

Green Extraction Techniques, Structural Analysis and Antioxidant Activities of β -Glucan Present in Oats



Engineering

KEYWORDS :

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ABSTRACT

High fiber products are generally considered to be healthy foods and food ingredients. Foods having water insoluble fiber are known to improve regularity and bulk formation. The extractions of active, high molecular, highly stable biologically active fibres in better yields from oats are the aims of our study. The study established the efficiency of different green (enzymatic) extraction methods employed in the isolation of soluble fibres (glucans) from oats. The studies showed that green extraction techniques are ideal for the separation of β -glucan of higher molecular masses with high yield and with better colloidal stability. Enzymatic extraction process appeared more efficient with least amount of protein in β -glucan extract. Yield of β -glucan on enzymatic extraction is found to be 13.9% whereas acidic and alkaline extraction has only 6.97 and 5% respectively. The activity of the β -glucan isolated by different extraction methods were studied by measuring the radical scavenging effects. β -glucan extracted by amylase at 0.2% concentration exhibits strong antioxidant activity (IC₅₀ 17.16 μ g/mL) by scavenging DPPH radicals. But β -glucan extracted by protease and lipase has low antioxidant activity (IC₅₀ 41.44 and 67.7 μ g/mL). The study has established that the molecular weight of β -glucan extracted by protease is high and is of 41.2 kDa and that by amylase and lipase is low. The zeta potential measurements showed that lipase and protease treatment of oats have positive effect on stability of β -glucan isolated. It is seen that the pH of the extracted β -glucan has profound effect on the zeta potential as well as the stability of the glucan. The particle size analysis showed that β -glucan extracted by enzymatic methods have 100 nm-1 μ m. Proton NMR was applied to estimate β -1 \rightarrow 3 and β -1 \rightarrow 6 linkage ratio of the β -glucans respectively. The SEM and TEM studies showed that the process of extraction of β -glucan from oats has profound effect on its shape and size.

INTRODUCTION

High fiber products are generally considered to be healthy foods and food ingredients. Foods having water insoluble fiber are known to improve regularity and bulk formation. Water-soluble fiber content in natural and processed foods have been linked to such beneficial effects as cholesterol reduction, blood sugar regulation in diabetics and prevention of colon cancer. Yet it is widely recognized that soluble fiber is lacking in the diet of most populations, which may be due to the taste, availability and difficulty in obtaining high fiber sources.

Accordingly there have been significant research and developments in food industry to create high fiber, multifunctional food additives, supplements, ingredients for use in the manufacture of processed foods. Commercial products enriched in physiologically functional ingredients are now available and are sometimes referred to as functional foods, (Sirtori *et al.*, 2009). Oats is an interesting food candidate, as it can lower the blood cholesterol level, as well as blood glucose (Wood *et al.*, 2007).

Although there are several studies on the molecular weight determinations, viscosity measurements, antioxidant properties of beta glucan present in oats, no systematic study is reported on the green extraction of beta-glucan from oats. This is the first report on the green techniques for the better extraction of high molecular weight β -glucan with considerable stability (Zeta Potential) from oats. Our studies paved way for the better understanding of morphological and structural features of enzyme extracted β -glucan on its activity. In vitro free radical scavenging effects of the enzyme extracted β -glucan were studied to analyze the effect of molecular weight, zeta potential and morphological features. The aim of the study is to extract high molecular weight (more active) β -glucans from oats with high yield by green extraction techniques for the production of value added functional foods. And also to correlate the activity of β -D glucans with its molecular weight, structure and morphological features.

MATERIALS AND METHODS

Chemicals: Gallic acid, Sodium carbonate (Fischer, India), 2,2-diphenyl 1-2-picryl hydrazyl (DPPH) (Sigma, Aldrich), hexane, ethyl acetate, Methanol (Merck), α -amylase (Sigma), protease enzyme (activity-1 μ mol/ml/mt), lipase enzyme (Sigma), activ-

ity-1.140 μ mol/ml/mt

Raw source

Oat grains were obtained from local market of Kerala. Grains were milled in high speed grinder and kept in paper bags at room temperature until further analysis of sample.

Preparation of Defatted oats

Oats were ground in a mixer to obtain a fine powder (110-120 mesh). The powder was defatted with hexane (1: 6 w/v) at room temperature for 16 hrs sequentially in a Soxhlet apparatus. The defatted powder was air dried for 18 hr and stored at 4 ° C for the later use.

Sample preparation

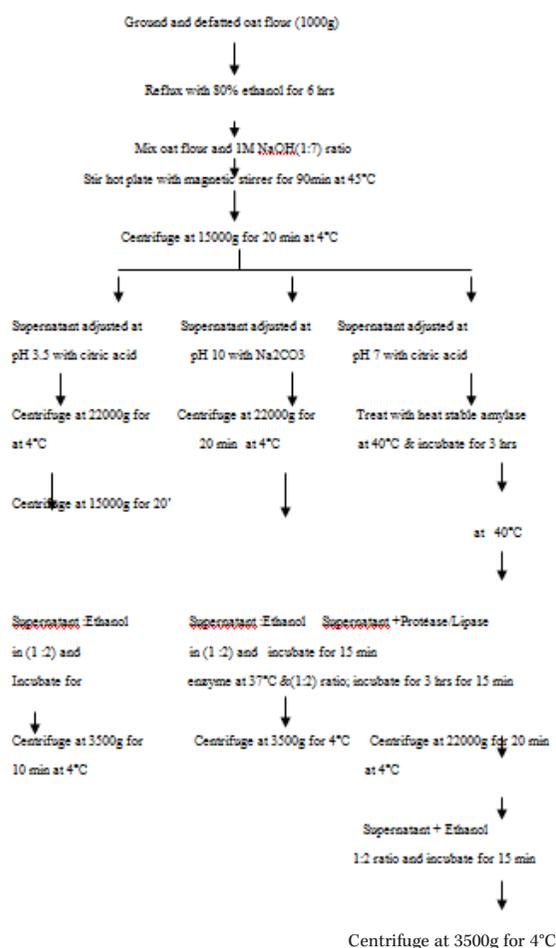
The defatted powder (1000 g) was blended with solvent ethanol. The solvent concentration, the extraction temperature, and the extraction times were set according to the requirement of the experiment. The extract was filtered using a sand core funnel then concentrated at 40°C using a rotary evaporator at 70 rpm. Light yellow syrup was obtained. The solution was cooled down to 15°C and then centrifuged with a high-speed centrifuge (Sigma, model 2-16PK) at 15000 rpm for 20 min to precipitate and remove water-soluble polysaccharides and proteins after filtering with a clean cloth and extracts were pooled and dried under vacuum for further removal of polysaccharides.

Extraction of oat β -glucan

Experiments have to be evaluated in small-laboratory scale and were intended to investigate the include ethanol extractions. The extracted oat brans were pooled by dry mixing extracted with amylase and protease, and centrifuged (8,000 g, 10 mts). After amylase and protease treatment, samples, together with the insoluble material were stored at -20°C before freeze-drying (-20°C for four days).

The samples of defatted whole oat flour was refluxed by 80% ethanol and were treated with 1M NaOH to inactivate the native enzymes. Further impurities were removed by treating the supernatant in three different ways. In the first method (M1) acidic condition was maintained using citric acid. In the second method (M2) impurities were removed in alkaline conditions by the use of Na₂CO₃ and in the 3rd method (M3) enzymes were used

for the extraction and purification of β -D-glucan. A schematic outline of the extraction procedure is presented in Fig. 1.



Molecular weight determination of extracted beta glucan by MALDI TOF Analysis

The molecular weight of chemically and enzymatically extracted samples were analysed by taking MALDI (Matrix assisted laser adsorption or desorption ionization technique), Shimadzu Biotech Axima CFR plus model no: 2.8.2.20080604. The extracted samples are treated with mixed bed cation and anion ion exchanger and then filtered through Millipore membrane (0.44 μ m). Mass spectrum of extracted samples were analysed by a mass spectrometer associated with a nitrogen laser (laser shot-20, laser energy-3.68eV) at 337nm in linear mode. The matrix (DHB: 2, 5-Dihydroxy Benzoic Acid). Molecular weights are obtained in terms of intensity against m/z ratios

For MALDI-TOF analysis, the samples were prepared by standard dried-droplet preparation on stainless steel MALDI targets using 2, 5-dihydroxybenzoic acid matrix. The non-derivatized and derivatized polysaccharides were mixed with 10 mM of 2, 5-DHB in the ratio of 1:1.

Zeta potential & Particle size measurements: Zeta potential represents the surface charge of particles. The Zetasizer NanoZS (Malvern, UK) was used to measure the zeta potential by using laser Doppler electrophoresis technique. The beta glucan solution was added into a disposable zeta potential cell and deionized water was used as a reference standard. The Particle size distribution defines the amount of particles present in the

solution. Malvern particle size analyzer was used to measure poly dispersity Index (PI) by using dynamic light scattering technique. Direct calculations from MALDI-TOF were also used for particle size determination. The poly dispersity index (PI) is a measure of the distribution broadness of the particle size, which was obtained by PCS analysis. Samples were diluted 10 times with distilled water before assessment.

Effects of Scanning electron microscopy: The surfaces of the chemically and enzymatically degraded natural glucan particles were observed by Scanning electron microscopy (SEM, JEOL JSM 5600 LV, Japan). The samples were filtered by using Millipore filter membrane (0.44 μ m). The samples were mounted on specimen stubs using electrically conducting adhesive carbon sheet (P/N, JEOL, Japan) and sputter-coated with gold by ionizing plasma. Imaging was performed with secondary electrons at an acceleration voltage of 10 kV.

Transmission Electron Microscopy studies of β -glucan particles

The surface morphology of the β -glucan particles isolated using various enzymatic methods were studied using TEM model no. FEI, Tecnai 30 G²S-TWIN microscope at an accelerating voltage of 100 kV. Samples were prepared by casting a drop produced under different conditions on to carbon coated copper grid and placed on a vacuum desiccator and the TEM pictures were obtained without staining.

NMR Spectroscopy studies of β -glucan particles: NMR spectra were recorded on Bruker Advance DPX_500 MHz FTNMR using deuterated water (D₂O) as solvent. Tetra methyl silane is used as internal standard and chemical shift is expressed in δ scale. Abbreviations used in ¹H NMR are s-singlet, d-doublet and m-multiplet, coupling constant J is reported in Hertz (Hz).

DPPH Radical Scavenging Activity: DPPH is a stable free radical that has been used to determine a compound's free radical scavenging activity. The DPPH scavenging effect was evaluated according to modified methods from Zou et al. Enzymatic extracted samples were prepared at different concentrations. The assay contained 1ml of 0.1mM DPPH in methanol and various concentrations of extract and standardized in methanol were made up to 3ml with DPPH. The contents were well mixed immediately and incubate for 20 minutes at room temperature. The degree of reduction of absorbance was recorded in UV visible spectrophotometer at 517 nm. (ShimadzuUV-2450). The percentage of scavenging activity was calculated as: % Scavenging activity = [AC-AS/AC] X100, Where AC is absorbance of control (without extract) and AS is absorbance of the sample. Percentage of radical scavenging activity was plotted against the corresponding concentration of extract to obtain IC₅₀ value. IC₅₀ is defined as the amount of the anti oxidant material required to scavenge 50% of the free radical in the assay system. IC₅₀ values are inversely proportional to antioxidant activity

RESULTS AND DISCUSSION

Evaluation of Extraction Method on Laboratory Scale

The laboratory-scale extraction method was performed in the steps typically used for extraction of β -glucans from cereals: 1) inactivation of endogenous enzymes, 2) extraction of β -glucans,

The yields of β -glucan at different extraction conditions were evaluated. Experiments include acidic extraction by boiling 0.192% citric acid (pH 3.5) at 100°C for 20 minutes. The alkaline extraction was carried out by boiling 0.04% NaOH, (pH 10) at 100°C for 20 minutes. Enzymatic extraction is carried for 3 hrs under ultrasonicator by inclusion of a protease and lipase after the combined hot-water extraction with amylase. The most important parameter is extractable β glucans. It is seen that the starting material have played an important role in the purity of

the product. High yield and high molecular weight of extractable β -glucans was more important in this study .

Table1: Yield of β -glucan on Hot and Cold Extractions

Extraction	Dry weight of sample(g)	Dry weight of sample after extraction (g)	Yield (%)
Cold extraction	500	290.57	23.2
Hot extraction	500	115.53	41.89
Acidic extraction on cold treatment	69.81	14.6	6.97
Acidic extraction on hot treatment	38.5	5.4	4.67
Alkaline extraction on cold treatment	69.81	10.4	5
Alkaline extraction on hot treatment	38.5	3.5	3.1
Enzymatic extraction (amylase+protease) on cold treatment	69.81	28.3	13.9
Enzymatic extraction (amylase+protease) on hot treatment	38.5	14.53	12

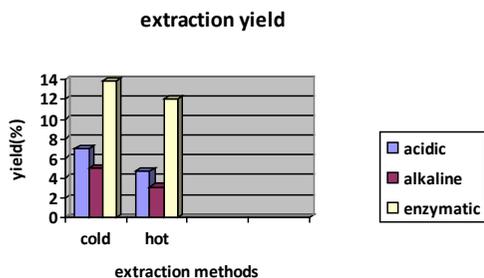


Table 1 reveals that cold extraction yield of 23.2% of starch whereas hot extraction yields 41.9%. Enzymatic extraction process appeared more efficient with least amount of protein in β -glucan extract. Yield of β -glucan on enzymatic extraction is found to be 13.9% on cold treatment and 12% on hot treatment. Green extraction gives poly-phenolic glycosides as well as polysaccharides. These glycosides help to deactivate natural enzymes which are responsible for slow deterioration, but cold extraction precedes enzyme deactivation. Enzymatic extraction process also removed more starch, fat and pentosans during the extraction of β -D-glucan gum. And hence the yield in the enzymatic extraction is lower in hot than in cold.

Table 1 also shows that alkaline extraction without mechanical agitation resulted in a lower yield of β -glucans .It was found that hot treatment yields only 3.1% of β -glucan. But the cold alkaline extraction yields 5 % of β -glucan. It is also seen that acidic extraction yields 4.67% under hot and 6.97 % under cold treatment respectively. The studies shows that the extraction pH has considerable effects on extraction kinetics. The extraction yield of β -glucans in all the experiments conducted is found to be between 3.1 and 13.9%. It is shown from the series of extraction studies that the enzymatic extraction on cold condition is an efficient technique for getting pure and active beta glucan from oats.

Table 2: Characterization of β -Glucan by MALDI-TOF

Extraction	Cold treatment	Hot treatment
Molecular weight of Beta-glucan by acidic extraction	2.73KDa-5.997 KDa	2.08KDa-5.334 KDa
Molecular weight of Beta-glucan by alkaline extraction	0.8880KDa-7.11KDa	0.146KDa--3.02 KDa
Molecular weight of Beta-glucan by enzymatic (amylase+protease) extraction	41.2KDa-127KDa	41.56KDa-124.8KDa

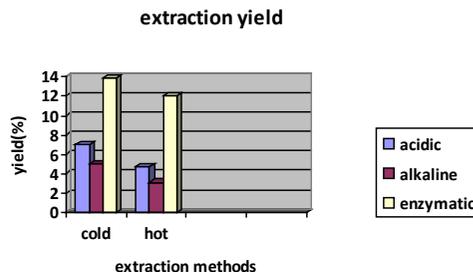


Table 2 reveals that cold and hot acidic extraction (fig 1a)with citric acid(0.192% , pH 3.5) yielded β -glucans of molecular mass 2.739 KDa and 2.08KDa.Under cold and hot alkaline extractions with Na_2CO_3 (pH 10) gave molecular masses of 880.18 Da and 146.76 Da. Maldi analysis has shown that the molecular weight of β -glucan extracted by alkaline conditions (fig I b) yielded low molecular weight than that in acidic extraction. Cold enzymatic extraction with amylase and subsequent treatment with protease gives high molecular weight of 41.18KDa . Hot enzymatic extraction gives molecular weight of 41.56KDa .The hot extraction of oats with enzymes yielded more of β -glucan with high molecular weights.

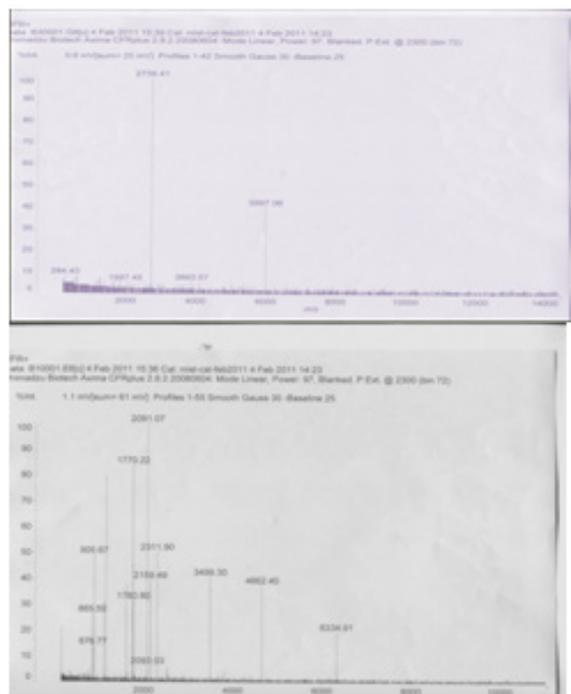
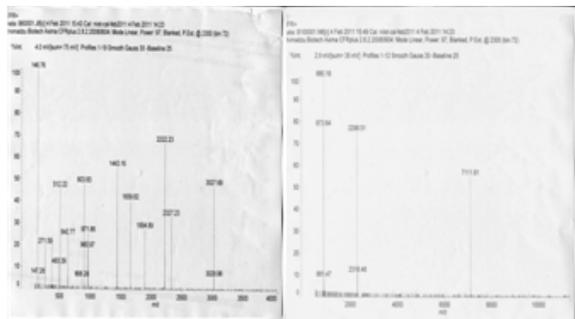


Fig 1a: MALDI-TOF Spectra of Acidic extraction under cold and hot treatments



0.15% amylase contains high molecular weight of 3.57KDa. The molecular weight data of 0.15M protease shows base peak at 3178.89Da. Protease extracted β -glucan shows zeta potential of +20mV around neutral pH and indicated incipient stability in solution. As pH of the β glucan extracted by lipase increased to 8, zeta potential increases to +45mV shows moderately stable and at pH 10 stability is increased and zeta potential of +53.2mV. It is established from the zeta potential analysis that the enzymatically (lipase) extracted β -glucans have better stability as pH increases.

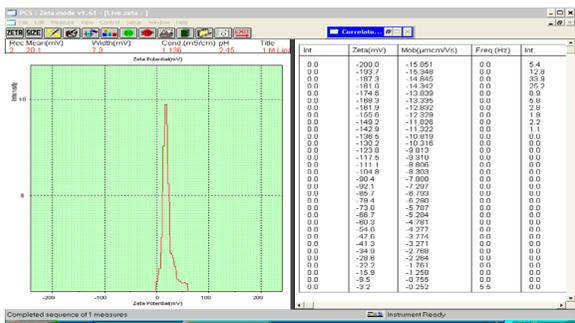


Fig 3a : Effect of Zeta potential on lipase extracted β -glucan

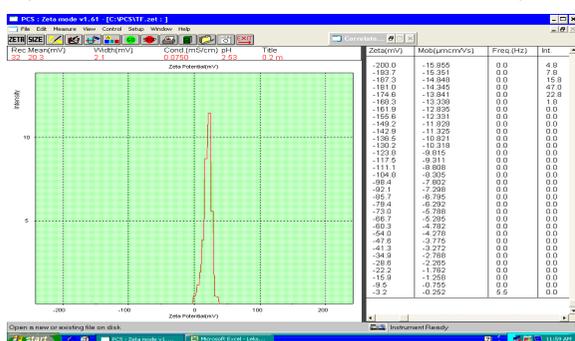


Fig 3b : Effect of Zeta potential on protease extracted β -glucan

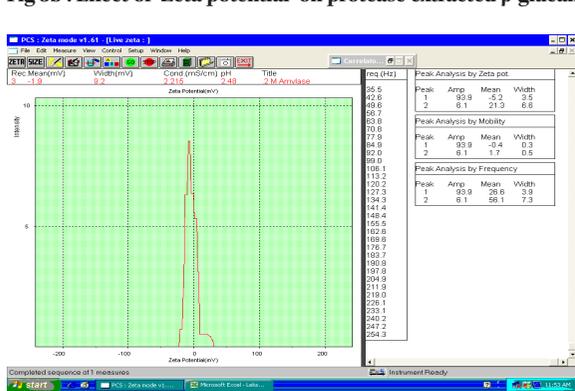


Fig 3c : Effect of Zeta potential on amylase extracted β -glucan

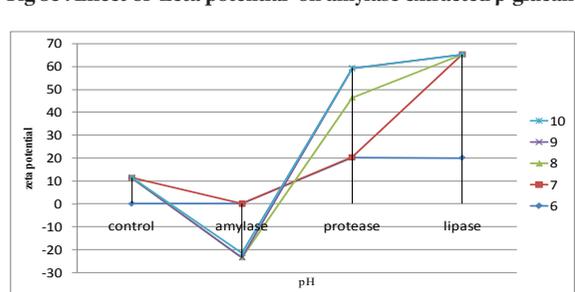
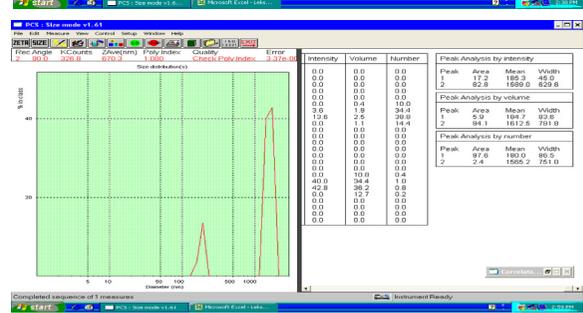
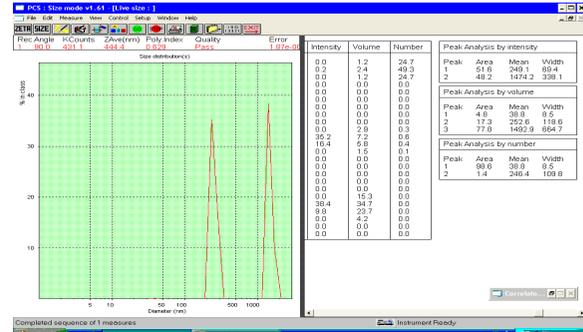


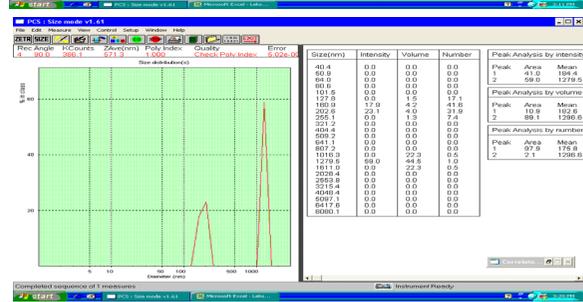
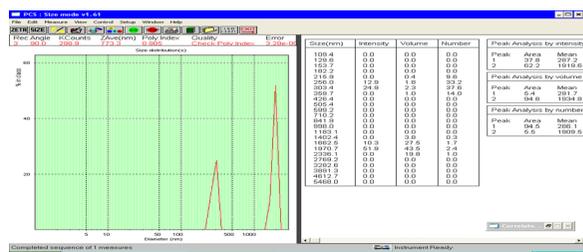
Fig showing effects of beta glucan extracted from different enzymes at different pH

Characterization of beta glucan particles

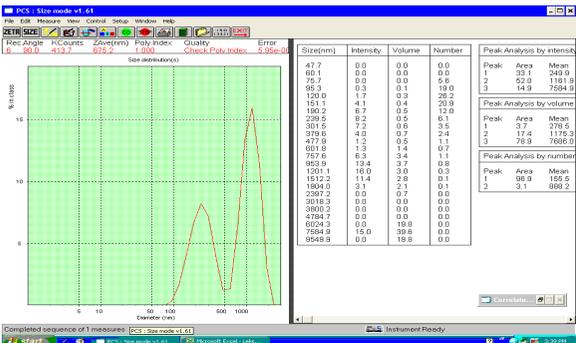
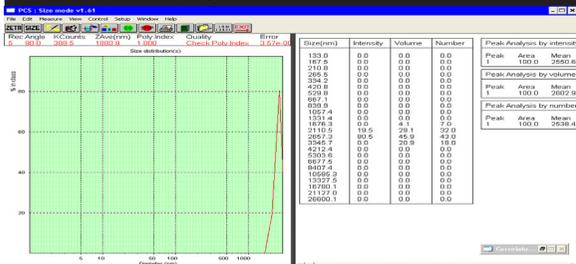
The particle diameters of β -glucan extracted by amylase at low concentration are 180 and 1565.2 nm and poly dispersity index (PI) value is found to be 1.00 and at high concentrations is 38.8 and 246.4 nm. The poly dispersity index (PI) value is found to be 0.629. The higher the PI value, the broader is the distribution of the particle sizes.



The particle diameters of β -glucan extracted by protease at low concentration are 175.8 and 1296.6 nm and poly dispersity index (PI) value is found to be 1.00 and at high concentrations are 286.1 and 1909.5 nm. PI is found to be 0.905



The particle diameters of β -glucan extracted by lipase at low concentration are 155.5 and 888.2 nm and poly dispersity index (PI) value is found to be 1.00 and at high concentrations are 2538.4 nm. PI is found to be 1.00.



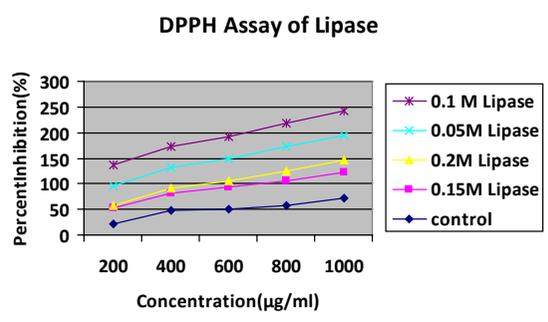
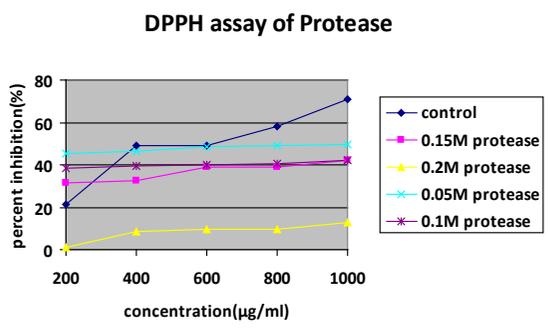
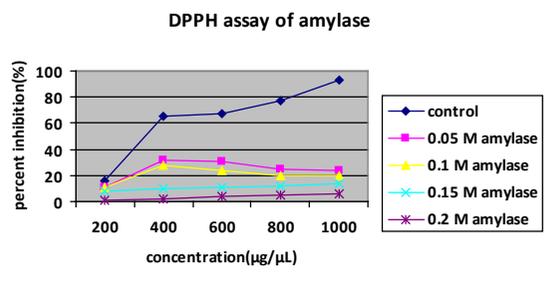
Effect on radical Scavenging activity of β-glucan isolated from oats under different concentrations of enzymes

Many natural foods contain active compounds that are antioxidants and have been used to prevent and/or help to cure diseases. In the present study, the antioxidant activities of enzymatically extracted β-glucan from oats were determined by DPPH free radical scavenging assay. The table 6 revealed that the concentrations of various enzymatic extracts of β glucan are required for the 50% inhibition of DPPH. It is seen that β glucan extracted by conventional method contains low scavenging activity of (IC₅₀) 248.516μg/μl. The addition of amylase at high concentration could achieve efficient free radical scavenging activity of (IC₅₀) 17.16μg/μl. High molarity protease extracted β -glucan should efficient free radical scavenging activity of 41.44 μg/μl .But low molarity protease extracted of β -glucan has IC₅₀ 184 μg/μl . β glucan extracted by Lipase at low concentration shows low scavenging activity of 208.8 μg/μl. On comparing β glucan extracted by amylase, protease and lipase it is seen that amylase at high concentration yields high scavenging activity. The mechanism of action of DPPH scavenging may take place rapidly with higher concentration of enzymatic extracts.

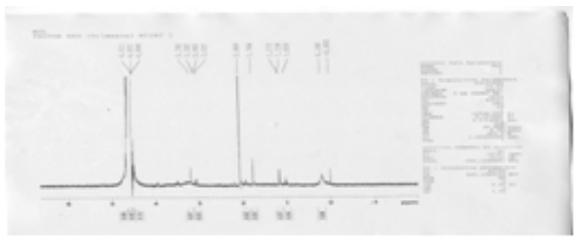
Effects of radical Scavenging effects of enzyme extracted β-glucan

Sl No	Name of the sample	Concentration (M)	IC ₅₀ values (μg/μL)
1	Gallic acid		248.5
2	β glucan extracted by Amylase	0.05	84.3
3	β glucan extracted by Amylase	0.1	61.6
4	β glucan extracted by Amylase	0.15	54.29
5	β glucan extracted by Amylase	0.2	17.16
6	β glucan extracted by Protease	0.05	195.8
7	β glucan extracted by Protease	0.1	187.5
8	β glucan extracted by Protease	0.15	184
9	β glucan extracted by Protease	0.2	41.44
10	β glucan extracted by Lipase	0.05	235.5
11	β glucan extracted by Lipase	0.1	211.2

12	β glucan extracted by Lipase	0.15	208.8
13	β glucan extracted by Lipase	0.2	67.7



NMR measurements for determination of structure of β glucan



¹H₁ NMR spectra of protease action of oats which contains β glucan were determined. ¹H₁ signals were 4.511, 4.48, 3.1 and 2.06 ppm were assigned to be H-1 of glucopyranose were attribute to the β 1→3 linkage, β 1→6 linkage, α-anomer of the reducing end and β anomer of reducing end respectively. Based on the respective peak areas at 4.48 & 4.51 ppm on ¹H₁ NMR spectrum, the ratio of β-1, 3 to β -1, 6 linkages is 1:12

Comparison in micro structure of beta glucan after Enzymatic Extraction: In order to analyze the reason for the higher yield obtained by enzymatic extraction, the surfaces of ultrasound assisted extraction from Beta glucan were imaged with a scanning electron microscope

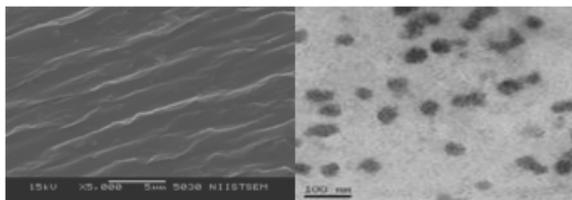
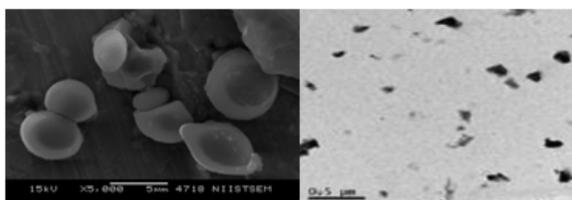


fig 5a SEM and TEM images of β -glucan extracted by conventional method

The SEM micrograph fig 5a shows the long fibrous nature of β glucan extracted by conventional method. It is easily seen from the micrographs at 5000 magnification the fibers of β glucan were broken down into smaller fibres by ultrasonication. The TEM micrograph shows that spherical globules were dispersed form, sizes range to 100nm.

The surface morphology of β glucan extracted by amylase at lower concentrations (fig5b) contains cobble like stone particles.

fig5b : SEM and TEM images of β -glucan extracted by amylase at 0.1M concentration



The particles were in clustered form and the size of particles is approximately 1 μ m. As the concentration of amylase increases morphological changes also occurs. The surface morphology of β glucan extracted by amylase at higher concentrations were flake like particles of clusters and isolated form, sizes ranging 1 to 10 μ m (fig 5c).

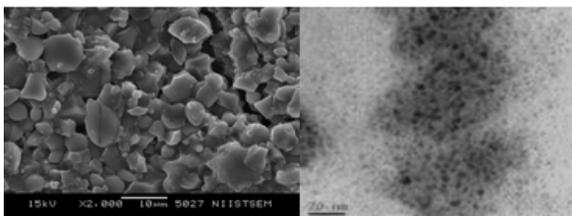


fig5c : SEM and TEM images of β -glucan extracted by amylase at 0.15M concentration

The SEM micrograph showed that the effect of concentration of protease on the morphology of the extracted β -glucan. The protease (0.05M) extracted β glucan of oats are needle shaped fibrous clusters which are dispersed in nature (fig 5d) The protease (0.15M) extracted β glucan of oats are fibrous in nature and entangled as networks (fig 5e). As concentration of protease (0.2M) increases size of the particles decreases, fibres are broken down and changes into self assembled network structure as shown in (fig 5f).

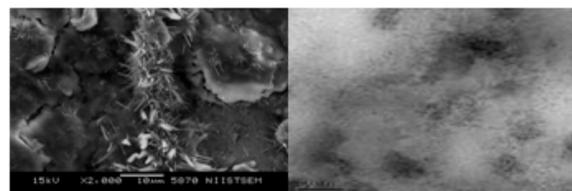


Fig 5d: SEM and TEM images of β -glucan extracted by protease at 0.05M concentration

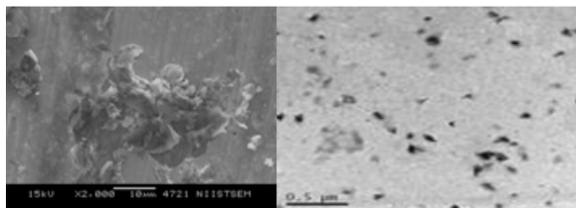


Fig 5e : SEM and TEM images of β -glucan extracted by protease at 0.15 M concentration

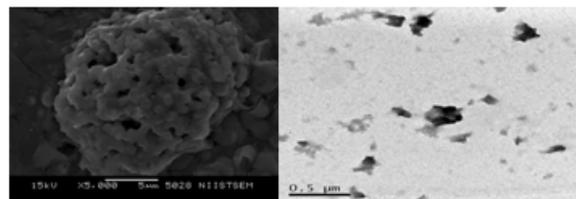


Fig 5f : SEM and TEM images of β -glucan extracted by protease at 0.2 M concentration

The SEM micrograph showed that the effect of concentration of lipase on the morphology of the extracted β -glucan. The lipase (0.05M) extracted β glucan of oats are spherical and flower shaped particles which are clustered in nature. The surface morphology of the β -glucan extracted by lipase at low concentrations (fig5h) contains spherical shaped lipid particles. The size of the particles is approximately 1 μ m. The surface morphology of β glucan extracted by lipase at higher concentrations (fig5i) were in the form of dispersed round fibres. The TEM micrograph showed that the effect of concentration of lipase on the morphology of the extracted β -glucan. The surface morphology of β glucan extracted by lipase at higher concentrations were in the form of dispersed particles ranging from 100-500nm sizes.

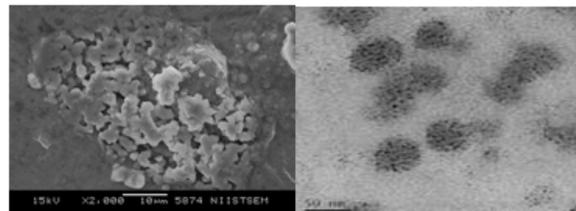


Fig 5g: SEM and TEM micrograph showing β -glucan extracted by Lipase at 0.05M concentration

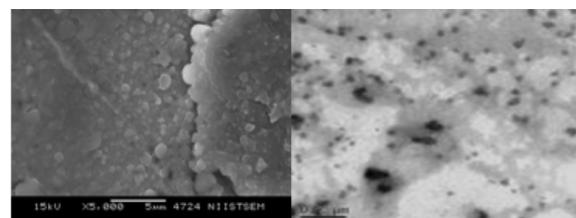


Fig 5h: SEM & TEM micrograph showing β -glucan extracted by Lipase at 0.15 M

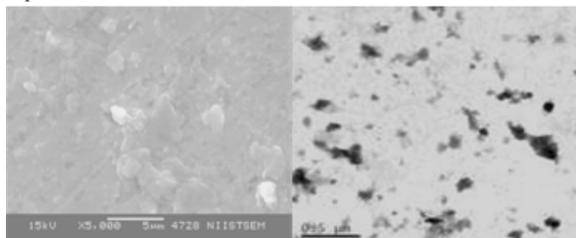


Fig 5i: SEM & TEM micrograph showing β -glucan extracted by Lipase at and 0.2 M concentration

Conclusions

The study established that green extractions techniques are better in comparison with chemical and mechanical extractions processes. Enzymatic extraction can increase yield and quality of β -glucan separated from oats. The quality of the β -glucan isolated by different extraction methods were studied by measuring the radical scavenging effects. The study gave effects of invitro antioxidant activity of β -glucan extracted by various enzymes. β -glucan extracted by amylase at high (0.2%) concentration exhibits strong antioxidant activity. (IC_{50} 17.16 μ g/mL) by scavenging DPPH radicals. It is seen that amylase have redox properties which allows them to act as reducing agents. The studies showed that green extraction technique are ideal for the separation of β -glucan of higher molar masses with high yield and with better colloidal stability. And also the stability of these particles increases with increase in pH. The study has established that the molecular weight of β glucan extracted by protease is high

and is of 41.2 KDa. Zeta potential measurements exhibited that β -glucan extracted by lipase has better stability (+53mV) maintained at alkaline pH. The studies showed that the process of extraction of β -glucan from oats has profound effect on its shape and size of the particles.

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