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Chemical and physical characterization of a proline-rich polypeptide from sheep colostrum

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A proline-rich polypeptide isolated from sheep colostrum is described. The molecular weight of the polypeptide determined by gel filtration is 17200. However, in the presence of guanidinium chloride the molecular weight found is about 6000. The polypeptide contains about 22% of proline, a high proportion of non-polar amino acids, a low percentage of glycine, and no alanine, arginine and cysteine residues. The only *N*-terminal amino acid found is leucine. C.d. spectra in water and in 50% (v/v) trifluoroethanol suggest the presence of block sequences of proline residues forming helices of polyproline II type. The proline-rich polypeptide is soluble at 4°C but is reversibly precipitated on warming to room temperature. Maximal precipitation is observed at pH4.6 and at ionic strength above 0.6. The precipitation depends on the concentration of the polypeptide. No effect of other proteins, Ca^{2+} and Zn^{2+} ions on the precipitation of the polypeptide was found. The proline-rich polypeptide is not an amphipathic protein. The lack of effect of the polypeptide on proteolytic enzymes ruled out the possibility that it is an inhibitor of proteinases.

During our studies on sheep colostral immunoglobulins it was found that IgG2 immunoglobulins isolated by chromatography on DEAE-cellulose were contaminated by a protein containing a large proportion of proline residues. The contaminating material was separated and identified as a proline-rich polypeptide. The amount of proline residues in this polypeptide is 22% of all amino acid residues (Janusz *et al.*, 1974). A comparatively low content of glycine, absence of alanine and a high content of hydrophobic amino acids makes the proline-rich polypeptide different from collagen-like proteins. Studies on a possible biological role of the proline-rich polypeptide showed that it increases the permeability of skin vessels and that it has a regulatory activity, stimulating or suppressing the immune response (Wieczorek *et al.*, 1979).

In the present paper the physicochemical properties of the proline-rich polypeptide are described. It was found that this polypeptide is different from other proline-rich proteins isolated from other sources.

Abbreviation used: IgG, immunoglobulin G.

Materials and methods

Materials

Sephadex G-200, G-75 and G-25 and Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. Dowex AG-501 (X8) was purchased from Bio-Rad Laboratories, St. Albans, Herts., U.K. Agarose was obtained from International Enzymes, Windsor, Berks., U.K., and purified agar powder was obtained from Difco, Detroit, MI, U.S.A. Acylamide for gel electrophoresis was purchased from Serva, Heidelberg, Germany. Cytochrome *c*, α-chymotrypsinogen, myoglobin, cetyltrimethylammonium bromide, Coomassie Brilliant Blue and trifluoroethanol were also products of Serva. Insulin A-chain, crystalline bovine serum albumin and the crystalline enzymes pepsin, papain and trypsin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Glucagon was purchased from Calbiochem, Los Angeles, CA, U.S.A. All other reagents used were of analytical grade. Polyamide sheets were obtained from Schleicher und Schuell, Dassel, Germany. Antiserum against sheep serum IgG was purchased from Miles, Elkhart, IN, U.S.A. Antisera

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against sheep colostrum, sheep colostral IgG Fab fragment and sheep colostral IgG Fc fragment were prepared by immunization of rabbits with the respective proteins. To prevent bacterial growth, all buffer and protein solutions contained NaN_3 at a final concentration of 0.03%.

Isolation of proline-rich polypeptide

Sheep colostral IgG2 immunoglobulins were prepared by the procedure described by Janusz *et al.* (1973). The separation of IgG2 and the proline-rich polypeptide was performed by gel filtration on Sephadex G-100 as described by Janusz *et al.* (1974). For further purification, the proline-rich polypeptide was applied to the column of Sepharose 4B conjugated with rabbit IgG anti-(sheep colostral IgG2). The fraction containing unretarded proline-rich polypeptide was concentrated by ultrafiltration with Amicon PM 10 membranes to a protein concentration of 10 mg/ml, and applied to a column (1.6 cm × 75 cm) of Sephadex G-75 in 0.1 M-sodium phosphate buffer, pH 7.2. A single symmetical protein peak was obtained. The proline-rich polypeptide was pooled, desalted on Sephadex G-25 and freeze-dried. All purification steps were performed at 4°C.

Immunoelectrophoresis

Immunoelectrophoresis was performed by the method of Scheidegger (1955) as modified by Hirschfeld (1960) in a 1.5% (w/v) agar gel in 50 mM-sodium barbital buffer, pH 8.6. Rabbit antisera against sheep colostrum, sheep colostral IgG2, sheep IgG Fab fragment and sheep IgG were used for precipitation.

Determination of protein content

Protein content was measured spectrophotometrically at 280 nm. The value of the absorption coefficient, $A^{1\%}_{280,1cm}$, determined for the proline-rich polypeptide was 7.82.

Determination of molecular weight

The molecular weight of the proline-rich polypeptide was determined by gel filtration and by polyacrylamide-gel electrophoresis at 4°C and at room temperature.

Gel filtration was performed on Sephadex G-100 in 0.1 M-Tris/HCl buffer, pH 7.4, containing 0.2 M-NaCl. Gel filtration of non-reduced and reduced proline-rich polypeptide and protein standards in the presence of 6 M-guanidinium chloride was performed on Sephadex G-200. Reduction, in the presence of 6 M-guanidinium chloride, was performed by using 0.15 M-dithiothreitol.

Polyacrylamide-gel electrophoresis in 15% (w/v) gel in the presence of 0.1% sodium dodecyl sulphate was performed as described by Laemmli (1970). Staining and destaining of the gels were performed by the method of Fairbanks *et al.* (1971).

For both methods, the following standard proteins were used: insulin A-chain (mol.wt. 2500), glucagon (mol.wt. 3500), myoglobin (mol.wt. 17800), α-chymotrypsinogen (mol.wt. 25000) and cytochrome *c* (mol.wt. 12400).

Amino acid composition

Samples of the proline-rich polypeptide were hydrolysed in 6 M-HCl for 24 h and 72 h at 110°C in ampoules sealed *in vacuo*. The hydrolysates obtained were analysed in a Locarte automatic amino acid analyser. Cysteine content was determined by oxidation to cysteic acid with 0.21 M-dimethyl sulphoxide by the method of Spencer & Wold (1969). The amino acid content was extrapolated to zero time of hydrolysis (Moore & Stein, 1963) and expressed in mol of amino acid/mol of polypeptide subunit of mol.wt. 6000.

Determination of phosphorus content

Samples of the proline-rich polypeptide were deionized on Dowex AG-501 (X8). The content of phosphorus was determined by the method of Chen *et al.* (1956).

Solubility properites of the proline-rich polypeptide

The precipitation of the proline-rich polypeptide was monitored by measurement of turbidity at 400 nm in a Shimadzu MPS spectrophotometer with 10 mm cuvettes. The required temperature was maintained by circulating water through a jacketed cell holder.

To assess the effect of temperature, turbidity of 0.8% solutions of the polypeptide in 10 mM-sodium phosphate buffer, pH 7.5, was measured at various temperatures. All other measurements were performed at 20°C.

To test the effect of pH, polypeptide solutions in 10 mM-sodium phosphate buffer, pH 7.5, were adjusted to various pH values by addition of 1.5 M-H_3PO_4 or 1.5 M-KOH. The final concentration of the polypeptide was 0.25%.

To assess the effect of ionic strength, the turbidity of 0.25% solutions of the polypeptide in 10 mM-sodium phosphate buffer, pH 7.5, was measured in the presence of NaCl at concentrations in the range 0.01–0.8 M.

For testing the effect of concentration of the proline-rich polypeptide, 0.03%, 0.062%, 0.125%, 0.25%, 0.5% and 0.73% solutions were prepared in 10 mM-sodium phosphate buffer, pH 7.5.

In all experiments the absorbance was recorded 10 min after the cuvettes were placed in the spectrophotometer.

Effects of the proline-rich polypeptide on proteinases and on proteolysis of sheep IgG2

The effects of the proline-rich polypeptide on

pepsin, trypsin and papain were studied by incubation of the enzymes with the polypeptide and measurement of the proteolysis of haemoglobin and casein at 37°C.

To find out whether the polypeptide affects the proteolysis of sheep colostral IgG2, a mixture of IgG2 and the polypeptide (molar ratio 1:30) was treated with pepsin, trypsin and papain. As controls, IgG2 or proline-rich polypeptide alone was used.

The extent of proteolysis was analysed by a ninhydrin method (Moore & Stein, 1954).

Interaction of the proline-rich polypeptide with sheep IgG2

^{125}I-labelled proline-rich polypeptide was obtained by iodination by the procedure of Unkeless & Eisen (1975). Interaction of the ^{125}I-labelled polypeptide with IgG2 was studied by gel filtration on Sephadex G-200 in 10mM-sodium phosphate buffer, pH7.2, containing 150mM-NaCl and in 10mM-sodium phosphate buffer, pH7.5, containing 50mM-NaCl.

Charge-shift electrophoresis

Amphiphilic properties of the proline-rich polypeptide were studied by using the charge-shift electrophoresis technique of Helenius & Simons (1977).

Circular-dichroism studies

C.d. spectra of aqueous 0.13% solutions of the proline-rich polypeptide in the range 190–300nm were recorded at 26°C in 2mm and 10mm cuvettes. The effect of trifluoroethanol on the conformation of the polypeptide was studied by recording the c.d. spectra of the polypeptide in aq. 50% (v/v) trifluoroethanol after preincubation for 2.5h at room temperature. The absorbance of the polypeptide solutions was below 2.0 in the range studied. Data are presented as reduced mean ellipticity, $[\theta]$, in degrees·cm^2·dmol^{-1}, a residue weight of 110 being used.

Determination of N-terminal amino acids

The proline-rich polypeptide was dansylated by the method described by Hartley (1970). Dansyl-amino acids were identified by t.l.c. on polyamide sheets (7.5cm × 7.5cm) in solvent systems used by Flensgrud (1976).

Results

Purification of the proline-rich polypeptide

The sheep colostral IgG2 preparation obtained after chromatography on DEAE-cellulose was subsequently fractionated on Sephadex G-200 or Sephadex G-100, and two protein peaks were obtained (Janusz *et al.*, 1974). The first fraction was IgG2 and the second was proline-rich polypeptide contaminated with IgG2. For additional purification, this second fraction was applied to an immunoadsorbent prepared from Sepharose 4B and rabbit IgG anti-(sheep colostral IgG) immunoglobulins. The purified proline-rich polypeptide did not interact with the antisera used.

The yield of proline-rich polypeptide varied between 400 and 800mg/litre of sheep colostrum collected within 6h after delivery. The content of the polypeptide in the sheep colostrum collected several days after delivery was much lower than in early colostrum.

Determination of the molecular weight of the proline-rich polypeptide

The molecular weight of the proline-rich polypeptide was determined with preparations both untreated and treated with dithiothreitol and iodoacetamide, in the absence and in the presence of 6M-guanidinium chloride. In gel filtration, in the absence of guanidinium chloride, the molecular weight found was 17200. In the presence of guanidinium chloride a molecular weight of about 6000 was found both for non-treated and for reduced and alkylated proline-rich polypeptide.

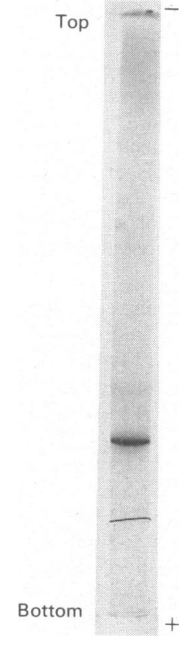

Fig. 1. *Polyacrylamide-gel electrophoresis of the proline-rich polypeptide in the presence of 0.1% sodium dodecyl sulphate*

Electrophoresis was performed as indicated in the Materials and methods section. The gel was stained with Coomassie Brilliant Blue.

Molecular weights determined at room temperature were the same as those determined at 4°C. In all gel-filtration experiments the presence of only one symmetrical protein peak was observed. After electrophoresis of the proline-rich polypeptide in polyacrylamide gel in the presence of 0.1% sodium dodecyl sulphate, a major protein band with a molecular weight of 5800 was found (Fig. 1). Two weak bands with higher molecular weights (12400 and 18200) were non-dissociated aggregates of the polypeptide.

Chemical composition of the proline-rich polypeptide

The amino acid composition of the proline-rich polypeptide is presented in Table 1. Proline residues comprise 22% of the all amino acid residues. We conclude that the polypeptide does not contain alanine, arginine or cysteine, since only trace amounts of these amino acids were found. No neutral carbohydrates, sialic acid or hexosamines were found in the polypeptide (Janusz *et al.*, 1974). The polypeptide contains 0.18% of phosphorus, determined after deionization of samples.

Studies of the solubility of the proline-rich polypeptide

The proline-rich polypeptide is soluble at 4°C but is reversibly precipitated on warming to room temperature.

The dependence of precipitation of the polypeptide on pH is presented in Fig. 2. Ionic strength above 0.6 strongly facilitated the precipitation. The higher the temperature the greater the amount of precipitate was formed, reaching a maximum at 48°C. At higher concentrations of the polypeptide the precipitation was stronger and started at a lower temperature compared with more-dilute solutions. The presence of other proteins, e.g. sheep colostral IgG2 or bovine serum albumin, or of Ca^{2+} or Zn^{2+} had no effect on the amount of the precipitate formed.

Effect of the proline-rich polypeptide on proteolytic enzymes and on proteolysis of sheep colostral IgG2 immunoglobulins

It was suspected that the proline-rich polypeptide might play a protective role against proteolysis of colostral immunoglobulins in the digestive tract. For this reason, proteolysis of sheep colostral IgG1 and IgG2 and of bovine serum albumin by trypsin, papain and pepsin was studied in the absence and in the presence of the polypeptide. No protective effect of the polypeptide was observed. It was also found that preincubation of proteolytic enzymes with the polypeptide had no effect on the activity of these enzymes.

Interaction of the proline-rich polypeptide with sheep colostral IgG2

Interaction of ^{125}I-labelled proline-rich polypeptide with sheep colostral IgG2 was studied by using

Table 1. *Amino acid composition of the proline-rich polypeptide*

Amino acid analysis was performed as described in the Materials and methods section. The values presented are averages of five determinations after extrapolation to zero time of hydrolysis. The results are expressed in mol of an amino acid/mol of polypeptide subunit, assuming the subunit molecular weight to be 6000.

	Amino acid composition (mol/mol)	
Amino acid	Found	Nearest integer
Lysine	2.41 ± 0.03	2
Histidine	1.47 ± 0.15	1
Arginine	0.23 ± 0.04	0
Aspartic acid	2.11 ± 0.01	2
Threonine	3.65 ± 0.21	4
Serine	3.05 ± 0.01	3
Glutamic acid	6.33 ± 0.02	6
Proline	11.23 ± 0.01	11
Glycine	1.75 ± 0.01	2
Alanine	0.45 ± 0.04	0
Valine	5.34 ± 0.01	5
Methionine	1.60 ± 0.07	2
Isoleucine	1.64 ± 0.12	2
Leucine	5.59 ± 0.01	6
Tyrosine	0.77 ± 0.16	1
Phenylalanine	2.57 ± 0.06	3
Cysteine	0.34 ± 0.01	0

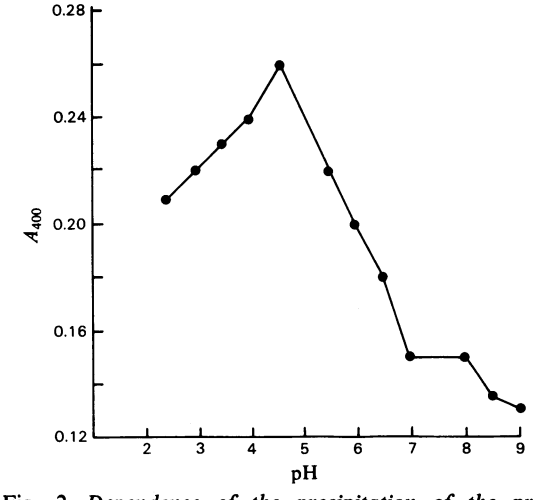

Fig. 2. *Dependence of the precipitation of the proline-rich polypeptide on pH*

The precipitation was monitored by measurement of turbidity at 400nm. For details see the Materials and methods section.

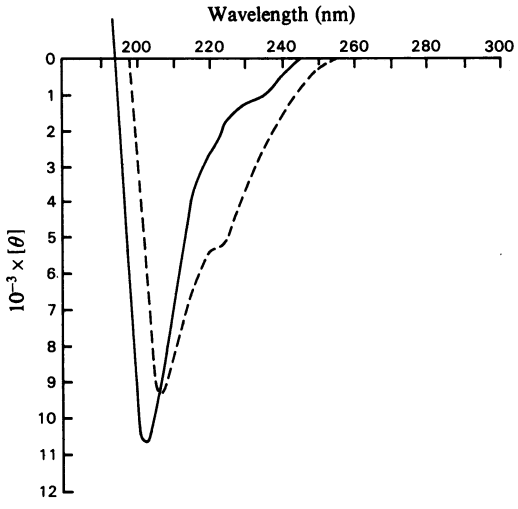

Fig. 3. *Circular-dichroism spectra of the proline-rich polypeptide in water* (——) *and in 50% (v/v) trifluoroethanol* (– – – –)
For details see the Materials and methods section.

a gel-filtration technique. It was found that at ionic strength above 0.05 no interaction between IgG2 and the polypeptide occurred (results not shown). This was confirmed by studying u.v. difference spectra and c.d. spectra (results not shown).

Charge-shift electrophoresis

To check whether the proline-rich polypeptide has amphiphilic character facilitating its precipitation, charge-shift electrophoresis was performed. No change in the electrophoretic mobility was observed when the polypeptide was applied without or with Triton X-100, or with Triton X-100 mixed with sodium deoxycholate or cetyltrimethylammonium bromide.

Circular-dichroism measurements

The shape of c.d. spectrum of the proline-rich polypeptide is similar to that of poly-L-proline II (Carver *et al.*, 1966), with a strong negative ellipticity (−10500) at 202nm (Fig. 3). This suggested that the polypeptide may contain block sequence(s) of proline residues. This assumption was strengthened by recording the c.d. spectrum of the polypeptide in 50% (v/v) trifluoroethanol, which is a helix-promoting solvent. In 50% trifluoroethanol the band at 202nm was no longer present, and bands at 207nm and 222nm characteristic of polypeptide containing helical structures appeared.

N-Terminal amino acids

T.l.c. of hydrolysis products of dansylated proline-rich polypeptide showed that the only *N*-terminal amino acid was leucine.

Discussion

The present modified purification of the proline-rich polypeptide gave pure preparations free of traces of immunoglobulins and containing only one *N*-terminal amino acid, leucine. Purified proline-rich polypeptide did not react with antisera against sheep colostral whey. Attempts to obtain anti-(proline-rich polypeptide) antibodies by various techniques of immunization were unsuccessful.

Determination of the molecular weight of the proline-rich polypeptide both untreated and treated with dithiothreitol and iodoacetamide under dissociating and non-dissociating conditions showed that the polypeptide is a non-covalent oligomer (mol.wt. 17200) of subunits of molecular weight about 6000. The apparent heterogeneity in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis may be explained by assuming that sodium dodecyl sulphate did not dissociate the polypeptide completely. Incomplete dissociation was also found in experiments on gel filtration of the proline-rich polypeptide in buffers containing sodium dodecyl sulphate. The polypeptide contains an exceptionally high proportion of proline residues (22%). The absence of alanine and the low content of glycine make the proline-rich polypeptide different from collagen-like proteins. The non-polar amino acids valine, leucine and isoleucine comprise 24% of the total amino acids determined.

The c.d. spectrum of the proline-rich polypeptide has a minimum at 202nm with a mean residue ellipticity of −10500 at 25°C. A c.d. band near this wavelength is observed only with unordered or extended polypeptide chains or polypeptides containing a large amount of proline, i.e. poly-L-proline II (Tiffany & Krimm, 1973). This type of c.d. spectrum could be also obtained for a protein in which parts of the polypeptide chain, containing at least four proline residues (Okabayashi & Isemura, 1968; Sakai *et al.*, 1969; Deber *et al.*, 1970), were folded into a polyproline helix, whereas other parts contain a low percentage of ordered structure. Comparison of c.d. spectra of the proline-rich polypeptide in the absence and in the presence of trifluoroethanol indicates a possibility of the presence of sequences of four or more proline residues. If proline residues, which are strong helix-breaking residues (Chou & Fasman, 1974), were randomly distributed along the polypeptide chain, then the shape of the c.d. spectrum would not be changed in the presence of trifluoroethanol, a solvent inducing a helical structure in most polypeptides and proteins (Brown *et al.*, 1969; Knof & Engel, 1974; Wong *et al.*, 1979). In our experiments the c.d. spectrum of

the proline-rich polypeptide in 50% trifluoroethanol was different from that of the polypeptide in water. The final answer must await amino acid sequencing of the polypeptide. An evaluation of the conformation of the proline-rich polypeptide based on the equations of Krigbaum & Knutton (1973) or on the computer c.d. spectra given by Greenfield & Fasman (1969) or Chen *et al.* (1974) gave erroneous results and was not in agreement with the shape of the c.d. spectrum of the polypeptide that we obtained.

The proline-rich polypeptide is soluble at 4°C but is reversibly precipitated on warming to room temperature. The results obtained suggest that hydrophobic interactions may be important in the precipitation of the polypeptide. This could be due to the high content of non-polar amino acids in the polypeptide. However, the results of charge-shift electrophoresis showed that the polypeptide is not an amphipathic protein and cannot form micellar structures. With the concentrations of the proline-rich polypeptide used in gel-filtration studies in the absence of dissociating agents the same molecular weight, 17200–17500, was found at 4°C and at room temperature. When higher concentrations of the polypeptide were used, the protein was precipitated. Attempts to study the aggregation of the polypeptide by spectral methods, c.d. and u.v. difference spectra, were also unsuccessful, because of low concentrations of the peptide. Moreover, the absence of observable aggregates may indicate that insoluble aggregates are formed rapidly.

The problem of the possible biological activity of the proline-rich polypeptide was intriguing. One possibility that we considered was that the polypeptide prevents proteolysis of colostral immunoglobulins. However, the results obtained showed that it did not inhibit proteolytic enzymes. At ionic strength above 0.05 the polypeptide did not react with sheep IgG immunoglobulins. Another possibility was that the polypeptide could affect the immune response. The results of our previous studies (Wieczorek *et al.*, 1979) showed that the polypeptide did in fact have a regulatory activity stimulating or suppressing the immune response. It also increased the permeability of skin vessels.

The origin of the proline-rich polypeptide is rather obscure. The possibility that it is a degradation product derived from immunoglobulins must be ruled out, since the polypeptide did not react with anti-immunoglobulin antibodies. Besides, the amino acid composition of the polypeptide is also against this possibility. The polypeptide cannot be a degradation product of the C1q component of complement because it contains no alanine and a very low amount of glycine residues. Another possibility is that the polypeptide is one of the degradation products of casein, which are known to be proline-rich proteins (McKenzie, 1971). To prove this, however, the determination of a complete amino acid sequence of the proline-rich polypeptide will be necessary.

Proline-rich proteins have also been found in dog colostrum (Swanson & Sanders, 1974), in human milk (Schade & Reinhart, 1970) and in bovine milk (Seto *et al.*, 1975; Seto & Ito, 1976). Although they showed some similarity to the present proline-rich polypeptide, e.g. they were reversibly precipitated and they increased the permeability of skin vessels (Seto & Ito, 1976), their physicochemical properties were different. Proline-rich proteins were found also in parotid-gland saliva (Henkin *et al.*, 1978; Isemura *et al.*, 1979; Kousvelari & Oppenheim, 1979; Wong *et al.*, 1979; Muenzer *et al.*, 1979). The conformation of some of these proteins resembles the conformation of the present polypeptide. However, they have much higher contents of alanine and glycine residues and higher molecular weights.

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