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Hydrolysis of some Casein Fractions with Plasmin

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Derechin (1961) showed that, when the proteolysis of whole casein is followed by estimating the ultraviolet-absorbing material remaining in solution after treatment of the reaction mixture with perchloric acid, a curve was obtained showing a lag period before a linear portion. The slope of this linear region is proportional to the amount of enzyme present and on this basis an assay for human plasmin was devised. However, when different batches of casein were used for the assay of a single plasmin preparation, apparent differences in activity up to 15% were obtained. These results necessitated a further study of casein to establish firstly the causes for these differences from batch to batch, secondly the reasons for the curve with a lag period and finally to seek for a more suitable protein substrate for plasmin assay.

Casein consists of several electrophoretically distinguishable components (Mellander, 1939), and a procedure for separating these in the presence of urea was described by Hipp, Groves, Custer & McMeekin (1952). From electrophoretic observations made on the protein fractions obtained by this procedure it was suspected that the α -casein precipitable in 4.7M-urea differed from that remaining with the β - and γ -casein in the supernatant solution, and further experiments confirmed

this. Both the components of α -casein were purified and the action of plasmin on each of them was examined.

EXPERIMENTAL

Buffers. Phosphate buffers, pH 8 (65 mM- Na_2HPO_4 -4 mM- KH_2PO_4) and pH 7.6 (63 mM- Na_2HPO_4 -9 mM- KH_2PO_4), were used to dissolve the casein for electrophoresis and for hydrolysis with plasmin respectively. Phosphate-urea: 6.6M-urea in phosphate buffer, pH 8. Phosphate-NaCl soln.: 10% (v/v) phosphate buffer, pH 7.6, in 0.2M-NaCl.

pH determination. Values of pH were determined with a MacInnes & Belcher (1933)-type glass electrode. Measurements were made at room temperature with a valve potentiometer and standardized with HCl-KCl mixture, pH 2.10 (0.01M-HCl-0.09M-KCl).

Electrophoresis. Analyses were carried out in the Tiselius apparatus at 0°, the diagonal schlieren optical system, with monochromatic light λ 546 m μ , being used. Protein concentrations were between 1.0 and 1.75 g./100 ml. The proportion of each protein in a mixture was calculated from its relative area in the electrophoresis pattern.

Ultracentrifugal analysis. Protein solutions in phosphate buffer, pH 8, containing in addition 0.15M-NaCl, were subjected to 250 000g in the Svedberg oil-turbine ultracentrifuge (Svedberg & Pedersen, 1940), with a 12 mm. cell. Optical observations by the diagonal schlieren method (Philpot, 1938) were photographically recorded on Ilford rapid-process panchromatic plates, a high-pressure Hg arc being used as a light source, from which a monochromatic light, λ 546 m μ , was isolated with a suitable filter.

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Purification of whole casein. A solution of casein (40 g. casein; Hammarsten, British Drug Houses Ltd.) was boiled and treated with perchloric acid as described previously (Derechin, 1961). The resulting precipitate was washed four times with water (2 l.) and used as starting material for the preparation of the fractions.

Preparation of α_1 -casein. The purified casein from 40 g. of whole casein was dissolved by addition of water and solid urea to give a concentration of 6.6M-urea in a volume of about 1.5 l. The solution was then diluted to 4.7M-urea with water and the pH adjusted to 4.5 by the slow addition of 0.2M-NaOH. The precipitate that formed was spun down, redissolved in 100 ml. of 6.6M-urea and reprecipitated by dilution to 4.7M-urea; this procedure was carried out three times.

Preparation of α_2 -casein and mixtures with β -casein. The supernatant solution remaining after the initial separation of the α_1 -casein was filtered through a Seitz EK filter to remove traces of suspended material, and then diluted with water to a urea concentration of 1.7M. The precipitate which formed consisted of a mixture of α_2 - and β -casein in the proportions of 55% and 45% respectively. It also contained some γ -casein but the amount of this was small and difficult to estimate.

The α_1 - and β -casein were separated by making use of the difference in their solubility in water at 0–2°, a method used by Hipp *et al.* (1952) to prepare β -casein free from α -casein. The precipitate obtained with 1.7M-urea was dissolved in 50 ml. of phosphate-urea and dialysed at 2–4° for 3 days against three daily changes of 500 ml. of distilled water. The solution was then made up to 400 ml. with water, cooled to 0–2° and the pH adjusted to 4.5 by the slow addition of 0.1M-HCl. The precipitate was removed by centrifuging for 10 min. at 500g at 0°, dissolved in phosphate-urea, dialysed and reprecipitated as described above. In this way, preparations containing an increased proportion of α_2 -casein were obtained. Two preparations of α_2 -casein were made: one reprecipitated five times contained 9% of β -casein and the other reprecipitated four times contained 12% of β -casein.

Preparation of β -casein. The protein remaining in solution in 1.7M-urea was precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to a concentration of about 1.6M. The supernatant solution was siphoned off and discarded. The precipitate contained 75% of β -casein, 15% of α_1 -casein and 10% of γ -casein. This fraction was dissolved in 400 ml. of water by the slow addition of 0.2M-NaOH and then the pH adjusted to 4.5 at 0–2° with 0.1N-HCl. The precipitate that formed was removed by centrifuging and discarded. The supernatant solution was warmed to 40°, giving a precipitate which was dissolved and reprecipitated as described above. The preparation contained 97% of β -casein and 3% of α_2 -casein.

Proteolysis with plasmin. Plasminogen prepared as described by Derechin (1961) was activated to plasmin with a streptokinase (Varidase, Lederle) solution containing 200 units/ml. at room temperature for 10 min. and then added to the casein solution at 40°. At convenient intervals, samples (4 ml.) of this reaction mixture were withdrawn and pipetted into 2 ml. of 1.7M-HClO₄ at room temperature, and shaken at intervals for 20–30 min. until most of the precipitated protein settled spontaneously to the bottom of the tube. The suspension was filtered and the extinction of the filtrate read at 276 m μ in 1 cm. cells with a Unicam

spectrophotometer model SP. 600. A blank was prepared for each curve by taking a sample from the reaction mixture within 1 min. of adding the plasmin to the casein.

RESULTS

Electrophoresis patterns of fractions. The fraction precipitated from casein in 4.7M-urea when examined electrophoretically shows a single peak, and has a mobility at pH 8.0 higher than that of the other components of casein; it is designated here α_1 -casein. The supernatant solution displays two electrophoretic components; the mobility of the faster, designated here α_2 -casein, is slightly less than that of the α_1 -casein (Fig. 1). When these fractions are mixed in suitable proportions three components can clearly be distinguished (Fig. 1d). The difference in mobility between the α_1 - and α_2 -casein run separately is clear (Fig. 1a, b).

In mixtures of the two purified components the separation is less than in mixtures of α_1 -casein with the crude α_2 -, β -casein fraction obtained at 1.7M-urea. This effect may be due to a stronger interaction between α_1 - and α_2 -casein when little or no β -casein is present. A small amount of an unidentified leading component was noticed, but its significance is obscure (Fig. 1d).

Further physical characterization of the α_1 - and α_2 -casein. The α_1 - and α_2 -casein were examined in

Fig. 1. Electrophoresis patterns of casein fractions and mixtures. (a) α_1 -Casein; (b) α_2 -casein; (c) fraction precipitated with 1.7M-urea; (d) mixture of (a) (30%) and (b) (70%); (e) mixture of (a) (30%) and (c) (70%). Run in phosphate buffer, pH 8, *I* 0.2. Photographs were taken after 1 hr. at 10 ma and then 3 hr. at 15 ma (a–c) or 4 hr. at 15 ma (d, e). Protein concentration, 1.0, 1.5, 1.0, 1.5 and 1.5 g./100 ml., respectively.

the ultracentrifuge. Although some impurities were present in the preparations, the main component of α_1 -casein in a solution containing 1 g./100 ml. had a sedimentation coefficient $S_{20,w}$ 4.85, whereas that of the main component of the α_2 -casein (0.95 g./100 ml.) was $S_{20,w}$ 10.6 (Fig. 2). Solutions of α_2 -casein above a concentration of about 3 g./100 ml. are yellowish whereas those of α_1 -casein are colourless.

Proteolysis with plasmin. The interaction of plasmin with various casein fractions is illustrated in Fig. 3. With whole casein the curve with a lag period was obtained and with β -casein a very flat curve, linear at the beginning. A steep straight line passing through the origin was obtained with α_2 -casein and a sigmoid curve with α_1 -casein. In the

Fig. 2. Ultracentrifuge patterns of α_1 - and α_2 -casein. (1) α_1 -Casein (1 g./100 ml.); (2) α_2 -casein (0.95 g./100 ml.). Photographs were taken after 50 min. at 54 000 rev./min. (about 250 000g).

Fig. 3. Proteolysis by plasmin of some casein fractions. $\blacktriangle$, Whole purified casein; $\bullet$, α_1 -casein; $\blacksquare$, α_2 -casein; $\triangle$, β -casein. Protein concentration, 2 g./100 ml. in the reaction mixture. Plasminogen solution (0.12 mg. of N/ml.) was activated as described.

latter case the maximum slope of the curve, which occurs at the inflexion point, was taken as a basis for comparison with other casein preparations.

With mixtures of α_2 - and β -casein, when the proportion of β -casein was greater than 30 %, reaction curves showing a lag period were obtained (Fig. 4). The lag period of these curves is short, probably because of the rather low protein concentration used (2 g./100 ml.). Also, the measurements of extinction were made against a blank taken about 1 min. after addition of the plasmin to casein, a procedure which also shortens the apparent lag period. The slope of the linear portion of these curves decreases linearly as the proportion of β -casein increases (Fig. 4b). The extrapolation of

Fig. 4. Proteolysis by plasmin of mixtures of α_2 - and β -casein. (a) Reaction curves of mixtures containing these percentages of β -casein: $\circ$, 9; $\bullet$, 32; $\triangle$, $\blacktriangle$, 45; $\blacksquare$, 75 and 97. Protein concentration 2 g./100 ml. in the reaction mixture, except for $\blacktriangle$, which contained 3 g./100 ml. Plasminogen solution (0.12 mg. of N/ml.) was activated as described. (b) Plot of the slope of the linear section of the curves/percentage of β -casein. Symbols as identified for (a).

this straight line to the ordinate suggests the slope that the reaction curve of pure α_2 -casein should have with the same enzyme preparation. The extrapolation to the abscissa suggests the proportion of β -casein necessary to block α_2 -casein as a substrate for plasmin. The curves obtained with preparations containing 75% and 97% of β -casein could be superimposed on each other (Fig. 4a). The slope of the linear part of the curve obtained with a mixture of α_2 - and β -casein does not change when the concentration of the total protein in the reaction mixture is raised from 2 to 3 g./100 ml.

The proteolysis of mixtures of α_1 - and α_2 -casein in various proportions gives the same type of results as α_2 - and β -casein mixtures, the slope of the curves decreasing as the proportion of α_1 -casein is raised (Fig. 5). The extrapolation to the abscissa of the linear part of the curve obtained by plotting slope against percentage of α_1 -casein suggests that 75% of α_1 -casein is necessary to block α_2 -casein as a substrate for plasmin. The slope of the reaction mixture containing 75% of α_1 -casein is the same as the maximum slope observed for α_1 -casein alone. However, no lag period was observed, all mixtures of α_1 - with α_2 -casein giving straight lines through the origin.

DISCUSSION

Casein was long considered to be a pure protein until Linderström-Lang (1925) obtained evidence of its chemical heterogeneity. Mellander (1939) showed that it was composed of at least three electrophoretic components which he designated α -, β - and γ -casein in the order of their decreasing electrophoretic mobility in phosphate buffer, pH 8.0.

Warner (1944) first devised the procedure for fractionating these components by chemical means. Since then other fractionation procedures have been devised and further components recognized in casein, designated α_1 -, α_{11} -, β -, γ - and δ -casein (Cherbuliez & Baudet, 1950), α_3 -, β -, k- and m-casein (Waugh & Hippel, 1956; Waugh, 1958), λ -casein (Long, Van Winkle & Gould, 1958), α_1 -, α_2 -, α_3 - and β -casein (McMeekin, Hipp & Groves, 1959). Information is lacking to establish with certainty the correspondence between the fractions purified or defined by the various workers and those described above. Waugh & Hippel (1956) showed that the k-casein has a mobility very near that of α_5 -casein, suggesting that these two proteins correspond respectively to our α_2 - and α_1 -casein.

The form of the reaction curves indicates that the linear portion of the curve obtained with whole casein is due to the α_2 -casein present, and suggests that the lag period before the linear part occurs is determined predominantly by the presence of β -casein. Differences in the activity observed with a single plasminogen preparation when assayed with different batches of casein might therefore be due to variations in the relative proportions of the α_1 - and β -casein to α_2 -casein, and the lag period to a higher affinity for, and lower rate of hydrolysis of, β -casein by plasmin in comparison with α_2 -casein. An analogous situation was described by Willstätter, Kuhn, Lind & Memmen (1927) in the lag period in the hydrolysis by liver ali-esterase of ethyl mandelate in the presence of its keto-ester.

At constant α_2 -casein/ β -casein ratio the slope of the linear portion of the hydrolysis curve is independent of the total protein concentration. This implies that the decrease in the slope which occurs when the proportion of β -casein in the mixture is increased cannot be attributed to lack of α_2 -casein substrate. The effect may be due to a qualitative change in the properties of the mixtures because of interaction between the various protein species present. Solubility measurements in the presence of calcium, and ultracentrifugal measurements, indicate that strong protein-protein interactions occur between the components of casein (Hippel & Waugh, 1955; Waugh & Hippel, 1956; Waugh, 1958). Such complex formation might interfere

Fig. 5. Proteolysis by plasmin of mixtures containing different proportions of α_2 - and α_1 -casein. (a) Reaction curves of mixtures with these percentages of α_1 -casein added: $\circ$, 0; $\blacktriangle$, 25; $\blacksquare$, 50; $\bullet$, 75; $\triangle$, 100. Protein concentration 2 g./100 ml. in the reaction mixture. Plasminogen solution (0.06 mg. of N/ml.) was activated as described. (b) Plot of the slope of the curves/concentration of α_1 -casein (%). Symbols as identified for (a).

with the progress of the proteolysis of casein either sterically or by specifically blocking sites susceptible to enzyme action. Complex formation might occur not only with the intact protein but also with the products of the hydrolysis by enzyme. It is possible therefore that the presence of β -casein in the mixtures gives rise to two effects: an effect on the lag period due to a higher affinity for the enzyme and lower rate of reaction; and a change in slope of the linear part of the hydrolysis curve due to complex formation between the hydrolysed β -casein and the other components of the casein mixture.

The slope of the hydrolysis curve of a mixture of α_2 - with β - or α_1 -casein cannot satisfactorily be represented in terms of an average of the hydrolysis rate of the component proteins. Although β - and α_2 -casein alone give linear plots through the origin (Fig. 3), their mixtures show a lag period (Fig. 4), and, whereas mixtures of α_1 - with α_2 -casein with up to 75% of the former give straight lines through the origin, α_1 -casein alone yields a sigmoid curve (Fig. 5).

The extrapolation to the ordinate of the curve in Fig. 4 (b) suggests a slope of 103 for the digestion curve of pure α_2 -casein. This value does not agree with that obtained with another α_2 -casein preparation. Here, the same enzyme preparation at half the concentration of that in the previous experiment gave a slope of 100, a value twice that expected. This difference might arise from uncertainties in the relative proportion of β - to α_2 -casein, which was determined from their areas in the electrophoresis patterns. It cannot be excluded that the linear extrapolation to the ordinate of curves in Fig. 4 (b) and Fig. 5 (b) might not be valid for α_2 -casein containing less than 10% of β - or α_1 -casein. The extrapolation of the curves in Fig. 4 (b) and Fig. 5 (b) to the abscissa suggests that 63% of β -casein or 75% of α_1 -casein respectively is necessary to block α_2 -casein as a substrate for plasmin. On the assumption that the molecular weights given by Waugh (1958) for the k - and α_3 -casein are correct, it can be calculated from these results that at least one molecule of β -casein or two molecules of α_1 -casein are necessary to block α_2 -casein as a substrate for plasmin. However, since the proteins used in preparing the mixtures were impure these values are probably lower than would be found for pure preparations.

The different patterns of the curves obtained with α_2 - and α_1 -casein alone may be due to differences in the mechanism of hydrolysis of the two proteins, such as the splitting of small peptides throughout, and the primary splitting of intermediate products of high molecular weight which then are further degraded. The former mechanism would result in a steady liberation of acid-soluble

material from the start of the reaction and the latter in the formation of large fragments initially, and smaller acid-soluble products only as the hydrolysis progressed further.

Linderström-Lang, Hotchkiss & Johansen (1938) suggested that proteolytic enzymes digested proteins only in their denatured form. On this basis, the curve obtained with α_1 -casein alone might represent in fact the denaturation curve of this protein, the rate of denaturation increasing as the proteolysis progresses. α_2 -Casein alone, which gives a straight line through the origin, might be completely denatured from the start of the reaction.

The method for following the proteolysis depends on the liberation of tyrosine and tryptophan, and peptides containing these amino acids, which are soluble in 0.57 M-perchloric acid, and not adsorbed on the protein precipitated. Thus the apparent rate of attack on the various fractions of casein will be much affected by the tyrosine and tryptophan content of these fractions, when the reaction is followed by the method described. Different results might be obtained if a method estimating the number of peptide bonds split (such as a ninhydrin reaction or a formol titration) were used.

Christensen (1954) showed that whole casein gives non-linear curves when the proteolysis with plasmin is measured by the liberation of acid-soluble material. His curves for the proteolysis of some α - and β -casein preparations suggest that possibly his α -casein preparations were α_1 -casein.

Northrop (1922) showed that the rate of the digestion of a mixture of casein and gelatin is equal to the sum of the rates of hydrolysis of the two substances alone, and suggested that this fact is inconsistent with the addition-compound hypothesis and the saturation hypothesis of enzyme action. Castañeda-Agulló (1956) confirmed Northrop's results with mixtures of casein or haemoglobin with gelatin, which were digested with trypsin, papain and pepsin.

The results presented here suggest that the behaviour of mixtures of proteins during enzymic hydrolysis differs from that to be expected from the behaviour of the individual proteins, possibly because of interactions between the substrate proteins in the mixtures. The conclusions of Northrop (1922) and Castañeda-Agulló (1956), that their kinetic studies are inconsistent with the conception of the saturation of enzyme with substrate, may not therefore be entirely justified.

SUMMARY

1. Whole casein and casein fractions were purified and their proteolysis by plasmin was examined.

2. With whole casein a curve showing a lag period before a linear portion is obtained and with β -casein a very flat curve is given. A steep straight line was obtained with α_2 -casein and a sigmoid curve with α_1 -casein.

3. With mixtures of α_2 - and β -casein or α_1 -casein the slope of the curves decreased linearly as the proportion of β - or α_1 -casein increased, and a lag period was observed with some mixtures of α_2 - and β -casein only.

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The Effect of Development on the Composition of a Long Bone of the Pig, Rat and Fowl

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The earlier investigators of the chemistry of bone growth (Weiske, 1889; Hammett, 1925*a-c*; Kramer & Shear, 1928; Swanson & Iob, 1937) worked largely on whole bones and were therefore concerned with bones as organs. More recently, those interested in the chemistry of bone growth have directed their attention to the cortex. The matrix of this tissue consists of the substances that constitute the connective tissue of skin and muscle. Bone differs from the connective tissue of these organs, however, in its characteristic 'hardness'. This property is attributed to the deposition of calcium salts within the matrix. Whereas earlier workers paid little attention to the organic matrix, the more recent investigators have studied its composition and its relationship to the bone minerals, and particularly the calcium.

Burns & Henderson (1936*a*) studied the influence of age from birth to adult life on the concentrations of calcium and carbonate in the cortical

bone of kittens and puppies. The work of Baker, Butterworth & Langley (1946) on the calcium:nitrogen ratio of carefully cleaned bone tissue was extended by Weidmann & Rogers (1950) in a comparative study of the degree of calcification of cortical bone from adult rats, rabbits and cats. These investigators subsequently showed (Rogers, Weidmann & Parkinson, 1952) that 89–97% of the nitrogen in the bone tissue of the adult rabbit, ox and man is present as collagen. The value for ox bone was later confirmed by Eastoe & Eastoe (1954). In later work Weidmann & Rogers (1958) found an increase in the calcium:nitrogen ratio in the cortical bone of the rabbit from about the fifth day of life and in that of the cat from about the ninth day. No similar work appears to have been done on the cortical bone of the pig, rat or fowl.

One of the characteristic signs of maturation in mammalian long bones is the appearance and growth of the secondary centres of ossification in the