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Recovery of chitosan from aqueous acidic solutions by salting-out: Part 1. Use of inorganic salts ☆

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Abstract

We describe a novel and easy-to-use approach based on salting-out to efficiently recover chitosan from aqueous acidic solutions. Inorganic salts of the Hofmeister series salted-out chitosans of 30 kiloDaltons (kDa), 240 kDa (35 cP) and a 70 cP form of the biopolymer dissolved in dilute HCl or acetic acid. The efficiency generally followed the ranking order of the Hofmeister's salts ($SO_4^{2-} > H_2PO_4^- \cong HPO_4^{2-} > NO_3^-$). Temperature (4 °C, room temperature, 50 °C) did not markedly affect the process. Ammonium sulfate was the most efficient salting-out agent of chitosans over a wide range of molecular sizes (2300 Da to >240,000 Da). The solubility (dilute HCl or acetic acid) of the salted-out chitosans varied from readily soluble to nearly insoluble. The choice of the Hofmeister's salt(s) for salting-out chitosans must take into account, (1) the efficiency of recovery, and (2) the molecular size of the chitosans to be recovered.

Keywords: Chitosan; Salting-out; Hofmeister's salts; Chitosan hydrolysates

1. Introduction

Chitosan is a partially *N*-acetylated linear polymer of 2-amino-2-deoxy-β-D-glucopyranose that occurs at low levels in some microorganisms (Arcidiacono & Kaplan, 1992; Hang, 1990). Industrial production of chitosan uses base-catalyzed hydrolysis of chitin obtained from the exoskeleton of crustaceans that is a by-product of the seafood industry (Kurita, 2006; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). The conditions of hydrolysis will influence the characteristics of the chitosan product. For instance, commercial chitosans vary largely in molecular sizes (typically 70 kDa to more than 1000 kDa) and degrees of deacetylation (typically 50–100%). Chitosan possesses a wide variety of commercial and biomedical applications (Macchi, 1996; Muzzarelli, 1999; Singla & Chawla, 2001; Ylitalo, Lehtinen, Wuolijoki, Ylitalo, & Lehtimaki, 2002) which are related to the physical properties of the biopolymer. For example, the hypocholesteroleamic efficicacy of chitosan increases in an inverse relationship to its molecular size and degree of *N*-acetylation (LeHoux & Grondin, 1993; Sugano, Watanabe, Kishi, Izume, & Ohtakara, 1988, 1992). Other studies have reported that chitosan molecules of 25–50 kDa are efficient in the treatment of stomach ulcers (Ito, Ban, & Ishihara, 2000) and the prevention of tumor growth in a mouse model through the activation of intestinal immune functions (Maeda & Kimura, 2004). Chitosan molecules of 28 kDa have been used as nanoparticles for the controlled release of drugs (medications) (Chen, Leem, & Park, 2003) whereas low molecular weight chitosans (≈2 kDa) have been shown to possess anti-fungal properties for

Abbreviations used: cP, centipoise; kDa, kiloDaltons.
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application to agriculture (Beaulieu, Lacasse, & Leclerc, 2003). Chitosan of 400 kDa is a suitable vehicle in a DNA vaccination approach of desensitization to peanut allergens in mice (Roy, Mao, Huang, & Leong, 1999).

The extended treatment of chitin with hot aqueous base solutions leads to deacetylation to various degrees. In addition, base-catalyzed hydrolysis generates a distribution of fragments of the biopolymer, a property known as polydispersion. Therefore, chitosans of low polydipersity and defined molecular sizes can only be efficiently and reproducibly prepared by controlled enzymatic hydrolysis using chitosanase (unpublished data) (Brzezinski, 1996, 2000; Masson, Denis, & Brzezinski, 1994). These conditions include, (1) a quick stop of the enzymatic digestion to prevent the generation of a polydispersed product, (2) an easy-to-use methodology to isolate the product of digestion rapidly and in a physical form that facilitates drying, ideally into a powder, (3) a product of digestion that is free of enzyme and, (4) conditions of isolation of the hydrolyzed product that make it fit for human uses, especially when applications to the biomedical field are sought. Furthermore, it may be desirable that the product of hydrolysis dissolves easily in an aqueous acidic milieu such as the one of the stomach.

Chitosan is a cationic polyelectrolyte. Its solubility in aqueous media should follow rules similar to those that apply to the solubility of proteins. Factors that influence polyelectrolytes solubility are pH, temperature and ionic strength of the dissolving medium. Salts of the Hofmeister series (Collins & Washabaugh, 1985; Hofmeister, 1988; Kunz, Henle, & Ninham, 2004) produce a salting-out effect that is defined as a decrease in the solubility of the solute (polyelectrolyte) resulting from an increase in the organization of water molecules around the ions instead of the solute. The ranking of anions with respect to their ability to precipitate proteins is, $SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^- > NO_3^-$ (Collins & Washabaugh, 1985; Hofmeister, 1988; Kunz et al., 2004). This salting-out effect results in the dehydration of the solute and its precipitation from solution (Collins & Washabaugh, 1985; Kunz et al., 2004) without alteration of the chemical structure of the solute.

Here, we report an innovative procedure that is based on the addition of inorganic salts of the Hofmeister series to aqueous solutions of chitosan. We show by way of examples that chitosans dissolved in dilute aqueous acids (e.g. acetic acid or hydrochloric acid) can be recovered with varied efficiencies by the addition of salts of sulfuric, phosphoric or nitric acids. The salted-out chitosans are recovered after removal of excess salt and can be obtained as a solid after drying.

2. Materials and methods

2.1. Chemical and reagents

The 30 kDa chitosan polymer (2 cP) and custom-made chitosan oligomers were purchased from Magistral Biotech Inc. (Blainville, QC). These chitosan samples were 93% deacetylated products and had been generated by chitosanase hydrolysis of commercial chitosan obtained from Marinard Biotech Ltée (Rivière-au-Renard, Gaspésie, QC). The molecular sizes of the 30 kDa, 240 kDa and chitosan oligomers were determined with a triple detector array instrument (Viscotek Corporation, Houston, TX) equipped with a low angle light scattering device. The triple-detector allowed integration of sample concentration, molecular mass, and viscosity measurements obtained by way of differential refractive index and light-scattering (right- and low-angle) detectors, and a bridge viscometer. The eluent consisted of a mixture of ammonium acetate (200 mM) and acetic acid (300 mM) buffered to pH 4.5. The SEC column was a TSK-gel G2500 PWXL (TOSOH, South San Francisco, CA). The 30 kDa chitosan product had a polydispersity of 1.2, as determined with the Viscotek instrument. This product is sold under the trade name Cholestol™/HEP-30™ or Libracol™/HEP-30™ and has been reported to possess hypocholesterolaemic properties in humans (LeHoux, Dupuis, Kelly, Brzezinski, & Radwan, 2005). The 240 kDa (35 cP, 82% deacetylated) and 70 cP chitosans (92% deacetylated) were from Vanson HaloSource (Redmond, WA). Reagents used in this study were of analytical grade. They were obtained from Sigma–Aldrich (St. Louis, MO), Fisher Scientific (Montreal, QC) or VWR Canlab (Montreal).

2.2. Determination of viscosity

Viscosities of the 30 kDa and 240 kDa chitosans were determined using a ViscoPro 2000 instrument equipped with a SPL-322 Big Dipper 2000 Viscometer model BCC-323 (Cambridge Viscosity Inc., Medford, MA). Values were corrected to 25 °C. Chitosan concentration (oven dried at 80 °C for 72 h, to a constant weight) was 1% (w/v) in 1% (w/v) aqueous acetic acid.

2.3. General procedures for salting-out

The general procedure for recovery of chitosan from aqueous solutions was performed as follows. To a solution containing 1–10% by weight, usually 1%, of chitosan in dilute aqueous hydrochloric acid (0.2 N) or dilute aqueous acetic acid (5%) was added, by portions, the salting-out agent as a solid or, preferably as a concentrated solution. The operations were conducted at various temperatures, as illustrated in the figures. The resulting suspension of salted-out chitosans were stirred for 30 min or, according to the amounts of chitosan to be recovered, for an extended period of time. The suspensions were centrifuged (10,000*g*, 30 min) and the amount of chitosan remaining in the supernatant was quantitated by colorimetry using the Cibacron brilliant red 3B-A dye (Muzzarelli, 1998). The pH of the initial solutions of chitosans was 2.9 and the pH of the supernatants were 3.2–4.2, depending on the salts used. Data were obtained from duplicate experiments done in

triplicates. Profiles of the percentage of salted-out chitosan as a function of the mass ratio of salt-to-solute are shown. Data were fitted using the SigmaPlot® computer software (Systat Software Inc., Point Richmond, CA) with built-in non-linear regression equations. Usually, 100 iterations were performed. A list of the mathematical equations used are shown in Appendix A.

2.4. Solubility assays

Salting-out agents were added at a 4:1 ratio with respect to solutes as described under the general procedures. The salted-out chitosans were washed five times with water by resuspension and centrifugation. A volume of 0.2 N HCl or 0.8 N acetic acid equivalent to the initial volume of solution was added. The tubes were placed on a rotary mixer (LabQuake, Barnstead/Thermolyne, Dubuque, IA), at room temperature, and solubility was determined by visual examination.

3. Results

3.1. Salting-out chitosans using sulfate salts

3.1.1. Ammonium sulfate

Sulfate anions rank as some of the most stabilizing and strongly hydrated anions of the Hofmeister series (Collins & Washabaugh, 1985; Hofmeister, 1988; Kunz et al., 2004). Among these, ammonium sulfate has been widely used as the reagent of choice to salt proteins out of solution (Green & Hughes, 1955). We investigated whether ammonium sulfate would show a similar effect in the case of chitosan and whether the salting-out effect would be influenced by temperature. Results showed in the case of a 30 kDa chitosan that increasing the mass ratio of ammonium sulfate in relationship to solute readily induced chitosan to be salted-out when the experiments were done at 4 °C (Fig. 1a). The relationship was characterized by a rapid rise followed by a plateau corresponding to salting-out 95% of dissolved chitosan at a mass ratio $\geqslant$1.5:1. The curve was fitted with a 3-parameter single rectangular hyperbolic equation (correlation coefficient (R) = 0.99) The profile of 30 kDa chitosan salting-out was similar when experiments were done at room temperature (Fig. 1d). Data were fitted with the same 3-parameter hyperbolic equation (R = 0.99). In this case, the mass ratio of ammonium sulfate needed to salt-out 95% of dissolved chitosan was decreased to 0.5:1. Ammonium sulfate was also found to be efficient to salt-out chitosan of 240 kDa at 4 °C (Fig. 1b) and room temperature (Fig. 1e). In these instances, a mass ratio of 1:1 ammonium sulfate to dissolved chitosan salted-out 95% of the polysaccharide whether experiments were done at 4 °C (Fig. 1b) or room temperature (Fig. 1e). The general use of ammonium sulfate as an efficient salting-out agent of chitosan was further demonstrated in the case of a 70 cP form of chitosan. Profiles identical to the 240 kDa chitosan product were observed. In this instance, a mass ratio of 1.0 salt-to-solute was sufficient to salt-out 95% of chitosan at 4 °C (Fig. 1c) and at room temperature (Fig. 1f).

We next investigated the relationship between the efficiency of ammonium sulfate to salt-out chitosans of a wide range of molecular sizes generated by chitosanase-based hydrolysis, at 4 °C. Results showed a progressive efficiency of ammonium sulfate to salt-out the low molecular weight chitosan oligomers (Fig. 2a). The profile was fitted with a 3-parameter single rectangular hyperbolic equation (R = 0.99) for chitosans samples ranging from 2300 to 240,000 Da. The efficiency of salting-out was at least 95% for chitosan samples of 26,000 Da and higher, including the 70 cP product (Fig. 2a). Temperature did not appreciably affect the efficacy of salting-out as shown by experiments performed at room temperature (Fig. 2b). In these instances, the relationship between molecular sizes and

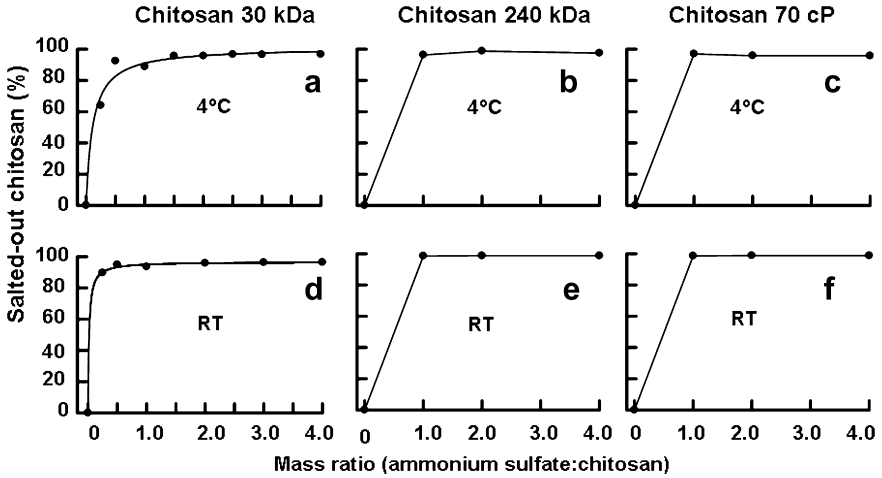

Fig. 1. Profiles of the efficiency of ammonium sulfate as a salting-out agent of chitosans of various molecular sizes dissolved in aqueous acetic acid. Results are shown for experiments performed at 4 °C and room temperature (RT) using increasing salt-to-solute ratio, as indicated. The quantity of chitosans remaining in solution in each assay was determined with a colorimetric test.

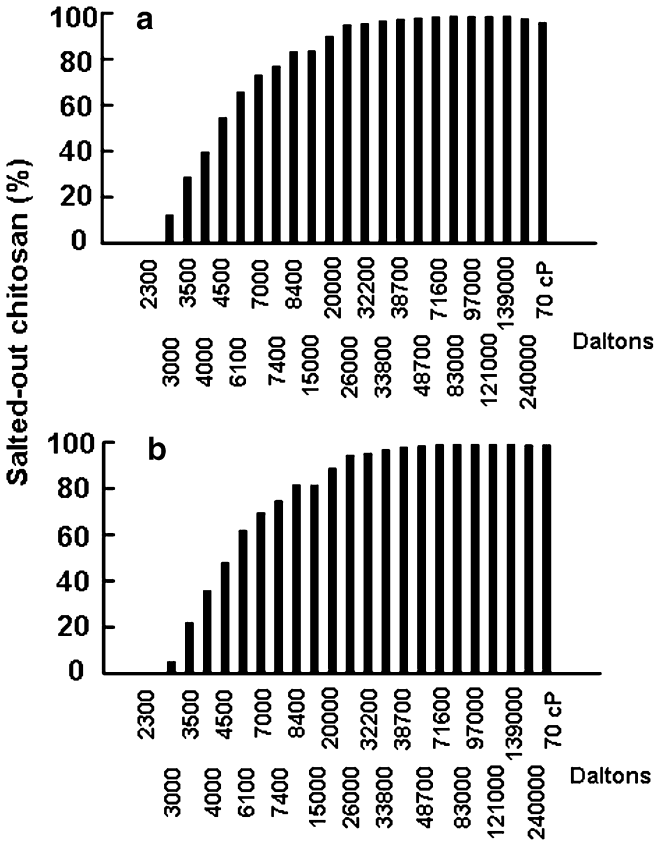

Fig. 2. Profiles of salting-out chitosans of various molecular sizes as a function of temperature using ammonium sulfate at a 5:1 salt-to-solute ratio. Experiments were performed, (a) at 4 °C and (b) at room temperature. The chitosan oligomers were generated by hydrolysis using chitosanase. The 240,000 Daltons and 70 cP chitosans were obtained from a commercial supplier. The quantity of chitosan remaining in solution in each case was determined with a colorimetric assay.

percentage of salted-out chitosans was also fitted with a 3-parameter single rectangular hyperbolic equation ($R = 0.99$). The efficiency of salting-out was at least 95% for chitosan samples of 26,000 Da and higher, including the 70 cP product.

3.1.2. Sodium sulfate

Sodium sulfate efficiently salted-out chitosan (30 kDa) in the case of experiments done at 4 °C (Fig. 3a). The process of salting-out showed a hyperbolic profile ($R = 0.99$) and it was found that a mass ratio of 0.5:1 salt-to-solute was sufficient to salt-out 95% of solute. Experiments performed at room temperature showed a similar hyperbolic relationship ($R = 0.99$) and a mass ratio 0.5:1 to salt-out at least 95% of dissolved 30 kDa chitosan (Fig. 3). However, sodium sulfate was found to be less efficient in the case of the 240 kDa chitosan in experiments performed at 4 °C (Fig. 3b). In this case, the hyperbolic profile ($R = 0.99$) revealed that a mass ratio of 4.0:1 salt-to-solute was needed to salt-out 96% of solute. An effect of temperature was noted as shown by experiments performed at room temperature (Fig. 3e). In this instance, the process of salting-out rapidly reached a plateau corresponding to 99% of dissolved chitosan salted-out at a 1:1 mass ratio of salt-to-solute. The effect of salting-out 70 cP chitosan was similar to the results obtained in the case of the 240 kDa compound. In this case, the hyperbolic profile ($R = 0.99$) of experiments performed at 4 °C revealed that a mass ratio of 4.0 was needed to efficiently (97%) salt-out this chitosan sample (Fig. 3c). A temperature effect was also noted. Experiments performed at room temperature showed that 99% of the solute was salted-out at a mass ratio of 1:1 salt-to-solute (Fig. 3f).

3.2. Salting-out chitosans using phosphate salts

3.2.1. Monobasic sodium phosphate

Phosphate salts rank closely to sulfate anions in the decreasing salting-out effects of anions of the Hofmeister series (Collins & Washabaugh, 1985; Kunz et al., 2004).

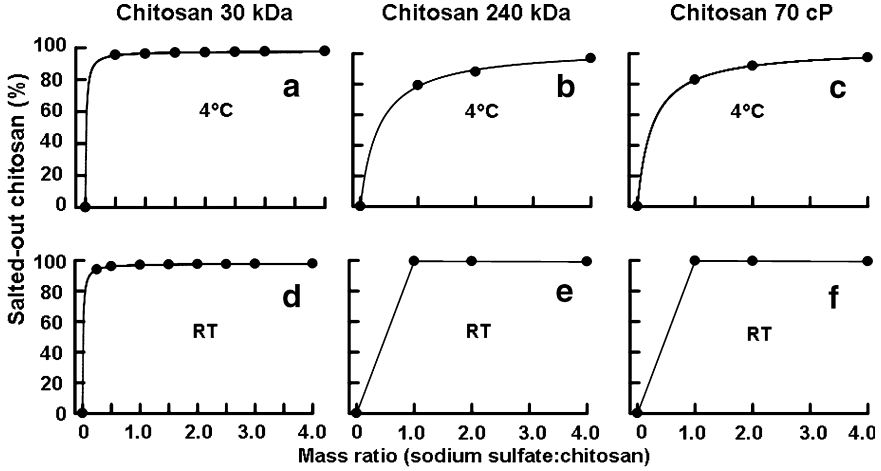

Fig. 3. Profiles of the efficiency of sodium sulfate as a salting-out agent of chitosans of various molecular sizes dissolved in aqueous acetic acid. Results are shown for experiments performed at 4 °C and room temperature (RT) using increasing salt-to-solute ratio, as indicated. The quantity of chitosans remaining in solution in each assay was determined with a colorimetric test.

We investigated the efficiency of monobasic and dibasic sodium phosphate to salt chitosan out of acidic solutions. In a first series of experiments, monobasic sodium phosphate was added in varied mass ratio to a solution of 30 kDa chitosan, at 4 °C. Results showed a salting-out profile characterized by a rapid rise that reached a plateau corresponding to 95% of chitosan salted-out at a 1.5:1 ratio of salt-to-solute (Fig. 4a). Data were fitted with a Chapman sigmoidal equation ($R = 0.99$). A similar profile was fitted with the same mathematical equation ($R = 0.99$) when the experiments were performed at room temperature (Fig. 4d). In this case, a 1.5:1 ratio effected the salting-out of 95% of solute. In marked contrast, monobasic sodium phosphate was poorly efficient to salt-out the 240 kDa chitosan, at 4 °C (Fig. 4b). Data were fitted with a 3-parameter sigmoidal equation ($R = 0.99$). Only 40% of the solute was salted-out at a ratio of 4:1. The efficiency of monobasic sodium phosphate was also low in the case of experiments done at room temperature (Fig. 4e). Data showed that 34% of dissolved chitosan was salted-out under these conditions. These data were consistent with the interpretation that monobasic sodium phosphate was relatively inefficient at salting-out chitosans of molecular sizes higher than 30 kDa. This interpretation was further tested using 70 cP chitosan. When experiments were done at 4 °C, the profile was fitted with a 2-parameter exponential equation ($R = 0.99$) and data revealed that 60% of 70 cP chitosan was salted-out at a 4:1 ratio of salt-to-solute (Fig. 4c). Experiments performed at room temperature did not improve the efficiency of monobasic sodium phosphate to act as a salting-out agent. In this instance, the profile was fitted with a 3-parameter sigmoidal equation ($R = 0.99$). Data showed that 40% of dissolved 70 cP chitosan was salted-out at a ratio of 4:1 salt-to-solute (Fig. 4f).

3.2.2. Dibasic sodium phosphate

In a next series of experiments, dibasic sodium phosphate was added in various mass ratio to an acidic solution of 30 kDa chitosan, at 4 °C. Results showed a salting-out profile that was characterized by a rapid rise to a plateau that was reached at a 2.5:1 ratio of salt-to-solute which corresponded to 95% of salted-out chitosan (Fig. 5a). The curve was fitted with a 4-parameter sigmoidal equation ($R = 0.99$). A similar profile was fitted with a 3-parameter single rectangular hyperbolic equation ($R = 0.99$) when the experiments were performed at room temperature (Fig. 5c). In this case, the ratio of salt-to-solute needed to bring down 95% of dissolved chitosan was 2.5:1. We next investigated whether dibasic sodium phosphate was more efficient than the corresponding monobasic form to salt-out chitosan of high molecular sizes. The 70 cP chitosan was tested for that purpose. Results showed that dibasic sodium phosphate was poorly efficient, as only 25% of solute was salted-out at a ratio of 4.0:1 (Fig. 5b). Increasing the temperature only resulted in a slightly improved efficiency. Data showed that 41% of chitosan was salted-out at a ratio of 4.0:1 salt-to-solute (Fig. 5d). The 240 kDa chitosan product was not salted-out by dibasic sodium phosphate (not shown).

3.3. Salting-out chitosans using sodium nitrate

Nitrate salts rank as weakly hydrated and low efficient salting-out agents according to the Hofmeister scale (Collins & Washabaugh, 1985; Kunz et al., 2004). We carried out a series of experiments to determine the salting-out efficiency of sodium nitrate on a combination of parameters, including chitosans of various molecular sizes and, temperature. Results showed that sodium nitrate salted-out 30 kDa chitosan efficiently when experiments were performed at 4 °C (Fig. 6a). A ratio of 2.5:1 salt-to-solute was needed to salt-out 95% of dissolved chitosan. Data were fitted with a 3-parameter sigmoid curve ($R = 0.99$). Performing the same experiments at room temperature revealed a similar relationship (Fig. 6d) that was fitted in the same way ($R = 0.99$). However, the kinetic of the process was slow and 90% of dissolved chitosan was salted-out

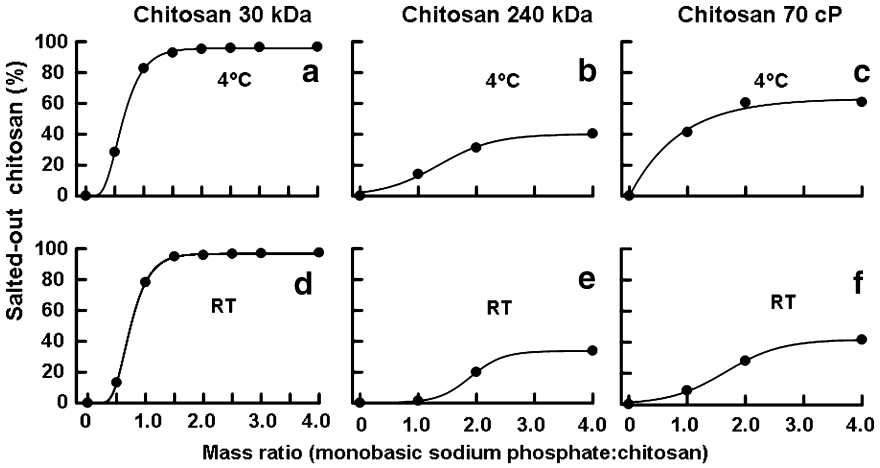

Fig. 4. Profiles of the efficiency of monobasic sodium phosphate as a salting-out agent of chitosans of various molecular sizes dissolved in aqueous acetic acid. Results are shown for experiments performed at 4 °C and room temperature (RT) using increasing salt-to-solute ratio, as indicated. The quantity of chitosans remaining in solution in each assay was determined with a colorimetric test.

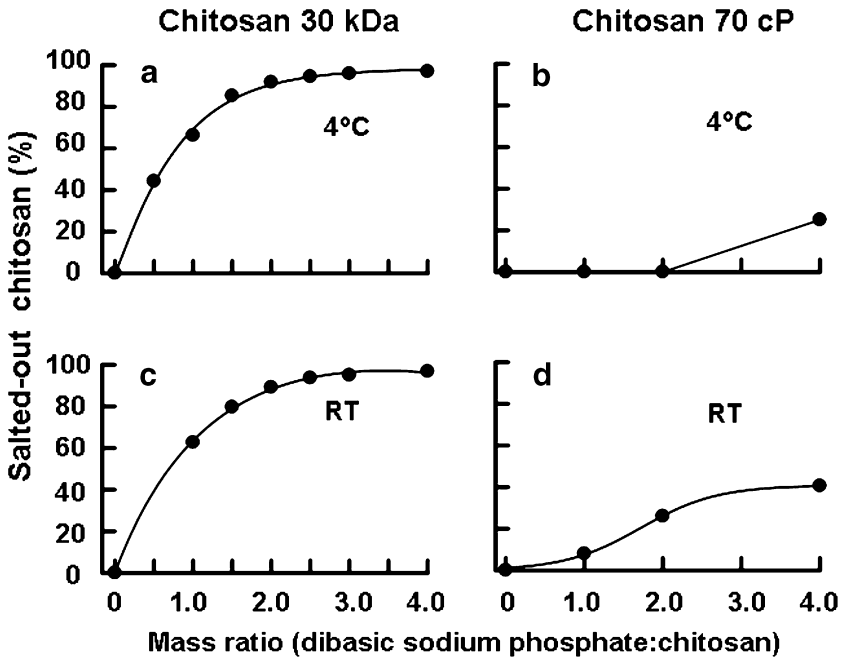

Fig. 5. Profiles of the efficiency of dibasic sodium phosphate as a salting-out agent of chitosans of various molecular sizes dissolved in aqueous acetic acid. Results are shown for experiments performed at 4 °C and room temperature (RT) using increasing salt-to-solute ratio, as indicated. The quantity of chitosans remaining in solution in each assay was determined with a colorimetric test.

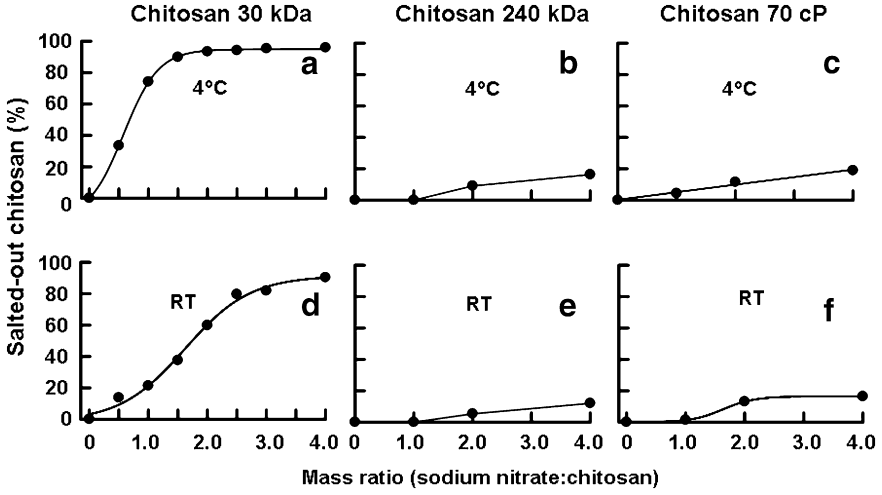

Fig. 6. Profiles of the efficiency of sodium nitrate as a salting-out agent of chitosans of various molecular sizes dissolved in aqueous acetic acid. Results are shown for experiments performed at 4 °C and room temperature (RT) using increasing salt-to-solute ratio, as indicated. The quantity of chitosans remaining in solution in each assay was determined with a colorimetric test.

at a ratio of 4.0:1 salt-to-solute. Whereas sodium nitrate performed well as a salting-out agent in the case of 30 kDa chitosan, it was poorly efficient in the case of chitosans of higher molecular sizes. In the case of 240 kDa chitosan, experiments performed at 4 °C (Fig. 6b) and room temperature (Fig. 6e) showed that a ratio of 4.0:1 of sodium nitrate-to-solute salted-out 16% and 12% of solute, respectively. Similar observations were made in the case of the 70 cP chitosan sample. In this instance, 19% (Fig. 6c) and 12% (Fig. 6f) of solute were salted-out at a ratio of 4.0:1.

3.4. Salting-out 30 kDa chitosan at 50 °C using various inorganic salts of the Hofmeister series

We next investigated the efficiency of salting-out chitosan of 30 kDa, chosen as an example, in experiments performed at 50 °C. Results showed that sulfate anions salted-out 95% of dissolved chitosan at a 1:1 ratio of salt-to-solute (Fig. 7a and b). The profiles resembled those observed in the case of experiments done at 4 °C and room temperature (Fig. 1 and 2). The profiles of salting-out 30 kDa chitosan using monobasic sodium phosphate

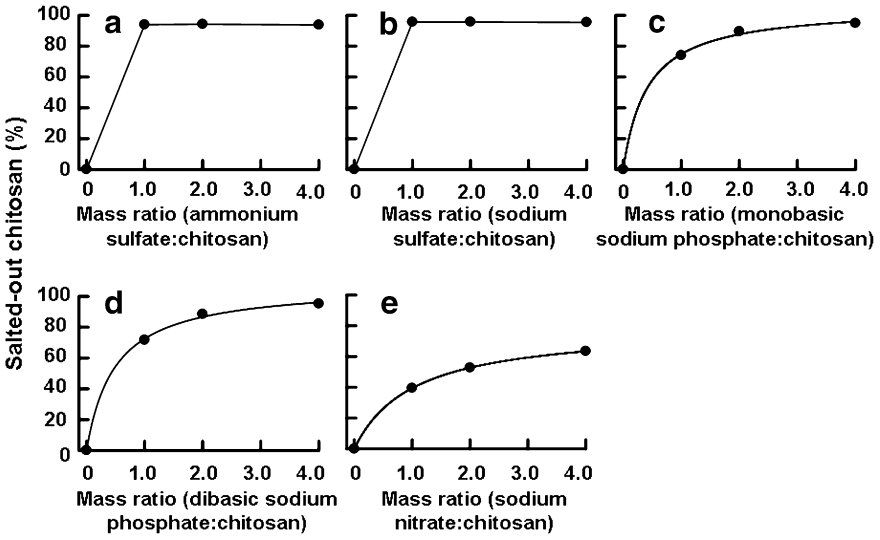

Fig. 7. Profiles of salting-out 30 kDa chitosan at 50 °C using various salts in different salt-to-solute ratio, as indicated. The quantity of chitosans remaining in solution in each assay was determined with a colorimetric test.

(Fig. 7c) and dibasic sodium phosphate (Fig. 7d) were respectively fitted with a 2-parameter single rectangular hyperbolic equation ($R = 0.99$). Data showed that a ratio of 4:1 was needed to salt-out 95% of dissolved 30 kDa chitosan. The efficiency of salting-out induced by the nitrate anion was found to be affected by temperature. In this instance, the profile fitted with a 2-parameter single rectangular hyperbolic equation ($R = 0.99$). Data revealed that 63% of dissolved 30 kDa chitosan was salted-out at a ratio of 4:1 (Fig. 7e), in contrast to 95% or better in the case of experiments done at 4 °C and room temperature (Fig. 6).

3.5. Ranking the efficiencies of various anions to salt-out 30 kDa chitosan: Effects of temperature and salt-to-solute ratio

Fig. 8 provides a summary of the efficiencies of the various anions used in this study to salt-out the 30 kDa chitosan product, as an example. Results clearly indicated that the anions used in this study (sulfate, phosphate and nitrate) displayed the same efficiency to salt-out 30 kDa chitosan when used as a 4:1 salt-to-solute ratio (Fig. 8b). The process of salting-out appeared to be independent of the temperature within the range 4–50 °C, except in the case of the nitrate anion. When a ratio of 1:1 salt-to-solute was used, it was quite clear that the efficiency of salting-out was, $SO_4^{2-} = H_2PO_4^{2-} > HPO_4^{2-} > NO_3^-$ in the case of experiments performed at room temperature and 50 °C (Fig. 8a). Our observations are in general agreement with the ranking order of salts of the Hofmeister series (Collins & Washabaugh, 1985; Hofmeister, 1988; Kunz et al., 2004). It can be noted that in the case of experiments done at 4 °C, the efficiency was $HPO_4^{2-} \cong NO_3^-$ (Fig. 8b). There was no significant effects of the cation in the case of sulfate salts or other salts (sodium or potassium, data not shown) use in the present study.

3.6. Solubility of chitosans recovered by salting-out

A key issue in the use of salts of the Hofmeister series to recover chitosans from acidic solutions is to afford a product that is soluble in acidic aqueous media. This is of

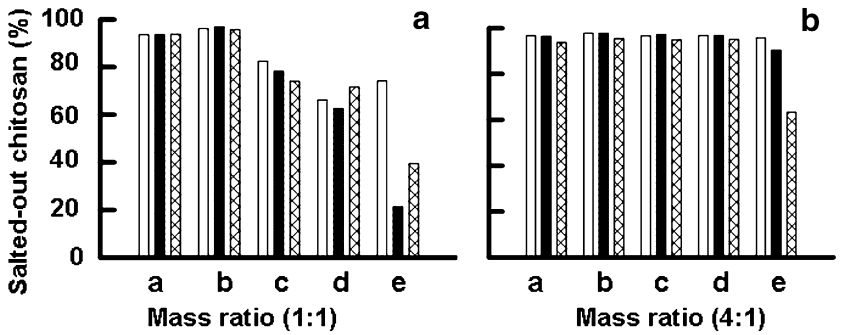

Fig. 8. Profiles of salting-out 30 kDa chitosan at 4 °C (empty columns), room temperature (filled columns) and 50 °C (cross-hatched columns) using 1:1 and 4:1 salt-to-solute ratio, as indicated. The salts used were, (a) ammonium sulfate, (b) sodium sulfate, (c) monobasic sodium phosphate, (d) dibasic sodium sulfate, and (e) sodium nitrate. The quantity of chitosan remaining in solution in each assay was determined with a colorimetric test.

Table 1
Solubility in dilute aqueous hydrochloric or acetic acids of chitosans precipitates obtained by salting-out

Salt used for salting-out	Solubility					
	30 kDa (93% DDA)		Chitosans 240 kDa (82% DDA)		70 cP (92% DDA)	
	HCl	Acetic acid	HCl	Acetic acid	HCl	Acetic acid
Ammonium sulfate	ns	ns	1–5 min	ns	⩽1 min	ns
Sodium sulfate	ns	ns	⩽1 min	ns	ns	ns
Sodium phosphate monobasic	⩽1 min	ns	Soluble	Soluble	ns	ns
Sodium phosphate dibasic	⩽1 min	ns	Soluble	Soluble	Soluble	Soluble
Sodium nitrate	Soluble	Soluble	Soluble	Soluble	Soluble	Soluble

Salting-out agents were added at a 4:1 ratio with respect to solutes, at room temperature. The precipitates were washed with water, a volume of dilute acid (HCl, 0.2 N; acetic acid, 0.8 N) equivalent to the initial volume of solution was added and time was recorded. Experiments were done in duplicates. Soluble, readily dissolved; ns, not soluble; DDA, degree of deacetylation.

importance in the case of applications of chitosans to industrial and biomedical fields. We selected two different aqueous environments for assays, dilute acetic acid (0.8 N) and dilute HCl (0.2 N) which was chosen to mimic the acidic environment found in the human stomach. Qualitative analysis performed at room temperature revealed that the patterns of solubilities varied according to the nature of the salt used for salting-out, the molecular sizes of chitosans and the aqueous acidic environment (Table 1). All chitosan samples salted-out with sodium nitrate were readily soluble. In the case of phosphate salts, most of the salted-out chitosans were soluble, except when dilute aqueous acetic acid was used as a dissolving medium (30 kDa chitosan) or when 70 cP chitosan was tested. In contrast, the sulfate salts produced a chitosan precipitate that was poorly soluble, except in the case of the 240 kDa and 70 cP products which were soluble in dilute hydrochloric acid but not in dilute acetic acid.

4. Discussion

A major challenge in the chitosan industry is to recover chitosan from aqueous solutions (e.g. hydrolysates) under conditions that are simple and that can be applied to an industrial scale. The most common technique is to decrease the solubility of chitosan by raising the pH through the addition of an inorganic base (for example, sodium or potassium hydroxide). This procedure is efficient but it suffers from the drawback that the resulting mixture is highly viscous, making the isolation of chitosan difficult by conventional techniques of separation. Furthermore, the desired product must be free of excess alkali. This can be accomplished by neutralization or by extensive washings. These are time-consuming and may result in an appreciable loss (mechanically or by dissolution) of chitosan. Polyphosphoric acid (Shu & Zhu, 2002) or polyphosphate salts (Chiou & Li, 2003) can also be used for recovery of dissolved chitosan but the precipitates are poorly soluble in aqueous media, thus limiting the use of this technique.

Chitosan of desired molecular sizes is reliably prepared by chitosanase hydrolysis. However, chitosanase rapidly degrade chitosan (unpublished data) (Brzezinski, 1996, 2000; Masson et al., 1994) and the reaction of hydrolysis must be rapidly stopped to limit polydispersity. The action of chitosanase can be stopped by raising the temperature of the hydrolysis vat. However, this approach is not practical for two reasons. First, the temperature ought to be raised quickly and this may represent a challenge when enzymatic hydrolysis is carried on a large scale. Second, the inherent stability of chitosanase to heat denaturation requires raising the temperature of the reaction to 60 °C or more (Pr. Ryszard Brzezinski, personal communication). These conditions favor the well-described Maillard reaction (Ikan, 1996; O'Brien, Nursten, Crabbe, & Ames, 1998) which leads to partial decomposition of chitosan and the generation of colored products resulting from the reaction of the primary amino groups of the chitosan molecules. This behavior is highly undesirable since these same amino groups are essential for the characteristic properties of chitosan. Two additional points are of interest. First, heat-denatured chitosanase may precipitate and be carried over in the subsequent steps of isolation of chitosan (e.g. by precipitation). Second, the partial resistance of chitosanase to heat denaturation may allow its renaturation and partial recovery of activity, adding to the possibility of further digestion of chitosan. Overall, the current methods of precipitation and isolation of chitosan from chemical or enzymatic hydrolysates or acidic solutions in general are therefore not adequate. Easy-to-use methodologies enabling high yields of a product suitable for commercial uses, and especially of biomedical uses are of the utmost interest. Therefore, there remains a need to provide a simple, reliable, reproducible method for retrieving chitosan from chemical or enzymatic hydrolysates as well as to give a product free of contaminants (e.g. chitosanase, undesirable salts) that can be used in the food and biomedical industries.

Here, we showed that inorganic salts of the Hofmeister series induced the salting-out of chitosans of various molecular sizes from acidic solutions at mild pH. In that respect, our data clearly indicated that the polysaccharide chitosan behaved in a manner similar to that has been originally described in the case of proteins. The efficiency of salting-out the chitosans investigated in the present study varied according to the nature of the salt used and, in some cases, the molecular sizes of chitosans. Sulfate salts (ammo-

nium sulfate and sodium sulfate) were found to be the most efficient salting-out agents as compared to other inorganic salts of the Hofmeister series. Ammonium sulfate effected quantitative or near-quantitative salting-out of 30 kDa, 240 kDa and 70 cP chitosans, whether experiments were performed at 4 °C, room temperature or 50 °C (Figs. 1, 7 and 8). Sodium sulfate was as efficient as ammonium sulfate (Figs. 3, Fig. 7 and 8) under similar conditions, except for experiments performed at 4 °C in the case of the 240 kDa and 70 cP chitosans (Fig. 3). Our data regarding the salting-out of chitosans were in general agreement with the Hofmeister scale, in which case the sulfate anion ranks as one of the most efficient agent (Collins & Washabaugh, 1985; Hofmeister, 1988; Kunz et al., 2004).

Phosphate anions are also considered effective salting-out agents, although they rank as less potent than sulfate anions (Collins & Washabaugh, 1985; Hofmeister, 1988; Kunz et al., 2004). This observation held true in the case of salting-out the 30 kDa chitosan compound using the monobasic and dibasic forms of sodium phosphate at 4 °C, room temperature and 50 °C (Figs. 4, 5, 7 and 8). In marked contrast, the phosphate salts were not able to efficiently salt-out chitosan polymers of 240 kDa (Fig. 4) or a 70 cP form of the biopolymer (Figs. 4 and 5) whether experiments were done at 4 °C or at room temperature. These observations suggested a selective effect of the phosphate anion as a general salting-out agent of chitosans. These findings could be of interest in the case of selective recovery of low molecular weight chitosans from solutions containing chitosans of high molecular sizes.

The nitrate anion is weakly hydrated and its property as a salting-out reagent ranks low on the Hofmeister scale (Collins & Washabaugh, 1985; Hofmeister, 1988; Kunz et al., 2004). Our data showed that the efficiency of sodium nitrate as a salting-out agent of chitosan was related to the molecular sizes of chitosans and the temperature at which the experiments were performed. For example, a 2–3:1 ratio of salt-to-solute were sufficient to salt-out the 30 kDa chitosan compound at 4 °C (Fig. 6) but a 4:1 ratio of salt-to-solute was needed when experiments were performed at room temperature (Fig. 6). Furthermore, there was a marked decrease in the kinetic of the salting-out process (Fig. 6d). Of interest, sodium nitrate was inefficient in its ability to salt-out chitosans of 240 kDa and 70 cP whether experiments were performed at 4 °C or room temperature (Fig. 6). These observations could find practical applications for a selective recovery of chitosans from mixtures of high and lower molecular weight hydrolysates.

Chitosans retrieved from acidic media using Hofmeister's salts should easily dissolve in dilute aqueous acidic media, especially when they are intended for biomedical applications. We used two aqueous acidic test media to investigate the solubility of 30 kDa exposed to various salts (Table 1). Data showed opposite results using sulfate anions or phosphate anions. Whereas the chitosan precipitate was poorly soluble in the former case, it was soluble only in dilute HCl in the latter case. In contrast, the 30 kDa chitosan product proved to be soluble in both test media when it had been salted-out using sodium nitrate. Preferential solubility behavior was also noted in the case of the 240 kDa chitosan and the 70 cP chitosan salted-out by exposure ammonium or sodium sulfate. These observations suggested that, in this case, the cation played a role in the solubility properties of salted-out chitosans. Furthermore, this observation was in agreement with the fact that the ammonium cation is more weakly hydrated than the sodium cation (Collins & Washabaugh, 1985). Salting-out using phosphate salts also produced mixed results with respect to the solubility of 240 kDa and 70 cP chitosans in the test media. Salting-out the 240 kDa chitosan product using monobasic and dibasic sodium phosphate produced a precipitate that was soluble in both media. However, only the precipitate of 70 cP chitosan generated by salting-out with dibasic sodium phosphate was soluble in both test media. Salting-out using sodium nitrate produced a soluble precipitate.

The explanation for the discriminant effects of the Hofmeister's salts used here with respect to solubility of the chitosan precipitates is not obvious. One possibility is related to the basic principle of the process of salting-out, that is a dehydration of the solute that causes its precipitation (Green & Hughes, 1955). Our data suggested that the nature of the Hofmeister's salt influenced the ability of chitosans to rehydrate following precipitation by salting-out. In this respect, it has been suggested that solvation effects are important in explaining the salting-out of solutes (Green & Hughes, 1955). This suggestion may also be of importance in the case of chitosan since it is a strongly hydrated polyanion that can bind two water molecules per monomer in the amorphous phase (Despond, Espuche, Cartier, & Domard, 2004). In addition, the possibility could not be excluded that the salting-out anions may form salts with the cationic primary amino groups of the chitosan polymer. This is a likely possibility since chitosans can behave as weak anionic exchangers (Roy, Todd, & Glassner, 1998). The formation of salts may differently affect the ability of chitosans to be rehydrated following precipitation by salting-out.

In summary, data shown in this report present evidence that chitosans could be recovered from mild acidic solutions thereof by applying the principle of salting-out using anions of the Hofmeister series. Our data further indicated that the choice of the Hofmeister's salt(s) must take into account the parameters of, (1) efficiency of recovery, (2) molecular size of the chitosans to be recovered and, to some extent, (3) temperature. The degree of acetylation of chitosan may be an important factor as well. Additional experiments would be required to evaluate the contribution of this parameter on the salting-out efficiency of inorganic salts of the Hofmeister series.

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Appendix A. List of the mathematical equations used for curve fitting

(a) 2-Parameter exponential. $y = a * (1 - \exp(-b * x))$.
(b) 3-Parameter sigmoid. $y = a/(1 + \exp(-(x - x_0)/b))$.
(c) 3-Parameter Chapman sigmoid.
$y = a * (1 - \exp(-b * x))\hat{}c$.
(d) 4-Parameter sigmoid.
$y = y_0 + a/(1 + \exp(-(x - x_0)/b))$.
(e) 2-Parameter single rectangular hyperbola.
$y = a * x/(b + x)$.
(f) 3-Parameter single rectangular hyperbola.
$y = a * x/(b + x) + c * x$.

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