Immunology 1983 **49** 685

New synthetic inhibitor to the alternative complement pathway

N. IKARI, Y. SAKAI, Y. HITOMI & S. FUJII *Division of Regulation of Macromolecular Function, Institute for Protein Research, Osaka University, Suita, Osaka, Japan*

Accepted for publication 14 March 1983

Summary. The inhibitory effects of 6-amidino-2-naphthyl 4-guanidinobenzoate (FUT-175) on the activities of factor B, factor D̄ and cobra venom factor (CVF)·Bb were examined. FUT-175 bound specifically to the Bb fragment of factor B or CVF·Bb. FUT-175 was a non-competitive inhibitor of the esterolysis of L-leucyl-L-alanyl-L-arginine naphthylester by factor B and CVF·Bb. FUT-175 also inhibited the haemolytic activity of factor B, the C3 convertase activity of CVF·Bb and the factor B-cleaving activity of factor D̄. The concentration of FUT-175 causing 50% inhibition of these activities was 10^{-5}–10^{-4}M.

INTRODUCTION

There are many reports describing inhibitors of the alternative complement pathway. These inhibitors have many different action mechanisms. Some protease inhibitors act on complement components to inhibit their activities.

DPF inhibits some serine proteases in the alternative complement pathway. At a concentration of 1 mM it inhibits factor D̄ irreversibly (Fearon, Austen & Ruddy, 1974; Dieminger, Vogt & Lynen, 1976) and at a concentration of 10 mM it inhibits factor B (Medicus, Götze & Müller-Eberhard, 1976), which is also a serine protease (Christie, Gagnon & Porter, 1980; Mole & Niemann, 1980). However, at a concentration of 1 mM it does not inhibit the haemolytic activity or esterolytic activity of factor B (Fearon *et al.*, 1974; Vogt *et al.* 1977). At a concentration of 10 mM it also inhibits CVF·Bb (Medicus *et al.*, 1976), a stable analog of C3bBb (Cooper, 1973).

Leupeptin inhibits the esterolytic activity of CVF·Bb ($Ki = 4 \times 10^{-5}$ M; Caporale, 1981). The inhibition is competitive or mixed type, as suggested by the structure of leupeptin, which has a similar sequence to the substrate and an inhibitory aldehyde group. However, Takada *et al.*, (1978) reported that, even at a concentration of 1 mM, leupeptin does not inhibit complement-mediated haemolysis in the alternative pathway.

Previously, we reported that 6-amidino-2-naphthyl 4-guanidinobenzoate (FUT-175) is a protease inhibitor of $C\overline{1r}$, C1 esterase, thrombin, plasmin, kallikrein and trypsin, and that it also inhibits complement-mediated haemolysis in the classical pathway or alternative pathway (Fujii & Hitomi, 1981). In this work, we examined the inhibitory effects of FUT-175

Abbreviations: FUT-175, 6-amidino-2-naphthyl 4-guanidinobenzoate; CVF, cobra venom factor; DPF, diisopropyl phosphofluoridate; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl) maleimide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid; MES, 2-(*N*-morpholino)-ethane-sulphonic acid; EDTA, ethylenediamine tetracetic acid disodium salt; EGTA, ethyleneglycol *bis* (β-aminoethylether)-*N*, *N*, *N*′, *N*′-tetracetic acid.

Correspondence: Dr Setsuro Fuju, Division of Regulation of Macromolecular Function, Institute for Protein Research, Osaka University, Suita, Osaka, 565 Japan.

0019-2805/83/0800-0685$02.00

on the protease activities of factor B, CVF·Bb and factor D̄ in the alternative complement pathway.

MATERIALS AND METHODS

Enzymes and reagents

Fresh frozen human plasma was obtained from Japan Red Cross Ltd. 6-Amidino-2-naphthyl 4-guanidinobenzoate (FUT-175; Fig. 1) and L-leucyl-L-alanyl-L-arginine napthylester (LeuAlaArgNE) were prepared in the Research Laboratories of Torii & Co. Ltd., Tokyo, Japan. *Naja naja kauthia* cobra venom and *n*-(7-dimethylamino-4-methyl-3-coumarinyl) maleimide (DACM) were obtained from Wako Pure Chemical Industries, Japan. DEAE–cellulose DE-52 was purchased from Whatman Ltd., England. Sephacryl S-300 and Sephadex G-25 were purchased from Pharmacia Fine Chemical Co., Sweden.

Factor B was purified as described in the previous report (Ikari *et al.*, 1983). Cobra venom factor (CVF), C3 and factor D̄ were purified by the methods of Pepys, Tompkins & Smith (1979), Tack & Prahl (1976) and Lesavre *et al.*, (1979), respectively. CVF·Bb was prepared from purified enzymes as follows: 15 mg of CVF, 8·8 mg of factor B and 0·4 mg of factor D̄ were incubated at 37° for 2 hr in 50 mM HEPES buffer (pH 7·4) containing 12 mM $MgCl_2$, and then EDTA was added at a final concentration of 24 mM. After dialysis against 5 mM potassium-phosphate buffer (pH 8·0), the dialysate was applied to a DEAE–cellulose column (DE-52, 2 × 10 cm). The column was washed with the same buffer and then the material was eluted with 400 ml of a linear gradient of 5–200 mM potassium-phosphate buffer (pH 8·0), as described by Vogt *et al.*, (1974). Fractions of CVF·Bb were collected, concentrated and applied to Sephacryl S-300 (2·6 × 76 cm) equilibrated with 20 mM sodium-phosphate buffer (pH 7·4) containing 0·15 M NaCl and 2 mM EDTA. The purity of the preparation of CVF·Bb was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and high-speed gel permeation chromatography (Ikari, Niinobe & Fujii, 1981).

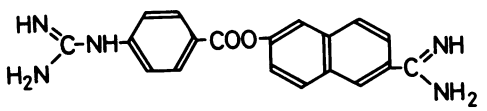

Figure 1. Structural formula of 6-amidino-2-naphthyl 4-guanidinobenzoate (FUT-175).

All enzymes were dialysed against 50 mM HEPES buffer (pH 7·4).

Experiment on inhibition of esterolytic activities of factor B and CVF·Bb

A sample of 58 μg of factor B was incubated for 5 min at 37° with FUT-175 in 50 mM HEPES buffer (pH 7·4) in a final volume of 200 μl. Then 700 μl of 50 mM MES buffer (pH 6·5) containing 0·15 M NaCl, and 100 μl of LeuAlaArgNE solution (1 μmol/ml) were added successively. The subsequent procedure was carried out as described previously (Ikari *et al.*, 1983). As a control, the esterolytic activity of factor B in the absence of FUT-175 was measured. The control activity was taken as 100% and the percent activity of enzyme with FUT was calculated. For measurement of the Ki value, LeuAlaArgNE solutions at concentrations of 0·5 μmol/ml and 1·5 μmol/ml were used. Inhibition of esterolytic activity of CVF·Bb was examined in the same manner using 90 μg of CVF·Bb.

Experiment of inhibition of C3 convertase activity of CVF·Bb

A sample of 0·9 μg of CVF·Bb was incubated for 5 min at 37° with FUT-175 in a total volume of 10 μl. Then 71 μg of C3 was added and incubation was continued for 30 min at 37° in a total volume of 110 μl. The reaction was stopped by adding 100 μl of 15% SDS solution, and 700 μl of 40 mM sodium-phosphate buffer (pH 7·0) was added. Then 100 μl of DACM solution (84 nmol/ml in acetone) was added and the mixture was stood for 30 min at room temperature. The relative fluorescence intensity was measured in a Hitachi 650-10M fluorescence spectrophotometer using excitation and emission wavelengths of 385 and 480 nm, respectively (Yamashita, Nagasawa & Koyama, 1981).

As a control, C3 was incubated with CVF·Bb in the absence of FUT-175, and the relative fluorescence intensity was measured under the same conditions. As a blank, C3 was incubated without CVF·Bb and FUT-175. CVF·Bb and FUT-175 at the concentrations used in this experiment had no measurable fluorescence. C3 convertase activity was determined as follows:

$$\text{Activity (\%)} = 100 \times \frac{I - Io}{Ic - Io}$$

where I is the relative fluorescence intensity of the sample, Io is the relative fluorescence intensity of the blank and Ic is the relative fluorescence intensity of the control.

Experiment on inhibition of haemolytic activity of factor B

Factor B was incubated for 5 min at 37° with various concentrations of FUT-175 in 50 mM HEPES buffer (pH 7·4). The mixture was applied to Sephadex G-25 equilibrated with 50 mM HEPES buffer (pH 7·4) and material was eluted with the same buffer.

Haemolytic activity of factor B was measured using rabbit erythrocytes (Pepys *et al.*, 1979; Platts-Mills & Ishizaka, 1974) as follows: Factor B-depleted serum (RB) was obtained by heating (50°, 15 min) fresh human serum. Rabbit erythrocytes (5×10^7 cells/ml) were suspended in isotonic veronal-buffered saline (pH 7·4) containing 0·1% gelatin, 10 mM EGTA and 2 mM $MgCl_2$. All haemolytic assays were performed in the same buffer. Test solution containing factor B was incubated for 17 min at 37° with 250 μl of rabbit erythrocyte suspension and 50 μl of RB reagent in a final volume at 1 ml. Then 2·5 ml of cold saline containing 0·4% sodium citrate was added and the mixture was centrifuged. The haemolytic activity of factor B in the test solution was measured at 412 nm.

The amount of factor B causing 50% haemolysis was determined. The reciprocal of the value was taken as the activity of factor B, the activity of the original factor B was taken as 100%, and the percent activity of factor B treated with FUT-175 was determined.

Incorporation of [^{14}C]-FUT-175 into factor B derivatives

[^{14}C]-FUT-175 labelled at the carbon of the guanidine group was used. Incorporation of [^{14}C]-FUT-175 was measured under the following different conditions: (i) 86 μg of factor B and 42 μg of [^{14}C]-FUT-175 (1·32 μCi/mg) were incubated for 5 min at 37°; (ii) 86 μg of factor B, 96 μg of CVF and $MgCl_2$ at a final concentration of 3·7 mM were incubated with 42 μg of [^{14}C]-FUT-175; (iii) 86 μg of factor B, 96 μg of CVF and 0·1 μg of factor $\bar{D}$ were incubated for 2 hr at 37° in the presence of $MgCl_2$ at a final concentration of 3·7 mM, and then 42 μg of [^{14}C]-FUT-175 was added and incubation was continued for 5 min at 37°. These three samples were mixed with 5% SDS solution containing 8 M urea and applied to SDS polyacrylamide gel (7%). Each gel was sliced into 2-mm sections, which were placed in scintillation vials containing 1 ml of piperidine and incubated overnight at 37°. Then, 0·5 ml of protosol® was added and the mixtures were incubated overnight at 60°. Radioactivity was measured in Aloka liquid scintillation counter model LSC-903 in 10 ml of scintillator.

Experiment on inhibition of factor B cleaving activity of factor $\bar{D}$

A sample of 0·2 μg of factor $\bar{D}$ was incubated for 5 min at 37° with FUT-175, and then 72 μg of CVF, 58 μg of factor B and $MgCl_2$ at a final concentration of 7 mM were added and incubation was continued for 2 hr at 37°. The reaction was stopped by adding 10 μl of 5% SDS containing 8 M urea, and SDS-PAGE was performed. Cleavage of factor B was determined densitometrically (Toyo digital densitorol DMU-33C). In the absence of FUT-175, *ca* 80% cleavage of factor B was observed. This factor B-cleaving activity of factor $\bar{D}$ was taken as 100% and the percentage activity of factor $\bar{D}$ in the presence of FUT-175 was calculated.

RESULTS

Inhibition of esterolytic activities of factor B and CVF·Bb

Previously, we reported that LeuAlaArgNE is a good substrate for factor B or its derivatives, such as Bb and CVF·Bb, and that FUT-175 inhibits the esterolytic activities of factor B and CVF·Bb (Ikari *et al.*, 1983). Figure 2 shows the inhibitory effect of FUT-175 on esterolytic activities of factor B and purified CVF·Bb. The concentrations of FUT-175 causing 50% inhibition of esterolysis by factor B and CVF·Bb were $6{\cdot}2 \times 10^{-5}$ M and $3{\cdot}2 \times 10^{-5}$ M, respectively (Fig. 2a). Ki values were determined from Dixon plots (Figs 2b, c). The Ki values of FUT-175 for factor B and CVF·Bb were 6×10^{-5} M and 4×10^{-5} M, respectively and results indicated that the inhibition was non-competitive.

Previously we found that FUT-175 at a concentration of 5×10^{-4} M causes 50% inhibition of the esterolytic activity of factor B (Ikari *et al.*, 1983). This difference from the present value was due to a difference in pH, since we found that FUT-175 is more inhibitory at pH 7·4, as used here, than at pH 6·5, as used previously (data not shown).

Next, we examined the effect of dialysis and gel filtration on the inhibitory effect of FUT-175. When factor B was incubated with FUT-175 and passed through Sephadex G-25, before measuring its esterolytic activity, the concentration of FUT-175 required for 50% inhibition of esterolysis was $1{\cdot}2 \times 10^{-4}$ M. Dialysis also gave similar results. This phenomenon suggests that the inhibitory effect of FUT-175 is not irreversibly

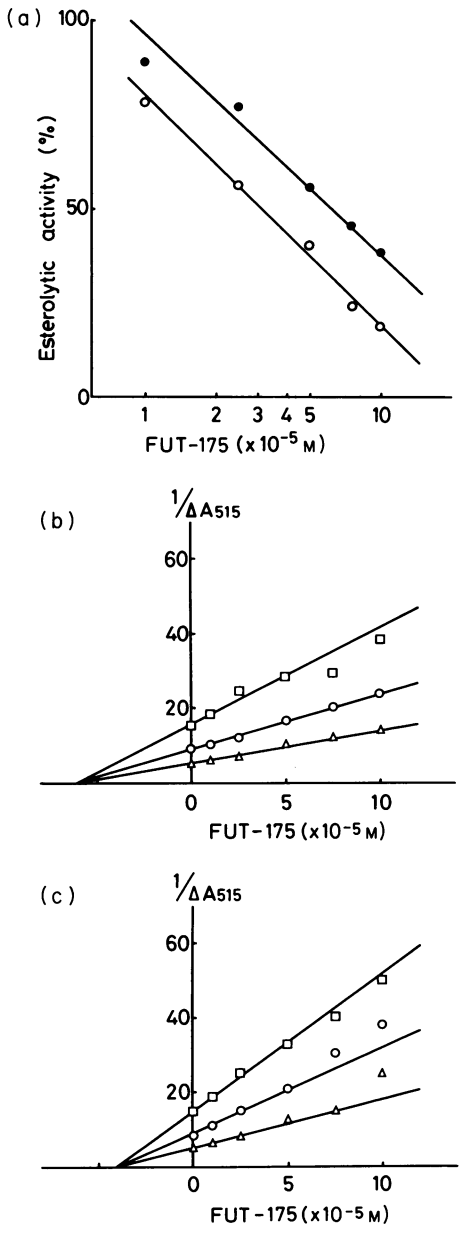

Figure 2. Inhibition by FUT-175 of esterolysis of Leu AlaArgNE. (a) Inhibitions of esterolytic activities of factor B (●) and CVF·Bb (○). Substrate was used at a final concentration of 1×10^{-3} M. (b) Dixon plot for inhibition of factor B at three different concentrations of substrate: $1{\cdot}5 \times 10^{-3}$ M (□), 1×10^{-3} M (○), 5×10^{-4} M (△). In this figure, the value of V is replaced by the value of ΔA515, the optical density of the sample at 515 nm minus that of the blank. (c) Dixon plot of inhibition of CVF·Bb at the same three different concentrations of substrate as for (b).

(Fujii & Hitomi, 1981) and that the activity of factor B is partially restored by dialysis or gel filtration.

Inhibition of C3 convertase activity of CVF·Bb

C3 is cleaved by C3 convertase to fragments C3b and C3a (Götze & Müller-Eberhard, 1971). Since fluid-phase C3b has an SH group, the amount of C3b formed can be determined by measurement of the SH content (Janatova *et al.*, 1980). Use of the DTNB or the other reagents reacting with SH groups generally used for determination of SH groups requires much protein for the measurement. Recently, Nagasawa showed that DACM is useful for measuring small amounts of C3b (Yamashita *et al.*, 1981). Therefore, we used DACM to determine the formation of C3b by C3 convertase.

CVF·Bb was incubated with FUT-175 first and then further with C3. After the incubation DACM was added. Since reaction of DACM with SH groups results in fluorescence, the C3 convertase activity, expressed as the C3b-forming activity, was measured as fluorescence. The percent activity of CVF·Bb was determined as described in the 'Materials and Methods'. As shown in Fig. 3, a concentration of 8×10^{-5} M FUT-175 caused 50% inhibition of the C3 convertase activity of CVF·Bb.

Inhibition of haemolytic activity of factor B

Previously FUT-175 was shown to inhibit comple-

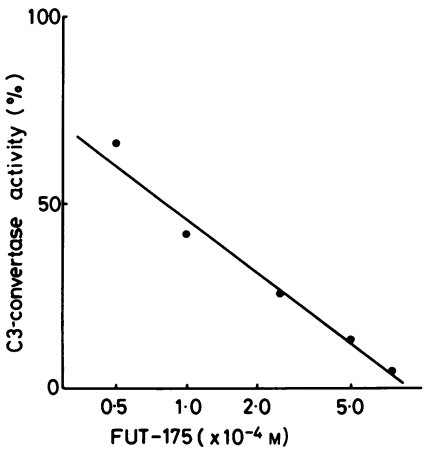

Figure 3. Inhibition of C3-convertase activity of CVF·Bb. C3-convertase activity was determined as described in 'Materials and Methods'.

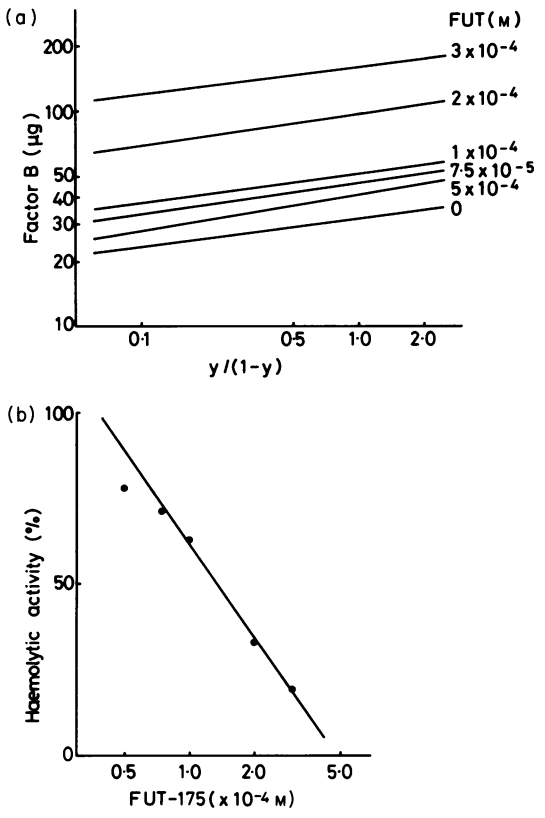

Figure 4. Inhibition by FUT-175 of haemolytic activity of factor B. (a) The haemolytic activity of factor B with or without pretreatment with various concentrations of FUT-175 was assayed. The degree of lysis (y) was calculated in each experiment and $y/(1-y)$ values were plotted against the amount of factor B on a log–log scale. (b) Haemolytic activity of factor B was determined as described in the Materials and Methods.

ment-mediated haemolysis in the alternative pathway (Fujii & Hitomi, 1981). In this work we examined the effect of FUT-175 on the haemolytic activity of factor B, which is a key enzyme in the alternative pathway (Fig. 4).

In the preceding sections, we showed that some inhibitory effect of FUT-175 on the esterolytic activity of factor B still remained after dialysis or gel filtration on Sephadex G-75, although the effect was decreased. This finding suggested that excess FUT-175 was mostly removed by passage through Sephadex G-25, and some FUT-175 bound to factor B still remained. This preparation was used for investigating the inhibitory effect of FUT-175 on factor B in complement-mediated haemolysis, because excess FUT-175 may also inhibit other proteases such as factor D̄.

Thus, in experiments, factor B was incubated with various concentration of FUT-175 and then passed through Sephadex G-25 before use. Haemolytic activity was measured using RB reagent and rabbit erythrocytes. The activity of factor B treated with FUT-175 was estimated by comparison of the amount of this enzyme causing 50% haemolysis (determined from Fig. 4a) with that of the original factor B not treated with FUT-175. As shown in Fig. 4b, on pretreatment with FUT-175, the haemolytic activity of factor B decreased with increase in the concentration of FUT-175. The concentration of FUT-175 causing 50% decrease in activity of factor B was $1{\cdot}4 \times 10^{-4}$ M. This value is consistent with that for the inhibitory effect of FUT-175 on the LeuAlaArgNE hydrolytic activity of factor B after dialysis or gel filtration of the enzyme–inhibitor complex.

Incorporation of [^{14}C]-FUT-175 into factor B and its derivatives

Factor B was incubated with [^{14}C]-FUT-175 in various conditions and then subjected to SDS-PAGE (Fig. 5). Incorporation of [^{14}C]-FUT-175 into factor B was observed when the two were incubated together (Fig. 5a). In the presence of CVF and magnesium ion, radioactivity was found in factor B, but not in CVF (Fig. 5b). The calculated molar ratio of FUT-175 bound to factor B was about one inhibitor molecule per factor B. When factor B was incubated with CVF, factor D̄ and magnesium ion to allow formation of the CVF·Bb complex, and was then incubated with [^{14}C]-FUT-175, radioactivity was incorporated into the Bb fragment, but not into CVF or the Ba fragment (Fig. 5c).

These results indicate that FUT-175 acts on factor B in the presence or absence of CVF, and that its site of action is the Bb fragment.

Inhibition of factor B-cleaving activity of factor D̄

Factor B is cleaved by factor D̄ in the presence of CVF and magnesium ion to form CVF·Bb (Cooper, 1973). The effect of FUT-175 on cleavage of factor B by factor D̄ was examined (Fig. 6).

Factor D̄ was preincubated with FUT-175, then factor B, CVF and magnesium ion were added, and the mixture was incubated further. After the incubation, materials were applied to SDS-PAGE, and the

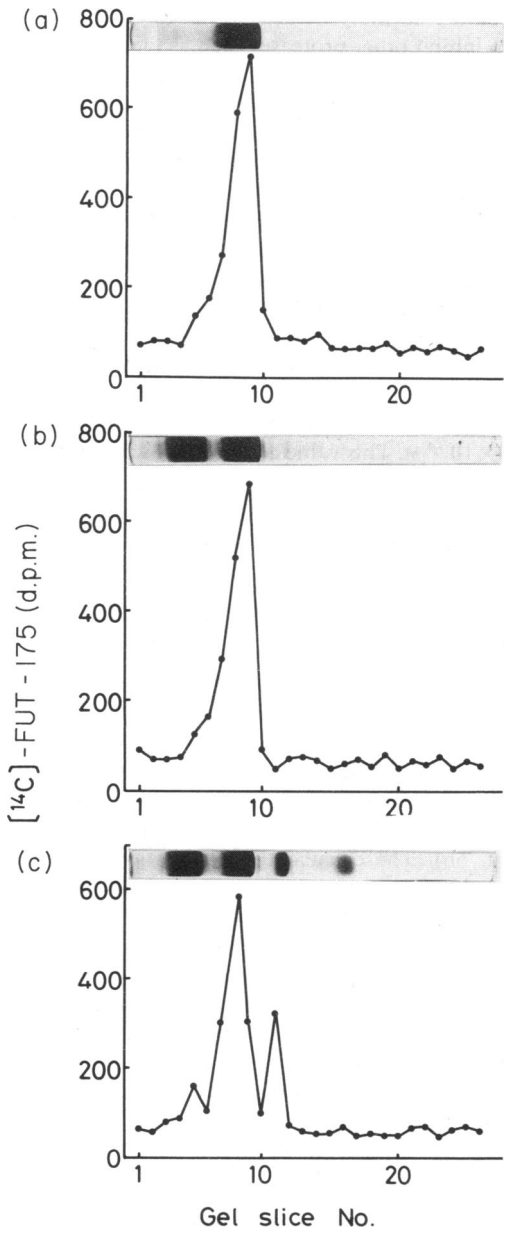

Figure 5. Incorporation of [^{14}C]-FUT-175 into factor B and its derivatives. [^{14}C]-FUT-175 was incubated with factor B as described in (a)–(c): (a) factor B and [^{14}C]-FUT-175 were incubated for 5 min at 37°, (b) factor B, CVF, Mg^{2+} and [^{14}C]-FUT-175 were incubated for 5 min at 37°, (c) factor B, CVF, factor D̄ and mg^{2+} were incubated for 2 hr at 37° to allow formation of CVF·Bb, and then [^{14}C]-FUT-175 was added, and incubation was continued for 5 min at 37°. The upper panels show SDS polyacrylamide gels stained for protein with Coomassie Brilliant Blue. The lower panels show the radioactivities of gel slices.

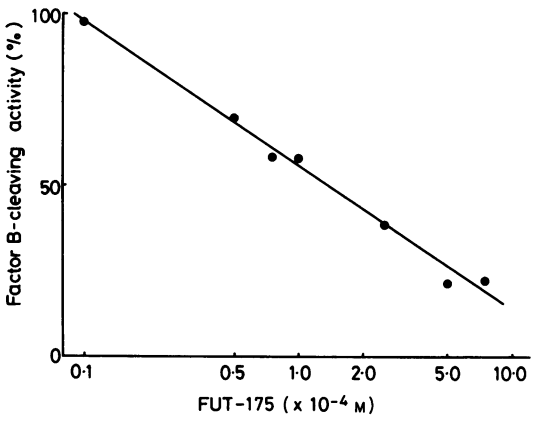

Figure 6. Inhibition of factor B cleaving activity of factor D̄. Cleavage of factor B by factor D̄ in the presence of CVF and Mg^{2+} was monitored by SDS-PAGE as described in 'Materials and Methods'.

cleavage of factor B by factor D̄ was measured densitometrically. The factor B-cleaving activity of factor D̄ in the absence of FUT-175 was taken as 100%. As shown in Fig. 6, the concentration of FUT-175 required for 50% inhibition of the cleaving activity of factor D̄ was $1{\cdot}4 \times 10^{-4}$ M.

DISCUSSION

In this work, the inhibitory effects of FUT-175 on factor B, CVF·Bb and factor D̄ were examined in a system with purified components.

FUT-175 inhibited the esterolytic activity and haemolytic activity of factor B, and its site of action on factor B was found to be in the Bb fragment. The inhibitory effect of FUT-175 on the esterolytic activity of factor B was non-competitive, and FUT-175 still remained on factor B after dialysis or gel filtration.

The inhibitory effect of FUT-175 on the haemolytic activity of factor B may be explained by supposing that when factor B is inhibited by FUT-175, it does not generate C3 convertase, C3bBb or it generates C3bBb with no C3 convertase activity.

FUT-175 inhibited both the esterolytic and the C3 convertase activity of CVF·Bb. Presumably, it also inhibits C3bBb, a human alternative C3 convertase. FUT-175 also inhibited the cleavage of factor B by factor D̄ in the presence of CVF and magnesium ion.

Previously it was found that $5{\cdot}1 \times 10^{-7}$ M FUT-175 caused 50% inhibition of complement-mediated hae-

molysis in the alternative pathway (Fujii & Hitomi, 1981). It is not clear whether this strong inhibitory effect of FUT-175 was due only to effects on the activities of factor B, factor D and C3 convertase or whether it was also due to the presence of another inhibition mechanism or to use of a different amount of enzyme.

REFERENCES

CAPORALE L.H. (1981) Leupeptin inhibits the C3/C5 convertase CVFBb of the complement system. *Biochim. biophys. Acta*, **660,** 151

CHRISTIE D.L., GAGNON J. & PORTER R.R. (1980) Partial sequence of human complement component factor B: novel type of serine protease. *Proc. natn. Acad. Sci. U.S.A.* **77,** 4923.

COOPER N.R. (1973) Formation and function of a complex of the C3 proactivator with a protein from cobra venom. *J. exp. Med.* **137,** 451.

DIEMINGER L., VOGT W. & LYNEN R. (1976) Purification and some properties of factor D of the human properdin system. *Z. Immun. Forsh.* **152,** 231.

FEARON D.T., AUSTEN K.F. & RUDDY S. (1974) Properdin factor D: characterization of its active site and isolation of the precursor form. *J. exp. Med.* **139,** 355.

FUJII S. & HITOMI Y. (1981) New synthetic inhibitors of C1r, C1 esterase, thrombin, plasmin, kallikrein and trypsin. *Biochim. biophys. Acta*, **661,** 342.

GÖTZE O. & MÜLLER-EBERHARD H.J. (1971) The C3-activator system: an alternate pathway of complement activation. *J. exp. Med.* **134,** 90s.

IKARI N., HITOMI Y., NIINOBE M. & FUJII S. (1983) Studies on esterolytic activity of alternative complement component factor B. *Biochim. biophys. Acta*, **742,** 318.

IKARI N., NIINOBE M. & FUJII S. (1981) Proteolysis of factor B by plasma kallikrein and plasmin. *FEBS Letters*, **131,** 143.

JANATOVA J., LORENZ P.E., SCHECHTER A.N., PRAHL J.W. & TACK B.F. (1980) Third component of human complement: appearance of a sulfhydryl group following chemical or enzymatic inactivation. *Biochemistry*, **19,** 4471.

LESAVRE P.H., HUGLI T.E., ESSER A.F. & MÜLLER-EBERHARD H.J. (1979) The alternative pathway C3/C5 convertase: chemical basis of factor B activation. *J. Immunol.* **123,** 529.

MEDICUS R.G., GÖTZ O. & MÜLLER-EBERHARD H.J. (1976) The serine protease nature of the C3 and C5 convertases of the classical and alternative complement pathway. *Scand. J. Immunol.* **5,** 1049.

MOLE J.E. & NIEMANN M.A. (1980) Structural evidence that complement factor B constitutes a novel class of serine protease. *J. biol. Chem.* **255,** 8472.

PEPYS M.B., TOMPKINS C. & SMITH A.D. (1979) An improved method for the isolation from *Naja Naja* venom of cobra factor (CoF) free of phospholipase A. *J. immunol. Meth.* **30,** 105.

PLATTS-MILLS T.A.E. & ISHIZAKA K. (1974) Activation of the alternate pathway of human complement by rabbit cells. *J. Immunol.* **113,** 348.

TACK B.F. & PRAHL J.W. (1976) Third component of human complement: purification from plasma and physicochemical characterization. *Biochemistry*, **15,** 4513.

TAKADA Y., ARIMOTO Y., MINEDA H. & TAKADA A. (1978) Inhibition of the classical and alternative pathways by amino acids and their derivatives. *Immunology*, **34,** 509.

VOGT W., DAMES W., SCHMIDT G. & DIEMINGER L. (1977) Complement activation by the properdin system: formation of a stoichiometric, C3 cleaving complex of properdin factor B with C3b. *Immunochemistry*, **14,** 201.

VOGT W., DIEMINGER L., LYNEN R. & SCHMIDT G. (1974) Formation and composition of the complex with cobra venom factor that cleaves the third component of complement. *Hoppe-Seyler's Z. physiol. Chem.* **355,** 171.

YAMASHITA N., NAGASAWA S. & KOYAMA J. (1981) Seikagaku **53,** 674 (authors' abstract in Japanese).