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Chitosan functional properties

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Chitosan is a partially deacetylated polymer of *N*-acetyl glucosamine. It is essentially a natural, water-soluble, derivative of cellulose with unique properties. Chitosan is usually prepared from chitin (2 acetamido-2-deoxy *β*-1,4-D-glucan) and chitin has been found in a wide range of natural sources (crustaceans, fungi, insects, annelids, molluscs, coelenterata etc.) However chitosan is only manufactured from crustaceans (crab and crayfish) primarily because a large amount of the crustacean exoskeleton is available as a by product of food processing. Squid pens (a waste byproduct of New Zealand squid processing) are a novel, renewable source of chitin and chitosan. Squid pens are currently regarded as waste and so the raw material is relatively cheap. This study was intended to assess the functional properties of squid pen chitosan. Chitosan was extracted from squid pens and assessed for composition, rheology, flocculation, film formation and antimicrobial properties. Crustacean chitosans were also assessed for comparison. Squid chitosan was colourless, had a low ash content and had significantly improved thickening and suspending properties. The flocculation capacity of squid chitosan was low in comparison with the crustacean sourced chitosans. However it should be possible to increase the flocculation capacity of squid pen chitosan by decreasing the degree of acetylation. Films made with squid chitosan were more elastic than crustacean chitosan with improved functional properties. This high quality chitosan could prove particularly suitable for medical/analytical applications.

***Keywords*: chitosan, squid, crustacean, viscosity, flocculation, film, solubility, functional, properties, composition**

1. Introduction

Chitosan is a partially deacetylated polymer of acetyl glucosamine (2 acetamido-2-deoxy b-1,4-D-glucan). It is essentially a natural, water-soluble derivative of cellulose with unique properties.

Chitosan be used as a flocculant, clarifier, thickener, fibre, film, affinity chromatography column matrix, gas-selective membrane, plant disease resistance promotor, anti-cancer agent, wound healing promoting agent and antimicrobial agent. It can be used in pet food and GRAS (generally regarded as safe) status has been applied for. It is used as a processing aid and is being trialled for applications in fruit preservation, wound dressings, cosmetics, artificial organs and pharmaceuticals [1].

Chitosan is usually prepared from chitin and chitin has been found in a wide range of natural sources (crustaceans, fungi, insects, annelids, molluscs, coelenterata etc.) [2]. However chitosan is only manufactured from crustaceans (crab, krill and crayfish) primarily because a large amount of the crustacean exoskeleton is available as a by product of food processing.

Squid pens (a byproduct of squid processing) are also a good source of chitin and hence chitosan (deacetylated chitin) [3]. Squid pens are removed from the squid during processing and are currently regarded as waste so the raw material is cheap. Crustacean sources of chitin must be treated with acid to remove calcium but squid pens are very low in calcium so the acid extraction step is not required. This change in the chitin extraction procedure should reduce the cost of processing and may reduce acid-hydrolysis of the chitin during processing. Thus chitin extracted from squid pens could be cheaper and better quality than chitin extracted from other sources. High quality chitosan could prove particularly suitable for the medical/analytical applications.

Structural work on squid pen chitin from *Loligo sp.* and *Ommastrephes bastrame*, has shown that these chitins are beta-chitins which have a much more open structure (parallel chain alignment) than the alpha-chitin (anti-parallel chain alignment) found in crustacean exoskeletons [3–5]. Very little work has been published on the functional properties of the chitosan prepared from squid although it was noted that chitosan extracted from *Ommastrephes bastrame* was more hygroscopic than chitosan extracted from crustacean [4, 5].

Previous confidential work at Industrial Research Limited has concentrated on the development of processes

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for producing chitosan. In this paper a sample of chitosan was prepared from New Zealand Arrow squid (*Nototodarus sloani*) pens and the functional properties of this squid chitosan were evaluated and compared with chitosan extracted from crustacean sources.

2. Materials and methods

2.1. Chitosans

The aim of this study was to evaluate the potential of squid pens as a resource for the production of chitosan. In this study we examined the chitosan from Arrow squid pens (*Nototodarus sloani* – Batch 1, NSOF-NPP6 (IRL)). The following chitosans from crustacean sources were also studied for comparison:
Chitosan from shrimp/crab – Seacure 443 (Pronova)
Chitosan from crab – Practical grade, C-0792, Lot 110HO105 (Sigma)
Chitosan from shrimp/crab – Profloc 340 (Pronova)

2.2. Preparation of a sample of squid chitosan

Frozen squid pens were defrosted by washing with water, dried for 18 h at 60 °C and milled using a Wiley knife mill. The damp, milled squid pens were soaked (35% w/w) in 2 M hydrochloric acid (HCl) for 18 h at room temperature and pressure (rtp) and then rinsed to neutrality in a Buchner funnel. The acid treated pens were soaked in 2 M sodium hydroxide (35% w/w) for 18 h (rtp) and then rinsed until the filtrate was colourless and neutral. Acid and alkali treated pens were tumbled for 4 h at 87 °C in 2 M sodium hydroxide, rinsed to neutrality and dried at 85 °C for 18 h. The resultant white material was chitin (yield 11.8%).

Chitin (9.3% %w/w) was tumbled in 15.6% (w/w) sodium hydroxide in propan-2-ol, under nitrogen for 12 h 15 min. The temperature was raised gently from room temperature to 87 °C for 30 min, maintained at 87 °C for 1 h and cooled for 45 min. The alkali-treated chitin was rinsed to neutrality as described previously and then dried at 85 °C for 18 h. The resultant off-white solid was chitosan (yield 79.1% of chitin). The yield of chitosan was 9.4 g per 100 g of frozen squid pens (maximum approx. 16%).

2.3. Preparation of chitosan powders

Homogenous, granular chitosan powders were prepared by grinding chitosan samples for 15 min in a Braun coffee grinder. These chitosan powders were used for all the experiments described in this report.

2.4. Determining chitosan solubility

A series of sodium acetate buffers (pH 2.5–7.5, 0.04 M) was prepared following a response surface experiment design (Figure 1). Chitosan (0.1 g) was weighed into duplicate disposable screw-cap test tubes (2 cm × 10 cm) and 9.9 ml of buffer was added. The suspensions were mixed and allowed to hydrate for 30 min at room temperature. Test tubes were placed in a boiling water bath for 20 min, mixed, cooled to room temperature and centrifuged at 4000 rpm (3434 relative centrifugal force) for 15 min (20 °C) in a Hereus Minifuge T. The physical appearance was observed. The supernatant and precipitate were separated, frozen and freeze-dried using a FTS Systems bench top freeze-drier. Solubility was measured as the % of the dry material recovered in the dry supernatant.

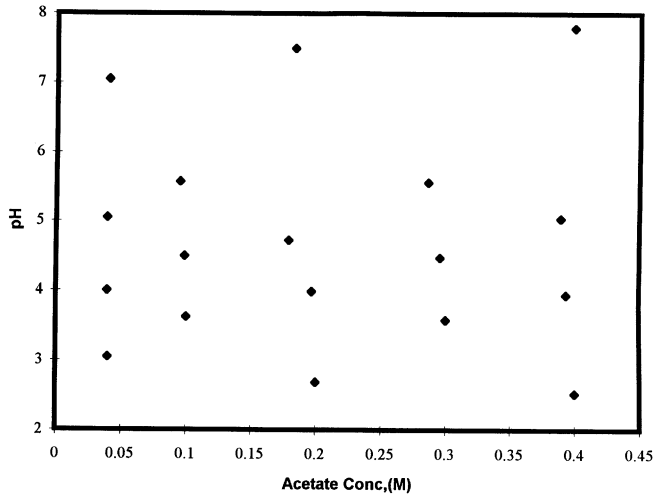

Figure 1. The distribution of sample points used in the chitosan solubility study.

It should be noted that other researchers dissolve chitosan in acetic acid and adjust the concentration and pH to the desired values. It was felt that the method used in this study was more accurate and a better representation of how chitosan might be used in a practical application.

2.5. Protein assay

A bovine serum albumin standard curve (0–1000 mg ml^{-1}) was prepared using Pierce standard BSA solution, (2 mg ml^{-1}, fraction V, in 0.9% sodium chloride and sodium azide, cat.no. 23209) and 1% (0.167 M) sodium acetate buffer (pH 4.5). Chitosan (0.1–1%) was dissolved in 1% acetate buffer pH 4.5.

BioRad Protein Assay Dye Reagent (0.1 ml) was added to 0.8 ml of protein sample/standard solution, the solutions were mixed and the absorbance at 595 nm was measured after 5 min using a Shimadzu UV 160 spectrophotometer.

2.6. Calculation of degree of acetylation

The degree of acetylation was calculated from the following equation.

Degree of acetylation =

[(8.69 − % nitrogen content)/(8.69 − 6.89)]*100

Where it is assumed that all the nitrogen detected is due to the chitosan, the nitrogen content of a glucosamine polymer is 8.69% (0% acetylation) and the nitrogen content of an *N*-acetyl glucosamine polymer is 6.89% (100% acetylation).

2.7. Rheological analysis

Chitosan solutions (0.1, 0.5, 1, 1.5 and 2%) were made up in 0.4 M acetate buffer pH 2.5 and assessed for apparent viscosity at 25 °C using a Haake VT500 Viscometer. An NV double concentric cylinder was used. The NV sensor cannot be used for viscosities greater than 1300 mPa·s. Apparent yield stress was calculated by fitting a linear model to the data from a flow curve (0 to 100 s^{-1}, 5 min.). Statistical analysis showed that R2 = 0.97 to 1.0 for all the chitosan solutions. Ostwald and Herschel-Buckley models had R2 values in the range 0.99 to 1 for the chitosan solutions. The apparent viscosity is the gradient of the flow curve at a shear rate of 40 s^{-1}.

2.8. Determining flocculation properties

This method was adapted from the method of Takagi and Kadowaki [6]. Chitosan solutions (0.1% w/w) and a Sigma Type III kappa-carrageenan solution (0.1% w/w) were prepared in 0.4 M acetate buffer (pH 4.5). A microtitre plate was filled (100 ml) with serial dilutions (1 in 2) of the four chitosans and kappa carrageenan (100 µl) was added. Flocculation was measured at 490 nm using a Bio-Tek Instruments Micro plate reader EL310. The flocculation factor is lowest dilution of chitosan that still flocculated the carrageenan.

2.9. Determining film forming properties

The appearance, thickness and tensile properties of chitosan films were assessed. Solutions of chitosan (1%) were prepared by dispersing chitosan in the 0.4 M acetic acid, allowing it to hydrate for 30 min with regular agitation and heating for 30 min in a boiling water bath. Chitosan solution (57.5 ml) was poured into a teflon coated loaf tin (85 mm width × 190 mm length × 50 mm height) and excess buffer was added (117 ml) to remove air bubbles. Each loaf tin was placed in a 50 °C oven for 18 h.

The chitosan films were carefully removed from the tins and placed on a flat surface. A sharp scalpel was used to cut strips 20 mm × 142 mm in length. Each strip was weighed and film thickness was measured using a SMIEC 0. 0.01 mm micrometer (Shanghai, China). Strips of paper (Copyright A480) and Parafilm "M" (American National Can, Greenwich, CT 06836) were used as controls. Flexibility was examined by folding a 20 mm × 142 mm film latitudinally and then releasing the force used to fold. Each strip was clamped into an Instron 1122 fitted with 200 N load cell. The chart speed was set at 50 mm min^{-1}, the gauge was 12.4 mm (1 mm gap) and the displacement speed was 5 mm min^{-1}. The scale was set at 5 (0–50 N) for all the strips except the paper strips for which the scale was 20 (0–200 N).

3. Results and discussion

3.1 The composition of squid pen chitosan and other chitosans

Squid pen chitin and chitosan were visibly cleaner than samples of chitin and chitosan from crab and crayfish. The crab and crayfish samples were contaminated with small fragments of red-pink exoskeleton that proved difficult to remove. Squid pens do not contain significant amounts of carotenoids whereas crab and crayfish exoskeletons contain large amounts of carotenoids.

The squid pen chitosan was similar in composition to commercial chitosan but it contained a substantially lower amount of ash (Table 1). Crustacean exoskeletons contain large amounts of calcium carbonate which must be extracted from the chitin whereas squid pen chitosan does not contain large amounts of mineral material. The low ash content shows that despite the milder alkali conditions used for the extraction of squid pen chitosan the squid pen chitosan is still cleaner (in terms of ash content) than the commercial chitosans. The squid pen chitosan had a higher degree of acetylation than crustacean chitosans.

Table 1. The ash, moisture, protein, nitrogen and acetyl content of selected chitosans.

Sample	*Source*	*%Ash*	*%Moisture*	*%Protein*	*%Nitrogen*	*%Acetyl*
Squid pen chitosan	Squid (IRL)	0.17	2.1	1.3	7.5 (7.8)	49.4
Seacure 443 chitosan	Crab/shrimp (Pronova)	0.58	11.2	1.3	7.2 (8.2)	27.2
Sigma chitosan	Crab (sigma)	0.51	4.8	1.3	7.1 (8.2)	27.2
Profloc 340 chitosan	Crab/shrimp (Pronova)	0.40	12.9	1.4	7.1 (8.2)	27.2

(No.) – %Nitrogen after adjusting for ash and moisture.

3.2. A comparison between the solubility of squid pen chitosan and other chitosans

Chitosan can be dissolved in aqueous solutions at above pH 7 but the solubility of chitosan will depend on the degree of acetylation, molecular structure and physical state (conformation, particle size etc). In this experiment the effect of acetate concentration and pH on chitosan solubility was examined. The results are shown in Table 2 and Figures 2–5.

Seacure 443 was soluble over the widest range of concentrations (it was the only one still soluble at pH 5) followed closely by Sigma chitosan and Profloc 340. Squid pen chitosan formed soft gels that proved difficult to centrifuge. It was less soluble than the commercial chitosans, it was 3.5% soluble at pH 7.05, 0.04 M acetate buffer as compared to approx 9% for commercial chitosans. This reduced solubility was consistent with the higher degree of acetylation and could be consistent with a higher molecular weight for squid pen chitosan.

3.3. The rheological properties of squid pen chitosan and other chitosans

Flow measurements for the four chitosans (squid pen chitosan, Seacure 443, Sigma chitosan and Profloc 340) were carried out and used to determine apparent viscosity and yield stress. The Seacure 443, Sigma chitosan and Profloc 340 solutions were nearly Newtonian with some shear thinning whereas the squid pen chitosan solutions exhibited distinct shear thinning. The effect of concentration on apparent viscosity and yield stress is presented in Figures 6 and 7.

Squid pen chitosan solutions were more viscous and had much larger yield stresses than the other chitosans. The higher yield stress of squid pen chitosan solutions was sufficient to prevent flow when a test tube of chitosan solution was inverted. The high viscosity and yield stress of squid pen chitosan could have important practical applications. Squid pen chitosan could be used as a thickening and suspending agent for acid products *eg* medical, cosmetic and food applications).

Table 2. The effect of acetate concentration and pH on the solubility of four chitosans.

Buffer	*Acetate conc. (M)*	*pH*	*IRL squid pen chitosan (%)*	*Seacure 443 chitosan (%)*	*Profloc 340 chitosan (%)*	*Sigma chitosan (%)*
1	0.40	2.53	(100) (Soft Gel)	100	100	100
2	0.20	2.69	(100) (Soft Gel)	100	100	100
3	0.04	3.06	(<100) (Soft Gel)	80.4	64.7	51
4	0.30	3.59	(100) (Soft Gel)	100	100	100
5	0.10	3.63	(<100) (Soft Gel)	100	100	81.6
6	0.39	3.95	(<100) (Soft Gel)	100	100	100
7	0.2	3.99	(<100) (Soft Gel)	100	99.9	100
8	0.04	4	(<100) (Soft Gel)	69.1	53.9	31.9
9	0.30	4.48	(<100) (Soft Gel)	100	96.9	100
10	0.10	4.5	(<100) (Soft Gel)	87.9	78.3	47.4
11	0.39	5.05	(<100) (Soft Gel)	100	86.1	67.7
12	0.18	4.73	(<100) (Soft Gel)	95.2	84.1	63.6
13	0.04	5.05	7.9	18.3	21.7	15.6
14	0.29	5.57	0	14.1	22.9	8
15	0.09	5.58	0	7.5	11.7	11.4
16	0.40	7.79	10	0	0	1.6
17	0.18	7.5	–	3.6	2.8	6.5
18	0.04	7.05	3.5	9.7	9	9.9

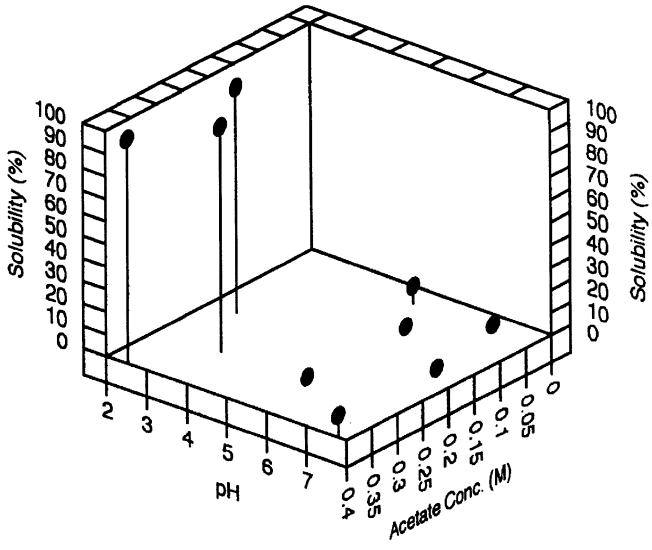

Figure 2. The effect of pH and acetate concentration on squid pen chitosan solubility.

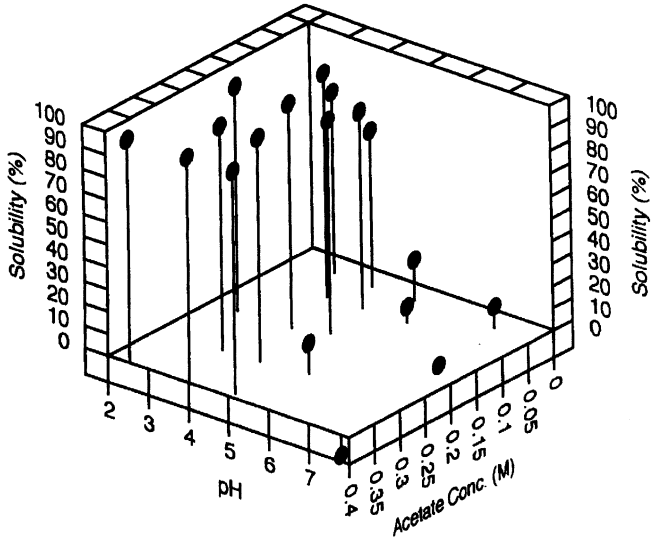

Figure 3. The effect of pH and acetate concentration on Seacure 443 chitosan solubility.

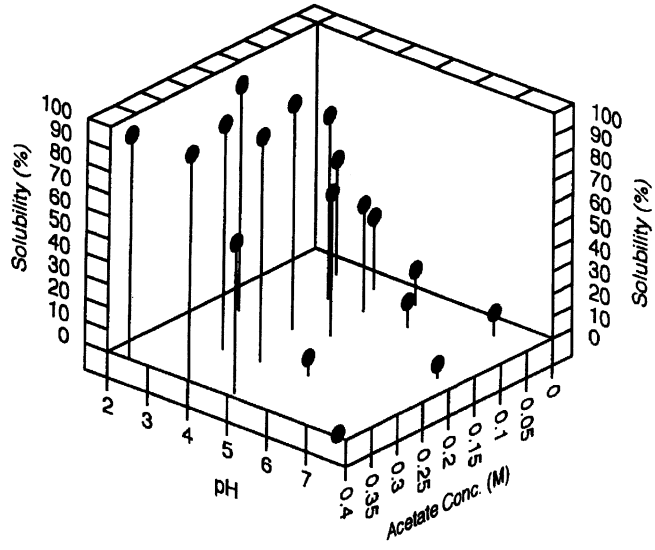

Figure 4. The effect of pH and acetate concentration on Sigma chitosan solubility.

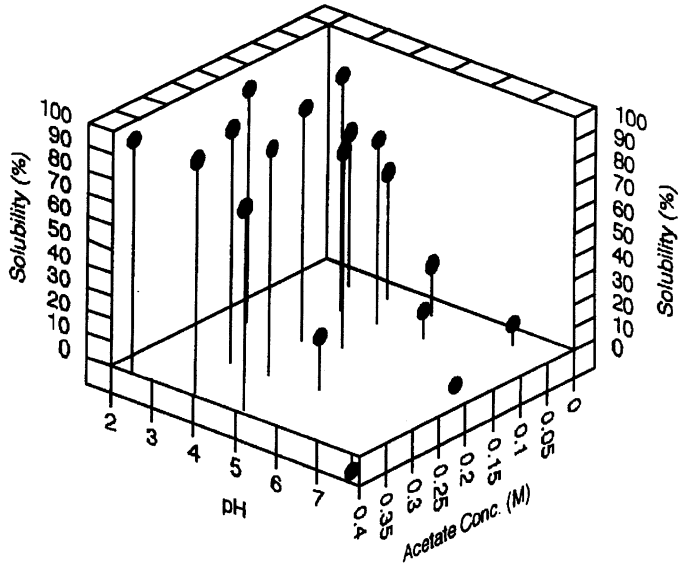

Figure 5. The effect of pH and acetate concentration on Profloc 340 chitosan solubility.

3.4. Flocculation properties

Chitosan is a positively charged polysaccharide and it can be used to precipitate negatively charged molecules (*eg* proteins) and particles (*eg* phospholipid complexes). In this assay for flocculation the negatively charged molecule carrageenan was precipitated from a clear solution. The flocculation factor is the minimum dilution of 0.1% chitosan that can flocculate the carrageenan.

Seacure 443 and Profloc 340 had similar flocculation factors, Sigma chitosan was not quite as effective as a flocculant and squid pen chitosan was a weak flocculant. These results may have been a consequence of differences in molecular weight. The squid pen chitosan had a higher degree of acetylation and so the amount of positively charged groups available for flocculating a negatively charged material was lower than in the crustacean

Table 3. The relative flocculation properties of four chitosans.

Flocculant	*Flocculation factor (1 = 0.1%)*
Squid pen chitosan	2
Seacure 443 chitosan	8
Sigma chitosan	4
Profloc 340 chitosan	8

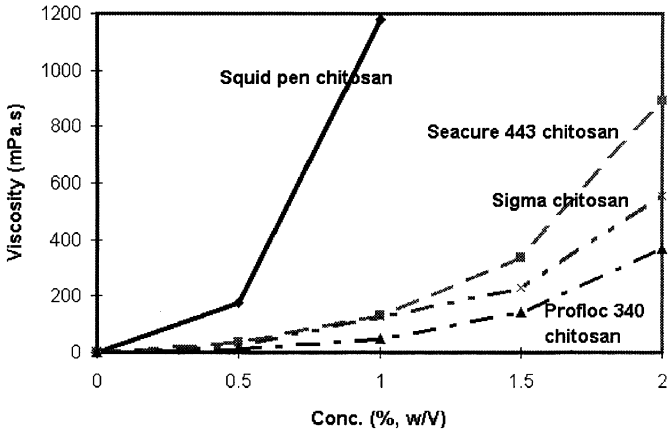

Figure 6. Effect of concentration on chitosan viscosity.

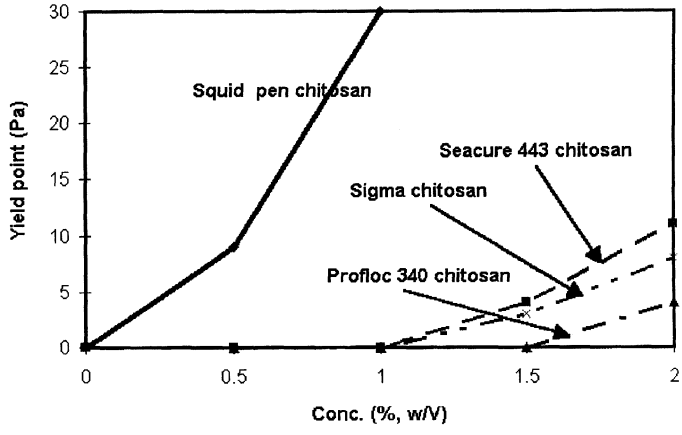

Figure 7. Effect of concentration on the yield point of chitosan solutions.

Table 4. The physical appearance, flexibility and thickness of chitosan and control films.

Film matrix	*Description*	*Thickness (m)*
Squid pen chitosan	A clear, transparent film with a crinkly surface similar in appearance to a crumpled cellophane wrapping. The film was flexible, it could be easily folded, but it quickly unfolded when the force was removed.	8×10^{-5} ($0.06–0.1 \times 10^{-3}$)
Seacure 443 chitosan	A yellow-tinted, transparent film with a smooth, shiny surface. The film was flexible, it could be folded and it remained folded when the force was removed.	6.5×10^{-5} ($0.025–0.07 \times 10^{-3}$)
Sigma chitosan	A yellow-tinted, transparent film with a smooth, shiny surface. The film was flexible, it could be folded and it remained folded when the force was removed.	6.5×10^{-5} ($0.025–0.07 \times 10^{-3}$)
Profloc 340 chitosan	A yellow-tinted, transparent film with a smooth, shiny surface. The film was flexible, it could be folded and it remained folded when the force was removed.	5.5×10^{-5} ($0.04–0.07 \times 10^{-3}$)
Parafilm ''M''	A soft opalescent film with a smooth, matt surface. The film was flexible, it could be folded and it remained folded once the force was removed with a slight tendency to unfold.	2.8×10^{-6}
Paper	A white, opaque sheet with a matt surface. It was less flexible than the other films but it could be folded. It quickly infolded when the force was removed.	1.2×10^{-6}

Table 5. Tensile properties of chitosan and control films.

Film matrix	*Yield stress (megaPa)*	*Yield strain*	*Elastic modulus (megaPa)*	*Break stress (megaPa)*	*Break strain*	*% Elongation*
Squid pen chitosan	22*	0.232*	114	16	0.402	43
Seacure 443 chitosan	21	0.127	218	(13)	0.877	78
Sigma chitosan	18	0.107	225	24	0.641	78
Profloc 340 chitosan	15	0.133	132	19	0.766	80
Parafilm ''M''	0.7	0.157	3.8	0.7	3.303	465
Paper	45	0.220	255	46	0.220	26

* Not a true yield point (see Figure 8).
( ) Unreliable.

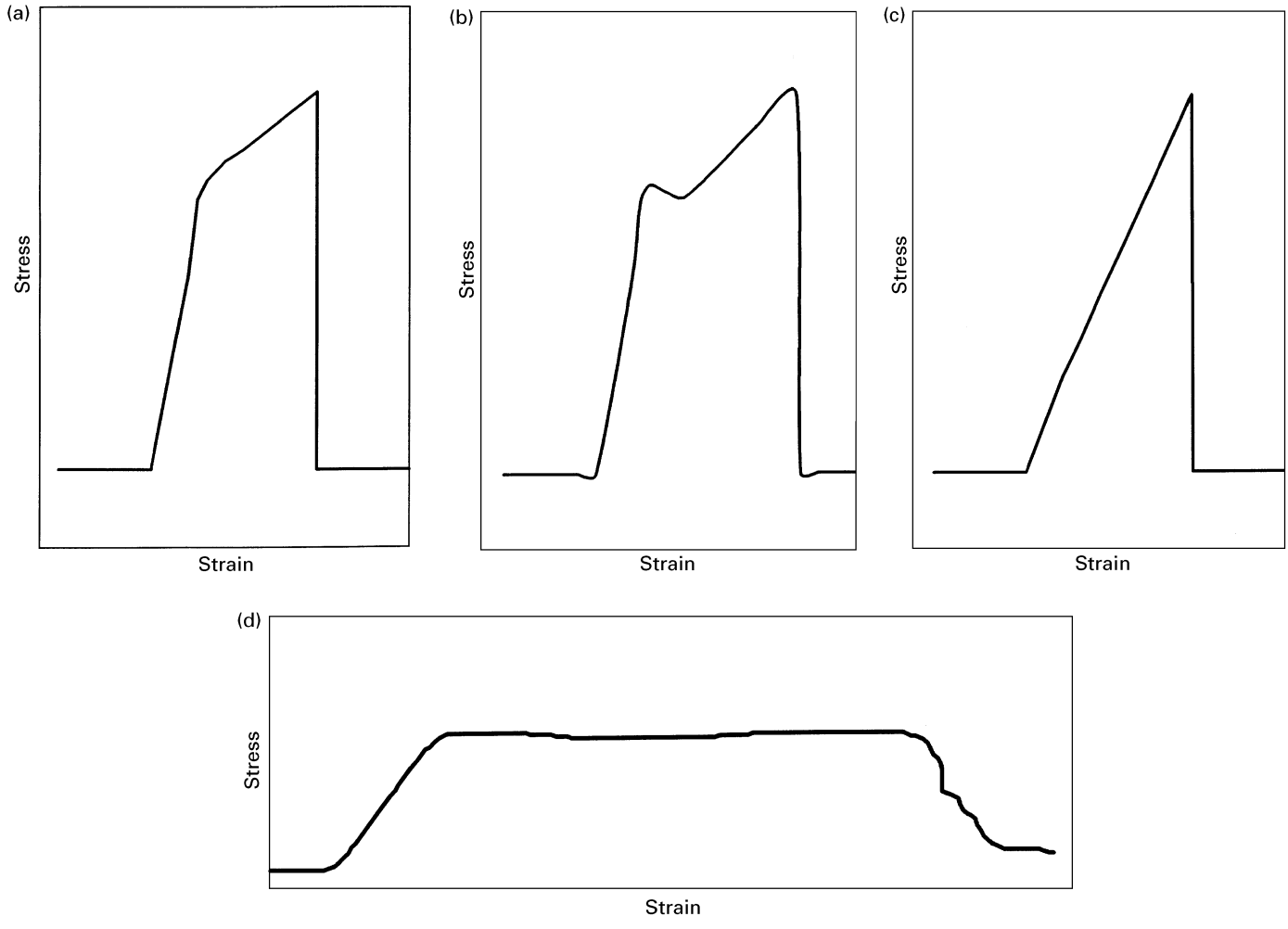

Figure 8 (a) Typical tensiometer chart record for squid pen chitosan (b) Typical tensiometer chart record for Seacure and Profloc chitosan (c) Typical tensiometer chart record for paper (d) Typical tensiometer chart record for Parafilm.

chitosan. Decreasing the degree of acetylation should increase the flocculation factor and this will be examined in future work.

3.5. Film forming properties

Chitosan films were prepared and assessed for appearance, flexibility, thickness and tensile strength. Parafilm "M" was selected as a control with low break stress and high break strain (stretchable). Paper was selected as a control with high break stress and low break strain (strong and brittle). The results are summarized in Tables 4 and 5 and Figure 8a and b.

In appearance the Seacure 443, Sigma chitosan and Profloc 340 films were identical. The Squid pen chitosan film was completely different from the other chitosans and Parafilm "M". It was similar to paper but it was much more elastic, had a shiny, slightly crinkled surface and was transparent. Seacure 443 and Sigma chitosan films were essentially identical. Profloc 340 films were very similar to Seacure 443 and Sigma chitosan films but Profloc 340 films were marginally weaker and more elastic (yield stress and elastic modulus lower). Squid pen chitosan films were more elastic (lower elastic modulus and higher yield strain) but did not stretch as much as the other chitosan films.

4. Conclusions

Squid pen chitosan has considerable potential. It is inherently purer than crustacean chitosans, it does not contain large amounts of calcium carbonate and carotenoids. It does contain large amounts of protein but these can be removed easily and should not present any problems with allergenicity. The purity of squid pen chitosan makes it particularly suitable for the high quality sector of the

chitosan market particularly medical and cosmetic applications. Solutions of squid pen chitosan have very interesting rheological properties, they have much higher viscosities and yield points than other chitosans. Squid pen chitosan solutions should prove useful as thickening and/or suspending agent in low pH applications. Squid pen chitosan can act as a flocculant although it will be necessary to reduce the degree of acetylation to produce effective flocculation activity. Films made from squid pen chitosan are different from films made using other chitosans. The fact that they are colourless is a major advantage, the textured effect could be useful where a more natural effect is desired but in any case it is likely that it will be possible to alter the texture by varying the film formulation. The increased elasticity may prove useful in applications where stretch without permanent deformation is required.

Work on the influence of acetylation on the properties of squid pen chitosan and on the molecular weight of squid pen chitosans is continuing [7]. At this stage it seems likely that a process for producing chitosan from squid pens will be cheaper than the current processes for producing crustacean chitosan [8].

5 Acknowledgements

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