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Inhibition of collagen glycation and crosslinking *in vitro* by methanolic extracts of Finger millet (*Eleusine coracana*) and Kodo millet (*Paspalum scrobiculatum*)

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Abstract

The present investigation was carried out to study the effects of methanolic extracts of Finger millet (*Eleusine coracana*) and Kodo millet (*Paspalum scrobiculatum*) on glycation and crosslinking of collagen. Tail tendons obtained from rats weighing 200–225 g were incubated with glucose (50 mM) and 3 mg of extracts of the above millets in methanol under physiological conditions of temperature and pH for 10 days. Early glycation was estimated by phenol-sulfuric acid method and the crosslinking was assessed by pepsin digestion, cyanogen bromide peptide map and viscosity measurements. Tendon collagen incubated with glucose (50 mM) showed 65% solubility on pepsin treatment; poor resolution of bands in the cyanogen bromide peptide map, and intrinsic viscosity of 0.84 dl/g. The collagen incubated with Finger millet and Kodo millet extracts inhibited glycation; 89% and 92% solubility in pepsin; good resolution of bands in the cyanogen bromide peptide map and intrinsic viscosity of 0.46 and 0.58 dl/g respectively. The study implicates the potential usefulness of the above millets in protection against glycation and crosslinking of collagen.

Keywords: Glycation; Collagen crosslinking; Diabetes; Antioxidant; Finger millet; Kodo millet; *Eleusine coracana*; *Paspalum scrobiculatum*

1. Introduction

The chemical reaction between the aldehyde group of reducing sugars and the amino group of proteins termed nonenzymatic glycosylation is a major factor responsible for the complications of diabetes and aging [11]. Proteins like collagen with a long half life and slow turn over are at an increased risk of undergoing glycation *in vivo* [13]. Earlier reports have shown the role of oxygen in crosslinking and chemical modification of collagen by glucose. The role of free radicals in nonenzymatic glycosylation of collagen and crosslinking is well known [2]. Antioxidative conditions and free radical scavengers inhibit these reactions [4].

Finger millet (*Eleusine coracana*) is cultivated and consumed widely in Africa and Asia, whereas Kodo millet (*Paspalum scrobiculatum*) is confined to India and is mainly consumed by the tribals and lower socio-economic class [21]. Both have high primary nutrient potential comparable to other major cereals. In addition, finger millet has a high content of dietary fiber–19.1 g, Ca–334 mg, phytate–209 mg, tannins–360 mg, Fe–2.4 to 6.4 mg, β-carotene–42 μg/100 g and total minerals–2.7% [5,21]. Kodo millet has high content of dietary fiber–37.8 g and phytate–135 mg/100 g [5]. The tiny finger millet grain has a dark brown seed coat, rich in polyphenols like phenolic acids and its derivatives, flavonoids and tannins [7,17]. Some flavonoids, have radical scavenging activity and therefore serve as antioxidants [23].

We have reported potent antioxidant activity in finger millet compared to other cereals and attributed it to the phenolics in the seed coat of the grain [16]. On screening related millets for antioxidant activity the methanolic extract of Kodo millet showed higher antioxidant activity than extract of Finger millet. The present work reports the effect of methanolic extracts of the above millets on glycation and crosslinking of rat tail collagen *in vitro*.

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2. Materials and methods

2.1. Materials

Pepsin, Chloramine T, hydroxyproline and butylated hydroxyanisole (BHA) were obtained from Sigma–Aldrich Chemical Company USA. Glucose and sodium dodecyl sulfate (SDS) was purchased from Sisco Research Laboratories, Mumbai India. All other chemicals were of analytical grade commercially available.

2.2. Preparation of methanolic extracts of finger millet and kodo millet

Twenty five grams of flour was defatted overnight with 125 ml of hexane. The hexane was decanted and the dry flour was refluxed with 100 ml methanol for 2 h at 60°C. The extraction was repeated with 50 ml methanol as described above. The extracts were pooled, filtered (Whatman No 1) and concentrated to dryness in a rotary evaporator. A portion of the extract was weighed and dissolved in known volume of methanol before use.

2.3. Incubation procedure

Tail tendons obtained from male Wistar rats weighing 200–225 g were washed thoroughly in saline at 4°C, and cut into approximately 5 cm long segments and used for the incubations. About 200 mg wet weight of tendons was incubated in 40 ml of 0.2 M Sodium phosphate buffer (pH 7.4) at 37°C in a shaking water bath for 10 days, with 50 mM glucose and 3 mg of extracts of finger millet or kodo millet in methanol, 125 mg of aminoguanidine and 1 mg of butylated hydroxyanisole. Sodium azide up to 3 mM final concentration was added to prevent contamination from microbes.

The amount of collagen in the rat tail tendons after incubation, were analyzed by estimating the hydroxyproline content at 557 nm (LKB Pharmacia spectrophotometer, England) using L-hydroxyproline as standard [19].

2.4. Glycation of collagen

The early glycation of collagen was assessed by the modified phenol-sulfuric acid method [12]. Immediately after the incubation period, the tendons were extensively washed in phosphate-buffered saline to remove unbound materials and dialyzed against 0.2 M phosphate buffer, pH 7.4, for 24 h. To 1 ml of deionized water containing about 3.0 mg collagen, 3.0 ml of concentrated sulfuric acid was added and vortexed. The solution was then cooled in ice, 0.05 ml of 80% phenol added, incubated at 37°C for 30 min, and absorbance measured at 485 nm (LKB Pharmacia spectrophotometer, England), using glucose as standard.

2.5. Pepsin digestion

Limited pepsin digestion was carried out [3]. About 20 mg tendon was treated with 400 μg pepsin in 40 ml of 0.5 M acetic acid for 3 h at 37°C in a shaking water bath. After the incubation period, the contents were centrifuged at 18,000 rpm for 30 min. Both the supernatant and pellet fractions were separated and digested with 6 N HCl for 24 h at 110°C. Then the amount of hydroxyproline was estimated as described above. From this the percentage of collagen digested by pepsin was calculated.

2.6. Viscosity measurements

Limited pepsin digestion of the rat tail collagen was carried out as described above. After incubation with pepsin, the viscosity was measured using Ostwalds viscometer [6]. Specific viscosities (η_{sp}) of the control (without glucose) and treated tail tendons were measured, between 15°C–45°C, in comparison with 0.5 M acetic acid (solvent). The denaturation profiles were obtained by plotting intrinsic viscosity (η), dl/g versus temperature (°C), where the concentration of collagen content is expressed as mg/ml.

2.7. Cyanogen bromide (CNBR) digestion and peptide map

About 10.0 mg of tendon was treated with 100 μl of 1.0 M sodium borohydride in 0.1 M sodium hydroxide overnight at room temperature and washed in deionized water. Cyanogen bromide (20/1 w/w) in 70% formic acid was added and incubated for 24 h at 30°C under nitrogen in the dark [1].

Peptide map was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of CNBr peptides under reducing conditions using a 4% stacking and 10% separating gel [9].

2.8. Statistical analysis

All experiments were conducted in triplicate. Data are presented as the means ± S.D. of 3 independent estimations. Statistical analysis was done using Single way Anova and Newman-Keuls Multiple-Range Test. P value of less than 0.05 was considered significant.

3. Results

Glucose content in collagen was 8.92 ± 0.361 μg/mg collagen, in collagen treated with 50 mM glucose it was 16.04 ± 0.583, in the collagen treated with glucose and methanolic extract of Finger millet it was 10.19 ± 0.718, in collagen treated with Kodo millet it was 9.11 ± 0.355, collagen treated with glucose and BHA it was 9.43 ± 0.009

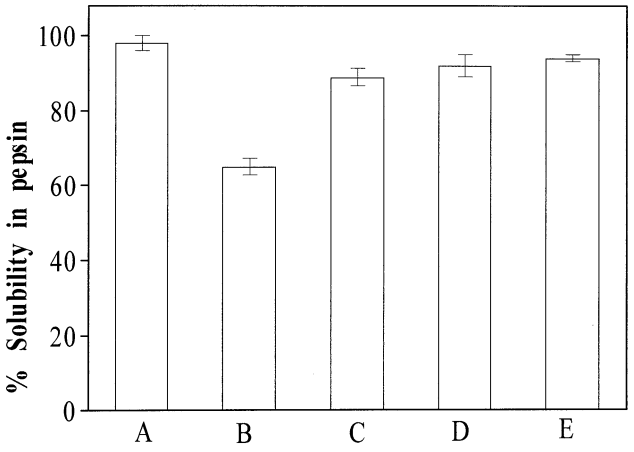

Fig. 1. Pepsin solubility of tendon collagen incubated under different conditions for 10 days. A–collagen alone, B–with glucose (50 mM), C–with glucose (50 mM) and methanolic extract of finger millet (3 mg), D–with glucose (50 mM) and methanolic extract of kodo millet (3 mg), E–with glucose (50 mM) and Aminoguanidine (125 mg). Values are mean ± S.D. of 3 individual experiments. All the treated groups had $p < 0.05$ vs. control.

and in collagen treated with aminoguanidine it was 9.49 ± 0.670 μg/mg collagen.

Fig. 1 shows the solubility criteria of collagen in pepsin after an incubation period of 10 days. Collagen incubated with glucose showed 65% solubility in pepsin, whereas on incubation with methanolic extracts of Finger millet, Kodo millet and aminoguanidine the solubility increased to 89%, 92% and 94% respectively.

Fig. 2 shows the intrinsic viscosity measurements of collagen incubated for 10 days at different temperatures. Intrinsic viscosity of collagen alone at 35°C was 0.082 ± 0.002 dl/g, with glucose it increased to 0.84 ± 0.01, with methanolic extracts of Finger millet it was 0.46 ± 0.01 and with Kodo millet 0.58 ± 0.02 respectively.

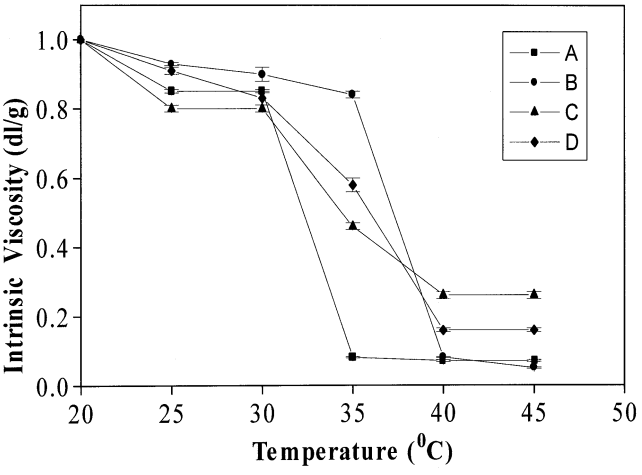

Fig. 2. Viscosity measurements of pepsin solubilized tendon collagen incubated under different conditions for 10 days. A–collagen alone, B–with glucose (50 mM), C–with glucose (50 mM) and methanolic extract of finger millet (3 mg), D–with glucose (50 mM) and methanolic extract of kodo millet (3 mg). Values are mean ± S.D. of 3 individual experiments.

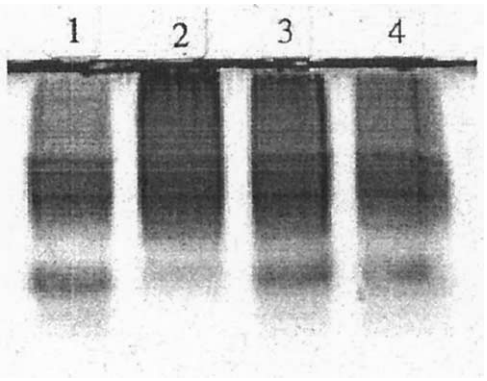

Fig. 3. SDS-PAGE of CNBR peptides of rat tail tendon collagen incubated under different conditions for 10 days. Lane 1–collagen with glucose (50 mM), lane 2–with glucose (50 mM) and methanolic extract of finger millet (3 mg), lane 3–with glucose (50 mM) and methanolic extract of kodo millet (3 mg), lane 4–collagen alone.

Fig. 3 shows peptide map of collagen after CNBr treatment after an incubation period of 10 days. Collagen incubated with glucose (50 mM) showed less resolution and spreading of bands compared to collagen treated with the methanolic extracts of Finger millet, Kodo millet and untreated collagen. This was indicative of protection against crosslinking by the extracts.

4. Discussion

Oxidation reactions play a critical role in the chemical modification and crosslinking of collagen by glycation [3,4]. Crosslinking of collagen under air depended on glucose concentration but was inhibited under antioxidative conditions (nitrogen atmosphere with transition metal chelators). It also depended on phosphate buffer concentration, but this effect was eliminated by addition of chelators, identifying trace metal ions in the buffer as catalysts of oxidative crosslinking reaction. Glycation and crosslinking are two processes and metal catalyzed oxidation couples both. Glycation may be a prerequisite for glucose induced crosslinking, but it may not determine the extent of crosslinking [13]. Oxidation of glycated collagen or glyoxidation may be the critical factor responsible for collagen crosslinking and attendant complications in diabetes mellitus [20].

In the present work 3 mg of the methanolic extracts of Finger millet and Kodo millet, significantly inhibited glycation similar to 125 mg of the antiglycating agent aminoguanidine and 1 mg of the well known synthetic antioxidant butylated hydroxyanisole.

The study on crosslinking of collagen, analyzed by pepsin digestion (Fig. 1) and CNBr digestion (Fig. 3) strongly suggested the protective role of the methanolic extracts of Finger millet and Kodo millet.

Viscosity measurements on pepsin solubilized collagen showed a significant decrease in intrinsic viscosity at 35°C, (which is the melting temperature of collagen) (Fig. 2).

Collagen treated with glucose showed decrease at only 40°C, which is expected as crosslinking results in lesser susceptibility to thermal degradation. Collagen treated with the methanolic extracts of Finger millet and Kodo millet showed a good decrease in intrinsic viscosity at 35°C, similar to untreated collagen indicating less crosslinking (Fig. 2).

A possible explanation for the above could be that natural antioxidants primarily of polyphenolic nature and other phytochemicals were extracted from the seed coats of the millet grains by methanol. Phenolics are multifunctional and can act as reducing agents (free radical terminators), metal chelators, and singlet oxygen quenchers [14]. More investigations are required to understand the role of phenolics in preventing glycation and crosslinking.

The diet of the rural or tribal population in India is predominantly cereals and millets (coarse cereals) which provide 80% of the total energy [21,22]. A clinical study was done in India with non-insulin dependent diabetes mellitus (NIDDM) patients on the glycaemic index (GI) of millet based foods, information on which is scanty [10]. The GI of kodo millet was 68 ± 8 and that of finger millet 104 ± 13. Another independent study on the glycaemic response and lipemic index on feeding rice, finger millet, tapioca and wheat diet on normal humans for 15 days did not alter the glycaemic index [8]. However, the plasma cholesterol profile was benefited significantly by finger millet and tapioca. Finger millet reduced total serum cholesterol, LDL by 9%, triglycerides by 15% and increased HDL [8]. The role of dietary fiber in lowering post prandial serum glucose is well known [15]. Both finger millet and kodo millet have a high fiber content as stated earlier and therefore they may be expected to influence health parameters on prolonged dietary intake.

Finger millet and Foxtail millet having phenolic seed coat (the red varieties) showed antioxidant activity by ESR measurements, β-carotene bleaching, lipid peroxidation and rat liver mitochondrial lysis [16,18]. Sripriya et al., [16] also showed by ESR study that 50% of DPPH free radicals was quenched by Vitamin C (1.708 $\mu m/ml^{-1}$), Vitamin E (0.7197 $\mu m/ml^{-1}$), BHA (1.720 $\mu m/ml^{-1}$) compared to 100 μg of finger millet extract in methanol. Further, the major antioxidant principle in the methanolic extract of finger millet was identified as catechin [18]. Catechin is known for its therapeutic properties as in green tea and black tea extracts [25]. Recently Watanabe et al., [24] showed antioxidants in Barnyard millet.

Against this background of increasing attention to the beneficial phytochemical and nutraceutical components in millet grains and coarse cereals, the present work assumes importance as the first to draw attention to the salutary effect of dietary millet seeds as a potential anti-aging and antidiabetic agent. Methanolic extracts of both kodo and finger millet containing mainly phenolics protected against glycation and crosslinking of tendon collagen significantly. It would be of interest to extend the study to other types of collagen since diabetes causes complications like neuropathy, nephropathy, retinopathy and atherosclerosis.

5. Conclusion

This is the first report to show the potential usefulness of Finger millet and Kodo millet in preventing glycation and crosslinking of collagen. The above millets could have potent therapeutic role as dietary supplements for the prevention of glycation induced complications, as in diabetes or aging after subjecting to further *in vivo* studies.

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