Studying Some Properties of Amylases Extracted from 'd'Anjou' Pears

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Abstract. Several characteristics of amylases involved in starch degradation were studied in extracts from immature (30 days before harvest) 'd'Anjou' pears (*Pyrus communis* L.). Enzyme activity was not detected until after at least 60 minutes of incubation in frozen or lyophilized tissues. Activity increased significantly after 90 minutes and increased linearly after 2 to 12 hours of incubation. Activity was greater, however, in frozen than in lyophilized tissues. Three buffers (acetate, tris-HCl, and imidazole-HCl) were used at a range of pH levels (4.6-8.2) to ascertain the optimum assay system. Highest specific activity was recorded with acetate buffer at pH 5.6. The K_m value in this system was 1.43×10^{-3} g·ml⁻¹. Specific activity increased as Ca concentration in the reaction mixture increased from 1 to 15 mM CaCl₂, but did not change as Ca concentration increased from 15 to 25 mM CaCl₂. The 'd'Anjou' pear amylases were purified 5.7-fold using ammonium sulfate fractionation.

Although various amylases from diverse origins have been purified and their physical and kinetic properties characterized, little is known about these enzymes in fruit. In plants, starch is degraded by at least two enzymes—alpha and beta amylases. Chitarra and Lajolo (1981) reported that, in 'Marmelo' bananas (*Musa acuminata* colla × *Musa balbisiana* colla ABB group), in vitro starch degradation had a different temperature coefficient than that of α-amylase. This result raised doubts that only α-amylase was involved. In maturing apples (*Malus domestica* Borkh.) and pears, changes in starch concentrations (mainly rated by visible differences after being stained with iodine) are used to determine optimum harvest maturation (Beattie et al., 1972; Kvale, 1986). Li (1991) observed that 'd'Anjou' pears with cork spot, a Ca-related disorder (Raese and Stahly, 1982), have little starch at harvest according to the starch-iodine test. This lack of starch suggests that either decreased starch synthesis or increased starch degradation may be involved in cork spot development.

Our paper reports results of experiments in which amylases in crude protein extracts from immature 'd'Anjou' pears were partially purified and characterized. We did not separate the various enzymes involved in starch degradation, since we were interested in establishing methodologies to investigate starch changes in normal and cork-spotted pears.

Sample preparation, extraction, and enzyme assay. Fruit that seemed normal were

collected from six mature 'd'Anjou' pear trees at the Mid-Columbia Agricultural Research and Extension Center in Hood River, Ore., on 2 Aug. 1990. Commercial harvest was on 6 Sept. 1990. The fruit were washed in a 0.1% low-phosphorus detergent solution (Liqui-Nox, Alconox, New York), rinsed with distilled water, and wiped dry. The calyx half of the fruit was cut into cubes, which were covered quickly with a cloth that had been wetted with 0.5% (w/v) sodium metabisulfite solution to minimize oxidative browning. About 40 g of tissue was sampled from 10 fruit, placed in cheesecloth bags, frozen in liquid nitrogen, and stored at -74°C. About 400 g of frozen tissue was accumulated and bulked for analysis. From the same fruit, a second sample was prepared in the same manner but was lyophilized for 7 days. About 400 g of tissue was accumulated by thoroughly mixing the ground lyophilized tissue, and all procedures were carried out on this bulked sample at 0 to 5°C. There were three replicated extractions and analyses for each data point.

Lyophilized tissue (15 g) was homogenized with 7.5 g insoluble polyvinylpyrrolidone and 7.5 g white quartz sand using a mortar and pestle. Preliminary extractions showed that at least 15 g dry weight or 100 g fresh weight of pear tissue was needed to obtain sufficient activity. The sample was ground for 5 min, and then another 10 min after adding 50 ml of the appropriate extraction buffer (Table 1). The slurry then was squeezed through two layers of Miracloth (CAL Biochem, LaJolla, Calif.), the supernatant was collected in a chilled beaker at 0°C, and the residue was reextracted twice more. All supernatants were combined and centrifuged at 5000× g for 20 min. The clear supernatant (designated as the crude enzyme extract) was transferred to a chilled beaker (0°C), and solid (NH₄)₂SO₄ was added to the crude enzyme extract to give specific saturation percentages. The solutions were stirred until the salt was completely dissolved

(40 min) and centrifuged at 10,000× g for 20 min, and the supernatant was discarded. The precipitate was redissolved in 3.0 ml of a solution consisting of the appropriate enzyme buffer (Table 1). The resulting enzyme solution was applied to an Econo-Pat 10 DC column packed with Bio-Gel P-6 desalting gel (Bio-Rad Laboratories, Richmond, Calif.) with a molecular weight exclusion of ≈6000 Da. The sample was allowed to run into the column after discarding the first 3.0 ml eluted. The higher-molecular-weight components were eluted by adding 4.0 ml of the above buffer and collecting a 4.0-ml fraction from the column into a chilled test tube at 0°C. All procedures were exactly the same for the frozen sample, except that the tissue was ground into a powder in a chilled mortar. Based on moisture content, equivalent weights of frozen and lyophilized tissues were extracted.

Amylase activity was assayed by incubating 0.25 ml of the enzyme solution for 2 or 4 h at 20°C with 0.25 ml of an assay buffer-soluble starch (1% w/v) solution (Table 1). The reaction was terminated by adding 0.5 ml dinitrosalicylic acid reagent. The mixture then was diluted 25-fold with distilled water. Optical densities (ODs) were read at 540 nm using a blank that was prepared with the same amount of enzyme, substrate, and reagent in which the enzyme activity was inactivated immediately in boiling water. OD_{540nm} values were converted to micromoles of maltose using a standard curve with maltose (1.0 to 5.0 μmol·ml⁻¹). Activity was expressed as micromoles of maltose liberated in 1 h of reaction at 20°C by 1 ml desalted crude extract. Specific activity was expressed as micromoles of maltose per hour per milligram of protein. Total activity was expressed as micromoles of maltose per hour.

Table 1. Extraction, enzyme, assay, and reaction buffer systems and resulting pH values used to determine activity of 'd'Anjou' pear amylases.

Buffer system	pH		
	Buffer		
	Tris-HCl	Acetate	Imidazole
Extraction ²	7.4	5.4	7.4
Enzyme ³	7.4	5.4	7.4
Assay ⁴	2.1	2.8	2.0
	2.2	5.4	4.0
	3.0	6.0	6.0
	7.4		7.4
	8.0		9.2
	9.0		
Final pH of reaction mixture	6.6	4.6	6.6
	6.9	5.4	6.8
	7.1	6.3	7.0
	7.4		7.4
	7.7		7.9
	8.2		

²0.1 M buffer: 5 mM CaCl₂, 10 mM mercaptoethanol, 5 mM ascorbic acid, and 3 mM Na₂S₂O₅.

³Enzyme buffer: 0.1 M buffer and 10 mM mercaptoethanol; refers to the buffer in which the precipitate was dissolved and passed through the desalting column.

⁴Assay buffer: 0.1 mM buffer and 0.1% (w/v) soluble starch; refers to the buffer in which amylase activity was determined.

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Soluble protein in the enzyme preparation was determined by the Bio-Rad (Bio-Rad Laboratories) microassay, using bovine serum albumin as the standard.

Optimum pH and buffer system. Three buffers were used for each part of the system. This procedure resulted in a range of pH values for the extraction, enzyme, assay, and final reaction mixture buffer combinations (Table 1). Lyophilized tissue (15 g) was extracted with the buffers as previously described. Final pH values resulting from the combination of assay and extraction buffers were 4.6 to 6.3 for acetate, 6.6 to 8.2 for tris-HCl, and 6.6 to 7.7 for imidazole-HCl.

Partial purification. 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. Tris- and imidazole-HCl extraction and assay buffers were pH 7.4. Acetate extraction and assay buffers were pH 5.4. Other chemical substances were the same in each buffer (Table 1). Lyophilized tissue (15 g) was extracted with each of the three buffers as previously described. Solid ammonium sulfate was added to the crude enzyme solution from each buffer to give 30%, 65%, or 80% saturation. The solutions were stirred for 40 min after each $(\text{NH}_4)_2\text{SO}_4$ addition and centrifuged at $10,000 \times g$ for 20 min, and the resulting precipitate was saved. These procedures were followed for each $(\text{NH}_4)_2\text{SO}_4$ fraction. Each precipitate was redissolved with the appropriate buffer and desalted by using an Econo-Pac DC column, as previously described, and amylase activity and protein concentration were determined.

Response to Ca. An acetate extraction buffer was used to study response to increasing Ca concentration in vitro. Other procedures used in extraction and partial purification were the same as described previously, except that a 65% $(\text{NH}_4)_2\text{SO}_4$ precipitated fraction was used. Enzyme solution (0.25 ml) was mixed with 0.25 ml of a soluble starch-sodium acetate buffer (pH 5.4) solution that contained 0, 8, 18, 28, 38, or 48, mM CaCl_2 , so that the final CaCl_2 concentration in the reaction mixture was 1 to 25 mM.

Kinetics. An acetate buffer extract from lyophilized tissue (15 g) was partially purified using the fraction precipitated with 65% $(\text{NH}_4)_2\text{SO}_4$. Enzyme solution (0.25 ml) was mixed with 0.25 ml of a soluble starch-sodium acetate buffer (pH 5.4) solution in which the soluble starch concentration was 0.5, 1, 2, 4, 6, 8, or 10 $\text{mg} \cdot \text{ml}^{-1}$.

Time course of amylase activity from 'd'Anjou' pears. Time course measurements on frozen and lyophilized tissues indicated that there was no discernible activity in either tissue until after 60 min of incubation. Lyophilized tissue released less maltose per milligram protein starting at 90 min incubation for up to 12 h (Fig. 1A) than frozen tissue. Response was nonlinear from 0 to 120 min, but was linear from 2 to 12 h (Fig. 1B).

Effect of pH and buffer systems. Optimum enzyme activity occurred at pH 5.6 in acetate

Fig. 1. Time course relationship of maltose production by amylases extracted from fresh and lyophilized 'd'Anjou' pear tissue from (A) 0 to 120 min and (B) 2 to 12 h. Pears were sampled 2 Aug. 1990, ≈ 30 days before commercial harvest.

and 7.4 in tris- and imidazole-HCl (Fig. 2). Activity was about twice as high in acetate than in tris-HCl.

Partial purification, Ca, and kinetics. The crude enzyme extracts obtained with the three buffer systems were partially purified bypassing the extracts through Bio-Gel P-6 desalting

columns followed by step-wise fractionation with $(\text{NH}_4)_2\text{SO}_4$. Analysis of variance indicated that there were significant differences between all criteria with respect to buffer, $(\text{NH}_4)_2\text{SO}_4$ fraction, and their interactive effects (Table 2). Because of the significant interaction and the fact that the experimental

Fig. 2. Interaction of three buffer systems, pH, and specific activity of amylases extracted from 'd'Anjou' pear tissue. See text for details concerning buffers.

Table 2. Protein concentration, activity, specific activity, total activity, and purification of 'd'Anjou' pear amylases in three buffer systems and four ammonium sulfate fractions.

Buffer, pH	Stage [% saturated (NH ₄) ₂ SO ₄ solution]	Protein dry wt (mg·g ⁻¹)	Activity (μmol maltose/h per 1 ml desalted crude extract)	Specific activity (μmol maltose/h per mg protein)	Total activity (μmol maltose/h)	Purification (based on 0% to 80% as 1.00)
Tris-HCl, 7.4	0-80	0.19 aA [*]	1.54 aA	2.14 aA	6.17 aA	1.00
	0-30	0.11 bC	1.10 bC	2.69 aA	4.40 bB	1.25
	30-65	0.03 cD	0.97 bcC	8.72 bB	3.87 bcC	4.18
	65-80	0.03 cD	0.77 cD	7.72 bB	3.07 cC	3.60
Acetate, 5.4	0-80	0.16 aB	1.41 aAB	2.43 aA	5.65 aAB	1.00
	0-30	0.10 bC	1.23 abBC	3.20 aA	4.92 abB	1.32
	30-65	0.02 cD	0.76 bD	12.91 bC	4.23 bB	5.30
	65-80	0.02 cD	0.00	0.00	0.00	0.00
Imidazole, 7.4	0-80	0.16 aB	1.35 aB	2.19 aA	5.40 aAB	1.00
	0-30	0.10 bC	1.23 aBC	3.40 bA	4.91 abB	1.54
	30-65	0.03 cD	1.04 bC	8.84 cB	4.29 bB	4.01
	65-80	0.02 cD	0.93 bC	10.41 dBC	3.71 cC	4.07

^{*}Means followed by the same lowercase letter were not significantly different at $P = 0.05$ within a buffer. Means followed by the same uppercase letter were not significantly different at $P = 0.05$ across buffers.

design was a split plot, LSD values were calculated to compare means within buffers (Table 2, lowercase letters) and across buffers (Table 2, uppercase letters). Enzyme activity was highest in the 0% to 80% fraction in all buffers. No activity was detected in the 65% to 80% (NH₄)₂SO₄ fraction from the acetate buffer extraction. The specific activity was highest in the 30% to 65% (NH₄)₂SO₄ fraction using the acetate buffer. The highest degree of purity was found in the acetate buffer 30% to 65% (NH₄)₂SO₄ fraction. For all buffers, the 30% to 65% and 65% to 80% fractions were the most pure. Based on the highest specific activity and degree of purity, the 30% to 65% (NH₄)₂SO₄ fraction of the acetate buffer extraction system

was the most favorable system in 'd'Anjou' pears.

Specific activity increased nonlinearly at Ca concentrations of 1 to 15 mM CaCl₂ in the reaction mixture (Table 3). Because of the rapid increase in specific activity between 5 and 10 mM CaCl₂, more data points must be examined to determine a more precise pattern. There was no further change at concentrations of 15 to 25 mM CaCl₂.

A Lineweaver-Burk plot (activity as a function of soluble starch concentration) was constructed using starch concentrations of 0.5 to 10 mg·ml⁻¹. As calculated from Fig. 3, K_m was 1.43×10^{-3} g·ml⁻¹ with a V_{max} of 1.3 μmol maltose/h.

Many plant tissues have alpha and beta amylases, but the assay methods used in this study were not specific for either one. Plant α-amylases usually require Ca for their activity (Okamoto and Akazawa, 1978; Preiss, 1982). We found that enzyme activity was enhanced by Ca. This result suggests that α-amylase probably was a constituent of the enzyme complex isolated. The long incubation time and the low K_m value (1.43×10^{-3} g·ml⁻¹) showed that the amylases in 'd'Anjou' pears have a low affinity for starch. In comparison, Fan (1975) observed that the K_m value of 5.45×10^{-3} g·ml⁻¹ for potato α-amylase was in the high range of values reported for other amylases (0.6×10^{-3} to 3.3×10^{-3} g·ml⁻¹) (Depinto and Campbell, 1968; Manning and Campbell, 1961). The incubation time (>90 min) was similar to that used for determining amylase activity in banana pulp (Chitarra and Lajolo, 1981) but shorter than that used for barley (*Hordeum vulgare* L.) (Greenwood et al., 1965).

We found that acetate buffer at pH 5.4 gave the highest specific activity compared to the tris- and imidazole-HCl buffer systems. This result agrees with those of other reports showing that acetate buffers at pH values of ≈5.5 resulted in maximum activities, mainly of α-amylase, in other crops (Dehaas et al., 1976; Greenwood et al., 1965; Koshiba and Minamikawa, 1981; MacGregor et al., 1974).

Literature Cited

- Beattie, B.B., B.L. Wild, and G.G. Coote. 1972. Maturity and acceptability of early picked Granny Smith apples for export. Austral. J. Expt. Agr. Animal Husbandry 12:323-327.
- Chitarra, A.B. and F.M. Lajolo. 1981. Phosphorylase, phosphatase, α-amylase activity, and starch breakdown during ripening of 'Marmelo' banana [(*Musa acuminata* Colla) × (*Musa balbisiana* Colla) AAB group] whole fruit and thin slices. J. Amer. Soc. Hort. Sci. 106:579-584.
- Dehaas, B.W., D.W. Chapman, and K.J. Goering. 1976. An investigation of the α-amylase from self-liquefying barley starch. Cereal Chem. 55:127-137.
- Depinto, J.A. and L.L. Campbell. 1968. Purification and properties of amylase of *Bacillus macerons*. Biochemistry 7:114-117.
- Fan, M.L. 1975. Purification and properties of potato α-amylase. Tawania 20:71-76.
- Greenwood, C.T. and A.W. MacGregor. 1965. The isolation of α-amylase from barley and malted barley, and a study of the properties and action-patterns of the enzymes. J. Inst. Breweries 71:405-417.
- Koshiba, T. and T. Minamikawa. 1981. Purification by affinity chromatography of α-amylase in cotyledons of germinating *Vigna mungo* seeds. Plant Cell Physiol. 22:979-987.
- Kvale, A. 1986. Maturity indexes for pears. Acta Agr. Scandinavia 36:301-304.
- Li, S.J. 1991. Determination of α-amylase activity and soluble starch content in normal and cork spotted 'd'Anjou' pear fruit. MS Thesis, Oregon State Univ., Corvallis, Ore.
- MacGregor, A.W. and R.G. Thompson. 1974. α-Amylase from immature barley: Purification and properties. J. Inst. Breweries 80:181-187.
- Manning, G.B. and L.L. Campbell. 1961. Thermostable α-amylase of *Bacillus stearothermophilus*. I. Crystallization and some general properties. J. Biol. Chem. 236:2952-2957.
- Okamoto, K. and T. Akazawa. 1978. Purification of α- and β-amylase from endosperm tissues of germinating rice seed. Agr. Biochem. 42: 1379-1384.
- Preiss, J. 1982. Regulation of the biosynthesis and degradation of starch. Annu. Rev. Plant Physiol. 33:431-454.
- Raese, T.T. and E.A. Stahly. 1982. Calcium sprays to control physiological disorders of 'd'Anjou' pears. Acta Hort. 124:119-122.

Table 3. Specific activity of amylases extracted from premature 'd'Anjou' pears at different Ca concentrations in the reaction mixture.

CaCl ₂ (mM)	Specific activity (μmol maltose/h per mg protein)
1	2.58
5	2.88
10	5.32
15	5.34
20	5.32
25	5.33

Fig. 3. Lineweaver-Burk plot of soluble starch(s) and amylase activity (1/v) in 'd'Anjou' pears. Tissue was extracted with acetate buffer and assayed at pH 5.4.