EFFECT OF MODIFICATION OF LYSINE RESIDUES OF COW COLOSTRUM TRYPSIN INHIBITOR ON ITS ANTITRYPTIC AND ANTICHYMOTRYPTIC ACTIVITY

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1. Introduction

The trypsin inhibitor from cow colostrum belongs to naturally-occurring polyvalent inhibitors. Besides trypsin, to which this inhibitor binds with a ratio of 1:1 [1], it also inhibits chymotrypsin to a lesser degree [2]. It is known that the primary structure of inhibitor B from cow colostrum partly resembles that of Kunitz's basic pancreatic trypsin inhibitor (BPTI) [3] and that lysine 18 plays a role in the active site of the inhibitor during its binding to trypsin [4]. In this paper, evidence is presented which supports the hypothesis that inhibitor B from cow colostrum has an active site responsible for the inhibition of α-chymotrypsin different from the active site responsible for the inhibition of trypsin.

2. Material and methods

Trypsin, a crystalline product of Léčiva, Prague, was recrystallized three times with magnesium sulfate. α-Chymotrypsin was obtained by the activation of chymotrypsinogen A (Léčiva, Prague) which had been recrystallized seven times with ammonium sulfate and twice with ethanol [5]. The trypsin inhibitor from cow colostrum was prepared by a procedure described elsewhere [6]. Methods for the preparation of completely or partly carbamylated and guanidinated derivatives of the trypsin inhibitor and the determination of its amino acid sequence have been described in the preceding paper [4].

The inhibitory activity of the native inhibitor and of its derivatives was determined in terms of differences in the activity between intact and inhibited enzymes. The inhibition of the esterase activities was measured by colorimetry of the uncleaved substrate [7]. For the determination of the inhibition of tryptic activity, 0.1 ml of inhibitor solution or of water (enzyme standard) and 0.1 ml (10 μg) of the trypsin solution were added to 0.5 ml of 0.1 M tris buffer, pH 7.8. After 3 min, 0.5 ml of 0.02 M *p*-toluenesulfonyl-arginine methyl ester (TAME) in water was added. The reaction was discontinued after 5 min of incubation at 25° by the addition of 2 ml of a mixture of aqueous solutions of 2 M hydroxylamine and 3.5 M sodium hydroxide (1:1, mixed together one hour before use). After 2 min, 1 ml of 6 N HCl and 1 ml of aqueous solution of 0.7 M $FeCl_3$ were added. Immediately after the samples had been mixed with the reagents, their absorbance at 540 nm was measured using water as a blank. The color standard was prepared from 0.5 ml of the buffer, 0.5 ml of water. The percentage of hydrolysis of the substrate was read from the calibration curve obtained with 0.5–10 μg of the enzyme. The inhibition of the esterase activity of chymotrypsin was measured by a similar procedure. To 0.5 ml of 0.25 M phosphate buffer, pH 6.45, 0.1 ml (0.1 μg) of chymotrypsin in 0.001 N HCl and 0.1 ml of inhibitor in water were added. After 5 min at 37°, 0.5 ml of 0.012 M L-tyro-

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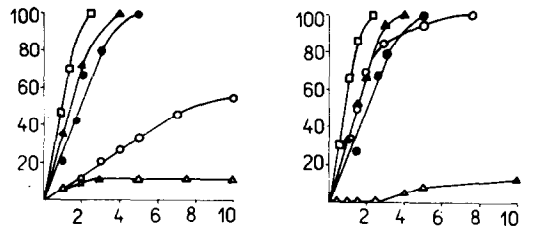

Fig. 1. Effect of concentration of native inhibitor and of modified inhibitors from cow colostrum on (a) proteolytic and (b) esterase activity of trypsin.

Abscissa: quantity of inhibitor in μg; *ordinate:* percentage inhibition. □—□ native inhibitor, △—△ fully carbamylated inhibitor, ▲—▲ CTI with carbamylated lysine 66 and *N*-terminal amino group, ○—○ CTI with both lysine residues guanidinated, ●—● CTI with lysine 66 guanidinated.

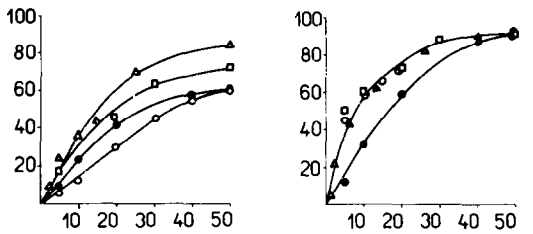

Fig. 2. Effect of concentration of native inhibitor and of modified inhibitors from cow colostrum on (a) proteolytic and (b) esterase activity of trypsin. The symbols are identical with those given in the legend to fig. 1.

sine ethyl ester (TEE) in 0.25 M phosphate buffer, pH 6.45 was added. All solutions were preheated at 37°. The incubation was allowed to proceed 20 min at 37°. The subsequent procedure was the same as that employed for the measurement of tryptic activity.

The inhibition of peptidase activity of trypsin was measured in terms of the inhibition of the hydrolysis of casein [8]. To 1.8 ml of 0.1 M tris buffer, pH 7.6, at 37°, 0.1 ml (10 μg) of trypsin or chymotrypsin in 0.001 M hydrochloric acid and 0.1 ml of the native or modified inhibitor were added. After 1 min of incubation, 1 ml of preheated casein (1 g/100 ml of 0.1 M tris buffer, pH 7.6) was added and the incubation was allowed to proceed 20 min at 37°. After this period, the reaction was discontinued by precipitation of the remaining casein by 3 ml of 5% trichloroacetic acid. The mixture was left for 20 min at room temperature, filtered, and the absorbance of the filtrate measured at 280 nm using a blank in which trypsin and the inhibitor had been replaced by the buffer. The values of the inhibition were obtained by subtraction of the measured values from those of the enzyme standard (in which the inhibitor had been replaced by the buffer).

3. Results and discussion

Inhibitor B from cow colostrum contains two lysine residues in its molecule, namely in position 18 and 66. By modification of the intact inhibitor, derivatives were obtained in which both lysine residues were converted into homocitrulline or homoarginine residues. Derivatives with only one modified lysine residue, in position 66, were prepared by modification of the inhibitor bound in the complex with trypsin and subsequent dissociation of the complex. With these two types of derivatives, the effect of the modification of the inhibitor on the inhibition of esterase and proteolytic activity of trypsin and chymotrypsin was examined. CTIB with both lysine residues converted into homocitrulline residues and *N*-terminal end group carbamylated loses more than 90% of its ability to inhibit both the esterase and the proteolytic activity of trypsin. The inhibitory activity toward the esterase activity of chymotrypsin remained unaltered and toward the proteolytic activity was even slightly higher after the modification. The antitryptic activity of the inhibitor with modified lysine 66 and *N*-terminal end group remained unchanged. The inhibitor derivative with homoarginine in position 18, i.e. with an amino acid in position 18 sensitive to the specificity of trypsin, inhibited the proteolytic activity of trypsin to the same degree as the native inhibitor and the esterase activity of trypsin considerably less. This phenomenon is most likely caused by the elongation of the side chain of the amino acid residue in position 18. The derivative retained full antichymotryptic activity.

It is thus obvious that lysine 18, which is essential for antitryptic activity, plays no role in the inhibition of chymotrypsin. The active site responsible for antichymotryptic activity is necessarily different from the active site for antitryptic activity. The problem

whether these sites are located in different regions of the molecule, as is the case of e.g. certain avian ovomucoids [9], or whether they overlap, will require further investigation. Our results are in agreement with the recent results with the pancreatic trypsin inhibitor which have shown that the antichymotryptic activity is retained after acetylation [10] or carbamylation [11] of the active site.

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References

[1] D.Čechová-Pospíšilová, V.Švestková and F.Šorm, in: Abstr. 6th FEBS Meeting, Madrid 1969 (Publ. Spanish Biochem. Soc. Madrid) p. 87.
[2] F.C.Wu and M.Laskowski, J. Biol. Chem. 213 (1955) 609.
[3] D.Čechová, V.Švestková, B.Keil and F.Šorm, FEBS Letters 3 (1969) 155.
[4] D.Čechová and G.Muszynska, FEBS Letters 8 (1970) 84.
[5] M.Kunitz, J. Gen. Physiol. 32 (1949) 265.
[6] D.Čechová, V.Jonáková-Švestková and F.Šorm, J. European J. Biochem. (1970) submitted for publication.
[7] S.Hestrin, J. Biol. Chem. 180 (1949) 249.
[8] M.Kunitz and J.H.Northrop, J. Gen. Physiol. 19 (1936) 991.
[9] R.E.Feeney, F.C.Stevens and D.T.Osuga, J. Biol. Chem. 238 (1963) 1415.
[10] N.Sevilla and M.Rigbi, Israel J. Chem. 7 (1969) 130 p.
[11] H.Fritz, H.Schult, R.Meister and E.Werle, Z. Physiol. Chem. 350 (1969) 1531.