Volume 265, number 1,2, 137–140 FEBS 08488 June 1990

Substrate specificity of two kallikrein family gene products isolated from the rat submaxillary gland

A. Elmoujahed, N. Gutman, M. Brillard and F. Gauthier

University François Rabelais, Faculty of Medicine, CNRS URA1334, 2bis Bd Tonnellé, 37032 Tours, France

Received 9 April 1990

Two proteinases which belong to the tissue kallikrein family were purified from rat submaxillary glands. These proteinases correspond to the products of the RSKG-7 and the rGK8 genes, as shown by the comparison of their partial amino-acid sequence with that deduced from nucleotide sequences. These two proteinases, kallikrein k7 and kallikrein k8, exhibit a marked preference for cleavage after arginyl residues. However, their overall specificities towards synthetic fluorogenic substrates differ significantly from each other and from that of true tissue kallikrein. Kallikrein k7 is strongly inhibited by soybean trypsin inhibitor, whereas kallikrein k8 is not. These data, demonstrating the individual specificity of these kallikrein-like proteinases, suggest that they could be involved in the processing of peptides other than kinins.

Kallikrein; Submaxillary gland; Kinetics

1. INTRODUCTION

The kallikrein gene family comprises a series of closely related genes, the number of which varies greatly from one species to another (see [1] for a review). The close relationship between the genes in this family may explain why only a few of the proteins they encode have been clearly identified. For example, only two protein products, tissue kallikrein and tonin, have been unambiguously assigned to members of the gene family in the rat [2,3]. Several other kallikrein-like proteinases have been reported, but none has been correlated with a cloned gene or mRNA [4–10]. The fact that kallikrein-like proteinases could be involved in the processing of peptides other than kinin-derived peptides prompted us to purify and analyse these proteinases. We will focus here on the purification and characterization of two of these proteinases from rat submaxillary glands. These enzymes appear to be the translation products of the RSKG-7 and rGK8 genes [11,12]; they have been provisionally called kallikrein k7 and kallikrein k8, but their biological function is, as yet, unknown. As a first step in this direction, we have investigated the enzymatic properties, particularly the cleavage specificity, of these proteinases and compared them to those of true tissue kallikrein, reported here as kallikrein k1.

Correspondence address: F. Gauthier, University François Rabelais, Faculty of Medicine, CNRS URA1334, 2bis Bd Tonnellé, 37032 Tours, France

Abbreviations: Boc, t-butyloxycarbonyl; Bz, benzoyl; NHMec, 7-amido-4-methylcoumarin; Z, benzyloxycarbonyl; SBTI, soybean trypsin inhibitor; pNPGB, *p*-nitrophenyl-*p*'-guanidinobenzoate

2. MATERIALS AND METHODS

Kallikreins k7 and k8 were purified from a homogenate of about 20 g of submaxillary glands excised from male Wistar rats after complete exsanguination. This homogenate was treated with 0.5% (w/v) sodium deoxycholate, equilibrated with 0.01 M phosphate buffer, pH 6.0, 0.1 M sodium chloride and applied to a DEAE-A50 column essentially as described by Brandtzaeg et al. [4]. Unbound fractions were divided into 5 pools, the third and the fifth were used as sources of kallikrein k7 and kallikrein k8, respectively. These two pools, denoted A and B in Fig. 1a, were concentrated and passed through an aprotinin-Sepharose column equilibrated in 0.05 M Tris/HCl buffer, pH 8.3, 0.025 M calcium chloride, 0.15 M sodium chloride. The material retained on the column was eluted with 0.05 M formic acid pH 2.4, dialysed against the equilibration buffer, then fractionated by reverse phase chromatography on a C4 cartridge using a 0–60% acetonitrile gradient in 0.075% TFA (Fig. 1b). Eluted fractions were immediately dialysed against 0.05 M Tris/HCl buffer pH 8.3, 0.15 M NaCl on a PD 10 column, concentrated, analysed by isoelectric focusing [13] or SDS polyacrylamide gel electrophoresis [14], and stored at −70°C.

N-terminal sequence analyses were performed on an Applied Biosystems 477A protein sequencer and the PTH derivatives were identified using an on-line associated model 120A analyser. Samples were reduced, pyridylethylated and fractionated by reverse phase chromatography on a C8 cartridge using a 0–60% acetonitrile gradient in 0.075% TFA, to separate the light and heavy chains of both proteinases. About 100 pmol of product was used for N-terminal analysis.

Active site titrations of kallikreins k7 and k8 were performed as described previously [14] using the bovine lung inhibitor aprotinin, first titrated by bovine trypsin.

The active site concentration of trypsin was determined as described by Chase and Shaw [15] using pNPGB. Briefly, proteinases (final concentrations in the 0.1 μM range) were incubated for 20 min with increasing amounts of aprotinin before starting the reaction with 10 μl 0.2 mM Z-Phe-Arg-NHMec and recording the fluorescence at 460 nm.

The efficiency of kallikreins k1, k7 and k8 towards synthetic fluorogenic substrates was determined by calculating the values of the second order rate constant k_{cat}/K_m. These values were determined

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

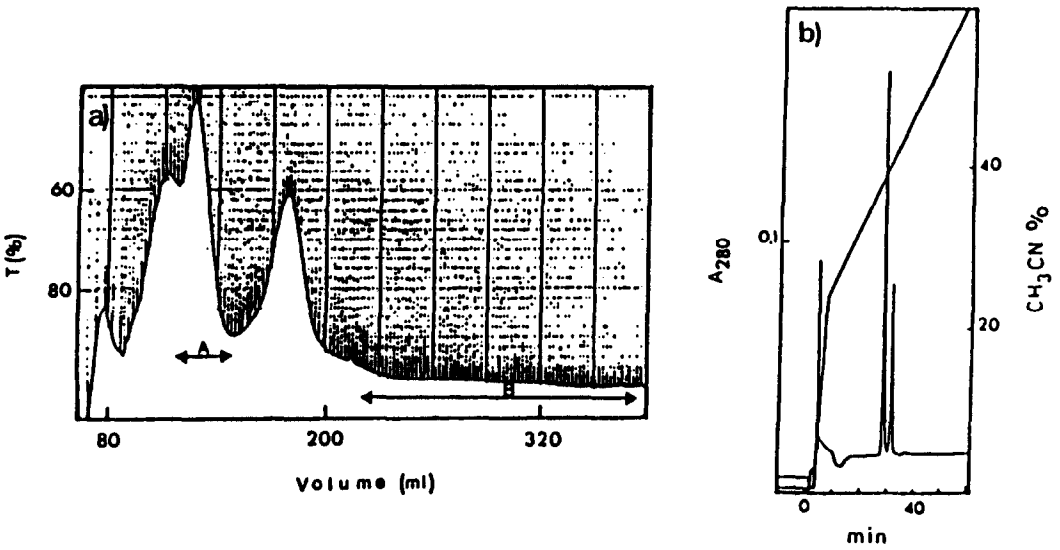

Fig. 1. Purification of kallikreins k7 and k8 from rat submaxillary glands: (a) Unbound material eluted from DEAE-A50 chromatography. Pools A and B were used to further purify kallikrein k7 and kallikrein k8 on aprotinin-Sepharose. (b) Reverse phase chromatography on a BU300 cartridge of the aprotinin-bound material corresponding to DEAE-A50 fraction B. Kallikrein k8 was eluted first; the second peak corresponds to kallikrein k7. The relative sizes of the peaks were reversed when fraction A was used as starting material. Other experimental details are given under section 2.

under first order conditions, i.e. with initial concentrations of substrates low enough in front of K_m to be neglected in the lower part of the Michaelis equation, which therefore reduces to: $-dS/dt = (k_{cat}/K_m)\, E_t\, S$ [16]. The reaction was monitored up to completion and the above equation was integrated to determine the half-time of the reaction, which is related to k_{cat}/K_m as follows: $k_{cat}/K_m = 0.693/E_t\, t_{1/2}$. About four experimental $t_{1/2}$ values could be measured from a single recording of the rate of hydrolysis of a given substrate. Their mean value was used to calculate k_{cat}/K_m. E_t values were calculated from the titration studies. Preliminary studies on the inhibition of kallikreins k7 and k8 by the soybean trypsin inhibitor were done, working with final enzyme concentrations in the 0.1 micromolar range and a 10-fold molar excess of inhibitor. Mixtures were incubated for 10 min before starting the reaction by adding fluorescent substrate (1 μM final).

3. RESULTS AND DISCUSSION

The great similarity among proteinases of the kallikrein family seriously hampers their identification and characterization. They often share common epitopes, which makes them difficult to differentiate with polyclonal antisera [17]. Their molecular weight is also very similar, but the net electric charge of some of them is rather different so that whole submaxillary gland extract can be fractionated by anion exchange chromatography. This method, originally reported by Brandtzaeg et al. [4], was used as the initial step from which at least five different kallikrein-like proteinases may be purified [unpublished]. Kallikreins k7 and k8 were purified as described above; the main steps are shown in Fig. 1. Both appear as two bands upon SDS gel electrophoresis. The bands from k7 had apparent M_r of 19,000 and 9,000 for the former, while those for k8 were 18,000 and 10,000 (Fig. 2). Kallikrein k1 appeared as a single band of 38,000 under the same conditions (not shown). The separation of kallikreins k7 and k8 into two chains probably results from an internal, possibly autolytic, cleavage in the region of the so called "kallikrein loop" [18]. However, such a cleavage in tissue kallikrein would not significantly modify the kininogenase activity [19]. The net electric charges of kallikreins k7 and k8 also clearly differ from that of tissue kallikrein. Isoelectric focusing gave a pH_i of 4.0 for kallikrein k1, whereas values of 5.2 and 5.1 were found for kallikreins k7 and k8 respectively.

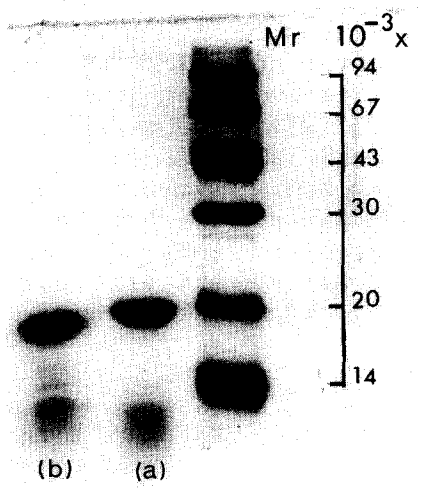

Fig. 2. SDS gel electrophoresis (15%) of kallikrein k7 (a), and k8 (b) after reduction. Each well contained about 3 μg protein. Numbers indicate $Mr/10^3$ of markers.

Table I

Amino acid sequences of the light and heavy chains of kallikrein k7 and kallikrein k8

```
                         KALLIKREIN K7:
               1           10                   20
light chain:  [V]-I-G-G-Y-K-C-E-K-N-S-Q---------
               88        93
heavy chain:  K-P-G-[D]-D-[H]-S-N-D-L-M-L-L-H-L-[S]-[Q]-P-A-D-I-T-D-G-V-K-V-I-------

                         KALLIKREIN K8:
               1           10
light chain:  [I]-I-G-G-F-N-C-E-K-N-S-Q-P-W-Q-V-A-V-Y--------
               88        93
heavy chain:  K-P-G-[N]-D-[Y]-S-N-D-L-M-L-L-K/H-L-[K]-[T]-P-A-D-I-T-D-G-V-K-V-------
```

Residues which differ from one proteinase to the other are boxed

The material eluted from C4 reverse phase chromatography was used for sequence analysis after reduction/pyridylethylation and reverse phase chromatography on a C8 cartridge. About 100 pmol of light and heavy chains were analysed and the sequences are given in Table I. Clearly, these sequences represent the products of the RSGK-7 gene described by Chen et al. [11], and of the rGK8 gene described by Brady et al. [12], since there was complete sequence homology between the partial amino acid sequences reported here and those deduced from the nucleotide sequence [11,12]. Kallikrein k7 could be also similar to the Proteinase A described by Kato et al. [8], the partial sequences of which differ by only two residues from that deduced from the RSKG-7 nucleotide sequence. The amino acid sequences of kallikreins k7 and k8 are very similar to that of true kallikrein. All three possess the main determinants which define serine proteinases and the specificity for a preferential cleavage after arginyl residues. Whether kallikreins k7 and k8 also exhibit the secondary specificity which characterizes true kallikrein [20] is of major importance for understanding their biological function. This restricted specificity, which explains why tissue kallikrein specifically excises the vasoactive peptide kallidin from kininogen without further proteolysis, is mainly due to the residue in P2 position (according to the nomenclature of Schachter and Berger [21]) adjacent to the arginyl residue [22]. The presence of a bulky hydrophobic residue such as phenylalanyl or leucyl at this position in the substrate is most favourable for interaction with tissue kallikrein [22]. We therefore investigated the kinetic properties of kallikreins k7 and k8, as well as those of rat tissue kallikreins k1, towards synthetic fluorogenic substrates in which the nature of the residue in P2 position varied while the P1 Arg residue was conserved. Titrated enzymes were used to provide a direct comparison of proteinase reactivity towards a given substrate. The results are shown in Table II. As previously reported for porcine tissue kallikrein, rat submaxillary kallikrein k1 preferentially accommodates those substrates with a Phe in position P2 and reacts poorly with substrates which have polar residues in this position. A similar restricted specificity was found for kallikrein k8 which cleaved substrates with Ser or Gly in position P2 poorly.

Table II

k_{cat}/K_m values for the hydrolysis of fluorogenic substrates by kallikreins k1, k7 and k8 (1/(mM · s))

	Kallikrein k1	Kallikrein k7	Kallikrein k8
Pro-Phe-Arg-NHMec	171	474	34
Z-Phe-Arg-NHMec	57	424	56
Boc-Phe-Ser-Arg-NHMec	<5	223	<5
Z-Arg-Arg-NHMec	<5	<5	<5
Boc-Leu-Gly-Arg-NHMec	<5	129	6
Bz-Phe-Val-Arg-NHMec	17	709	33
Boc-Val-Pro-Arg-NHMec	<5	345	20

However, this enzyme differs from true kallikrein in that it preferentially cleaves Z-Phe-Arg-NHMec rather than the kininogen-like kallikrein substrate Pro-Phe-Arg-NHMec. Its specificity as a kininogenase may thus be questionable. At variance with these results, the specificity of kallikrein k7 is not as limited as that of kallikrein k1 or kallikrein k8, since it can accommodate substrates with Ser, Gly or Pro in position P2 (Table II). Pro-Phe-Arg-NHMec, though quickly hydrolysed by kallikrein k7, is not the best substrate of this proteinase which preferentially accommodates a valyl residue in P2 (Table II). These results agree with those from structural analyses of porcine tissue kallikrein defining the critical residues involved in substrate binding [23]. Tyr-93 and Tryp-205 are key residues which give kallikrein its P2 specificity by forming a hydrophobic sandwich that traps nonpolar aminoacids in the P2 position [18]. Both are found in true kallikrein and in kallikrein k8 but not in kallikrein k7 [11]. The presence of a histidyl residue in postion 93 of kallikrein k7 could explain its broader specificity. This structural change in kallikrein k7 could also be related to the fact that this enzyme, but not kallikreins k1 and k8, is inhibited by SBTI. It may be therefore concluded that, in spite of their obvious structural and functional similar-

ities, these enzymes have sufficiently different catalytic properties for each to have unique substrate specificities. The tissue localization of these proteinases together with their different levels of expression probably help define the specificity of each enzyme. P1 mRNA, coding for kallikrein k8, has been found only in the rat prostate and submaxillary glands [12], whereas the RSKG7 gene encoding kallikrein k7, is expressed in the kidney and submaxillary glands [11]. Definition of the precise substrate specificities of these proteinases using protein substrates, together with a knowledge of their tissue and cell distributions, will provide a basis for identifying their physiological substrate.

Acknowledgements: The authors thank Dr J. Hoebeke for his critical reading of the manuscript and helpful suggestion and C. Bourdin for skillful technical assistance. This work was supported in part by Grant CRE 895006 from INSERM.

REFERENCES

[1] MacDonald, R.J., Margolius, H.S. and Erdös, E. (1988) Biochem. J. 253, 313-321.
[2] Swift, G.H., Dagorn, J.C., Ashley, P.L. Cummings, S.W. and MacDonald, R.J. (1982) Proc. Natl. Acad. Sci. USA 79, 7263-7267.
[3] Lazure, C., Seidha, N.G., Thibault, G., Genest, J. and Chrétien, M. (1981) in: Peptides Synthesis, Structure and Function. Proceedings of the VIIth American peptide Symposium (Rich, D.H. and Gross. M.R. eds) Pierce chemical, Rockford, IL, pp. 517-520.
[4] Brandtzaeg, P., Gautvik, K.H., Nustad, K. and Pierce J.V. (1976) Br.J. Pharmacol. 56, 155-167.
[5] Chao, J. (1983) J.Biochem. 258, 4434-4439.
[6] Khullar, M., Scicli, G., Carretero, O.A. and Scicli, G. (1986) Biochemistry 25, 1851-1857.
[7] Ikeno, K., Ikeno, T., Kuzuya, H. and Ishiguro, I. (1986) J. Biochem. 99, 1219-1226.
[8] Kato, H., Nakanishi, E., Enjyoji, K., Hayashi, I., Oh-Ishi, S. and Iwanaga, S. (1987) J. Biochem. 102, 1389-1404.
[9] Johansen, L., Bergundhaugen, H. and Berg, T. (1987) J. Chromatogr. 387, 347-359.
[10] Gutman, N., Moreau, T., Alhenc-Gelas, F., Baussant, T., El Moujahed, A., Akpona, S. and Gauthier, F. (1988) Eur. J. Biochem. 171, 577-582.
[11] Chen, Y., Chao, J. and Chao, L. (1988) Biochemistry 27, 7189-7196.
[12] Brady, J.M., Wines, D.B. and MacDonald, R.J. (1989) Biochemistry 28, 5203-5210.
[13] Schellenberg, F. and Weill, J. (1987) Drug Alc. Depend. 19, 181-191.
[14] Laemmli, U.K. (1970) Nature, 227, 680-685.
[15] Chase, T. and Shaw, E. (1970) Methods Enzymol. 19, 20-27.
[16] Fersht, A (1985) in: Enzyme structure and mechanism, 2nd ed, Freeman, New York, pp. 98-120.
[17] Chao, J. and Chao, L. (1987) Biochim. Biophys. Acta, 910, 233-239.
[18] Bode, W., Chen, Z. and Bartels, K. (1983) J. Mol. Biol. 164, 237-282.
[19] Dittmann, B. and Wimmer, R. (1979) in: Current Concepts in Kinin Research (Haberland, G.L. and Hamberg, V. eds) Pergamon Press, Oxford, pp. 121-126.
[20] Fiedler, F. (1976) in: Chemistry and biology of the kallikrein-kinin system in health and disease (Pisano, J.J. and Alisten, K.F. eds) US Government printing office, Washington, pp. 93-95.
[21] Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157-162.
[22] Fiedler, F. (1987) Eur. J. Biochem. 163, 303-312.
[23] Tschesche, H., Mair, G., Godec, G., Fiedler, F., Ehret, W., Hirschauer, C., Lemon, M., Fritz, H., Schmidt-Kastner, G. and Kutzbach, K. (1979) Adv. Exp. Med. Biol. 120A, 245-260.