Production and Application of Tannin Acyl Hydrolase: State of the Art

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I. Introduction

Tannin acyl hydrolase (E.C. 3.1.1.20), commonly called tannase, catalyzes the hydrolysis of ester and depside bonds in such hydrolyzable tannins as tannic acid, thereby releasing glucose and gallic acid (Dykerhoff and Ambruster, 1933). Tannase acts only on the ester linkages present in hydrolyzable tannins (Dykerhoff and Ambruster, 1933) and does not act on condensed tannins (George and Sen, 1960). This enzyme finds widespread application in food and beverage processing. Tannase also finds extensive use in the manufacture of instant tea (Coggon *et al.*, 1975). It is also used as a sensitive analytical probe for determining the structure of naturally occurring gallic acid esters (Haslam and Tanner, 1970).

However, the practical use of this enzyme is at present limited by its high cost, as well as by a lack of sufficient knowledge about its properties, optimal production, and large-scale application. The available data on tannase are scattered, and there is no review that covers all of its aspects. The present chapter covers all the existing literature on tannase and presents a unified picture of the state of knowledge on the topic, with suggestions for new areas of research.

II. Historical Highlights

The series of events that led to the discovery and an understanding of tannase provides interesting insight into the economical and technological factors involved in the development of a new product.

Teighem (1867) was the first to report the formation of gallic acid by two fungi, which occurs naturally when an aqueous solution of tannin or a filtered infusion of gall nut solution is exposed to air. These organisms, identified as *Penicillium glaucum* and *Aspergillus niger*, were able to grow with tannic acid as a the sole source of carbon, hydrolyzing it to gallic acid and glucose.

Fernback (1901) showed that the hydrolysis of tannin is brought about with the help of a particular enzyme—tannase. This enzyme was isolated from *A. niger*, and the potential application of tannase in the manufacture of gallic acid using tannin-containing substances as raw materials was discovered. Pottevin (1901) studied the properties of tannase and reported that the enzyme is inducible.

The first report on the isolation of tannase produced by *A. oryzae* in a wheat bran medium by solid-state fermentation was published by Kita

(1917). Further studies on the properties and reaction specificity of the enzyme were described by Dykerhoff and Ambruster (1933), Mikhlin and Gulkina (1932), and Thom and Raper (1945).

Fang (1940) reported the use of tannase in the production of gallic acid by fermentation of tannic acid and a new method for cultivation of *A. niger* on a solid substrate. Toth (1944) showed that tannase is composed of two enzymes: gallic acid esterase (which acts on ester linkages) and depsidase (which acts on depside bonds).

Madhavakrishna and Bose (1961) purified and crystallized tannase from a plant source: divi-divi (*Caesalpinia coriaria*) pods. Tannase from *A. niger* was purified and characterized by Dhar and Bose (1964). A new method for determining the activity of tannase was described by Iibuchi *et al.* (1967) based on the change in optical density of the substrate, tannic acid, at 310 nm. Tannase produced by *A. flavus* (Yamada *et al.*, 1968) and *A. oryzae* (Iibuchi *et al.*, 1968) was also purified and characterized.

A patent was assigned to Tenco Brooke Bond Ltd. (1971) for enzymic solubilization of tea cream using tannase. Yamada and Tanaka (1972) reported the use of tannase in the treatment of grape juice and wine. Kimura *et al.* (1973) were issued a patent for precipitation of tannase using polyethylene glycol and reported on the characteristics of the enzyme immobilized on an inorganic support by covalent attachment. Coggon *et al.* (1975) described a continuous-column treatment for the production of cold water-soluble tea using tannase immobilized on glass beads. The presence of low levels of tannase in the rumen of cattle was reported by Begovic and Duzic (1976), who also purified bovine tannase from the mucosal membrane of the rumen and small intestine of cattle for the first time (Begovic and Duzic, 1977). A simple rapid method for detection of tannase on polyacrylamide gel was described by Aoki *et al.* (1979a).

Jean *et al.* (1981) developed a gas-chromatographic method for assay of tannase activity. Katwa *et al.* (1981) described a procedure for the assay of immobilized tannase and studied the kinetic parameters of the enzymatic reaction. Deschamps *et al.* (1983) demonstrated the production of tannase by bacterial strains with chestnut bark as the sole source of carbon. Rajkumar and Nandy (1983) purified and characterized tannase produced by *P. chrysogenum*. Weetal (1985a) described enzymatic gallic acid esterification with soluble and immobilized tannase using a wide range of alcohols and diols.

Tsai (1985) described a process for the manufacture of instant tea by treating black tea leaves with tannase and cell wall lytic enzymes. Gathon *et al.* (1989) studied tannase entrapment in reverse micelles for the production of the antioxidant propyl gallate from tannic acid.

Beverini and Metche (1990) purified the two isozymes of tannase, tannase I and tannase II, from *A. oryzae*. Barthomeuf *et al.* (1994) reported that an *in situ* immobilized tannase from *A. niger* could be obtained by harvesting the mycelium after growth, followed by freeze-drying, which could then be used in the hydrolysis of tannic acid to gallic acid.

III. Tannin-Hydrolyzing Enzymes

The term "tannin" has been used in a wide sense in the botanical literature. Tannins are defined as water-soluble phenolic compounds with molecular weights ranging from 500 to 3000 that have the property of combining with proteins, cellulose, gelatin, and pectin to form an insoluble complex (Swain and Bate-Smith, 1962). Many authors speak of tannin as if it were a single entity. Poor terminology can confuse readers, particularly that found in the older literature. Tannic acid has frequently been used incorrectly as a general term for tannins (Haslam, 1966). Tannins can be classified into two distinct groups: (i) hydrolyzable tannins and (ii) condensed tannins.

Hydrolyzable tannins consist of a polyhydric alcohol esterified with gallic acid or derivatives of gallic acid (Nishira and Joslyn, 1968) (Fig. 1). The hydrolyzable tannins can be subdivided into two types: (a) gallotannins and (b) ellagitannins. Upon hydrolysis, gallotannin yields glucose and phenolic acids, gallic acid being predominant among the latter, for example, Chinese gallotannin (*Rhus semilata*) and sumac tannin (*Rhus coriaria*). When hydrolyzed, ellagitannins yield glucose and ellagic acid together with gallic acid, and frequently other acids structurally related to gallic acid, for example, myrobolan (*Terminalia chebula*) tannin and divi-divi (*Caesalpinia coriaria*) tannin (Haslam *et al.*, 1961).

Condensed tannins are made up of phenols of the flavone type and are often called flavolans because they are polymers of such flavan-3-ols as catechin or such flavan-3,4-diols as leucocyanidins. In contrast to the hydrolyzable tannins, they do not contain sugar residues (Goodwin and Mercer, 1983). A typical condensed tannin can be represented by the dimer procyanidin, to which molecules of flavan can be added as indicated (Fig. 2). No individual high-molecular-weight condensed tannin polymer has ever been isolated in the pure state (Grant, 1976). Examples of condensed tannins include wattle (*Acacia mollisima*) tannin and quebracho (*Schinopsis lorentzii*) tannin. Condensed tannins are less susceptible to microbial and chemical attack (Lewis and Starkey, 1962).

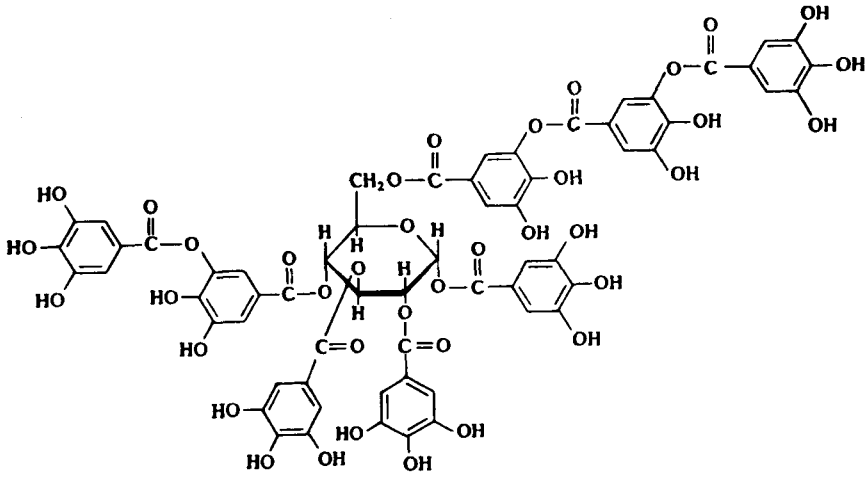


FIG. 1. Hydrolyzable tannin (e.g., sumac tannin).

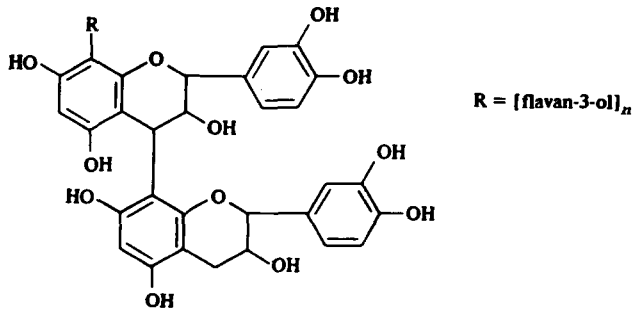
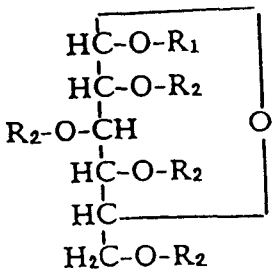


FIG. 2. Condensed tannin.

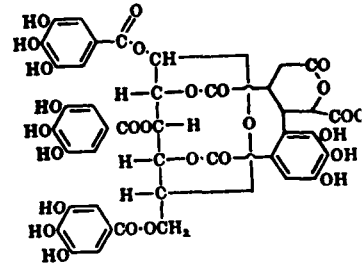
A. ENZYMES THAT ACT ON HYDROLYZABLE TANNINS

Tannase cleaves ester and depside linkages in such hydrolyzable tannins as tannic acid and chebulinic acid (Fig. 3). It also acts on the ester and depside linkages in methylgallate and *m*-digallic acid, respectively. Tannase hydrolyzes only those substrates that contain at least two phenolic OH groups in the acid component. The esterified COOH group must be on the oxidized benzene ring and must not be *ortho* to one of the OH groups (Dykerhoff and Ambruster, 1933).

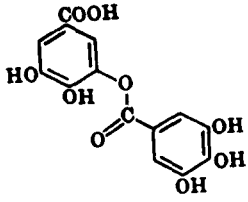
Chlorogenic acid, a depside of caffeic acid and quinic acid, is resistant to tannase, despite the fact that caffeic acid contains two phenolic OH groups. This is attributed to the presence of a double bond in the



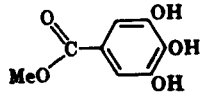
Tannic acid
R_1 – Gallic acid
R_2 – m-Digallic acid



Chebulinic acid



m-Digallic acid



Methyl gallate

FIG. 3. Substrates of tannin.

side-chain carrying the esterified COOH group (Dykerhoff and Ambruster, 1933). However, Yamada and Tanaka (1972), in a patent for the use of tannase in wine making, reported that tannase hydrolyzes the chlorogenic acid present in grape juice to yield caffeic acid and quinic acid. Tannase is also reported to hydrolyze (–)-epicatechin gallate and (–)-epigallocatechin-3-gallate (Nierenstein, 1936; Bradfield and Penny, 1948), which are condensed tannins present in tea.

B. ENZYMES THAT ACT ON CONDENSED TANNINS

Only a very limited number of microorganisms have been reported to degrade condensed tannins and catechins. The mechanism and the

enzymes involved in such degradation are unknown (Grant, 1976; Lewis and Starkey, 1962; Deschamps and Leulliette, 1984; Galiotou-Panayatou and Macris, 1986). Galiotou-Panayatou *et al.* (1988) isolated a novel enzyme from *Calvatea gigantea* that hydrolyzes catechin, the building block of condensed tannin. This enzyme has been purified and characterized. The enzyme had an optimal pH of 8 and a temperature optimum of 35°C (Galiotou-Panayatou *et al.*, 1988). Sambandam (1983) isolated catechin-2,3-dioxygenase from *Chaetomium cupreum*, which cleaved catechin, releasing protocatechuic acid, catechol, and phloroglucinol carboxylic acid. The enzyme was active in the pH range of 2 to 8, with optimal pHs at 2.8 and 7.0. The enzyme was stable up to 50°C and had a molecular mass of 40,000 daltons. Catechin oxygenase was a glycoprotein exhibiting two isoelectric points.

Tannase does not act on condensed tannins (George and Sen, 1960). The biodegradation of condensed tannin by microorganisms remains an area that has not been understood or explored in detail. A lack of complete information on the enzymes involved in the hydrolysis of condensed tannin suggests that there is a need for further and continuing research in this area.

C. Significance of Tannins in Plants

Tannins are believed to occur in the vacuoles of intact plant cells (Forsyth, 1964). Some plants accumulate tannin, particularly in the bark and heartwood. The exact function of tannins in plants is not clear. At the intracellular level of metabolism, tannins are of little value to the plant, although they have functions like wound healing or act structurally as pigments (White, 1957). Such accumulated tannins protect the vulnerable parts of the plants from microbial attack by inactivating viruses and invasive extracellular enzymes of microbes by direct tanning action (White, 1957). Enzymes secreted by attacking microorganisms are wholly or partially inactivated by complex formation with tannins (Goldstein and Swain, 1965), while such microbial substrates as polysaccharides and nonenzyme proteins, present in the plants, become highly resistant to microbial attack after binding to a tannin molecule (Betnoit *et al.*, 1968). The plant, however, appears to protect itself from high concentrations of toxic phenols by the use of such specialized structures as the "glands" of gossypol (Singleton and Kratzer, 1969). The inhibitory action of tannins on the growth of bacteria (Sivaswamy, 1982; Henis *et al.*, 1964), fungi (Mur, 1953; Lewis and Papavizas, 1968), yeast (Jacob and Pignal, 1975), and viruses (Cadman, 1960) is well established.

IV. Source of Tannase

A. Microorganisms

A number of microorganisms—including bacteria, fungi, and yeasts—have been reported to produce tannase (Table I). Extensive screening studies have been conducted to select potent cultures for tannase production.

Ganga *et al.* (1977, 1978) screened a number of fungi belonging to *Penicillia* and *Aspergilli* for production of tannase by submerged and liquid-surface fermentation. Among the different *Aspergillus* strains, *A. oryzae*, *A. flavus*, and *A. japonicus* were found to produce high titers of tannase compared to the other strains. Among the *Penicillium* spp., *P. islandicum* was reported to produce maximum tannase (Ganga *et al.*, 1978). Tannase production by *Penicillium* strains was lower than that produced by *Aspergillus* species (Ganga *et al.*, 1978). Yamada *et al.* (1967) conducted screening of about 80 fungal strains for tannase production and reported that two strains belonging to *A. oryzae* produced maximum tannase activity. The only commercial source of tannase available at present is produced by *A. oryzae* strain ATCC 9362.

Reshetnikova *et al.* (1984) screened five species of *Ascochyta* (*A. cucumeris*, *A. pisi*, *A. biochemica*, *A. boltshauseri*, and *A. viciae*) for tannase production using a sucrose–mineral medium containing gallotannin. The highest tannase activity was produced by *A. boltshauseri* and *A. viciae*, whereas the lowest tannase activity was observed with *A. pisi*.

Deschamps *et al.* (1983) isolated bacterial strains belonging to *Bacillus*, *Corynebacterium*, and *Klebsiella* that were able to degrade tannic acid and related compounds from decaying bark of pine (*Pinus maritima*) and oak (*Quercus pedunculata*). Among these strains, the best tannase production (0.064 U/ml) was observed in the case of *Corynebacterium* sp. Q 40 after 5 h of fermentation. These strains were able to produce tannase only in the presence of tannic acid in the medium (Deschamps *et al.*, 1983). A similar strategy was adopted by Deschamps and Leulliette (1984) to isolate potent tannin-degrading yeasts. Aoki *et al.* (1976, 1979a,b) isolated several yeast-like strains from soil for tannase production. A strain identified as a *Candida* produced extracellular as well as intracellular tannase only in the presence of tannic acid.

It is well known that product titers obtained in submerged fermentation need not be the same in solid-state fermentation. This has been observed in the cases of citric acid and α-amylase production by two different fermentation methods (Shankaranand *et al.*, 1992), thereby

TABLE I

MICROORGANISMS CAPABLE OF PRODUCING TANNASE

Microorganism	Reference
Bacteria	
Bacillus pumilis	Deschamps *et al.*, 1983
B. polymyxa	Deschamps *et al.*, 1983
Corynebacterium spp.	Deschamps *et al.*, 1983
Klebsiella pneumoniae	Deschamps *et al.*, 1983
Yeast	
Candida sp. K 1	Aoki *et al.*, 1976, 1979a,b
Fungi	
Ascochyta boltshauseri	Reshetnikova *et al.*, 1984
A. pisi	Reshetnikova *et al.*, 1984
A. biochemica	Reshetnikova *et al.*, 1984
A. viciae	Reshetnikova *et al.*, 1984
Aspergillus carneus	Ganga *et al.*, 1977
A. flaviceps	Ganga *et al.*, 1977
A. flavus	Chae and Yu, 1973
A. fumigatus	Lewis and Starkey, 1962
A. japonicus	Ganga *et al.*, 1977
A. leuchensis inui	Rhind and Smith, 1922
A. nidulans	Ganga *et al.*, 1977
A. niger	Nishira and Mugibayashi, 1960
A. oryzae	Yamada *et al.*, 1967
A. oryzae pseudoflavus	Seiji *et al.*, 1973
A. parasiticus	Ganga *et al.*, 1977
A. tamari	Vandamme *et al.*, 1989
A. terreus	Ganga *et al.*, 1977
A. ustus	Ganga *et al.*, 1977
Chaetomium lobosum	Nishira and Mugibayashi, 1960
Mucor pranii	Nishira and Mugibayashi, 1960
Myrothecium verrucaria	Nishira and Mugibayashi, 1960
Neurospora	Nishira and Mugibayashi, 1960
Rhizopus	Nishira and Mugibayashi, 1960
Trichothecium roseum	Nishira and Mugibayashi, 1960
Penicillium chrysogenum	Rajkumar and Nandy, 1983
P. fellutanum	Ganga *et al.*, 1978
P. islandium	Ganga *et al.*, 1978
P. notatum	Ganga *et al.*, 1978
P. variable	Ganga *et al.*, 1978

indicating a need for intensive screening to select a potent culture particularly suitable for a solid-state fermentation system.

Eight strains of *Penicillium*, five strains of *Aspergillus*, three strains of *Neurospora*, and one strain each of *Trichothecium roseum*, *Mucor pranii*, *Myrothecium verrucaria*, and *Chaetomium lobosum* were screened for tannase production in a wheat bran medium containing 4% tannic acid as an inducer. Strains belonging to *Aspergillus* and *Penicillium* were found to be potent producers of tannase (Nishira and Mugibayashi, 1960). Lekha *et al.* (1993) also conducted extensive screening of fungal cultures for tannase production by solid-state fermentation and reported *A. niger* isolated by a baiting method to be the best tannase producer.

Microorganisms used in the industrial production of food processing enzymes should be listed as GRAS (generally recognized as safe) (Fordham and Block, 1987). GRAS microorganisms are nonpathogenic, nontoxic, and should generally not produce antibiotics (Walsh and Headon, 1994).

B. Plants and Animals

Tannase has been reported to be present in many tannin-rich plant materials, such as myrobolan (*Terminalia chebula*) fruits, divi-divi (*Caesalpinia coriaria*) pods, dhawa (*Anogeissus latifolia*) leaves and the bark of konnam (*Cassia fistula*), and babul (*Acacia arabica*) and avaram (*Cassia auriculata*) trees (Madhavakrishna *et al.*, 1960).

Its presence is also recorded in the rumen mucosa of cattle (Begovic and Duzic, 1976). However, the levels reported are very low. Tannase was purified from bovine mucosa membrane of the rumen and small intestine (Begovic and Duzic, 1977). The gall larvae that undergo development in plant galls produce tannase to hydrolyze the tannic acid abundant in plant galls (Nierenstein, 1930).

Although many enzymes are obtained from animal and plant sources (Godfrey, 1985), microorganisms are becoming the favored source for production of industrial enzymes because of their biochemical diversity and their technical and economic advantages (Underkofler, 1976). Microorganisms can be cultured in large quantities in a short time by established methods of fermentation. Thus, they can produce an abundant and regular supply of the desired enzyme. Microbial enzymes are more stable than analogous proteins obtained from plant or animal sources. Furthermore, microbes can be subjected to genetic manipulation more readily than animals and plants (Headon and Walsh, 1994; Walsh and Headon, 1994).

Genetic manipulation by mutation and selection has played a central role in increasing the yield of many enzymes produced by microorganisms (Walsh and Headon, 1994). Advances in the area of recombinant DNA technology have facilitated the development of an entirely new strategy to increase the levels of expression of specific enzymes (Walsh and Headon, 1994). For example, cellulase genes from *Cellulomonas uda* have been efficiently cloned and expressed in *Zymomonas mobilis* (Misawa *et al.*, 1988). Large-scale fermentation of such recombinant organisms can yield appreciable quantities of any enzyme. Many commercially available enzymes are produced by this method (Cullen, 1987). However, no such studies have been conducted to improve tannase production.

C. Physiological Significance of Tannase in Plants, Animals, and Microorganisms

1. *Plants*

Tannase has been isolated from such hydrolyzable tannin-containing plant materials as myrobolan (*Terminalia chebula*) fruits, divi-divi (*Caesalpinia coriaria*) pods, and dhawa (*Anogeissus latifolia*) leaves, as well as from condensed tannin-containing plant materials like avaram (*Cassia auriculata*), babul (*Acacia arabica*), and konnam (*Cassia fistula*) bark (Madhavakrishna *et al.*, 1960). Sometimes hydrolyzable and condensed tannin are produced in the same plant, but generally in separate tissues (Haslam, 1981; Haslam and Lilley, 1988). The physiological significance of tannase in plants has been discussed by Madhavakrishna *et al.* (1960). It was suggested that, together with large quantities of sugars, plants synthesize chebulinic acid, gallic acid, and hexahydroxyphenic acid during growth. As the fruit ripens, these acids may become esterified with glucose, with the help of tannase to form complex tannins. After abscission, the esterase hydrolyzes the tannins (Madhavakrishna *et al.*, 1960). The structure of the condensed tannin molecule is such that it cannot be hydrolyzd by tannase (Dykerhoff and Ambruster, 1933). In the case of condensed tannins, tannase helps to synthesize, at one stage or another, some intermediates or precursors, which in turn undergo transformation into the complex tannin molecules (Madhavakrishna *et al.*, 1960).

2. *Animals*

Tannins are present in a variety of plants utilized as feed (Salunkhe *et al.*, 1982). The deleterious nutritional effects of dietary tannins have

been reviewed (Price and Butler, 1980). They exert negative effects on protein, fat, and carbohydrate utilization of food (Salunkhe *et al.*, 1982). Tannins complex with all the digestive enzymes and affect the digestion and availability of proteins. The secretions of the digestive tract contain mucoproteins (Buddecke, 1972), and tannins are known to react with these mucoproteins (Mitjavila *et al.*, 1968). Any tannin that escapes this reaction can react with the proteins of the outer cellular layer of the gut and can reduce the passage of nutrients through the gut (Hand *et al.*, 1966). The low levels of tannase reported to be present in the rumen mucosa of cattle (Begovic and Duzic, 1976) probably hydrolyze the tannic acid present in the diet to phenols and sugar, which can be readily absorbed (Glick and Joslyn, 1970), thereby alleviating the toxicity exerted by these compounds.

3. Microorganisms

Tannins are the fourth most abundant plant constituent, after cellulose, hemicellulose, and lignin (Swain, 1965). High tannin content in plant materials is associated with resistance to microbial attack, and the durability of certain long-lived trees and their wood has been ascribed to their high tannin content (White, 1957). Tannin accumulation in bark is particularly effective in preventing germination of spores of attacking fungi and penetration of fungal hyphae or such bacteria that rely on the action of extracellular enzymes to open up a pathway for the organism (White, 1957). Tannase is known to be produced by a number of microorganisms, including bacteria, yeasts, and fungi. Of these, the fungi are known to be the most potent enzyme producers (Lekha, 1996). Species of *Ascochyta*, phytopathogenic fungi, are also reported to produce tannase (Reshetnikova *et al.*, 1984). Tannase produced by these microorganisms probably serves as a mode of invasion into the host plant by hydrolyzing these complex polyphenolic materials present in the bark of plants, which are known to confer protection against the attacking pathogenic fungi.

Tannase present in soil microorganisms probably plays an active role in the decomposition and recycling of plant materials containing tannin.

V. Tannase Assay

Tannase acts on the ester and depside linkages present in tannic acid, liberating glucose and gallic acid (Dykerhoff and Ambruster, 1933). Therefore, the activity of tannase can be measured by estimating the residual tannic acid (Deschamps *et al.*, 1983) or gallic acid (Nicolson *et*

al., 1931) formed due to enzyme action. Most of the old methods were based on the titration of the gallic acid liberated by enzyme action (Freudenberg *et al.*, 1927; Nishira, 1961; Haslam and Stangroom, 1966) and hence do not yield correct results due to the problem of determining the endpoint accurately (Madhavakrishna *et al.*, 1960).

Numerous photometric (Chen, 1969), colorimetric (Haslam and Tanner, 1970), and UV-spectrophotometric (Parmentier, 1970) methods have been described in the literature for the assay of tannase. The colorimetric and photometric methods are not specific (Jean *et al.*, 1981). Madhavakrishna *et al.* (1960) described a procedure for quantitative estimation of tannase by determining the glucose liberated from tannic acid by the action of tannase. The reaction time used in this method is 24 h, so that it is not suitable for routine analysis of tannase.

The spectrophotometric method developed by Iibuchi *et al.* (1967) has been used by most workers (Rajkumar and Nandy, 1983; Aoki *et al.*, 1976; Iibuchi *et al.*, 1968). This method is based on the change in optical density of the substrate tannic acid at 310 nm. According to this method, one unit of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 μmol of ester bond in tannic acid per minute. Yamada *et al.* (1968) used a modified version of this method using methyl gallate as the substrate.

Haslam and Tanner (1970) were critical of the spectrophotometric method of Iibuchi *et al.* (1967), as it is based on an extremely narrow difference in UV absorption of gallic acid and its methyl ester or tannic acid, both of which have absorbance in the UV region. They developed a new spectrophotometric assay utilizing *p*-nitrophenyl esters of gallic acid as the substrate. However, this method did not find wide acceptance, probably due to the nonavailability of these substrates.

Sanderson *et al.* (1974) described a method for tannase estimation that was based on a slight modification of the method of Iibuchi *et al.* (1967). This method is highly sensitive and is ideal for routine analysis of a large number of samples due to its short reaction time and continuous, direct monitoring of the reaction in the spectrophotometer. A major drawback of the method is that the enzyme activity cannot be expressed in SI units. Jean *et al.* (1981) developed a gas-chromatographic method for estimation of tannase activity by determining the gallic acid released as a result of enzymatic hydrolysis of methyl gallate. This method is said to be rapid, specific, reliable, and reproducible (Jean *et al.*, 1981). Thomas and Murtaugh (1985) described a method for assay of tannase activity using an extract of black tea leaf as the substrate by a pH stat method.

VI. Production of Tannase

There are two main fermentation types that are generally used for production of commercial enzymes. These are submerged fermentation and solid-state fermentation (Frost and Moss, 1987). A literature survey indicates that tannase has been produced by liquid-surface, submerged, and solid-state fermentation, though production of tannase has been most extensively carried out in a submerged fermentation system.

A. Tannase Production by Submerged Fermentation

Submerged fermentation involves the growth of the microorganism as a suspension in a liquid medium in which various nutrients are either dissolved or suspended as particulate solids in many commercial media (Frost and Moss, 1987). Submerged fermentation is the preferred method for production of most of the commercially important enzymes, principally because sterilization and process control are easier to engineer in these systems (Aunstrup *et al.*, 1979).

Such details as media, fermentation time, temperature, and the location of tannase produced by different microorganisms in submerged fermentation are given in Table II. No details on optimization of the processes described in Table II have been published, but it seems likely that fairly extensive optimization will be required to arrive at the medium and the conditions described.

1. Media Composition

The fermentation medium must meet the nutritional requirements of the microorganism (Frost and Moss, 1987). It basically contains sources of carbon, nitrogen, minerals, and some growth factors, such as essential amino acids and vitamins (Volesky and Luong, 1985).

Carbon Source. As tannase is an inducible enzyme, tannic acid itself was used as the sole carbon source as well as an inducer (Yamada *et al.*, 1968; Aoki *et al.*, 1976). Additional carbon sources, like glucose (1%) and sucrose (3%), were used along with tannic acid for tannase production by *A. oryzae* (Fumihiko and Kiyoshi, 1975) and *A. niger* in submerged fermentation (Dhar and Bose, 1964), respectively. In fact, tannic acid concentration was found to be the crucial factor influencing the levels of enzyme. In submerged fermentation the concentration of tannic acid used ranged from 0.1 to 10% (Yamada *et al.*, 1968; Nishira and Mugibayashi, 1956). In the case of *A. oryzae*, growth and tannase activity decreased when the tannic acid in the medium was increased from 0.5 to 1% (Ganga *et al.*, 1977). A crude extract of gall nut powder

TABLE II

FERMENTATION CONDITIONS USED FOR TANNASE PRODUCTION BY SUBMERGED FERMENTATION

Micro-organisms	Media	Concentration (%)	Time (h)	Temperature (°C)	Location of enzyme	Reference
A. flavus	Tannic acid $NaNO_3$ $MgSO_4 \cdot 7H_2O$ KCl pH 6.0	0.1 0.2 0.05 0.005	96	30	Extracellular	Yamada *et al.*, 1968
A. niger	Tannic acid Sucrose $NaNO_3$ K_2HPO_4 $MgSO_4$ KCl $FeSO_4$ pH 6.5	2 3 0.3 0.1 0.05 0.05 0.001	144	28		Dhar and Bose 1964
A. oryzae	Tannic acid NH_4Cl $KHPO_4$ $MgSO_4 \cdot 7H_2O$ $AlCl_3 \cdot 6H_2O$ pH 6.0	2 0.2 0.2 0.1 0.001	70–120	30		Yamada *et al.* 1967
A. oryzae IAM 2636	Tannic acid Glucose $NH_4H_2PO_4$ K_2HPO_4 $MgSO_4 \cdot 7H_2O$ pH 5.5	2 1 1.4 0.2 0.05	48	30	Extracellular	Fumihiko and Kiyoshi, 1975
A. niger LCF.8	Tannin extract from gall nut powder 72.6% pH 4.5		29	33	Intracellular	Barthomeuf *et al.*, 1994
P. chrysogenum NCIM 722	Czapek–Dox medium + tannic acid 2%		120	28	Intracellular	Rajkumar and Nandy, 1983
Bacillus spp.	Commercial chestnut extract pH 6.8	1	6	40	Extracellular	Deschamps *et al.*, 1983
Candida sp. K 1	Tannic acid $Na_2HPO_4 \cdot 12H_2O$ K_2HPO_4 $MgSO_4 \cdot 7H_2O$ Monosodium glutamate	3 0.3 0.3 0.05 1	144	35	Extracellular	Aoki *et al.*, 1976

containing 72.6 g/liter of tannin, adjusted to pH 4.5, was also used as the culture medium for tannase production by *A. niger* in submerged fermentation (Barthomeuf *et al.*, 1994).

Nitrogen Source. The nitrogen sources used for tannase production include sodium nitrate (Yamada *et al.*, 1968; Dhar and Bose, 1964), ammonium chloride (Vandamme *et al.*, 1989), ammonium oxalate (Reshetnikova *et al.*, 1984), and ammonium sulfate (Fumihiko and Kiyoshi, 1975). Reshetnikova *et al.* (1984) also reported that ammonium chloride was inhibitory for tannase production by *Ascochyta* spp. Organic nitrogen sources like monosodium glutamate, glutamic acid, and casein hydrolyzate have also been used for tannase production (Aoki *et al.*, 1976; Lippitsch, 1961).

Minerals. Traces of Fe, Zn, and Cu have been reported to be essential for the production of tannase by *A. niger* (Lippitsch, 1961), whereas Fe had no influence on tannase production in the case of *Penicillium* (Nishira, 1961).

2. *pH*

Enzymes, being proteins, contain ionizable groups; consequently, the pH of the culture medium affects their structure and function (Frost and Moss, 1987). Most microbial extracellular enzymes are produced in greatest yield at a growth pH, somewhere near the pH for maximum enzyme activity (Volesky and Luong, 1985). In the case of tannase produced by submerged fermentation, the optimum initial pH used was in the acid range of 4.5–6.5 (Yamada *et al.*, 1968; Fumihiko and Kiyoshi, 1975; Barthomeuf *et al.*, 1994). However, most of the published work relates to shake-flask cultures run under uncontrolled conditions of pH, and the final pH values are rarely recorded. In the case of tannase production by *A. niger* in a 20-liter fermentor, pH was adjusted throughout fermentation by automatic regulation using aqueous ammonia or orthophosphoric acid (Pourrat *et al.*, 1982). Maximum enzyme activity was obtained at pH 7. However, Barthomeuf *et al.* (1994) reported that, for tannase production by *A. niger,* at pH values below 3.5, the enzyme was unstable, and that substrate hydrolysis and diffusion of enzyme into the medium occurred at pH values above 5.5.

3. *Sterilization*

The most common method of sterilization for liquid, as well as for solid, media is by heat under pressure (Beckhorn *et al.*, 1965). For liquid media in flasks and tubes, this is usually done at 120°C for 20 min. Tannic acid is thermolabile, so that the other medium components are usually dissolved in distilled water and sterilized at 120°C for 20 min.

Tannic acid dissolved in a minimal amount of water was filter sterilized and aseptically added to the rest of the autoclaved and cooled medium (Deschamps and Leulliette, 1984), or a tannic acid-containing medium was instantaneously sterilized separately at 110°C (Aoki *et al.*, 1976).

4. Inoculum Preparation

A highly developed production strain must be protected against the risk of degeneration, contamination, or loss of viability (Volesky and Luong, 1985). On a laboratory scale, spores are prepared by inoculating agar slants on suitable media by the microorganism, incubating the slants, and harvesting spores by adding 3–5 ml of water containing a non-ionic detergent (0.01%), while aseptically scraping the spores from the agar surface (Mudgett, 1986). Tannase being an inducible enzyme, the maintenance medium for tannase-producing strains usually contains low levels of the inducer, tannic acid, along with other media components (Yamada *et al.*, 1967; Aoki *et al.*, 1976). It has been shown that the yield of tannase can be increased by growing the fungus for several successive asexual generations on gallotannin (Nicolson *et al.*, 1931). Nishira (1961) also reported that the length of precultivation of *Penicillium* had an influence on the adaptation of the mold to the substrate tannin, when grown in a Czapek–Dox medium containing 0.5% tannin.

For tannase production by *P. chrysogenum*, the inoculum was prepared by growing the culture on potato dextrose agar slants for 10 days (Rajkumar and Nandy, 1983). In the case of tannase production by *Aspergillus* and *Penicillium* strains in submerged fermentation, 2% inoculum was used (Ganga *et al.*, 1977, 1978).

A liquid medium was also used for inoculum production by Pourrat *et al.* (1982). Mycelia of *A. niger* weighing about 0.5 g were grown in 500 ml of Karrow medium (Prescott and Dunn, 1959) in which sucrose was replaced by 3% tannic acid and the pH was adjusted to 3.5. The medium was sterilized for 30 min at 110°C, incubated at 30°C, and left undisturbed to form the mycelial mat. After 10 days, mycelium was harvested under sterile conditions, ground, and homogenized in 50 ml of sterile water. Two percent of this spore suspension, which had a spore density of 3×10^{10} spores/ml, was used as inoculum for tannase production by submerged fermentation (Pourrat *et al.*, 1982). A similar methodology was used for inoculum preparation by Barthomeuf *et al.* (1994) for tannase production by *A. niger*.

5. Incubation Temperature

Enzyme fermentation is governed by the temperature, but the optimum for synthesis of a particular enzyme may differ from the optimum

for growth (Frost and Moss, 1987). The optimum temperature for tannase production in most of the cases was found to be 30°C (Table II), except in the case of bacteria and yeast, which required slightly higher temperatures of 40 and 35°C, respectively (Deschamps *et al.*, 1983; Aoki *et al.*, 1976).

6. Aeration and Agitation

In laboratory-scale tannase production, shaken cultures in flasks (120 oscillations/min) were employed to ensure proper aeration and agitation (Yamada *et al.*, 1968; Aoki *et al.*, 1976). In the case of tannase production by *A. niger* in a 6-liter fermentor, the initial stirring rate was maintained at 300 rpm and then increased to 450 rpm after 24 h to offset an increase in the viscosity of the medium due to mycelial growth. The dissolved oxygen level was regulated at 30–40% by means of an Ag–Pb electrode (Barthomeuf *et al.*, 1994). They also reported that insufficient aeration impeded growth, while excessive aeration favored oxidation of tannins and had an inhibitory effect on the biosynthesis of tannase by *A. niger*. Small-jar fermentors provided with mechanical agitators (400 rpm) and air sparkers were used by Pourrat *et al.* (1982) for tannase production by *A. oryzae*. Constant airflow at 0.4 VVM was found to be better than dissolved oxygen at 30%, both in terms of growth and enzyme activity (Pourrat *et al.*, 1982). In the cases of *A. flavus* and *A. oryzae*, a static condition enhanced tannase production, but stirred conditions favored enzyme production in the case of *A. japonicus* (Ganga *et al.*, 1977).

7. Harvesting Time

Depending on the organism and the amount of tannase produced, fermentation may take 1 to 10 days. For tannase production by submerged fermentation, the fermentation time varied from 48 to 120 h in the case of *A. oryzae* (Okamura *et al.*, 1988; Yamada *et al.*, 1967; Fumihiko and Kiyoshi, 1975). In the case of *Candida* and *A. niger*, the fermentation was continued for 6 days (Aoki *et al.*, 1976; Dhar and Bose, 1964). A shorter fermentation time of 29 h was also reported for tannase production by *A. niger* (Barthomeuf *et al.*, 1994). Tannase produced by bacteria was released into the medium during the active growth phase, and maximum tannase was obtained after 5–6 h of fermentation (Deschamps *et al.*, 1983).

8. Recovery of the Enzyme

In submerged fermentation, the next step after termination of fermentation is separation of microbial cells and suspended solids from the

liquid medium (Aunstrup, 1977). This is usually achieved by filtration in the case of mycelial organisms, or by centrifugation in the case of bacteria and yeast (Volesky and Luong, 1985).

In the case of tannase from *A. niger* (Barthomeuf *et al.*, 1994) and *P. chrysogenum* (Rajkumar and Nandy, 1983), the enzyme produced being intracellular, an additional step of sonication or grinding of the mycelium to release the enzyme is required.

Barthomeuf *et al.* (1994) reported that, at maximum production (29 h), tannase was strongly bound to the mycelium, and not more than 5% of the enzyme could be released by chemical and physical methods (i.e., grinding the mycelium with sand and glass beads, polytrol grinding, osmotic shock, and sonication). Hence, 90-hour-old mycelium was used because, with the beginning of autolysis, the cell wall became fragile and recovery of enzyme was greatly increased. Tannase was obtained by physical disruption of the mycelium by congelation–decongelation and addition of concanavalin A into the medium. Addition of con A to the medium after decongelation increased tannase recovery from 22.5 to 34.5% by facilitating desorption of the enzyme from its binding site (Barthomeuf *et al.*, 1994).

Commercially, tannase is produced by submerged fermentation (Okamura and Yuasa, 1987). Table III gives a list of patents for tannase production using submerged fermentation.

B. Tannase Production by Liquid-Surface Fermentation

Liquid-surface fermentation involves the growth of culture on the surface of a liquid medium at a shallow depth and held in a suitable container (e.g., stainless steel trays) (Mitchell and Lonsane, 1992).

Only three preliminary reports (Pourrat *et al.*, 1982; Ganga *et al.*, 1977, 1978) are available on the production of tannase by liquid-surface fermentation. In the case of tannase production by *A. niger*, the liquid medium containing all the necessary minerals along with carbon, nitrogen, and inducer was inoculated and left undisturbed so that a mycelial mat was formed on the surface. The enzyme was found to be intracellular, and tannase production was high when the initial pH of the medium was 7. The fermentation time was as long as 8 days (Pourrat *et al.*, 1982). In the case of tannase production by different strains of *Aspergillus*, 25 ml of the medium was used in 100-ml Erlenmeyer flasks. Two percent tannic acid was used in the medium, and fermentation was continued for 7 days at 30°C (Ganga *et al.*, 1977). The inoculum level used was 2%.

TABLE III

PATENTS FOR TANNASE PRODUCTION BY SUBMERGED FERMENTATION

1. Tannase produced by *Aspergillus* (Y. Fumihiko and M. Kiyoshi, Jpn. Pat. 72,25, 786, 1975).
2. Manufacture of tannase with *Aspergillus* (S. Okamura [Kikkoman Corp.] and K. Yuasa [Inabata and Co. Ltd.], Jpn. Pat. 62,272,973, 1987).
3. Fermentative manufacture of tannase (S. Okamura, K. Mizusawa, K. Takei, Y. Imai, and S. Ito [Kikkoman and Inabata], Jpn. Pat. 63,304,981, 1988).
4. Process for preparation of tannase intended for the production of gallic acid with *Aspergillus* (E. Vandamme, M. Jerome, A. Vermiera, and M. Maria, Eur. Pat. 339,011, 1989).

In the case of tannase produced by *Penicillium* spp. in liquid-surface fermentation, 0.5% tannic acid was used in the medium and fermentation was continued for 8 days (Ganga *et al.*, 1978). More tannase was produced in liquid-surface fermentation as compared to submerged fermentation by *A. flavus*, *A. oryzae*, and *P. islandicum* (Ganga *et al.*, 1977, 1978). However, the fermentation time required in all these cases was 7–10 days.

These preliminary studies indicated that liquid-surface fermentation was not suitable for tannase production due to the longer fermentation time required and the intracellular nature of the enzyme. Other reasons cited for not adopting liquid-surface fermentation for production of other microbial metabolites include higher handling costs, the risk of infection, and the difficulty involved in applying modern methods of parameter and process control (Kumar and Lonsane, 1989).

C. TANNASE PRODUCTION BY SOLID-STATE FERMENTATION

The essential feature of solid-substrate fermentation is the growth of microorganisms on an insoluble substrate without a free liquid phase (Mitchell and Lonsane, 1992). The moisture level in solid-substrate fermentation may be between 30 and 80%; for production of most enzymes it is typically in the region of 60% (Laukevics *et al.*, 1984).

The production of enzymes using solid-state fermentation has developed from the "koji process." The traditional Japanese koji process involves growth of filamentous fungi (e.g., *A. oryzae*) on moist mixed substrates (e.g., rice, wheat, soybeans) to produce a mixture of extracellular amylolytic and proteolytic enzymes that were used in food pres-

ervation, flavoring, and texture modifications (Hesseltine and Wang, 1967; Yamada, 1977; Steinkraus, 1984).

The literature on microbial production of tannase by solid-state fermentation is meager. Except for a few exploratory reports (Kita, 1917; Nishira, 1959a,b; Nishira and Mugibayashi, 1960) on production of tannase by solid-state fermentation using wheat bran, there are no data available on the effect of media parameters on tannase production by solid-state fermentation.

The first report on tannase production by *A. oryzae* in a solid-state fermentation process was by Kita (1917). Tannase production increased with tannin content in the medium. Very high concentrations of tannin (20% of wheat bran) were found to inhibit the growth of the fungus (Kita, 1917).

Nishira (1959a,b) reported the production of tannase by *Penicillium* spp. grown on wheat bran in the presence of gallotannin. The enzyme was produced only in the presence of tannic acid or gallic acid, and production was not growth-related. Gallotannin was completely degraded to gallic acid. Gallic acid decarboxylase was found to be co-produced along with tannase.

Nishira and Mugibayashi (1960) studied the formation of tannase by different fungi in a wheat bran medium. Various strains of *Penicillium*, *Aspergillus*, *Rhizopus*, *Neurospora*, *Trichothecium roseum*, *Mucor pranii*, *Myrothecium verrucaria*, and *Chaetomium lobosum* were cultured in a wheat bran medium containing 4% tannin and incubated at 25–27°C for 3–10 days. *Aspergillus* and *Penicillium* species were found to produce higher titers of the enzyme. Tannase was leached from fermented bran using 1% sodium chloride, allowing a contact time of 24 h (Nishira and Mugibayashi, 1960).

Work on tannase production by solid-state fermentation was initiated at the Central Food Technological Research Institute (CFTRI) in Mysore, India. This research yielded very promising results. Extensive screening of fungal cultures from a culture collection, as well as those isolated from soil and different tannin-rich plant materials, was carried out. *A. niger* PKL 104, a potent culture for tannase production, was isolated by a baiting method (Lekha *et al.*, 1993).

The physicochemical parameters for tannase production by this culture in solid-state fermentation was optimized by a response surface methodology (Lekha *et al.*, 1994). Wheat bran was used as the solid substrate, and 6% tannic acid was added to the medium for maximum induction of tannase (Lekha, 1996). Addition of nitrogen and mineral salts did not have an effect on tannase production. This is because wheat bran provides mixed carbon, nitrogen, and other growth factors

that can meet the nutritional requirements of the organism (Mudgett, 1986). The optimum pH and temperature for tannase production were 6.5 and 28°C, respectively. Moisure content is one of the critical parameters that affects enzyme production in solid-state fermentation. The optimum moisture content for tannase production was determined to be 62% (Lekha *et al.*, 1994). The inoculum level used was 5% (w/w), and the maximum amount of tannase was produced on day 3. Increased bed height was found to have a negative effect on tannase production, probably due to reduced aeration and increased heat buildup (Lekha, 1996).

Relevance of SSF for Tannase Production

Solid-state fermentation offers a number of economic advantages over conventional submerged fermentation for enzyme production (Mudgett, 1986). The production medium is often simple, using agroindustrial by-products like wheat bran, rice bran, or wheat straw as the substrate (Mitchell and Lonsane, 1992). Because the moisture level is low, the volume of medium per unit weight of substrate is low; hence, enzyme activity is usually very high (Deschamps and Huet, 1985). Thus, to achieve a given enzyme productivity, fermentor volumes can be much smaller than in submerged fermentor systems (Mitchell and Lonsane, 1992). Additionally, effluent treatment requirements are also reduced (Moo-Young *et al.*, 1983).

Direct spore seeding is usually sufficient (Mitchell and Lonsane, 1992). Extraction of the enzyme from fermented bran with a small amount of water yields a relatively concentrated enzyme product (Frost and Moss, 1987). The inexpensive media, the high enzyme productivity, the easy enzyme recovery, and the reduced effluent treatment requirements involved in solid-state fermentation help in bringing down the cost of enzyme production (Ramakrishna *et al.*, 1982).

Solid-state fermentation has been largely neglected since World War Two in the West. Consequently, negligible research and developmental efforts have been put into the techniques involved in solid-state fermentation (Mitchell and Lonsane, 1992). No comparison of the economics was made between solid-state and submerged fermentation before submerged fermentation was selected for intensive development in the 1940s (Ralph, 1976). The technique of submerged fermentation in aerated stirred tanks, mainly developed for antibiotics, gained wide acceptance and was soon applied to the manufacture of industrial enzymes (Frost and Moss, 1987). It has been estimated that enzyme production by solid-state fermentation at present accounts for about only 5% of world total market enzyme sales (Frost, 1986).

Comparative studies of tannase production by *A. niger* using three different fermentation methods revealed that enzyme production with solid-state fermentation was 2.5 and 4.8 times higher compared to that for submerged and liquid-surface fermentation, respectively (Lekha and Lonsane, 1993). The fermentation time required to produce maximum enzyme was only 3 days, compared to 6 days for submerged and liquid-surface fermentation. The enzyme was completely extracellular in solid-state fermentation compared to the partly intracellular nature of the enzyme obtained from submerged and liquid-surface fermentation (Lekha and Lonsane, 1993). Tannase produced by solid-state fermentation was found to be more thermostable compared to the extra- and intracellular tannase produced by submerged fermentation (Lekha and Lonsane, 1993).

These studies clearly indicate the advantages of solid-state fermentation for tannase production. A number of commercial extracellular enzymes (e.g., protease, amylase, pectinase, amyloglucosidase, and rennin) are successfully produced by solid-state fermentation in Japan (Cannel and Moo-Young, 1980).

A resurgence of interest has occurred in Western and European countries in response to the ever-rising demand for economy in the fermentation process (Steinkraus, 1984). Proper exploitation of this technique could lead to significant cost reductions in tannase production. The use of agroindustrial wastes as solid substrates will not only help to combat environmental pollution, but will also eliminate their treatment by cost-intensive waste-disposal techniques (Lonsane and Ghildyal, 1992).

VII. Regulation of Tannase Biosynthesis

There are three modes of genetic regulatory mechanisms that affect the synthesis and secretion of an enzyme, viz., induction, catabolite regulation, and feedback regulation (Demain, 1971).

A. Regulation by Induction

In the case of inducible enzymes, an enzyme is synthesized only when the substrate or substrate analogue is present in the medium (Demain, 1971). Tannase is an inducible enzyme produced only in the presence of tannic acid or its end-product, that is, gallic acid (Knudson, 1913; Nishira and Mugibayashi, 1953). The minimum structural requirement for adaptive tannase formation is gallic acid (Nishira, 1959a). Kita (1917) reported that *A. oryzae* secretes tannase even when it is cultured on a substrate containing no tannin. However, in all the media

reported for tannase production, tannic acid was present as an inducer. The minimum concentration of tannic acid that could stimulate formation of tannase was found to be 0.1% (Knudson, 1913).

However, the mechanism of induction by tannic acid is not known. The large size and reactivity of tannic acid prevent uptake of the molecule through the cell membrane. The reaction of tannic acid with the cell wall has been reported to impair permeability (Herz and Kaplan, 1968). These facts suggest that tannic acid cannot be the inducing agent. The mechanism of induction is probably similar to that of cellulase (Singh and Hayashi, 1995), that is, the microorganism produces a basic level or a constitutive amount of tannase that hydrolyzes tannic acid to glucose and gallic acid, which can enter the microbial cell and function as an inducer. In fact, the rate of induction was faster when gallic acid was used as an inducer (Seiji *et al.*, 1973).

B. Regulation by Catabolite Repression

This involves the inhibition of the formation of certain enzymes by the catabolic products of the readily utilizable carbon source (Demain, 1971). The strongest repression is observed in a medium containing glucose (Eveleigh and Montenecourt, 1979). The repressed enzyme can be constitutive or inducible, but in most cases inducible enzymes are involved (Demain, 1971). However, in the case of tannase, no catabolite repression was observed. Knudson (1913) reported that the presence of 10% sucrose did not inhibit the secretion of tannase by *A. niger*, but the secretion of tannase was inhibited to a certain extent in the case of *Penicillium* spp. In addition, the presence of glucose did not repress tannase formation (Seiji *et al.*, 1973). Another interesting observation was the shortening of the time lag for tannase production by glucose, ribose, and glycerol with tannic acid as the inducer. Transfer of the induced mycelium in a glucose-containing medium, without the inducer, caused an almost immediate cessation of tannase formation, indicating that the tannase mRNA of this strain is very unstable. Production of tannase resumed on addition of gallic acid (Seiji *et al.*, 1973).

C. Regulation by Feedback Inhibition

Feedback inhibition is the phenomenon by which the final metabolite of a pathway inhibits the synthesis of an enzyme, usually the first enzyme (Demain, 1971). No reports on feedback inhibition have been published in the case of tannase. In fact, gallic acid, which is the end-product, has been reported to induce tannase synthesis (Seiji *et al.*,

1973). Pyrogallol, which is also an end-product of tannin hydrolysis, does not stimulate enzyme formation (Knudson, 1913; Nishira, 1960).

VIII. Location of Tannase

Tannase, when produced by submerged fermentation, has been reported to be intracellular in most fungi, for example, *P. chrysogenum* (Rajkumar and Nandy, 1983), *A. flavus* (Yamada *et al.*, 1968), *A. niger* (Pourrat *et al.*, 1982), and *Aspergillus tamari* (Vandamme *et al.*, 1989). In the case of *A. oryzae pseudoflavus*, during growth in an inorganic salt medium containing an inducer, accumulation of both intra- and extracellular tannase was reported (Seiji *et al.*, 1973). Okamura *et al.* (1988) reported that tannase produced by *A. oryzae* was extracellular. In yeasts and bacteria, it has been reported to be extracellular (Aoki *et al.*, 1976; Deschamps *et al.*, 1983).

Tannase produced by *A. niger* PKL 104 in submerged fermentation was completely intracellular when produced by submerged and liquid-surface fermentation during the initial 48 h of growth. Subsequently, the enzyme was secreted into the medium with progress of the fermentation (Lekha and Lonsane, 1993). However, tannase produced by the same culture in solid-state fermentation was completely extracellular throughout the course of fermentation (Lekha and Lonsane, 1993).

IX. Purification of Tannase

Tannase has been purified from a variety of fungi, namely, *A. flavus* (Yamada *et al.*, 1968), *A. oryzae* (Iibuchi *et al.*, 1968; Fumihiko and Kiyoshi, 1975), *Candida* spp. (Aoki *et al.*, 1976), *P. chrysogenum* (Rajkumar and Nandy, 1983), and *A. niger* (Barthomeuf *et al.*, 1994). The starting material was either culture filtrate (Fumihiko and Kiyoshi, 1975) or mycelial extract obtained by sonication of the mycelial cells (Yamada *et al.*, 1968), depending on the localization of the enzyme. Tannase purification schemes have generally used standard column chromatographic techniques, mainly ion-exchange and gel filtration.

The first step is usually ammonium sulfate (Yamada *et al.*, 1968) or acetone precipitation (Beverini and Metche, 1990), which resulted in an initial concentration as well as purification. Aoki *et al.* (1976) reported the failure of ammonium sulfate to precipitate yeast tannase. Various other precipitating agents were tested, and it was found that rivanol (2-ethoxy-6,9-diaminoacridinium lactate) was suitable for precipitation of tannase from the culture broth. Precipitation of tannase using such

polymers (1–90%) as polyethylene glycol, polyvinyl alcohol, and dextran has also been reported (Kazuo *et al.*, 1973).

The second step employed in most cases was ion-exchange chromatography (Rajkumar and Nandy, 1983; Yamada *et al.*, 1968). Tannase is known to be an acidic protein (Adachi *et al.*, 1968), and so an anion exchanger was used in all reported cases. DEAE-sephadex/cellulose was used for purification of tannase from *A. flavus*, *A. oryzae*, and *P. chrysogenum* (Yamada *et al.*, 1968; Fumihiko and Kiyoshi, 1975; Rajkumar and Nandy, 1983). In the case of yeast tannase, the enzyme was adsorbed so strongly to DEAE-cellulose that, even with the use of a 0.9-*M* phosphate buffer, the enzyme could not be eluted from the column. Therefore, ECTEOLA-cellulose, with a less exchangeable capacity, was used instead of DEAE-cellulose (Aoki *et al.*, 1976).

The enzyme was adsorbed to the ion-exchange column at an acidic pH (pH 5) and eluted from the column using gradient elution with increasing ionic strength of the buffer or salt (Iibuchi *et al.*, 1968; Rajkumar and Nandy, 1983). The last step employed in tannase purification was gel-filtration chromatography (Rajkumar and Nandy, 1983; Yamada *et al.*, 1968; Iibuchi *et al.*, 1968). Since tannase is a high-molecular-weight protein (~200,000 daltons), sephadex G-200 was used by most workers (Yamada *et al.*, 1968; Rajkumar and Nandy, 1983; Aoki *et al.*, 1976). Iibuchi *et al.* (1968) used sephadex G-100 in the final tannase purification step.

Affinity chromatography offers an extremely high-resolution method for purifying proteins and has been widely used in the laboratory (Volesky and Luong, 1985). It can significantly reduce the number of steps required to purify a protein due to its biospecificity (Cuatrecasas *et al.*, 1968) and has an excellent possibility of becoming a large-scale recovery technique (Clonis *et al.*, 1986). However, this technique was not explored for tannase purification, except in one report on the separation of isozymes of tannase from *A. oryzae*. Tannase I, which has strong esterase activity, and tannase II, which has strong depsidase activity, were separated on Con-A Ultrogel by elution with methyl-D-glucose at concentrations of 10 and 50 m*M*, respectively (Beverini and Metche, 1990).

Barthomeuf *et al.* (1994) used a different protocol for purification of tannase from *A. niger*. Crude enzyme was obtained by physical disruption of the mycelium, employing congelation–decongelation and addition of concanavalin A into the medium. Insoluble materials were eliminated by centrifugation, and the supernatant was filtered through a 0.45-μm membrane. The filtrate was subjected to tangential ultrafiltration with a 200,000-dalton threshold membrane, followed by filtra-

TABLE IV

SPECIFIC ACTIVITIES OF TANNASE BEFORE AND AFTER PURIFICATION

Source	Location	Specific activity[a]		Final purification step	Yield, %	Reference
		Crude extract	Purified enzyme			
A. niger	Mycelium	68	1980	High pressure size exclusion chromatography	15.1	Barthomeuf *et al.*, 1994
A. oryzae IAM 2636	Culture broth	6.8	77	Sephadex G-100	4.0	Fumihiko & Kiyoshi, 1975
A. oryzae	Culture broth	ND	78	Sephadex G-100	11.5	Iibuchi *et al.*, 1967
A. flavus	Mycelium	4.3×10^3	135×10^3	Sephadex G-200	48.2	Yamada *et al.*, 1968
P. chrysogenum	Mycelium	3.6	86	Sephadex G-200	18.5	Rajkumar & Nandy, 1983
Candida spp.	Culture broth	ND	92	Sephadex G-200	7.3	Aoki *et al.*, 1976

[a]Specific activity in U/mg protein.

tion through a membrane with a molecular weight cutoff of 100,000 daltons. Addition of con A to the medium increased tannase recovery from 22.5 to 34.5%, probably by facilitating desportion of enzyme from its binding site (Barthomeuf *et al.*, 1994). Table IV summarizes the recovery and specific activities of tannase purified from different microorganisms.

X. Properties of Tannase

Tannases from many fungi have been extensively characterized. These results have led not only to an understanding of how these enzymes operate and are regulated, but also to an appreciation of their vastly different physicochemical properties.

A. pH Optimum and Stability

In general, tannase is an acidic protein with an optimum pH around 5.5 (Table V). In the case of *A. niger* and *P. chrysogenum*, the optimum pH was 6 (Barthomeuf *et al.*, 1994; Rajkumar and Nandy, 1983). Tannases from yeast (Aoki *et al.*, 1976) and *A. oryzae* (Iibuchi *et al.*, 1968) was stable in a broad pH range (3.5–8), whereas tannases produced by *P. chrysogenum* (Rajkumar and Nandy, 1983) and *A. oryzae* (Yamada *et al.*, 1968) were stable in the narrow ranges of 4.5–6 and 5–5.5, respectively.

B. Temperature Optimum and Stability

The temperature optimum reported for tannase activity was around 30°C in the case of *A. oryzae* (Iibuchi *et al.*, 1968), *P. chrysogenum* (Rajkumar and Nandy, 1983), and *A. niger* (Barthomeuf *et al.*, 1994), except for 60 and 50°C in the cases of *A. flavus* (Yamada *et al.*, 1968) and yeast tannase (Aoki *et al.*, 1976), respectively. Tannase from *A. niger*, *A. oryzae*, and *P. chrysogenum* were stable up to 30°C (Table V), and *A. flavus* tannase was stable up to 60°C for 10 min (Yamada *et al.*, 1968).

C. Molecular Mass

Tannase is a high-molecular-weight protein whose molecular weight is reported to vary from 186,000 to 300,000 daltons, depending on the strain (Table V). The native enzyme consists of two different polypeptide chains (subunits) of similar molecular size (Adachi *et al.*, 1968; Aoki *et al.*, 1976; Rajkumar and Nandy, 1985).

Amino acid analysis of tannase from *Candida* revealed that the enzyme consisted of 786 amino acid residues per molecule (Aoki *et al.*, 1976). Alanine and arginine were identified as the N-terminal amino acids in the case of tannase from *A. flavus* (Yamada *et al.*, 1968) and *P. chrysogenum* (Rajkumar and Nandy, 1985). The C-terminal amino acids lysine and glutamic acid were present only in *P. chrysogenum* tannase (Rajkumar and Nandy, 1985).

The enzyme is a typical serine esterase and was completely inactivated by phenyl methyl sulfonyl fluoride (Rajkumar and Nandy, 1983). Radio-isotope studies using diisopropyl-^{32}P-phosphoryl (DI^{32}P) tannase revealed that the tannase molecule contains one essential serine (Adachi *et al.*, 1971). The amino acid sequence around active serine was

similar to that of subtilisin, composed of –Thr–Ser–Meth– (Adachi *et al.*, 1971).

D. Enzyme Inhibition

Tannase from *A. niger* was inactivated by O-phenanthroline, phenyl methyl sulfonyl fluoride, ethylene diamine tetraacetic acid (EDTA), 2-mercaptoethanol, and sodium thioglycolate (Barthomeuf *et al.*, 1994). Tannase from *A. oryzae* was also completely inactivated after dialysis against an EDTA solution (Iibuchi *et al.*, 1968), whereas no inhibition was observed with such chelating agents as EDTA and O-phenanthroline and such SH agents as parachloromercuric chloride in the case of tannase from *A. flavus*, but it was completely inactivated by diisopropyl fluorophosphate (DFP) (Yamada *et al.*, 1968). Yeast tannase was also not inhibited by EDTA (Aoki *et al.*, 1976). Tannase from *A. oryzae* (Iibuchi *et al.*, 1968) and *P. chrysogenum* (Rajkumar and Nandy, 1983) was most inhibited by Zn^{2+}, Cu^{2+}, and Fe^{2+}. Tannase from *A. niger* was strongly inhibited by copper and to a lesser extent by ferric and zinc ions at concentrations of 20 m*M*. This inhibition could be strongly attenuated by adding 20 m*M* EDTA into the reaction medium (Barthomeuf *et al.*, 1994).

Tannase from *A. oryzae* was inhibited competitively by substrate analogues like *n*-propyl gallate and isoamyl gallate, which have phenolic hydroxyl groups, except for 2,6-dihydroxy benzoic acid, which inhibits noncompetitively. Therefore, the binding site of tannase may be able to react with any phenolic hydroxyl group, although the substrate forming a true enzyme–substrate complex must be an ester compound of gallic acid (Iibuchi *et al.*, 1972). Pyrogallol, gallic acid, gallaldehyde, and gallamide were reported to be competitive inhibitors for tannase produced by *A. niger*, with K_i values of 217, 661, 1510, and 175 μ*M*, respectively (Parmentier and Verbruggen, 1973).

E. K_m of Tannase

The K_m of tannase produced by *P. chrysogenum* was reported to be 0.48×10^{-4} *M* when tannic acid was used as the substrate (Rajkumar and Nandy, 1983). The K_m values of tannase produced by *A. flavus* were 0.5×10^{-4} *M* for tannic acid, 1.4×10^{-4} *M* for glucose-1-gallate, and 8.6×10^{-4} *M* for methyl gallate (Yamada *et al.*, 1968). The K_m values for different substrates revealed that natural tannic acid is the best substrate for the enzyme and that methyl gallate is less reactive than tannic acid (Yamada *et al.*, 1968).

TABLE V

PROPERTIES OF PURIFIED TANNASE

Micro-organism	pH		Temperature, °C		Properties	
	Opt.	Stab.	Opt.	Stab.	IEP	Mol. wt.
A. flavus IFO 5839	5.5	5–5.5	50–60	60	4	192,000
A. niger LCF 8	6.0	3.5–8	35	4–45	4.3	186,000
A. oryzae	5.5	3–7.5	30–40	30		200,000
Asp. sp. AN 11	5.5	5–6.5	30–40	30		200,000
Penicillium chrysogenum	5–6	4.5–6	30–40	30		300,000
Candida sp. K 16	6	3.5–7.5	50	40		250,000

Opt = optimum; Stab = stability; IEP = isoelectric point.

F. CARBOHYDRATE CONTENT

All the fungal tannases reported thus far are glycoproteins. The carbohydrate content of tannase is relatively high, primarily consisting of such neutral sugars as mannose, galactose, and hexosamines (Aoki *et al.*, 1976). *P. chrysogenum* tannase contained 66.2% neutral sugars and

TABLE V

CONTINUED

Inhibitors (% inhibition)	Inactivators	Protein content	Carbohydrate	Reference
DFP (98%)		80%	25.4% hexose	Yamada *et al.*, 1968 Adachi *et al.*, 1971
$CuSO_4$ (68%) $ZnCl_2$ (39%) $FeCl_3$ (13%) DFP (83%)	O-phenanthroline PMSF, EDTA 2 mercaptoethanol sodium thioglycolate $MgSO_4$, $CaCl_2$ $MnCl_2$, $CoCl_2$		43% sugar	Barthomeuf *et al.*, 1994
Zn (70%) Cu (70%)	EDTA			Iibuchi *et al.*, 1968, 1972
$CuCl_2$ $ZnCl_2$				Chae *et al.*, 1983
Cu (53%) Zn, Fe (45%) Mn (22%) Mg (17%)	PMSF	32%	66.2% neutral sugar, 1–9% hexosamine	Rajkumar and Nandy, 1983, 1985
		35%	61.9% neutral sugars hexosamine 2.2%	Aoki *et al.* 1976

1.9% hexosamine (Rajkumar and Nandy, 1985). Yeast tannase contained 61.9% hexose and 2.2% hexosamine (Aoki *et al.*, 1976), whereas tannase from *A. flavus* had a comparatively smaller (25.4% hexose) carbohydrate content (Adachi *et al.*, 1968).

The polypeptide moiety was relatively small and varied from strain to strain, ranging from 12.5% (nitrogen) in the case of *A. flavus* (Yamada

et al., 1968) to 38% (protein) in the case of yeast tannase (Aoki *et al.*, 1976).

The biological significance of such a high carbohydrate content is not known. It is well known that tannins associate strongly with proteins by hydrogen bond formation between the phenolic hydroxyl group of the tannin and the carboxyl group of the protein peptide bonds, forming insoluble precipitates (Canon, 1955). Fungal tannases not only escape the inhibitory effects of hydrolyzable tannins, they also render tannin innocuous by cleaving it into inert products like glucose and gallic acid (Strumeyer and Malin, 1970). It was suggested that the carbohydrate coating most probably protects the polypeptide backbone, which would then be less accessible to the tannin molecule. The carbohydrate coating on tannase not only protects the enzyme but also directs the substrate to the limited, but accessible, active-site region, where cleavage to non-tannin products can be accomplished (Strumeyer and Malin, 1970).

XI. Isozymes of Tannase

Toth and Barsony (1943) reported that gallotannin-decomposing tannase contains two separate enzymes—an esterase and a depsidase—with specificities for ester linkage and *m*-digallic acid ester linkages, respectively (Fig. 4). Tannase is composed of a mixture of both (Toth, 1944). Among these enzymes, gallic acid esterase is predominant. The identities of these two enzymes were subsequently confirmed by Haslam *et al.* (1961). The esterase and depsidase were fractionated using column chromatography, and the two enzymes differed slightly with respect to their pH optimum and stability (Haslam and Stangroom, 1966).

Beverini and Metche (1990) fractionated the two isozymes of tannase by affinity chromatography on Con-A Ultrogel. Tannase I has esterase activity, while tannase II had a strong affinity for *m*-digallic acid and pyrogallol derivatives containing depside groups. The values of K_m for *m*-digallic acid and methyl gallate were 0.7 and 6.2 m*M*, respectively, for tannase II, whereas the affinities of tannase I for ester and depside substrates were very similar (2.0 m*M* for *m*-digallic acid and 1.7 m*M* for methyl gallate). The two isozymes also differed in terms of their carbohydrate and polypeptide contents (Beverini and Metche, 1990).

XII. Mode of Action of Tannase

An extensive study on the pathway of hydrolysis, substrate specificity, and inhibition of tannase produced by *A. oryzae* was carried out by

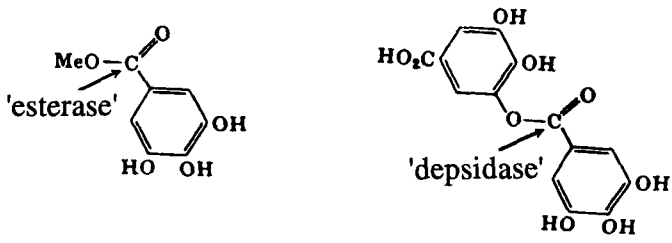


FIG. 4. Esterase and depsidase activities of tannase.

libuchi *et al.* (1972). Tannic acid (Fig. 5(I)) was found to be completely hydrolyzed by the enzyme to gallic acid and glucose through 2,3,4,6-tetragalloyl glucose (Fig. 5(III)) and two kinds of monogalloyl glucose (Fig. 5(IV)). The pathway of hydrolysis of tannic acid by the enzyme is discussed later in this chapter.

The position of gallic acid in the two kinds of monogalloyl glucose has not been determined. Nishira (1962) reported the formation of an intermediate compound, that is, glucose–gallic acid, which on hydrolysis produced gallic acid and glucose.

Deschamps *et al.* (1983) observed the formation of two intermediate products during degradation of tannic acid by *Bacillus pumilis*. These intermediate products were considered to be di- and trigallic acids, probably bonded to glucose. However, these intermediates were not found when other strains (e.g., *Bacillus polymyxa*, *Klebsiella pneumoniae*, and *Corynebacterium* spp.) were employed.

Rajkumar and Nandy (1986) reported that *P. chrysogenum* metabolizes tannic acid and chebulinic acid with the formation of an intermediate compound, which is subsequently converted to gallic acid. The intermediate product had an absorbance at 277 nm and produced gallic acid on hydrolysis with sulfuric acid.

P. chrysogenum metabolizes gallic acid, producing oxalic acid as the final product (Rajkumar and Nandy, 1986). It was found that pyrogallol, an inhibitor of *P. chrysogenum*, was not metabolized by the organism, but tannic acid and gallic acid were metabolized after 3 and 4 days, respectively, in the presence of pyrogallol. Watanabe (1965) studied the decomposition of gallic acid by *A. niger* using fluoroacetate as the inhibitor. He reported that citric acid, *cis*-aconitic acid, and α-keto glutaric acid were the degradation products.

Inhibition studies using 0.002 *M* fluoroacetate revealed that *P. chrysogenum* can metabolize gallic acid following a different metabolic path-

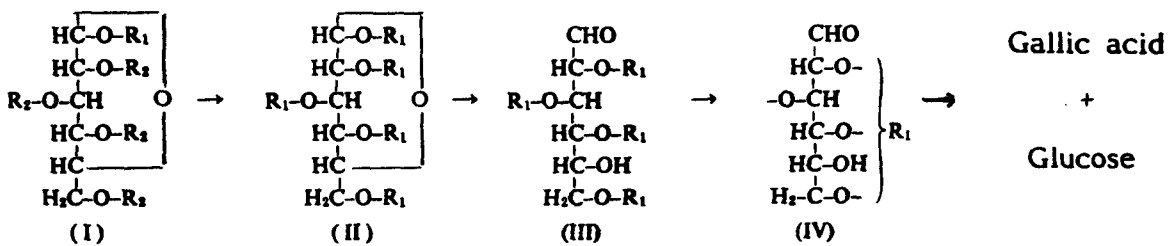


FIG. 5. Hydrolyzing pathway of tannic acid by tannase.

way, where citric acid is the only product of metabolism (Rajkumar and Nandy, 1986).

XIII. Immobilization of Tannase

With an understanding of the nature of industrial enzymes and their catalytic potential, the use of these enzymes has gradually been extended to a variety of fields, including food processing, brewing, pharmaceuticals, medicine, textiles, and detergents (Kennedy *et al.*, 1987). Despite the advantages of using enzymes, their commercial use has been limited because (a) most of them are relatively unstable, (b) the cost of enzyme isolation and purification is still high, and (c) it is technically expensive to recover active enzyme from the reaction mixture after completion of the catalytic process (Kennedy *et al.*, 1987). These problems can be solved by immobilizing the enzyme. Indeed, immobilized enzymes bypass many of the shortcomings that frequently make soluble enzymes commercially impractical (Trevan, 1980). Their superiority comes from cheaper cost due to reuse, improved stability, prevention of allergenic responses, or other undesirable interactions between the enzyme and specific components of the reaction mixture, including contamination of the final product by the enzyme (Trevan, 1980).

The first report on the immobilization of tannase was by Weetal and Detar (1974). Tannase from a commercial source was covalently attached to alkylamine porous silica activated with glutaraldehyde. A comparative study on kinetic parameters, pH, temperature, and operational stability was carried out. The optimum pH of tannase was slightly shifted to the alkaline region and thermal stability was increased after immobilization (Weetal and Detar, 1974). Tannase from *A. niger* was immobilized according to this method and used for enzymatic gallic acid esterification in organic solvents (Weetal, 1985a). A comparison of the synthesis catalyzed by soluble and immobilized tannases indicated a difference in obtained yields. In the enzymatic synthesis of propyl gallate using immobilized tannase, the yield of propyl gallate was 41.4% with respect to gallic acid, compared to 3.5% when soluble tannase was used (Weetal, 1985b). In a soluble system, maximum synthesis was observed with methanol (40%), while in an immobilized system maximum synthesis (85%) was observed with amyl alcohol (Weetal, 1985a).

In a patent on green tea conversion using tannase and natural tea enzymes, Sanderson *et al.* (1974) used immobilized tannase prepared by a technique employing diazo-coupling onto glass beads. Coggon *et al.* (1975) also described a process for production of instant tea using

tannase immobilized on glass beads by diazo-coupling. The immobilized tannase was capable of solubilizing between 60 and 80% of the tea cream in tea extracts using either a batchwise or continuous-column treatment process. The immobilized tannase was shown to undergo little loss of enzyme activity on extended use. The enzyme can be readily separated from the reaction mixture in this technique, used again, and provides a means for complete removal of enzyme from the material undergoing treatment (Sanderson *et al.*, 1974).

Katwa *et al.* (1981) described a procedure for the assay of immobilized tannase with polyacrylamide, collagen, and Duolite as matrices, based on spectrophotometric determination of gallic acid formed in the enzymatic hydrolysis of tannic acid. The kinetic parameters of the enzymatic reaction have been studied and an assay procedure formulated. This method was found to be much more accurate than those reported previously.

XIV. Applications of Tannase

Tannase is extensively applied in the food, feed, beverage, brewing, pharmaceutical, and chemical industries.

A. Instant Tea

This is the most promising application of tannase (Sanderson *et al.*, 1974). An important requirement of instant tea is cold-water solubility, as instant tea is mostly used to prepare iced tea (Coggon *et al.*, 1975). The presence of tea cream, a cold water-insoluble precipitate that forms naturally in brewed tea beverages when allowed to stand for a few hours at or below 4°C, is therefore a major problem in instant tea manufacture (Sanderson, 1972).

Tea cream is a hydrogen-bonded complex of the polymeric black tea polyphenols (i.e., thearubigins and theaflavins) with caffeine. The galloyl groups on the black tea phenols are involved in complexation with caffeine (Wickremansinghe, 1978). In the conventional process for preparing instant tea, the hot-water extract of tea is subjected low temperatures with agitation, followed by centrifugation of the tea cream (Nagalakshmi *et al.*, 1985). The tea cream is usually discarded, which represents a considerable loss of the major flavor components (Coggon *et al.*, 1975).

The chemical method for solubilizing tea cream involves treatment with sulfite and molecular oxygen together with an alkali (Sanderson, 1972). Instant tea powder produced by chemical methods, when recon-

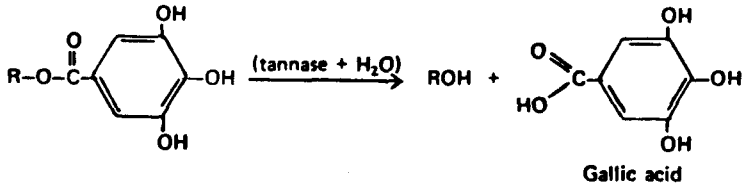


FIG. 6. Deesterification of tea polyphenols by tannase.

stituted as a hot beverage, reacts very badly on addition of milk by taking on a dull-blackish, unpleasant coloration (Coggon *et al.*, 1975). A patent assigned to Tenco Brooke Bond Ltd. (1971) describes the process for the preparation of instant tea from black tea. The enzyme-treated sample at 5°C was slightly hazy and had an undissolved solid content of 1.35%, while an untreated sample at 5°C was visually opaque and had an undissolved solid content of 7.5%.

A process for the preparation of cold water-soluble instant tea from fresh green tea flush that uses tannase in a preconversion treatment was described by Sanderson *et al.* (1974). Black tea of high quality and good color that yields a good milk reaction and a substantially reduced level of cold water-insoluble solids was obtained by contacting green tea with tannase, followed by conversion of the green tea to black tea.

Action of Tannase on Tea Polyphenols

Tannase catalyzes the hydrolysis of the ester linkages between galloyl groups and various compounds present in unconverted tea leaves (Sanderson *et al.*, 1974). The reaction is a deesterification (Fig. 6), where R–OH can be epicatechin and epigallocatechin. This deesterification enhances the natural levels of gallic acid and epicatechin in nonconverted green tea leaf material. This favors the formation of large amounts of epitheaflavic acid during the tea conversion process on the tea leaf material, which has undergone preconversion tannase treatment. Epitheaflavic acid is responsible for the bright reddish-black tea-like color and has very good cold-water solubility (Nagalakshmi *et al.*, 1985). Further deesterification of green tea leaf constituents prevents the formation of any gallated tea oxidation products by eliminating the precursors of these compounds, which are normally present in black tea infusions.

Therefore, elimination of such poorly soluble compounds is probably important for producing instant tea with good color and solubility, and for obtaining a good yield when the green tea conversion process is

carried out after preconversion tannase treatment (Sanderson *et al.*, 1974). A continuous process for solubilizing tea cream by passing a hot-water extract of black tea through a column containing immobilized tannase was also described by Coggon *et al.* (1975). A number of patents have been granted for manufacture of instant tea (Table VI).

B. Beer Chillproofing

Masschelein and Batum (1981) reported that tannase produced by a strain of *A. flavus* significantly reduced chill haze formation in beer. Discoloration and haze development during beer storage could be prevented by enzymic hydrolysis of wort phenolics with tannase and lactase (Rossi *et al.*, 1988).

C. Wine Making

Yamada and Tanaka (1972) described the use of tannase in wine making. The enzyme hydrolyzed chlorogenic acid to caffeic acid and quinic acid, which favorably influenced taste. Chae *et al.* (1983) explored the potential of tannase in the manufacture of acorn wine. Korean acorns (*Quercus* spp.) contain 6.5–7.5% tannic acid (Chae and Yu, 1973). Acorn wine was produced from a koji of rice powder and acorn powder (1:1) using an *Aspergillus* strain. The final wine had an ethanol content of 10%, a reducing sugar content of 7%, and a pH of 4.0 (Chae *et al.*, 1983). Tannase was used along with lactase to treat grape juice and grape musts so as to remove phenolic substances for stabilization of the beverage (Cantarelli, 1986; Cantarelli *et al.*, 1989).

D. Production of Gallic Acid

Tannase hydrolyzes tannic acid to glucose and gallic acid (Iibuchi *et al.*, 1972). As early as 1901, Fernback implicated the industrial application of the enzyme in the manufacture of gallic acid using tannin-containing substances as raw materials. Gallic acid is mainly used as a synthetic intermediate for the production of pyrogallol and gallic acid esters used in the food and pharmaceutical industries (Deschamps and Lebeault, 1984). In terms of pharmaceutical production, gallic acid is used in the synthesis of trimethoprim.

At present, gallic acid is made industrially by chemical hydrolysis of naturally occurring gallotannins. Deschamps and Lebeault (1984) reported the production of gallic acid from tara tannin by using *Klebsiella pneumoniae* and *Corynebacterium* spp., where the yield of gallic acid

TABLE VI

PATENTS FOR TEA CREAM SOLUBILIZATION USING TANNASE

1. Tenco Brooke Bond Ltd., U.K. Pat. 1,249,932, 1971.
2. G. W. Sanderson, N. J. Englewood, P. Coggon, and N. Y. Orangeburg, Green tea conversion using tannase and natural tea leaves, U.S. Pat. 3,812,266, 1974.
3. P. Coggon, H. N. Graham, and G. W. Sanderson, Cold water-soluble tea, U.K. Pat. 1,380,135, 1975.
4. P. Coggon, H. N. Graham, A. C. Hoefler, and G. W. Sanderson (Unilever NV), Tea leaves extractable in cold water, Ger. Pat. 2,610,533, 1976.
5. Y. Takino (Coca-Cola), Enzymic solubilization of tea cream, U.S. Pat. 3,959,497, 1976.
6. P. Coggon, H. N. Graham, A. C. Hoefler, and G. W. Sanderson, Tea, U.K. Pat. 1,546,508, 1979.
7. C. H. Tsai (Procter and Gamble), Enzymic treatment of black tea leaf, Eur. Pat. 135,222, 1985.

was 55%. Pourrat *et al.* (1985) described a method for production of gallic acid from tara tannin and sumac tannin using a strain of *A. niger*. The total yield of gallic acid was 30% at 45 h in the case of tara tannin and 9.7% in the case of sumac tannin, in terms of raw material weight (Pourrat *et al.*, 1987). The low yield was due to biodegradation of the gallic acid produced by these strains. Use of mutant strains lacking the capacity to degrade gallic acid or use of immobilized tannase will result in high yields of gallic acid (Deschamps and Lebeault, 1984). Further studies in this direction are needed because, from an economical point of view, as the chemical process is not expensive, a biotechnological process has to be more selective and produce better yields.

Gallic acid is also used in the enzymatic synthesis of propyl gallate, which is mainly used an an antioxidant in fats and oils, as well as in beverages (Weetal, 1985a). A patent was granted (Weetal, 1985b) for the synthesis of propyl gallate using tannase immobilized on porous silica.

E. ANIMAL FEED ADDITIVES

The use of a number of enzymes in animal feed is gaining in importance (Berry and Paterson, 1990). The antinutritional effects of tannins are well known (Singleton and Kratzer, 1969). They are present in a variety of plant materials that are used as feed (Bate-Smith and Rasper,

1969). The use of tannase in the pretreatment of tannin-containing feed may prove beneficial in removal of these undesirable compounds and also improved digestibility.

Tannins form insoluble complexes with proteins (Swain, 1965) and are present in a wide variety of plant materials that are used as foods and feed (Bate-Smith and Rasper, 1969). The interaction of tannins with protein plays an important role in nonruminants (Tamir and Alumot, 1970). They exert their antinutritional effects by complexing with dietary and endogenous proteins, as well as with digestive enzymes, thereby interfering with normal digestion, leading to a drain of high-quality proteins from the body (Salunkhe, *et al.*, 1982). Tannins are also known to interfere with the absorption of iron (Lauren and Lee, 1988). Other deleterious effects of tannins include damage to the mucosal lining of the gastrointestinal tract, alteration in excretion of certain cations, and increased excretion of certain proteins and essential amino acids (Singleton and Kratzer, 1969). The use of tannase as an ingredient of animal feed would improve the digestibility of the feed.

F. MISCELLANEOUS

Tannase may also find use in cosmetology to eliminate the turbidity of plant extracts and in the leather industry to homogenize tannin preparation for high-grade leather tannins (Barthomeuf *et al.*, 1994). Tannase may also find potential application in the food industry for improving food quality by removing undesirable substances (Barthomeuf *et al.*, 1994). Tannase could possibly find application in various fields, including animal nutrition and bioconversion of plant materials.

XV. Conclusions and Future Prospects

Laboratory-scale studies on the production of tannase by solid-state fermentation showed several advantages over conventional submerged fermentation (Lekha *et al.*, 1994). Proper exploitation of this technique would enable a significant reduction in the cost of enzyme production. Scale-up studies are essential to establish the feasibility of the process at an industrial scale.

The advent of recombinant DNA technology has revolutionized research in the field of enzymology. Although tannase has been produced and purified from a number of microbial sources, isolation and characterization of tannase genes would be essential to understand the molecular biology of tannase biosynthesis. The role of the structural and

regulatory genes involved in tannase biosynthesis is also worth investigating in order to successfully clone and overproduce this enzyme in the desired host.

All the tannases reported so far are inducible enzymes, and enzyme production was found to increase with increased tannic acid concentrations. Consequently, high concentrations of tannic acid have to be used for maximum enzyme induction. Genetic improvement of the strain to produce constitutive mutants that can synthesize tannase in the absence of tannic acid would be highly desirable.

From an applications point of view, it would be desirable to immobilize the enzyme to enable its widespread use in food and beverage processing. An equally important aspect would be improvement in the thermostability of tannase.

Tannase is known to have synthetic ability in nonaqueous media (Weetal, 1985a,b). This could be exploited to synthesize very specific gallic acid esters, which are used as antioxidants in the food processing industry.

Some potential applications mentioned in the literature still require further research to emphasize their worth and economic feasibility so as to encourage the use of tannase on a commercial scale.

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