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Bradykinin degradation pathways in human blood plasma

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The kinetics of proteolysis of bradykinin and its metabolites in human blood plasma was studied, the limiting stages were determined and each stage of the degradation was correlated with the appropriate plasma enzyme. A scheme of the pathways of major and minor degradation processes has been proposed.

Blood plasma; Bradykinin; Mass spectrometry

1. INTRODUCTION

The study of the proteolysis of bradykinin (BK) and related peptides in human blood plasma and serum is of particular interest for the investigation of enzyme regulation of the kallikrein–kinin system, as well as of various pathological states of the organism associated with regulation malfunctions [1]. During BK digestion in human blood plasma or serum, several products of cleavage are observed simultaneously. Some of them have specific biological activities that differ from that of the initial BK [2], while others are known to reduce the rate of proteolysis of BK or its metabolites by various types of inhibition [3]. Consequently, a scheme of the BK digestion pathways in such a multi-enzyme and multi-substrate system should be necessarily based on the data of a direct kinetic study of proteolysis. This scheme can be very helpful for improving understanding of the mechanisms of transformation and termination of BK-induced biological effects and for their further pharmacological corrections.

2. MATERIALS AND METHODS

Bradykinin was from Serva. The individual metabolites were isolated chromatographically from hydrolysates of BK in native or salt-free plasma.

Fresh human blood plasma containing 0.55% sodium citrate was frozen and stored at −20°C before use.

Digestion products were separated by reverse-phase HPLC on columns packed with Nucleosil 5 C_{18} (Macherey-Nagel). Mass spectra were recorded on a KhZh-MKh 3303 mass spectrometer equipped with an SIE AP ion source (V/O, Scientific Instrumentations, Russia).

To study the kinetics of digestion, peptide with an initial concentration of 0.1–1 mM was incubated in plasma. At certain intervals 5–10 μl aliquots were taken from the reaction mixture, de-proteinized by the addition of an equal volume of methanol, and analyzed by HPLC. Concentrations of the degradation products were estimated using the coefficients of extinction calculated from extinctions of Phe and peptide bonds in accordance with the additive scheme [4].

Abbreviations: BK, bradykinin; (1-j)BK, fragment of the BK sequence from 1 to j amino acid residues; SIE AP, soluble ions extraction at atmospheric pressure; ACE, angiotensin-I-converting enzyme.

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3. RESULTS AND DISCUSSION

The reaction mixtures obtained by BK incubation in blood plasma or serum were chromatographically separated (Fig. 1), 5–10 μl aliquots of the eluate were collected near peak maxima, and introduced directly into the mass spectrometer. Products of proteolysis were easily identified by the m/z values of their quasimolecular ions (Table I). The concentrations were calculated from the peak area data taking into account their extinctions. The dynamics of concentration changes are shown in the form of kinetic curves (Fig. 2).

It can be seen that the initial stage of BK hydrolysis results in the accumulation of (1–8)BK and (1–7)BK. In parallel experiments it was shown that (1–8)BK is hydrolyzed in plasma directly to (1–5)BK (Fig. 3). Consequently (1–8)BK and (1–7)BK both originate from BK. Judging from the known specificities of plasma enzymes (1–8)BK and (1–7)BK are formed due to kininase I (carboxypeptidase N) and kininase II (ACE), respectively [3,5]. Estimates of the initial rate of (1–8)BK accumulation (V_a) and the rate of its decomposition at the late stage of reaction (V_d) corresponds to its ratio $V_a/V_d \sim 2$. With this ratio the expected maximum yield of (1–8)BK should not exceed 30%, however, the maximal (1–8)BK concentration observed was 65%. This means that, at the beginning of the process, (1–8)BK is not decomposed. This result is in accordance with the known K_m values for (1–8)BK and BK hydrolysis by

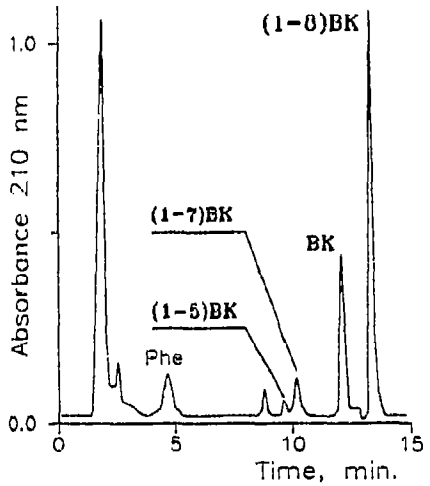


Fig. 1. Chromatogram of the reaction mixture obtained by BK incubation in human blood plasma. Elution was by a 0–25% gradient of acetonitrile in an acetonitrile/0.05 phosphoric acid mixture (pH 2). Unmarked peaks are the background peaks of plasma.

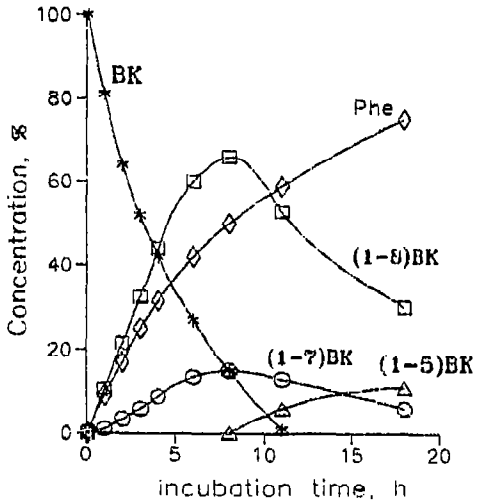


Fig. 2. Kinetic curves of BK degradation in human blood plasma. Concentrations are expressed as a percentage of the initial BK molar concentration.

purified ACE. K_m for (1–8)BK is 150-fold higher than that for BK, which is explained by the presence of Pro at position 6 which is known to completely block dipeptidase activity of ACE [6]. The main product of the (1–8)BK cleavage is (1–5)BK, which originates from the tripeptidase ACE activity.

Yet it is not impossible that carboxypeptidases are involved in the hydrolysis of (1–8)BK [5]. For example, in salt-free plasma the products of (1–8)BK degradation are (1–5)BK, (1–6)BK and (1–7)BK (Fig. 3). The process is accompanied by a 7- and 1.5-fold drop in the hydrolysis rate, respectively, for (1–8)BK and (1–7)BK, which triggers the activities of the postproline-cleaving enzyme and a dipeptidylcarboxypeptidase other than ACE (marked by "X" in Scheme 1). The composition of the degradation mixture is confirmed by a mass spectrum of the de-proteinized hydrolysate applied directly to the mass spectrometer (Fig. 4). The respective products of (1–7)BK and (1–6)BK proteolysis in plasma are (1–5)BK and (1–4)BK, which is in agreement with direct mass spectrometric data.

It is noteworthy that we found only N-terminal products of cleavage. The absence of the corresponding C-terminal products may be due to their further degradation, which is confirmed by the concentration ratio of Phe and N-terminal products (Fig. 3).

The data obtained enabled us to elucidate minor and major processes in plasma and to make a scheme of proteolysis pathways (Scheme 1, dotted and solid lines, respectively). In accordance with the scheme all the previously found metabolites [2,7–9] derived from BK by C-terminal cleavage, in principle, may be formed, but their actual concentration depends on the ratio of rates of certain sequential and parallel processes. In particular the absence of (1–7)BK as a product of BK cleavage in serum [9], in contrast to plasma, is due to activation of carboxypeptidase U during blood clotting [10].

The scheme leaves pathways of further (1–5)BK and (1–4)BK proteolysis unclear. Mass spectrometry of their hydrolysates revealed only free amino acids.

In conclusion, it should be mentioned that the study of proteolysis in such multi-enzyme systems as plasma

Table I

Identification of the chromatographic peaks of the products of bradykinin degradation in human blood serum

Peak number	Mass spectrum	Identification
1	166(35)$[M+H]^+$,120(100)	Phe
2	573(100)$[M+H]^+$	Arg-Pro-Pro-Gly-Phe
3	757(100)$[M+H]^+$	Arg-Pro-Pro-Gly-Phe-Ser-Pro
4	1060(30)$[M+H]^+$,530(100)$[M+2H]^{2+}$	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
5	904(100)$[M+H]^+$	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe

The numbering of peaks corresponds to the chromatogram of Fig. 1.

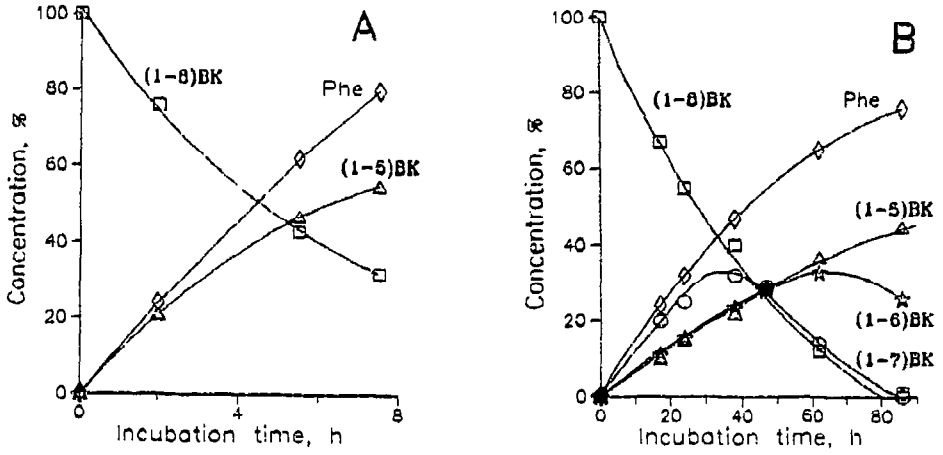


Fig. 3. Kinetic curves of (1–8)BK degradation in plasma (A) and salt-free plasma (B). Concentrations are expressed as a percentage of the initial (1–8)BK molar concentration.

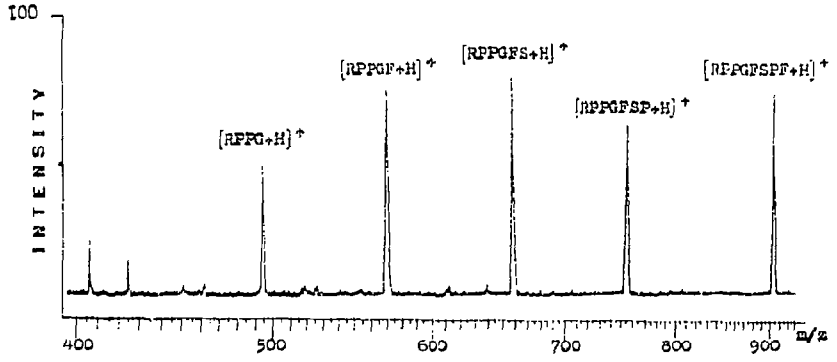
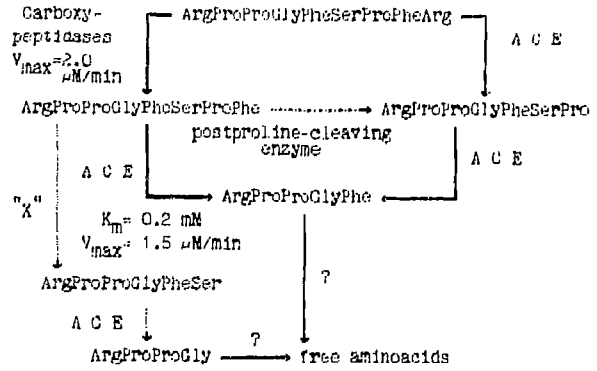


Fig. 4. Mass spectra obtained after direct introduction of de-proteinized hydrolysate of (1–8)BK in salt-free plasma. Peaks intensities are expressed as a percentage of the intensity of the maximal peak. Symbols of the amino acids are given in one-letter code.



Scheme 1. Degradation pathways of bradykinin in blood plasma. Note that the proteolysis pathways marked by dotted lines are detected in salt-free plasma only.

is efficiently facilitated by the combination of SIE AP mass spectrometry [11] and HPLC. Such a technique allows the possibility of identifying metabolites without preliminary synthesis of standards, and determining the composition of hydrolysates without fractionation, thus opening new perspectives for similar research.

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