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Research article

Carboxypeptidase I from triticale grains and the hydrolysis of salt-soluble fractions of storage proteins

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ABSTRACT

Carboxypeptidase I was purified from triticale grains (×*Triticosecale* Wittm.) by a 5-step purification procedure including gel filtration, cation-exchange chromatography and affinity chromatography. The enzyme was purified 595.9 fold with a 1.58% recovery. Triticale carboxypeptidase I is a homodimer with a molecular weight of 124.2 kDa and a subunit weight of 55.2 kDa. Each subunit is composed of two polypeptide chains (33.4 and 21.3 kDa). Serine was found in the active site of triticale carboxypeptidase I; DFP (diisopropylflourophosphate) and other applied inhibitors of serine proteases inhibited the enzyme activity. Triticale carboxypeptidase I hydrolyzes N-CBZ-dipeptide (N-carbobenzoxy-dipeptide) substrates at low pH. N-CBZ-Phe-Ala, N-CBZ-Phe-Leu and N-CBZ-Ala-Met were hydrolyzed with the highest rates. The lowest K_m value and the highest k_{cat}/K_m ratio were observed for hydrolysis of N-CBZ-Phe-Ala. Studies on the amino acid sequence revealed that the purified enzyme is homologous to carboxypeptidase I from barley. Analyses of conserved regions in the sequence of triticale carboxypeptidase I revealed the presence of Ser, Asp and His that compose the catalytic triad. Intact storage proteins were poor substrates for carboxypeptidases. Carboxypeptidase I together with carboxypeptidase III effectively degraded albumins proteolytically modified by endopeptidase EP8. Modified globulins were degraded at a slower rate, and all three carboxypeptidases were required for a significantly increased activity. Studies of the expression of the carboxypeptidase I gene revealed that the synthesis of the enzyme occurs mainly in the scutellum of the grain. The enzyme is also expressed in the aleurone layer of the grains, although its function in this tissue is unknown.

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1. Introduction

The mobilization of storage proteins during germination of cereal grains is a complex process. The process rate depends on the pH, the degree of reduction of disulphide bonds in the storage proteins, the amount of inhibitors, and in particular on the co-operation between endopeptidases and carboxypeptidases [1]. The mobilization process is commenced by endopeptidase-catalyzed hydrolysis of some peptide bonds in the storage protein substrate, leading to an increase in its solubility. Prolamins constitute the major group of storage proteins. One of the enzymes that hydrolyze native prolamins is cysteine endopeptidase EP8 from germinating triticale grains, a homolog of endopeptidase EP-A from barley [2]. Both enzymes are secreted by the aleurone layer in response to gibberellins [3,2]. Until now, it has been unknown whether these enzymes also contribute to the hydrolysis of other types of storage proteins. Following preliminary hydrolysis, storage proteins are hydrolyzed to short peptides by cysteine endopeptidases [4], and the short peptides are subsequently hydrolyzed to amino acids. Serine carboxypeptidases are responsible for releasing the amino acids [5,6]. These enzymes catalyze hydrolysis of peptide bonds at the C-termini of peptides at a low pH [7,8]. Only two carboxypeptidases (IV and V) hydrolyze substrates containing proline at the penultimate position [7]. Carboxypeptidases I, II and III do not have this ability, but they hydrolyze wide range of peptide substrates, mainly hydrophobic, but also with positive charge (carboxypeptidase II) [1,6]. Carboxypeptidases are inhibited specifically by DFP, which indicates that these enzymes have a serine in the active site [9,10]. Previous studies on the hydrolysis of storage proteins from cereal

Abbreviations: CABS-Sepharose 4B, [*N*-(ε-aminocaproyl)-*p*-aminobenzyl] succinyl-Sepharose 4B; CP, carboxypeptidase; DFP, diisopropylfluorophosphate; E-64, N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-Leucyl]-agmatine; EDTA, disodium ethylenediaminetetraacetate; EP, endopeptidase; N-CBZ, *N*-carbobenzoxy; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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grains by carboxypeptidases have focused on prolamins [2]. These studies revealed that carboxypeptidases catalyze the hydrolysis of native prolamins at a low rate; however, protein modifications remarkably affect the activity of these enzymes. Moreover, the joint action of carboxypeptidase and aspartyl endopeptidase from dry grains may cause an increased hydrolysis rate of gliadins compared to the hydrolysis rate in the presence of individual enzymes [11].

Five carboxypeptidases that have different site of syntheses and substrate specificities have been detected in grains of barley and wheat [10,7]. Two of them, carboxypeptidase I and III, appear in the starch endosperm during germination due to *de novo* synthesis. The location of the enzymes synthesis has been studied mostly at the activity or protein level [12,13], but the results do not provide unambiguous answers because these enzymes are transported between different tissues of the cereal grain. However, additional insights could be provided by studying the transcript level. The location of the synthesis of carboxypeptidase I mRNA has been studied in barley [14,15]. Although the scutellum is likely the main site of synthesis, the expression of the gene in the endosperm is not certain. There is less doubt concerning the location of carboxypeptidase III synthesis. However, these enzymes, due to secretion, appear in the starchy endosperm as the products of *de novo* synthesis in the scutellum and aleurone layer, respectively [7,14,16], where they participate in the mobilization of storage proteins [14,2]. Other carboxypeptidases are already present in dry grains [7]. During the degradation of storage proteins in barley, wheat and triticale grains, carboxypeptidases I, II and III are the most active carboxypeptidases [7,10,12]. On the fourth day of imbibition, the activities of carboxypeptidases I and III are dominant in starchy endosperm [12]; hence, these enzymes are proposed to participate in efficient hydrolysis of the products of storage proteins degradation by endopeptidases. In this context, purification and characterization of both enzymes became immensely important. The results of the characterization of carboxypeptidase III have been presented earlier [17], we now describe the properties of carboxypeptidase I.

2. Materials and methods

2.1. Plant materials

Grains of the triticale (×*Triticosecale* Wittm.) cultivar Fidelio (Plant Breeding Station, Laski, Poland) were washed, surface sterilized with 0.5% hypochlorite and germinated for 3 days at 23 °C in the dark. Selected seedlings were then divided into shoots, roots and endosperms with seed coats and scutellum. Endosperms with seed coats and scutellum were used to isolate total RNA. Endosperms with seed coats were used to purify carboxypeptidase I. Water-soluble and salt-soluble fractions of the storage proteins were obtained from dry triticale grains.

2.2. Isolation of storage proteins from triticale grains

The isolation was performed according to Sauvaire et al. [18]. 10 g of triticale flour was suspended in 50 ml of a 50 mM acetate buffer at pH 5.2. The extraction was performed at 4 °C for 30 min with magnetic stirring. The extract was centrifuged at 25,000 *g* for 30 min at 4 °C, and the pellet was subjected to another round of extraction and centrifugation. Supernatants containing soluble fractions of albumins and globulins were pooled and dialyzed against deionised water for three days. The preparation was then centrifuged, and albumins (soluble in water) and globulins (insoluble in water) were dried with a vacuum evaporator and stored at −20 °C.

2.3. Enzyme preparations

Endopeptidase EP8 was isolated and purified according to Prabucka and Bielawski [19] with modifications [2]. Carboxypeptidases were isolated as described previously [2].

2.4. Assays of peptidases activities and the hydrolysis of storage proteins

The endopeptidase activity during the purification of endopeptidase EP8 was determined according to Casano et al. [20] with modifications [2]. One unit of endopeptidase activity is defined as the amount of enzyme that causes a 0.01 increase of A_{340} during 1 h.

Hydrolysis of albumins and globulins by EP8 was conducted as described previously [21] with modifications [2]. Reaction mixture contained 0.85 U of EP8, 0.5% (w/v) solution of substrate (triticale albumins or globulins). In order to assay activity of the enzyme samples were incubated for 2 h at 37 °C, then reaction was terminated by TCA (trichloroacetic acid in final concentration 6% w/v), samples were centrifuged and absorbance was measured at 280 nm. To subject the products of hydrolysis of the storage proteins by EP8 to SDS-PAGE analysis and to obtain substrates for carboxypeptidases, the samples were incubated for 24 (SDS-PAGE and hydrolysis by carboxypeptidases) and 48 h (SDS-PAGE) at 37 °C. The reaction was terminated by addition of E-64 in final concentration 5 μM (inhibitor did not influence carboxypeptidases activities).

Determination of the carboxypeptidase I activity with synthetic substrates (N-CBZ-dipeptides) was performed according to Mikola and Kolehmainen [22] with modifications [2]. One unit of carboxypeptidase I activity is defined as the amount of enzyme that releases 1 μM of the C-terminal amino acid during 1 min at 30 °C at the optimum pH.

The activities of the carboxypeptidases with intact albumin and globulin as well as the products of endopeptidase EP8 digestion were assayed according to Drzymała et al. [2]. The reaction mixture contained 0.058 U of carboxypeptidase activity (single or in the mixture containing two or three carboxypeptidases), 200 mM acetate buffer, pH 4.6 and substrate (albumins or globulins, or products of their hydrolysis by EP8). The samples were incubated for 24 h, then the reaction was terminated by TNBS (trinitrobenzosulfonic acid) solution in 5% (w/v) disodium tetraborate. Further steps were performed as during determination of activity of carboxypeptidase against synthetic substrates.

2.5. Purification of carboxypeptidase I

Isolation and purification of carboxypeptidase I were performed as described by Drzymała and Bielawski [17] with modifications. Samples were homogenized in a 50 mM acetate buffer at pH 4.6, supplemented with 100 μM iodoacetamide. The carboxypeptidases were extracted by incubation at 4 °C for 1 h at a magnetic stirrer. The extract was filtered through cheesecloth and centrifuged at 25,000 *g* for 30 min at 4 °C. The supernatant was then fractionated with ammonium sulfate (35–80% saturation) and centrifuged under the same conditions. The pellet was dissolved in a 10 mM acetate buffer at pH 4.6 and dialyzed overnight against the same buffer. The dialyzed preparation was subjected to cation-exchange chromatography on CM-cellulose (1.5 cm × 30 cm) pre-equilibrated with a 0.1 M acetate buffer at pH 4.6. Proteins bound to the resin were eluted with a linear gradient of NaCl (0–0.6 M). Fractions with carboxypeptidase I activity were pooled, concentrated with a PM30 membrane (Amicon) and subjected to gel filtration with a Sephadex G-100 column (1.5 × 50 cm) and a 10 mM acetate buffer at pH 4.6. The active fractions were collected and loaded on a SP8HR column.

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