# The vasoactive proteins in human urine

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Received 9 November 1984

We have purified vasoactive polypetides from the urine of normotensive humans by gel filtration on Sephadex G150 superfine ionic exchange chromatography on DEAE-cellulose and isoelectric focusing in polyacrylamide gels using a pharmalyte pH range of 2.5–5.0 The purified polypetide fraction yielded four bands by isoelectric focusing with isoelectric points at pH 4 15, 4 05, 3 9 and 3 8 On dodecyl sulphate/polyacrylamide gel electrophoresis (page) two bands appeared, one small band with a $M_r$ of 29 000 and one broad band ranging from $M_r$ 49 000 to $M_r$ 42 500 The polypetides lower the blood pressure of rabbits in a bioassay and cleave D-valyl-L-leucyl-L-arginine-4-nitroanilide with a specific activity comparable to that of kallikrein

*Human urine* *Vasoactive polypeptide* *Kallikrein*

## 1. INTRODUCTION

The urine of normotensive human beings contains a polypeptide fraction with blood pressure lowering activity [1–4]. It is of particular interest that the vasoactivity of this polypeptide fraction was shown to be impaired in patients with primary hypertension [5,6]. Furthermore, it was found that the urine fraction from patients with primary hypertension and from normotensive individuals with a family history of hypertension failed to lower blood pressure in a rabbit bioassay system [6]. In the previous study from this laboratory [6] it was concluded that the vasoactive principle was distinct from kallikrein based on enzymatic measurements of kallikrein-like activity. As deficient excretion of kallikrein also has been implemented in the pathogenesis of primary hypertension [7] it was felt of particular importance to isolate and characterize further the polypeptide fraction from the urine of normotensive individuals and to see whether, indeed, it can be distinguished from urinary kallikrein.

## 2. MATERIALS AND METHODS

### 2.1. *Isolation of urinary vasoactive polypeptides*

Purification of the urinary vasoactive polypeptides was as follows: the protein of 30 l of the 24-h urine from normotensive human subjects was precipitated with ammonium sulphate (650 g/l urine). The precipitate was dissolved in 600 ml of doubly distilled water, containing 7 mM 2-mercaptoethanol. Portions of 60 ml were desalted by passing the samples over a Sephadex G25 medium (Pharmacia) (5 × 60 cm) column. After testing the vasodilating activity of the salt-free fraction in a rabbit bioassay (see below) it was passed through a DEAE-cellulose (Whatman DE 52) (1.5 × 20 cm) column equilibrated with 10 mM $NH_4$-acetate, 7 mM 2-mercaptoethanol, pH 5.5. The retained proteins were eluted with 1 M $NH_4Cl$, 7 mM 2-mercaptoethanol, pH 5.5. Both fractions, the run-off fraction and the eluate, were tested in the rabbit bioassay. Portions (20 ml) of the eluted vasoactive fraction containing acidic proteins were chromatographed on a Sephadex

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00145793/85/$3 30 

G150 superfine (Pharmacia) (5 × 100 cm) column in 10 mM $NH_4$-acetate, 7 mM 2-mercaptoethanol, pH 5.5. Fractions (5 ml) were tested in the rabbit bioassay. The vasoactive fractions were pooled and the material was passed through a DEAE-cellulose (Whatman DE 52) (1.5 × 20 cm) column equilibrated with 10 mM $NH_4$-acetate, 7 mM 2-mercaptoethanol, pH 5.5. The retained polypeptides were eluted with a linear 5–500 mM $NH_4Cl$ equilibration buffer gradient. Fractions (4 ml) were tested in the rabbit bioassay. The vasoactive fractions were pooled and the material was exhaustively dialysed against doubly distilled $H_2O$ containing 7 mM 2-mercaptoethanol. Dialysed material was lyophilised and the powder was dissolved in 1 ml of a 1 in 15 dilution of pharmalyte covering a pH range from 2.5 to 5 and 15% sucrose. Portions of 0.5 ml were applied to a polyacrylamide gel column (1.6 × 10 cm) and further fractionated by isoelectric focusing in a Pharmacia Gel Electrophoresis Apparatus GE-2/4LS (see [9]). The gel was cut in pieces according to pH and the proteins were eluted from the gel by electrophoresis at 250 V for 12 h in a Pharmacia chamber GE-2/4LS using 50 mM Tris, 350 mM glycine. The eluates were dialysed against doubly distilled water, containing 7 mM 2-mercaptoethanol. The samples were then assayed for activity (see below) and analysed for protein by electrophoresis according to [9] and [12]. Protein concentrations were determined using bovine serum albumin as standard [10].

### 2.2. *Biological assay*

The fractions were tested for activity in a rabbit bioassay as described [6].

### 2.3. *Cleavage of D-valyl-L-leucyl-L-arginine-4-nitroanilide*

Cleavage of the synthetic substrate D-Val-Leu-Arg-PNA was assayed according to [11].

## 3. RESULTS

Fig.1 summarizes the elution profiles of the various chromatographic steps. The hatched

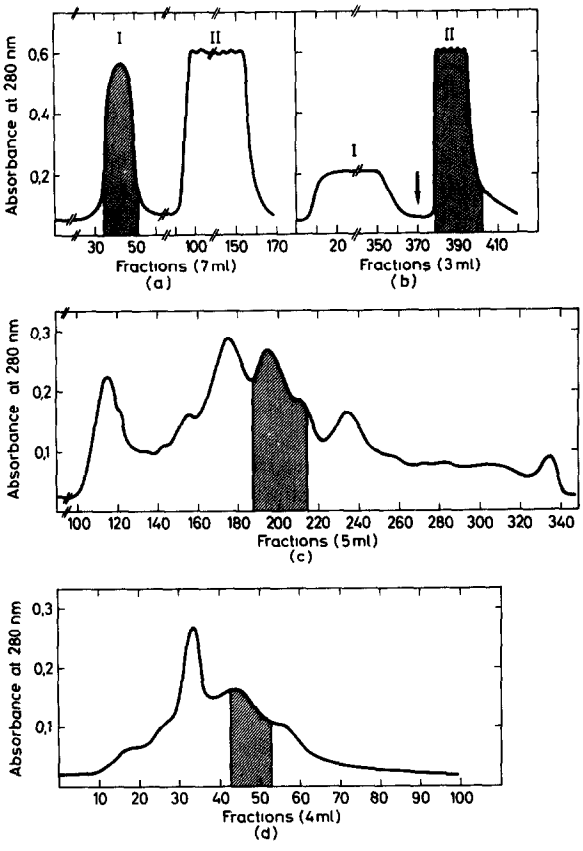


Fig.1. The chromatographic purification of human urinary vasoactive polypeptides (a) Desalting. The protein in 30 l urine was pelleted with ammonium sulphate and redissolved in $H_2O$, 7 mM 2-mercaptoethanol as described in section 2. Portions (60 ml) were applied to a Sephadex G25 medium (5 × 60 cm) column and eluted with the same solution. Hatched peak I (protein peak) contains the activity. (b) Separation of neutral-basic from acidic urinary proteins. The desalted protein in 1050 ml of 10 mM $NH_4$-acetate, 7 mM 2-mercaptoethanol, pH 5.5, was passed through a DEAE-cellulose (1.5 × 20 cm) column and eluted with 1 M $NH_4Cl$, 7 mM 2-mercaptoethanol, pH 5.5 Start of elution is indicated by the arrow. Hatched peak II (acidic proteins) contains the activity. (c) Fractionation of acidic urinary proteins. The proteins eluted in (b), (20 ml) in 1 M $NH_4Cl$, 7 mM 2-mercaptoethanol, pH 5.5, were applied to a Sephadex G150 superfine (5 × 100 cm) column and eluted with 10 mM $NH_4$-acetate, 7 mM 2-mercaptoethanol, pH 5.5. The hatched region of the elution profile contains the activity. (d) Fractionation of active acidic urinary proteins. Proteins from hatched region of the elution profile (c) in 450 ml of 10 mM $NH_4$-acetate, 7 mM 2-mercaptoethanol, pH 5.5, were passed through a DEAE-cellulose (1.5 × 20 cm) column and then eluted with a linear 5–500 mM $NH_4Cl$ equilibration buffer gradient. The activity was found in the hatched region of the elution profile.

regions represent the vasoactive fractions.

1 ml of the peak I-fraction in (a) lowered the blood pressure in the rabbit bioassay by 35%. 50 $\mu$l of the acidic protein fraction in (b) was equally effective, while 1 ml of the neutral-basic proteins in the run off (peak I) was inactive. The acidic proteins were further fractionated on Sephadex G150 superfine (c). 400 $\mu$l of the pooled vasoactive material lowered the blood pressure of rabbits by 36%. The vasoactive material from the Sephadex G150 column was fractionated on DEAE-cellulose (d). 40 $\mu$l of the vasoactive material lowered the blood pressure by 30% in the rabbit bioassay.

The vasoactive urinary polypeptides (from (d)) were fractionated in polyacrylamide gel by isoelectric focusing using a pharmalyte pH gradient. The vasoactive material was found in the pH range of 3.5–4.5. As seen in fig.2(a) it gives four diffuse bands with isoelectric points 4.15, 4.05, 3.9 and 3.8. Fig.2(b) shows a dodecyl sulphate–PAGE separation of the purified vasoactive polypeptides (from the pH range of 3.5–4.5) on a 15% polyacrylamide slab gel. The broad band A covers the $M_r$ range from 49000 to 42500. A smaller band B has an apparent molecular mass of 29 kDa.

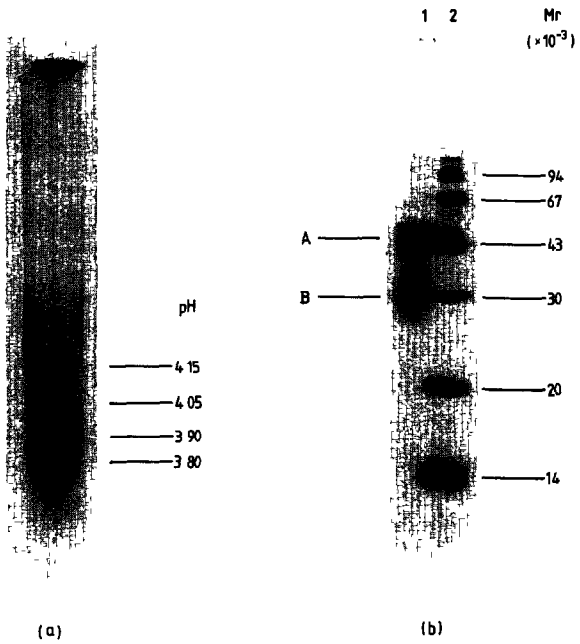


Fig.2. (a) Isoelectric focusing of the isolated vasoactive human urinary proteins (from the pH range 3.5–4.5) in polyacrylamide gel using a pharmalyte pH range from 2.5 to 5 according to [9]. (b) SDS–PAGE of the purified vasoactive human urinary proteins (found in the pH range 3.5–4.5) by the procedure in [12] Slot 1, vasoactive proteins. Slot 2, standard proteins, phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and $\alpha$-lactalbumin.

0.6 $\mu$g of the vasoactive material (from the pH range of 3.5–4.5) lowered the blood pressure in the rabbit bioassay by 32%. This polypeptide preparation had a specific activity of 1.6 $\mu$mol·min$^{-1}$·mg$^{-1}$ protein measured with the model substrate D-Val-Leu-Arg-PNA.

## 4. DISCUSSION

As seen in fig.2(a) the vasoactive polypeptides from the urine of normotensive humans of both sexes (described [1–6]) are found in 4 major bands with isoelectric points 4.15, 4.05, 3.9 and 3.8. Human urinary kallikrein, on isoelectric focusing in an ampholine pH gradient from 3 to 5, yields 6 bands with isoelectric points 4.25, 4.05, 4.00, 3.9, 3.8 and 3.75 [8]. Thus, the vasoactive proteins purified and characterised in this work and kallikrein are similar with respect to their behavior on focusing. The vasoactive proteins studied by us give, on dodecyl sulphate–PAGE [12], a very broad badly resolved band A spanning the molecular mass region from 49 to 42.5 kDa. A smaller diffuse band B had an apparent $M_r$ of 29000 (fig.2(b)). Pure human urinary kallikrein, on dodecyl sulphate–PAGE [13], gives two bands with $M_r$ values of 41000 and 34000 [8]. The differences in $M_r$ of the vasoactive proteins studied by us and kallikrein might be due however to the fact that analyses were carried out in different electrophoretic systems. Such differences were noted before [14].

0.6 $\mu$g of the vasoactive polypeptide preparation studied lowered the blood pressure in the rabbit bioassay by 32%. This preparation cleaved 1.6 $\mu$mol D-Val-Leu-Arg-PNA·min$^{-1}$·mg$^{-1}$ protein. When human urinary kallikrein was tested in the rabbit bioassay and in cleavage of D-Val-Leu-Arg-PNA the same amount, 0.6 $\mu$g, lowered the blood pressure by 35%. Kallikrein cleaved 1.8 $\mu$mol substrate·min$^{-1}$·mg$^{-1}$ protein. Accordingly, the vasoactive proteins isolated in this work and kallikrein have comparable biological and enzymatic activities.

The vasoactive proteins from the urine of normotensive humans were isolated and characterised in this work as a group (fig.2(a)). It remains to characterise the components of the group separated from each other.

## ACKNOWLEDGEMENTS

This work was supported by the Ministry of Research and Technology, FRG. We thank Professor H. Fritz, University of Munchen, for providing us with a sample of human urinary kallikrein.

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