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 grains. 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 prolaminides [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-carbobenzyo-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 μM 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 acetic acid buffer, pH 4.6, supplemented with 100 μM 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 × 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 × 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-(α-aminocaproyl)-p-aminobenzyl]succinyl-Sepharose 4B (CABS-Sepharose 4B) column (1 cm × 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. BioLogic 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 acetic acid 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 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-leucylamide-(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$^+$ (AgNO₃) in final concentrations of 0.5 mM were used. The samples were pre-incubated for 30 min at 30°C in 0.05 M acetic acid 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
The 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$^{-2}$1 mM$^{-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 ($k_{cat}$=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.

**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 $\text{Al}^{3+}$, $\text{Pb}^{2+}$, or $\text{Mn}^{2+}$. 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.

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**Table 1: Purification of triticale carboxypeptidase III**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>95.2</td>
<td>993</td>
<td>0.095</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ precipitation (35–80%)</td>
<td>75.5</td>
<td>440</td>
<td>0.173</td>
<td>80.5</td>
<td>1.81</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>74.4</td>
<td>34</td>
<td>2.19</td>
<td>78.3</td>
<td>22.8</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>26.6</td>
<td>9.1</td>
<td>2.9</td>
<td>31</td>
<td>30.3</td>
</tr>
<tr>
<td>SP8HR</td>
<td>3.4</td>
<td>0.24</td>
<td>14.2</td>
<td>3.6</td>
<td>149.5</td>
</tr>
<tr>
<td>CABS-Sepharose 4B</td>
<td>0.66</td>
<td>0.016</td>
<td>41.2</td>
<td>0.7</td>
<td>434.2</td>
</tr>
</tbody>
</table>

**Fig. 1 SDS–PAGE of purified triticale carboxypeptidase III**
**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.

![Fig. 2 Identification of triticale carboxypeptidase III by mass spectrometry](image)

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

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

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

†Standard error ± 0–5%.

‡Standard error ± 5–10%.

§Standard error ± 10–20%.
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 \( k_{\text{cat}}/K_m \) ratio. The maximum value of \( k_{\text{cat}}/K_m \) 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 \( k_{\text{cat}} \) value (9873 min\(^{-1}\)) at pH 5.5 after which the value of this constant decreased.

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.

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

<table>
<thead>
<tr>
<th>Agent</th>
<th>Agent concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control –</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>96.0</td>
</tr>
<tr>
<td>1,10-Phenantroline</td>
<td>0.01</td>
<td>114.1</td>
</tr>
<tr>
<td>E-64</td>
<td>0.0014</td>
<td>107.0</td>
</tr>
<tr>
<td>E-64</td>
<td>0.01</td>
<td>98.7</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>0.05</td>
<td>100.4</td>
</tr>
<tr>
<td>DFP</td>
<td>0.1</td>
<td>49.7</td>
</tr>
<tr>
<td>DFP</td>
<td>1.0</td>
<td>10.1</td>
</tr>
<tr>
<td>pHMB</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>pHMB</td>
<td>0.01</td>
<td>6.3</td>
</tr>
<tr>
<td>PMSF</td>
<td>1.0</td>
<td>18.5</td>
</tr>
<tr>
<td>CuCl(_2)</td>
<td>0.5</td>
<td>13.5</td>
</tr>
<tr>
<td>PbCl(_2)</td>
<td>0.5</td>
<td>76.0</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>0.5</td>
<td>109.8</td>
</tr>
<tr>
<td>FeCl(_2)</td>
<td>0.5</td>
<td>110.5</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>0.5</td>
<td>103.9</td>
</tr>
<tr>
<td>BaCl(_2)</td>
<td>0.5</td>
<td>106.6</td>
</tr>
<tr>
<td>AlCl(_3)</td>
<td>0.5</td>
<td>81.3</td>
</tr>
<tr>
<td>MnCl(_2)</td>
<td>0.5</td>
<td>75.4</td>
</tr>
<tr>
<td>AgNO(_3)</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>0.5</td>
<td>103.2</td>
</tr>
<tr>
<td>HgCl(_2)</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Mercurochrome</td>
<td>0.5</td>
<td>0</td>
</tr>
</tbody>
</table>

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

Isolation and characterization of carboxypeptidase III

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

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
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

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

**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
by investigating the effect of DFP, a specific inhibitor of
site of carboxypeptidase III was also detected chemically
were identified. The occurrence of serine in the active
forming the catalytic triad of serine carboxypeptidases
P21529). All the peptides containing amino acids

The 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 carboxypepti-
dase III from barley malt [8] and yeast carboxypeptidase
Y [32], whereas the presence of other groups are con-
sidered to cause the inhibition of barley malt carboxypepti-
dases I and II by mercuric compounds [5].

Triticale carboxypeptidase III prefers substrates con-
taining 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 carboxy-
peptidase 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 isoleu-
cine, 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

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**Fig. 7 Thermal stability of triticale carboxypeptidase III**
The pre-incubation temperature 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 triti-
cale 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 carboxy-
peptidase 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 pre-
sence 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 mercuro-
chrome. Such inhibition and the results of mass spectrometry
identification indicate the occurrence of a free
sulphydryl 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 carboxypepti-
dase III from barley malt [8] and yeast carboxypeptidase
Y [32], whereas the presence of other groups are con-
sidered to cause the inhibition of barley malt carboxypepti-
dases I and II by mercuric compounds [5].
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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