ON THE PROPERTIES OF TRYPSIN INHIBITORS FROM HUMAN AND BOVINE COLOSTRUM

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1. Introduction

Colostral trypsin inhibitor plays an important role in the mechanism of absorption of immune bodies by a new-born mammal [1, 2].

In human colostrum the content of the trypsin inhibitor is much lower than in the case of other mammals. This can be explained by different pathways of antibody transfer: predominantly via placenta in man, via colostrum in other mammals [2]. The highest concentration of the inhibitor occurs in human colostrum between the 2nd and 3rd day [1, 3].

Laskowski and Laskowski obtained a pure trypsin inhibitor preparation from bovine colostrum [4]. To facilitate the procedure of its isolation, they recommended later [1] as the first step a precipitation of the bulk of proteins with 2.5% trichloroacetic acid at 80°, which causes a loss of approximately 75% of the total inhibitor. The inhibitor resulting from this method has a molecular weight of 10,500, it is highly stable in acid media and at elevated temperature [1, 5].

Čechová et al. have determined its partial primary structure and found that it is structurally related to the basic inhibitor from bovine pancreas [6]. Recently the same authors have shown that the crude inhibitor preparation from bovine colostrum which has been pre-treated with trichloroacetic acid contained several active components, a group of iso-inhibitors [7]. The question remained open whether this micro-heterogeneity is inherent or acquired during the initial stages of the isolation process.

To compare the human and bovine inhibitors, we tried to isolate the inhibitor from human colostrum in an analogical way to that described for the bovine inhibitor. Difficulties in the early stage of the isolation procedure prompted us to reinvestigate some properties of proteins responsible for trypsin inhibition in the colostrum prior to its treatment with trichloroacetic acid. This study revealed substantial qualitative differences between the human and bovine colostrum as regards trypsin inhibition.

2. Experimental

The inhibitor was determined by the spectrophotometric method of Kunitz [8] in 0.1 M phosphate buffer pH 7.6 at 37° with casein Merck "For Biochemistry" as substrate and β-trypsin isolated from the commercial Worthington trypsin TRL [9] as enzyme. The concentration of trypsin was determined spectrophotometrically using the absorption coefficient 16.1 [10].

The inhibitor content of human colostrum from 2nd and 3rd day varied between 20 and 40 μg of trypsin inhibited per ml and it was constant over a month in a refrigerator. The inhibitor content of bovine colostrum from 1st day was 500–600 μg of trypsin inhibited per ml. It was constant for at least

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Abbreviations:
TCA : trichloroacetic acid
BPTI: basic pancreatic trypsin inhibitor (Kunitz).

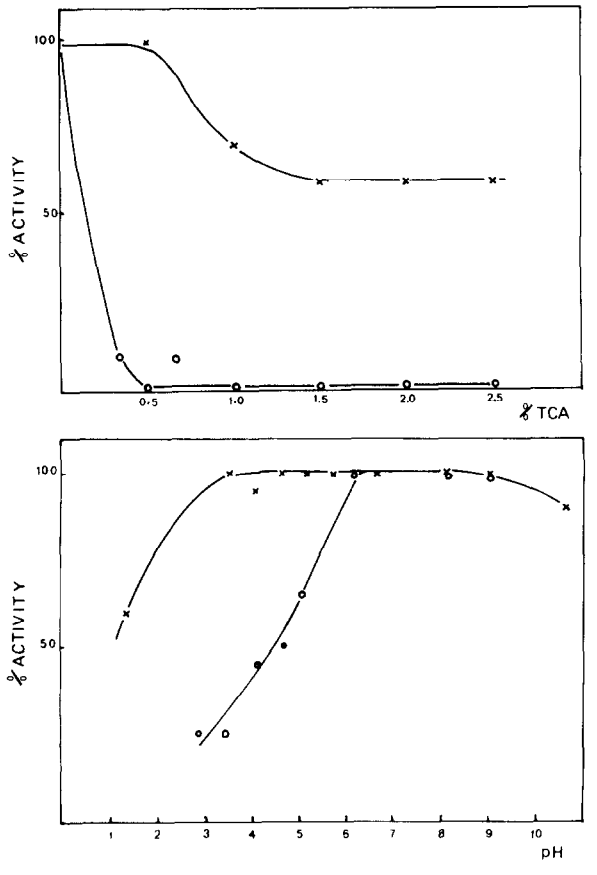

Fig. 1. Stability of the colostrum inhibitors towards trichloroacetic acid (a) and at different pH values (b). X——X bovine, o——o human colostrum. Room temperature, incubation time 30 min (a) and 1 hr (b).

4 months when the colostrum was stored in a refrigerator. The inhibitor content of the serum from a woman in labor was 1.9 mg of trypsin inhibited per ml.

Incubation with trichloroacetic acid: the colostrum was mixed with the same volume of TCA of appropriate concentration, samples were evaluated for inhibitor content after 30 min of incubation at room temperature. In the case of bovine colostrum in the range of 1.5–2.5% TCA concentration, filtration has been found necessary prior to sample withdrawal.

Incubation at different pH-values: the colostrum was adjusted to an appropriate pH and left for 1 hr at room temperature. 100 μl of the solution was withdrawn for the assay. Bovine colostrum was diluted after pH adjustment ten times with a buffer of the same pH.

Incubation at different temperature: colostrum (bovine 10 X diluted) was adjusted to an appropriate pH and kept at different temperatures. Samples were withdrawn at given times and kept at 0° prior to the assay.

For the gel chromatography on a Sephadex G-100 column, samples of the human and bovine colostrum were centrifuged for 30 min at 2000 rpm at room temperature and left for 2 hr in the refrigerator. The fat layer was discarded and in the case of human colostrum the lower liquid was concentrated three times by ultrafiltration. The ultrafiltrate was devoid of activity. Serum sample was chromatographed directly.

3. Results

3.1. *Stability of the inhibitors towards trichloroacetic acid*

Preliminary experiments have shown, that the treatment of the human colostrum with 2.5% trichloroacetic acid destroys its antitryptic activity completely. The comparison of the residual inhibitor activity with that of the bovine colostrum at different TCA concentrations is shown in fig. 1a.

In the human colostrum the presence of the inhibitor can no more be detected even at a 0.5% TCA concentration which changes the pH value of the colos-

Table 1
Residual antitryptic activity of human colostrum incubated at different temperatures for 1 hr and at 56° for different intervals. At the same conditions the activity of bovine colostrum remained unaltered.

conditions	activity	
	pH 4.5	pH 7.6
22°, 60 min	50	100
37°, 60 min	25	95
56°, 60 min	20	60
10 min, 56°	25	80
20 min, 56°	25	65
40 min, 56°	25	70
60 min, 56°	20	60

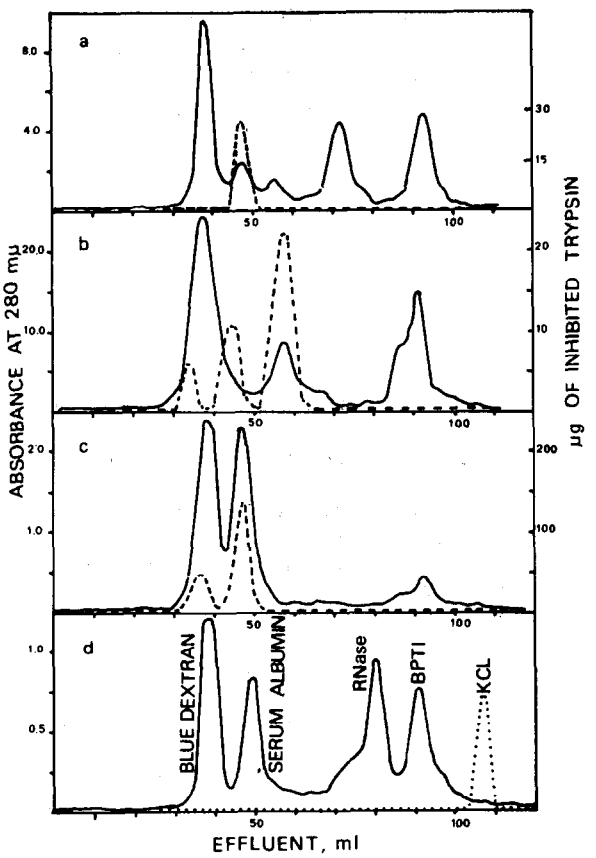

Fig. 2. Elution patterns of human (a) and bovine (b) colostrum, of serum from pregnant woman (c) and of standard mixture (d). Sephadex G-100 column (1.1 × 110 cm) was eluted with a 0.01 M ammonium carbonate buffer pH 8.5, fractions 2.2 ml in volume were taken at 45 min intervals. Full line, absorbance at 280 nm; dashed line, activity in μg of trypsin inhibited by 1 ml of the eluate; dotted line, conductivity.

trum to 3.5. Only one third of the inhibitor disappears from the bovine colostrum at about 1% TCA, the residual activity does not change at higher TCS concentrations. It follows that in the bovine colostrum there exist at least two inhibitors, differing in their stability towards TCA.

3.2. *pH and temperature dependence*

To find whether the loss of the inhibitor is due specifically to trichloroacetic acid or to the pH change, a series of pH dependence assays was undertaken (fig. 1b). The bovine inhibitor is quite unaffected over a broad pH range. In human colostrum its concentration decreases below pH 6.0. Still 25% of antitrypsin activity remains at pH 3.5 and room temperature after 1 hr; a 0.5% TCA concentration at this pH caused a total loss of the activity in 30 min.

The influence of temperature at different time intervals and two pH values is summarized in table 1.

3.3. *Gel chromatography*

Three different fluids containing trypsin inhibitors were chromatographed under the same conditions on a Sephadex G-100 column (fig. 2): bovine and human colostrum and the serum from a woman in labor, containing very high antitrypsin activity [11, 13].

Only one peak of antitrypsin activity was found in the human colostrum. It had the elution volume close to that of serum albumin. This volume did not change on rechromatography of the pooled peak. The activity disappeared entirely in 2.5% trichloroacetic acid.

Bovine colostrum gave three active peaks. The first appeared with the dead volume of the column, the second was close to the elution volume of serum albumin, and the third at a volume corresponding approximately to a molecular weight of 22,000.

In 2.5% TCA at room temperature, the original activity of the first and second peak decreased to 10, resp. 25%, the third peak remained fully active. After the TCA treatment, this peak was submitted to rechromatography. It emerged from the column at the same elution volume as before.

The serum from a pregnant woman gave two peaks of activity, the major one was eluted simultaneously with serum albumin.

4. Discussion

Both physico-chemical and chemical studies [1, 6, 13, 14] have assigned the colostrum inhibitors to the class of heat and acid stable inhibitors of low molecular weight (6000–10,000). All these studies dealt with inhibitors, which have survived the treatment of the parent colostrum with trichloroacetic acid at elevated temperatures.

From the results presented in this work it follows,

that in native bovine colostrum the acid stable inhibitor is only one of at least three active components of different stability and that in human colostrum the only active component is destroyed by both trichloroacetic acid and heat.

One of the surprising findings was the low elution volume of the TCA stable inhibitor from bovine colostrum which emerged before the peak of ribonuclease and corresponded to a molecular weight of trypsin. There was no activity found in the range between the elution volume of ribonuclease and basic pancreatic trypsin inhibitor, which served as standard proteins in a parallel run. The relation of our TCA stable inhibitor to the bovine colostrum inhibitors which have been isolated previously by the TCA treatment [1, 7, 8] deserves further investigation.

The second inhibitor from the bovine colostrum and the only active peak from the human colostrum were both inactivated by TCA. The emerged with an elution volume close to that of serum albumin and of the major inhibitor peak from serum.

The trypsin inhibitor of human serum, known as α_1-antitrypsin, has a slightly lower molecular weight than serum albumin [15]. It is destroyed by TCA [1, 16] and has a low stability below pH 5.5 [1, 15–17]. It occurs in high concentration in the blood of women in labor [11].

All the results indicate, that the human colostrum inhibitor and the second high molecular weight bovine colostrum inhibitor resemble more the α_1-antitrypsin from human serum than the group of the heat and acid stable low molecular weight bovine inhibitors.

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References

[1] M. Laskowski, Jr. and M. Laskowski, J. Biol. Chem. 190 (1951) 563.
[2] L. Schneider and J. Szathmary, Z. Immunitätsforsch. 98 (1940) 24.
[3] J. Hyánek, R. Nosková and Z. Cafourková, Ann. Paediat. 204 (1965) 125.
[4] M. Laskowski, Jr. and M. Laskowski, Federation Proc. 9 (1950) 194.
[5] B. Kassel and M. Laskowski, J. Biol. Chem. 219 (1956) 203.
[6] D. Čechová, V. Švestková, B. Keil and F. Šorm, FEBS Letters 4 (1969) 155.
[7] D. Čechová, V. Jonáková-Švestková and F. Šorm, Collection Czech. Chem. Commun. 35 (1970) 3085.
[8] M. Kunitz, J. Gen. Physiol. 30 (1947) 291.
[9] D.D. Schroeder and E. Shaw, J. Biol. Chem. 243 (1968) 2943.
[10] A. D'Albis and J.J. Bechet, Biochim. Biophys. Acta 140 (1967) 435.
[11] E.S. Duthie and L. Lorenz, Biochem. J. 44 (1949) 167.
[12] F.C. Moll, S.F. Sunden and J.R. Brown, J. Biol. Chem. 233 (1958) 121.
[13] M. Laskowski, Jr., P.H. Mars and M. Laskowski, J. Biol. Chem. 198 (1952) 745.
[14] M. Laskowski, B. Kassel and G. Hagerty, Biochim. Biophys. Acta 24 (1957) 300.
[15] H.E. Schultze, K. Heide and H. Haupt, Klin. Wochschr. 40 (1962) 427.
[16] H.F. Bundy and J.W. Mehl, J. Biol. Chem. 234 (1959) 1124.
[17] R.J. Peanasky and M. Laskowski, J. Biol. Chem. 204 (1953) 153.