INFLUENCE OF THE CARBOHYDRATE MOIETY ON ROTATIONAL CORRELATION TIME AND HYDRODYNAMIC VOLUME OF COW COLOSTRUM TRYPSIN INHIBITOR

Petr ŠTROP[a] and Věra JONÁKOVÁ[b]

[a] *Department of Protein Chemistry, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, and*
[b] *Institute of Molecular Genetics, Czechoslovak Academy of Sciences, 166 37 Prague 6*

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A large polysaccharide moiety of the cow colostrum trypsin inhibitor was removed by enzymatic cleavage; fully active protein was obtained by affinity chromatography. Rotational correlation times of the inhibitor labelled by dansyl chloride obtained from fluorescence depolarization measurements and hydrodynamic volumes from gel-permeation chromatography for protein with and without polysaccharide were compared with those of homologous Kunitz inhibitor. The mean rotational correlation time 105 ns and apparent mol. weight 16 000 were obtained for the colostrum inhibitor with polysaccharides; these values are considerably reduced to 44 ns and 8 700 dalton after removal of the polysaccharide moiety. For the pancreatic inhibitor an about four times shorter correlation time of 24 ns was obtained.

The cow colostrum trypsin inhibitor secreted by the lactogenic system of cow[1-3] has a large carbohydrate part, which for some fractions of the inhibitor accounts for more than a half of the total molecular weight[4]. It is bound to a single residue, Asn 27 (ref.[5]). In analogy to the pancreatic trypsin inhibitor[6] it is localized opposite to the reactive site at the base of the pear-shaped molecule and elongates the protein part by the bulky and flexible polysaccharidic chain. The high resolution NMR spectra have proved that the carbohydrate moiety has only a limited effect on the spatial structure of the protein part but causes a pronounced restriction of the overall mobility[6]. In this paper the polysaccharide moiety was split off and the influence of the carbohydrate on the hydrodynamic size and the rotational correlation time of the molecule was studied by fluorescence depolarization and gel-permeation chromatography.

EXPERIMENTAL

Materials and Methods

Cow colostrum trypsin inhibitors were isolated as described[2,3]. The cow colostrum inhibitor

(40 mg) of fraction CTI-B2 (ref.[2]) was cleaved with 20 mg of Emulsin (22 830 Serva) and 1 ml of a Neuramidinase solution (30 294 Serva) in 5·3 ml of 0·05 mol l^{-1} sodium acetate buffer pH 5·5. The solution was covered with 1 ml of toluene and kept at 37°C for 60 hours. Then the water solution was applied on a 12 × 1·5 cm Sepharose CL 4B column and eluted with 0·05 mol l^{-1} acetate buffer pH 6·3 containing 0·3 mol l^{-1} NaCl and 0·02 mol l^{-1} $CaCl_2$. The eluent (10 ml) was applied on a 10 ml Trypsin-Sepharose column and washed with the same buffer. The desorption was performed with 0·01 mol l^{-1} HCl in 0·3 mol l^{-1} NaCl and 0·02 mol l^{-1} $CaCl_2$, pH 2. After rechromatography on the Trypsin-Sepharose column (Fig. 1) the protein was desalted on a Sephadex-G25 column. The carbohydrate composition is given in Table I.

Preparation of the dansyl derivatives. 12 mg of the basic pancreatic trypsin inhibitor (Trasylol, Bayer, BRD) was stirred 5 hours at 5°C with 60 µl of an acetone solution of 5-dimethylamino--1-naphthalene sulfonyl chloride (dansyl chloride, 100 mg/mol, Pierce, USA). The reaction mixture was filtered and dansyl chloride removed on a Sephadex-G25 column. Spectrophotometrically 1 mol of dansyl group per mol of the pancreatic inhibitor was found. When 50 µl of the dansyl solution was mixed for 2·5 hours at pH 7·5 with the same protein solution, 0·1 mol of the dansyl group per mol of the inhibitor was determined. At higher degrees of substitution

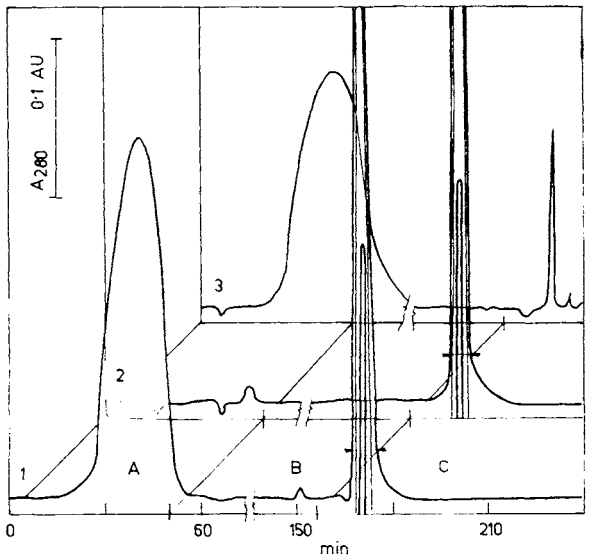

FIG. 1

Affinity chromatography of the cow colostrum trypsin inhibitor on Trypsin-Sepharose after cleavage of the polysaccharide part. 1 chromatography after cleavage of the carbohydrate, 2 rechromatography, 3 rechromatography of non-retained material from the run shown on Fig. 1/1. A sample application and washing at pH 6·3, flow rate 40 ml/hour, B washing of the column, pH 6·3, flow rate 150 ml/hour, C desorption at pH 2, flow rate 40 ml/hour. For other conditions see Methods

than 1 mol/mol most of the pancreatic inhibitor precipitates. The modification of the cow colostrum inhibitor was performed with 8 mg of the inhibitor in 1 ml of 0·1 sodium phosphate buffer pH 8·5, 50 μl of the dansyl chloride solution was used and the mixture was stirred at 6°C for 1·5 hours. 1·9 mol of the dansyl group were bound to 1 mol of the colostrum inhibitor. GLC analysis of carbohydrates was done on 2 000 × 3 mm Gas Chrom Q column at 160—250°C. The hydrolysis of the samples was done in 1·5 mol l^{-1} HCl at 80°C for 24 hours; acetylation was performed 1 : 1 w/w pyridine-acetonitrile. The amino acid analysis was made routinely after 20 hour hydrolysis on Durrum D 500 analyzer; glucosamine and galactosamine were determined after hydrolysis with methanesulfonic acid[7]. The fluorescence measurements were made on the Perkin–Elmer Spectrofluorometer 44A with temperature variation, rotational correlation times were calculated according to ref[8].

RESULTS

Removal of carbohydrates of the colostrum inhibitor: The carbohydrate content was reduced after the treatment with Emulsin, Neuraminidase and after affinity chromatography to 1/16 of its original value, *i.e.* from 19 to app. 1·2 mol of carbohydrates per mol of the protein. The protein was obtained in 50% yield. When wheat germ lectin-Sepharose was used to remove the rest of the glycoprotein, less than 0·3 mol of saccharides per mol of the inhibitor was found.

TABLE I

Carbohydrate composition of cow colostrum trypsin inhibitor B before and after carbohydrate cleavage. I inhibitor before treatment, II isolated inhibitor after treatment with Emulsin, III maximum and minimum values given by Tschesche and coworkers[4]. AAA data from amino acid analyzer, GLC data from gas–liquid chromatography

Monosaccharides mol/mol protein	I		II		III
	GLC	AAA	GLC	AAA	
Fuc	0·2		0·05		0·6— 1·5
Man	7·3		0·4		6·1—11·9
Gal	1·8		0		1·6— 5·1
Glc	1·8		0·5		0·8— 1·8
GalN	1·6	1·7	0·2	0·5	4·1— 7·1
GlcN	4	4	0·5	0·3	7·3—14·8
NeuN	2·3		0		0·9— 3·4
Total	19		1·2		21·9—40[a]

[a] This value is slightly different from the sum of the minimal and maximal carbohydrate content given in column III. The number gives the carbohydrate content of the two fractions of the cow colostrum inhibitor (CTI AIV and CTI BII) with the minimal and maximal carbohydrate content given by Tschesche and coworkers[4].

TABLE II

Rotational correlation times τ_r, apparent and calculated molecular weights for cow colostrum trypsin inhibitor with and without polysaccharides and for basic pancreatic trypsin inhibitor. Rotational correlation times calculated using 12 ns as the fluorescence life time of the dansyl group[10]. Apparent molecular weights are taken from experiments shown on Fig. 3, molecular weights calculated from amino acid and carbohydrate analysis. Activity was measured on N-benzoyl-D, L-arginin-*p*-nitroanilide according to ref.[12]. Stokes radii [Å] were calculated from the gel-permeation chromatography data using an equation given by Laurent and Killander[13]

Protein	Activity AU/mg	τ_r 20°C	Mol. weight		Stokes radius
			apparent	calc.	
CTI A			16 000		20·6
CTI B	3·3	105 ns	16 000	11 000	20·2
CTI C			16 000		19·2
CTI B without polysaccharides	7·5	44 ns	8 700	8 000	15·7
BPTI		24 ns	6 300	5 300	12·4

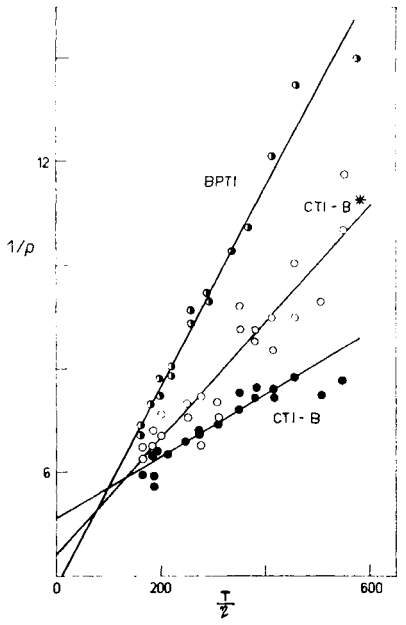

FIG. 2

Perrins plots for the basic pancreatic trypsin inhibitor (BPTI), and for the B isoinhibitor from cow colostrum with polysaccharide moiety (CTI-B) and after the cleavage of the polysaccharide (CTI-B)*. *P* is the polarization of fluorescence, *T* is temperature and η is the viscosity, 0·01M phosphate buffer, pH 6·5

Modification of inhibitors with dansyl chloride: Samples of the basic pancreatic trypsin inhibitor with 1/10, 1/1, 2/1 and 3·3/1 molar ratios of dansyl chloride to inhibitor were prepared. Samples with 1/10 and 1/1 ratios were soluble and gave linear Perrins plots. An increasing degree of substitution has resulted in precipitation and non-linear Perrins plots. The cow colostrum trypsin inhibitor can be modified to a higher extent without precipitation. A linear Perrin plot was obtained for the protein modified with 2 dansyl groups per mol of the colostrum inhibitor (Fig. 2).

Fluorescence depolarization studies: Table II gives rotational correlation times for the basic pancreatic trypsin and cow colostrum trypsin inhibitors. The shortest correlation time of 24 ns was obtained for the pancreatic inhibitor, while the homologous natural colostrum inhibitor with the polysaccharide chain gave a more than four times higher value (105 ns). After the cleavage of the most of the attached carbohydrates the rotational correlation time is reduced to only 44 ns. Measurements of the apparent molecular weight (Table II, Fig. 3) using gel-permeation chromatography coincide very closely with the fluorescence measurements. A linear plot

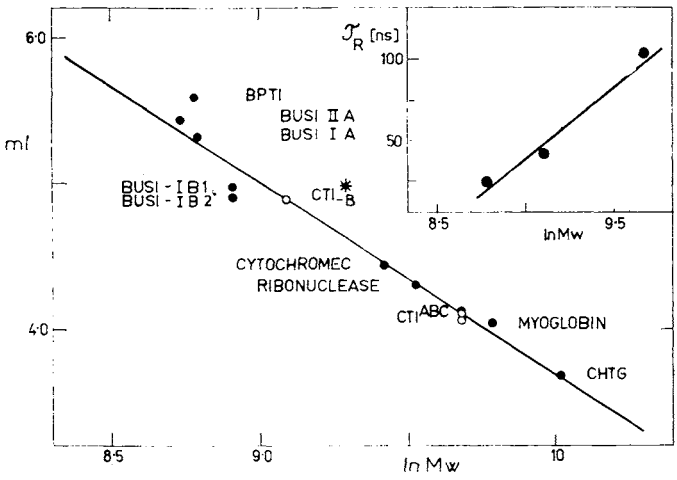

FIG. 3

Determination of the apparent molecular weights for trypsin isoinhibitors from cow colostrum by gel-permeation chromatography. Dependence of the elution volumes on molecular weight. CTI — A.B.C trypsin isoinhibitors from cow colostrum with a polysaccharide moiety. CTI B* isoinhibitor B after cleavage of the polysaccharide moiety, BPTI basic pancreatic trypsin inhibitor, BUSI IIA basic trypsin inhibitor from bull seminal plasma, BUSI IA, BUSI IB2 isoinhibitors of trypsin from bull seminal plasma, CHTG chymotrypsinogen. 80 × 0·4 cm Sephadex G-50 Superfine column, 0·05 mg l^{-1} phosphate buffer, 0·15 mol l^{-1} NaCl, pH 6·5.

of the rotational correlation time *vs* ln of the molecular weight was obtained. Molecular weights of 16 000 and 8 700 were obtained for untreated and treated colostrum inhibitor.

DISCUSSION

The method used earlier for cleavage of small flexible glycopeptides[9] was proved to be applicable to larger glycoproteins with a bulkier compact protein part. In the colostrum inhibitor Asn 27 was found to be the carbohydrate binding site[5]. The cleavage of the Asn–Glcn bond is not always complete, even in the case of a small peptide, perhaps due to a sterical hindrance[10]. We have found residual 0·05–0·3 mol of Glcn per mol of protein. Thus number is well below unity even when a correction for a partial destruction is taken into account. The material obtained after the treatment with Emulsin is heterogeneous; a portion of the protein is completely devoid of carbohydrates; such material was obtained in a low yield when a lectin-Sepharose column was used.

For the measurement of the rotational correlation times the material with 1·2 mol of carbohydrates per mol of the inhibitor was used. Its apparent molecular weight determined by gel-permeation chromatography is 8 700, only about 9% higher than the molecular weight calculated from the amino acid and carbohydrate composition. Much larger differences were found for the untreated colostrum inhibitor (mol. weights 16 000 and 11 000) suggesting that the polysaccharide moiety of the inhibitor is much bulkier than the polypeptide part. This is also manifested by the 2·4 times larger rotational correlation time compared to the protein without polysaccharides, and 4·4 times larger than that for Kunitz inhibitor. This coincides with the observations that the NMR line widths of the backbone amide resonances of the colostrum inhibitor are about four times larger than for the Kunitz inhibitor[6]. A good correlation of the apparent molecular weights obtained from gel-permeation chromatography and rotational correlation times suggests that the influence of the binding of the dansyl label to the three proteins measured is mostly compensated. When we used the fluorescence lifetime of the dansyl group 12 ns as given by Steiner and McAlister[11] we obtained the rotational correlation time 24 ns for the Kunitz inhibitor, 44 ns for the colostrum inhibitor without carbohydrates and 105 ns for the colostrum inhibitor with the polysaccharide moiety. These data fall into the range of correlation times reported in literature: 13 ns for ribonuclease, 25 ns for Soybean trypsin inhibitor, 30 ns for the insulin dimer and 26 ns for the growth hormone[8].

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