Interactions of dextranucrase purified from *Streptococcus mutans* 890 with plant polyphenols

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**A B S T R A C T**

Plant polyphenols have been extensively studied for their chemopreventive properties for human health. Dextranucrase plays an essential role in synthesizing exopolysaccharides from its exclusive substrate sucrose in *Streptococcus mutans*. In the present study, the effect of polyphenols gallic acid and tannic acid was investigated on the dextranucrase activity. The enzyme was purified by ethanol precipitation followed by column chromatography by Sephadex G-200 gel chromatography, followed by PEG-400 treatment. The purified enzyme exhibited 52 fold enrichment with 17.5% yield and specific activity of 3.54 Units/mg protein. On SDS-PAGE enzyme protein gave a single band with a molecular weight of 160 kDa. Dextranucrase activity was inhibited 80–90% by 0.04 mM tannic acid (TA) or 0.4 mM gallic acid (GA) suggesting that tannic acid has 10-fold more inhibitory potential than gallic acid on the activity of dextranucrase. CD/ORD studies revealed modifications in the tertiary structure of enzyme protein in presence of tannic acid and gallic acid, which were further confirmed by fluorescence spectra of the protein in presence of tannic acid. These results suggest that inhibition of dextranucrase activity in *S. mutans* by polyphenols may have potential applications in the prevention and control of dental caries.

1. **Introduction**

Polyphenols are the secondary metabolites of plants [1] which have been the subject of growing interest because of their possible beneficial effects on human health. It has been reported that they offer protection against the development of cardiovascular diseases, cancers, osteoporosis, diabetes, and neurodegenerative diseases [2–4]. The role of polyphenols have also been reported in the inhibition of mammalian disaccharides in intestine [5]. Polyphenols also inhibit the growth of many bacteria such as *Streptococcus mutans*, *Streptococcus sobrinus* *Porphyromonas gingivalis*, *Prevotella intermedia* which are responsible for the development of periodontal diseases [6]. The *in vivo* studies with polyphenols extracted from green tea, cocoa and grape seeds and cranberry are reported to have potential anticariogenic properties, such as inhibition of cell surface hydrophobicity and biofilm formation by *S. mutans* [7,8]. In this study we describe the effect of polyphenols gallic acid and tannic acid on the purified dextranucrase (EC 2.4.1.5) from *S. mutans*, an enzyme involved in the formation of dental caries. This enzyme is present both in extracellular and intracellular locations and its glucosyltransferase activity results in the formation of exopolysaccharides (dextrans) by transfer of glucose units to the polymer chain which are involved in the attachment of microbes on the tooth surfaces leading to their proliferation [9,10] and in the genesis of infection [11]. The data reported herein suggested that enzyme activity was inhibited 80–90% by polyphenols by modulating its tertiary structure in *S. mutans*.

2. **Materials and methods**

2.1. **Chemicals**

Chemicals of analytical grade were used in this study. Brain Heart Infusion Broth (BHI), Agar, peptone, yeast extract, sucrose, glucose, dextrose, NaCl, maleic acid and ethanol were obtained from HiMedia Pvt. Ltd. (Mumbai, India). Gallic, tannic acids and Sephadex G-200 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were obtained from E. Merck Pvt. Ltd. (Mumbai, India) or Sisco Research Lab Pvt. Ltd. (Mumbai, India).

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**Abbreviations:**

GA, Gallic acid; TA, Tannic acid; PEG, Polyethylene glycol; CD, Circular Dichroism; ORD, Optical rotatory dispersion; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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2.2. Bacterial strain and growth conditions

Bacterial strain *Streptococcus mutans* (MTCC 890) was obtained in lyophilized from MTCC (microbial type culture collection) Institute of Microbial Technology Chandigarh, India. Bacterial cells were cultured in a growth medium containing 0.5% BHI, 1% of peptone, dextrose, yeast extract, 0.05% Tween-80 at pH 7.2–7.5 incubated at 37 °C for 20 h. Purity of culture was checked by microscopic examination.

2.3. Cell free extracts and cell disruption

*S. mutans* were grown in batch culture and the cells were collected by centrifugation at 12,000 rpm for 15 min at 4 °C. The cells were washed with saline (0.9% NaCl) three times. Bacterial cells suspended in 10 mM sodium maleate buffer, pH 6.8 were disrupted in a Branson sonifier, (Model 575) with three 30 s periods of sonic treatment at 6 amps. The suspension was kept on ice bath during this procedure. The cell suspension was centrifuged at 10,000 rpm for 15 min and the supernatant fluid was used as source of the enzyme, after passing through Sephadex 250 column. The enzyme activity was eluted in void volume. Unless otherwise stated all the procedures were carried out at 4 °C.

2.4. Purification of dextransucrase

Dextransucrase enzyme was purified from *S. mutans* using the modified method as described previously Kumada et al., 1987 [12]. The culture supernatant was precipitated by treating with 50% (v/v) ethanol for 1 h below 0 °C. The preparation was centrifuged at 12,000 rpm for 30 min and the precipitate collected was dissolved in 10 mM sodium maleate buffer (pH-6.0) added with 0.1 mM PMSF and 1 μM APMSF. The solubilized preparation was subjected to Sephadex G-200 column chromatography, using (1.5 × 15 cm) column previously equilibrated with the same buffer. The column was eluted with 10 mM sodium maleate buffer (pH-6.0) with a flow rate of 40 ml/h and 30 elution fractions with 3 ml per tube was collected. Elution fractions were checked for enzyme activity and the fractions containing dextransucrase activity were pooled. The pooled fractions containing the enzyme were treated with PEG-400 to a final concentration of 33% (v/v) and incubated for 12 h at 4 °C for precipitation. To separate the dextransucrase fraction the mixture was centrifuged at 12,000 rpm for 20 min. The pellet was suspended in 10 mM sodium maleate buffer (pH 6.0). The enzyme preparation was dialyzed overnight using 10 kDa cutoff membrane. The dialysate was analyzed for enzyme activity and protein concentration. Except otherwise stated, all procedures were carried out at 4 °C.

2.5. SDS- PAGE analysis of purified enzyme

Purified enzyme was analyzed on 8% Sodium dodecyl sulfate polyacrylamide gel electrophoresis following the method of Laemmli (1970) [13]. Electrophoresis was started initially at 50 V until the dye front reached separating gel and then continued at 100 V. A pink plus pre-stained ladder ranging from 175 to 10 kDa purchased from Gene Direccion was used as the protein standards for SDS-PAGE. The protein was visualized by staining the gel in Coomassie brilliant blue.

2.6. Enzyme assay

Dextransucrase activity was assayed following the method described by Dahlqvist (1984) [14]. Reaction mixture containing 0.05 M sodium maleate buffer (pH 6.0), 0.1 M sucrose, and the enzyme in the presence or absence of aqueous extracts of gallic acid or tannic acid in total volume of 0.5 ml was incubation for 30 min at 37 °C. The samples were assayed for glucose using Glucostat Kit (Reckon Diagnostic Pvt. Ltd.). The enzyme activity was determined as μmol sucrose hydrolyzed per min/mg of protein. Product formation was linear in the 30 min incubation period under the assay conditions. One unit of dextransucrase activity was defined as the amount of enzyme required to release 1 μmol of glucose per min under standard assay conditions.

2.7. Protein estimation

Protein was measured by the procedure of Lowry et al. (1951) [15] using bovine serum albumin as the standard.

2.8. Kinetic studies

The effect of polyphenols on kinetic parameters of dextransucrase was evaluated by assaying the enzyme activity at different substrate concentrations (2–40 mM) in the absence or presence (2 mM gallic acid or 0.2 mM tannic acid) of polyphenols. The data were analyzed by Line weaver-Burk plot and from the straight lines obtained, the kinetic parameters $K_m$, $V_{max}$ and $K_i$ were determined using programmable calculator. The effect of temperature was studied by assaying enzyme activity at different temperatures (4–55 °C). From the curve activity vs. temperature, optimum temperature was determined. The effect of pH was determined by assaying the enzyme activity at pH 4.5–8.5. The data was plotted activity vs. pH to determine the optimum pH.

2.9. Reversibility of enzyme inhibition

The enzyme preparation (protein of 1.98 mg/ml) containing 0.3 mM gallic or 0.03 mM tannic acids, respectively was dialyzed against 10 mM sodium maleate buffer (pH 6 or 6.8) for 16 h with constant stirring at 4 °C. Mock preparations without inhibitors were run simultaneously. Dextransucrase activity was determined as described above.

2.10. UV-CD spectroscopic studies

CD measurements were determined using Jasco spectropolarimeter (Model J-720) equipped with a microcomputer. The instrument was calibrated with D-10-camphorsulfonic acid (10-CSA). All the CD measurements were carried out at 25 °C with a thermostatically controlled cell holder attached to a Neslab RTE-110 water bath with an accuracy of ±0.1 °C. Far UV-CD spectra were measured at a wavelength of 200–250 nm and at protein concentration of 167 μg/ml. The concentrations of gallic acid and tannic acid used were 0.30 mM and 0.03 mM respectively. The path length was 1 mm. Near UV-CD spectra were measured between 250 and 300 nm.

2.11. Fluorescence spectroscopic studies

Fluorescence measurements were performed on Shimadzu spectrofluorimeter (Model RF-540) equipped with a data recorder DR-3. The fluorescence spectra were measured at 25 ± 0.1 °C with a 1 cm path length cell. The excitation and emission slits were set at 5 and 10 nm respectively. Intrinsic fluorescence was measured by exciting the protein solution at 295 or 280 nm and emission spectra was recorded in the range of 300–500 nm in the presence of gallic acid (0.3 mM) or tannic acid (0.03 mM) at a protein concentration of 167 μg/ml.

2.12. Statistical analysis

Statistical analysis of the data was performed using SPSS. All the results were expressed as mean ± S. D except otherwise stated. A p-value <0.05 was considered significant.

3. Results

As shown in Table 1, dextransucrase activity in *S. mutans* was mainly located as an extracellular protein. Cell associated dextransucrase activity was quite low (17%) as compared to that present in the extracellular medium (73%).
Values are mean ± SD (n = 4).

Dextran sucrose was purified from S. mutans, using 50% ethanol precipitation followed by Sephadex G-200 gel chromatography and PEG-400 fractionation (Table 2). The culture obtained after growing S. mutans for 16–18 h in liquid media was centrifuged and the cells were discarded. The cell free extract was used as the source of extracellular dextran sucrose which was subjected to ethanol (50% v/v) fractionation. The enzyme preparation was subjected to gel filtration chromatography using Sephadex G-200 column. The enzyme was eluted with 10 mM sodium maleate buffer (pH 6.0). Dextran sucrose activity was eluted in (15–30) fractions, which were pooled and concentrated. The enzyme was enriched by 14 fold with a yield of 55.8%. Pooled enzyme fractions (15–30) fractions, which were pooled and concentrated. The enzyme protein marker run simultaneously with enzyme preparation (Fig. 1). To further characterize dextran sucrose activity, the effect of pH was studied by measuring enzyme activity in different pH buffers ranging from pH 4.0–8.5. Dextran sucrose activity exhibited a broad pH vs. activity curve and showed optimum activity between pH 5.0 and 7.0 with a maximal activity near 5.5–6.0. The effect of temperature on dextran sucrose activity showed that optimum temperature was near 37 °C. There was a rapid decline in enzyme activity above the optimum temperature (data not shown).

### 3.1. Effect of gallic and tannic acids on dextran sucrose activity

The effect of gallic acid (0–0.6 mM) and tannic acid (0–0.06 mM) was studied on the purified dextran sucrose activity from S. mutans in vitro. These results are shown in Fig. 2. Addition of 0.1 mM gallic acid (Fig. 2a) or 0.01 mM tannic acid (Fig. 2b) to the assay system reduced the enzyme activity from 0.340 ± 0.02 μmol/min/mg protein in the control to 0.308 ± 0.002 and 0.282 ± 0.003 μmol/min/mg proteins respectively. 0.5 mM gallic acid or 0.05 mM tannic acid further reduced the enzyme activity to 0.034 ± 0.03 μmol/min/mg protein and 0.068 ± 0.03 μmol/min/mg protein respectively.

Using Line weaver-Burk plot, kinetic analysis revealed mixed type of enzyme inhibition by gallic acid or tannic acid (Table 3). There was a significant increase in $K_m$ of the enzyme from 5 mM in the absence of gallic or tannic acid to 10 mM and 20 mM in the presence of gallic or tannic acids respectively (Table 3). $V_{max}$ of the enzyme was reduced from 2.32 in the control to 0.620 and 0.742 units/mg protein in the presence of gallic or tannic acids respectively. The value of $K_i$ of enzyme inhibition by gallic acid was 0.11 mM and for tannic acid was 0.013 mM under these conditions.

As shown in Table 4, the addition of 0.3 mM gallic acid or 0.03 mM tannic acid to the enzyme produced 80–90% enzyme inhibition. However, dextran sucrose activity was restored to almost control levels upon removal of compound by dialysis. These findings suggested that dextran sucrose inhibition by gallic or tannic acid is reversible in nature.

### 3.2. Biophysical characterization of dextran sucrose

The interactions between polyphenols and dextran sucrose were studied by ORD/CD spectra using purified dextran sucrose in the absence and presence of gallic acid or tannic acid. There was essentially no difference in the far-UV CD spectra of the enzyme protein (Fig. 3) in presence or absence of tannic acid, which gave a peak of negative ellipticity around 208 nm. However, in presence of gallic acid the observed ellipticity was reduced and peak $\lambda_{max}$ shifted to 203 nm under the experimental conditions. These findings suggest that there was essentially no change in secondary structure of protein molecule in presence of tannic acid or gallic acid except that the absorption peak was reduced in presence of gallic acid which implies that polyphenol binding hinders the CD spectra under the far-UV region.

Near-UV CD spectra of dextran sucrose showed a peak around 260 nm and another peak at 270 nm with the native protein molecule (Fig. 4). However, the presence of gallic acid or tannic acid induced marked changes in the near UV-CD spectra with a peak at 257 nm and another peak at 268 nm in presence of tannic acid. Gallic acid binding to dextran sucrose protein showed considerable changes in the CD spectra. In addition to a peak at 257 nm and 260 nm, another peak at 275 nm was also observed.

These observations may indicate that the binding of polyphenols to dextran sucrose results in modifications in the tertiary structure of protein molecule. There were also changes in the shape of CD spectra curve with an appearance of another peak around 275 nm.

Fig. 5 shows the intrinsic fluorescence spectra of dextran sucrose in presence and absence of polyphenols. When the protein was excited at 280 nm and the emission spectra was recorded between 300 and 500
nm, the fluorescence spectrum of the protein was essentially similar to that in presence of gallic acid. However, the addition of tannic acid to purified dextransucrase protein gave a strong peak around 350 nm with a shoulder around 330 nm and another small peak at 460 nm.

These observations suggest considerable change in the tertiary structure of protein molecule in presence of tannic acid which was not apparent in presence of gallic acid. Thus, the underlined mechanism of mode of interactions of polyphenols is presumably different resulting alterations in the tertiary structure of dextransucrase in presence of gallic acid or tannic acid.

4. Discussion

In the present study dextransucrase was purified to homogeneity
from *S. mutans* using 50% ethanol precipitation followed by Sephadex G-200 gel chromatography and PEG-400 fractionation. The purified protein yielded 17.5% recovery and 52.05 fold enrichment of enzyme activity. SDS-PAGE revealed a single band of the enzyme protein corresponding to a molecular weight of 160 kDa. These results in general, are in agreement to those reported from several other bacterial species [16]. Fukui et al. (1982) [17] have reported the molecular weight of 180 kDa for the purified dextranucrase from *Streptococcus mutans*, reidentified as *S. sobrinus* by Sales et al., 2018 [18]. Tsumori et al. (1985) [19] have reported molecular weight of 158 kDa of the enzyme isolated from *Streptoccocus mutans* HS6 (serotype a).

These findings suggest some variations in the molecular weight of the enzyme isolated from different species of *S. mutans*. The observed differences among different species could be attributed to several factors such as: (a) the amount of enzyme protein present (b) differences in the degrees of enzyme purification procedures, and (c) the technical difficulties encountered in analyzing large molecular weight proteins [20]. Nevertheless, the purified protein gave a single band on SDS-PAGE, thus indicating the homogeneity of the isolated enzyme.

The dextranucrase activity exhibited a broad activity vs. pH curve, with an optimum pH between pH 5.5–6.0. The experiments using insoluble and particulate dextranucrase essentially showed similar degree of temperature and pH optima. These results are similar to those reported by Kuramitsu (1974) [11] for dextranucrase in *Streptococcus mutans* GS-5.

Aqueous extracts of certain plant chewing sticks contain considerable amounts of polyphenols including gallic and tannic acids, which are reported to inhibit dextranucrase activity in *S. mutans* [21]. The present data further reinforces the contention that gallic and tannic acids inhibited 80–90% dextranucrase activity in *S. mutans*, tannic acid being nearly 10-fold more effective in suppressing the enzyme activity than gallic acid. The exact mechanism of inhibition of dextranucrase activity by polyphenols is unknown, although the data presented here provide insights to the mode of their inhibitory action. It is likely that the inhibition of dextranucrase by polyphenols is determined by molecular structure of the phenolics and physical state of the enzyme. pKa for gallic acid is 4.5. Thus, ionized molecule contains a lone pair of electrons at >C=O in the carboxyl group and be active at pH 5 and above. This indeed explains the basis of the inhibitory activity of the polyphenols, which

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**Table 3**

| Enzyme preparation     | $K_m$ (mM) | $V_{max}$ (μmol/mg protein) | $K_i$ (mM) |
|------------------------|------------|-----------------------------|------------|
| Control                | 5 ± 0.03   | 2.32 ± 0.015                | 0          |
| Gallic acid            | 10 ± 0.02  | 0.62 ± 0.012                | 0.11       |
| Tannic acid            | 20 ± 0.14  | 0.742 ± 0.019               | 0.013      |

Values are mean ± SD; (n = 4).

**Table 4**

| Preparation               | Activity* (U/mg protein) | %age Activity |
|---------------------------|--------------------------|---------------|
| Control (Dialysed)        | 0.334 ± 0.012            | 100           |
| Enzyme + GA (0.3 mM) (Undialysed) | 0.083 ± 0.025          | 25            |
| Enzyme + GA (0.3 mM) (Dialysed) | 0.329 ± 0.002          | 98.5          |
| Enzyme + TA (0.03 mM) (Undialysed) | 0.698 ± 0.015         | 30            |
| Enzyme + TA (0.03 mM) (Dialysed) | 0.329 ± 0.003          | 98.8          |

* activity was determined in triplicates; the median values are indicated.
  * GA indicates gallic acid.
  * TA indicates tannic acid.
  * $P < 0.05$.

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**Fig. 3.** Far-UVCD spectra of dextranucrase in the absence and presence of gallic acid or tannic acid. CD spectra was measured as described in above mentioned method.

**Fig. 4.** Near-UVCD spectra of dextranucrase in the absence and presence of gallic acid or tannic acid. CD spectra was measured as described in above mentioned method.

**Fig. 5.** Intrinsic fluorescence emission spectra of dextranucrase in the absence and presence of gallic or tannic acids.
interacts with –NH group in the active site of the enzyme as a function of pH as described earlier [22].

Both gallic acid and tannic acid showed mixed type of enzyme inhibition. The observed inhibition of dextranase by gallic or tannic acids was similar to that reported for mammalian disaccharides in rat intestine. However, kinetically the inhibition of intestinal sucrase by polyphenol was competitive-type, in contrast to non-competitive inhibition of dextranase observed in the present study. Thanjavur et al. (1982) [23] have also described uncompetitive-type of dextran- sucase inhibition by pyridine analogues.

In the present study, the fluorescence spectra produced a marked increase in emission spectra in presence of tannic acid which was not observed in presence of gallic acid. This corroborates the contention, that gallic acid and tannic acid interactions with enzyme protein are distinct. Fluorescence spectra generally reflect the confirmation of tryptophan residues in protein molecule. When 295 nm is selected as the excitation wavelength, only tryptophan residues are attributed to the intrinsic fluorescence of a protein [24]. Thus, it is apparent that binding of tannic acid to dextranase exposes a tryptophan residue which was not the case in presence of gallic acid. The binding of polyphenols to enzyme protein indicate modifications in tertiary structure of the protein under the experimental conditions. Gupta et al. (2009) [8] have also reported changes in tertiary structure of brush border sucrase and exposure of tryptophan residues by CD spectra and fluorescence analysis in presence of gallic or tannic acid.

In the far-UV region, there was essentially no difference in the CD/OR spectra of dextranase in the absence or presence of gallic acid or tannic acid. However in presence of gallic acid, the negative ellipticity was reduced or \( \lambda_{\text{max}} \) shifted to 203 nm under experimental conditions. This suggest that secondary structure of the protein molecule is not affected by the interactions with the gallic acid or tannic acid. However in the near-UV region CD/OR spectra showed considerable changes that suggests modifications in the tertiary structure of the dextranase in presence of tannic acid and gallic acid.

Tsumori et al. (1997) [25] have also reported that Asp, Trp and His amino acid residues are essential for the catalytic activity of dextranase/GTFs. Asp \(^{113} \) was identified as sugar binding acceptor sub-sites in the enzyme [26]. It was suggested that a nucleophilic site (-COO\(^{-} \) group of Asp) attacks the C-1 of the glucosyl moiety of sucrose, resulting in glucosyl-enzyme complex formation. The other acidic group might facilitate the release of fructose moiety that gives a proton to the oxygen atom of the glucosidic linkage. It is likely that the side chain of aspartic acid (CH\(_2\)COOH) acts as a nucleophile and reacts with flavo- noids, causing enzyme inhibition. The presence of –COO\(^{-} \) group in the gallic acid molecule, which contains a lone pair of electrons at C = O group, may interact more efficiently with protonated residues X: H in the active site of the enzyme, as explained above, thus inhibit the enzyme activity [23]. This may also be responsible for alterations in the tertiary structure of the enzyme protein by polyphenols as analyzed by bio-physical techniques. However, the site of interaction with polyphenols with dextranase is not known.

In conclusion, the present data shows that plant phenolics, which are the naturally occurring constituents of edible fruits and vegetables, are potent inhibitors of dextranase/glucosyltransferases activities in \( S. \) mutans and it might be useful in the control of dental caries.

Author contributions statement

DG has performed experiments and wrote the manuscript, S.A.R helped in writing the main manuscript and reviewed the article. A.M contributed in concept design and/or analysis and interpretation of data and revising it critically for important intellectual content and S.C.S provided culture facilities and reviewed the article for important inputs. The article was reviewed for final submission by all authors.

Declaration of competing interest

All authors declare that there is no conflict of interest with the contents of this article.

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