ISOLATION OF ISOINHIBITORS FROM COW COLOSTRUM BY AFFINITY CHROMATOGRAPHY ON COLUMN OF TRYPSIN-SEPHAROSE 4B

D. ČECHOVÁ

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received March 27th, 1973

The new, simple method of isolation of trypsin inhibitors from cow colostrum is based on affinity chromatography on a column of trypsin-Sepharose 4B. The inhibitor isolated was purified on a column of DEAE-cellulose and thus resolved into 7 isoinhibitors of which 4 are present in dominant quantities. The analysis of the obtained products by disc-electrophoresis at pH 8·3 has shown that each of these dominant inhibitors migrates as one zone and is therefore highly pure. The migration rates of the isoinhibitors slightly differ, as well as their amino acid composition and specific activity.

Since the time the first preparation of insoluble trypsin[1] was obtained and shown to retain its ability to form complexes with its naturally occurring inhibitors[2], experiments have been made to use these findings for an effective isolation of trypsin inhibitors. Fritz and coworkers[3,4] were the first who developed the methods of isolation of the inhibitors from different sources by affinity chromatography on a column of trypsin attached to an insoluble support. Kassell and Marciniszyn[5], and Chauvet and Acher[6] have used for an effective isolation of the trypsin inhibitor from bovine organs Sepharose which was activated by cyanogen bromide, in analogy to the procedure used first by Porath[7] for the attachment of chymotrypsin. An advantage of Sepharose is its polydextran character and the fact that it has no ion-exchange effects as the supports used originally.

In this paper are described the isolation of the trypsin inhibitor from cow colostrum on trypsin-Sepharose, the final purification of the inhibitor and its resolution into isoinhibitors on DEAE-cellulose. The earlier described methods of isolation of this inhibitor[8,9] were more elaborate and the final yields were lower; these isolation procedures involved moreover precipitation by 2·5% trichloroacetic acid which could impair the non-protein moiety of the inhibitor molecule.

EXPERIMENTAL

Material. Sepharose 4B was a product of Pharmacia, Uppsala, Sweden. DEAE-Cellulose DE 11 was from Whatman, England. Bovine trypsin was a gift of the Institute for Clinical Chemistry and Biochemistry, Munich. Cow colostrum was obtained from the Collective farm, district Nymburk, Bohemia.

Preparation of affinity column. The attachment of trypsin to Sepharose 4B was carried out according to the method described for the isolation of trypsin inhibitor from bovine organs[5]. Sepharose (70 g) was washed several times with distilled water on a glass filter. Distilled water (50 ml) was added to the wet product. The activation was effected by cyanogen bromide (9 g) dissolved in distilled water (100 ml). The solution of cyanogen bromide was added to Sepharose with stirring and the pH of the mixture was maintained at pH 11 by 5M-NaOH for 6 to 7 min. Sepharose was subsequently washed with 4 l of distilled water (0°C) and by 4 l of cooled 0·05M sodium borate, pH 9, on a glass filter. Trypsin (2 g) to be attached to activated Sepharose was dissolved in 100 ml of cooled borate buffer at pH 9 immediately before the activation and the solution was then maintained at 0°C. Activated Sepharose was added to the trypsin solution with stirring. The mixture was stirred overnight in the cold room, then placed on a column (diameter 3·5 cm) and washed stepwise with 4 l of cooled buffer (0·05M sodium borate, 0·01M-$CaCl_2$, pH 9) and with 2 l of 0·5M-NaCl containing 0·01M-$CaCl_2$ at a rate of 20 ml/h. The column was then washed with 0·1M sodium acetate, pH 4, containing 0·3M-$CaCl_2$ and with 0·01M-$CaCl_2$ until the pH of the effluent was constant. When not used, the column was stored in the cold room at 4°C. The quantitative amino acid analysis of a sample of trypsin-Sepharose showed that approximately 14 mg of protein was attached to 1 ml of wet Sepharose. The whole column could retain a quantity of inhibitor corresponding to 800 inhibitory units.

The quantitative amino-acid analysis of the inhibitors and the determination of antitryptic activity were described in detail in the preceding paper[9]. The activity of the inhibitor is given in inhibitory units. One inhibitory unit inhibits 2 μg of trypsin by 50%. The specific activities are expressed as the number of inhibitory units in a quantity of protein which dissolved in 1 ml would show an absorbance of 1 000 at 280 nm.

Disc electrophoresis was carried out in 15% polyacrylamide gel and in Tris-glycine buffer at pH 4·5 (ref.[10]). The protein was stained by 1% amido black and the gel was destained in 7·5% acetic acid. Samples of 0·5 mg of protein per one gel column were used.

RESULTS AND DISSCUSSION

Preparation of Sample for Affinity Chromatography

The colostrum from the first day after the delivery was frozen and kept at −20°C until treated further. The colostrum was thawed before the treatment and centrifuged at 2000 rev./min, 30 min, and the upper lipid layer was removed. One ml of the colostrum contained 400−700 units of antitryptic activity. A volume of 1 l of colostrum was diluted (1 : 2) with water and allowed to stand 24 h at room temperature. The precipitate formed was centrifuged off and the clear supernatant was kept separately. The precipitate was extracted by 1 volume of water, and the mixture was again centrifuged. The supernatant was pooled with the supernatant from the

preceding step and the pH of the solution was adjusted to 7·5. The contaminating proteins precipitated after the pH-adjustment were removed by filtration over an asbestos filter coated by a thin kieselguhr layer. The filtrate was used for subsequent purification by affinity chromatography.

Chromatography on Sepharose Column with Attached Trypsin

The trypsin-Sepharose column (3·5 . 10 cm) was washed with 0·1M sodium acetate containing 0·3M-NaCl and 0·01M-$CaCl_2$ before the application of the sample. The sample (2—4 l) was allowed to flow through the column at a rate of 100 ml/10 min and 200 ml fractions were collected. The absorbance at 280 nm and the antitryptic activity were measured continuously. After the capacity of the column had been exhausted, *i.e.* after the appearance of activity in the effluent fractions, the applica-

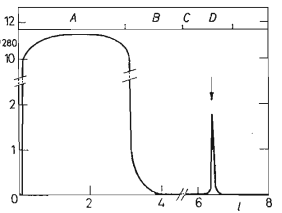

FIG. 1

Chromatography on Column of Trypsin-Sepharose 4B (3·5 . 8·5 cm)

A application of sample, *B* 0·1M sodium acetate pH 4·0, containing 0·01M-$CaCl_2$ and 0·3M-NaCl, *C* 0·01M-$CaCl_2$ in 0·3M-NaCl, *D* 0·01M-HCl containing 0·01M-$CaCl_2$ and 0·3M-NaCl.

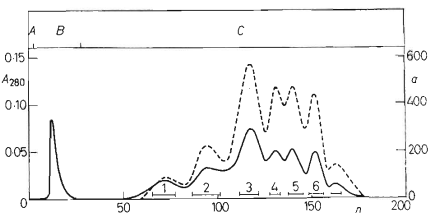

FIG. 2

Chromatography of Inhibitors on DEAE-Cellulose at pH 7·3

Full line, absorbance at 280 nm, dashed line activity (*a*) expressed as the number of inhibitory units in 1 ml of sample. *A* application of sample, *B* washing with 0·03M-Tris-HCl buffer *C* elution by a linear gradient of 0—0·02M-NaCl in 0·03M-Tris-HCl buffer at a flow rate 4·5 ml/15 min (one fraction). *n* number of fractions. 1—7 pooled fractions.

tion of the sample was discontinued and the column was washed with the buffer at pH 4 until the absorbance at 280 nm dropped below 0·010 (approximately 2 l).

TABLE I

Isolation of Inhibitor from Colostrum

Degree of purification	% of total activity	Specific activity[a]
Colostrum	100	—
Filtrate after precipitation of colostrum	83	34
Material after purification by affinity chromatography	70	7 600
Material after purification of DEAE-cellulose	43	9 250[b]

[a] The unit of specific activity is defined in the experimental section. [b] Average specific activity of material from peaks 2 through 7.

TABLE II

Quantitative Amino Acid-Analysis of Material from Peaks 3 through 6

Hydrolysis 20 h. No corrections for losses of labile amino acids during the hydrolysis were made. The content of half-cystine was determined as cysteic acid in a sample oxidized by performic acid.

Amino acid	Material from peak			
	3	4	5	6
Lysine	3·0	2·1	2·0	2·0
Arginine	3·0	3·0	3·0	3·1
Aspartic acid	8·5	8·3	8·1	8·1
Threonine	5·1	5·8	5·7	5·7
Serine	3·1	2·9	3·0	3·1
Glutamic acid	10·2	10·2	10·1	10·5
Proline	6·9	6·7	6·7	6·5
Glycine	4·0	4·0	4·0	4·0
Alanine	4·1	4·1	4·0	4·0
Half-cystine	6·0	6·0	6·0	6·0
Methionine	1·0	1·0	1·0	1·0
Isoleucine	0·8	0·8	0·8	0·8
Leucine	5·2	5·0	5·0	5·2
Tyrosine	2·6	2·7	2·5	2·7
Phenylalanine	3·5	4·0	3·8	3·8

TABLE III
Specific Activity of Material Purified on DEAE-cellulose

Material from peak	Specific activity[a]	Material from peak	Specific activity
1	5 000	5	9 600
2	9 800	6	9 500
3	7 600	7	9 600
4	9 400		

[a] The unit of specific activity is defined in the experimental section.

The inhibitor attached was displaced by 0·01M hydrochloric acid containing 0·3M-NaCl and 0·01M-$CaCl_2$; the flow rate was 20 ml/10 min (one fraction) (Fig. 1). The inhibitor-containing fractions were pooled according to the measurement of antitryptic activity and the pH of the solution was adjusted to 6 by 0·2M sodium hydroxide. The solution was desalted on a column of Sephadex G-25 (3·7 . 26 cm), equilibrated with 0·01M ammonium carbonate and freeze-dried. The amino acid analysis of this product showed that the sample contained traces of valine and histidine which are not present in the molecule of the inhibitor. The inhibitor was therefore subjected to additional purification on a column of DEAE-cellulose. The specific activity of the sample was more than 200-times higher after the affinity chromatography step (Table I).

Chromatography on DEAE-Cellulose

The lyophylised sample (50 mg) after affinity chromatography on trypsin-Sepharose was dissolved in distilled water (10 ml). The conductivity and the pH of this solution were adjusted to equal the conductivity and the pH of the equilibrating buffer. The solution was applied to a column of DEAE-cellulose (1·9 . 28·5 cm) equilibrated with 0·03M-Tris-HCl buffer at pH 7·3. The inhibitor was eluted from the column by a linear gradient of 0 – 0·2M-NaCl in 0·03M-Tris-HCl buffer at pH 7·3 (600 + 600 ml). As can be seen in Fig. 2, the inhibitor emerges from the column in 7 peaks of which 4 are dominant. The amino-acid analyses of the pooled and desalted fractions contained in the dominant peaks are almost identical (Table II). A difference in the number of lysine and perhaps also threonine residues can be observed in peak 3. The purity of the inhibitor can be judged by the absence of valine and histidine in the analyzed samples. These amino-acids are namely absent in the molecule of the trypsin inhibitor from cow colostrum[9]. Another proof of purity of the individual inhibitors is the fact that each of them moves as one zone on disc electrophoresis. Two zones were

observed when the mixture of material from all main peaks was analyzed by disc electrophoresis. The specific activity of the material from peak 2, and 4 through 7 is almost identical (Table III). The specific activity of the material from peak 3 is lower, yet neither the amino-acid analysis nor disc eletrophoresis indicate a contamination. The low specific activity of the material from peak 1 is probably caused by its contamination. The quantity of this component in cow colostrum was low and therefore it was not purified further.

The described method of isolation of inhibitors from cow colostrum permits the material to be obtained very quickly and under mild conditions. The results show that the trypsin inhibitor from cow colostrum is in fact heterogeneous as has been shown earlier[9], and that this heterogeneity is most likely not the result of improper isolation procedure only.

I am indebted to Dr H. Fritz, Institute of Clinical Chemistry and Biochemistry, Munich, for the sample of trypsin. I thank Mrs Y. Černá for skillful technical assistance and to Mr J. Zbrožek for the amino-acid analyses.

REFERENCES

1. Levin Y., Pecht M., Goldstein L., Katchalski E.: Biochemistry *3*, 1905 (1964).
2. Fritz H., Schult H., Neudecker M., Werle E.: Angew. Chem. Intern. Ed. *5*, 735 (1966).
3. Fritz H., Schult H., Hutzel M., Wiedemann M., Werle E.: Z. Physiol. Chem. *348*, 308 (1967).
4. Fritz H., Gebhardt M., Fink E., Schramm W., Werle E.: Z. Physiol. Chem. *350*, 129 (1969).
5. Kassell B., Marciniszyn M. B.: Proc. of the International Research Conference on Proteinase Inhibitors, Munich 43, 1970.
6. Chauvet J., Acher R.: FEBS Letters *23*, 317 (1972).
7. Porath J., Axén R., Ernback S.: Nature *215*, 1491 (1967).
8. Laskowski M. jr, Laskowski M.: J. Biol. Chem. *190*, 563 (1951).
9. Čechová D., Jonáková-Švestková V., Šorm F.: This Journal *35*, 3085 (1970).
10. Davis B. J.: Ann. N. Y. Acad. Sci. *121*, 104 (1964).

Translated by V. Kostka.