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THE undersigned, acting as a committee of the Graduate School, have read the accompanying thesis submitted by Inez Everett
for the degree of Master of Science.
They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science.

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Enzymes in Apples

A thesis submitted to the Faculty of the
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by

Inez Everett

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Enzymes in Apples

- Introduction -

A study of the enzymes of apples affords an interesting field for investigation from a chemical standpoint. The changes in appearance, palatability and composition which apples undergo during the process of ripening are supposed to be due chiefly to enzyme action. It was with the idea of gaining further information concerning the kinds of enzyme activity in apples, that this systematic study was made.

In all of the investigations the apples used were secured from the state of Washington, and were of the following varieties: Yellow Newtown Pippin, Rome Beauty, Arkansas Black and King David, all of which are known to be good keeping apples. In one or two experiments when fresh pulp was desired, Jonathan apples were used. In all the determinations of enzyme activity, a constant temperature was secured by means of Freas' electric incubator.

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- Chemical Changes During Ripening of Apples -

Bulletin No. 94 on "Studies on Apples" by Bigelow, Gore and Howard, (1), explains the effect of various gases on the keeping qualities and palatability of fruits. Some experiments were carried on with apples stored in sealed vessels. The following facts were noted: large quantites of carbon dioxide were evolved during long periods of time; large quantities of alcohol were found, also. Yeast cells were discovered in the parenchyma cells of the sound apples. This latter observation is modified, however, by the statement that no alcohol or yeast occurred in apples whose skins were prefectly intact.

In experiments with growing apples it was determined that crude fiber, ash, protein, sugar, acid, water, pectin, and dextrin all increased during growth. In growing fruit the acid content gradually became less and the starch increased by degrees until the fruit began to ripen, then it decreased. Sucrose and invert

sugar increased steadily up to the last analysis. A portion of each sample was left in darkness and analyzed at intervals, with the final results that starch was changed into sucrose, sucrose was inverted and the invert sugar consumed in respiration.

The observations on apples in common storage showed a gradual diminution of all constituents except in two cases where acid and starch decreased but total sugar increased. When apples were allowed to sweat in piles, starch was converted into sugar in two or three weeks.

In a study of the respiratory quotient of apples, it was discovered that unripe apples breathe more rapidly at 30° and 33° than at 18° C.; apples relatively high in acid respire more rapidly and have a higher respiratory quotient than apples low in acid.

-Literature Concerning Enzymes in Apples -

In regard to the relation of enzymes to the changes occurring in the ripening of fruits, G. Warcollier has published two articles - "Invertase in Apple Must and

Cider", (2), and "Cause of the Presence of Abnormal Amounts of Starch in Bruised Apples",(3). In the first article, he states that in neutralizing apple must with sodium hydroxide and keeping it at 56° for one hour, no change occurs in the amount of sucrose or of reducing sugars. Therefore invertase does not occur in apple must. The preliminary inversion of sucrose which occurs in the change of apple must to cider, is due to the action of invertase secreted by the yeast cells, and that the ferment diffuses from the latter into the must where it is capable of existing for considerable periods. Invertase must occur naturally in apples, since sucrose is inverted during the ripening of the fruit. It was suggested that the ferment is destroyed during the pressing of the apples, perhaps as the result of the precipitation of tannin.

In the second article he explains the presence of abnormal amounts of starch in bruised apples by the fact that tannin coagulates the amylase and thereby prevents

the transformation of starch into fermentable sugars.

Lindet,(4), reported a soluble ferment in the juice of apples, which causes coloration of the tissues by absorption of oxygen and the evolution of carbon dioxide. It can be precipitated from the juice by alcohol and its activity is destroyed by boiling. He concluded that the coloration is due to oxidation of tannin by a soluble ferment of the kind designated by Bertrand as laccase (now called oxidase).

Aside from these references, no mention of work on enzymes in apples could be found in the literature available for consultation.

- Experimental Work -

- The Preparation of Material for Examination -

To obtain material in a suitable form for examination was the first problem to consider. It was necessary to secure an extract of the cell contents which would contain the enzyme in an active form. Since it was not known whether the enzymes are intracellular or extracellular, the first method that naturally suggested

itself was the rasping of the apples to shreds in the horse radish grater, to completely rupture the cells; after which the pulp was pressed in a hand press. The juice thus obtained was full of suspended particles so that an attempt was made to filter it. Filtration was impossible owing to the clogging of the filter, so this method was abandoned. The second method consisted in treating the apples in the above manner but decanting instead of trying to filter the juice, after the must stood until the apple pulp separated out, leaving a supernatant liquid comparatively free from pulp. Tests for some of the enzymes were made using this juice which was pipetted off. The third method was an attempt to completely dehydrate the apple pulp. The apples were cut in very thin slices, after removing the cores, and placed in a vacuum desiccator over concentrated sulphuric acid. After drying for several days an unsuccessful attempt was made to grind them to a powder; they were subjected to another similiar drying and ground in

a mortar. This was not successful, owing to the large amount of sugar and pectin bodies in the tissue. The slices were gummy and impossible to grind to a powder even after several weeks in the desiccator. The fourth method consisted in treating thin slices of apple by the acetone ether method, first used by Buchner,(5),. After this treatment and drying in air over night they could be readily ground to a powder in a mortar or in a mill. Yellow Newtown Pippin, Rome Beauty, Arkansas Black and King David varieties of apples were prepared in this manner and used in all subsequent tests for enzymes. Juice concentrated by Gore's freezing process, (6), was also used in several tests.

In the tables showing the experimental work, all references to "fresh apple juice" means the juice obtained by mixing fresh, pared and cored apples with an equal weight of fine quartz sand and gently grinding in a mortar until reduced to a uniform pulp, then straining this pulp through bolting silk. This gave a fairly

clear liquid and was used in all experiments where tests were made on fresh apple juice.

-Examination of the Different Preparations for Enzymes -

Diastases:- According to Thatcher and Koch,(7), diastases are readily diffusible in water. Therefore if any enzymes of this type were present in the apple flesh it may be assumed that they would appear in the juice extracted from the pulp, after complete rupturing of the cells. In testing for diastases the material was prepared by rasping the apples and permitting the pulp to settle out, then decanting the clear supernatant liquid. Three varieties of apples were used and four separate mixtures prepared for each variety of juice. The first contained 10 c.c. of active juice, 10 c.c. of soluble starch, prepared by the Lintner method,(8), and 12 c.c. of distilled water. The second contained the same amounts of soluble starch and water and 10 c.c. of juice that had been boiled for ten minutes and made up to its original volume with water. The third contained 10 c.c.

of active juice, and sufficient tenth normal sodium hydroxide to exactly neutralize the 10 c.c. of juice, (determined by a preliminary titration, using phenolphthalein as an indicator), and 10 c.c. of soluble starch and sufficient distilled water to make the volume 32 c.c. The fourth contained water and starch as a check on the reagents. The contents of the flasks were mixed and placed in the incubator at 40°C. for one hour. At the end of that time they were cooled and made up to 100 c.c. with a mixture of sulphuric acid, salt and phosphotungstic acid, which stopped the action and precipitated the proteins. An aliquot part was drawn off for determining its reducing sugar content according to the method suggested by Thatcher and Koch,(7). The results are shown in Table I.

At a later date when material was obtained by other methods another series of tests was made, following the same general operation as before. The results are shown in Table II.

Table I. Tests for Diastase in Flesh of Apples.

Material Used	Reducing Sugars before action	Reducing Sugars after action
Jonathan Apples:		
Decanted juice	0.0192	0.0183
Decanted Juice boiled	0.0207	0.0212
Decanted juice neutralized	0.0197	0.0192
Control (Water only)	none	none
Yellow Newtown Pippen		
Decanted juice	0.0113	0.0113
Decanted juice boiled	0.0217	0.0212
Decanted juice neutralized	0.0103	0.0113
Control	none	none
Rome Beauty		
Decanted Juice	0.0103	0.0098
Decanted Juice boiled	0.0207	0.0202
Dedanted juice neutralized	0.0113	0.0103
Control	none	none

Table II. Tests for Diastases in Various Preparations from Flesh of Apples.

Material Used	Reducing Sugars found after action	
	Active Extract	Boiled Extract
Water Extract of Acetone-dried pulp	0.0202	0.0207
Juice concentrated by Gore's process	0.0356	0.0351
Fresh Apple juice	0.0316	0.0316

From the results obtained it appears that there are no diastases in apple flesh.

Invertase:- In testing for invertase, the same method of preparation was used as in diastases except that 10 c.c. of a ten per cent solution of cane sugar was used instead of starch as the substrate material. After acting at 40° for twenty hours, aliquot portions were withdrawn and tested for reducing sugars using Fehling's solution. The results are given in Table III.

The above tests were also carried out using concentrated juice and fresh apple juice. The results are shown in Table IV.

From the foregoing results it appears there are no invertases in apple flesh. If the sucrose is inverted, during the ripening of the apple, it must be hydrolyzed by some means other than the enzyme invertase; or else the action must be completed before the stage of ripeness of the apples which were studied.

Tannase:- First a determination was made of the amount

Table III. Tests for Invertase in Flesh of Apples.

Material Used	Reducing Sugar before action	Reducing Sugar after action
Yellow Newtown Pippen		
Decanted juice	0.0113	0.0113
Decanted juice boiled	0.0212	0.0217
Decanted juice neutralized	0.0113	0.0103
Control	none	none

Table IV. Tests for Invertase in Various Preparations from Flesh of Apples.

Material Used	Reducing Sugar after action	
	Active Extract	Boiled Extract
Water Extract of acetone-dried pulp	0.0207	0.0207
Juice concentrated by Gore's process	0.0396	0.0396
Juice concentrated by Gore's process neutralized	0.0376	Lost
Fresh Apple Juice	0.0192	0.0187
Fresh Apple Juice neutralized	0.0192	0.0192

of tannin in the different varieties of apples, according to Proctor's modification of Lowenthal's Method,(9). The following results were obtained:

Yellow Newtown Pippin	0.208% of tannin
Rome Beauty	0.208% of tannin
Arkansas Black	0.192% of tannin
King David	0.132% of tannin

After proving the presence of tannin, tests were made for the tannin splitting enzyme, tannase. With each of the four varieties of apples the four following mixtures were made. First, 10 c.c. of active apple juice, 2 c.c. of a one per cent solution of tannin, 18 c.c. of water; second, 10 c.c. of boiled juice, 2 c.c. of tannin, 18 c.c. of water; third, 10 c.c. of active juice, 20 c.c. of water; fourth, 10 c.c. of boiled juice, 20 c.c. of water. These were incubated at 40°C for twenty four hours. At the end of this time each solution was tested with a few drops of ferric chloride and the colors developed in the boiled and unboiled

solutions compared. In no case could any difference in intensity of color be observed. This leads to the conclusion that tannase is not present in the flesh of apples.

Glucosidases:- In attempting to determine the presence of emulsase in apple pulp the following tests were made. First, 10 c.c. of active juice and 2 c.c. of one percent solution of amygdalin, a typical glucoside; second, 10 c.c. of boiled apple juice and 2 c.c. of one percent solution of amygdalin; third, 10 c.c. of neutralized juice and 2 c.c. of one percent solution of amygdalin; fourth, 10 c.c. of boiled juice and water. These mixtures were allowed to act at 40° C. for twenty-four hours. In no case could an oder of benzaldehyde be detected at the end of this time. Check tubes containing emulsin and amygdalin produced a strong oder of benzladehyde in a few minutes. The results lead to the conclusion that apples contain no glucoside splitting enzyme.

Esterases:- In testing for esterases the fresh apple

juice was used and the following series of mixtures prepared. The first contained 5 c.c. of active juice and 2 c.c. of ethyl malonate plus 5 c.c. of water; the second, 5 c.c. of active juice, 2 c.c. of ethyl malonate and 5 c.c. of tenth normal NaOH ; the third contained 5 c.c. of juice boiled for ten minutes, cooled, and made to its original volume, 2 c.c. of ethyl malonate, and 5 c.c. of water. The fourth, fifth, and sixth tubes were prepared as the first, second and third respectively, except that a one tenth percent solution of steapsin was used in each case instead of the apple juice. These mixtures were kept at 40° C. for twenty hours, after which aliquot parts were removed and titrated with n/100 NaOH using phenolphthalein as an indicator. The results are shown in Table V.

From the results shown in this table, the presence of an esterase is indicated. In alkaline medium the activity is greatly increased, and is almost identical with the activity produced by 0.1 % solution of steapsin.

Table V. Test for Esterases in Flesh from Apples.

Material Used	N/100 Alkali Required
Fresh Apple juice	9.2 cc
Fresh Apple juice boiled	7.5 cc
Fresh Apple juice with excess of N/10 alkali	39.8 cc
Steapsin solution	7.3 cc
Steapsin solution boiled	none
Steapsin solution with excess of N/10 alkali	40.9 cc

Oxidases:- A qualitative test for oxidases was made by using a solution of guiac directly on the freshly cut surface of an apple. Deep blue colorations appeared around the outer edges, near the skin, and around the core portion. The color was increased when hydrogen peroxide was added. These facts give evidence of the presence of oxidases and peroxidases.

Oxidases in apple flesh were also determined by the phenolphthalin method,(11). The material, which was the fresh apple juice, was titrated with n/20 NaOH, using phenolphthalein as an indicator, to find the exact amount of NaOH required to give a faint pink then the phenolphthalin was added. After standing several hours 2 c.c. samples were taken and one c.c. of n/20 NaOH added. The test depends upon the development of a pink color upon the addition of the alkali, showing that an oxidation of the phenolphthalin to phenolphthalein has taken place. Table VII shows the results.

The development of the pink color in the unboiled

Table VII. Tests for Oxidases in Flesh of Apples using Phenolphthalin Test.

Material Used	Results
Water Extract of acetone - dried pulp	+
Water Extract of acetone - dried pulp boiled	-
Reagents only	-
Fresh apple juice	+
Fresh apple juice boiled	-
Reagents only	-

extracts, and its failure to appear in the boiled extracts, prove the presence of an active oxidase in the apple juice.

Proteases:- In testing for proteases the following mixtures were made: first tube, 5 c.c. of fresh apple juice, 5 c.c. of egg albumin (saturated solution), and 10 c.c. of water; second, 5 c.c. of apple juice boiled, 5 c.c. of albumin, and 10 c.c. of water; third, 5 c.c. of pepsin, 5 c.c. of albumin, and 10 c.c. N/10 HCl; fourth, 5 c.c. of pepsin boiled, 5 c.c. of albumin, and 10 c.c. N/10 HCl; fifth, 15 c.c. of water and 5 c.c. of albumin. The above mixtures were duplicated, substituting peptone for albumin as the substrate. The tubes were kept in the incubator at 40° C. for twenty-four hours. Then aliquot parts of the solutions were pipetted off and tested by triketohydrinene-hydrate, a method recently proposed by Harding and McLean, (10). A one percent solution of glutamic acid, containing the equivalent of 0.1 mgm. of nitrogen in the amino acid form

Table VI. Tests for Proteases in Flesh of Apples.

Material Used	Results
5 cc apple juice	amino acid equivalent
Apple juice and albumin	.12 mgm of Nitrogen
Apple juice boiled and albumin	.07 mgm of Nitrogen
Water and albumin	none
Apple juice and peptone	.10 mgm of Nitrogen
Apple juice boiled and peptone	.10 mgm of Nitrogen
Water and peptone	.03 mgm of Nitrogen

per c.c., was used to serve as the standard for color. The following table, VI, shows the results obtained.

This table shows that a protease is present in apple flesh, since a greater amount of amino acid is developed in the active than in the boiled extract.

Pectinases:- No tests were made for pectinases in the flesh of apples, owing to the fact that, as yet, no satisfactory method for their determination has been discovered. Literature regarding pectinases in fruits is reviewed by Cooley,(12). It is probable that the softening of apples when they become thoroughly ripe is due to pectinase action, but, as has been stated, no method for determining accurately whether this enzyme is present in apple tissue could be found.

- Tests for Enzymes in Apple Seeds -

At the same time that tests were being made for enzymes in the flesh of apples, parallel tests were made for enzymes in seeds. The preparation of the seeds for examination was quite simple. They were taken from the

apples, their brown outer coat removed and then they were dried in a vacuum desiccator for two or three weeks. At the end of that time they could be quite readily ground up with fine quartz sand. The following tests were made on the water extract of seeds ground up with equal parts of sand. In each case two grams of the seed and sand mixture, which is equivalent to one gram of seeds, was digested with 100 c.c. of distilled water for at least thirty minutes at room temperature, and aliquot portions of the water extract used for the tests.

Diastases:- In testing for diastases in apple seeds, a water extract of the seed and sand mixture was made. Two grams of the mixture, which was equivalent to one gram of seeds was digested with 100 c.c. of distilled water. It was then filtered and aliquot parts pipetted off for investigation. The following combinations were made: first, 20 c.c. of active seed extract and 10 c.c. of ten percent solution of soluble starch; second, 20 c.c.

of boiled sedd extract and 10 c.c. of ten percent solution of soluble starch. After incubating at 40° C. for twenty hours the reducing sugars were estimated. The tube containing active extract dontained 0.00247 grams and the boiled extract contained 0.001247 grams, showing 0.001223 grams difference, which is probably due to enzyme action. Another set of tests were run using larger amounts of seed extract, and using a control of seed extract and water. The active extract showed 0.1018 grams of reducing sugar and the boiled extract showed 0.0022 grams, indicating 0.0996 grams of reducing sugar due to enzyme activitiy. The control gave no reducing sugars.

Invertase:- An active water extract of apple seeds was filtered and test prepared the same as in the examination for diastases, excppt sucrose in stead of starch was used as a substrate. The action took place at 40° C. for twenty-four hours. The active extract produced 0.001768 grams of reducing sugar and the boiled extract

produced 0.001689 grams. The difference of 0.000079 is probably due to a slight hydrolysis of the sucrose by the action of the water at 40° C. during that length of time or due to experimental error. It was concluded that there was no invertase present in apple seeds.

Emulsase:- Active apple seed extract and water when allowed to act at 40° C. for several hours, produced a strong oder of benzaldehyde. This is explained by the fact that during the grinding of the seeds with fine quartz sand, the cells were thoroughly ruptured and the enzyme mixed with the substrate in the seeds. An action evidently took place, developing benzaldehyde. In another test, amygdalin was added to the apple seed extract. A strong oder of benzaldehyde was evolved, due to the action of the enzyme, emulsase, in the seeds upon the amygdalin. Furthermore, qualitative tests showed the presence of hydrocyanic acid, the other product of hydrolysis of amygdalin, in both the preparations used. It is an amygdalin-splitting enzyme similar to or identical with emulsin.

Esterases:- A test for esterases in seeds was made in the following manner: first tube contained 5 c.c. of apple seed extract, 2 c.c. of olive oil, and 5 c.c. of water; second, 5 c.c. of apple seed extract, boiled, 2 c.c. of olive oil, and 5 c.c. of water; third, 5 c.c. of apple seed extract, made alkaline with 5 c.c. of N/10 NaOH, and 2 c.c. of olive oil. A duplicate of the above tests was also made on the commercial enzyme, steapsin, in place of the apple seed extract. In each case a control of 10 c.c. of water and 2 c.c. of olive oil was used. Aliquot parts of the solutions were pipetted off and titrated with N/10 NaOH. The results obtained are shown in Table VIII.

Judging from the results an exterase which hydrolyses olive oil may be present in minute quantities.

Proteases:- Proteases in seeds were investigated by incubating the following mixtures for twenty-four hours at 40° C.: first, 5 c.c. of seed extract, 5 c.c. of albumin, and 10 c.c. of water; second, 5 c.c. of boiled

Table VIII. Tests for Esterases in Apple Seeds

Olive oil used as a substrate.

Material Used	N/100 alkali required
Water Extract of seeds	15 cc
Water Extract of seeds boiled	20 cc
Water Extract of seeds with excess of N/10 alkali	44 cc
Water Extract of seeds boiled with excess of N/10 alkali	37.4 cc
Steapsin solution	25 cc
Steapsin solution boiled	36.2 cc
Steapsin solution with excess of N/10 alkali	37.4 cc
Control	5 cc

extract, 5 c.c. of albumin, and 10 c.c. of water; third, 5 c.c. of seed extract, 5 c.c. of albumin, and 10 c.c. N/10 HCl. The above tests were repeated using peptone as the substrate. After incubating at 40° C. for twenty four hours, aliquot parts were tested by the ninhydrin method,(10). Table IX shows the results obtained.

The results show that a protein splitting enzyme is present in the seeds. Since it attacks both albumin and peptone it is of the trypsin type.

Oxidases:- In testing for oxidases in seeds the phenolphthalin test,(11), was used. This depends upon the development of a pink color when the phenolphtalin is oxidized to phenolphthalein in the presence of an alkali. Twenty-five c.c. of seed extract and 5 c.c. of phenolphthalin, 25 c.c. of boiled seed extract and 5 c.c. of phenolphthalin, and water controls were the mixtures made. These were tested at frequent intervals with a few drops of N/10 NaOH. No color developed even after incubation for as long as thirty-six hours. It

Table IX. Tests for Proteases in Apple Seeds.

Material Used	Results
5 cc of apple seed extract	amino acid equiszalent
apple seed extract and albumin	0.013
apple seed extract boiled and albumin	none
apple seed extract and excess of N/10 acid	0.003
apple seed extract and peptone	0.017
apple seed extract boiled and peptone	none
apple seed extract and excess of N/10 alkali	0.006

appears therefore that no oxidases are present in apple seeds.

- Conclusions -

The results obtained during this investigation may be summarized as follows:

Enzymes present in the flesh of apples:

Diastases	absent
Invertases	absent
Tannases	absent
Glucosidases	absent
Esterases	present
Proteases	present
Oxidases	present

Enzymes present in seeds of apples:

Diastases	present
Invertases	absent
Glucosidases (Emulsin)	present
Esterases	present
Proteases	present
Oxidases	absent

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