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Action of Degradative Enzymes on the Light Fraction of Bovine Septa Protein Polysaccharide

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1. Fragments from enzymic degradation of protein–polysaccharide light fraction (PPL) have been analysed. 2. The time-course of action of some proteolytic enzymes and of hyaluronidase on PPL has been followed by viscometric techniques. 3. It is suggested that papain acts to produce single polysaccharide chains, whereas other proteolytic enzymes tried give evidence of twin-chain residues. 4. The molecular weight of the fragments derived from complete enzyme action on PPL supports this postulate. 5. A structure of the PPL complex is suggested.

In a previous paper (Luscombe & Phelps, 1967) information on the size, shape and homogeneity of the PPL* fraction of nasal-septa chondromucoprotein was advanced. The present paper sets out to record kinetically the response to degradation of PPL when acted on by proteolytic and mucolytic enzymes. The principal method used was the measurement of the index of viscosity on a Couette viscometer (Ogston & Stanier, 1953). The results indicate that this method furnishes a simple and accurate measure of the extent of degradation of the complex.

From these results, certain conclusions could be postulated about the structure of the complex that received confirmation by the determination of weight-average molecular weight on the residues after extended enzymic degradation. A diagrammatic representation of the PPL particle can be presented that accounts for most of the properties of the complex.

MATERIALS AND METHODS

The preparation of PPL from ox nasal septa and analytical methods apart from those described below have been given by Luscombe & Phelps (1967).

Materials

Uronic acid. Allowance was made for the contribution to the extinction by the galactose present. Galactose (100 μg.) gave a colour equivalent to 8·4 μg. of uronic acid.

Amino acids. These were determined by the ninhydrin method of Yemm & Cocking (1955). The action of neuraminidase and galactose oxidase on PPL was followed according to the method of Robinson & Pierce (1964).

Enzymes. The following enzymes were used: chymotrypsin (EC 3.4.4.5) and trypsin (EC 3.4.4.4), crystalline from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex; galactose oxidase (EC 1.1.3.9), type 1, and neuraminidase (EC 3.2.1.18), type 5, from Sigma Chemical Co., St Louis, Mo., U.S.A.; hyaluronidase (EC 3.2.1.35), ex ovine testes, from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; papain (EC 3.4.4.10), twice recrystallized, from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; Pronase (grade B) from Calbiochem, Los Angeles, Calif., U.S.A.

Papain. This was activated by incubation with 10mM-cysteine hydrochloride or 50mM-KCN–50mM-EDTA for 30min. at 45°.

Hyaluronidase. This was purified by fractionation on a DEAE-Sephadex 50 column (Sora & Ionescu-Stoian, 1963). Over 90% of the hyaluronidase activity was present in the first protein peak eluted from the column. This peak was asymmetrical with a break occurring when half the total volume of the peak had been eluted. The activity of the enzyme recovered from the two halves was estimated by the flocculation technique described by Dorfman (1955). The first half, which was used in this work, had an activity after freeze-drying of 1900–3000 units/mg.; the second half had only 20% of this activity. The purified material was unstable.

Buffers. The composition of the buffers was taken from Dawson, Elliott, Elliott & Jones (1959). Buffers were used to give a final concentration 0·04M for enzymic incubations or 0·01M for viscometric measurements. Citrate–phosphate buffer at the given pH was used with the following enzymes: chymotrypsin, pH7·4; trypsin, pH7·9; Pronase, pH7·4; papain, pH5·5; hyaluronidase, pH5·6.

Preparation of residues

Alkali residues. PPL (500mg.) was treated with 100ml. of 0·5N-NaOH at 4° for 19hr. The degraded material was then precipitated and treated by the Sevag technique according to the method of Anderson, Hoffman & Meyer (1965). The final precipitate was taken up in water, dialysed and freeze-dried to give a white powder.

* Abbreviation: PPL, protein–polysaccharide light fraction.

Trypsin and papain residues. PPL in the appropriate buffer was incubated for 16hr. with either trypsin at 37° or papain at 45° at a substrate:enzyme ratio 1:50. Tryptic activity was independent of the addition of NaCl but a concentration of 0·2M was necessary for maximum activity of papain. At the end of the incubation the mixtures were clarified by centrifugation and the supernatant was treated with 2vol. of ethanol. The precipitate was centrifuged down, dissolved in water and dialysed overnight against distilled water. A small amount of precipitate usually formed at this stage and was centrifuged off before freeze-drying. Before analysis extraneous protein was removed by the Sevag technique.

Hyaluronidase residues. PPL (1g.) was incubated at 37° with 10mg. of hyaluronidase (3000units/mg.) in 80ml. of buffer with NaCl to give a 0·2M solution. The reaction was followed by the flocculation technique. After 24hr. the percentage transmission of an 0·02ml. sample of the incubation mixture, in 12ml. of flocculation medium, had risen from 23 to 98%. The mixture after centrifuging off the small amount of precipitate that formed was put on to a Sephadex G-75 column (52cm. × 3cm.) and eluted with the same saline buffer as used for the incubation. The first 45ml., after the void volume, contained the protein peak (measured at 280mμ). This was dialysed against constant changes of distilled water for 24hr. The small amount of precipitate that formed (probably denatured enzyme) was removed by centrifugation and the supernatant freeze-dried.

Physical methods

Viscometry. Viscosities were measured at 25° in a Couette viscometer similar to that of Ogston & Stanier (1953). After the addition of enzyme to a solution of PPL in the viscometer, the decrease in the angle of deflexion of a beam of light reflected from a mirror attached to the top of the bob was recorded as it passed across a 180° semicircular scale of radius 1m. Angular deflexions on the scale were calibrated against angular rotation of the torsion head, at any given shearing rate. Deflexions could be read to 0·05° on the torsion head, and the sensitivity, with water, was 4·5°/unit velocity gradient with no. 40 Eureka as suspending wire.

Approach to sedimentation equilibrium. Runs were performed at 20° on a Beckman model E analytical ultracentrifuge operated under standard conditions. The concentration of samples was determined with a synthetic-boundary cell, and calculations followed the method described by Schachman (1957).

Partial specific volume. These values were determined from density measurements of the alkali and hyaluronidase residues with a 10ml. density bottle at 20°. These values together with the value for collagen of 0·706 (Lewis & Piez, 1964) were used to calculate those of the proteolytically prepared residues.

RESULTS

The chemical analysis of PPL and residues from enzymic degradation is given in Table 1. The trypsin-digested fragment contained 37% of the original protein whereas the papain-treated material contained 20% as estimated by the biuret reaction. Alkali removed essentially all the protein. The lower value obtained for the alkali-residue hexosamine component is thought to be due to the destructive action of alkali on the amine group.

The hyaluronidase residues have an increased percentage of galactose. This was shown by Gregory, Laurent & Rodén (1964) to be due to the presence of keratosulphate. Removal of protein lowers the percentage of galactose remaining with the chondroitin sulphate.

Weight-average molecular weights for the residues (Table 1) represent those calculated at the meniscus of the approach-to-equilibrium run. With alkali residues and proteolytic residues little variation was observed in the calculated molecular weights at different times during the run, but those of the hyaluronidase residues decreased with time, indicating polydispersity. The values in Table 1 for the hyaluronidase residues were obtained during the first 10min. of the run at 7928 rev./min. A high-speed sedimentation run (59780rev./min.) of this material gave a single asymmetric peak, which after 50min. at speed showed signs of a slight double peak, confirming the polydispersity of the material. A similar run with alkali-degraded material showed a single symmetrical peak even after 2hr. at speed, indicating no great variation in the size of the population.

Electrophoresis for proteins on cellulose acetate strips with a constant current of 1·2mA/5cm. strip

Table 1. *Analysis of protein–polysaccharide light fraction and residues from its enzymic and alkaline degradation*

Results of chemical analysis are expressed as % dry wt. of the sodium salt.

	Protein (Folin–phenol)	Protein (biuret)	Uronic acid	Hexosamine	Molar ratio uronic acid:hexosamine	Galactose	Partial specific volume (ml./g.)	$10^{-4}\times$ Weight-average molecular weight
PPL	14·8	19·7	27·0	27·2	1·11	4·9	—	—
Papain residues	2·3	3·9	37·4	34·4	1·02	3·2	0·537	2·11
Trypsin residues	3·7	7·3	34·7	32·4	1·03	4·2	0·544	4·33
Alkali residues	0·02	—	36·7	31·4	0·94	1·8	0·53	2·17
Hyaluronidase residues	33·6	42·2	5·1	11·5	2·48	13·0	0·61	29–33

and veronal acetate buffer, pH 8·6 and I 0·1, when applied to the hyaluronidase residues, showed no protein material but a typical Alcian blue polysaccharide staining band.

Action of neuraminidase and galactose oxidase on PPL. Neuraminidase acts only on terminal sialic acid residues (Gottschalk, 1960) and removed over 90% of the sialic acid present in the complex, so that these groups must be terminal and not involved in other linkages. When PPL was first incubated with neuraminidase and then with galactose oxidase, there was a release of residues acting as galactose. The molar ratio of sialic acid removed by neuraminidase to galactose oxidized was 1·0:0·97. (This ratio would result if galactose is the linkage sugar for sialic acid.) Though galactose oxidase was shown not to react with neuraminidase controls, colours were developed with *N*-acetylgalactosamine and galactosamine, giving 91 and 82% of an equivalent weight of galactose respectively.

Chromatograms of the sugars released after treating PPL with 0·1 N-sulphuric acid for 1 hr. at 80° or 16 hr. at 20° always showed definite galactose spots but never more than a trace of hexosamine.

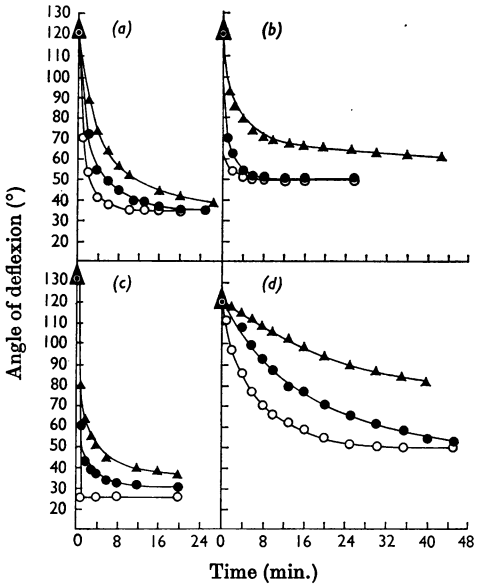

Fig. 1. Viscosity of PPL solutions during enzyme action. The angle of deflexion was recorded in the apparatus of Ogston & Stanier (1953). PPL (50 mg. in 12 ml. of buffer) was incubated with enzymes at various concentrations. The pH and composition of buffers are given in the text. The temperature was 25°. Amounts of enzyme added to each 12 ml. of solution were as follows. (*a*) Pronase: ○, 40 μg.; ●, 20 μg.; ▲, 10 μg. (*b*) Trypsin: ○, 100 μg.; ●, 50 μg.; ▲, 10 μg. (*c*) Papain: ○, 100 μg.; ●, 10 μg.; ▲, 5 μg. (*d*) Hyaluronidase: ○, 1000 μg.; ●, 500 μg.; ▲, 100 μg.

Effect of enzyme degradation on the viscosity of the complex. The decrease in the angle of deflexion of the light-beam reflected from the mirror, for constant shear rate, plotted against time for the course of the reaction with hyaluronidase, trypsin, papain and Pronase is shown in Fig. 1. Chymotrypsin gave curves similar to trypsin. β-Glucuronidase and neuraminidase had no measurable effect on the viscosity. The shapes of the curves obtained from the action of the proteolytic enzymes are similar, although the amount of protein they may remove from the complex differs. The final viscosities of the complex recorded as a percentage of the original value for various enzymes were as follows: trypsin, 40%; papain, 18%; chymotrypsin, 36%; Pronase, 29%; hyaluronidase, 40%.

Presumably the number of protein bonds broken by trypsin in the first few minutes, when a rapid fall of viscosity takes place, is very small compared with the number that are finally broken with longer time, as measured by the increase in extinction of ninhydrin-reacting groups liberated. With a substrate: enzyme ratio 500:1 the viscosity falls to its final value in 4 min.; with half this amount of enzyme, the final viscosity is reached in 8 min. However, with a substrate: enzyme ratio 100:1 the increase in extinction in the ninhydrin reaction is still evident at 3 hr. (Fig. 2), and shows that proteolysis is continuing. The bonds broken in this longer period have no further effect on the viscosity of the residue, and it is assumed that trypsin is attacking the protein core, and any liberated peptides that may have been produced early in the reaction. This point is discussed below.

The results from the viscosity experiments have been plotted as rate curves following first-order

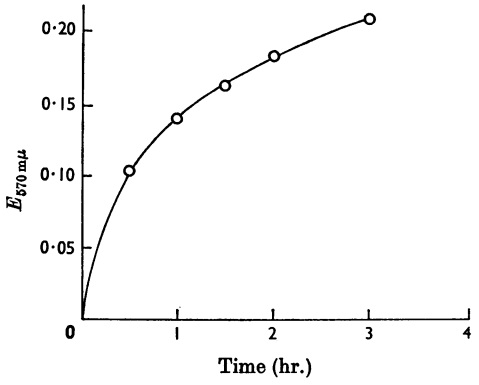

Fig. 2. Release of ninhydrin-reacting groups from PPL by trypsin. The enzyme: substrate ratio was 1:100. The temperature was 25°. Details of method and buffer are given in the text.

reaction kinetics. The formula has been used in the form:

$$t = \frac{2{\cdot}303}{k}\log(\eta_0-\eta_\infty) - \frac{2{\cdot}303}{k}\log(\eta_t-\eta_\infty)$$

where η_0 is the initial value of the relative viscosity and η_t and η_∞ are the relative viscosities of the sample at time t and time ∞. A plot of $\log(\eta_t-\eta_\infty)$ against t should be linear if the reaction is first-order and the slope should equal $-k/2{\cdot}303$, so that a decrease in slope will indicate a decrease in reaction constant. The values obtained from the results relating to 100mg. of complex are plotted in Fig. 3.

The three proteolytic enzymes trypsin, chymotrypsin and Pronase all give straight lines over the main part of the reaction period. The slopes of the lines of trypsin, chymotrypsin and Pronase are similar, so that the number of bonds of the protein moiety contributing to the viscosity that are broken in unit time is similar, although the specificity of the enzymes differs. The first-order equation is obeyed so that the rate depends on the concentration of the substrate present.

Papain initially shows the same rate as the other enzymes, but after 4min. the slope of the line changes abruptly to indicate a changed rate constant. A similar decrease after 2–3min. was observed when the same amount of enzyme was used with 50mg. of substrate.

Papain contains active thiol groups as well as those remaining from the cysteine used for activation, and the possibility existed that this extra effect was due to oxidoreductive depolymerization. However, similar rapid falls in viscosity were obtained when the papain was activated by potassium cyanide and a glass Ostwald viscometer used. The decrease in viscosity is therefore thought not to be due to cysteine or to the catalytic effect of ions from the stainless steel of the Couette viscometer.

With 200μg. of hyaluronidase (1900units/mg.) the curve is at first linear until 7min. after an initial lag period; this slow start was observed repeatedly with similar enzyme:substrate ratios. After 7min. the rate of reaction increases and further increases are seen with time. Hyaluronidase acts on chondroitin chains by random interior attack along the chain followed by transglycosidation, and thus the number of substrate units, contributing to the viscosity, available to the enzyme might be expected to increase during the early stages of enzymic attack.

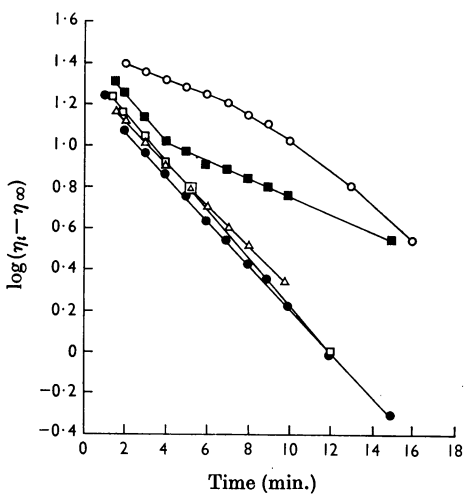

Fig. 3. Application of first-order reaction kinetics to viscosity data for enzymic attack on PPL. PPL (100mg. in 12ml. of buffer) was used for each run. The temperature was 25°. The pH and composition of buffers are given in the text. Amount of enzyme added to 12ml. of solution: ○, 200μg. of hyaluronidase; ●, 20μg. of trypsin; □, 20μg. of Pronase; ■, 5μg. of papain; △, 50μg. of chymotrypsin.

DISCUSSION

The work reported here began as an attempt to follow viscometrically the action of various enzymes on PPL complex. The discrepancy between the rate curves for papain and the other proteolytic enzymes suggested that papain was capable of some further action on the complex. A natural corollary of this was to determine the molecular weights of the finally changed residues prepared in sufficient quantity so that ultracentrifugal measurements and chemical analysis could be conducted.

The results of this work suggest that the weight-average molecular weight of the trypsin-digested complex is $4{\cdot}3\times10^4$ and that of the papain-digested complex is $2{\cdot}1\times10^4$ (Table 1). Mathews (1956) and Buddecke, Kröz & Lanka (1963) reported values of $5{\cdot}0\times10^4$–$5{\cdot}5\times10^4$ for the trypsin-digested remnant. Both sets of results were obtained from light-scattering data, which would tend to place emphasis on the high-molecular-weight components of a polydisperse system. Our values for the papain-digested complex are in the lower range of those reported severally by Mathews (1956), Buddecke *et al.* (1963) and Partridge, Davis & Adair (1961) as $2{\cdot}0\times10^4$–$2{\cdot}8\times10^4$. The papain fragment and low-temperature alkali-digested fragments have similar molecular weights, though the latter preparation contains no protein. It is thus unlikely that the polysaccharide chain can be further dissected by proteolytic treatment. These values support the contention of Anderson *et al.* (1965) that papain-digested remnants have only one polysaccharide chain whereas the trypsin-digested fragment has two. Such a hypothesis would fit the evidence

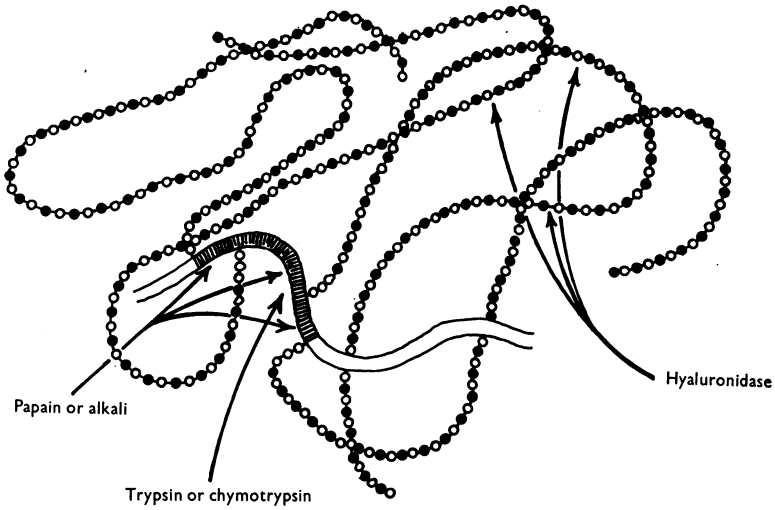

Fig. 4. Diagram to illustrate enzymic degradation of PPL. A protein core and four polysaccharide chains are shown.

obtained from viscosity measurements during enzymic degradations where trypsin left 40% of the initial viscosity and papain left only 18%, and where the rate curves suggest that initially papain cleaves the same functional type of bond as the other proteolytic enzymes, but is additionally able to break further linkages (Fig. 4). To construct a model of the probable structure of PPL it is necessary to obtain some idea of the size of the protein core. Our results for the residues left after hyaluronidase degradation are similar to those reported by Gregory *et al.* (1964) and indicate a weight-average molecular weight for the protein moiety, after subtracting the weight due to carbohydrate, of 12×10^4–14×10^4. Partridge *et al.* (1961), using osmotic-pressure data on a chondromucoprotein preparation, reported corrected values of 12×10^4. Luscombe & Phelps (1967) showed that the composition of the complex in terms of neutral sugars was consistent with the presence of keratosulphate and glycoprotein intimately associated with the structure. Additional support for the presence of glycoprotein is seen in the proportions of galactose to sialic acid obtained after enzymic treatment of PPL. Gregory *et al.* (1964) have shown that the major part of the glucosamine and galactose associated with hyaluronidase-treated PPL residues is due to the presence of keratosulphate. Nevertheless the chondroitin sulphate–protein complex forms a large percentage of PPL and the structure here proposed will be minimally affected by the presence of this extra material.

By using the average molecular weight $3{\cdot}2 \times 10^6$ determined from sedimentation data and a protein content of 20% (Luscombe & Phelps, 1967) the weight due to the carbohydrate moiety will be $2{\cdot}55 \times 10^6$. Approx. 14% of this weight will be due to carbohydrate other than chondroitin sulphate chains, which will then have a weight $2{\cdot}2 \times 10^6$. If the chain weight is $2{\cdot}1 \times 10^4$ there would be on average 100 chains/molecule. The difficulty in apportioning the protein has already been mentioned, but the error is not likely to be large if it is taken in the same ratio as chondroitin sulphate to other carbohydrate. The average molecular weight of protein associated with the chondroitin sulphate would then be $5{\cdot}65 \times 10^5$. With a core weight 12×10^4–14×10^4 an average of four or five cores would be present in each structure, each core forming a unit carrying 25–20 chondroitin sulphate chains.

A plot of the decrease of viscosity caused by hyaluronidase with time is initially almost linear and fits a model where the oligosaccharide units are removed from the surface of a sphere or cylinder. The more rapid decrease in viscosity caused by proteolytic enzymes points to the necessity of the integrity of the protein moiety in maintaining viscosity. Also, the cleavage of relatively few bonds causes the splitting of the large unit into many smaller units, which may themselves be aggregates. The question why keratosulphate and glycoprotein occur in mildly extracted material is presumably connected with the tendency of this complex to form spherical aggregates in solution. That this is so is suggested by the lack of anomalous viscosity normally associated with long chains such as hyaluronic acid. However, an ultrafiltrate of a

homogenate of cartilage that still contained soluble collagen was shown to have a much higher viscosity than PPL and exhibited pronounced anomalous viscosity (Luscombe & Phelps, 1967). Evidence for collagen–mucopolysaccharide interaction has been given by Mathews (1965). Larger aggregates may thus form by an end-to-end arrangement with the participation of randomly threaded strands of keratosulphate and glycoprotein to give a longer more filamentous structure such as those demonstrated by Mathews & Lozaityte (1958) in a water-extracted complex. Similarly, low-molecular-weight material will be obtained by the use of extraction procedures that cause the breakdown or removal of protein. The molecular weights determined on the protein core of hyaluronidase-treated material revealed polydispersity. Thus, quite apart from methods of extraction and the age of the cartilage, there will be differences in the size of the sub-units.

It is concluded that the average molecule isolated by the present method of extraction consists of several cores of protein, on average four or five, associated with 100 chains of chondroitin sulphate each of 40 disaccharide units. The chondroitin sulphate chains will be randomly coiled around the protein cores with a tendency to lie towards the outside of the unit near the water solvent. Because of the polyelectrolyte nature of the protein polysaccharide, the separate cores and associated chains will tend to aggregate and the keratosulphate and other glycoprotein present will also form part of the aggregation. This process, controlled only by steric considerations, will tend to a spherical or slightly ellipsoidal shape in aqueous extracts of cartilage and the preponderance of chondroitin sulphate chains lying externally will give the low apparent isoelectric point of the complex. The spaces created by random aggregation would enable the easy penetration of proteolytic enzymes and a few bonds broken would result in a rapid disintegration of the whole unit. A diagram of the proposed structure is given in Fig. 5.

The hypothesis of aggregation of small units could account for (*a*) the polydispersity observed by small differences in the number and size of units in each aggregate and (*b*) the alteration of constituents with age. Variations could also occur in the amount of 'trapped' protein. The concept of aggregates has also been proposed by Partridge (1966).

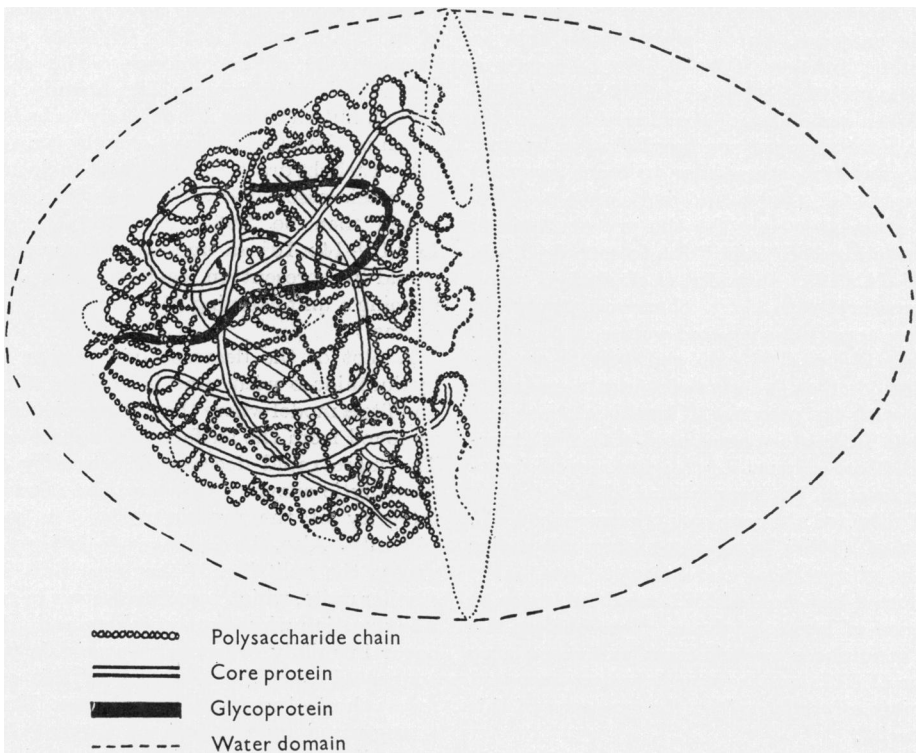

Fig. 5. Three-dimensional diagram of the suggested structure of water-extracted PPL.

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