Journal of Bioscience and Bioengineering
VOL. 120 No. 3, 252–256, 2015

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Interaction of carbohydrates with alcohol dehydrogenase: Effect on enzyme activity

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Received 3 July 2014; accepted 7 January 2015
Available online 7 February 2015

Alcohol dehydrogenase was covalently conjugated with three different oxidized carbohydrates *i.e.*, glucose, starch and pectin. All the carbohydrates inhibited the enzyme. The inhibition was studied with respect to the inhibition rate constant, involvement of thiol groups in the binding, and structural changes in the enzyme. The enzyme activity decreased to half of its original activity at the concentration of 2 mg/mL of pectin, 4 mg/mL of glucose and 10 mg/mL of starch within 10 min at pH 7. This study showed oxidized pectin to be a potent inhibitor of alcohol dehydrogenase followed by glucose and starch. Along with the aldehyde-amino group interaction, thiol groups were also involved in the binding between alcohol dehydrogenase and carbohydrates. The structural changes occurring on binding of alcohol dehydrogenase with oxidized carbohydrates was also confirmed by fluorescence spectrophotometry. Oxidized carbohydrates could thus be used as potential inhibitors of alcohol dehydrogenase.

[**Key words:** Alcohol dehydrogenase; Carbohydrates; Inhibition; Thiol groups; Covalent binding]

Proteins and carbohydrates are natural polymers that are abundantly found in most complex biological systems. These two polymers interact by forming a complex through covalent and/or non-covalent binding. Among the various non-covalent interactions, hydrogen bonding and electrostatic interactions play an important role in enhancing the functional attributes of the polymer complex (1). For covalent interactions, the polysaccharides are oxidized to get aldehyde groups which then react with the amino groups of proteins. Enzymes show different kinds of interactions with bio-macromolecules in the cell. Such interactions affect the enzyme activity as well as its stability (2). Carbohydrates are one of the important macromolecules with which enzymes interact. Hence enzyme–polysaccharide interactions attract attention with respect to enzyme stability. Covalent interactions between enzyme and carbohydrate can be either glycosylation or glycation. Although both the processes involve attachment of glycan to enzyme (protein), glycosylation is an enzymatic process while glycation is a non-enzymatic process. Glycosylation attaches the glycan part to nitrogen of asparagine or arginine and hydroxyl oxygen of serine, threonine or tyrosine. However glycation attaches the glycan part to nitrogen of lysine via Schiff's base formation (3).

Many authors have reported covalent as well as non-covalent interactions between the enzymes and polysaccharides (4–6). These interactions enhance the stability of enzymes at extreme temperatures and pH (7,8). Non-covalent interactions between enzymes and polysaccharides in a mixture have also been reported to enhance structural as well as functional properties of proteins (5). Conjugation of polysaccharide with an enzyme can either enhance or inhibit the activity and/or stability depending on the structural changes occurring in the enzyme. Based on the structural and functional properties, enzymes could respond differently to the same modification method. For instance, microencapsulation of glucose oxidase and laccase using poly(ethyleneimine) have shown completely opposite effects on thermal stability of the said enzymes. At high temperature, poly(ethyleneimine) chelates copper from active site of the laccase and hence decrease its thermal stability, whereas same microencapsulation enhances the thermal stability of glucose oxidase (9). Synthetic polysaccharide prepared using glucal has shown inhibition of amylase (10), whereas basic polysaccharides are reported to be inhibitory for lipase activity (11).

Alcohol dehydrogenase belongs to the zinc metalloenzymes that catalyse the oxidation of alcohols to aldehydes and vice versa. Inhibition of alcohol dehydrogenase may prove to be advantageous to study the alcohol metabolism as well as treatment for methanol poisoning in humans (12). The susceptibility of humans to methanol toxicity is dependent upon folate metabolism. It is an uncommon but hazardous intoxication characterized by visual impairment and formic acidaemia. Alcohol dehydrogenase metabolizes methanol to formate which accumulates in body and thereby causes toxicity. The therapy of methanol toxicity is based on the inhibition of alcohol dehydrogenase (13,14). Alcohol is metabolized in the liver by alcohol dehydrogenase to acetaldehyde. Acetaldehyde is further broken down by aldehyde dehydrogenase to acetic acid and water. Inhibitors of alcohol dehydrogenase may

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.01.008

help to study this pathway of alcohol metabolism (15). The inhibitors of alcohol dehydrogenase have also been used in cosmetics to avoid the effect of added alcohol on the skin (16). Many compounds including thiol compound (2), 4,4′ dithiodipyridine (17), flavonoids (18), pyrazoles (12) and 2,2,2-trifluoroethanol (19) have been reported to act as inhibitors of alcohol dehydrogenase.

In the present study, we report the inhibitory effect of covalent binding between carbohydrates (glucose, starch and pectin) and alcohol dehydrogenase on the enzyme activity. Glucose (monosaccharide), starch (glucose containing polysaccharide) and pectin (anionic polysaccharides) were selected for the study. The inhibition of alcohol dehydrogenase by oxidized carbohydrates was thoroughly studied by determining the inhibition rate constant and evaluating the involvement of thiol groups on the inhibition.

MATERIALS AND METHODS

Materials Alcohol dehydrogenase from *Saccharomyces cerevisiae* (46 U/mg as determined experimentally), sodium metaperiodate, nicotinamide adenine dinucleotide (NAD) and sodium pyrophosphate were purchased from Sigma Aldrich, St. Louis, MO, USA. Glucose, starch and pectin (MW 75,000 Da and degree of esterification 65–70%) were purchased from Himedia, Mumbai, India.

Preparation of conjugate of carbohydrate with alcohol dehydrogenase The enzyme-carbohydrate conjugate was formed by using a previously reported method (8). Sodium metaperiodate (0.1 M) solution was prepared in 0.1 M sodium acetate buffer of pH 5.0 and used as the oxidizing solution. Carbohydrates (glucose, starch and pectin) 100 mg each was oxidized in 10 mL of oxidizing solution in dark at room temperature ($25 \pm 2^{\circ}C$) for 90 min, after which the oxidation was stopped by adding 0.3 mL of ethylene glycol, and kept in dark for 1 h. Oxidized carbohydrates (except glucose) solutions were dialysed against 0.01 M sodium phosphate buffer of pH 7.5 at 4°C overnight. Alcohol dehydrogenase solution (1 mg/mL) was prepared in 0.01 M sodium phosphate buffer of pH 7.5 and mixed with equal volume of each oxidized carbohydrate solution and kept for conjugate formation at room temperature ($25 \pm 2^{\circ}C$). These conjugates were used for analysing the activity of alcohol dehydrogenase.

Alcohol dehydrogenase activity assay Assay mixture (800 μL) consisted of 0.05 M sodium pyrophosphate buffer of pH 8.8 (260 μL), 50 mM ethanol (220 μL), 15 mM NAD (300 μL) and reaction was started immediately by addition of enzyme solution (20 μL, 0.1 mg/mL). Reduction of NAD to NADH was followed for 4 min by taking absorbance at 340 nm. The assay mixture for the blank (800 μL) consisted of 0.05 M sodium pyrophosphate buffer of pH 8.8 (260 μL), 50 mM ethanol (220 μL), 15 mM NAD (300 μL) and distilled water (20 μL). Extinction coefficient (ε) value of 6.22 mM^{-1} cm^{-1} for NADH at 340 nm was used to calculate activity (20). One unit of enzyme activity was defined as millimoles of NAD reduced per min at room temperature and pH 8.8. Protein concentration in the sample was analysed using Folin-Lowry method (21) and it was calculated using bovine serum albumin as the standard in the range of 40–200 μg/mL. The protein concentration of enzyme was 0.1 mg/mL which was used for calculation of specific activity of an enzyme.

Stability of the enzyme and progress of inhibition Each oxidized carbohydrate (glucose, starch and pectin) in the range of 2–10 mg/mL was mixed with equal volume of the enzyme solution (1 mg/mL) and incubated at room temperature. Samples were withdrawn after regular time intervals (5–30 min for glucose and pectin, 10–60 min for starch), and assayed for the residual enzyme activity as described above. Control enzyme solution was maintained without addition of inhibitors and used as a reference of the original enzyme activity.

A semi-log plot of the percent residual activity vs time was plotted for each enzyme-carbohydrate conjugate. The slope of the inhibition rate constant (k) was calculated, and the time required for the activity to decrease to half of its original activity ($t_{1/2}$) in the presence of inhibitor was calculated as 0.693/k.

The effect of pH on the covalent interaction of carbohydrate with alcohol dehydrogenase The enzyme solution was prepared in buffer of pH 7, 8 and 9 (sodium phosphate buffer of pH 7 and 8, glycine-NaOH buffer of pH 9). The oxidized carbohydrates (4 mg/mL) were mixed with equal volume of enzyme solutions (1 mg/mL) at pH 7, 8 and 9. The enzyme was incubated for 10 min with glucose, 20 min with starch and 5 min with pectin at room temperature. Each inhibitor had different inhibition rate constants; hence time of incubation was varied accordingly to get maximum inhibition for better comparison of residual activity. The residual enzyme activity was calculated using the assay method described above. The inhibition rate of enzyme by carbohydrates at each pH was calculated as percent of the control activity.

Evaluation of thiol groups in free and carbohydrate conjugated alcohol dehydrogenase Free thiol content of alcohol dehydrogenase before and after the conjugation with oxidized carbohydrates was determined using Ellman's method (22) with slight modifications. The enzyme (0.5 mL, 1 mg/mL) was incubated with the oxidized carbohydrate (0.5 mL, 10 mg/mL) for 15 min to prepare inhibited enzyme sample. The free and inhibited enzyme sample (700 μL) were added to the mixture of 50 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (50 μL), 1 M Tris buffer of pH 8.0 (100 μL) and distilled water (150 μL). The assay mixture was incubated for 5 min and absorbance was taken at 412 nm. Blank was prepared similarly except that distilled water was used instead of sample. The standard curve of cysteine was prepared using 20–100 μM of cysteine. The thiol groups present in the enzyme before and after the conjugation with oxidized carbohydrates were calculated in terms of cysteine equivalent thiol groups.

Fluorescence spectrophotometer analysis Fluorescence spectrophotometer analysis was done using fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies, Inc., Santa Clara, CA, USA) to investigate structural changes in the enzyme after its covalent interaction with carbohydrates. The excitation wavelength was set at 280 nm and the emission spectrum was recorded from 300 to 500 nm. Free and conjugated enzymes were used as samples. Conjugated enzymes were prepared by mixing enzyme and oxidized carbohydrates and incubating for 30 min. Fluorescence measurements were carried out in 0.01 M sodium phosphate buffer of pH 7.5.

RESULTS AND DISCUSSION

Stability of the enzyme and progress of inhibition The covalent interaction of oxidized carbohydrate with alcohol dehydrogenase was studied by preparing enzyme-carbohydrate conjugates. All the three carbohydrates under study inhibited alcohol dehydrogenase after binding with it covalently. Inhibition kinetics was studied by using different concentrations of each carbohydrate from 2 to 10 mg/mL for various time intervals (Fig. 1). The inhibition kinetics was evaluated by a semi-log plot of percent residual activity vs time in terms of inhibition rate constant, k and half-life $t_{1/2}$. The inhibition rate constant of alcohol dehydrogenase in the presence of all oxidized carbohydrates increased with an increase in the concentration of carbohydrates. At low concentration (2 mg/mL) of oxidized carbohydrate, inhibition rate constants of the enzyme were 0.030, 0.014 and 0.052 for glucose, starch and pectin, respectively. At higher concentration (10 mg/mL) of oxidized carbohydrate, inhibition rate constants of the enzyme were 0.214, 0.052 and 0.409 for glucose, starch and pectin, respectively. It is evident from these results that pectin had highest inhibition rate constant followed by glucose and starch. The half-life of the enzyme after covalent conjugation with the carbohydrates was calculated using the inhibition rate constant (Fig. 2). At 2 mg/mL of carbohydrates, the half-life of enzyme-starch conjugate was approximately four times higher than that of the enzyme-pectin conjugate and two times higher than that of the enzyme-glucose conjugate. The concentration of oxidized carbohydrates at which the enzyme activity reduced to half of its original activity can be a good indicator of the efficiency of oxidized carbohydrates as an inhibitor of alcohol dehydrogenase. Pectin (2 mg/mL), glucose (4 mg/mL) and starch (10 mg/mL) were able to decrease the activity of enzyme to half of its original activity within 10 min. These results indicated that oxidized carbohydrates acted as potent inhibitors of alcohol dehydrogenase. However, pectin was the most potent inhibitor followed by glucose and starch. The oxidation of carbohydrate generates reactive aldehyde groups which can bind to active site of the enzyme. Alcohol dehydrogenase has binding sites for aldehyde. Alcohol dehydrogenase can covert aldehyde to alcohol and vice versa. However, carbohydrate is not a substrate for alcohol dehydrogenase; it binds to enzyme due to the presence of reactive aldehyde group. Once the carbohydrate is bound to the active site of the enzyme, it may act as its inhibitor. Pectin is structurally very complex which generates more free aldehyde groups and has additional carboxyl groups which can bind to imine groups of enzyme forming carbodiimide bond. The structural complexity and presence of additional carboxyl groups in pectin as compared to starch may be responsible for strong binding of oxidized pectin to enzyme, and which resulted in high