cannot be concluded from the manometric measurements alone (*5*). This study shows that the hydrogen peroxide–induced oxidation of ascorbic acid practically ceases after the added peroxide has been decomposed by this activity in fresh juice. Consequently, this induced oxidation is of more concern in heat-treated passion fruit juice than in fresh or frozen juice. The magnitude of this "protective" action was shown by an experiment in which 100 ml. each of boiled and unboiled juice containing 1m*M* hydrogen peroxide was aerated by sintered glass aerators for 10 minutes. Forty-seven per cent of the initial ascorbic acid content remained in the boiled juice and 78% in the unboiled juice.

The iron content of fresh passion fruit juice (*12*) is in the range (2 to 5 p.p.m.) found for most common fruit juices (*39*). As iron from salts in this concentration range is much more active (Table III) than passion fruit juice in promoting the hydrogen peroxide–induced oxidation of ascorbic acid, an inhibitor of the reaction is suspected to be present in the juice. Another indication of this possibility is the rapid leveling off of initial rates for the greater juice concentrations (Figure 3) in a semilogarithmic relationship (Figure 5). For the iron salt solutions, the increase of initial rates with increasing metal ion concentration (Table III) is steep and linear over the range of rates observed for juice dilutions from $^1/_{48}$ to $^1/_2$. Exploratory experiments do show a pronounced inhibitory action of passion fruit juice on the initial aerobic rate of oxidation of ascorbic acid in solutions containing ferrous ion and hydrogen peroxide.

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PROTEIN FRACTIONATION

Fractionation of Commercial Papain by Ion Exchange

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THE PRACTICALITY OF FRACTIONATION of relatively low molecular weight and basic proteins by ion exchange chromatography is well established. Such functionally diverse proteins as cytochrome c (*9*), ribonuclease (*4, 5*), lysozyme (*5, 11*) egg white fraction (*10*), and calf thymus histone (*2*) have been fractionated.

Commercial papain has been fractionated by ion exchange with a carboxylic acid resin into substantially purified papain and chymopapain fractions; the process is suitable for adaptation to large quantities of material. The enzyme proteins were exchanged from unbuffered aqueous solution and were eluted with different concentrations of sodium chloride as reasonable pH control was achieved by pretitration of the resin.

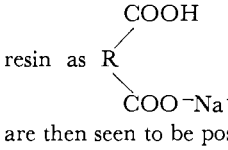
As papain, too, is a basic protein of relatively low molecular weight, the fractionation of commercial papain by ion exchange chromatography seemed feasible. This was attempted to achieve a purification of the proteases found in commercial papain rather than an analytical protein fractionation. Results from this laboratory indicate that substantially purified papain and chymopapain fractions can be obtained from commercial papain by ion exchange chromatography with a carboxylic acid resin.

Preliminary experiments indicated that the papain fraction was not retarded on passage through columns of resin pretitrated to pH values in the range of 5.5 to 7.0, if exchange was attempted from salt solutions. This lack of exchange occurred with sodium chloride solutions as dilute as 0.01*M*—lower concentrations were not investigated. When exchange was attempted from aqueous solution, the papain fraction, as well as the chymopapain fraction, was retained by the column, and inert protein could be removed by a water wash. The author's procedure, accordingly, involved exchange of enzymatically active protein from aqueous solution followed by a water wash to remove inert protein and then elution of a papain fraction and a chymopapain fraction with sodium chloride.

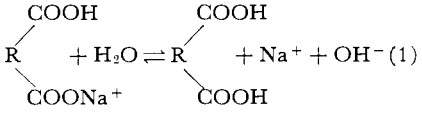
Experimental

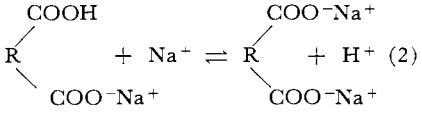
Conditioning of Resin. The resin employed in this work was Amberlite IRC-50, a weak carboxylic acid resin, mesh size 20–50. The resin as obtained is in the H form and was treated as follows.

One hundred and twenty grams of dry resin in a 3-liter beaker were stirred with 500 ml. of 2*N* hydrochloric acid for 4 hours. The resin was then washed with sufficient distilled water to remove adhering acid. The washed resin was stirred and titrated with 4*N* sodium hydroxide in the presence of 1 liter of 1.72*M* sodium chloride to the desired pH. As the resin is slow to equilibrate, an extended period of time (24 hours) is required for the titration. The acid treatment and titration steps were then repeated; the titration was carried out to a pH of 7.0. A resin titrated in this manner to a pH of 7.0 exhibited the following column behavior. On passage of water through a 1 × 30 cm. glass chromatographic column containing 5 grams of wet resin at a rate of 0.3 ml. per minute, the effluent pH increased to 8.7. On passage of 1% sodium chloride solution, the effluent pH remained substantially constant, initially being 6.9 and then dropping to 6.6. On passage of 10% sodium chloride solution, the effluent pH was initially 6.3 and then dropped to 6.0. With intermediate salt concentrations, the effluent pH assumed intermediate pH ranges. Presumably, this behavior of the resin can be explained on the basis that there are residual free carboxyl groups on the resin. Formulating the



are then seen to be possible,





Reaction 1 constitutes a hydrolysis and Reaction 2 an exchange, and the two reactions, in conjunction, seem to provide the degree of pH control observed.

Chromatographic Experiments. Purification Procedure. In a typical run, 15 grams of wet resin (pretitrated to pH 7.0) was packed into a 1 × 30 cm. glass chromatographic column equipped with a stopcock to control the effluent flow. Packing was accomplished by suspending the moist resin in 200 ml. of water, pouring the suspension into the column, and allowing the water to run out at a rapid rate. By repeated addition and draining of water (the liquid level always being above the resin level), the moist resin compacted to an even column, free of channels. After forming the column in this fashion, the liquid level was allowed to drain to the top of the resin column, and the stopcock was closed preparatory to carrying out the protein exchange.

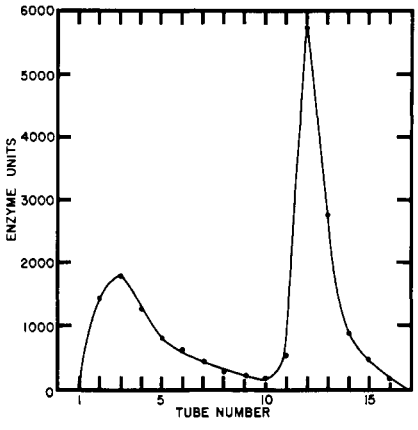


Figure 1. Typical purification procedure of commercial papain on Amberlite IRC-50

Ninety-five milliliters of a filtered 1% solution (pH 5.0) of a commercial papain preparation was passed through the column at a flow rate of 0.3 ml. per minute. All of the enzyme was retained by the column—the effluent pH not rising above 7.5. Inert protein

Table I. Purification Run[a]

	NaCl, 1%										NaCl, 10%					
Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
pH	8.5	7.5	7.1	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.9	6.8	6.3	6.2	6.3	6.2
Units[b]	0	1420	1780	1240	830	650	480	270	230	195	530	5700	2750	935	480	160
Units/mg. N	0	495	620	460	490	420	410	...	...	...	171	470	430	346	250	...

[a] 17,000 enzyme units, 215 units per mg. of nitrogen were exchanged.
[b] 6000 units recovered with 1% sodium chloride; 11,000 units recovered with 10% sodium chloride.

was then washed off the column with 10 ml. of distilled water. A more extensive water wash did not affect the results. After the water wash, elution was started with 1% sodium chloride and then followed with 10%. Five-milliliter fractions were automatically collected at a flow rate of 0.3 ml. per minute and each fraction was analyzed for enzyme and nitrogen content. Results of this procedure are shown in Table I and Figure 1.

Analytical Procedures. Enzyme assays were performed on suitable aliquots of chromatographic fractions by a modified milk clotting procedure (*3*) and clotting times in seconds, t, were converted to enzyme units, E, by the relationship $E = (1000/t - 10) \times$ (total vol./aliquot vol.). The inhibitory effect of sodium chloride on enzyme activity was corrected for by dividing enzyme units by the correction factors 0.85 for 1% sodium chloride solutions and 0.75 for 10% sodium chloride solutions (*3*).

Nitrogen analyses were carried out by the Kjeldahl procedure.

Protein contents were measured spectrometrically at 284 mμ; 1-ml. aliquots of chromatographic fractions were diluted with suitable quantities of 0.13*M* sodium hydroxide. Under these conditions Beer's law is obeyed by filtered solutions of the various papain preparations used in this study.

pH determinations were carried out with the Beckman Model G pH meter.

Results

Figure 1 and Table I present the results of a typical purification procedure; 17,100 enzyme units are placed on the column and 17,000 units are eluted—6000 units by 1% sodium chloride and 11,000 units by 10% sodium chloride.

The enzyme activity in the 1% sodium chloride eluates was rapidly lost at low pH but could be precipitated from solution at pH 8.6 by sodium chloride. These properties correspond to the papain fraction of papaya latex (*1*) and accordingly elution with 1% sodium chloride gives a papain-containing eluate. From the 10% sodium chloride eluate, enzyme protein could be precipitated at pH 2.5 by saturation with sodium chloride. These properties correspond to the chymopapain-containing, high salt fraction of papaya latex (*7*) and, accordingly, elution with 10% sodium chloride yields the chymopapain-containing fraction.

Commercial papain products are prepared directly from dried papaya latex (*6*), and fractionation should yield, at least, a papain-containing and a chymopapain-containing fraction.

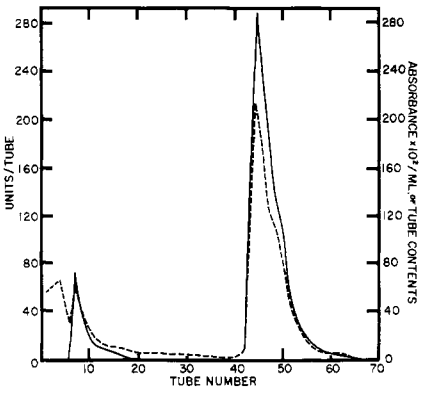


Figure 2. Fractionation of commercial papain on Amberlite IRC-50

———Enzyme activity
– – – – –Protein concentration

An idea of the degree of purification achieved by the ion exchange fractionation can be obtained from the data of Table I. The original enzyme preparation contained 215 units per mg. of nitrogen [both papain and chymopapain show the same milk clotting activity (*7*)], and tubes containing papain in quantity showed a two- to threefold increase in units per milligram of nitrogen. Tubes containing chymopapain in quantity showed up to a twofold increase in units per milligram of nitrogen. These enrichment factors are in line with those previously reported for the crystallization of papain (*1*) and chymopapain. Of course, the inconstancy of the units per milligram of nitrogen ratios for both papain- and chymopapain-containing fractions indicates the inhomogeneity of both fractions. This was corroborated by electrophoretic runs on the combined middle tubes of each fraction. The electrophoretic results indicate two and possibly a third component in the papain fraction and a major and minor component in the chymopapain fraction.

To study this question further, several attempts at analytical fractionation were carried out as described. Figure 2 presents the results of a typical experiment in which 50 grams of wet resin (pretitrated to pH 7.0) was packed in a 2 × 30 cm. glass column; 2700 units of enzyme activity were added to the column by passage of 15 ml. of 1%, filtered, enzyme solution. After a 5-ml. water wash, elution with 1% sodium chloride was started prior to collection of tube 1 and with 10% sodium chloride solution prior to tube 37. Three-milliliter fractions were collected.

The 1% sodium chloride fraction shows the presence of two distinct proteins and possibly, if tailing is not responsible, a third protein. Enzyme activity is associated with one of the two distinct proteins and with the possible third protein. The 10% sodium chloride elution pattern shows the presence of two only slightly resolved proteins both showing enzyme activity. The results of these fractionations bear out the above electrophoretic data. Electrophoretic data (*8*) on a commercial papain preparation similar to the one studied in this work indicate the presence of five protein components.

While fractionation of papain and chymopapain has been attained by the above procedures, the fact that application of the appropriate salt solution to the column results in emergence of protein at the holdup volume of the column seems to indicate that displacement of protein rather than exchange is occurring. Better chromatographic resolution could possibly be obtained by the use of slower flow rates and a smaller particle size resin. These points were not investigated.

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