PRO-XAN Process: Stability of Proteins and Carotenoid Pigments in Freshly Expressed Alfalfa Juice

Donald de Fremery,* E. M. Bickoff, and George O. Kohler

The relative stabilities of carotene, xanthophyll, and protein have been determined in freshly expressed juice from alfalfa (*Medicago sativa*). About 6% of the carotene and 29% of the xanthophyll are lost during incubation at 50° for 4 hr at pH 5.5. Under these same conditions, more than half of the initial protein is hydrolyzed. Its stability is increased markedly by raising the pH and lowering the temperature. Proteolysis is most active at pH 5.5 and 50–60°.

Alfalfa (*Medicago sativa*) is an excellent source of protein and xanthophyll for poultry nutrition. However, its high level of fiber limits its use in high-energy rations. To overcome this limitation, a wet fractionation process has been developed at our laboratory which separates a portion of the alfalfa protein and carotenoid pigments as a dry product (PRO-XAN) that is essentially devoid of fiber (Knuckles *et al.*, 1970, 1972; Lazar *et al.*, 1971; Miller *et al.*, 1972; Spencer *et al.*, 1970, 1971). The bulk of the alfalfa is recovered in a form which still meets industry standards for high-quality dehydrated alfalfa meal.

The incorporation of the PRO-XAN process into an existing alfalfa dehydration operation requires capital expenditures that might be prohibitive to the small dehydrator. The idea occurred to us that a group of dehydrators could pool their resources into a joint venture wherein each processor would express alfalfa juice and dehydrate the pressed residue in his own plant, and then ship the juice to a central processor for production of PRO-XAN (the protein–xanthophyll concentrate). Such an operation requires a knowledge of the stability of the proteins and carotenoid pigments under ambient conditions of time, temperature, and pH. Previous work in other laboratories has demonstrated the enzymatically induced instability of proteins (Brady, 1961; Macpherson, 1952; Singh, 1962; Tracey, 1948) and carotenoids (Booth, 1960; Walsh and Hauge, 1953) in juice or crushed plant material. In our experiments, the stability of proteins, carotene, and xanthophyll in alfalfa juice was determined systematically at three pH values (5.5, 7.0, and 8.5) and six temperatures (0–50° in 10° increments) for periods of time from 0.25 to 7.0 hr. Additional pH and temperature conditions were studied to ascertain pH and temperature optima for endogenous protein hydrolysis.

EXPERIMENTAL SECTION

Preparation and Incubation of Samples. Juice was expressed from freshly-chopped alfalfa by the method of Knuckles *et al.* (1970), except that ethoxyquin and ammonia were not added. The juice was passed through a 40-mesh screen to remove a small amount of residual fiber and then held in ice until incubation could be started (less than 1 hr). For incubation, individual samples were brought to the desired temperature, the pH was adjusted by the addition of 1 *N* HCl or 1 *N* NaOH with stirring, and the samples were placed in a constant-temperature water bath in open beakers. During incubation, the pH was maintained at the initial level by the dropwise addition of 1 *N* NaOH. Samples were removed periodically for the determination of protein, carotene, and xanthophyll.

Analytical Methods. Total nitrogen was determined in all samples by the standard Kjeldahl method. Nonprotein–nitrogen was assumed to be that nitrogen which was soluble in 10% trichloroacetic acid, and protein–nitrogen was determined by difference. Volume changes during incubation of the samples due to evaporation and/or addition of 1 *N* NaOH resulted in minor changes in the concentration of total nitrogen; calculations included a correction for these changes. In our experiments, the initial concentration of protein–nitrogen in alfalfa juice varied from 3.0 to 7.5 mg/ml. Liquid samples for carotene and xanthophyll analysis were frozen rapidly in thin layers (~3 mm) in metal trays at −60°, dried in a freeze-dryer at a shelf temperature of 20°, ground to a fine powder in a mortar and pestle, stored temporarily at −23°, and analyzed in duplicate for carotene and total xanthophyll by the method of Quackenbush *et al.* (1970). Results are expressed as mg of carotenoid/lb of juice solids. The moisture content was determined as the loss in weight of samples held in a forced-draft oven for 2 hr at 110°.

RESULTS AND DISCUSSION

Carotene Stability. At 50° (the highest temperature used), the loss of carotene is only 6% during 4 hr at pH 5.5 (Figure 1). At the higher pH values, carotene is even more stable. When samples are incubated at lower temperatures (between 0 and 40°), carotene loss during storage for 4 hr is 2% or less, regardless of pH. These results indicate a higher degree of stability in juice samples than has been reported in whole homogenates of leaf tissue. Walsh and Hauge (1953) report carotene losses of 70 and 85% in homogenates incubated in the dark and light, respectively, at 40° for 6 hr at pH 6. Booth (1960) reports a 15% loss of carotene during incubation of leaf homogenates for 30 min at room temperature. The greater stability of carotene in filtered (or screened) juice is an important consideration in the possible shipment of juice prior to heat coagulation.

Xanthophyll Stability. Xanthophyll is markedly less stable than carotene during storage of alfalfa juice. About 30% of the initial amount is lost during incubation at 50° for 4 hr at pH 5.5 (Figure 2). As with carotene, breakdown is inhibited at the higher pH levels. At 40°, the rate of breakdown is retarded, being 2, 6, and 10% during 4 hr at pH 8.5, 7.0, and 5.5, respectively. At temperatures of 30° or below, xanthophyll loss is less than 5% in 4 hr, regardless of pH.

The relative stabilities of carotene and xanthophyll in alfalfa

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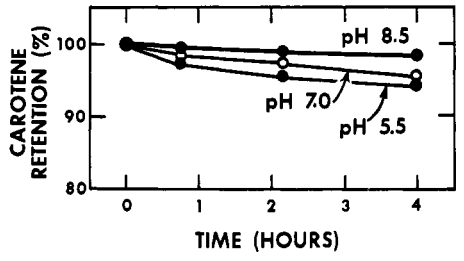

Figure 1. Carotene retention during storage of freshly expressed alfalfa juice at 50°. Initial level = 191 mg/lb (dry basis)

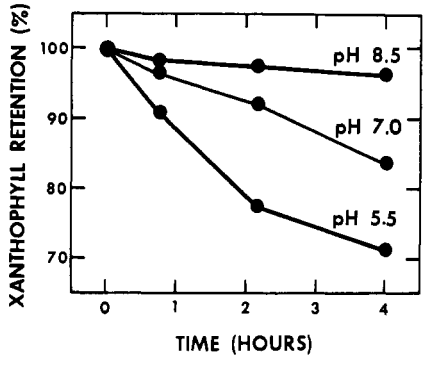

Figure 2. Xanthophyll retention during storage of freshly expressed alfalfa juice at 50°. Initial level = 395 mg/lb (dry basis)

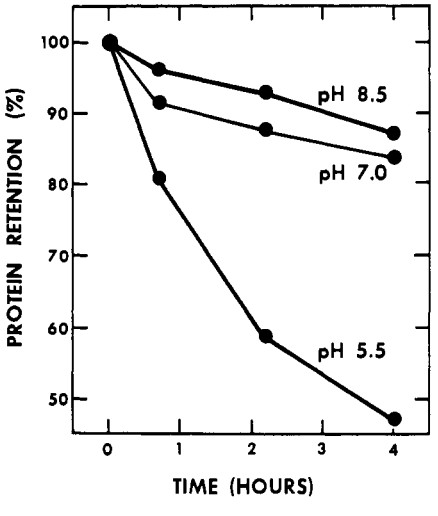

Figure 3. Protein retention during storage of freshly expressed alfalfa juice at 50°

juice are directly reversed from their stabilities in dry alfalfa products. Witt *et al.* (1971, 1972) have shown that xanthophyll is more stable than carotene in PRO-XAN. Livingston *et al.* (1955) and Knowles *et al.* (1968) have reported similar data on carotenoid stability during storage of dehydrated alfalfa meal. Heat inactivation of enzymes during preparation and/or drying of these products, in addition to their low levels of moisture, has essentially eliminated enzymatic activity. Although our data show that xanthophyll is oxidized enzymatically more rapidly than carotene, the relative rates of nonenzymatic oxidation of these carotenoids are apparently reversed. Since nonenzymatic oxidation is many times slower than enzymatic oxidation, the half-life of carotenoids in these dried products is measured in weeks or months instead of hours.

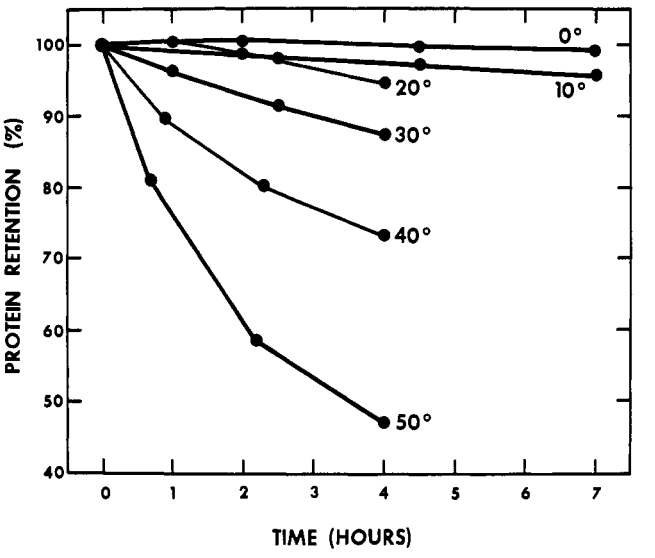

Figure 4. Protein retention during storage of freshly expressed alfalfa juice at pH 5.5

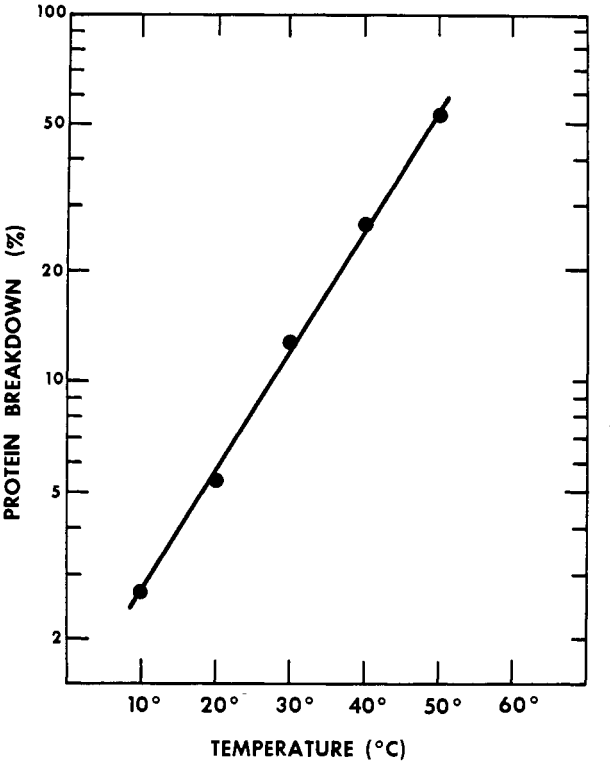

Figure 5. Protein breakdown during storage of freshly expressed alfalfa juice for 4 hr at pH 5.5

Protein Stability. Proteins are the least stable of the three constituents that were studied. Singh reported that "on a hot day, up to 40% of proteins in an extract from young wheat leaves could be lost in 2 hr," (Singh, 1962). We have found that more than half of the initial protein is hydrolyzed at 50° after 4 hr at pH 5.5 (Figure 3). As with the carotenoids, stability is increased at higher pH levels.

When the temperature is lowered, the rate of hydrolysis progressively decreases. This is most evident at pH 5.5 (Figure 4). There is essentially a halving in rate of breakdown with each 10° decrease in temperature. Thus, if the percentage breakdown of protein (4 hr at pH 5.5) is plotted log-

Table I. Protein Breakdown during 3 Hr

Temp, °C	pH 5.5, %	pH 7.0, %	pH 8.5, %
0	<1	<1	<1
10	2	2	2
20	3	4	3
30	10	6	5
40	23	6	5
50	47	14	10

arithmically against the incubation temperature, a straight line is obtained (Figure 5).

Of interest to alfalfa processors is the proteolysis that can be expected during 3 hr when juice is held under various pH-temperature combinations (Table I). If juice can be held at 20° or below, maintenance of an alkaline pH becomes unnecessary. If, on the other hand, temperatures rise to 30° or above (as can happen during summertime operation), pH maintenance becomes necessary to prevent an appreciable loss of protein. This same argument applies to losses of the carotenoid pigments, although with less urgency due to their greater stability.

Our method of assay measures protein nitrogen as that nitrogen which is insoluble in 10% trichloroacetic acid. Since this method of determining protein might not correlate with the amount of protein determined by heat coagulation, we compared the hydrolytic protein loss by these two different techniques in a sample of alfalfa juice that had undergone extensive proteolysis. The protein breakdown measured by these two methods was comparable—42% breakdown as measured by acid precipitation and 35% by heat coagulation. Thus, we feel that the laboratory results reported in this paper are applicable to a commercial process using heat coagulation to precipitate the proteins.

Some Factors Affecting Proteolysis. Since the proteins demonstrate such a high degree of instability, the effects of temperature and pH were investigated more fully. The effect of incubation temperatures greater than 50° at pH 5.5 is shown in Figure 6. The proteolytic activity that is present in juice samples held for 6 hr at 50° is arbitrarily assigned a value of 1.0. At 70°, this activity is rapidly lost. At 60°, the activity is appreciable, and it is even greater than the activity at 50° during the initial phase. These results are consistent with the results of Brady (1961), who also observed a high degree of proteolytic activity at 60° in juice from white clover. Tracey (1948), however, noted rapid inactivation of proteolysis at 60° in the juice from tobacco leaves.

In samples of alfalfa juice incubated at 50° for 1 hr at different pH levels, the maximum rate of proteolysis occurs at pH 5.5 (Figure 7). The proteolytic activity that is present in juice samples held at this pH is arbitrarily assigned a value of 1.0. The pH optimum determined in this study agrees with the observations of Tracey (1948), Brady (1961), and Singh (1962), all of whom report pH optima in the range of pH 5–6 in several plant species. The decrease in activity in the alkaline range emphasizes the importance of proper pH adjustment to minimize loss of proteins and carotenoids (Figures 1–3).

Other workers have observed that antimicrobial agents do not inhibit proteolysis, thus establishing the fact that endogenous plant enzymes are effecting protein breakdown (Brady, 1961; Macpherson, 1952; Singh, 1962; Tracey, 1948). Our experiments agree with this previous work in that the extent of proteolysis is essentially the same in the presence or absence of either toluene or 0.005 *M* sodium azide.

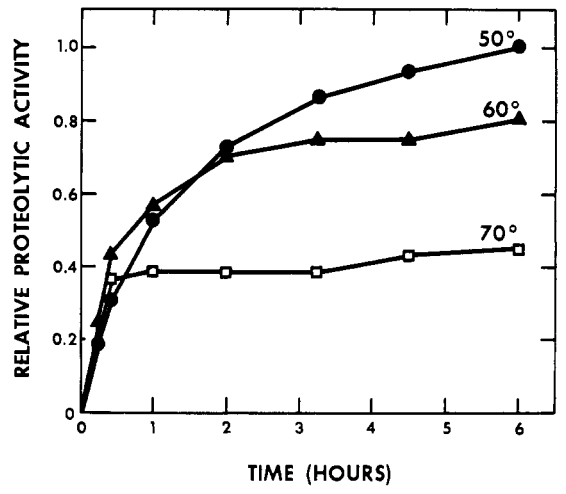

Figure 6. Relative proteolytic activity during storage of freshly expressed alfalfa juice at pH 5.5

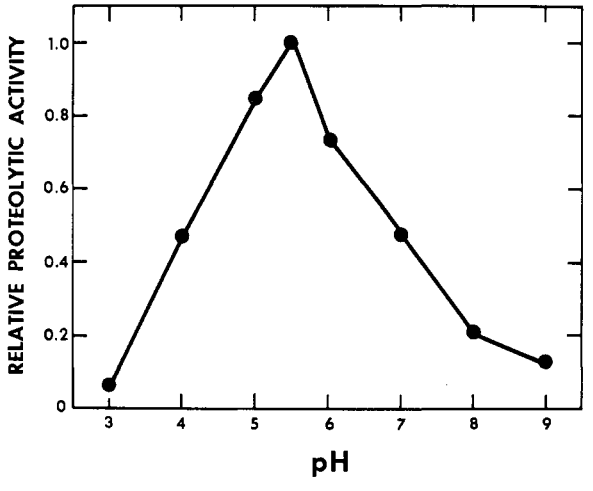

Figure 7. Relative proteolytic activity during storage of freshly expressed alfalfa juice for 1 hr at 50°

The experiments reported in this paper have measured the relative stability of protein, xanthophyll, and carotene in freshly expressed alfalfa juice. Although a large amount of protein, the least stable component, can be hydrolyzed during storage of juice, proper control of temperature and pH should hold this hydrolysis to a very low level during the time necessary for collection, transport, and processing of the juice. Provided that there were enough interested small alfalfa processors in a relatively restricted geographic area, it appears that the type of operation envisioned in the introduction to this paper is entirely feasible.

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Effect of High-Pressure Pulsation of Some of the Physical–Chemical Properties of Ovalbumin

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The effect of pulsating pressure (2500–5000 atm) on some of the physical–chemical properties of ovalbumin was examined. Conditions reducing electrostatic repulsion promoted extensive aggregation of the pressure-treated protein. Ovalbumin subjected to pressure treatment at pH 4 or 5 in salt-free solution exhibited a rapid equilibrium between a monomer and *n*-mer. Electrophoretic studies and phosphorus analysis revealed a pressure-induced hydrolysis of a phosphate ester at pH $\leq$ 4.0. Chemical analysis did not show any change in the accessibility of sulfhydryl groups resulting from treatment at any pH. However, small conformational changes resulting from pressure treatment were reflected by changes in the reactivity of tyrosyl residues with *N*-acetylimidazole and the accessibility of tyrosyl and tryptophanyl residues to various perturbants.

Numerous studies have shown that proteins are denatured by high-pressure treatments above 1000 atm (Brandts *et al.*, 1970; Bridgeman, 1914; Hawley, 1971; Ikkai and Ooi, 1966; Miyagawa, 1965; Suzuki, 1958, 1963; Suzuki *et al.*, 1962). However, the effect of rapid pulsating high pressures, *i.e.*, high intensity shock waves, on protein structure has not been investigated. The present investigation was undertaken to examine the changes in a protein's structure resulting from electrohydraulic (EH) shock waves. Ovalbumin, which is known to be affected by hydrostatic pressures, was selected for this study.

EXPERIMENTAL PROCEDURES

Materials. Crystalline ovalbumin (Grade III) obtained from Sigma Chemical Co. was used without further purification. For studies of unbuffered solutions, the protein was exhaustively dialyzed against deionized–distilled water followed by adjustment of the pH to the desired value with 0.1 *N* NaOH or 0.1 *N* HCl. Both the untreated (native) and the EH-treated ovalbumin solutions were centrifuged at 13,500 $\times$ *g* and 2° for 15 min; the supernatants were filtered through sterile 0.45-μ Millipore filters and stored in sterile screw-capped vials at 3°.

The A_1 component of ovalbumin was prepared from fresh chicken egg white by the procedure of Rhodes *et al.* (1958) using CM–cellulose chromatography. Analysis showed that the preparation contained 0.13% phosphorus and 5.1 SH groups per 45,000 g. Disk electrophoresis revealed two bands, with the faster migrating component corresponding to ovalbumin-A_1 and representing 90% of the total material.

Electrohydraulic Equipment. The electrohydraulic apparatus was supplied by General Electric Co., Chemical Systems Branch of Research and Development Center, Schenectady, N. Y. Alternating current at line voltage (220 V) was transformed and rectified to a high voltage direct current which was used to charge the capacitors. The capacitor bank consisted of four 6-microfared (μF) capacitors connected in parallel to yield a total of 24 μF. A charging voltage of 13 kV was used throughout this study. The charged capacitors were made to discharge rapidly by means of a secondary switching circuit and an ignition, which is essentially a quick electronic switch. The high-intensity electric field developed across the electrode gap causes ionization of the water molecules and the formation of a gaseous plasma. Since the inertia of the surrounding water exerts an almost rigid opposition to the expansion of the plasma, intense high-pressure shock waves are generated. Martin (1960) has estimated the shock wave pressures to be in the range of 2500–5000 atm and the pressures rise and fall within microseconds. The rate of discharge was held at one discharge per second and a total of 200 discharges was delivered for each treatment in this study. Using the relationship $E = {}^1/_2\, CV^2$, where E is the energy in joules, C the capacitance in μF, and V the voltage in kV, an energy output of 2028 J per discharge or a total of 405,600 J per treatment was calculated.

The electrode consisted of a stainless steel inner core separated from an aluminum outer tube by epoxy-glass insulation

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