PROTEOLYTIC AND ESTEROLYTIC ACTIVITY OF A MILK-CLOTTING PROTEASE (MCP) FROM *BACILLUS MESENTERICUS* STRAIN 76

St. P. STOEVA and B. K. MESROB

Department of Organic Chemistry, Institute of Chemical Technology, Sofia-1156, Bulgaria

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1. Introduction

The MCP has been isolated from *Bacillus mesentericus* strain 76 [1] and purified in analytical scale [2]. Some of its properties have been described: molecular weight, pH and temperature stability, effect of ions, and N-terminal amino acid sequence to the 15th step [3]. The proteolytic action of the MCP on whole casein was investigated and compared with those of calf rennin, pepsin and the commercial milk-clotting preparation of Pharmachim Sofia [4].

The kinetic studies reported in this paper were performed in order to obtain more information about the proteolytic and esterolytic action of the MCP and to determine the *K*m values for the hydrolysis of BAEE and TEE.

2. Materials and methods

2.1. *Enzyme preparation*

Microbial enzyme from *Bacillus mesentericus* strain 76 was obtained after Emanouilov [1] and purified according to Ivanov et al. [2] by three-fold precipitation with ethanol isoelectric focussing. The pooled active fraction was passed through gel column of Sephadex G-25 (20 × 410 mm), equilibrated with 1 mM calcium chloride.

Abbreviations: MCP, milk-clotting protease from *Bacillus mesentericus* strain 76; TEE, L-tyrosine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; BAEE, *N*-α-benzoyl-L-arginine ethyl ester

*Soxhlet units is the measure for the MCP quantity used by hydrolysis of all the substrates. The MCP was used directly after gel filtration to avoid inactivation during liophylisation.

2.2. *Protein substrates*

Casein powder, according to Hammarsten, was purchased from E. Merck (Germany).

Hemoglobin was kindly donated by Dr P. Nyshanyan (Institute of Epidemiology and Microbiology, Sofia).

2.3. *Synthetic substrates*

TEE and BAEE were purchased from Fluka AG (Basel, Switzerland). All other reagents used were commercial products of the highest available quality.

2.4. *Milk-clotting activity*

The milk-clotting activity was determined according to Iwasaki et al. [5] using fresh pasteurized skim-milk as a substrate.

2.5. *Substrate hydrolysis*

The enzyme-catalysed hydrolysis of the substrates were followed using Radiometer pH-stat model TTT2 (SBR3) coupled with autotitrator ABU 12 (Radiometer Denmark), equipped with a thermostated vessel.

Proteolytic activity was determined using 2% casein solution in 0.1 M KCl + 5 mM calcium chloride and 2% solution of urea-denaturated hemoglobin, prepared according to Anson [6] without adding the buffering agent. The presence of urea in the hemoglobin solution does not change the activity of the MCP. The hydrolysis of casein and hemoglobin was determined in the pH region 2.8–11.0 and 3.1–11.0, respectively, except the pH regions near the isoelectric points of the substrates (pH 4.5–6.2 for casein and 5.2–7.0 for hemoglobin). To each substrate solution was added 50 μl MCP solution in 1 mM calcium chloride, containing 30 Soxhlet units milk-clotting activity*.

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The optimum pH for the hydrolysis of TEE and BAEE was determined using 1 ml of 100 mM solution of the substrate in 0.1 M kalium chloride + 5 mM calcium chloride. To the substrate solution was added 75 μl MCP solution, containing 45 Soxhlet units for the hydrolysis of TEE and 100 μl MCP solution (60 Soxhlet units) in the case of BAEE. The Michaelis constants (K_m) of the enzyme reaction were estimated by the Lineweaver-Burk plot [7]. The concentrations of the substrates were varied from 0.01–0.04 M in 0.1 M kalium chloride + 0.05 M calcium chloride.

Proteolytic and esterolytic activity was determined as follows: 1 ml substrate solution was thermostated in the pH-stat vessel for 5 min at 35°C. Then the pH was adjusted to the desired value and the reaction was initiated by introducing the enzyme solution. The temperature of the reaction vessel was maintained constant at 35°C. Titrations were performed with 0.01 N potassium hydroxyde as titrating agent.

3. Results

The pH-activity curves for the hydrolysis of casein and urea-denatured hemoglobin by the action of the MCP were presented in fig.1. The MCP showed an optimal pH at 8.5–9.0 for the hydrolysis of casein and pH 8.5–9.2 for the hydrolysis of urea-denatured hemoglobin. There is a difference in the activity pH curves for both substrates in the pH region 6.3–6.9

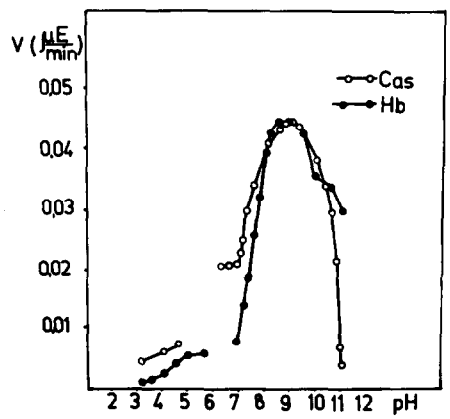

Fig.1. pH-Optimum of casein and hemoglobin hydrolysis by the action of the MCP.

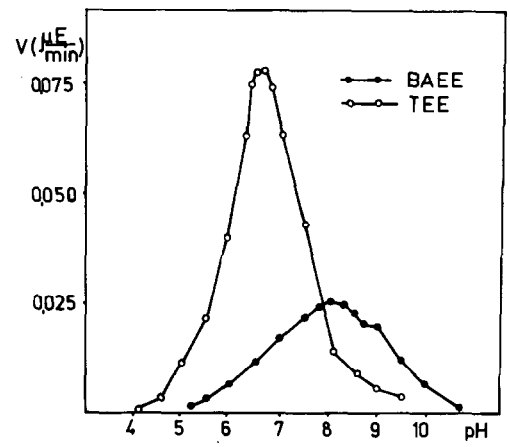

Fig.2. pH-Optimum of TEE and BAEE hydrolysis by the action of the MCP.

where casein hydrolysis is about 1.5-times greater than hemoglobin hydrolysis under the same conditions.

Hydrolytic activity of the MCP on synthetic substrates examined using the titration method is shown in fig.2. The MCP showed an optimal pH 6.6, for the hydrolysis of TEE and pH 8.0, for the hydrolysis of BAEE. The rate of the TEE hydrolysis is 4-times higher than of BAEE under the same conditions (calculate per unit introduced enzyme activity).

The K_m for TEE and BAEE hydrolysis determined at the optimal pH for each substrate (TEE-6.6 and BAEE-8.0) are 83 mM for TEE and 26.6 mM for BAEE.

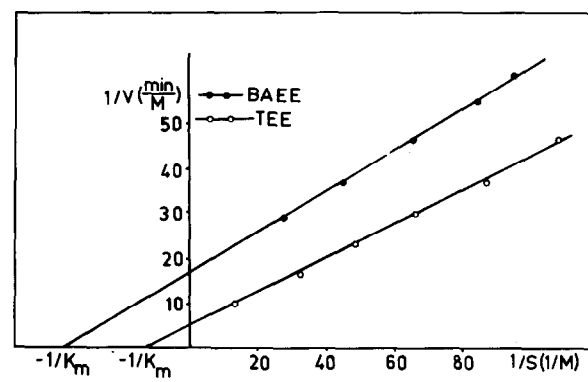

Fig.3. K_m-Values for the hydrolysis of TEE and BAEE by the action of the MCP.

4. Discussion

A number of proteases obtained from microorganisms (*Bacillus subtilis, Streptomyces fradiae, Asperigillus orizae*–bacterial and mold origin) show an alkaline pH optimum for casein hydrolysis [8]. Our results indicate that the MCP is a typical alkaline protease with a pH optimum toward casein and hemoglobin 8.5–9.0 However, in the pH region 5.8–6.5 its proteolytic action decreases markedly and under appropriate conditions the MCP may be used as a milk-clotting enzyme. These results are in good agreement with our previous investigations [4]. The higher rate of casein hydrolysis than of hemoglobin hydrolysis in the pH region 6.2–6.6 probably is due to the joined action of both phosphoesterolytic and proteolytic action [9].

The MCP is capable of hydrolyzing both esters of aromatic amino acids (TEE) and esters of basic amino acids (BAEE). Such a broad specificity toward the synthetic ester substrates is typical for the alkaline proteases of *Bacillus* group [10]. The K_m for the hydrolysis of ester substrates by the bacterial protease (MCP) are much higher in comparison with the K_m for the hydrolysis of the same substrates by animal enzymes (chymotrypsin and trypsin) [11–13]. (The comparison with the hydrolysis of tyrosin substrate is not quite correct because they use ATEE.) This suggests a difference between the esterolytic properties of the MCP and of the animal enzymes.

The difference between the pH optimum of proteolytic action (pH 8.5–9.0) and esterolytic action (pH 6.6 and pH 8.0) on the one hand and the difference between the pH optima of esterolytic activity of MCP toward TEE and BAEE (TEE-6.6 and BAEE-8.0) on the other hand, are explained by the adaptability of the bacterial protease to the substrates.

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