

Isolation and characterization of carboxypeptidase III from germinating triticale grains

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Carboxypeptidase III from germinating triticale grains was purified 434.2-fold with a six-step procedure including: homogenization, ammonium sulfate precipitation, cation-exchange chromatography on CM-cellulose, gel filtration chromatography on Sephadex G-150, cation-exchange chromatography on SP8HR column (HPLC), and affinity chromatography on CABS-Sepharose 4B. Triticale carboxypeptidase III is a monomer with a molecular weight of 45 kDa, which optimally hydrolyzes peptides at temperature 30–50°C and pH 4.6. *N*-CBZ-Ala-Phe, *N*-CBZ-Ala-Leu, and *N*-CBZ-Ala-Met are hydrolyzed at the highest rates. Amino acids with aromatic or large aliphatic side chains are preferred in position P1', whereas the presence of these types of groups in position P1 of the substrate results in a lower rate of hydrolysis. Peptides containing glutamic acid in positions P1 are poor substrates for the enzyme. This phenomenon suggests the hydrophobic substrate-binding sites S1 and S1'. The active site contains serine since diisopropylfluorophosphate and phenylmethanesulfonyl fluoride reduce the activity by 89.9% and 81.5%, respectively. Moreover, the activity of triticale carboxypeptidase III is reduced by mercury ions and organomercurial compounds, which suggests the presence of a sulfhydryl group adjacent to the active site of the enzyme. Identification of purified enzyme by mass spectrometry method demonstrated that the enzyme is a homolog of barley carboxypeptidase III.

Keywords carboxypeptidase III; enzymatic properties; germination; triticale

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Introduction

Plant carboxypeptidases belong to the group of serine peptidases. They have a catalytic triad composed of

serine, histidine, and aspartic acid. They catalyze the hydrolysis of peptide, ester, and amide bonds, releasing amino acids, alcohols, and ammonia groups from the C-termini of peptides, esters, and amides, respectively [1]. In 1983 and 1986, Mikola [2,3] isolated and partially characterized the carboxypeptidases of barley and wheat grain. Five carboxypeptidases were distinguished differing in molecular weight and substrate specificity. Three of them, carboxypeptidase I, II, and III, were purified and their primary structure was determined [4–9]. The spatial structure of wheat carboxypeptidase II was also reported [10,11]. These studies demonstrated that carboxypeptidases were a diverse group of enzymes with regard to structure and tissue location as well as functions. It was shown that carboxypeptidase I and II were dimers, whereas carboxypeptidase III from barley and wheat was a homolog of yeast carboxypeptidase Y, with monomeric structure [9,12]. The activity of serine carboxypeptidases is inhibited by diisopropylfluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF), but the enzymes are not sensitive to disodium ethylenediaminetetraacetate (EDTA), or other chelating agents [13–15]. The basic substrates of these enzymes—peptides—are hydrolyzed at low pH.

Carboxypeptidase III from barley is a single polypeptide chain composed of 411 amino acids, with a molecular mass of 48 kDa. It shows higher homology to yeast carboxypeptidase Y than carboxypeptidases I and II of the same species [9]. A similar phenomenon was observed in the case of carboxypeptidase from wheat [12]. The enzyme is synthesized in cells of the aleurone layer in response to gibberellins [16–18] and secreted to the starchy endosperm [14], where it participates, together with other proteases, in the mobilization of storage proteins in the germinating grains of cereals [19]. Recently, other functions of this enzyme have been proposed. The expression of carboxypeptidase III was

observed in the course of the development of wheat grains [20] and in the course of differentiation of vessel elements in the leaves and roots of wheat seedlings. The expression of this gene was correlated with the process of genomic DNA fragmentation [21].

Our earlier studies showed that the activity of carboxypeptidase III appeared in germinating triticale grains 2 days after the onset of imbibition, to constitute 3 days later ~65% of the total carboxypeptidase activity pool in the starchy endosperm [18]. The enzyme took part in the degradation of peptides formed as a result of the action of endopeptidases on triticale prolamines [19]. In addition, its expression in the course of processes occurring in programmed cell death (PCD) in wheat [20,21] makes this enzyme an interesting subject of study.

Materials and Methods

Plant materials

Triticale (*xTriticosecale* Wittm.) cv. Fidelio grains were obtained from Plant Breeding Station in Laski, Poland. Grains were washed with water and surface-sterilized with 0.5% sodium hypochlorite for 30 min and germinated for 3 days at 23°C in humid chamber in the dark. Selected seedlings were divided into root, coleoptile, scutellum, and endosperm-containing seed coat. Endosperms with seed coats were homogenized and used in the purification procedure.

Determination of carboxypeptidases activities and protein concentration

In order to assay carboxypeptidases activities, dipeptides with blocked N-terminus α -amine group [*N*-carbobenzoxy-dipeptides (*N*-CBZ-dipeptides)] were used as the substrates. The *N*-CBZ-Ala-Phe was used to study the pH and temperature influence on activity and stability of triticale carboxypeptidase III. All substrates were obtained from Sigma-Aldrich, Fluka, and Bachem. Assays were performed according to the method of Mikola and Kolehmainen [22] with modifications as described previously [19]. One unit of carboxypeptidase activity was defined as the amount of enzyme that can release 1 μ M of C-terminal amino acid per minute at 30°C and at optimal pH.

Purification of carboxypeptidase III

Endosperm isolated from triticale grains was homogenized with blade homogenizer in 0.05 M acetate buffer, pH 4.6, supplemented with 100 μ M iodoacetamide to inhibit cysteine endopeptidases activity (4 ml of buffer

per gram of fresh weight). Carboxypeptidases were extracted for 1 h at 4°C. Extract was filtered through two layers of cheesecloth and centrifuged for 30 min at 4°C at 25,000 g. The supernatant was fractionated with 35–80% saturation of ammonium sulfate and centrifuged for 30 min at 25,000 g at 4°C. The precipitate was collected and dissolved in 0.01 M acetate buffer, pH 4.6, and dialyzed overnight against the same buffer. Desalted preparation was centrifuged for 30 min at 35,000 g at 4°C and then the supernatant was loaded onto a CM-cellulose column (1.5 cm \times 30 cm) pre-equilibrated with 0.01 M acetate buffer, pH 4.6. The column was eluted with increasing gradient of 0–0.6 M NaCl. Pooled fractions with carboxypeptidase II and III activities were concentrated with Amicon Ultrafiltration system (membrane cut-off 30,000) and then subjected to gel filtration chromatography on the Sephadex G-150 column (2.5 cm \times 100 cm) with 0.01 M acetate buffer, pH 4.6, at 4°C. Active fractions were pooled and loaded onto SP8HR column pre-equilibrated with 0.01 M acetate buffer, pH 4.6, and eluted with linear gradient of 0–0.35 M NaCl. Fractions with carboxypeptidase III activity were then applied to affinity chromatography on the [*N*-(ϵ -aminocaproyl)-*p*-aminobenzyl]succinyl-Sepharose 4B (CABS-Sepharose 4B) column (1 cm \times 5 cm) pre-equilibrated with 0.05 M NaH₂PO₄, pH 4.4, containing 0.1 M NaCl. Carboxypeptidase III was eluted with the gradient (0–100%) of 0.05 M Na₂HPO₄, pH 7.5. Bio-Logic low-pressure system (Bio-Rad) was used in all chromatography steps except performing cation-exchange chromatography on SP8HR-HPLC. Protein content was measured according to Bradford method [23].

Determination of molecular weight by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and identification of protein

Homogeneity and molecular weight of purified carboxypeptidase III was analyzed by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) method according to Laemmli [24]. Gels were stained with Coomassie brilliant blue or silver nitrate according to Blum *et al.* [25]. In order to determine molecular weight, SDS–PAGE molecular weight standards of low range were used (Bio-Rad). Purified protein with carboxypeptidase III activity after SDS–PAGE was subjected to identification by mass spectrometry (electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry—ESI-FTICR) in the Laboratory of Mass Spectrometry, IBB, PAS, Warsaw, Poland.

Determination of molecular weight by gel filtration

Molecular weight of carboxypeptidase III was analyzed by gel filtration on the Sephadex G-150 column (2.5 cm × 100 cm) equilibrated with 0.075 M phosphate buffer, pH 6.5, containing 0.1 mM DL-dithiothreitol according to Mikola [2].

Temperature and pH optima and stability of carboxypeptidase III

In order to determine the temperature optimum for carboxypeptidase III, samples were pre-incubated (3 min) and incubated (15 min) at 0–60°C. The reaction was terminated with TNBS solution and further steps of activity assay were performed according to the method of Mikola and Kolehmainen [22] with some modifications [19]. Stability of the enzyme was analyzed as the function of loss of activity of triticale carboxypeptidase III during 20, 40, and 60 min of pre-incubation at 0, 20, 30, 40, 50, and 60°C.

Optimum pH of carboxypeptidase III was determined in 0.05 M acetate buffer, in pH 3.6–5.6 range, and in 0.05 M phosphate buffer, in pH 5.6–7.0 range. Influence of pH on K_m and k_{cat} was analyzed in 0.15 M McIlvain buffer, pH 3.0–7.0. Stability of the enzyme was analyzed in 0.05 M acetate buffer, in pH 3.6–5.6 range. Samples were pre-incubated for 48 h in buffers of different pH values at 4°C, then pH was adjusted to 4.6 with 0.05 M acetic acid or 0.05 M sodium acetate, and activity was assayed with *N*-CBZ-Ala-Phe as the substrate.

Influence of inhibitors and metal ions on triticale carboxypeptidase III activity

The influence of various inhibitors and metal ions on carboxypeptidase III activity was studied. 0.01 M EDTA, 0.01 mM 1,10-phenanthroline, 0.0014 and 0.01 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 0.05 mM pepstatin A, 0.1 and 1 mM DFP, 0.01 and 0.1 mM *p*-hydroxymercuribenzoate (pHMB), 1 mM PMSF, and chlorides of various metals, except Ag^+ ($AgNO_3$) in final concentrations of 0.5 mM were used. The samples were pre-incubated for 30 min at 30°C in 0.05 M acetate buffer, pH 4.6, supplemented with inhibitor or metal ion, and then activity assay was performed with *N*-CBZ-Ala-Phe as the substrate.

Substrate specificity and kinetic properties of triticale carboxypeptidase III

Substrate specificity and K_m values were determined with various N-blocked dipeptides. The activity of the

enzyme was assayed in 0.05 M acetate buffer, pH 4.6. K_m and V_{max} values were calculated from Lineweaver and Burk plot [26] and used to calculate k_{cat} and k_{cat}/K_m ratio.

Data analyses

The data presented in the tables and in the figures are means calculated from three independent replications; the standard error is presented in the figure in the form of vertical bars at each mean. The standard error of the results placed in the tables did not exceed 5%, except the kinetic properties of triticale carboxypeptidase III. Analysis of carboxypeptidase III sequence was carried out using the BLASTP program on the NCBI server and ClustalW on EMBL-EBI server. Alignments were analyzed by Jalview.

Results

Purification of triticale carboxypeptidase III

Carboxypeptidase III was purified in a six-step procedure including: homogenization and extraction, ammonium sulfate precipitation, ion-exchange chromatography on CM-cellulose, gel filtration chromatography on Sephadex G-150, cation-exchange chromatography on SP8HR column (HPLC), and affinity chromatography on CABS-Sepharose 4B (Table 1). The purification fold and recovery were monitored by assay of activity against *N*-CBZ-Ala-Phe, the most specific substrate for triticale carboxypeptidase III of all used substrates. A high purification fold was achieved by application of two steps of cation-exchange chromatography on CM-cellulose and the column SP8HR (HPLC) as well as affinity chromatography on CABS-Sepharose 4B. As a result of this procedure, a protein with specific activity of 41.2 U/mg was obtained. The purification fold was 434.2, whereas the recovery was 0.7%.

Determination of molecular weight and identification by mass spectrometry

Purified preparation of the enzyme was analyzed with 12% polyacrylamide gel under denaturing condition (Fig. 1). The single band of purified protein with a molecular mass of 45 kDa was cut out, digested with trypsin, and identified by the mass spectrometry method at the Environmental Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, PAS, Warsaw, Poland. The protein is a homolog of carboxypeptidase III from barley (CP-MIII) and wheat (CP-WIII) and the product of the rice gene Os02g0114200 (Fig. 2). About 57% coverage of the obtained sequence with the amino

Table 1 Purification of triticale carboxypeptidase III

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	95.2	993	0.095	100	1
(NH ₄) ₂ SO ₄ precipitation (35–80%)	75.5	440	0.173	80.5	1.81
CM-cellulose	74.4	34	2.19	78.3	22.8
Sephadex G-150	26.6	9.1	2.9	31	30.3
SP8HR	3.4	0.24	14.2	3.6	149.5
CABS-Sepharose 4B	0.66	0.016	41.2	0.7	434.2

acid sequence of barley carboxypeptidase III was observed. In addition, the molecular mass of carboxypeptidase III determined by the gel filtration method was 40.4 kDa, which indicates that under physiological conditions the enzyme is a monomer.

Substrate specificity of triticale carboxypeptidase III

Studies on the substrate specificity of the enzyme at the activity and kinetic constants indicate that *N*-CBZ-Ala-Met, *N*-CBZ-Ala-Leu, and *N*-CBZ-Ala-Phe are the best substrates for triticale carboxypeptidase III (Table 2). The values of the k_{cat}/K_m ratio for these substrates are similar, 10277, 9209, and 10928 min⁻¹ mM⁻¹, respectively. The presence of the aromatic side chain of phenylalanine in position P1 of the substrate (*N*-CBZ-Phe-Met, *N*-CBZ-Phe-Leu) results in a decrease of the activity of

the enzyme, averaging ~80%. Carboxypeptidase III demonstrates low activity against substrates containing valine, isoleucine, or glutamic acid in this position. The K_m values for these substrates are 0.78, 0.81, and 0.79 mM, respectively. The values of k_{cat} are similar for hydrolysis of *N*-CBZ-Ile-Leu and *N*-CBZ-Val-Phe, while the glutamic acid at the penultimate position unfavorably interacts with the active site of the enzyme ($k_{\text{cat}}=262$ min⁻¹). The enzyme does not hydrolyze substrates with glycine in the P1 position. The amino acids with large side chain (methionine, leucine, or phenylalanine) are preferred in the C-terminal position of the substrate. The presence of a side chain with negative charge in this position causes a 96% decrease of the activity of triticale carboxypeptidase III. Substrates containing glycine in position P1' are hydrolyzed at a low rate, whereas triticale carboxypeptidase III does not hydrolyze substrates containing proline in the penultimate or C-terminal positions.

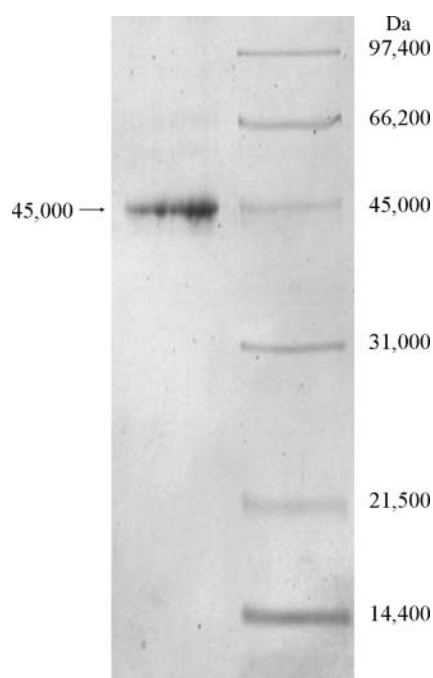


Fig. 1 SDS-PAGE of purified triticale carboxypeptidase III

Influence of inhibitors and metal ions on the activity of triticale carboxypeptidase III

The activity of the enzyme is inhibited by 89.9% by 1 mM DFP, 81.5% by 1 mM PMSF, and 100% by 0.1 mM pHMB (Table 3). E-64, a diagnostic inhibitor of cysteine peptidases, pepstatin A, and EDTA do not affect the activity of carboxypeptidase III. The 1,10-phenanthroline causes a slight activation of the enzyme.

Studies on the effect of ions of various metals on carboxypeptidase III activity revealed that the presence of zinc and iron ions activates the enzyme by 10% and 9%, respectively. A slight decrease of activity was observed in the case of samples pre-incubated with Al³⁺, Pb²⁺, or Mn²⁺. Copper and silver ions inhibited the enzyme activity by 86.5% and 98.5%, respectively, whereas pre-incubation of carboxypeptidase III with mercury ions or mercurochrome resulted in a complete inhibition of the enzyme activity.

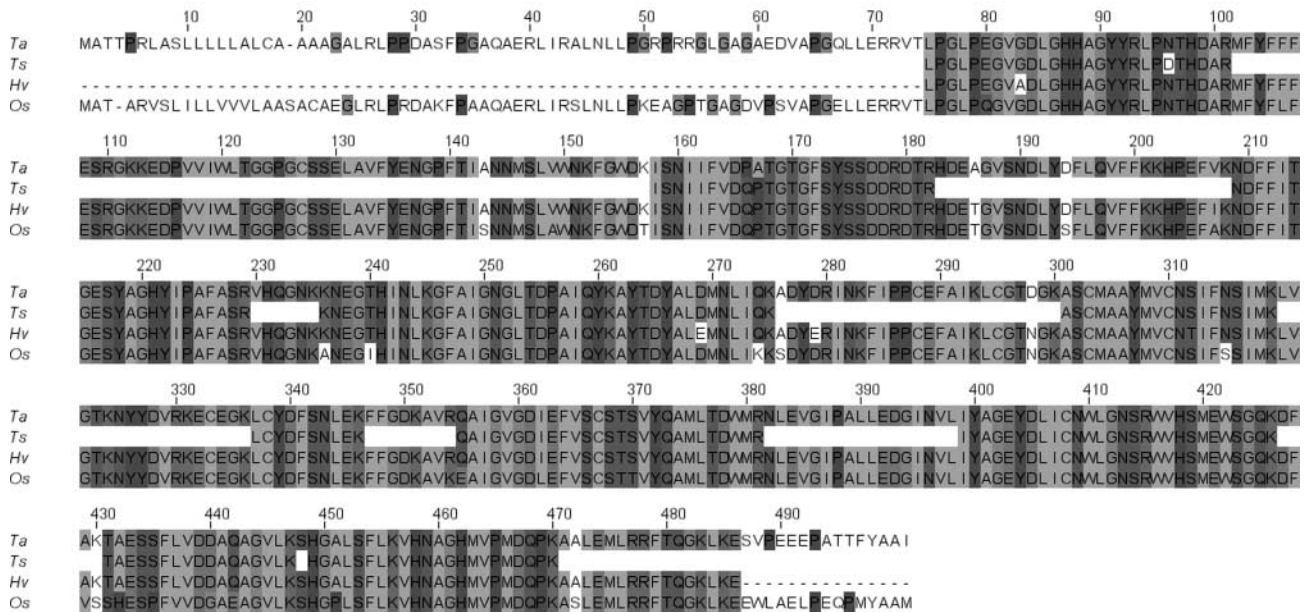


Fig. 2 Identification of triticale carboxypeptidase III by mass spectrometry Hv, *Hordeum vulgare* carboxypeptidase III, CP-MIII, accession number P21529; Ta, *Triticum aestivum* carboxypeptidase III, CP-WIII, accession number P11515; Os, *Oryza sativa* Os02g0114200, accession number NP_001045667; Ts, *xTriticosecale* carboxypeptidase III, regions not covered by mass spectrometry sequencing spaces.

Table 2 Substrate specificity and kinetic parameters of peptide substrates' hydrolysis by triticale carboxypeptidase III

Substrate	Relative activity (%)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\text{min}^{-1} \text{mM}^{-1}$)
<i>N</i> -CBZ-Ala-Phe	94.3 [†]	0.37 [‡]	3802 [†]	10277 [‡]
<i>N</i> -CBZ-Ala-Met	100 [†]	0.36 [‡]	3934 [‡]	10928 [‡]
<i>N</i> -CBZ-Ala-Leu	98.4 [†]	0.42 [‡]	3868 [†]	9209 [‡]
<i>N</i> -CBZ-Ala-Glu	2.1 [†]	1.48 [‡]	1061 [§]	417 [§]
<i>N</i> -CBZ-Phe-Ala	19.4 [†]	0.59 [‡]	761 [‡]	1289 [‡]
<i>N</i> -CBZ-Phe-Met	28.8 [†]	0.55 [§]	955 [‡]	1737 [‡]
<i>N</i> -CBZ-Phe-Leu	24.3 [†]	0.23 [‡]	551 [‡]	2397 [‡]
<i>N</i> -CBZ-Phe-Gly	0.7 [†]	2.03 [‡]	416 [‡]	204 [‡]
<i>N</i> -CBZ-Glu-Tyr	6.0 [†]	0.78 [‡]	262 [‡]	336 [‡]
<i>N</i> -CBZ-Val-Phe	7.6 [†]	0.81 [‡]	782 [§]	965 [§]
<i>N</i> -CBZ-Ile-Leu	5.9 [†]	0.79 [†]	602 [‡]	762 [‡]
<i>N</i> -CBZ-Gly-Ala	0	—	—	—
<i>N</i> -CBZ-Gly-Gly-Ala	0	—	—	—
<i>N</i> -CBZ-Gly-Pro	0	—	—	—
<i>N</i> -CBZ-Pro-Phe	0	—	—	—
<i>N</i> -CBZ-Pro-Ala	0	—	—	—

Activity of the enzyme towards *N*-CBZ-Ala-Met is assumed as 100%; —, not determined.

[†]Standard error \pm 0–5%.

[‡]Standard error \pm 5–10%.

[§]Standard error \pm 10–20%.

pH optimum and stability

The pH optimum for carboxypeptidase III activity was determined using two buffers: 50 mM acetate buffer in

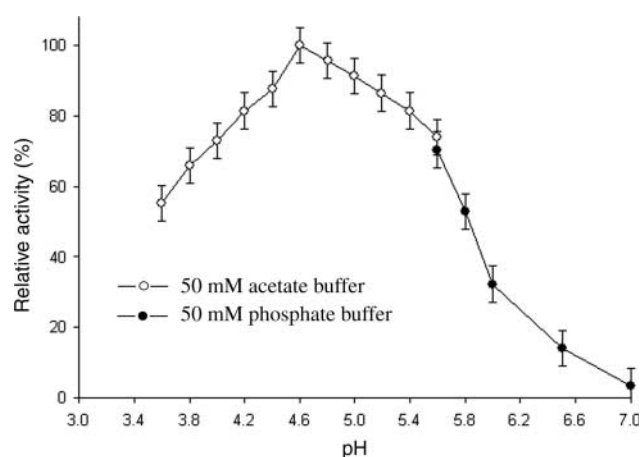
the pH 3.6–5.6 and 50 mM phosphate buffer in the buffering range 5.6–7.0. *N*-CBZ-Ala-Phe was used to determine the activity of triticale carboxypeptidase III.

Table 3 Influence of inhibitors and metal ions on carboxypeptidase III activity

Agent	Agent concentration (mM)	Relative activity (%)
Control	–	100
EDTA	10	96.0
1,10-Phenanthroline	0.01	114.1
E-64	0.0014	107.0
E-64	0.01	98.7
Pepstatin A	0.05	100.4
DFP	0.1	49.7
DFP	1.0	10.1
pHMB	0.1	0
pHMB	0.01	6.3
PMSF	1.0	18.5
CuCl ₂	0.5	13.5
PbCl ₂	0.5	76.0
ZnCl ₂	0.5	109.8
FeCl ₂	0.5	110.5
MgCl ₂	0.5	103.9
BaCl ₂	0.5	106.6
AlCl ₃	0.5	81.3
MnCl ₂	0.5	75.4
AgNO ₃	0.5	1.5
CaCl ₂	0.5	103.2
HgCl ₂	0.5	0
Mercurochrome	0.5	0

Activity of the enzyme towards *N*-CBZ-Ala-Phe in standard conditions is assumed as 100%.

The results showing relative activity of the enzyme are presented in **Fig. 3**. The pH optimum for the activity of triticale carboxypeptidase III was in the pH 4.2–5.4, with the highest activity of the enzyme being observed at pH 4.6. Below the optimal pH values, the activity of the enzyme at pH 3.6–4.2 remained at the level of 55–80%. At pH above 5.6, the enzyme activity showed a sharp decrease, only 3.4% activity left at pH 7.0. Similar phenomenon was observed when studying the influence of pH on k_{cat}/K_m ratio. The maximum value of k_{cat}/K_m was reached at pH 4.5. The value of the constant K_m for *N*-CBZ-Ala-Phe persisted at a constant level in the pH 3.0–5.0 (**Fig. 4**). The affinity of the enzyme towards *N*-CBZ-Ala-Phe was reduced above pH 5, and the value of K_m increased to 17.52 mM at 7.0. The turnover number of triticale carboxypeptidase III at pH 3.0–4.5 was within the range 2208–3802 min⁻¹. The enzyme reached maximal k_{cat} value (9873 min⁻¹) at pH 5.5 after which the value of this constant decreased.

**Fig. 3 Effect of pH on activity of triticale carboxypeptidase III.**

Studies on the stability of carboxypeptidase III from triticale revealed that the enzyme was most stable at pH 4.4 (**Fig. 5**). About 48-h pre-incubation in the pH caused only 2% decrease in the activity. Increasing or lowering the pH in the course of pre-incubation resulted in a regular decrease in the activity of the enzyme in the pH range studied.

Temperature optimum and stability

Triticale carboxypeptidase III demonstrated maximum activity after 15-min incubation at 45°C (**Fig. 6**). An increase in temperature of 5°C compared to the optimum resulted in an 8% decrease of activity in the presence of the substrate *N*-CBZ-Ala-Phe but further increasing of the temperature to 55°C resulted in the denaturation of the enzyme. At 30–50°C, only relatively small changes in the activity were observed (~10%).

The thermal stability of triticale carboxypeptidase III was studied as a function of time-dependent decrease of the enzyme activity, at the various temperatures (**Fig. 7**). The activity was determined following 20, 40, and 60 min pre-incubation at temperatures: 0, 20, 30, 40, 50, and 60°C. The stability of carboxypeptidase III at 20, 30, and 40°C was similar. A slight difference in the activity of the enzyme was observed after 20, 30, and 40 min pre-incubation at 20 and 40°C, with only 15, 17, and 22% difference, respectively. Higher temperatures (50 and 60°C) resulted in considerably reduced stability of the enzyme. After 20 min pre-incubation at these temperatures, a 40% and 55% decrease, respectively, in the activity was observed. Further pre-incubation did not cause such evident changes, and the activity of

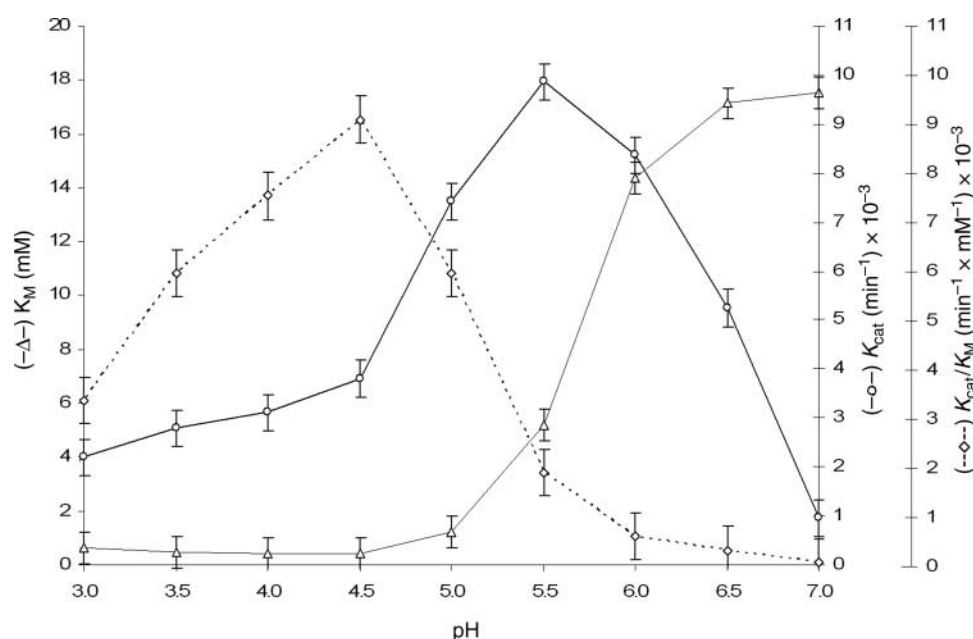


Fig. 4 Effect of pH on kinetic parameters of *N*-CBZ-Ala-Phe hydrolysis by triticale carboxypeptidase III

the enzyme was 31 and only 1.5%, respectively, of the initial level after 60 min pre-incubation at 50 and 60°C.

Discussion

The primary structure, functions, and the regulation of the synthesis of carboxypeptidase III in various species were characterized at the nucleic acid level [12,20,21,27–29], but attempts of purification of carboxypeptidase III and characterization of its catalytic properties were rare. Carboxypeptidase III from barley malt has been purified

1454-fold, with a recovery of 0.5%, using ammonium sulfate precipitation, ion-exchange chromatography on DE-52 cellulose, and affinity chromatography on GYBS-Sepharose 4B [8]. In this study, triticale carboxypeptidase III was purified to homogeneity, although the purification fold (434.2) was lower. The recovery and specific activity of purified enzyme was similar to the procedure used by Breddam and Sørensen [8]. To obtain homogeneous triticale carboxypeptidase III, CABS-Sepharose 4B was applied as an affinity chromatography step. Moreover, two ion-exchange chromatography steps on CM-cellulose and

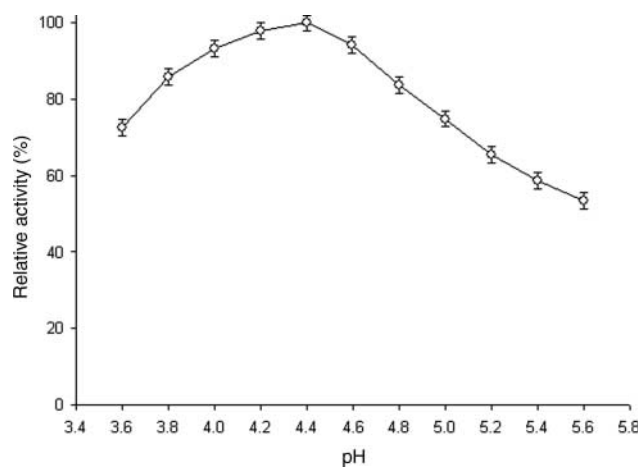


Fig. 5 pH-dependent stability of triticale carboxypeptidase III. Activity of the enzyme after 48 h pre-incubation in pH 4.4 was assumed as 100% of the activity.

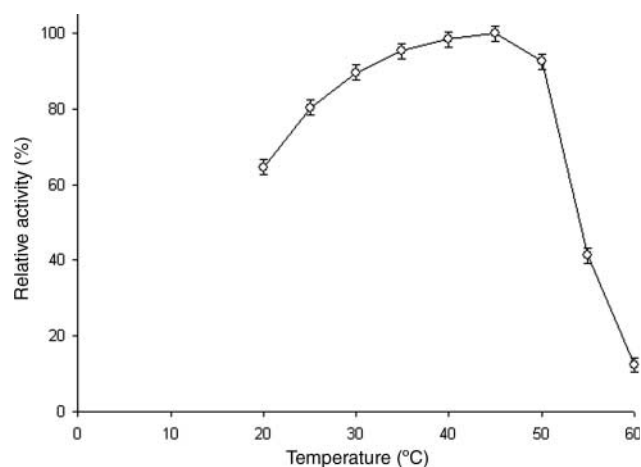


Fig. 6 Effect of temperature on hydrolysis rate of *N*-CBZ-Ala-Phe by triticale carboxypeptidase III

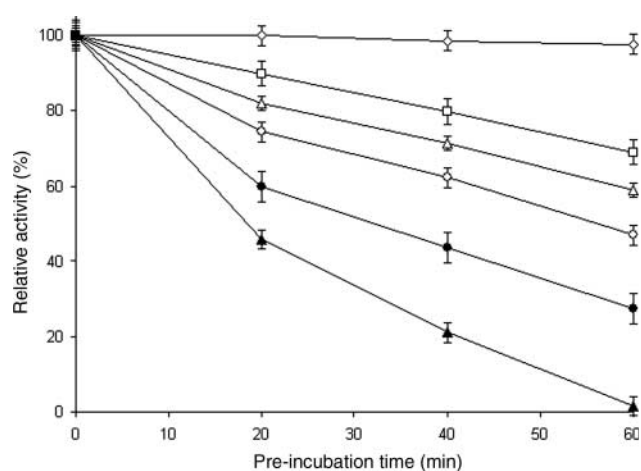


Fig. 7 Thermal stability of triticale carboxypeptidase III The pre-incubation temperatures is 0°C (◇), 20°C (□), 30°C (△), 40°C (○), 50°C (●), and 60°C (▲).

SP8HR and gel filtration were applied. Cation-exchange chromatography as one of the purification steps was also used by Preston and Kruger [30,31], who isolated an enzyme from wheat grains (carboxypeptidase B) which hydrolyzed *N*-CBZ-Ala-Phe with the highest rate. They also applied affinity chromatography on a bed coupled with hemoglobin, chromatography, and re-chromatography on CM-cellulose followed by electrofocusing which resulted in 140-fold purification of the enzyme and an 11% recovery.

The molecular mass of triticale carboxypeptidase III determined by gel filtration was 40.4 kDa. A similar mass for carboxypeptidase III from barley and wheat (43 and 40 kDa, respectively) was obtained by Mikola in 1983 and 1986 [2,3]. The molecular mass of the enzyme from barley, determined by the SDS-PAGE method was higher by 11% [8] (48 kDa). Determination of the amino acid sequence of the enzyme [9] and molecular mass estimated on this basis (46.25 kDa) allow assuming the results obtained by the gel filtration method are lowered. A similar phenomenon was observed in the case of triticale carboxypeptidase III. The molecular mass of the enzyme determined by SDS-PAGE was 45 kDa and was 10% higher than the mass obtained by gel filtration. Analysis of band 45 kDa by the mass spectrometry method revealed that the protein is a homolog of serine carboxypeptidase III precursor (accession number: P21529). All the peptides containing amino acids forming the catalytic triad of serine carboxypeptidases were identified. The occurrence of serine in the active site of carboxypeptidase III was also detected chemically by investigating the effect of DFP, a specific inhibitor of

serine peptidases on the activity of the enzyme. The inhibitor was found to reduce the activity of the enzyme by ~90%. Similar results were obtained for barley carboxypeptidase III [2,8,14]. Simultaneously, PMSF, non-specific inhibitor of serine peptidases, inhibited the activity of the enzyme by 81.5%. Strong inhibition was also observed after pre-incubation of triticale carboxypeptidase III with 0.1 mM pHMB. At the same time, the diagnostic inhibitor for cysteine peptidases—E-64—did not significantly affect the rate of *N*-CBZ-Ala-Phe hydrolysis. This phenomenon would suggest the presence of cysteine in the active site of the enzyme, since the enzyme was also inhibited non-specifically by other mercuric compounds used, such as HgCl₂ or mercurochrome. Such inhibition and the results of mass spectrometry identification indicate the occurrence of a free sulfhydryl group close to the active site of triticale carboxypeptidase III. The effect of mercuric compounds on the activity of serine carboxypeptidases was demonstrated earlier [2,5,8]. This phenomenon concerns carboxypeptidase III from barley malt [8] and yeast carboxypeptidase Y [32], whereas the presence of other groups are considered to cause the inhibition of barley malt carboxypeptidases I and II by mercuric compounds [5].

Triticale carboxypeptidase III prefers substrates containing amino acids with short hydrophobic side chain in the penultimate position and amino acids with aromatic or long aliphatic side chain in position P1'. Similar results were obtained in studies on the substrate specificity of barley [14] and wheat [3] carboxypeptidases III. The values of k_{cat}/K_m ratio for this type of substrates (*N*-CBZ-Ala-Met, *N*-CBZ-Ala-Phe, and *N*-CBZ-Ala-Leu) were the highest among all the substrates studied, but lower, however, than the k_{cat}/K_m values obtained for barley carboxypeptidase III [8]. The reason for this phenomenon may be a lower strength of the binding of peptide substrates in the active site of triticale carboxypeptidase III relative to barley carboxypeptidase III and yeast carboxypeptidase Y [8,33], although K_m value for *N*-CBZ-Phe-Ala hydrolysis by wheat carboxypeptidase B is higher than that of triticale carboxypeptidase III [31]. A more detailed analysis of the substrate specificity of triticale carboxypeptidase III shows that the preferred amino acids in position P1 of the peptide substrates are alanine, followed by phenylalanine, valine, and isoleucine, whereas in position P1': methionine, phenylalanine, leucine, and alanine. Substrates with glutamic acid and glycine in the both penultimate and terminal positions are poorly hydrolyzed (~3%). The preferences of the enzyme towards hydrophobic substrates, low activity

against substrates with negative charge or a positive one in the case of carboxypeptidases III from barley and wheat [3,8] suggest a strongly hydrophobic character of substrate binding sites.

The temperature optimum of carboxypeptidase III from triticale grains is in the 30–50°C range, with maximum activity at 45°C. Studies on the thermal stability of the enzyme demonstrated that carboxypeptidase III shows similar stability throughout the 20–40°C temperature range, whereas 60 min pre-incubation at 60°C results in a 98.5% decrease of the activity of the enzyme. Data on the stability of barley carboxypeptidases indicate that enzymes that hydrolyze *N*-CBZ-Phe-Phe demonstrate higher stability at higher temperatures than those that hydrolyze *N*-CBZ-Phe-Ala [34].

Serine carboxypeptidases hydrolyze peptides at low pH [1]. The optimum pH for the hydrolysis of *N*-CBZ-Ala-Phe by crude extracts from wheat grains after 3 days of germination is 4.8 [3], whereas the optimum pH determined by Breddam and Sørensen [8] for carboxypeptidase III from barley was 4.5. Similar results were obtained for triticale carboxypeptidase III. The enzyme optimally hydrolyzed the peptide substrates *N*-CBZ-Ala-Phe and *N*-CBZ-Ala-Met at pH 4.6. Increasing the pH to above 5.6 resulted in a rapid decrease of the activity of the enzyme. The reduced activity of triticale carboxypeptidase III is most probably caused by weaker binding of the substrate which can be testified to rapid increase of the value of K_m for *N*-CBZ-Ala-Phe at pH above 5.5. Similar results were obtained earlier when studying the effect of pH on the kinetics of barley carboxypeptidase III and it was ascribed to changes in the protonation of the group involved in substrate binding [1] and also to possible conformational changes of the enzyme [8]. The course of pH-dependent changes of k_{cat} value suggests the occurrence, in catalytic domain of the enzyme, of two side chains of amino acids essential in hydrolysis reactions. The first, with pK about 4, may function as proton acceptor (general base), while the second, with pK equal to 6.5, as proton donor (general acid). The presence of a group with pK 4 was also found in the active site of barley carboxypeptidase III [8], barley carboxypeptidase II [35], and carboxypeptidase Y [36], while the pK of the latter group in the active site of barley carboxypeptidase III was slightly higher [8].

The high activity of triticale carboxypeptidase III and the high stability of the enzyme at low pH are of considerable importance in the aspect of the functions of the enzyme in the course of degradation of storage proteins.

The pH value in the starchy endosperm of barley grains from day 3 to day 5 after the onset of imbibition is maintained in the range 4.0–5.0 [16,37]. At the same time, the most intense mobilization of storage proteins is observed in the grains of many cereals. As a result of low pH optimum and high stability at such conditions, carboxypeptidase III is highly effective in the process.

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