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Commercial and Potential Utilization of Lipoxygenase

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ABSTRACT

The high specificity and activity of lipoxygenase (EC 1.13.1.13) have not been widely exploited in commercial applications. An analytical application of substrate specificity is exemplified by the Canadian Food and Drug Official Method FA 59 for determining unisomerized linoleic acid in hydrogenated fats. An attractive potential application of lipoxygenase is the lipoxygenase oxidation of linoleic acid for conversion of a renewable resource into a valuable chemical intermediate. Hydroxy-conjugated octadecadienoic acids (HCD) have been prepared by oxidation of a 10% soybean soapstock solution with an aqueous soy flour extract followed by reduction of the hydroperoxide. High yields and a 20-min reaction time are features of this procedure. These laboratory-scale experiments indicate that the processing cost to produce hydroxy-conjugated octadecadienoic acids can be estimated at 21 cents per lb. This cost does not include the cost of the soapstock. The combined hydroxy, conjugated diene, and fatty acid groups in HCD give it the potential of being a versatile chemical intermediate. HCD is readily converted to hydroxystearate or conjugated triene and can compete directly with tung oil acids or hydrogenated castor oil acids. Other reactions can be visualized based on functional group modification to yield products with potential application in the formulation of coatings, lubricants, emulsifiers, and plasticizers.

TABLE I

Plant Sources of Lipoxygenase

Apple (8)	Peppers (12)
Alfalfa (9)	Potatoes (11,25,26)
Barley (10)	Pumpkin (12,27)
Cauliflower (11,12)	Rapeseed (27)
Eggplant (12,13)	Red beans (16)
Flaxseed (14,15)	Snap beans (22)
Greenbeans (16)	Soybean seeds (12,28,29)
Lima beans (16)	Squash (12,30)
Maize (17-19)	Sunflower seeds (27)
Mustard (20)	Tomatoes (12,31)
Navy bean (16)	Watermelon (32)
Peas (12,21,22)	Wheat (33)
Peanut (16,23,24)	

INTRODUCTION

The enzyme lipoxygenase (EC 1.13.1.13) in the presence of oxygen rapidly oxidizes linoleic acid to a hydroperoxy-conjugated octadecadienoic acid. Lipoxygenase oxidation and related areas have been the subject of several reviews (1-6). This paper will cover selected aspects related to the commercial importance of the enzyme, lipoxygenase, and will amplify its possible role in production of industrially useful oxygenated fatty acids.

Lipoxygenase Sources

Soybean lipoxygenase has the highest activity of the various lipoxygenases and is the most economical source of lipoxygenase. Lipoxygenase has been isolated from many other plant sources (2), and lipoxygenase activity has been reported in platelets (7). Many of the plant sources apparently have their own individual variety of lipoxygenase. Commercially important plants or seeds which contain lipoxygenase are summarized in Table I. Additional references as well as references to some exotic plants containing lipoxygenase are given by Axelrod (2). The activities and selectivities of lipoxygenases vary and are dependent on the pH of the reaction mixture and nature of the substrate (2,16,17,19,23,24). For example, corn germ lipoxygenase (17) yields almost pure 9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid whereas lipoxygenase from flaxseed (14) yields 80% 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid.

In constrast, soybean lipoxygenase generally is reported to give a mixture consisting of about 70% 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid and 30% 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid. However, this ratio is dependent on the pH of the reaction, oxygen content, and presence of calcium ion. More recently separate enzymes have been isolated (34,36) from soybean lipoxygenase. These isoenzymes each give different ratios of the 9- and 13-hydroperoxide isomers and are probably responsible for much of the varying results reported by different workers for soybean lipoxygenase. The pH at which these isoenzymes demonstrate maximum activities varies from pH 6.8 to 9.0. This difference in the pH profiler of the isoenzymes results in product formation being dominated by various isoenzymes at different pH's.

REACTION PRODUCTS

The various lipoxygenases have been studied under

TABLE II

Some Oxygenated Fatty acids Associated with Lipoxygenase Oxidation of Linoleic Acid

9-D-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid
13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid
9-oxooctadeca-10,12-dienoic acid (38)
13-oxooctadeca-9,11-dienoic acid (38)
9-hydroxy-*cis*-12,13-epoxy-*trans*-10-octadecadienoic acid (40,41)
13-hydroxy-*cis*-9,10-epoxy-*trans*-11-octadecadienoic acid (40,41)
9,12,13-trihydroxy-*trans*-10-octadecadienoic acid (40,41)
9,10,13-trihydroxy-*trans*-11-octadecadienoic acid (40,41)
12-oxo-13-hydroxy-*cis*-9-octadecenoic acid (42)
10-oxo-9-hydroxy-*cis*-12-octadecenoic acid (42)
9,10-dihydroxy-*cis*-12-octadecenoic acid (3,43,44)
12,13-dihydroxy-*cis*-9-octadecenoic acid (43,44)
12-oxo-9,10-dihyroxy-*trans*-11-octadecenoic acid (43,44)
9-oxo-12,13-dihydroxy-*trans*-10-octadecenoic acid (43,44)
9,12-dihydroxy-*trans*-10-octadecenoic acid (43,44)
10,13-dihydroxy-*trans*-11-octadecenoic acid (43,44)
11-hydroxy-12,13-epoxy-9-oxtadecenoic acid (39)

aerobic conditions where the other substrate, oxygen, is supplied in excess and under anaerobic conditions where the oxygen supply is depleted. Under anaerobic conditions, the product hydroperoxy-conjugated octadecadienoic acids undergo rearrangement to 9-oxooctadeca-10,12-dienoic acid, 13-oxooctadeca-9,11-dienoic acid, and many other products (37-39). Table II summarizes oxygenated fatty acids derived from hydroperoxy-conjugated octadecadienoic acids by rearrangement or subsequent reactions. Decomposition products such as n-pentane and dimers of oxygenated octadecadienoic acids are also found. Other products such as hexenal and pentane (45,46) illustrate the complex nature of this reaction. These products are thought to result from homolytic cleavage of the hydroperoxide followed by rearrangement or scission of a carbon-carbon bond or by the reaction of the hydroperoxide with other components in the reaction mixture. The decomposition and rearrangement products of hydroperoxy octadecadienoic acids are postulated to be responsible for the development of off-flavors in many food products (46) which are derived from some of the plants listed in Table I.

KINETICS

The kinetics of lipoxygenase oxidation have been well studied (47,48,83). This reaction has many puzzling features which were finally accounted for in the kinetic sequence recently published by Lagocki et al. (49). The kinetic scheme in Figure 1 accommodates all the features of the reaction and the competitive inhibition which is enhanced by the large affinity of the free enzyme for product. The sequence also provides excellent agreement between experimental and calculated reaction rate constants. The scheme shown in Figure 1 assumes that random binding of both substrate (Lo) and product (Lo-OOH) molecules occurs and that hydroperoxide or product formation occurs only when both hydroperoxide and substrate (Lo) are attached to the same lipoxygenase molecule. In addition, it is assumed that both binding sites for Lo-OOH and Lo are catalytically the same and that the reaction is being carried out in the presence of excess oxygen. The lipoxygenase oxidation mechanism, its relation to reaction features such as stereospecificity and iso-lipoxygenases, fluorescence of the lipoxygenase-O_2 complex, and the role of iron have been discussed in a recent review (2).

ANALYTICAL USE

Besides its importance in the development of off-flavors in foods, the specificity of lipoxygenase has been utilized in a procedure for determining linoleic acid (50,51) and has

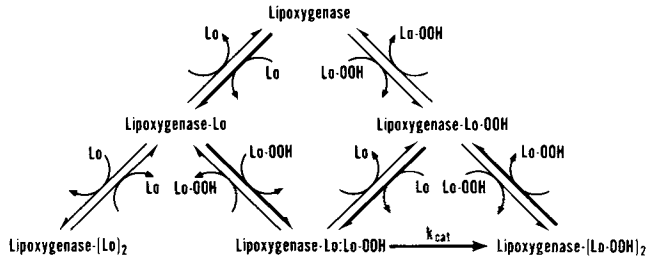

FIG. 1. Kinetic scheme for lipoxygenase oxidation of linoleic acid. Lipoxygenase-Lo and lipoxygenase-Lo-OOH are used to indicate the complex between liposygenase and linoleic acid or lipoxygenase and linoleic hydroperoxide. Lo = linoleic acid and Lo-OOH = linoleic-hydroperoxide. The interaction of oxygen in the scheme is not shown. The kinetic parameters are k_{cat} = 6.26 x 10^{-5} moles/sec $(mg/ml)^{-1}$ and K_M = 7.7 x 10^{-6} moles/liter.

TABLE III

Substrates[a] Oxidized by Lipoxygenase (52-54)

Fatty acid[b]	
6,9,12-16:3	6,9,12-18:3
9,12-17:2	9,12,15-18:3
5,8,11-17:3	6,9,12,15-18:4
5,8-18:2	10,13-19:2
6,9-18:2	10,13,16-19:3
7,10-18:2	11,14-20:2
8,11-18:2	11,14,17-20:3
9,12-18:2	5,8,11,14-20:4
10,13-18:2	5,8,11,14,17-20:5
11,14-18:2	9,12,15-21:3
12,15-18:2	6,9,12,15-21:4
13,16-18:2	10,13,16-22:3
9,15-18:2	4,7,10,13,16,19-22:6
5,8,11-18:3	Crepenynic acid

[a]Geneva numbering: Numbers separated by commas indicate the position of the double bond. Last two numbers separated by colon indicate number of carbon atoms and number of double bonds.

[b]Double bonds in fatty acids must all have *cis* configuration for lipoxygenase oxidation.

been adopted for use in the Canadian Food and Drug Official Method FA 59. This method is used for determining unisomerized linoleic acid in hydrogenated fat. Since the enzyme is specific for methylene interrupted 1,4 *cis,cis*-octadecadienoic acids, it is well suited for this role. However, the enzyme will oxidize fatty acids other than those having the double bonds in the 9,12-positions. Some of these substrates which are known to be oxidized by lipoxygenase are shown in Table III (52-54). In hydrogenated fats, this is not a serious deficiency since positional isomerization is normally accompanied by geometric isomerization of the *cis* double bond to the *trans* configuration which is not susceptible to lipoxygenase oxidation. The main advantage of the lipoxygenase method is the requirement of only simple reagents and an ultraviolet (UV) spectrophotometer for measuring conjugated diene absorption. The reagents and instrumentation are available in most quality control laboratories or can be readily obtained. A collaborative study is in progress by the AOCS Biochemical Methods Committee to confirm the applicability and limitations of this assay procedure.

BLEACHING OF WHEAT FLOUR

The only example of commercial utilization of lipoxygenase is in the bleaching of wheat dough. The process is extensively used in the U.S. and England in the baking of bread (4,55).

Bleaching of wheat dough with lipoxygenase is accomplished by adding between 0.5 to 1% enzyme active soy

TABLE IV

Compounds Oxidized by Coupling with Lipoxygenase Oxidation of Linoleic Acid

Carotene (56,68,71-74)
Xanthophyll (2)
Vitamin A (2)
Phytofluoene (2)
Thyroxine (69)
Cholesterol (70,71)
Bixin (72)
Luminol (73,75)
Cytochrom C (73,75)
Diphenylisobenzofuran (73,75)
Tetracyclone (73,75)
Chlorophyll (76)
Violaxanthin (77)

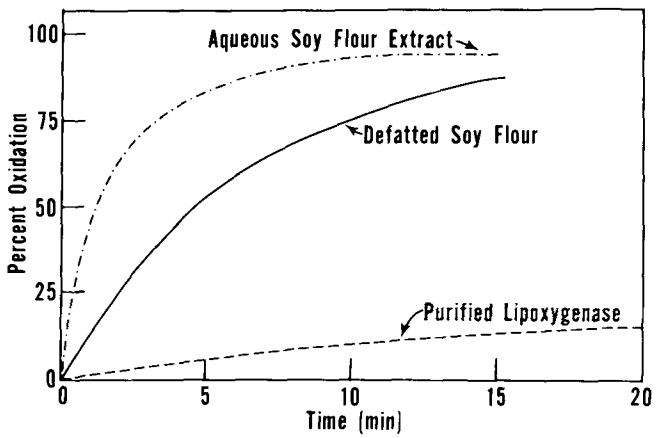

FIG. 2. Activity of lipoxygenase from various sources in 10% aqueous ethanol. Substrate concentration = 4 mg/ml safflower oil soaps, substrate to lipoxygenase ratio = 80.

flour to wheat flour and was first described by Haas and Bohn (56) in a series of patents filed in 1934. The method is still used as it was originally described. Addition of soy flour results in a very white bread having a fine crumb and expanded loaf volume. Additional benefits include an improved nutty flavor and better rheological properties such as ease of mixing, resistance to deformation, and improved mastication (2,57-64).

Lipoxygenase oxidation of linoleic and linolenic acids in wheat dough (65) is the first step in the bleaching of carotenoid pigments in wheat flour (66-68). The second step in the oxidation of pigments is the result of secondary "coupled" oxidation between hydroperoxy octadecadienoic acids and the pigments in wheat flour. Surprisingly, similar pigments in soy flour are not bleached by this technique.

The coupled oxidation of pigments and improvement in

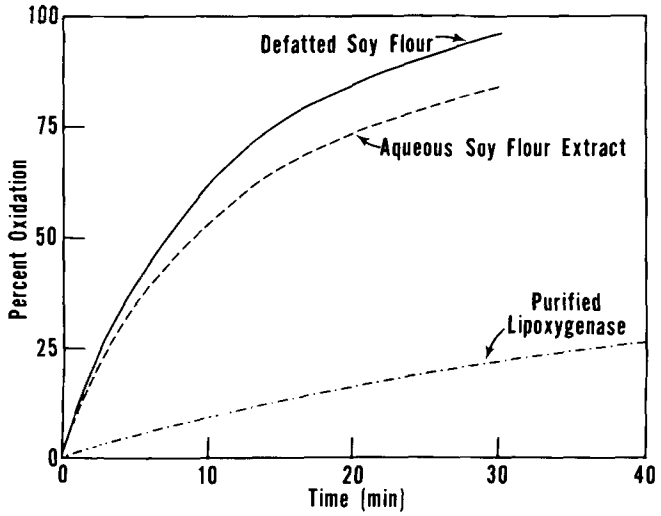

FIG. 3. Activity of lipoxygenase from various sources in 20% aqueous DMSO. Substrate concentration = 100 mg/ml safflower oil soaps, substrate to lipoxygenase ratio = 500.

rheological properties are the result of various possible reaction sequences. The carotene pigments are thought to be destroyed simultaneously during the formation of linoleic hydroperoxide. Another possibility is that pigments react immediately with the linoleic hydroperoxide after it is formed. Many other compounds which are also destroyed when coupled with lipoxygenase oxidation are listed in Table IV. A further possibility exists that these compounds are destroyed by subsequent reactions with other enzymes present in soy flour which use the compound in question plus linoleic hydroperoxide as substrates. This theory is supported by the isolation of a variety of oxygenated fatty acids which have been identified in lipoxygenase-wheat flour-water suspensions (see Table II). These oxygenated fatty acids can also be the result of a reaction catalyzed by a complex formed between lipoxygenase and wheat gluten (40,41,43).

Some of the products listed in Table II are speculated to arise by a reaction with the sulfhydryl or thiol groups in cereal proteins (59,60). These reactions are suggested as being responsible for the observed improvements in the rheological properties as well as for the development of nutty flavors (58-60).

PRODUCTION OF HYDROXY-CONJUGATED OCTADECADIENOIC ACIDS

Lipoxygenase oxidation of soybean oil soapstock has been explored as a means of producing hydroxy-conjugated octadecadienoic acids (78,79). In this process, soapstock

TABLE V

Analysis of Oxidized Soybean Soaps[a] in 20% Aqueous DMSO[b] Containing 0.75% Butylated Hydroxy Toluene

Enzyme preparation	S/E ratio	Product composition, %		Extent of oxidation, %[d]	Total fatty acid recovery, %
		HOCD[c]	Lo[c]		
Purified lipoxygenase	250	12.5	63	15	93
Soy flour extract	500	48	18	76	90
Defatted soy flour	500	59	7.3	90	90

[a]Concentration of soapstock, 100 mg/ml of 20% DMSO. Reaction time, 45 min.

[b]DMSO, dimethyl sulfoxide; S/E, substrate to enzyme ratio.

[c]HOCD, hydroxy-conjugated octadecadienoic acids; Lo, linoleic acid.

[d]Percent oxidation is based on the percent linoleic acid remaining in the sample.

TABLE VI

Estimated Processing Cost for Production of HCD

Item	Cents per lb
Soybean whey	0.2
Solvents (DMSO, n-butanol)	5.0
Chemicals ($NaHSO_3NaOH$, H_3SO_4 H_3BO_3)	9.0
Fuel and energy	1.8
Labor (batch process)	5.0
Total estimated cost[a]	21.0

[a]Estimated cost does not include cost of the soybean soapstock or other substrate source or the cost of plant maintenance and interest on capital investments.

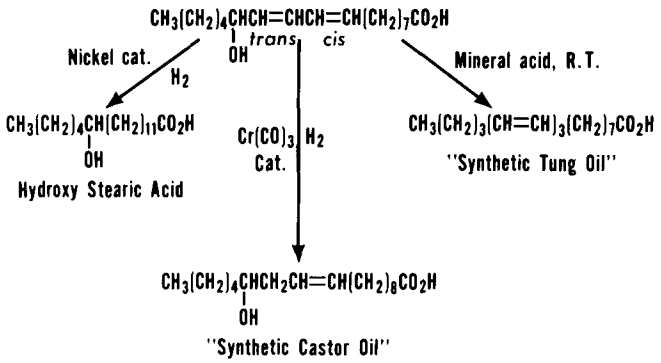

FIG. 4. Hydrogenation and dehydration of HCD.

was oxidized with soy flour extract, and the hydroperoxy dienoic acids were then reduced to hydroxy-conjugated octadecadenoic acids in situ with bisulfite or sodium borohydride.

Aqueous soy flour extract was investigated as a low-cost source of lipoxygenase, and its activity was compared to purified lipoxygenase and defatted soy flour.

Figure 2 shows the activity of these various lipoxygenase sources at a substrated level of 4 mg/1 in 10% aqueous ethanol. The high activity of the aqueous soy flour extract was especially encouraging since soy whey, also an aqueous extract, could probably be utilized as a commercial lipoxygenase source. Soy whey is a by-product and pollutant from soluble soy protein manufacturing and would be an extremely economical source of lipoxygenase.

The 4 mg/ml substrate concentration was the largest that could be effectively used in a 10% aqueous ethanol solvent system. Higher substrate concentrations of safflower oil (SFO) soaps were not completely soluble, and the insoluble soaps were not oxidized by the enzyme. Higher ethanol concentrations resulted in denaturation of the enzyme.

This problem was solved by using a 20% aqueous DMSO solvent system. Figure 3 shows the activity of various lipoxygenase sources in 20% aqueous DMSO at a 10% substrate level. The activity of both the soy flour and the soy flour extract was high under these conditions in spite of the high solvent concentrations.

Addition of small amounts of antioxidants to the reaction mixture was discovered to increase the yield of hydroxy-conjugated dienes from the oxidation of soybean soapstock. The data in Table V show the analysis of oxidized soybean soaps to which 0.75% butylated hydroxy toluene (BHT) has been added. Total oxidation is not increased by the addition of BHT, but about a 10% increase in the isolated yield of hydroxy-conjugated dienoic acids was observed. This is probably because the antioxidant inhibits further hydroperoxide reaction or breakdown.

Lipoxygenase oxidation reactions using these high substrate concentrations in 20% aqueous DMSO were also

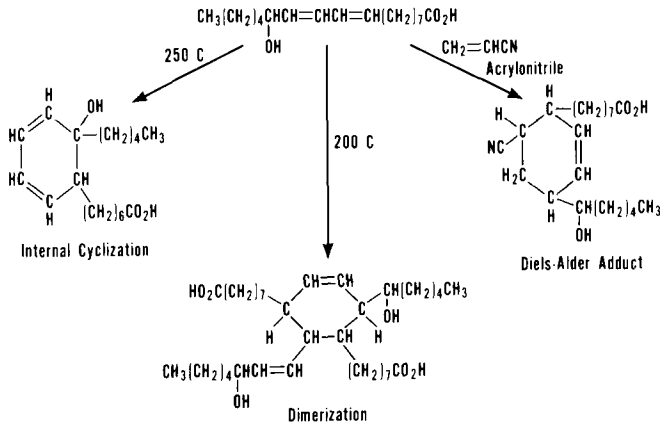

FIG. 5. Diels-Alder reactions possible with HCD.

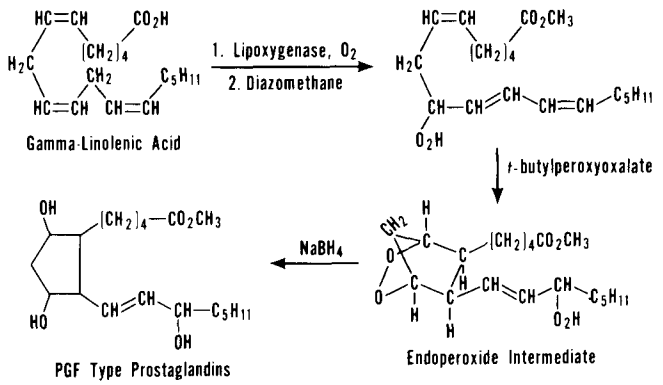

FIG. 6. Prostaglandin synthesis from γ-linolenic acid hydroperoxide.

dependent on several other parameters. The reaction mixture must be kept at a pH of about 10 in order to prevent decomposition of the linoleic acid hydroperoxide. A very efficient stirring rate was necessary in order to incorporate sufficient oxygen into the reaction mixture and to maximize yields of hydroxy-conjugated dienoic acids.

The novelty and utility of use of DMSO in an enzyme system for solubility of substrate opens the possibility of commercialization. The feasibility of using inexpensive by-product enzyme source, atmospheric oxygen as one substrate and soybean soapstock as another, prompted an estimate of processing cost as shown below.

PROCESSING COST

The processing cost of producing hydroxy-conjugated octadecadienoic acid was estimated at 21 cents/lb based on laboratory experiments. Table VI shows a breakdown of this estimated processing cost. Solvent recoveries were assumed to be 90% for this estimation. The figures in Table VI do not include the cost of the linoleic acid substrate source which varies considerably depending on the current fat and oil situation. It also does not include the cost of such items as plant maintenance, overhead, and cost of the capital investment.

POTENTIAL USE AND REACTIONS OF HYDROXY-CONJUGATED OCTADECADIENOIC ACID

The combined hydroxy, conjugated diene, and fatty acid groups in HCD give it the potential of being a versatile chemical intermediate. HCD is easily dehydrated by treating it with mineral acids such as HCl or H_2SO_4. The

resulting product is a conjugated triene which should substitute for the dwindling supply of tung oil type fatty acids. Complete hydrogenation of HCD would produce a hydroxystearic acid which is similar to that obtained by hydrogenation of ricinoleic acid, the main component of castor oil acids. Partial selective hydrogenation of HCD with chromium carbonyl (80) as the catalyst could be used to produce "synthetic" castor oil acids. The above reactions are summarized in Figure 4. These products would compete for part of the 100 million lb market which is currently supplied by imported tung and castor oils.

Diels-Alder reactions can give a variety of products which would have potential application in the formulation of coatings or plasticizers. Figure 5 illustrates typical cyclization and Diels-Alder reactions and gives possible applications for the reaction products. Many other products can be visualized by varying the dienophile used in this reaction.

Oxidation, halogenation, and esterification are other obvious reactions in which HCD could participate. Lipoxygenase has also been used for the laboratory preparation of stereospecific hydroperoxides of arachidonic and gamma-linolenic acids (81). These hydroperoxides were isolated and purified by high-pressure liquid chromatography and then used in the biosynthesis of prostaglandins (82). Figure 6 illustrates prostaglandin synthesis form the gamma-linolenic acid hydroperoxide. The reactions shown in Figures 4-6 suggest products which probably can be prepared from HCD or the hydroperoxide. Much work is still needed to determine the commercial feasibility of these laboratory procedures.

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