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ISOLATION OF ACETYLESTERASE FROM CITRUS WASTE

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This application is made under the act of March 3, 1883, as amended by the act of April 30, 1928, and the invention herein described and claimed if patented in any country, may be manufactured and used by or for the Government of the United States of America throughout the world for governmental purposes without the payment to us of any royalty thereon.

This invention relates to utilization of waste materials discarded in the food processing industry for the production of an enzyme useful in chemical industry. In particular the invention relates to the isolation of acetylesterase from citrus waste, i. e., orange peel, grapefruit peel, and lemon peel.

Another aspect of this invention lies in the method of purifying crude acetylesterase contaminated with pectinesterase. It has been found that the crude acetylesterase contaminated with pectinesterase obtained by salt precipitation can be purified by a dialysis technique whereby acetylesterase is retained by the semi-permeable membrane whereas pectinesterase is destroyed. An additional object of this invention, therefore relates to the purification of acetylesterase contaminated with pectinesterase.

Further objects and advantages of this invention will be obvious from the following description:

The inventors have found that citrus fruits contain a novel enzyme. This enzyme has the property of hydrolyzing esters and is most effective on esters of acetic acid. For this reason it has been named acetylesterase. Acetylesterase is not to be confused with lipase which is most effective against esters of the higher molecular weight acids such as oleic, stearic, etc. Further, acetylesterase is not to be confused with cholinesterase. Whereas both of these enzymes will hydrolyze acetylcholine, cholinesterase is inhibited by the presence of eserine whereas acetylesterase is not.

Analyses of sections of citrus fruit have indicated that the acetylesterase is largely concentrated in the peel and its concentration diminishes toward the center of the fruit. In the peel itself, the flavedo contains about twice the concentration of acetylesterase as in the albedo. Therefore the flavedo is the preferred raw material for our process. However, since the albedo contains some acetylesterase, one can use the entire peel as the raw material.

Briefly described, the isolation process involves pressing the juice from citrus peel, salting-out the acetylesterase from the press juice, and dialysis of the precipitate if a purer product is desired.

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The first step in the isolation involves pressing the peel to separate the juice containing acetylesterase from the solid materials (cellulose, protopectin, etc.). For this purpose any of the usual types of apparatus employed for pressing juices out of materials can be used. It is preferable to comminute the peel before or during pressing to obtain a higher yield of juice. Another expedient which has the effect of increasing the yield of press juice involves mixing a solid, finely divided, insoluble, inert material with the peel prior to the pressing operation. For this purpose one can use sand, diatomaceous earth, silicon carbide, emery, aluminum oxide, pumice, fuller's earth, bentonite, barite, quartz, commercially available filter aids, and other materials in this category. These materials merely act as abrasives and cause rupturing of the tissues whereby more juice is released. They also keep the mass in the press in a porous state so that the juice can run out. The yield of acetylesterase can also be increased by incorporating an inorganic salt such as sodium chloride in the mass before pressing. This alternative has the disadvantage that although the yield of acetylesterase is increased, the product is contaminated to a great extent with pectinesterase. However, if a pure product is not desired and the presence of pectinesterase can be tolerated then this alternative method may be of interest.

The next step in the process involves isolation of acetylesterase from the press juice. This is readily accomplished by addition of an inorganic salt. Immediately after obtaining the press juice, it is preferable to add an oxalate salt such as ammonium oxalate, potassium oxalate or sodium oxalate. This reagent prevents oxidative darkening of the press juice and thus protects the enzyme against deterioration. Only a small amount of oxalate is required, i. e., from about 1 to about 5 parts per 100 parts of solution. After stabilization, the press juice is filtered if any insoluble material is present and is then ready for the salt precipitation. Although ammonium sulphate is preferred as the precipitant, other inert, water-soluble inorganic salts can be used as, for example, sodium chloride, magnesium sulphate, sodium sulphate, potassium sulphate, potassium chloride, ammonium chloride, etc. The amount of salt to be added should be from about 25 to about 50 parts of salt per 100 parts of juice. The smaller proportions of salt are preferable if a pure product is desired while the higher proportions of salt give larger yields of less pure material, i. e., con-

taminated with pectinesterase. After addition of the salt the solution is allowed to stand to obtain complete precipitation of acetylesterase. The solution is preferably kept cool, although this is not essential. The precipitate formed contains acetylesterase, ammonium oxalate, and some pectinesterase and can be used in this form in hydrolysis reactions.

If a purer product is desired a dialysis is carried out. To this end the precipitate is dissolved in aqueous oxalate solution of about 1 to about 5% concentration (any of the aforementioned oxalate salts are suitable). This solution is then placed in a semi-permeable membrane of a suitable type (regenerated cellulose film, for instance) and is dialyzed against an oxalate solution of the concentration and type described directly above. The acetylesterase does not pass through the membrane and the final product will be acetylesterase dissolved in the oxalate solution. This dialysis effects a considerable purification as pectinesterase is completely destroyed. The resulting pure solution of acetylesterase in oxalate is stable and can be stored and shipped in this form. It can also be directly employed in many hydrolytic processes.

If a solid product is desired this solution can be dialyzed against water. The resulting dialysate can then be lyophilized (i. e., dried in vacuum while in the frozen state) to obtain the solid material. During this dialysis against water some deactivation occurs and therefore the yield of active material is somewhat less than when the process is only taken to the previous step.

The stabilization technique mentioned above involves addition of an inorganic oxalate to the acetylesterase or solutions or juices containing the same. The oxalate prevents oxidative darkening and deterioration of the enzyme. Whereas a press juice containing acetylesterase will be practically completely inactivated by standing for several days, addition of the oxalate will allow the juice to retain its activity for months. Since acetylesterase deteriorates quite rapidly, it is advantageous that the oxalate be present in all steps of the isolation. For this reason the oxalate is added to the press juice immediately after it is obtained and is also used in the solutions for dialysis (both within and without the membrane). The finished product can be conveniently kept in oxalate solution whereby its activity will be retained for months. As stated above, the solutions or juices containing acetylesterase should contain from about 1% to about 5% of the oxalate. Although sodium oxalate is preferred, other soluble inorganic oxalates such as potassium oxalate, ammonium oxalate, etc. are suitable. Obviously, the particular cation is irrelevant, the point is to have the oxalate ions in solution.

As mentioned heretofore, the precipitated acetylesterase formed in the salting-out step is generally contaminated with pectinesterase. By dialysis a purification can be obtained. In such a process the acetylesterase is retained in the membrane while the pectinesterase is destroyed. To prevent deterioration of the acetylesterase during the dialysis, it is preferable to maintain the acetylesterase in oxalate solution and to use an oxalate solution for the liquid surrounding the membrane. The inorganic oxalates and concentrations thereof set forth in the paragraph immediately above are equally applicable to this step in the process.

The following examples illustrate the techniques of this invention. It is understood that these examples are submitted only by way of illustration and not limitation.

Example 1

3712 grams of navel orange flavedo was ground, mixed with 2400 grams of sand and pressed. To the press juice obtained (2000 ml.) was added 80 grams of sodium oxalate. By filtering the press juice, 1800 ml. of clear juice was obtained. To 1600 ml. of the clear juice, 388 grams of ammonium sulphate was added to give 0.4 saturation and the solution allowed to stand overnight in a refrigerator. The large crystals of ammonium oxalate occluded with acetylesterase which deposited were filtered off and dissolved in 380 ml. of 0.1 M sodium oxalate solution. This solution after filtration was dialyzed against 0.1 M sodium oxalate for 10 days in the cold to yield 415 ml. of a clear, pale yellow solution of acetylesterase in approximately 0.1 M sodium oxalate. This solution was assayed using triacetin as a substrate and it was found that it contained 40% of the acetylesterase activity as compared with the original press juice.

Example 2

To 1440 grams of fresh orange flavedo was added 21.1 grams of solid sodium chloride followed by grinding in a food mill and mixing with 144 grams of a refined diatomaceous earth filter aid. This mixture was then pressed in a conventional laboratory press and 875 ml. of press juice was obtained. The press juice was saturated with sodium oxalate, filtered and sufficient ammonium sulphate added to bring the concentration to 0.7 saturation (about 429 grams $(NH_4)_2SO_4$). The precipitate formed was removed by filtration, dissolved in 175 ml. of 0.1 M sodium oxalate solution. This solution was dialyzed against 0.1 M sodium oxalate solution and finally against water. The resulting solution was dried by lyophilization to yield 1.04 grams of acetylesterase as powder. Assay against triacetin showed that 41% of the acetylesterase activity had been recovered.

Example 3

2770 grams of ground grapefruit flavedo was mixed with 2271 grams of sand and pressed in a conventional laboratory press. To the 1570 ml. of press juice obtained was added 50 grams of sodium oxalate and the juice filtered. 360 grams of ammonium sulphate was added to 1490 ml. of the clear juice and let stand for 2 hours. The insoluble material was filtered off and dissolved in 285 ml. of 0.1 M sodium oxalate solution. This solution was dialyzed against 0.1 M sodium oxalate solution for several days to yield a solution of acetylesterase which retained its activity after storage in a refrigerator for more than eight months.

The acetylesterase prepared in accordance with this invention is useful to bring about controlled hydrolysis of compounds. Because of its particular activity against acetic acid esters, it can be used to hydrolyze acetic ester groups in compounds while leaving intact other ester groups. For instance if the product is applied to ethyl para-acetyloxy-benzoate, ethyl para-hydroxybenzoate and acetic acid will be produced. The acetic acid ester group will be hydrolyzed while the benzoic acid ester group will not be affected. Likewise the product can be used for the resolution of mixtures of esters whereby the esters of

the lower acids particularly acetic acid will be hydrolyzed while the esters of higher molecular weight acids will not. The acetylesterase can also be applied to compounds containing both acetic ester groups and amide groups whereby only the acetic ester groups will be hydrolyzed. Further extensions of this principle will be obvious to those skilled in the art.

Experiments were conducted on the hydrolytic activity of the acetylesterase produced according to Example 2 herein. The following table indicates the results obtained. The activities given represent the relative hydrolytic activity of the acetylesterase against each ester. These activities are relative and based on an arbitrary selection of 1.0 for monoacetin.

Ester	Activity
Monoacetin	1.0
Monopropionin	0.36
Monobutyrin	0.009

This data means that if the three esters are subjected to acetylesterase under the same conditions the amount of carboxyl radical liberated in each case will be in the molar ratio of 1, 0.36, and 0.009. These experiments clearly illustrate the highly selective activity of the enzyme.

Having thus described our invention, we claim:

1. The process of isolating acetylesterase which comprises pressing citrus peel to separate the juice from the solid material, stabilizing the juice by dissolving an inorganic oxalate therein, precipitating the acetylesterase from the stabilized solution by salting-out with inorganic salt and separating the precipitated acetylesterase.

2. The process in accordance with claim 1 wherein the citrus peel is orange peel.

3. The process in accordance with claim 1 wherein the citrus peel is grapefruit peel.

4. The process in accordance with claim 1 wherein the citrus peel is lemon peel.

5. The process of isolating acetylesterase which comprises pressing citrus peel to separate the juice from the solid material, stabilizing the juice by dissolving about 1% to about 5% of an inorganic oxalate therein, precipitating the acetylesterase from the stabilized solution by salting-out with an inorganic salt, and separating the precipitated acetylesterase.

6. The process of isolating acetylesterase which comprises pressing citrus peel to separate the juice from the solid material, stabilizing the juice by dissolving an inorganic oxalate therein, precipitating the acetylesterase from the stabilized solution by salting-out with about 25 to about 50% of an inorganic salt, and separating the precipitated acetylesterase.

7. The process of isolating acetylesterase which comprises pressing citrus peel to separate the juice from the solid material, stabilizing the juice by dissolving therein about 1% to about 5% of an inorganic oxalate therein, precipitating the acetylesterase from the stabilized solution by salting-out with about 25% to about 50% of an inorganic salt, and separating the precipitated acetylesterase.

8. The process of isolating acetylesterase which comprises pressing citrus peel to separate the juice from the solid material, stabilizing the juice by dissolving an inorganic oxalate therein, precipitating the acetylesterase from the stabilized solution by salting-out with an inorganic salt, separating the precipitated acetylesterase, dissolving it in an aqueous solution of an inorganic oxalate and dialyzing it.

9. The process of isolating acetylesterase which comprises pressing citrus peel to separate the juice from the solid material, stabilizing the juice by dissolving an inorganic oxalate therein, precipitating the acetylesterase from the stabilized solution by salting-out with an inorganic salt, separating the precipitated acetylesterase, dissolving it in an aqueous solution of an inorganic oxalate and dialyzing it against aqueous inorganic oxalate.

10. The process of isolating acetylesterase which comprises pressing citrus peel to separate the juice from the solid material, stabilizing the juice by dissolving about 1% to about 5% of an inorganic oxalate therein, precipitating the acetylesterase from the stabilized solution by salting-out with about 25% to about 50% of an inorganic salt, separating the precipitated acetylesterase, dissolving it in an inorganic oxalate solution of about 1% to about 5% concentration and dialyzing this solution against an inorganic oxalate solution of the same range of concentration.

11. The process of isolating acetylesterase which comprises pressing citrus peel to separate the juice from the solid material, stabilizing the juice by dissolving about 1% to about 5% of sodium oxalate therein, precipitating the acetylesterase from the stabilized solution by salting-out with ammonium sulphate in a concentration of about 24%, separating the precipitated acetylesterase, dissolving it in a sodium oxalate solution of about 1% to about 5% concentration and dialyzing this solution against a sodium oxalate solution of substantially the same range of concentration.

12. The process of purifying acetylesterase contaminated with pectinesterase which comprises dissolving the contaminated acetylesterase in aqueous inorganic oxalate solution and dialyzing this solution against aqueous inorganic oxalate solution.

13. The process of purifying acetylesterase contaminated with pectinesterase which comprises dissolving the contaminated acetylesterase in aqueous inorganic oxalate of about 1% to about 5% concentration and dialyzing the resulting solution against an aqueous inorganic oxalate of approximately the same range of concentration.

14. The process of purifying acetylesterase contaminated with pectinesterase which comprises dissolving the contaminated acetylesterase in aqueous sodium oxalate of about 1% to about 5% concentration and dialyzing this solution against an aqueous sodium oxalate solution of approximately the same range of concentration.

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