Isolation and Characterisation of Microhelices from Lutoids of Hevea Latex

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When the ionic concentration of B serum from the lutoids is lowered by either dilution with water or dialysis against water it always gives a precipitate. This precipitate frequently contains structures known as microhelices. The precipitate can be resolved by column chromatography on Sephadex or Bio-Gel into four components all of which have been found to be glycoproteins. Their molecular weights are approximately 160 000, 74 000, 22 000 and 5000. Only two of these components, those of molecular weights 160 000 and 22 000 when combined, give microhelices. These are referred to as the 'assembly factor' and the 'pro-helical protein' respectively. These results show that microhelices are made up of a combination of two glycoproteins. These two components are required to combine in a certain stoichiometric ratio for the formation of microhelices.

When B serum is subjected to ultracentrifugation at 314 000 g for 4 h, a gelatinous sediment and a clear supernatant are obtained. Neither the sediment nor the supernatant gives microhelices after dialysis. Microhelices are obtained only if the sediment and the supernatant are recombined and dialysed. These observations confirm that microhelices require at least two components for their formation. The gelatinous sediment contains the large molecular weight assembly factor.

Microhelices have been observed either as single helices or in bundles. The reason for this has been elucidated.

The variation of occurrence of microhelices between individual samples within a clone as well as between clones observed previously has been attributed to an inherent deficiency of the pro-helical protein in the B serum of the samples which are deficient in microhelics.

The lutoids in *Hevea* latex first discovered by Homans *et al.*[1] have been of much interest as they have a considerable influence on the latex flow characteristics[2] as well as having the properties of vacuoles[3-6] or lysosomes[7,8]. Interesting ultrastructural features of the lutoids have been described by electron microscopy: in lutoids from latex vessels in young tissue, Dickenson[3] and Archer *et al.*[9] observed bundles of tightly coiled helical structures — the *microfibrils* — which were isolated and characterised as a single protein by Audley[10, 11, 12]. This microfibrillar protein was absent from lutoids of mature tissues. In studies of centrifuged latex from mature trees Archer *et al.*[9] observed 'a second type of microfibril' with a zig-zag configuration that appeared to be derived from the lutoids. The significance of this zig-zag particle was not known at that time.

During an investigation on the long-term effect of ethephon, bark samples from

mature trees which had been treated with ethephon showed, under electron microscope, lutoids which contained numerous particles which resembled the 'second type of microfibril' of Archer *etal*.[9] These were termed *microhelices* by Gomez and Yip[13, 14, 15] who observed these particles more frequently in lutoids from tissues repeatedly treated with ethephon than in untreated tissues. Occasionally the microhelices were also observed within the lutoids in bottom fraction of ultracentrifuged latex.

Most of the lutoids in latex can be sedimented in a fraction (the bottom fraction) by ultracentrifugation[16, 17]. The fluid obtained by freezing and thawing the bottom fraction is termed B-serum[18], which consists mainly of the contents of lutoids. During an investigation of the action of dialysed B-serum on rubber particles *in vitro*, Southorn and Yip[19] encountered structures similar to the microhelices and speculated that these particles were probably proteins. Subsequently, it was discovered that the microhelices could also be precipitated by dialysing B serum against water[20]. They are a fundamental feature of lutoid composition which deserves detailed investigation.

A recent study[21] on the occurrence and distribution of microhelices in clones of *Hevea* showed that there was a great variation in the occurrence of microhelices between clones. There was also variation between individual samples in the occurrence of microhelices but no seasonal trend could be discovered.

The present work was undertaken to isolate and characterise the microhelices and to determine the conditions which affect their formation.

EXPERIMENTAL

Latex was collected under refrigerated conditions from RRIM 600, RRIM 501 and Tjir 1 in *Field 14D* at the RRIM Experiment Station and ultracentrifuged in prechilled rotors at $0°C-5°C$ in either a Spinco Model L or Model L2 — 65B ultracentrifuge as described earlier[17]. B-serum was prepared as described in a previous study[21] and aliquots of 5 ml were dialysed against 1 litre of deionised water at approximately $5°C$. The resulting precipitate was recovered by centrifugation at 15 000 r.p.m. (20 000 g max) for 30 minutes.

Column Chromatography

The precipitate was redissolved in 5 ml of 2% (weight/volume) NaCl (approximately $0.35M$ NaCl). Ultrasonication at 20 KHZ for 20 s aided in redissolving the precipitate; the solution was kept in an ice-water bath throughout this operation.

The salt solution was chromatographed on a column (50 × 2.5 cm) of either Sephadex G-150 (Pharmacia, Uppsala, Sweden) or Bio-Gel P-100 (BioRad Laboratories, U.S.A.) which was made in $0.35M$ NaCl and elution was carried out in the same solvent; 3 ml fractions were collected and the optical densities of each fraction at 260 nm and 280 nm were measured. A graph of the optical density against the fraction number was prepared. Several such column chromatography runs were made to recover substantial quantities of the components eluted from the column.

Concentration of the Eluted Components

The fractions under the corresponding peaks obtained from several experiments were pooled and concentrated to 5 ml by ultrafiltration in an AMICON 'DIAFLO' ultrafiltration cell equipped with a

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UM-2 membrane having a molecular weight cut off property at 1000 daltons.

Ultracentrifugation of B-serum

Some samples of the original B serum were also ultracentrifuged at 60 000 r.p.m. (approximately 314 000 g max) for 4 h using a rotor type 65 in the Spinco Model L2-65B ultracentrifuge. The resulting gelatinous sediment and a supernatant liquid were separately recovered.

Hydrolysis and Paper Chromatography

Freeze-dried samples (usually 1−2mg), were mixed with 3 ml of 6N HCl and hydrolysed in evacuated and sealed glass tubes at 105°C for 24 hours. The acid was then removed under vacuum and the hydrolysates were redissolved in n-butanol-acetic acid-water (62-15-26). Paper chromatography for 20 h in the same solvent was done using a Whatman No. 1 sheet of chromatography paper. The paper was dried at 100°C for 20 min and the amino acid spots were located with 0.2% ninhydrin in acetone containing 2% pyridine. Sugar spots were located with reagents containing $AgNO_3$ and NaOH.

RESULTS AND DISCUSSION

Dialysis of B serum against water always gave a precipitate. The precipitate usually showed microhelices accompanied by a background material *(Figure 1)* If B serum was dialysed against 0.35M NaCl, precipitation was negligible. Dilution of B serum with water also produced a precipitate containing microhelices. Again, if the precipitate was redissolved in 0.35M NaCl and the solution diluted with water or dialysed against water, microhelices were reprecipitated. These results are consistent with the explanation that the precipitation of microhelices was due to the lowering of the ionic concentration

Figure 1. Microhelices in a precipitate obtained by dialysis of B serum (Tjir 1) against water. Mag. × 18 000

in B serum on dialysis against water or on dilution with water.

Since microhelices were observed in the precipitate on lowering the ionic concentration it was suggested that they were formed from a soluble precursor in B serum. Judging from their dimensions, as observed by electron microscopy, it was thought that their molecular weight was very high. In this connection it may be relevant to mention here that one of the methods of recovering the microfibrillar protein from the serum from young lutoids was by centrifugation of the serum as described by Audley[10]. Therefore during the early stages of the present work, the feasibility of recovering the microhelices by sedimentation from B serum in the ultracentrifuge was investigated.

The result obtained by ultracentrifugation of B serum at 314 000 g max was unexpected but provided further infor-

mation about the chemical structure of the microhelices: neither the gelatinous sediment nor the supernatant showed microhelices after dialysis. (Occasionally, a few microhelices were noticeable in the gelatinous sediment and also in the original whole B serum *before* dialysis. This, however, is due to preformed microhelices which are sometimes present in the lutoids *in vivo* and are recovered in B serum.)

When the supernatant from the ultracentrifugation of B serum was recombined with the sediment and the mixture was homogenised and dialysed against water, numerous microhelices were observed in the resulting precipitate. This interesting observation suggested that the microhelical material required at least *two* components of B serum for its formation. One of these components was sedimented by high-speed centrifugation of B serum in the gelatinous sediment and was therefore of a large molecular weight. The other component remained in solution in the supernatant. On recombination and dialysis the two components combined to form the microhelices.

Parallel experiments in which salt solutions of the precipitate containing microhelices were ultracentrifuged gave similar results: a gelatinous pellet showing occasionally a single microhelix but mainly non-descript amorphous material and a supernatant showing no microhelices after dialysis. Again on recombination of the pellet with the supernatant, numerous microhelices were obtained after dialysis.

Although the identity of the microhelices was uncertain at this stage of the work, they were presumed to be proteinaceous in view of the observation that acid hydrolysates of the precipitate containing microhelices showed a large number of amino acid spots with paper chroma-

tography. It was conceivable therefore that the microhelices were proteins formed by a combination of at least two proteins during the dialysis of B serum. For simplicity, the large molecular weight component which sedimented in the pellet was termed the *assembly factor* and the other component the *pro-helical protein*[22]. The process of formation of microhelices was tentatively visualised as the assembly of the relatively small pro-helical protein molecules on the large molecules of the assembly factor as the ionic strength in B serum was lowered on dialysis or by dilution with water.

Column Chromatography

The precipitate containing microhelices, after redissolving in 0.35M NaCl and subjecting to column chromatography on either Sephadex G-150 or Bio-gel P-100 was resolved into four peaks *(Figure 2)*. Since these column chromatography systems are based on the principle of gel filtration, the materials from the peaks were eluted in the order of their decreasing molecular weights, *i.e.* the material in the first peak (A or A' in *Figure 2*) was of the largest molecular weight (most probably the assembly factor). Indeed, when the gelatinous pellet from the ultracentrifugation of B serum was redissolved in salt and subjected to gel filtration on Sephadex G-150 column, it gave a very large peak, A with very small peaks, B, C and D[22] confirming that the pellet was largely made of component A (the assembly factor).

Re-formation of Microhelices in vitro *and their Characterisation*

The materials recovered from the pooled and concentrated peaks were recombined in pairs to find which two components formed microhelices. (Thus, combinations of 1 ml each of A + B, A + C and A + D
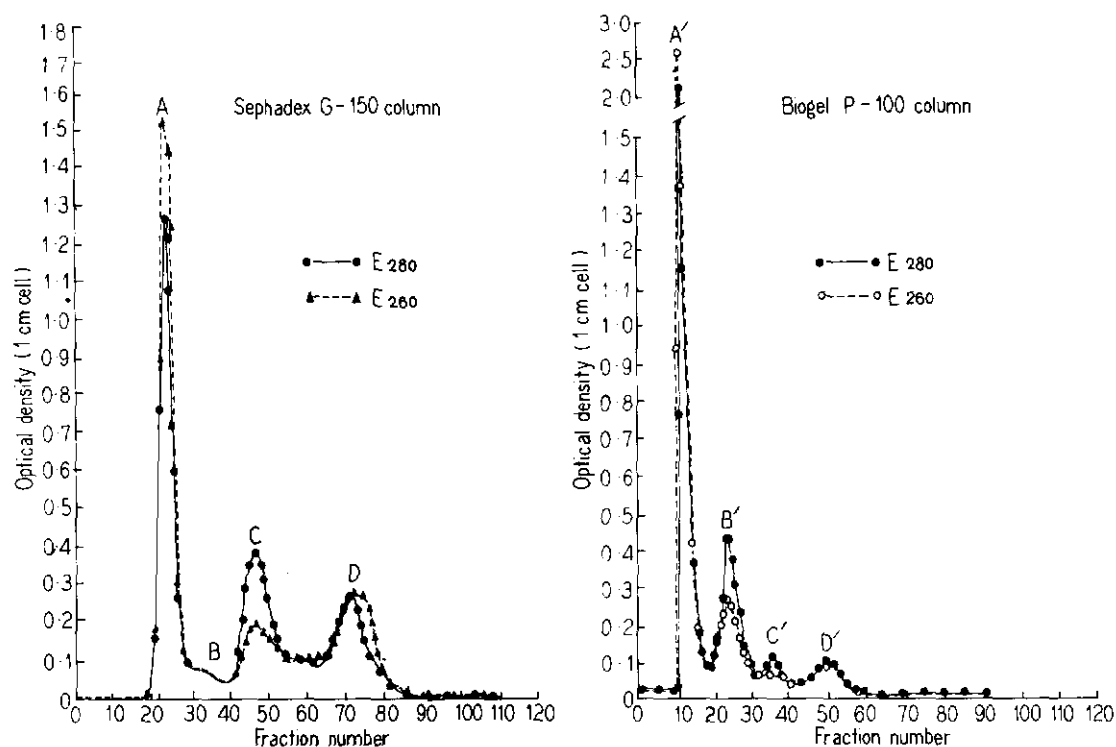
Figure 2. Column chromatography on columns of Sephadex G-150 and Biogel P-100 of a salt solution of the precipitate from dialysed B-serum, containing microhelices.

were made from the Sephadex column materials and of $A' + B'$, $A' + C'$ and $A' + D'$ were made from the Bio-Gel column materials.) After combining, each mixture was dialysed against water and the precipitate (if any) was recovered and examined by electron microscopy.

Only mixtures $A + C$ and $A' + C'$ showed microhelices(*Figure 3*).The other mixtures showed only an amorphous, nondescript material.

These results confirmed the previous observation that microhelices were made from two components. If component A (or A') is the assembly factor, component C (or C') should be the pro-helical protein.

The approximate molecular weights of the four components were estimated[22] from their gel filtration behaviour and were found to be: $A = 160\,000; B = 74\,000; C = 22\,000$ and $D = 5000$. After acid hydrolysis and paper chromatography, all the four components showed several amino acid spots staining with ninhydrin. They also showed spots due to sugar after hydrolysis and chromatography. From these results it is concluded that microhelices are made by a combination of two glycoproteins in the lutoids and are themselves glycoproteins. The presence of carbohydrate also in the microfibrillar protein has been reported by Audley[11], and it is likely that the microfibrillar protein is also a glycoprotein. However, it is not yet known whether the microfibrils and

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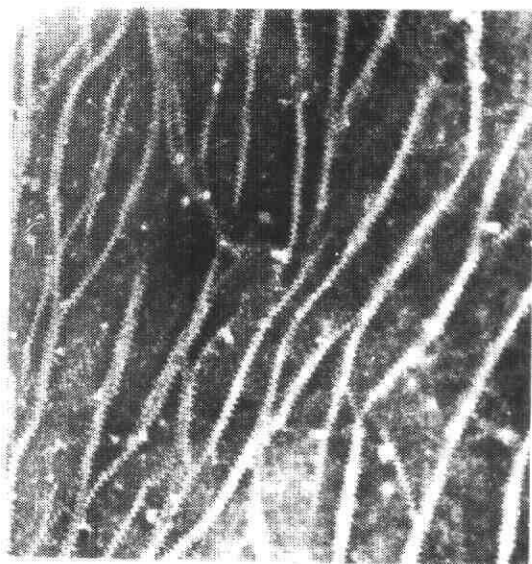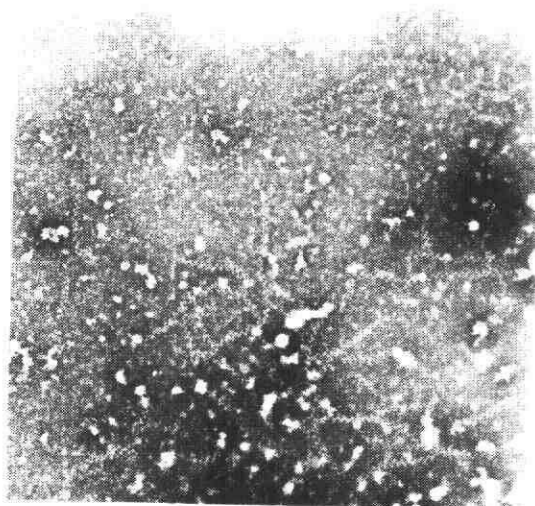
From peaks A and C

From peaks A' and C'

Figure 3. Microhelices formed by re-combination of materials from peaks A and C (profuse bundles) from the Sephadex G-150 column and peaks A' and C' (single helices) from the Bio-Gel P-100 column. Mag. × 18 000

the microhelices bear any relationship with each other.

Paper electrophoresis[23] in veronal buffer at pH 8.6 showed that component A (or A') was anionic, having the same mobility as that of the band (vi) of B serum. Component C had the same mobility as band (v) and components B and D the same mobility as bands (iii) and (iv) respectively (Figure 4); A is therefore an acidic protein and C a basic protein. These results indicated that only the minor protein bands of B serum, bands (v) and (vi) were involved in the formation of microhelices. The major proteins of B serum i.e. the major basic protein represented by band (i)[24] and the major acidic protein hevein represented by band (viii)[25] were not involved in the formation of microhelices.

Microhelices in Bundles and Singles

Microhelices occur as profuse bundles or as many single helices (Figure 3) and sometimes as a mixture of both. The bundles are considered as single helices pressed together laterally.

It has been observed that if B serum was first dialysed against 0.35M NaCl, negligible precipitation resulted. A second dialysis against water resulted in considerable precipitation with large numbers of *single* microhelices. This precipitate, after redissolving in the salt solution and subjecting to gel-filtration chromatography, showed the four peaks but the fourth peak (component D or D') was greatly reduced[26]. Component D (or D') being of a low molecular weight (5000) was obviously lost partially during the first dialysis of the serum against salt solution. Being insoluble in water, the remainder precipitated out along with the other three components during the second dialysis against water.
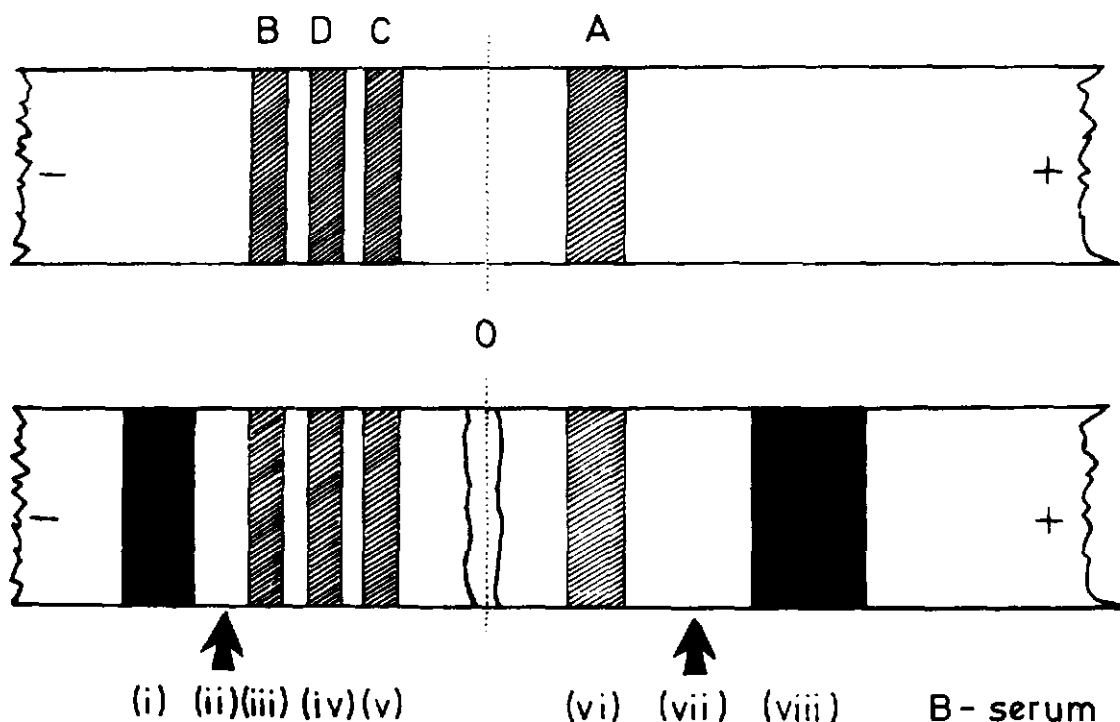
Figure 4. Diagram illustrating paper electrophoresis of the four components and B serum. The numbering of the bands in the electropherogram of B serum is according to Moir and Tata [23].

In further experiments, component D was added (in solution) to the preparation containing single microhelices (also in solution) and the mixture was dialysed against water. The resulting precipitate showed bundles of microhelices.

Protein D (or D') therefore appears to have a role in the formation of bundles of microhelices and its concentration in B serum may be critical for the formation of single or bundles of microhelices. The bundles illustrated in *Figure 3* resulted from the combination of components A and C which had been recovered from the Sephadex column whereas the single helices were obtained by combining A' and C' recovered from the Bio-Gel column. This discrepancy can be explained in the light of the results described above: the resolution between components C' (mole weight 22 000) and D' (mole weight 5000) has been more efficient on the Bio-Gel column than that between components C and D on the Sephadex column. Component C therefore contained some component D and the mixture of A with C (contaminated with some D) resulted in the formation of bundles of microhelices.

Occurrence and Distribution of Microhelices in Clones of Hevea

As mentioned above, dialysis of B serum against water always produced a precipitate. This precipitate usually

showed microhelices but variations were observed between individual samples within a clone as well as between clones[21] : microhelices were rarely observed in RRIM 501 but were profuse in Tjir 1 and were also frequently observed in RRIM 600.

To investigate the cause for this variation precipitates obtained by dialysis of B sera from these clones were redissolved in 0.35M NaCl and resolved by column chromatography on Sephadex G-150. Four peaks were obtained irrespective of whether the microhelices were present or not in the original precipitate. Protein estimations on the material recovered from the four peaks showed that when microhelices were not observed in the original precipitate, there was a deficiency of component C (the pro-helical protein)[26]. This was clearly noticeable in RRIM 501 where the recombination of the components A and C (1 ml of each) gave a precipitate after dialysis which did not show microhelices. However, when 1 ml of component A from this experiment (RRIM 501) was combined with 1 ml of component C which had been previously isolated from RRIM 600, microhelices were observed after dialysis (*Figure 5*). (The reason for the formation of bundles of microhelices has been explained above.) Throughout all these experiments, the method had been so designed that the resolution of the precipitate on the column, the concentration of each peak material eluting from the column to a fixed volume (5 ml) and finally the recombination of the components A and C in equal volumes (1 ml each) maintained the stoichiometry of the original concentrations of the two essential components of microhelices. These experiments clearly indicated that the formation of micro-

helices required the combination of the two components, (both of which are glycoproteins) in a certain stoichiometric ratio, irrespective of the clone from which the proteins were isolated.
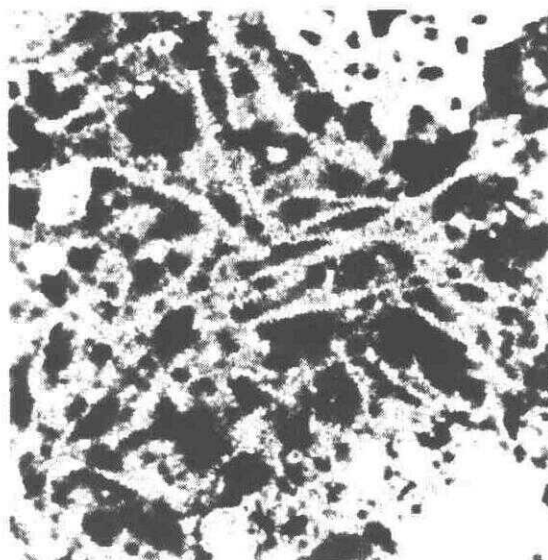

Figure 5. Microhelices formed by the combination of component A *from RRIM 501 and component* C *from RRIM 600. Mag.* × *18 000*

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