Visualizing Enzyme Infusion into Apple Tissue

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Enzymes traditionally used in food processing are applied to ground or macerated tissue with little or no retention of cellular structure. More recently developed applications use enzymes to selectively alter tissue properties while retaining some structure. Process development has been hindered by the lack of conclusive evidence showing that enzyme infusion into plant tissue pieces is possible. This study provides direct evidence that such infusion is possible by using fluorescence microscopy to monitor vacuum infusion of fluorescein-labeled α-amylase into apple cubes. This method is generally applicable to any plant or animal tissue and to any macromolecule capable of derivatization.

Keywords: *Infusion; enzyme;* α*-amylase*

INTRODUCTION

Enzymes have been used for centuries to alter the properties of fruits and vegetables. Endogenous grain enzymes are essential for the production of beer and bread. Exogenous enzymes are widely used to increase juice yield and clarity. In these cases, enzymes are applied to ground or macerated tissue with little or no retention of cellular structure. In other applications, enzymes are used to selectively alter tissue properties while retaining some cellular structure. These applications have been the subject of several reviews (Baker and Wicker, 1996; McArdle and Culver, 1994).

In theory, macromolecules such as enzymes should be able to reach substrate sites through intercellular spaces and cell wall pores. Studies with cultured soybean cells showed that succinylated wheat germ agglutinin (36 kDa, 2.5 nm Stokes' radius) and bovine serum albumin (67 kDa, 3.6 nm Stokes' radius) penetrated cell walls. Treatment of these cultured cells with pectinase increased the rate of infusion, presumably by increasing cell wall pore size (Baron-Epel et al., 1988). In other work, carrots were infused with dextrans (<40 kDa) during a blanching treatment (Mudahar et al., 1991).

Several processes have been developed for selectively altering fruit and vegetable properties by enzyme infusion. Blanched peach halves infused under vacuum with pectin methylesterase and calcium chloride showed significantly increased firmness after thermal processing (Javeri et al., 1991). Infusion of scored citrus fruits with pectic enzymes under various combinations of pressure and vacuum readily separated citrus peels from the segmented fruit (Adams and Kirk, 1991; Berry et al., 1988; Ben-Shalom et al., 1986; Bruemmer, 1981; Bruemmer et al., 1978). Numerous infusion processes have been developed to improve wet milling properties and brewing efficiencies of grains (Steinke and Johnson, 1991; Caransa et al., 1988; Toth et al., 1984; Roushdi et al., 1981).

All of these applications require development of an infusion process to place enzymes in contact with substrate sites within tissues. Movement of enzymes within plant tissues can be detected by assaying for enzymatic activity or by searching for evidence of enzyme activity, such as solubilization of grapefruit albedo. Development of other applications could be facilitated by a rapid method to track the rate of enzyme infusion. The current work demonstrates a general method for monitoring macromolecule infusion into plant tissue through use of a fluorescently labeled enzyme. This work also provides direct evidence that such infusion is possible under selected conditions.

MATERIALS AND METHODS

Preparation of Fluorescently Labeled Enzyme. α-Amylase (Sigma, St. Louis, MO) was derivatized with a fluorescent probe using a procedure supplied by Molecular Probes (Eugene, OR). The enzyme was dissolved to 10 mg/mL in 0.1 M sodium bicarbonate. The succinimidyl ester of 6-[fluorescein-5(and-6)-carboxamido]hexanoic acid (Molecular Probes) was dissolved to 10 mg/mL in dimethylformamide (DMF). The fluorescent reagent solution (1 volume) was slowly added to the enzyme solution (10 volumes) with stirring at room temperature. The reaction was terminated after 1 h by the addition of 1 volume of 1.5 M hydroxylamine hydrochloride, pH 8−8.5.

Excess fluorescent reagent was removed from the solution by chromatography on Sephadex G-25. The protein concentration of the infusion solution was determined according to the Lowry method (Lowry et al., 1951). The fluorescent label

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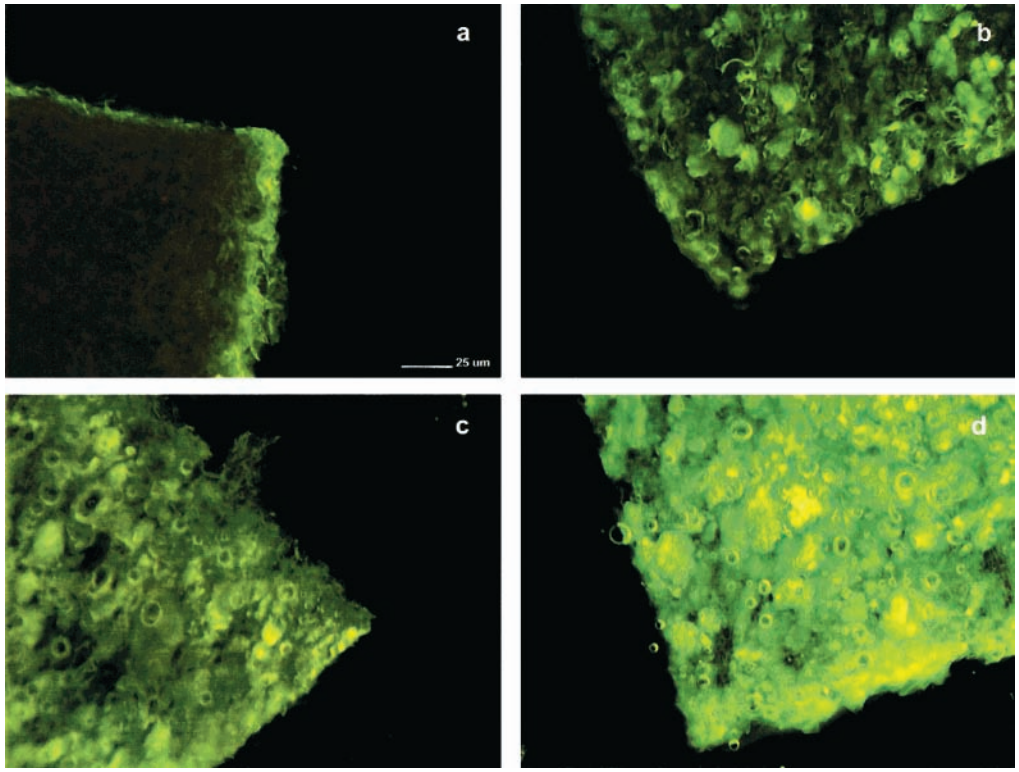

Figure 1. Fluorescence microscopy of apple cubes infused with fluorescein-labeled α-amylase for (a) 0, (b) 15, (c) 30, and (d) 60 min.

content of the derivatized protein was determined using the approximate extinction coefficient ($\epsilon_{494} = 68000$).

Sample Infusion. Granny Smith apples were purchased from a local grocer in July 1999 (Washington state fall 1998 crop) and cut into 1 cm cubes; caution was taken to avoid any visible vascular tissue. Freshly cut tissue cubes were placed in a solution of fluorescein-labeled α-amylase (4 mg/mL protein; 1 mol of dye/mol of protein) and held under vacuum (25 in Hg) for up to 60 min. After infusion, the vacuum was slowly released over ~30 s. Samples were rinsed with distilled water, hand sectioned through the center of the cube with a razor blade, and immersed in glycerol on a slide.

Fluorescence Microscopy. Enzyme infusion was monitored using an Olympus light microscope (BH-2, Leeds Precision Instruments, Minneapolis, MN) fitted with epifluorescence optics (Mercury-100, CHIU Technical Corp., Kings Park, NY) and an Olympus 2X objective lens (SPlanFL2, Leeds Precision Instruments). Fluorescein-labeled α-amylase was visualized using an excitation wavelength of 494 nm and an emission wavelength of 515 nm. Images of enzyme-infused tissue were captured using a Sony CCD digital camera module (DKC-5000, Meyer Instruments, Houston, TX).

RESULTS AND DISCUSSION

Fluorescence micrographs in Figure 1 show the presence of fluorescein-labeled α-amylase as it infused into 1 cm apple cubes. Apple sections did not fluoresce when infused with buffer. When cubes were dipped into a solution of labeled protein, rinsed, and sectioned, cross sections showed binding of the protein to outer tissue layers (Figure 1a). Such binding would be expected from ionic and hydrophobic interactions between the labeled protein and cell wall constituents. After 15 min of infusion, fluorescein-labeled α-amylase penetrated far into the tissue (Figure 1b). The most intense fluorescence was located in the outer 0.04 mm of the cube, but fluorescence was present throughout the tissue. Focal adjustment throughout the depth of the sample plane showed the presence of labeled protein between and within individual apple cells. After 30 min of infusion, the level of fluorescence increased throughout the sample, indicating higher levels of infused protein (Figure 1c). After 60 min of infusion, the entire cross section showed heavy infiltration by infused protein (Figure 1d).

Vacuum was critical for this infusion process. In the absence of vacuum, the infusion rate was so slow as to be undetectable. Varying the ionic strength by using 5−500 mM phosphate buffer did not alter the vacuum infusion rate. The labeled protein aggregated in higher ionic strength buffers. Varying the infusion pH had no discernible effect on the vacuum infusion rate but tended to promote protein aggregation under acidic conditions.

The presence of infused protein in intercellular spaces is not unexpected. These spaces, largely filled with air, occupy ~25% of mature apple tissue volume. These spaces are ~50−100 μm × 3000 μm and are radially oriented from the apple core (Khan and Vincent, 1990). The size and orientation of these spaces readily permitted infusion of an enzyme solution. The rate of infusion increased significantly under vacuum because of partial removal of this trapped air.

The presence of labeled protein within apple cells is more surprising. Intact and functional cell membranes should prevent nonspecific protein uptake. These results indicate loss of membrane integrity. This loss could result from mechanical or oxidative damage during harvest and storage. It has been shown that hydrogen peroxide accumulates in stored apples because of decreased levels of superoxide dismutase and catalase (Masia, 1998). Lipid peroxidation could cause changes in cell membrane permeability.

The degree of maturity and time since harvest also cause changes in apple cell walls, altering the rate of

infusion. As apples mature, endogenous pectinolytic enzymes begin to break down pectins in the cell walls; cellulose and hemicellulose components remain unchanged (Bartley, 1976). Intercellular spaces expand and cellular adhesion decreases as fruit matures, creating larger spaces for infusion (Khan and Vincent, 1990).

α-Amylase was selected for use in this study because of its commercial significance and because of its lack of unusual structural features. Apple tissue was chosen for this study because it is readily available and contains relatively little vascular tissue in the pericarp. Vascular tissue should increase the infusion rate by providing a channel for fluid movement.

The use of fluorescently labeled compounds to monitor infusion is not limited to apple tissue or to α-amylase. This method has been used to follow the infusion of bovine serum albumin and β-glucuronidase into potato and apple tissue (Culver et al., 1995). This technique could easily be adapted for monitoring infusion of any macromolecule capable of derivatization, such as polysaccharides, and with any plant or animal tissue.

It is hoped that this method will facilitate development of commercial processes for selectively altering tissue properties by macromolecule infusion. Development of appropriate infusion conditions will require methods to detect successful infusion. Once developed, infusion conditions may require modification due to variation in properties of the infused tissue. Use of fluorescently labeled macromolecules will permit rapid assessment of the extent and reproducibility of tissue infusion.

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