Pectinesterase and Pectin Complexes Inhibit Ion Exchange Membrane Separation

R. W. Chen, K. A. Sims, and L. Wicker*

Department of Food Science and Technology, Food Process Research and Development Laboratory, The University of Georgia, Athens, Georgia 30602

Ion exchange membrane disks (FMC Co., Pine Brook, NJ) equilibrated at pH 5.0, 6.0, 7.2, and 8.0 were tested for separation of pectinesterase (PE) extracted from Marsh grapefruit (MGF) pulp. Anionic exchange disks (QUAT, DEAE, and PEI) bound less PE and protein than cationic exchange disks (SP and CM). Less than 20% of PE was bound to SP membrane at pH 6.0, 7.2, and 8.0. Low PE binding ability in the ion-exchange system may be attributed to the presence of PE−pectin complexes, which act as a competitor with the inorganic ions of the membranes. Water washing the pulp prior to PE extraction lowered the pectin content by 86%, but did not substantially improve PE binding to SP membrane.

Keywords: *Marsh grapefruit; cloud stability; clarification; pectin; pectinesterase*

INTRODUCTION

Quality and aesthetic properties of citrus juice center on cloud stability. The major contribution to cloud loss is thermostable pectinesterase (PE) activity. This enzyme is firmly associated with the cell wall and is abundant in the pulp and juice sac tissue (Joslyn and Pilnik, 1961). Upon juice extraction, the enzyme de-esterifies the pectin. Interaction of pectic acid with cations (Stevens et al., 1950) or with other colloidal cloud constituents (Shomer et al., 1991) causes cloud loss. To maintain the quality and cloud stability of citrus juice products, it is necessary to inactivate or separate PE from citrus juice during processing. Currently, commercial practice to inactivate thermostable PE is achieved by heat treatment, such as evaporation or high temperature, short time (HTST) pasteurization. Eagerman and Rouse (1976) reported that the inactivation time for citrus pectinesterase in juice is 1 min at 90 °C. Heat destroys spoilage bacteria, inactivates enzymes, and can be used to produce concentrates (Varsel, 1980). However, concentration by evaporation removes a portion of the low molecular weight materials required for good flavor and aroma. The heat involved in the evaporation process can caramelize juice solids and introduce an undesirable taste.

Membrane separations offer processing and product quality advantages over traditional separation techniques (Drioli et al., 1981). Membranes in citrus processing were used to preconcentrate juice before evaporation and minimize energy consumption required during evaporation (Watanabe et al., 1979; Braddock and Sadler, 1989). Low-temperature separation of juice serum, which contains most oxygenated aroma components, can be obtained by ultrafiltration (UF) (Hernandez et al., 1992). No PE activity was detected in the permeate using UF membrane of 500 kDa molecular weight cutoff (MWCO) in orange juice (Hernandez et al., 1992). Köseoglu et al. (1990) reported no PE permeation in citrus juice after UF with 50 and 100 kDa MWCOs. UF was used to clarify passion fruit prior to concentration (Yu and Chiang, 1986), and ion exchange was used for deacidification (Lue and Chiang, 1989). UF in apple juice processing saves energy, cuts enzyme usage, increases juice yield, and enhances product quality (Hackert and Swientek, 1986). Compared to concentration processes based on evaporation, freezing, or sublimation, reverse osmosis provides gentler treatment of flavor and aroma components of juices and requires significantly less energy (Robe, 1983).

UF permeation and removal of PE is inhibited by formation of a PE−pectin complex in model systems (Snir et al., 1995) and in citrus juice (Hernandez et al., 1992; Snir et al., 1996). The permeability of PE in model systems increased with pH and/or cation concentration and was maximum at either pH 8.0 with low salt or pH 3.8 with high salt (Snir et al., 1995). Since the PE−pectin complex is too large for efficient separation and removal of PE by UF, separation of PE based on charge warrants evaluation. Since PE exists as a complex with pectin in citrus juice and at juice pH, binding of PE or PE−pectin to a membrane surface may be an alternative method to remove PE from a juice stream. Inorganic, ion exchange membranes tolerate harsh processing, cleaning, and sterilizing environments and have excellent resistance and hydrodynamics. Ion-exchange membranes which consist of a microporous plastic sheet of silica and poly(vinyl chloride) (PVC) have advantages in protein separation (Goldberg et al., 1989). They also have potential in fluid food processing, particularly for removal of charged compounds which are detrimental to product quality. The objective of this study was to evaluate binding of PE from Marsh grapefruit (MGF) pulp. Cationic and anionic laboratory scale ion-exchange membranes were evaluated for permeation of PE, PE−pectin, and total protein at different pH, ionic strength, and residual pectin content.

MATERIALS AND METHODS

Sample Preparation. PE extraction followed a modified procedure of Wicker et al. (1988). Unless stated otherwise,

* Corresponding author [telephone (706) 542-1055; fax (706) 542-1050; e-mail lwicker@uga.cc.uga.edu].

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chemicals used were analytical grade and were obtained from Fisher Scientific (Atlanta, GA). Frozen MGF pulp (Citrus World, Lake Wales, FL) was homogenized in 0.25 M Tris-HCl, 0.3 M NaCl, pH 8.0, buffer in a ratio of 1:5 (w/v) in a Sorvall Omni Mixer (Ivan Sorvall Inc., Newtown, CT). The homogenate was stirred for 1 h at room temperature and the supernatant collected by centrifugation for 20 min at 8000*g* and filtered through a Miracloth (Calbiochem, La Jolla, CA). The pH was monitored and corrected throughout the extraction process. After collection of the supernatants by centrifugation, PE extract was dialyzed against buffer (1:40 w/v) for 6 h at 4 °C in order to remove NaCl before undergoing membrane separation procedures. Dialysis tubing was boiled in 10% acetic acid for 10 min and then rinsed in deionized water (Wicker, 1992). Dialyzing buffers used in this study included 0.01 M sodium phosphate, pH 7.2 and 6.0; 0.02 M sodium acetate, pH 5.0; and 0.02 M Tris-Cl^{-}, pH 8.0.

Adsorption Study on Tubing. Adsorption of protein and PE to tubing was determined in a preliminary study. Norprene and silicone formulated tubings (size 14, 70 cm in length) (Masterflex, Nile, IL) were cleaned by running deionized water (65−70 °C) for 1 h, followed by 2 N NaOH for 15 min. Crude PE extract was passed through each tubing at room temperature in recycle mode for 30 min. The flow rate was controlled at 5 mL/min. Samples before and after circulation were collected for protein and PE activity determination.

Ion-Exchange Systems. Three anionic-exchange Acti-disks (QUAT, FMC #81210 and Arbor Tech #6872-1101; DEAE, FMC #81110 and Arbor Tech #6872-1001; and PEI, FMC #80110 and Arbor Tech #6872-0101) and two cationic-exchange Acti-disks (SP, FMC #80120 and Arbor Tech #6872-0201; and CM, FMC #80130 and Arbor Tech #6872-0301), supplied by FMC Co. (Pine Brook, NJ), were used in this study. Each disk cartridge consists of a microporous plastic sheet material hermetically sealed inside a leakproof polypropylene housing, which holds 3 mL of solution. The cartridge was connected to a peristaltic pump (Model 7520-20 with pump head Model 7014-20; Cole-Palmer, Chicago, IL) with a silicone formulated tubing (Masterflex, size 14, 70 cm in length). Each Acti-disk was flushed with 50 mL of 70% ethyl alcohol to reduce microbial contamination and then equilibrated with low ionic strength buffer before loading the sample. Samples were loaded at a flow rate of 5 mL/min at room temperature. Bound protein was eluted using 0.3 M NaCl in start buffer, and detected qualitatively by reading the absorbance at 280 nm. Both single-pass and recycle modes were conducted in order to evaluate the effectiveness of separation. Single-pass trials were conducted by passing the PE extract through the membrane disks one time, and the permeate was collected for analysis. In recycle mode, the permeate stream was recirculated back to the reservoir for 3 h. The bound protein was eluted using salt-containing buffer in the reverse direction.

Membrane Cleaning. The cleaning protocol suggested by FMC Co. was followed. Reverse flushing of the ion-exchange membrane disks with water and 70% ethyl alcohol at room temperature was used before storage. Water flushing removes the salt which damages the membranes, and the use of alcohol reduces microbial growth. The remaining fluid in the membrane cartridges was flushed with methanol, and the residual methanol was forced out with a syringe. The membrane was air-dried and stored at room temperature.

Ultrafiltration (UF) System. The UF membrane used in this study was a hollow-fiber module made of polysulfone, obtained from A/G Technology Corp. (Needham, MA). The membrane had a 30 000 MWCO (Snir et al., 1995). Crude PE extract (pH 8.0) was passed through the membrane in recycle mode either at 24 °C with 10 psig inlet pressure or at 10 °C with 15 psig inlet pressure. Three hours of total recycle mode was conducted. Samples of feed, clarified serum, and retentate were collected at different time intervals for analysis.

Pulp Washing. MGF pulp was washed with water to remove most carbohydrates and pectic substances. Preweighed MGF pulp was mixed with water (approximately 1:10 w/v) and stirred in a beaker. Pulp was collected with cheesecloth, and the washing procedure was repeated 3 additional times. Excess water was squeezed from the pulp with cheesecloth, and pulp was then weighed for extraction. Weight gain from water washing was negligible.

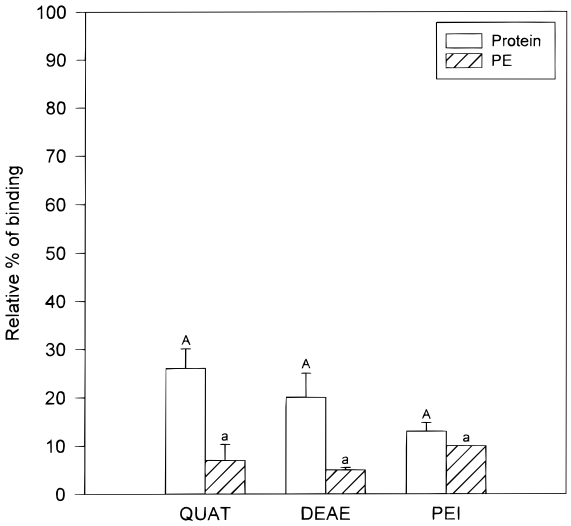

Figure 1. Relative protein and PE binding to anionic membrane Acti-Disks, QUAT, DEAE, and PEI, at pH 7.2 in 0.01 M sodium phosphate buffer. Standard error is less than symbol size if error bar is invisible. Means of triplicates are reported. Means marked by same upper or lower case letters are not significantly different ($\alpha = 0.05$).

Analytical Methods. Pectinesterase activity was determined by titrimetric assay described by Rouse and Atkins (1955) in 1% high methoxyl pectin (Citrus Colloids, Hereford, U.K.), 0.1 M NaCl, pH 7.5 at 30 °C. The amount of standardized NaOH required to neutralize the release of carboxylic groups was recorded with a Brinkman 614 Impulsomat automatic titrator (Westbury, NY). Pectinesterase units (PEU) were defined as the amount of PE that releases 1 μmol of carboxyl groups per minute. Protein was determined by a dye-binding protein assay (Bradford, 1976) using 2−20 μg/mL bovine serum albumin (BSA) as standard. The anhydrogalacturonic acid (AGA) content of each pectic fraction was determined by the method of Blumenkrantz and Asboe-Hansen (1973). Anhydrogalacturonic acid (2.5−12.5 μg in 200 μL of water) was used as a standard.

Statistical Analysis. Experiments were replicated in triplicate. Results were analyzed using the Statistical Analysis System program (SAS, 1987). The Duncan's Multiple Range Tests were conducted to compare the differences between sample means at a significance level of 0.05.

RESULTS AND DISCUSSION

Effects of Membrane Binding on PE and Protein. A preliminary study of protein and PE binding to the tubing used indicated that a negligible amount (less than 3%) was bound to tubing. Relative PE and protein binding to the anionic membrane disks (QUAT, DEAE, and PEI) is shown in Figure 1. At pH 7.2, anionic exchange disks had little binding for PE. The percentage of PE bound to QUAT, DEAE, and PEI Acti-Disks was 7.3% ± 5.7, 5.0% ± 0.9, and 10.0% ± 0, respectively. More total protein than PE was bound to anionic disks. The percentage of protein bound to QUAT, DEAE, and PEI Acti-Disks was 26.3% ± 7.0, 20.3% ± 8.6, and 12.7% ± 3.1, respectively. There was no significant ($\alpha = 0.05$) difference between these three anionic membranes in either PE or protein binding abilities.

Plant PE shows a broad activity optimum between pH 6.5 and 9.5 (Lineweaver and Ballou, 1945). The isoelectric point of MGF PE (both thermolabile and

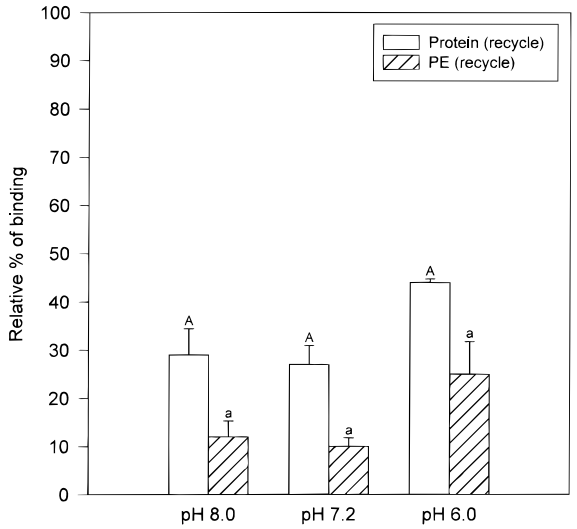

Figure 2. Relative protein and PE binding to cationic SP Acti-Disk at pH 8.0 in 0.02 M Tris-HCl buffer, and at pH 7.2 and 6.0 in 0.01 M sodium phosphate buffer. Time for recycle mode was 3 h. Means of triplicates are reported. Means marked by same upper or lower case letters are not significantly different ($\alpha = 0.05$).

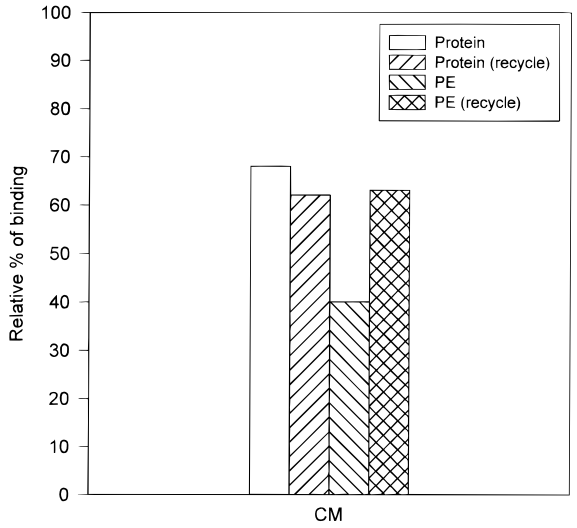

Figure 3. Relative protein and PE binding to cationic CM Acti-Disk at pH 5.0 in 0.02 M sodium acetate buffer. Time for recycle mode was 3 h. A single replication is reported.

thermostable PE) is above 10 (Seymour et al., 1991). Thus, at pH <10, PE should be positively charged and should bind cationic membranes. Our results show that less than 30% of PE is bound to cationic SP membrane at pH values less than the apparent p*I*. The percentage of PE bound to cationic SP membrane was 12.0% ± 5.7, 9.5% ± 2.5, and 24.5% ± 9.5 at pH 8.0, 7.2, and 6.0, respectively (Figure 2). More than 60% of total protein also permeated without treatment. The percentage of protein bound at pH 8.0, 7.2, and 6.0 was 29.3% ± 9.4, 26.5% ± 5.5, and 44.0% ± 1.0, respectively (Figure 2). In addition, at pH 6.0, when the recycling time was increased to 3 h, the SP membrane fouled severely. The flow rate decreased from 5 to 2 mL/min, and the eluate (first 15 mL collected) contained 200 μg/mL pectin.

At pH 5.0, cationic disks (Figure 3) had better binding ability of PE and total protein than anionic disks at pH 7.2 (Figure 1), or cationic disks at pH 6.0, 7.2, or 8.0 (Figure 2). In a single trial on CM membrane disk at pH 5.0, 40.7% of PE in the single-pass mode and 63.2% of PE in the recycle mode were bound to the membrane disk. The amount of protein bound to the CM disk in single and recycle modes was 67.8% and 62.1%, respectively (Figure 3). However, PE was not stable at pH 5.0. After dialysis at pH 5.0, a precipitate formed, which was removed by filtration through Miracloth before membrane separation. For 100 mL of dialyzed PE extract, the PE activity decreased from 30.5 to 23.4 PEU/mL in one experiment. The precipitate was resuspended with 15 mL of water, and the total PE activity was 180 units. Protein concentration also decreased from 0.22 to 0.059 mg/mL. Similar decreases in PE and protein concentrations at pH 5.0 were also observed in the subsequent experiments. For SP Acti-Disk, PE and protein binding abilities were significantly different ($\alpha = 0.05$) at pH 5.0 (either single pass or recycle mode) from those at pH 6.0, 7.2, or 8.0.

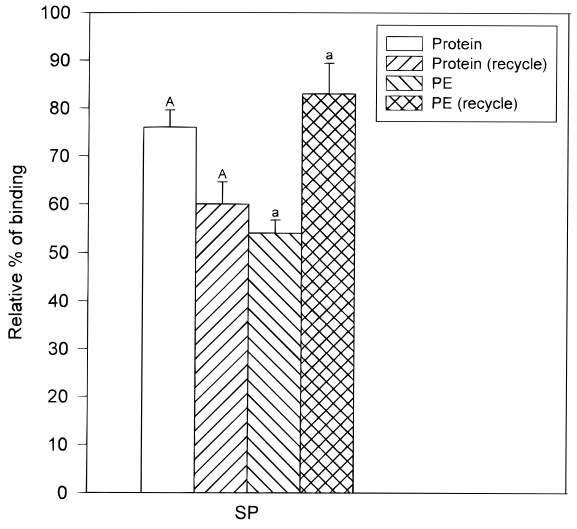

Figure 4. Relative protein and PE binding to cationic SP Acti-Disk at pH 5.0 in 0.02 M sodium acetate buffer. Time for recycle mode was 3 h. Means of triplicates are reported. Means marked by same upper or lower case letters are not significantly different ($\alpha = 0.05$).

At pH 5.0, the cationic SP membrane disk had the greatest percentage of PE binding (Figure 4). PE binding was not significantly different in recycle mode compared to single-pass mode on SP membrane disk (Figure 4). In recycle mode, 84.7% ± 11.1 of PE bound to SP membrane disk, and 53.7% ± 4.7 of PE was bound in single pass. Recycling did not significantly improve protein binding (76.3% ± 6.2 in single-pass mode vs 60.0% ± 8.0 in recycle mode). The specific activity was determined before membrane treatment and after elution for SP membrane. Results indicated (3.5 ± 1.2)-fold and (6.3 ± 3.5)-fold increases in purity from the original extract for single-pass and recycle modes, respectively.

Effect of Pectin on PE Binding. The combined application of pulp washing, additional centrifugation, and UF was evaluated for pectin removal and PE binding. PE extract prepared from previously washed and centrifuged pulp had decreased pectin content while the PE activity was unaffected (Table 1). Pectin content was reduced by 34% by an additional centrifugation (treatment A+C). Pulp washed with water prior to extraction (treatment B) lowered the pectin by 50%. The combination of these two procedures (treatment B+C) had the best result in removing most pectin (87%). The washing procedure did not affect PE activity. PE is

Table 1. Results of PE Activity and AGA Concentration by Different Pulp Treatments[a]

	treatment A	treatment A+C	treatment B	treatment B+C
PE (units/mL)	20.8 ± 1.0	19.8 ± 1.0	21.7 ± 2.6	22.0 ± 1.9
AGA (μg/mL)	730 ± 115	483 ± 57	367 ± 57	97 ± 13

[a] Treatment A: PE extract prepared from original pulp with centrifugation at 8000*g* for 20 min. Treatment B: PE extract prepared from prewashed pulp with centrifugation at 8000*g* for 20 min. Treatment C: PE extract prepared from pulp with additional centrifugation at 8000*g* for 20 min.

ionically bound to the cell wall and can only be solubilized by alkaline pH and high ionic strength buffer (MacDonnell et al., 1945). Wicker et al. (1988) reported that PE is not water-soluble unless the cell wall is enzymatically degraded.

PE extract prepared from washed pulp was passed through the UF system to evaluate pectin removal and effect on PE activity. The data in Table 2 show that approximately 50% (from 105.0 to 52.8 μg/mL) of pectin in the crude extract was removed by ultrafiltration at 24 °C, 10 psig. The PE activity decreased from 19.8 to 1.58 units/mL in the permeate as well. Most pectin and PE were removed during the first 30 min of UF. At 10 °C with 15 psig, the pectin content decreased approximately 85% (from 97.3 to 14.9 μg/mL) after 3 h total recycle by ultrafiltration. The PE activity was also lowered from 18.6 to 0.57 units/mL. It was not possible to completely remove pectin by polysulfone UF membrane of 30 000 MWCO under either condition (24 °C with 10 psig or 10 °C with 15 psig inlet pressure), and loss of PE activity occurred. The pectin-reduced permeate collected after UF was dialyzed against 0.01 M sodium phosphate buffer at pH 7.2 and passed through the cationic SP membrane. Less than 40% of PE was bound to SP membrane. An increase in recycling time did not improve PE binding (Table 2).

The partitioning of pectin, protein, and PE after filtration on anionic QUAT followed by filtration on cationic SP membrane was evaluated. Negatively charged pectin was removed by QUAT anionic exchange membrane disk. After 3 h in recycle mode, 66.0% ± 2.2 of pectin was decreased, while 10.7% ± 1.7 of PE and 54.0% ± 2.2 of protein were bound (Figure 5). After filtration of the QUAT permeate onto SP membrane disk, 18% ± 2.8 of PE and 30.7% ± 3.3 of protein were bound onto SP. Also, 27.7% ± 6.9 of pectin was bound to SP membrane (Figure 5).

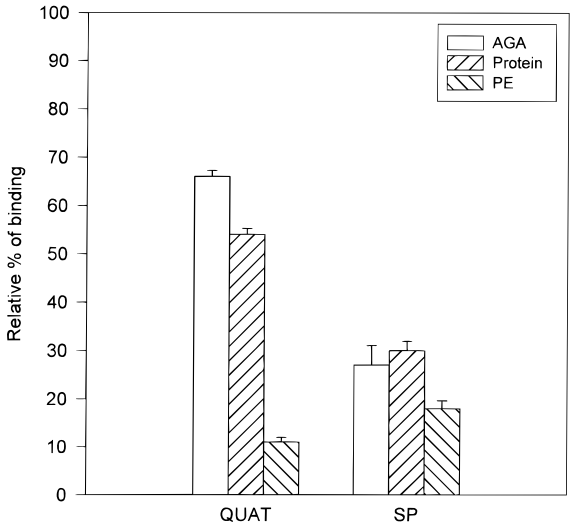

Figure 5. Relative AGA, protein, and PE binding to anionic QUAT, followed by cationic SP Acti-Disks at pH 7.2 in 0.01 M sodium phosphate buffer. Means of triplicates are reported.

Interactions between proteins and an ion exchanger depend on several factors, including the net charge and surface charge distribution of the protein. Other factors include pH, ionic strength, and the nature of the ions and other additives to the solvent, such as organic solvents etc. (Karlsson et al., 1989). One of the most important parameters which determine protein binding to ion exchange membranes is pH, since it affects the effective charge on both the protein and the ion exchanger. At pH values far away from the p*I*, proteins bind strongly and do not desorb at low ionic strength. Near the p*I*, the net charge of a protein is less, and consequently it binds less strongly.

PE is less protonated at higher pH values, and has less tendency to form an inactive complex with an anion. Theoretically, PE is positively charged at pH 8.0, 7.2, 6.0, or 5.0 while pectin is negatively charged at pH values above 4.0. Under such conditions, positively charged PE can combine with pectin at the ionized carboxylic acid groups. At lower concentrations, cations displace PE from an inactive PE−pectin complex and stimulate activity, whereas at higher concentrations cations competitively bind carboxylic acid groups and act as a competitive inhibitor of PE activity (Lineweaver and Ballou, 1945; Charnay et al., 1992). The competitive displacement theory is probably not the only factor

Table 2. PE Activity and AGA Concentration of the Permeate in Polysulfone Ultrafiltration (30 000 MWCO), at Different Running Times[a]

	time (min)						
	0	30	60	90	120	150	180
			24 °C, 10 psig				
PE (units/mL)	19.8	1.8	1.75	1.44	1.5	1.42	1.58
AGA (μg/mL)	105	51.1	51.8	53.4	51.1	52.8	52.8
			10 °C, 15 psig				
PE (units/mL)	18.6	1.03	0.95	0.74	0.86	0.69	0.57
AGA (μg/mL)	97.3	15.0	11.6	11.6	13.2	14.9	14.9

	PE Activity after SP Acti-Disk Membrane Filtration after Ultrafiltration[a]					
	permeate[b]	initial[c]	single pass	recycle 1 h	recycle 2 h	recycle 3 h
			24 °C, 10 psig			
PE (units/mL)	3.39	2.84	2.03	1.80	1.96	2.53
			10 °C, 15 psig			
PE (units/mL)	1.42	0.65	0.41	0.41	0.55	0.46

[a] Data presented are from a single replication. [b] Total permeate collected after ultrafiltration. [c] Initial PE activity after dialyzing against 0.01 M sodium phosphate buffer at pH 7.2.

involved in binding and release of PE from pectin complexes (Wicker, 1995). Complete solubilization and release of PE was not observed by extraction at pH 10, at pH 8.0 in the presence of EGTA, Triton, or urea, or after cell wall degradation (Wicker et al., 1988). Macdonald et al. (1994) reported incomplete separation of PE−pectin complexes after continuous-flow electrophoresis and column separation. They proposed that minor isozymes of lemon PE may actually be due to complex formation between major PE isozymes with pectin. Furthermore, the magnitude and extent of PE activation and inhibition were influenced by the type and concentration of cation (Leiting and Wicker, 1997), and the effect was unique at low pH compared to high pH (Snir et al., 1995). The heterogeneity of pectin, including total degree of esterification (% DE), distribution of DE, neutral sugar branching, and neighboring methoxyl groups, may be factors in PE binding and release. Nari et al. (1991) proposed noncooperative and cooperative cation binding. Cooperative cation binding was influenced by the carboxylic acid group near the methoxyl ester and by cation concentration. The mechanism of de-esterification and distribution of de-esterified groups by lemon PE isozymes resulted in different pectin gel structures induced by cations (Macdonald et al., 1993).

The FMC Acti-disks maintain the specified charge characteristics over the pH range used in this study. The incomplete binding of positively charged PE to the cationic exchange membrane may be attributed to the existence of PE in a pectin complex. In addition, since PE was not sufficiently mobile within the carbohydrate−protein complex (Glover and Brady, 1995) and considered to be strongly bound with pectin (Macdonald et al., 1994), the PE which bound the anionic or cationic membranes was probably in the form of a PE−pectin complex rather than free enzyme.

CONCLUSIONS

Anionic exchange disks (QUAT, DEAE, and PEI) did not bind PE or protein in substantial quantities. Greater binding of PE to SP cationic exchange disk was observed at pH 5.0, but less than 20% of PE was bound to SP membrane at pH 6.0, 7.2, and 8.0. Formation of PE−pectin complexes may "mask" the individual surface charge character of PE and pectin and result in poor binding abilities of either to the membranes. Although it is beyond the scope of this study, PE and pectin probably form heterogeneous complexes with varying numbers of binding sites and binding constants. In calcium pectate gelation, the number and sequence of carboxyl groups, cation concentration, and inhibition of junction zone formation by methoxyl groups influence gel formation (Axelos and Thibault, 1991). An analogous situation between calcium pectate gelation and PE−pectin complex formation probably exists.

ABBREVIATIONS USED

QUAT, quaternary amine strong anionic exchanger; DEAE, diethylaminoethyl weak anionic exchanger; PEI, polyethylenimine weak anionic exchanger; SP, sulfopropyl strong cationic exchanger; CM, carboxymethyl weak cationic exchanger; MGF, Marsh grapefruit; PE, pectinesterase; BSA, bovine serum albumin; UF, ultrafiltration; MWCO, molecular weight cutoff.

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