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IMMUNOLOGICALLY ACTIVE NONAPEPTIDE FRAGMENT OF A PROLINE-RICH POLYPEPTIDE FROM OVINE COLOSTRUM: AMINO ACID SEQUENCE AND IMMUNOREGULATORY PROPERTIES*

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Abstract—It has been previously found that a proline-rich polypeptide (PRP) isolated from ovine colostrum has a regulatory effect on the immune response. To study the relationship between the structure of PRP and its immunomodulatory properties, the polypeptide was digested by chymotrypsin. Products of the proteolysis were separated by gel filtration and three fractions were obtained: PRP-1, PRP-2 and PRP-3. The activity of the fractions was compared with the activity of the untreated PRP. It was found that PRP-1 was inactive, whereas PRP-2 and PRP-3 showed an activity in the regulation of the immune response assayed by measurement of PFC, and by studying effects on delayed hypersensitivity, formation of autologous rosette-forming cell, and sensitivity of thymocytes to hydrocortisone. The activity of PRP-2 and PRP-3 was comparable to the activity of PRP. The PRP-3 fraction of low mol. wt was further purified and a pure nonapeptide of mol. wt 1000 (PRP-3b) was isolated. The amino acid sequence of PRP-3b was: Val—Glu—Ser—Tyr—Val—Pro—Leu—Phe—Pro. The nonapeptide showed the full spectrum of biological activities of PRP. Comparison of terminal amino acid suggested that PRP-3b was neither the NH_2- nor the COOH-terminal fragment of PRP. The amino acid sequence of the nonapeptide indicated that PRP-3b is different from other known immunomodulators.

INTRODUCTION

During our studies on ovine colostral immunoglobulins, it was found that IgG2 immunoglobulins isolated by chromatography on DEAE-cellulose were contaminated with a proline-rich polypeptide (PRP).§ The polypeptide was isolated and characterized (Janusz *et al.*, 1974, 1981). The amount of proline residues in PRP is 22% of all amino acid residues present. It also contains a high number of nonpolar amino acids. A relatively low content of glycine and the absence of alanine makes PRP different from collagen-like proteins. The polypeptide also is different from proline-rich proteins isolated from other sources. In the absence of dissociating agents, the apparent mol wt. of PRP is 18,000. It is composed of subunits of apparent mol. wt 6000. Studies on a possible biological role of PRP 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; Zimecki *et al.*, 1978, 1982, 1983).

To obtain information on the relationship between the structure of PRP and its activity, experiments on fragmentation of PRP were performed. The results presented in this paper showed that by digestion of PRP with chymotrypsin a nonapeptide fragment of mol. wt 1000 could be isolated. The fragment showed biological activity similar to PRP. The amino acid sequence of the fragment was determined.

MATERIALS AND METHODS

Reagents and miscellaneous materials

Guanidine hydrochloride was purchased from Fluka, Switzerland. Sephadex G-10, G-25 and G-100 were products of Pharmacia, Sweden. Carboxypeptidase B (EC 3.4.17.2), type I, treated with DFP;

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§Abbreviations; ARFC, autologous rosette-forming cells; dansyl chloride, 5-dimethylaminonaphthalene-1-sulfonyl chloride; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; DTH, delayed-type hypersensitivity; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HBSS, Hanks' balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; OSA, ovine serum albumin; PBS, 0.02 *M* phosphate buffer containing 0.15 *M* NaCl, pH 7.2; PFC, plaque-forming cells; PNA, peanut agglutinin; PRP, proline-rich polypeptide; TNBS, 2,4,6-trinitrobenzenesulfonic acid; SRBC, sheep red blood cells; HC, hydrocortisone.

α-chymotrypsin (EC 3.4.21.1) attached to carboxylmethyl cellulose (580 units per gram of solid); standards of dansyl amino acids and ninhydrin spray reagent were obtained from Sigma, St. Louis, MO. Hydrazine (anhydrous) dansyl chloride, phenyl isothiocyanate, trifluoroacetic acid, trifluoroacetic anhydride, butyl acetate, butanol and propanol were purchased from Pierce Eurochemie, Rotterdam, Holland. Pyridine, plates of silica gel G for TLC, were obtained from Merck, Darmstadt, F.R.G. Glycine methyl ester hydrochloride was a product of Sigma. Plates of polyamide for TLC were purchased from Schleicher-Schuell, F.R.G. Rabbit anti-mouse IgG (IgG fraction) was obtained from Miles International, Vienna, Austria. RBC were stored in an Alsever solution. Before use, they were washed 3 times with PBS. All other reagents were of analytical grade. To prevent any bacterial growth, all buffers and protein solutions contained sodium azide (whenever it was possible to use it) at a final concn of 0.03%.

Mice

Eight-to-sixteen-week old mice, male and female hybrids F_1 (129/A Boy × B6), were purchased from the Animal Farm of our Institute.

PRP

The polypeptide was prepared from ovine colostrum according to the method of Janusz *et al.* (1981).

Determination of NH_2-terminal amino acids

Peptides were dansylated by the method described by Hartley (1970). Dansyl amino acids were identified by TLC on polyamide sheets (7.5 × 7.5 cm) in solvent systems used by Flengsrud (1976).

Determination of COOH-terminal amino acids

(A) COOH-terminal amino acids were released by treatment of peptides with carboxypeptidase B (treated with DFP) according to the procedure described by Sajgó (1974). (B) Hydrazinolysis was performed at 115°C for 6–16 hr, as described by Sajgó (1974). COOH-terminal amino acids were identified by TLC on silica-gel plates in various solvent systems (Hartley, 1970).

Determination of protein content

The concn of PRP was measured spectrophotometrically at 280 nm using an absorption coefficient, $A^{1\%}_{280,1\,cm}$, value of 7.82 (Janusz *et al.*, 1974). Protein or peptide content in products of proteolysis was measured spectrophotometrically at 280 nm or by the Bradford (1976) method, using BSA as a standard, or by a dry-wt determination.

Determination of the mol wt. of PRP-3b

The mol. wt of PRP-3b was determined in a mass spectrometer type WSM-2 (Poland) using a low-pressure head.

Digestion of PRP with chymotrypsin

Insoluble chymotrypsin (16.5 mg corresponding to 10 units of activity) was washed with water to remove borate buffer salts present in the preparation and added to 50 mg of PRP in 5 ml of water. The reaction mixture was adjusted to pH 8.0 and was incubated with gentle stirring, for 20 hr at 30 C. The pH of the reaction mixture (8.0) was kept constant in a pH-stat using 0.02% ammonia solution. At the end of the digestion, the reaction mixture was centrifuged at room temp for 10 min at 2800 *g*. The precipitate was washed with 1 ml of water (adjusted with ammonia solution to pH 8.0). The supernatants were pooled and adjusted with conc. formic acid to 1 *M* in formic acid. The products of proteolysis were separated by gel filtration on Sephadex G-10 in 1 *M* formic acid. Three fractions were obtained (designated as PRP-1, PRP-2 and PRP-3). The fractions were lyophilized to remove water, formic acid and ammonium formate. In the case of incomplete removal of salts, the preparation was desalted by gel filtration and again lyophilized. The number of peptide bonds cleaved by proteolysis was determined with a ninhydrin reagent, using leucine as a standard.

Amino acid composition

Samples of PRP or PRP-3b (1 mg) were hydrolyzed in 6 *M* HCl for 24 hr at 110°C in ampoules sealed *in vacuo*. The hydrolysates obtained were analyzed in a Locarte automatic amino acid anlayser.

Preparative paper chromatography

Fraction PRP-3 (4 mg) obtained after chymotryptic digestion of PRP was applied to a Whatman 3 MM (9.5 × 27.5 cm) strip in a narrow uniform zone across the width of the paper (3.5 cm). Separation of fractions was performed in 1-butanol–acetic acid–water (4:1:5, v/v, upper phase). To locate the position of the peptides, guide strips (3 mm in width) were cut out from the paper and sprayed with ninhydrin. Each peptide fraction detected with ninhydrin was eluted first with the upper phase of the solvent system used (5 × 6 ml × 5 min) and then with 10% formic acid (5 × 6 ml × 5 min). The eluates were pooled and dried in a vacuum rotary evaporator.

Amino acid sequence determination

The amino acid sequence was determined by the manual method as described by Hartley (1970). The NH_2-terminal amino acid was identified according to the Edman procedure whereas the remaining amino acids were identified as dansyl derivatives using modifications described by Peterson *et al.* (1972) and Weiner *et al.* (1972).

Bioassay systems

Antibody assay (PFC-19S and PFC-7S). The effect of PRP on the primary and secondary immune responses was studied by determination of PFC-19S

and PFC-7S to SRBC in mice (Mishell and Dutton, 1967) as described by Wieczorek *et al.* (1979).

DTH. The effect of PRP on DTH was studied according to the procedure of Lagrange *et al.* (1974) as described by Wieczorek *et al.* (1979).

Autologous rosette formation. This assay was performed according to the procedure of Charreire and Bach (1975) with minor modifications (Zimecki *et al.*, 1983). Rosettes with at least four erythrocytes per one viable thymocyte were counted.

Sensitivity to HC. The effect of PRP on the sensitivity of thymocytes to HC was studied according to the procedure described by Zimecki *et al.* (1982).

RESULTS

Digestion of PRP with chymotrypsin

To facilitate the separation of the products of proteolysis and salts, insoluble chymotrypsin was used for digestion of PRP. It was found that under the conditions used 13 peptide bonds in a PRP subunit (mol. wt 6000) were cleaved during the proteolysis. The products of proteolysis were separated by gel filtration in 1 *M* formic acid. Three fractions (monitored by absorption at 280 nm) designated as PRP-1, PRP-2 and PRP-3 were obtained (Fig. 1). Assuming the quantity of PRP used for proteolysis was 100% (by weight), 45–50% of the material was recovered in fraction PRP-1, 30% in PRP-2, and 10–15% in PRP-3. It was observed that after fractions PRP-1–PRP-3 had been eluted from Sephadex G-10 (Fig. 1), a ninhydrin-positive material (presumably small peptides and/or free amino acids) appeared in the eluate.

The biological activity of the fractions obtained was compared with the activity of the untreated PRP. Fraction PRP-1 as well as the ninhydrin-positive low mol wt. material were inactive in all bioassays used

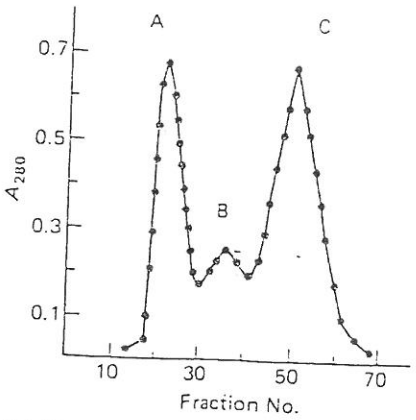

Fig. 1. Gel filtration of products of chymotryptic digestion of PRP on Sephadex G-10 in 1 *M* formic acid. Digestion products prepared from 250 mg of PRP were applied (10 ml) to the column (3.4 × 32.5 cm). The flow rate was 24 ml/hr and 5-ml fractions were collected. (A) PRP-1 (tubes 18–28). (B) PRP-2 (tubes 32–38). (C) PRP-3 (tubes 44–60).

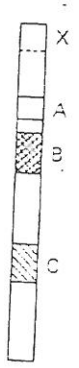

Fig. 2. Preparative paper chromatography of PRP-3 on Whatman 3 MM paper. Peptides were localized with a ninhydrin reagent. Intensity of the spots: (□) weak, PRP-3a (A); (▩) very strong, PRP-3b (B); and (▨) strong, PRP-3c (C). For details, see Materials and Methods. (×) Application of the sample.

and were of no further interest for studies described in this paper. At the same time, fractions PRP-2 and PRP-3 displayed an activity, as immunomodulators, comparable with the activity of the untreated PRP (in respect to the wt of the dose). Our further studies concentrated on the PRP-3 fraction, since it contained an active peptide(s) of presumably lower mol wt. than that present in PRP-2.

Properties of the PRP-3 fraction

Determination of terminal amino acids in PRP-3 showed that the fraction is composed of several peptides. Attempts to purify these peptides by fractionation on ion-exchangers were not fully satisfactory. Separation of peptides was achieved by preparative paper chromatography on Whatman 3 MM paper (Fig. 2). Three fractions were obtained. Assuming the quantity of PRP-3 applied to the paper was 100%, 10–15% of the material was recovered in fraction PRP-3a, 60–70% in PRP-3b, and 15–25% in fraction PRP-3c. Determination of terminal amino acids showed that valine was the only NH_2-terminal amino acid found in PRP-3a and PRP-3b, and valine and leucine were found in PRP-3c. The only COOH-terminal amino acid found in PRP-3b was proline. For comparison, terminal amino acids were also determined in PRP. It was confirmed that leucine was the NH_2-terminal amino acid (Janusz *et al.*, 1981), and isoleucine was the COOH-terminal amino acid in PRP.

Biological assays showed that the activity of the PRP-3 fraction was fully related to the PRP-3b peptide. It should be mentioned here that treatment of PRP or PRP-3 with 1 *M* formic acid and solvents used for paper chromatography of PRP-3 showed no effect on the biological activity of the preparation. The presence of only one NH_2- and COOH-terminal amino acid indicated that PRP-3b was pure. The yield of PRP-3b, in respect to PRP used for proteolysis, was 7–9%.

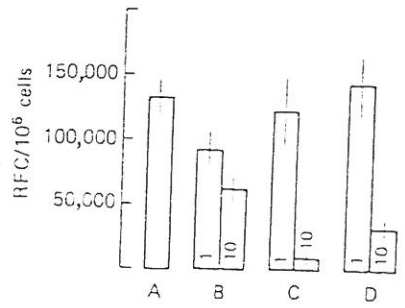

Fig. 6. Effect of PRP and its proteolytic fragments on ARFC. Thymocytes were incubated with various doses of PRP (1 or 10 μg) and the number of ARFC was evaluated. The values presented are averages of three determinations. (A) Control (PBS). (B) PRP. (C) PRP-2. (D) PRP-3b. Bars denote SE.

tions. It was found that, in the case of the humoral immune response, PRP stimulated low or suppressed high immune responses against SRBC in mice (Wieczorek *et al.*, 1979). The polypeptide generated cells capable of inducing the GvH reaction from T-cell precursors, induced transformation of the HC-sensitive thymocyte pool into a HC-resistant one, and vice versa. PRP had an effect on the maturation of thymocytes and the generation of suppressor T-cells (Zimecki *et al.*, 1982). The polypeptide changes the number of ARFC. It lowered the number of ARFC among HC-resistant glass-adherent thymocytes and increased ARFC in HC-sensitive non-adherent cells (Zimecki *et al.*, 1983). PRP increased the number of antigen θ-positive cells in T-cell precursors from the spleen (unpublished results). However, the exact mechanism of its activity as an immunomodulator is still unknown and is under investigation in our laboratories. The results we have obtained hitherto suggest that PRP interacts with T-cell precursors inducing both T suppressor and T helper cells. The net effect of PRP depended on the actual immunological status of animals studied (Wieczorek *et al.*, 1979).

The effects of PRP on the immune response resemble, in many respects, the effects of substances isolated from the thymus. It was found, for example,

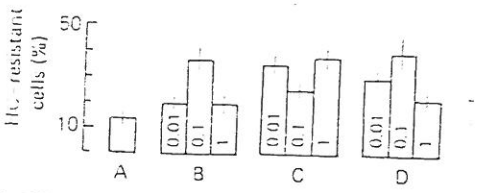

Fig. 7. Effect of PRP and its proteolytic fragments on the sensitivity of thymocytes to HC. For experiments, thymocytes agglutinated by PNA were used. The resistance of thymocytes is expressed as percent of the control (cells not treated with HC). (A) Thymocytes treated with HC in the presence of PRP. (B), (C) and (D) cells treated with HC in the presence of 0.01, 0.1 or 1 μg of PRP, PRP-2 and PRP-3b, respectively. Bars denote SE. The values presented are averages of five determinations.

that PRP and thymic hormones display similar effects on autoimmunity (Zimecki *et al.*, 1983; Lau *et al.*, 1980) and T-cell maturation (Zimecki *et al.*, 1982; Low *et al.*, 1979; Pahwa *et al.*, 1980; Bach *et al.*, 1979; Auti and Wigzell, 1980). Thymopoietein, similarly to PRP, can return the immune balance toward normal whether the initial immune deviation was in the direction of enhanced immune responsiveness or suppressed immune responsiveness (Wieczorek *et al.*, 1979; Goldstein and Lau, 1980).

It is interesting that some proteolytic fragments of PRP retain their biological activity. The results of proteolysis of PRP were quite reproducible in respect to the quantity and activity of fragments obtained. The most interesting fragment obtained was the nonapeptide PRP-3b. It showed the full spectrum of biological effects of PRP at similar wt doses. However, on a molar basis, the activity of the nonapeptide (mol. wt 1000) was about six-fold lower than the activity of untreated PRP (subunit mol. wt 6000). The fact that biological activity of PRP and its fragments was resistant to a prolonged treatment with 1 *M* formic acid or organic solvents used for chromatography facilitated separation and purification of PRP-3b.

Determination of terminal amino acids in PRP and PRP-3b suggests that PRP-3b is neither an NH_2- nor a COOH-terminal fragment of PRP.

It is worth mentioning here that, although chymotrypsin exhibits a rather broad specificity, hydrolysis of the peptide bond at the carboxyl group of proline was unexpected. There is a possibility that the lysine residue in PRP which follows the COOH-terminal proline of PRP-3b (our unpublished results) affects the suspectibility of this peptide bond to chymotrypsin. Markland *et al.* (1966) noticed that residues of basic amino acids (lysine, arginine) adjacent to a weakly susceptible bond enhance cleavage at that site. The action of chymotrypsin frequently is difficult to predict accurately, since it also depends upon factors outside the intermediate environment of the bond cleaved (Kasper, 1970).

Comparison of the amino acid sequence of PRP-3b with sequences of other immunomodulatory factors showed a low homology with β_2-thymosin (Low and Goldstein, 1979) and β_4-, β_8- and β_9-thymosins (Low and Goldstein, 1982; Hannapel *et al.*, 1982). There is no similarity to the thymic peptide (FTS) from pig serum (Bach *et al.*, 1977), to thymic humoral factor (Kook *et al.*, 1975) or to tuftsin (Nishioka *et al.*, 1972). The sequence Tyr—Val or Tyr—Leu is present in thymopoietin (Folkers *et al.*, 1981). These sequences and also Val—Pro, Pro—Leu, Val—Glu, Val—Phe, Phe—Pro and Leu—Phe are also present in many peptide hormones and proteins with various biological activities, it is rather difficult to ascribe any essential role of these sequences in the biological activity of PRP. An analogue of the sequence Val—Glu—Ser in PRP-3b is present in α_1-thymosin, in the form of Val—Asp—Thr— (Low and Gold-

Table 1. Amino acid composition of PRP and PRP-3b peptide

Amino acid	Amino acid composition (moles/mole) PRP Found	PRP Nearest integer	PRP-3b Found	PRP-3b Nearest integer
Lysine	2.3	2	N.D.	N.D.
Histidine	1.4	1	N.D.	N.D.
Arginine	N.D.	N.D.	N.D.	N.D.
Aspartic acid	2.3	2	N.D.	N.D.
Threonine	3.9	4	N.D.	N.D.
Serine	3.2	3	1.4	1
Glutamic acid	6.3	6	1.3	1
Proline	11.3	11	1.8	2
Glycine	1.8	2	N.D.	N.D.
Alanine	N.D.	N.D.	N.D.	N.D.
Valine	5.2	5	2.3	2
Methionine	1.8	2	N.D.	N.D.
Isoleucine	1.8	2	N.D.	N.D.
Leucine	5.8	6	1.2	1
Tyrosine	0.8	1	1.0	1
Phenylalanine	2.8	3	1.4	1
Cysteine	N.D.	N.D.	N.D.	N.D.

Amino acid analysis was performed as described in Materials and Methods. The values presented are averages of two determinations. The results are expressed in moles of amino acid/mole of PRP subunit of mol. wt 6000 (Janusz *et al.*, 1981), or in moles of amino acid/mole of PRP-3b of mol. wt 1000. N.D.: not detected.

Structure and properties of PRP-3b peptide

The mol. wt of PRP-3b, determined in a mass spectrometer, is 1000. Comparison of the amino acid composition of PRP and PRP-3b is presented in Table 1. PRP-3b contains nine amino acid residues only, in comparison to 50 amino acids present in the subunit of PRP (mol. wt 6000).

Determination of the amino acid sequence showed the following alignment of amino acids in PRP-3b: Val—Glu—Ser—Tyr—Val—Pro—Leu—Phe—Pro.

Treatment of PRP or PRP-3b with 6 *M* guanidine hydrochloride for 6 hr at room temp had no effect on the biological activity of the preparations.

Effects of PRP and its proteolytic fragments on the immune response

The activity of PRP-3b as an immunomodulator was compared with the activity of the untreated PRP

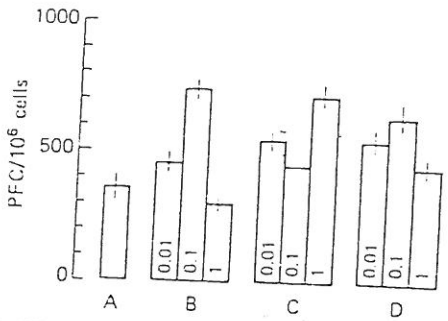

Fig. 3. Effect of PRP and its proteolytic fragments on the primary humoral immune response measured by determination of PFC-19S. Mice were injected with PRP (0.01, 0.1 or 1 μg/mouse) 48 and 24 hr before application of the antigen (SRBC). (A) Control (PBS). (B) PRP. (C) PRP-2. (D) PRP-3b. Each experimental group of mice contained 10 animals. Bars denote SE.

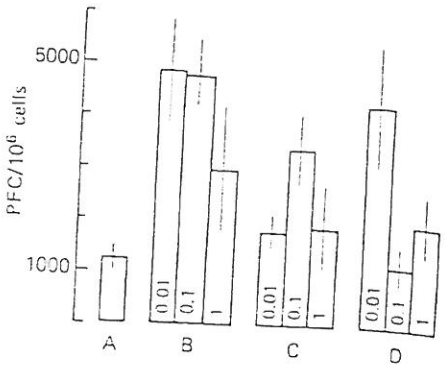

Fig. 4. Effect of PRP and its proteolytic fragments on the secondary immune response to SRBC PFC-7S. Mice were sensitized with SRBC, then treated with PRP and SRBC (for details see Materials and Methods; for other explanations see Fig. 3).

(effect on PFC-19S and 7S, DTH, autologous rosette formation, sensitivity to HC). For the sake of comparison, the results of studies of the activity of fraction PRP-2 obtained after chromatography on Sephadex G-10 of digestion products of PRP are also included. As is presented in Figs 3–7 PRP-3b practically showed, in all bioassays used, the same activity as untreated PRP or fraction PRP-2 (in respect to the wt dose of the preparation).

Control mice were treated with PBS. It has been previously found that control values were similar irrespective of the use of PBS, BSA, OSA or poly-L-proline.

DISCUSSION

During our previous studies we have shown that PRP had regulatory effect on the immune response. PRP showed various effects on immunological reac-

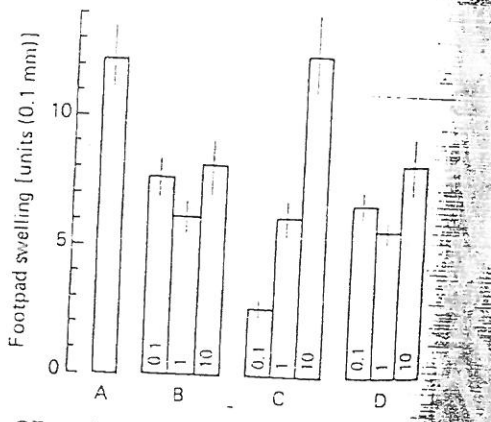

Fig. 5. Effect of PRP and its proteolytic fragments on cellular immune response. Mice were treated with PRP (0.1 or 10 μg/mouse) and SRBC as described in Materials and Methods. Each experimental group contained 10 animals. Ordinates: swelling of foot-pads of mice in units (1 unit corresponds to 0.1-mm increase in swelling). (A) Control (PBS). (B), (C) and (D) PRP, PRP-2 and PRP-3b respectively. Bars denote SE.

stein, 1979). However, there is no information on the role of this sequence in the activity of α-thymosin. Nevertheless, it is interesting that peptide substances with such different amino acid sequences as PRP-3b and thymic hormones display similar immunoregulatory activity. Chemical synthesis of PRP-3b and its analogues (under studies in our laboratory) will be of great help in elucidating the mechanism of action of the peptide and the relationship between the structure and immunoregulatory activity of PRP-3b.

PRP-3b, which is a fragment of a substance isolated from a natural source, is one of a few peptides of known structure with immunomodulatory properties. Therefore, it could be of interest in studies on the mechanism of the immune response.

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