A METHOD FOR MEASURING THE LIPASE ACTIVITY OF CHEDDAR CHEESE

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SUMMARY

A method is described for the direct estimation of lipase activity in cheddar cheese, using the Conway microdiffusion method to measure the fatty acid liberated by hydrolysis of tributyrin.

I. INTRODUCTION

A method was described by Peterson, Johnson, and Price (1948) for the direct estimation of the lipase activity of cheddar cheese. However, other workers (Lubert, Smith, and Thornton 1949; Stadhouders and Mulder 1957) have failed to reproduce the results of Peterson and his co-workers. No satisfactory method for the direct measurement of cheese lipase activity has been published, and, because of this, workers studying hydrolysis in ripening cheese have used the changes in fatty acid concentration to indicate lipase activity. It is considered that this approach may lead to false conclusions, and it was the purpose of this work to develop a method for the direct estimation of the lipase activity of cheddar cheese.

II. METHODS AND RESULTS

(a) Estimation of Lipase Activity

Ten grams of the cheese to be analysed was ground with a pestle and mortar, and mixed to a smooth suspension with 20 ml of cold distilled water. This suspension was centrifuged for 5 min at 3000 r.p.m. in a "Christ" centrifuge (Junior 0 model), using a 6 x 20 ml angle-head. The fat layer and the aqueous layer were removed and discarded. The protein sediment was weighed wet in the tube. No attempt was made to dry the sediment. The supernatant liquid was simply poured out of the tube and the tube shaken to remove any remaining drops of liquid. The wet protein sediment was then suspended in twice its weight of cold distilled water. Vigorous shaking was avoided, since it may inactivate the enzymes. For the measurement of lipase activity, a volume of the protein suspension was taken which was, in millilitres, numerically equal to the weight, in grams, of the original protein sediment (wet). This sample was transferred to a glass-stoppered 100 ml Erlenmeyer flask. To the flask were added 0·1 ml tributyrin (British Drug Houses Ltd.), 15 ml 1 : 5,000 "Merthiolate" solution (Lilly & Co.), and distilled water to bring the total

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volume to about 12 ml. The contents of the flask were transferred to a beaker, and, using a magnetic stirrer and a Jones model B pH meter, the pH of the mixture was brought to 7·5 with 0·1N NaOH. The mixture was returned to the glass-stoppered flask and the volume made up to 15 ml with distilled water. The flasks were incubated at 30°C for 24 hr.

After incubation, the butyric acid liberated by hydrolysis of the tributyrin was estimated by microdiffusion. The contents of the flasks were transferred to centrifuge tubes and acidified with 2 ml of 20% (v/v) H_2SO_4. The tubes were closed with rubber stoppers and centrifuged for 3 min at 3000 r.p.m. in the "Christ" centrifuge previously described. For microdiffusion, 0·5 ml samples of the supernatant liquid were used. These estimations were done in duplicate.

The method used for the estimation of butyric acid by microdiffusion was that of Conway (1950, p. 246). The Conway units were incubated at 37°C for 18 hr. The contents of the outer chamber were then transferred to a 10 ml beaker, using a Pasteur pipette. The chamber was washed twice with about 1 ml distilled water, and the washings added to the beaker, making a total volume of about 4 ml for titration. Three drops of neutral phenolphthalein (0·1% in alcohol) were added, and the solution titrated with $\frac{N}{50}$ NaOH, using an "Agla" micrometer syringe (Burroughs Wellcome).

The difference between the titration of the test sample and the sum of the titrations for the two blanks allows calculation of the amount of butyric acid liberated by enzyme action on the tributyrin.

Note.—Modifications have been made to this method, viz. (*a*) the pestle and mortar used for the preparation of the sample have been replaced by a tissue grinder, this being a rotating plunger in a ground glass tube, and (*b*) the "Christ" centrifuge has been replaced by a high-speed refrigerated centrifuge. However, sufficient results using these modifications are not available to report at present.

(b) Effects of Variations in the Method

(i) *Lipase Activity of Various Cheese Fractions.*—In order to obtain some degree of concentration of the enzyme, an experiment was performed comparing the lipase activity of the three fractions which are produced by centrifugation in the "Christ" centrifuge at 3000 r.p.m. for 5 min. The results for the three fractions are given in Table 1.

It is clear from these results that the lipase activity is concentrated in the protein sediment. This fraction, therefore, was used throughout the work for the measurement of lipase activity in cheese.

TABLE 1

COMPARISON OF LIPASE ACTIVITY OF THREE FRACTIONS OBTAINED BY CENTRIFUGATION OF CHEESE SUSPENSION AT 3000 R.P.M. FOR 5 MIN

Fraction	Lipase Activity*
Fat	0·0031
Supernatant	0·0000
Protein	0·0240

* ml $\frac{N}{50}$ NaOH 24 hr at 32°, pH 7·0.

The weight of protein sediment from a constant weight of cheese varies with the age of the cheese. Therefore it was necessary to take a variable sample, depending on the weight of the protein sediment, such that it always represented a constant weight of cheese. The sample taken as described in the method represents 3·33 g of cheese.

(ii) *Effect of Preservatives.*—Since bacteria could develop and cause appreciable hydrolysis during the long incubation period, it was necessary to find a bacteriostatic agent which did not affect the lipase activity. A comparison was made of the efficiency, in this respect, of thymol and merthiolate. The results are given in Table 2.

TABLE 2

VIABLE MICROBIAL COUNTS ON DIGESTION MIXTURE BY POUR-PLATE METHOD USING GLUCOSE TRYPTONE YEAST EXTRACT AGAR INCUBATED AT 30° FOR 48 HR

Agent	Viable Count per ml		
	Zero	8 hr	24 hr
Thymol	7×10^5	6×10^5	24×10^7
	8×10^5	8×10^5	32×10^7
	4×10^5	2×10^5	18×10^7
Merthiolate 1 in 10,000 ..	54×10^4	16×10^3	35×10^2
	46×10^4	26×10^3	13×10^3
Merthiolate 1 in 50,000 ..	27×10^5		8×10^5
	12×15^5		15×10^5
	84×10^4		92×10^4

The results show that thymol is not satisfactory, while merthiolate, at a final concentration of 1:50,000, is just adequate to maintain the microbial population at a constant level.

It should be pointed out that the common bacteriostatic agents, such as chloroform and toluene, could not be used due to their solvent properties, which would interfere with the substrate availability.

(iii) *Effect of pH and Temperature of Incubation.*—Although a complete "optimum pH" experiment was not made, it was shown that the lipase activity was higher at pH 7·5 than at 6·5 or 8·5. The results are given in Table 3.

TABLE 3

EFFECT OF pH ON LIPASE ACTIVITY
(Incubated for 24 hr at 32°)

pH	Lipase Activity*
6·5	·0073
7·5	·0177
8·5	·0113

* ml $\frac{N}{50}$ NaOH.

Since the lipase activity of cheese is very low, it was deemed necessary to measure it under optimal conditions, rather than under the conditions existing in the cheese. For this reason, also, the incubation temperature used was 30°C.

(iv) *Butyric acid Estimation.*—Using the microdiffusion technique of Conway (1950), it was found possible to recover, in the final titration solution, 97–100 per cent. of amounts of butyric acid added to suspensions of cheese. However, preliminary experiments showed that, in order to obtain these recoveries, it was necessary to remove the protein from the mixture by centrifugation. The results are given in Table 4. Butyric acid was added to give a final concentration of 0·001 M.

TABLE 4

EFFECT OF THE PRESENCE OF PROTEIN (INSOLUBLE) ON DIFFUSION OF BUTYRIC ACID (CONWAY METHOD)

—	Volume of Suspension (ml)	Recovery (%)
Protein removed ..	6	94·1
	3	98·8
	1	98·8
Protein present ..	6	51
	2	89
No cheese added ..	..	100

Therefore, the digestion mixture was centrifuged after acidification, and 0·5 ml samples taken from the supernatant for estimation of butyric acid by the Conway technique. The Conway units were incubated at 37°C, rather than at room temperature, in order to get complete diffusion within 18 hr. At this temperature a fixative of firmer consistency is required. This was prepared by mixing equal quantities of soft white paraffin and solid paraffin wax.

(c) Lipase Activity of Ripening Cheddar Cheese

The method described was used to measure the lipase activity of several cheddar cheeses of various ages. The results are expressed as ml x 10^2 of 0·02N NaOH. Some of these results are presented in Table 5.

TABLE 5

CHANGES IN LIPASE ACTIVITY OF RAW AND PASTEURIZED MILK CHEDDAR CHEESE DURING RIPENING

Pasteurized Milk Cheese			Raw Milk Cheese		
Age (weeks)	No. of Samples	Average Values*	Age (weeks)	No. of Samples	Average Values*
3–5	7	0·04	3–5	4	0·78
9–10	4	0·40	9–10	4	1·68
13–14	6	2·00	15–16	4	4·22
17–18	6	1·31	19–20	4	1·77
20–22	4	1·64	25–26	3	1·40
26–28	3	1·25			

* Expressed in ml x 10^2 of 0·02N NaOH. Represents mean of the lipase activities of the cheeses in each age group.

The results show variations in the lipase activity of cheddar cheese during the ripening period. The average lipase activity throughout the first 100 days of ripening is approximately 1 unit as measured by this method.

III. DISCUSSION

The object of this study was to develop a method for the direct estimation of lipase activity in cheddar cheese. The hydrolysis produced by the method of Peterson, Johnson, and Price (1948) within an incubation period of 1 hr would suggest that a very high concentration of free fatty acids would result over a ripening period of several months. Since this is not the case, and since other workers could not reproduce the results using this method, it would appear that it is not a reliable measure of lipase activity in cheese. Lubert, Smith, and Thornton (1949) were unable to detect any lipase activity in cheese, and, since then, studies of lipolysis in ripening cheese have been restricted to, or based upon, measurements of the free fatty acids present. The failure to detect any lipase activity has probably been due to insufficiently long incubation periods, and to methods lacking the sensitivity required to estimate very small amounts of fatty acid.

The method described in this present report is capable of measuring concentrations of butyric acid below 0·5 mM. Using incubation periods of 24 hr, it has been possible to detect lipase activity in samples of cheddar cheese. The amounts of free fatty acid which an activity of the degree observed in this study would be expected to produce may be estimated by assuming an average activity, throughout the ripening period, of 1 unit. In 1 g of cheese, this would yield free fatty acid equivalent to 0·1 ml of 0·02N NaOH in 24 hr. When extended over a ripening period of 100 days, this would produce, in 1 g of cheese, amounts of free fatty acid equivalent to c. 2 ml of 0·1N NaOH. This concentration is of the order of that which is actually present in ripened cheddar cheese.

It is concluded, therefore, that the method described in this paper is more satisfactory for the estimation of lipase activity in cheese than is that of Peterson, Johnson, and Price (1948). Using this method it should be possible to measure the lipase activity of a cheese at any stage during ripening, and to determine the characteristics of the enzyme systems involved, and further work in this regard is now being undertaken.

IV. ACKNOWLEDGEMENT

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