Lipolytic Activity of White Pepper Powder at High Temperatures in a Nonaqueous System

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ABSTRACT: White pepper is added as a spice to goose fat in the food industry (particularly in the liver pâté industry) and causes the development of undesirable flavors. In the present study, we demonstrate that white pepper exhibits lipolytic activity when reacted with goose fat in a hydrophobic system. Temperature and pH dependence measurements suggested that two enzymes or isoenzymes with optimal temperatures at 55 and 85°C, and with optimal pH at 7 and 9, are involved. Wet sterilization of the white pepper irreversibly inactivates its lipolytic activity. Dehydration, destroyed while remoistening, recovered the catalytic activity of the pepper. The reaction obeys classical Michaelis-Menten kinetics at 85°C.
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KEY WORDS: Enzyme, goose fat, lipolytic activity, organic solvent, white pepper.

White pepper powder, used in the food industry as a spice, is prepared from the kernels of the fruit *Piper nigrum.* The kernels are gathered and dried after they have just turned slightly yellow. White pepper can yield about 1.5% volatile oil and about 7% oleoresin (1). This spice, in its powder form, is added as a flavor to goose fat, which serves as the pasteurizing medium in the liver pâté industry. Pasteurizing is performed at 70°C for approximately 30 min. There were many complaints regarding the development of off-flavors and the release of free fatty acids from stored goose liver pâté pasteurized in goose fat that contains white pepper. A previous report (2) indicated that lipolysis of goose fat is the main cause for the undesirable flavors, and that the source of the enzyme may be in the added white pepper.

A rational approach raises the obvious question: Does lipolytic activity take place at these extreme conditions of temperatures and water content? In nature, enzymes operate in an aqueous environment. The chemical and physical properties of enzymes depend on water, which participates either directly or indirectly in the enzymatic reaction, as well as in stabilizing the enzyme conformation (3). Water is required for most enzyme inhibition processes, in particular those that involve thermal inactivation. However, there is some experimental evidence that some enzymes are catalytically active in organic solvents (4–6). Thus, the controversy is not whether water is needed, but how much water is needed.

In the present study, we described the catalytic activity possessed by white pepper at different conditions of temperature and pH when added to the goose fat that provided the organic environment.

MATERIALS AND METHODS

Commercial white pepper kernels were purchased in a local food market. The kernels were grained through a 25-mesh sieve. Water content in the powdered white pepper was determined by toluene distillation (7). Lipid content of the white pepper was determined gravimetrically by hexane extraction in a Soxhlet apparatus.

Frozen abdominal goose fat tissue was purchased in a local butcher shop. The fat was cut into small pieces and melted over boiling water. The melted fat was kept at 95°C for 15 min and then filtered twice through cotton cloth and finally through Whatman No. 1. filter paper. The fat was then frozen and kept at –20°C until use. Water content in goose fat was determined gravimetrically by drying a sample in an oven at 30°C overnight under vacuum.

Fatty acid composition of the goose fat and the white pepper lipid was determined by gas–liquid chromatography (GLC) after methylation according to Mehlenbacher (8). The analysis was carried out on a Hewlett-Packard (Avondale, PA) gas chromatograph with a flame-ionization detector. The detector and injection port temperatures were maintained at 250 and 230°C, respectively. A stainless-steel column, 200 cm × 4 mm (inside dimensions), packed with 10% SP-2330 on 100/120 mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA) was used to quantitate the individual fatty acid methyl esters. The carrier gas was nitrogen with a flow rate of 34 mL/min. The column temperature was maintained for the first 16 min at 200°C and then raised to 220°C (40°C/min) for 26 more min. Peak areas were measured with a Hewlett-Packard 3696 A integrator. Peaks identifications were made by comparing the retention times of the samples with those of methyl ester standards (Sigma Chemical Co., St. Louis, MO) examined under identical conditions.

Goose fat, before and after lipolysis, and white pepper lipids were fractionated to their lipidic components by thin-

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layer chromatography (TLC) on a silica gel plate with a mixture of petroleum ether, ether, and acetic acid (80:20:1.5) as the running solvent. The lipidic components were scraped off the TLC plates, dissolved in chloroform, filtered through a Whatman No. 1 filter paper, evaporated to dryness, and methylated for fatty acid composition determination.

Measuring the lipolytic activity of white pepper in goose fat at different temperatures was carried out as follows: 5 g of goose fat and 0.5 g of powdered white pepper were placed in a 125-mL stoppered Erlenmeyer flask and heated in a shaking bath (200 strokes/min) for 2 h. At the end of the incubation period, 25 mL ethanol was added, and the solution was titrated immediately with 0.1 mol/L NaOH and phenolphthalein as an indicator. The concentration (%) of free fatty acids (acid value) formed during incubation was used to express lipolytic activity. Goose fat treated under the same conditions, but without added white pepper, served as the control. The influence of incubation time was studied following the determination of the optimal temperature for lipolytic activity.

To examine the influence of pH on white pepper lipolytic activity, 5 g of white pepper was suspended in 10 mL of buffer at different pHs and stirred for 1/2 h. Buffers ranging from pH 3–7 were prepared by mixing 0.1 mol/L citric acid with 0.1 mol/L dibasic sodium phosphate solutions. Buffers ranging from pH 7.5–10 were prepared from 0.1 mol/L Tris–HCl. After thorough mixing, the suspension was dehydrated in an oven at 30°C overnight under vacuum. Distilled water was added to the dehydrated pepper to obtain the original water content of the untreated white pepper powder. Five grams of fat and 0.5 g of treated white pepper were used for the determination of enzyme lipolytic activity at 85°C, as described previously. Kinetic parameters were obtained by reacting various amounts of goose fat with 0.5 g white pepper in 50 mL isooctane at 85°C for 2 h.

All the experiments were carried out in triplicate, and the results are the mean of three determinations.

RESULTS AND DISCUSSION

The natural lipolytic reaction of lipase is hydrolysis of carboxylic esters of fats to give free fatty acids, partial glycerides, and glycerol. This reaction involves water as a substrate. The reaction is reversible, and the enzyme can catalyze transesterification (which does not involve water as substrate) of glycerol and free fatty acids to form triglycerides (9). Under conditions in which the amount of water in the reaction system is restricted, hydrolysis of the fat can be minimized so that lipase-catalyzed interesterification becomes the dominant reaction (9). Enzymatic reactions in hydrophobic conditions should fulfill the following requirements: (i) the enzyme should be cofactor-independent because common cofactors are insoluble in organic solvents; (ii) substrates should be soluble in the organic media; and (iii) water should be a nonreactant in the enzymatic process (10). Lipase meets all these conditions.

In this work we studied the lipolytic activity of white pepper under hydrophobic conditions. In this reaction system, the only available water is that which occurs naturally in the fat and in the white pepper. Water levels in white pepper and goose oil were 11.5 and 0.12 g/100 g, respectively. Water content in the studied white pepper is in agreement with previous reports (1).

TLC lipid analysis of the goose fat revealed that most of the fat is composed of triglycerides. Traces of mono- and diglycerides were also observed. No free fatty acids were observed in the fresh goose fat or in the fat control samples. Fatty acid composition of the fresh goose fat and its lipid components is presented in Table 1. A similar fatty acid profile was observed by Nir *et al.* (11). Following lipolytic activity of white pepper, a fraction of free fatty acids was observed after TLC fractionation. The composition of the free fatty acid and triglyceride fractions of goose fat after lipolysis at 85°C for 2 h are also presented in Table 1. The main fatty acid in goose fat and its fractions is oleic acid (18:1), which accounts for approximately 50% of the fatty acids. Following lipolytic activity, the composition of the free fatty acid fraction differed from that of the TG fraction. The main fatty acids found in the free fatty acid fraction were oleic (18:1), 34 g/100 g; linoleic (18:2), 30 g/100 g; and linolenic (18:3), 17 g/100 g. Because unsaturated fatty acids are more susceptible to oxidative deterioration, development of undesirable off-flavors and tastes in industrial goose fat may be explained by the oxidation and rancidity processes of unsaturated fatty acids produced by the lipolytic activity of white pepper. The lipid content of white pepper was 4.5 g/100 g as measured by hexane extraction. GLC analysis of the oil indicated that myristic acid (C:14) was the longest fatty acid chain. Thus, we concluded that the lipids of the white pepper contributed little to the fatty acid composition of the goose fat.

The effect of temperature on the formation of free fatty acids, when white pepper is added to goose fat, is shown in Figure 1. No reaction took place in the absence of white pepper at any temperature. The main lipolytic activity was found in the range of 70–90°C with a peak at 85°C. Minor activity was also observed at 55°C. Measuring the lipolytic activity at

TABLE 1
Fatty Acid Composition (g/100 g) of Goose Fat and Its Fractions Before and After Lipolytic Activity of White Pepper[a]

	Fresh			After lipolysis	
	Whole	TG	MG + DG	TG	FFA
14:0	0.27	0.36	0.60	0.43	0.49
16:0	19.70	19.50	24.40	20.57	7.29
16:1	4.02	4.25	—	3.58	8.61
18:0	5.05	5.27	4.25	5.16	1.47
18:1	54.64	53.85	51.03	52.03	34.36
18:2	12.90	12.43	19.70	14.66	30.86
18:3	3.41	4.32	—	3.55	16.90

[a]For 2 h at 85°C. TG, triglycerides; MG, monoglycerides; DG, diglycerides; FFA, free fatty acids.

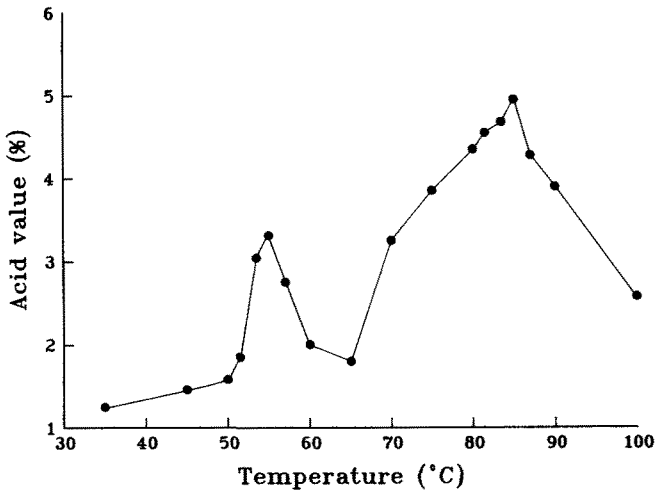

FIG. 1. The effects of temperature on the lipolytic activity of white pepper in goose fat. The ratio of white pepper to goose fat was 1:10 (w/w) and reaction time was 2 h.

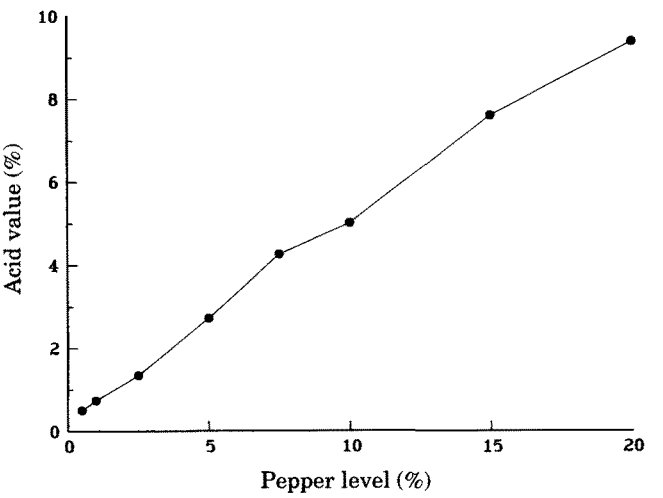

FIG. 3. The effect of white pepper levels on the lipolysis of goose fat at 85°C for 2 h.

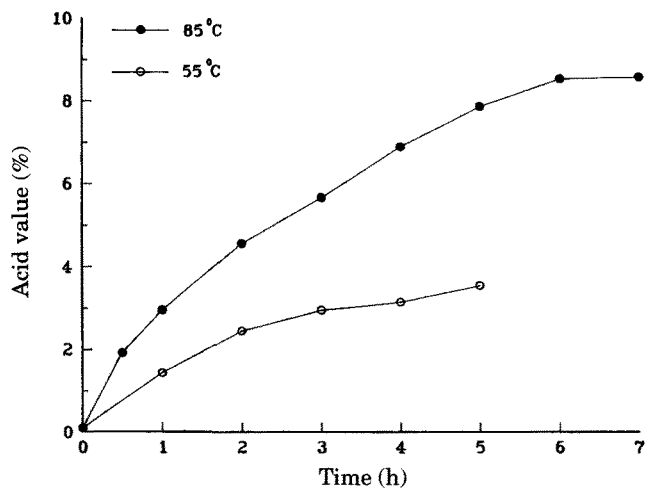

FIG. 2. The time course of white pepper lipolytic activity in goose fat at 55 and 85°C. The ratio of white pepper to goose fat was 1:10 (w/w).

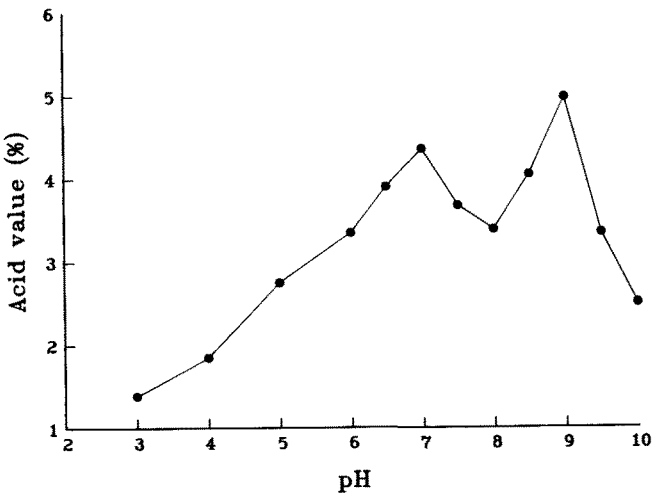

FIG. 4. The dependence of lipolytic activity of white pepper in goose fat on the pH of the buffer in which the white pepper was suspended. The ratio of white pepper to goose fat was 1:10 (w/w), and the reaction was performed at 85°C for 2 h. The white pepper was treated as described in the Materials and Methods section.

these temperatures demonstrated that enzymatic activity was present even after 7 h at 85°C (Fig. 2). This prolonged duration of enzymatic activity was also observed in pancreatic lipases operating in organic solvents (12).

Examining the influence of increasing the concentration of white pepper powder in goose fat on the formation of free fatty acids revealed that lipolytic activity increased linearly from 0.5 up to 20 g white pepper/100 g oil (Fig. 3).

The lipolytic activity at 55°C disappeared after heating the white pepper in goose fat for 2 h at 85°C. Furthermore, no lipolytic activity at either temperature was observed after steam sterilization of the white pepper at 121°C for 20 min. Removing the water from the white pepper by vacuum dehydration reduced its lipolytic activity by 75% at both temperatures. However, catalytic activity was almost completely restored by remoistening the powder to its original water content.

All enzymatic reactions in aqueous solutions are strongly pH-dependent. This phenomenon raises important aspects concerning the involvement of pH in the activity of enzymes in an almost anhydrous organic system. In the present investigation, we exposed the white pepper to various buffers of different pHs. The pepper was then dehydrated under vacuum and remoistened to its original water content. The enzymatic activity of the treated pepper was assayed in goose fat as a function of pH at 85°C for 2 h. The catalytic activity of the white pepper depends greatly on the pH of the buffer with which the white pepper was treated. Figure 4 demonstrates that maximum activity was found at pH 9, although a minor activity peak was also observed at pH 7. It appears that the enzyme memorizes the pH of the last buffer to which it has been exposed. Similar results were obtained by Zaks and Klibanov (10) with pancreatic lipase activity in organic sys-

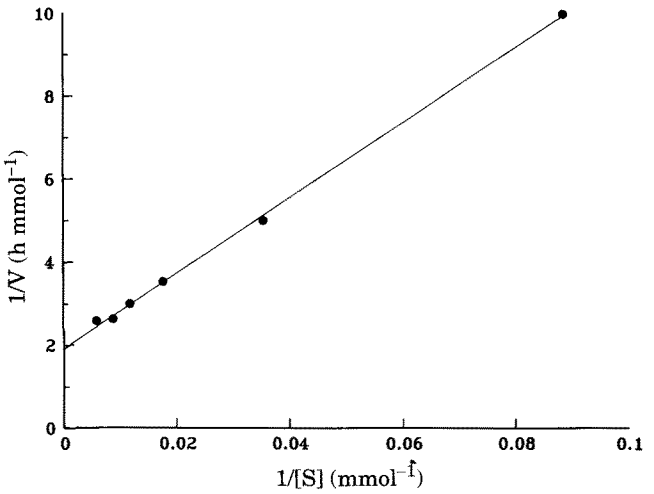

FIG. 5. Kinetic behavior of the lipolytic reaction catalyzed by white pepper in goose fat dissolved in isooctane. Conditions are described in the Materials and Methods section.

tems. These authors suggested that the enzyme's ionogenic groups acquire the corresponding ionization states, which then remain both in the solid state and in organic solvent (10). Our observation points out that the white pepper contains a neutral and an alkaline lipolytic enzyme. Alkaline lipases are known to exist in different plants and germinating oilseeds (13,14), and acidic lipases have been found in palms (15).

Anhydrous conditions, such as those that exist in organic systems, impose unnatural environments upon enzymes. Thus, we wondered whether enzymes in these extreme conditions exhibit the well-known kinetics that follow Michaelis-Menten equations. Figure 5 presents the kinetic pattern of the lipolytic activity of white pepper in isooctane containing goose fat as substrate. Because goose fat contains almost exclusively triglycerides and the predominant fatty acid is oleic acid, we considered goose fat as a substrate to have a molecular weight of 885.4. The kinetic performance of white pepper in isooctane at 85°C followed the Michaelis-Menten kinetics with calculated constants of Km = 47.55 mmol/L and V_{max} = 0.84 mmol/(L • h).

These observations suggest that an enzymatic activity is involved in the production of free fatty acids in goose fat, and that at least two enzymes or isoenzymes with different optimal pH and temperature may exist in white pepper. Commercial manufacturing of white pepper powder involves soaking of the kernels in water for about a week to soften and loosen their outer skin (1). This procedure might serve as a useful tool to decrease the pepper lipolytic activity if a buffer with a low pH is used instead of water. In addition, inactivation of the lipolytic of white pepper can be achieved by wet sterilization. Reducing the water content of the white pepper is less effective because a residual catalytic activity is still present.

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