Involvement of Proteins in Cloud Instability of Valencia Orange [*Citrus sinensis* (L.) Osbeck] Juice

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The present study examined the involvement of proteins in cloud flocculation of Valencia orange juice. Marked differences in cloud instability were found between juices of different harvest dates. Heating of enzymatic pectin degraded juice from April and June harvests resulted in development of clumps and their precipitation. Although the juice from both harvesting dates remained hazy, the juice of April harvest was more turbid than that from June. Usually clarification increases as the temperature increases from ambient to 125 °C. Clarification occurred at pH 2.5−4.5 and was maximal at pH 3.5. The clarification of the April harvest juice was markedly lower than that of the June harvest. The fresh juice contained about 5.2 and 1.7 mg mL^{-1} insoluble cloud matter (ICM) and alcohol-insoluble serum solids (AISS), respectively. The ICM and the AISS, respectively, contained: proteins (244.5 ± 8.7 and 132 ± 1.8 μg mg^{-1}), galacturonic acid (40 ± 0 and 120 ± 0 μg mg^{-1}) and neutral sugars (270 ± 39 and 329 ± 23 μg mg^{-1}). Enzymatic pectin degradation resulted in removal of a marked portion of the pectin, and was accompanied by partial removal of neutral sugars (mainly glucose and galactose) and some proteins from the pectic polymer in both AISS and ICM. Proteins of the AISS included major bands at 10−14, 20, and 28 kDa and those of the ICM bands at 22, 24, 26, and 45 kDa.

Keywords: *Citrus; cloud; neutral sugars; orange juice; pectin; protein*

INTRODUCTION

Protein coagulation and its role in cloud instability have been studied previously and discussed in relation to the roles of pectin, calcium pectate, and pectin methyl esterase (Shomer, 1988, 1991; Shomer et al., 1982, 1991, 1999). Cloud flocculation is usually facilitated by enzymatic pectin degradation (EPD), either in aqueous peel extract (PEX) (Shomer, 1988) or in juice sacs extract, i.e., Shamouti orange juice (Shomer et al., 1999). Coagulation of soluble proteins can be detected visually because of the appearance of suspended particles during the heating of clear EPD serum. This phenomenon results in the formation of turbid serum, as has been reported to occur in sera of various plant tissue extracts such as those of PEX, potato tuber, and wheat flour (Shomer, 1988; Shomer et al., 1982, 1995). Some tissue extracts do not contain soluble coagulable proteins, and it is important to understand whether protein coagulation is involved in the flocculation of insoluble cloud matter (ICM). In citrus fruit, the flavedo extract includes soluble coagulable proteins. Proteins of the albedo extract were not found to be heat coagulable (Shomer, 1991), and in juice sacs the heat-coagulable proteins are the ICM proteins (Shomer et al., 1999).

In Shamouti orange juice, the ICM consists of about 18% proteins (as N × 6.25), and the alcohol-insoluble solids of the serum (AISS) include about 12% proteinaceous matter (Shomer et al., 1999). When the content of the ICM (∼6.5 mg mL^{-1}) and the AISS (∼1.8 mg mL^{-1}) are considered in the juice, it appears that most of the proteins are ICM composites (∼1.2 mg mL^{-1}), and a very small amount is found as AISS matter (∼0.18 mg mL^{-1}). Furthermore, although heat-coagulable proteins were not found in the serum, the AISS includes proteinaceous matter (detected as N × 6.25). It is assumed that the serum proteinaceous matter consists of small amount of polypeptides and either peptides or free amino acids bound to polysaccharides (Shomer et al., 1999).

The turbidity and color intensity of Valencia orange juice are higher than those of Shamouti orange juice because of the relatively high content of insoluble constituents, mainly pigment bodies, i.e., chromoplasts and their pigment constituents, and essential oil (Gross et al., 1972; Merin and Shomer, 1984; Shomer, 1996). In addition, in Israel, the harvest season of Shamouti orange is from December to February and that of Valencia orange is from early spring to early summer.

The present study dealt with the involvement of protein coagulation in cloud instability of Valencia orange juice from fruits harvested at mid-April and early-June. Coagulation/flocculation and cloud instability were identified under various conditions of temperature, EPD, and pH. Proteins, pectin, and neutral sugars were analyzed in both the AISS and the ICM.

MATERIALS AND METHODS

Preparation of Juice. Valencia orange (*Citrus sinensis* L. Osbeck) juice was squeezed from fruits harvested in April and June 1996. The juice preparation and analyses of proteins, pectin, neutral sugars and the statistical analysis were done as described by Shomer et al. (1999). The various treatments were as follows: Cloud flocculation by EPD of the juice and heating at 90 °C for 5 min (EPD heated) and five control treatments in order to assess the clarification due to heat

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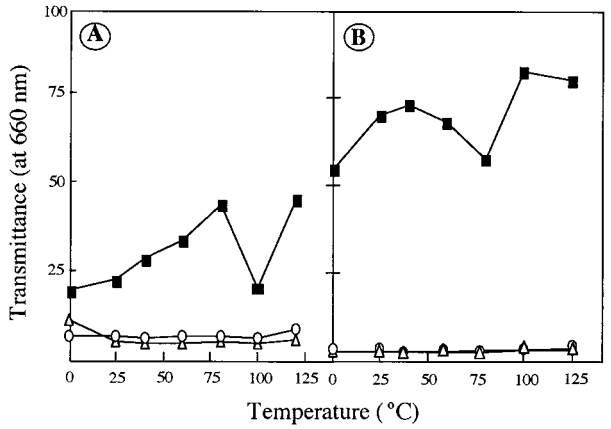

Figure 1. Turbidity of the upper zone of a column of natural Valencia orange juice, as affected by coagulation/flocculation at various heating temperatures. Treatments marked by different letters are significantly different ($p < 0.05$) according to Friedman ANOVA. (A) Juice from fruits harvested in April. (B) Juice from fruits harvested in June. ○, Control[a]; △, inactivated EPD[a]; ■, EPD heated[b].

coagulation: (1) juice (control); (2) juice heated at 90 °C for 5 min (control heated); (3) juice after EPD (EPD); (4) juice after incubation with inactivated Ultrazym [a solution of 1% (w/v) Ultrazym in distilled water was boiled under reflux for 5 min, cooled to ambient temperature, and added to the juice in ratios adjusted to the required concentration (inactivated EPD)]; (5) juice after incubation with inactivated Ultrazym (same as 4) and heated at 90 °C for 5 min (inactivated EPD heated).

RESULTS AND DISCUSSION

Marked differences in cloud instability were found between juices from different harvest dates. EPD-heated juice from both April and June resulted in the development and precipitation of cloud clumps, although the upper zone of the juice columns from both harvesting dates remained relatively turbid (Figure 1). The precipitated clumps in both juice samples were obvious. The upper zone of the April harvest juice column (Figure 1A) was more turbid (~6 to ~40% transmittance) than that of June (~1 to over 75% transmittance, Figure 1B). Usually, after EPD, clarification increases as the temperature increases from ambient to 125 °C. It should be noted that in the April juice clarification was markedly lower after heating at 100 °C.

Decrease in turbidity as a result of cloud flocculation was observed following heating of EPD April juice at pectinase concentrations ranging from 50 to 1000 μg mL^{-1} (Figure 2A). Different results were obtained in June harvest juice in which clarification increased up to somewhat more than 50% transmittance. As with Shamouti orange juice (Shomer et al., 1999), in April juice, clarification was higher in EPD-heated juice (Figure 2A). Nevertheless, in the June harvest of Valencia juice, high clarification by EPD was visible either with or without heating (Figure 2B). The June harvest juice was obtained from overripe fruit. Hence, it can be hypothesized that in overripe fruit the cloud proteins are partially denatured and tend to coagulate and to catalyze cloud flocculation as a result of EPD, similar to what occurs by heating.

As in the case of Shamouti orange juice (Shomer et al., 1999), clarification of EPD-heated juice was affected by pH; it occurred at pH 2.5−4.5 and was maximal at pH 3.5 (Figure 3A,B). As in the other experiments (Figures 1 and 2), the clarification of the April harvest

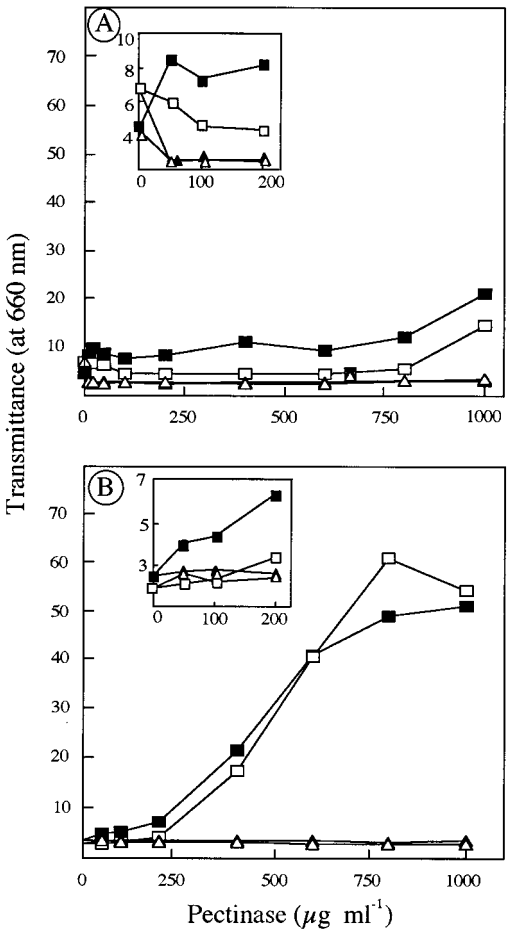

Figure 2. Turbidity of the upper zone of a column of natural Valencia orange juice as affected by coagulation/flocculation as a result of enzymatic pectin degradation at various pectinase concentrations at 100 °C. Treatments marked by different letters are significantly different ($p < 0.05$) according to Friedman ANOVA. (A) Juice from fruits harvested in April: △, inactivated EPD[a]; ▲, inactivated EPD heated[a]; □, EPD[b]; ■, EPD heated[c]. (B) Juice from fruits harvested in June: △, inactivated EPD[a]; ▲, inactivated EPD heated[a]; □, EPD[b]; ■, EPD heated[b]. Insert: Low concentrations of pectinase.

juice was markedly lower than that of the June juice. It appears that the highest clarification depends on the isoelectric point of the cloud proteins, which is around pH 3.5.

Marked differences in clarification due to protein coagulation are noted in juice of different fruit batches. However, despite the variability among juice samples, the same trend of cloud instability is obvious with regard to the effect of EPD (Figure 1A,B), heating (Figure 2A,B), and pH (Figure 3A,B). These results are in agreement with those obtained for Shamouti orange juice in relation to the role of insoluble cloud proteins in coagulation/flocculation and the resulting clarification (Shomer et al., 1999). As in the case of Shamouti, also in Valencia orange juice, cloud flocculation depends on temperature and pH that determine the extent at which cloud proteins undergo heat coagulation.

Unlike the temperature effect (Figure 1), the effects of pH and pectinase concentrations on clarification were done at a single temperature level of 100 °C. The results of these experiments (Figures 2 and 3) are comparable with the juice clarification observed at 100 °C in the temperature effect experiment (Figure 1). The above explains the relatively low clarification level of the April

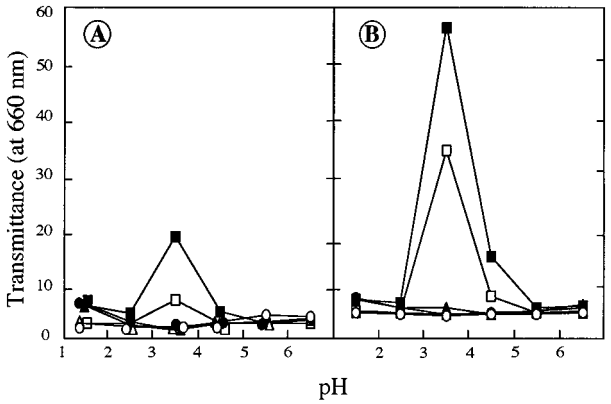

Figure 3. Turbidity of the upper zone of a column of natural Valencia orange juice, as affected by coagulation/flocculation at various pH levels at 100 °C. Treatments marked by different letters are significantly different ($p < 0.05$) according to Friedman ANOVA. (A) Juice from fruits harvested in April: ○ control[a]; ●, control heated[a]; △, inactivated EPD[a]; ▲, inactivated EPD heated[a]; □, EPD[a]; ■, EPD heated[b]. (B) Juice from fruits harvested in June: ○, control[a]; ●, control heated[a]; △, inactivated EPD[a]; ▲, inactivated EPD heated[a]; □, EPD[b]; ■, EPD heated[c].

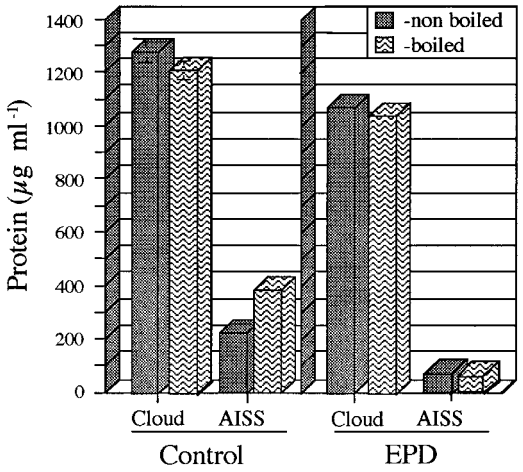

Figure 4. Protein content of insoluble cloud matter (cloud) and alcohol-insoluble serum solids (AISS) of Valencia orange juice, untreated and after enzymatic pectin degradation (EPD) and heat coagulation/flocculation. Error bars indicate ± SD of triplicates.

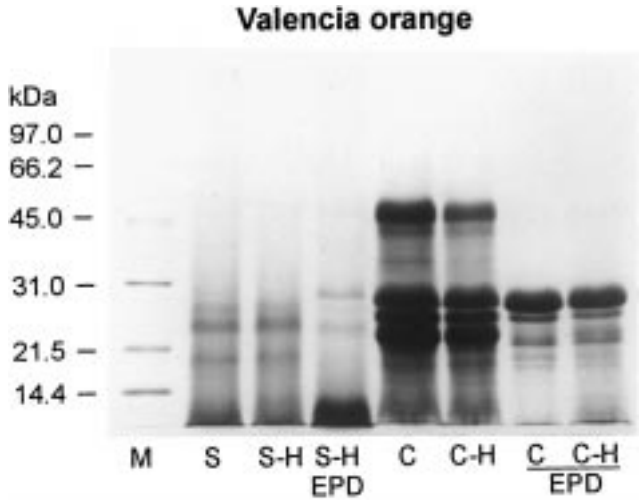

Figure 5. Protein electrophoresis profiles of insoluble cloud matter and serum alcohol-insoluble solids of natural Valencia orange juice after heating to boiling point and enzymatic pectin degradation. C, insoluble cloud matter; C-H, insoluble cloud matter following heating at 100 °C; EPD, enzymatic pectin degraded juice; molecular weight markers; S, alcohol-insoluble serum solids; S-H, alcohol-insoluble serum solids following heating at 100 °C; alcohol-insoluble solids of the serum extracted from pectinase-degraded juice.

juice at pH 3.5 (Figure 3A). The clarification levels of the June harvest juice at pH 3.5 (Figure 3B) in both the EPD and the EPD-heated juice were also in agreement with the same treatments in nonheated and heated juices at 100 °C (Figure 1). The same phenomena were observed with respect to the effect of enzyme concentration (Figure 2) and the 100 °C treatment (Figure 1).

Despite of the obvious formation of precipitated cloud clumps in Valencia orange juice upon heat coagulation of proteins, the upper zone of the column was less clarified than that of Shamouti. In this context, Valencia orange juice contains larger quantities of essential oil and carotenoid pigments (Gross et al., 1972). The predominant component (~90%) of the essential oil is *d*-limonene, which is an organic solvent that dissolves part of the carotenoids in the juice (Shomer, 1996; Shomer et al., 1985). Both the carotenoids and the essential oil are biosynthesized and secreted by the chromoplasts in the juice sac tissue (Shomer, 1996). Upon squeezing and cell breakdown, the secreted oil with some proportion of dissolved carotenoids is dispersed in the juice as emulsified droplet bodies. The essential oil and its dissolved carotenoids interact with the coagulated proteins and flocculate with the other cloud constituents (Shomer, 1988). However, as far as cloud proteins are concerned, the extent of clarification depends on the interactions of the flocculated cloud protein with nonproteinaceous cloud constituents such as emulsified oil droplets, dissolved carotenoids, and polysaccharides.

Generally, clarification of Valencia orange juice under the effects of the various conditions coincide with that of Shamouti orange juice, even for the moderate clarification in juice of overripe fruits. Thus, the present study strengthens the hypothesis about clarification as a result of PME activity and the binding of cloud proteins to demethoxylated pectic substances (Shomer et al., 1999).

It is suggested that Valencia orange juice contains more nonproteinaceous cloud matter than the maximum amount that can coprecipitate with the coagulated cloud protein. The noninteracted cloud matter remains suspended and confers turbidity to the upper juice column, despite the precipitation of the clumps that form. In this context, it seems that clarification due to such interactions is a complicated process and deserves further elucidation, particularly when conjunction with PME activity is considered.

Cloud and AISS Proteins. The ICM and the AISS account for about 5.2 and 1.7 mg mL^{-1}, respectively, of the fresh juice. Analyses of the ICM and the AISS revealed the presence of proteinaceous matter: ~1281 μg mL^{-1} protein (244.5 ± 8.7 μg mg^{-1} in the ICM dry matter) and ~222 Fg mL^{-1} protein (132.3 ± 1.8 μg mg^{-1} in the AISS dry matter) (Figure 4). The cloud protein content is in agreement with reported data for reconstituted orange juice (Klavons et al., 1991). It is somewhat higher than those reported for commercial orange and lemon juice (Klavons and Bennett, 1985; Klavons et al., 1991) and also somewhat higher than that of

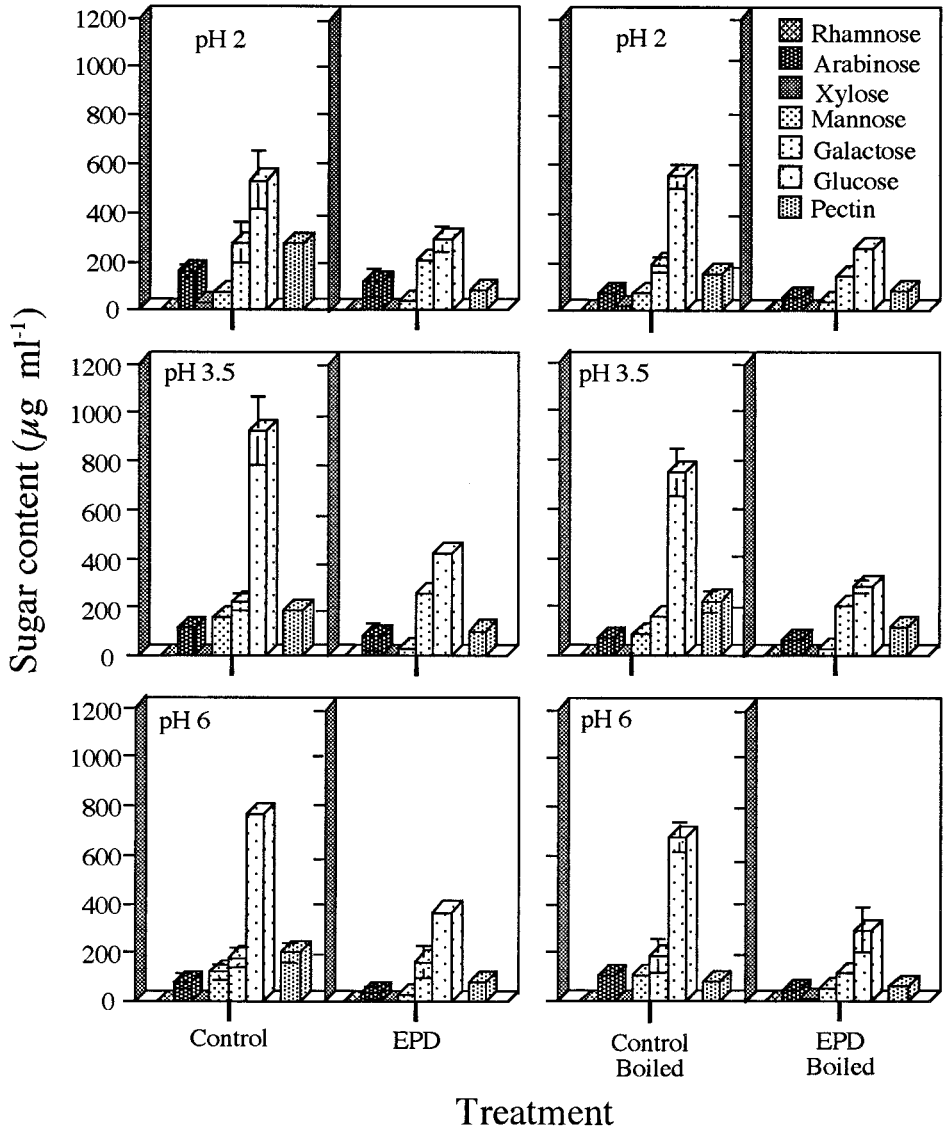

Figure 6. Content of galacturonic acid and neutral sugars in insoluble cloud matter of natural Valencia orange juice at three pH levels [2, 3.5 (natural level), and 6] and after heat coagulation/flocculation and enzymatic pectin degradation (EPD). Error bars indicate ± SD of triplicates.

Shamouti orange juice (Shomer et al., 1999). As in Shamouti orange juice (Shomer et al., 1999), EPD resulted in a slight decrease in the ICM protein and reduced the protein content of the AISS by more than 50%. Heat treatment resulted in a slight decrease of the protein contents of the ICM and in a marked increase in the AISS in the control. No marked differences were noted in the protein content among the treatments of the EPD juice.

SDS−PAGE profiles revealed major differences between the protein bands of the ICM and those of the AISS (Figure 5). The AISS proteins are seen indistinctly, indicating traces of proteinaceous matter with observable bands in the vicinity of 10−14, 20, and 28 kDa. This AISS proteinaceous matter was not heat coagulable. Following EPD of the AISS, one additional band appeared at 31 kDa despite the reduction of the total AISS proteinaceous matter by >50% of the nonboiled juice and by >80% in the heated juice (Figure 4). These results show that, after EPD, one band of the exogenous pectinase is found in the residual AISS but not in the ICM. This trace is less obvious than that found in the AISS of the Shamouti orange juice (Shomer et al., 1999), perhaps because of differences in the juice components and their interactions with the Ultrazym proteins. As a result of EPD, the pectin bound proteinaceous matter and the degraded pectins were not precipitated as AISS. It appears that most of the pectin-bound proteinaceous matter is soluble and is washed away from the AISS as a result of EPD.

The ICM proteins reveal polypeptides in the range of 17−50 kDa, similar to those of the Shamouti orange juice (Shomer et al., 1999), with distinct bands at 22, 24, 26, and 45 kDa. As in the cloud of Shamouti orange juice, no residual exogenous pectinase was found in the ICM of the EPD-heated juice. Therefore, also in this case it seems that the cloud flocculation was not affected by the exogenous pectinase.

Nevertheless, following EPD, polypeptides of molecular weight over 30 kDa were not found in the electrophoresis pattern of the ICM (Figure 5), and the amounts of total cloud proteins were reduced by 17% in both the control and the EPD treatments (Figure 4). These differences correspond to those found in Shamouti orange juice and have been explained and discussed elsewhere (Shomer et al., 1999) in light of the abnormal

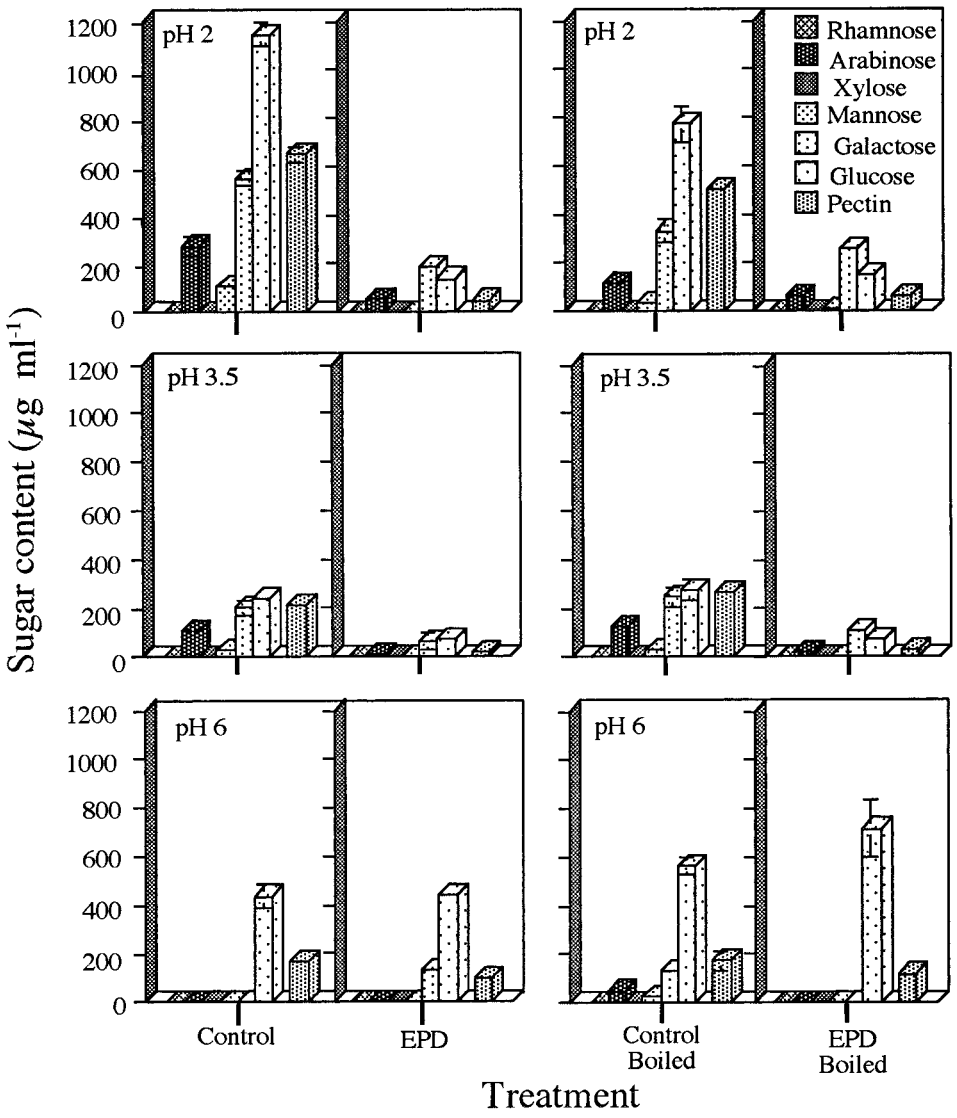

Figure 7. Contents of galacturonic acid and neutral sugars in alcohol-insoluble serum solids of natural Valencia orange juice at three pH levels [2, 3.5 (natural level), and 6] and after heat coagulation/flocculation at 100 °C and enzymatic pectin degradation (EPD). Error bars indicate ± SD of triplicates.

SDS binding, shape, and charge of the glycoproteins before the enzymatic treatment (See and Jackowski, 1990).

Pectin and Neutral Sugars. Polysaccharides of both the serum and the ICM include pectin (analyzed as galacturonic acid) and the neutral sugars: rhamnose, arabinose, xylose, mannose, galactose, and glucose (Figures 6 and 7). The galacturonic acid contents in the AISS and the ICM were 120 ± 0 and 40 ± 0 μg mg^{-1}, respectively. The total neutral sugar contents in the AISS and in the ICM were 329 ± 23 and 270 ± 39 μg mg^{-1}, respectively. The amount of galacturonic acid in the AISS (~211 ± 4.7 μg mL^{-1}) was higher than that of the ICM (~184 ± 9 μg mL^{-1}). Glucose and galactose were the predominant neutral sugars in the AISS (240 ± 2.4 and 203 ± 31 μg mL^{-1}, respectively). In the ICM, glucose was found in much higher relative abundance (923 ± 136 μg mL^{-1}), and the galactose was somewhat higher (221 ± 33 μg mL^{-1}) than in the AISS. These relative amounts are different from those previously reported for PEX (Shomer, 1991), in which the predominant sugars were glucose and arabinose: 258 and ~40 μg mL^{-1}, respectively, in the AISS and 21.6 and 10.5 μg mL^{-1} in the coagulate, respectively.

EPD was accompanied by reductions of glucose contents in the ICM (to ~423 ± 13 μg mL^{-1}) and of glucose and galactose in the AISS (to 75 ± 2.5 and 63.6 ± 35 μg mL^{-1}, respectively). These results are in agreement with those previously reported for PEX (Shomer, 1991) and for Shamouti orange juice (Shomer et al., 1999), in which the contents of part of the neutral sugars (mainly glucose) were found to be in accordance with those of galacturonic acid. It appears that the used exogenous pectinase (consisting mainly of EC 4.2.2.10) is not fully capable of degrading all of the pectic substances and neutral sugars.

As shown in Figures 6 and 7, there were differences between the control and the EPD treatments, where in the AISS the total amount of pectin decreased from 211.0 ± 4.7 to 21.0 ± 0.28 and the neutral sugars decreased from 587.7 ± 39.7 to 153 ± 38.9. In the ICM, the EPD resulted in decreasing pectin content from 183.6 ± 9.1 to 103.2 ± 4.5 and of total neutral sugars from 1414.7 ± 204.1 to 783 ± 83.2. As a result of heating of EPD juice, the total neutral sugars content in the ICM decreased from 783.5 ± 83.2 to 573.2 ± 70.2, perhaps by β-elimination. On the other hand, in the AISS, the amount of pectin increased from 21.0 ± 0.3 to 27.4 ±

0.3 and that of neutral sugars increased from 153.1 ± 38.9 to 204.3 ± 19.0. It is assumed that EPD resulted in removal of pectin-bound neutral sugars, and the relative high molecular weight residues removed from the ICM appear as AISS matter.

CONCLUSIONS

The present study supports the results of previous and parallel studies of both peel extracts (Shomer, 1988; Shomer et al., 1991) and natural juice (Shomer et al., 1999) about the role of proteins in cloud flocculation and the resulted clarification. Although precipitated cloud clumps were formed in the EPD-heated juice, similar to those of Shamouti orange juice (Shomer et al., 1999), the upper zone of the column was less clarified. EPD juice of overripe fruit (June harvest) was clarified to an extent similar to that of EPD-heated juice, perhaps because of the overripe character of the fruit of June harvest.

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