

## Pharmacological and physicochemical properties of collagen breakdown-products

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**Abstract.** It has been found that the pharmacologically active, low molecular products obtained by digestion of telopeptides-deprived type I collagen with bacterial collagenase is a heterogenous mixture of at least 21 peptides of different molecular weight. They contain 3 to 15 amino acid residues. About 80% of them are tripeptides of the sequence Gly-Pro-X. The most abundant are two peptides: Gly-Pro-Hyp and Gly-Pro-Ala. The peptides injected into the lateral cerebral ventricle of the rat evoked some behavioral effects. They decreased the psychomotoric activity (evaluated with Lat's test) and increased the cataleptic action of haloperidol. On the other hand, they did not exert any effect on amphetamine - induced stereotypy and did not counteract the apomorphine - induced stereotypy.

**Key words:** collagen, peptides, dopaminergic system, central nervous system

## INTRODUCTION

Collagen constitutes about 30% of total mammalian proteins. It is present in every organ. Both primary and secondary structures of collagen are distinctly different in comparison to other proteins. It contains large amounts of glycine, proline and very low quantities of aromatic and sulphuric amino acids. The characteristic feature of collagen is the presence of hydroxyproline and hydroxylysine, which very rarely occur in other animal proteins. At least 14 different collagen types have been detected till now. Type I is the most abundant form of this protein in mammalian tissues (Bańkowski 1982, Mayne and Burgeson 1987, Bańkowski and Pałka 1989, Van der Rest 1990).

The collagen molecule (tropocollagen) consists of 2 parts of various structure. The central, triple-helical part of this protein is composed of repetitive triplet, (Gly-X-Y)<sub>n</sub>, whereas the N- and C-terminal fragments (telopeptides) demonstrate a different amino acid composition. They contain less glycine and proline and more aromatic amino acids. Hydroxyproline does not occur in these fragments.

Collagen is resistant to the action of most non-specific proteolytic enzymes. It is specifically digested by tissue and bacterial collagenases. Products of collagenolysis exert several biological effects (Telejko et al. 1990a, b). It was previously found that the biological activity of collagen-degradation products depends on their molecular weight. Peptides of the molecular weight about 3,000 Da, injected in high doses increased the haloperidol-induced catalepsy, whereas in low doses they enhanced the apomorphine-induced stereotypy. Peptides of low molecular weight (1,200 Da) given in various doses enhanced the catalepsy but did not affect the stereotypy. The investigated products of proteolysis were a mixture of peptides released both from triple-helical parts and telopeptide fragments of collagen molecules (Telejko et al. 1990c).

The aim of the present studies was the evaluation of pharmacological properties of peptides released from triple-helical parts of type-I collagen mole-

cules and of physicochemical characteristics of these products.

## METHODS

### Preparation of pharmacologically active peptides

Preparation and purification of type I collagen have been performed according to the method described by Chung and Miller (1974) with slight modifications. Wistar rat tail tendons were cut into small pieces and suspended in 0.5 M acetic acid in the ratio of 1:50 (w/v) and then homogenized at 0°C. The homogenate was extracted with the same solution for 24 h with continuous mixing and then centrifuged at 15,000 x g, at 4°C. The supernatant was collected and the sediment was submitted to further extraction with 0.5 M acetic acid and centrifuged in the same conditions. Both supernatants were combined. Collagen was precipitated from acidic solution by the addition of NaCl to final concentration of 0.9 M and then redissolved in 0.05 M Tris-HCl, containing 1 M NaCl. The proteins contained in this solvent were fractionated by a further increase of NaCl concentration. The protein precipitated by 2.6 M NaCl contained pure type I collagen as evaluated by the amino acid analysis and electrophoretic studies. Lyophilized type I collagen was dissolved in 0.5 M acetic acid to obtain concentration of 5 mg/ml. The collagen solution was supplemented with pepsin (Serva): 0.1 mg/ml and incubated at 4°C in order to separate the low-molecular weight products of digestion (telopeptides) from high molecular (triple helical) fragments of collagen molecules. The telopeptides-deprived type I collagen was dialysed against 0.05 M NH<sub>4</sub>HCO<sub>3</sub> for 72 h with several changes of the dialysing solution. Protein was determined by the biuret method (Light and Bailey 1980). Hydroxyproline was determined by the method of Bergman and Loxley (1963). Nitrogen was estimated according to Kjeldahl method.

The digestion of collagen with bacterial collagenase (Boehringer) was performed at 37°C, for 6 h.

The substrate : enzyme ratio was 20:1. Products of digestion were centrifuged at  $15.000 \times g$  for 45 min, at  $4^{\circ}\text{C}$ . The low molecular weight products, soluble in  $0.05 \text{ M NH}_4\text{HCO}_3$ , were lyophilized and stored at  $-20^{\circ}\text{C}$ . The molecular-sieve chromatography of collagen-degradation products was performed on a column ( $2.4 \times 60 \text{ cm}$ ) with Sephadex G-25 "coarse". A sample of the volume of 2ml was applied on the column and eluted with water at the flow-rate of 30 ml/h. 3 ml fractions were collected and absorbance at 254 nm was measured.

### Behavioural studies

The experiments were performed on male Wistar rats of the body weight 180-200 g. The animals were kept in a room lighted for 12 h per day (from 6 to 18 h). They were fed with standard granular diet and water ad libitum. All studies were performed at the same hours, between 10 and 14 h. The investigated peptides were dissolved in physiological saline and injected into the lateral cerebral ventricle (icv) in a volume of  $10 \mu\text{l}$  with the use of Hamilton microsyringe. The psychomotoric activity of rats was evaluated by the Lat's test (1965). Catalepsy was evoked with haloperidol and evaluated by the method of Simons et al. (1969). Haloperidol (HAL, Richter, Budapest) was administered intraperitoneally (ip) in a dose of 0.5 mg/kg. Stereotypy was induced by apomorphine (APO, Sandoz, 2 mg/kg) and amphetamine (AMPH, Warsaw, Pharmacentical Laboratories Polfa; 5 mg/kg) and evaluated by the method of Kennedy and Zigmond (1978). Statistical analysis was performed with the use of the following methods: psychomotoric activity was evaluated with the Student "t" test, catalepsy and stereotypy with the analysis of variance and Duncan test.

### Methods used for the physicochemical characteristic of pharmacologically active peptides

1.  $\alpha$ -amino nitrogen was determined by the ninhydrin method (Derenyi and Gergely 1974).

2. Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis of collagen has been performed according to Laemmli (1970). 7.5% acrylamide and 0.1% SDS were used.

3. Electrophoretic separation of the investigated peptides was performed by the method of Swank and Munkers (1971). 13.8% acrylamide and 0.1% SDS were used.

4. Estimation of the molecular weight of the peptides was performed by the molecular sieve chromatography on Bio-Gel P-2 column ( $1.6 \times 80 \text{ cm}$ ). The sample of 1ml was applied on the column and eluted with water at the flow rate of 18 ml/h. 2 ml fractions were collected and absorbance at 210 nm was measured. The following standards of different molecular weight were used: angiotensin (1,250 Da), bradykinin (1.060 Da) and arginine (174 Da).

5. The assay of the amino acid composition of collagen and its degradation products was performed by the use of amino acid analyser: AAA 881 - Microtechna, Praha. The proteins and peptides were hydrolysed in 6 N HCl, with the addition of phenol (1mM) under nitrogen at  $110^{\circ}\text{C}$  for 16 h.

6. Ion-exchange chromatography of peptides was performed with the use of long column of amino acid autoanalyser (AAA 881 - Microtechna, Praha) filled with a strong cation-exchange resin (Ostion K-7527). The lyophilized sample was dissolved in citrate buffer, pH 2.2 to obtain concentration of 10 mg/ml. A sample of  $200 \mu\text{l}$  was applied on the column. The peptides were eluted with the citrate buffer at the flow rate of 75 ml/h, using the gradients of pH and ionic strength. The following elution conditions were applied: 0-68 min: pH 3.25 -  $0.2 \text{ M Na}^+$ , 69-102 min: pH 4.2 -  $0.2 \text{ M Na}^+$ , 103-132 min: pH 5.28 -  $0.2 \text{ M Na}^+$ , 133-164 min: pH 7.9 -  $1.1 \text{ M Na}^+$ . 2 ml fractions were collected and -amino nitrogen was estimated.

7. Desalting of peptides was performed by the method of high performance liquid chromatography (HPLC) with the use of Du Pont chromatography : model 8500. The peptides were applied on a column ( $4 \times 250 \text{ mm}$ ) with an absorbent Zorbaks ODS and eluted with 0.2 M buffer : trimethylamine/acetic acid, pH 3.2, with a linear gradient (0-

20%) of 2-propanol. The position of peptides in the eluate was estimated by the assay of absorbance at 254.4 nm. The peptide-containing fractions were evaporated in vacuum.

8. N-terminal amino acids were identified as described by Narita (1970). The dinitrophenol (DNP) - derivatives of N-terminal amino acids were separated and identified by the method of thin-layer chromatography on the Silica-Gel plates (Merck). The chromatograms were developed in two systems as described by Walz et al (1963).

9. C-terminal amino acids were identified by the method described by Akabori. The material was submitted to hydrazinolysis at 100°C for 10 h. The DNP-derivatives of C-terminal amino acids were identified by the same method as in the case of N-terminal amino acids.

10. Analysis of amino acid sequence was performed according to the method described by Edman and modified by Tarr (1982). The 3-phenyl-2-thiohydantoin derivatives of amino acids (PTH-amino acids) were dissolved in 25% acetonitrile solution. Separation of PTH-amino acids was performed by the method described by Lottspeich (1980) with the use of isocratic chromatograph, type 310, produced by IChF PAN Warsaw. Column of the size 2 x 250 mm was filled with LiChrosorb RP-18 (5 µm) produced by Merck. Elution was performed with a mixture of 0.01 M acetate buffer pH 5.3, water, acetonitrile and 1,2-dichloroethane in the ratio 0.9:67:33:0.7 (v/v/v/v) with the flow rate 40 µl per min at 63°C. The derivatives of amino acids were identified by the assay of retention time in comparison to standards, produced by Sigma.

## RESULTS

### Preparation of collagen peptides

The purity of isolated type I collagen was evaluated by electrophoretic and chemical methods. It was found that the sample of collagen submitted to electrophoresis on SDS - polyacrylamide gel demonstrates only bands corresponding to type I collagen subunits:  $\alpha_1(I)$ ,  $\alpha_2(I)$ ,  $\beta_{11}$ ,  $\beta_{12}$  and  $\gamma$ . No

contaminating proteins were found. The hydroxyproline : nitrogen ratio was equal to 0.82, which corresponds to highly purified preparations of type I collagen. The collagen-degradation products, obtained by collagenase digestion, divided during molecular sieve chromatography into 2 fractions. The first one elutes close to void volume ( $V_0$ ), the second between void and total ( $V_t$ ) volumes of the column (not shown in the figure). Pharmacological activity of the last fraction was investigated. In this paper the investigated fraction is named "collagen peptides" (CP).

### The effect of collagen peptides on the psychomotoric activity

The investigated peptides significantly reduced the psychomotoric activity of the rats as evaluated by the Lat's test. The peptides given in 3 different doses (2.5 ; 5.0 and 15.0 µg) evoked the decrease of walking time in comparison to control. Statistically significant differences were observed in the case of doses 5.0 and 15.0 µg ( $P < 0.001$ ). Also the time of immobility was significantly reduced by the same doses of the peptides ( $P < 0.01$  and  $P < 0.05$  respectively). Only a slight effect of peptides on the washing time was observed. The number of standing-up reactions was significantly reduced after an injection of 5.0 µg ( $P < 0.05$ ) or 15.0 µg ( $P < 0.01$ ) of the peptides (Fig.1).

### The effect of collagen peptides on the haloperidol-induced catalepsy

The peptides given in all doses increased the cataleptic action of haloperidol. It was evaluated by the analysis of variance that significant differences are apparent between the mean values of control and investigated groups, at various times of observation: in the 105th min ( $P < 0.05$ ), in the 120th min ( $P < 0.01$ ), in the 150th min ( $P < 0.005$ ), in the 180th min ( $P < 0.001$ ), in the 240th min ( $P < 0.05$ ) and in the 300th min ( $P < 0.01$ ). The mathematical analysis performed with the use of Duncan test demonstrated that the peptides, in the dose of 2.5 µg, only

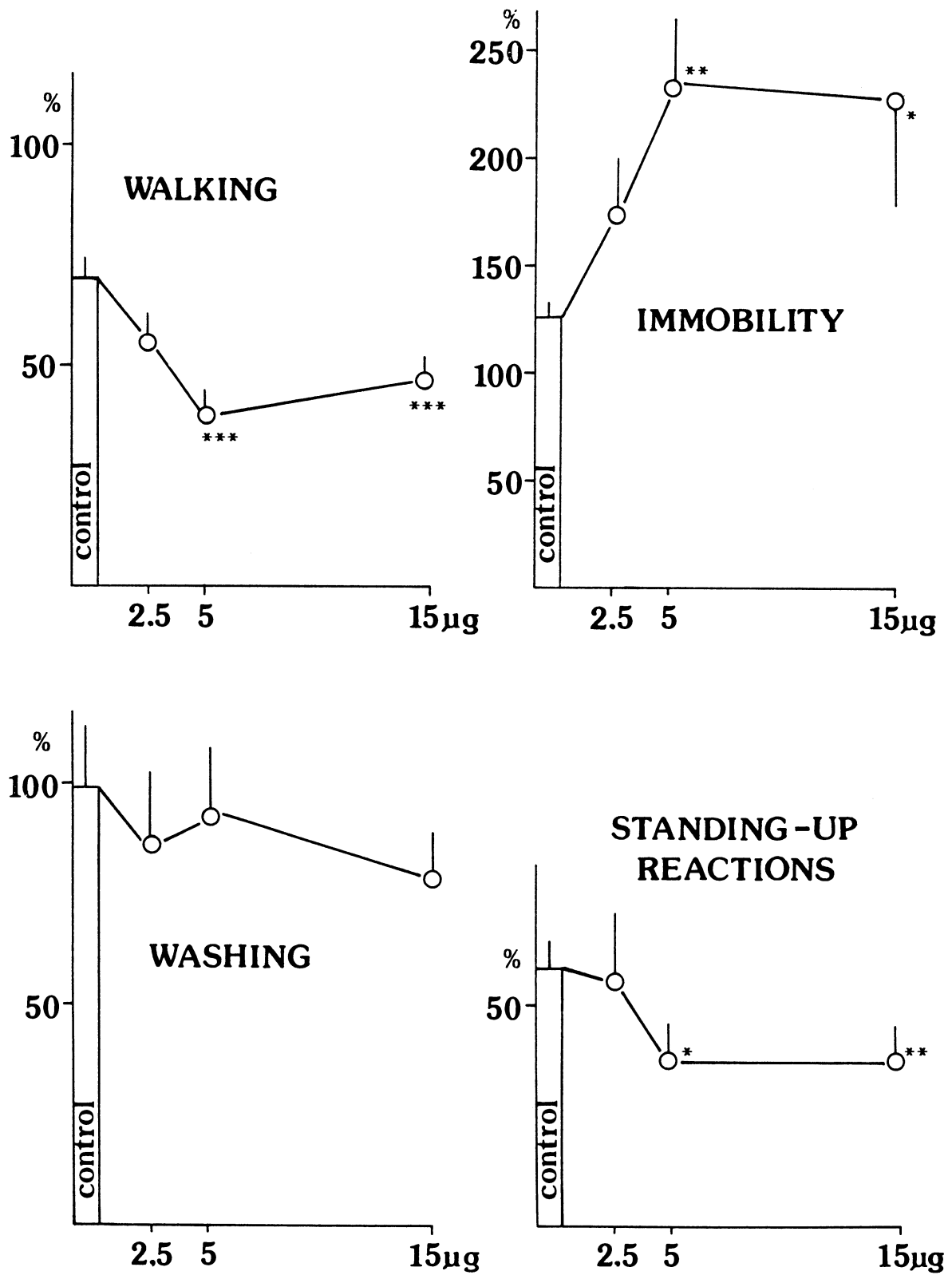


Fig. 1. The effects of various doses of CP (icv 15 min before) on the psychomotoric activity of the animals in Lat's test. Each point is the mean of 10 results + SD. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  as compared with controls.

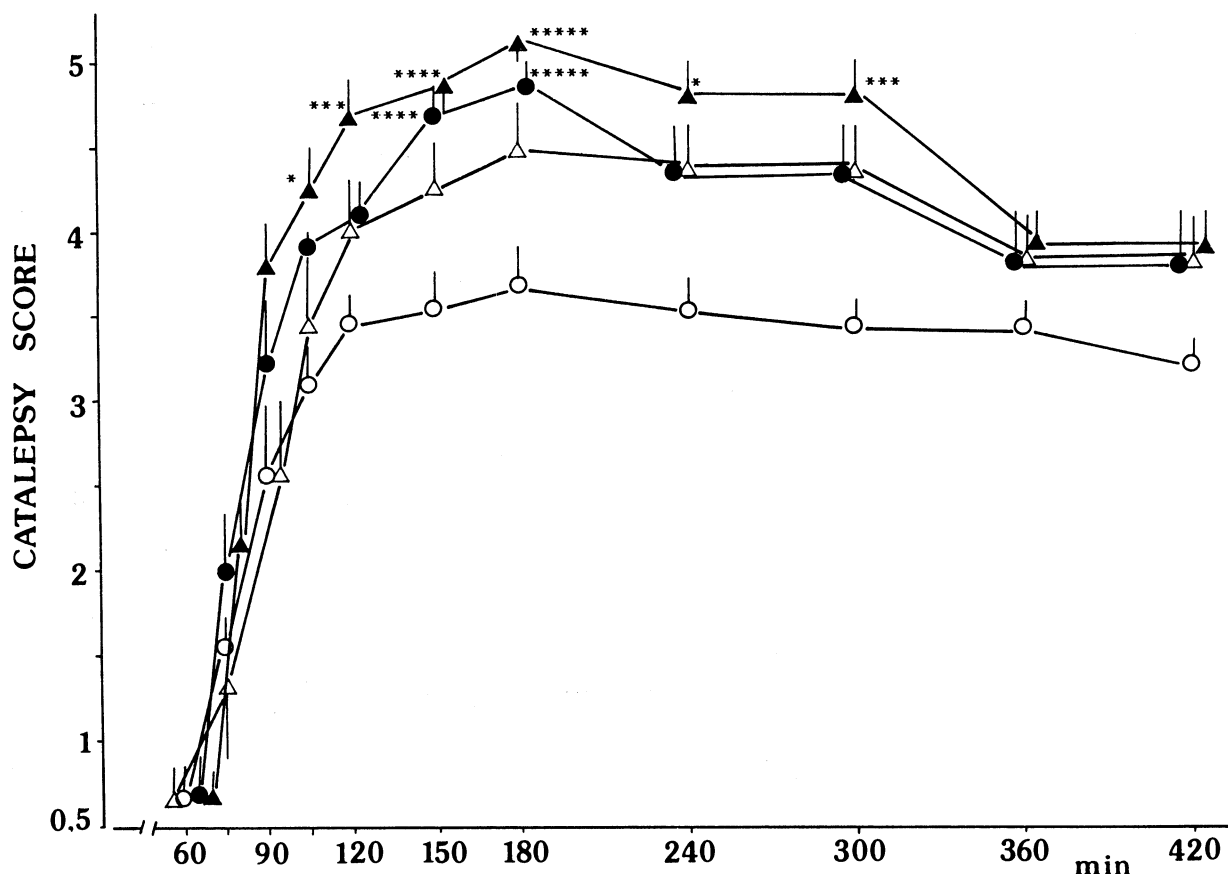


Fig. 2. The effects of various doses of CP on haloperidol - induced catalepsy in rats. Open circles - control (HAL 0.5 mg/kg, ip + saline, icv 45 min after); open triangles, CP (2.5 µg icv); dark circles, CP (5.0 µg icv); dark triangles, CP (15.0 µg icv). Each point is the mean of 10 results + SD. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.005$ ; \*\*\*\*  $P < 0.001$  as compared with controls.

insignificantly increase the cataleptic action of haloperidol. A higher dose (5.0 µg) evoked the statistically significant effects in comparison to control in the 150th min ( $P < 0.005$ ) and the 180th min ( $P < 0.001$ ) of observation. The highest dose (15.0 µg) enhanced the catalepsy beginning from the 105th min of observation ( $P < 0.005$ ). The most significant effects were apparent in the 180th min of the experiment ( $P < 0.001$ ) (Fig. 2).

#### The effect of collagen peptides on the stereotypy

##### APOMORPHINE - INDUCED STEREOTYPY

The peptides given in all the investigated doses enhanced the apomorphine-induced stereotypy, but

statistically significant effects were apparent in the 25th min of observation after the administration 5.0 µg of the peptides ( $P < 0.001$ ) (Fig. 3).

##### AMPHETAMINE - INDUCED STEREOTYPY

The investigated peptides did not evoke significant effect on amphetamine-induced stereotypy. Slight effects were observed at 1 min of observation only (not shown in the figure).

##### Amino acid composition of collagen peptides

The collagen peptides contain high amounts of proline and glycine and low amounts of aromatic amino acids. The ratio of acidic to basic amino acids equals about 1.0:0.8 (not shown in the table).

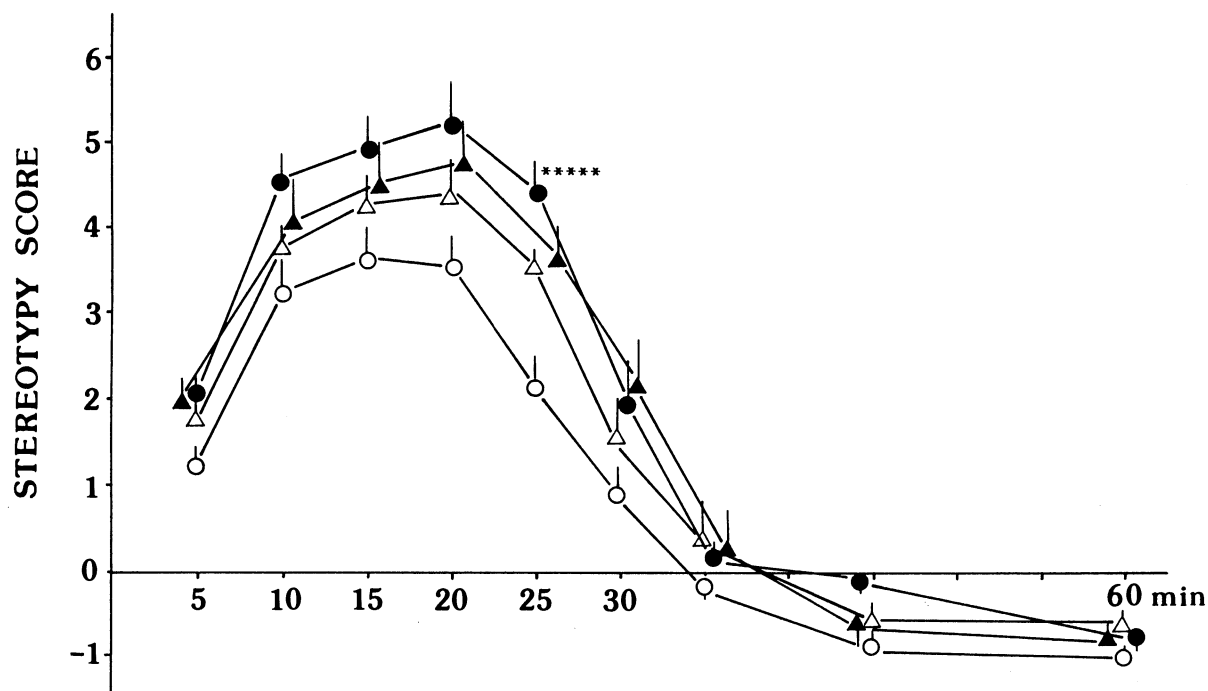


Fig. 3. The effects of various doses of CP on the apomorphine - induced stereotypy in rats. Open circles, control (APO 2.0 mg/kg, ip + saline, icv 15 min before); open triangles, CP (2.5 g icv); dark circles, CP (5.0 g icv); dark triangles, CP (15.0 g icv). Each point is the mean of 10 results + SD. \*\*\*\*  $P < 0.001$  as compared with controls.

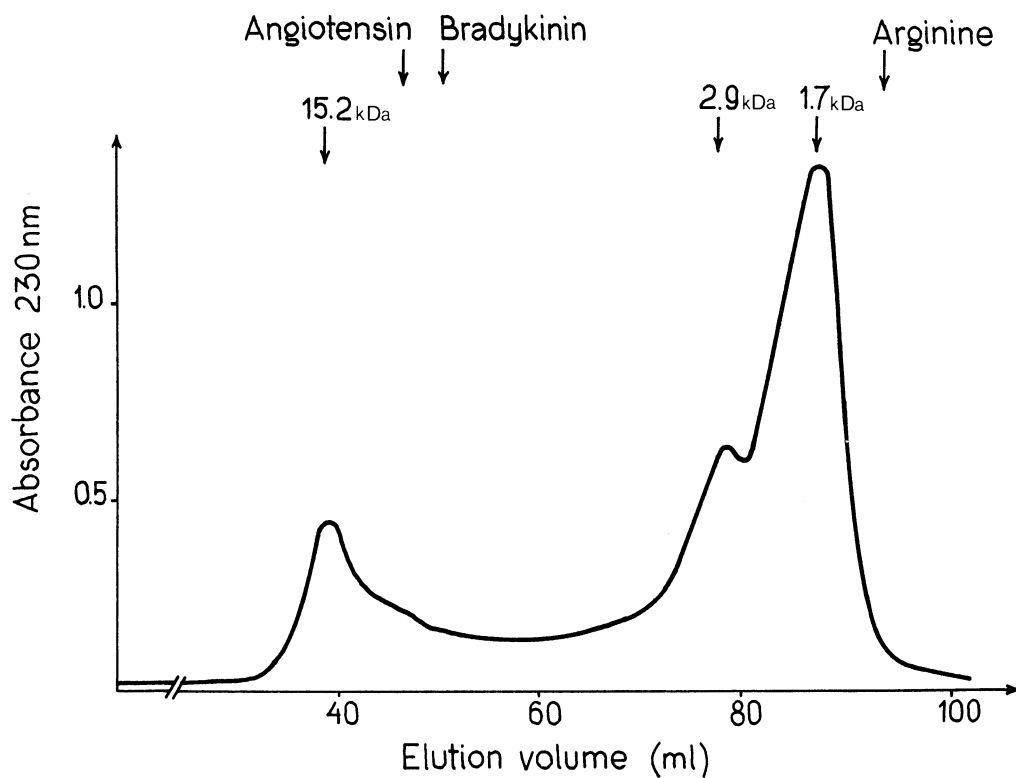


Fig. 4. Molecular sieve chromatography of CP on Bio-Gel P-2 column (1.6 x 80 cm).

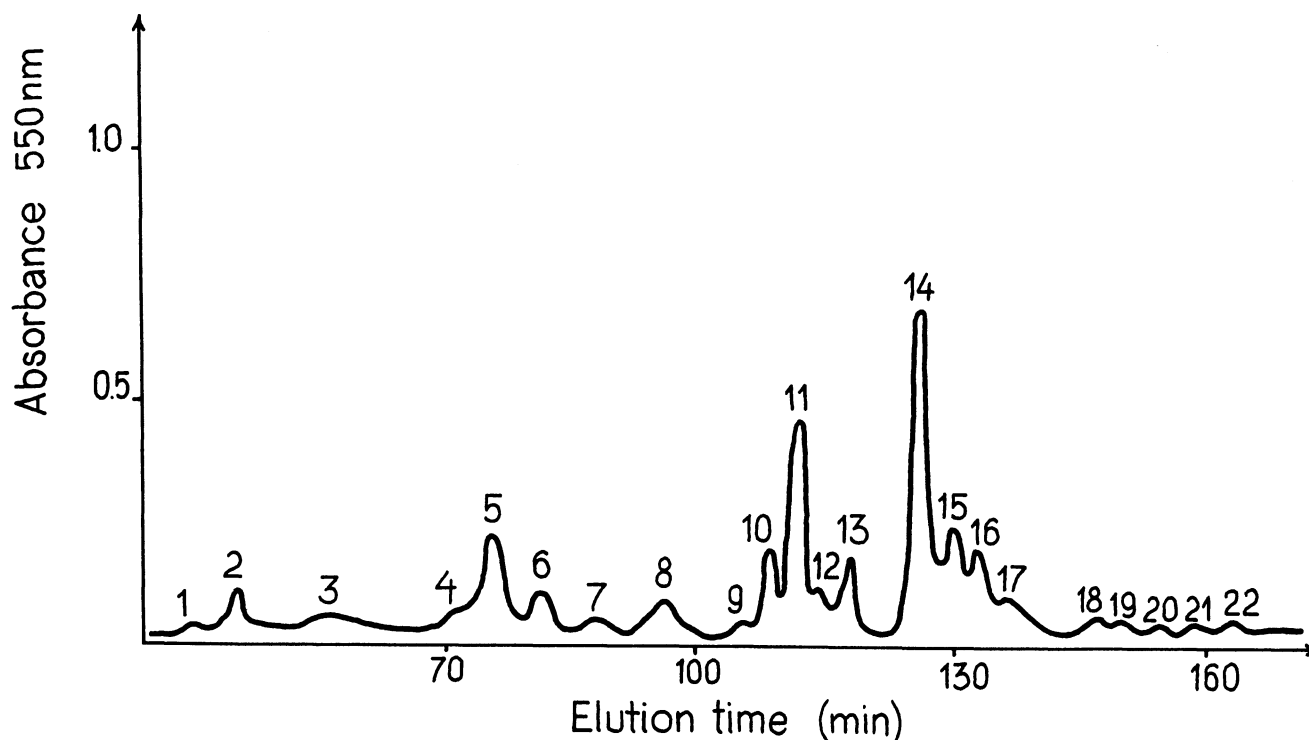


Fig. 5. Ion-exchange chromatography of CP on Ostion K-7527 column.

### Molecular weight of collagen peptides

Unfortunately, it was not possible to evaluate the molecular weight of collagen peptides by the electrophoresis on SDS - polyacrylamide gel. The collagen peptides did not bind the Coomassie Brilliant Blue. It allows us to conclude that their molecular weight is lower than 2 kDa.

TABLE I

Molar percentage of the main peptides fractions obtained by ion-exchange chromatography and their amino acids sequences, Gly- glycine, Pro- proline, Thr- threonine, Hyp- hydroxyproline, Ser- serine, Ala- alanine, Val- valine, Met- methionine

| Number of fraction | Molar percentage of peptides | Amino acids sequence |
|--------------------|------------------------------|----------------------|
| 8                  | 6.1                          | Gly-Pro-Thr          |
| 11                 | 36.2                         | Gly-Pro-Hyp          |
| 13                 | 8.9                          | Gly-Pro-Ser          |
| 14                 | 21.9                         | Gly-Pro-Ala          |
| 15                 | 4.1                          | Gly-Pro-Val          |
| 16                 | 3.6                          | Gly-Pro-Met          |

The approximate molecular weight of these products was measured with the use of molecular sieve chromatography on Bio-Gel P-2 and estimation of  $\alpha$ -amino nitrogen before and after the acidic hydrolysis. As can be seen from Fig.4, three main peaks of peptides were eluted from the column. The ratio of  $\alpha$ -amino nitrogen before and after total acidic hydrolysis varied from 1.0:15.2 for the first peak to 1.0:1.7 in the last one. It allows us to conclude that the investigated peptides contain from 2 to 16 amino acid residues and their molecular weight varies from 0.15 to 1.5 kDa.

### Heterogeneity of collagen peptides and their amino acid sequence

During the ion-exchange chromatography the investigated peptides were separated into 21 fractions (Fig.5). In each case the glycine was N-terminal amino acid. The amino acid sequence of the most abundant 6 peptides was estimated. The results are presented in the Table 2. All of them are tripeptides of the sequence Gly-Pro-X. The follow-



ing amino acids: Thr, Hyp, Ser, Ala, Val, Met were found at the C-terminal (X) position.

## DISCUSSION

We have found that the peptides released from type I collagen are the final products of collagen digestion. They are resistant to further action of bacterial collagenase. About 10.7% of total peptide bonds number are cleaved under conditions described in this paper.

The digestion of collagen was performed in 0.05 M  $\text{NH}_4\text{HCO}_3$ , which is not a good solvent for collagen but it creates proper pH for the action of bacterial collagenase. On the other hand, it is easily destroyed in vacuum to gas products ( $\text{CO}_2$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{O}$ ) during the lyophilization procedure. It makes possible to obtain collagen degradation products, free of any salts which may disturb the pharmacological studies. Some high molecular weight peptides released from collagen are not soluble in these conditions and they are removed from the incubation medium by centrifugation. The peptides soluble in  $\text{NH}_4\text{HCO}_3$  were divided during the molecular sieve chromatography into 2 fractions. Only one of them exerted pharmacological effects.

It has been found that the investigated peptides decreased the psychomotoric activity in rats. The same effects were found if the peptides were given 15 or 30 min before observation (Telejko et al. 1990c). Significant shortening of walking time and prolongation of immobility time were observed. Significant decrease of standing up reactions was apparent. According to some authors (Telejko et al. 1990c), this phenomenon is an index of "explorative" activity of the animals.

The action of peptides on the central nervous system at least partially depends on dopaminergic mechanisms. They increased the cataleptic action of haloperidol - an antagonist of dopaminergic receptor. On the other hand, small doses of these peptides increased the stereotypy induced with apomorphine-agonist of dopaminergic receptors. The peptides did not affect the amphetamine - induced stereotypy.

It was found that the investigated fraction is heterogeneous. During the molecular sieve chromatography on Bio-Gel P-2 this material divided into 3 peaks which correspond to substances of the molecular weight from 200 to 1,500 Da. As can be concluded from the assay of  $\alpha$ -amino nitrogen before and after acidic hydrolysis, these peptides were composed from 3 to at least 15 amino acid residues. Chromatographic studies have shown that 21 peptides are present in the investigated material. About 80% of them are tripeptides of the sequence Gly-Pro-X. The most abundant are Gly-Pro-Hyp (36.2%) and Gly-Pro-Ala (21.9%).

The variety of effects exerted by collagen peptides seems to be connected with the heterogeneity of the investigated material. Different peptides may evoke different biological responses. Since the low molecular collagen breakdown products obtained by the action of bacterial collagenase are a mixture of many peptides, we are not able to say which of them are responsible for their pharmacological activity. The described effects may be result of combined action of various peptides or only some of them. It was reported by other authors (Wiśniewski 1979) that various protein degradation products exert significant biological effects. Barczak-Osińska et al. (1983) found that fibrinogen degradation products enhance the action of drugs stimulating the CNS. Sobaniec et al. (1975) found that albumin degradation products enhance the effects of drugs acting depressively on CNS. It may be concluded that the products of proteolysis significantly modify the effects of various drugs acting on CNS.

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