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# Post-fermentation processing conditions and solution properties of an extracellular fungal polysaccharide isolated from the culture filtrate of *Schizophyllum commune*

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The solution properties of schizophyllan samples, isolated from native and thermally treated culture broths of *Schizophyllum commune*, have been investigated using static and dynamic laser light scattering, rheological and size exclusion chromatographic measurements. An attempt was made to correlate the variability in the physicochemical properties of the biopolymer with the processing conditions of the culture broths.

## INTRODUCTION

The native water-soluble non-ionic polysaccharide schizophyllan produced by the fungus *Schizophyllum commune* (Kikumoto *et al.*, 1970, 1971) dissolves as a rigid triple helix in aqueous solution up to pH 12·8 (Kashiwagi *et al.*, 1981) whereas a single coiled chain prevails at higher pH, in the presence of DMSO and/or at temperatures above 135°C as a result of breaking of intermolecular H bonds responsible for the ordered conformation (Rinaudo & Vincendon, 1982; Sato *et al.*, 1983; Kitamura & Kuge, 1989).

Many industrial applications, e.g. the control of rheology of aqueous phases in oil recovery, take advantage of the large viscosity associated with the triple helical conformation of schizophyllan. As for other fermentation polysaccharides, product variations are observed among samples from one manufacturer and among samples from several manufacturers. Such variations could strongly affect the solution properties and are a concern for large scale applications. They could arise from the fermentation and/or post-fermentation processing. In the case of schizophyllan, post-fermentation operations involve alkali treatment followed by a thermal treatment. In a previous paper the authors reported that denaturation and/or degradation could result from the combined effect of pH and temperature (Zentz *et al.*, 1992).

The first objective of this work was to examine the solution properties of broth-form schizophyllan samples obtained from the same manufacturer. The second objective was to determine if a relation between the observed differences in solution properties and the post-fermentation processing conditions exist.

## EXPERIMENTAL

The schizophyllan samples were supplied by Sanofi Bio-Industries (Baupte, France) as diluted fermentation broths containing $NaN_3$ or Kathon CG as a bactericide.

Four different samples from three different fermentations were examined. S1 is a clarified unpasteurized fermentation broth protected with $NaN_3$. S2T is a clarified thermally treated fermentation broth protected with Kathon CG.

S3 and S3T only differ by the post-biosynthesis processing. Both of them came from the same fermentation operation; only S3T was thermally treated after the fermentation step (100°C, 30 min). Both samples were protected by Kathon CG.

Bioreactors of different sizes were used for these three batches.

Dilute solutions of schizophyllan were obtained by

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diluting the clarified fermentation broth with 20 g litre$^{-1}$ NaCl containing 0·4 g litre$^{-1}$ $NaN_3$ (step 1) followed by sequential filtration through Millipore filters (8, 1·5, 0·45 and 0·22 $\mu$m) for removing macro-impurities (bacterial cells) (step 2). The clarified dilute solutions were then purified by extensive ultrafiltration at constant volume against 20 g litre$^{-1}$ NaCl/0·4 g litre$^{-1}$ $NaN_3$ to remove low molecular weight impurities (proteins, small sugars, etc.) (step 3). Finally, a new sequential filtration was carried out under the same conditions as for step 2 (step 4).

Polymer concentrations were determined using a Dohrman DC80 carbon analyser. All stock solutions were stored at 6°C before use.

Lower $M_w$ fractions were obtained by sonication. Analytical high performance size exclusion chromatography (HPSEC) on Toyosoda 6000 PW packings was used for monitoring the progress of the sonication. Schizophyllan samples were detected with a refractive index detector (Shimadzu, model 6A). The results are expressed as the partition coefficient $K'_d = (V_e - V_0)/(V'_e - V_0)$, where $V_e$ is the elution volume, $V_0$ the total exclusion volume and $V'_0$ the total permeation volume (salt peak).

A Contraves low shear LS30 viscometer was used for viscosity measurements in the Newtonian regime.

Absolute scattered light intensities were measured in 20 g litre$^{-1}$ NaCl/0·4 g litre$^{-1}$ $NaN_3$ with a Chromatix KMX6 (Milton Roy, USA) low angle laser light scattering (LALLS) photometer at a forward angle of 5° and with a Macrotron (Amtec, Villeneuve Loubet, France) photometer in an angular range 20–150°. The radii of gyration $(R_g)_z$ were evaluated from the angular dependence in the low angle limit by use of classical static Zimm plots. The solutions were made optically clear by filtration through 0·22 $\mu$m Millipore filters. A value of $dn/dc = 0{\cdot}142$ ml g$^{-1}$ was used for the refractive index increment of schizophyllan.

Dynamic light scattering measurements were performed with a KMX6/Malvern multibit correlator K7025 equipment (homodyne detection type) at low polymer concentration and low scattering angle so that only translational diffusion is of importance.

## RESULTS AND DISCUSSION

The results of light scattering and low shear viscosity measurements are reported in Table 1. HPSEC elution profiles are given in Fig. 1. In all cases, the removal of low molecular weight impurities by ultrafiltration at constant volume (step 4) results in biopolymer solutions with lower molecular weight, less angular dependence of the scattered light and lower aggregation tendency as shown by the decrease in the Huggins constant ($k'$).

Variations in $M_w$ and viscosity are observed not only among samples from different fermentations but also among samples from the same fermentation, but differing by the nature of post-fermentation processing (S3 and S3T). Moreover, the comparison between S2T

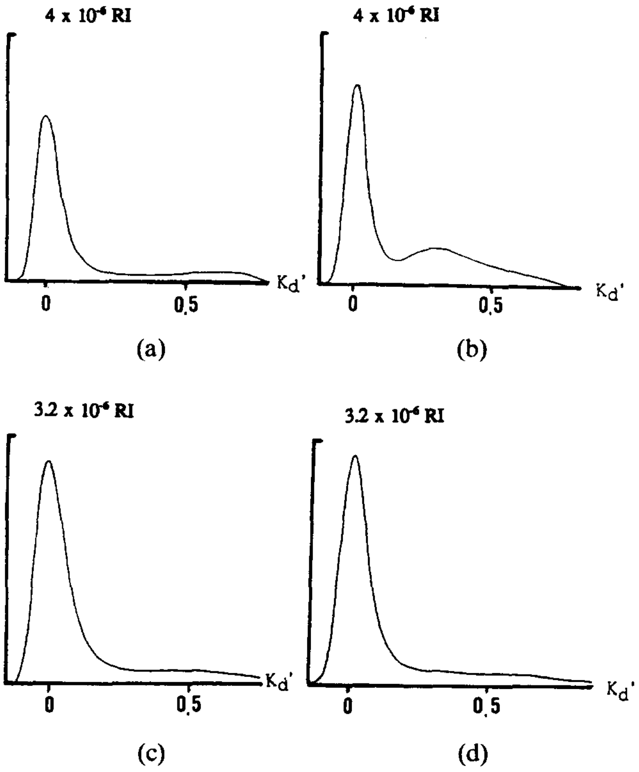

**Fig. 1.** HPSEC elution profiles of purified and diluted commercial batches. (a) S1; (b) S2T; (c) S3; (d) S3T.

**Table 1. Results of light scattering and low shear viscosity measurements**

| Sample: | S1 | S2T | | S3 | | S3T |
|---|---|---|---|---|---|---|
| Treatment$^a$ | After UF (step 4) | After UF (step 4) | Before UF (step 2) | After UF (step 4) | Before UF (step 2) | After UF (step 4) |
| $\overline{M}_w$ (dalton) | 5·5–6·8 × 10$^6$ | 3·7 × 10$^6$ | 6·7 × 10$^6$ | 5 × 10$^6$ | 8 × 10$^6$ | 6·9 × 10$^6$ |
| $A_2$ (g ml$^{-2}$) | — | ≃2 × 10$^{-4}$ | ≃2·5 × 10$^{-4}$ | ≃2·5 × 10$^{-4}$ | ≃4 × 10$^{-5}$ | ≃2·5 × 10$^{-4}$ |
| $[\eta]_0$ (ml g$^{-1}$) | 9000, 9500 | 6800 | 9000 | 8000 | 8500 | 9100 |
| $k'$ | 0·55–1·6 | 0·45 | 1·4 | 0·47 | 3 | 0·75 |
| Solvent | 20 g litre$^{-1}$ NaCl/0·4 g litre$^{-1}$ $NaN_3$ filtration through 0·22 $\mu$m filters | | | | | |

$^a$See experimental section.
UF, Constant volume ultrafiltration.

and S3T show large differences in solution properties although the post-fermentation thermal treatment was identical in both cases. The lowest molecular weight is measured for sample S2T the chromatographic profile of which greatly differs from that of sample S3T although both samples have undergone the same thermal post treatment. The elution profile of S2T (Fig. 1) shows the presence of two different macromolecular species which could be ascribed to the coexistence of triple helical (TH) and single chains (SC), respectively, in the proportion 2:1. This probably originates from alkaline and thermal post-fermentation processing which could induce partial denaturation and/or degradation as previously reported (Zentz *et al.*, 1992).

The comparison between S3 and S3T indicates that the post-fermentation thermal treatment unfavourably affects the 'quality' of solutions (higher $M_w$ and $k'$; more pronounced angular dependence of the scattered light.

Finally, data of poor reproducibility were observed with sample S1, possibly as a consequence of an inadequate protection to bacterial growth by sodim azide.

Figure 2 shows the variation of the 'zero shear rate' specific viscosity of S3, S2T and S3T versus the overlap parameter $C\,[\eta]_0$ which is characteristic of the degrees of occupancy of space by the polymer molecules. The general flow behaviour of schizophyllan samples agrees well with that reported for a wide range of polysaccharides, e.g. xanthan (Cuvelier, 1988), CMC (Castelin *et al.*, 1987) and galactomannans (Pezron, 1988).

For all samples studied, the slopes of log $\eta_{sp}$ versus log($C\,[\eta]_0$) are almost similar in the dilute regime (1·4–1·7 at $C < C^*$), while at higher concentration ($C > C^*$) a somewhat lower slope is observed for the thermally treated sample S2T as compared to the untreated one (Fig. 3).

A plausible explanation for such a difference could be due to a somewhat larger overall flexibility of S2T as a consequence of a partial denaturation of the native ordered rigid conformation as evidenced from chromatographic profiles (Fig. 1). Although such an explanation should be advanced with great caution due to the lack of a theoretical basis for the scaling laws in the intermediate concentration domain, this could be supported by the data reported by Morris *et al.* (1980) on the flow behaviour of hyaluronate molecules as a function of added salt.

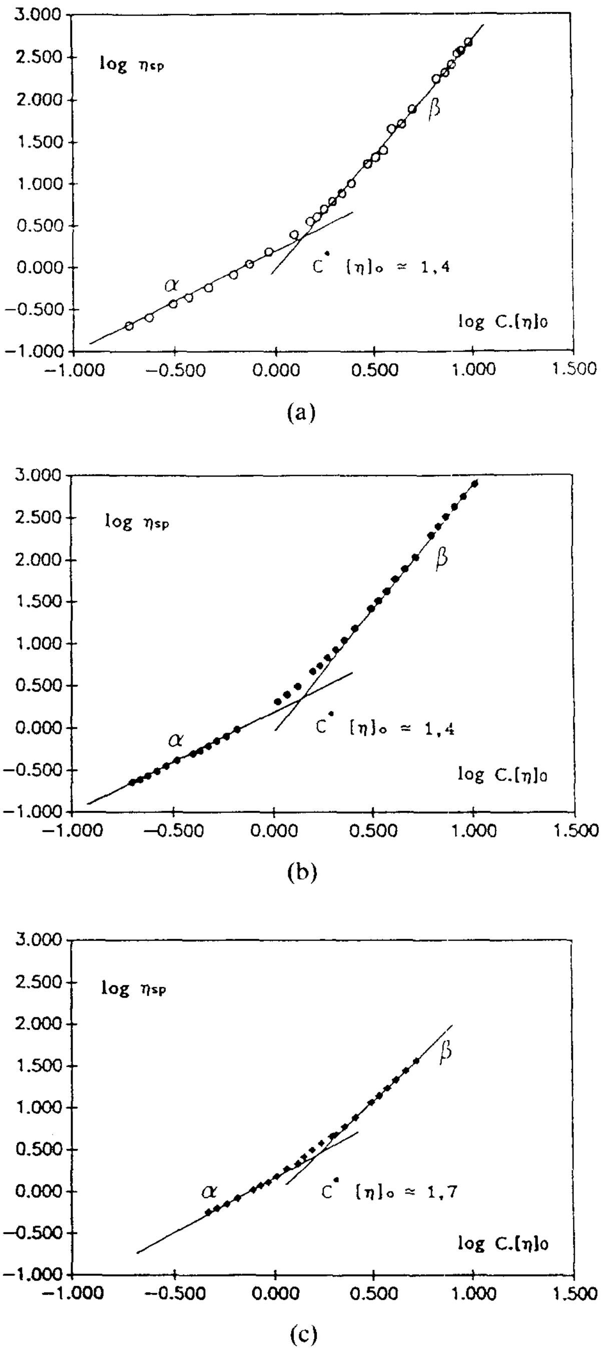

**Fig. 2.** Variation of the 'zero shear rate' specific viscosity versus the overlap parameter $C\,[\eta]_0$. (a) Purified S3; (b) purified S3T; (c) purified S2T.

For long rod-like macromolecules, the analysis of the scattering behaviour in the high angle asymptotic domain (Holtzer, 1955) shows that a constant plateau should be reached at large scattering factor $\mu = \sin(\theta/2) \times 4\pi n/\lambda$. From the magnitude of the asymptote one may obtain the linear mass density, i.e. the mass per unit length $M/L$. The Holtzer plots of purified S2T, S3 and unpurified S3T are shown in Fig. 3. As expected for rigid rods, an asymptotic limit is approached for both S3 and S2T giving $M/L$ in the range 1900–2200 dalton nm$^{-1}$ and 1400–1700 dalton nm$^{-1}$ for S3 and S2T, respectively. The value found for S3 is not so far from the values expected for a triple helical schizophyllan molecule ($M/L$ = 2000–2300 dalton nm$^{-1}$) (Yanaki *et al.*, 1980; Kashiwagi *et al.*, 1981).

The lower mass per unit length for S2T should be interpreted by considering the presence of a single

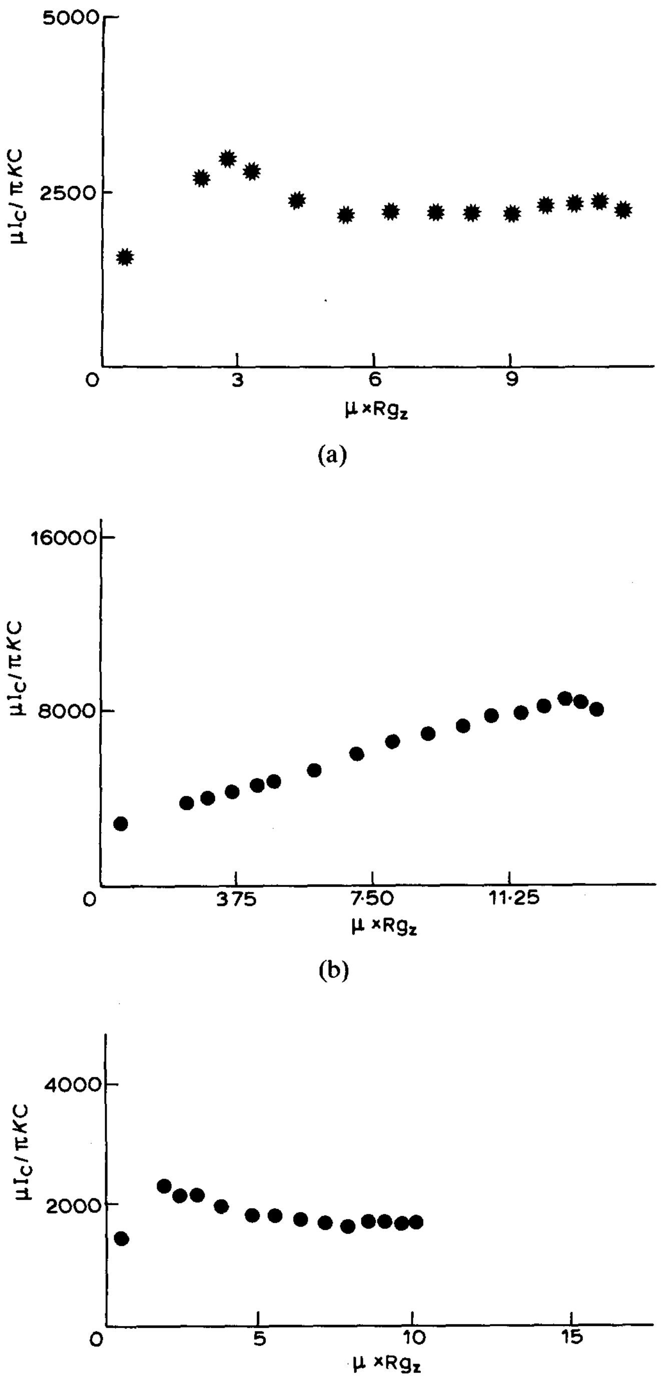

**Fig. 3.** Holtzer plots for schizophyllan samples. (a) Purified S3; (b) unpurified S3T; (c) purified S2T.

chain coming from the partial denaturation of the native structure and is in agreement with the chromatographic profile reported in Fig. 1.

In contrast, no asymptote could be reached for unpurified S3T and a much higher $M/L$ is found which could be ascribed to the presence of aggregates.

Further evidence in support of an overall higher flexibility of S2T can be inferred from the analysis of dynamic light scattering of varying molecular weight fractions of purified S3, S3T and S2T.

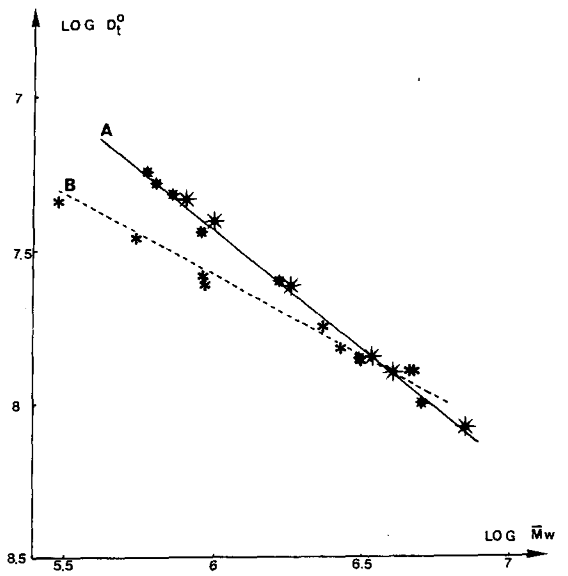

**Fig. 4.** Power laws for the translational diffusion coefficients versus molecular weight. (a) Purified S3 ( ✱ ) and purified S3T (✳); (b) purified S2T ( ∗ ).

The power laws for the translational diffusion coefficients against molecular weight are reported in Fig. 4. In any case, $D_t$ scales as $M_w^\gamma$ but it appears that the exponent ($\gamma$) is lower for S2T, thus indicating a less stiff overall conformation.

As suggested by the elution profile (Fig. 1(B)), a plausible explanation could be that S2T is a mixture of two types of population namely triple helix and single coil.

In support of this, the authors have examined the solution behaviour of a mixture which contained $\frac{2}{3}$ triple helical conformation (sample S3, $\overline{M}_w = 5 \times 10^6$ dalton and $\frac{1}{3}$ of single coil (denaturated S3, $\overline{M}_w = 1{\cdot}6 \times 10^6$ dalton).

The molecular characteristics and the elution profile (Fig. 5) of such a sample are very close to those reported for the sample S2T (Fig. 1(B)).

Data reported in Fig. 5 show that the industrial thermal treatment does not necessarily result in a partial denaturation of the native triple helical conformation as indicated by the fact that the $\overline{M}_w$ dependence of the translational diffusion coefficient is identical for S3 and S3T ($D_t = 1{\cdot}86 \times 10^{-3}\,\overline{M}_w^{-0{\cdot}78}$).

The same is observed with regard to the molecular weight dependence of $[\eta]_0$ as shown by Fig. 6. Both samples S3 and S3T can be reasonably well modelled by the Yamakawa-Fujii theory (1974) and a good agreement with reported literature data is found as indicated by data reported in Table 2.

Bohdanecky (1983) showed that the Yamakawa-Fujii theory for $[\eta]_0$ can be expressed approximately as:

$$(\overline{M}_w^2/[\eta]_0)^{1/3} = A_\eta + B_\eta \overline{M}_w^{1/2}$$

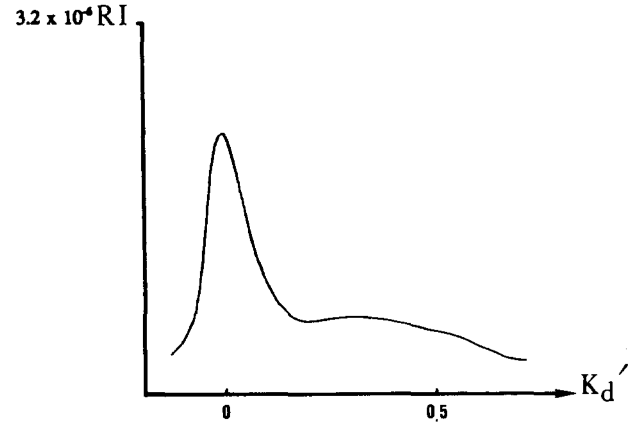

**Fig. 5.** HPSEC profile of a schizophyllan sample ($D_t$ = 1·25 · $10^{-8}$ cm$^2$ s$^{-1}$, $\bar{M}_w$ = 3·7 · $10^6$ dalton) containing a 2:3 triple helix/single chain mixture.

with

$$A_\eta = 1{\cdot}516 \times 10^{-8} A_0 (M/L)$$

$$B_\eta = 1{\cdot}516 \times 10^{-8} B_0 (<h^2>/\bar{M}_w)^{-1/2}$$

$A_0$ and $B_0$ are known functions of $d/2q$ (the reduced diameter) and have been tabulated by Bohdanecky (1983).

As can be shown from the data reported in Table 3, the values found for S3 and S3T agree rather well with those reported by other authors for the triple helical schizophyllan. Only a slight tendency for aggregation is observed for S3T as indicated by the measured $M/L$ value (in agreement with higher $k'$). This could have some importance in more a concentrated solution where polymer/polymer interactions are made more favourable.

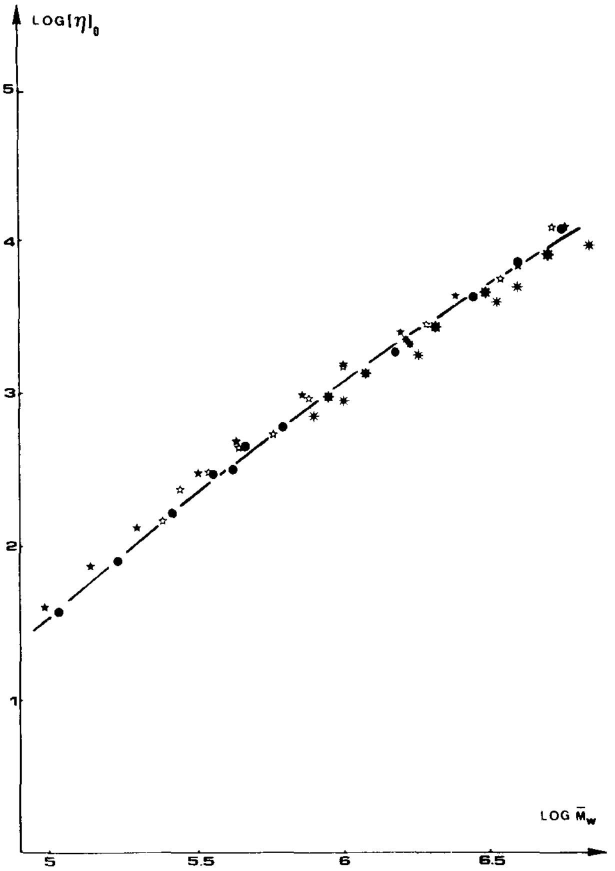

**Fig. 6.** Molecular weight dependence of $[\eta]_0$ for schizophyllan samples in 20 g litre$^{-1}$ NaCl. (★) Yanaki *et al.* (1980); (☆) Yanaki *et al.* (1983); (●) Kashiwagi *et al.* (1981); (✱) S3; (✳) S3T. Solid line: curve calculated for an unperturbed worm-like cylinder with $M/L$ = 2200 dalton nm$^{-1}$, $q$ = 180 nm and $d$ = 2·6 nm.

Good agreement between the experimental radii of gyration and theoretical ones calculated for an unperturbed worm-like chain with $q$ = 200 nm and $M/L$ = 2000 dalton nm$^{-1}$ (Benoit & Doty, 1953) is observed for sample S3 as illustrated in Fig. 7.

**Table 2. Mark Houwink relations for schizophyllan; $[\eta]_0 = K \times \bar{M}_w^{\,a}$ (for $\bar{M}_w > 5 \times 10^5$ dalton)**

| Sample | Solvent | Method of determination ($\bar{M}_w$) | $K$ | $a$ |
|---|---|---|---|---|
| Yanaki *et al.* (1980) | $H_2O$ | Sedimentation equilibrium | 1·04 × $10^{-4}$ | 1·19 |
| Yanaki *et al.* (1983) | $H_2O$ | HPLC-LALLS couplage | 4·65 × $10^{-5}$ | 1·24 |
| Kashiwagi *et al.* (1981) | NaOH $10^{-2}$ N | LALLS | 6·33 × $10^{-6}$ | 1·37 |
| S3 | NaCl 20 g litre$^{-1}$ | LALLS | 4·14 × $10^{-5}$ | 1·24 |
| S3T | NaCl 20 g litre$^{-1}$ | LALLS | 5·15 × $10^{-5}$ | 1·20 |

**Table 3. Results of graphical resolutions by method of Bodhanecky (1983)**

| Fractions of schizophyllan | Solvent | $A_\eta$ | $B_\eta$ | $M/L$ (dalton nm$^{-1}$) | ($<h^2>/\bar{M}_w$) (nm$^2$ dalton$^{-1}$) | $q$ (nm) | $d$ (nm) |
|---|---|---|---|---|---|---|---|
| Yanaki *et al.* (1980) | $H_2O$ | 490 | 0·39 | 2050 | 0·176 | 180 | 2·7 |
| Kashiwagi *et al.* (1981) | NaOH $10^{-2}$ N | 540 | 0·40 | 2250 | 0·167 | 190 | 2·8 |
| Purified (S3) | NaCl 20 g litre$^{-1}$ | 540 | 0·42 | 2250 | 0·152 | 170 | 2·6 |
| Purified (S3T) | NaCl 20 g litre$^{-1}$ | 600 | 0·44 | 2500 | 0·138 | 170 | 2·6 |

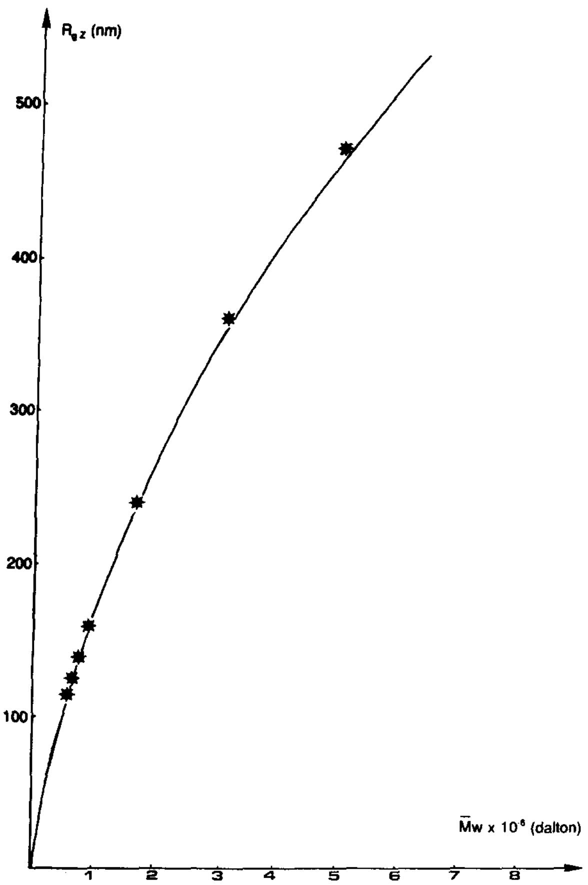

**Fig. 7.** Comparison between experimental and theoretical radii of gyration (✱) experimental points (purified S3). Solid line: unperturbed worm-like chain with $M/L$ = 2000 dalton nm$^{-1}$ and $q$ = 200 nm.

## CONCLUSION

It is generally admitted that microbial polysaccharides can be obtained in a reproducible way from the culture of carefully selected strains. On the other hand, the methods for recovering the biopolymers from the fermentation broths are generally mild. For these reasons the microbial polysaccharides have a consistent structure and therefore consistent properties, making them of great interest for industrial applications. In contrast, the properties of natural polysaccharides are dependent on the generally more drastic methods used for their extraction and also on climatic conditions. Nevertheless, evidence exists that microbial polysaccharides from the same bacterial strain but produced under different cultural conditions and/or subjected to different post-fermentation treatments may show large changes in solution properties. In this respect, the authors recently reported experimental evidence showing that xanthan originating from the same fermentation exhibited different solution properties according to the nature of the thermal post-fermentation treatment (Wehrhahn *et al.*, 1990). Depending on the thermal history, the variability of the xanthan properties could be a consequence of a partial denaturation of the native conformation. In the case of purified schizophyllan, the authors have shown (Zentz *et al.*, 1992) that degradation and/or denaturation could result from the combined effect of pH, temperature and oxygen. Probably a partial denaturation of the native conformation as a result of thermal treatment could be at the origin of the gel permeation profile observed for S2T (Fig. 1(B)) and the lower measured value of the mass per unit length $M/L$ (Fig. 3(C)) could indicate the presence of a significant number of single strands. The comparison between the solution properties of samples originating from the same fermentation broth (S3 and S3T) shows that aggregation of the polysaccharide molecule can result from the thermal treatment of the unpurified fermentation broth (higher $M/L$ and $k'$). This emphasizes that the impurities from the fermentation broth can unfavourably affect the solution properties of schizophyllan as is also the case with scleroglucan (Rivenq *et al.*, 1989; Kalpacki *et al.*, 1990). Finally, among many factors that can affect the solution properties of fermentation polysaccharides, those relevant to the nature and conditions of post-treatment of fermentation broths seem to be of major importance in the variability of the biopolymer solution properties.

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