Proteolytic degradation of barley ribulose-1,5-bisphosphate carboxylase/oxygenase and recognition of the fragments by monoclonal antibodies

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Abstract

Limited proteolysis of barley ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) was effected by treatments with trypsin and *Staphylococcus aureus* strain V8 protease. Treatment of native RuBPCO with proteases resulted in the degradation of the large subunit (LS) of the enzyme. Trypsin cleaved three fragments from the LS but the *S. aureus* strain V8 protease cleaved only one. The small subunit (SS) was not affected. In the presence of 0.5 % sodium dodecyl sulfate, RuBPCO degraded into several fragments; some of them were fairly stable. Monoclonal antibodies (Mabs) against barley RuBPCO were applied in immunoblotting analysis to distinguish which of the fragments were recognized. All the Mabs recognized the fragments with molecular masses close to those of the LS. Differences among Mabs were observed in the fragments with low molecular mass.

Additional key words: *Hordeum vulgare* L.; limited proteolysis; monoclonal antibodies; RuBPCO.

Introduction

Limited proteolysis is one of the methods for characterization of proteins and polypeptides and identification of some individual proteins in complex mixtures (Cleveland *et al.* 1977, Bottomley 1982), for investigation of the structural changes related to some enzymes' functions (Gutteridge *et al.* 1986, Houtz and Mulligan

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Abbreviations: ATP - adenosine-5'-triphosphate; DEAE - diethylaminoethyl; EDTA - ethylene diamine tetraacetic acid; LS - large subunit; Mab - monoclonal antibody; PMSF - phenyl-methyl sulphonyl fluoride; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; SDS-PAGE-sodium dodecyl sulphate polyacrylamide gel electrophoresis; SS - small subunit.

1991), and for characterization and location of specific regions and sites in the primary protein structure (epitopes) recognized by monoclonal antibodies (Sueyoshi *et al.* 1988, Xu and Van Huystee 1991). However, little is known about the fragmentation of RuBPCO by endogenous and exogenous proteases. More sensitive to proteolysis is RuBPCO LS, and the characteristic pattern of *in vivo* degrading fragment from RuBPCO is different depending on experimental conditions (Mae *et al.* 1989, Mitsuhashi *et al.* 1992, Hildbrand *et al.* 1994, Desimone *et al.* 1996). Under limited proteolysis of native spinach RuBPCO with trypsin, two short peptides were released from the N-terminus and one from the C-terminus of the LS (Mulligan *et al.* 1988). Denatured RuBPCO from spinach is digested by different proteases (chymotrypsin, papain, and *S. aureus* V8 protease), and the large subunit of the enzyme generates about 15–25 bands (Bottomley 1982). Less attention has been paid to the fragmentation of RuBPCO from barley. Barley RuBPCO is less sensitive to trypsinolysis compared to wheat and spinach RuBPCO (Gutteridge *et al.* 1986). The characterization of Mabs against RuBPCO applying limited proteolysis of the enzyme has not been made so far. Monoclonal antibodies against RuBPCO from barley leaves have been produced and partially characterized (Mladjova *et al.* 1992). All the Mabs recognize only the LS in Western blotting after SDS electrophoresis, suggesting sequential but not conformational type of the recognized epitopes.

In the present paper an attempt was made to more thoroughly characterize the epitopes on RuBPCO molecule recognized by the Mabs, using the limited proteolysis.

Materials and methods

Plants: Pre-soaked seeds of *Hordeum vulgare* L. were germinated and grown under a 12 h day with 200 μmol(photon) m^{-2} s^{-1} at 27 °C during day and 25 °C at night. After 10 d, when the primary leaves had fully expanded, they were harvested, and rapidly frozen in liquid nitrogen.

RuBPCO extraction and purification was done by a modified method described in Demirevska-Kepova and Simova (1989). Leaves were ground to a fine powder with a pre-chilled mortar and a pestle, and homogenized (1:4 m/v) in an ice-cold 100 mM Tris-HCl (pH 8.0) buffer containing 20 mM $MgCl_2$, 20 mM $NaHCO_3$, 1 mM EDTA, 20 mM β-mercaptoethanol, 1 mM PMSF, 3 % m/v *Polyclar AT*, 1 % m/v ovalbumin, and 12.5 % v/v glycerol. The homogenate was centrifuged at 16 000×*g* for 30 min. The supernatant was fractionated with 35-55 % $(NH_4)_2SO_4$. The pellet was redissolved in 20 mM Tris HCl (pH 8.0) buffer containing 20 mM $MgCl_2$, 20 mM $NaHCO_3$, 1 mM EDTA, 20 mM β-mercaptoethanol, 5 mM ATP, and 12.5 % v/v glycerol, and desalted on a column of *Sephacryl S-200*. The RuBPCO enriched fraction was purified on a DEAE-*Sephacel* column. RuBPCO was eluted with 400 mM KCl, and further purified on a *Sephacryl S-300* column. All these procedures were carried out at 4 °C. Purified RuBPCO was stored under liquid nitrogen, and was characterized electrophoretically on 15 % SDS-PAGE.

Proteolytic degradation of RuBPCO: The purified enzyme (native RuBPCO) was activated by 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ for 30 min at 30 °C. RuBPCO was semi-denatured by 0.5 % m/v SDS in 0.1 M Bicine-NaOH (pH 8.2) with 20 mM β-mercaptoethanol and 10 % v/v glycerol. Native or semi-denatured RuBPCO was then proteolyzed with trypsin (500:1, m/m) or protease from *Staphylococcus* or *S. aureus* strain V8 (100:1, m/m) in 0.1 M Bicine-NaOH (pH 8.2) buffer containing 20 mM $MgCl_2$, 20 mM $NaHCO_3$, 20 mM β-mercaptoethanol, and 10 % v/v glycerol for 15-90 min at 30 °C. Proteolytic degradation was stopped by boiling the reaction mixture for 5 min after addition 1:1 (v/v) of 100 mM Tris-HCl buffer (pH 6.8) containing 2 % m/v SDS, 2 % v/v β-mercaptoethanol, 0.2 M EDTA, 10 % v/v glycerol, and 0.2 % m/v bromphenolblue in ethanol, and stored until electrophoresis at -20 °C.

Soluble protein content was determined by the method of Bradford (1976).

SDS-PAGE and immunoblotting: Polyacrylamide gel electrophoresis using 15 % separating and 5 % concentrating slab gels (1 mm thick) was performed according to Laemmli (1970). The resulting polypeptides were stained with Coomassie Brilliant Blue *G 250*. After 15 % SDS-PAGE, non-stained polypeptides were electrophoretically transferred onto nitrocellulose membranes. Immunoblotting was made as described by Mladjova *et al.* (1992). Polypeptides were visualized with 0.2 % 3,3'-diaminobenzidine in 100 mM Tris HCl (pH 7.2) buffer containing 150 mM NaCl, and after adding 0.02 % H_2O_2 (final concentration).

Results and discussion

Additional information about the limited proteolysis of RuBPCO from barley was obtained. Two proteases with different specificity (trypsin cleaving peptide bonds on the carboxyl side of lysine and arginine residues; *S. aureus* V8 protease attacking peptide bonds on the carboxyl side of aspartic and glutamic acid residues) were applied expecting different polypeptide profiles after proteolysis.

Degradation of native RuBPCO: After incubation of native RuBPCO with trypsin (Fig. 1*A*), bands corresponding to three fragments of the LS were obtained: one wide band with almost unchanged electrophoretic mobility, and two thinner bands with molecular masses between 57.2 and 42.9 kDa. After incubation of native RuBPCO with protease from *S. aureus* strain V8 (Fig. 1*B*) only one fragment with molecular mass near 42.9 kDa was found. It was slightly stained, and situated more distant from LS than the fragments obtained with trypsin treatment. The control was kept under the same conditions but without protease. The electrophoretic profiles of both control and variants contained additional polypeptide bands, probably cross-linked aggregates of LS and SS with molecular masses of about 110 and 26 kDa. Susceptibility of the RuBPCO LS to both protease actions was established. The SS in native RuBPCO remained apparently intact under the assay conditions. Evidently, the SSs were discriminated from proteolytic degradation when assembled into

holoenzyme. Such a differential susceptibility to proteolysis was also observed by Otto and Feierabend (1994). RuBPCO as a multimeric protein complex cannot be simply subjected to proteolytic degradation. Modification or denaturation of RuBPCO is the possible mechanism triggering protein degradation (Moreno *et al.* 1995, Desimone *et al.* 1996).

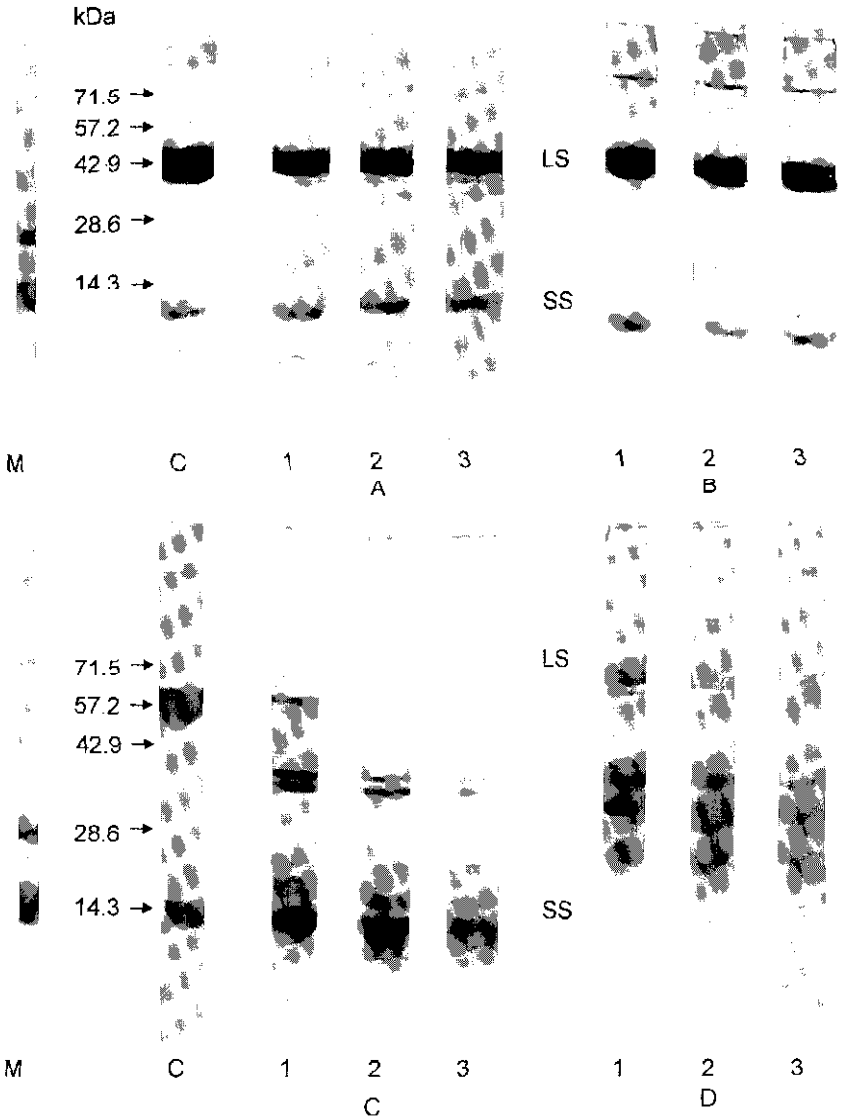

Fig. 1. SDS-PAGE analysis of native (*top*) and semi-denatured (*bottom*) RuBPCO after proteolysis with trypsin (500:1, m/m) for 15-60 min at 30 °C (*A, C*), and with *S. aureus* V8 protease (100:1, m/m) for 30-90 min at 30 °C (*B, D*). The polypeptides were stained with Coomassie brilliant blue G-250. The positions of the LS and SS of RuBPCO are indicated. Lanes: *C* - control without protease; *1, 2* and *3*(*A*) - digestions for 15, 30 and 60 min, (*B*) - digestions for 15, 30 and 90 min, respectively. *M* - molecular mass markers 14.3-71.5 (*Fluka*).

Degradation of semi-denatured RuBPCO: RuBPCO denatured by boiling tended to aggregate and in the presence of proteases the electrophoretic profiles contained poorly distinguishable polypeptide bands (results not shown). More distinct electrophoretic patterns were obtained using semi-denatured RuBPCO in the presence of 0.5 % SDS (Fig. 1*C,D*). Some of the fragments were stable during the incubation. After incubation of semi-denatured RuBPCO with trypsin (Fig 1*C*) two relatively stable fragments with molecular masses close to those of LS, two fragments with molecular masses between 57.2 and 42.9 kDa, and many overlapping fragments below 28.6 kDa were observed. Using the protease from *S. aureus* strain V8 (Fig. 1*D*) relatively stable fragments were also obtained but with different electrophoretic mobility (four fragments with molecular masses between 57.2 and 42.9 kDa, and four fragments between 42.9 and 28.6 kDa). Semi-denatured RuBPCO LS degraded into several fragments; some of them were relatively stable. Some bands with molecular masses lower than 14.3 kDa were observed, but we had no immunochemical evidences that they were cleaved from LS or SS. SDS seems to change the susceptibility of RuBPCO to proteases by changing its conformation (Mae *et al.* 1989). The relative stability of fragments obtained by exogenous proteases is similar to that of fragments produced by endogenous proteases (Hildbrand *et al.* 1994). When RuBPCO was in native state it was more stable towards the proteases used than the semidenatured one.

Immunoblotting of degraded RuBPCO: The obtained fragments were transferred onto nitrocellulose sheet by Western blotting, and were exposed to the four monoclonal antibodies (2G2, 2F4, 2D11, and 2H5) against RuBPCO (Fig. 2). When native RuBPCO was used in the presence of trypsin for 20 min, all the Mabs interacted with one wide polypeptide band related to LS and with RuBPCO LS aggregates with larger molecular masses (Fig. 2*A*). We suggest that the wide polypeptide band includes non-degraded LS and LS without two short peptides released from the N- and C-termini. Hence, the determinant groups could not be situated in those termini. Mabs 2F4 and 2D11 recognized seven fragments from semi-denatured RuBPCO with the same electrophoretic mobility for both Mabs (Fig. 2*B*, lanes *2* and *4*). These were likely stable determinant groups during proteolysis. Mab 2G2 recognized the same fragments as above except for one with molecular mass close to 28.6 kDa, which was missing (Fig. 2*B*, lane *1*). Mab 2H5 recognized two fragments close to LS, and only one fragment with molecular mass between 28.6 and 14.3 kDa (Fig. 2*B*, lane *3*).

All Mabs recognized only the LS when native RuBPCO was used in the presence of protease from *S. aureus* strain V8 (Fig. 2*C*). The results were similar to those obtained with native RuBPCO digested by trypsin. But the semi-denatured RuBPCO was recognized differently by the four Mabs (Fig. 2*D*). Mab 2G2 recognized four fragments with molecular masses between 28.6 and 14.3 kDa (Fig. 2*D*, lane *1*), Mab 2D11 reacted with three of them (Fig. 2*D*, lane *4*), Mab 2H5 with the two situated in the middle fragments (Fig. 2*D*, lane *3*), and Mab 2F4 with the two fragments closer to 28.6 kDa (Fig. 2*D*, lane *2*). Differences among Mabs appeared in the fragments with lower molecular masses as was the case of trypsin-digested semi-denatured RuBPCO.

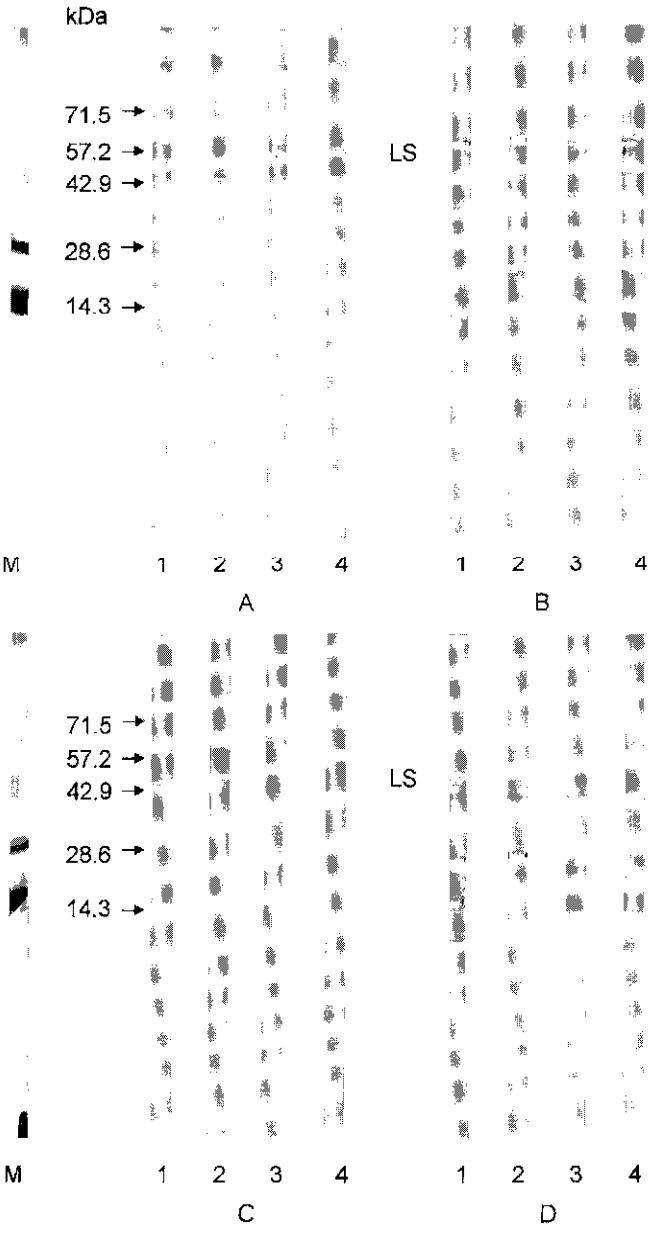

Fig. 2. Immunoblotting analysis of native (*A, C*) and semi-denatured (*B, D*) RuBPCO fragments obtained after proteolysis with trypsin (500:1, m/m) for 20 min (*A, B*) or with *S. aureus* V8 protease (100:1, m/m) for 40 min (*C, D*) at 30 °C revealed by Mabs against barley RuBPCO. The positions of the molecular mass markers 14.3-71.5 (*Fluka*) (*M*) are indicated at the left side. Lanes: *1* - Mab 2G2; *2* - 2F4; *3* - 2H5; *4* - 2D11. The recognized polypeptides were visualized with 3,3'-diaminobenzidine.

Our earlier studies have shown that Mabs 2G2, 2F4, 2H5, and 2D11 produced against the RuBPCO holoenzyme from barley recognize only the LS, and that the epitopes are partially overlapping as established by the ELISA additivity test (Mladjova *et al.* 1992, Demirevska-Kepova *et al.* 1996). In the present study all the four Mabs recognized many fragments obtained from the LS of RuBPCO by treatment with two exogenous proteases of different specificity. Probably, these determinant groups were relatively stable during proteolysis. Differences among Mabs about the recognized epitopes appeared only in the fragments with low molecular masses, which further supported the finding that these epitopes overlapped.

References

Bottomley, W.: Characterization of chloroplast proteins by partial proteolytic digestion. - In: Edelman, M., Hallick, R.B., Chua, N.-H. (ed.): Methods in Chloroplast Molecular Biology. Pp. 1055-1061. Elsevier Biomedical Press, Amsterdam - New York - Oxford 1982.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W., Laemmli, U.K.: Peptide mapping by limited proteolysis in SDS and analysis by gel electrophoresis. - J. biol. Chem. **252**: 1102-1106, 1977.

Demirevska-Kepova, K., Simova, L.: [Isolation and purification of ribulose 1,5-bisphosphate carboxylase/oxygenase from barley leaves.] - Fiziol. Rast. (Sofia) **15**(3): 3-10, 1989. [In Bulg.]

Demirevska-Kepova, K., Simova-Stoilova, L., Kyurkchiev, S., Juperlieva-Mateeva, B.: Epitope mapping of RuBPCO from barley leaves. - Compt. rend. Acad. Sci. Bulg. **49**: 93-96, 1996.

Desimone, M., Henke, A., Wagner, E.: Oxidative stress induces partial degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. - Plant Physiol. **111**: 789-796, 1996.

Gutteridge, S., Millard, B.N., Parry, M.A.J.: Inactivation of ribulose-bisphosphate carboxylase by limited proteolysis: Loss of the catalytic activity without disruption of bisphosphate binding or carboxylation. - FEBS Lett. **196**: 263-268, 1986.

Hildbrand, M., Fischer, A., Feller, U.: Protein catabolism in bean leaf discs: accumulation of a soluble fragment of ribulose-1,5-*bis*phosphate carboxylase/oxygenase under oxygen deficiency. - J. exp. Bot. **45**: 1197-1204, 1994.

Houtz, R.L., Mulligan, R.M.: Protection of tryptic-sensitive sites in the large subunit of ribulosebisphosphate carboxylase/oxygenase by catalysis. - Plant Physiol. **96**: 335-339, 1991.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Mae, T., Kamei, C., Funaki, K., Miyadai, K., Makino, A., Ohira, K., Ojima, K.: Degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in the lysates of the chloroplasts isolated mechanically from wheat (*Triticum aestivum* L.) leaves. - Plant Cell Physiol. **30**: 193-200, 1989.

Mitsuhashi, W., Craft-Brandner, S.J., Feller, U.: Ribulose-1,5-bisphosphate carboxylase/oxygenase degradation in isolated pea chloroplasts incubated in the light or in the dark. - J. Plant Physiol. **139**: 653-658, 1992.

Mladjova, N., Demirevska-Kepova, K., Kehayov, I., Kyurkchiev, S.: Production and characterization of monoclonal antibodies to barley ribulose 1,5-bisphosphate carboxylase/oxygenase. - Plant Physiol. Biochem. **30**: 523-529, 1992.

Moreno, J., Peñarrubia, L., Garcia-Ferris, C.: The mechanism of redox regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase turnover. A hypothesis. - Plant Physiol. Biochem. **33**: 121-127, 1995.

Mulligan, R.M., Houtz, R.L., Tolbert, N.E.: Reaction-intermediate analogue binding by ribulose bisphosphate carboxylase/oxygenase causes specific changes in proteolytic sensitivity: The N-terminal residue of the large subunit is acetylated proline. - Proc. nat. Acad. Sci. USA **85**: 1513-1517, 1988.

Otto, S., Feierabend, J.: Assay and comparative characterization of the proteolytic degradation of isolated small subunit and holoenzyme of ribulose-1,5-bisphosphate carboxylase/oxygenase in chloroplasts from rye leaves. - J. Plant Physiol. **144**. 26-33, 1994.

Sueyoshi, K., Kubo, Y., Yamagishi, K., Ogura, N., Ochiai, K., Fukushima, K., Ikeda, T., Nakagawa, H.: Monoclonal antibodies specific for NADH-ferricyanide reductase domain of spinach leaf nitrate reductase. - Plant Cell Physiol. **29**: 975-980, 1988.

Xu, Y., Van Huystee, R.B.: Identification of an antigenic determinant on anionic peanut peroxidase by monoclonal antibodies. - J. exp. Bot. **42**: 935-945, 1991.