INHIBITION OF SUCROSE SYNTHETASE CLEAVAGE ACTIVITY BY PROTEIN FACTORS

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1. Introduction

Sucrose synthetase (UDP-glucose: D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13) catalyzes the synthesis and cleavage of sucrose. It is the only transglycosylation reaction involving sugar nucleotides that is readily reversible. The enzyme shows a wide specificity for the nucleoside base, unlike most enzymes of sugar nucleotide metabolism which are specific for a particular base [1]. The enzyme exhibits differences in some properties between the forward and backward reaction: different cleavage and synthesis pH optima[2–5], buffers [2–6], temperature [6] and trypsin action [7] as well as activation or inhibition by mercaptoethanol [5], metal ions [5–8,9], phenylglycosides [10] and ATP [9–11].

Evidence has been presented indicating that when the enzyme was preincubated with oxidized glutathione or oxidized thioredoxin, sucrose cleavage was inhibited whereas sucrose synthesis was still proceeding at a normal rate [12]. Sucrose cleavage could be restored by incubation with dithiothreitol or reduced glutathione. This did not necessarily indicate that either glutathione or thioredoxin were the natural agents affecting the activity of the enzyme.

This paper reports the isolation of protein factors from wheat seeds that inhibit sucrose synthetase cleavage activity without affecting the sucrose synthesis activity. They have been named cleavage inhibitory proteins (CIP) I, II and III. One of them was further purified and some of its properties were studied. CIP-III affects sucrose synthetase by changing its affinity for UDP. Sucrose synthetase in the presence on CIP-III shows sigmoideal instead of hyperbolic saturation curves. The protein factor behaves like a negative modulator.

2. Materials and methods

All chemicals were purchased from Sigma (St Louis MO). Wheat seeds (*Triticum aestivum* L. cv. 'San Agustín INTA') were supplied by the Instituto Nacional de Tecnología Agropecuaria (Balcarce). Sucrose synthetase from wheat germ was obtained completely free of sucrose phosphate synthetase activity as in [13] and was further purified by passage through a Bio-Gel A-1.5 m column [12].

2.1. *Determination of enzyme activities*

Sucrose synthetase was assayed in the direction of sucrose synthesis by incubating in 0.05 ml total vol. 0.5 μmol fructose, 0.25 μmol UDP-Glc, 5 μmol Tris–HCl buffer (pH 8.0), enzyme and fractions to be tested at 30°C. The reaction was stopped by adding 0.2 ml 0.5 M NaOH and heating in a waterbath at 100°C for 10 min [14]. The sucrose formed was determined by the thiobarbituric acid method in 0.9 ml total vol. [15]. Sucrose cleavage (fructose formation) was assayed by incubating in 0.05 ml total vol., 10 μmol sucrose, 0.25 μmol UDP, 5 μmol Hepes buffer (pH 6.5), enzyme and fractions to be tested at 30°C. The reaction was stopped by adding 0.2 ml 0.1 M Tris–HCl buffer (pH 8.0) and heating in a waterbath at 100°C for 1 min. The fructose formed was measured by coupling hexokinase, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase, and following spectrophotometrically the appearance of NADPH [1]. The coupled assay was not affected by the presence of any of the inhibitory protein factors. Activity measurements were carried out under conditions where velocity was linear with time and amount of enzyme.

One unit of enzyme (synthesis direction) is defined

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as the amount which catalyzes the formation of 1 μmol sucrose/min at pH 8.0. One unit of enzyme (cleavage direction) is defined as the amount which catalyzes the formation of 1 μmol fructose or UDP-Glc/min, at pH 6.5.

Because of unspecific inhibitory substances present during the early stages of purification and because of the enzyme was affected differently by each inhibitory protein factor, it has not been feasible to quantitate in absolute terms the activity of the 3 cleavage inhibitory proteins through the purification procedure. Accordingly no specific activity measurements of the protein factors are included below.

2.2. *Isolation of protein factors*

Unless otherwise stated, all manipulations were carried out around 4°C and centrifugations were performed at 15 000 × *g*. Wheat seeds (200 g) soaked overnight in distilled water, were homogenized in 350 ml 25 mM Tris–HCl buffer (pH 8.0) in a Waring blendor for 2 min. The homogenate was filtered through 4 layers of cheese cloth and was adjusted to pH 4.5 by addition of 2 M formic acid. After centrifugation, the precipitate formed was removed and the supernatant was neutralized to pH 7.6 with 1 M ammonia. Solid ammonium sulfate was added to give 40% saturation. The precipitate formed was removed by centrifugation and the ammonium sulfate concentration of the supernatant increased to 80%. The precipitate was allowed to settle 5 h and collected by centrifugation for 20 min. The precipitate was dissolved in 25 mM Tris–HCl buffer (pH 8.0) and dialyzed overnight against 10 mM Tris–HCl buffer (pH 8.0), 1 mM EDTA. The dialyzed protein solution was applied to a DEAE-cellulose column 2 × 8 cm equilibrated with 100 mM potassium phosphate buffer (pH 7.0) and then washed with 1 mM EDTA. The proteins were eluted with 400 ml of a linear gradient of potassium phosphate buffer (pH 7.0) from 20–200 mM containing 1 mM EDTA. Fractions of 2.5 ml were collected every 12 min. All fractions were analyzed for sucrose synthetase cleavage inhibitory activity. The fractions with this activity were pooled and concentrated in an Amicon ultrafiltration cell with a PM-10 membrane. Gel filtration was carried out in columns of Bio-Gel P-150 1 × 43 cm equilibrated with 10 mM Hepes buffer (pH 6.5). Proteins were eluted with the same buffer and fractions (0.45 ml) were collected every 30 min and analyzed as for the DEAE-cellulose chromatography. Fractions with cleavage inhibitory activity were pooled, lyophilized and finally dissolved in water.

3. Results and discussion

Seed extracts were submitted to acid precipitation and ammonium sulfate fractionation as in section 2. Chromatography of the ammonium sulfate fraction on DEAE-cellulose columns produced an elution profile that indicated the presence of 3 peaks with cleavage inhibitory activity (fig.1A). The pattern was very reproducible and was not modified if the protein solution applied to the column was preincubated with 10 mM mercaptoethanol or the elution was carried in the presence of the same concentration of mercaptoethanol, a procedure used to avoid the formation of aggregates [16]. The pooled active fractions under each peak were analyzed for their capacity to inhibit sucrose cleavage and for their influence in sucrose synthesis. At this stage of the purification, the inhibition

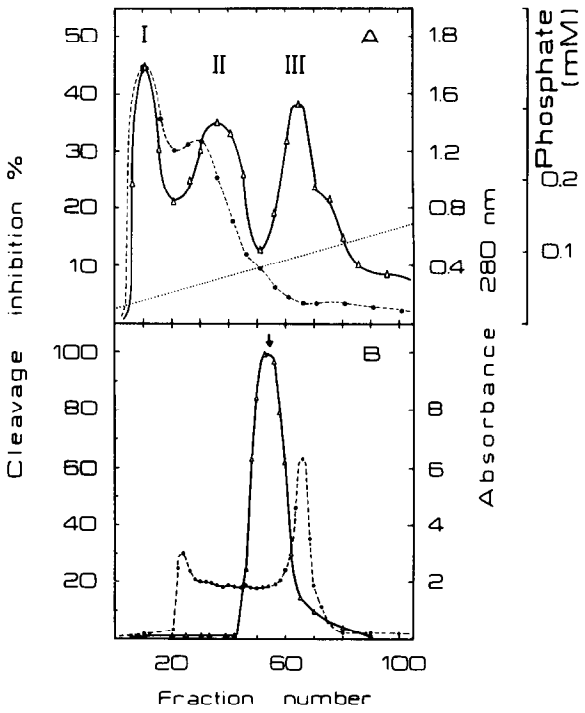

Fig.1. (A) Chromatography of ammonium sulfate fraction (40–80%) on DEAE-cellulose: sucrose synthetase cleavage inhibition (△——△); absorbance at 280 nm (●– – –●); phosphate gradient (. . .). (B) Gel filtration of peak III from DEAE-cellulose chromatography on BIO Gel P-150. The arrow indicates the position of cytochrome *c*; details in section 2.

Table 1
Effect of the pooled fractions under each peak of the DEAE-cellulose column on sucrose synthetase activity (sucrose synthetase, 600 μg/assay)

Additions	Cleavage (μmol fructose formed/10 min)	Inhibition (%)	Synthesis (μmol sucrose formed/10 min)	Activity (%)	Cleavage / Synthesis
None	45	0	28.5	100	1.6
Pool I					
220 μg	41	9	30	105	1.4
440 μg	36.3	19.4	29	102	1.2
Pool II					
36 μg	34.4	23.6	33	116	1.0
72 μg	22.8	49.4	33	116	0.7
Pool III					
16 μg	37.6	16.4	31	109	1.2
32 μg	28.9	35.8	30	105	0.9

produced was linearly related to the protein amount present under each peak, provided that the inhibition did not exceed 50% (table 1). The data indicated that the substances not only did inhibit cleavage but also produced a slight activation (10–15%) of sucrose synthesis. These changes are more clearly seen observing the cleavage/synthesis ratio. The substances were hereafter named CIP I, II and III. CIP-III, as judged by the protein elution profile, was the purest and it was selected for further purification. Gel filtration on Bio-Gel P 150 (fig.1B) resulted in the isolation of a substance with an elution behavior similar to that of cytochrome *c* (M_r 13 000). Fractions with the highest inhibitory activity were pooled and concentrated.

The effect of CIP-III on sucrose cleavage and synthesis can be seen in fig.2. A complete suppression of cleavage activity was obtained when sufficient amount of CIP-III was added to the enzyme. Under this condition, a small activation of synthesis was always observed. The amount of CIP-III was linearly related to the rate of cleavage inhibition up to an inhibition of ~40%, but no absolute relationship could be established between the amount of CIP-III and of sucrose synthetase, on account that the enzyme used was not homogenous.

The inhibitory effect of CIP-III on sucrose cleavage could be eliminated if it was heated for 10 min at 100°C, or treated with 2 M trifluoroacetic acid, or submitted to the action of trypsin for 20 h. The effect of CIP-III on sucrose cleavage is similar to the effect reported to be produced by oxidized thioredoxin or oxidized glutathione [12]. However, the effect of CIP-III was affected neither by preincubation with the enzyme nor by the incubation with sucrose or UDP. This is a clear difference between the effect of thioredoxin on the enzyme activity that required preincubation to obtain full inhibition, and the action of CIP-III.

The changes reported here, affecting the reaction catalyzed by sucrose synthetase, would require modification in both V_{max} and K_m for substrates and products so that the equilibrium constant remained invariable. An investigation of the effect of CIP-III on

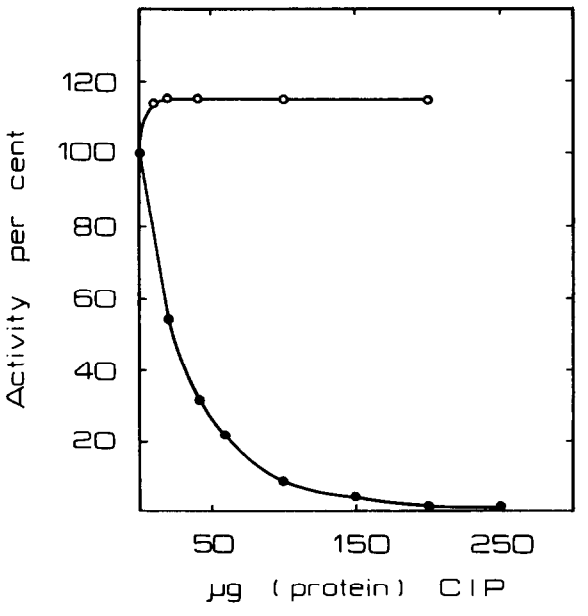

Fig.2. Inhibition of cleavage and stimulation of synthesis by CIP III on sucrose synthetase: sucrose synthetase, 350 μg; synthesis (o——o); cleavage (•——•).

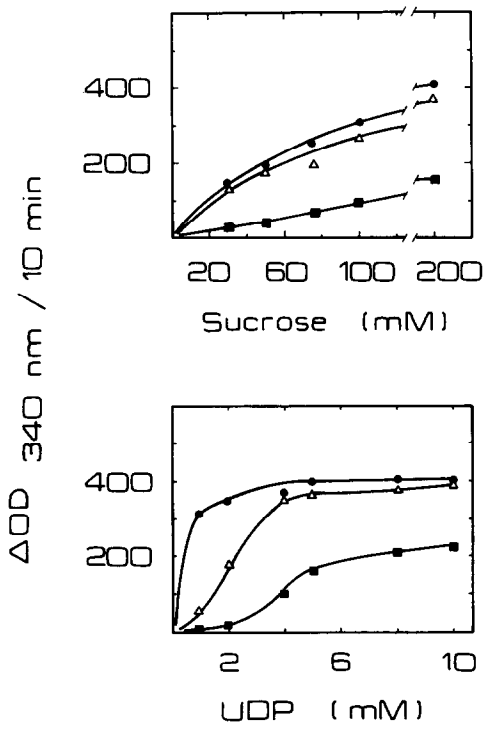

Fig.3. The effect of CIP III on the affinity of sucrose synthetase for sucrose (A) and for UDP (B): No addition (•); CIP-III 12 μg (△); CIP-III 30 μg (■).

the affinity of the enzyme for UDP and sucrose can be seen in fig.3. CIP-III changed the enzyme affinity for UDP only.

In the presence of CIP-III the activity of sucrose synthetase fitted a sigmoidal curve (Hill coefficient 3, $S_{0.5}$ for UDP 20 mM) while the uninhibited enzyme exhibited hyperbolic kinetics (Hill coefficient 0.95, K_m for UDP 0.6 mM). CIP-III did not affect K_m for sucrose but did modify V_m. This is the first report of a protein factor affecting the activity of sucrose synthetase.

These findings agree with the few cases reported of reversible reactions that are unidirectionally inhibited [17]. In the cases of glutamate dehydrogenase from *Blastocladia emersonii* [18] and formate kinase from *Clostridium cylindrosporum* [19], various ligands inhibit the direct reaction but have no effect on the initial rate of the reverse reaction. The apparent irreversibility is probably achieved by allosteric effects of the various ligands which lead to a decreases affinity of the enzyme for one substrate [18].

The inhibition of sucrose cleavage by CIP III fits closely this pattern and indicates the existence of a regulatory mechanism controlling the activity of sucrose synthetase. Many queries are raised by the discovery of the cleavage inhibitory proteins, especially regarding the metabolic role of sucrose synthetase. Work on this problem, on the full characterization of CIP-III, as well as on the purification of the other cleavage inhibitory proteins and their interrelation is in progress.

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