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Identification of a Major Soluble Protein in Senescent Broccoli Head.

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ABSTRACT

The major soluble protein of broccoli head (C band protein) was broken down and protease activity was increased during storage, but this phenomenon was inhibited by heat treatment (45 ℃ for 14 min). We purified this C band protein by means of ammonium sulfate fractionation, chromatographies on DEAE-cellulose and Shepharose 6B column.

Molecular weight of C band protein was calculated to be 525 kDa. The C band protein was composed of two types of subunits. Molecular weight of large subunit was 48.5 kDa and that of small subunit was 17 kDa. The C band protein subunits reacted with rice leaf anti-RuBisCo polyclonal antibody. And C band protein showed the RuBisCo activity. These results indicated that C band protein was RuBisCo.

Previously, authors reported that the heat treatment (45 ℃ for 14 min.) was beneficial to keep broccoli head fresh during storage. And we speculated that heat treatment, cited above, was effective for maintaining broccoli head to counteract with senescence (1). C band protein was a major soluble protein of broccoli head. The C band protein is degraded and protease activity is increased during storage (2). This phenomenon is inhibited by heat treatment (2). Therefore, we were interested in the interrelationship between the behavior of the C band protein and senescence.

Broccoli head consisted of a mass of flower buds. There is a few reports about the degradation of flower proteins during senescence (3,4). The characterization of C band protein and protease is required to clarify the mechanism of C band protein degradation. According to this line of work, we partially purified C band protein and measured its molecular weight.

Broccoli head (f.w.=100 g) was homogenized in 100 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM *p*CMB, 0.2 mM PMSF, 10 mM EDTA and 4 % (w/v) polyclar AT in a blender for 5 min. The homogenate was filtered through a nylon mesh. The filtrate was centrifuged at 15,000 x g for 10 min and the supernatant was obtained (crude extract, 150 ml). The crude extract was dipped in water bath at 60 ℃ for 5 min in order to inactivate protease in the extract. Protease of the extract was completely inactivated by this treatment (data not shown). And, C band protein was seemed to be intact after this treatment. Then, the crude extract was cooled in ice bath and centrifuged at 10,000 x g for 10 min. Solid ammonium sulfate was added to the supernatant and the precipitate formed between 50 to 85 % saturation was collected. The precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM *p*CMB, 0.2 mM PMSF, 10 mM EDTA (buffer A) and dialysed against the same buffer. The

Abbreviations:
EDTA, ethylendiaminetetraacetic acid; RuBisCo, riburose-1,5-bisphosphate carboxylase/oxigenase; *p*CMB, *p*-chloromercuribenzoate; PMSF, phenylmethyl sulphonyl fluoride; 2-ME, 2-mercapto ethanol; polyclar AT, polyvinylpyrrolidone; PAGE, polyacrylamide gel electrophoresis; LSU, large subunit; SSU, small subunit

dialysed enzyme solution was applied to a DEAE-cellulose column (2.2 x 24 cm) equilibrated with buffer A and eluted with a liner concentration gradient from 0-0.5 M NaCl in buffer A. Each fraction was put on to 5 % PAGE, and fractions containing C band protein were collected. The collected fraction was brought to 50% saturation with ammonium sulfate and centrifuged as described above. The precipitate was discarded, and the supernatant was brought to 85% saturation with ammonium sulfate. The fraction precipitated between 50 and 85% ammonium sulfate was dialysed against buffer A, and applied to a Sepharose 6B column (1.8 x 88 cm) equilibrated with buffer A and eluted with the same buffer. The fractions containing C band protein was detected by PAGE. The molecular weight of the C band protein was estimated to be 525 kDa by gel filtration on Sepharose 6B (Fig. 1). All above procedure was carried out at 4 ℃.

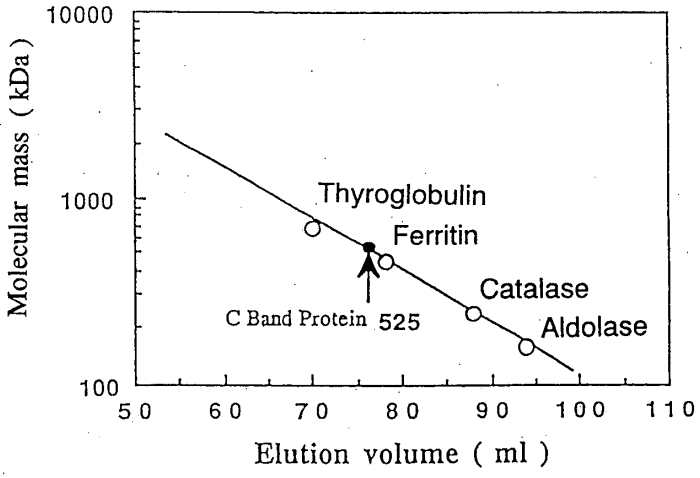

Fig. 1　Molecular weight measurement of C band protein with Sepharoce 6-B gel filtration

Thyroglobulin (650,000), ferritin (450,000), catalase (240,000), aldolase (158,000) were used as molecular weight markers.Five mg of each marker proteins were applied to the column of Sepharoce 6-B

The molecular weight also was estimated by disc electrophoresis according to the method of Hedrick and Smith (5). Partially purified C band protein, thyroglobulin, ferritin, catalase, or aldolase was applied to 3.4, 4, 5 and 6 % PAGE. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) and Rm values of each proteins were calculated. The molecular weight of C band protein was calculated to be 525 kDa. This value was similar to that obtained by gel filtration.

To investigate the structure of C band protein, fraction of C band protein was cut off from the column of the 5% PAGE gel. After soaking in SDS buffer (125 mM Tris - HCl (pH 6.8), 20 % glycerol, 5% 2-Me, 1% SDS), the band was applied to 12.5 % SDS PAGE. Two bands were detected on the gel. Authors could confirm that C band protein was composed of two subunits. Molecular weight of large subunit was calculated to be 48.5 KDa and that of small subunit was calculated to be 17 KDa (Fig. 2).

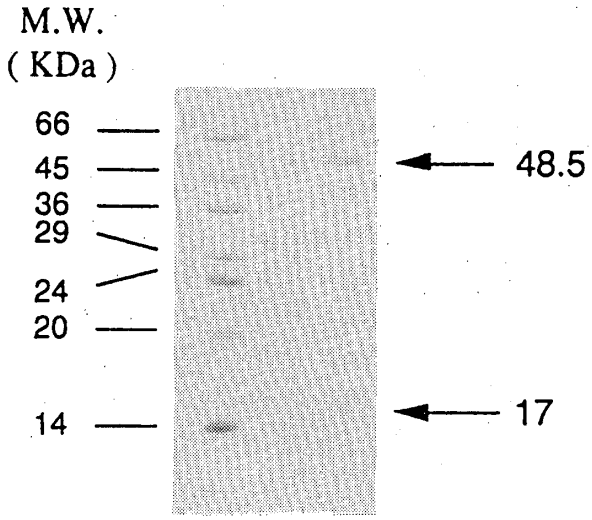

Fig. 2　Subunit composition of C band protein

C band protein band was cut out and treated with SDS buffer for 30 min at 40 ℃. The band was applied to 12.5 % SDS PAGE. Bovine albumin (660,000), egg albumin (450,000), glyceraldhyde-3-phosphate dehydrogenase (360,000), carbonic anhydrase (290,000), trypsinogen (240,000), trypsin inhibitor (20,000), and α-lactalbumin (140,000) were used as molecular weight markers.

The ratio of the C band protein in the crude extract was also measured by the method of A.

Makino et al.(6). SDS treated crude extract was put on to SDS PAGE. After the electrophoresis, the gel was stained with CBB solution for 1.5 hours and destained. All of the protein bands and the two subunits bands were cutting out and incubated with 1 ml of formamide for 5 hours at 50 ℃. The concentration of CBB dye eluted from the gels was measured at 595 nm. The C band protein composed about 30 % of total broccoli head protein.

These results indicated a close relationship between the C band protein and riburose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). RuBisCO is known to be a key enzyme of photosynthesis and major protein of the leaf tissue. After two dimensional electrophoresis, the C band protein was treated by immuno blotting (7) with rice leaf anti-RuBisCO polyclonal antibody. Both of the C band protein subunits immunoreacted with rice leaf anti-RuBisCO polyclonal antibody (Fig. 3). And we confirm the presence of RuBisCO activity, measured according to the method of Lorimer et al. (8), in partially purified C band protein.

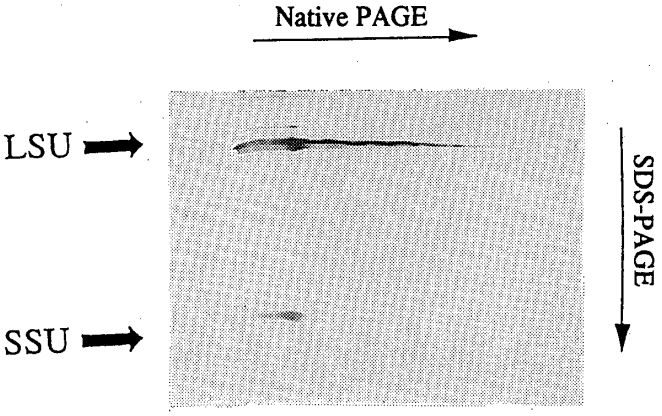

Fig. 3 Two dimensional gel electrophoresis and immuno blotting of C band protein

The sample containing C band protein was applied to 5 % native PAGE in disk and developed secondly on 12.5 % SDS-PAGE. Rice leaf anti-RuBisCO polyclonal antibody was used as a first antibody. LSU, large subunit; SSU, small subunit.

These results indicated that C band protein was RuBisCo and major soluble protein of broccoli head, whose degradation during senescence was inhibited by heat treatment.

Acknowledgement

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老化に伴って減少するブロッコリー花蕾中の可溶性タンパク質の同定

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摘　要

ブロッコリー花蕾を高温処理（45℃の温水に14分間浸漬）すると貯蔵性が高まり，老化に伴い上昇するプロテアーゼ活性および主要な可溶性タンパク質（Cーバンドタンパク質）の減少が抑制された。　このCーバンドタンパク質を同定するため硫安分画，DEAEセルロースおよびSepharose 6Bカラムクロマトグラフィーを用い分離した。Cーバンドタンパク質の分子量は525 kDaと大きく，SDS-PAGEでは分子量48.5 kDaと17kDaの2つのサブユニットに分離した。

タンパク質およびサブユニットはイネのRuBisCoポリクロ-ナル抗体と反応し，RuBisCo活性を示した。以上の結果から，このタンパク質はRuBisCoであると同定された。