TWO TRYPSIN ISOINHIBITORS FROM COW COLOSTRUM DIFFERING IN PRIMARY STRUCTURE

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Received March 17th, 1976

Several chromatographically different forms of the low molecular weight trypsin inhibitor have been isolated from cow colostrum by ion-exchange chromatography. A systematical comparison of the peptide maps of tryptic digests of the oxidized forms, quantitative amino acid analysis of the peptides, and their sequential analysis provided evidence of two isoinhibitors differing in amino acid composition, namely by a replacement of lysine by threonine. The N-terminal amino acid sequence of isoinhibitor B is $\overset{1}{\text{Phe}}$-Gln-Thr-..., and of isoinhibitor C $\overset{1}{\text{Phe}}$-Gln-Lys-....

The low molecular weight trypsin inhibitor from cow colostrum exists in several chromatographically different forms[1,2]. Its molecule consists of a protein chain of 67 amino acids[3], crosslinked by three disulfide bonds[4], and of a sugar moiety. It was not therefore certain whether differences in the protein structure or in the sugar moiety are responsible for the occurrence of multiple inhibitor forms. The sugar analysis of the larger number of these forms[5] has demonstrated differences in the sugar moieties. This study provides evidence showing that the structure of the protein moiety is not uniform either. We have isolated two isoinhibitors differing in structure of the protein moiety and determined the site of the amino acid replacement.

EXPERIMENTAL

Material

Cow colostrum was obtained on the first day after the delivery from collective farm of district Nymburk, Bohemia. It was stored at $-20°C$ until treated further. CM-Sephadex C-25 and Sephadex G-25 were products of Pharmacia Fine Chemicals (Uppsala, Sweden). DEAE-Cellulose DE 11 was from Whatman Biochemicals (Maidstone, Kent, England). Bovine trypsin, twice crystallized and TPCK-trypsin were from Worthington Biochemical Corporation (Freehold, N. J., U.S.A.). Benzoyl-D,L-arginine *p*-nitroanilide hydrochloride (BAPA) used as substrate was prepared in the Service Laboratory, Czechoslovak Academy of Sciences, Lysolaje.

Methods

Determination of inhibitor activity. The activity of the inhibitor was measured in terms of its reaction with trypsin[6]. The activity of trypsin was estimated by the method of Nagel and cowor-

kers[7] by measurement of absorbance increase at 405 nm resulting from the hydrolysis of benzoyl--D,L-arginine *p*-nitroanilide. HCl (BAPA). The inhibitory activity is expressed in mg of active trypsin inhibited by one ml of the solution tested. The quantity of active trypsin was calculated on the basis of the titration of active centers in the trypsin preparation used[8].

Quantitative amino acid analyses of the inhibitor (hydrolyzed in 6M-HCl, 20 h, at 110°C) were carried out by the technique of Spackman and coworkers[9] in an amino acid analyzer manufactured by the Instrument Development Workshops, Czechoslovak Academy of Sciences. Samples containing cystine and methionine were oxidized in performic acid[10]; cystine was determined as cysteic acid and methonine as methionine sulfone.

Starch gel electrophoresis was performed in 10·5% starch gel in 0·2M formic acid, pH 2·8 (ref.[11]). The sample applied contained 2 mg of protein. Electrophoresis was allowed to proceed at 120 V for 16 h at room temperature. At the end of the run the gel layer was fixed in methanol--water-acetic acid (5 : 4 : 1). The fixed gel was dipped in 1% bromophenol blue and stained 2h. Destaining was effected in the fixative solution.

N-*Terminal end group analysis.* N-Terminal amino acids were determined by the dansyl technique[12]; the modification of Gross and Labouesse[13] was used. Dansyl-(1-dimethylamino-naphthalene-5-sulfonyl)amino acids were identified on silica gel thin layers. One-dimensional repeated development was carried out in the following systems: chloroform-n-butyl alcohol--toluene-acetic acid (7 : 1 : 1 : 1, by vol.), n-butyl alcohol-toluene-25% NH_4OH (8 : 1 : 1 by vol.), ethanol-toluene-25% NH_4OH (7 : 2 : 1, by vol.), tert-amyl alcohol-pyridine-toluene--n-butyl alcohol (6 : 2 : 1 : 1, by vol.)[14].

Determination of N-*terminal amino acid sequence of form* C2. Form C2 (10 mg) was subjected to sequential degradation according to Niall and Edman[15]; the modified detection[16] described earlier was used.

Preparation of inhibitor. A concentrate of the inhibitor was prepared from colostrum by a method[1] involving the precipitation of contaminating proteins in colostrum by 2·5% trichloroacetic acid at 60°C and precipitation of the inhibitor in the filtrate by ammonium sulfate (80% saturation). The material obtained was purified further by chromatography on CM-Sephadex at pH 3·5. The material showing inhibitory activity was resolved into three fractions designated A, B, and C in order of their emergence from the column under the conditions described. These fractions were treated separately as described elsewhere[1].

Chromatography on DEAE-*cellulose.* Fraction B (1 000 mg) was dissolved in 0·005M phosphate buffer, pH 7·2. The pH and conductivity of the sample were adjusted to the same values as those of the equilibrating buffer. The sample was then applied onto a DEAE-cellulose column (3 × 45 cm) equilibrated with 0·01M phosphate buffer. Four chromatographically different forms, designated B1 — B4 were isolated after the application of linear and stepwise gradient of ionic strength. A detailed description of the chromatography has been presented elsewhere[1]. Individual forms were desalted on Sephadex G-25 medium in 0·2% formic acid and freeze-dried.

Fraction C (3 800 mg) was dissolved in 0·002M phosphate buffer, pH 7·0, and applied after the adjustment onto a column of DEAE-cellulose. The course and the description of the chromatography are obvious from Fig. 1. Chromatographic forms designated C1 — C5 were desalted as described above and freeze-dried. Forms C1 (70 mg) and C5 (60 mg) were purified on a column of DEAE — cellulose (Fig. 2), desalted and freeze-dried.

Purification of form C2 by descending chromatography. Form C2 was subjected to descending chromatography on Whatman No 3 paper in system S1, n-butyl alcohol-pyridine-acetic acid--water, (15 : 10 : 3 : 12). The purified sample was eluted by distilled water and freeze-dried.

Tryptic digestion of oxidized forms B1, B3—B4, C1—C5. Forms B1, B3—B4, C1—C4 (15-mg samples) and C5 (2 mg) were individually oxidized in performic acid[10]. The digests were adjusted to pH 8·3 (0·1M ammonium carbonate) and digested with trypsin (1 : 50, w/w). The digestion was

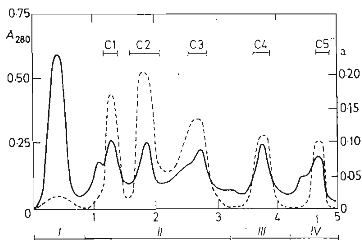


FIG. 1

Chromatography of Fraction C on DEAE-Cellulose at pH 7·0

The column (4·4 × 38 cm) was equilibrated with 0·005M phosphate buffer. After application of the sample inactive proteins were displaced by the equilibrating buffer (*I*). The chromatographic forms of the inhibitor were eluted by 0·1M phosphate buffer (*II*), 0·01M phosphate buffer containing 0·01M-NaCl (*III*), and 0·01M phosphate buffer containing 0·04M-NaCl (*IV*); full line absorbance at 280 nm, broken line, activity (*a*) in mg of active trypsin inhibited by 1 ml of the solution examined. Volume of effluent in liters (l), C1—C5 individual forms.

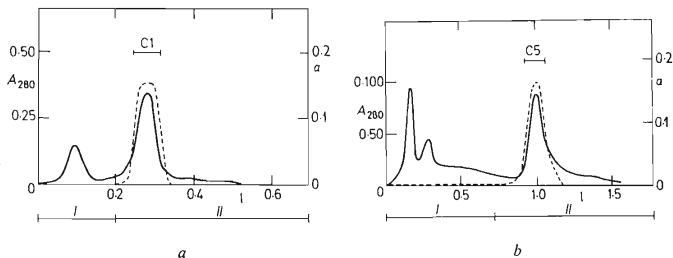


FIG. 2

Rechromatography of Form C1 (*a*) and C5 (*b*) on DEAE-Cellulose at pH 7·0

The column (2·5 × 28 cm) was equilibrated with 0·005M phosphate buffer. After application of the sample, inactive material was displaced (*I*) by the equilibrating buffer (*a*) and by 0·01M phosphate buffer containing 0·01M-NaCl (*b*), respectively. The displacement of the forms (*II*) was effected by 0·01M phosphate buffer (*a*) and by 0·01M phosphate buffer containing 0·025M-NaCl (*b*), respectively. Full line, absorbance at 280 nm, broken line, activity (*a*) in mg of active trypsin inhibited by 1 ml of the solution examined. Volume of effluent in liters (l), C1 and C5 individual forms.

allowed to proceed 2 h at 37°C and was discontinued by acidification of the samples to pH 3·0 by formic acid and by taking the samples to dryness.

Fractionation of tryptic digest of forms B1, B3—B4, C1—C5. In orienting experiments peptide maps were prepared with 2 mg samples of all digests. High voltage electrophoresis was carried out at 4000 V and 4°C on Whatman No 3 paper in system S2, formic acid–acetic acid–water (1 : 3 : 16) at pH 1·9. At right angles to the electrophoresis run descending chromatography was carried out in system S1. Individual peptides were stained with 0·2% ninhydrin in acetone. The peptide maps of the individual forms are given in Fig. 3.

The remaining material (forms B1, B3, B4, C1—C4, 10-mg portions) was separated (on 5-cm bands) by high voltage electrophoresis at 4000 V in system S 2 on Whatman No 3 paper. The digests of tryptic peptides from forms B1, B3, B4, C3, and C4 were resolved into 4 zones, peptides from forms C1 and C2 into 5 zones. Individual zones were detected at the margin with 0·2% ninhydrin solution in acetone. The zones were subsequently cut out, stitched to Whatman No 3 paper and subjected to descending chromatography in system S1, n-butyl alcohol–pyridine–acetic acid–water (15 : 10 : 3 : 12). Individual peptides were again detected at the margin with 0·2% ninhydrin solution in acetone; the zones were cut out, eluted by distilled water, the eluates dried and subjected to quantitative amino acid analysis.

RESULTS

The low molecular weight trypsin inhibitor of cow colostrum was obtained in several forms by ion-exchange chromatography of the starting material. Three forms with antitryptic activity, designated A(15%), B(50%), and C(35%) were isolated by chro-

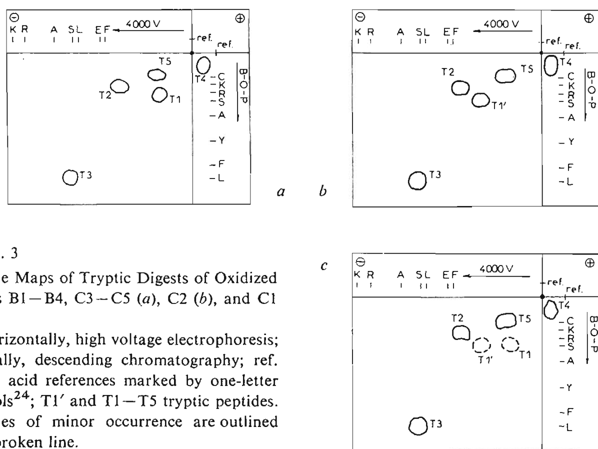


FIG. 3

Peptide Maps of Tryptic Digests of Oxidized Forms B1—B4, C3—C5 (*a*), C2 (*b*), and C1 (*c*)

Horizontally, high voltage electrophoresis; vertically, descending chromatography; ref. amino acid references marked by one-letter symbols[24]; T1′ and T1—T5 tryptic peptides. Peptides of minor occurrence are outlined by a broken line.

matography on CM-Sephadex, as described in the ealier paper[1]. Fraction B, which was dominant, was chromatographed on DEAE-cellulose DE 11 and afforded 4 forms designated B1, B2, B3, and B4 in order of their emergence from the column. The amino acid sequence[3] as well as the disulfide bonds[4] of dominating form B2 have been determined. In this study the remaining forms of fractions B and C were examined. Contrary to form B2(64%), forms B1(8%), B3(12%), and B4(16%) were obtained in lower yields. The amino acid composition of all these forms was identical with the amino acid composition of form B2 (Table I). The peptide maps of the tryptic digests of oxidized forms B1, B3, and B4 showed the presence of 5 ninhydrin positive peptides designated T1 – T5 and located in identical positions as in the map of form B2. The peptide map, which is identical for forms B1, B2, B3, and B4 is given in Fig. 3*a*. The quantitative amino acid analyses of all peptides isolated from the tryptic digests of forms B1, B3, and B4 were likewise identical with those of tryptic peptides of form B2 whose amino acid sequences have been determined[3]. There is therefore no doubt at all that the amino acid sequences of forms B1, B3, and B4 are identical with the known amino acid sequence of form B2, given in Table V.

Fraction C was resolved into 5 forms (C1 to C5) by chromatography on DEAE-cellulose, as shown in Fig. 1. An examination of inhibitory activity of these forms has

TABLE I

Amino Acid Composition of Forms B1 – B4 of Cow Colostrum Trypsin Inhibitor

The values were obtained with 20-h hydrolysates and are not corrected.

Amino acid	Number of residues per mol			
	B1	B2	B3	B4
Lysine	2·01	2·00	1·96	1·92
Arginine	3·00	3·02	2·95	3·08
Aspartic Acid	7·92	8·00	8·00	7·80
Threonine	5·88	5·98	6·18	5·84
Serine	3·10	3·01	3·00	2·92
Glutamic Acid	10·12	10·05	9·87	9·66
Proline	6·87	7·12	6·65	6·67
Glycine	4·00	4·00	4·00	4·08
Alanine	4·02	4·00	4·04	4·08
Cysteic acid	5·98	5·94	5·92	6·08
Methionine	0·94	0·99	1·09	1·08
Isoleucine	0·98	1·00	0·96	0·92
Leucine	5·20	5·04	5·40	5·33
Tyrosine	2·85	3·01	2·96	2·95
Phenylalanine	4·00	4·00	3·96	3·80

shown that form C1 accounts for 18%, form C2 for 31%, form C3 for 27%, form C4 for 14%, and form C5 for 10% of the total inhibitory activity of fraction C. According to amino acid analysis, starch gel electrophoresis, and N-terminal end group analysis (phenylalanine as the only N-terminal residue), forms C3 and C4 were obtained in pure state. Forms C1, C2, and C5 required additional purification. Forms C1 and C5 were rechromatographed on DEAE-cellulose. The final yields of the pure forms were very low. The total quantity of form C5 obtained was 2 mg and was used for the peptide map of the tryptic digest of oxidized material. Form C2 was purified by descending chromatography in system S2.

The amino acid composition of forms C3 and C4 (Table II) is identical with the amino acid composition of form B2 and thus also with the amino acid composition of forms B1, B3, and B4. Likewise the peptide maps of the tryptic digests of oxidized forms C3 to C5 show 5 peptides in positions identical with those in the peptide maps of forms B1 to B4 (Fig. 3*a*). The amino acid compositions of peptides isolated from the tryptic digests of oxidized forms C3 and C4 were completely identical with those of peptides derived from form B2 and reported earlier[3]. The yields of the peptides after isolation also correspond to the values given for form B2 (Table IV). These findings provide evidence showing that the protein moiety of forms C3 to C5 does not differ from the protein moiety of forms B1 to B4.

TABLE II

Amino Acids Composition of Forms C1—C4 of Cow Colostrum Trypsin Inhibitor

The values were obtained with 20-h hydrolysates and are not corrected.

Amino acid	Number of residues per mol			
	C1	C2	C3	C4
Lysine	2·72	3·00	1·99	2·00
Arginine	2·90	2·85	2·97	3·00
Aspartic acid	7·55	7·60	7·68	7·67
Threonine	5·40	5·05	5·84	5·80
Serine	2·90	2·95	2·72	3·02
Glutamic acid	10·40	10·32	10·30	10·25
Proline	7·20	6·95	7·11	7·10
Glycine	3·94	3·94	3·95	4·05
Alanine	4·20	4·00	4·00	3·95
Cysteic acid	5·90	5·97	6·01	5·95
Methionine	0·89	0·90	0·94	1·00
Isoleucine	1·03	0·85	1·09	0·95
Leucine	4·75	4·90	5·00	5·10
Tyrosine	2·83	2·60	2·65	2·70
Phenylalanine	4·05	4·00	4·10	3·94

Forms C1 and C2 (Table II) differ in the number of lysine and threonine residues from forms C3, C4. Form C2 has one lysine residue more and one threonine residue less; likewise, form C1 shows a lower threonine content and a higher lysine content (Table II). The peptide map of the tryptic digest of oxidized form C2 (Fig. 3*b*) differs from the corresponding map of form B2 (Fig. 3*a*) in the position of one peptide T1'. We isolated all tryptic peptides and determined their amino acid composition; the peptide showing different electrophoretic mobility is the N-terminal peptide which unlike all the N-terminal peptides of forms identical with form B2, does not contain threonine yet lysine. The amino acid analyses of the N-terminal peptides (designated T1, T1') of all the forms examined are given in Table III. We have shown in our earlier studies[3] that threonine is the third residue from the N-terminus of the molecule of form B2 and also in the corresponding tryptic peptide (Table V). We determined the sequence of the first four N-terminal amino acids as Phe.Gln.Lys.Pro. This finding demonstrates that the third amino acid residue from the N-terminus is in the molecule of form C2 a lysine residue.

There are 6 ninhydrin-positive peptides on the peptide map of oxidized form C1 (Fig. 3*c*). The quantitative amino acid analyses of the peptides isolated show that the N-terminal peptide is present in two forms in the tryptic digests: as peptide T1 containing threonine and as peptide T1' containing lysine. The ratio of the yields of

TABLE III

Amino Acid Composition of N-Terminal Peptides T1 and T1' from Tryptic Digests of Form B2 and Forms C1 – C4

The values were obtained with 20-h hydrolysates and are not corrected.

Amino acid	Number of residues in peptide					
	B2	C1		C2	C3	C4
	peptide T1	peptide T1	peptide T1'	peptide T1'	peptide T1	peptide T1
Alanine	0·96	1·10	1·00	1·00	0·95	1·00
Aspartic acid	1·00	1·20	1·13	1·07	1·00	1·07
Arginine	0·95	0·90	0·91	0·88	1·00	0·96
Cysteic acid	1·07	1·00	1·01	1·00	1·00	1·02
Glutamic acid	2·98	3·00	2·96	2·89	2·85	3·03
Leucine	2·07	1·90	1·87	1·97	2·02	2·04
Lysine	—	—	1·04	0·90	—	—
Phenylalanine	0·68	0·70	0·73	0·71	0·85	0·72
Proline	3·07	2·90	3·14	3·00	2·93	2·96
Threonine	0·96	1·00	—	—	1·00	0·96

both peptides is 1 : 1 and the sum of the yields of both peptides corresponds to the yield of peptide T1 obtained with forms B1 to B4 and C3 to C4 or to the yield of peptide T1′ obtained with form C2 (Table IV). Form C1 obviously contains both the inhibitor with threonine as the third amino acid from the N-terminus and the inhibitor with lysine in the corresponding position. The structures of both inhibitors are given in Table V. The quantity of isoinhibitor C in colostrum is considerably lower than that of isoinhibitor B and represents about 14% of the total inhibitory activity of the low molecular weight inhibitor if we take into account the fact that it is not present in fraction A (obtained by chromatography on CM-Sephadex and not yet analyzed).

DISCUSSION

The low molecular weight cow colostrum trypsin inhibitor has been obtained in several forms[1,2]. The inhibitor contains besides the protein moiety a firmly bound sugar component. It has been demonstrated that the sugar component is not uniform and differs with individual inhibitors in the content of sugar units and thus in its molecular weight[5]. It remained to be shown whether the protein moieties differ or not. In this study we isolated 9 chromatographically different forms and demonstrated that these forms represent two isoinhibitor types differing in the structure of the protein moiety, namely in the third amino acid residue from the N-terminus of the molecule. This position is occupied by threonine in type B and by lysine in type C (Table V).

The existence of a microheterogeneity in the amino acid composition of naturally occurring inhibitors has been observed with several inhibitors[17–21]. These inhibitors are mostly proteins lacking a sugar moiety. The situation with the cow colostrum trypsin inhibitor forms is more complicated. Both the sugar components and the

TABLE IV

Yields of Peptides Isolated from Tryptic Digests of Form B2 and Forms C1—C4

The yields of the peptides in μmol were calculated from the values obtained by amino acid analysis. The tryptic digests were prepared with 10-mg portions of the proteins.

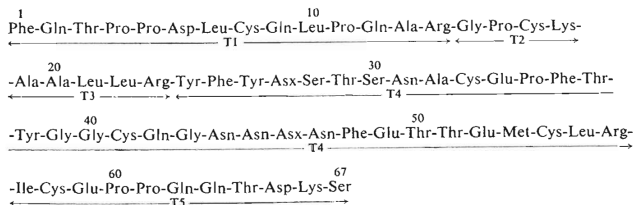
Peptides	Peptides, mmol				
	B2	C1	C2	C3	C4
T1	0·225	0·100	—	0·230	0·240
T1′	—	0·110	0·225	—	—
T2	0·260	0·270	0·296	0·300	0·280
T3	0·285	0·210	0·245	0·260	0·300
T4	0·300	0·230	0·240	0·240	0·275
T5	0·245	0·220	0·236	0·282	0·290

protein moieties differ. We therefore use on purpose the term "chromatographic form" when speaking of the material isolated from one peak by ion exchange chromatography. The sugar moiety is as a rule labile and it is likely that several forms are formed during the isolation procedure as a result of the liberation of sialic acid. We use the term „isoinhibitor" only in cases were we mean amino acid replacements. Several chromatographically different forms with identical amino acid sequence we regard as one isoinhibitor. Isoinhibitor B thus involves forms B1 through B4, C3 to C5, and partly also form C1. Isoinhibitor C involves form C2 and partly also form C1. The fact that form C1 contains both isoinhibitors at a 1 : 1 ratio shows that this form does not represent one isoinhibitor contamined with the other one but a mixture of two isoinhibitors. This supports the view that differences in sugar moieties rather than a microheterogeneity in amino acids are responsible for the occurrence of chromatographically different forms.

The conclusion that trypsin inhibitors exist in both cow and pig colostrum as inhibitors differing in lysine content has been made also in the laboratory of Laskowski[22,23]. These authors regard the forms isolated from cow colostrum as falling into three groups containing isoinhibitors with one, two, or three lysines. In this study we isolated isoinhibitors with two and three lysines only. We have not been able as yet to obtain the forms contained in fraction A in sufficiently pure state. It looks likely that this fraction contains the isoinhibitor with one lysine residue. The lower number

TABLE V

Primary Structure of Isoinhibitor B and N-terminal Amino Acid Sequence of Isoinhibitor C



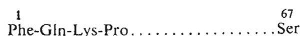
Isoinhibitor B

1 ... 10
Phe-Gln-Thr-Pro-Pro-Asp-Leu-Cys-Gln-Leu-Pro-Gln-Ala-Arg-Gly-Pro-Cys-Lys-
← T1 → ← T2 →

20 ... 30
-Ala-Ala-Leu-Leu-Arg-Tyr-Phe-Tyr-Asx-Ser-Thr-Ser-Asn-Ala-Cys-Glu-Pro-Phe-Thr-
← T3 → ← T4

40 ... 50
-Tyr-Gly-Gly-Cys-Gln-Gly-Asn-Asn-Asx-Asn-Phe-Glu-Thr-Thr-Glu-Met-Cys-Leu-Arg-
T4 →

60 ... 67
-Ile-Cys-Glu-Pro-Pro-Gln-Gln-Thr-Asp-Lys-Ser
← T5 →



Isoinhibitor C

1 ... 67
Phe-Gln-Lys-Pro. . . . . .Ser

of asparagine residues observed with most forms we ascribe to the interference of the sugar moiety with the acid hydrolysis rather than by amino acid replacements.

The occurrence of two isoinhibitors in cow colostrum described here is obviously due to genetic variation. The replacement of a lysine residue by a threonine residue is made possible by the replacement of one base in the triplet responsible for the synthesis of the third amino acid residue from the N-terminus of the molecule. This replacement does not affect the antitryptic activity of the inhibitor. Isoinhibitor B, however, contains 6 threonine residues. It remains to be shown why one of these residues can undergo mutation. The substitution of one threonine residue by a lysine residue in the N-terminal sequence of the molecule does not affect the activity of the inhibitor whereas an amino acid replacement inside the molecule might lead to undesired alterations of activity. The structure of inhibitor B is by 40% homologous with the structure of Kunitz pancreatic trypsin inhibitor[24]. At one site only a threonine residue is replaced by a lysine residue in both structures. The two alanine residues located at both sides of lysine 26 in the Kunitz inhibitor are replaced by two serines in the neighborhood of threonine 29 in the colostrum inhibitor. These are one-point mutations, too. This finding also supports the hypothesis that the replacement of threonine by lysine inside the molecule is undesirable from the viewpoint of survival of the species.

The existence of two homologous trypsin inhibitors in cow colostrum, representing a mixed product of several animals, can be the result of allelomorphism. Allelomorphism connected with the replacement of one amino acid residue has been reported in detail with carboxypeptidase A, where homozygotes of both isozymes were obtained[25].

The occurrence of several isoinhibitors in cow colostrum can be the result of the existence of two genes controlling the synthesis of the inhibitor. These genes can originate in gene duplication and subsequent mutation.

We thank Mrs Y. Černá and Mrs M. Rysová for careful technical assistance. The Edman degradation experiments were carried out by Mr K. Grüner, the amino acid analyses by Mr J. Zbrožek, Miss V. Himrová, and Mrs E. Dršková.

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Translated by V. Kostka.