Enzymic Hydrolysis of Naringin in Grapefruit

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Naringin, a bitter principle of grapefruit and shaddock, was found to be hydrolyzable by an enzyme occurring in a commercial pectinase. The hydrolytic activity of the enzyme was most effective at 50° C. and pH 4. Varying the citrate ion concentrations had no effect on the enzyme activity, but the hydrolytic end products decreased the rate of hydrolysis. The hydrolysis of naringin from the various parts of grapefruit was studied. Applications of the enzymic hydrolysis for "debittering" various grapefruit products were suggested as well as the use of this enzyme in connection with a more specific colorimetric determination of naringin in grapefruit products with sodium hydroxide.

NARINGIN, the 7-rhamnoglucoside of the flavanone naringenin, found in some varieties of citrus—mainly grapefruit (*Citrus paradisi*) and shaddock (*Citrus grandis*)—is the characteristic bitter principle of these fruits (*1, 7*).

Acid hydrolysis of naringin, one of the methods for the preparation of rhamnose (*9*), yields rhamnose, glucose, and the aglycon, naringenin. Rabate (*10*) was unsuccessful in attempting the hydrolysis of naringin with rhamnodiastase, a crude enzyme preparation from the seeds of *Rhamnus* species that will remove rutinose from its glycoside. Hall (*4*) reported a glycosidase type of enzyme, extracted from celery seeds using Tauber's technique (*12*), which will hydrolyze naringin in vitro at pH 7 and 37° C. to naringenin and a new disaccharide. He also found the occurrence of this enzyme in the leaves of the tree, *Citrus grandis*, Osbeck and in smaller amounts in regions between the flavedo and albedo of the fruit.

Attempts by the author to extract this enzyme from the leaves of shaddock trees using Tauber's technique did not prove successful. The activity of the enzyme extracted was very small when determined under the conditions described by Hall (*4*).

In treating grapefruit juice with Pectinol (Rohm & Haas Co.) to facilitate filtering, the total glycoside content of the juice was greatly reduced as determined by the Davis alkaline-diethylene glycol procedure (*2*). Naringin in aqueous solution was also completely hydrolyzed by Pectinol 100-D yielding glucose, rhamnose, and naringenin as detected by filter paper chromatography. Pectinol, therefore, contains an enzyme or enzymes capable of hydrolyzing naringin.

Experimental

Enzyme Preparation. The enzyme preparations used in the experiments were Pectinol 10-M and Pectinol 100-D. Dextrose is the diluent in 10-M and filter aid (diatomaceous earth) in 100-D.

Preparation of Standard Substrate. Commercial naringin (Eastman Kodak P. 3699) was recrystallized from water and dried at 60° C. for 24 hours (melting point 82° C.). A 0.05% solution of naringin was prepared by dissolving 0.500 gram of the recrystallized naringin in approximately 850 ml. of water at about 60° C. Fifteen grams of citric acid monohydrate was added lowering the pH to about 2. It was cooled, adjusted to pH 4 with the addition of 4*N* sodium hydroxide, and diluted to 1 liter. This solution contained 50 mg. of naringin per 100 ml. and was used as the standard substrate solution. When kept in cold storage, there was a tendency for the naringin to crystallize out of solution. It could be redissolved by slightly warming the solution in a water bath.

Effect of Temperature on Rate of Hydrolysis. The temperatures used in this experiment were 0°, 10°, 20°, 30°, 40°, 50°, 60°, and 70° C. Duplicate 25-ml. aliquots of the standard substrate solution were treated with 0.375 gram of Pectinol 10-M and placed in constant temperature baths. Naringin content was determined at hourly intervals by the Davis method. The results are shown in Figure 1. The rate of hydrolysis was highest at 60° C. At temperatures between 50° and 60° C., nearly complete hydrolysis was obtained in 5 hours. At 70° C., the activity of enzyme was definitely inhibited.

Effect of Hydrogen Ion Concentration. In studying the effect of hydrogen ion concentration on the hydrolysis of naringin by Pectinols, a series of 0.05% naringin solutions in citrate buffer adjusted to different pH was prepared. An amount of Pectinol 10-M, equivalent to 1.5%, was added to duplicate samples at each pH level. The mixtures were then immersed in a water bath maintained at 50° C. and the naringin content of each sample was determined (*2*) after 2 hours and again after 4 hours. The results as calculated in per cent hydrolysis are shown in Figure 2. The enzymic activity seemed to be the greatest at pH 4. However, within the range of pH 3.5 to 4.5, the differences were negligible.

Effect of Enzyme Concentration. In this study varying amounts of Pectinol 10-M were added to standard substrate solutions prepared according to the method described previously. The concentrations of enzymes varied from 0.2 to 1.4% in 0.2% increments. Duplicate samples at each enzyme concentration were incubated at 50° C. and naringin determinations made at hourly intervals. The results plotted with the enzyme concentrations as abscissa and the per cent hydrolysis as ordinate are shown in Figure 3. At low enzyme concentrations, the rate of hydrolysis was proportional to time. At higher enzyme concentrations, there was a decrease in the rate with time.

Influence of Concentration of Substrate on Rate of Hydrolysis. A series of substrate solutions containing 0.01, 0.02, 0.04, 0.05, 0.06, 0.08, and 0.10 gram of naringin per 100 ml. was prepared with 1.5% citric acid solution adjusted to pH 4 with sodium hydroxide. Duplicate samples of each concentration were incubated at 50° C. with 1.5%

Pectinol 10-M. The naringin content was determined in each sample at hourly intervals and the per cent hydrolysis was calculated. The results are shown in Figure 4. Up to a concentration of 0.10%, more than 94% of the original substrate was hydrolyzed in 5 hours under this condition.

Effect of Citrate Ions. Naringin solutions (0.05%) with varying amounts of citric acid monohydrate were adjusted to pH 4 with sodium hydroxide. Duplicate samples of each citrate concentration were treated with 1.5% Pectinol 10-M and incubated at 50° C. Citrate concentrations varying from 0.025 to 0.100 *M* seemed to have little effect on the hydrolytic activity of Pectinol on naringin. There was a lower hydrolytic activity in the control which contained no citrate buffer. This decrease was due to a change of pH of the sample after the addition of enzyme preparation as this solution had little buffer action.

Effect of End Products. The hydrolytic products of naringin as described previously are rhamnose, glucose, and naringenin. Among these substances, glucose is a naturally occurring sugar in grapefruit juice (*1*). Little, if any, of the other two compounds have been found in grapefruit juice. In the study of the effect of glucose, Pectinol 100-D was used instead of Pectinol 10-M because the latter has glucose as the carrier. To a series of standard substrate solutions, crystalline glucose was added to make concentrations of 0, 1.5, 3.0, and 5.0%. Duplicate samples of each glucose concentration were treated with 1.5% Pectinol 100-D and incubated at 50° C. for 2 hours. In the case of rhamnose and naringenin, 0.02% of each substance was added to the standard substrate solutions in duplicate, and the mixtures were treated with 1.5% Pectinol 10-M. The results on the effect of glucose in various concentrations and of rhamnose and naringenin on the activity of the enzyme are shown in Table I. The high concentration of glucose slightly inhibited the activity of this enzyme. The aglycon, naringenin, also had a slight inhibitory effect even at a very low concentration. However, the effect due to rhamnose at the concentration tested was not significant.

Heat Inactivation of Enzyme Hydrolyzing Naringin. A series of solutions containing 3% Pectinol 10-M was prepared and heated at 70°, 80°, and 90° C. for various time intervals. After heating, equal volumes of substrate solutions containing 100 mg. of naringin per 100 ml. were added so that the resultant solution contained 50 mg. naringin per 100 ml. of citrate buffer at pH 4. The mixtures were then incubated at 50° C. in triplicate for 2 hours. At the end of the incubation period, the naringin content of the samples was determined and

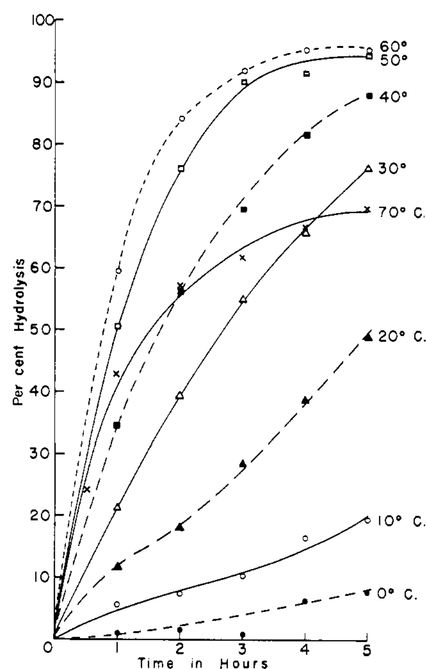


Figure 1. Effect of temperature on the rate of hydrolysis on naringin

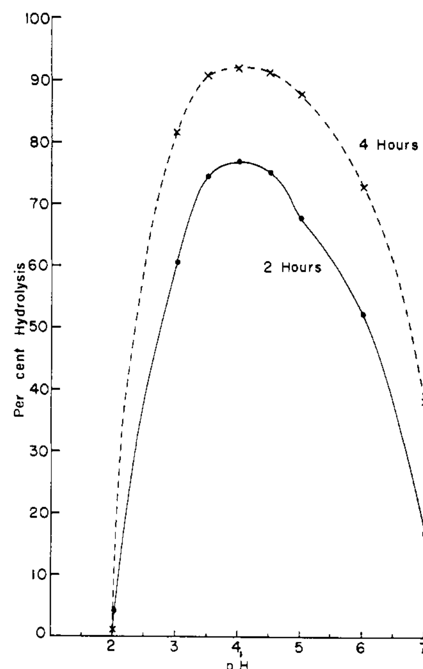


Figure 2. Effect of pH on the hydrolysis of naringin

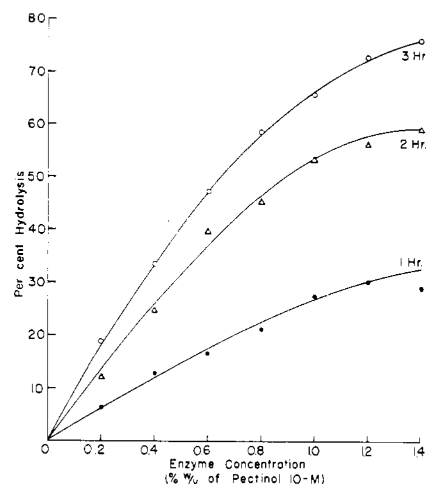


Figure 3. Effect of enzyme concentration on the hydrolysis of naringin

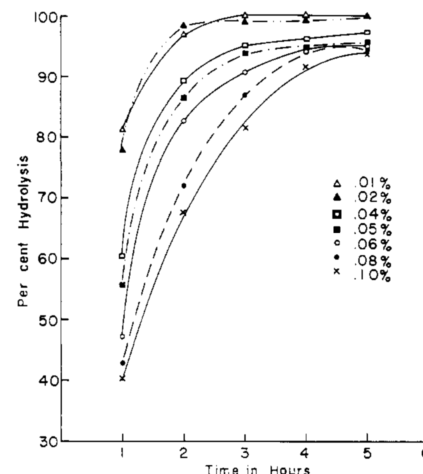


Figure 4. Effect of concentration of substrate on the rate of hydrolysis of naringin

Table I. Effect of Hydrolytic End Product on the Activity of Pectinol on Naringin at 50° C. for 2 Hours

Type and Concentration of End Product	Hydrolysis in 2 Hours, %	
Glucose		
0%[a]	79.5	78.8
1.5%[a]	72.6	73.1
3.0%[a]	66.8	65.4
5.0%[a]	62.2	61.1
Naringenin 0.02%[b]	68.0	68.9
Rhamnose 0.02%[b]	74.5	72.8
Check (Pectinol 10-M)	75.8	76.4

[a] Pectinol 100-D used.
[b] Pectinol 10-M used.

Table II. Heat Inactivation of Enzymes Hydrolyzing Naringin

Temperature, ° C.	Time, Min.	Replicates 1	2	3
		% Inactivation		
70	10	8.0	6.4	7.2
	20	23.5	25.3	24.4
	30	33.5	37.0	35.2
80	1	95.8	91.8	93.8
	3	100.0	100.0	100.0
	5	100.0	100.0	100.0
90	0.25	100.0	100.0	100.0
	0.5	100.0	100.0	100.0
	1.0	100.0	100.0	100.0

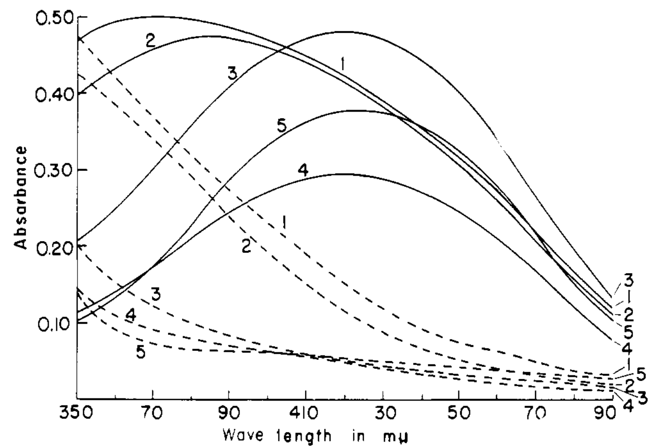
the per cent inactivation calculated. The results are shown in Table II. Temperatures above 80° C. seriously inactivated this enzyme.

The Glycoside in Grapefruit. Naringin has been the only glycoside identified in grapefruit. In most reports the presence of naringin has been shown only in the peel and rag where the concentration of this glycoside is high. Its presence in the juice was inferred from the characteristic bitter taste of the juice and was identified only by the relatively new chromatographic technique. The action of alkali on naringin produces a yellow color which is the basis of a method for determining flavanone glycoside (*2*). The yellow color thus produced by naringin when dispersed in diethylene glycol has a maximum absorption peak at 420 mμ.

The possibility of substances in grapefruit juice—other than naringin—forming yellow color with alkaline diethylene glycol was shown by spectral studies of this color, which were made on a Beckman Model B spectrophotometer. The absorption maxima varied from 380 to 410 mμ with the color produced by the juices. Extracts of peel and rag, when tested with alkaline diethylene glycol gave colors which have absorption maxima close to 420 mμ.

Four Duncan grapefruit were carefully peeled so as to remove the albedo completely. The juice sacs were separated from the section membrane, the seeds were removed, and the contents were put in two layers of cheese cloth and squeezed to remove as much juice as possible without unnecessary maceration of the tissue. All components—the rind (including flavedo and albedo), the rag (section membrane), and juice sac membrane—were macerated in a Waring Blendor with various amounts of distilled water. The extracts and juice were filtered, after addition of filter aid, on a prepared filter pad and diluted to appropriate concentration for analysis within the range of the Davis alkaline diethylene glycol procedure. Spectral curves of these colors are shown in Figure 5. Peel and rag extracts both showed their maximum absorption peaks close to that of naringin; while the colors produced by the juice, and juice sac plus juice, had their maxima different from that of naringin. The shift in the absorption maxima indicates that, in the juice, this color is not produced by naringin and alkaline diethylene glycol only.

Hydrolysis of Naringin in Grapefruit. Twenty-five milliliters of the filtered juice or the filtered extracts of the peel, rag, and juice sac membrane used in the previous experiment were treated with 0.375 gram of Pectinol 10-M (equivalent to 1.5%). The hydrogen ion concentration of the solution was adjusted to pH 4 with 4*N* sodium hydroxide added



- - - - Original sample
— Hydrolyzed sample
1. Juice only
2. Juice with juice sacs
3. Rag extract
4. Peel extract
5. Naringin solution

Figure 5. Spectral curves of color produced by alkaline diethyleneglycol (original and hydrolyzed samples) on various components of grapefruit

dropwise; usually four to six drops were needed for juice and considerably less for the extracts. The mixture was then transferred to a 23 × 200 mm. tube, capped lightly with a cork stopper and placed in a water bath at 50° C. for 5 hours. The glycoside content was determined using the alkaline diethylene glycol method. The spectral curves of these samples, made on a Beckman Model B spectrophotometer, are presented in Figure 5 (hydrolyzed sample). These curves showed a rapid decrease in absorbance between 350 and 440 mμ and a gradual decrease up to 490 mμ with no prominent absorption peaks. At 420 mμ (used in the Davis method), the juice and juice sac membrane were higher in absorbance than the peel and rag extracts, which closely resembled authentic naringin solution. In addition, chromatographic studies indicated that naringin had been removed from these samples by hydrolysis.

Figure 6 shows spectral curves obtained on glycosides of grapefruit juice extracted with various pressures on a commercial extractor. In this instance, the total glycosides and the unhydrolyzed portion both increased with increasing extracting pressures. The absorption maxima of the total glycosides also had a tendency to vary depending on the extracting pressure. When the absorbance of the hydrolyzed sample at each measured wave length was subtracted from corresponding absorbance of the total glycosides at similar wave length, and when the differences were plotted, the resultant curves, for all three samples, showed maximum absorption peaks at about 420 mμ, which was closer to that of naringin.

Discussion

The presence of nonpectic enzymes in commercial pectic enzymes has been frequently reported in the literature. This is not surprising, as the source of the commercial enzymes is usually fungal in nature. Thus, Fish and Dust-

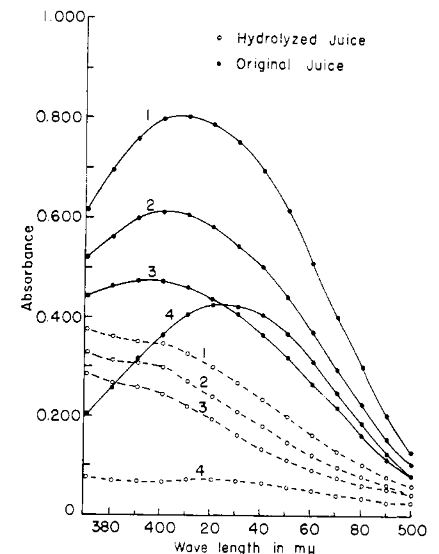


Figure 6. Spectral curve of color produced by alkaline diethyleneglycol on grapefruit juice extracted with different pressures on a commercial juice extractor

1. High pressure, approximately 70 p.s.i.g.
2. Medium pressure, approximately 50 p.s.i.g.
3. Low pressure, approximately 30 p.s.i.g.
4. 0.05% naringin solution

man (*3*) showed that Pectinol-A contained enzymes capable of hydrolyzing maltose, sucrose, starch, and inulin. The hydrolysis of modified cellulose (carboxymethylcellulose) by this enzyme was indicated by the work of Lineweaver, Jang, and Jansen (*8*). Pectinol was also known to contain enzymes which hydrolyze protein and xylan (*6*). According to Huang (*5*), enzyme preparations made from *Aspergillus* species hydrolyze certain anthocyanins. The enzyme or enzymes found in Pectinol which hydrolyze naringin may be similar to the "anthocyaninase" described by Huang, and it can safely be assumed to be a β-glycosidase, although emulsin was inactive on anthocyanins (*5*). Hall (*4*) also found that emulsin did not hydrolyze naringin, and this fact was also substantiated in the author's

laboratory. Not all commercial pectic enzymes contain this naringin hydrolytic activity. A sample of Takamine pectic enzyme was tried and was ineffective, but three sources of diastase—viz., Parke-Davis Takadiastase, Wallerstein Laboratory pharmaceutical Mylase, and Clarase diastic enzyme, produced by Takamine Laboratory—showed some degree of activity.

β-Glycosidase from sweet almonds was reported to have an optimum activity at pH 4.4 (*11*). The optimum action of the glycosidase reported in the present paper is about pH 4 with only small differences within the range of pH 3.5 to 4.5. This enzyme has very little action at pH 2. Hall (*4*) used the enzyme from celery seed at pH 7. He did not report the activity at any other pH and failed to ascertain the optimum hydrogen ion concentration for the enzyme. He also reported that the end products contained a disaccharide. Chromatographic studies in this laboratory showed only rhamnose and glucose separately. In general, the end products of an enzyme-catalyzed reaction exert a mass action effect which was shown by the experiment on the decreasing of enzymic activity with increasing concentrations of glucose. Although naringenin is insoluble in water, a 0.02% suspension had inhibitory action, while rhamnose at that concentration had little effect.

The enzymic activity was definitely reduced at 70° C. Complete inactivation of the enzyme was obtained in 3 minutes at 80° C. and in 15 seconds at 90° C. The time recorded in Table II did not take into consideration the time required for the samples to reach the desired temperature—for 80° C., about 1 minute and for 90° C., 30 seconds. As the experiments on heat inactivation were carried out at pH 4, varying the pH may change the critical temperatures.

The effect of citrate ion on the enzymic activity was studied as, in any practical applications, citrate will be the main buffering medium. The concentration of the citrate ions had no critical significance on the enzymic activity.

Enzyme Efficiency. The efficiency of the naringin-hydrolyzing enzyme in the commercial pectinase preparations was not uniform in every lot, and a more or less arbitrary unit of efficiency must be established in order to measure the activity of various preparations. The activity was measured by determining the velocity constant, k, for hydrolysis of a 0.05% naringin (melting point, 82° C.) by 1 gram of an enzyme preparation in 100 ml. of the reaction solution in the first 60 minutes.

$$k = \frac{2.303}{t_2 - t_1} \log \frac{C_1}{C_2}$$

where k is the velocity constant, t_1 and t_2 are time, and C_1 and C_2 are concentrations at time t_1 and t_2, respectively. The use of a time limit and a definite initial substrate concentration is necessary as k varies with these factors.

Possible Applications of Enzymic Hydrolysis of Naringin. The discovery of a commercially available enzyme capable of hydrolyzing naringin may have various applications. Although a slight bitterness is characteristic of all grapefruit products, excessive bitterness is objectionable. The application of this enzymic hydrolysis when used in appropriate conditions can reduce the naringin content of grapefruit products. Of special interest is bland sirup. During the concentration of the expressed "peel juice" the bitter principle, naringin, tends to concentrate and to render the products excessively bitter. By treating the liquor with Pectinol or with another source of this enzyme before or during concentration, it is possible to remove all the naringin.

The utilization of this process in the "debittering" of grapefruit juice is hindered by the association of this enzyme with large quantities of pectic enzymes which readily clarify the juice. The isolation of the naringin-hydrolyzing enzyme from the bulk of pectic enzymes is a prerequisite to the practical application of enzymic hydrolysis for the "debittering" of grapefruit juice. However, the loss of colloidal suspension of the juice may be remedied by the use of various commercially available protective colloids.

Also, as naringin can be quantitatively removed by this enzyme, the determination of total flavanone glycoside with the Davis procedure, before and after hydrolysis, shows the amount of naringin in the sample.

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VITAMIN A IN EGGS

Seasonal Variations in the Vitamin A Content of Hens' Eggs

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The vitamin A content of fresh and stored shell eggs was determined by means of techniques not previously used with eggs. A study of stored shell eggs indicated there might be seasonal changes in the vitamin A content of eggs from any one hen. Data from eggs from a group of hens over a 10-month period and the method used in the determinations are presented.

Determination of Vitamin A

Reagents. Skellysolve B. Petroleum naphtha with a boiling point of 60–71° C. obtained from Skelly Oil Co., Chicago, Ill.

Florisil. 60/100 mesh obtained from The Floridin Co., Tallahassee, Fla.

Activated glycerol dichlorohydrin. Preparation and recovery of used glycerol