EFFECT OF COLD ADAPTATION OF PUMA RYE ON PROPERTIES OF RUDP CARBOXYLASE[1,2]

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Summary

The enzyme from leaves of cold-hardened rye plants was: (1) more stable between pH 7.75 and 8.3 (2) more stable at -25°C (3) less sensitive to SDS and (4) more sensitive to urea than the same enzyme from unhardened plants. The molecular weights of the purified enzymes from the two sources were the same but their charges differed.

Introduction

In an electrophoretic survey of soluble proteins and membrane polypeptides extracted from chloroplasts of hardened and unhardened plants of cold-hardy and cold-sensitive cultivars of wheat and rye, we observed that the RUDP carboxylase (EC 4.1.1.39) fraction from a Sephadex G-50 column contained one protein band in all cold-hardened material and two in all unhardened material (1). Changes in the invertase isozyme complement of wheat in response to cold-hardening have been reported by Roberts (2) and support his hypothesis that isozymic substitution is important in the cold-hardening process (3). We now report several preliminary steps taken to elucidate the change in RUDP carboxylase that occurs during cold hardening.

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[2] Abbreviations: RUDP carboxylase = Ribulose-1,5-diphosphate carboxylase; Tris = Tris (hydroxymethyl) amino methane; RH = hardened Puma rye; RNH = unhardened Puma rye; Vo = void volume; Ve = elution volume; LDH = lactate dehydrogenase; SDS = sodium dodecyl sulfate.

Materials and Methods

Rye (Secale cereale L. cv. Puma) was used throughout this work as the source of RUDP carboxylase. Conditions used for germination, growth at cold-hardening and non-cold-hardening temperatures and the extraction of soluble proteins of chloroplasts from Puma rye were described previously (1). The cold-hardened plants survived temperatures down to -30°C, in contrast with the unhardened sensitivity to -6°C, according to tests in which the temperature was lowered 1°C per hour (4).

Crude RUDP carboxylase was prepared by macerating leaves in 20 mM Tris-HCl (pH 8.1) buffer containing 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol (5 ml buffer/gm fresh wt) at 0°C in a Servall Omnimixer at full speed for 30 seconds. The brei was removed by filtration through 8 layers of cheesecloth after which the filtrate was centrifuged twice at 35,000 x g for 15 minutes. All the RUDP carboxylase activity of the supernatant precipitated at 0°C for 30 min. in the 25-50% $(NH_4)_2SO_4$ fraction (5,6) and the precipitate was stored as a precipitate at -25°C.

For further purification, the 25-50% $(NH_4)_2SO_4$ fraction was dissolved in buffer containing 20 mM Tris-HCl (pH 8.1), 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol. The sample was dialyzed against the same buffer, applied to the top of a Sephadex G-100 column (1.5 x 100 cm) and eluted with the above buffer. Fractions containing RUDP carboxylase activity were pooled, concentrated by ultrafiltration (1), applied to the top of a Sephadex G-200 column (1.5 x 100 cm), and eluted as described for the G-100 column. Fractions containing RUDP carboxylase activity from the second column were pooled and stored as a 50% $(NH_4)_2SO_4$ precipitate at -25°C.

The molecular weights of RUDP carboxylase from hardened and unhardened rye were compared by Sephadex G-200 chromatography (1.5 x 100 cm) using 20 mM Tris-HCl (pH 8.1) containing 1 mM $MgCl_2$ and 1 mM β-mercaptoethanol as the eluting buffer. The elution volumes of the two enzymes were compared to those of proteins of known molecular weights. The void volume was 37.0 ml as determined by using Blue Dextran 2000 (7).

Table I. Effect of pH of the Extraction Medium on Ribulose-1,5-Diphosphate Carboxylase Activity

Extraction Medium	Final pH	RuDP Carboxylase Activity (μmoles HCO_3^- fixed/mg protein/hr.) RH	RNH
*20mM Tris-HCl	8.30	2.48	2.40
*20mM Tris-HCl	7.75	2.10	0.48
Distilled H_2O	7.75	2.04	0.30

*Tris-HCl extraction medium also contained 1mM $MgCl_2$ and 1mM β-mercaptoethanol.

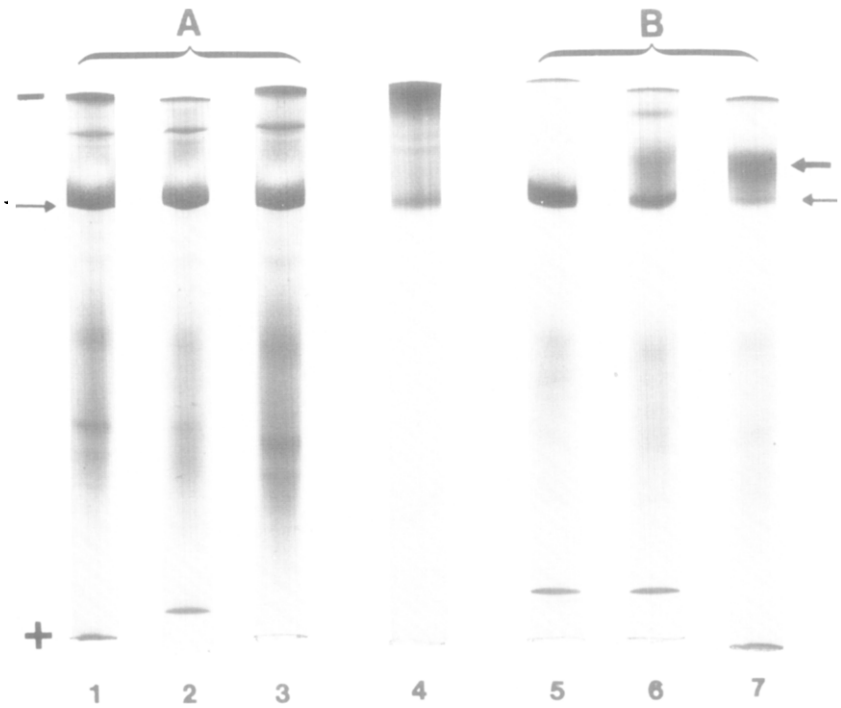

Figure 1. Photograph illustrating the results obtained when the soluble extracts of chloroplasts from hardened (A) and unhardened (B) Puma rye were electrophoresed on 6% polyacrylamide gels. Protein was stained with amido black. Small arrow marks position of RUDP carboxylase. Large arrow marks the position of the large diffuse band. The gels represent the electrophoretic patterns of the soluble extracts obtained when chloroplasts were extracted in distilled H_2O (final pH 7.75) (1 and 7), 20 mM Tris-HCl (pH 7.75) - 1 mM $MgCl_2$ - 1 mM β-mercaptoethanol (2 and 6), and 20 mM Tris-HCl (pH 8.3) - 1 mM $MgCl_2$ - 1 mM β-mercaptoethanol (3 and 5). Gel 4 represents purified RUDP carboxylase from unhardened rye.

RUDP carboxylase was assayed at 25°C in the presence and absence of denaturants by the incorporation of $|^{14}C|$ $NaHCO_3$ into acid stable products (8). The reaction was linear for at least ten minutes from which initial rates were calculated.

Electrophoresis was performed on polyacrylamide gels according to the method of Davis (9) and the proteins were stained with Amido Black.

Protein was determined by the method of Lowry (10).

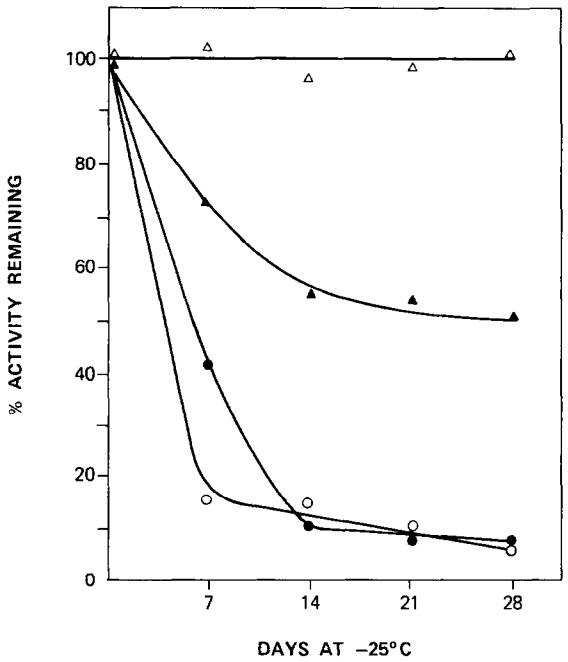

Figure 2. Stability of RUDP carboxylase activity to -25°C. The 25-50% $(NH_4)_2SO_4$ fraction was prepared from hardened and unhardened Puma rye leaves as described in Methods. This fraction from hardened rye was stored as a precipitate (Δ) and in solution (o) at -25°C; similarly for unhardened rye: as a precipitate (▲) and in solution (●). The solutions contained the 25-50% $(NH_4)_2SO_4$ fraction dissolved in 20 mM Tris-HCl (pH 8.1) -1 mM $MgCl_2$ -1 mM β-mercaptoethanol. Enzyme activity was determined as described in Methods.

Results

When RUDP carboxylase was assayed in the total soluble extracts form isolated chloroplasts from hardened and unhardened Puma rye, highest activity was obtained with 20 mM Tris-HCl, pH 8.3 as the extracting buffer (Table 1). The enzyme from hardened rye extracts lost 18% of its activity when extracted into distilled water (final pH of 7.75) and 10% when extracted into 20 mM Tris-HCl at pH 7.75. However, RUDP carboxylase from unhardened rye extracts lost 80-90% of its activity when extracted into either distilled water or 20 mM Tris-HCl buffer at pH 7.75.

Electrophoresis of all hardened rye extracts on 6% polyacrylamide gels

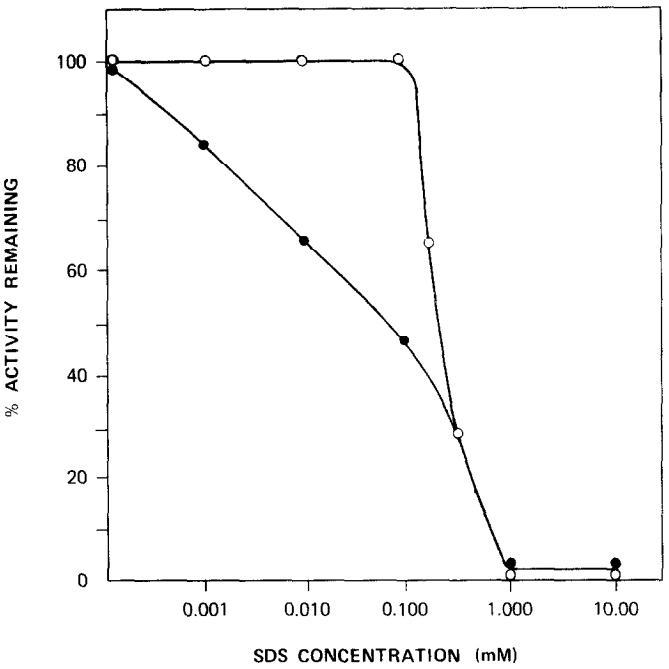

Figure 3. The effect of SDS on RUDP carboxylase activity. The 25-50% $(NH_4)_2SO_4$ fractions from hardened (o) and unhardened (•) Puma rye were dissolved in 20 mM Tris-HCl (pH 8.1) -1 mM $MgCl_2$ -1 mM β-mercaptoethanol and RUDP carboxylase activity assayed, in the presence of varying SDS concentrations, as described in Materials and Methods. In each case, 0.040 mg of protein was used.

showed one protein band for RUDP carboxylase (Fig. 1A, small arrow). Unhardened rye chloroplasts extracted into 20 mM Tris-HCl, pH 8.3 showed one protein band for RUDP carboxylase (Fig. 1B, 5). However, a large diffuse band (Fig. 1B, large arrow) similar to the one described previously (1) preceded the RUDP carboxylase band (Fig. 1B, small arrow) only in unhardened samples extracted from chloroplasts either in distilled water or 20 mM Tris-HCl buffer at pH 7.75. This band, therefore, correlates with the loss in RUDP carboxylase activity.

The activity of crude RUDP carboxylase was measured during storage as a 50% $(NH_4)_2SO_4$ precipitate at -25°C. The activity of the preparation from the hardened source remained constant over a period of 28 days (2.8 μmoles HCO_3^- fixed/mg protein/hr) while that from the unhardened source decreased by 50%

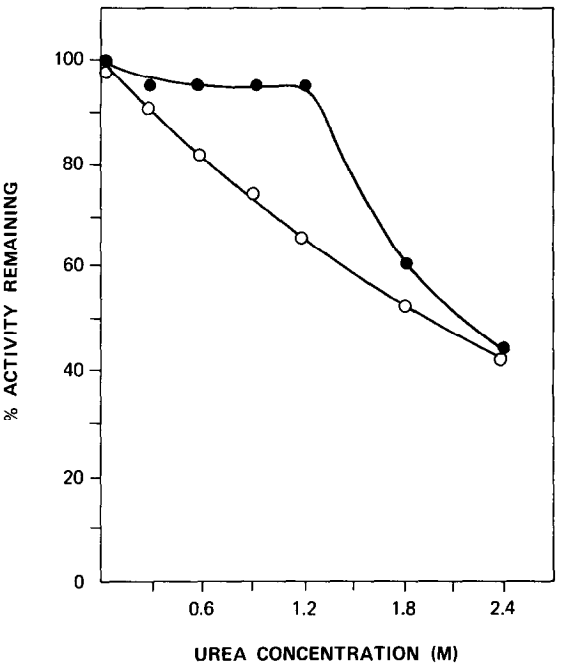

Figure 4. The effect of urea on RUDP carboxylase activity. The 25-50% $(NH_4)_2SO_4$ fractions from hardened (○) and unhardened (●) Puma rye were dissolved in 20 mM Tris-HCl (pH 8.1) -1 mM $MgCl_2$ -1 mM β-mercaptoethanol and RUDP carboxylase activity assayed, in the presence of varying urea concentrations, as described in Materials and Methods. In each case, 0.044 mg of protein was used.

(2.48 to 1.24 μmoles HCO_3^- fixed/mg protein/hr) in this time (Fig. 2). When the crude enzymes from both sources were dissolved in 20 mM Tris-HCl, pH 8.1 containing $MgCl_2$ and β-mercaptoethanol at 1 mM, they lost 85% of their activities after 14 days (Fig. 2). Therefore, RUDP carboxylase from hardened rye is more stable as a precipitate at -25°C than that from the unhardened source. Furthermore, Fig. 2 shows that the greater stability of the former was not due to the presence of a natural cryoprotectant (11) since both hardened and unhardened enzymes showed similar sensitivity to freezing at -25°C when dissolved in buffer alone.

The stabilities of the crude RUDP carboxylase preparations towards two denaturing agents were also tested (Fig. 3). In the presence of 0.1 mM SDS,

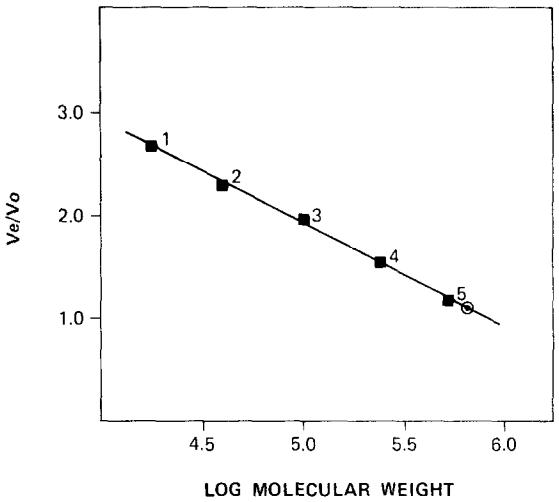

Figure 5. Molecular wt. determination of hardened (o) and unhardened (•) Puma rye RUDP carboxylase was by Sephadex G-200 chromatography as described in Materials and Methods. Standard proteins ( ) used were 1, myoglobin (MW 17,800); 2, ovalbumin (MW 45,000); 3, hexokinase (MW 99,000); 4, catalase (MW 240,000); 5, apoferritin (MW 480,000).

when SDS/protein was 0.23, the crude enzyme from hardened rye retained full activity but that of unhardened rye was 55% less. When SDS/protein was greater than 0.23, crude RUDP carboxylase from hardened rye lost considerable activity and at 1 mM SDS (SDS/protein = 2.3) both preparations were completely inhibited. Therefore, the enzyme from the hardened source was more stable towards low concentrations of SDS.

The hardened preparation was, however, less stable towards urea (Fig. 4). RUDP carboxylase activity from the hardened source decreased steadily as the concentration of urea was increased up to 2.4 M (urea/protein = 655) at which point 58% of the original activity had been lost. The enzyme from unhardened rye was relatively insensitive to urea up to a concentration of 1.2 M (urea/protein 327.5) above which activity fell off to 55% at 2.4 M.

Preliminary experiments on the physical properties of the enzymes were performed. After purification of the crude enzymes as described in Materials

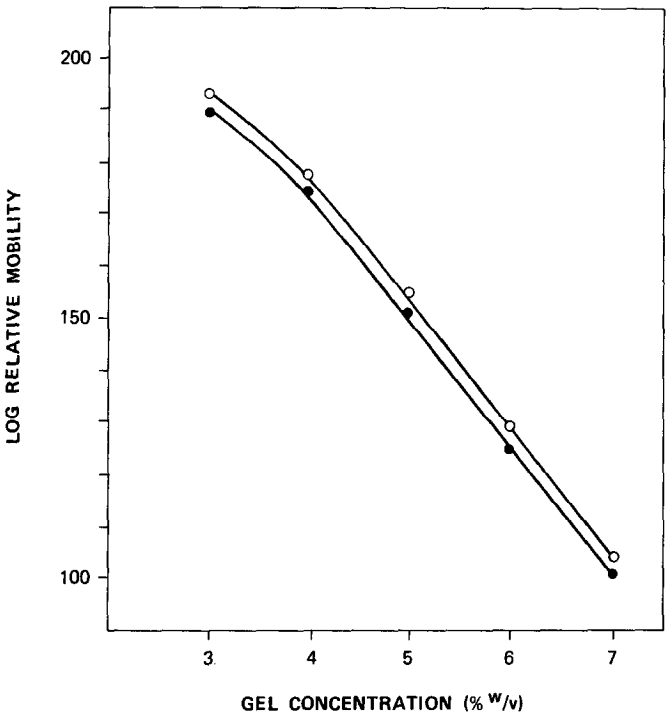

Figure 6. Determination of relative size and charge of RUDP carboxylase from hardened (o) and unhardened (•) Puma rye. The 25-50% $(NH_4)_2SO_4$ fractions were dissolved in 20 mM Tris-HCl (pH 8.1) -1 mM $MgCl_2$ -1 mM β-mercaptoethanol and electrophoresed (120 μg protein/gel) on 3,4,5,6 and 7% gels as described in Materials and Methods. Proteins were stained with amindo black. Relative mobility was measured as described by Hendrick and Smith (13).

and Methods, the molecular weights of RUDP carboxylase from hardened and unhardened rye were found to be the same by Sephadex G-200 chromatography (Fig. 5). The molecular weights were estimated to fall in the range of 500,000 to 600,000 in agreement with that found for the same enzyme from other sources (12).

According to the criteria established by Hendrick and Smith (13) RUDP carboxylase from hardened and unhardened rye was of the same molecular weight but differed in charge (Fig. 6).

Discussion

The previously reported extra band of protein in the RUDP carboxylase

Table II. Comparison of the Properties of RUDP Carboxylase from Hardened and Unhardened Rye

Conditions	RUDP Carboxylase from hardened rye
pH < 8	more stable
Precipitate, -25°C	more stable
SDS denaturation	more stable
urea denaturation	less stable
Molecular weight	same
charge	more negative

fraction from chloroplasts of unhardened rye and wheat was apparently an inactive artefact of extraction at pH less than 8. The fact that the stability of the same enzyme was changed by the temperature at which the plants were grown was established by several additional criteria (Table II). There was a change in charge with no concommitant change in the size of RUDP carboxylase during adaptation to low temperature. This was in contrast to Roberts' observations (3,14,15) that changes occur in the ratios of isozymes (that differ considerably in molecular weight according to Sephadex chromatography) of several enzymes from wheat leaves as a result of cold-hardening. Isozymes which differ in charge but not size are already known (13). Of particular pertinence is the observation of changes similar to those reported here in LDH from the foot of fresh water clams during cold adaptation (Hulbert, Moon and Huner, unpublished results). Until further measurements reveal whether or not we are dealing with varying degrees of folding of a single native protein our results indicate that RUDP carboxylase exists as at least two charge isomers the presence of which depends on the temperature at which it was synthesized.

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