AN ABSTRACT OF THE THESIS OF

Shiao Jing Li for the degree of Master of Science in Horticulture presented on June 7, 1991.

Title: Determination of α-Amylase Activity and Soluble Starch Content in Normal and Cork Spotted 'D'Anjou' Pear Fruit

Abstract approved: _____
Dr. Tim Facteau

There was a linear relationship of α-amylase activity from 2 to 12 hours incubation in both freeze-dried and fresh 'd'Anjou' pear fruit tissue. Activity was greater, however, in fresh as compared with freeze-dried tissues. α-Amylase activity from both types of samples was not detected until 60 minutes incubation. After 90 minutes, activity increased significantly in both freeze-dried and fresh tissue extracts.

Three different buffers (acetate, Tris-HCl and imidazole-HCl) were used at varying pH levels (from 4.60 to 8.23) to ascertain the optimum assay system. Highest specific activity was recorded with an acetate buffer at pH 5.64. The Km value in this system was 1.43 $mg.ml^{-1}$.

Specific activity of α-amylase increased as Ca

concentration in the reaction mixture increased from 1 to 15 mM $CaCl_2$. No changes in specific activity were found as the Ca concentration increased from 15 to 25 mM $CaCl_2$.

Alpha-amylase from 'd'Anjou' pear fruit was purified 5.68 fold using ammonium sulfate fractionation and a desalting column (Bio-Gel p-6).

Activity of α-amylase and protein, calcium and starch concentration were measured in preharvest calcium-treated normal and corkspotted 'd'Anjou' pear fruit at commercial harvest maturity. Activity and specific activity of α-amylase extracted from corkspotted fruit were higher as compared to α-amylase extracted from normal tissue. Protein concentration was greater in corkspotted fruit. Starch levels were less in corkspotted than in normal fruit as evidenced either by an iodine stain visual technique or by quantitative analysis of hot-water soluble starch. No interaction were found with any measured parameter between calcium treatment and fruit condition. Activity of α-amylase extracted from calcium-treated pears was greater than that extracted from normal pears. Corkspotted fruit had less calcium as compared to normal fruit.

Determination of α-Amylase Activity and Soluble Starch Content in Normal and Cork Spotted 'D'Anjou' Pear Fruit

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed June 7, 1991

Commencement June 1992

APPROVED:

Professor of Horticulture in charge of major

Head of Department of Horticulture

Dean of Graduate School

Date thesis presented June 7, 1991

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Tim Facteau, my major professor, for his support, encouragement and guidance during the course of this study.

My appreciation is extended to Dr. Pat Breen for his help. Special thanks to Dr. Paul Chen. and Dr. David Thomas for serving on my graduate committee and their invaluable help.

At the Mid-Columbia Agricultural Research and Extension Center I am also grateful to the staff and my other friends for their assistance.

Table of Contents

List of Figures

List of Tables

List of Appendix Table

DETERMINATION OF α-AMYLASE ACTIVITY AND SOLUBLE STARCH CONTENT IN NORMAL AND CORK SPOTTED 'D'ANJOU' PEAR FRUIT

Chapter 1

INTRODUCTION

Cork spot is a physiological disorder of 'd'Anjou' pear fruit. It is characterized by a bumpy, uneven appearance of the surface with brown, dry, corky tissue in the flesh when peeled. The lesions are usually most common near the calyx end.

It has been reported that cork spot in pear is associated with low fruit calcium levels (Mason and Welsh, 1970; Woodbridge, 1971; Richardson, 1976; Vaz and Richardson, 1984). Bevacqua (1989), however, reported that there was no difference in calcium between normal and disordered fruit. He also found no association with pyruvic kinase activity and cork spot, but affected fruit had higher protein concentration than clear fruit.

Chen (personal communication) noted that 'd'Anjou' pears with cork spot had less starch at harvest than normal pear when stained with iodine. The lack of starch in cork spotted pears suggested that starch degradation might be involved in the development of the disorder in the pulp tissue. Furthermore, since α-amylase is part of enzyme system that degrades starch, changes in activity might be

used as an indicator of susceptibility to cork spot.

Even though cork spot and bitter pit of apple are similar disorders, Farmer (1906) suggested that bitter pit in apple was related to increase starch content. Carne (1927) put forth the theory that bitter pit in apple was necrosis of immature starch-filled cells, resulting from excessive transpiration, followed by osmotic action between the starch-filled cell and those in which the starch has been largely or completely changed to sugar.

Alpha-amylase, an endoglycosidase, randomly hydrolyzes α-(1-4) linkages of amylose and the side chain of glycogen and amylopectin. It can cleave on either side of a branch point except in very highly branched regions, but does not hydrolyze α-(1-6) linkages at branch points (Rawn, 1989). Alpha-amylase is very widely distributed in nature. Its presence can be expected in plant tissues that actively metabolize starch such as germinating cereals and the leaves of many species of plant.

In higher plants, α-amylase has been studied and characterized mainly in cereal crops. Macgregor and Thompson (1974) reported that the specific activity of α-amylase from immature barley was increased 750 fold after purification. Alpha-amylase from immature hard red spring wheat, potato, bean seed and pearl millet have also been purified and characterized (Marchylo et al., 1974; Fan, 1975; Yamasaki and Suzuki, 1979; Abdul-Hussain and Varriana-

Marston, 1982). Although a number of α-amylases from different origins have been purified and characterized according to their physical and kinetic properties, very little is known about α-amylase from fruit crops (Chitarra, 1981).

The starch iodine test is used to asses maturity of apples for common and controlled atmosphere storages (Reid et al., 1982; Kvale, 1986; Lau, 1988). Preliminary research on the iodine test as a maturity indice for 'd'Anjou' pears (Chen, personal communication) suggests that it is more variable than for apples and may not be suitable. However, Fidder and Mann (1972) and Kvale (1986) both believed that the test was a reliable maturity index for the pear cultivars they tested.

The objectives of this study were as follows:

1. To isolate, purify and characterize some properties of α-amylase from 'd'Anjou' pear fruit.
2. To determine if there are differences in activity of α-amylase between normal and cork spotted 'd'Anjou' pear fruit at commercial harvest.
3. To determine if there are differences in starch content between normal and cork spotted 'd'Anjou' pear fruit at commercial harvest.

Chapter 2

LITERATURE REVIEW

Physiological Studies of Pear Cork Spot

Cork spot is a physiological disorder of 'd'Anjou' pear fruit and is similar to bitter pit in apple. It is characterized by a bumpy, uneven appearance of the surface with brown, dry, corky tissue in the flesh when peeled. The lesion are usually most common near the calyx end (Mason and Welsh, 1970; Woodbridge, 1971).

Most research has focused on the relationships of fruit mineral levels, mainly nitrogen, calcium and boron to the incidence of the disorder. It has been reported that cork spot in 'd'Anjou' pear is associated with a low calcium level (Mason and Welsh, 1970; Woodbridge, 1971; Richardson, 1976; 1979). Vaz and Richardson (1984) found that the symptoms increased with decreasing fruit calcium concentration and increasing storage duration.

Al-Ani and Richardson (1984) concluded that cork spotted tissue had higher nitrogen concentration. Excess nitrogen fertilization has been shown to increase the severity and incidence of cork spot (Raese 1982).

Increasing N:Ca ratio has been significantly correlated to cork spot at harvest and after storage although N alone was not correlated (Richardson and Al-Ani, 1982). They

showed that severity of cork spot was not correlated with B, N, K, P, Mg or any other minor element. Similarly, cork spotted and normal pear fruit had different Ca and N : Ca ratios in all types of subsamples, based on either dry or fresh weight. The peel and the cortex tissue showed the greatest difference using Ca concentration and N : Ca ratio. Corkspot was absent in 1985 and 1986, with a peel N : Ca ratio below 6.3 (Curtis et al., 1990). However, Woodbridge (1971) believed that affected fruit usually had higher levels of K and B than did normal fruit. Bevacqua (1989) also reported that fruit with cork spot had higher concentrations of potassium, calcium and magnesium than normal fruit at harvest in one year (1988), but there was no difference in mineral content between normal and disordered fruit in 1987. In his studies, soil tests and leaf analysis did not show any differences between normal trees and trees prone to cork spot. He concluded that cork spot might be related to high concentration of potassium, since difference were more consistent than with other minerals.

Researchers have noted several other differences between normal and cork spotted fruit. Wang and Mellenthin (1973) found that affected 'd'Anjou' pears had higher and accelerated rates of ethylene production and respiration when compared with unaffected fruits. Higher levels of chlorogenic acid level also were associated with d'Anjou' pear tissues affected with cork spot. Since chlorogenic

acid is the principal phenolic compound in pears and is a primary substrate in the enzymatic browing of pear tissue, they concluded that chlorogenic acid was related to the browning of cork spot tissues. They also found that chlorogenic acid levels were higher in sun exposed fruit and suggested that the temperature increase by sunlight might injure cells thus increasing pereability to oxygen and allowing the oxidation of chlorogenic acid.

Citrus leaves deficient in calcium have been shown to have increased activity of pyruvic kinase (PK) (Bar-Akiva et al., 1976). Pyruvic kinase activity is regulated by K, Mg and Ca (Lavon et al., 1988), three cations that have been shown to be involved in cork spot. These relationships led to the speculation that activity of PK could be an indicator of cork spot. Bevacqua (1989) found no consistant association between PK activity and corkspot of 'd'Anjou' pear over two seasons. He did find that cork spotted fruit had higher levels of soluble protein than did normal fruit. Activity of PK has been correlated with bitter pit development in apple (Witney and Kushad, 1990). It is not clear why pears and apples differ with respect to PK activity because cork spot and bitter pit appear to be similar disorders.

The amount and distribution of starch in affected tissues is another difference between bitter pit of apples and cork spot of pears. Pitted areas have elevated

levels of starch (Farmer, 1906; Carne, 1972), which led to the hypothesis that bitter pit was caused by rupture of starch-filled cells. Pears with corkspotted area, however, have less starch when stained by the iodine test (Chen, personal communication).

α-Amylase

α-Amylase, an endoglycosidase, randomly hydrolyzes α-(1-4) linkages of amylose and the side chains of glycogen and amylopectin. It can cleave on either side of a branch point except in very highly branched regions (Rawn, 1989). α-amylase is very widely distributed in nature. Its presence can be expected in plant tissues that actively metabolize starch, such as in germinating cereals and in the leaves of many species of plants. Animal and microbial source also contain α-amylase. An important property of α-amylase is its ability to degrade whole starch granules (Manners,
1985).

β-amylase, an exoglycosidase, sequentially removes β-maltose from the free hemiacetal ends or nonreducing ends of the outer branches. β-amylase stops cleavage before branch point are reached. Neither α-amylase nor β-amylase hydrolyzes the α-(1-6) linkages at branch points. The compounds remaining after hydrolysis by α- and β-amylase are known as limit detrins (Rawn, 1989).

pH and temperature have effects on α-amylase activity and stability. α-amylase usually requires Ca^{2+} for its activity and heat stability. Calcium also plays an important role by regulating the differential production of α-amylase isozyme and intracellular transport and secretory

release of molecules (Beck and Ziegler, 1989).

α-amylase has been purified from a wide range of plant, animal and microbial sources. Many methods are now available for the purification and analysis of α-amylase, including specific precipitation as a glycogen complex, affinity chromotography, ion exchange chromatography and gel filtration chromatography.

pH and α-Amylase

The activity of α-amylase varies with pH. However, α-amylases extracted from different plants have different pH optimas. Greenwood et al., (1965) reported that the pH/activity curves for both barley and malted α-amylase were similar with maximum activity occuring at pH 5.5. The activity of barley α-amylase fell off rapidly at pH values higher than 6 and it was rapidly and irreversibly deactivated at pH values below 4. Both the barley and malted barley α-amylase were completely and irreversibly inactivated by pre-incubation at pH 3.6 for 2 hours. Other cereal α-amylases have been repeated to have optimum pH's of 5.5 (Macgregor and Thompson., 1974; Macgregor, 1978; Dehaas et al., 1976).

Marchylo et al., (1975) found no loss in activity from red spring wheat was found after 30 minutes of incubation at any pH with the exception of pH 3.0. At this pH, the enzyme

was completely inactivated in 2 minutes.

Beleia and Varriano-Marston (1981) reported that the optimum pH for pearl millet α-amylase purified by starch column chromatography was between 4.4 and 4.8. This is lower than that shown for cereal α-amylases. In pearl millet, optimum pH for the glycogen complex α-amylase was 4.4. The activity of the glycogen complex α-amylase was not so drastically altered by changes in pH as was the activity of α-amylase purified by the starch column procedure, particularly between pH values of 6.0 and 7.2. α-amylase from immature wheat also had a maximum activity in that pH region (Marchylo et al., 1975).

For malted millet, Abdul-Hussain and Varriana-Marston (1982) reported that the optimum pH for α-amylase was 4.0-4.5. This value was slightly lower than the 4.4-4.8 range reported by Beleia and Varriano-Marston (1981).

In maturing grains, optimal pH values for α-amylase activity were in the range of 5.5 to 6.0. According to Fan (1975), purified α-amylase activity from potato tubers appeared to be sensitive to acid condition lower than pH 5.0. There seemed to be no marked effects of pH in the range of 5.0-8.5. The optimum, however, was at pH 5.5.

Several reports have appeared in the literature describing activity of α-amylase derived from microbial sources. It was shown that α-amylase from Thermophiles exhibited a rather broad pH optimum in the range of pH 5.5

to 6.5, with half maxima at pH 4.8 and 7.5. The purified enzyme was rapidly inactivated at pH values below 7.0 when held at 65^{0}C (Glymphy and Stutzenberger, 1977). Menzi et al., (1957) found that the pH optimum of α-amylase from β. subtilis was relatively broad with activity not changing significantly between pH 5.0 and 7.0. However Dondero and Montgomery (1978) reported a broad pH optimum with a maximum at pH 7.0 for the hydrolysis of starch in a whole potato slurry by β. subtilis α-amylase. Almost no liquefaction was observed at pH 5.0.

In a recent years, pH optimum of α-amylases extracted from yeast have been reported. Moranelli et al., (1987) showed an optimum in the range from 3.75 - 5.5, but Wilson and Ingledew (1982) and Simoes-Mendes (1984) found an optimum at 6.3 from Schwanniomyces alluvius. The optimum pH of 4.8-6.0 for the α-amylase of Sacc.fibuligera differs from the optimum pH of 5.5 reported for α-amylase of L. kononenkoae (Spencer-Martins and Van Uden, 1979).

With respect to α-amylases extracted from animals, hog pancreatic α-amylase displayed a broad pH optimum with essentially no change in activity between pH 7.0 and pH 8.0. However, the immobilized enzyme showed a pH optimum at pH 7.0 while at pH 8.0 there was only 80% activity compared to the soluble enzyme (Strumeyer et al., 1974). The pH-activity profile of chicken pancreatic amylase was shown to be a bell-shaped curve with a maximum activity at pH 7.5.

The enzyme activity was unaffected at alkaline pH values even after a 24 hr treatment, but it readily decreased after 30 min at pH values below 7 (Buonocore et al., 1977).

In summary, α-amylase extracted from different sources show varying pH optimum. No literature was found reporting pH-activity relationship of α-amylase from tree fruit.

Buffer Systems and α-Amylase

Although the most common buffer used to isolate α-amylase from different sources is an acetate buffer (Greenwood et al., 1965; Fan, 1975; Warchalewski and Tkachuk, 1976; Yamasaki and Suzuki, 1979; Beleia and Varriano-Marston, 1981), other authors have suggested that Tris-HCl buffer could be used to extract this enzyme (Macgregor, 1978; Koshiba and Minamikawa, 1981). Leung and Thorpe (1984) reported that a potassium phosphate buffer (pH 6.85) also could be used to extract the α-amylase isozyme from tobacco callus. Although these reports show that acetate, Tris-HCl and phosphate buffers can be used to extract α-amylase, direct comparison of various buffer system (Koshiba and Minamikawa, 1981; acetate, Tris-HCl and glycine-NaOH; Gogoi et al., 1987; acetate, phosphate and Tris-HCl) showed that acetate buffer in the pH range of 4.8-6.0 resulted in greatest activity.

Swain and Dekker (1966) and Koshiba and Minamikawa

(1981) reported that citrate buffer was an inhibitor of α-amylase. Pre-incubation of enzyme extracts with 0.05 M citrate led to almost 50% inhibition of the amylase activity present (Leung and Thorpe, 1984).

Kinetic Parameters of α-Amylase

The Michaelis constant, Km is associated with the strength of binding of substract to an enzyme. The value of Km is the substrate concentration that gives half maximal reaction velocity. The enzyme is half saturated when [S] = Km (Rawn, 1989). The Km is an important kinetic parameter for several reasons. First, the value of Km characterizes the interaction of enzyme with a given substrate; second, the Km value of many enzymes are near the physiological concentrations of their substrates; third, the allosteric effectors of regulatory enzymes can alter the rate of reaction by changing the value of Km for a substrate (Rawn, 1989).

Fan (1975) observed that the Km value of 5.45 $mg \cdot ml^{-1}$ of soluble starch for potato α-amylase was in the high range of values reported for other α-amylase ($0.6x10^{-3}$ $g \cdot ml^{-1}$ to $3.3x10^{-3}$ $g \cdot ml^{-1}$) (Manning and Campbell, 1961; Depinto and Campbell, 1968). Koshiba (1981) reported that the Km value from the germinating Vigna mungo seeds was found to be approximately 1.1 $mg \cdot ml^{-1}$.

It has been reported that some anions (Cl^{-1}, Br^{-1}) affected the maximum velocity of α-amylase, but that the Km was not dependent on the nature of the anion present (Levevizki and Steer, 1974; Vincenzo and Poerio, 1976).

Calcium and α-Amylase

It has been reported that α-amylase is a calcium-dependent enzyme (Vallee et al., 1959; Applebaum, 1964; Doane, 1969; Terra et al., 1977; Buonocore et al., 1977). Some authors have proposed that plant α-amylases usually require Ca^{2+} for their activity and heat stability and that EDTA inhibits the activity (Swain and Dekker, 1966; Okamoto and Akazawa, 1978; Koshiba and Minamikawa, 1981).

Irshad and Sharma (1981) found that plant α-amylases were quite sensitive to high calcium ion concentration in vitro but in contrast, hog pancreatic and bacterial enzymes were not affected. However, Leung and Thorpe (1984) reported the same level of amylase activity was observed in the presence or absence of 45 mM $CaCl_2$. Thus it appears difficult to classify various α-amylases based on their responses toward high calcium concentration in vitro.

On the other hand, calcium is involved in the release of α-amylase from gibberellic acid (GA_3)-stimulated barley aleurone cells (Chrispells and Varner, 1967). Calcium, in addition to GA_3 was necessary to obtain a high rate of α-

amylase production in aleurone layer removed from the starchy endosperm. Jocobsen et al., (1970) showed that the additional α-amylase activity appearing when Ca^{2+} was added to the incubation medium of isolated aleurone layers was largely the consequence of the appearance of B group isozyme.

Additional evidence that calcium affects the synthesis of α-amylase in cereal grain tissue was obtained using rice. The rate of α-amylase synthesis was saturated at about 0.5 mM $CaCl_2$ while the secretion rate continue to rise at 10 mM $CaCl_2$ (Mitsui et al., 1984). In a study of α-amylase secretion from single aleurone layers, Varne and Mense (1972) noted that calcium enhanced α-amylase release from aleurone layers and hypothesized that ion binding to cell wall was necessary for the passage of amylase through the wall. Moll and Jone (1981) also studied the secretion of α-amylase from single aleurone layers and concluded that the calcium effect is mediated at the plasma membrane.

Purification of α-Amylase

Heat treatment has been used by several workers to partially purify α-amylase. Greenwood and Milne (1968) suggested that a high protein concentration in the enzyme extract during heat treatment was critical for removing β-amylase and recovering α-amylase. Warchalewski and Tkachuk

(1976) also reported heat treatment at 70^{0}C destroyed all of the β-amylase. Immature wheat α-amylase in crude extracts was reasonably stable to heat and a heat-treatment step was advantageous, therefore, in removing a large amount of contaminating heat-labile proteins (Marchylo et al., 1975).

However, Beleia and Varriana-Marston (1981) reported that heat treating a crude extract from pearl millet increased specific enzyme activity but reduced amylase activity. Abdul-Hussain and Varriana-Marston (1982) also showed that α-amylase activity of a crude extract was substantially reduced as a result of heat treatment.

The most often used method of protein precipitation is by addition of inorganic salts such as ammonium sulfate. Ammonium sulfate precipitation has been used to purify α-amylase resulting in increased specific activity (Yamasaki and Suzuki, 1979; Koshiba and Minamikawa, 1981; 1983; Sakano et al., 1981; Beleia and Varriano-Marston, 1981; Hayashida and Teramoto, 1986).

The absorption of α-amylase on starch granules has been used for many years as a step in the isolation of the enzyme. However, this method has suffered because starch granules are complex aggregates which are neither pure nor homogenous. A study carried out by Schwimnner and Ball (1949) indicated that glycogen in 40% ethanol was superior to starch granules as an absorbent of malt α-amylase.

Loyter and Schramm (1962) first demonstrated the efficiency of the method by which α-amylases from various sources were specifically prepitated from crude extracts forming a glycogen-enzyme complex. These glycogen-enzyme complexes can yield essentially pure enzyme (Macgregor and Thompson, 1974; Marchylo et al., 1975; Fan, 1975; Schanovich and Hill, 1976; Warchalenski and Tkachuk, 1976; Beleia and Varriano-Marston, 1981; Abdul-Hussain, 1982; Fursov et al., 1986).

α-Amylase can be further purified by the following three methods:

Ion-exchange Chromatography

Ion exchange is defined as the reversiable exchange of ions in solution with ions electrostatically bound to some sort of insoluble support medium. The value of this technique in the isolation and separation of charged compounds is that conditions can be found under which some compounds are electrostatically bound whereas others are not (Cooper, 1989).

Macgregor et al., (1974) found that ion exchange chromatography of a α-amylase-glycogen dextrin mixture on carboxymethyl cellulose (CMC) gave a single, sharp peak of α-amylase activity. Other methods such as DEAE-Cellulose and Dowex anion-exchange resin column were effective for

further purifying α-amylase (Glymph and Stuzenberger, 1977; Yamasaki and Suzuki, 1979; Hayashida and Teramoto, 1986; Moraneill et al., 1987; Gogoi et al., 1987).

Affinity Chromatography

The basis of affinity chromatography is to covalently affix certain molecules to an immobile solid matrix such as an agarose bead. The vast majority of molecules have no affinity for the bound molecule or ligand and flow through the matrix unretained. The desired macromolecules, however, recognizes the bound molecule, bind to it and, thus, are retarded (Cooper, 1989).

Silvanovich and Hill(1976) have demonstrated that β-cyclllodextrin sepharose 6B has a specific affinity for α-amylase but not β-amylase. Koshiba and Minamikawa (1981; 1983) used this affinity chromatography technique to purify the α-amylase which increases in the cotyledon of germinating Vigna mungo seeds. Dextrin was used as a ligand, since it was found to be a competitive inhibitor of amylase and also capable of binding to an activated epoxy-derivative matrix (Turchi and Becher, 1987).

A carbose-AH -Sepharose 4B affinity chromatography was successfully applied to the purification of α-amylase (De Mot and Verachtert, 1987). As compared to other described affinity matrices for amylase, the use of acarbose allows

the efficient separation of a mixture of amylolytic enzymes while ligand degradation is avoided.

Gel Permeation Chromatography

When a mixture of different sized molecules are placed on top of a column containing spherical beads, the large molecules cannot easily diffuse into the pores and are eluted from the column with little or no resistance. The small molecules diffuse into the pores of the gel beads and are, thereby, effectively removed from eluting buffer (Cooper, 1989).

Fan (1975) isolated α-amylase with a Sephadex G75 column. Fractions 6 and 7 had most of the enzyme activity and showed a 30 fold level of purification. The subsequent gel filtration step was found to be more efficient for separating the enzyme from its contaminants than charcoal-celite chromatography which has been used by other researchers. Gel filtration on a Bio-Gel P-100 column was adopted for the last step to purify the enzyme after the second DEAE-cellulose column.

Sakano et al., (1982) and Bhella and Altosaar (1984) reported that the ethanol precipitated crude enzyme extract showed a major protein peak that corresponded to the α-amylase activity when passed through a Sephacryl-200 column.

Starch in Apple and Pear Fruit

Starch, the reserve carbohydrate of the majority of higher plant, occurs as water-insoluble granules that vary in size and shape, depending on the species and maturity of the plant (Manners, 1985). These granules have diameters ranging from 3 to 100um and are comprised of amylose, a linear polysaccharide, and amylopectin, a branched polysaccharide (Rawn, 1989).

Amylose is an unbranched, water-soluble polymer of 100 to 300 D-glucose residues connected by α-(1-4) glucosidic bonds. Amylose has a helical conformation that contains six residues per turn. Iodine molecules can bind in the central core of the amylose helix to form a starch-iodine complex that produces a characteristic deep blue color (Rawn, 1989).

Amylopectin is a branched, water-insoluble polymer comprised of thousands of D-glucose residues. The main chain of amylopectin consists of D-glucose residues attached to the main chain at branch points that occur about every twenty-fifth residue. These side chains are linked to the main chain by α-(1-6) glycosidic bonds, each side chain contains 15 to 25 glucose residues joined by α-(1-4) glycosidic bonds (Rawn, 1989).

Starch in Maturing Apple and Pear

The starch iodine test has long shown promise as a simple means of assessing apple maturity. The method is based on the hydrolysis of starch to sugar as apple fruits ripen. This occurs initially in the core area and then proceeds in the cortical tissue in a pattern characteristic of the variety. Because of the reaction of starch with iodine on a cut surface, the relative degree of hydrolysis can be assessed.

Beattie et al., (1972) and Beattie and Wild (1973) developed a starch pattern index for apple and used regression analysis to determine the optimal harvest dates in different districts. Reid et al., (1982) used the starch iodine pattern as a maturity index for Granny Smith apples. This index had the least variation and could be a reliable parameter for predicting harvest dates. Lau (1988) reported that the starch index was correlated negatively with starch content and positively with internal ethylene concentration in apples.

The starch degradation patterns have been also applied to pear fruit. Fidley and Mann (1972) believed that changes in starch content of the fruit was the most reliable one among the maturity indexs. Kvale (1986) also indicated the close relationship between starch content and its degradation pattern was a reliable maturity index for pear

cultivars. Both of these studies used the iodine test to evaluate starch content. However, Chen (personal communication) found no association between starch content and firmness of 'd'Anjou' pears. Firmness is the common maturity index for 'd'Anjou' pear (Hansen and Mellenthin, 1979).

Bitter Pit in Apple and Starch

Bitter pit of apple and cork spot of 'd'Anjou' pear are assumed to be similar disorders primarily because both are believed to be caused by fruit mineral inbalance. There are several important differences, however. Bitter pit symptoms usually do not become evident until the fruit is in storage even though it is initiated while the fruit is still on the tree. The term cork spot is used in apples to distinguish on tree corking from that appearing in storage (termed bitter pit) (Faust and Shear, 1968). In 'd'Anjou' pears, cork spot usually is descernible 3-4 weeks prior to harvest. Richardson and Al-Ani (1982) reported that up to 50% of cork spot showed up in storage whereas Facteau (Personal communication) found few instances over a several year period when significant amounts of cork spot appeared in stored 'd'Anjou' pear fruit.

Another important difference between cork spot of pears and bitter pit of apples is the occurrence of starch in

affected tissues. Pears affected with cork spot have less starch as measured by the iodine stain test (Chen, personal communication). Apples, however, have more starch. Farmer (1907) first noted that starch content of the discolored pit tissue of bitter pit was much higher than in the unaffected tissue. Carne et al., (1927) suggested that bitter pit was caused by the rupture of starch-filled immature cells because of the differences in osmototic potential between starch-filled and sugar-filled cells. Suit et al., (1932) showed that affected tissue was low in sucrose but high in glucose and fructose. They postulated that metabolic process resulting in sucrose synthesis could be disrupted; this would cause an accumulation of glucose and fructose, resulting in a slow down of starch hydrolysis. However, Atanasoff (1934) suggested that the difference between the starch content of the affected tissue and healthy tissue was too small to create a large osmotic difference.

But Smock (1936) and Smock and Van Doren (1937) thought that persistence of starch could not be taken as cogent evidence of Carne's theory (1927), and that starch accumulation in the affected tissue of corking disorders may be concomittant with the result rather than a causal factor.

Smock and Van Doren (1937) and Macarthur (1940) also reported that starch content in the affected tissue in all types of corking disorder was higher than in the surrounding unaffected tissue.

Chapter 3

SOME PROPERTIES AND PURIFICATION OF α-AMYLASE FROM 'D'ANJOU' PEAR FRUIT

Abstract

There was a linear relationship of α-amylase activity from 2 to 12 hours incubation in both freeze-dried and fresh 'd'Anjou' pear fruit tissue. Activity was greater, however, in fresh as compared with freeze-dried tissues. α-Amylase activity from both types of samples was not detected until 60 minutes incubation. After 90 minutes, activity increased significantly in both freeze-dried and fresh tissue extracts.

Three different buffers (acetate, Tris-HCl and imidazole-HCl) were used at varying pH levels (from 4.60 to 8.23) to ascertain the optimum assay system. Highest specific activity was recorded with an acetate buffer at pH 5.64. The Km value in this system was 1.43 $mg \cdot ml^{-1}$.

Specific activity of α-amylase increased as Ca concentration in the reaction mixture increased from 1 to 15 mM $CaCl_2$. No changes in specific activity were found as the Ca concentration increased from 15 to 25 mM $CaCl_2$.

Alpha-amylase from 'd'Anjou' pear fruit was purified 5.68 fold using ammonium sulfate fractionation and a desalting column (Bio-Gel p-6).

Introduction

Alpha-amylase, an endoglycosidase, randomly hydrolyzes α-(1-4) linkages of the side chain of glycogen and amylopectin. It can cleave on either side of a branch point except in very highly branched regions, but does not hydrolyze α-(1-6) linkages at branch points (Rawn, 1989). Alpha-amylase is very widely distributed in nature. Its presence can be expected in plant tissues that actively metabolize starch, such as, in germinating cereals and the leaves of many species of plant.

In higher plants, α-amylase has been studied and characterized mainly in cereal crops. Margregor and Thompson (1974) reported that the specific activity of α-amylase from immature barley was increased 750 fold after purification. Alpha-amylase from immature hard red spring wheat, potato, bean seed and pearl millet have also been purified and characterized (Marchylo and Kruger, 1974; Fan, 1975; Yamasaki and Suzuki, 1979; Abdul-Hussain and Varriana-Marston, 1982).

Although a number of α-amylase from different origins have been purified and characterized according to their physical and kinetic properties, very little is known about α-amylase from fruit crops. Chitarra (1981) reported that α-amylase activity from whole 'Marmelo' banana fruit increased during the climacteric as starch degradation

proceded, but he did not purify and characterize this enzyme. No published information was found concerning the α-amylase from pear fruit.

The objectives of the present study was to isolate, purify and characterize some properties of α-amylase from 'd'Anjou' pear fruit.

Materials and Methods

Sample Preparation

Normal appearing fruit were collected from six mature 'd'Anjou' pear trees at the Mid-Columbia Agricultural Research and Extension Center in Hood River on 2 Aug 1990. Commercial harvest was on 6 Sept 1990. The fruit were washed in a 0.1% solution of a low phosphorus detergent (Liqui-Nox, Alconox, N.Y., N.Y.), rinsed with distilled water and wiped dry. The calyx half on the fruit was cut into cubes, which were quickly transferred onto a cloth wetted with 0.5% (W/V) sodium metabisulfite solution. A second treated cloth was used to cover the samples as they were accumulated. Approximately 30 g of tissue were sampled, placed in cheesecloth bags, frozen in liquid nitrogen, then stored at -74^{0}C. From the same fruit, a second sample was prepared in the same manner but was lyophilized for 7 days. Approximately 400 g tissue were accumulated by thoroughly mixing the ground lyophilized tissue and all methodology procedures were carried out on this bulked sample. Percent dry weight was calculated from the lyophilized tissue.

Extraction and Partial Purification and Time Course of α-Amylase Activity

All procedures were followed at 0-5^{0}C. Fifteen g lyophilized tissue were homogenized with 7.5 g insoluble polyvinyl polypyrrolidone (PVPP) and 7.5 g white quartz sand with a mortar and pestle. The sample was ground for 5 minutes. The sample was ground for an addition 10 minutes after addition of 50 ml of an extraction buffer consisting of 0.1 M Tris-HCl (pH 7.4), 5 mM $CaCl_2$, 10 mM mercaptoethanol, 5 mM ascorbic acid and 3 mM $Na_2S_2O_5$. The preparation was then squeezed through two layers of miracloth. The extraction step was carried out three times. The resulting supernatant was centrifuged at 5,000x g for 20 minutes. To the crude enzyme solution, solid $(NH_4)_2SO_4$ was added to give 80% saturation. The solution was stirred until it was completely dissolved (40 minutes) and then centrifuged at 10,000x g for 20 minutes. The precipitate was resuspended in 3.0 ml of a solution consisting of 0.1M Tris-HCl (pH 7.4) with 5 mM $CaCl_2$ and 10 mM mercaptoethanol. The resulting extract was applied to an Econo-Pac 10 DC column packed with Bio-Gel p-6 desalting gel. The sample was allowed to run into the column by discarding the first 3.0 ml eluted. The higher molecular components in the extract were eluted by adding 4.0 ml of the above buffer and collecting a 4.0 ml fraction from the column. All the

procedures were exactly the same for the fresh frozen sample except that 100 g tissue was ground into a powder in a chilled mortar. Based on the moisture content, equivalent weights of fresh and lyophilized tissues were extracted.

α-Amylase Assay

Alpha-amylase activity was assayed by incubating 0.25 ml of the enzyme preparation for 2 and 4 hours at 20^0C with 0.25 ml of a soluble starch (1%, W/V, soluble starch, 0.1 M sodium acetate at pH 5.4)-sodium acetate mixture. The reaction was interrupted by the addition of 0.5 ml dinitrosalicylic acid reagent (dissolve at room temperature 1 g. of 3,5-dinitrosalicylic acid in 20 ml. of 2 N NaOH and 50 ml. H_2O; add 30 g. of Rochelle salt, and make to 100 ml. with distilled H_2O), heated for 5 minutes in boiling water, then cooled in ice water. The mixture was then diluted 25 times with distilled water. Optical values were read at 540 nm using a blank which was prepared with the same amount of enzyme, substrate and reagent in which the enzyme activity was inactivated immediately. Optical density readings were converted to umole maltose using a standard curve with maltose (1.0 to 5.0 umole$\cdot$ml^{-1}). Activity was expressed as umole maltose liberated per hour at 20^0C by 1 ml desalted crude extract (DCE). Specific activity was expressed as umole maltose$\cdot$hr$^{-1}\cdot$mg^{-1} protein.

Protein Assay

Soluble protein in the enzyme preparation was determined by the Bio-Rad microassay with bovine serum albumin (BSA) as the standard. Three volumes (0.01, 0.02 and 0.03 ml) enzyme preparation were each diluted to 0.8 ml in clean, dry test tubes. To each, 0.2 ml of the Bio-Rad dye reagent was added then vortexed and/or mixed several times by gentle inversion of the tubes. Optical density readings were taken at 595 nm from 5 to 60 minutes since the color was stable over this period of time. A standard curve was constructed with BSA (1 to 16 $ug \cdot ml^{-1}$) and OD readings were converted to ug protein.

Optimum pH and Buffer System

Table 3.1 gives an outline of the three buffer systems tested and resulting pH values from the extraction and assay buffers used. Lyophilized tissue (15 g) from the bulked sample taken on 2 Aug 1990 were used and extracted. The extraction buffers had concentrations of chemicals described in the extraction and partial purification section. Extraction and desalting procedures were followed as previously discussed. Final pH values resulting from the combination of assay and extraction buffers ranged from 4.60 to 7.60 for acetate, 6.59 to 8.23 for the Tris-HCl and 6.60

to 7.86 for imidazole-HCl buffers, respectively.

Purification of α-Amylase from Pear Fruit

Tris-HCl, acetate and imidazole-HCl buffer systems were used to study the effects of step wise fractionation with ammonium sulfate. The best buffer combinations found in the pH-buffer system experiment were used. For the three systems, the extraction buffer was at pH 7.4. For the assay buffer, Tris-HCl and imidazole-HCl buffers were at pH 7.4 while the acetate buffer was at pH 5.4. Other chemical substances were the same in each buffer as previous described. Lyophilized tissue (15 g) was extracted with each of the three buffers as previously described. To the crude enzyme solution from each buffer, solid ammonium sulfate was added to give 30, 65 and 80% saturation. The solutions were stirred for 40 minutes after each $(NH_4)_2SO_4$ addition, centrifuged at 10,000x g for 20 minutes and the resulting precipitate saved. These procedures were followed for each $(NH_4)_2SO_4$ fraction. Each precipitate was resuspended with the appropriate buffer then desalted. Alpha-amylase activity was determined in each fraction.

Calcium Concentration and Activity of α-Amylase

An extraction buffer consisting of 0.1 M acetate

buffer (pH 7.4), 2 mM $CaCl_2$, 10 mM mercaptoethanol, 5 mM ascorbic acid, 3 mM $Na_2S_2O_5$ was used to study response to increasing Ca^{++} concentration in vitro. Other procedure of the extraction and partial purification were the same as described previously except that a 65% $(NH_4)_2SO_4$ precipitated fraction was used. 0.25 ml enzyme solution was mixed with 0.25 ml of a soluble starch-sodium acetate buffer (pH 5.40) solution which contain 0, 8, 18, 28, 38, and 48 mM $CaCl_2$ respectively, so the final $CaCl_2$ concentration in the reaction mixture ranged from 1 to 25 mM.

Kinetics of α-Amylase Activity

The response of 'd'Anjou' pear fruit α-amylase to increasing substrate concentration was studied. Lyophilized tissue (15g) was extracted with an extraction buffer consisting of 0.1 M sodium acetate (pH 7.4), 5 mM $CaCl_2$, 10 mM mercaptoethanol, 5 mM ascorbic acid and 3 mM $Na_2S_2O_5$. The enzyme was partially purified by using the fraction precipitated with 65% $(NH_4)_2SO_4$ saturation. 0.25 ml enzyme solution was mixed with 0.25 ml of a soluble starch-sodium acetate buffer (pH 5.40) solution where the concentration of soluble starch concentration was 0.5, 1, 2, 4, 6, 8, and 10 $mg \cdot ml^{-1}$, respectively.

Results and Discussion

Time Course of α-Amylase Activity from 'd'Anjou' Pear Fruit.

Initially, both fresh and lyophilized tissues were extracted to determine how much tissue was required and to insure that enzyme activity would be found. No α-amylase activity was detected in fresh samples when 10-75 g were extracted. Activity was found in samples weighing 100 g fresh weight. These results showed that at least 15 g dry weight was needed and that the activity of α-amylase in pear fruit was low in comparison to that reported from cereal crops (Marchylo et al., 1975; Warchalewski and Tkachuk, 1976; Macgregor, 1978). Time course measurements on both fresh and lyophilized tissues indicated that there was no discernible activity in either tissue until 60 min incubation (Fig. 1). Lyophilized tissue released less maltose per mg protein starting at 60 min incubation (Fig. 1) for up to 12 hours (Fig. 2) as compared to the fresh sample. Response was non-linear from 0 to 120 minutes but was linear from 2 to 12 hours (Figs. 1 and 2).

Activity of α-amylase extracted from the midgut of R. americana was linear for three hours (Terra et al., 1977). α-amylase from pear tissue appears to linear over a longer time span than from other biological material.

Effect of pH and Buffer Systems on the Specific Activity of α-Amylase.

The effect of pH on the specific activity of α-amylase extracted from lyophilized pear tissue was determined using Tris-HCl, acetate, and imidazole-HCl buffers. The pH-activity curves for three buffers are shown in Fig. 3. Optimum activity occurred at pH 5.64 in the acetate, and 7.4 in Tris-HCl and imidazole-HCl buffers, respectively. There were significant differences between all three buffers. It was obvious that acetate buffer was superior and significantly different. Activity was about two-fold higher in the acetate as compared to the Tris-HCl buffer. The pH/activity curves for both barley and malted α-amylase were similar with maximum activity at pH 5.5 using a acetate buffer (Greenwood et al., 1965). Macgregor et al., (1974) and Dehaas et al., (1976) reported similar results for other cereal α-amylase's. Koshiba and Minamikawa (1981) and Gogoi et al., (1987) found that maximum activity was in an acetate buffer. In general, the most common buffer used to isolate α-amylase from various sources is an acetate buffer (Fan, 1975; Warchalewski and Tkachuk, 1976). The optimum pH found in pear, 5.64 in acetate buffer, is very close to cereal values. Acetate buffer appears to be the best system, agreeing with most published literature.

Purification of α-Amylase from 'd'Anjou' Pear Fruit.

Purification of the crude enzyme extract was done by passing through a Bio-Gel p-6 desalting column and by stepwise fractionation with $(NH_4)_2SO_4$. The desalting gel excluded molecules with molecular weights greater than 6,000 daltons. Since most proteins are heavier than this, most smaller molecules were removed.

Stepwise addition of $(NH_4)_2SO_4$ was used to purify the crude enzyme extract in the three buffer systems. Table 3.2 gives results for protein concentration, activity, specific activity, total activity and purification level in the acetate, Tris-HCl and imidazole-HCl buffers for the four fractions that were separated. Table 3.3 shows the analysis of variance table for the buffer-$(NH_4)_2SO_4$ experiment. ANOVA indicated that significant differences were found between all parameters with respect to buffer, $(NH_4)_2SO_4$ fraction and their interactive effects (Table 3.3). Because of the significant interaction and the fact that the experimental design was a split-plot, LSD values were calculated to compare means within buffers (small letters in Table 3.2) and across buffers (large letters in Table 3.2). α-amylase activity was highest in the 0-80% fraction in all buffers and lower in other fractions. No activity was detected in the 65-80% $(NH_4)_2SO_4$ fraction from the acetate buffer extraction. The specific activity of α-amylase was

highest in the 30-65% $(NH_4)_2SO_4$ fraction using the acetate buffer. This fraction had higher specific activity values in both the Tris and imidazole buffers than the other salt fractions. The highest purification was also found in the acetate buffer- 30-65% $(NH_4)_2SO_4$ fraction. For all buffers, the 30-65 and 65-80% fraction had the greatest purification. Based on the highest specific activity and greatest purification, the 30-65% $(NH_4)_2SO_4$ fraction of the acetate buffer extraction system was the most favorable system for α-amylase in 'd'Anjou' pear fruit.

Calcium Effects on the Specific Activity of Pear α-Amylase

The specific activity of α-amylase was increased in a non-linear manner as calcium concentration increased from 1 to 15 mM $CaCl_2$ in the reaction mixture (Table 3.4). There was no further change as the concentration increased from 15 to 25 mM $CaCL_2$, the highest level tested.

Plant α-amylases usually require calcium for their activity (Okamoto and Akazama, 1978; Preiss, 1982). Okamoto and Akazama (1978) reported that ethylenediaminetetraacetic acid (EDTA) inhibited amylase activity possibly because calcium was complexed from the system. Irshad and Sharma (1981) observed that plant α-amylase from several sources were quite sensitive to high calcium ion concentration in

vitro. However, Leung (1984) noted that no additional activity was observed using tobacco callus extracts with 2.25 or 5 mM $CaCl_2$.

Results presented in this study suggest that α-amylase from 'd'Anjou' pear fruit is sensitive to low levels of calcium but not to high levels.

Kinetics of α-Amylase Activity

The effect of concentration of soluble starch on activity of α-amylase is shown in Fig. 4. A Lineweaver-Burk plot was constructed using starch concentration ranging from 0.5 to 10 $mg \cdot ml^{-1}$. As calculated from Fig 4, the Km of pear α-amylase was 1.43 $mg \cdot ml^{-1}$ with a Vmax of 1.3 umole maltose$\cdot hr^{-1}$. This Km value from pear was similar to that reported from Vigna mungo cotyledons (Koshiba and Minamikawa, 1981). The pear Km value is higher than that reported for human saliva and hog pancreas (Fischer and Stein, 1961) but lower than α-amylase extracted from potato (Fan, 1975).

The value of Km characterize the interaction of enzyme with a given substrate. The higher the Km value, the lower is the affinity between enzyme and substrate. This means that pear has a lower affinity compared to that of human saliva and hog pancreas; in contrast, the lower the Km value, the higher is the affinity between enzyme and substrate. Pear has a higher affinity than that of potato.

Protein Differences between Fresh and Lyophilized Pear Tissue

One of the initial objectives in this study was to determine whether fresh or lyophilized tissues could be used to extract α-amylase. This was necessary because of the time required to make extractions and run the assays and the need to store samples. It was found that, when expressed as umole maltose·mg protein^{-1}, activity from fresh samples was greater than that from lyophilized tissue (Figs. 1 and 2). The reason for this difference was because protein yields from fresh frozen but not lyophilized tissue were approximately 4 times less than protein levels extracted from lyophilized tissue (Table 3.5). Fresh tissue yielded 0.025±0.023 whereas lyophilized tissues yielded 0.1074±0.053 mg protein·g dry weight^{-1}, respectively. In spite of the differences, lyophilized samples were used in all studies primarily because activity of α-amylase was judged to be sufficient. Differences in protein yields were probably because of more cell disruption during lyophilization.

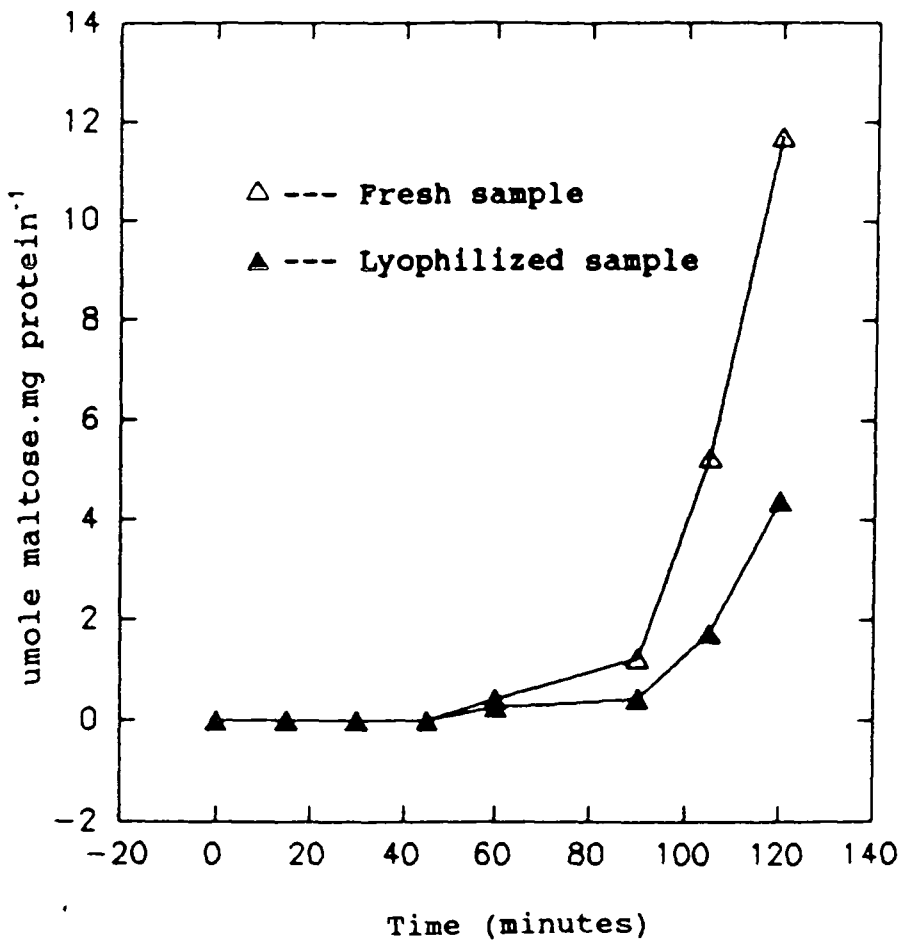

Figure 1. Time course relationship from 0 to 120 minutes showing the production of maltose by α-amylase extracted from fresh and lyophilized 'd'Anjou' pear tissue. Pears were sampled 2 Aug 1990, approximately 30 days prior to commercial harvest

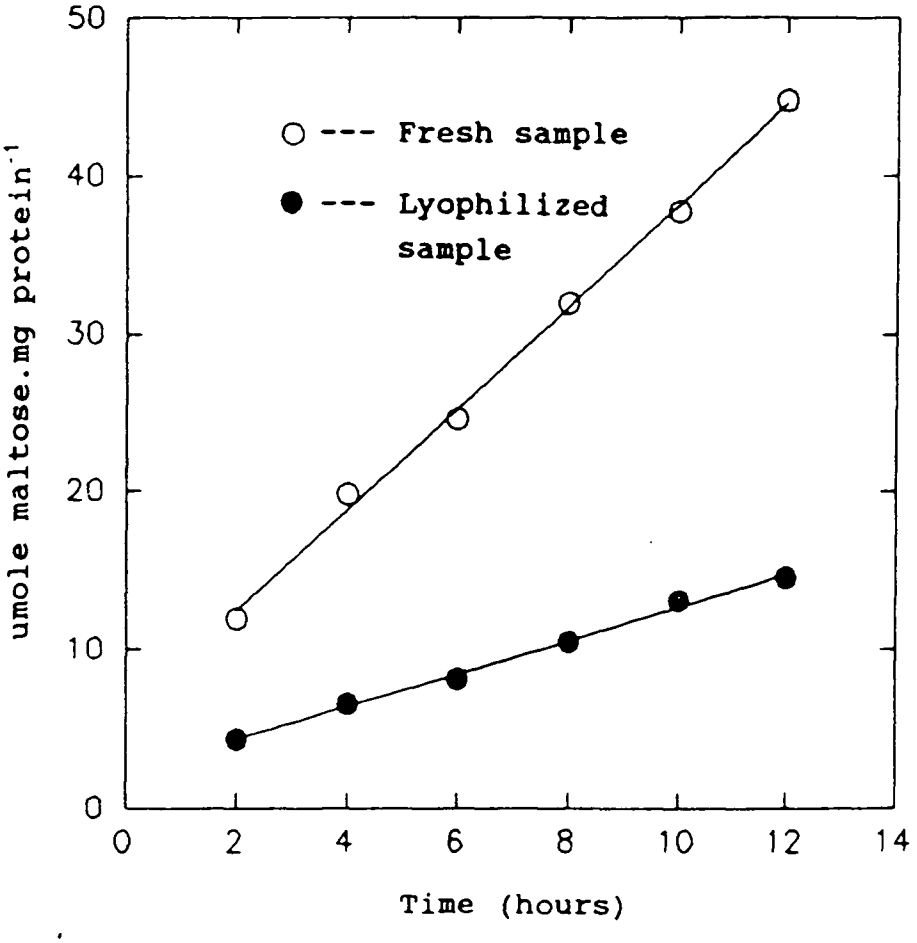

Figure 2. Time course relationship from 2 to 12 hours showing the production of maltose by α-amylase extracted from fresh and lyophilized 'd'Anjou' pear tissue. Pears were sampled 2 Aug 1990, approximately 30 days prior to commercial harvest

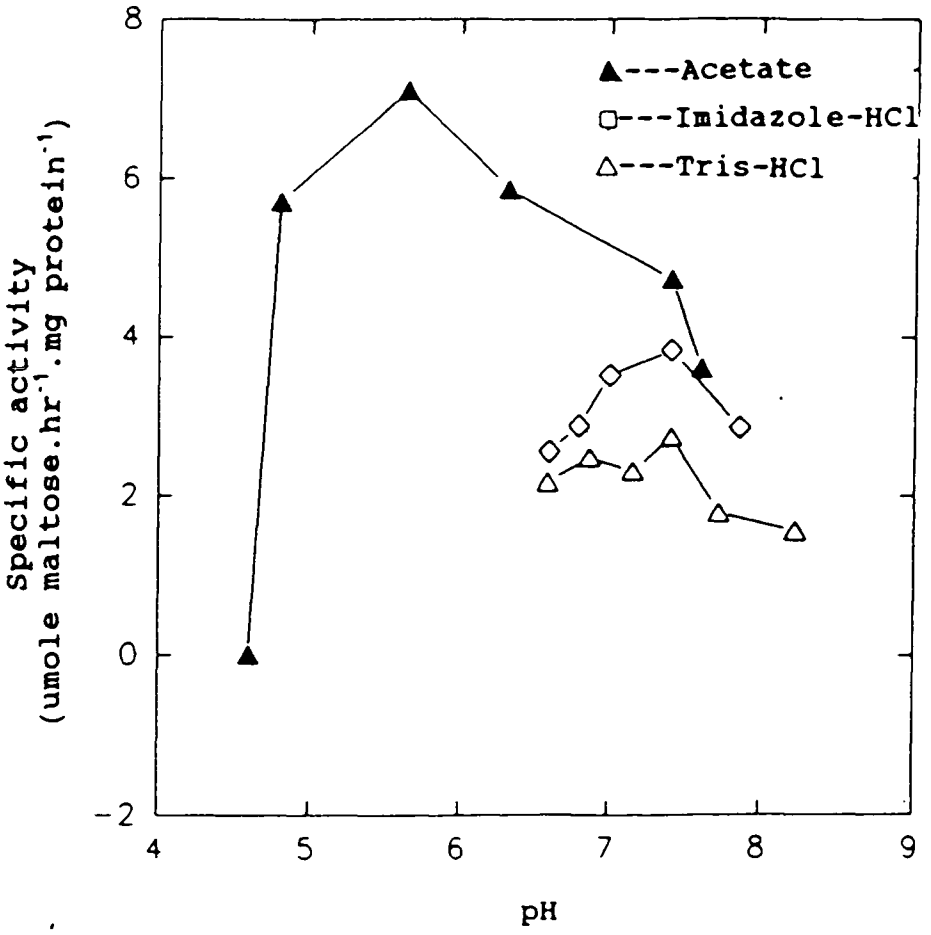

Figure 3. Interaction of three buffer systems, pH and specific activity of α-amylase extracted from normal 'd'Anjou' pear tissue. See text for details concerning buffers

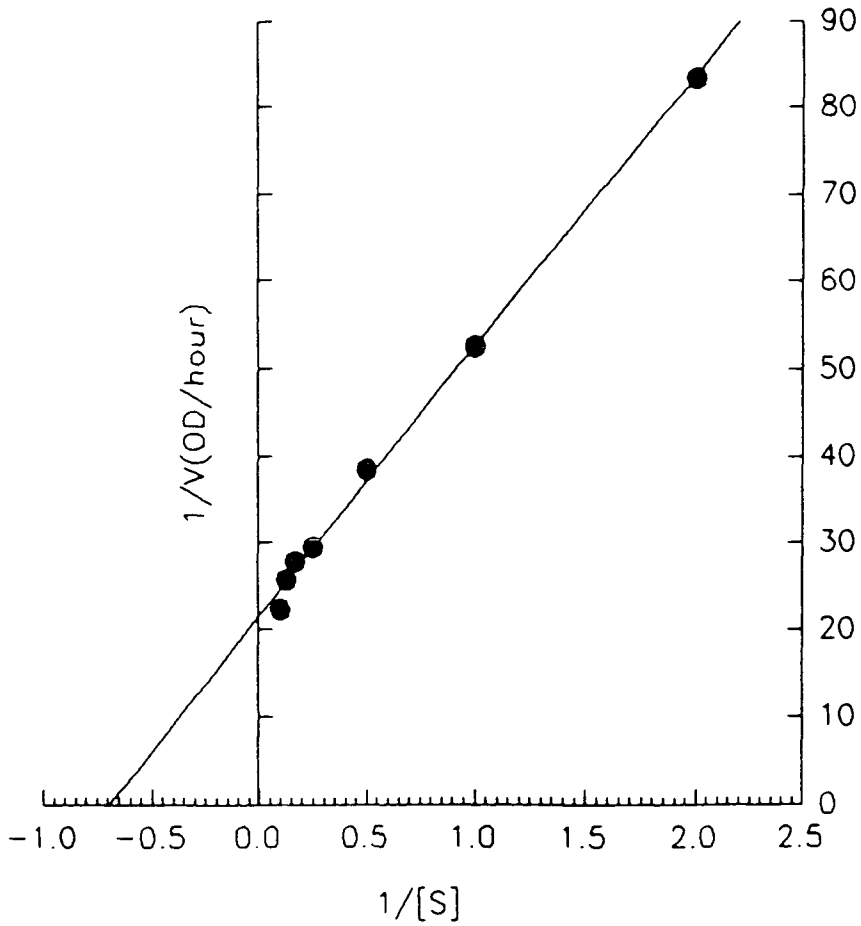

Figure 4. Lineweave-Burk plot of soluble starch and activity of α-amylase extracted from 'd'Anjou' pear fruit. Tissue was extracted with an acetate buffer and assayed at pH 5.40

Table 3.1 Outline of extraction, enzyme, assay and reaction buffer systems and resulting pH values used to determine activity of α-amylase from 'd'Anjou' pear fruit.

Buffer	Tris-HCl	Acetate	Imidazole
Extraction buffer (0.1M, 5mM $CaCl_2$, 10mM mercaptoethanol, 5mM ascorbic acid, 3mM $Na_2S_2O_5$)	7.40	7.40	7.40
Enzyme buffer[z] (0.1M, 5mM $CaCl_2$, 10mM mercaptoethanol)	7.40	7.40	7.40
Assay buffer (0.1 M, 5mM $CaCl_2$)	2.10	2.80	2.00
	2.20	5.40	4.00
	3.00	6.00	6.00
	7.40	7.40	7.40
	8.00	8.15	9.20
	9.00		
Reaction mixture buffer (0.1M, 5mM $CaCl_2$)	6.59	4.60	6.60
	6.87	5.64	6.80
	7.15	6.31	7.00
	7.40	7.40	7.40
	7.72	7.60	7.86
	8.23		

[z] Enzyme buffer refers to buffer in which the precipitate was dissolved and pass through the desalting column.

Table 3.2 Protein concentration, activity, specific activity, total activity and purification of α-amylase from 'd'Anjou' pear fruit in three systems in four ammonium sulfate fractions.

Buffer	Stage[z]	Protein ($mg \cdot g\ DW^{-1}$)	Activity[y]	Specific[y] activity	Total[y] activity	Purification[x]
	0-80	.193a[w]A[v]	1.543a A	2.143a A G	6.173a A	1.00
Tris-HCl	0-30	.109b B	1.100b B	2.693a A	4.400b B	1.25
pH 7.4	30-65	.030c C	.967 c C G	8.717b B	3.867c C	4.18
	65-80	.026d D	.767 d D	7.716c C	3.067d D	3.60
	0-80	.155a E	1.413a A F	2.432a A G	5.653a E	1.00
Acetate	0-30	.103b F	1.230b B	3.201b D	4.920b F	1.32
pH 5.64	30-65	.022c G	.763 c D	12.907cE	4.227c B	5.30
	65-80	.017d H	0 d E	0 dF	0 d G	0
	0-80	.164a I	1.347a F	2.190a G	5.397a H	1.00
Imidazole	0-30	.097b J	1.227b B	3.396b D	4.907b F	1.54
pH 7.40	30-65	.032c C	1.037c C	8.839c B	4.293c B	4.01
	65-80	.024d D G	.927 d G	10.409dH	3.707d C	4.77

[z] Stage refers to percent saturated solution with $(NH_4)_2SO_4$.

[y] Activity refers to $umole\ maltose \cdot hr^{-1} \cdot ml\ desalting\ crude\ extraction^{-1}$.
Specific activity refers to $umole\ maltose \cdot hr^{-1} \cdot mg\ protein^{-1}$.
Total activity refers to $umole\ maltose \cdot hr^{-1}$.

[x] Value were calculated using 0-80% value as 1.00.

[w] Means followed by a small letter were not significantly different using a LSD value at 5% within a buffer.

[v] Means followed by a large letter were not significantly different using a LSD value at 5% across buffer.

Table 3.3 Analysis of variance table for stepwise $(NH_4)_2SO_4$ purification of α-amylase from 'd'Anjou' pear fruit in three buffer systems.

Factor	Df	Protein ($mg \cdot g\ DW^{-1}$)		Activity[z]		Specific[y] Activity		Total[x] Activity	
		MS	F	MS	F	MS	F	MS	F
Rep	2	$.210x10^{-4}$	4.094	$.17x10^{-3}$	.0425	.1479	.675	$.281x10^{-1}$	.436
Buffer	2	$.731X10^{-3}$	142.3**	.1574	39.35**	7.473	34.12**	2.519	39.11**
Rep x Buffer (Error A)	4	$.513x10^{-5}$		$.40x10^{-2}$		.2190		$.644x10^{-1}$	
Stage	3	.0442	17198.4**	1.207	804.33**	114.68**	609.99**	19.30	824.79**
Buffer x Stage	6	$.234x10^{-3}$	91.05**	.2117	141.13**	32.55	170.87**	3.387	144.74**
Error B	18	$.257x10^{-5}$		.0015		.1905		.0234	

* Significant at < 0.05 level, ** Significant at < 0.01 level.

z Activity unit is umole maltose$\cdot hr^{-1} \cdot$ ml DCE^{-1}.

y Specific activity is umole maltose$\cdot hr^{-1} \cdot$ mg $protein^{-1}$.

x Total activity unit is umole maltose$\cdot hr^{-1}$.

Table 3.4 Specific activity of α-amylase extracted from 'd'Anjou' pear fruit at different concentrations of calcium in the reaction mixture

$CaCl_2$ (mM)	Specific activity (umol maltose·hr^{-1}·mg $protein^{-1}$)
1	2.579
5	2.878
10	5.319
15	5.341
20	5.341
25	5.341

Table 3.5 Protein yield extracted from the fresh and lyophilized 'd'Anjou' pear fruit tissue

Rep	Fresh (mg protein•g DW^{-1})	Lyophilized (mg protein•g DW^{-1})
I	.0262	.1072
II	.0272	.1040
III	.024	.1111
Average	.0250	.1074
S.D	.023	.053

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Chapter 4

DETERMINATION OF α-AMYLASE ACTIVITY, PROTEIN CONCENTRATION AND STARCH CONTENT IN NORMAL AND CORK SPOTTED 'D'ANJOU' PEAR FRUIT.

Abstract

Activity of α-amylase and protein and starch concentrations were measured in preharvest calcium-treated normal and corkspotted 'd'Anjou' pear fruit at commercial harvest maturity. Activity and specific activity of α-amylase extracted from corkspotted fruit were higher as compared to α-amylase extracted from normal tissue. Protein concentration was greater in corkspotted fruit. Starch levels were less in corkspotted than in normal fruit as evidenced either by an iodine stain visual technique or by quantitative analysis of hot-water soluble starch. No interaction was found with any measured parameter between calcium treatment and fruit condition. Activity of α-amylase extracted from calcium-treated pears was greater than that extracted from normal pears.

Introduction

Cork spot is a physiological disorder of 'd'Anjou' pear fruit, which is similar to bitter pit in apple. It is characterized by a bumpy, uneven appearance of the surface with brown, dry, corky tissue in the flesh when peeled. The lesion are usually most common near the calyx end.

It has been reported that cork spot in pear is associated with low fruit calcium levels (Mason and Welsh, 1970; Woodbridge, 1971; Richardson, 1976; Vaz and Richardson, 1984). Bevacqua (1989), however, reported that there was no difference in calcium between normal and disordered fruit. He also found no association with pyruvic kinase activity and cork spot, but affected fruit had a higher protein concentration than clear fruit.

Farmer (1906) suggested that the bitter pit in apple was related to the starch content. Carne (1927) put forth the theory that bitter pit in apple was necrosis of immature starch-filled cells, resulting from excessive transpiration, followed by osmotic action between the starch-filled cell and those in which the starch had been largely or completely changed to sugar.

Chen (personal communication) noted that 'd'Anjou' pears with cork spot had less starch at harvest than normal pear when stained with iodine. The lack of starch in

corkspotted pears suggested that starch degradation might be involved in the development of disorder in the pulp tissue. Furthermore, since α-amylase is part of enzyme system that degrades starch, changes in activity might be used as an indicator of susceptibility to cork spot.

The starch iodine test is used to assess maturity of apples for common and controlled atmosphere storages (Reid et al., 1982; Kvale, 1986; Lau, 1988). Preliminary research on the iodine test as a maturity indice for 'd'Anjou' pears (Chen, personal communication) suggests that it was more variable than for apples and may not be suitable. However, Fidder and Mann (1972) and Kavie (1986) both believed that the test was a reliable maturity index for the pear cultivars they tested.

The objectives of this study were as follows:

1. To determine if there are difference in activity of α-amylase between normal and cork spotted 'd'Anjou' pear fruit at commercial harvest.
2. To determine if there are difference in starch content between normal and cork spotted 'd'Anjou' pear fruit at commercial harvest.

Materials and Methods

Plant Material and Calcium Treatment

'D'Anjou' pear fruit were collected at commercial harvest maturity (6 September 1990, 62.3 N pressure) from a 4 acre block at the Mid-Columbia Agricultural Research and Extension Center, Hood River, Oregon. The trees were approximately 60 years old, mostly on 'Bartlett' seedling rootstock. Normal appearing and fruit with visible cork spot were sampled from similar locations on trees. However, because of the sample size required for enzyme analysis, fruit were combined from units of approximately 88 trees. Trees sampled were divided into 2 blocks. Each block consisted of approximately 220 'd'Anjou' pear trees (88 'Bartlett' pollenizers), half which received 3 applications of 3 lbs $CaCl_2$/ 100 gal with 2 oz B-1956 (Surfactant) at the rate of 400 gal/acre. Applications were made on 13 June 1990, 16 July 1990 and 17 August 1990. These treatments had been applied at similar timings yearly since 1988. At harvest, approximately 100 fruit were examined for cork spot from 24 individual trees in each block.

Sample preparation

The fruit were washed in a 0.1% solution of a low

phosphorus detergent (Liqui-Nox, Alconox, N.Y., N.Y.), rinsed with distilled water and wiped dry. The calyx half on the fruit was cut into cubes, which were quickly transferred onto a cloth wetted with 0.5% (W/V) sodium metabisulfite solution. A second treated cloth was used to cover the samples as they were accumulated. Approximately 30 g of tissue were sampled, placed in cheesecloth bags, frozen in liquid nitrogen, then stored at -74^0C and lyophilized for 7 days. Percent dry weight was calculated from the lyophilized tissue.

Extraction and Partial Purification

All procedures were followed at $0-5^0$C. Fifteen g lyophilized tissue were homogenized with 7.5g insoluble polyvinyl polypyrrolidone (PVPP) and 7.5g white quartz sand with a mortar and pestle. The sample was ground for 5 minutes. The sample was ground for an additional 10 minutes after addition of 50 ml of an extraction buffer consisting of 0.1 M acetate (pH 7.4), 5 mM $CaCl_2$, 10 mM mercaptoethanol, 5 mM ascorbic acid and 3 mM $Na_2S_2O_5$. The preparation was then squeezed through two layers of miracloth. The extraction step was carried out three times. The resulting supernatant was centrifuged at 5,000x g for 20 minutes. To the crude enzyme solution, solid $(NH_4)_2SO_4$ was added to give 65% saturation. The solution was stirred

until it was completely dissolved (40 minutes) and then centrifuged at 10,000x g for 20 minutes. The precipitate was resuspended in 3.0 ml of a solution consisting of 0.1 M acetate (pH 7.4) with 5 mM $CaCL_2$ and 10mM mercaptoethanol. The resulting extract was applied to an Econo-Pac 10 DC column packed with Bio-Gel P-6 desalting gel. The sample was allowed to run into the column by discarding the first 3.0 ml eluted. The higher molecular components in the extract were eluted by adding 4.0 ml of the above buffer and collecting a 4.0 ml fraction from the column.

α-Amylase Assay

Alpha-amylase activity was assayed by incubating 0.25 ml of the enzyme preparation for 2 and 4 hours at 20^0C with 0.25 ml of a soluble starch (1%, W/V, soluble starch, 0.1 M sodium acetate at pH 5.4)-sodium acetate mixture. The reaction was interrupted by the addition of 0.5 ml dinitrosalicylic acid reagent (dissolve at room temperature 1g. of 3,5-dinitrosalicylic acid in 20 ml of 2 N NaOH and 50 ml H_20; add 30 g of Rochelle salt, and make to 100 ml with distilled H_20), heated for 5 minutes in boiling water, then cooled in ice water. The mixture was then diluted 25 times with distilled water. Optical values were read at 540 nm using a blank which was prepared with the same amount of enzyme, substrate and reagent in which the

enzyme activity was inactivated immediately. Optical density readings were converted to umole maltose using a standard curve with maltose (1.0 to 5.0 umole$\cdot ml^{-1}$). Activity was expressed as umole maltose liberated per hour at 20^0C by 1 ml desalted crude extract (DCE). Specific activity was expressed as umole maltose$\cdot hr^{-1}\cdot$mg protein^{-1}.

Protein Assay

Soluble protein in the enzyme preparation was determined by the Bio-Rad microassay with bovine serum albumin (BSA) as the standard. Three volumes (0.01, 0.02 and 0.03 ml) enzyme preparation were each diluted to 0.8 ml in clean, dry test tubes. To each, 0.2 ml of the Bio-Rad dye reagent was added then vortexed and/or mixed several times by gentle inversion of the tubes. Optical density readings were taken at 595 nm from 5 to 60 minutes since the color was stable over this period of time. A standard curve was constructed with BSA (1 to 16 ug$\cdot ml^{-1}$) and OD readings were converted to ug protein.

Iodine-Starch Test

'd'Anjou' pears with and without corkspot were stained with iodine at harvest. A 5 mm transverse section was removed from the point of the greatest diameter from 10

pears with and without corkspot and dipped into an iodine solution. The sections were removed and, after 5 minutes, were rated for the amount of blue color using a scale developed by P. Chen (personnal communication) where 0 equaled no starch clearing and 5 equaled complete disappearance of starch.

Starch Analysis

The method was according to modification of Potter and Breen (1980). Two g lyophilized fruit tissue was ground with a mortar and pestle in 30 ml 80% ethanol. The mixture was boiled for 2 minutes then centrifuged at 12,000x g for 5 minutes. The supernatant was decanted and discarded. These operations were performed twice. The resulting pellet was dried at 60^0C. The dried pellet was dispersed in 20 ml acetate buffer at pH 5.4, boiled for 10 minutes, then made up to 20 ml. A 1.0 ml aliquot was digested with 0.1 ml amyloglucosidase (20 mg$\cdot$1 ml acetate buffer^{-1}) at 55^0C for 90 minutes. The digest was centrifuged at 12,000x g for 5 minutes and a 0.10 ml aliquot of the supernatant fraction was analyzed for glucose. The samples were diluted to 0.5 ml in test tube, and mixed thoroughly with 5.0 ml of Combined Enzyme-Color Reagent (Sigma Kit, 510-A) solution and incubated at 37^0C for 30 minutes. The absorbance was read at 450 nm. A glucose standard curve was

established using 1 $mg \cdot ml^{-1}$ from 0 to 1.0 $mg \cdot ml^{-1}$. The soluble starch suspension (1 $mg \cdot ml^{-1}$) was used to generate a standard curve from 0 to 1 mg $starch \cdot ml^{-1}$, relating starch to glucose.

Calcium Analysis

0.5 g lyophilized fruit tissue was ashed at 500^0C for 5 hours. The residue was dissolved in increments of 0.1 N HCl with $SrCl_2$, filtered through Whatman #1 filter paper, then brought up to 100 ml. Calcium was determined on a Perkin-Elmer atomic absorption spectrophotometer.

Statistical Analysis

The split design for ANOVA was used to test for significant differences between the α-amylase activity, protein concentration, and specific activity and starch content. Calcium treatment was the whole plot and the types of fruit were the subplot.

Results

Table 4.1 shows the activity, specific activity and protein and starch concentration in normal and corkspotted 'd'Anjou' pear tissue. Results of the effects of calcium treatment on activity, specific activity and protein and starch concentration from calcium and non-calcium treated fruit are shown in Table 4.2. Table 4.3 gives the analysis of variance for the measured parameters. No interactions between calcium and pear disorder were found for any parameter measured; therefore only main effects will be discussed. The foliar calcium treatments increased the activity of α-amylase but did not affect the other parameters (Table 4.2). Calcium spray did not affect the percentage of cork spot or peel levels of calcium (Table 4.4), but calcium content in normal fruit was higher than that of corkspotted fruit (Table 4.5). Pears with corkspot had increased activity and specific activity of α-amylase. Corkspotted pears also had higher protein levels but decreased concentration of starch (Table 4.1). At harvest, pears with cork spot had much less starch as determined by the iodine staining method as did normal fruit (Figure 5).

Discussion

Results presented here show that 'd'Anjou' pears with corkspot differed significantly from normal pears at harvest. Corkspotted fruit had greater activity of α-amylase, less starch, both by a visual iodine stain and by quantitative analysis, and higher concentration of protein. Increased activity of α-amylase and the lack of starch suggest that corkspotted fruit were devoid starch. However, it was possible that starch synthesis was affected. Data presented here only reveal levels at one point in time, commercial harvest. Sequential sampling will be necessary to show changes occurring over the entire maturation period to see whether starch was present or not.

In general, cork spot appears on the exposed part of the tree on the exposed part of the fruit. Wang and Mellenthin (1973) found that sun-exposed fruit surfaces of 'd'Anjou' pears had increased levels of chlorogenic acid. They suggested that levels might be high enough to cause tissue browning, one of the visual symptoms of cork spot. They also suggested that respiratory products could accumulate since cork spotted pears had higher and accelerated rates of both ethylene production and respiration, cause cell injury, and increase cell permeability to oxygen. This, along with the higher levels

of chlorogenic acid, could result in tissue browning. The increased rates of ethylene production and respiration suggest that corkspotted pears ripen at a faster rate than normal fruit. If so, disappearance of starch and increased activity of α-amylase might be results, not causes of cork spot. However, light induced de novo synthesis of β-amylase in mustard cotyledons (Sharma and Schofer, 1987). It may be that intense sunlight has a similar effect in 'd'Anjou' pear fruit. Results presented here were not designed to separate the time course of the different responses in the tissues. According to Faust and Shear (1968) increased production of ethylene was the " very first sign" of cork spot in apple. No literature was found showing the first changes in corkspotted pear but starch clearing might occur.

Differences in activity of α-amylase between the corkspotted and normal fruit could arise because of : 1) more actual α-amylase·mg protein^{-1}, 2) an actual difference in the enzyme and 3) either inhibitors or cofactors being extracted to a greater extent in one tissue as compared to the other.

Preharvest calcium treatment inhanced the activity of α-amylase extracted from pear tissue. Others have shown that calcium increases α-amylase activity in other plants (Irshad and Sharma, 1981). In chapter 3, data were presented to show that calcium also increased pear α-amylase activity in vitro. These observations suggest that activity

of α-amylase should be lower in cork spotted pears since calcium levels have been shown to be lower in pears with cork spot (Mason and Welsh, 1970; Woodbridge, 1971; Richardson and Al-Ani, 1982; Vaz and Richardson, 1984), or, the opposite, that pears high in calcium (such as normal fruit) have increased activity of α-amylase. No differences were found in the calcium content of the Ca-treated pears (peel Ca levels; Ca-treated, 1.128 mg·g dry weight^{-1}; non-treated, 1.130 mg·g dry weight^{-1}). However, corkspotted pears had significantly lower levels of calcium, agreeing with most published literature. Since the actual activity in corkspotted fruit was higher than in normal fruit, total Ca content does not appear to be related. It may be that total Ca content is not a controlling factor and that metabolically available Ca is critical.

Increased levels of protein in corkspotted tissues have been reported (Bevacqua, 1989) and data presented here confirm this observation. Faust and Shear (1968) reported increased protein N in apples with cork spot and others have found increased protein N in bitter pit tissue (Feucht and Aranciba, 1965). Chen (personal communication) has found that 'd'Anjou' pears with a physiological disorder called " black speck" also have higher protein levels as compared to normal fruit. Increased protein levels, thus, may be a result of some type of stress and not a causal factor.

Fig. 5. Iodine starch test on 'd'Anjou' pears with (rows 2 and 4) and without (rows 1 and 3) cork spot, harvest, 1990. Starch index values were 1.3 for normal and 4.4 for corkspotted fruit.

Table 4.1 Activity and specific activity of α-amylase and protein and starch concentration in normal and corkspotted 'd'Anjou' pear tissue. Fruit were harvested on 5 Sept 1990 at 62.3 N firmness (commercial maturity).

Fruit type	Activity [z]	Specific activity[y]	Protein (mg•g DW^{-1})	Starch (g•100g FW^{-1})
Normal	0.913	2.954	.082	.509
Corkspotted	2.314	5.454	.113	.195
Significance level	< .01	< .01	< .01	< .01

[z] Activity expressed as umole maltose•hr^{-1}•ml DCE^{-1}.

[y] Specific activity expressed as umole maltose•hr^{-1}•mg $protein^{-1}$.

Table 4.2 Activity and specific activity of α-amylase and protein and starch concentration in preharvest Ca-treated 'd'Anjou' pear tissue.

Calcium[z] treatment	Activity [y]	Specific activity[y]	Protein (mg•g DW^{-1})	Starch (g•100g FW^{-1})
No	1.506	3.961	.098	.356
Yes	1.721	4.491	.098	.348
Significance level	<.01	N.S	N.S	N.S

[z] $CaCl_2$ applied at rate of 3 lbs/100 gal, 400 gal/acre on 13 June, 16 July and 17 August 1990 with B-1956 at rate of 2 oz/100 gal.

[y] Activity expressed as umole maltose•hr^{-1}•ml DCE^{-1}. Specific activity expressed as umole maltose•hr^{-1}•mg $protein^{-1}$.

Table 4.3 Analysis of variance table for protein concentration, activity and specific activity of α-amylase and starch content in normal and corkspotted 'd'Anjou' pear fruit with calcium and no calcium treatment

Factor	Df	Protein ($mg \cdot g\ DW^{-1}$)		Activity[z]		Specific[y] Activity		Starch ($g \cdot 100g\ FW^{-1}$)	
		MS	F	MS	F	MS	F	MS	F
Block	1	1.602×10^{-4}	2.403	$.158 \times 10^{-1}$	65.560	$.202 \times 10^{-1}$	.553	3.060×10^{-3}	.662
Calcium	1	$.060 \times 10^{-5}$	.009	.276	1145.228**	1.983	54.33	$.442 \times 10^{-4}$	.009
Error A (Block x calcium)	1	6.667×10^{-5}		$.241 \times 10^{-3}$		$.365 \times 10^{-1}$		4.620×10^{-3}	
Type	1	$.789 \times 10^{-1}$	1797.27**	11.766	1376.140**	37.520	1075.072**	.594	1262.68**
Calcium x Type	1	8.817×10^{-5}	2.008	$.334 \times 10^{-1}$	3.906	$.338 \times 10^{-1}$	.968	$.150 \times 10^{-4}$	.032
Error	18	4.390×10^{-5}		$.855 \times 10^{-2}$		$.349 \times 10^{-1}$		4.708×10^{-4}	

* Significant at < 0.05 level, ** Significant at < 0.01 level.
[z] Activity unit is umole maltose$\cdot hr^{-1} \cdot$ml DCE^{-1}.
[y] Specific activity is umole maltose$\cdot hr^{-1} \cdot$mg $protein^{-1}$.
[x] Total activity unit is umole maltose$\cdot hr^{-1}$.

Table 4.4 Effect of preharvest Ca-treatment on cork spot and peel Ca levels of 'd'Anjou' pear fruit at commercial harvest (5 Sept 1990). Values are means from 48 trees per treatment, 100 fruit per tree.

Treatment	Cork spot %	Peel Ca($mg \cdot g\ DW^{-1}$)
With Ca	3.3	1.13
No Ca	5.2	1.13
Significance Level	N.S	N.S

Table 4.5 Calcium content in normal and corkspotted 'd'Anjou' pear tissue. Fruit were harvested on 5 Sept 1990 at 62.3 N firmness (commercial maturity).

Fruit type	Calcium content ($mg \cdot g\ DW^{-1}$)
Normal	.382
Corkspotted	.274
Significance level	<.01

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Chapter 5

SUMMARY

The time required for an extract from 'd'Anjou' pear tissue containing α-amylase to release maltose from a starch solution was 60 minutes. This was considerable longer than that reported in the literature for other plant tissues, most notable cereals. A large increase in activity was found after 90 minutes and activity was linear from 2 to 12 hours incubation. Activity of α-amylase remained consistent from 30 days before to commercial harvest.

A comparison of three buffer systems (Tris-HCl, imidazole-HCl and acetate) at pH's ranging from 4.60 to 8.23 showed that an acetate buffer at pH 5.64 gave maximum activity.

Alpha-amylase was purified in each buffer system by ammonium sulfate fractionation and by passing the extracts through desalting columns. Maximum purification was found in a 30-65% ammonium sulfate fraction when tissues were extracted with an acetate buffer in the reaction mixture at pH 5.64. Alpha-amylase was purified 5.30 fold with a specific activity of 12.907 umole maltose$\cdot hr^{-1}\cdot$ mg protein^{-1} and a yield of 68.47%.

The specific activity of pear α-amylase increased <u>in vitro</u> as the calcium concentration increased from 1 to 15 mM

$CaCl_2$ in the reaction mixture but did not change from 15 to 25 mM.

The Km of pear tissue α-amylase was 1.43 $mg \cdot ml^{-1}$ with a Vmax of 1.3 $umole \cdot maltose^{-1}$. The low Km indicates that pear α-amylase has a low affinity for starch and that the reaction rate is slow as compared with cereal α-amylase.

No interactions were found between activity and specific activity and protein and starch concentrations in preharvest calcium-treated pears with and without cork spot. At commercial harvest, activity and specific activity of α-amylase extracted from 'd'Anjou' pears with cork spot were higher than that from normal pears. Protein levels were greater whereas starch concentrations were lesser in corkspotted fruit. Calcium-treated pears had greater activity of α-amylase than non-treated fruit but specific activity of α-amylase, protein concentration and soluble starch content were not different. Corkspotted fruit had less calcium as compared to normal fruit.

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APPENDIX

Table A.1 OD readings at 540nm within 2 hours incubation with fresh and lyophilized 'd'Anjou' pear sample.

Time (min)	Fresh				lyophilized			
	I	II	III	average	I	II	III	average
0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0
60	.002	.002	.001	.0016	.003	.002	.003	.0027
90	.003	.003	.002	.0026	.004	.003	.005	.0040
105	.012	.011	.010	.011	.017	.015	.018	.017
120	.024	.025	.023	.024	.048	.052	.058	.053

Table A.2 Data of the α-amylase activity (umole maltose•mg protein^{-1}) from 2 to 12 hours incubation with fresh and lyophilized 'd'Anjou' pear fruit.

Time (hours)	Fresh				Lyophilized			
	I	II	III	average	I	II	III	average
2	11.70	12.06	12.16	11.97	4.37	4.26	4.51	4.38
4	20.04	18.63	20.97	19.88	6.66	6.62	6.53	6.60
6	24.92	23.33	25.72	24.66	8.21	8.21	8.16	8.19
8	32.55	28.63	34.77	31.98	10.57	10.51	10.44	10.51
10	37.34	36.77	39.18	37.76	13.10	13.31	12.79	13.07
12	44.25	43.63	46.36	44.75	14.67	14.41	14.42	14.50

Table A.3 Specific activity of α-amylase extracted from 'd'Anjou' pear fruit with a Tris-HCl buffer at different pH's

pH of reaction mixture	Specific activity (umole maltose•hr^{-1}•mg $protein^{-1}$)				
	I	II	III	Average	S.D
6.59	2.382	2.150	2.064	2.200	.165
6.87	2.568	2.358	2.520	2.482	.110
7.15	2.352	2.219	2.352	2.308	.077
7.40	2.760	2.704	2.760	2.741	.032
7.72	1.992	1.664	1.728	1.795	.174
8.23	1.800	1.317	1.600	1.572	.243

Table A.4 Specific activity of α-amylase extracted from 'd'Anjou' pear fruit with a acetate buffer at different pH's

pH of reaction mixture	Specific activity (umole maltose•hr^{-1}•mg $protein^{-1}$)				
	I	II	III	Average	S.D
4.60	0	0	0	0	0
4.80	5.659	5.641	5.750	5.694	.055
5.64	8.068	6.904	6.354	7.109	.875
6.31	6.465	5.607	5.479	5.950	.536
7.40	4.784	4.309	5.083	4.725	.390
7.60	4.008	3.475	3.333	3.605	.356

Table A.5 Specific activity of α-amylase extracted from 'd'Anjou' pear fruit with a imidazole-HCl buffer at different pH's

pH of reaction mixture	Specific activity (umole maltose•hr^{-1}•mg $protein^{-1}$)				
	I	II	III	Average	S.D
6.60	2.537	2.517	2.623	2.559	.056
6.80	2.914	2.891	2.843	2.883	.036
7.00	3.600	3.518	3.418	3.512	.091
7.40	3.771	3.826	3.892	3.830	.061
7.86	2.949	2.891	2.758	2.866	.098

Table A.6 Ammonium sulfate fractionation of α-amylase extracted with a Tris-HCl buffer at pH 7.40 from normal 'd'Anjou' pear fruit at commercial maturity.

Stage[z]	Volume (ml)	Protein ($mg \cdot g\ DW^{-1}$)	Activity[y]	Specific[y] activity	Total[y] activity	Purification (fold)	Yield (%)
	4	0.192	1.50	2.106	6.00	1	100
0-80%	4	0.192	1.55	2.153	6.20	1	100
	4	0.194	1.58	2.170	6.32	1	100
	4	0.109	1.12	2.738	4.48	1.30	74.7
0-30%	4	0.108	1.08	2.671	4.32	1.24	69.7
	4	0.111	1.10	2.640	4.40	1.22	69.6
	4	0.028	0.98	9.414	3.92	4.80	65.3
30-65%	4	0.030	0.95	8.482	3.80	3.94	61.3
	4	0.031	0.97	8.255	3.88	3.80	61.4
	4	0.026	0.700	7.143	2.80	3.40	46.7
65-80%	4	0.027	0.800	7.843	3.20	3.64	51.6
	4	0.026	0.800	8.163	3.20	3.76	50.6

[z] Stage refers to saturated solution with $(NH_4)_2SO_4$.

[y] Activity refers to u mole maltose$\cdot hr^{-1} \cdot$ ml DCE^{-1}.
Specific activity refers to umole maltose$\cdot hr^{-1} \cdot$ mg $protein^{-1}$.
Total Activity refers to umole maltose$\cdot hr^{-1}$.

Table A.7 Ammonium sulfate fractionation of α-amylase extracted with a acetate buffer at pH 7.40 from normal 'd'Anjou' pear fruit harvested 2 Aug 1990.

Stage[z]	Volume (ml)	Protein ($mg \cdot g\ DW^{-1}$)	Activity[y]	Specific[y] activity	Total[y] activity	Purification (fold)	Yield (%)
	4	.150	1.38	2.456	5.52	1	100
0-80%	4	.161	1.45	2.409	5.80	1	100
	4	.155	1.41	2.431	5.64	1	100
	4	.101	1.30	3.448	5.20	1.404	94.2
0-30%	4	.106	1.17	2.958	4.68	1.228	80.7
	4	.102	1.22	3.196	4.88	1.315	86.5
	4	.022	1.13	13.951	4.52	5.680	81.9
30-65%	4	.023	.98	11.574	3.92	4.804	67.6
	4	.023	1.06	13.196	4.24	5.428	75.2
	4	.016	0	0	0	0	0
65-80%	4	.017	0	0	0	0	0
	4	.017	0	0	0	0	0

[z] Stage refers to saturated solution with $(NH_4)_2SO_4$.

[y] Activity refers to u mole maltose$\cdot hr^{-1} \cdot$ ml DCE^{-1}.
Specific activity refers to umole maltose$\cdot hr^{-1} \cdot$ mg $protein^{-1}$.
Total Activity refers to umole maltose$\cdot hr^{-1}$.

Table A.8 Ammonium sulfate fractionation of α-amylase extracted with a imidazole-HCl buffer at pH 7.40 from normal 'd'Anjou' pear fruit.

Stage[z]	Volume (ml)	Protein ($mg \cdot g\ DW^{-1}$)	Activity[y]	Specific[y] activity	Total[y] activity	Purification (fold)	Yield (%)
	4	.163	1.33	2.180	5.32	1	100
0-80%	4	.166	1.35	2.174	5.40	1	100
	4	.164	1.36	2.215	5.44	1	100
	4	.095	1.18	3.327	4.72	1.526	88.7
0-30%	4	.098	1.25	3.466	5.00	1.565	92.6
	4	.098	1.25	3.394	5.00	1.532	91.9
	4	.030	1.02	9.003	4.08	4.130	86.6
30-65%	4	.033	1.10	8.943	4.40	4.114	80.1
	4	.034	1.10	8.571	4.40	3.869	80.9
	4	.023	.87	10.235	3.84	4.694	65.4
65-80%	4	.024	.96	10.667	3.84	4.960	71.1
	4	.024	.95	10.326	3.80	4.661	69.8

[z] Stage refers to saturated solution with $(NH_4)_2SO_4$.

[y] Activity refers to u mole maltose$\cdot hr^{-1} \cdot$ ml DCE^{-1}.
Specific activity refers to umole maltose$\cdot hr^{-1} \cdot$ mg $protein^{-1}$.
Total Activity refers to umole maltose$\cdot hr^{-1}$.

Table A.9 Data on the protein concentration, activity and the specific activity of alpha-amylase in cork spotted and normal d'Anjou' pear fruit. Fruit was harvested on 5 Sep 1990.

Sample	Activity[z]	Protein (mg•g FW)	Specific activity[y]
Clear			
A-E[x](1)	0.950	0.080	3.146
A-E (2)	0.926	0.081	3.036
A-E (3)	0.910	0.082	2.955
F-J[x](1)	0.920	0.084	2.911
F-J (2)	0.880	0.082	2.848
F-J (3)	0.910	0.084	2.890
K-O[x](1)	1.046	0.083	3.342
K-O (2)	0.997	0.081	3.280
K-O (3)	1.070	0.082	3.462
P-U[x](1)	0.750	0.083	2.419
P-U (2)	0.800	0.082	2.606
P-U (3)	0.800	0.084	2.548
Cork Spot			
A-E(1)	2.500	0.115	5.814
A-E(2)	2.510	0.114	5.878
A-E(3)	2.393	0.112	5.711
F-J(1)	2.073	0.110	5.019
F-J(2)	2.133	0.111	5.140
F-J(3)	1.948	0.109	4.751
K-O(1)	2.430	0.112	5.772
K-O(2)	2.421	0.113	5.698
K-O(3)	2.495	0.115	5.802
P-U(1)	2.200	0.113	5.176
P-U(2)	2.327	0.114	5.424
P-U(3)	2.333	0.118	5.266

[z] Activity refers to umole maltose•hr^{-1}•ml DCE^{-1}.
[y] Specific activity refers to umole•hr^{-1}•mg $protein^{-1}$.
[x] A-E refers to $CaCl_2$ applied at rate of 3 lbs/100 gal, 400 gal/acre on 13 June, 16 July and 17 August 1990 with B-1956 at rate of 2 oz/100 gal within block 1.
F-J refers to no $CaCl_2$ treatment within block 1.
K-O refers to $CaCl_2$ treatment within block 2.
P-U refers to no $CaCl_2$ treatment within block 2.

Table A.10 Data on the soluble starch content in cork spotted and normal d'Anjou' pear fruit. Fruit was harvested on 5 Sep 1990.

Sample	Starch($g \cdot 100\ g\ FW^{-1}$)
Clear	
A-E[x](4)	.502
A-E(5)	.528
A-E(6)	.468
F-J[x](4)	.534
F-J(5)	.548
F-J(6)	.578
K-O[x](4)	.536
K-O(5)	.490
K-O(6)	.510
P-U[x](4)	.458
P-U(5)	.464
P-U(6)	.494
Cork Spot	
A-E(4)	.192
A-E(5)	.190
A-E(6)	.190
F-J(4)	.196
F-J(5)	.216
F-J(6)	.216
K-O(4)	.206
K-O(5)	.179
K-O(6)	.180
P-U(4)	.184
P-U(5)	.194
P-U(6)	.192

[x] A-E refers to $CaCl_2$ applied at rate of 3 lbs/100 gal, 400 gal/acre on 13 June, 16 July and 17 August 1990 with B-1956 at rate of 2 oz/100 gal within block 1.
F-J refers to no $CaCl_2$ treatment within block 1.
K-O refers to $CaCl_2$ treatment within block 2.
P-U refers to no $CaCl_2$ treatment within block 2.