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**Membrane Processing of Citrus Extracts: Effects
on Pectinesterase Activity and Cloud Stability**

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Membrane Processing of Citrus Extracts: Effect on Pectinesterase

Activity and Cloud Stability

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C. Abstract - Membrane Processing of Citrus Extracts: Effect on Pectinesterase Activity and Cloud Stability

The U.S. team studied the role of cations and pH on thermolabile (TL-PE) and thermostable (TS-PE), permeation in ultrafiltration (UF) membranes, affinity to ion exchange membranes, mechanism of cation and pH activation, and effect on PE stability. An optimum pH and cation concentration exists for activity and UF permeation, which is specific for each cation type. Incomplete release of PE from a pectin complex resulted in low PE binding to cationic and anionic membranes. Incubation of PE at low pH increases the surface hydrophobicity, especially TL-PE, but the secondary structure of TL-PE is not greatly affected.

The Israeli team showed that stable cloud colloidal constituents flocculate following the conversion of soluble to insoluble biopolymers. First, formation of pectic acid by pectinesterase activity is followed by the formation of calcium pectate gel. This process initiates a myriad of poorly defined reactions that result in juice clarification. Second, protein coagulation by heat resulted in flocculation of proteinaceous bound cloud constituents, particularly after enzymatic pectin degradation. Pectinesterase activity is proposed to be an indirect cause for clarification; whereas binding of cloud constituents is the primary event in clarification by pectate gel and coagulated proteins. Understanding the mechanism of interaction of protein and pectic polymers is key to understanding cloud instability. Based on the above, it was hypothesized that the structure of pectin-protein coagulates plays a key role in cloud instability.

D. Objectives of Original Research Proposal - Membrane Processing of Citrus Extracts:

Effect on Pectinesterase Activity and Cloud Stability

The overall objectives of this project were to elucidate the underlying mechanism(s) of citrus cloud stability, while simultaneously developing alternative methods to remove, inhibit, or inactivate thermostable PE. The specific objectives to be addressed were:

1. To evaluate partitioning of thermostable pectinesterase (PE) and to determine if PE activators and/or inhibitors are solubilized during extraction.
2. To develop affinity matrixes and/or immobilized ligands that preferentially bind thermostable PE and develop surface modified membranes that will preferentially bind or inactivate thermostable PE.
3. To fractionate and identify citrus cloud constituents and evaluate the attractive and repulsive forces that contribute to cloud stability.

Note: The original objectives remained the same. The proposed methodology and experimental design changed as new information was obtained. It was learned that the tight affinity of PE-pectin complexes precluded separation of PE by the proposed means.

E. Relevant data, methodology, results and discussion

The research by the U.S. team addressed partitioning of TSPE and the role of activators and/or inhibitors of TLPE and TSPE activity. The U.S. team also studied the role of cations and pH on permeation of PE in ultrafiltration, affinity to ion exchange membranes, mechanism of cation activation, and effect on PE structure and denaturation.

Pectinesterase (PE) is a cell wall bound enzyme and is solubilized by high salt, high pH extractant buffer. In the competitive displacement model, metal cations interact with the carboxyl groups of

pectin, releasing PE from an inactive pectin complex, but higher cation concentrations competitively displace PE and result in inhibition (MacDonnell et al., 1945; Nari et al., 1991; Charnay et al., 1992). The first indication of the complexity of the influence of cations and pH on PE activity became evident from our early results. Cations and pH influence not only the solubilization of PE from a pectin-PE complex, but also uniquely influence activity, inhibition, permeation through ultrafiltration and ion exchange membranes, and structure.

The effects of membrane fouling and molecular weight cutoff (MWCO) on partitioning of pectinesterase (PE) isolated from Marsh grapefruit could be expressed by a logarithmic function. Statistically higher fouling rates were observed for Marsh grapefruit extracts on 30 K and 100 K polysulfone UF membranes than on a 10 K membrane. Permeability of PE decreased 30-60% during processing regardless of the MWCO of the membrane. About 40-50% of the total activity in the permeate of the 30 K UF membrane was thermostable PE.

At pH values of 3.8 and no added salt, pectinesterase (PE) was retained by a 30K MWCO UF membrane. Permeability increased with increasing pH and/or cation concentration. Flux was not increased at higher pH and/or cation concentration. Divalent cations were more effective than monovalent cations at stimulating activity and permeability of PE. Greatest PE permeability was obtained at pH 8.0 and 0.15M NaCl, pH 6.0 and 0.15M NaCl, pH 3.8 and 0.4M NaCl or pH 3.8 with 0.1M CaCl₂. The results suggest that PE is bound to soluble pectin and is retained by UF membranes. Adding cations or raising the pH releases PE from the electrostatic binding to pectin and enables the enzyme to permeate UF membranes.

PE activity increased 281%, 183%, and 130% with lead acetate (6.8 mM), ferric chloride (0.2 mM), and calcium chloride (20 mM), respectively. Naturally occurring polyamines,

putrescine, spermidine, and spermine had negligible stimulating effect. All cations tested inhibited PE activity at higher concentrations. Solubilization of total PE was decreased and thermostable PE (TS-PE) was not affected by polyamines. Loss of activity upon solubilization suggests that the TL-PE-pectate complex stabilized TL-PE. No change in TS-PE activity suggests that it may be bound with greater affinity, physically entrapped, or non-electrostatically bound.

PE has high pI, (greater than 9.0) and pectin has an apparent pK of 3.5- 4.0. Thus, PE and pectin should have high affinity for ion exchange (IEX) membranes at a wide range of pH values and should be separated by IEX. Thus we looked at anionic and cationic membranes for separation of PE and pectin. Ion exchange membrane disks (FMC Co., Pine Brook, NJ) were tested at pH 5.0, 6.0, 7.2, and 8.0 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. Binding or permeation is not selective for TS-PE or TL-PE.

If competitive displacement were the only factor involved in activation and solubilization of PE, then the same magnitude of activation should have been observed with sufficient amounts of cation, but this was not the case. The type and concentration of cation influenced the magnitude and extent of PE activation and inhibition. We observed similar maximum stimulation of PE activity with sodium chloride and calcium chloride at pH 6 or pH 8, but not at pH 3.8. The

peak height, peak shape, and concentration of cation at maximal stimulation of activity was unique for each cation studied. Furthermore, total and TS-PE activities were influenced differently depending on the cation used to dissociate PE from the pectin complex. Although the competitive displacement theory is well documented, the unique effect of cations suggests that this theory alone does not explain the role of cations on PE activity, solubilization, and pectin complexes.

After the research with cations and pH, it became clear that covalent or other types of crosslinks to the cell wall, although possibilities, were not the major concern in solubilizing, binding, or removing PE from the juice stream. Further research was directed towards understanding the mechanism of action of cations and pH on PE activity and structure.

Using the selective extraction procedure and column chromatography, the highest reported purity of TS-PE was obtained from Marsh grapefruit pulp. The catalytic rate constants for TS-PE were determined at pH 7 at temperatures ranging from 25 to 60 °C under sodium added or calcium added conditions. The isokinetic temperature for sodium and calcium added conditions was 327.8 K and 312.4 K, respectively. The different isokinetic temperatures and compensation parameters suggest that sodium and calcium uniquely affect TS-PE catalysis. Under all conditions, $\Delta S^\ddagger$ was negative, which is the indication of structural changes that the reactants undergo while forming the activated complex. Because $\Delta S^\ddagger$ is negative, formation of PE - pectin activated complex is a process of rotational and vibration freedom decrease.

The effect of pH on stability and solvent accessible hydrophobicity of TL-PE and TS-PE were studied using hydrophobic fluorescence spectroscopy with a hydrophobic probe, 8-anilino-1-sulfonate (ANS). ANS fluoresces only in hydrophobic environments. At neutral pH, ANS fluorescence intensity was an order of magnitude lower for both pectinesterase (PE) isozymes than for bovine serum albumin (BSA), a hydrophobic protein. At pH values less than 3.5, ANS

fluorescence of both PE isozymes increased and, at pH 2.0, was higher than ANS=BSA fluorescence. Fluorescence increase at low pH was greater in ANS=TL-PE than ANS=TS-PE. Activity loss and increase in fluorescence after pH 2 treatment was irreversible for TL-PE, but nearly fully recovered for TS-PE. Resistance to conformational change at low pH by TS-PE probably contributes to enhanced stability. It was not possible to purify enough TS-PE to do further structural studies. The secondary structure of TL-PE was studied at low pH and high heat. TL-PE was inactivated completely after 8 hr at pH 3.3 but retained activity at pH 4.1 and pH 7.0. Initial studies show that circular dichroism (CD) spectra and secondary structural analysis no great secondary structural differences between pH 7.0 and pH 4.1. Heating TL-PE at pH 7.0 and pH 4.1 resulted in irreversible protein precipitation and loss of CD signal. Destabilization of TL-PE structure occurs at a lower temperature at pH 4.1 than at pH 7.0. An increase of surface hydrophobicity resulting from disturbance of tertiary structure most likely contributes to decreased heat stability. At low pH, TL-PE may be in a molten globule state, where the secondary structure is largely intact, but the structures between the secondary structure is changed to expose more hydrophobic regions.

Undesirable separation of cloud matter is a major quality and aesthetic problem of industrial processed orange juice particularly in reconstituted products. Membrane filtration of orange juice, although it avoids elevated heating, results in hard to reconstitute juice of stable cloud from concentrate. In order to develop high quality reconstituted natural juices, it is of basic importance to understand the mechanisms responsible for the irreversible cloud flocculation in accordance with the filtration efficiency.

The research carried out by the Israeli team included two major parts: 1. The role of proteins in cloud flocculation as related to juice clarification in order to understand their impact on processing, quality and shelf life. 2. Study of the involvement of heat protein coagulation and the resultant flocculation of cloud components with filtration properties of synthetic membranes during

processing.

The research was carried out on four main sections: (a) Involvement of proteins in cloud instability of Shamouti orange juice; (b) Protein Coagulation/Flocculation in Valencia orange (*Citrus sinensis* L. Osbeck) juice and its relation to cloud instability; (c) Ultrafiltration performance of heat treated Shamouti [*Citrus Sinensis* (L.) Osbeck] orange juice; (d) Pectinesterase and heat treatment effects on UF of Valencia orange (*Citrus sinensis* L. Osbeck) juice.

The Israeli research was performed in accordance with previous findings, which showed that stable cloud colloidal constituents tend to flocculate following the conversion of soluble biopolymers into insoluble ones. Two main factors cause to such phenomenon: **1.** The formation of pectic acid by pectinesterase activity followed by the formation of calcium pectate gel. This process initiates a myriad of poorly defined reactions that result in juice clarification. **2.** Protein heat coagulation. This phenomenon resulted in flocculation of proteinaceous bound cloud constituents, particularly after enzymatic pectin degradation.

Pectinesterase activity is proposed to be an indirect cause for clarification; whereas binding of cloud constituents is the primary event in clarification by pectate gel and coagulated proteins (Stevens et al. 1950; Shomer et al. 1982; 1991; Shomer, 1988; 1991). The interaction of protein and pectic polymers is not clear. This aspect is a key point in the understanding of cloud instability. Based on the above, it was hypothesized that the structure of pectin-protein coagulates plays a key role in cloud instability. However, the binding affinity between pectin-protein coagulates and cloud constituents is not known.

F. Description of the Cooperation

The problems associated with membrane processing and cloud stability of citrus products were addressed by the two research teams. The Israeli scientists investigated parameters of stability of cloud constituents as affected by membrane processing. The US scientists evaluated the conditions to separate PE using ultrafiltration and ion exchange membranes. The role of cations and pH on PE activity, permeation and structure is critical. The teams communicated via FAX and electronic mail primarily. Both teams took advantage of the opportunity to visit and discuss research at jointly attended scientific meetings. For each team, it was possible to schedule additional visits in conjunction with other responsibilities. The 7-21 day visits and joint research project greatly facilitated cooperation and discussion of differing view points. It promoted a very rewarding exchange of information and ideas.

G. Evaluation of the research achievement with respect to the original research proposal and its objectives

The overall objectives of this project were to elucidate the underlying mechanism(s) of citrus cloud stability, while simultaneously developing alternative methods to remove, inhibit, or inactivate thermostable PE. The U.S. team evaluated the partitioning of thermostable pectinesterase (PE) and the role of cations to solubilize TL-PE and TS-PE. They also determined that cations not only activated PE, but also that cations had a specific effect on activity and inhibition of PE. They showed that PE-pectin complexes were best disrupted at high pH and salt concentrations, which precludes use of ion exchange affinity matrix to preferentially bind thermostable PE. Research on the effect of pH and heat on structure, including surface hydrophobicity and secondary structure has provided information on how and why PE denatures and forms complexes with pectin. The Israeli team focused on citrus cloud

constituents and forces that contribute to cloud stability. They showed the involvement of protein coagulation in cloud instability of natural Shamouti orange juice, i.e., in the juice sacs' extract. They reported no soluble heat coagulable proteins in the natural juice. Nevertheless, the behavior and composition of the cloud constituents and instability were similar to coagulation and flocculation behavior of turbid extracts, which contain soluble coagulable proteins.

Since Valencia orange is one of the major citrus cultivars for juice production in both Israel and the USA, and since it differs in its properties from Shamouti orange juice, the second section of the research was carried out on Valencia orange juice. Based on the results obtained in the previous sections about cloud instability of the juice itself, these instability factors were studied in relation to membrane filtration. In this context, a major problem with membrane produced reconstituted juices is the irreversible loss of cloud stability. The hypothesis was that since in membrane filtration, where the soluble and insoluble constituents undergo fractionation in the absence of PE activity because of heat inactivation, the reconstituted juice supposed to be more stable. However, despite the elimination of PE activity, under these conditions the balance between the various solids and solutes of the suspensions are disturbed, thus reducing colloidal stability. Since the previous research sections pointed to protein-pectin-cloud interactions under heating to be responsible for cloud instability, this point was studied with regard to juice filtration. The role of protein-pectin-cloud interactions in both the cloud instability and membrane fouling during juice filtration experiments. These findings led to the assumption that PE activity also influences the above interactions upon heating. Therefore, higher concentrations of PE and heat treatment were employed to Valencia orange juice to examine its role in membrane filtration.

H. Conclusions

Understanding the mechanism of interaction of proteins, PE, cations and pectic polymers is key to understanding cloud instability. First, formation of pectic acid by pectinesterase activity is followed by the formation of calcium pectate gel. Factors which influence PE activity, inhibition and structure will likely influence cloud stability. A specific optimum pH and cation concentration exists for solubilization of PE, activity, and inhibition. Incubation of PE at low pH increases the surface hydrophobicity, especially TL-PE, but the secondary structure of TL-PE is not greatly affected. An optimum pH and cation concentration exists for thermolabile (TL-PE) and thermostable (TS-PE) permeation in ultrafiltration (UF) membranes and affinity to ion exchange membranes. The mechanism of cation and pH activation, and effect on PE stability, activity and UF permeation is specific for each cation type. Incomplete release of PE from a pectin complex resulted in low PE binding to cationic and anionic membranes.

PE activity and formation of pectic acid initiates a myriad of poorly defined reactions that result in juice clarification. The second key factor in cloud stability is protein coagulation by heat resulted in flocculation of proteinaceous bound cloud constituents, particularly after enzymatic pectin degradation. Pectinesterase activity is proposed to be an indirect cause for clarification; whereas binding of cloud constituents is the primary event in clarification by pectate gel and coagulated proteins. Stable cloud colloidal constituents flocculate following the conversion of soluble to insoluble biopolymers. Based on the above, it was hypothesized that the structure of pectin-protein coagulates plays a key role in cloud instability.

I. List of Publications resulting from the Research Project

- Snir, R., Koehler, P.E., Sims, K.A., and Wicker*, L. 1994. pH and Cations Influence Permeability of Marsh Grapefruit Pectinesterase on Polysulfone Ultrafiltration Membrane. *J. Agric. Food Chem.* 43: 1157-1162.
- Snir, R., Koehler, P.E., Sims, K.A. and Wicker, L. 1996. Total and thermostable pectinesterases in citrus juices. *J. Food Sci.* 61: 379-382.
- Snir, R., Wicker, L., Koehler, P.E., and Sims, K.A. 1996. Membrane fouling and molecular weight cutoff effects on the partitioning of pectinesterase *J. Agric. Food Chem.* 44: 2091-2095.
- Sun, D. and Wicker, L. 1996. pH affects Marsh grapefruit pectinesterase stability and conformation. *J. Agric. Food Chem.* 44: 3741-3745.
- Leiting, V.A. and Wicker, L. 1997. Inorganic cations and polyamines moderate pectinesterase activity. *J. Food Sci.* 62(2):1-4.

Theses:

- Leiting, V.A. 1995. The Effect of Polyamines on Activation and Ultrafiltration of Pectinesterase, M.S. Thesis, University of Georgia, Athens, GA.
- Daqing Sun, 1997. Pectinesterase Activity, Stability, and Structure, Ph.D. Thesis, University of Georgia, Athens, GA
- Chen, R. 1997. Membrane Separation of Pectinesterase from Marsh Grapefruit Pulp, M.S. Thesis, University of Georgia, Athens, GA.

J. Patents

No patents were filed.