

On the Process of the Formation of Lower Carbonyl Compounds in the Enzymatic Oxidation of Linoleic Acid

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In the previous report,¹⁾ it was confirmed that most of volatile carbonyl compounds found in fish flesh during storage was produced by the atmospheric oxidation of lipid fraction in the flesh. Studies on the autoxidation of fatty materials have revealed the facts that the primary products of autoxidation are the peroxide or hydroperoxide (PV), and that by the transformation or decomposition of the peroxide and the interaction of the peroxide with other materials of autoxidation and further oxidation of the peroxide, the secondary products may be formed, including various aldehydes, ketons and acids etc., some of which are responsible for rancid flavour and odor.²⁾³⁾ However, there has been probably little experimental evidence about the mechanism by which the lower products are formed.

And then, in order to obtain some information on the process of the formation of lower carbonyl compounds by the oxidation, a preliminary experiment was carried out in considering the relationship between the formation of water-soluble carbonyl compounds (SC) and the quantity of the peroxide and between the formation of water-soluble and non-water-soluble carbonyl compounds (NC), adopting the oxidative action of soybean lipoxidase for linoleic acid.

Experiments

Oxidation substrate: Linoleic acid (Tokyo Chemical Industry Co., I. V. 153.3) was used after being purified by urea fractionation.⁴⁾ The substrate emulsion was prepared each experiment day as described below: a known quantity of linoleic acid was suspended in 0.8% span 20 solution. The mixture was warmed shaking and immediately homogenized. The emulsion was arranged to contain 5.0 mg of fatty acid per cc.

Lipoxidase: Crude lipoxidase preparations, obtained by the following procedure, were used after being diluted 10 times with water: defatted soybean meal was homogenized with 10 volumes of distilled water, and was centrifuged and filtered through defatted cotton.

Oxidation reaction: The reactions were generally performed in the ordinary test tubes at 20°C or 25°C, and those, under anaerobic condition, in Fig. 1, were made in thunberg tubes. The reaction mixture was made of 0.5 cc or 1.0 cc of the substrate emulsion, and of 0.5 cc of the enzyme preparation and was added with sufficient water to give a final volume of 5.5 cc. The enzymatic reaction was stopped by adding 0.5 cc of 1N HCl or that with the co-existence of Mohr's salt by 0.3% when the estimation of the peroxide was intended.

Analysis: The reaction mixture was vigorously shaken with 1 g of NaCl and filtered, and it was filtered after being placed in boiling bath exactly for 2 min. and cooled in water when the filtrate was taken for the estimation of peroxide. PV was determined by rhodanate method.⁵⁾ In order to obtain the higher reproducibility, coloration was made in ice bath. Peroxide value was expressed as γ Fe⁺⁺⁺ in the mixture. SC was determined as in the previous report¹⁾ on the filtrate obtained by treating with NaCl. The estimation value will be referred to as the approximate quantity of volatile carbonyl

compounds. Total carbonyl compounds (TC) was determined by the modified procedure of the same method: coloration was made by the addition of KOH ethanol solution, after the reaction mixture being heated with dinitrophenylhydrazine solution and cooled. Carbonyl value was expressed as γ CH_3CHO in the mixture.

Preparation of NC fraction: To 5 cc of the substrate (containing 10 mg of linoleic acid per cc.), 40 cc of water and 2 cc of the enzyme solution were added and the mixture was allowed to stand at 20°C for about 5 hr. The mixture was vigorously shaken with 10 g of NaCl, and was filtered. Residual matters were dried in dessicator under vacuum, after being washed with 20% NaCl in 0.1N HCl until no amount of SC was found in the filtrate. Dried matters were treated with carbonyl-free ether twice and ether was removed from the solution by the evaporation under reduced pressure. To residue obtained, 20 cc of 40% NaHSO_3 was added, and the solution was placed in ice bath with occasional shakings for about 2 hr. After some amount of NaHSO_3 crystallized out in the solution by cooling, was dissolved by the addition of about 5 cc of water, the solution was again treated with ether several times. To the aqueous solution, 8 cc of H_2SO_4 (1:1) was added. SO_2 generated was removed by passing CO_2 through the solution at about 40°C. The resultant solution was further treated with ether twice, and ether solution was evaporated to dryness after being washed with water. The product was dissolved in 10 cc of 0.2 % Span 20 solution.

Results and Discussion

Formation of SC in relation with PV

The formation of SC and PV in two reaction mixtures containing different quantities of the enzyme was shown in Fig. 1. In the case of the mixture containing the enzyme preparation of full strength, from the outset of the reaction, much PV was produced and SC also increased gradually. However, in the case of that of half strength, the formation of SC was scarcely appreciable irrespective of steady increase of PV. These facts suggest that appreciable formation of SC

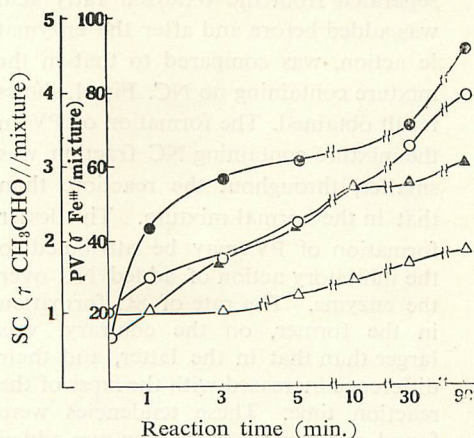


Fig. 1. Formation of SC and PV in the oxidation of the linoleic acid-emulsion by the enzyme preparation of full strength (SC ○, PV ●), and of half strength (SC △, PV ▲).

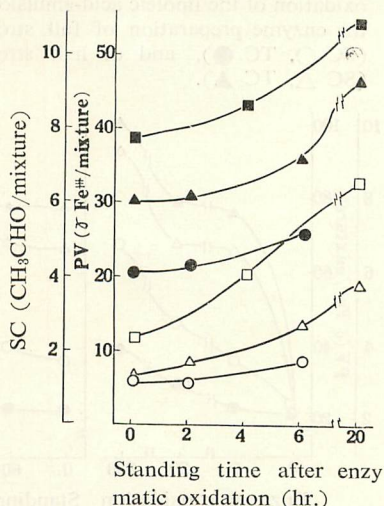


Fig. 2. Formation of SC and PV in the emulsion kept under anaerobic condition after the enzymatic oxidation of 1 min. (SC ○, PV ●) 3 min. (SC △, PV ▲) and 5 min. (SC □, PV ■).

may be due to the presence of PV in a certain quantity or more and SC may not be probably a product following directly after the formation of PV. Changes similar to those mentioned above were perceived in the reaction-mixture kept under anaerobic condition after being subjected to the enzymatic action. As seen in Fig. 2, the increasing rate of the products varied according to its initial values, and that of larger initial value was more steady than that of smaller value, and in the latter case both PV and SC showed scarcely appreciable change even after 4 hr. standing. These results may confirm the assumptions mentioned above.

Formation of SC and TC

The formation of SC and TC in the course of the oxidation of the reaction mixture containing different quantity of the enzyme was investigated. Results obtained were shown in Fig. 3. In the case of the mixture containing the enzyme preparation of full

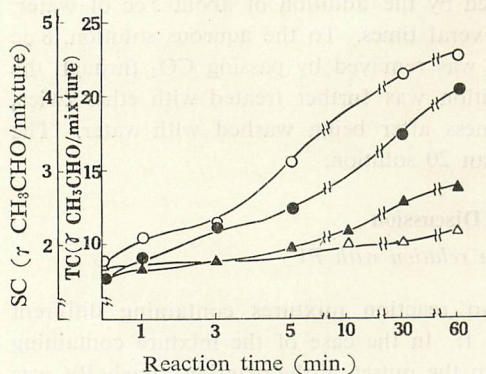


Fig. 3. Formation of SC and TC in the oxidation of the linoleic acid-emulsion by the enzyme preparation of full strength (SC ○, TC ●), and of half strength (SC △, TC ▲).

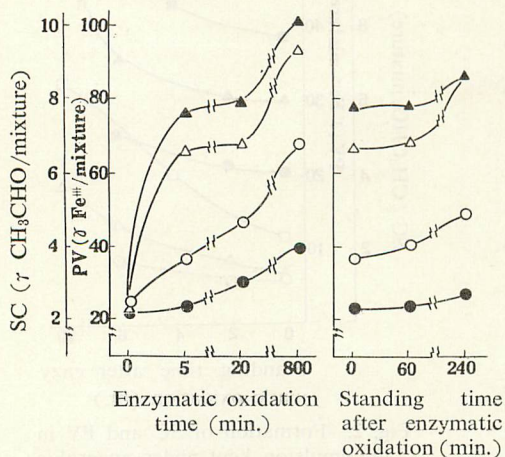


Fig. 4. Formation of SC and PV in the enzymatic oxidation of the linoleic acid-emulsion with NC fraction (SC○, PV△), and without NC fraction (SC●, PV▲).

strength, both SC and TC concurrently increased with the increase of PV, while in the case of half strength the formation of SC was to be hardly appreciated, and that of TC showed gradual increase from the outset of the reaction. From these facts, it was anticipated that the formation of SC precedes to that of NC, and furthermore, that NC may probably be a precursor of SC.

Formation of SC from NC

In order to ascertain whether SC is a product derived from NC or not, the formation of SC and PV in the reaction mixture to which the fraction of NC separated from the oxidized fatty acid was added before and after the enzymatic action, was compared to that in the mixture containing no NC. Fig. 4. shows result obtained. The formation of PV in the mixture containing NC fraction was smaller, throughout the reaction, than that in the normal mixture. This lesser formation of PV may be attributed to the inhibitory action of added NC over the enzyme. The rate of SC formation in the former, on the contrary, was larger than that in the latter, and their difference increased with the lapse of the reaction time. These tendencies were found out when NC fraction was added to the mixture after the enzymatic action, and the difference of SC produced was somewhat larger than that in the preceding case.

Some variations of the substrate concentration showed almost no influence on the formation of SC, whereas it was considerably influential on that of PV. Accordingly, the presence of some unoxidized fatty acids which might have been remained in NC fraction, may not be responsible for much formation of SC. Thus it was presumed that SC was likely to be the later product derived from NC consequent on the formation of PV. This presumption may be applied to the case of autooxidation, since the pattern of the enzymatic oxidation has been admitted to be almost similar to that of the autooxidation.⁶⁾ The details of process of formation of lower carbonyl compounds from unsaturated fatty materials will be researched with the investigation of each carbonyl compounds.

Summary

The formation of SC by the enzymatic oxidation of linoleic acid could hardly be perceived under the presence of smaller quantity of PV, while that of TC was appreciable in the same case. The addition of NC fraction to the mixture resulted in the acceleration of SC-formation and the inhibition of PV-formation. By some variations of the substrate concentration, the rate of PV-formation was considerably affected, while that of SC was hardly affected. Thus, SC produced by the oxidation of linoleic acid, was supposed to be a later product derived from NC consequent on the formation of PV.

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