Isolation and Characterization of a Chlorogenic Acid Esterase from *Aspergillus niger*

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Chlorogenic Acid Esterase, *Aspergillus niger*, High Performance Thin Layer Chromatography

The isolation and characterization of a specific chlorogenic acid esterase is described. The enzyme activity is measured by determination of the hydrolysis product caffeic acid. The enzyme had been concentrated by means of ultrafiltration and column-chromatography. The pH- and temperature optimum were 6.5 and 45 °C respectively. Divalent cations were not required for the enzyme activity. As other esterases, this enzyme is inhibited by di-isopropyl-phosphorofluoridate. The $K_m$-value is 0.70 mM chlorogenic acid, the molecular weight 240000. The described enzyme is specific for chlorogenic acid.

On the other hand a typical unspecific esterase like the pig liver esterases does not split chlorogenic acid.

The isoelectric focusing reveals several isoenzymes of chlorogenase within a pH-range of 4.0–4.5.

**Introduction**

Extracts of *Aspergillus niger* cultures contain several enzymes such as pectinases, proteases, cellulases and esterases. The range of application for these enzymes is mainly confined to viticulture and fruit juice manufacturing industries.

Quite early a small chlorogenic acid splitting activity has been observed during other investigations and – although incompletely – reported [1–4]. As described by Freudenberg [5], chlorogenic acid is a depside of caffeic acid and quinic acid (3,4-Dihydroxy-cinnamoyl-l-quinic acid). Chlorogenic acid is a very common ester in plants where it plays a role in disease resistance [6–8] and browning reactions [9–11].

Up to now it was not known whether or not the chlorogenic acid splitting activity is due to a side effect of unspecific esterases or to a novel specific enzyme [12, 13].

**Materials and Methods**

Pectinolytic enzyme preparations, prepared from culture medium of aspergillus niger, have been used. Because of the low chlorogenic acid splitting activity in these materials a larger batch was necessary. This was kindly prepared by C. H. Boehringer & Sohn, Ingelheim according to the method described by Beckhorn, Labbee and Underkofler [14]. Moreover several purification steps were necessary. At first, a further concentration was made by ultrafiltration through Amicon ultrafilters XM 50 and XM 100 A. By means of this procedure low molecular weight substances were separated such as the great excess of pectinases.

The second purification method was simpler but more effective: a fractional ethanol precipitation of the spray-dried raw material. The fraction with the highest enzyme activity was then further purified by chromatography on a Pharmacia column (100 × 1.5 cm) with Aca 34 polyacrylamide gel (LKB) using 0.1 m citric buffer pH 6.5.

The evidence of the enzymatic activity was demonstrated using chlorogenic acid (puriss., Roth) as substrate and measuring the amount of caffeic acid (purum, Roth) as a criterion for the degree of enzyme activity.

The enzyme activity was quantitatively measured by two different methods:

1. **High-performance-thin-layer-chromatography (HPTLC)**. 2.5 ml 0.2% chlorogenic acid were incubated for 15 min at 45 °C with 2.0 ml 0.1 m citric buffer, pH 6.5 and 0.5 ml test solution. 200 nl of the incubation mixture were applied to the HPTLC plates (Merck, Darmstadt) by means of the Nano-
Applicator (CAMAG). In addition standard solutions of caffeic acid were applied; development was performed in a solvent system of ethylacetate: formic acid (85%): water (100:5:3). The determination was carried out using the Zeiss DC-Spectrophotometer KM 3 at the absorption maximum of caffeic acid on the plate (322 nm). Quantification was accomplished by integrating the peaks with the Autolab Minigrator (Spectra Physics).

2. Autotitrator-system, indicating the formation of caffeic acid or generally free carboxylic groups in enzyme containing samples by consumption of NaOH. For autotitration the Titrigraph, Titrator and Auto-Burette (Radiometer, Copenhagen) instruments were used. The reaction mixture (3 ml 0.2% chlorogenic acid and 0.1 ml test solution) was incubated at 45 °C.

The autotitration-system has been mainly used for testing the substrate specificity of the chlorogenase with several esters such as acetylthiocholine (acetylcholinesterase test kit, Boehringer Mannheim); ethyl butyrate, methyl butyrate, naphthyl-(1)-butyrate, naphthyl-(2)-butyrate, naphthyl-(1)-acetate, naphthyl-(2)-acetate, glyceryl triacetate (Merck, Darmstadt); DL-p-hydroxy-amygdalic-acid-ethylster (EGA Chemie) and sunflower oil.

The pig liver esterase from Boehringer Mannheim (104698) was employed as an unspecific carboxylic-ester hydrolase (EC 3.1.1.1). The protein concentration was determined by measuring the absorption of the test solution at 280 nm, where the absorption of a 0.1% albumin solution $A_{280} = 1$ was taken for 1 mg protein/ml.

All chemicals used were of analytical grade.

Furthermore, the enzyme was separated into several bands by isoelectric focusing on polyacrylamide gel plates (LKB 2103 Ampholine PAG plates) with the pH range 4.0–6.5, anodic buffer 0.1 M glutamic acid in 0.5 M $H_2PO_4^-$, cathode electrode solution 0.1 M $\beta$-alanine; 8 °C, 15 mA, 1200 V; 2.5 h.

Before starting the isoelectric focusing experiment with the samples, the gel plates were prefocussed for one hour (1200 V, 6 mA, 8 °C). Then aliquots of the test solution were applied to the gel surface by small pieces of filter paper which had to be removed after 30 minutes of focusing to avoid tailing. The experiment was continued for two hours. At the end of the isoelectric focusing procedure, the pH-gradient on the gel was measured by a special micro-electrode. The marginal strips (of 3 cm width) to the left and right of the gel were cut off and after fixation with trichloroacetic acid and sulfosalicylic acid — stained with Coomassie Brilliant Blue R 250 according to the LKB manual. The unstained middle part of the gel was cut into small strips, using the stained bands of protein at the gel border as a measure for the width of the strips. The gel strips then were incubated for 20 min at 45 °C with citric buffer pH 6.5 and 0.2% chlorogenic acid as substrate. This was followed by a high performance thin-layer chromatography indicating which bands contained chlorogenase and which not. The protein content of the band fraction was determined by the absorption at 280 nm after separation on a G-25 Sephadex column (1.5 x 20 cm).

Results

Isolation

The fractional ethanol precipitation has proved to be the most acceptable method for concentrating the chlorogenase in the ultrafiltrated (PM 30 filters) raw material.

Starting from 15 g in 150 ml distilled water — as to the O. D. $A_{280} = 13.5$ g protein — fraction 6 (42% ethanol) and 7 (46% ethanol) gave the highest chlorogenase activity. The activity measured by the caffeic acid produced (HPTLC method) was 61 mU/mg (fraction 6). Compared to the aqueous culture extract, this means a 12.7-fold increase in the enzyme activity. 4.5% of the 13.5 g protein were found in fraction 6.

For further purification fraction 6 was chromatographed on the AcA 34 column: 2 ml of this fraction (61 mg protein) and 0.5 ml of dextran blue were fractionated and volumes of 3 ml were collected. The exclusion limit of AcA 34 gel for molecular weight is about 350000. Substances with such a molecular weight or higher would be eluated after 65 ml/void volume (e. g. dextran blue). The enzymatic activities of the column eluates are 873 mU/mg for fraction 98–101 ml, 1050 mU/mg in 101–104 ml and 880 mU/mg in 104–107 ml eluate. The unspecific esterase starts from 107 ml elution volume (Fig. 1).

Characterization

By calibration of the AcA 34 column with rabbit muscle aldolase (MV 158000), cattle liver catalase
Elution vol. [ml]

Substrate: chlorogenic acid, glyceryl triacetate

Fig. 1. Separation of chlorogenase and unspecific esterases on a AcA 34 column (1.5 x 100 cm, 2 ml ethanol fraction 6 corresponding to 60 mg protein). The weak dotted line means O. D. at 280 nm.

(MG 240 000) and ferritin (MG 450 000), the molecular weight of the chlorogenase could be determined and was found to be 240 000 (elution volume 104 ml). A cochromatography of the test solution with catalase confirmed this finding. This corresponds also to the ultrafiltration experiments performed with Amicon Ultrafilters PM 30, XM 50 and XM 100 A. In all cases the enzyme activity was found in the retentate, indicating a molecular weight higher than 100 000.

The incubation experiments showed a relative broad temperature range for the enzyme activity. Considerable activity was evident even at room temperature and below, whereas inactivation over 50 °C was rather strong. The maximum activity was found at around 45 °C.

The pH-optimum was 6.5. Half maximal enzyme activity was found at pH 3.0 and 8.0. The pH-range 1.5–12.5 has been tested.

Divalent cations are not essential for the enzyme activity. This was proved by adding 1.0 mM EDTA to the incubation mixture.

After addition of di-isopropyl-phosphorofluoridate (DFP, 10⁻⁴ M) to an incubation mixture, a complete inhibition of the enzyme activity was evident. This reaction is well known for other esterases as an inactivation by phosphorylation of the serine in the active site of the enzyme.

Using chlorogenic acid concentrations ranging from 0.25 mg/ml to 8.0 mg/ml, a $K_m$ value of 0.70 mM was determined.

For characterization of an enzyme preparation moreover the substrate specificity is most important. The ethanol precipitate No. 6 was tested with the following substrates: acetylthiocholine, ethyl butyrate, methyl butyrate, naphthyl-(1)-acetate, naphthyl-(2)-acetate, DL-p-amyladalic-acid-ethylester and sunflower oil. In no case was an esterhydrolysis found. In comparison, the unspecific esterase from pig liver showed a high activity for methyl- and ethyl butyrate, glyceryl triacetate and DL-p-hydroxy-amyladallic-acid-ethylester, but did not split chlorogenic acid.

Besides the small chlorogenase activity, the raw material (PM 30) possessed a strong glyceryl triace-
Table I. Fractional precipitation of 15 g starting product (as to O. D. 580nm 13.5 g protein) in 150 ml water with ethanol. Each precipitate was dissolved in 20 ml distilled water. Chlorogenase and unspecific esterases (glyceryl triacetate splitting) activities were determined.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ethanol concentration</th>
<th>Protein [g]</th>
<th>Chlorogenase [mU/mg]</th>
<th>Glyceryl triacetate splitting [mU/mg]</th>
<th>Glyceryl triacetate splitting/chlorogenase ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6%</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>17%</td>
<td>0.36</td>
<td>-</td>
<td>168</td>
<td>&gt;</td>
</tr>
<tr>
<td>3</td>
<td>25%</td>
<td>0.21</td>
<td>-</td>
<td>243</td>
<td>&gt;</td>
</tr>
<tr>
<td>4</td>
<td>32%</td>
<td>0.33</td>
<td>20</td>
<td>303</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>38%</td>
<td>0.44</td>
<td>49</td>
<td>679</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>42%</td>
<td>0.61</td>
<td>61</td>
<td>820</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>46%</td>
<td>0.51</td>
<td>58</td>
<td>706</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>50%</td>
<td>0.32</td>
<td>28</td>
<td>886</td>
<td>32</td>
</tr>
<tr>
<td>Supernatant (250 ml)</td>
<td>50%</td>
<td>10.1</td>
<td>-</td>
<td>62</td>
<td>&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Starting solution (150 ml)</td>
<td>13.5</td>
<td>15</td>
<td>285</td>
<td>19</td>
</tr>
</tbody>
</table>

tate splitting activity (285 mU/mg). As can be seen from Table I, there is an increase of the chlorogenase compared to the glyceryl triacetate hydrolysis by fractional ethanol precipitation. The ratio of glyceryl triacetate activity to chlorogenase decreased from 19 (raw material) to 13 (fraction 6) resp. 12 (fraction 7). This indicates the possibility of separating these different enzyme activities. Fig. 1 demonstrates the clear cut separation of both activities on the AcA 34 column. The elution volumes 95–107 ml with the highest chlorogenase content are completely free of a glyceryl triacetate esterase. This enzyme activity has its maximum after 116–122 ml with a chlorogenase content which can be ignored.

For further characterization, the ethanol precipitate No. 6 was studied by the isoelectric focusing method. Beside two strong bands without chlorogenase activity, several chlorogenase active bands were detected within a pl-range of 4.0–4.5. The highest specific activity was found with 8.250 mU/mg in a middle band between anode and cathode. This means an increase in activity by a factor of 1.719 compared to the aqueous culture extract.

As to the lack of need for divalent metal ions and as to the selective inhibition by di-isopropyl-phosphorofluoridate, the chlorogenase behaves similarly to other carboxylesterases. The $K_m$ value of 0.70 mM chlorogenic acid is almost the same as with the mammalian liver esterase (0.50 mM ethyl butyrate) [15].

As to the molecular weight and pH-optimum, chlorogenase differs from most other esterases.

However the main characteristic feature is the high substrate specificity. Therefore we would recommend a separate EC number within the group of carboxylesterases (EC 3.1.1.1).