The leaves of tomato, banana and red beet show low levels of waxiness and cutin (<50 μg./cm.²). Cauliflower and cabbage leaves show little cutin, but are considerably waxy. The leaves of different apple varieties carry 30–70 μg./cm.² of surface waxy substances and 70–120 μg./cm.² of cutin. Rhododendron and laurel leaves show higher degrees of waxiness and cutin deposition of about 250 and 450 μg./cm.² respectively. The ornamental *Euonymus japonicus* leaf is exceptional in having little surface wax (20 μg./cm.²) and 600 μg./cm.² of cutin. More cutin has invariably been found in the upper than in the lower leaf surface.

The blackcurrant fruit cuticle carries about 150 μg./cm.² of waxy materials and 150 μg./cm.² of cutin. Apple fruits of different varieties differ considerably in skin thickness; surface waxy deposits vary from 600 to 1200 μg./cm.² and cutin is present to the extent of 750–1000 μg./cm.² As the tomato fruit develops, outer layers of cells become progressively impregnated with cutin and in this way substantial skins, containing about 1000 μg./cm.² of cutin, are formed.

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STUDIES ON CASEIN. III.*—Preparation of a Carbohydrate-rich Fraction and a Calcium-sensitive Fraction from α-Casein†

By M. E. Q. PILSON, G. O. HENNEBERRY and B. E. BAKER

α-Casein was fractionated by the addition of calcium chloride to an α-casein solution (pH 7) which had previously been kept for 45 min. at pH 12. One fraction (Fraction A) accounted for 13·4% and another (α-casein — Fraction A) for 56·7% of the original α-casein.

The sugar contents (anthrone) of Fraction A, and α-casein — Fraction A, were 3·94 and 0·68 mg./g., respectively, and the hexosamine contents were 4·58 and 0·68 mg./g., respectively.

The product α-casein — Fraction A was precipitated from aqueous solution (pH 7) by the addition of calcium ion whereas Fraction A was not precipitated under the same conditions. No precipitate was formed on addition of 0·1M-calcium chloride solution to a solution containing 3 parts of α-casein — Fraction A to one part of Fraction A but a precipitate was formed if Fraction A was pretreated with rennin.

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Introduction

In 1939 Mellander[1] applied the classical Tiselius electrophoretic technique[2, 3] to the separation of the components of the casein complex. He obtained three distinct electrophoretic components designated α-, β- and γ-casein in order of decreasing mobility. Warner[4] described a method for the isolation of α- and β-casein which is based on the higher solubility of the latter in water at pH 4·4 and at a temperature of 2°.

Hipp *et al.*[5] described two methods for the separation of the three components of casein in quantity, viz., (1) based on differences in their solubility in 50% alcohol in the presence of salts, and (2) based on the solubility in aqueous urea solution at the isoelectric point of casein.

Von Hippel & Waugh[6] described a procedure for the preparation at constant pH and at reduced temperature, of a mixture of α- and β-casein. The method involved the addition of calcium chloride to skim milk, the isolation of the micelles by high-speed centrifugation and the removal of calcium from the micelle by precipitation with potassium oxalate followed by dialysis. The casein was recycled through the same process and the resultant second-cycle casein was a mixture of α- and β-casein in approximately the same proportions in which they occur in skim milk.

MacRae & Baker[7, 8] separated the α-, β- and γ-components of the casein complex by filter-paper electrophoresis. The papers were stained for carbohydrates according to the method of Koiw & Gronwall[9] and staining was observed only at the position of the α-casein band. In a subsequent paper, Reynolds *et al.*[10] reported that the total sugar contents of α-, β- and γ-casein were 1·5, 0·8 and 0·9 mg./g. respectively. Individual sugars were determined by filter-paper chromatography and it was observed that α-casein contained mannose and galactose and β- and γ-casein contained only galactose. These sugars accounted for about half the sugar content of the three casein fractions as determined by the anthrone method.

The present paper deals with the isolation of a fraction from α-casein (Warner), which is relatively high in sugar content and possesses some of the properties of the κ-casein prepared by Waugh & Von Hippel.[11]

Experimental

Materials

Whole casein.—This was prepared from a composite sample of milk (Macdonald College herd) by the method of Warner.[4]

α-Casein.—This was prepared from whole casein by the method of Warner.[4]

Fraction A.—α-Casein (33·5 g.) was suspended in distilled water (1250 ml.). Aqueous NaOH (1N) was added slowly to the suspension until pH 12 was reached. The resultant solution was kept at 25° for 45 min. and then HCl (3N) was added slowly until the pH was 7·0. The total volume of the casein solution at this point was approximately 2000 ml. Sufficient calcium chloride solution was added to the casein solution to adjust the solution to 0·25M with respect to calcium ion. The mixture was set aside for 14 h. at 4° and the precipitate (P_1) then recovered by centrifugation. The supernatant was dialysed for 5 h. against five changes each of 2000 ml. of distilled water at 4°. HCl (0·1N) was added to the dialysed solution until the pH was 4·5. The product, which was recovered by centrifugation, was washed with water and then with alcohol and with acetone. The weight of air-dried material (P_2) was 6·0 g.

The product (P_2) was suspended in distilled water (400 ml.) and NaOH (1N) added until the pH was 12. The resultant solution was kept at 25° for 45 min. and then HCl (3N) was added slowly until the pH was 7·0. Sufficient calcium chloride was then added to adjust the solution at 0·25M with respect to calcium ion. The resultant solution, which was opalescent, was filtered and then dialysed for 5 h. against four changes of distilled water (each 12,000 ml.) and then for 5 h. against 2000 ml. of 0·5% Versene solution (Bersworth Chemicals Co., Framingham, Mass.) (pH 8·5, 4°) and against two changes of distilled water each of 12,000 ml. at 4°. HCl (0·1N) was added to the dialysed solution to give pH 4·5. The precipitate was recovered by centrifugation and was washed with water and then with alcohol and with acetone. The weight of air-dried material (Fraction A) was 4·5 g. (13·9% N).

α-Casein — Fraction A.—The precipitate P_1, which was obtained in the preparation of

Fraction A, was suspended in distilled water (1000 ml.). Versene (Bersworth Chemicals Co., Framingham, Mass.) (10 g.) was added and then NaOH (0·2N) to give pH 8·5. The resultant solution was dialysed for 5 h. against four portions of distilled water (12,000 ml. each, 4°) which had been adjusted to pH 8·5 with NaOH, and against two changes (12,000 ml. each, 4°) of distilled water. The dialysed solution had pH 8·0 and this was adjusted to pH 4·5 by addition of 0·1N-HCl. The precipitated casein was recovered by centrifugation and then washed with water, alcohol and acetone. The weight of the air-dried product (P_3) was 22·0 g.

The precipitate (P_3) was subjected to the same treatment as had been used in the preparation of Fraction A and the resultant product was further purified by the method used for precipitate P_1. The weight of air-dried material (α-casein — Fraction A) was 19·0 g. (13·9% N).

Results and discussion

Carbohydrate and phosphorus contents of the casein fractions

Total sugars, galactose, mannose and glucose, and hexosamine, were determined by the methods described by Reynolds *et al.*,[10] and phosphorus by the method of Martin & Doty.[12] Table I gives the results of the analyses.

Table I

Carbohydrate and phosphorus contents of α-casein fractions[a]

Sample	Phosphorus	Total sugar (anthrone)	Hexosamine	Galactose	Mannose	Glucose
	%	mg./g.	mg./g.	mg./g.	mg./g.	mg./g.
Fraction A	0·92	3·94	4·58	2·75	0·51	0·00
α-Casein — Fraction A	1·38	0·68	0·68	0·00	0·00	0·00
α-Casein	1·22	1·51	2·11	0·75	0·48	0·00

[a] Based on 15% N

It will be observed that the total sugar figure for Fraction A was about six times that for α-casein — Fraction A as determined by the anthrone method, and the hexosamine content of Fraction A was nearly seven times that of α-casein — Fraction A. The fact that no glucose or mannose was detetected in α-casein — Fraction A by filter-paper chromatography, whereas the anthrone method revealed the presence of 0·68 mg./g. of sugar, would suggest that α-casein — Fraction A contained sugars other than galactose or mannose.[13]

Paper electrophoresis of the casein fractions

Samples of α-casein, Fraction A and α-casein — Fraction A were subjected to filter-paper electrophoresis.[7] Single bands of identical mobilities were obtained with the separate fractions, and with a mixture of Fraction A and α-casein — Fraction A (1 part Fraction A to 3 parts α-casein — Fraction A).

Behaviour of casein fractions towards calcium ions

1% solutions of Fraction A and α-casein — Fraction A were prepared by the slow addition of 0·2N-NaOH to aqueous suspensions of the protein until pH 7·0 was reached. The solutions were then diluted with distilled water to the appropriate volumes. The solutions were then examined in order to ascertain the behaviour of the casein fractions toward calcium ions.

When equal volumes of Fraction A solution and 0·1M- or 0·5M-calcium chloride solutions were mixed, an opalescent solution was formed but there was no precipitate. α-Casein — Fraction A solution gave an immediate, heavy, white, stringy precipitate on addition of calcium chloride. The addition of trichloroacetic acid to the supernatant gave no further precipitation.

A mixture of 3 parts of α-casein — Fraction A solution and 1 part of Fraction A solution turned milky when mixed with an equal volume of 0·1M-calcium chloride solution, but with 0·50M-calcium chloride a precipitate formed and the supernatant was opalescent. A mixture of α-casein — Fraction A solution and Fraction A solution (4 parts α-casein — Fraction A to 1 part Fraction A) gave a milky solution and slight precipitation when mixed with an equal volume of 0·1M-calcium chloride solution.

Waugh & Von Hippel[11] showed that the addition of calcium ion to second-cycle casein, at concentrations markedly lower than those found in skim milk, led to formation of a coarse heavy precipitate. It will be noted that α-casein — Fraction A behaved in a similar manner. Also it may be noted that the fraction designated in the present paper as Fraction A yielded an interaction product with α-casein — Fraction A which formed stable micelles on the addition of calcium ion as did κ-casein.[11]

Behaviour of casein fractions towards rennin

These experiments were performed with 1% protein solution at pH 6·0 and commercial rennin extract (Canada Packers Ltd.). The rennin extract was added to the protein solutions in amounts to give a dilution of 1 part in 5000.

Equal volumes of Fraction A solution and 0·1M-calcium chloride solution were mixed and then rennin was added. No change was observed for the first 10 min. and then a white precipitate slowly separated in the reaction mixture. No change was observed when rennin was added to the α-casein — Fraction A solution in the absence of calcium chloride.

A solution containing 3 parts of α-casein — Fraction A to 1 part of Fraction A was mixed with an equal volume of 0·1M-calcium chloride solution. Rennin was then added to the resultant milky solution. In 2–5 min. a precipitate began to form and after 10 min. most of the protein had precipitated. The experiment was repeated except that the calcium chloride solution was added to the solution of α-casein — Fraction A and Fraction A which had been set aside for 10 min. after the addition of rennin. A precipitate appeared immediately on the addition of the calcium chloride.

A mixture of α-casein — Fraction A solution and rennin was kept for 10 min. A sufficient volume of Fraction A solution was then added to give a ratio of α-casein — Fraction A to Fraction A of 3 : 1, followed by an equal volume of 0·1M-calcium chloride solution. A precipitate began to form after 3–5 min. This experiment was repeated, except that rennin was allowed to act on Fraction A for 10 min. before the addition of the α-casein — Fraction A and the calcium chloride solution. A large precipitate formed immediately on the addition of the α-casein — Fraction A and the calcium chloride to Fraction A which had been treated with rennin.

These experiments show that the stable micelle formed when calcium ion is added to a mixture of α-casein — Fraction A and Fraction A will precipitate in the usual way on the addition of rennin. If Fraction A is first treated with rennin and then added to α-casein — Fraction A in an amount which would normally prevent the precipitation of α-casein — Fraction A by 0·1M-calcium chloride, it will be found that Fraction A has lost its stabilising effect.

It is of interest to note that the fractions designated in the present paper as Fraction A and α-casein — Fraction A are similar in their behaviour to Fraction S and second-cycle casein described by Waugh & Von Hippel.[11]

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PHOSPHOLIPID HYDROLYSIS IN COD FLESH STORED AT VARIOUS TEMPERATURES

By JUNE OLLEY and J. A. LOVERN

Cod were stored at 20, 0, −14, −22 and −29°, samples being withdrawn at intervals, the lipids extracted and analysed for free fatty acids (FFA) and phosphorus. Chloroform or toluene vapour ultimately inhibited enzymic liberation of FFA, hence studies at 0 and 20° were with small pieces of flesh obtained aseptically and stored without preservative. Tests with cooked fish showed that non-enzymic hydrolysis is negligible. Comparison of sterile fish at 0° with non-sterile iced fish showed that all phospholipid breakdown in iced cod can be attributed to autolysis. At 0°, but not at the other temperatures, there is a marked initial lag before rapid hydrolysis begins. Only rough comparison is possible from the data available, but the rate of hydrolysis at −14° is about 10 times that at −22°. At 0° hydrolysis proceeds little faster than at −14°, while the rate at 20° is about 3 times that at 0°. Data at −29° are too few even for rough comparison. At all temperatures studied the only products accumulating from phospholipid degradation are FFA and water-soluble phosphorus derivatives. The similar course of phospholipid hydrolysis and of protein denaturation in frozen cod was confirmed and its possible significance is discussed.

Introduction

During prolonged storage of cod in crushed ice, phospholipids in the flesh are degraded to free fatty acids and water-soluble fragments.[1] Under such conditions hydrolysis might be due to enzymes of either bacterial or fish tissue origin, with perhaps some non-enzymic hydrolysis[2, 3] also occurring.

The work reported here was designed partly to provide more information on the cause of phospholipid breakdown in iced cod, and partly to amplify published findings[4] on lipid hydrolysis in frozen cod. Data were also obtained on the course of phospholipid hydrolysis in cod flesh at a relatively high temperature (20°).

Experimental

Bacterial sterility is commonly maintained in enzyme studies by means of chloroform or toluene, but the incubation period seldom exceeds 24 hours or so. It is unlikely that small concentrations of these substances would inhibit non-enzymic hydrolysis, but chloroform is known to produce slow inhibition of phospholipases A[5] and C.[6] Gradual loss of activity in a mixed preparation of phospholipases A and B in the presence of a chloroform–toluene mixture has also been noted,[7] although not attributed to the preservative.

Sterile storage at 0° and 20°

Strict comparison with previous work on iced fish demands periodical withdrawal of three whole fish at a time. It is impossible to set up a storage experiment on this scale in which sterility is obtained and maintained solely by aseptic preparation and precautions. Accordingly chloroform–toluene was used. Small cod, of about 1 kg. when gutted, headed and tailed, were placed three at a time in a series of 12 large cans with well-fitting but not air-tight lids. Six cans were placed completely inside steam baths for 75 min. To each can were added 100 ml.