Carbohydrate Polymers 87 (2012) 2202–2210

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Methodologies for the extraction and analysis of konjac glucomannan from corms of *Amorphophallus konjac* K. Koch

Melinda Chua [a], Kelvin Chan [a,b,c], Trevor J. Hocking [a], Peter A. Williams [d], Christopher J. Perry [a], Timothy C. Baldwin [a,*]

[a] School of Applied Sciences, University of Wolverhampton, Wulfruna Street, Wolverhampton WV1 1SB, United Kingdom
[b] HMREC, Faculty of Pharmacy, University of Sydney, NSW2006, Sydney, Australia
[c] CompleMED, College of Health and Science, University of Western Sydney, NSW2560, Sydney, Australia
[d] Glyndwr University, Plas Coch, Mold Road, Wrexham LL11 2AW, United Kingdom

ARTICLE INFO

Article history:
Received 4 August 2011
Received in revised form 17 October 2011
Accepted 18 October 2011
Available online 28 October 2011

Keywords:
Amorphophallus konjac
Konjac glucomannan
Ethanol extraction
3,5-Dinitrosalicylic acid
Gel permeation chromatography
Zero shear viscosity

ABSTRACT

Here we present a comparison of commonly used methodologies for the extraction and quantification of konjac glucomannan (KGM). Compositional analysis showed that the purified konjac flour (PKF) produced using a modified extraction procedure contained 92% glucomannan, with a weight average molecular weight (M_w), polydispersity index (PDI) and degree of acetylation (DA) of $9.5 \pm 0.6 \times 10^5$ g mol^{-1}, 1.2 and 2.8 wt.%. These data, plus Fourier-transform infrared spectral (FTIR) and zero shear viscosity analyses of the extract (PKF) were all consistent with the literature. Comparison of three existing methodologies for the quantitative analysis of the KGM content of the PKF, namely 3,5-dinitrosalicylic acid (3,5-DNS), phenol–sulphuric acid and enzymatic colorimetric assays; indicated that the 3,5-DNS colorimetric assay was the most reproducible and accurate method, with a linear correlation coefficient of 0.997 for samples ranging from 0.5 to 12.5 mg/ml, and recoveries between 97% and 103% across three spiking levels (250, 500 and 750 μg/g) of starch. These data provide the basis of improved good laboratory practice (GLP) for the commercial extraction and analysis of this multifunctional natural polymer.

1. Introduction

Amorphophallus konjac has long been used in China and Japan as a food source and as a traditional medicine. The corm tissues of this species are known to be a valuable source for glucomannan, a soluble, non-cellulosic polysaccharide. Traditionally, this polysaccharide (which has no calorific content), commonly known as KGM, is extracted from corm tissues in the form of flour from which foodstuffs (e.g. noodles) are prepared (Chua, Baldwin, Hocking, & Chan, 2010). Such foodstuffs promote satiety and are used as an anti-obesity agent in the Orient. Over the past two decades, KGM has been introduced on a relatively small scale into the United States and Europe, both as a food additive (Zhang, Xie, & Gan, 2005) and a dietary supplement in the treatment of obesity-related dyslipidemia (Keithley & Swanson, 2005; Vasques et al., 2008) and diabetes (Vuksan et al., 2000, 2001).

Chemically, KGM is composed of a linear chain of β-1,4-linked D-glucose and D-mannose residues in a molar ratio of 1:1.6, with side branches through β-1,6-glucosyl units. The degree of branching is estimated at approximately 3 branches for every 32 sugar residues. The acetyl groups along the KGM backbone are located, on average, every 9–19 sugar units at the C-6 position (Maeda, Shimahara, & Sugiyama, 1980; Shimahara, Suzuki, Sugiyama, & Nishizawa, 1975).

In recent decades, methods for the extraction and purification of KGM have been studied and developed. KGM is extracted either by mechanical means (dry processing), or by wet (chemical) processing methods. The dry processing method involves milling of dried konjac chips into crude konjac flour (CKF), which is subsequently purified via wind-sifting (Parry, 2010; Takigami, 2000). Konjac flour produced using such methods is of low purity and is sold as food commodity at a low price.

Wet processing methods include the use of lead acetate (Wu, Meng, Chai, & Wang, 2002), salt (e.g. aluminium sulphate) (WIPO, 1993), 2-propanol coupled with starch hydrolyzing enzymes (Wootton, Luker-Brown, Westcott, & Cheetham, 1993) and ethanol (Ogasawara, Yamazaki, & Nunomura, 1987; Sugiyama, Shimahara, & Andoh, 1972) to extract KGM from CKF. KGM extracted by lead

Abbreviations: CKF, crude konjac flour; CMA, Chinese Ministry of Agriculture; DA, degree of acetylation; 3,5-DNS, 3,5-dinitrosalicylic acid; FCC, Food Chemicals Codex; FTIR, Fourier-transform infrared spectra; GLP, good laboratory practice; GPC-MALLS, gel permeation chromatography coupled to multiangle light scattering detectors; KGM, konjac glucomannan; LVKF, low-viscosity konjac flour; NKF, nutraceutical-grade konjac flour; PDI, polydispersity index; PHP, potassium hydrogen phthalate; PKF, purified konjac flour; RI, refractive index; RSD, relative standard deviation; DI, deionised water.

* Corresponding author. Tel.: +44 1902 32 2142; fax: +44 1902 32 1093.
E-mail address: T.Baldwin@wlv.ac.uk (T.C. Baldwin).

0144-8617/$ – see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2011.10.053

acetate is not edible, thus limiting its applications in the non-food industries, whilst enzymatic purification, which involves degradation of starch (regarded as an impurity) by hydrolyzing enzymes is not selective. KGM may be depolymerised by contaminating β-mannanase activity in the starch hydrolyzing enzymes (Gong et al., 2002).

Given the deficiencies mentioned above, to date, the most common konjac flour purification methods involve ethanol extraction of KGM, due to its simplicity and high efficiency (Takigami, 2000). Considerable success in producing high quality PKF have been reported by Ogasawara et al. (1987), Sugiyama et al. (1972), using the ethanol extraction method. Such methods are, however, time-consuming, taking 147 h (Sugiyama's method) to 384 h (Ogasawara's method); and producing low-yields of PKF.

The content of glucomannan is a key indicator for evaluating the quality of konjac flour (Byrne, 2001; Liu et al., 2002; U.S. FCC, 2003). However, at present there is no globally agreed regulatory standard for konjac flour and therefore, harmonization of commercial standards is needed to assess and ensure the quality of existing KGM products (Chua et al., 2010), especially if these products are to be introduced more widely into Western markets.

With this in mind, the objectives of the current study were twofold: Firstly, to develop an improved processing methodology for konjac flour based on the working principles of both Sugiyama's and Ogasawara's methods. Secondly, to compare three existing methodologies for the quantitative analysis of the glucomannan content in konjac flour samples to identify the most reliable assay method. These methods included the 3,5-DNS colorimetric assay, which has been adopted by the Chinese Ministry of Agriculture (CMA) for the classification of konjac flour (Liu et al., 2002); the phenol–sulphuric acid colorimetric assay, which has been used for several decades for the determination of reducing sugars (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956); and an enzymatic colorimetric assay using a commercially available glucomannan assay kit, which has been used in Western food industries for the analysis of KGM content in foodstuffs. The overall objective was to provide an improved protocol for the extraction, purification and analysis of KGM which could be used in the establishment of GLP for this useful and important foodstuff.

2. Materials and methods

2.1. Materials

Two reference samples were used in the current study: (1) nutraceutical-grade konjac flour (NKF) (>99% glucomannan) obtained from M&S Colloid Technology Ltd., Hong Kong and (2) low-viscosity konjac flour (LVKF) (>98% glucomannan) purchased from Megazyme International Ireland Ltd., Ireland.

3,5-DNS reagent was prepared by mixing solutions A and B. Solution A was prepared by mixing phenol (0.70 g), 10% (w/w) sodium hydroxide (1.50 ml), deionised (DI) water (5 ml) and sodium bisulphite (0.70 g). Solution B was prepared by mixing potassium sodium tartrate (22.50 g), 10% sodium hydroxide (30 ml) and 1% (w/w) 3,5-DNS (88 ml).

2.2. Preparation of crude konjac flour from fresh corm material

The corm was weighed, washed, with its epidermis removed and sliced into pieces, 2–3 mm in thickness. The corm slices were then immersed in 1% (w/v) sodium bisulphite for 1 min, followed by oven-drying at 120 °C for 40 min. The drying process was continued at 60 °C until a constant weight was obtained. The dried corm slices were subsequently ground and the resultant flour sieved (425 μm aperture) to produce CKF.

2.3. Purification of crude konjac flour

2.3.1. Method 1

CKF (2.00 g) was stirred in 50% (v/v) ethanol (200 ml) for 30 min at room temperature, followed by centrifugation (5000 × *g*, 30 min, 25 °C) to remove the aqueous ethanol. This procedure was repeated twice under the same conditions by stirring the pellet obtained in freshly prepared 50% (v/v) ethanol after each centrifugation. Subsequently, the resultant pellet was stirred in DI water (200 ml) for 2 h at room temperature followed by centrifugation (9000 × *g*, 30 min, 25 °C). This procedure was repeated twice under the same conditions by stirring the insoluble materials obtained in DI water, with the supernatants retained after each centrifugation. The supernatants collected were mixed and the volume was reduced to ~1/3 the original volume by rotary evaporation. Glucomannan present in the solution was precipitated overnight with 95% (v/v) ethanol (600 ml) at 4 °C, followed by centrifugation (9000 × *g*, 40 min, 25 °C). The resultant pellet was washed twice with anhydrous ethanol (dehydration process) and subsequently isolated by vacuum filtration, before being freeze-dried for 48 h. The dried material was ground and sieved to produce PKF (referred to as PKF1 hereafter).

2.3.2. Method 2

CKF (2.00 g) was stirred in 50% (v/v) ethanol (200 ml) for 90 min at room temperature, followed by centrifugation (5000 × *g*, 30 min, 25 °C) to remove the aqueous ethanol. The resultant pellet was added to DI water (200 ml) and stirred for 3 h at room temperature. The solution was then diluted to 400 ml with DI water, prior to centrifugation (9000 × *g*, 30 min, 25 °C) to remove the insoluble materials. Subsequently, rotary evaporation was performed to reduce the volume of the filtrate to ~1/3 the original volume, followed by KGM precipitation with 95% (v/v) ethanol (450 ml), centrifugation, dehydration, filtration and freeze drying, as described in method 1 to produce PKF (referred to as PKF2 hereafter).

2.4. Comparison of methods for assay of glucomannan content

The reproducibility and accuracy of the 3,5-DNS, phenol–sulphuric acid and enzymatic colorimetric assays were first compared by analysing CKF and LVKF samples in triplicate on different dates under the same experimental conditions. The absorbance readings were determined using a Helios α UV-visible spectrophotometer (Thermo Electron Corp., Rochester). Both glucose and mannose calibration curves were constructed for the 3,5-DNS and phenol–sulphuric acid assays, in order to compare the sensitivity of the assay systems to each reducing sugars. The 3,5-DNS method was further validated before being used for the determination of glucomannan content of PKFs.

2.4.1. 3,5-DNS colorimetric assay

2.4.1.1. Construction of standard D*-glucose and* D*-mannose calibration curves.* D-Glucose stock solution (1 mg/ml) was placed (0.40, 0.80, 1.20, 1.60 and 2.00 ml) into 25 ml volumetric flasks, respectively (using DI water as a blank). DI water was then added to the volume of 2.00 ml, followed by the addition of 3,5-DNS (1.50 ml) to each flask. Each mixture was heated for 5 min in a boiling water bath and cooled to room temperature before being diluted to 25 ml with DI water in a volumetric flask. Absorbance was then measured at 550 nm and a plot of the measured absorbance against the glucose content (mg) constructed. A D-mannose standard curve was constructed using the procedure as described for glucose.

2.4.1.2. Preparation of KGM sample solutions and colorimetric reactions. Konjac flour (0.2000 g) was added to a magnetically stirred

formic acid–sodium hydroxide buffer (0.1 mol/L; 50 ml) and mixed for 4 h at room temperature. The mixture was then diluted with formic acid–sodium hydroxide buffer to 100 ml in a volumetric flask, followed by centrifugation (4500 × *g*, 40 min, 25 °C). The KGM sample solution (5.00 ml) obtained was added to a 25 ml volumetric flask followed by the addition of 3 M sulphuric acid (2.50 ml). The resultant solutions were stirred and hydrolyzed for 90 min in a boiling water bath and allowed to cool to room temperature before the addition of 6 M sodium hydroxide (2.50 ml). The solution was then made up to 25 ml with DI water to form the KGM hydrolysate. Both the KGM sample solution and KGM hydrolysate were subjected to colorimetric reactions (using DI water as a blank), as described in Section 2.4.1.1. The glucomannan content was determined by evaluation of Eq. (1),

$$\text{GMcontent}\,(\%) = \frac{5000f(5T - T_0)}{m} \quad (1)$$

where f = correction factor, T = glucose content of KGM hydrolysate (mg), T_0 = glucose content of KGM sample solution (mg) and m = mass of konjac flour (200 mg).

2.4.1.3. Method validation study. The precision of the 3,5-DNS colorimetric assay, expressed as the relative standard deviation (RSD), was evaluated by assaying four replicate of the NKF sample, each at six different concentrations (0.5, 1.0, 2.5, 5.0, 10.0 and 12.5 mg/ml). Accuracy was assessed by spiking the NKF samples (0.2000 g) with a possible interfering substance, *i.e.* starch (VWR BDH Prolabo, UK) at three levels: 250, 500 and 750 μg/g (in 4 replications).

2.4.2. Phenol–sulphuric acid colorimetric assay

2.4.2.1. Construction of standard D*-glucose and* D*-mannose calibration curves.* Glucose standard solutions (16, 32, 48, 64 and 80 μg/ml), 2.00 ml each, were added into test tubes (using DI water as a blank), followed by the addition of 5.0% (w/w) phenol reagent (1.00 ml). Concentrated sulphuric acid (5.00 ml) was then delivered rapidly into each tube and allowed to stand for 10 min at room temperature, prior to incubation at 25 °C for 20 min. Absorbance was then measured at 490 nm and a plot of the measured absorbance against the glucose concentration (μg/ml) was constructed. A mannose calibration curve was constructed using the procedures as described for glucose.

2.4.2.2. Preparation of KGM sample solutions and colorimetric reactions. Konjac flour (0.0500 g) was added to magnetically stirred DI water (40.00 ml) and mixed for 4 h at room temperature. The solution was then diluted with DI water to 50 ml in a volumetric flask, followed by centrifugation (4000 × *g*, 40 min, 25 °C). The supernatant (3 ml) was subsequently diluted with DI water to 50 ml to form the KGM sample solution, which was then subjected to colorimetric reaction, as described in Section 2.4.2.1 (using DI water as a blank). Absorbance was then measured at 490 nm and the glucomannan content was determined by evaluation of Eq. (2),

$$\text{KGMcontent}\,(\%) = \frac{100fC_1}{C_2} \quad (2)$$

where f = correction factor, C_1 = concentration of reducing sugar (glucose) in the KGM hydrolysate (μg/ml) and C_2 = concentration of KGM sample solution (μg/ml).

2.4.3. Enzymatic colorimetric assay

This was performed using a glucomannan assay kit purchased from Megazyme International Ireland Ltd. (catalogue number: K-GLUM). *N.B.* This experiment was carried out during 2007, following the procedures cited in the kit instruction booklet (K-GLUM 10/04). Unfortunately, an error in the final extraction volume (FEV) for the KGM content calculation (page 7 of instruction booklet) was noted. It was observed that the FEV value used in the given protocol (*i.e.* 100 ml) was not correlated with the value (*i.e.* 250 ml) employed in the final calculation of KGM content (page 10 of instruction booklet). We have contacted the company to clarify this matter and it has responded by issuing a revised instruction booklet (K-GLUM 08/10) for this kit; with the FEV being amended to 250 ml in the protocol. The results presented in the current study were thus based upon 100 ml of FEV. The deviation in results obtained due to the possible use of an incorrect multiplier is discussed.

2.5. Determination of starch content

This was performed using a total starch assay kit purchased from Megazyme International Ireland Ltd. (catalogue number: K-TSTA).

2.6. Determination of protein content

Nitrogen analysis was performed using the Kjeldahl method (AOAC, 1999). The protein content was calculated by applying the nitrogen conversion factor of 5.7, as proposed by the U.S. Food Chemicals Codex (FCC) and European Commission (Byrne, 2001; Parry, 2010; U.S. FCC, 2003).

2.7. Determination of the degree of acetylation (DA)

DA, defined as the wt.% of acetyl-substituted residues in the KGM backbone (Gao & Nishinari, 2004) was determined by a back-titration method. Two solutions were used: solution A (~0.45 M NaOH) and solution B (~0.2 M HCl). Potassium hydrogen phthalate (PHP) was used as the primary standard for all solutions. The standardisation procedure is as follows: firstly, PHP (1.00 g) was dissolved in DI water and titrated with solution A, using phenolphthalein indicator. The titre was recorded as V_a (ml) and the mass of PHP as m_{PHP} (g). Secondly, solution B (10.00 ml) was titrated with solution A using phenolphthalein indicator and the titre was recorded as V_b (ml). Thirdly, the KGM sample (m_{KGM} = 1.000 g) was stirred in DI water (250 ml) for 3 h at room temperature and 2 drops of phenolphthalein indicator added. The solution was titrated with ~0.1 M sodium hydroxide to a permanent pink colour (pre-neutralization). Solution A (10.00 ml) was then added and the mixture stirred for 3 h at room temperature. The remaining alkali was back titrated with solution B and the titre recorded V_c (ml). The DA was determined by evaluation of Eq. (3),

$$\text{DA} = \frac{4300m_{PHP}(100 - V_cV_b)}{2042.3V_am_{KGM}} \quad (3)$$

2.8. FTIR analyses

IR spectra were generated directly from the powdered samples using a Mattson Genesis II spectrometer (Thermo Electron Corp., Madison, WI) at room temperature, using an ATR stage.

2.9. Determination of the molecular mass distribution by aqueous gel permeation chromatography coupled to multiangle light scattering detectors (GPC-MALLS)

The GPC-MALLS analysis was undertaken in collaboration with Professor P.A. Williams at the Centre for Water Soluble Polymers at Glyndwr University. The preparation of sample solutions, conditions employed for the GPC-MALLS apparatus and the interpretation of light scattering data were based upon the protocols previously described by Ratcliffe, Williams, Viebke, and Meadows (2005).

2.10. Determination of the zero shear viscosity (η_0)

Shear flow measurements were performed using AR500 and AR2000 rheometers (TA Instruments, New Castle, DE) at 25 °C. Stock solutions of PKF1, PKF2 and NKF (2%, w/w, each) were prepared by adding each sample (2.00 g) to the vortex of DI water (98.00 g) created by an overhead electric stirrer. After 30 min mixing at room temperature, the samples were stirred for 2 h in an 80 °C water bath, followed by the addition of DI water to compensate for evaporative loss. To appropriate aliquots of the 2% (w/w) stock solutions, DI water was added to achieve concentrations of 0.2%, 0.3%, 0.4%, 0.5%, 0.75%, 1.25%, 1.5% and 1.75% (w/w). The diluted solutions were mixed on a roller mixer overnight and allowed to equilibrate to room temperature for 30 min before measurement. Sample concentrations of 0.2–0.75% were measured on an AR2000 rheometer using single concentric cylinder geometry, whilst sample concentrations of 1–2% were measured on an AR500 rheometer using 4 cm diameter cone geometry, typically in the shear rate range of 1–1000 s^{-1}. Zero shear viscosity was determined using the Cross model based on Eq. (4):

$$\eta = \eta_\infty + \left[\frac{\eta_0 - \eta_\infty}{1 + (\lambda\dot{\gamma})^m}\right] \quad (4)$$

where η = viscosity at a given shear rate ($\dot{\gamma}$); η_0 = zero shear viscosity, η_∞ = infinite shear-rate viscosity; λ = arbitrary constant and m = power law exponent.

2.11. Statistical analysis

Data were subjected to a paired t-test at 5% level of probability ($P < 0.05$) using the SPSS 16.0 software.

3. Results and discussion

3.1. Purification of CKF

The working principle and procedures involved in both Ogasawara's and Sugiyama's methods for the extraction and purification of KGM are similar (Ogasawara et al., 1987; Sugiyama et al., 1972), with differences in the duration of ethanol extraction and hydration treatment of CKF. In both methods, the CKF was stirred continuously (3–10 days) in different ethanol concentrations ranging from 50 to 100% (v/v), followed by oven-drying (60–90 °C) of the resultant flour before being hydrated (3–12 h) to form a sol. As the sol produced was highly viscous, it was diluted up to 10 fold before being dialyzed (72 h). The dialyzed solution was subsequently freeze-dried to form the PKF. As mentioned previously, although the resultant flour produced by both methods was found to retain the physicochemical properties of KGM, these methods are, however, time consuming and produce low yields of PKF.

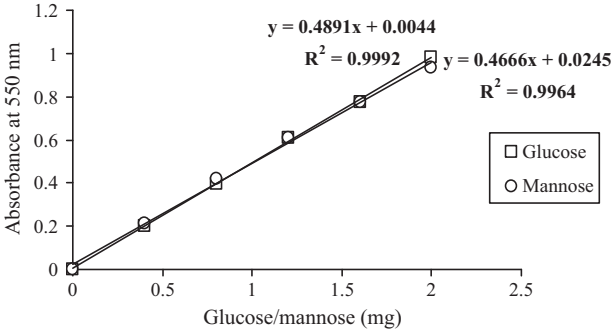

Fig. 1. Glucose and mannose standard calibration curves for the 3,5-DNS colorimetric assays ($n = 3$).

Since it is important to employ a method that ensures proper purification without compromising the yield of the purified flour, two methods with different frequency/duration of ethanol washing and hydration treatments were hence established in the current study. Both methods are characterized by consecutive steps of: (1) ethanol (50%, v/v) washings to remove impurities such as soluble starch and low molecular weight sugars; (2) dispersing the flour in water with agitation to extract the KGM; (3) precipitation of the KGM by 95% (v/v) ethanol and (4) drying and grinding the KGM precipitate. Method 2 comprised of an ethanol washing treatment (90 min) and a hydration step (3 h), and is less laborious compared to method 1 which involved three ethanol washing (30 min each) and three hydration steps (2 h each).

A 38 ± 2% and 53 ± 4% yield of PKF was obtained from the CKF using purification method 1 and 2, respectively. The yield of PKF from both methods is considerably higher than those reported in Ogasawara's (22%) and Sugiyama's (35%) methods (Ogasawara, Yamazaki, & Nunomura, 1987; Sugiyama et al., 1972). The lower yield of PKF using method 1 is expected as more ethanol washing and hydration steps were involved, which may result in the production of PKF of higher purity, but a concomitant lower yield.

3.2. Comparison of assays to determine glucomannan content in KGM samples

Determination of the glucomannan (or reducing sugar) content using the 3,5-DNS (Farhoosh & Riazi, 2007; Lindsay, 1973; Miller, 1959), phenol–sulphuric acid (Cuesta, Suarez, Bessio, Ferreira, & Massalsi, 2003; Dubois et al., 1956) and enzymatic (Tekinsen & Guner, 2010) colorimetric assays have been previously reported in the literature. The current study compared the reproducibility and accuracy of these three methods, followed by further validation studies to determine the best assay method for quantification of the glucomannan content of KGM samples.

Both glucose and mannose standard curves were constructed for the 3,5-DNS (Fig. 1) and phenol–sulphuric acid (Fig. 2)

Table 1
Glucomannan content (%) of CKF and LVKF determined by the 3,5-DNS, phenol–sulphuric acid and the enzymatic colorimetric assays on two occasions ($n = 3$).

	3,5-DNS		Phenol–sulphuric acid colorimetry		Enzymatic colorimetry	
	CKF	Colorimetry	CKF	LVKF	CKF	LVKF
Replicate 1	53.7	89.9	51.4	85.6	30.3	45.4
	52.1	93.2	55.6	95.2	31.5	42.4
Replicate 2	52.5	90.0	45.4	91.7	28.7	43.2
	50.9	97.9	59.2	97.9	30.2	41.7
Replicate 3	53.9	90.6	53.2	84.5	31.6	41.7
	53.2	95.5	58.7	99.6	30.2	41.1
Mean ± SE	53.4 ± 0.4[a]	90.2 ± 0.2[a]	50.0 ± 2.4[a]	83.9 ± 2.3[a]	30.2 ± 0.9[a]	43.4 ± 1.1[a]
	52.1 ± 0.7[a]	95.5 ± 1.4[a]	57.8 ± 1.2[b]	97.7 ± 1.4[b]	30.6 ± 0.5[a]	41.7 ± 0.4[a]

Different letters following mean values within the same column indicate significant differences at the $P < 0.05$ level.

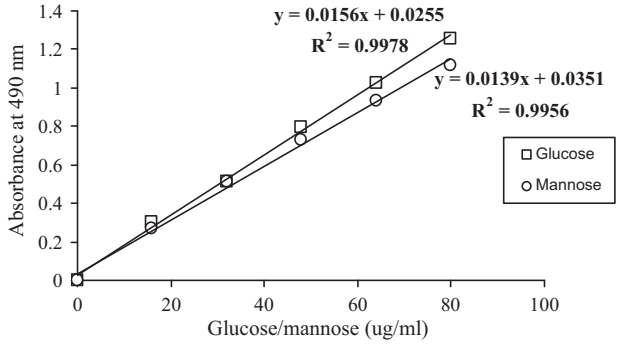

Fig. 2. Glucose and mannose standard calibration curves for the phenol–sulphuric acid colorimetric assays ($n = 3$).

colorimetric assays, in order to compare the sensitivity of the assay systems to each sugar. From the gradient of the calibration curves, it was found that mannose has a slightly lower sensitivity compared to glucose in both assay systems. Therefore, a correction factor for mannose sugar, *i.e.* 1.03 [1/2.6 + (1.6/2.6 × 0.4891/0.4666)] and 1.07 [1/2.6 + (1.6/2.6 × 0.0156/0.0139)] was determined for each method and employed in Eqs. (1) and (2), respectively. These correction factors were derived from the gradient of the constructed standard curves for both sugars and the typical glucose to mannose ratio (1:1.6) reported in KGM (Maeda et al., 1980; Shimahara et al., 1975).

Table 1 shows the glucomannan content of both samples as determined by the 3,5-DNS, phenol–sulphuric acid and enzymatic colorimetric assays, ranged from 50.9 to 53.9% (CKF) and 89.9 to 97.9% (LVKF); 45.4 to 59.2% (CKF) and 84.5 to 99.6% (LVKF); and 28.7 to 31.6% (CKF) and 41.1 to 45.4% (LVKF), respectively. There were no significant differences in the mean glucomannan values obtained from each of the 3,5-DNS and enzymatic colorimetric assays between the two occasions, indicating that both assay methods are more reproducible compared to the phenol–sulphuric acid colorimetric assay. Cuesta et al. (2003) has previously reported that the reproducibility of the phenol–sulphuric acid assay is highly dependent on the modality of sulphuric acid addition, either directly over the liquid surface or over the side of the tube. Furthermore, the speed of acid addition has also been shown to be critical. Saha and Brewer (1994) demonstrated that rapid addition of acid generates sufficient heat to break all the glycosidic bonds in complex carbohydrates. Clearly, the reproducibility of this method is an issue given the difficulty in the precise addition of the acid to produce consistent results, as shown in the current study, which was performed according to Dubois's protocol.

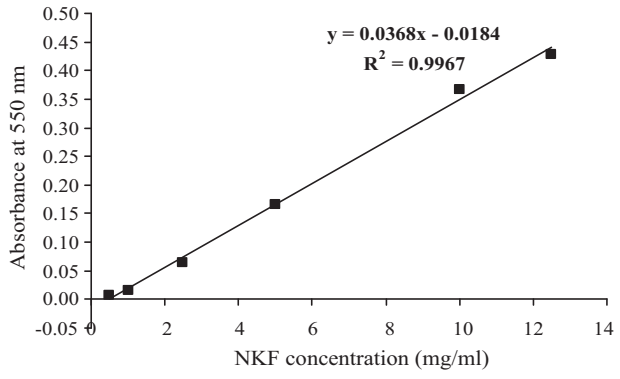

Fig. 3. Linearity of 3,5-DNS colorimetric assay at six NKF concentrations, ranging from 0.5 to 12.5 mg/ml ($n = 4$).

In terms of accuracy, the glucomannan contents of both CKF and LVKF determined by the 3,5-DNS and phenol–sulphuric acid assays were in reasonable agreement to the previously reported values for both samples (49–60% and >98%, respectively), compared to the enzymatic colorimetric assay which yielded a considerable lower result for LVKF (~43%), despite its high reproducibility. As mentioned in Section 2.4.3, an error for the FEV value involved in the final calculation of KGM content using the glucomannan assay kit was encountered in the current study. If the incorrect FEV value (*i.e.* 250 ml), as cited in the kit instruction booklet was applied to our data, the resultant glucomannan content will be 2.5 fold (*i.e.* around 100%) higher than the current results. The reason for the low results obtained using this assay kit is unclear, and it is possible that the enzymatic reactions involved were incomplete, and therefore, further studies are needed to investigate and optimise the experimental conditions employed in this assay method.

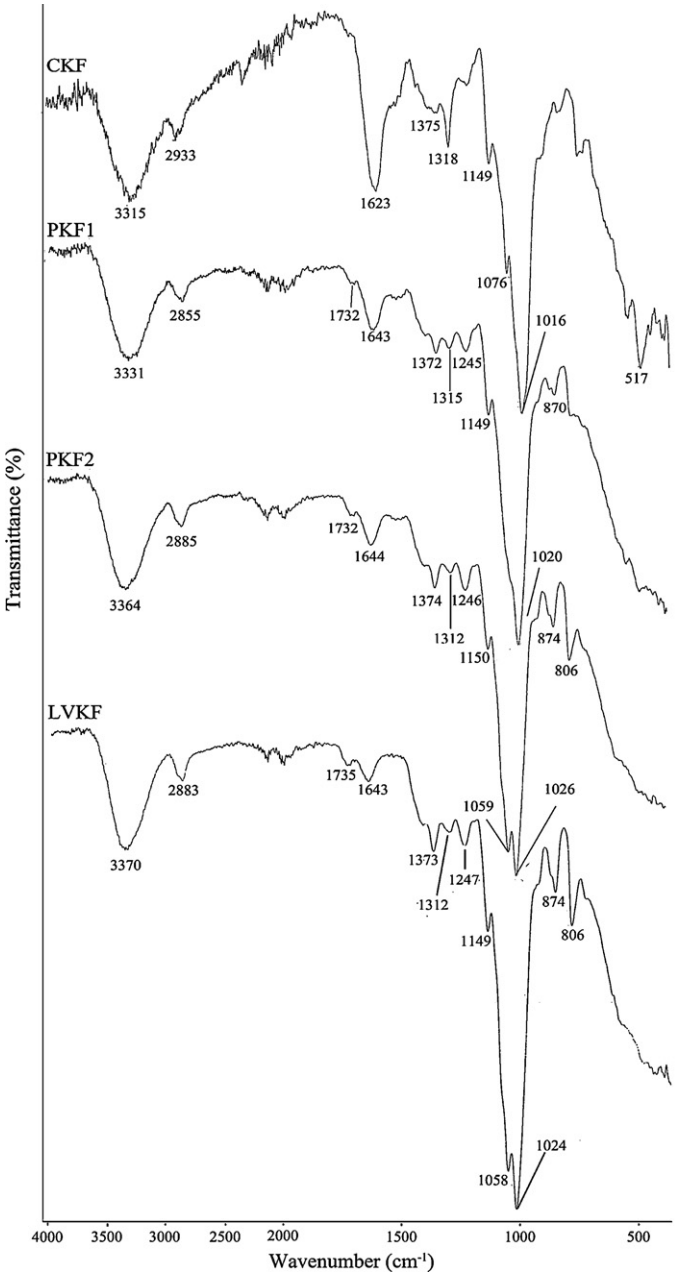

Fig. 4. FTIR spectra of CKF, PKF1, PKF2 and LVKF samples.

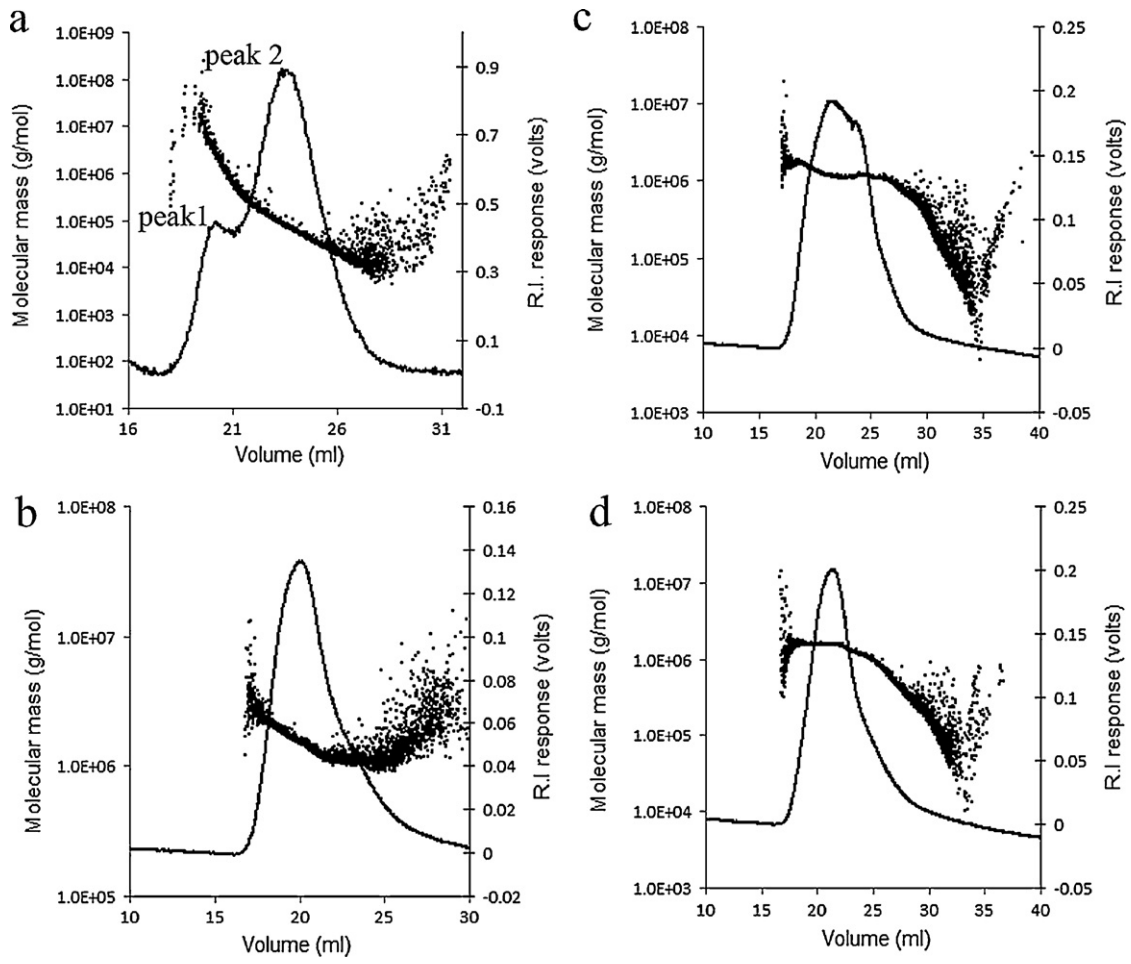

Fig. 5. Molecular mass and RI elution profiles of (a) LVKF, (b) NKF, (c) PKF1 and (d) PKF2 generated by GPC-MALLS.

Table 2
Repeatability of the 3,5-DNS colorimetric assay at six NKF concentrations ($n = 4$).

NKF (mg/ml)	Minimum	Median	Maximum	Mean	SD	RSD (%)
0.5	0.006	0.007	0.007	0.007	0.001	8.9
1.0	0.013	0.015	0.016	0.015	0.002	10.2
2.5	0.056	0.069	0.071	0.065	0.006	9.9
5.0	0.162	0.165	0.172	0.166	0.004	2.6
10.0	0.364	0.367	0.368	0.366	0.002	0.6
12.5	0.420	0.428	0.438	0.429	0.008	1.8

Based upon the analysis as mentioned above, it was hence concluded that the 3,5-DNS colorimetric assay was the most reproducible and accurate among the three methods used and further validation studies were performed subsequently to investigate its accuracy and precision in more depth. A plot of mean absorbance against the NKF concentrations, as shown in Fig. 3, was linearly dependent on the concentration in the range from 0.5 to 12.5 mg/ml with a correlation coefficient of 0.997 and RSD ranging from 0.6 to 10.2% (Table 2). These data demonstrate a high precision of results obtained using the 3,5-DNS colorimetric assay. Moreover, the overall recoveries for glucomannan were found to be between 97 and 103% across the three spiking levels (250, 500 and 750 μg/g) of starch, with RSD ranged from 5.6 to 8.1%. From these data, it was confirmed that the 3,5-DNS colorimetric assay was the method of choice both in terms of reproducibility, accuracy and precision for the determination of the glucomannan content of KGM samples in the later stages of the study.

3.3. Glucomannan content of PKFs determined by the 3,5-DNS assay

According to the CMA, European Commission and the U.S. FCC, the acceptance limit of glucomannan content for konjac flour is between 70 and 75%, and for KGM is between 90 and 95% (Byrne, 2001; Liu et al., 2002; U.S. FCC, 2003). As shown in Table 3, the glucomannan content of CKF has increased from ~53% to ~91% in PKF1 and ~92% in PKF2, with no significant differences compared to the glucomannan content of both reference samples, LVKF and NKF. Hence, the glucomannan content of both PKFs fall within the acceptance limits for KGM, but are slightly lower compared to the PKF produced by the Sugiyama's method (Sugiyama et al., 1972), which was shown to be 95% as D-glucose.

3.4. Starch content

Starch, constituting 10–30% of the dry matter of fresh corm (Li, Xia, Wang, & Xie, 2005), is regarded as the common impurity of KGM samples. The starch content of both PKF1 and PKF2 (0.6% and 0.9% of the dry weight, respectively) fall within the European standards for KGM, *i.e.* <1% (Byrne, 2001).

3.5. Protein content

The protein content of both PKF1 and PKF2 (4.4% and 3.8% of the dry weight, respectively) fall within the U.S. acceptance limit (<8%) (U.S. FCC, 2003), but is slightly higher under the European standards

Table 3
The glucomannan content, starch content, protein content, molecular mass distribution[a], polydispersity index (PDI), zero shear viscosity (η_0)[b] and the degree of acetylation (DA) of five konjac flour samples.[c]

Sample	Glucomannan (%)	Starch (%)	Protein (%)	M_n (g mol^{-1})	M_w (g mol^{-1})	M_z (g mol^{-1})	PDI (M_w/M_n)	η_0 (Pa s)	DA (%)
CKF	52.7 ± 0.5[a]	35.7[a]	7.5	–	–	–	–	–	–
PKF1	90.3 ± 0.7[b]	0.6[b]	4.4	$4.4 \pm 1.1 \times 10^5$	$9.1 \pm 0.6 \times 10^5$[a]	$1.4 \pm 1.3 \times 10^6$	2.1[a]	702.6	2.4[a]
PKF2	91.4 ± 0.5[b]	0.9[b]	3.8	$8.2 \pm 0.8 \times 10^5$	$9.5 \pm 0.6 \times 10^5$[a]	$1.1 \pm 1.2 \times 10^6$	1.2[b]	815.6	2.8[a]
LVKF	92.9 ± 1.3[b]	0.8[b]	0.4	$1.3 \pm 1.8 \times 10^4$	$5.5 \pm 0.8 \times 10^4$[b]	$1.6 \pm 1.9 \times 10^4$	4.2[c]	–	1.8[a]
NKF	96.9 ± 1.3[b]	0.5[b]	0.4	$1.4 \pm 0.4 \times 10^6$	$1.4 \pm 0.4 \times 10^6$[a]	$1.5 \pm 0.9 \times 10^6$	1.0[b]	886.9	2.0[a]

[a] Mean ± percentage relative error; number-average (M_n), weight-average (M_w), z-average (M_z) molecular weights.
[b] Sample solution = 2% (w/w).
[c] Different letters following mean values within the same column indicate significant differences at the $P < 0.05$ level.

for KGM (<1.5%) (Byrne, 2001). The percentage reduction of protein content achieved after purification using method 1 (41.2%) and method 2 (49.7%), however, was comparable to those reported by Takigami (47–50%), who compared the composition of commercial konjac flour produced from the Japanese and Chinese konjac cultivars, before and after purification using the ethanol extraction method (Takigami, 2000). As the protein content of CKF in the current study (Table 3) is ~3 times higher than those reported by Takigami (2000), this indicates that an additional deproteinization procedure e.g. via Sevag method or alkaline protease (Wang et al., 2007), needs to be performed during the extraction and purification process if konjac cultivars with high protein content are used for the KGM production.

3.6. Degree of acetylation (DA)

The DA determined for the PKF1, PKF2, LVKF and NKF (Table 3) are similar to previously reported values: 1.6% (Huang, Takahashi, Kobayashi, Kawase, & Nishinari, 2002), 1.98% (Maekaji, 1978) and 3.7% (Dea et al., 1977), the observed differences may be due to the purity of sample used and differences in the origin and genotype of the starting material. The DA values for both PKF1 and PKF2 indicate that a significant proportion of acetyl-substituted residues, which are believed to confer solubility of KGM in aqueous solution (Gao & Nishinari, 2004; Huang et al., 2002; Maekaji, 1978), have not been removed from the polymer chain during the extraction and purification process.

3.7. FTIR analyses

The spectra obtained for PKF1 and PKF2 (Fig. 4) are in agreement with the reference sample (LVKF) and with those previously reported (Takigami, 2000; Xiao, Lu, & Zhang, 2001; Yu, Huang, Ying, & Xiao, 2007). For PKF1, PKF2 and LVKF, the broad peak at ~3300 cm^{-1} results from the stretching vibration of O–H groups. The peaks at ~2900 cm^{-1}, ~1370 cm^{-1} and ~1050 cm^{-1} are assigned to –CH_2– stretching vibration, and two C–H bending modes, respectively. The small peak at ~1730 cm^{-1} is due to C=O stretching vibration (Jacon, Rao, Cooley, & Walter, 1993; Maekaji, 1974; Zhang, Nishinari, Williams, Foster, & Norton, 2001). The peaks at ~1150 cm^{-1} and ~1030 cm^{-1} are usually cited as C–O–C stretching modes from ether groups in the pyranose rings. Peaks attributed to β-glucosidic and β-mannosidic linkages are observed at ~870 cm^{-1} and ~800 cm^{-1}.

The most striking difference between the PKFs and the CKF is the dramatic increase in the intensity of the whole IR spectrum as a result of the purification process. This indicates that the majority of common impurities are broadly IR inactive, suggesting that impurities such as starch and protein are present at low levels. Moreover, there is a significant reduction in the intensity of the band in the region 1623–1644 cm^{-1} which is consistent with a fall in protein content after purification. This band can be sensibly attributed to

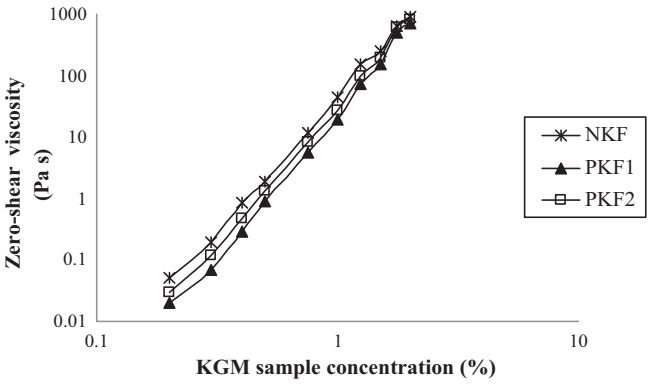

Fig. 6. Double logarithmic plot of the zero shear viscosity and the concentration of konjac flour sample solutions.

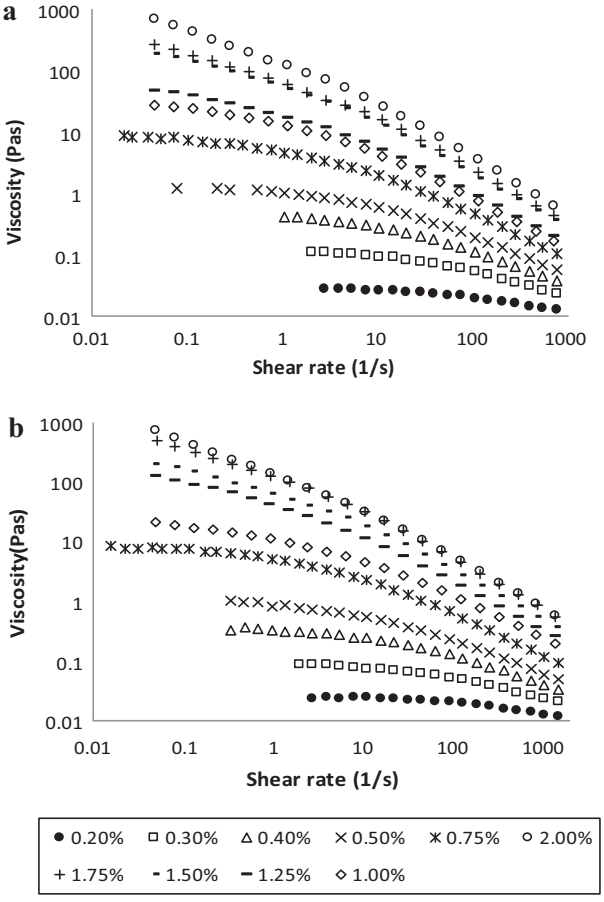

Fig. 7. Viscosity–shear rate profiles for different concentrations of konjac flour sample solutions at 25 °C. (a) PKF1, (b) PKF2.

the amide C=O stretch of protein backbone, with LVKF having the lowest intensity band and the lowest protein content.

3.8. Molecular mass distribution

Fig. 5 shows the molecular mass and RI elution profiles of the konjac flour samples generated from GPC-MALLS analysis. The M_w of both PKF1 ($9.1 \pm 0.6 \times 10^5$ g mol^{-1}) and PKF2 ($9.5 \pm 0.6 \times 10^5$ g mol^{-1}), as shown in Table 3, are in close agreement with those determined by Ratcliffe et al. (2005) (8.5×10^5 g mol^{-1}) for similar samples and show reasonable agreement with those reported by Kohyama, Iida, and Nishinari, (1993) (6.9×10^5 g mol^{-1}). The polydispersity index of PKF2 (1.2) is comparable to the reference sample, NKF (1.0) and to the values previously reported by Ratcliffe et al. (2005), ranging from 1.1 to 1.5. The higher polydispersity index recorded for PKF1 (2.1) was due to its low M_n value, suggesting that degradation of KGM polymer chains had occurred during the 6 h hydration treatment to which the CKF was subjected using purification method 1. In the case of LVKF, the differences observed in molecular weight parameters, including the lower M_w and wider polydispersity compared to other konjac flour samples were expected, and these differences were most probably due to the processing method(s) employed to degrade/hydrolyze the KGM polymer chains during the preparation the konjac flour sample.

3.9. Zero shear viscosity

A double logarithmic plot of sample concentration against the zero shear viscosity for NKF and both PKFs are shown in Fig. 6. Collectively, the zero shear viscosity increased linearly with increasing sample concentration, ranging from 0.2 to 2% (w/w). At 2%, the zero-shear viscosity of NKF, PKF1 and PKF2 is 886.9 Pa s, 702.6 Pa s and 815.2 Pa s, respectively (Table 3). The trend in the data presented in Fig. 7, essentially a shift in the onset of shear thinning to lower shear rates with increasing sample concentration, is consistent with the literature for KGM (Wientjes, Duits, Jongschaap, & Mellema, 2000) and other polysaccharides such as galactomannans, chitosan and gellan (Hwang & Shin, 2000; Miyoshi & Nishinari, 1999).

4. Conclusions

The data presented has demonstrated that purification of CKF using method 2 which involved an ethanol wash of CKF (90 min), a hydration treatment (3 h), followed by KGM precipitation and freeze-drying, not only produced PKF with a high glucomannan content (91.4 ± 1.3%) and physicochemical properties consistent with the literature, but also generated a higher yield of PKF (53%, w/w) than that reported elsewhere. It has also been shown that in comparison to two other widely used assays, the 3,5-DNS colorimetric assay is the most reliable and accurate method of choice with which to monitor the glucomannan content present in konjac-derived products. Given the increasing demand for KGM in the food industry and the promising data for the use of KGM in the treatment of various medical conditions e.g. obesity, diabetes, constipation, the methodologies outlined here represent the basis of an improved GLP for this product and as such may also assist in the establishment of KGM monographic standards, leading to an industrial production guidelines.

Acknowledgements

We would like to thank Prof. H.C. Guo (Yunnan Agricultural University, P.R. China) for providing some of the corm materials used in this study. We would also like to acknowledge the valuable support of Dr. Ian Ratcliffe (Glyndwr University) for the use of GPC-MALLS and rotational rheometers.

References

AOAC. (1999). *Official methods of analysis method 988.05*. Gaithersburg, MD: AOAC International.

Byrne, D. (2001). Commission directive 2001/30/EC of 2001, amending Directive 96/77/EC laying down specific purity criteria on food additives other than colours and sweeteners [online]. Brussels: European Commission. http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2001:146:0001:0023:EN:PDF Accessed 4.07.2011.

Chua, M., Baldwin, T. C., Hocking, T. J. & Chan, K. (2010). Traditional uses and potential health benefits of *Amorphophallus konjac* K. Koch ex N.E. Br. *Journal of Ethnopharmacology*, *128*, 268–278.

Cuesta, G., Suarez, N., Bessio, M. I., Ferreira, F. & Massalsi, H. (2003). Quantitative determination of pneumococcal capsular polysaccharide serotype 14 using a modification of phenol–sulphuric acid method. *Journal of Microbiological Methods*, *52*, 69–73.

Dea, I. C. M., Morris, E. R., Rees, D. A., Welsh, E. J., Barnes, H. A. & Price, J. (1977). Associations of like and unlike polysaccharides: Mechanism and specificity in galactomannans, interacting bacterial polysaccharides, and related systems. *Carbohydrate Research*, *57*, 249–272.

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.

Farhoosh, R. & Riazi, A. (2007). A compositional study on two current types of salep in Iran and their rheological properties as a function of concentration and temperature. *Food Hydrocolloids*, *21*, 660–666.

Gao, S. & Nishinari, K. (2004). Effect of degree of acetylation on gelation of konjac glucomannan. *Biomacromolecules*, *5*, 175–185.

Gong, X. M., Yan, R. W., Xu, H. Y., Li, C., Guang, S. Y. & Fang, M. (2002). Study of the extraction methods and properties of konjac glucomannan. *Fine Chemicals*, *19*, 486–488 (in Chinese)

Huang, L., Takahashi, R., Kobayashi, S., Kawase, T. & Nishinari, K. (2002). Gelation behaviour of native and acetylated konjac glucomannan. *Biomacromolecules*, *3*, 1296–1303.

Hwang, J. K. & Shin, H. H. (2000). Rheological properties of chitosan solutions. *Korea–Australia Rheology Journal*, *12*, 175–179.

Jacon, S. A., Rao, M. A., Cooley, H. J. & Walter, R. H. (1993). The isolation and characterization of a water extract of konjac flour gum. *Carbohydrate Polymers*, *20*, 35–41.

Keithley, J. & Swanson, B. (2005). Glucomannan and obesity: A critical review. *Alternative Therapies in Health and Medicine*, *11*, 30–34.

Kohyama, K., Iida, H. & Nishinari, K. (1993). A mixed system composed of different molecular weights konjac glucomannan and kappa-carrageenan: Large deformation and dynamic viscoelastic study. *Food Hydrocolloids*, *7*, 213–226.

Li, B., Xia, J., Wang, Y. & Xie, B. J. (2005). Grain-size effect on the structure and antiobesity activity of konjac flour. *Journal of Agricultural Food Chemistry*, *53*, 7404–7407.

Lindsay, H. (1973). A colorimetric estimation of reducing sugars in potatoes with 3,5-dinitrosalicylic acid. *Potato Research*, *16*, 176–179.

Liu, P. Y., Zhang, S. L., Zhu, G. H., Chen, Y., Ouyang, H. X., & Han, M. (2002). *Professional standard for the classification, requirements and test methods of konjac flour*; *Technical Report NY/T 494* [online]. Sichuan: Chinese Ministry of Agriculture. http://www.konjacfoods.com/pdf/NY494-cn.pdf Accessed 4.07.11.

Maeda, M., Shimahara, H. & Sugiyama, N. (1980). Detailed examination of the branched structure of konjac glucomannan. *Agricultural and Biological Chemistry*, *44*, 245–252.

Maekaji, K. (1974). The mechanism of gelation of konjac mannan. *Agricultural and Biological Chemistry*, *38*, 315–321.

Maekaji, K. (1978). Determination of acidic component of konjac mannan. *Agricultural and Biological Chemistry*, *42*, 177–178.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*, 426–428.

Miyoshi, E. & Nishinari, K. (1999). Rheological and thermal properties near the sol–gel transition of gellan gum aqueous solutions. *Progress in Colloid and Polymers Science*, *114*, 68–82.

Ogasawara, S., Yamazaki, H. & Nunomura, W. (1987). Electrophoresis on konjac mannan gel. *Seibutsu Butsuri Kagaku*, *31*, 155–158 (in Japanese)

Parry, J. (2010). Konjac glucomannan. In A. Imeson (Ed.), *Food stabilisers, thickeners and gelling agents* (pp. 198–215). Singapore: Blackwell Publishing Ltd.

Ratcliffe, I., Williams, P. A., Viebke, C. & Meadows, J. (2005). Physicochemical characterization of konjac glucomannan. *Biomacromolecules*, *6*, 1977–1986.

Saha, A. K. & Brewer, C. F. (1994). Determination of the concentrations of oligosaccharides, complex type carbohydrates, and glycoproteins using the phenol–sulfuric acid method. *Carbohydrate Research*, *254*, 157–167.

Shimahara, H., Suzuki, H., Sugiyama, N. & Nishizawa, K. (1975). Isolation and characterization of oligosaccharides from an enzymic hydrolysate of konjac glucomannan. *Agricultural and Biological Chemistry*, *39*, 293–299.

Sugiyama, N., Shimahara, H. & Andoh, T. (1972). Studies on mannan and related compounds. I. The purification of konjac mannan. *Bulletin of the Chemical Society of Japan*, *45*, 561–563.

Takigami, S. (2000). Konjac mannan. In G. O. Phillips, & P. A. Williams (Eds.), *Handbook of hydrocolloids* (pp. 413–424). FL: CRC Press.

Tekinsen, K. K. & Guner, A. (2010). Chemical composition and physicochemical properties of tubera salep produced from some *Orchidaceae* species. *Food Chemistry*, *121*, 468–471.

U.S. Food Chemicals Codex, & Food and Nutrition Board. (2003). (5th ed.). *Food chemicals codex* WA, U.S.: National Academy of Sciences., pp. 238–239.

Vasques, C. A. R., Rossetto, S., Halmenschlager, G., Linden, R., Heckler, E., Fernandez, M. S. P., et al. (2008). Evaluation of the pharmacotherapeutic efficacy of *Garcia cambogia* plus *Amorphophallus konjac* for the treatment of obesity. *Phytotheraphy Research*, *22*, 1135–1140.

Vuksan, V., Sievenpiper, J. L., Owen, R., Swilley, J. A., SpadaforaA, P., Jenkins, D. J. A., et al. (2000). Beneficial effects of viscous dietery fiber from konjac-mannan in subjects with the insulin resistance syndrome. *Diabetes Care*, *23*, 9–14.

Vuksan, V., Sievenpiper, J. L., Xu, Z., Wong, E. Y. Y., Jenkins, A. L., Zdravkovic, U. B., et al. (2001). Konjac-mannan and American ginsing: emerging alternative therapies for type 2 diabetes mellitus. *Journal of the American College of Nutrition*, *20*, 370S–380S.

Wang, X., Yuan, Y., Wang, K., Zhang, D., Yang, Z. & Xu, P. (2007). Deproteinization of gellan gum produced by *Sphingomonas paucimobilis* ATCC 31461. *Journal of Biotechnology*, *128*, 403–407.

Wientjes, R. H. W., Duits, M. H. G., Jongschaap, R. J. J. & Mellema, J. (2000). Linear rheology of guar gum solutions. *Macromolecules*, *33*, 9594–9605.

WIPO. (1993). *Clarified konjac glucomannan* [online]. Geneva, Switzerland: World Intellectual Property Organization. <http://www.sumobrain.com/patents/wipo/Clarified-konjac-glucomannan/WO1993003047A1.pdf> Accessed 11.07.11.

Wootton, A. J., Luker-Brown, M., Westcott, R. J. & Cheetham, P. S. J. (1993). The extraction of a glucomannan polysaccharide from konjac corms (Elephant Yam, *Amorphophallus rivierii*). *Journal of the Science of Food and Agriculture*, *61*, 429–433.

Wu, Y. J., Meng, W. N., Chai, J. T. & Wang, J. F. (2002). Extraction of glucommanan from *Amorphophallus konjac*. *Science and Technology of Food Industry*, *23*, 41–43 (in Chinese).

Xiao, C., Lu, Y. & Zhang, L. (2001). Preparation and physical properties of konjac glucomannan-polyacrylamide blend films. *Journal of Applied Polymer Science*, *81*, 882–888.

Yu, H., Huang, Y., Ying, H. & Xiao, C. (2007). Preparation and characterization of a quaternary ammonium derivative of konjac glucomannan. *Carbohydrate Polymers*, *69*, 29–40.

Zhang, H., Yoshimura, M., Nishinari, K., Williams, M. A. K., Foster, T. J. & Norton, I. T. (2001). Gelation behaviour of konjac glucomannan with different molecular weights. *Biopolymers*, *59*, 38–50.

Zhang, Y. Q., Xie, B. J. & Gan, X. (2005). Advance in the applications of konjac glucomannan and its derivatives. *Carbohydrate Polymers*, *60*, 27–31.