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INACTIVATION OF POLYPHENOL OXIDASE IN STANLEY PLUM JUICE USING AN IMMOBILIZED PROTEASE ENZYME

By

Joseph F. Arnold

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ABSTRACT

INACTIVATION OF POLYPHENOL OXIDASE IN STANLEY PLUM JUICE USING AN IMMOBILIZED PROTEASE ENZYME

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In this research proteases from *Rhizopous*, papaya (papain), and *Aspergillus niger* (7107) were immobilized via formation of a Schiffs base (covalent aldimine linkage) to amino-alkylsylil glass using glutaraldehyde. The immobilized protease was then used in an attempt to inhibit PPO activity and minimize ACY degradation.

The initial study showed that protease immobilized through covalent coupling to 400-500 A diameter controlled pore glass (CPG) beads significantly reduced the activity of a commercial PPO preparation.

The second project phase consisted of exposing an acetone extract of PPO, from Stanley plums, to immobilized food grade proteases, papain and 7107, along with a control (plain CPG beads without protease). These columns were stored at 5 and 22°C. The activity

and stability of the immobilized proteases at these two temperatures was monitored for 7 days. The results showed that papain at 5°C exhibited the most consistent inactivation capability.

The final research phase consisted of exposing Stanley plum juice, with two soluble solids concentrations (14 and 16 °Brix), to immobilized papain in a fluidized bed reactor. Two controls were used consisting of a raw, untreated juice sample and a pasteurized (High Temperature Short Time (HTST), 88°C/1min) juice sample. The least amount of ACY degradation, as shown by spectrophotometric measurement of ACY in acidified ethanol at 535 nm, occurred in the pasteurized, control sample. The inhibition of ACY degradation in the remaining samples, other than pasteurized, was minimal. This may be due to the protein concentrations present in the juice sample which overloaded the immobilized protease. Other components of the juice, such as sugars, organic acids, or ACY, may also cause this effect by protecting the active sites or physically interfering with the ability of the enzyme and substrate to react together.

Microbial analyses indicate that additional measures must be taken to reduce the microbial counts in the treated juice sample. The pasteurized sample, however, showed acceptable microbial levels indicating good industrial scale-up potential.

Total protein analysis, using the Kjeldahl procedure, indicated that minimal differences exist between the untreated, pasteurized and treated samples. This is probably due to the low, initial protein concentrations in the juice and that enzyme content is only a small portion of the total protein content. Even if the PPO was inactivated, the initial concentration may have been so small that it would not be reflected in the results. The same holds true for the plum PPO extract before and after it had been exposed to uncoated CPG beads. It may also be due to the fact that enzymes inactivated via proteolytic digestion can still be measured by the Kjeldahl method.

Rank preference tests indicated that no significant ($p < 0.05$) preference existed between the 14 °Brix samples and 16 °Brix samples respectively. An extended triangle test showed that no significant difference ($p < 0.05$) existed between the 16 °Brix sample and the pasteurized control sample. The test also showed no significant preference ($p < 0.05$) between the two samples. However, only one replication was performed resulting in a relatively high type II error. Consequently, the sensory results should only be interpreted as a general indication of what the sensory characteristics may be in the plum juice samples.

This work is dedicated to James and Eva Arnold
for their eternal love and support.

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LITERATURE REVIEW

In recent years, purple plums have become an integral part of Michigan agriculture. Plums have been found to be an excellent "filler crop" because they can be harvested between the cherry and apple crops thereby increasing the efficiency of the local processing facilities. At present time, approximately one-half of the plums go to fresh market while the other half are processed, mainly canned.

This research has two unique aspects. First it will attempt to produce plum juice from Stanley plums, a hopefully desirable product due to the plums' high anthocyanins (ACY), good flavor and juicing characteristics. Secondly, it will attempt to permanently inactivate polyphenol oxidase (PPO) by exposing the juice to an immobilized protease enzyme.

The prevention of enzymatic browning, primarily catalyzed by the action of PPO, has been shown to be a major concern in the storage and processing of fruit and vegetable products. Kader (1985) states that good appearance is one of the most important attributes consumers consider prior to purchasing fruit and vegetable products. Therefore, much research has, and is being done to determine the mechanisms that cause enzymatic browning and ways to prevent this phenomena.

This review will cover the biochemistry of PPO, effects of PPO on anthocyanin degradation, current means of inhibiting enzymatic browning and protease enzyme immobilization. Protease enzyme immobilization and its use to inhibit or destroy PPO is the basis of the present effort to reduce enzymatic browning in plum juice products.

Polyphenol oxidase

Enzyme Nomenclature

Polyphenol oxidases (PPO) belong to a group of substances called oxidoreductases. This group of enzymes catalyze the oxidation of phenolic compounds in the presence of molecular oxygen (Vamos-Vigyazo, 1981). For many years the two enzymes classified as PPO were catechol oxidase or o-diphenol oxygen reductase (EC 1.10.3.1) and laccase or p-diphenol oxygen oxidoreductase (EC 1.10.3.2). Subsequently, in 1973 the subclass "10" was abolished and all the phenolases were categorized as "monophenol monooxygenases" (EC 1.14.18.1) with catechol oxidase and laccase being combined as monophenol dihydroxy-L-phenylalanine oxygen oxidoreductase (EC 1.14.18.1) (Anon., 1973). However, Mayer (1987) reports that the international nomenclature has again been changed with monophenol monooxygenase (tyrosinase) being referred to as 1.14.18.1, diphenol

oxidase (catechol oxidase, diphenol oxygen oxidoreductase) as 1.10.3.2 and laccase as 1.10.3.1. Regardless of their grouping, these enzymes are quite different in their substrate specifity.

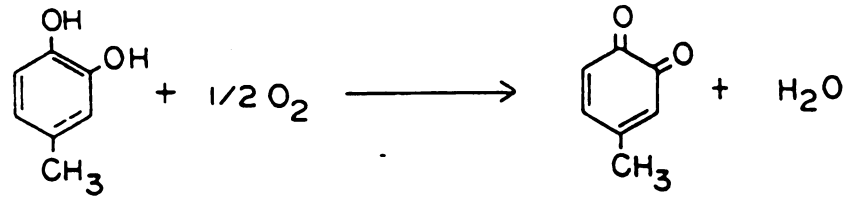
Catechol oxidase, now generally referred to as PPO, oxidizes phenolic compounds with ortho- and vicinal (3,4,5) trihydroxy OH-groups (Zaprometov, 1977). This enzyme is responsible for two specific reactions. First the hydroxylation of monophenols (like p-cresol) to o-diphenols (like 4-methylcatechol). This is referred to as cresolase activity since p-cresol is often used as a substrate (Figure 1.1). The second reaction involves the oxidation or dehydrogenation of the o-diphenols to o-benzoquinones which is referred to as catecholase activity since catechol is often used as a substrate in the assay of PPO activity on o-diphenols (Figure 1.2)(Sanchez-Ferrer et al., 1988). Trivial names of this enzyme include phenolase, polyphenolase, catechol oxidase and o-diphenol oxidase (Vamos-Vigyazo, 1981).

The second enzyme, laccase, oxidizes o- and p-dihydroxy phenols but does not hydroxylate monophenols (Walker, 1975). This enzyme occurs less frequently than PPO in fruits and vegetables but has been found in some peach cultivars (Mayer and Harel, 1968), mushrooms (Brown, 1967; Turner et al., 1975) and tomatoes (Filner et al., 1969).

p-Cresol

4-Methylcatechol

Figure 1.1. Hydroxylation of monophenols to o-diphenols, ie. cresolase activity.



4-Methylcatechol

4-Methyl *o*-benzoquinone

Figure 1.2. Oxidation of o-diphenols to o-benzoquinones, ie. catecholase activity.

This review and research, however, will primarily deal with the catecholase activity of PPO since it has been determined by Siddiq et al. (1992) to be the dominant enzymatic reaction to cause browning in Stanley plums (cv. *Prunus domestica L.*).

Incidence of PPO

PPO is found in all plants, some fungi and some animal organs (Brown, 1967). However, PPO content varies widely, depending on species or cultivar and stage of maturation. Because of this, PPO has been studied in a wide variety of fruits such as bananas (Palmer and Whitaker, 1963), pears (Rivas and Whitaker, 1973), grapes (Cash et al., 1976), peaches (Jen and Kahler, 1974), green olives (Ben-Sholam et al., 1977), mango (Park et al., 1980) and apples (Coseteng and Lee, 1987).

Voigt and Noske (1966) found that clarified apple and pear juice were practically devoid of PPO activity which remained almost entirely in the pulp. Dang (1971) found that PPO activity was much higher in the skins of plums when compared to the flesh and sap. Seventy seven to ninety six percent of the acivity was present as particulate, insoluble enzyme that decreased slightly during ripening.

Role of PPO

In nature, PPO plays many roles. Most importantly, the quinones formed by PPO action undergo secondary polymerization which yield dark, insoluble polymers that act as a barrier to the spread of microbial and viral infections (Rubin and Artsikovskaya, 1960). It has also been found that plants resistant to adverse climatic conditions generally have higher PPO activities than susceptible varieties (Vamos-Vigyazo, 1981). For example, Khrushcheva and Krehin (1965) found higher levels of PPO activity in the leaves of the frost-resistant plum tree *Prunus ussuriensis* when compared to respective plants susceptible to frost damage. Furthermore, PPO has been reported in phenol biosynthesis (Walker, 1975) and auxin biosynthesis (Gordon and Paleg, 1961).

However, the PPO reaction food scientists are primarily concerned with is enzymatic browning. The o-quinones, which are formed as primary products of the oxidative reaction catalyzed by the enzyme, (a) react with each other to form high molecular weight polymers, (b) form macromolecular complexes with amino acids or proteins and (c) oxidize compounds of lower oxidation-reduction potentials (Mathew and Parpia, 1971). Reactions (a) and (b) lead to the formation of brown pigments or melanins; the higher the molecular mass the darker the color. In the manufacture of black tea

(Takeo, 1966), sultana grapes (Grncarevic and Hawker, 1971) and prunes (Moutounet and Mondies, 1976) enzymatic browning is desirable and necessary for an acceptable final product. The action of PPO can also be beneficial for taste and flavor in fermented beverages. In most cases, however, enzymatic browning is undesirable. This includes browning caused by bruising during handling and transportation, exposure to air in cut, sliced or pulped states, or when thawed (cell breakage after freezeing).

Biochemistry of PPO

PPO requires molecular oxygen to catalyze the hydroxylation of monophenols and oxidation of o-diphenols. Mason et al. (1955) determined that the oxygen for hydroxylation came directly from atmospheric oxygen, not water. He labeled atmospheric oxygen as 18-O_2 and the oxygen in water as H_2-16O and visa versa (19-O_2 and H_2-18O). When the experiment was run using 18-O_2 and H_2-16O the end product (o-diphenols) contained 18-O_2. Conversely, when the experiment was run using 16-O_2 and H_2-18O there was no 18-O_2 incorporated in the final product. This demonstrates that one atom of oxygen is incorporated into the phenol and the other into the water that is formed. Thus PPO acts as a monooxygenase or monophenol oxidase in this reaction.

The mechanisms of the second reaction (oxidation or dehydroxylation) are not known with great certainty but

most probably occur according to an ordered, sequential mechanism (Whitaker, 1972). Eskin et al. (1971) did show that oxygen binds to the enzyme first which then reacts with the o-diphenol. The resulting o-benzoquinones then rapidly polymerize to form brown pigments or melanins.

Substrate Specifity

PPO from different tissues utilize different phenolic substrates to varying degrees. The evidence indicates that PPO from all sources studied thus far exhibit activity toward o-diphenols. PPO from apples (Cosetang and Lee, 1987) has activity on both mono- and diphenols. However, PPO from bananas (Palmer and Whitaker, 1963), tea leaf (Takeo, 1966) and peaches (Jen and Kahler, 1974) shows exclusive activity on o-diphenols and no ability to hydroxylate monophenols. This is also the case for Stanley plums. Siddiq et al. (1992) have shown that the concentration of monophenols is not significant and that Stanley plum PPO only reacts with o-diphenols. This information forms the basis for the use of catechol as a PPO substrate in the present study. Other substrates found in plums (cv. d'Ente) are chlorogenic acid, catechin, caffeic acid and DOPA (Moutounet and Mondies, 1976).

K_M and V_{max}

Vamos-Vigyazo (1981) reported that the affinity of PPO towards a given substrate may vary widely, even if isoenzymes of the same origin are considered. He also suggested that these differences might be due to steric factors connected to differences in the protein structure.

No relationship could be found between K_M and V_{max} values obtained for different substrates with a given PPO preparation (Lavollay et al., 1963; Soler et al. 1966; Vamos-Vigyazo and Gajzago, 1978). However, the efficiency of a specific substrate for a specific PPO preparation was established as V_{max} at $2K_M$ substrate concentration (Lavollay et al, 1963). Moutounet and Mondies (1976) reported that the K_M for plums (cv. d'Ente), with catechol as the substrate, was 13.0 mM.

pH Optima

The optimum pH for PPO activity varies with the source of the enzyme and the substrate. The range is relatively wide, generally between pH 4.0 and 7.0 (Aylward and Haisman, 1969). Moutounet and Mondies, (1976) reported that PPO from d'Ente plums had optimum activity at pH 4.25 but maintained a high percentage of activity at pH 3.8, which was the normal pH of the plum tissue. However, most of the activity was lost at pH 7.0.

Heat Stability

Vamos-Vigyazo (1981) stated that of all the stone fruits, plums generally have the most active and heat stable PPO. Dang and Vankov (1970) found that PPO was more heat stable in the juice than in the pulp of a given fruit. They also reported that the temperatures for inactivation of PPO ranged from 89.5 to 110°C. In addition, no relationship could be established between pH and heat tolerance for PPO in plums (Jankow, 1963).

ANTHOCYANINS

Structure

The water-soluble ACY pigments, which usually range in color from red to blue, are one of the major flavenoid classes (Gross, 1987a). Their basic nucleus consists of two aromatic rings linked together by a three-carbon unit (Fig. 1). An ACY pigment is composed of an aglycone (an anthocyanidin) esterfied to one or more sugars. These sugars consist of glucose, rhamnose, galactose, xylose and arabinose. ACY may also be "acylated" with one or more molecules of p-coumeric, ferulic, caffeic, malonic, vanillic or acetic acids esterfied to the sugar molecule (Francis, 1985). Markakis (1974) and Timberlake (1980) have shown that the color of ACY's are pH dependent. They appear to be

red in acidic media, blue or purple in alkaline media and almost colorless at intermediate hydrogen ion concentrations.

Location in Plums

ACY's are located mainly in the skin of plums. They accumulate in the vacuoles of the epidermal and subepidermal tissue (Gross, 1987b). Timberlake (1980) states that the ACY contained in plums (no cv. given) are cyanidin-3-rutoniside, peonidin-3-rutoniside and 3-glucosides. Druetta et al. (1985) reported that the major ACY's in plums (*Prunus salicina* cv. Carmesin) were cyanidin-3-glucoside and cyanidin-3-sambubioside.

Degradation of ACY

There are many ways ACY can be degraded since they are very unstable (Shirkhande, 1976). This is especially true for ACY in fruit juices, like Concord grape, because of the influences of pH, metal complexes, enzymes and other chemical constituents present both in the grape and other conditions of processing and storage (Sastry and Fisher, 1952; Asen et al., 1969; Peng and Markakis, 1963; Cash et al., 1976). In general, ACY degradation can occur from high temperatures during processing and storage, oxidation, loss of ascorbic acid, high pH, complexing metals, sugars and sugar degradation products, light and sulphur dioxide.

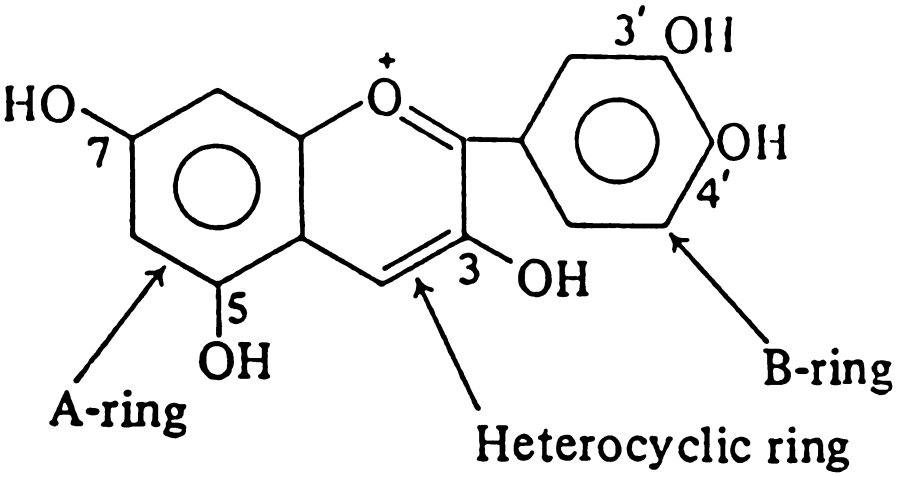


Figure 2. Flavon structure, basis of ACY molecule.

However, Sistrunk (1972) concluded that among all the enzymatic and nonenzymatic reactions occurring in Concord grape juice, the PPO enzyme was the most destructive to the ACY pigment.

Sakamura et al. (1965) was among the first to suggest that the enzyme which destroys ACY's in eggplants was a metal-containing oxidase. He also found that ACY losses were distinctly accelerated with the addition of chlorogenic acid, a substrate of PPO. Peng and Markakis (1963) have shown that ACY's alone were a poor substrate for mushroom PPO, but they were quickly decolorized enzymatically with the addition of better substrates such as catechol. It has been reported that in strawberries, the ACY pigments were destroyed either by direct oxidation by the quinones formed from the breakdown of D-catechin by PPO or by copolymerization into tannin formed via D-catechin-quinone polymerization (Wesche-Ebeling, 1984). In more recent research, Wesche-Ebeling and Montgomery (1990) state that the quinones and intermediary compounds formed during oxidation of D-catechin by PPO may be responsible for the destruction of ACY's either through oxidation or co-polymerization. Co-polymerization led to the formation of polymeric pigments responsible for the red colors observed in food products in which ACY's are no longer present. It is clear that PPO plays an active part in

the degradation of ACY but the exact fate of the pigment is still not known for certain.

POLYPHENOL OXIDASE INHIBITION

There are several, general methods of inhibiting the ACY degradation and browning caused by PPO. These include: 1) Specific inactivation of the enzyme itself, 2) Elimination of the substrate that reacts with PPO, 3) Interaction of a chelating agent with the copper (Cu^{++}) prosthetic group, and 4) Elimination of the oxygen required for the reaction to occur.

Golan-Goldhirsh et al. (1984) suggest that in the presence of a substrate with a fast k_{cat} (catalytic rate constant) inactivation and an excess of reductant, the enzymatic reaction would proceed with no color formation until the enzyme is completely and irreversibly inactivated. This may be due to the fact that the PPO would react with that substrate first rather than with the mono and o-di phenols which would result in dark color formation.

Sulfites

The first reductants to be used extensively in the prevention of browning were sulfites (Ponting, 1960; Diemair et al., 1960; Mayer et al., 1964; Sistrunk, 1972). Sulfites act as a reductant that forms a

compound by reacting with the o-benzoquinones to form a colorless complex (LuValle, 1952). The formation of this compound prevents the condensation of the o-quinones to form dark pigments and this will continue until either the sulfites or the PPO is used up (Embs and Markakis, 1965). There is some evidence that shows that sulfites may also inhibit PPO itself. Sayavedra-Soto and Montgomery (1986) suggest that the major mode of direct, irreversible inhibition of PPO was modification of the protein structure with retention of its molecular unity. Despite the positive capabilities of the sulfites to prevent browning and PPO activity, the future use of bisulfites is questionable.

On August 8, 1986, the Food and Drug Administration (FDA) banned the use of sulfite preservatives in fresh fruit and vegetables (Anon., 1986; FDA, 1986) because sulfites have been linked to adverse health reactions in some individuals. These adverse effects have mainly occurred among asthmatics (Langdon, 1987). The rule was modified on January 9, 1987, stating that any foods containing greater than 10 parts per million (ppm) bisulfites must have this information listed on the package label (Anon., 1986; FDA, 1986).

Ascorbic Acid and Other Inhibitors

In reaction to the FDA rulings concerning sulfites, many alternative methods to control enzymatic browning

have and are being investigated. A great number include formulations of ascorbic acid, erythorbic acid or their sodium salts with citric acid (Anon., 1977; Labell, 1983; Andres, 1985; Duxbury, 1986; Langdon, 1987; Hsu et al., 1988; Santerre et al. 1988; Sapers et al., 1989). However, Taylor et al. (1986) showed that most of these methods are not as effective as sulfites because they do not penetrate the cellular matrix sufficiently. Moreover, Ponting and Joslyn (1948) reported that ascorbic acid is easily oxidized by endogenous enzymes or by iron- or copper catalyzed reactions. As the concentration of ascorbic acid drops, due to the previously described reactions or by way of reducing o-quinones, Mahoney and Graf (1986) showed that ascorbic acid may actually have a prooxidant effect. Borenstein (1965) and Sapers and Ziolkowski (1987) have shown that erythorbic acid oxidizes more quickly than ascorbic acid making it even less effective.

In 1987, Seib and Liao reported that ascorbic acid-2-phosphate and ascorbic acid-2-triphosphate are stable against oxidation by H_2O_2 and release ascorbic acid when hydrolyzed by phosphatase. Sapers et al. (1989) stated that ascorbic acid-2-phosphate and -triphosphate showed promise as inhibitors of enzymatic browning on cut surfaces of raw apples but were ineffective in apple juice.

A fat soluble analog of ascorbic acid, ascorbyl palmitate, was shown to be an effective antioxidant for vegetable oils (Cort, 1974). Subsequently, in 1989, Sapers et al. showed that ascorbyl palmitate, as well as other ascorbic acid-6-fatty acid esters exhibited some anti-browning activity in apple juice but were of limited value when applied to the surface of cut apple slices.

Diethyldithiocarbamate, 2-mercaptobenzothiazole, cyanide, EDTA and azide are chelating agents that inhibit PPO by interacting with its copper prosthetic group (Mayer and Harel, 1979; Vamos-Vigyazo, 1981). Sporix, an acidic polyphosphate, is an effective chelating agent for PPO (Friedman, 1986) and has been shown to be an effective anti-browning treatment in apple juice (Sapers et al., 1989) but it is not FDA approved for use in food products. Beta-cyclodextrins, cyclic oligosaccharides composed of 6 or more glucose units with alpha-1,4 linkages and beta-cyclodextrin combinations with ascorbic acid or ascorbic acid derivatives formed inclusion complexes with the PPO substrates thereby preventing their oxidation to o-quinones and subsequent polymerization to brown pigments (Sapers et al., 1989). Cinnamic and benzoic acids have also been shown to inhibit PPO in apple juice but lose their effectiveness after approximately 7 hours (Walker, 1976).

Ozmianski and Lee (1990) attempted to prevent browning in grape juice (cv. Niagra) by the addition of honey (5%). Their rationale for using honey was that sugar solutions reduce the concentration of dissolved oxygen and the rate of diffusion of the oxygen from air into the fruit tissue (Joslyn and Ponting, 1951). Their results suggest that honey contains a low molecular weight peptide which interacts, as a chelating agent, with PPO's copper prosthetic group. However, the effect of honey on polyphenol oxidase in grape juice was significantly lower when compared to that of ascorbic acid.

Most of these sulfite substitutes, including reducing agents, chelating agents, acidulants, inorganic salts and ascorbic acid complexes have been proven to be successful against enzymatic browning but their effectiveness is usually short-term. They are also additives. This research proposes to inhibit PPO without adding anything to the product, which may be important in this label-conscious society.

ENZYME IMMOBILIZATION

Immobilized enzymes, and their applications for use in food and medical processes, have been researched extensively, beginning in the early 1960's (Richardson, 1974). Immobilized enzymes can offer certain advantages

over soluble enzymes in areas such as the study of enzymes, analytical biochemistry, preparative pharmacology and industrial applications, including food processing (Taylor et al., 1976). Some advantages and disadvantages of using this form of enzyme are listed below (Taylor et al., 1976):

Advantages of immobilized enzymes:

1) Enzyme is reusable.
2) Reaction is easily terminated by separating substrate from enzyme.
3) More precise control.
4) Less product inhibition.
5) Greater pH and temperature stability.
6) Can use enzymes presently unusable for various reasons.
7) Potential operation over greater pH range by modifying charge characteristics of support.
8) Continuous or batch use.
9) Greater reactor design flexibility.

Disadvantages:

1) Inactivation with continuous operation.
2) Cost of support.

In principle, enzymes that perform single or sequential reactions can be immobilized by one of five general methods (Mosbach, 1980):

1) Covalent attachment of enzyme to an insoluble matrix. Porous glass and ceramics, stainless steel, sand, charcoal, cellulose, synthetic polymers and metallic oxides have been utilized.
2) Adsorption of enzymes on solid supports such as ion exchangers. This would also include hydrophobic and affinity binding.
3) Inclusion of enzyme within a polymeric, organic or biological gel lattice.
4) Cross-linking of enzymes with a bifunctional reagent. Among the most popular cross-linkers are glutaraldehyde, dimethyladipimidate, dimethylsuberimidate and aliphatic diamines.
5) Encapsulation of enzymes so that the enzymes are enveloped within various forms of membranes that are impermeable for enzymes, and other macromolecules, but permeable for low molecular weight substrates.

There are a variety of organic and inorganic supports to choose from. However, controlled pore glass (CPG) beads will be used for the present research because the enzyme can be covalently attached to the beads. In addition the use of silica based carriers offers the advantages of chemical/microbial stability and incompressability which allows for high pressures and flow rates. CPG is prepared by heating borosilicate

glass to around 600°C where it undergoes phase separation. Subsequent acid leachings of the borate component produces a support with well defined porosity (Kennedy and White, 1985). The enzyme can be coupled to the beads via formation of a Schiffs' base (aldimine linkage) to amino-alkylsilyl-glass using the bifunctional reagent glutaraldehyde (Gusek et al., 1990).

Many applications are being explored for utilizing immobilized enzymes in the food industry. Immobilized lactase may hydrolyze the lactose in milk or whey resulting in an increase in sweetness, solubility and carbohydrate sugars, resulting in broader fermentation possibilities, more ready fermentation of these sugars and diminished possibility of lactose crystallization (Pitcher, 1980(a)). Trypsin, which has an antioxygenic effect on milk; ie. inhibits the development of oxidized flavor (Lim and Shipe, 1972), has been covalently attached to porous glass (Weetall, 1969) and used to retard the development of these oxidized flavors (Shipe et al., 1972). However, there are relatively few processes where immobilized enzymes are being used on a commercial basis because of the cost involved in support preparation. Yet there are some examples of immobilized enzymes in commercial processes. Sato et al. (1975) and Skinner (1975) developed a method of producing L-aspartic acid by immobilizing *Escherichia coli* cells in

fixed beds. The immobilized enzyme selectively removed the acetyl group from the optically active L-isomer of a racemic mixture of the aceylated amino acid. The resulting free L-form was easily separated from the aceylated D-form, which was then racemized chemically to regenerate more L-amino acid. Converting to this continuous process from the previous batch process reduced costs approximately 40% (Taylor et al., 1976). Alpha-galactosidase (alpha-D-galactoside galactohydrolase, EC 2.1.22), which is immobilized using mycelial pellets of *Morteriella vinacea* containing the enzyme is being used to hydrolize raffinose (O-alpha-D-glucopyranoside) in sugar beet molasses to galactose and sucrose (Pitcher, 1980(b)). Glucose isomerase may be immobilized using diethylamino-ethyl cellulose (DEAE), an ion exchanger, or covalently coupled to CPG beads (Habiba, 1989) which is then used to produce high fructose corn syrup (HFCS). The commercial process involves liquefying raw starch, saccharifying to dextrose, isomerizing to fructose and refining (Aschengreen, 1975; Barker, 1975; Skinner, 1975).

However, this research deals with the immobilization of protease. Proteolytic enzymes such as ficin, rennin and papain have been immobilized using collagen as a carrier (Venkatasubramanian et al., 1975). Immobilized papain was then used to chill-proof beer by hydrolyzing residual proteins that would otherwise

percipitate and cloud the beverage when it was placed in cold storage. Bliss and Hultin (1977) immobilized a filamentous prokaryote protease (*Streptomyces griseus*) which was subsequently used in a plug flow reactor to inactivate fungal glucose oxidase in solution at low concentrations. This process proved to be effective when compared to only silanized glass with no protease bound to it. However, these researchers also found that less tomato pectin methylesterase was inactivated by glass-bound protease than by plain glass. It was concluded that this was most likely due to the masking of the adsorption sites by the immobilized protease.

OBJECTIVE

The objectives of this research will consist of the following:

1) Determine if the immobilized protease will inactivate pure PPO.
2) Assess column stability, storage conditions, and maximum flow rate while retaining PPO inactivation.
3) Comparing PPO inactivation between CPG with immobilized protease and CPG without immobilized protease, and comparing a fungal protease to papain.
4) Expose plum juice to the predetermined food grade protease that has been immobilized on CPG beads.
5) Store treated and control plum juices at refrigeration temperature (5°C) and evaluate quality objectively (ACY degradation and microbial analysis) and subjectively (sensory analysis). Two controls will be used, a raw untreated juice sample and a pasteurized juice sample.

MATERIALS AND METHODS

The Stanley variety plums were harvested at maturity in September, 1991 from orchards in Alma, Michigan and immediately frozen. The plum samples were stored at -20°C at Michigan State University until further processing was required.

Plum Juice Production

One hundred pounds of Stanley plums were removed from -20°C storage and allowed to thaw overnight at 5°C. Debris (ie. stems, leaves, shrivelled fruit) were removed. The plum samples were heated to 65°C and macerated in double jacketed, stainless steel kettles. The macerated plums were cooled to 49°C and a commercial grade pectinase was added (1 g pectinase per 10 lbs crushed fruit). After holding 6 hours at room temperature the crushed fruits were pressed to obtain juice using a rack and cloth press. The yield of plum juice was approximately 59 lbs. The soluble solids content and pH of the juice was determined using an Abbe-3L refractometer (Bausch & Lomb Optical Co.) and a Corning 610 A pH meter. This juice was subsequently stored at -20°C until required.

Extraction of Polyphenol Oxidase (PPO) from Plums

Extraction of PPO enzyme was carried out using a modification of the method of Cash et al. (1976). All extraction materials were maintained at refrigeration temperatures (2-5°C) to reduce losses of enzymatic activity during extraction. A representative sample of 100 g of tissue from 9-10 uniform sized plums was blended in a pre-chilled blender with 2x volume of 5°C, 0.1M Tris hydroxymethyl aminomethane buffer (pH 9.5) for 2 minutes. The homogenate was filtered through 8 layers of cheesecloth and the filtrate was precipitated with 4x volume of -20°C acetone. When precipitation was complete (approximately 30 seconds), the precipitate was collected by straining through 1 layer of 35 micron nylon cloth. The precipitate was suspended in 100 ml of 5°C, 0.1M sodium acetate, pH 7.0. Pectic substances were precipitated by the addition of 16 mls of 5°C, 0.05M calcium chloride. The solution was centrifuged in a refrigerated centrifuge at 4400 x G for 10 minutes and the supernatant was used as crude enzyme extract for the enzyme assays.

Assay of PPO Enzyme Activity

The standard reaction mixture, for enzyme assay, consisted of 3.4 mls 0.1M sodium acetate buffer, pH 6.0, 0.4 ml 0.3M catechol, and 0.2 ml PPO extract. A Lambda Perkin Elmer spectrophotometer, equilibrated at 30°C

with enzyme kinetics software package, was used to monitor change in absorbance at 420 nm per minute for 3 minutes. One unit of enzyme activity was calculated from the slope of the curve which determined optical density (O.D.) at $420_{nm/min}$ due to the oxidation of catechol. The assays of PPO enzyme activity were performed in duplicate.

Immobilization of Protease on CPG Beads

Three different types of protease enzyme were immobilized on CPG beads. In the initial study, a nonfood-grade protease (fungal type 18, Rhizopous species; Sigma) was immobilized using a modification of the method described by Gusek et al. (1990).

Ten grams of CPG beads (400-500 A; Sigma) were mixed with 2.7 g zirconium chloride ($ZrCl_4$) in 30 mls of 1,2 dimethoxy ethane and held for 2 hours at 25°C. The slurry was transferred to a rotary evaporator and held under a partial vacuum for 10 hours at 86°C. The CPG beads were then dried in a vacuum oven for 3 hours at 30°C and calcined to the oxide in a muffle furnace for 16 hours at 350°C. Residual $ZrCl_4$ was hydrolyzed with successive washings of water, dilute sulphuric acid, water, and acetone.

Derivitization by silanization (Weetall, 1976) was accomplished by combining 18 mls distilled water with 2 mls gamma-aminotriethoxy silane, adding CPG beads, and

adjusting pH to between 3 and 4 using 6N HCl. The solution was placed in a 75°C water bath for 2 hours, filtered through a No. 1 filter paper in a Buchner funnel and washed with 20 mls/g distilled water.

The CPG beads were then dried for at least 2 hours at 115°C. Activation was accomplished using the methods of Stolzenbach and Kaplan (1976). A combination of 1 ml of 5% glutaraldehyde (in 0.1M phosphate buffer, pH 8.0) per 0.5 g CPG beads was held for 1 hour at 25°C and then successively washed with 450 mls cold distilled water and 50 mls cold phosphate buffer, pH 8.0. For immobilization, a solution consisting of 1 mg protease/1 ml distilled water/0.5 g CPG beads was allowed to react for 24 hours at 5°C with gentle agitation using an orbital water bath. Unbound protease was removed with successive washings of cold 0.1M phosphate buffer, pH 8.0, cold 1.0M NaCl, and 0.1M phosphate buffer, pH 6.5 (storage buffer).

Two food-grade proteases, papain (papaya) and 7107 (*Aspergillus niger*), obtained from ROHM Enzyme Technology, were also immobilized using the same procedure described above.

Viscosity Analysis of Plum Juice

Difficulties were encountered in passing the plum juice through the columns. The density of the CPG beads was too great resulting in a "drop by drop" flow rate.

To overcome this problem we attempted to reduce the viscosity of the juice allowing a more efficient flow rate.

Two treatments were employed, pectinase and honey, to reduce the viscosity of the juice. Honey, along with pectinase has been found to be an effective treatment for inactivating the pectin structure in apple juice (McLellan et al., 1983) Four 500 ml samples of juice were prepared containing 0.5, 1.0, 1.5, and 2.0 g pectinase respectively. An additional four 500 ml samples of juice were prepared containing 5, 10, 15, and 20% honey. A control sample, 500 mls of pure juice, was also tested. All samples were held at 25°C for one hour prior to testing.

The viscosity of the treated and control plum juice was tested with a Haake RV 12 Rotoviscometer using an MV cup with MV-1 sensor and an M 500 measuring head. A Hewlett Packard Processor and a Hewlett Packard Data Acquisition/Control Unit was used to process the results. Ten measurements were observed, ranging from 0 to 500 revolutions per minute (rpm) with torque ranging from 0 to .003. These measurements were plotted and the slope of the curve equaled the viscosity of the samples analyzed.

Inactivation of Plum PPO by Immobilized Protease

In the initial study, 1 g of CPG beads immobilized with the nonfood-grade protease (Rhizopous) was packed into a 10x1 cm column (Bio-Rad). A 2 ml solution containing 1 mg commercial PPO (Sigma)/1 ml distilled water was passed through the column on a daily basis, for 5 days, and again for 3 days after 2 months had passed. The eluent from the column was assayed for PPO enzyme activity to determine the effectiveness of the immobilized protease in inactivating the pure PPO enzyme. This activity was then compared with the activity of the control PPO enzyme which had not been passed through the column.

Subsequently, 1 g of CPG beads, with immobilized papain and 7107 respectively, were packed into 50x1 cm columns (Bio-Rad). These columns were stored at 5°C and 25°C. Five mls of crude PPO enzyme extract from Stanley plums was passed through each of the columns ,once, on a weekly basis for seven weeks, and assayed for enzyme activity using the method described earlier. The control columns contained CPG beads with no protease immobilized to them.

Based on the results of the above study, immobilized papain was chosen to test the efficacy of immobilized protease in maintaining plum ACY's by inactivating the PPO enzyme in the plum juice. However, the flow rate was found to be unacceptable, even with

the previously described viscosity manipulations. So instead of the columns, a fluidized bed reactor was used. The juice and the immobilized protease were combined in a 3 L beaker and allowed to react together, with gentle stirring, for 30 minutes.

Untreated plum juice was exposed to immobilized papain, in two concentrations, for this study. The first concentration contained 0.25 g of CPG beads with immobilized papain which was exposed to 3 L of juice. The second contained 0.50 g of CPG beads with immobilized papain which was also exposed to 3 L of juice. Each 3 L batch of juice was subsequently divided into two, 1.5 L parts. One part remained at the initial, 14, ^{o}brix while the other was sweetened to 16 ^{o}brix with sucrose.

Two control juices were used for comparison. The first was prepared by pasteurizing plum juice to 88°C/1 min using a Cherry-Burrel No Bac Spiratherm and adjusting its sweetness to 16 ^{o}brix, to resemble a commercial juice product. The second control consisted of the raw, untreated plum juice.

All the juice samples were frozen until further objective and subjective evaluations were performed.

Protease Assay

The effluent from the immobilized protease exposure, from both plum PPO solutions and plum juice

(not commercial PPO solution), was assayed for the presence of any protease enzyme, which may have eluted, using a protease substrate gel tablet kit from Bio-Rad. This was done to determine if the protease enzyme was effectively immobilized on the CPG beads.

Anthocyanin (ACY) Degradation

Some problems were encountered in the attempt to extract PPO from the plum juice using the methods employed in the extraction of PPO from whole plums described earlier. In this technique the protein-pectin complex was percipitated with acetone. Subsequently, the PPO is solubilized using sodium acetate buffer. In the plum juice, however, pectin concentrations were greatly reduced by the addition of pectinase during the juice extraction process. This inhibited the protein-pectin complex from forming and percipitating when acetone was added. So for this research we will relate the rate of ACY degradation to the concentration of PPO in the plum juice samples.

The methods of Cash et al. (1976) were used to determine ACY concentration and degradation spectrophotometrically at 535 nm. Sample volumes (9 mls) consisted of one part juice to two parts 0.025M citrate buffer. This solution was kept in a 30°C water bath to maintain a constant temperature for the accelerated (temperature abused) ACY degradation

measurement. Total ACYS were extracted by mixing 1 ml of sample with 19 mls of extracting solvent consisting of 95% EtOH-1.5 N HCl in an 85:15 ratio (Skalski and Sistrunk, 1973). These samples were allowed to stand at room temperature for one hour before reading the absorbance at 535 nm. ACY pigment changes were followed at hourly intervals for the first seven hours and then a final sample was taken at 24 hours. For the long term (15 days) ACY degradation measurements, the water bath portion of the procedure described above was omitted.

Microbial analysis

Raw and processed (juice exposed to immobilized papain and also pasteurized) juice samples were tested, in triplicate, for total (standard) plate count (SPC), coliform counts, and yeast and mold counts, at 5 day intervals, using the following procedures (FDA,1990):

Dilution Water

A stock phosphate buffer was prepared by combining 34 g KH_2PO_4/liter distilled water and adjusting to pH 7.2 with sodium hydroxide. The dilution water was prepared by combining 1.25 mls stock phosphate buffer/liter distilled water, dispensed out in 9 and 99 ml aliquots, and autoclaved for 15 minutes.

Standard Plate Count

SPC agar was prepared according to package directions, brought to a boil, separated into 100 ml increments, autoclaved for 15 minutes, and cooled to 46°C. One ml of CPG bead treated sample diluted to 1:10,000 and one ml of pasteurized sample diluted to 1:100, was plated, incubated at 32°C for 48 hours, and the colonies were counted using a Quebec colony counter. These dilutions/counts reflect the 30-300 CFU/plate rule.

Coliform Count

Violet Red Bile (VRB) agar was prepared according to package directions, brought to a boil, and cooled to 46°C. A 1:100 dilution, reflecting the 30-300 CFU/plate rule, was used for all samples. The plates were incubated at 32°C for 24 hours. A Quebec colony counter was used to count colonies. Representative colonies were inoculated, using a flamed loop, into Brilliant Green Bile (BGB) agar and incubated at 32°C for 24-48 hours for confirmation.

BGB agar was prepared according to package directions. A test tube, containing a Durham tube, was then filled with 10 mls of BGB agar, autoclaved for 15 minutes, and allowed to cool to room temperature.

Yeast and Mold Counts

An antibiotic solution was prepared containing 100 mls of phosphate buffer and 500 mg each of chlortetracycline (Sigma) and chloramphenicol (Sigma). Two mls of antibiotic solution were mixed with 100 mls of SPC agar. Dilutions identical with the SPC procedure were used. The plates were incubated at 25°C for 5-7 days. The Quebec colony counter was used to count the colonies.

Total Protein Analysis

A total protein analysis was performed on the raw, treated, and pasteurized juice as well as the treated and untreated plum PPO extract. This was done to determine if any protein was lost from the plum juice during the immobilized protease exposure or pasteurization procedure. The analysis would also ascertain if any protein was lost from the plum PPO sample when exposed to CPG beads.

The protein determination procedure used was the Kjeldahl method and was executed in accordance with AOAC 24.038 (Crude protein and meat-block digestion method) (AOAC, 1984) and AOAC 47.021 (Micro-Kjeldahl method) (AOAC, 1984). The Kjeldahl method basically consisted of heating the sample in sulphuric acid and digesting until the carbon and hydrogen were oxidized and the

protein nitrogen was reduced and transformed to ammonium sulfate. Then concentrated sodium hydroxide (30% w/w) was added, and the digest heated to drive off the liberated ammonia into a known volume of a standard, boric acid solution (4% w/w). The unreacted acid was determined, via titration with HCl (0.1 N), and the results were transformed, by calculation, into a percentage of protein in the organic sample (Pomeranz and Meloan, 1987).

Protein Digest Preparation

It was assumed that the juice samples and the plum PPO extract contained approximately 2% protein. Three, 5 g juice samples, three, 3 g plum PPO samples and three, 5 g blanks (dd H_2O) were weighed into 100 ml Kjeldahl digestion tubes. One Kjeldahl tab and 5 mls of H_2SO_4 were added to each tube which were then placed in a Tecator 1016 Digestor digestion block with a heat setting of 1.5 and allowed to sit overnight. The heat setting was then increased by 0.5 every two hours until 3.5 was reached and again allowed to sit overnight. The heat setting was then increased by 0.5 every 30 min until a setting of 10 was reached and remained there for an additional 30 min. The slower than normal (1.5 for 1 hr and 0.5 increase every 30 min until setting 10) heat increase was necessary due to the excessive foaming

which occured because of the high sugar, carbohydrate and water content of the plum juice samples.

Distillation

After the samples turned from black to clear, they were placed, individually, into a distillation apparatus which consisted of a Buchi 322 Distillation Unit and a Buchi 342 Control Unit along with a Dosimat/655 Titration Unit, an Impulsomat/614, and a Brinkman/632 pH meter. The control unit controls were set as follows: H_2O = 1.9, NaOH = 2.1, Distllation time = 5.0 min, Distillation mode = 3, and Aspiration switch on. Manual instructions were followed for the pre-heating procedure.

Sixty mls of boric acid solution was poured into the receiving vessel. Its pH was entered into the Impulsomat/614 as the titration endpoint. The sample was subsequently distilled and titrated with the ml of HCl dispensed displayed on the Dosimat/665. This amount of HCl was then entered into the following equation to determine the % protein in the sample:

$$\% \text{ Protein} = \frac{(\text{HCl (mls)} - \text{Blank (mls)})(1.4007)(6.25)(\text{N HCl})}{\text{sample weight, g}}$$

RESULTS AND DISCUSSION

Plum Juice Yield and Characteristics

The yield of plum juice from 100 lbs of Stanley plums was approximately 59% (59 lbs of juice). The soluble solids content of the pure Stanley plum juice was 14 ^{o}Brix with a pH of 3.9.

PPO Inactivation

Initial Inactivation Study

An initial inactivation study was done using a pure, commercial PPO (fungal type 18, Rhizopous species; Sigma). The activity of the commercial PPO preparation was significantly reduced, by two log cycles, when passed through the immobilized, *Rhizopous* protease column (Fig. 3). The peak which occurs in the control enzyme on day 4 resulted from a fresh batch of commercial PPO. However, this did not influence or alter the inactivation capability of the immobilized protease. An attempt was made at regenerating the inactivation capabilities of the CPG beads on day 58. The CPG beads were allowed to react in a protease solution identical to that of the immobilization procedure described earlier. The inactivation capability was better on day 59 but

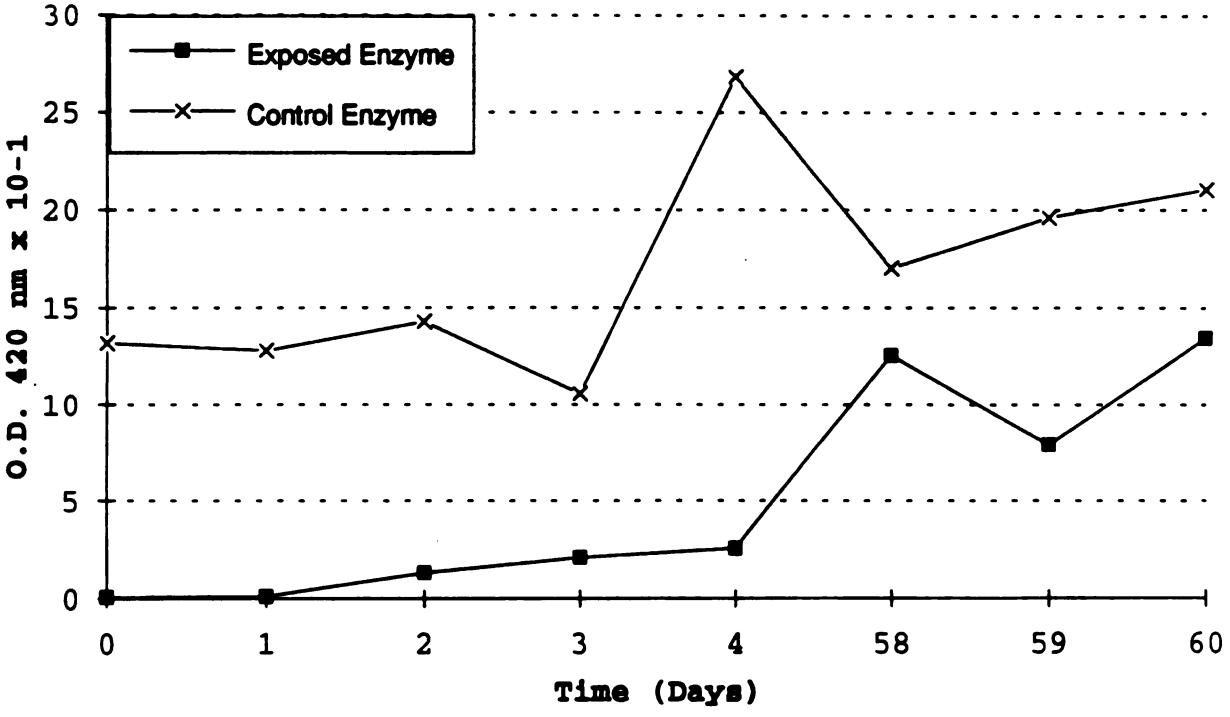


Figure 3. Commercial PPO exposed to immobilized *Rhizopous* protease vs. unexposed commercial PPO solution .

reverted back to day 58 levels, on day 60. This may be due to the protease adhering, but not permanently immobilized, to the beads from the reactivation process. This "non-immobilized" protease would then be responsible for the drop on day 59 but when it eluted, the inactivation capability of the immobilized protease returned to that of day 58. However, no protease analysis of the elluent was done for this portion of the study to confirm the previous statement.

Plum PPO Extract Inactivation

In this study, as indicated earlier, two types of proteases, papain and microbial (*Asp. niger*, 7107) were used to inactivate plum PPO. The immobilized enzyme columns were maintained at 5 and 22°C for the duration of the study. The initial activity of the PPO extracted from Stanley plums is given in Figure 4. It should be noted that some variability exists in the initial activitiy of the PPO extracts which may be reflected in the final activities. This crude extract was then passed through immobilized protease columns, as described earlier, to ascertain the inactivation capabilities of the specific protease at the above two storage environments.

Figure 5 shows the activity of the PPO enzyme extract after being passed through columns stored at 5°C and containing immobilized papain, 7107 and control

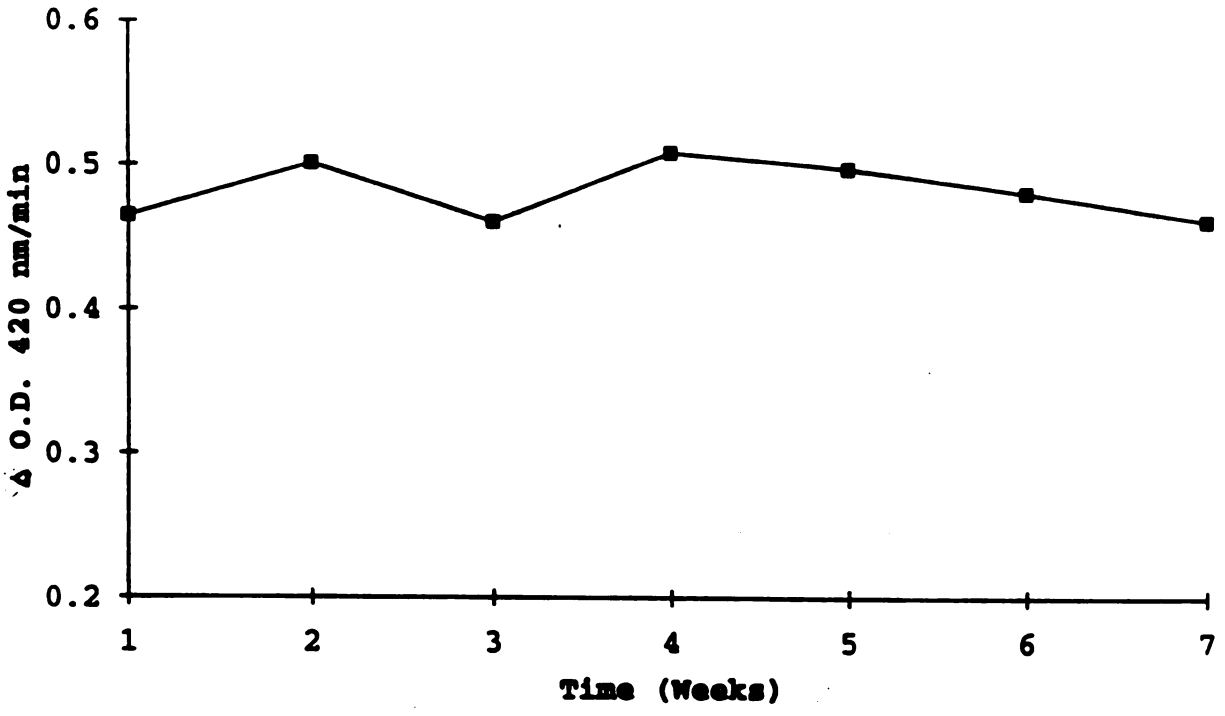


Figure 4. Initial activity of plum PPO extract prior to being passed through immobilized protease (papain and 7107) and control (uncoated CPG beads) columns.

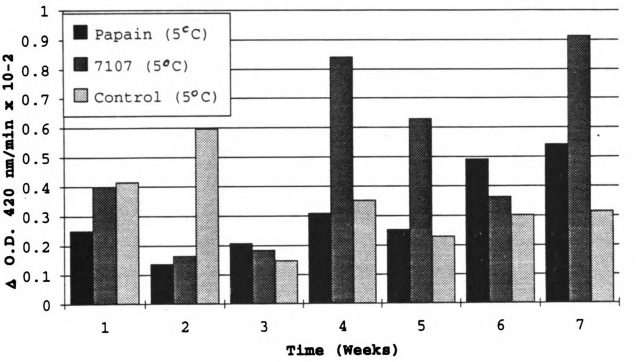


Figure 5. Inactivation capabilities of immobilized proteases and control, at 5°C, when exposed to plum PPO.

(without protease) CPG beads. Until weeks 5 and 6, the control columns also showed inactivation of the PPO enzyme. As mentioned earlier, Bliss and Hultin (1977) have suggested that some masking of adsorption sites by the immobilized enzyme could occur resulting in lower inactivation values when compared to plain CPG beads.

Figure 5 also shows the activity of PPO after being exposed to 7107 at 5°C. The results demonstrate the ineffectiveness of 7107 at this temperature.

Figure 6, with the only difference being storage temperature (22°C), also shows that the immobilized papain was generally more effective in inactivating the PPO extract. The immobilization capability of 7107 was better than control for the first three weeks at 22°C but then lost that ability. A reason for this may be that the immobilization process bound the protease so tightly to the CPG beads that relatively few active sites were made available to the PPO enzyme.

Figure 7 summarizes the data which shows that immobilized papain, stored at both 5 and 22°C, was more effective in inactivating PPO than 7107. However, the columns stored at 5°C exhibited better stability. This indicates that the immobilized papain retains higher levels of activity at 5°C.

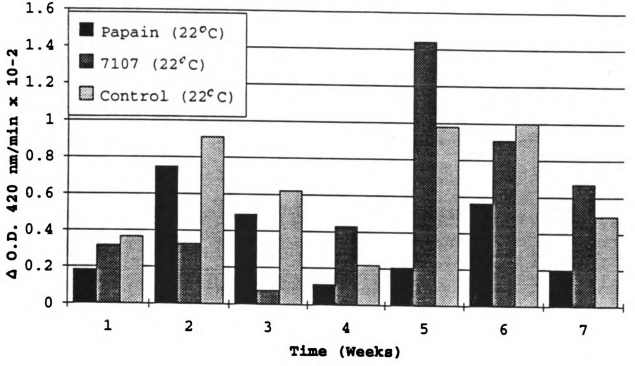


Figure 6. Inactivation capabilities of immobilized proteases and control, at 22°C, when exposed to plum PPO.

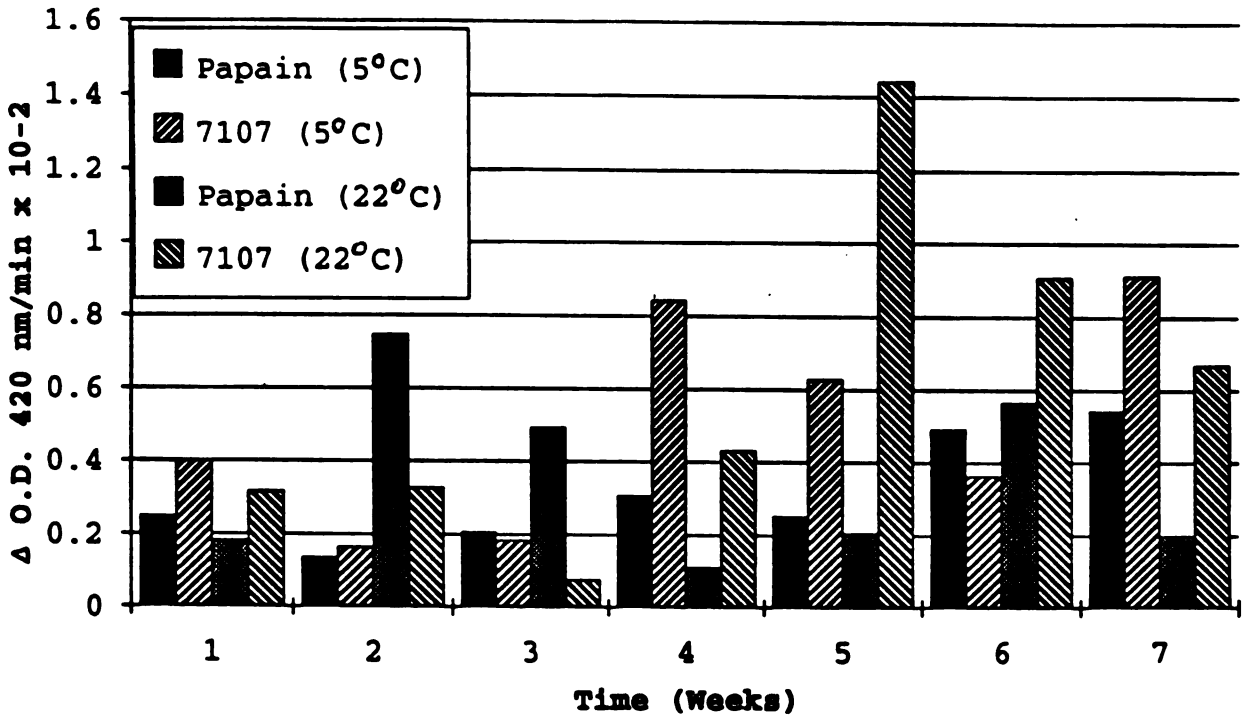


Figure 7. Inactivation capabilities of immobilized papain and 7107, at 5 and 22°C, when exposed to plum PPO.

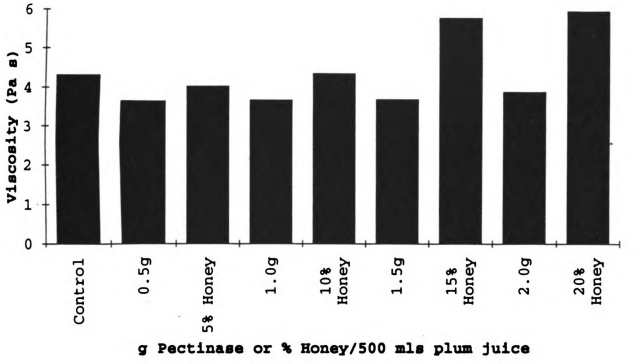


Figure 8. Change in viscosity of plum juice via addition of pectinase or honey.

Plum Juice Viscosity

An attempt was made at reducing the viscosity of the plum juice for this study to produce a clear cloud-free juice which could pass through the columns without clogging them. Figure 8 shows the results regarding the impact of pectinase and honey on the viscosity of plum juice. Even though honey can influence the viscosity of plum juice by inactivating the pectins (McLellan et al. 1983), it was not pursued because the flavor of the juice was significantly altered. The best results were obtained by adding 0.5 g pectinase/500 mls juice and this concentration was used for subsequent plum juice analysis.

Inactivation of PPO in Stanley Plum Juice

There were some problems when attempting to pump the plum juice through the columns containing the immobilized protease. The CPG beads would not allow the juice to pass through due to the high viscosity of the juice. So a fluidized bed reactor was used instead of the columns. Figure 9 shows the accelerated (temperature abused) rate of ACY degradation in the various juice samples over a period of 24 hours. It seems that the pasteurized sample retained the most ACY, ie. had the lowest PPO concentration. The ACY concentrations of the 14 ^{o}Brix juice samples were higher

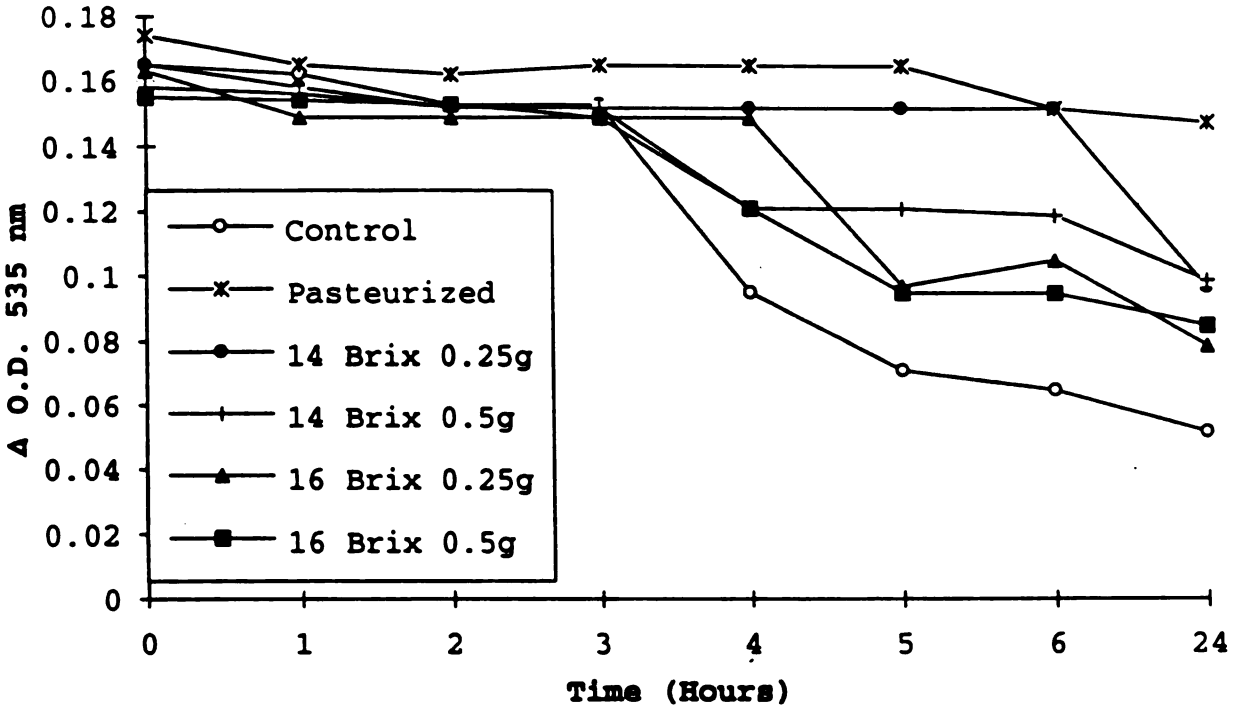


Figure 9. Accelerated degradation of ACY in treated and untreated plum juice samples measured over a 24 hour time interval.

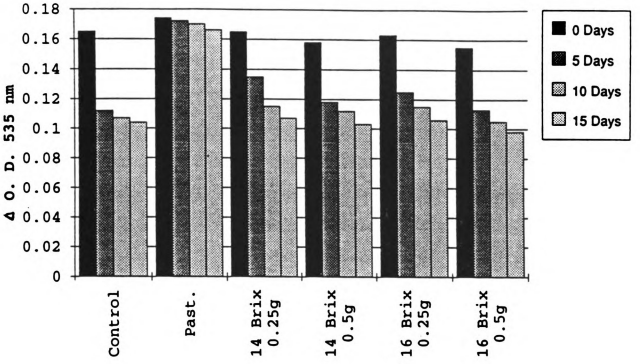


Figure 10.Degradation of ACY in treated and untreated plum juice samples stored at 5°C over a period of 15 days.

than the 16 ^{o}Brix and the juice samples exposed to the 0.25g CPG beads had higher ACY concentrations than those exposed to 0.5g CPG beads. However, all juice samples had higher ACY concentrations than the raw, untreated control sample. Figure 10 showsthe actual rate of ACY degradation at 5 day intervals over a period of 15 days.

PPO has been implicated in ACY pigment degradation in presence of the proper phenolic substrates such as catechol, chlorogenic acid, or the ACYs' themselves. It seems the samples, which had been exposed to immobilized protease, have lower ACY concentrations when compared to the pasteurized juice. Even though the immobilized protease readily inactivated PPO extracted from plums, the same is not true when juice was exposed to the immobilized protease. When the fluidized bed reactor was used, the contact between the protease and the PPO may have been reduced, as compared to the degree of contact attained when in a column, which may account for this result. Another reason for this may be that additional oxygen was incorporated into the juice as it was processed being in the fluidized bed reactor. Low oxygen concentrations, low temperature, and relatively low pH are required for optimum ACY stability (Markakis, 1974). Other components of the juice, such as sugars or organic acids, may cause this effect by protecting

active sites or physically interfering with the ability of the enzyme and substrate to react together.

Protease Analysis

No protease was detected in the plum PPO extract or the Stanley plum juice after it had been exposed to the immobilized protease. This shows that the protease was effectively immobilized on the CPG beads.

Microbial Analysis

No federal standards could be found for microbial levels in fruit juice products. We can, however, compare these results to the federal, maximum microbial levels of fluid milk (FDA, 1990), giving an indication of acceptable or unacceptable microbial levels in the plum juice.

Standard Plate Count

Table 1 shows the standard plate count for all the samples. The samples were analyzed at 5 day intervals for 15 days. All samples had relatively high counts, except for the pasteurized sample, although the control sample was the highest. These results were expected because the immobilized protease should not effect the level of the microbial population. FDA (1990) states that 100,000 CFU/ml is the maximum microbial level for

fluid milk SPC. This indicates that additional anti-microbial measures should be employed in the treated juice products. The pasteurized sample microbial results, however, fell well below this this level, making it commercially acceptable.

Coliform Count

Table 2 shows the coliform counts for all the samples. The only sample which contained any coliforms was the 14 ^{o}Brix, 0.25 g CPG bead sample. This sample may have been contaminated during processing since all other samples had negative indications (< 100 CFU/ml). FDA (1990) states that 1 CFU/ml is the acceptable coliform level. This level may be accomplished with proper sanitation during processing.

Yeast and Mold Count

Table 3 shows the yeast and mold counts for all the samples. The initial counts ranged from 5.8×10^5 to 7.1×10^5 CFUs'/ml juice, excluding the pasteurized sample. After 10 days at 5^{o}C, the counts ranged from 1.05×10^5 to 1.65×10^5 CFUs'/ml juice, again, excluding the pasteurized sample. These counts were so high that the mold actually became visible after 5 days. This shows that some form of microbial destruction, such as heat or pH manipulation is required for juice to have

Table 1. Standard plate count analysis of treated and untreated Stanley plum juice (CFU/ml x 10^{-4}).

			14 ºBrix		16 ºBrix	
Time (Days)	Untreated	Past.	0.25g	0.5	0.25	0.5
0	97	0.15	59	89	62	54
5	143	0.21	70	122	92	75
10	162	0.25	105	153	113	97

Table 2. Coliform count analysis of treated and untreated Stanley plum juice (CFU/ml x 10^{-2}).

			14 °Brix		16 °Brix	
Time (Days)	Control	Past.	0.25g	0.5	0.25	0.5
0	<1	<1	5	<1	<1	<1

Table 3. Yeast and mold analysis of treated and untreated Stanley plum juice (CFU/ml x 10^{-4}).

			14 ºBrix		16 ºBrix	
Time (Days)	Untreated	Past.	0.25g	0.5	0.25	0.5
0	65	0.24	69	71	61	58
5	148	0.40	131	148	98	75
10	165	0.52	152	171	122	105

an acceptable shelf-life. No federal standards could be found regarding yeast and mold counts.

Total Protein Analysis

Figure 11 shows the total protein analysis using the Kjeldahl procedure. The results indicate that minimal differences exist between the untreated, pasteurized and treated samples. This may be due to the low, initial protein concentrations of the juice and that enzyme content is only a small portion of the total protein content. This is reflected by Gebhardt et al. (1982) who state that raw and canned plums have an average protein content of 0.79% and 0.36% respectively. Even if the PPO was inactivated, the initial concentration may have been so small that it would not be reflected in the results. The same holds true for the plum PPO extract before and after it had been exposed to uncoated CPG beads because of the physical destruction of the PPO. Even though the enzyme is inactivated, it still contains nitrogen which is measured by the Kjeldahl method.

Conversely, the quantity of immobilized protease may not have been sufficient to inactivate all the enzymes, including PPO, in 3 L of juice. If this were the case, the enzymes in the juice could overwhelm the protease reducing PPO inactivation which results in an increase in the degradation of ACY in the juice samples.

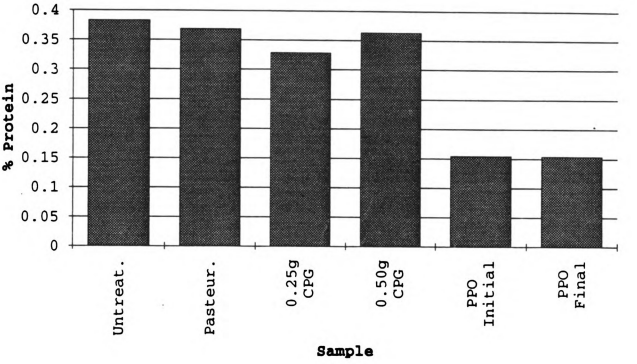


Figure 11. Total protein content (%) of the untreated, pasteurized, and treated juice samples (exposed to immobilized papain) and plum PPO enzyme before and after being exposed to uncoated CPG beads.

CONCLUSION

Protease enzymes immobilized on CPG beads seem to be an effective means for inactivating PPO solutions and extracts. A portion of this inactivation may be due to the CPG beads themselves because the control columns (CPG without protease) also exhibited inactivation capabilities. However, the final activities of the PPO (after exposure) were less consistent than that of PPO exposed to papain and 7107.

Subjecting raw plum juice to the immobilized papain had little effect on inhibiting ACY degradation. The best ACY retention over time occurred in the pasteurized juice sample.

Microbial results indicate that additional measures must be taken to reduce the microbial counts in the treated juice sample. The pasteurized sample, however, showed acceptable microbial levels indicating good industrial scale-up potential.

Rank preference tests (Sensory analysis, Appendix A) indicated that no significant ($p < 0.05$) preference exist between the 14 °Brix samples an 16 °Brix samples respectively. An extended triangle test showed that no significant difference ($p < 0.05$) exists between the 16 °Brix sample and the pasteurized control sample. The test also showed no significant preference ($p < 0.05$)

between the two samples. However, only one replication was performed resulting in a relatively high type II error. Consequently, the sensory results should only be interpreted as a general indication of what they may actually be.

The most significant result of this research was the impact of pasteurization on the plum juice. This process in a juice with relatively high ACY concentrations, relatively low microbial counts, and sensory studies indicate that there may not be any significant difference ($P < 0.5$) between the pasteurized sample and the raw, untreated juice. This result bodes well for future research for scaling up this process to industrial levels because the pasteurization process is similar to that currently being used for other juice products making it very cost effective.

Future research may also include variations on the protease carrier to maximize mass transfer and because CPG beads are not a cost effective option. Also, the combining of protease treatments with heat, ascorbic acid, citric acid, etc. treatments and the topical or surface applications of food grade protease solutions to prevent enzymatic browning on cut surfaces of fruit and vegetables could also be researched further.

APPENDIX A

APPENDIX A

Sensory Analysis Methodology

The objective of this sensory work was to determine which juice sample, for each CPG bead concentration, is preferred overall. Since no preference existed between these samples, an extended triangle test was used to confirm if any difference existed between a treated 16 ^{o}Brix sample (0.25g CPG) and the pasteurized sample (16^{o}Brix).

Two types of sensory evaluation test methods were employed to evaluate the five different juice samples. Rank tests (Larmond, 1977a), one for 0.25 g CPG and one for 0.5 g CPG were used to determine which juice was preferred overall. An extended triangle test (Jellinek, 1985) was used to determine if any difference existed between the treated plum juice and the control (pasteurized) plum juice. The extended portion of the triangle test ballot made provisions for panelists to express their preference for the odd and duplicate samples.

Sensory Test Methods

The rank test procedure, which was administered first, and analysis was followed according to the methods of Larmond (1977a). The panel consisted of 24

untrained students, faculty and staff from Michigan State University. Subjects evaluated 3 samples per test, consisting of 14 °Brix juice (0.25 g CPG), 16 °Brix juice (0.25 g CPG), and control (16 °Brix juice, pasteurized). The second rank test included 14 and 16 °Brix juice (0.5 g CPG) as well as the pasteurized juice.

To determine any difference between the 16 °Brix (0.25 and 0.5 g CPG) and the control juice, extended triangle tests were used and analyzed according to Larmond (1977b). The untrained panel consisted of 24 students, faculty and staff. Subjects evaluated 3 samples per test consisting of 16 °Brix juice (0.25 and 0.5 g CPG) and the control juice.

Environmental Conditions

All sensory tests were held in the sensory evaluation laboratory of the Department of Food Science and Human Nutrition at Michigan State University. This laboratory is equipped with fifteen isolated testing booths, temperature regulated positive airflow, and constant illumination. Panelists evaluated the juice samples under white fluorescent lighting.

Sample Preparation/Presentation

The juice samples were removed from refrigeration approximately one hour prior to sensory evaluation.

Samples consisted of approximately 20 mls of juice which was poured into one ounce plastic cups labeled with a three-digit random number for identification. The samples were allowed to come to room temperature prior to the sensory evaluations. All sample presentation orders were randomized with a total of 9 samples per panelist. Subjects were instructed to drink ambient temperature deionized water, as well as eat unsalted crackers, ad libitum prior to and between sample evaluations. Panelists were also allowed to swallow or expectorate the juice samples. The tests were held consecutively on one day lasting from mid-morning to mid-afternoon with a total of 9 samples per panelist.

Sensory Statistical Analysis

Two-way ANOVA was used to test the significance of main effects for the rank test (Larmond, 1977a). The statistical, triangle test, difference analysis chart in Larmond (1977c) was used to determine the significance of any differences for the triangle test.

Sensory Evaluation Results

Minimal sensory research has been reported for Stanley plum juice regardless of treatment or soluble solids content.

Preference Rank Test

The preference rank mean scores are shown in Table 4. There was no significant preference in plum juice treated with 0.25 g CPG and control (pasteurized) regardless of soluble solids content. The same conclusion holds true for plum juice treated with 0.50 g of CPG beads (Table 5).

Extended Triangle test

This test was conducted using a control (pasteurized plum juice at 16 ^{o}Brix with no exposure to immobilized protease), and 16 ^{o}Brix plum juice from the 0.25 g CPG bead treatment. Eleven of the 24 panelists were able to choose the correct (odd) sample. Thirteen out of 24 are required for a significant difference (P = 0.05) to exist. Therefore, no significant difference was detected between the samples.

Out of the 11 panelists who identified the correct (ood) sample, 6 preferred the pasteurized control sample and 5 preferred the treated sample. These results show that there was no significant preference (P = 0.05) between the two samples.

The descriptions of the two samples, from the previously mentioned panelists are reported in Table A.3. Both samples were found to be tart and sweet ,however, the protease treated juice exhibited a "less concentrate" flavor.

Table A.1. Two-way ANOVA for preference rank test juice treated with 0.25 g CPG beads.

Source	DF	SS	MS	F	Prob.
Samples	2	0.013	0.007	0.009	<0.1
Judges	23	0	0	0	
Error	47	34.67	0.74		
Total	72	34.68			

Table A.2. Two-way ANOVA for preference rank test juice treated with 0.5 g CPG beads.

Source	DF	SS	MS	F	Prob.
Samples	2	0.004	0.002	0.003	<0.1
Judges	21	0	0	0	
Error	43	31.78	0.74		
Total	66	31.79			

Table A.3. Descriptions from Extended Triangle Test

These descriptions are only from the 11 panelists who identified the odd sample correctly.

0.25g Papain/CPG-16 ºBrix, serving code = 265

- strong aftertaste
- more sour taste
- a bit more tart than others, different
- musty, stale but with strong fruit flavor
- more tart than odd sample
- strong flavor
- more tart, tangy
- more bitter, tastes more "concentrated"
- sweeter flavor, made juice more palatable
- more sweet, concentrate

Pasteurized-16 ºBrix, sample code = 512

- less concentrate flavor
- tart taste, smoother than odd
- a tart, smooth fruit flavor. a little musty taste
- mild, fruit flavor but not very flavorful
- not as tangy
- more tart flavor
- can taste flavor better
- smooth, sweet
- more dilute taste
- stale flavor, didn't do much for me
- more tart, less sweet

WORKSHEETS AND QUESTIONAIRES FOR SENSORY EVALUATION

Table A.4 Worksheet for Rank Preference (0.25 g CPG) Test

Date: _____ No.: ____

Type of samples: Stanley plum juice from 0.25g Papain/CPG and pasteurized.

Type of test: Rank Preference

Sample	Description	Serving code
14° Brix	0.25g Papain/CPG-14° Brix	533
16° Brix	0.25g Papain/CPG-16° Brix	721
16° Brix	Pasteurized-16° Brix-Control	872

Serving orders:

Subject #					
1-4	5-8	9-12	13-16	17-20	21-25
533	721	872	533	721	872
721	533	533	872	872	721
872	872	721	721	533	533

Notes:

-Container used: plastic cup (without cover)
-Amount of juice/container: 20 mls
-Juice samples were measured out using a graduated cylinder.
-Serving temperature: 25°C (room temperature)
-Use pitcher to get deionized water from laboratory
-Prepare napkins, unsalted saltines, water cups, spit cups, sample cups (labeled) and arrange on presentation trays.
-Serve samples according to the set number sequence from left to right.

Table A.5. Worksheet for Rank Preference (0.5 g CPG) Test

Date: _____ No.: ____

Type of samples: Stanley plum juice from 0.5g Papain/CPG and pasteurized.

Type of test: Rank Preference

Sample	Description	Serving code
14° Brix	0.5g Papain/CPG-14° Brix	479
16° Brix	0.5g Papain/CPG-16° Brix	168
16° Brix	Pasteurized-16° Brix-Control	331

Serving orders:

Subject #

1-4	5-8	9-12	13-16	17-20	21-25
479	168	331	479	168	331
168	479	479	331	331	168
331	331	168	168	479	479

Notes:

-Container used: plastic cup (without cover)
-Amount of juice/container: 20 mls
-Juice samples were measured out using a graduated cylinder.
-Serving temperature: 25°C (room temperature)
-Use pitcher to get deionized water from laboratory
-Prepare napkins, unsalted saltines, water cups, spit cups, sample cups (labeled) and arrange on presentation trays.
-Serve samples according to the set number sequence from left to right.

Table A.6. Worksheet for Extended Triangle Test

Date: _____ No.: ____

Type of samples: 16 °Brix Stanley plum juice from 0.25g Papain/CPG and pasteurized.

Type of test: Extended triangle

Sample	Description	Serving code
16° Brix	0.25g Papain/CPG-16°Brix	265 (238, 617)
16° Brix	Pasteurized-16° Brix	512 (325, 712)

Serving orders:

Subject #

1-4	5-8	9-12	13-16	17-20	21-25
265(238)	512(325)	265(238)	512(325)	512(325)	265(238)
265(617)	512(712)	512(325)	265(238)	265(238)	512(325)
512(325)	265(238)	265(617)	512(712)	265(617)	512(712)

Notes:

-Container used: plastic cup (without cover)
-Amount of juice/container: 20 mls
-Juice samples were measured out using a graduated cylinder.
-Serving temperature: 25°C (room temperature)
-Use pitcher to get deionized water from laboratory
-Prepare napkins, unsalted saltines, water cups, spit cups, sample cups (labeled) and arrange on presentation trays.
-Serve samples according to the set number sequence from left to right

Table A.7. Questionnaire for Rank Preference test

Name: _____ Date: _____ Test#: _____

Product: Stanley Plum Juice Panelist#: _____

INSTRUCTIONS:

1. Before tasting the samples **and** between each sample, rinse your mouth with water. You may also use the saltines to remove the flavor from your mouth at any time.

2. You have received three samples. Each sample is labeled with a 3-digit number.

3. Taste the samples in the order listed on your questionnaire. You may either swallow or spit out the samples (spit cup provided).

4. Rank the following samples for preference. The one you prefer most is ranked first. The one you prefer second is ranked second. The one you prefer least is ranked third.

Place the code numbers on the appropriate lines:

331 168 479

1: _____

2: _____

3: _____

Comments:

Table A.8. Questionnaire for Extended Triangle Test

Name: _____ Date: _____ Test#: _____

Product: Stanley Plum Juice Panelist#: _____

INSTRUCTIONS:

1. Before tasting the samples **and** between each sample, rinse your mouth with water. You may also use the saltines to remove any flavors from your mouth at any time.

2. You have received three samples. Two of the samples are identical and the other is different. Each sample is labeled with a 3-digit number.

3. Taste the samples in the order listed on your questionnaire. You may either swallow or spit out the samples (spit cup provided).

---

4. Please circle the number of the **odd** sample.

   Sample numbers: 238 325 712

---

5. Now **describe**, regarding overall flavor, the odd sample and the duplicate.

Odd sample:

Duplicate:

---

6. Lastly, list the sample you **prefer**, the odd or the duplicate.

Preference: _____

Comments:

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