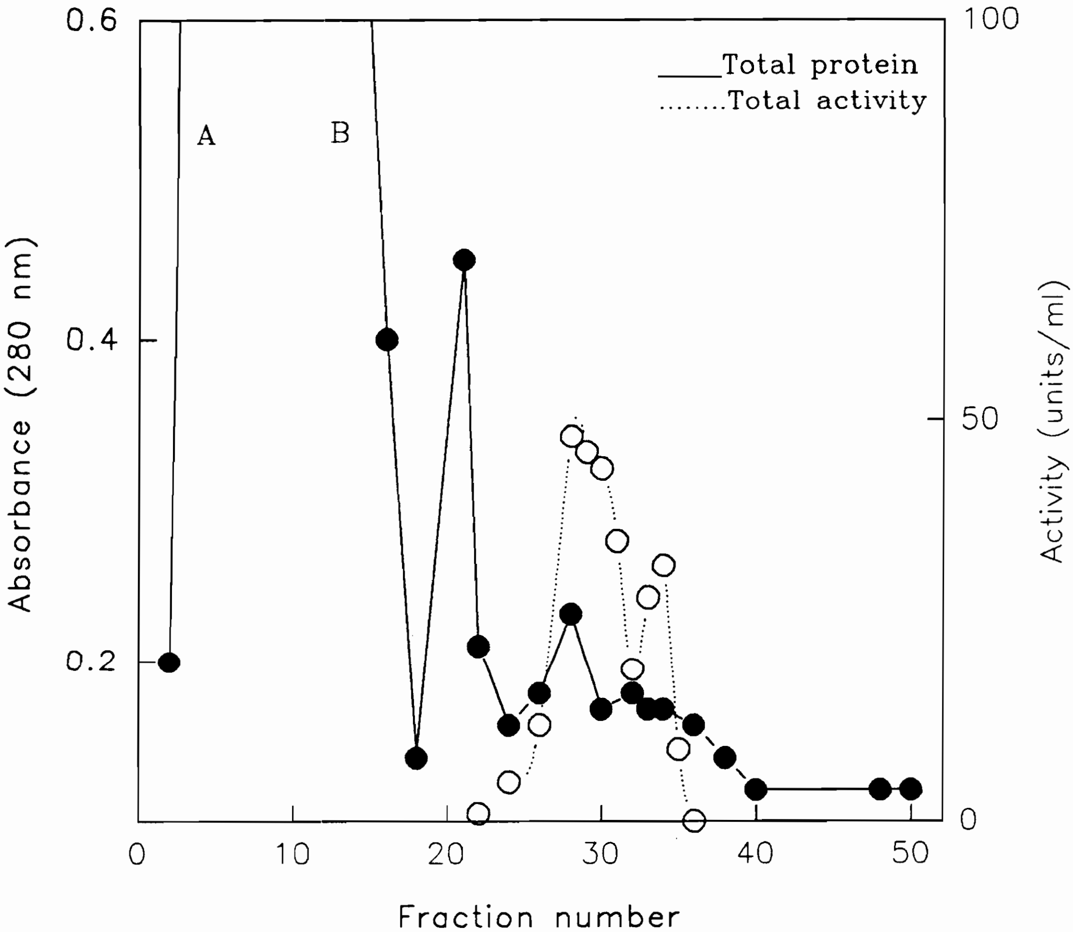
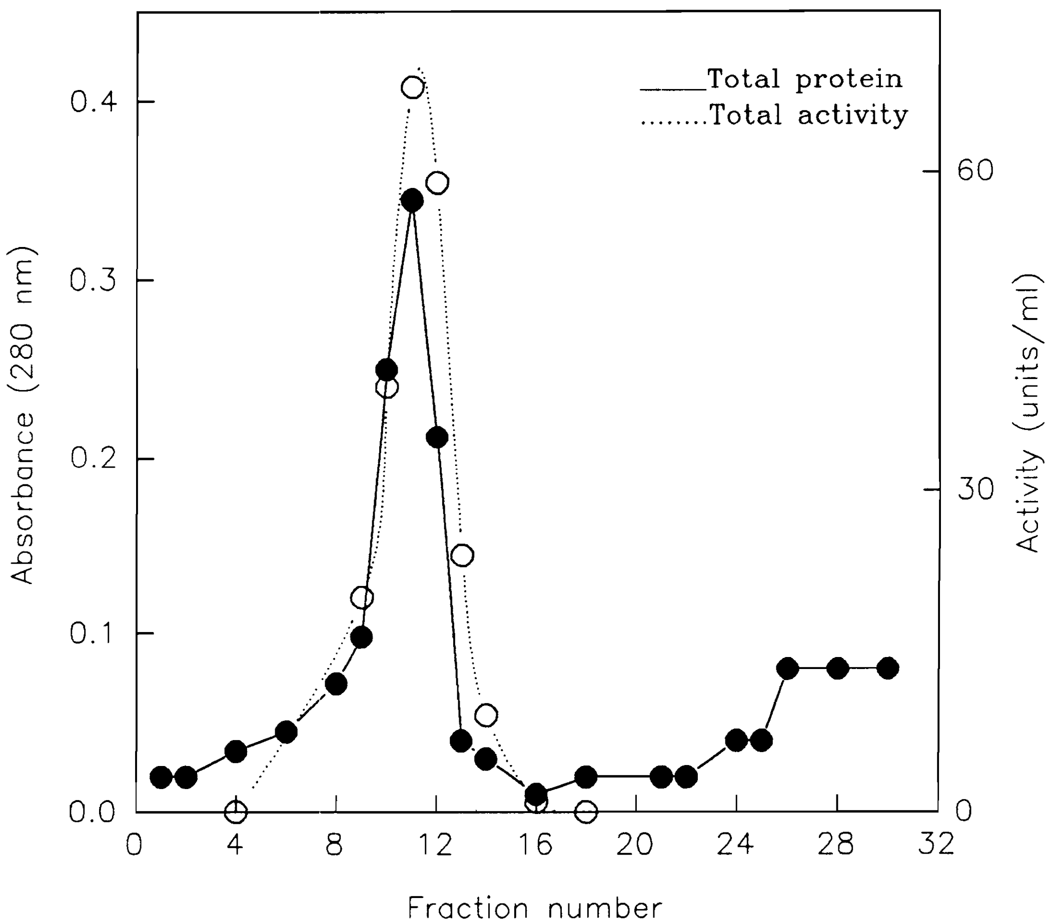
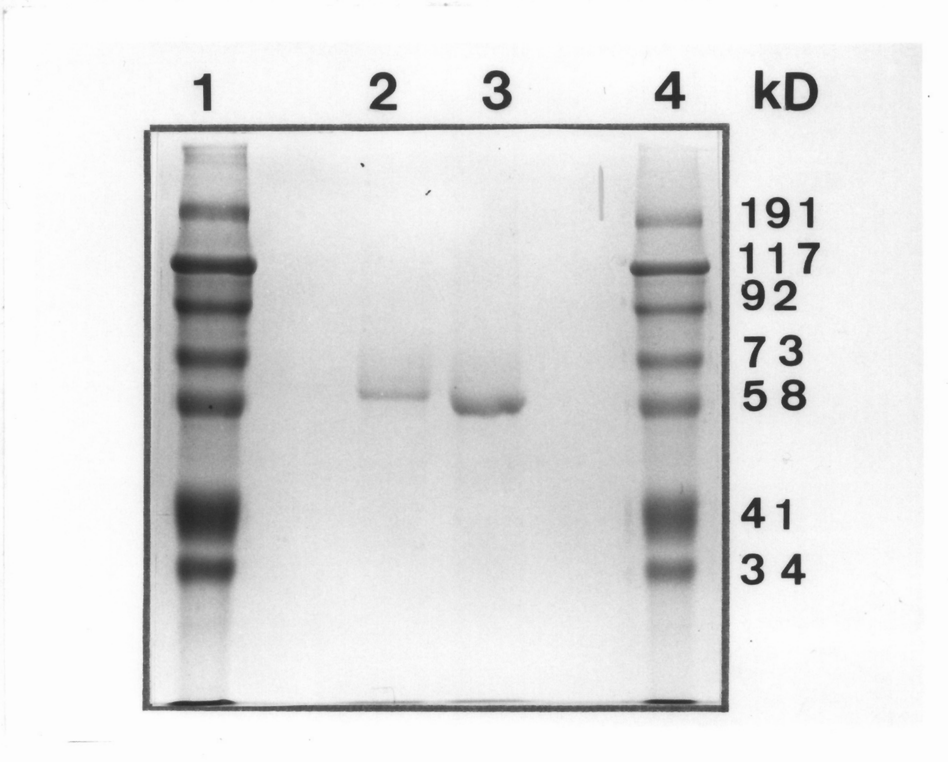

0.6
0.4
0.2
100
50
0
Absorbance (280 nm)
Activity (units/ml)
A
B
____Total protein
........Total activity
0
10
20
30
40
50
Fraction number

Figure 3.2. Superose 12 protein and activity profile of the tannase from E. parasitica. The active fractions from Mono Q chromatography were pooled and concentrated to 0.5 ml. The solutions were loaded onto a Superose 12 FPLC column and eluted with a 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 % NaCl. Fractions of 1 ml were collected and assayed spectrophotometrically for tannase activity. Protein concentration is indicated as absorbance at 280 nm, tannase activity is reported as units per ml. One unit = 1 μmol methyl gallate hydrolyzed per min.



____Total protein
........Total activity
Absorbance (280 nm)
Activity (units/ml)
Fraction number
0.0
0.1
0.2
0.3
0.4
0
30
60
0
4
8
12
16
20
24
28
32

Figure 3.3. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) of purified tannase from E. parasitica. Purified preparations of tannase were subjected to SDS-PAGE in the presence and absence of ß-mercaptoethanol. The purified protein (20 μg) was boiled in sample buffer containing 1 % SDS with and without 0.1 % ß-mercaptoethanol. The protein was applied to a 10 % gel and separated at a constant current of 25 mA. The samples were then stained with Coomassie Brilliant Blue. The lanes contain (1) molecular weight markers, (2) tannase without ß-mercaptoethanol, (3) tannase with ß-mercaptoethanol, (4) molecular weight markers.


1
2
3
4
kD
191
117
92
73
58
41
34

Properties of the purified tannase

Electrophoresis - The enzyme was purified to electrophoretic homogeneity. When analyzed by SDS/PAGE, the purified enzyme migrated as a single protein band which corresponded with the band that showed tannase activity in the native gel.

Molecular weight of the tannase - The molecular mass of the purified enzyme was found to be about 240,000 (Fig. 3.4 A). The subunit molecular weight of the enzyme was determined with SDS/PAGE to be M 58,000 (Fig. 3.4 B). Samples with and without ß-mercaptoethanol showed a single band at a similar molecular molecular weight (Fig. 3.3).

Isoelectric focusing - The tannase was separated into six bands after isoelectric focusing. They were observed between pH 4.7 and 5.1 (Fig. 3.5).

Effect of pH

pH stability - As shown in Fig. 3.6, the tannase was stable in pH range of 4.0 to 7.5 for 12 hr.

Optimum pH - The optimum pH was found to be 5.5. Minimal activity was recorded at pH 2.5 and 8.5 (Fig. 3.7).

Effect of temperature

Stability - The enzyme was stable at temperatures under 30 C: the activity was lost completely at 50 C in 20 min and at 60 C in 10 min (Fig. 3.8).

The enzyme was routinely stored at 4 C in 10 mM acetate buffer, pH 5.5. Under these conditions, the tannase was stable for 3 months without apparent loss of activity.

Optimum temperature - The optimum temperature for the enzyme activity was 30 C. Good enzyme activity was observed between 20 and 45 C. The activity was low at 60 C (Fig. 3.9).

Hydrolysis of methylgallate - Titrimetrically determined, initial rates for methyl gallate hydrolysis by the tannase were directly proportional to the concentration of the enzyme (Fig 3.10).

Relationship between the spectrophotometric method and the titration method for enzyme activity determination - The relationship between tannase assays at 310 nm using the spectrophotometer and the amount of liberated gallic acid is shown in Fig. 3.11. It is apparent from the figure that the two curves are similar and that there is a close relation between the two methods for tannase activity determination.

Figure 3.4. Molecular weight determination of purified tannase from E. parasitica. A. Gel filtration on Superose 12. Molecular weight standards were: 1) apoferritin, 2) yeast alcohol dehydrogenase, 3) cytochrome c, 4)albumin, 5) carbonic anhydrase. B. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Molecular weight standards were: 1) macroglobulin, 2) ß-galactosidase, 3) fructose-6-P-kinase, 4) pyruvate kinase, 5) fumarase, 6) lactic dehydrogenase, 7) triose phosphate isomerase. The arrows indicate the values obtained for tannase.

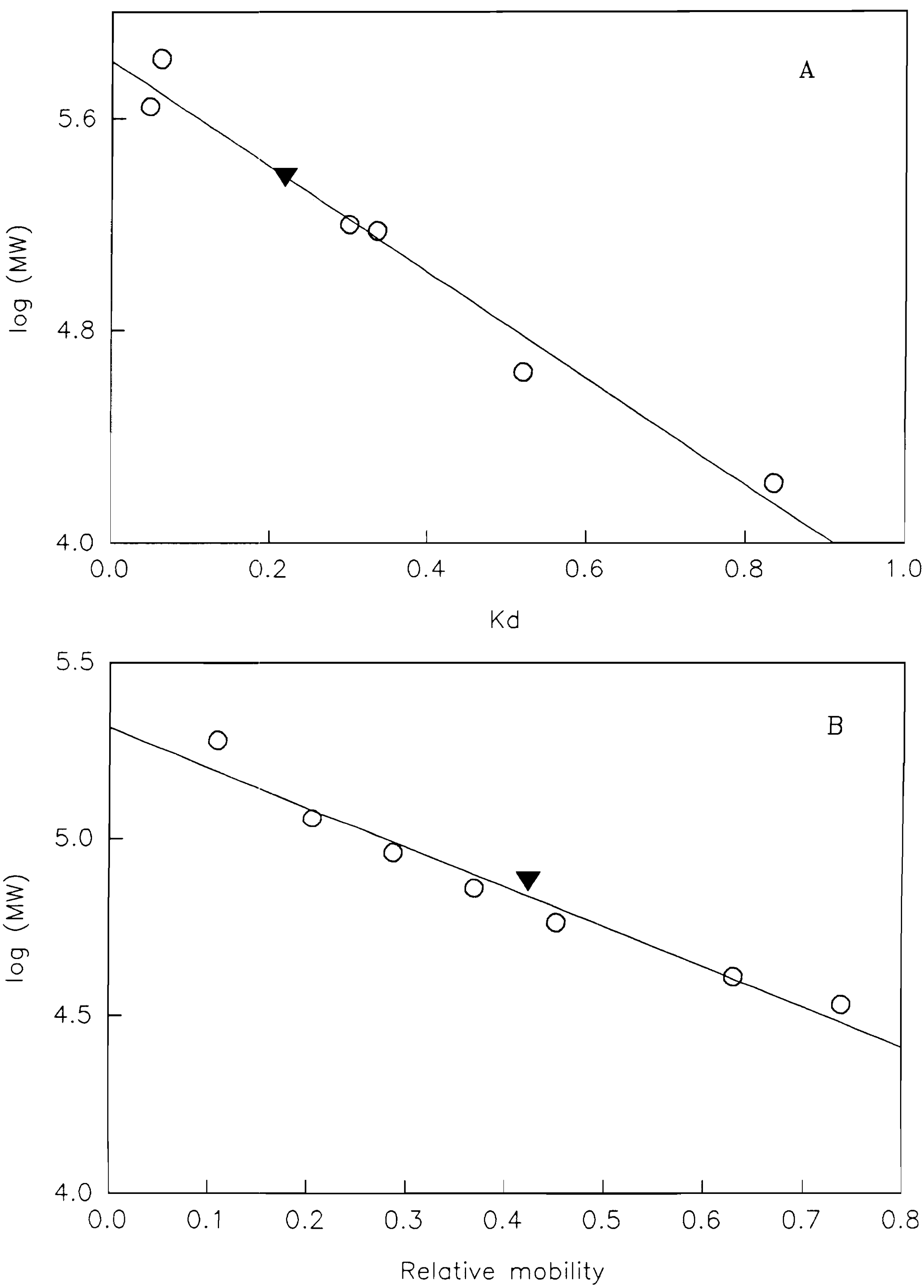

A
log (MW)
5.6
4.8
4.0
0.0
0.2
0.4
0.6
0.8
1.0
Kd
B
log (MW)
5.5
5.0
4.5
4.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Relative mobility

Figure 3.5. Isoelectric focusing of the purified tannase from *E. parasitica*. A. Isoelectric focussing (IEF) was performed with ampholytes 3 to 10 and 4 to 6. Gels (4 %) were prepared and run for 5 hr. The gels were stained with Coomasie Brilliant Blue. Lanes 1 and 2, pH standards. Lane 3, 20 μg of purified enzyme. B. Determination of pIs of the tannase. From the graphic representation (distance of migration vs observed pH), the values for the *E. parasitica* tannase (▲) were determined using linear regression analysis.

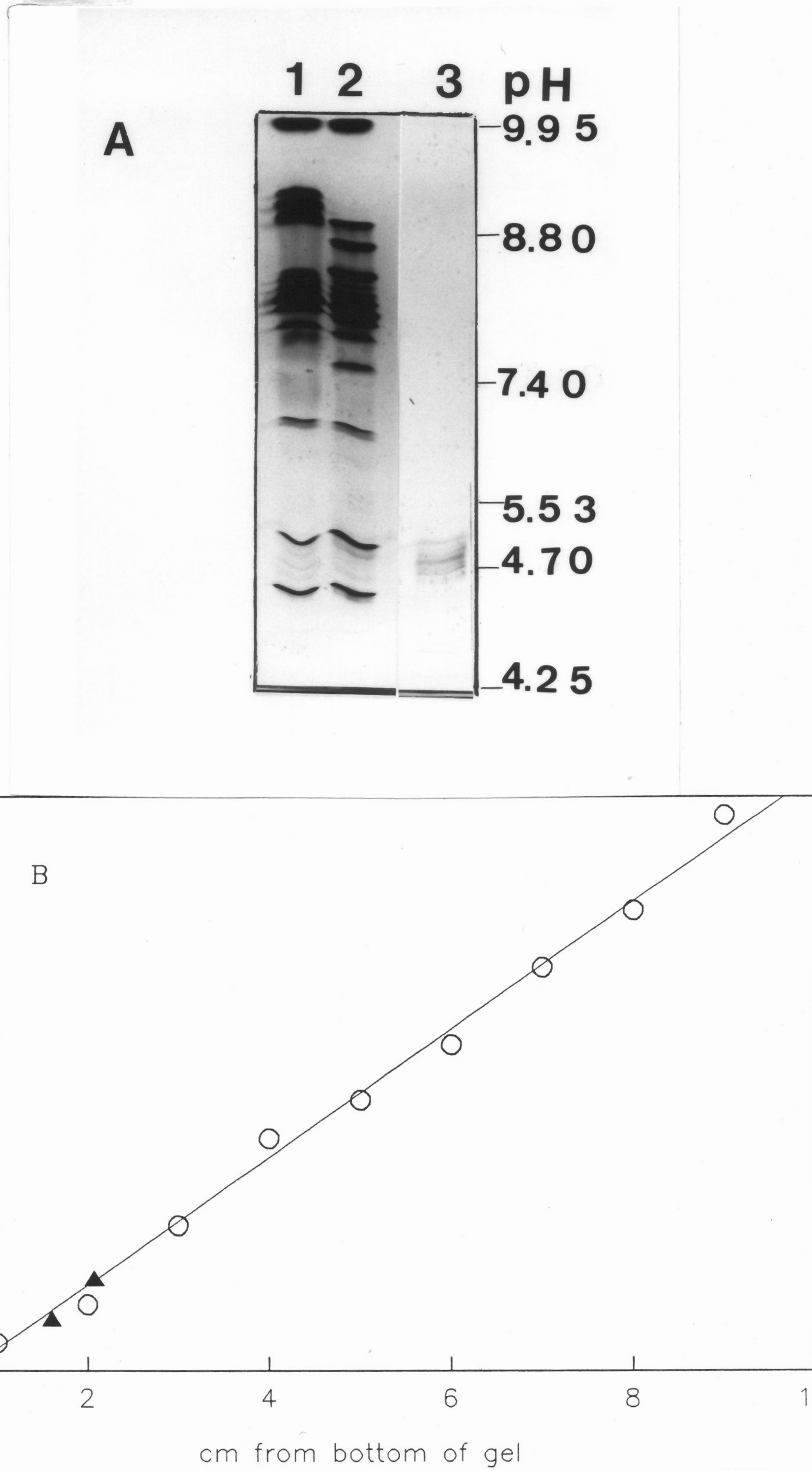

A
1 2 3 pH
9.95
8.80
7.40
5.53
4.70
4.25
B
pH
cm from bottom of gel
10
9
8
7
6
5
4
0
2
4
6
8
10

Figure 3.6. pH stability of purified tannase from *E. parasitica*. The tannase was maintained for 12 hr in the following buffers: 10 mM Glycine, 10 mM acetate, 10 mM citrate-phosphate, and 20 mM Tris. Relative activity in the figure is the remaining activity expressed as percentage of the activity before the treatment.

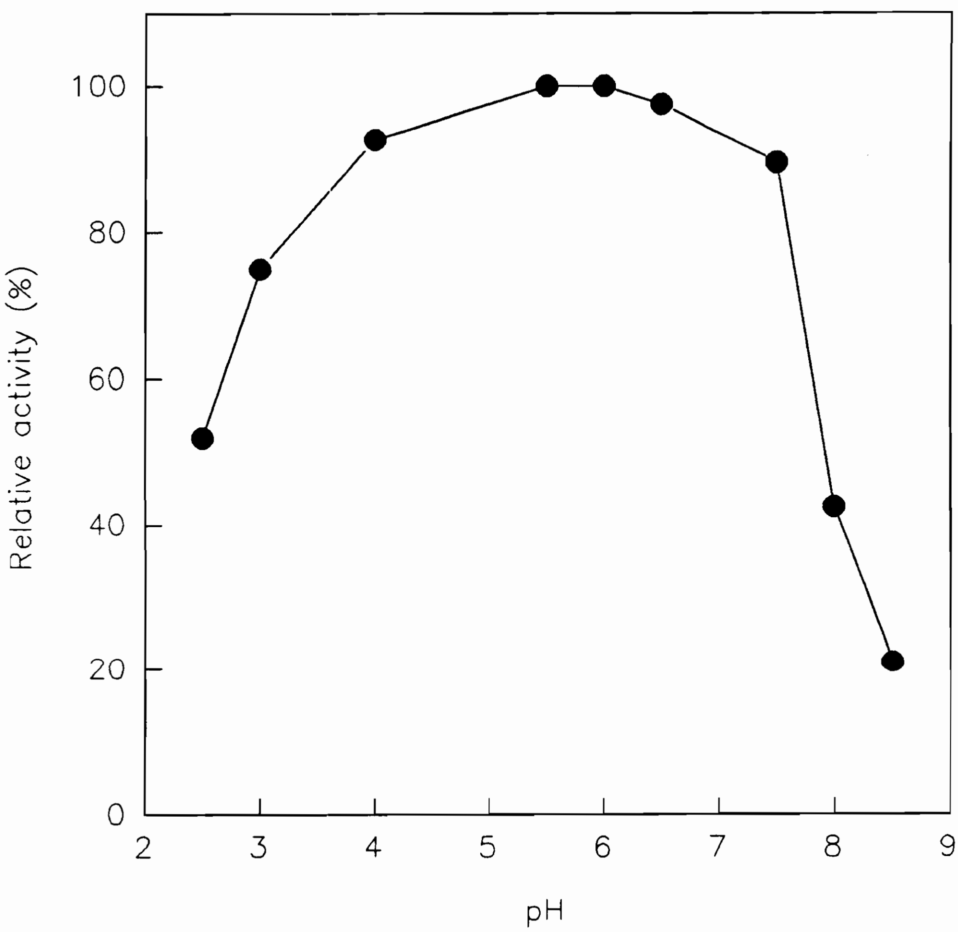
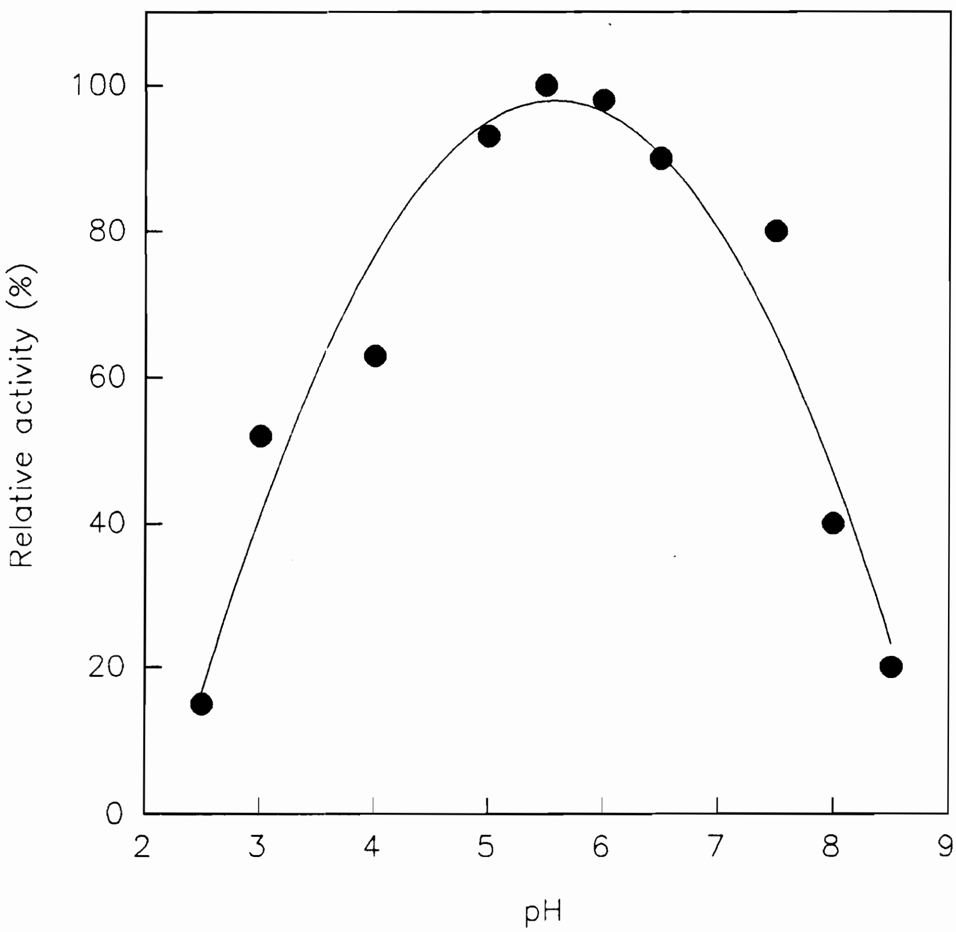

100
80
60
40
20
0
Relative activity (%)
2
3
4
5
6
7
8
9
pH

Figure 3.7. pH optimum of purified tannase from *E. parasitica*. The substrate methyl gallate was dissolved in buffer solutions of different pHs as indicated in fig. 3.6. Final concentration of the substrate in the reaction mixture (1 ml) was 20 mM. Reactions were initiated by addition of the purified enzyme to a final concentration of 5 μg/ml.



Relative activity (%)
0
20
40
60
80
100
2
3
4
5
6
7
8
9
pH

Figure 3.8. Temperature stability of purified tannase from <u>E. parasitica</u>. The incubation mixtures contained the purified enzyme 0.5 ml in 10 mM acetate buffer (pH 5.5). Samples were incubated at each temperature for 10 and 20 min and withdrawn for activity assayed with methyl gallate. Final concentration of methyl gallate in the reaction mixture (1 ml) was 20 mM and the enzyme concentration was 5 μg/ml.

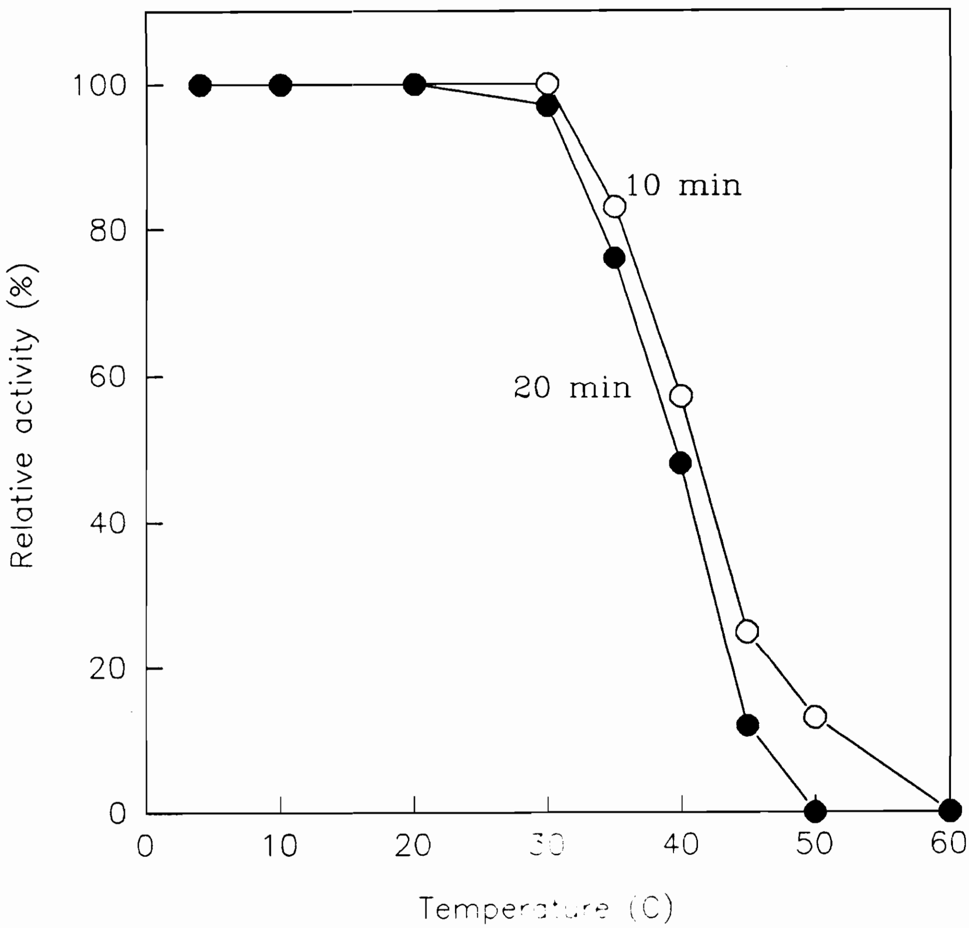

Relative activity (%)
100
80
60
40
20
0
10 min
20 min
0
10
20
30
40
50
60
Temperature (C)

Figure 3.9. Temperature optimum of purified tannase from *E. parasitica*. The substrate methyl gallate in 10 mM acetate buffer pH 5.5 at a final concentration of 20 mM was pre-equilibrated to the temperatures indicated above and then the purified enzyme was added to the reaction mixture (1 ml) at a final concentration of 5 μg/ml, in order to initiate the reaction. The activity was determined spectrophotometrically in a 10 min reaction.

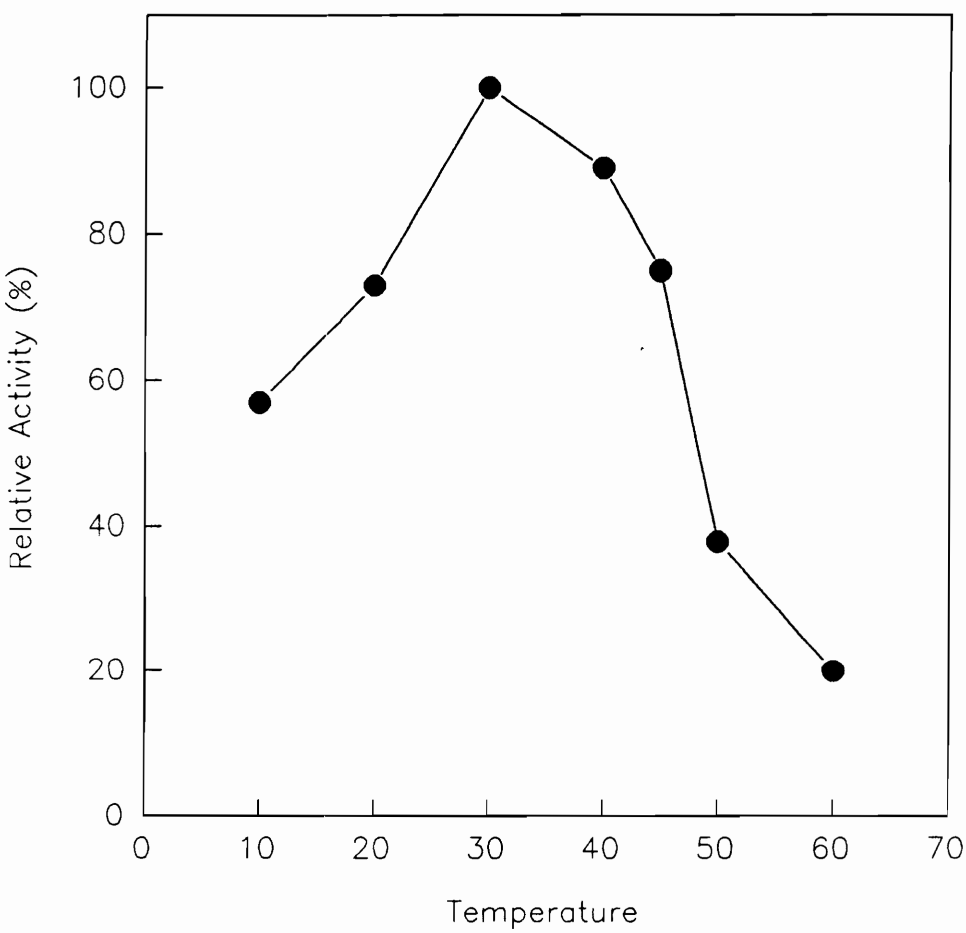
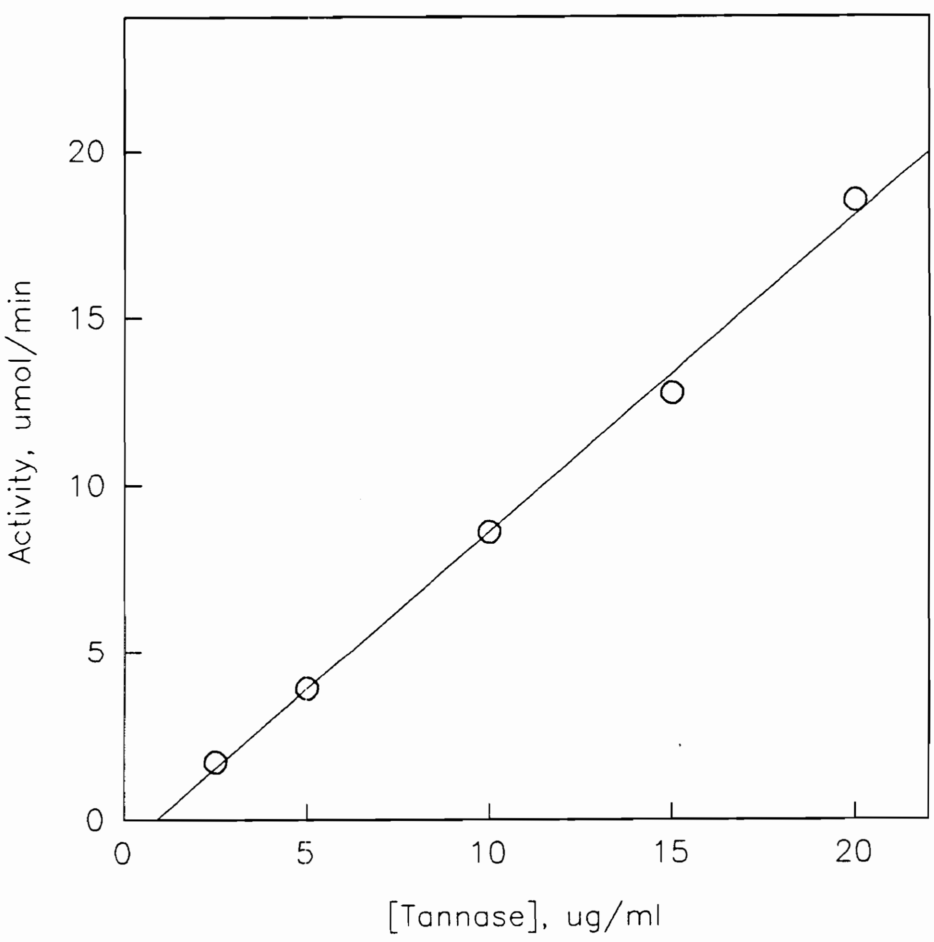
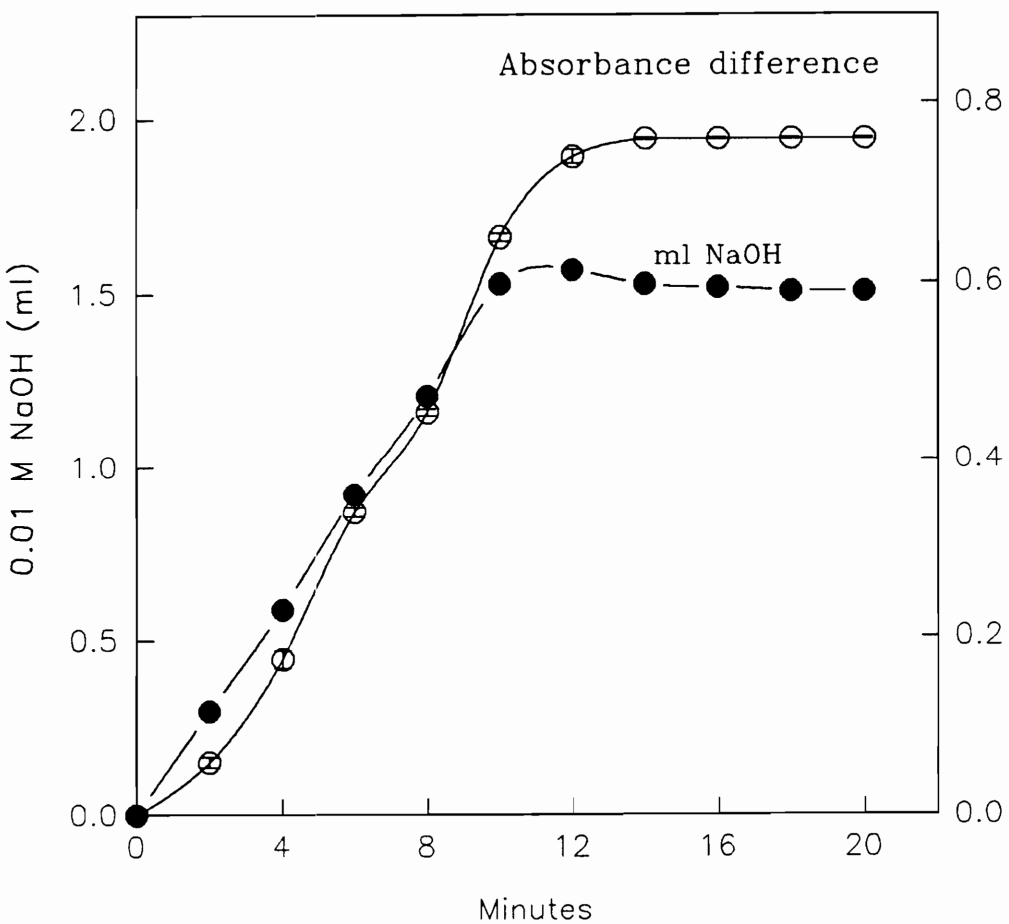

Relative Activity (%)
0
20
40
60
80
100
0
10
20
30
40
50
60
70
Temperature

Figure 3.10. Dependance of initial hydrolysis rates of methyl gallate on _E. parasitica_ tannase concentration. Various concentrations of the tannase diluted in 10 mM acetate buffer (pH 5.5) were assayed titrimetrically with methyl gallate as substrate. Final concentration of methyl gallate in the reaction mixture (1 ml) was 20 mM.



Activity, umol/min
20
15
10
5
0
0
5
10
15
20
[Tannase], ug/ml

Figure 3.11. Relation between spectrophotometric and titration methods of E. parasitica tannase activity determination. For the titration method, the reaction was started by the addition of the purified enzyme and the amount of 10 mM NaOH used was recorded at different time periods. The reaction was followed for 20 min with the autotitrator, using the procedure outlined in Materials and Methods. For the spectrophotometric method, the enzyme reaction was initiated with the addition of the enzyme and was stopped by the addition of 90 % methanol at different time periods (2-20 min) and the change in absorbance at 310 nm was determined, using the procedure described in Materials and Methods.


Absorbance difference
ml NaOH
0.01 M NaOH (ml)
Absorbance difference
Minutes
0.0
0.5
1.0
1.5
2.0
0.0
0.2
0.4
0.6
0.8
0
4
8
12
16
20

Enzyme activity on substrate analogues - The activity of the tannase on substrate analogues at equivalent concentration of gallic acid are summarized in Table 3.2. The highest tannase activity was observed when the substrate was 1,2,3,4,6-pentagalloyl glucose. On aleppo-tannic acid, the enzyme activity was slightly lower than that observed for pentagalloyl glucose. Methyl gallate and hamamelitannin were good substrates for the enzyme. In contrast, the ellagitannins vescalagin and castalagin were very poor substrates and the hydrolysis that occurred was minimal. The other substrates tested, 2,4,6-trigalloyl, and 1,2,4,6-tetragalloyl glucose, were good substrates for the tannase.

Substrate relationships of _E_. _parasitica_ tannase

Kinetics of methyl gallate hydrolysis - Titrimetrically determined initial rates for methyl gallate hydrolysis were directly proportional to tannase concentration. (Fig. 3.12 A) In a typical assay at 30 C, with a substrate concentration of 50 mM, a 10-min reaction time was sufficient to assay the tannase at concentration as low as 5 μg/ml.

Values for K_m were determined for methyl gallate by titrimetrically measuring initial velocities at varying substrate concentration. Graphics analysis (Fig. 3.12 A inset) yield values of K_m = 7.1 mM and V_{max} = 10.3 μm/min. These values were similar to those determined with Pennzyme. The value obtained with this program for K_m was 7.49 ± 0.61 mM and for V_{max} was 11.12 ± 0.42 μm/min.

The variation of initial reaction velocity with methyl gallate concentration yielded a hyperbolic curve (Fig. 3.12 A) consistent with simple Michaelis-Menten-type kinetics.

When gallic acid was tested as a potential inhibitor of methyl gallate, the inhibition constant was calculated with Pennzyme to be 11.0 mM ± 0.18 (Table 3.5). The mechanism of inhibition was found to be competitive. (Fig. 3.12 B)

Kinetics of Hamamelitannin hydrolysis - Values for K_m for tannase hydrolysis of hammamelitannin were determined as described for methyl gallate. Graphic analysis (Fig. 3.13 A inset) yielded values of K_m = 6.01 mM and V_{max} = 12.5 μm/min. The values calculated with Pennzyme were 5.07 ± 0.80 mM and 12.41 ± 0.84 μm/min, respectively.

The variation of initial reaction velocity with hamamelitannin concentration yielded a hyperbolic curve (Fig. 3.13 A) consistent with simple Michaelis-Menten-type kinetics.

Gallic acid was demonstrated to be an effective inhibitor of the tannase when hamamelitannin was used as a substrate. The inhibition constant was calculated with Pennzyme to be 13.09 ± 0.5 mM (Table 3.5). Both concentrations used were inhibitory (Table 3.4) and the type of inhibition was competitive (Fig. 3.13 B).

When the ellagitannins, vescalagin and castalagin, were used as possible inhibitors of the E. parasitica tannase, no inhibitory effect was observed with castalagin and a very low inhibition was recorded for vescalagin (Table 3.4).

Kinetics of Aleppo tannic acid hydrolysis - Values for K_m and V_{max} were determined as indicated for the other two substrates. Graphic analysis gave values of K_m = 1.31 mM and V_{max} = 12.3 µm/min (Fig 3.14 A inset). The calculated values with Pennzyme were 0.94 ± 0.12 mM and 10.50 ± 0.61 µm/min, respectively.

The variation of initial reaction velocity with aleppo-tannic acid concentration yielded a hyperbolic curve (Fig. 3.14 A) consistent with simple Michaelis-Menten-type kinetics.

The inhibitory effect of gallic acid at 25 mM and 50 mM is shown in Fig. 3.14 B. When tannic acid was used as substrate, gallic acid was a competitive inhibitor of the enzyme tannase (Fig 3.14 B) and the inhibition constant was calculated with Pennzyme to be 12.7 mM ± 0.05 (Table 3.5).

A summary of the kinetic parameters (calculated with Pennzyme) for the three compounds that functioned as substrates are shown in Table 3.3.

Table 3.2. Relative activities of *E. parasitica* tannase on substrate analogues

Substrate analogue	Enzyme activity (units/ml)	Relative activity (%)
1,2,3,4,6 pentagalloyl glucose	2.50±0.1[a]	100
Aleppo tannic acid	2.25±0.02	90
2,4,6 trigalloyl glucose	2.00±0.1	80
Hamamelitannin	1.90±0.04	76
Methyl gallate	1.85±0.05	74
1,2,4,6 tetragalloyl glucose	1.60±0.06	64
Vescalagin	0.21±0.09	8
Castalagin	0.10±0.002	4

Reaction mixture contained 50 mM gallic acid equivalents in the substrates. Purified tannase was added to initiate the reaction and the hydrolysis of different substrate was followed titrimetrically for 10 min. One unit = 1 μmol methyl gallate hydrolyzed per min.

[a] Values are an average of three replicates and are mean ± standard deviation.

Table 3.3. Michaelis constants and maximum velocities for three substrates of E. parasitica tannase.

Substrate	**K_m (mM)**	**V_{max} (units/ml)**	**V_{max}/K_m**
Aleppo tannic acid	0.94± 0.12[a]	10.50± 0.61	11.20
Hamamelitannin	5.07± 0.80	12.41± 0.84	2.44
Methyl gallate	7.49± 0.61	11.12± 0.42	1.48

Values for Km were determined by titrimetrically measuring initial velocity at varying substrate concentrations. One unit = 1 μmol methyl gallate hydrolyzed per min.

Values were calculated using Pennzyme and are the average of three replicates.

[a] Mean value ± standard deviation

Table 3.4. Degree of inhibition of E. parasitica tannase activity by different compounds using hamamelitannin as a substrate

Potential inhibitor	% Inhibition
25 mM Gallic acid	15
50 mM Gallic acid	24
50 mM Castalagin	0
50 mM Vescalagin	1

Initial rate of hydrolysis of hamamelitannin by the tannase at pH 5.5 and 30 C was measured titrimetrically in the presence of the inhibitors. Final concentration of hamamelitannin in the reaction mixture was 50 mM. Values are the average of three replicates.

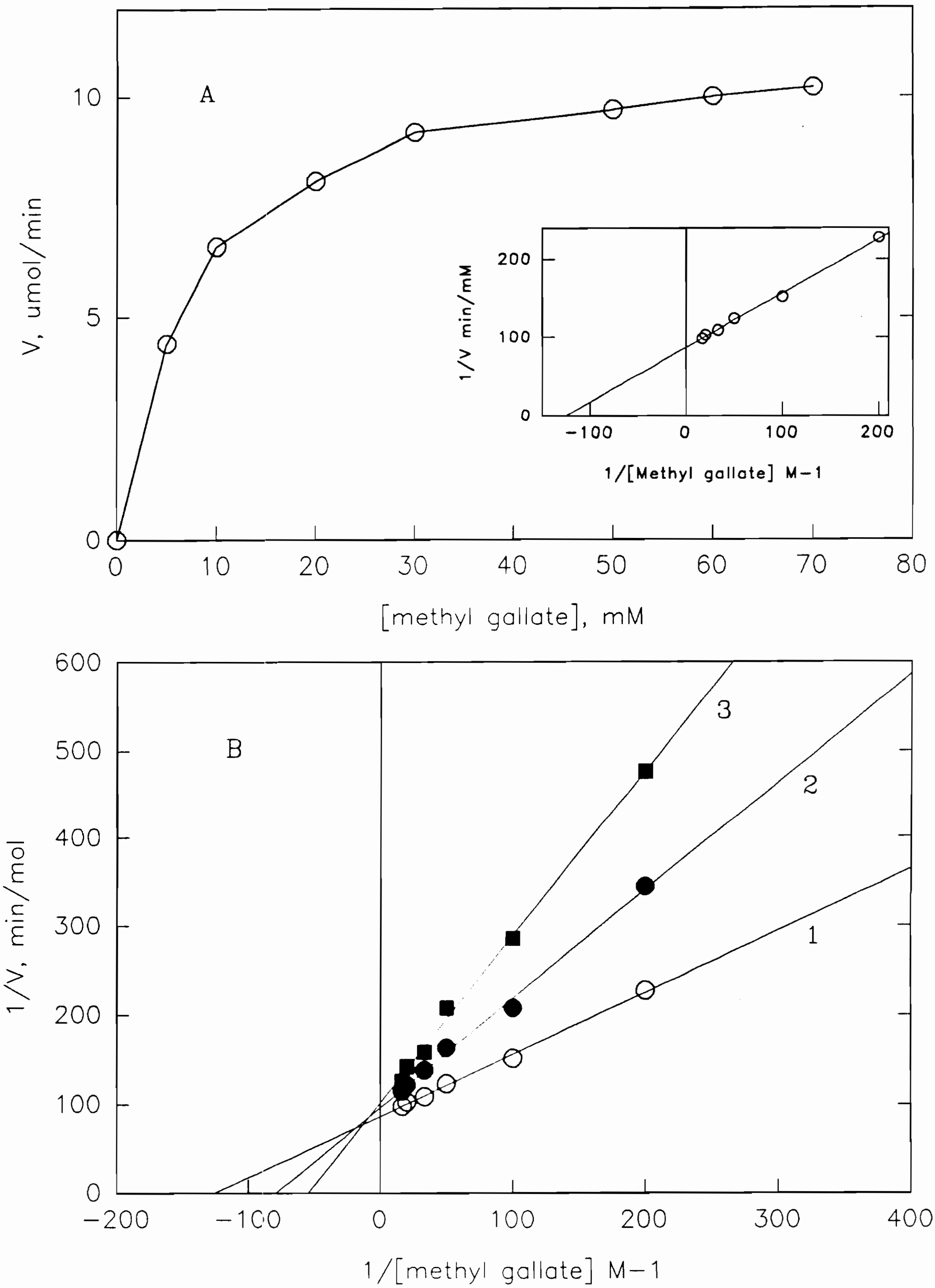
Table 3.5. Inhibition of *E. parasitica* tannase activity by two concentrations of gallic acid

Inhibitor	**Substrate**	**K_i (mM)**
Gallic acid	Aleppo tannic acid	12.7± 0.05[a]
Gallic acid	Methyl gallate	11.0± 0.18
Gallic acid	Hamamelitannin	13.1± 0.50

Final concentrations of gallic acid in the reaction mixture were 25 and 50 mM. The assay of tannase activity was initiated by the addition of purified enzyme and followed titrimetrically for 10 min at pH 5.5 and 30 C. The average values were calculated using Pennzyme for three replicates.

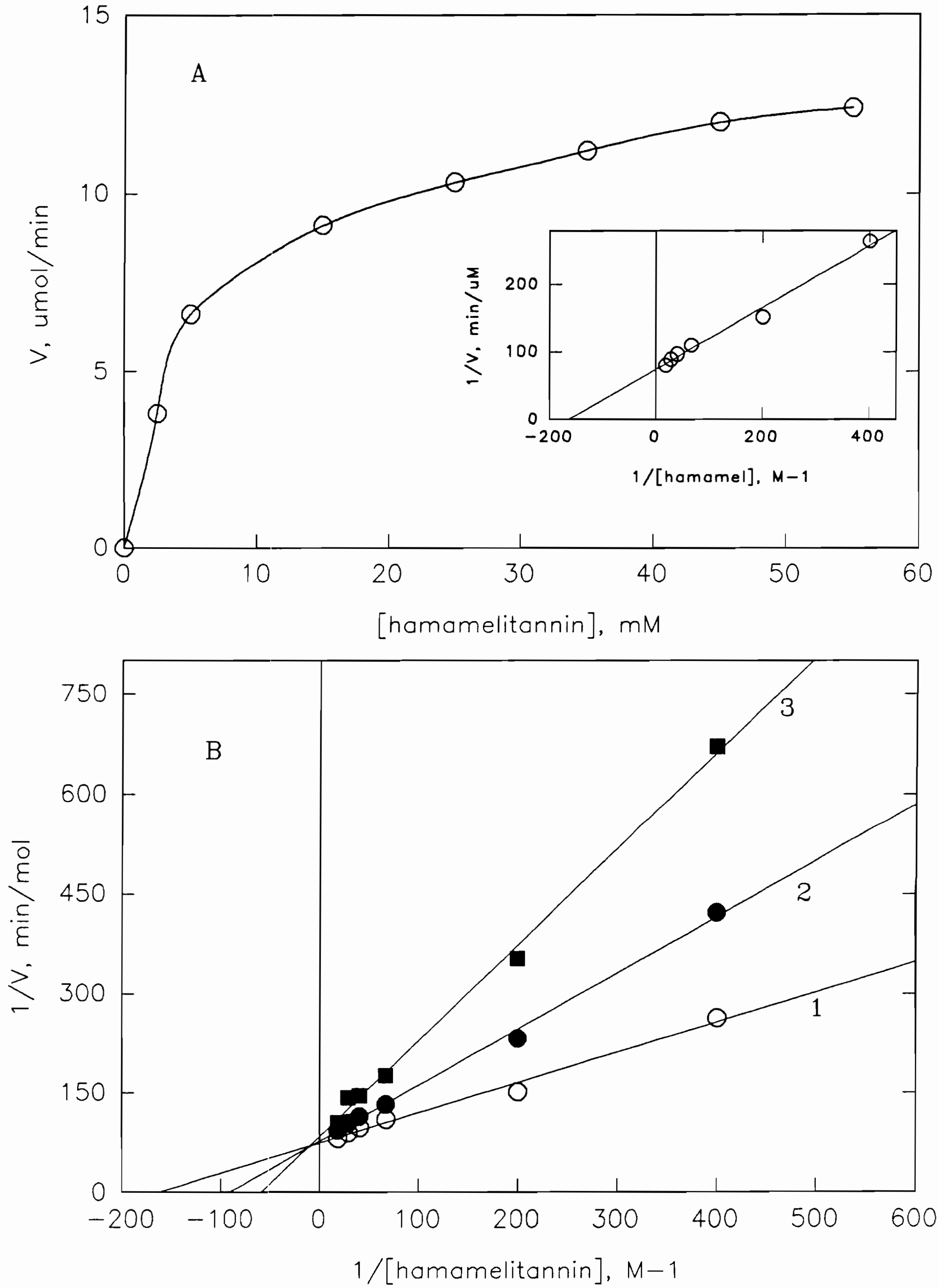
[a] Mean values ± standard deviation

Figure 3.12. Kinetics of methyl gallate hydrolysis and *E. parasitica* tannase inhibition by gallic acid. A. Dependence of initial velocity of hydrolysis on methyl gallate concentration. Initial velocities were determined titrimetrically. Reactions were at 30 C in 10 mM acetate buffer (pH 5.5). Concentration of tannase in the reaction solution was 10 μg/ml. Inset: Lineweaver-Burk double reciprocal plot of the same data. B. Effect of gallic acid concentration on tannase initial velocity. The final concentrations of methyl gallate in reaction mixture (2.5 ml) were 5, 10, 20, 30, 50, 60, and 70 mM at three concentrations of gallic acid. Final enzyme concentration was 10 μg/ml. The concentrations of gallic acid were: line 1, none; line 2, 25 mM, and line 3, 50 mM. The lines were drawn by linear least-squares regression analysis.



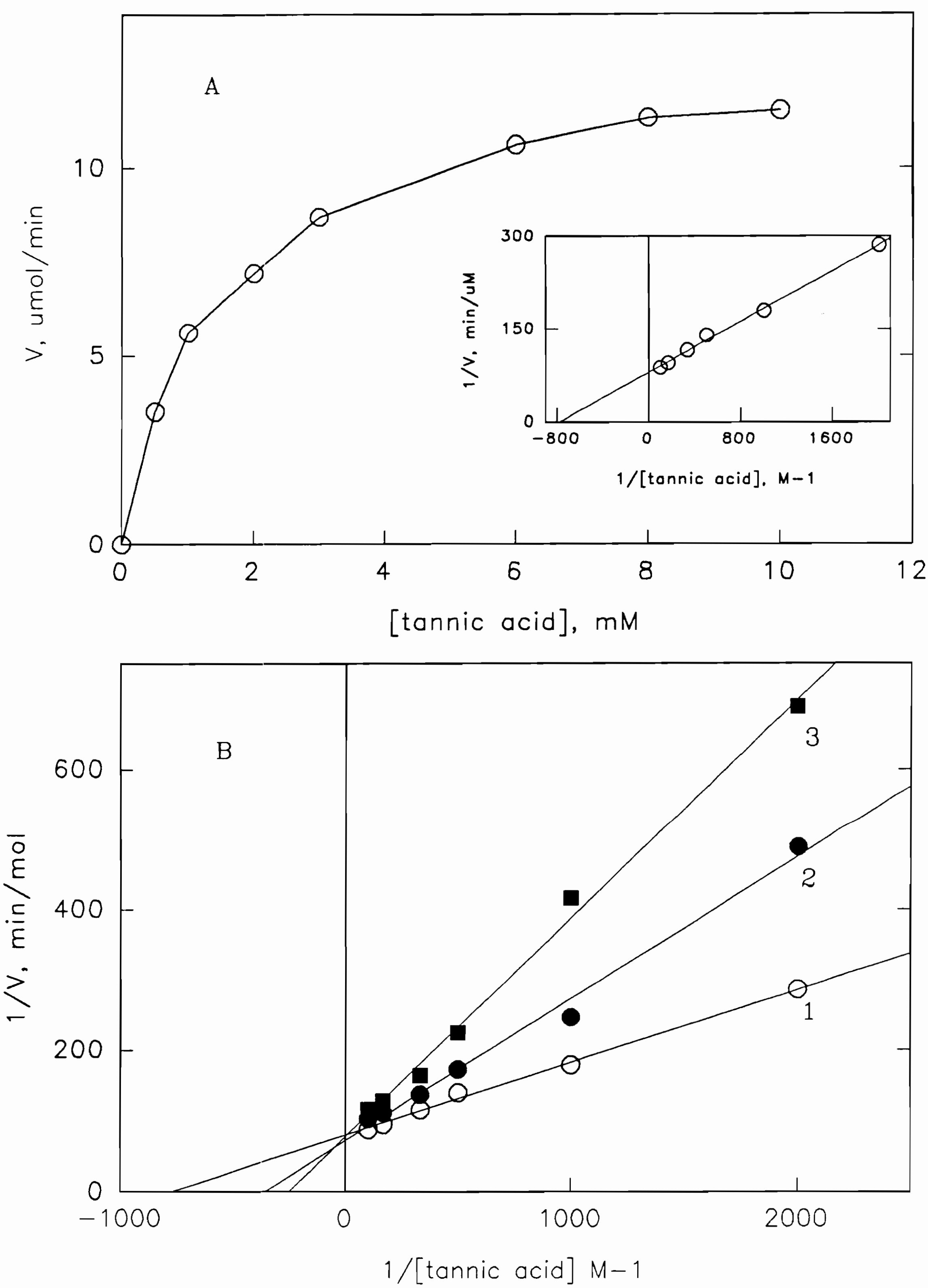
A
V, umol/min
[methyl gallate], mM
1/V min/mM
1/[Methyl gallate] M-1
B
1/V, min/mol
1/[methyl gallate] M-1
1
2
3

Figure 3.13. Kinetics of hamamelitannin hydrolysis and *E. parasitica* tannase inhibition by gallic acid. A. Dependence of initial velocity of hydrolysis on hamamelitannin concentration. Initial velocities were determined titrimetrically. Reactions were at 30 C in 10 mM acetate buffer (pH 5.5). Concentration of purified tannase in the reaction solution was 10 μg/ml. Inset: Lineweaver-Burk double reciprocal plot of the same data. B. Effect of gallic acid concentration on tannase initial velocity. The final concentrations of hamamelitannin in the reaction mixture (2.5 ml) were 2.5, 5, 15, 25, 35, 45, and 55 mM at three concentrations of gallic acid. Final enzyme concentration was 10 μg/ml. The concentrations of gallic acid were: line 1, none; line 2, 25 mM, and line 3, 50 mM. The lines were drawn by linear least-squares regression analysis.



A
V, umol/min
[hamamelitannin], mM
1/V, min/uM
1/[hamamel], M-1
B
1/V, min/mol
1/[hamamelitannin], M-1
1
2
3

Figure 3.14. Kinetics of aleppo tannic acid hydrolysis and tannase inhibition by gallic acid. A. Dependence of initial velocity of hydrolysis on aleppo-tannic acid concentration. Initial velocities were determined titrimetrically. Reactions were at 30 C in 10 mM acetate buffer (pH 5.5). Concentration of tannase in the reaction solution was 10 μg/ml. Inset: Lineweaver-Burk double reciprocal plot of the same data. B. Effect of gallic acid concentration on tannase initial velocity. The final concentrations of tannic acid in the reaction mixture (2.5 ml) were 0.5, 1, 2, 3, 6, 8 and 10 mM at three concentrations of gallic acid. Final enzyme concentration was 10 μg/ml. The concentrations of gallic acid were: line 1, none; line 2, 25 mM, and line 3, 50 mM. The lines were drawn by linear least-squares regression analysis.


A
V, umol/min
0
5
10
0
2
4
6
8
10
12
[tannic acid], mM
1/V, min/uM
300
150
0
−800
0
800
1600
1/[tannic acid], M−1
B
1/V, min/mol
600
400
200
0
−1000
0
1000
2000
1/[tannic acid] M−1
3
2
1

DISCUSSION

The tannase from _E_. _parasitica_ was purified to electrophoretic homogeneity through a four-step procedure involving salt fractionation, anion exchange chromatography, and gel filtration. Several other fungal tannases have been purified using DEAE Sephadex as an anion exchanger and Sephadex (G 100 and G200) for gel filtration, both packed in open columns (Iibuchi et al., 1968; Rajakumar and Nandy, 1983, Yamada et al., 1968). The procedure used for purification of the _E_. _parasitica_ tannase was quicker (2 days) than the previous procedure (5 days) due to the use of FPLC chromatography. The enzyme was purified 142-fold with a 10 % yield and a specific activity of 170 μmoles of methyl gallate hydrolyzed per minute per mg of protein. The specific activities of other purified fungal tannases show a wide variation in values from a high value of 135,000 for _A_. _flavus_ (Adachi et al., 1968), an intermediate value of 904 for _A_. _niger_ (Dhar and Bose, 1963) to lower values of 78 for _Aspergillus_ _oryzae_ (Iibuchi et al., 1968), and 86 for _P_. _chrysogenum_ (Rajakumar and Nandy, 1983). The values obtained for _E_. _parasitica_ tannase were similar to the latter.

The native molecular weight of the purified tannase was determined by Superose-12 (FPLC) chromatography. The enzyme was eluted from the column with an apparent molecular weight of 240,000. Most other fungal tannases have molecular weights around 200,000 (Iibuchi et al., 1968; Yamada et al., 1968); however, for yeast tannases, a molecular weight of 250,000 was reported (Aoki et al., 1976). The tannase from E. parasitica was found to be larger than most other fungal tannases. Under denaturing conditions of SDS-PAGE gel electrophoresis, a subunit molecular weight of 58,000 was observed in the presence and in the absence of ß-mercaptoethanol, suggesting that the enzyme was denatured by the treatment with SDS and dissociated into four subunits. Since mercaptoethanol reduces S-S covalent bonds, we assume that tannase subunits are not linked by covalent bonds. Although some literature on yeast tannases suggests a dimeric structure (Aoki et al., 1976), our results are more consistent with an enzyme with a tetrameric structure.

The purified enzyme from E. parasitica showed a broad pH dependence with optimum activity at 5.5 and 70 % of its activity ranging between 4.0 to 7.5. The optimum pHs for tannase activity were reported 5.0 to 6.0 for P. chrysogenum tannase (Rajakumar and Nandy, 1983); 5.0 to 5.5 in case of the enzyme isolated from A. flavus (Adachi et al.,1968), 5.5 for tannase from A. oryzae (Iibuchi et al., 1968), and 3.5 to 7.5 for yeast tannase (Aoki et al., 1976). The values reported here for E. parasitica tannase are similar to those reported for other fungal tannases.

The tannase from E. parasitica was stable within the pH range of 4.0 to 7.5. Tannase from P. chrysogenum (Rajakumar and Nandy, 1983) was found to be stable at pH range of 4.0 to 6.5 and the enzyme from A. flavus (Yamada et al., 1968) was stable within pHs 5.0 and 5.5. The values reported for A. oryzae tannase (Iibuchi et al., 1968), 3.0 to 7.5 for 12 hr, are similar to those obtained for the E. parasitica tannase.

The optimum temperature for activity of the purified tannase from E. parasitica was 30 C. The enzyme was stable up to 30 C with 50 % stability observed up to 40 C. Similar observations were reported for tannase from A. oryzae (Iibuchi et al. 1968). The tannase from P. chrysogenum (Rajakumar and Nandy, 1983) was stable up to 30 C with 95 % stability observed up to 45 C. The enzyme from E. parasitica was not very stable at high temperatures. In contrast, the tannase from A. niger had a temperature optimum for activity of 45 C and the critical inactivation temperature was found to be 80 C, indicating that the enzyme was quite stable to heating (Dhar and Bose, 1963).

Isoelectric focusing of the tannase from E. parasitica showed that the enzyme separated in six bands with isoelectric points in the range of pH from 4.6 to 5.1. The bands may represent isoenzymes or post-translational modifications. Working with the tannase of A. oryzae, Beverini and Metche (1990) reported the presence of seven isoenzymes with isoelectric points in the range 3.5 to 4.0. Haslam et al. (1961) reported that tannase is an acidic protein having isoelectric points around 5.0. The E. parasitica tannase showed its isoelectric points at pH values that are close to the value reported by Haslam et al. (1961).

The evaluation of the values of the Michaelis constants (K_m) for the three substrates suggests that aleppo tannic acid, with a K_m = 0.95 mM, is the best substrate for the enzyme. The V_{max}/K_m ratio was almost five times higher than for hamamelitannin and more than seven times higher than for methyl gallate. Hamamelitannin yielded a K_m value close to that for methyl gallate. The V_{max}/K_m ratio for hamamelitannin was 1.6-times higher than the value for methyl gallate. Similar behaviors were reported for the tannase from A. flavus (Yamada et al., 1968) toward different substrates. They used tannic acid, glucose-1-gallate and methyl gallate as substrates and, although the K_m values reported were lower than the values obtained for E. parasitica tannase, their ranking was similar with lowest K_m value for tannic acid (0.05 mM), followed by glucose-1-gallate (0.14 mM) and methyl gallate (0.86 mM). Beverini and Metche (1990), working with the tannase of A. oryzae, reported a K_m value of 6.2 mM for methyl gallate. This latter value is close to the one obtained for E. parasitica tannase. The differences reported for kinetic constants of tannase from different fungi are not surprising. As was indicated by Dhar and Bose (1963), microbial tannases have similar properties in many respects e.g., temperature-activity relationship, heat inactivation, and pH stability. He also suggested that tannases differed in many other respects e.g., enzyme-concentration-activity and substrate-concentration-activity relationships, and isoelectric points.

Iibuchi et al.(1972) found an increase of relative tannase activity with increase in the number of ester bonds for gallic acid esters of glucose when the substrates were used at equivalent ester bond concentration. Our results partially support these findings since aleppo tannic acid has seven ester bonds, hamamelitannin has two ester bonds, and methyl gallate one ester bond. Our data for tannase activity on substrate analogues supplied at equivalent ester bond concentration indicate that pentagalloyl glucose had a relative activity slightly higher than aleppo tannic acid. The composition of aleppo tannic acid corresponded to that of a ß-penta-m-digalloyl glucose with two gallic acids linked in form of a depside (Haslam et al., 1961). It was first suggested by Toth and Barsony (1943), later confirmed by Haslam and Stangroom (1966), and recently by Beverini and Metche (1990), that tannase from the fungus _A. oryzae_ has independent esterase and depsidase activities with different affinities for substrates containing ester bonds or depside groups. If we compared two substrates like aleppo tannic acid which has seven gallic acids and two of them linked in a form of a depside, with pentagalloyl glucose that has five gallic acids, with no depside groups, it may be possible that the tannase from E. parasitica could have a higher activity on the substrate containing only ester bonds with no depside groups. This possibility could explain its relatively lower activity for aleppo tannic acid than for pentagalloyl glucose. For the other substrates analogues, the relative activity generally decreased with decreasing number of ester bonds. The behavior of tetragalloyl glucose could not be explained and chemical confirmation of the tetragalloyl glucose sample used may be helpful.

When the ellagitannins, castalagin and vescalagin, were tested, very low relative tannase activities were observed at 30 C in a 10 min reaction. Several authors (Yoshida et al., 1985; Tanaka et al., 1986; Yoshida et al., 1989; Hatano et. al, 1989;) reported the use of the enzyme tannase to hydrolyze ellagitannins in order to analyze the products for structure determination of hydrolyzable tannins. Those ellagitannins had not only hexahydroxydiphenoyl (HHDP) groups but also galloyl groups in the molecule. The ellagitannins we used, castalagin and vescalagin, have only HHDP groups and no galloyl groups. As was indicated by Iibuchi et al. (1972), the tannase of A. oryzae showed no restriction on the substrate of the alcohol composing a substrate but that the acid must be gallic acid. The absence of galloyl groups in the molecules of castalagin and vescalagin may be the reason for the low tannase activity observed. Another observation regarding the use of tannase for hydrolysis of ellagitannins is that the reaction was allowed to continue for long periods of time, from 15 hr (Hatano et al., 1989), 49 hr (Yoshida et al., 1989) to 5 days (Yoshida et al., 1985). We tested the tannase hydrolysis of castalagin and vescalagin in a 10 min reaction. Haslam et al. (1961) suggested that when long reactions times are used, the liberated gallic acid may inhibit the enzyme or cause an alteration in the pH of the buffer.

The results of the kinetic studies are in general agreement with the _E. parasitica_ growth and tannase activity experimental data reported in Chapter 2. Hamamelitannin, a good substrate for the tannase, is absent in Chinese chestnut bark but present in American chestnut bark (Elkins et al., 1979; 1981). The presence of other galloyl esters in Chinese chestnut tannins (Hebard and Kaufman, 1978), especially, could explain the tannase activity observed on Chinese chestnut summer bark extracts as well as, in part, the good _E. parasitica_ growth that was reported for Chinese chestnut summer and winter bark extracts (Chapter 2). Our experiments indicated that galloyl esters such as trigalloyl-glucose and pentagalloyl-glucose were good substrates for the _E._

parasitica tannase.

Gallic acid was an effective inhibitor of the tannase of E. parasitica with all the substrates and concentrations tested. Haslam et al. (1961) indicated the gallic acid accumulation might inhibit tannase activity. The inhibition observed when hamamelitannin was used as the substrate may have an important biological consideration. Elkins found (Griffin and Elkins, 1986) that gallic acid accumulates in culture medium during hamamelitannin breakdown by E. parasitica. These levels not only could have the potential to kill chestnut tissue (Griffin and Elkins, 1986), but also could slow down or lower the activity of the tannase. The gallic acid could exert feedback control when the amount produced exceeds the needs of the organism or if gallic acid breakdown is very slow. With lower tannase activity (at constant tannase concentration), the fungus also may suffer from sub-optimal organic carbon supply and therefore, E. parasitica may need to turn on alternative metabolic pathways for organic carbon utilization, such as gallic acid utilization, or could produce more tannase. Gallic acid could be metabolized as an energy source. In preliminary experiments, using the autotitrator, we found that E. parasitica was able to metabolize gallic acid with acid generation from crude enzyme extracts. Elkins found (Griffin and Elkins, 1986) that gallic acid concentration began to decrease in the culture medium during hamamelitannin breakdown by E. parasitica after 3 days (Figure 1.1, Chapter 1). Several fungi and bacteria can utilize gallic acid as a carbon source and the pathway of gallic acid degradation has been determined. Aspergillus strain 64 converted gallic acid into pyrogallol (Yoshida et al. 1982). Beveridge and Hugo (1964) isolated α-ketoglutarate from Pseudomonas convexa grown on gallic acid. A. niger cleaved gallic acid into cis-aconitic, α-ketoglutaric and citric acids (Watanabe 1965). Gurujeyalakshmi and Mahadevan (1987) found that A. flavus produced an enzyme that oxidized gallic acid. They proposed a degradative pathway that went from gallic acid to muconic acid, oxadipidic acid, oxaloacetic acid and finally to pyruvic acid. Rajakumar and Nandy (1986) concluded that P. chrysogenum produces an oxidase that decomposed gallic acid into oxalic acid. Degradation of gallic acid by E. parasitica needs further study. If E. parasitica metabolizes gallic acid, its amount in chestnut tissues, available to kill tissues, would decrease.

The ellagitannins castalagin and vescalagin were tested as potential inhibitors of hamamelitannin and no inhibitory effect was detected at a relative concentration of the ellagitannins (inhibitors) that was higher than the relative concentrations that Elkins (Griffin and Elkins, 1986) found in sound bark tissue of American and European chestnuts.

According to the data reported, the ratios of (castalagin + vescalagin): hamamelitannin, are 1:4 for American chestnut and 1:2 for European chestnut. In our experiments, the ratio was 1:1. The results of these studies did not provide enough evidence to support the hypothesis that in European chestnut ellagitannins would tie up the E. parasitica tannase making it less available for hamamelitannin hydrolysis. Castalagin and vescalagin were not good substrates for the tannase so the possibility that they may compete with hamamelitannin to bind at the same active site of the enzyme is minimal. The other possibility is that the ellagitannins could bind at a different location on the surface of the enzyme other than the active site, causing conformational changes so that hamamelitannin can not bind at the active site. This hypothesis may not be feasible considering that tannases, according to Strumeyer and Malin (1970), have a carbohydrate coating that protects the polypeptide backbone, which would then be less accessible to tannin molecules. The same authors suggested that the carbohydrate coating may direct the substrate to the limited but accessible active-site region where cleavage to non-tannin products can take place. Protection of tannases from inhibition by condensed wattle tannins was reported by Beverini and Metche (1990). They concluded that protection may be dependent upon the carbohydrate content of the tannase and the concentration of the condensed tannin.

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SUMMARY AND CONCLUSIONS

The objectives of this dissertation were to study Endothia parasitica (Murr.) P.J.& H.E. Anderson (syn:Cryphonectria parasitica (Murr.) Barr) growth on tannins from blight-susceptible American (Castanea dentata) and blight-resistant Chinese (C. mollissima) chestnuts, to characterize the tannase (tannin acyl hydrolase) produced by the fungus, and to determine its tannin-substrate and-inhibitor relationships in order to clarify the role of the enzyme in the chestnut blight disease. The data from the E. parasitica growth study indicated that the fungus was able to grow in total aqueous and tannin extracts from blight-susceptible American chestnut bark as well as in total aqueous and tannin extracts from blight-resistant Chinese chestnut bark. Little differences in E. parasitica growth was noted between chestnut species. Conidium germination experiments data showed that germ tubes were longer at 18 h for the pathogen grown in American chestnut total aqueous summer bark extracts than that grown in Chinese chestnut extracts, and the final amount of growth was slightly higher in American chestnut total aqueous bark extract. The small differences in E. parasitica growth in chestnut extracts recorded in this study did not provide much evidence for the possible role of tannins in blight susceptibility.

The tannase was more abundant in the fungus intracellularly than extracellularly. The partially purified enzyme had a pH optimum of 5.5 and a temperature optimum for activity of 30 C. Less tannase activity was produced by E. parasitica grown in Chinese chestnut total aqueous and tannin extracts than was produced by growth in the respective American chestnut extracts for both winter and summer bark. Also tannase activity in fungus grown in American chestnut summer bark tannin extract was higher than when grown in aleppo tannic acid. It was suggested that differences in the number of ester bonds present in hamamelitannin, a prominent tannin in American chestnut, and aleppo tannic acid might explain the differential tannase activity found. It was concluded that tannin utilization by the fungus is likely to be important to the formation of the high-biomass mycelial fans that are important in pathogenesis. The expression of tannase by E. parasitica may serve as a marker for detecting sources of resistance for Chinese-American chestnut hybrids or American chestnut in early stages of a breeding program. Further research is required to clarify the reliability of this assumption.

To understand better the role that E. parasitica tannase plays in tannin metabolism, the enzyme was isolated, purified, and characterized. Its kinetics and inhibitory properties were also studied. The tannase was isolated from the mycelium of

E. parasitica and purified 142-fold with a 10 % yield and a specific activity of 170 units/mg protein. The estimated molecular weight was 240 kD. The tannase molecule may be a tetramer composed of four subunits with molecular weights of 58 kD. The pH optimum of the purified E. parasitica tannase was 5.5 and the purified enzyme was stable within the pH range of 4.0 and 7.5. The optimum temperature for activity was 30 C and the enzyme was stable up to 30 C with a 60 % stability up to 40 C for 10 min. Isoelectric focusing separated the tannase into six bands in the pH range of 4.6 to 5.1 that may represent isoenzymes or post-translational modifications.

Based on the Michaelis-Menten constant (K_m) of the tannase for different substrates, aleppo tannic acid was the best of the three substrates tested with a K_m = 0.95 mM. The V_{max}/K_m ratio was almost five-times higher than for hamamelitannin and seven-times higher than for methyl gallate. The results partially support previous findings, obtained with A. oryzae tannase. A greater relative tannase activity occured with substrates that contained a greater number of ester bonds than when the substrates were supplied at equivalent ester bond concentration. E. parasitica tannase activity on eight substrate analogues supplied at equivalent ester bond concentrations indicated that the activity of the enzyme was slightly higher on pentagalloyl glucose, a substrate with five gallic acids linked to glucose by ester bonds, than on aleppo-tannic acid, a substrate with seven gallic acids, but two of them linked in the form of a depside. It was suggested that ethe E. parasitica tannase may have a higher activity on substrates containing ester bonds with no depside groups. This assumption was based on previous reports that indicated that the tannase from A. oryzae has independent esterase and depsidase activities with different affinities for substrates containing ester bonds or ester bonds with some of them linked in the form of a depside.

The kinetic data presented in this study generally agree with the E. parasitica growth and tannase activity results. Hamamelitannin was a good substrate for the enzyme. As was reported, hamamelitannin is absent in Chinese chestnut bark but present in American chestnut bark. This difference may explain, in part, the lower tannase activity observed in Chinese chestnut bark extracts than in American chestnut bark extracts. The data collected in this study also indicated that galloyl esters were good substrates for the E. parasitica tannase. Previous studies have demonstrated that galloyl esters are present in Chinese chestnut tissues. These findings could explain the tannase activity observed in Chinese chestnut summer bark extracts as well as, in part, the good growth observed on Chinese winter and summer bark extracts.

The data presented in this study indicated that gallic acid was an effective inhibitor of the E. parasitica tannase with all substrates tested. The inhibition observed when hamamelitannin was used as a substrate may have important biological consequences. As was previously reported, gallic acid accumulated in culture medium during hamamelitannin breakdown catalyzed by E. parasitica tannase. Gallic acid accumulation could not only lower the activity of the tannase but also kill chestnut tissue. With lower tannase activity, the fungus may suffer from sub-optimal organic carbon supply and, therefore may require alternative metabolic pathways for organic carbon utilization or produce more tannase. Gallic acid metabolism may occur. From these data we could conclude that inhibition of the hamamelitannin by gallic acid is possibly an important regulatory mechanism in the growth and pathogenicity of the fungus E. parasitica on American chestnut. Further studies on the metabolism of gallic acid by E. parasitica could help the understanding of this consideration.

Our results also suggested that the ellagitannins vescalagin and castalagin were not good substrates for the tannase of E. parasitica, since very low relative tannase activity was recorded for both compounds. Galloyl groups are not present in the molecules of either castalagin or vescalagin. We think that the E. parasitica tannase activity, as well as A. oryzae tannase activity, may require gallic acid in the substrate. This study also indicated that castalagin and vescalagin were not inhibitors of hamamelitannin at the concentration tested. This concentration was higher than the relative concentrations that were reported in sound bark tissue of American and European chestnuts.

VITA

Graciela Maria Farias was born in Tucuman, Argentina on March 16, 1953 to Carlos A. Farias and Graciela G. de Farias. She graduated from Santa Rosa Catholic High School, Tucuman, Argentina in 1969. In 1970 she entered the National University of Tucuman and received a B. S. in Agronomy in 1975. She worked for several years as a Research Associate at the National Institute of Agricultural Technology in Tucuman, Argentina. In 1985 she enrolled in graduate school at Virginia Polytechnic Institute to pursue a Master degree in Plant Pathology which she obtained in 1987. In 1988 she entered the doctoral program of study in the Department of Plant Pathology. In May 19, 1990 she married George Santopietro.

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