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Polysaccharide determination in protein/polysaccharide mixtures for phase-diagram construction

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ABSTRACT

The effect of protein removal from protein–polysaccharide mixtures on the determination of total sugar concentration for phase-diagram construction was explored by response surface methodology. Phenol–sulphuric acid method was used to estimate the total sugar concentration whereas phase separation and microstructure of biopolymers in the mixtures were probed by means of fluorescent microscopy. All the factors studied (trifluoroacetic acid (TFA) volume, protein concentration, precipitation time) had significant influence on the polysaccharide recovery from the mixtures. The overall pattern of protein precipitation with TFA was also found to highly depend on protein–time interactions. Images taken at different mixture concentrations and with two different filter sets revealed a phase-separated system with the guar gum domains surrounded by a continuous protein phase. Experimental measurements as well as microscopical evidence suggests that, for greater than 60% polysaccharide recovery (±6% error), the protein content in the mixtures should be < ~5.5% (w/v) with less than 0.5 mL TFA combined with more than 30 min rest under the influence of TFA. The results of the present investigation showed that when attempting to construct phase diagram of mixed biopolymer systems the equilibrium phases must be diluted to a protein content < ~5% (w/v) before TFA treatment if the maximum polysaccharide concentration is to be determined.

1. Introduction

Phase separated biopolymer systems is an active research area owning to its applications in food, nutraceutical, and drug industry (Kasapis, 2008; Tolstoguzov, 2003). A first step to investigating such systems in the liquid state usually involves construction of phase diagrams of the constituent biopolymers at the specified conditions of interest in order to identify their compatibility region. Several methods can be used to construct a biopolymer phase diagram depending on the desired accuracy. Methods such as the phase-volume-ratio method (Polyakov, Grinberg, & Tolstoguzov, 1980; Schorsch, Clark, Jones, & Norton, 1999) or simple visual observation of the separated phases (Hemar, Tamehana, Munro, & Singh, 2001; Thaiudom & Goff, 2003) are frequently used to construct phase diagrams of binary biopolymer mixtures. However, the most detailed phase diagram is obtained when the concentration of biopolymers in the separated phases is determined analytically. The polysaccharide concentration in the phases can be determined with methods such as refractometry (Bourriot, Garnier, & Doublier, 1999b), or flow injection analysis (Kontogiorgos, Tosh, & Wood, 2009b), with the most popular apparently being the phenol–sulphuric method (Antonov, Dmitrochenko, & Leontiev, 2006; Ercelebi & Ibanoglu, 2007; Kim, Decker, & McClements, 2006; Lazaridou & Biliaderis, 2009; Li et al., 2008a; Perrechil, Braga, & Cunha, 2009; Zhang & Foegeding, 2003).

The original (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) as well as the microplate format of the method (Masuko et al., 2005) for total sugar determination have been developed for pure sugar, oligosaccharide or polysaccharide solutions free of interferences. In mixed biopolymer systems, however, interferences resulting from the protein component may significantly affect the absorption measurements. Addition of sulphuric acid to proteins results in various amounts of ammonium sulphate, sulphur and carbon dioxide. These products will interfere with the overall chemistry of the reactions making additionally dubious the use of the calibration curves. Furthermore, blank correction to alleviate signals that originate from sources other than the polysaccharide is also problematic since different blanks are needed as the protein concentration varies in the mixtures. Therefore, estimation of polysaccharide concentration is not a straightforward issue and removal of protein prior to analysis is required. The aforementioned reasons also create difficulties in other methods, such as total hydrolysis of the polysaccharide followed by quantitative HPLC, making these techniques laborious and difficult to be used in practice.

Response surface methodology (RSM) to achieve optimization provides an effective way to visualize how the system's response

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changes when one or more of its factors change. RSM is advantageous as it reduces the number of experimental trials needed to evaluate multiple parameters and their interactions and provides sufficient information for statistically acceptable results. RSM has also been successfully demonstrated as useful in optimizing process variables and widely applied for optimizing conditions in polysaccharides for food and drug industry (Gu, Abbas, & Zhang, 2009; Kshirsagar & Singhal, 2007; Lee, Lin Ye, Landen, & Eitenmiller, 2000; Qiao et al., 2009; Simsek, Poyrazoglu, Karacan, & Velioglu, 2007; XuJie & Wei, 2008; Yongjiang, Zhong, Jianwei, Minger, & Xueqian, 2009).

The objective of the present investigation, therefore, is to explore the effect of protein removal from the mixtures on the determination of total sugar concentration for phase-diagram construction in binary protein/polysaccharide mixtures using RSM and phenol–sulphuric method as analytical tool.

2. Materials and methods

2.1. Materials and sample preparation

Guar gum, α-D-mannose, trifluoroacetic acid (TFA) and sodium caseinate were purchased from Sigma (Sigma–Aldrich, St. Luis, MO). Distilled water and all chemicals used for the total sugar determination were of analytical grade. Guar gum and sodium caseinate were dispersed in distilled water at 0.2% (w/v) and 2, 11 and 20% (w/v), respectively under continuous stirring and mild heating for several hours. After the end of hydration period the stock solutions were centrifuged to remove any insoluble material. Stock solutions were subsequently mixed in 1:1 ratio yielding binary mixtures with 0.1% (w/v) guar concentration and 1, 5.5, or 10% (w/v) protein concentration as determined by the experimental design (Table 1).

2.2. Total sugar determination

TFA (0.1, 0.8, or 1.5 mL) was added in the mixtures and vortexed immediately. The resulting mixture was left to stand for 5, 32.5, or 60 min at room temperature and immediately centrifuged at 6000 rpm for 10 min (Beckman, TJ-6). An aliquot of the supernatant was taken and phenol–sulphuric acid method was then carried out to determine the total sugar concentration (Dubois et al., 1956). The absorbance readings of each tube were measured at 490 nm using a spectrophotometer (Shimazu UV-VIS 160A). A calibration curve was prepared using mannose as standard solution. All determinations were carried out in triplicate whereas the central point in the experimental design was repeated 18 times (Table 1). The blank consists of all reagents used in their respective amounts excluding mannose or guar gum. Each run had a different blank solution according to the levels of the independent variables in the experimental design.

Table 1
Experimental design and levels of factors in actual and coded values in the investigation of their effect on the determination of total sugar concentration in protein–polysaccharide mixtures.

Factors	Levels		
	−1	0	1
TFA volume (mL) (X_1)	0.1	0.8	1.5
Time (min) (X_2)	5	32.5	60
Protein concentration (%, w/v) (X_3)	1	5.5	10

Run	Factors		
	TFA volume (mL)	Time (min)	Protein concentration (%, w/v)
1	0.1	5	1
2	0.1	60	1
3	0.1	32.5	5.5
4	0.1	5	10
5	0.1	60	10
6	0.8	32.5	1
7	0.8	5	5.5
8[a]	0.8	32.5	5.5
9	0.8	60	5.5
10	0.8	32.5	10
11	1.5	5	1
12	1.5	60	1
13	1.5	32.5	5.5
14	1.5	5	10
15	1.5	60	10

[a] This is the central point in the experimental design that was repeated 18 times in total.

2.3. Experimental design and statistical analysis

For the investigation of the effect of TFA volume, time and protein concentration on the determination of total sugar concentration in protein–polysaccharide mixtures response surface methodology with a full factorial face central composite design (FCCD) ($\alpha = 1$) was employed using Minitab Statistical Software (v. 15, Minitab Inc., US). FCCD consists of 20 base runs that were performed in triplicate. This design yields 18 replicates of the central point and 42 replicates of the cube and axial points leading to a total of 60 replicates (Table 1). Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses, allowing each experimental response to be optimized. The range and centre point values of three independent variables were selected on the basis of previously published data on guar–protein phase diagrams (Antonov, Lefebvre, & Doublier, 1999; Bourriot, Garnier, & Doublier, 1999a; Neirynck, van Lent, Dewettinck, & van der Meeren, 2007).

A full second order polynomial response surface model was fitted to the experimental data that is given by the equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is the guar gum concentration, β_0, β_i, β_{ii} and β_{ij} are the regression coefficients estimated by the model and X_i, X_j are levels of the independent variables. The model includes, from left to right, an intercept, linear terms, squared terms and quadratic interaction terms.

2.4. Fluorescence microscopy

Polysaccharide solution, pre-stained with 0.01% (w/v) solution of fluorescent brightener 28 (Sigma–Aldrich, St. Luis, MO) was mixed with pre-stained with rhodamine B (0.02%, w/v) sodium caseinate solution at different ratios (1:1, 2:1, 1:2 guar:protein) yielding solutions with final biopolymer concentrations of 0.05–1, 0.05–2.5, 0.05–5, 0.1–1, 0.1–2.5 and 0.1–5% (w/v) (guar–protein). A drop of freshly prepared mixture was placed on a slide, covered with a cover slip and placed on the stage of the microscope at room temperature. Microscopy observations were carried out using an Olympus BX-41 epifluorescent microscope equipped with a mercury burner and an Olympus digital camera. Filter cubes with wide band UV excitation/emission filters 350/440 and 555/595 nm were used to observe the polysaccharide (fluorescent brightener 28) and the protein phase (rhodamine B), respectively. Images were captured with both filter sets and brightness/contrast, highlights, shadows and midtones ("levels") were subsequently adjusted using Creative Suite 8 (Adobe Systems Inc., CA, US).

Table 2
Estimated regression coefficients for the polynomial model used to describe the total sugar concentration (%, w/v). Analysis was performed using coded units.

Factor	Coefficient
Constant	0.050***
TFA	−0.003*
Time	0.004**
Protein	−0.0065***
TFA^2	0.0035 (NS)
Time^2	−0.004 (NS)
Protein^2	−0.003 (NS)
TFA × time	−0.002 (NS)
TFA × protein	0.002 (NS)
Time × protein	0.005**
r^2	0.546
Probability of lack-of-fit	0.213 (NS)

NS: not significant.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

3. Results and discussion

3.1. Investigation of phenol–sulphuric method in protein–polysaccharide mixtures

Preliminary findings showed that, the estimated total sugar concentration in protein/polysaccharides mixtures varies depending on the protein content in the mixtures. In particular, a significant downward trend ($p < 0.05$) in polysaccharide recovery was observed as the protein concentration in the mixtures increased (data not shown). This is indicative of interferences of the protein component on the phenol–sulphuric method that may affect the construction of phase diagrams. Therefore, the need for modification in the methodology seems necessary to optimize polysaccharide determination from the mixtures.

The actual guar concentration that could be determined with the phenol–sulphuric method in the stock solution was investigated first and compared with the nominal (dispersed polysaccharide powder). The actual guar concentration was estimated to be $0.094 \pm 0.007\%$ (w/v) when the nominal was 0.100% (w/v) indicating about a 6% deviation. This error is in close agreement with previously reported values on the phenol–sulphuric acid method in guar gum solutions (~5%) (Ng et al., 2009). These differences are attributed to the purity of the guar gum preparation and its hydration behaviour since it highly depends on the molecular weight and concentration of the dispersed galactomannan (Ng et al., 2009; Wang, Ellis, & Ross-Murphy, 2003; Parvathy, Susheelamma, & Tharanathan, 2007). Therefore some material is removed after the centrifugation step that was employed resulting in actual concentration that is lower than the nominal. The following discussion is done in terms of the actual concentration (0.094%, w/v), which was held constant for all mixtures throughout.

The effect of three parameters (TFA, time and protein concentration) on the estimation of total sugar concentration by the phenol–sulphuric method was investigated next by RSM. The coefficient estimates of model equation, along with the corresponding p-values, are presented in Table 2. p-Values were used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable. The constant (0.050, Table 2) shows that about half of the guar gum concentration in the mixtures can be determined if there was no influence from the factors. All the factors (TFA volume, protein concentration, time) had a significant influence on the polysaccharide recovery from the mixtures. The results showed that TFA had a significant ($p < 0.05$) linear contribution on guar concentration with the negative coefficient value (Table 2) implying that the higher the TFA volume the lower the resulting guar gum that can be estimated with the present methodology. This however is counteracted by the quadratic effect implying the overall pattern is also subject to interactions with protein and time that samples were left in the presence of the acid in the systems (Fig. 1 and Table 2). TFA is not expected to play direct role on the measurements since guar gum is a neutral polysaccharide and not influenced by changes of pH. Furthermore polysaccharide hydrolysis usually occurs at high temperatures and is unlikely to take place at room temperature as in the present investigation (Phillips & Williams, 2009). Therefore, the significant effect ($p < 0.05$) of TFA on the determination of guar concentration could be attributed to the effect of acid on the protein. As TFA is added in the mixtures pH drops and proteins denature as they reach their isoelectric point thus precipitating. However, at increased protein concentrations, protein precipitation results in removal of the polysaccharide due to its entrapment in the protein aggregates (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003) and therefore less sugars can be determined in the resulting supernatant. Therefore, as protein concentration increases in the mixtures less total sugars can be determined because more polysaccharide is co-precipitating entrapped in the protein matrix (de Kruif & Tuinier, 2001) (Fig. 1a). The linear effect of time as well as its interaction with protein concentration had both positive significant contributions to the total sugar concentration (Table 2). Fig. 1b shows that guar gum concentration relatively increases with increasing the time that the samples were left in the presence of TFA before phenol–sulphuric analysis. This implies that the longer the time lapse more proteins precipitate and therefore more polysaccharide can be estimated in the supernatant. The overall effect of the factors is also subject to their quadratic effect and interactions. The quadratic effects of each factor (X_i^2) was not significant for

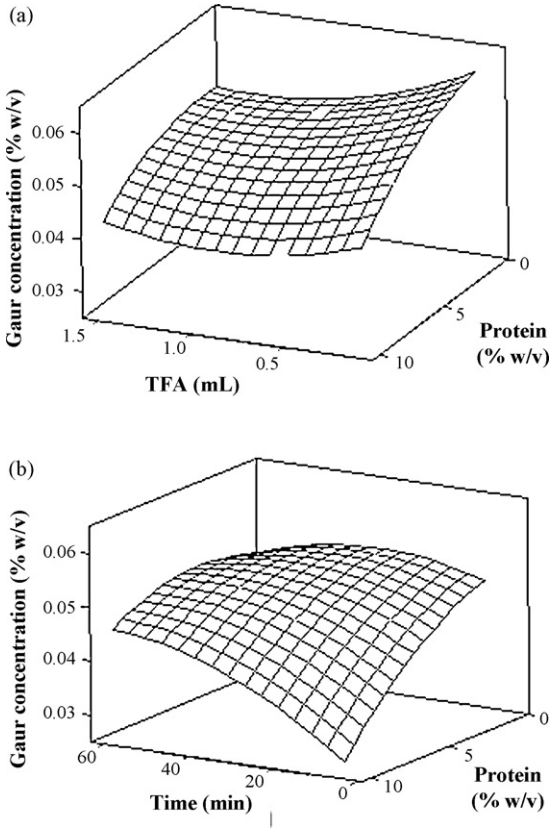

Fig. 1. Surface plots of total sugar concentration (%, w/v) versus (a) as a function of TFA and protein concentration and (b) as a function of time and protein concentration.

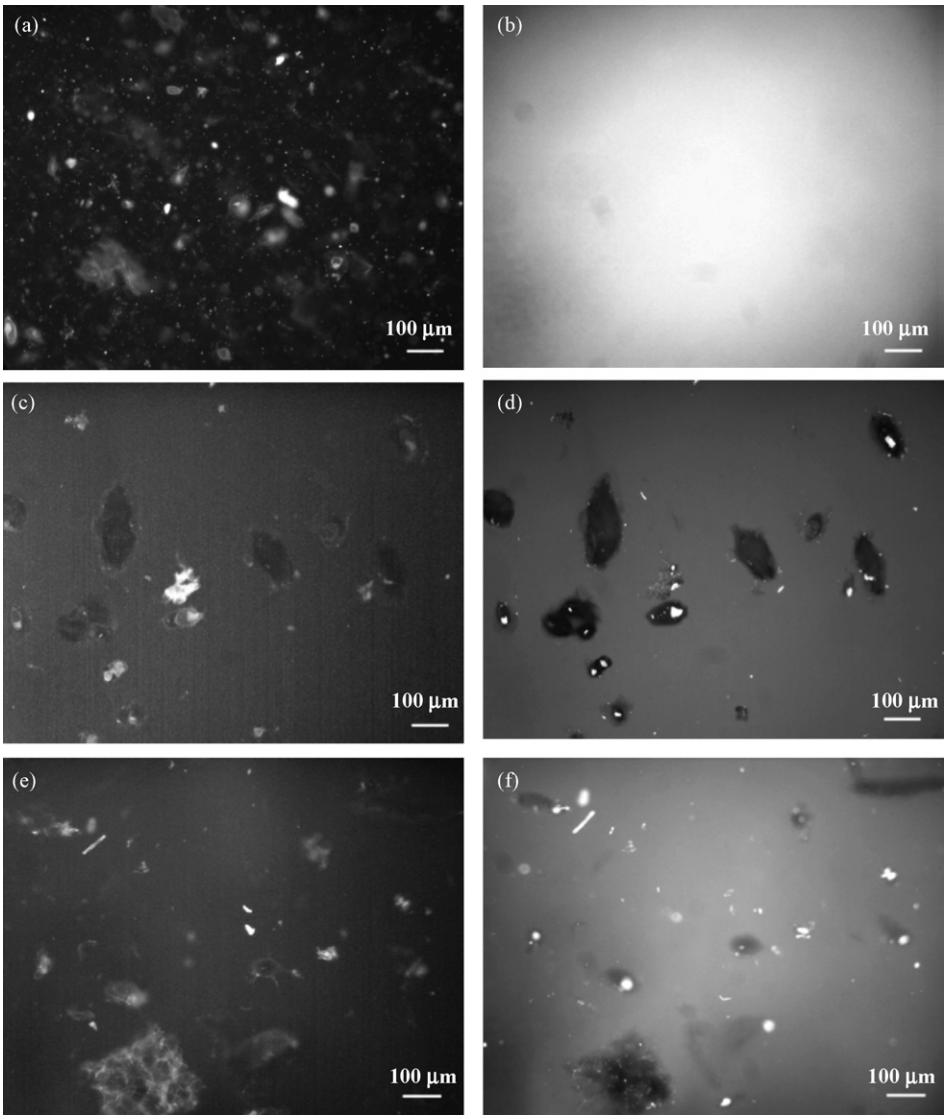

Fig. 2. Fluorescent images of the mixtures at different guar/protein concentrations (a) 0.1% (w/v) guar gum (b) 5% (w/v) sodium caseinate, (c and d) 0.1–2 and (e and f) 0.05–1 (%, w/v guar–protein). Images were taken using two different filter sets: left (a, c and e) for excitation of fluorescent brightener 28 (polysaccharide phase) and right (b, d and f) for excitation of rhodamine B (protein phase). Scale bar: 100 μm.

all factors studied something that indicates that it is not expected curvature in the response surface, that is to say, it will not be an optimum polysaccharide concentration (either maximum or minimum) by varying these factors.

By using the estimated regression coefficients, the response variable, Y (total sugar concentration) and the test variables X_1, X_2 and X_3 (representing TFA volume, time and protein concentration, respectively) can be related by the following equation: $Y = 0.050 - 0.003X_1 + 0.004X_2 - 0.0065X_3 + 0.0035X_1^2 - 0.004X_2^2 - 0.003X_3^2 - 0.002X_1X_2 + 0.002X_1X_3 + 0.005X_2X_3$. The lack-of-fit, which is a measure failure of the model to represent the data in the experimental domain (Qiao et al., 2009) was not significant ($p > 0.05$) relative to the pure error indicating that the fitted equation could describe the response under any combination of values of the variables tested. The obtained r^2 value (0.546) indicates the agreement between the experimental and predicted values of the determined guar gum concentration and although from analytical chemistry perspective seems to be low, it is satisfactory if we take into account the complexity of mixed biopolymer systems as well as the objective of the present investigation.

The mean maximum polysaccharide concentration that could be determined was about 0.06% (w/v) that represents about 64% of the mixture concentration. The unrecovered amounts can therefore be attributed to the negative effects of the test variables as well as losses possibly due to polysaccharide co-precipitation and interferences from proteins that remained in the polysaccharide phase. However, the method can be reliably used to estimate the polysaccharide concentration in mixed biopolymer systems, as the percentage error (±6%) is considered acceptable for the purposes of the present investigation. Taking all findings of the present investigation into consideration, it is possible to estimate about 60% of the initial guar gum concentration in the mixtures (0.094%, w/v), when the protein content in the mixtures ranges between 5 and 10% (w/v), about 0.5 mL TFA is added and left for 30 min rest. For greater than 60% guar gum determination, the protein content in the mixtures must be less than 5.5% (w/v) with less than 0.5 mL TFA and more than 30 min rest. However, these suggestions could work best for biopolymer mixed systems involving neutral polysaccharides since the charged ones may form complexes with proteins (Antonov et al., 1999) that cannot be removed with TFA precipitation thus further increasing the complexity of the system.

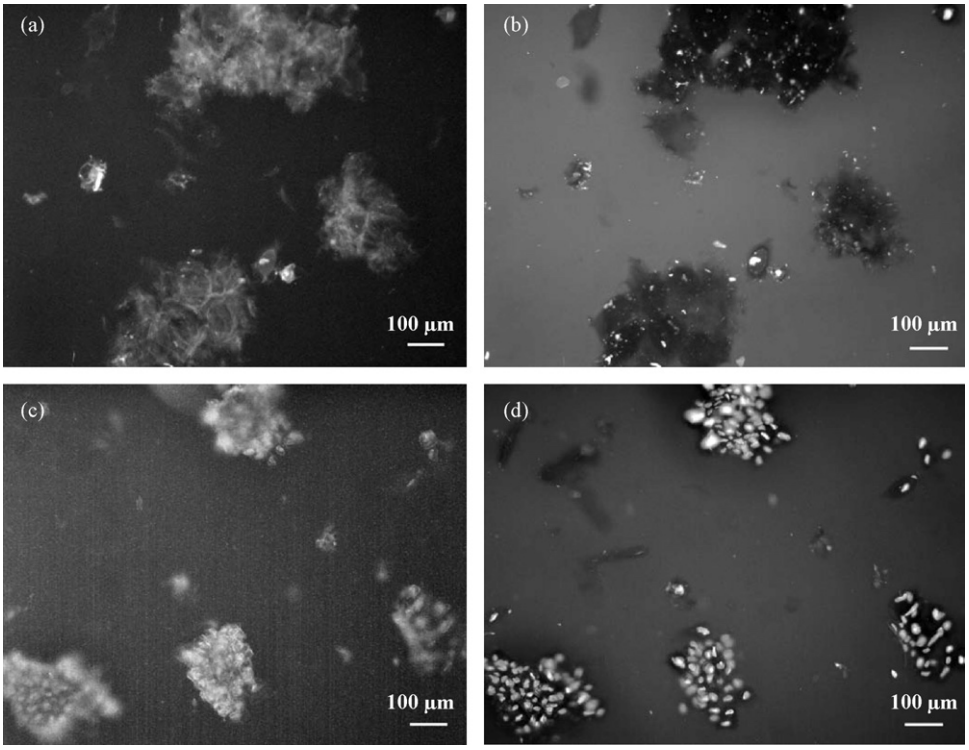

Fig. 3. Fluorescent images of the mixtures at (a and b) 1% (w/v) and (c and d) 5% (w/v) protein concentrations at the same guar concentration (0.1%, w/v). Protein inclusions can be formed within the polysaccharide phase (b and d) that are more evident at high protein concentrations. Scale bar: 100 μm.

The results provide strong evidence that when attempting to construct phase diagram of mixed biopolymer systems the equilibrium phases must be diluted to a protein content < ~5% (w/v) before TFA treatment if the maximum polysaccharide concentration is to be determined.

The incompatible nature of proteins and polysaccharide (Doublier, Garnier, Renard, & Sanchez, 2000; Neirynck et al., 2007; Tavares & da Silva, 2003; Turgeon et al., 2003) clearly influences the determination of the polysaccharide in the mixtures. Therefore, the microstructure of the mixtures was investigated next by fluorescence microscopy to seek for further support for the experimental results of total sugar concentration determination.

3.2. Morphology of mixed systems

Morphological features and topological arrangement of biopolymers in the mixtures were probed by means of fluorescent microscopy. Guar gum solutions revealed bright patterns scattered homogeneously throughout the sample (Fig. 2a). In contrast, sodium caseinate solutions in the absence of guar result in featureless patterns at all concentrations studied (1, 5 and 10%, w/v) (Fig. 2b). Phase separation in the system was evident with addition of guar gum into the protein phase. Images taken at various mixture concentrations and with two different filter sets revealed a phase-separated system with the guar domains surrounded by a continuous protein phase (Fig. 2c–f). Similar irregular-shaped morphological elements have been previously observed in high molecular weight β-glucan/whey protein mixtures (Kontogiorgos, Tosh, & Wood, 2009a; Kontogiorgos et al., 2009b), pea protein/κ-carrageenan (Musampa, Alves, & Maia, 2007), β-lactoglobulin/pectin (Girard, Sanchez, Laneuville, Turgeon, & Gauthier, 2004) or soy protein/κ-carrageenan (Li, Hua, Qiu, Yang, & Cui, 2008b). In contrast, mixtures of guar/micellar casein (Bourriot et al., 1999a; Norton & Frith, 2001; Schorsch et al., 1999), gelatin/maltodextrin (Kasapis, 2008), milk proteins/xanthan (Hemar et al., 2001) or low molecular weight β-glucan/whey protein mixtures (Kontogiorgos et al., 2009a) exhibited spherical morphology. Evidently, the morphological features of protein/polysaccharide mixtures vary depending on the properties of the solvent and constituent biopolymers. Visualization of mixtures using two different excitation/emission filter sets revealed new morphological characteristics as guar gum and protein increases. Guar aggregates are able to entrap protein as is evidenced by the intense bright spots within the guar aggregates (Fig. 3). Furthermore, as protein content in the mixture increases the protein that is entrapped in the polysaccharide aggregate also increase (Fig. 3b). These protein inclusions are difficult to be removed with TFA and may play important role on the polysaccharide quantification, as they will both interfere with absorption measurements and volume changes of the system leading to erroneous calculations. Taking everything into account, microscopical evidence as well as experimental measurements (Fig. 1) show that for greater polysaccharide estimation the protein concentration must be low (<~5%, w/v) before phenol–sulphuric method in mixed systems.

4. Conclusions

The effect of protein precipitation prior to phenol–sulphuric acid method in protein/polysaccharide mixtures was investigated by response surface methodology and fluorescent microscopy. Protein concentration, TFA volume as well as the time that the mixture was left in the presence of TFA, significantly influence the total sugar concentration that is estimated with this method. Fluorescent microscopy revealed a phase-separated system while some protein inclusions are formed within the polysaccharide phase, which provides further evidence of interferences in the determination of the total sugar concentration. Equilibrium phases must be diluted to a protein content < ~5% (w/v) before TFA treatment for maximum total sugar concentration determination in phase-diagram construction of mixed biopolymer systems.

References

Antonov, A. Y., Lefebvre, J., & Doublier, J.-L. (1999). On the one-phase state of aqueous protein-uncharged polymer systems: casein–guar gum system. *Journal of Applied Polymer Sciences*, *71*, 471–482.

Antonov, A. Y., Dmitrochenko, A. P., & Leontiev, A. L. (2006). Interactions and compatibility of 11S globulin from *Vicia faba* seeds and sodium salt of carboxymethylcellulose in an aqueous medium. *International Journal of Biological Macromolecules*, *38*, 18–24.

Bourriot, S., Garnier, C., & Doublier, J.-L. (1999a). Phase separation, rheology and microstructure of micellar casein–guar gum mixtures. *Food Hydrocolloids*, *13*, 43–49.

Bourriot, S., Garnier, C., & Doublier, J.-L. (1999b). Phase separation, rheology and structure of micellar casein–galactomannan mixtures. *International Dairy Journal*, *9*, 353–357.

de Kruif, C. G., & Tuinier, R. (2001). Polysaccharide protein interactions. *Food Hydrocolloids*, *15*, 555–563.

Doublier, J.-L., Garnier, C., Renard, D., & Sanchez, C. (2000). Protein–polysaccharide interactions. *Current Opinion in Colloid and Interface Science*, *5*, 202–214.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Ercelebi, E.-A., & Ibanoglu, E. (2007). Influence of hydrocolloids on phase separation and emulsion properties of whey protein isolate. *Journal of Food Engineering*, *80*, 454–459.

Girard, M., Sanchez, C., Laneuville, S. I., Turgeon, S. L., & Gauthier, S. F. (2004). Associative phase separation of β-lactoglobulic/pectin solutions: a kinetic study by small angle static light scattering. *Colloids and Surfaces B: Biointerfaces*, *35*, 15–22.

Gu, F., Abbas, S., & Zhang, X. (2009). Optimization of Maillard reaction products from casein–glucose using response surface methodology. *LWT—Food Science and Technology*, *42*, 1374–1379.

Hemar, Y., Tamehana, M., Munro, P. A., & Singh, H. (2001). Viscosity, microstructure and phase behavior of aqueous mixtures of commercial milk protein products and xanthan gum. *Food Hydrocolloids*, *15*, 565–574.

Kasapis, S. (2008). Phase separation in biopolymer gels: a low- to high-solid exploration of structural morphology and functionality. *Critical Reviews in Food Science and Nutrition*, *48*, 341–359.

Kim, H.-J., Decker, A. E., & McClements, D. J. (2006). Preparation of multiple emulsions based on thermodynamic incompatibility of heat-denatured whey protein and pectin solutions. *Food Hydrocolloids*, *20*, 586–595.

Kontogiorgos, V., Tosh, S. M., & Wood, P. J. (2009a). Kinetics of phase separation of oat β-glucan/whey protein isolate binary mixtures. *Food Biophysics*, *4*, 240–247.

Kontogiorgos, V., Tosh, S. M., & Wood, P. J. (2009b). Phase behaviour of high molecular weight oat β-glucan/whey protein isolate binary mixtures. *Food Hydrocoilloids*, *23*, 949–956.

Kshirsagar, A. C., & Singhal, R. S. (2007). Optimization of starch oleate derivatives from native corn and hydrolyzed corn starch by response surface methodology. *Carbohydrate Polymers*, *69*, 455–461.

Lazaridou, A., & Biliaderis, C. G. (2009). Concurrent phase separation and gelation in mixed oat β-glucans/sodium caseinate and oat β-glucans/pullulan dispersions. *Food Hydrocolloids*, *23*, 886–895.

Lee, J., Lin Ye, L., Landen, W. O., Jr., & Eitenmiller, R. R. (2000). Optimization of an extraction procedure for the quantification of vitamin E in tomato and broccoli using response surface methodology. *Food Composition and Analysis*, *13*, 45–57.

Li, X., Deng, F., Hua, Y., Qiu, A., Yang, C., & Cui, S. (2008). Effect of molecular weight of dextran on the phase behavior and mictrostructure of preheated soy protein/dextran mixtures. *Carbohydrate Polymers*, *72*, 160–168.

Li, X., Hua, Y., Qiu, A., Yang, C., & Cui, S. (2008). Phase behavior and microstructure of preheated soy proteins and κ-carrageenan mixtures. *Food Hydrocolloids*, *22*, 845–853.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol–sulfuric method in microplate format. *Analytical Biochemistry*, *339*, 69–72.

Musampa, R. M., Alves, M. M., & Maia, J. M. (2007). Phase separation, rheology and microstructure of pea protein–kappa-carrageenan mixtures. *Food Hydrocolloids*, *21*, 92–99.

Neirynck, N., van Lent, K., Dewettinck, K., & van der Meeren, P. (2007). Influence of pH and biopolymer ratio on sodium caseinate-guar gum interactions in aqueous solutions and in o/w emulsions. *Food Hydrocolloids*, *21*, 862–869.

Ng, D., Coventry, K., Lim, P., Wiltshire, J., Qiao, G. G., Boulton, A., et al. (2009). An improved technique for concentration measurement of galactomannan solutions by differential refractive index. *Carbohydrate polymers*, *77*, 150–153.

Norton, I. T., & Frith, W. J. (2001). Microstructure design in mixed biopolymer composites. *Food Hydrocolloids*, *15*, 543–553.

Parvathy, S. K., Susheelamma, N. S., & Tharanathan, R. N. (2007). Hydration characteristics of guar gum samples and their ractions. *Food Hydrocolloids*, *21*, 630–637.

Perrechil, F. A., Braga, A. L. M., & Cunha, R. L. (2009). Interactions between sodium caseinate and LBG in acidified systems: Rheology and phase behavior. *Food Hydrocolloids*, *23*, 2085–2093.

Phillips, O. G., & Williams, A. P. (2009). *Handbook of hydrocolloids*. Oxford: Woodhead.

Polyakov, V. I., Grinberg, V. Y., & Tolstoguzov, V. B. (1980). Application of phase-volume-ratio method for determining the phase diagram of water–casein–soybean globulins system. *Polymer Bulletin*, *2*, 757–760.

Qiao, D., Hu, B., Gan, D., Sun, Y., Ye, H., & Zeng, X. (2009). Extraction optimized by using response surface methodology, purification and preliminary characterization of polysaccharides from Hyriopsis cumingii. *Carbohydrate Polymers*, *76*, 422–429.

Schorsch, C., Clark, A. H., Jones, M. G., & Norton, I. T. (1999). Behaviour of milk protein/polysaccharide systems in high sucrose. *Colloids and Surfaces B: Biointerfaces*, *12*, 317–329.

Simsek, A., Poyrazoglu, E. S., Karacan, S., & Velioglu, Y. S. (2007). Response surface methodological study on HMF and fluorescent accumulation in red and white grape juices and concentrates. *Food Chemistry*, *101*, 987–994.

Tavares, C., & da Silva, J. A. L. (2003). Rheology of galactomannan–whey protein mixed systems. *International Dairy Journal*, *13*, 699–706.

Thaiudom, S., & Goff, H. D. (2003). Effect of κ-carrageenan on milk protein polysaccharide mixtures. *International Dairy Journal*, *13*, 763–771.

Tolstoguzov, V. B. (2003). Some thermodynamic considerations in food formulation. *Food Hydrocolloids*, *17*, 1–23.

Turgeon, S. L., Beaulieu, M., Schmitt, C., & Sanchez, C. (2003). Protein–polyssacharide interactions: phase-ordering kinetics, thermodynamic and structural aspects. *Current Opinion in Colloid and Interface Science*, *8*, 401–414.

Wang, Q., Ellis, P. R., & Ross-Murphy, S. B. (2003). Dissolution kinetics of guar gum powders. II. Effects of concentration and molecular weight. *Carbohydrate Polymers*, *53*, 75–83.

XuJie, H., & Wei, C. (2008). Optimization of extraction process of crude polysaccharides from wild edible BaChu mushroom by response surface methodology. *Carbohydrate Polymers*, *72*, 67–74.

Yongjiang, W., Zhong, C., Jianwei, M., Minger, F., & Xueqian, W. (2009). Optimization of ultrasonic-assisted extraction process of Poria cocos polysaccharides by response surface methodology. *Carbohydrate Polymers*, *77*, 713–717.

Zhang, G., & Foegeding, E. A. (2003). Heat-induced phase behavior of β-lactoglobulin/polysaccharide mixtures. *Food Hydrocolloids*, *17*, 785–792.