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Characterizing harmful advanced glycation end-products (AGEs) and ribosylated aggregates of yellow mustard seed phytocystatin: Effects of different monosaccharides

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ABSTRACT

Advanced glycation end products (AGEs) are at the core of variety of diseases ranging from diabetes to renal failure and hence gaining wide consideration. This study was aimed at characterizing the AGEs of phytocystatin isolated from mustard seeds (YMP) when incubated with different monosaccharides (glucose, ribose and mannose) using fluorescence, ultraviolet, circular dichroism (CD) spectroscopy and microscopy. Ribose was found to be the most potent glycating agent as evident by AGEs specific fluorescence and absorbance. YMP exists as a molten globule like structure on day 24 as depicted by high ANS fluorescence and altered intrinsic fluorescence. Glycated YMP as AGEs and ribose induced aggregates were observed at day 28 and 32 respectively. In our study we have also examined the anti-aggregative potential of polyphenol, resveratrol. Our results suggested the anti-aggregative behavior of resveratrol as it prevented the in vitro aggregation of YMP, although further studies are required to decode the mechanism by which resveratrol prevents the aggregation.

1. Introduction

Proteinases carry out a variety of specialized physiological processes in the biological system that are essential for sustainment of life such as digestion, activation of zymogens, complement activation and many more to follow. Proteinase inhibitors act as master regulators, uncontrolled action of proteinases can lead to several diseases like neurodegenerative disorders, emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, and muscular dystrophy. Cystatins are the regulators of cysteine proteinases keeping an eye on the activity of these so as to prevent the over-expression of these proteases. Cystatins are ubiquitously distributed throughout the living system. The cystatin superfamily is classified into four subfamilies stefins, cystatin, kininogens and phytocystatin. Stefins are smallest member of the superfamily. They are single chain proteins of about 11 kDa lacking both disulphide bonds and carbohydrate content [1]. Cystatins are also low molecular weight (13 kDa) protein containing two disulphide bonds but devoid of any carbohydrate content [2]. Kininogen family comprises the blood plasma kininogens, which are larger than the member of the two other families. Phytocystatins are class of reversibly binding natural cysteine proteinases inhibitors found in plants.

Cysteine proteinase inhibitors are also known to play a major role in defense against bacteria [3], viruses [4], plant eating insect [5], antigen presentation, programmed cell death, cell cycle and in tumorigenesis [6]. Cystatins like all other proteins are biologically active when in proper conformation. Many scientific reports suggests that under physiological conditions say chemical modifications such as glycation [7], phosphorylation [8] and sumoylation [9] results in unfolding of native proteins thereby leading to formation of aggregates and hence loss of the functional activity of these thiol protease inhibitors. These aggregates are of major concern in the pathology of several common neurodegenerative diseases such as Alzheimer, Parkinson's and in many other diseases.

Glycation is a spontaneous non-enzymatic multi-step process occurring in all cell types and organisms. It involves the addition reaction of nucleophilic groups of DNA, lipids and proteins with a reactive carbonyl compounds, including ribose, mannose, glucose, glyoxal, and methylglyoxal [10]. Glycation compromise the function of lipids [11–13], most importantly proteins [14–17] and also the nucleic acids [18]. The initial step of classic protein glycation involves the reaction of carbonyl group of reducing sugars with the N-terminal amino group and the side chains of lysine and arginine residues, leading to the formation of reversible Schiff base (an aldimine). Further this base through acid–base catalysis undergoes sequential rearrangements to yield more stable early glycation products known as Amadori product [19]. These amadori products later through a series of oxidation, dehydration and irreversible rearrangement forms fluorescent, yellow/brown advanced

Abbreviations: YMP, yellow mustard phytocystatin; AGEs, advanced glycation end-products; CD, circular dichroism; ANS, 8-Anilinonaphthalene-1-sulfonic acid.

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glycation end products (AGEs). These AGEs are capable of cross-linking proteins thereby forming aggregates and hence affecting their normal function [20]. Formation of aggregates is often irreversible, and aggregates commonly contain high levels of non-native conformations mainly inter-molecular beta-sheet structures.

In the present study, we have observed the in vitro aggregation and glycation pattern of phytocystatin isolated from yellow mustard seeds (YMP) in the presence of different monosaccharides (glucose, ribose and mannose). This work is a first of its kind as for the first time cystatin isolated from a plant source is being used as a model protein to explore the effect of aggregation and glycation. Also we have investigated the effect of polyphenol, resveratrol, on the aggregative and fibrillogenic behavior of phytocystatinin the presence of various monosaccharides. Polyphenols are being used extensively in the treatment of these AGEs and aggregates. The importance of our work stems from the fact that it will be helpful in designing potential drugs and a rational of treating various diseases associated with the protein aggregates and AGEs.

2. Materials and methods

2.1. Materials

Sodium phosphate monobasic and sodium phosphate dibasic were purchased from SRL (Mumbai, India). D-ribose, D-glucose, D-mannose, resveratrol (polyphenol), EtBr, Agarose, Histopaque 1077, RPMI 1640 and fluorescent dyes, viz., 8-anilino1-naphthalene sulphonic acid (ANS), Congo red (CR) as well as Thioflavin T (ThT) were purchased from Sigma (St. Louis, USA). Fresh human blood samples were taken in citrate dextrose preparations. All other reagents were of the best analytical grade.

2.2. Methods

2.2.1. Isolation of YMP

YMP was isolated from mustard seed in a simple two-step process including ammonium sulphate saturation and gel-filtration chromatography as reported earlier [21]. The isolated cystatin is called as yellow mustard phytocystatin (YMP).

2.2.2. Glycation of cystatin isolated from yellow mustard seeds

AGEs were prepared by incubating 10 μM YMP in sodium azide (0.02%), 50 mM phosphate buffer (pH 7.0) using ribose, glucose and mannose (25 mM) as modifiers for (0–32 days) at 37 °C. Aliquots were withdrawn after every 3rd day from incubated samples for further studies. Phytocystatin without monosaccharides was incubated under the similar conditions and was used as control. All solutions were filtered by 0.2 μm sterile filters under aseptic conditions before their use. After incubation, AGEs and their control were extensively dialyzed against phosphate buffer saline at 4 °C that was later stored in aliquot at −20 °C till further analysis.

2.2.3. Characterization of glycated YMP

- Activity assay: Inhibitory activity of control YMP and monosaccharides incubated YMP was measured as per the ability to inhibit papain by the method of Kunitz [22]. The activity of control was taken as a reference.
- UV spectroscopic measurement: UV absorption measurement of control YMP and monosaccharides incubated YMP was observed making use of spectrophotometer (Shimadzu, JAPAN) using a cuvette of 1.0 cm length. Spectra were recorded in the range of 190–400 nm.
- Non-tryptophan fluorescence measurement: The glycation specific fluorescence and total AGE fluorescence intensities were monitored with a RF-1501 spectrofluorophotometer (Shimadzu Co. Japan). The samples were excited at a wavelength of 335 nm which is specific for maldondialdehyde (MDA)-modified protein fluorescence and at 370 nm which is specific for pentosidine (P)-like-fluorescence. The emission was recorded in the range of 350–550 nm and 400–550 nm for maldondialdehyde (MDA)-modified protein and pentosidine (P)-like-fluorescence respectively [23]. The final protein concentration of protein was kept 4 μM. Both the excitation and emission slit widths were 5 nm with a path length of 1 cm.

2.2.4. Glycation induced tertiary structure

- Intrinsic fluorescence measurement: Intrinsic fluorescence of control YMP and monosaccharides incubated YMP was monitored on a RF-1501 spectrofluorophotometer (Shimadzu Co. Japan). The excited wavelength was 280 nm and emission spectrum was recorded in the range 300 to 400 nm.
- Extrinsic (ANS) fluorescence measurement:

ANS binding was measured by fluorescence emission spectra with excitation at 380 nm and emission range was taken from 400 to 600 nm. YMP concentration was 4 μM in 50 mM sodium phosphate buffer, pH 7.5 [24].

2.2.5. Glycation induced secondary structure

- Circular dichroism: CD analysis was carried out with a Jasco spectropolarimeter, model Jasco 815 and serial no B069061168, calibrated with ammonium D-10-camphorsulfonate. Spectra of control YMP and monosaccharides incubated YMP were taken in the range 190–250 nm. The concentration of YMP was 10 μM and path length was 1 mm. The spectra obtained were normalized by subtracting the base line recorded for the buffer having the same concentration of monosaccharides under similar conditions. All the measurements were carried out at room temperature. Each spectrum was an average of three scans.

2.2.6. Glycation induced amyloid structure formation (aggregation study)

- Precipitation reaction
  Interaction of YMP (4 μM) with different monosaccharides (ribose, glucose and mannose) was studied in 50 mM sodium phosphate buffer (pH 7.5) by turbidity method at 350 nm on Shimadzu UV-1700 spectrometer with the help of 1 cm path length cuvette [25]. For the analysis proper blank of control YMP was taken into account.
- Thioflavin T (ThT) assay:

ThT fluorescence measurements were performed by mixing sample and ThT dye at 25 °C on a RF-1501 spectrofluorophotometer (Shimadzu Co. Japan). The excitation wavelength was 440 nm and the emission range was 450–600 nm. The emission and the excitation slit widths were kept at 5 nm. The final concentration of YMP was 4 μM and of ThT was 20 μM [26].

- Congo red assay (CR):

Aggregation of YMP was further examined by red shift of CR upon binding with aggregates. Spectra was recorded in the range of 400–700 nm on Shimadzu UV-1700 spectrophotometer by using cuvette of path length 1 cm. Aliquots were prepared in the presence of different monosaccharides with YMP concentration of 4 μM and incubated for 4 h. 60 μl of each aliquot was added to 440 μl of a solution containing 10 μM CR in 20 mM sodium phosphate buffer and thus maintaining a ratio of 1:6. After 2–3 min of equilibration, absorbance was recorded [27].

- Scanning electron microscopy (SEM):

SEM was carried out to visualize morphological features of protein aggregates. The samples were air dried on the cover slip and then gold