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Biochemical profiling of mucilage extracted from seeds of different citrus rootstocks

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ABSTRACT

Hetero-polysaccharide mucilage was extracted from the seed coats of different citrus rootstocks viz. Rough lemon, Sachtion citrumelo and Yuma citrange for investigating its biochemical and molecular properties. Investigations showed that the mucilage contained (mg/g) starch 3.13–5.04; maltose 3.23–4.31; glucosamine 0.017–0.289; D-xylose 0.059–0.107 and total soluble sugars 8.13–11.82. Specific enzyme activities were 16.98–35.96, 30.60–98.45, 42.00–73.98, 660.98–738.35 and 7.660–19.27 IU mg^{-1} of protein for protease, amylase, catalase, peroxidase and superoxide dismutase, respectively. Proximate analysis showed 12.85–13.94% moisture, 11.25–14.06% crude protein, 0.31–0.86% crude lipid, 1.31–2.69% crude fibre, 2.95–3.45% ash and 81.48–91.49 kJ 100 g^{-1} energy. The comparative characterization of the extractable proteins was profiled by SDS-PAGE and quantified using Bradford assay. Structural properties of samples were analyzed and compared using Fourier transformation infrared (FT-IR) spectroscopy.

1. Introduction

Mucilage is a thick, gluey substance produced by most plants and some microorganisms. It is a polar glycoprotein and an exopolysaccharide. It occurs in various parts of nearly all classes of plant, usually in relatively small percentages, and is frequently associated with other substances, such as tannins and alkaloids. Mucilage in plants is thought to aid in water storage and seed germination, and to act as a membrane thickener and food reserve. Among the richest sources are cacti (and other succulents) and flax seeds. Mucilage has a unique purpose in some carnivorous plants. The plant genera *Drosera* (Sundews), *Pinguicula*, and others have leaves studded with mucilage-secreting glands, and use a "flypaper trap" to capture insects. Exopolysaccharides are the most stabilising factor for microaggregates and are widely distributed in soils. Therefore, exopolysaccharide-producing "soil algae" play a vital role in the ecology of the world's soils. The substance covers the outside of, for example, unicellular or filamentous green algae and cyanobacteria. Amongst the green algae especially, the group Volvocales are known to produce exopolysaccharides in a certain part of their life cycle (Silverman, 1992; Schlegel, 1985).

Mucilages are soluble hydrophilic polysaccharides and complex polymers of carbohydrate nature with branched structures and occur in non-storage form (Copeland & McDonald, 2005; Matsuhiro, Lillo, Saenz, Urzua, & Zarate, 2006). Mucilages consist of polyuronides and galacturonides that chemically resemble the pectic compounds (Western, Skinner, & Haughn, 2000) and hemicelluloses that are produced during development of various tissue types, common throughout the plant kingdom (Del-Valle, Hernandez-Munoz, Guarda, & Galotto, 2005). Physically, they are similar to the gums found in the bark and stem of many plants.

The "cisternae" of Golgi apparatus are believed to be involved in the synthesis of pectin and secretion of mucilages. Pectins are major component of primary cell walls and seed mucilages. Five stages are involved in the mucilage secretory cell development where different sets of genes play role in the development of seed coat (Western et al., 2001). The mucilages are present in dehydrated form within the epidermal cell. In hydrated state, the mucilage envelops the whole seed and forms a pectin hydrogel (Zwieniecki, Melcher, & Holbrook, 2001).

High concentration of hydroxyl groups in the polysaccharide has high water binding capacity. The complex polysaccharide is a part of dietary fibre which can absorb a large amount of water. It can dissolve and disperse, and can form viscous or gelatinous colloids (Dominguez-Lopez, 1995), and can also play an important role in the drought resistance of certain plant species (Nobel, Cavelier, & Andrade, 1992). Like the seed coat itself, the mucilage is believed

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to be important in many respects. It has been reported in the literature that mucilage plays role in seed germination and dispersal (Penfield, Meissner, Shoue, Carpita, & Bevan, 2001). Mucilage is used for fruit coatings to increase shelf life and has a vital role in food and cosmetics (Del-Valle et al., 2005); to purify drinking water (Saenz, Sepulveda, & Matsuhiro, 2004), for improving Chinese noodles (Mishra, Yadav, Agarwal, & Bajpai, 2004). It can affect both the metabolic and genetic structure of the bacterial community (Benizri, Nguyen, Piutti, Slezack-Deschaumes, & Philippot, 2007). It is also being used in house paint and tannery effluent for solid removal and has a role in textile and pharmaceutical industry (Charles et al., 2007). Moreover, mucilages are, being used for their binding, thickening, stabilizing and humidifying properties in medicine (Anroop, Ghosh, Parcha, & Vasanti, 2006). Several techniques are available for the elucidation of mucilage structure. In this study SDS-PAGE and FT-IR techniques have been used to explore the significant biochemical profiling of mucilage in citrus rootstocks seeds. The outcome of the results is being reported and discussed.

2. Materials and methods

2.1. Plants sampling

Healthy and mature fruits were harvested from three rootstock varieties; Rough lemon (Citrus jambheri Lush), Sachtion citrumelo (Citrus paradisi × Poncirus trifoliate) and Yuma citrange (Poncirus trifoliate × Citrus sinensis), planted in experimental fruit orchard square # 9, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. The fruits were stored at 4 °C ± 1 and seeds were extracted as per analysis requirement.

2.2. Chemicals and reagent

The chemicals used in the experiment were of analytical grade and were procured from the Sigma–Aldrich, Fluka, Merck, etc.

2.3. Extraction of mucilage

Mucilage was extracted in potassium phosphate buffer (pH 7.0) following the method reported by Charles et al. (2007). Testa of seeds was collected from three citrus rootstock cultivar i.e. Rough lemon, Sachtion citrumelo and Yuma citrange carefully by forceps and dried at ambient temperature. Dried testa of each cultivar were ground with adding double distilled water and the samples were centrifuged (Centrifuge U 32R, Hettich, Germany) at 10,000 × *g* at 4 °C for 5 min. The supernatants were collected. The extraction was repeated and a second supernatant was obtained. The two supernatants were combined and were stored at 4 °C in 100 mL sterilized bottles (Szentmihályi, Vinkler, Lakatos, Illés, & Then, 2002).

2.4. Carbohydrate analysis

Starch and total soluble sugars were determined by rapid and convenient anthrone reagent method as described by Thimmaiah (2004) and the concentrations of starch and total soluble sugars were determined with the help of starch and glucose standard curves respectively. The concentration of maltose was determined by the 3,5-dinitrosalicylic acid (DNS) method (Jayaraman, 1981) using maltose as standard. The amount of D-xylose was quantified following the method reported by Monsigny, Petit, and Roche (1988). The glucosamine was measured by the procedure as described by Thimmaiah (2004).

2.5. Proximate analysis

Seed moisture contents were determined following the standard method (AACC, 1980). The crude protein contents were calculated by converting the nitrogen content through micro-Kjeldahl method ($N \times 6.25$) (AOAC, 1984). Ash contents were determined by dry-ashing in a furnace at 525 °C for 24 h (ISO, 1977). Crude fibre (ISO, 1981) and crude lipids were also determined (AOAC, 1975). The energy contents were determined by multiplying the percentage of crude protein, crude lipid and carbohydrate by the factors 16.7, 37.7 and 16.7, respectively (Siddhuraju, Vijayakumri, & Janardhanan, 1992; Bais, Singh, & Sing, 1970).

2.6. Polyacrylamide gel electrophoresis of extractable protein from mucilage

Proteins were extracted using potassium phosphate buffer (100 mM, pH 7.7). The comparative pattern of the extractable proteins was performed by SDS-PAGE using Mini Protein system of BioRad (England) and prestained protein ladder of Fermentas (10–160 kDa) as described by Laemmli (1970). The total protein contents were determined spectrophotometrically (Spectrophotometer T 60 U, PG Instrument Ltd, UK) at 595 nm using Bradford reagent of (BioRad) bovine serum albumin (BSA) as standard (Bradford, 1976).

2.7. Enzyme analysis of seed mucilage of citrus rootstocks

The specific activity of α-amylase was determined by the modified DNS method reported by Varavinit, Chaokasem, and Shobsngob (2002). The specific activity of protease was calculated by casein (Hammarsten) digestion assay described by Drapeau (1974). Specific activities of catalases (CAT) and peroxidase (POD) were determined following the method of Chance and Maehly (1955). The activity of superoxide dismutase (SOD) was determined by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) (Giannopolitis & Ries, 1977; Wu, Li, Wang, Zhou, & Mao, 2010).

2.8. Fourier transformation infra red (FT-IR) spectral analysis of mucilage

Fourier transform infrared spectra of the mucilage were recorded with FT-IR (Thermonicolete FT-IR, 2000, USA) spectrometer with Encompass software in the range of 4000/500 cm^{-1} (Charles, Huang, & Chang, 2008). FT-IR scans were collected on completely dried thin films cast on KBr discs from N,N′-dimethylformamide (DMF) solution. The spectra covered the infrared region 4000–500 cm^{-1}, the number of scans per experiment was 100 and resolution was 6 cm^{-1} León-Martínez, Méndez-Lagunas, and Rodríguez-Ramírez (2010).

3. Results and discussions

Mucilaginous cells provide water-retaining barrier around the seeds when they burst during imbibitions. Such barriers may also restrict oxygen uptake, due to the presence of phenolic contents and other structural features in some seed coats that restrict exchange of gases between the embryo and environment. As seed starts to take up water, it releases sugars, organic acids, ions, amino acids, and proteins into the surrounding rapidly. These solutes might stimulate the growth of fungi and bacteria in the soil. Another reason may be the carbohydrates present in mucilages because it holds OH^{-1} group and it also contains glucosamine that is directly proportional to fungus growth. In other words, carbohydrates especially glucosamine, play a vital role to fulfill the nutritional

Table 1
Carbohydrate analysis of seed mucilages of different selected citrus rootstocks.

Rootstock	Starch (mg/g)[a]	Maltose (mg/g)[a]	Glucosamine (mg/g)[a]	D-Xylose (mg/g)[a]	TSS (mg/g)[a]
R. lemon[b]	3.13 ± 0.5	4.31 ± 0.5	0.017 ± 9.0[NS]	0.069 ± 5.6	11.82 ± 2.5
S. citrumelo[c]	4.19 ± 0.4	3.23 ± 0.5	0.289 ± 9.2[NS]	0.059 ± 4.0	8.13 ± 0.45
Y. citrange[d]	5.04 ± 1.4	3.61 ± 0.5	0.275 ± 9.4[NS]	0.107 ± 20.3	10.42 ± 1.3
Range	3.13–5.04	3.23–4.31	0.017–0.289	0.059–0.107	8.13–11.82
Mean	4.12	3.72	0.194	0.078	10.12

[NS] Non significant.
[a] Results are the averages of four determinations, ±, SD.
[b] R. lemon: (Rough lemon).
[c] S. citrumelo (Sachtion citrumelo).
[d] Y. citrange (Yuma citrange).

Table 2
Proximate composition and energy contents of seed mucilages of different selected citrus rootstocks.

Rootstock	Moisture (%)[a]	Crude protein (%)[a]	Crude lipid/fat (%)[a]	Crude fibre (%)[a]	Ash (%)[a]	Energy (kJ 100 g^{-1})[a]
R. lemon[b]	13.94 ± 0.99[NS]	11.38 ± 1.10[NS]	0.86 ± 0.06	1.31 ± 0.41	2.98 ± 0.25[NS]	81.48 ± 94.24[NS]
S. citrumelo[c]	12.85 ± 0.34[NS]	11.25 ± 0.59[NS]	0.60 ± 0.11	2.69 ± 0.24	3.45 ± 0.31[NS]	85.17 ± 89.72[NS]
Y. citrange[d]	13.31 ± 0.41[NS]	14.06 ± 0.86[NS]	0.31 ± 0.07	1.67 ± 0.21	2.95 ± 0.09[NS]	91.49 ± 124.45[NS]
Range	12.85–13.94	11.25–14.06	0.31–0.86	1.31–2.69	2.95–3.45	81.48–91.49
Mean	13.37	12.23	0.59	1.89	3.13	86.05

[NS] Non significant.
[a] Results are the averages of four determinations, ±, SD.
[b] R. lemon: (Rough lemon).
[c] S. citrumelo (Sachtion citrumelo).
[d] Y. citrange (Yuma citrange).

requirement of the pathogens. Previous reports confirmed that mucilage directly affects the fungal nourishment because more fungal growth was observed on the seed mucilage of different citrus rootstocks. Lilly and Barnett (1951) reported that carbohydrates and nitrogenous compounds are the major sources of energy and contribute to new protoplasm of the fungus.

3.1. Carbohydrates quantification

Yuma citrange proved to be the best having highest concentration (5.04 mg/g) of starch in the seed mucilage followed by Sachtion citrumelo (4.19 mg/g) and Rough lemon (3.13 mg/g) (Table 1). Rough lemon possessed highest concentration of maltose (4.31 mg/g) in the seed mucilage followed by Yuma citrange (3.61 mg/g), while the lowest concentration (3.23 mg/g) was noted in the seed mucilage of Sachtion citrumelo. Similarly, Rough lemon had the highest concentration of total soluble sugar in the seed mucilage. There were non-significance ($^{NS}p < 0.05$) differences in glucosamine contents of the three seed types. Yuma citrange was the best having highest concentration (0.107 mg/g) of D-xylose as compared to other two citrus rootstocks.

3.2. Proximate analysis

There was non-significant ($^{NS}p < 0.05$) difference among all the three citrus rootstocks for their moisture, ash, energy and crude protein contents. However in Rough lemon maximum amount (0.86%) of crude fat/lipids was noted, which is significantly ($^{*}p < 0.05$) different to the amount found in Yuma citrange, while, significant ($^{*}p < 0.05$) difference was observed between Sachtion citrumelo (2.96%) and other both the rootstocks (Rough lemon (1.31%), Yuma citrange (1.67%)) in case of crude fibre. All these result are reported in Table 2.

Seed chemical composition has been described as an important factor in maintaining the seed viability. Seed mucilage of *Prosopis flexuosa* DC showed moisture content of 12.19, 13.0 and 12.6%; ash contents 3.81, 3.72 and 2.21%; total proteins 10.9, 10.4 and 19.1%; crude fat/lipids 0.28, 0.37 and 0.0%; crude fibre from seed was 8.9% adopting different extraction procedures (Ibanez & Ferrero, 2003). Results are similar to that of proximate analysis of citrus seed mucilage. However, inverse results were found in case of proximate composition of five genotypes of *Manihot esculenta*.

Table 3
Enzymatic activity of seed mucilage of citrus rootstocks viz. rough lemon, sachtion citrumelo and yuma citrange (IU/mg of protein) and total soluble protein (μg/mL).

Rootstock	Protease (μg/mL)[a]	Amylase (μg/mL)[a]	Catalase (μg/mL)[a]	Peroxidase (μg/mL)[a]	SOD[b] (μg/mL)[a]	Protein (μg/mL)[a]
R. lemon[c]	16.98 ± 0.50	50.76 ± 17.92	73.98 ± 8.34	662.86 ± 62.26[NS]	19.27 ± 0.79	0.69 ± 0.07[NS]
S. citrumelo[d]	17.94 ± 1.30	98.45 ± 30.14	52.19 ± 2.41	660.98 ± 24.69[NS]	7.660 ± 0.56	0.66 ± 0.07[NS]
Y. citrange[e]	35.96 ± 8.61	30.60 ± 14.00	42.00 ± 3.40	738.35 ± 42.55[NS]	11.58 ± 1.11	0.73 ± 0.05[NS]
Range	16.98–35.96	30.60–98.45	42.00–73.98	660.98–738.35	7.660–19.27	0.66–0.73
Mean	23.63	59.94	56.06	687.40	12.84	0.69

[NS] Non significant.
[a] Results are the averages of four determinations, ±, SD.
[b] superoxide dismutase.
[c] R. lemon: (Rough lemon).
[d] S. citrumelo (Sachtion citrumelo).
[e] Y. citrange (Yuma citrange).

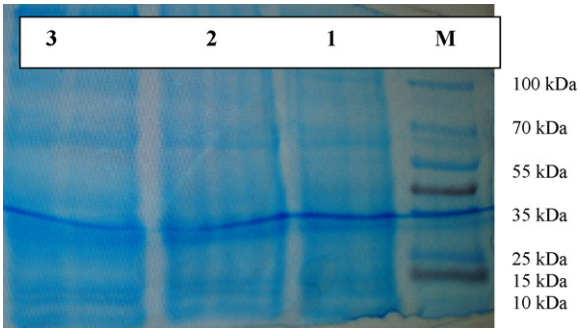

Fig. 1. Protein pattern of extracted protein from seeds mucilage of three citrus rootstocks in the optimized buffer after SDS-PAGE (12.5%). Lane M is the prestained protein marker; lanes 1, 2 and 3 are Rough lemon, Sachtion citrumelo, Yuma citrange, respectively.

3.3. Enzymatic activity

The analysis of variance of the data on enzyme showed significant ($^{*}p < 0.05$) differences in enzymatic activities on the studied three rootstocks. Sachtion citrumelo seed mucilage exhibited the highest α-amylase specific activity (98.45 IU/mg) of proteins. Similarly, highest activity of protease was noted in the seed mucilage of Yuma citrange. Non-significant difference was observed in all the three samples having peroxidase activities of 662.86, 660.98, 738.35 IU/mg of protein in Rough lemon, Sachtion citrumelo and Yuma citrange respectively. Rough lemon seed mucilage showed highest activity (19.27 IU/mg of protein) of superoxide dismutase.

Protein contents of seed mucilage of different citrus rootstocks are presented in Table 3. Highest protein contents were shown in the seed mucilage of Yuma citrange (0.73 μg/mL) followed by Rough lemon, (0.69 μg/mL) and Sachtion citrumelo (0.66 μg/mL).

Table 4
Frequencies of the FT-IR bands for seed mucilage of citrus rootstocks viz. rough lemon, sachtion citrumelo and yuma citrange.

Frequency (cm^{-1})	Assignment	Comment
684, 529	NH (amide)	Out of plane bending of NH (amide)
1035	COC	Stretching of ester associated with amide
1418	CH	
1640, 1637	Amide I: ν (C=O)	Amide (I) associated with carbonyl
2150, 2070		
2926	ν (CH)	Associated with CH_2
3335, 3330	ν (N–H)	Stretching with H-bonds
3510, 3495	ν (OH)	Stretching associated with N–H

3.4. Characterization of citrus seed mucilage proteins by SDS-gel electrophoresis

The protein patterns of seed mucilage from Rough lemon, Sachtion citrumelo and Yuma citrange are shown in lanes 1, 2, and 3, respectively in Fig. 1. In lanes 1–3, the position of the protein bands against the standard used in lane M suggests that the molecular weights ranged for 25 to 40 kDa. Visual analysis of the SDS-PAGE reveals that protein concentration is higher in lane 3 (Yuma citrange) followed by lane 2 (Sachtion citrumelo) and lane 1 (Rough lemon).

Proteases play a number of important roles in plants during germination they mobilize the stored protein. Seed storage protein may be hydrophilic in nature and it help to conserve water but lipids/fat may cause restriction between seed protein and water relation. Citrus seed mucilage has a sufficient amount of fat and this may also contribute in deteriorating seeds rapidly has already been reported that unsaturated fatty acid may cause loss of viability (Khan, Alam, Abbas, & Iqbal, 2003). In higher plants SOD is the first line of defence against oxygen radical mediated toxicity and is distributed in many cellular compartments. It is evident from the data that SOD activity in rough lemon is higher; it may help the seed to combat against lipid peroxidation.

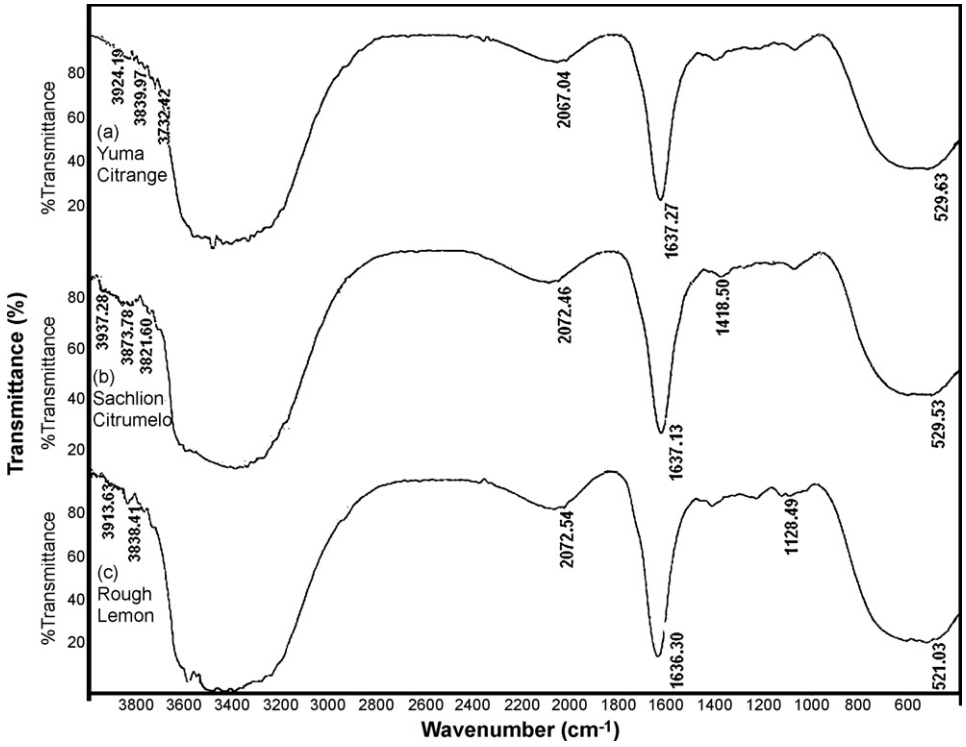

Fig. 2. Comparison of FT-IR spectra of (a) Yuma citrange, (b) Sachtion citrumelo and (c) Rough lemon.

To our knowledge, no report has been found from literature on SDS-PAGE for the mucilage of citrus species but the root mucilage of some other plant cassava (*Manihot esculenta* Crantz L.) showed that molecular weight of protein is ranged from 15 to 20 kDa (Charles et al., 2008).

3.5. Fourier transformation infrared (FT-IR) spectroscopy

FT-IR spectra of the seed mucilage of Yuma citrange, Rough lemon and Sachtion citrumelo are shown in Fig. 2 and the data is presented in Table 4. FT-IR spectra of the seed mucilage of Yuma citrange showed a broad OH stretching associated with N–H appeared at 3495 cm^{-1}. The NH stretching with H-bonds was observed at 3330 cm^{-1}. A short broad peak observed at 2067 cm^{-1} corresponds to diimide. The sample exhibited the characteristics IR absorption of polysaccharides at 1637 cm^{-1}, which specifically correspond to amide associated with carbonyl (C=O). The strong absorbance of thin peak indicated protein content in the samples. The spectra showed a broad peak ranging at 675–529 cm^{-1} assigned to out of plane bending of NH amide. FT-IR spectrum of the seed mucilage of Rough Lemon showed a characteristic band of OH stretching associated with N–H at 3510 cm^{-1} and N–H stretching with H-bonds was observed at 3335 cm^{-1}. The other peaks observed in the FT-IR spectrum of the sample were assigned as 2072 cm^{-1}, diimides; 1636 cm^{-1}, amide 1 associated with carbonyl (C=O); 1418 cm^{-1}, CH; 1250 cm^{-1}, C–O stretching band of complex polysaccharides; 1128 cm^{-1}, stretching of the C–O–C group of polysaccharides; 1030 cm^{-1}, stretching of CO and 715–522 cm^{-1}, out of plane bending of NH amide. Similarly, seed mucilage of Sachtion citrumelo exhibits characteristic bands of OH stretching at 3500 cm^{-1}, which support the structure of the studied sample. The peak related to N–H stretching was observed at 3348 cm^{-1}. In comparison with other two samples, a new peak located at 2926 cm^{-1} was assigned to CH associated with CH_2 group. The other peaks observed in this sample were assigned as: 1636 cm^{-1}, amide associated with carbonyl (C=O); 1409 cm^{-1}, C–H bending of alkane and 614 cm^{-1}, out of bending of NH amide. It was observed that the region ranging for 740–560 cm^{-1} was very broad clearly indicating that it was out of the plane of symmetry. The spectra of seed mucilage of all the three rootstocks were almost similar but in comparison with other sample, a new peak located at 2926 cm^{-1} was observed in Sachtion citrumelo, assigned to CH associated with CH_2 group. A very short peak in mucilage of Yuma citrange at 1540 cm^{-1} corresponds to stretching of –CN and bending of –NH also provides evidence about presence of protein contents in the sample (Charles et al., 2008). A small sharp peak observed at 1105 cm^{-1} attributed to C–O and ring vibration shows difference in α- and β-glucan spectra (Sandula, Kogan, Kacurakova, & Machova, 1999).

4. Conclusion

Seed coats of different citrus rootstocks viz. Rough lemon, Sachtion citrumelo and Yuma citrange were used fro the extraction of hetero-polysaccharide mucilage. The comparative characterization of the extractable proteins was profiled by SDS-PAGE and quantified using Bradford assay. Structural properties using Fourier transformation infrared (FT-IR) spectroscopy of the studied samples also supported the molecular behavior of the samples. The results showed that the mucilage contained starch 3.13–5.04; maltose 3.23–4.31; glucosamine 0.017–0.289; D-xylose 0.059–0.107 and total soluble sugars 8.13–11.82 mg/g. Specific enzyme and proximate analysis activities were also determined and reported. This study has unveiled the chemistry of citrus seed mucilage, which represents the variability in carbohydrates, crude fat, crude fibre, protease and superoxide dismutase (SOD) among all the citrus rootstocks species viz. Rough lemon, Sachtion citrumelo and Yuma citrange. This research will help the researchers to explore the agro-industrial potential of mucilage.

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