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Purification of sucrose synthase from thermotolerant wheat grains and its characterization

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Abstract

Aim : Wheat is common and popular cereal with wide utilization and acceptability. High temperature stress drastically affects crop productivity and quality by disturbing sucrose metabolism. Hence, the aim of present investigation was to purify and characterize of sucrose synthase from thermotolerant wheat for various physico-chemico-kinetic properties.

Methodology : The immature grains of thermotolerant wheat WH-1021 were used for purification of sucrose synthase by using traditional protein purification techniques viz. $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration through sephadex G-100 and DEAE-cellulose ion exchange chromatography. The purity was checked by native PAGE, while subunit by SDS PAGE.

Results : The enzyme was purified about 27 fold with approximately 37% recovery. The native enzyme had a molecular weight of 269 KDa and subunit molecular weight was found to be 63 KDa, indicating that enzyme is a homotetramer. The purified enzyme exhibited optimum activity at 37°C with thermostability upto 50°C and pH 6.5. Sucrose synthase showed K_m values of 14.28 and 1.18 mM for sucrose and UDP, respectively. Its activity was inhibited by Mn^{2+} (38.5%), while NO_3^- stimulated the activity by 20.8% at 2 mM concentration.

Interpretation : Among the various nucleotides tested, the enzyme was highly specific for UDP as substrate and NADP^+ and G-6-P were found to be the potent inhibitors. The kinetic studies revealed that sucrose synthase catalyzed the sucrose degradation by ping-pong mechanism. The results of the present study would help in planning the strategies for increased wheat productivity without compromising the quality under stress conditions.

Purification and characterization of sucrose synthase from grains of thermotolerant wheat genotype WH-1021

Tradition protein purification techniques viz. $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration through sephadex G-100 and DEAE-cellulose ion exchange chromatography was use, The purity was checked by native PAGE while subunit by SDS PAGE

The native enzyme had a molecular weight of 269 kDa and subunit molecular weight 63 kDa

Optimum activity at 37°C thermostable upto 50°C at pH 6.5.

K_m values of 14.28 and 1.18 mM for sucrose and UDP, respectively

Activity inhibited by Mn^{2+} (38.5%) while stimulated by NO_3^- (20.8%) at 2 mM

The enzyme was highly specific for UDP as substrate and NADP^+ and G-6-P were found to be the potent inhibitors

Sucrose synthase catalyzed the sucrose degradation by ping-pong mechanism revealed by kinetic studies

Introduction

Wheat is an important cereal crop due to its unique physio-chemical and nutritional properties that makes it irreplaceable staple food in the daily diet of modern man. Its production area is very wide of about 221.70 million ha with annual production of 659.9 million tonnes within a wide range of environments, which explains its huge involvement in human life around the whole world (FAO, 2015). However, wheat production and quality is greatly affected by climatic changes at either germination, growing or maturation stage that in turn directly affects the nutrition properties and quality of end products. From past few years the increased terminal heat stress has lead to terrible loss of crop productivity (Bita and Gerats, 2013) as cooling period for wheat is constantly reducing and high temperature beyond 30°C, which is usually encountered during later part of grain filling period, drastically reduces the yield and quality (Singh *et al.*, 2011; Zhao *et al.*, 2011).

The developing grains are active sites for the synthesis of a wide range of necessary metabolites and macromolecules such as starch and proteins that are essential for plant growth. Study of different level of sugar under various stresses play a crucial role in conferring the tolerance to plants by modulating several physiological processes (Rathinasabapathi, 2000; Sami *et al.*, 2016). Starch synthesis is one of the important process that is greatly offended by this high temperature chiefly after anthesis that leads to overall reduction in grain size (Tian *et al.*, 2006). Starch is the principal storage form of carbohydrates that forms about 70 to 85% of final dry weight of grain. Sucrose is the precursor of starch along with being the main mobile form for photosynthetic assimilates from leaves to endosperm. The ability of plant to transport sucrose is significantly correlated with temperature as unfavorable temperature can potential reduces crop productivity (Fitz Simons and Oosterhuis, 2016). Sucrose metabolism is one of the crucial processes as it is essential for the distribution of crucial carbon resources and for the initiation of various signals involving hexose based sugar signals (Koch, 2004). Sucrose synthase (UDP glucose: D-fructose-2-glucosyl transferase, EC 2.4.1.13) is the first and most important enzyme that catalyzes the first metabolic step in sucrose-starch conversion pathway, which catalyzes the reversible interconversion of sucrose and UDP to UDP-glucose and fructose (Baroja-Fernández *et al.*, 2003; Keeling and Myers, 2010). But sucrose synthase mainly contributes in the direction of sucrose degradation to provide sugar nucleotides for complex polymers synthesis (Chourney, 1981; Volpicella *et al.*, 2016).

The relationship between source and sink decides the yield of a crop. As it is highly active in growing sink organ, so can act as a marker for sink strength (Jie *et al.*, 2012). The characterization of this important interconversions between sucrose and starch in sink organs of thermotolerant wheat is very important and this prerequisite the detailed knowledge of the sucrose synthase. Hence, the aim of the present study was to

purify sucrose synthase from thermotolerant wheat and further characterize it for molecular weight, effect of temperature, pH, substrate concentration, ions and inhibitors.

Materials and Methods

Plant material : The immature grains (21 Days after anthesis) of thermotolerant wheat WH-1021 were harvested from the field of Wheat Section of the Department of Plant Breeding, CCS Haryana Agricultural University, Hisar. All the chemicals and biochemicals were of high purity analytical grade.

Extraction of sucrose synthase : Extraction of enzyme was as per the method described by Sharma and Chugh (2017) using ammonium fractionation (30-60%), column (sephadex G-100) and ion exchange chromatography (DEAE- cellulose) techniques.

Enzyme assay : Sucrose synthase activity in crude extract and in the preparations during purification process was assayed by the method of Shannon and Dougherty (1972). The reaction mixture (1 ml) that contain UDP (One μ mole), sucrose (25 μ moles) and MES buffer (pH 6.5, 60 μ moles) and enzyme preparation was incubated for 15 min at 37°C. The reaction was terminated by placing the reaction mixture in boiling water bath for 2 min. Released fructose was measured by Somogyi's modified method (Somogyi, 1945). The equivalent amount of fructose was calculated from a standard curve prepared by using glucose (200 μ g ml⁻¹). One unit of enzyme is defined as the amount of enzyme that catalyses the formation of one μ mole of product per hour.

Protein estimation : Protein in various fractions during purification was detected by measuring the extinction at 280 nm. Quantitative estimation of protein at each step of purification, however, was done by following the method of Lowry *et al.* (1951).

Characterization of sucrose synthase : The molecular weight of purified enzyme was estimated through Sephadex G-100. The column was calibrated with the following standard protein markers (2 mg ml⁻¹ each): cytochrome-C (12.4 kDa), carbonic anhydrase (29.0 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa) and β -Amylase (200 kDa). The void volume was calculated by using blue dextran. The log molecular weight of each standard protein was plotted against elution volume and the molecular weight of purified enzyme was calculated from the calibration graph. Subunit molecular weight was determined by carrying out polyacrylamide gel electrophoresis (10% gel) in the presence of SDS following the method of Laemmili (1970).

The purity of enzyme preparation was checked by native-PAGE using anionic system of Davis (1964). Different buffers of different pH values were used viz. for pH range 4.0–5.6, 0.06 M acetate buffer, for pH range 5.7–6.7, 0.06 M MES buffer, for pH range 5.8–7.2, 0.06 M phosphate buffer and for pH range 7.3–8.0,

0.06 M tris-HCl buffer and pH at which maximum activity was observed was taken as pH optima. The effect of temperature on the purified enzyme activity was evaluated at temperature ranging from 25 to 65°C. Except for the enzyme, all constituents of the reaction mixture were maintained at appropriate temperature in a water bath before starting the reaction. Thermostability of purified enzyme was tested by measuring the residual activity after incubating the enzyme for 30 min at temperature ranging from 25 to 65°C. The substrate specificity of enzyme sucrose synthase was determined by incubating different potential nucleotide substrates viz. UDP, ADP, CDP, TDP and GDP at a concentration of 1 mM with the enzyme at a saturating concentration of sucrose (25 mM). Activity was calculated by taking activity of control as 100%.

The activity of purified preparation was measured using UDP as substrate at a final concentration varying from 0.2 to 1.8 mM and at a constant concentration of sucrose (25 mM) and similarly using sucrose as substrate at a final concentration varying from 5 to 45 mM and at a constant concentration of UDP (1 mM). The K_m values were determined by using the Lineweaver-Burk plot method (1934). The mechanism of bisubstrate reaction catalyzed by purified enzyme was investigated by incubating the enzyme with saturating and sub-saturating concentrations of one substrate and varying the concentration of the other substrate.

The activity of purified preparation was measured in the presence of different metabolites at three different concentrations (1, 2 and 5 mM). The percentage of stimulation

and inhibition was expressed as relative to the enzyme activity without inhibitor. The effects of monovalent Na^+ , K^+ , Cl^- , HCO_3^- , CH_3COO^- and NO_3^- ; divalent Ca^{2+} , Mn^{2+} , Mg^{2+} , CO_3^{2-} and SO_4^{2-} ions were determined by carrying out enzyme assay at 2 mM concentration of these metal ions. Residual activity was calculated taking activity of control as 100%.

Results and Discussion

Sucrose synthase from wheat grains was purified through a traditional four-step purification procedure. The specific activity in crude extract and $(\text{NH}_4)_2\text{SO}_4$ fraction was 10.11 and 32.20 units mg^{-1} protein (30–60%), respectively (Table 1) with 3.18 fold purification. In further purification by molecular exclusion and ion exchange chromatography through Sephadex G-100 and DEAE-cellulose, about 27 fold purity was obtained with approximately 37% recovery. Sytykiewicz *et al.* (2008) also reported a 21-fold purification of sucrose synthase through the Sephadex G-150 and DEAE-cellulose column, in cherry leaves. In DEAE cellulose column the enzyme elution was carried out with 0.1–0.5 M NaCl gradient in 30 mM tris-HCl buffer (pH 7.5), which illustrates the anionic nature of the enzyme. The final enzyme preparation had specific activity of 270.41 units mg^{-1} protein, which is comparable to that reported for sucrose synthase from wheat grains (Anand and Singh, 1986).

The molecular weight of purified sucrose synthase as determined from gel filtration through sephadex G-100 column was 269 kDa (Fig. 1 and 2A), which was in accordance with the molecular weight calculated by the equation proposed by Squire

Fig. 1 : SDS-PAGE pattern of purified sucrose synthase from wheat grains

Table 1: Summary of purification of sucrose synthase from immature wheat grains

Fraction	Volume (ml)	Total activity (Units)	Protein (mg)	Specific activity (Units/mg ⁻¹ protein)	Fold purification	Recovery (%)
Crude extract	315	4200.00	415.30	10.11	-	100
(NH ₄) ₂ SO ₄ (30-60%)	20	3438.60	106.80	32.20	3.18	81.19
Sephadex G-100	33	2773.16	15.88	174.63	17.26	66.03
DEAE-cellulose	60	1541.32	5.7	270.41	26.72	36.70

Table 2 : Substrate specificity of sucrose synthase

Substrate	Concentration (mM)	Enzyme activity (% control)
UDP	1	100
ADP	1	34
TDP	1	12
CDP	1	21
GDP	1	3

Table 3 : Effect of various metabolites on purified sucrose synthase

Metabolites	Enzyme activity (% of control)		
	1 mM	2 mM	5 mM
AMP	100.0	102.9	73.7
ADP	103.6	100.0	88.4
ATP	100.9	100.0	100.0
ADPG	103.5	99.4	100.0
NAD ⁺	99.3	101.3	101.9
NADH	100.0	100.4	101.2
NADP ⁺	99.7	95.9	84.1
NADPH	100.0	99.6	99.2
G-1-P	102.5	101.0	100.0
G-6-P	100.8	86.6	66.4

Table 4 : Effect of various metal ions on purified sucrose synthase

Ions (2 mM)	Enzyme activity (% control)	Stimulation (%)	Inhibition (%)
Control	100.0		
K ⁺	111.9	11.9	-
Na ⁺	109.7	9.7	-
Mn ²⁺	61.5	-	38.5
Mg ²⁺	84.5	-	15.5
Ca ²⁺	98.7	-	01.3
Cl ⁻	98.7	-	01.3
HCO ₃ ⁻	112.9	12.9	-
CH ₃ COO ⁻	83.9	-	16.1
NO ₃ ⁻	120.8	20.8	-
CO ₃ ²⁻	109.9	9.9	-

(1964). The subunit molecular weight, as determined by SDS-PAGE, was estimated to be 63 kDa (Fig. 2B), indicating the tetrameric configuration of sucrose synthase and single band on SDS-PAGE suggested the homomeric structure of sucrose

synthase. The homomeric structure is generally connected with high stability of enzyme, which is turn is prerequisite for the proper functioning of the enzyme (Bergandahi and Marsh, 2017). The sucrose synthase purified from rice was homooctameric enzyme

Fig. 2 : Determination of molecular weight of purified sucrose synthase through A- Sephadex G-100, B- SDS-PAGE

from rice grains with molecular weight 235 kDa (Singal *et al.*, 2009). The variations reported in the observed molecular weight values may be due to different methods employed for these determinations and different plant species used.

The environment surrounding the enzyme is essential for its stability and function. The enzyme activity as determined over a range of pH 4.0-8.0 (Fig. 3A) increased from 84.77 Units mg^{-1} protein to 270.25 Units mg^{-1} protein at pH 4.0 and 6.5, respectively. Thereafter, enzyme activity decreased which clearly indicates that pH 6.5 is optimum purified sucrose synthase. This pH value is in conformity with the report for the enzyme from rice grains (Singal *et al.*, 2009), which has pH optima of 6.5 in the direction of UDP-glucose synthesis. The difference observed has been related to some conformational changes in enzyme, which alter its kinetic properties.

The sucrose synthase showed maximum activity at 37°C and lesser activity above and below this temperature (Fig. 3B). The enzyme purified during the present investigation was stable upto 50°C over a pre-incubation period of 30 min, and at higher temperature, the enzyme underwent rapid inactivation (Fig. 2C). At 65°C, the enzyme lost 75% of its activity. Similarly, the enzyme purified from tomato (Sun *et al.*, 1992), rice grains (Elling and Kula, 1995) and sugar beet (Klotz *et al.*, 2003) had thermostability between 50-60°C. The loss in the activity above the temperature range could be due to distortion of 3-D native structure of the enzyme.

Out of all different nucleotide substrates tested at 1 mM concentration and saturating concentration of sucrose (25 mM), sucrose synthase activity was highest with UDP (Table 2). None of the other nucleoside diphosphate tested was as active as UDP,

Fig. 3 : (A) pH Activity profile, (B) Temperature activity profile and (C) Temperature stability of purified sucrose synthase from wheat grains. Values are mean of three replicates $\pm$ SD from triplicate determinations

which is in consistent with earlier reports for the enzyme from wheat and rice grains (Huang and Wang, 1998; Singal *et al.*, 2009), showing higher affinity of enzyme for UDP as substrate. Although, ADP specific sucrose synthase have also been reported from spinach leaves (Pozueta-Romero *et al.*, 1991).

Fig. 4 : (A) Determination of Michaelis-Menten constant (K_m) for purified sucrose synthase as a function of sucrose concentration and (B) UDP concentration

Sucrose synthase followed typical Michaelis-Menten kinetics with K_m values of 14.28 mM for sucrose (Fig. 4A) and 1.18 mM for UDP (Fig. 4B). Similar, hyperbolic saturation curves and high K_m values for sucrose have been reported for the enzyme purified from wheat and barley (Martinez *et al.*, 1993). The

Fig. 5 : Effect of varying concentration of sucrose and UDP on sucrose synthase activity in the presence of three different concentration of UDP (A) and sucrose (B). Values are mean of three replications $\pm$ SD

effectiveness of sucrose cleavage reaction prominent at a higher concentration of sucrose and UDP is characteristic of developing grains. A plot of reciprocal concentrations of UDP vs reciprocal of reaction rates at fixed sub-saturating (10 mM and 15 mM) and saturating (25 mM) concentrations of sucrose gave a set of parallel lines (Fig. 5A), indicating that the enzyme follows ping-pong type of bisubstrate-biproduct mechanism. Similarly, a double reciprocal plot of sucrose concentrations vs reaction rates at sub-saturating (0.5 mM and 0.8 mM) and saturating (1 mM) concentration of UDP again gave a set of parallel lines (Fig. 5B), confirming the view that the enzyme follows ping-pong mechanism.

The effect of various metabolites was studied on sucrose synthase activity. The results presented in Table 3 indicate that the enzyme activity was negligibly affected by metabolites at low concentration (1 mM). At 5 mM concentration AMP, ADP, NADP⁺

and G-6-P inhibited the enzyme activity by approximately 26, 12, 16 and 34%, respectively. NADP⁺ and G-6-P also inhibited the enzyme activity by about 4 and 13% at 2 mM concentration. Similar result for NAD⁺, NADPH and G-1-P has also been found in soybean (Morell and Copeland, 1985). The activity of this enzyme *in vivo*, therefore, is more likely to be controlled by substrate availability than by other type of allosteric modulation.

The effect of various metal ions on sucrose synthase activity was studied by incubating the various cations and anions at a concentration of 2 mM each at pH 6.5 (Table 4). Mn²⁺, Mg²⁺, CH₃COO⁻ and SO₄²⁻ were found to be potent inhibitors, whereas, K⁺, Na⁺, HCO₃⁻, NO₃⁻ and CO₃²⁻ stimulated the enzyme activity. No particular effect of Ca²⁺ and Cl⁻ ions was observed on stimulation or inhibition of enzyme activity. The results compare well with the finding that has been reported for

sucrose synthase in cleaving direction (Tanase and Yamaki, 2000; Bruskova et al., 2009).

In the present study the purified sucrose synthase catalysed the sucrose cleavage by ping-pong mechanism with high specificity for UDP as substrate. Its activity is enhanced by metal ions such as K^+ , Na^+ , HCO_3^- , CO_3^{2-} and with maximum enhancement by NO_3^- , which represents the use of these metal ions for regulating the enzyme activity. The present study might help in understanding the function and properties of sucrose synthase that in turn would be beneficial for crop productivity in stress and signaling.

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