# PROTEOLYSIS OF FACTOR B BY PLASMA KALLIKREIN AND PLASMIN

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## 1. Introduction

Factor B (B), a complement component of the alternative pathway, is known to be a precursor of alternative C3/C5 convertase. B is cleaved by factor $\bar{D}$ ($\bar{D}$) in the presence of C3 and $Mg^{2+}$ into two fragments, Bb and Ba [1]. C3b can be replaced by cobra venom factor (CVF). The bimolecular complex of CVF and B is converted to the active form CVF–Bb by $\bar{D}$ [2]. Activation of B by trypsin, plasmin and pronase instead of $\bar{D}$ has been reported [3]. In [4] trypsin cleaved B to form the CVF–Bb complex. However, the effect of CVF on cleavage of B by protease is unknown.

Here we investigated the mode of cleavage of B in the presence and absence of CVF by Hageman factor fragment (HFf), plasma kallikrein and plasmin, which are generated during contact activation. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showed that plasma kallikrein cleaved B in the presence or absence of CVF into 2 fragments ($M_r$ 60 000, 38 000). A plasmin digest of B in the presence of CVF was similar to a kallikrein digest. In the absence of CVF, plasmin cleaved B to 4 fragments ($M_r$ 60 000, 48 000, 38 000, 30 000). HFf did not cleave B. Plasmin and kalikrein cleaved B and yielded the CVF–Bb complex in the presence of CVF, as shown by high-speed gel permeation chromatography.

## 2. Materials and methods

Human plasma was obtained from Japan Red Cross. Cobra venom (*Naja naja kauthia*) was purchased from Wako Pure Chemical Industries Ltd.

### 2.1. *Preparations of various proteases, C3 and CVF*

B, $\bar{D}$, C3, CVF, plasminogen and Hageman factor were purified as in [5–10]. Bb was isolated as in [1]. Plasmin was purified after activation with urokinase. HFf was isolated after incubation with Hageman factor and purified kallikrein as in [10]. Plasma kallikrein was prepared as in [11] from spontaneously activated crude kallikrein.

### 2.2. *SDS–PAGE*

This was done as in [12] using 7% gel under non-reducing conditions. A sample of 20 μg B with or without CVF (molar ratio 1:1) in 0.02 M sodium phosphate buffer containing 0.15 M NaCl and 0.01 M $MgCl_2$ was preincubated at 37°C for 5 min, then mixed with HFf, plasma kallikrein and plasmin, respectively, at an enzyme–substrate ratio of 1:30. After various incubation periods, samples of the mixture were subjected to SDS–PAGE.

### 2.3. *Analysis by high-speed gel permeation chromatography (GPC)*

The formation of a CVF–Bb complex was investigated by GPC (Hitachi 635A) on a column (0.75 × 60 cm) of TSKG 3000 SW (Toyo Soda Manufacturing Co.) with 0.05 M sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl and 0.005 M EDTA as eluant at room temperature. The flow rate was adjusted to 0.5 ml/min, and the absorbance at 254 nm was measured. The elution time of blue dextran in the void volume was 18.8 min and that of tyrosine in the bed volume was 46.6 min. A mixture of CVF (20 μg) and B (20 μg) was preincubated at 37°C for 5 min in the presence of 0.005 M $MgCl_2$, and then plasmin (1 μg), kallikrein (1 μg) of $\bar{D}$ (0.25 μg) was added, and incubation was continued at 37°C for 2 h. EDTA (final conc. 0.01 M) was added to the mixture (final vol. 70 μl) after incubation, and a 50 μl aliquot of the mixture was subjected to GPC.

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## 3. Results and discussion

The $M_r$-values of B and CVF were estimated to be 90 000 and 140 000, respectively, by SDS–PAGE (fig.1a,b). B was converted to Bb ($M_r$ 60 000) and Ba ($M_r$ 38 000) by $\bar{D}$ in the presence of CVF (fig.1c). CVF was resistant to plasma kallikrein and plasmin. When B was incubated with HFf at 37°C for 3 h in the presence and absence of CVF, no significant cleavage of B was detected (fig.2). On the other hand, B was cleaved by plasma kallikrein both in the presence and absence of CVF to two fragments of $M_r$ 60 000 and $M_r$ 38 000 corresponding to Bb and Ba, respectively, produced by $\bar{D}$ (fig.3). Both HFf and plasma kallikrein have amidolytic activity on the synthetic substrate D-Pro–Phe–Arg–*p*-nitroanilide [10], while the spectrum of the synthetic substrate for kallikrein differs from that for HFf [10,13]. This difference seems to be related to the differences in the behaviors of kallikrein and HFf with B. HFa, a high $M_r$ form of activated Hageman factor, is a more potent activator of factor XI than HFf [14]. It would be interesting to know whether HFa cleaves B.

Plasmin cleaved B to two fragments similar to Bb and Ba in the presence of CVF (fig.4B). However, in the absence of CVF, it cleaved B to 4 fragments (fig.4A). The apparent $M_r$-values of these fragments were 60 000, 48 000, 38 000 and 30 000, respectively.

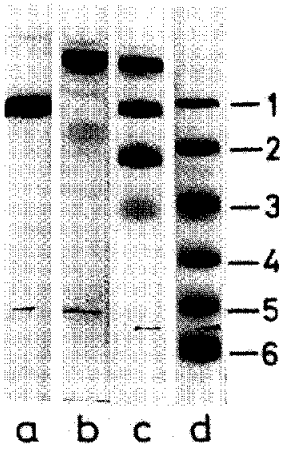


Fig.1. Cleavage of B by $\bar{D}$ in the presence of CVF on SDS–PAGE: 16 μg B was incubated with 15 μg CVF and 0.25 μg $\bar{D}$ at 37°C for 1 h; (a) B, (b) CVF, (c) B incubated with CVF and $\bar{D}$ (d); $M_r$ standards (1) phosphorylase *b*, (2) bovine serum albumin, (3) ovalbumin, (4) carbonic anhydrase, (5) soybean trypsin inhibitor, (6) α-lactalbumin.


Fig.2. SDS–PAGE of an incubation mixture of B and HFf in the absence (a) and presence (b) of CVF. The arrow shows the position of HFf.

Bb ($M_r$ 60 000) isolated from an incubation mixture of B, $\bar{D}$ and C3 was not cleaved by plasmin. The fragments of $M_r$ 60 000 and 38 000 corresponded to Bb and Ba, but the materials in the fragments of $M_r$ 48 000 and 30 000 derived from B are still unknown. The $NH_2$-terminal portions of the fragments must be determined.

B with CVF is likely to be cleaved at one point by plasmin. Without $Mg^{2+}$, B was cleaved to 4 fragments even in the presence of CVF. These results indicate that the points in B that are susceptible to cleavage are concealed when B forms a complex with CVF and $Mg^{2+}$.

B is cleaved by $\bar{D}$ in the presence of CVF to form a complex CVF–Bb, an active C3/C5 convertase [15]. As described above, in the presence of CVF, B is cleaved by kallikrein and plasmin to 2 fragments. The formation of a complex of CVF with the $M_r$ 60 000 fragment produced by kallikrein or plasmin was examined by GPC analysis. On GPC analysis, small amounts of protein were detected rapidly. A mixture of B and CVF gave two peaks in the presence of EDTA

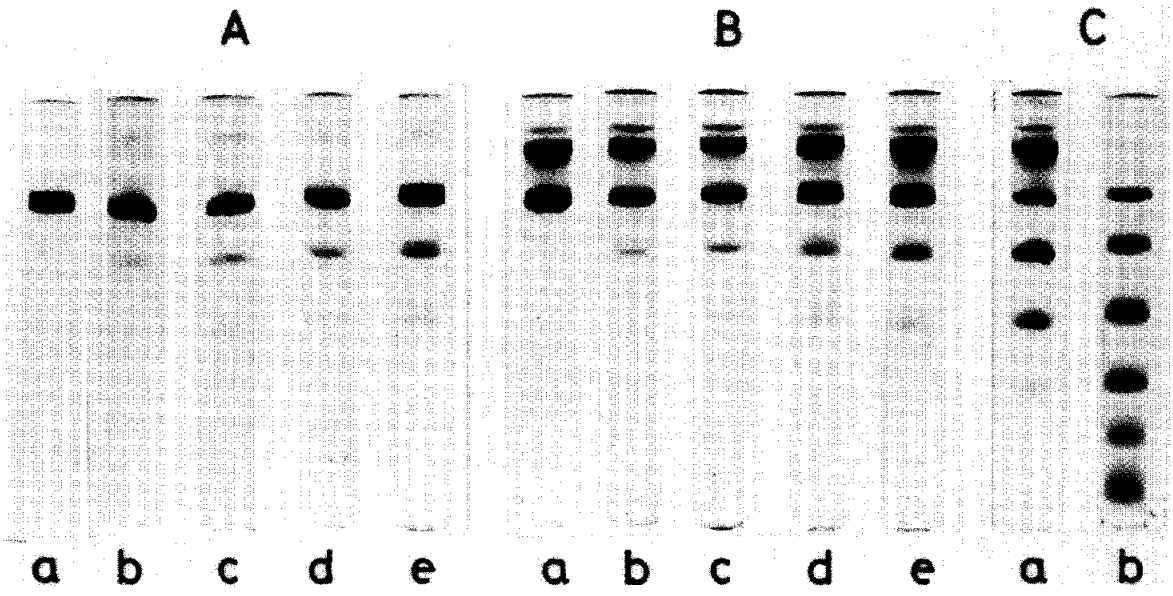


Fig.3. Cleavage of B by plasma kallikrein. (A) SDS–PAGE of B incubated for the following times with plasma kallikrein in the absence of CVF: (a) 0 min, (b) 30 min, (c) 60 min, (d) 120 min, (e) 180 min. (B) In the presence of CVF: (a) 0 min, (b) 30 min, (c) 60 min, (d) 120 min, (e) 180 min; $M_r$ standards as in fig.1.

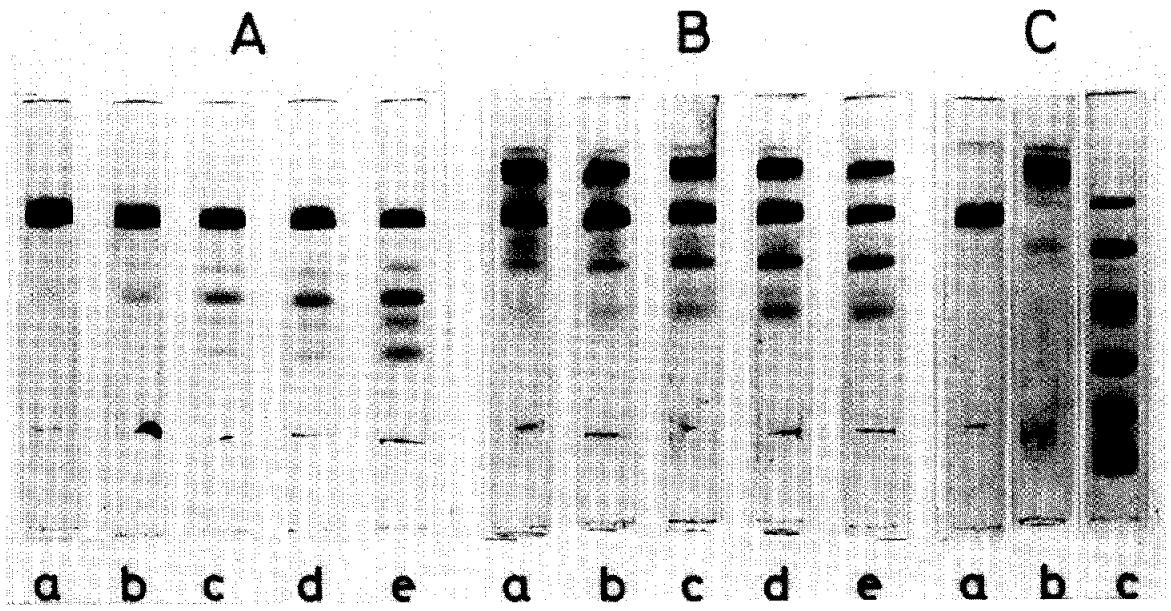


Fig.4. Cleavage of B by plasmin. (A) SDS–PAGE of B incubated for the following times with plasmin in the absence of CVF: (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min, (e) 180 min. (B) In the presence of CVF: (a) 15 min, (b) 30 min, (c) 60 min, (d) 120 min, (e) 180 min. (C) (a) B, (b) CVF, (c). $M_r$ standards as in fig.1.

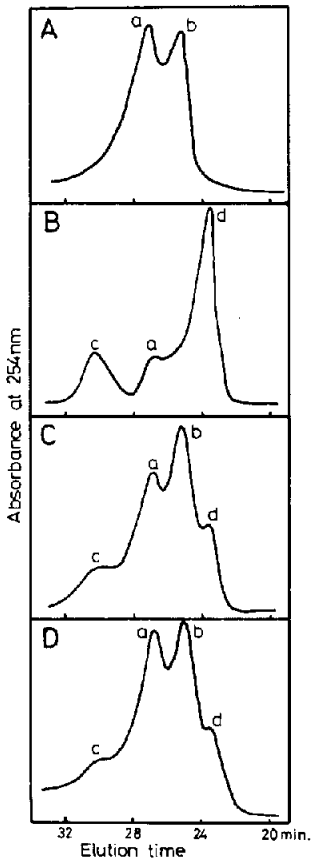


Fig.5. Elution pattern on high speed gel permeation chromatography (GPC). The reaction mixture was incubated for 2 h at 37°C and applied to GPC: (A) B + CVF; (B) B + CVF + $\bar{D}$; (C) B + CVF + plasmin; (D) B + CVF + plasma kallikrein; (a–d) were concluded to be B, CVF, Ba and CVF–Bb, respectively.

(fig.5A). The elution time of B was 27.0 min and that of CVF was 25.3 min. A mixture of B, CVF, $MgCl_2$ and $\bar{D}$ was incubated at 37°C for 2 h, and then EDTA was added, and an aliquot was subjected to GPC (fig.5B). The 3 peaks eluted at 23.6 min, 27.0 min and 31.5 min were concluded to be those of CVF–Bb, remaining B, and Ba, respectively. The elution patterns of mixtures of B, CVF, $MgCl_2$ and plasmin or kallikrein are shown in fig.5C,D. Each mixture was mixed with EDTA before GPC. Four peaks were eluted at 23.6 min, 25.3 min, 27.5 min and 31.5 min. This result shows the formation of a complex of CVF and B fragment produced by plasmin or kallikrein.

Here, plasmin and plasma kallikrein cleaved B in the absence of CVF. In the presence of CVF, B was cleaved by plasmin or kallikrein to a compound forming a complex with CVF, indicating activation of B in the purified system. However, in [16] low hemolytic activity of plasmin was shown in the alternative complement pathway. In [5] a $M_r$ 47 000 fragment of B was suggested in serum. This fragment seems to correspond with the $M_r$ 48 000 fragment obtained on digestion of plasmin without CVF here. Although it is unknown at present whether the participation of plasmin and kallikrein in the alternative complement pathway results in activation or degradation of B in the plasma, a significant change of B seems to occur on contact activation with the Hageman factor.

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