Eur. J. Biochem. *164*, 637–642 (1987)

Studies on the effect of serine protease inhibitors on activated contact factors

Application in amidolytic assays for factor XII_a, plasma kallikrein and factor XI_a

Guido TANS[1], Truus JANSSEN-CLAESSEN[1], Jan ROSING[1] and John H. GRIFFIN[2]

[1] Department of Biochemistry, University of Limburg, Maastricht
[2] Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California

(Received October 30, 1986/January 19, 1987) – EJB 86 1152

Amidolytic assays have been developed to determine factor XII_a, factor XI_a and plasma kallikrein in mixtures containing variable amounts of each enzyme. The commercially available chromogenic *p*-nitroanilide substrates Pro-Phe-Arg-NH-Np (S2302 or chromozym PK), Glp-Pro-Arg-NH-Np (S2366), Ile-Glu-(piperidyl)-Gly-Arg-NH-Np (S2337), and Ile-Glu-Gly-Arg-NH-Np (S2222) were tested for their suitability as substrates in these assays. The kinetic parameters for the conversion of S2302, S2222, S2337 and S2366 by β factor XII_a, factor XI_a and plasma kallikrein indicate that each active enzyme exhibits considerable activity towards a number of these substrates. This precludes direct quantification of the individual enzymes when large amounts of other activated contact factors are present. Several serine protease inhibitors have been tested for their ability to inhibit those contact factors selectively that may interfere with the factor tested for. Soybean trypsin inhibitor very efficiently inhibited kallikrein, inhibited factor XI_a at moderate concentrations, but did not affect the amidolytic activity of factor XII_a. Therefore, this inhibitor can be used to abolish a kallikrein and factor XI_a contribution in a factor XII_a assay. We also report the rate constants of inhibition of contact activation factors by three different chloromethyl ketones. D-Phe-Pro-Arg-CH_2Cl was moderately active against contact factors ($k = 2.2 \times 10^3$ M^{-1} s^{-1} at pH 8.3) but showed no differences in specifity. D-Phe-Phe-Arg-CH_2Cl was a very efficient inhibitor of plasma kallikrein ($k = 1.2 \times 10^5$ M^{-1} s^{-1} at pH 8.3) whereas it slowly inhibited factor XII_a ($k = 1.4 \times 10^3$ M^{-1} s^{-1}) and factor XI_a ($k = 0.11 \times 10^3$ M^{-1} s^{-1}). Also Dns-Glu-Gly-Arg-CH_2Cl was more reactive towards kallikrein ($k = 1.6 \times 10^4$ M^{-1} s^{-1}) than towards factor XII_a ($k = 4.6 \times 10^2$ M^{-1} s^{-1}) and factor XI_a ($k = 0.6 \times 10^2$ M^{-1} s^{-1}). Since Phe-Phe-Arg-CH_2Cl is highly specific for plasma kallikrein it can be used in a factor XI_a assay selectively to inhibit kallikrein. Based on the catalytic efficiencies of chromogenic substrate conversion and the inhibition characteristics of serine protease inhibitors and chloromethyl ketones we were able to develop quantitative assays for factor XII_a, factor XI_a and kallikrein in mixtures of contact activation factors.

Contact activation of human plasma results in the conversion of the zymogens factor XII, plasma prekallikrein and factor XI into the active serine proteases factor XII_a, kallikrein and factor XI_a (for reviews see [1, 2]. During the initial stages of contact activation the zymogens factor XII and prekallikrein participate in a so-called reciprocal activation mechanism in which factor XII_a activates prekallikrein to kallikrein, which in its turn converts factor XII into factor XII_a [3]. Activation of factor XII is further enhanced by autoactivation of the zymogen factor XII by its own active form α-factor XII_a [4, 5]. Factor XII_a is also the enzyme responsible for the activation of factor XI [6, 7]. Efficient contact activation requires the presence of negatively charged surfaces, such as sulfatides or dextran sulfate, and the non-enzymatic protein cofactor high-molecular-mass kininogen [8, 9]. Insight into the mode of action of these cofactors has been gained mostly by studying the separate zymogen activations. In such cases catalytic amounts of enzyme are added to follow the activation of excess of zymogen. Since in these reactions a number of positive and negative feedback mechanisms occur additional information about contact activation is obtained by studying the activation of combinations of zymogens. In these cases large amounts of more than one activated contact factor are formed and specific assays are required to quantify each factor without interferences by the other enzymes present.

In recent years a number of so-called chromogenic substrates have been introduced that can be used to set up such assays. Chromogenic substrates are, however, not fully specific for a single coagulation factor but can in many cases be converted by more than one coagulation factor. This prevents a direct and accurate quantification of factor XII_a, plasma kallikrein and factor XI_a in reaction mixtures that contain large amounts of each enzyme. In this paper we show chromogenic substrates can be used in combination which inhibitors to develop assays for the quantification of each

Correspondence to G. Tans, Capaciteitsgroep Biochemie, Biomedisch Centrum, Rijksuniversiteit Limburg, Postbus 616, NL-6200-MD Maastricht, The Netherlands

Abbreviations. Glp, pyroglutamic acid; -NH-Np, -*p*-nitroanilide; Dns, 5-dimethylaminonaphtalene-1-sulphonyl; S2302, D-Pro-Phe-Arg-NH-Np; S2366, Glp-Pro-Arg-NH-Np; S2222, Ile-Glu-Gly-Arg-NH-Np; S2337, Ile-Glu-(piperidyl)-Gly-Arg-NH-Np.

Enzymes. Plasma kallikrein (EC 3.4.21.34); coagulation factor XI_a (EC 3.4.21.27); coagulation factor XII_a (EC 3.4.21.38).

individual protein in the presence of other activated contact factors.

MATERIALS AND METHODS

Materials

All reagents used were of the highest grade commercially available. Chemicals used in the purification of factor XII, factor XI and kallikrein were obtained from sources described previously [10–14]. Ovalbumin, soybean trypsin inhibitor, lima bean trypsin inhibitor and benzamidine/HCl were from Sigma Chemicals (St Louis, MO). The chloromethyl ketones D-Phe-Pro-Arg-CH_2Cl, D-Phe-Phe-Arg-CH_2Cl and Dns-Glu-Gly-Arg-CH_2Cl were obtained from Calbiochem-Behring Corp. The chromogenic substrates Ile-Glu-Gly-Arg-NH-Np (S2222), D-Pro-Phe-Arg-NH-Np (S2302), Ile-Glu-(piperidyl)-Gly-Arg-NH-Np (S2337) and Glp-Pro-Arg-NH-Np (S2366) were purchased from AB Kabi Diagnostica (Stockholm, Sweden). Chromogenic substrate concentrations were determined from their absorbance at 316 nm using a molar absorption coefficient of 13000 M^{-1} cm^{-1} as indicated by the manufacturer. Concentrations of chloromethyl ketones were determined by nitrogen analysis according to Ward et al. [15] using an Antek digital nitrogen detector model 720 coupled to an Antek pyroreactor 771. 1.6 ml polyethylene snap-cap centrifuge tubes from Sarstedt were cleared of protein adsorption by incubation for at least 6 h with reaction buffer containing 5 mg/ml ovalbumin. After this incubation the tubes were washed twice with distilled water and dried before use. This treatment was necessary to entirely recover protein reactants from the reaction tubes.

Proteins

Human factor XII was purified using a modification of the original procedure of Griffin and Cochrane [10] described by van der Graaf et al. [11]. Factor XI was purified according to Bouma et al. [12] and plasma prekallikrein was purified as described by van der Graaf et al. [11]. β-Factor XII_a was prepared by incubation of factor XII with trypsin followed by DEAE-Sephadex chromatography [13]. Kallikrein and factor XI_a were prepared from prekallikrein and factor XI with β-factor XII_a as described by van der Graaf et al. [11, 14]. The protein preparations were homogeneous and pure as judged by gel electrophoresis in the presence of sodium dodecyl sulfate. β-Factor XII_a appeared as a closely spaced doublet (30 kDa) on non-reduced gels, which upon reduction resulted in a single band [16]. Kallikrein showed the characteristic closely spaced doublet (85 kDa) on non-reduced gels, which separated on reduced gels in one heavy (50 kDa) and two light chains (35 kDa and 33 kDa). Factor XI_a appeared as a single protein band at 160 kDa, which upon reduction resulted in heavy and light chains of 50 kDa and 30 kDa respectively.

Electrophoresis was performed on 10% (5% stack) polyacrylamide gels in the presence of sodium dodecyl sulfate as described by Laemmli [17].

Protein preparations were stored at −80 °C in storage buffer consisting of 4 mM sodium acetate, 2 mM acetic acid, 150 mM NaCl, 1 mM EDTA and 0.02% sodium azide at pH 5.3.

Protein concentrations were determined according to Lowry et al. [18] using bovine serum albumin as a standard. The molar concentrations of coagulation factors were calculated assuming the following molecular masses: 160 kDa for factor XI_a [6]; 85 kDa for kallikrein [13, 19] and 30 kDa for β-factor XII_a [16, 20, 21].

Chromogenic substrate hydrolysis

The rate of substrate hydrolysis by activated contact factors was routinely determined at 37 °C in 1-ml semimicro polystyrene Sarstedt cuvettes (1 cm light path) in a total volume of 500 µl buffer containing 100 mM Tris/HCl (pH 8.3 at 37 °C), 150 mM NaCl, 0.5 mg/ml ovalbumin and appropriate amounts of enzyme and substrate. From the rate of change in absorbance at 405 – 500 nm, determined using an Amino-DW2C spectrophotometer set in the dual-wavelength mode, the rate of *p*-nitroaniline formation was calculated using a molar absorption coefficient of 9900 M^{-1} cm^{-1} [22]. Further experimental details are given in the legends to the tables and figures.

RESULTS

Kinetic parameters of chromogenic substrate conversion by contact factors

A number of commercially available chromogenic substrates can be used to quantify activated coagulation factors. For contact activation proteins D-Pro-Phe-Arg-NH-Np (S2302) has been developed for kallikrein and Glp-Pro-Arg-NH-Np (S2366) appears to be a good substrate for factor XI_a. Although there are as yet no specific chromogenic substrates for factor XII_a it has been reported that this enzyme readily cleaves S2302 and the factor X_a substrates S2337 and S2222 [5, 23].

No complete data are available on the activities of each of the activated contact factors on each of these substrates. Therefore, we have determined the kinetic parameters of the hydrolysis of the above-mentioned chromogenic substrates by β-factor XII_a, factor XI_a and kallikrein at pH 7.2 and at pH 8.3 at 37 °C.

The kinetic parameters were obtained from Lineweaver-Burk plots of the rates of *p*-nitroaniline formation measured at substrate concentrations ranging from 50 µM to 1 mM. In those cases where the K_m was well above 1 mM only k_{cat}/K_m values are given. Table 1 summarizes the data obtained. In aggreement with the literature, S2302 was the best substrate for kallikrein and S2366 was most suitable for factor XI_a. The best substrate for factor XII_a, as judged by the k_{cat}/K_m value, was S2302. However, S2337, although less readily converted by factor XII_a, is to be preferred for factor XII_a determination since with this substrate the interference of kallikrein will be much less. Kallikrein has also a high activity towards S2366 and, therefore, poses a problem in the quantification of factor XI_a. As can be seen all three enzymes exhibited a higher catalytic efficiency (k_{cat}/K_m) at pH 7.2 than at pH 8.3. No differences in specificity were observed, however, between pH 7.2 and pH 8.3. Since the decrease in activity at pH 8.3 was the most pronounced for kallikrein chromogenic assays of factor XII and factor XI should preferably be carried out at pH 8.3. It can be seen from the data in Table 1 that although reasonable specificity can be obtained specific inhibitors are necessary in order to quantify the amounts of factor XII_a, factor XI_a and kallikrein present in reaction mixtures that contain substantial amounts of all three enzymes.

Inhibition by the reversible inhibitor benzamidine

The inhibitor constant (K_i) of inhibition of factor XII_a, factor XI_a and kallikrein by the reversible inhibitor

Table 1. *Kinetic constants of substrate conversion by factor XI_a, β-factor XII_a and plasma kallikrein*
The kinetic parameters (K_m and k_{cat}) for conversion of the chromogenic substrates S2302, S2222, S2337 and S2366 by β-factor XII_a, factor XI_a or plasma kallikrein were determined as follows. 20 µl enzyme in storage buffer containing 0.5 mg/ml ovalbumin was added to 430 µl reaction buffer in a 1-ml semimicro polystyrene Sarstedt cuvette (1 cm light path) at 37 °C. Reaction was started with the addition of 50 µl water containing varying amounts of chromogenic substrate. The hydrolysis of substrate was monitored using an Amino-DW2C spectrophotometer set in the dual-wavelenght mode at 405 – 500 nm. The final concentrations reached in the cuvette were: 100 mM Hepes (pH 7.2 at 37 °C) or 100 mM Tris/HCl (pH 8.3 at 37 °C), 150 mM NaCl, 0.5 mg/ml ovalbumin, 5 nM β-factor XII_a, 0.5 nM factor XI_a or 0.5 nM plasma kallikrein and varying amounts of chromogenic substrate. The rate of *p*-nitroaniline formation was calculated from the absorbance change at 405 – 500 nm using a molar absorption coefficient of 9900 M^{-1} cm^{-1} [22]. From the rates determined at varying substrate concentrations Lineweaver-Burk plots were constructed from which K_m and V_{max} were determined by statistical analysis according to Eisenthal and Cornish-Bowden [24]. k_{cat} was subsequently calculated by division of V_{max} with the concentration of enzyme present in the cuvette. In the case of factor XI_a k_{cat} was calculated per dimer of 160 kDa

pH	Chromogenic substrate		Factor XI_a			β-factor XII_a			Kallikrein		
			K_m	k_{cat}	$10^{-3} \times k_{cat}/K_m$	K_m	k_{cat}	$10^{-3} \times k_{cat}/K_m$	K_m	k_{cat}	$10^{-3} \times k_{cat}/K_m$
			mM	s^{-1}	M^{-1} s^{-1}	mM	s^{-1}	M^{-1} s^{-1}	mM	s^{-1}	M^{-1} s^{-1}
7.2	D-Pro-Phe-Arg-NH-Np	(S2302)	–	–	5.2	0.065	20.1	309	0.15	320	2133
	Glp-Pro-Arg-NH-Np	(S2366)	0.43	333	774	–	–	21.6	0.43	173	402
	Ile-Glu-Gly-Arg-NH-Np	(S2222)	–	–	56.6	0.44	22.4	51.4	–	–	16.3
	Ile-Glu-(piperidyl)-Gly-Arg-NH-Np	(S2337)	–	–	59.6	0.19	21.4	113	0.43	13.3	31.1
8.3	D-Pro-Phe-Arg-NH-Np	(S2302)	–	–	4.2	0.17	34	200	0.43	438	1019
	Glp-Pro-Arg-NH-Np	(S2366)	1.0	595	595	–	–	19.2	–	–	208
	Ile-Glu-Gly-Arg-NH-Np	(S2222)	–	–	38.0	0.53	24	45.2	–	–	8.8
	Ile-Glu-(piperidyl)-Gly-Arg-NH-Np	(S2337)	–	–	35.8	0.25	26	104.0	0.85	12	14.1

Table 2. *Inhibitor constant (K_i) for the inhibition of β-factor XII_a, factor XI_a and plasma kallikrein by the reversible inhibitor benzamidine*
The inhibitor constant (K_i) of benzamidine was obtained by measuring the rate of substrate hydrolysis by β-factor XII_a, factor XI_a or plasma kallikrein at varying concentrations of chromogenic substrate in the absence and presence of varying amounts of benzamidine. Rates of *p*-nitroaniline formation were dertermined in a total volume of 500 µl in 1-ml semimicro cuvettes at pH 7.2 or at pH 8.3 as described in the legend to Table 1. S2302 was used as a substrate for β-factor XII_a or kallikrein and S2366 was used for factor XI_a. From the rates of substrate hydrolysis thus determined at different substrate concentrations in the presence of varying benzamidine concentrations, Dixon plots [25] were constructed to calculate the K_i for inhibition by benzamidine

Enzyme	K_i	
	pH 7.2	pH 8.3
	mM	
Factor XI_a	0.22	0.43
β-Factor XII_a	1.12	0.85
Kallikrein	0.24	0.44

benzamidine was determined at 37 °C at pH 7.2 and pH 8.3. In all cases straight Dixon plots [25] were obtained from which K_i was determined (data not shown). Table 2 summarizes the K_i values obtained. At pH 7.2 kallikrein and factor XI_a had the same K_i for benzamidine (0.22 – 0.24 mM), which was about five times lower than the K_i for factor XII_a. At pH 8.3 the K_i values for factor XI_a and kallikrein increased twofold and there was a slight decrease in the K_i for β-factor XII_a. The differences in K_i between kallikrein, factor XI_a and β-factor XII_a were not large enough for selective inhibition of interfering enzymes in a chromogenic assay. However, the different sensitivities towards benzamidine may be of use to test whether factor XII_a or one of the other two enzymes is responsible for a certain observed enzymatic activity (cf. [5]).

Inhibition of contact activation factors by soybean and lima bean trypsin inhibitor

Soybean trypsin inhibitor is a well-known inhibitor of a number of serine proteases of the blood coagulation system. With respect to contact activation soybean trypsin inhibitor has been reported to inhibit kallikrein as well as factor XI_a [26, 27]. Fig. 1 shows the decrease of the amidolytic activity of activated contact factors upon incubation with varying amounts of inhibitor for 1 min at pH 8.3. Inhibition of kallikrein was complete at concentrations higher than 20 µg/ml. Inhibition of factor XI_a was significantly less than that observed for kallikrein. The amidolytic activity of factor XII_a was not affected by concentrations of inhibitor that completely blocked kallikrein and that inhibited Factor XI_a for more than 70%. At very high soybean inhibitor concentrations, however, some inhibition of factor XII_a was observed. Thus soybean trypsin inhibitor can be effectively used to inhibit the amidolytic activities of kallikrein and factor XI_a (partly) in a factor XII_a assay.

Lima bean trypsin inhibitor did not inhibit kallikrein, had a poor reactivity towards factor XII_a and readily inhibited the amidolytic activity of factor XI_a (Fig. 1B). The latter finding contrasts with earlier reports in literature that the clotting activity of factor XI_a is not affected by lima bean trypsin inhibitor [26, 27]. As yet we have no explanation for this discrepancy. The inhibition characteristics of our lima bean trypsin inhibitor preparation were such that it can be used to prevent factor XI_a from contributing in a kallikrein assay. For inhibition of factor XII_a such high concentrations of lima bean trypsin inhibitor are required that it is not of practical use.

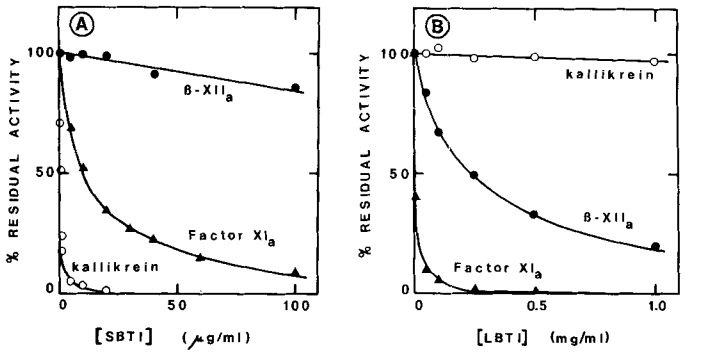

Fig 1. *Inhibition of activated contact factors by soybean and by lima bean trypsin inhibitor.* 430 μl buffer, containing 100 mM Tris/HCl (pH 8.3 at 37°C), 150 mM NaCl, 0.5 mg/ml ovalbumin and varying amounts of (A) soybean trypsin inhibitor (SBTI) or (B) lima bean trypsin inhibitor (LBTI), were preincubated at 37°C in a 1-ml semimicro cuvette (1 cm pathlength). After 4 min reaction was started with the addition of 20 μl storage buffer containing 2.25 pmol β-factor XII$_a$, 0.225 pmol factor XI$_a$ or 0.225 pmol plasma kallikrein. The final reaction conditions were 95 mM Tris/HCl (pH 8.3 at 37°C), 150 mM NaCl, 0.5 mg/ml ovalbumin, 5 nM β-factor XII$_a$, 0.5 nM factor XI$_a$ or 0.5 nM kallikrein and amounts of trypsin inhibitor as indicated in the figure. After 1 min 50 μl chromogenic substrate (5 mM in water) was added to determine the remaining amidolytic activity. From the initial rate of change in absorbance, determined at 405–500 nm using an Amino DW2C spectrophotometer set in the dual-wavelength mode, the residual amount of free enzyme was calculated and expressed as the percentage of the activity determined in a mixture without inhibitor. The chromogenic substrates used were S2302 for β-factor XII$_a$ (●—●) or kallikrein (○—○) and S2366 for factor XI$_a$ (▲—▲)

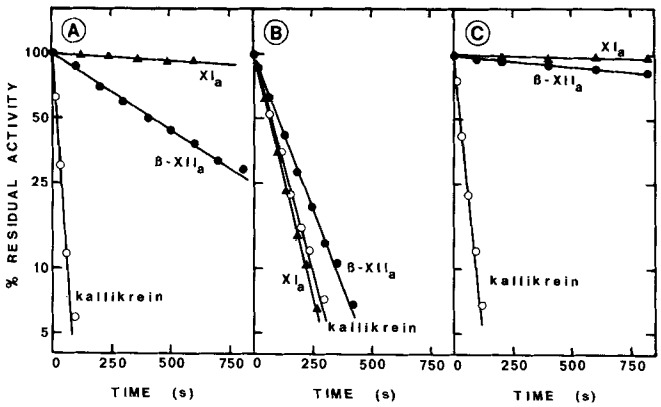

Fig. 2. *Inhibition of activated contact factors by chloromethyl ketones.* β-factor XII$_a$ (50 pmol), factor XI$_a$ (5 pmol) or plasma kallikrein (5 pmol) was incubated for 3 min at 37°C in a total volume of 190 μl in 1.6 ml Sarstedt snap-cap centrifuge tubes. Reaction was started with the addition of 10 μl chloromethyl ketone in 10 mM HCl. The final reaction conditions were 80 mM Tris/HCl (pH 8.3 at 37°C), 120 mM NaCl, 0.5 mM EDTA, 0.5 mg/ml ovalbumin, 0.02% sodium azide and 250 nM β-factor XII$_a$ (●—●), 25 nM factor XI$_a$ (▲—▲), 25 nM kallikrein (○—○) and (A) 2.5 μM Dns-Glu-Gly-Arg-CH_2Cl, (B) 5 μM D-Phe-Pro-Arg-CH_2Cl, (C) 200 nM D-Phe-Phe-Arg-CH_2Cl. At the time intervals indicated in the figure 10-μl samples were withdrawn and assayed for residual amidolytic activity in 1-ml semimicro cuvettes (1 cm pathlength) in a total volume of 500 μl buffer containing 100 mM Tris/HCl (pH 8.3 at 37°C), 150 mM NaCl, 0.5 mg/ml ovalbumin and 380 μM S2302 in the case of β-factor XII$_a$ or kallikrein and 500 μM S2366 in the case of factor XI$_a$. From the rate of change in absorbance, determined at 405–500 nm using an Amino-DW2C spectrophotometer set in the dual-wavelength mode, the percentage of residual free enzyme present in the reaction mixture was calculated. 100% was the activity determined in a similar incubation mixture in the absence of chloromethyl ketone

Inhibition by chloromethyl ketones

Recently a number of chloromethyl ketone inhibitors have become commercially available some of which have also been reported to be potent inhibitors of plasma kallikrein and factor XII$_a$ [28–30]. We have studied the inhibition of the active contact factors by D-Phe-Pro-Arg-CH_2Cl, Dns-Glu-Gly-Arg-CH_2Cl and D-Phe-Phe-Arg-CH_2Cl under pseudo-first-order conditions (excess inhibitor). Fig. 2 shows the pseudo-first-order plots obtained. The rate constants of inhibition, determined from the slopes of these lines, were proportional to the amount of inhibitor present (data not shown) indicating that second-order kinetics were obeyed throughout. Table 3 summarizes the second-order rate constants calculated from these plots. D-Phe-Phe-Arg-CH_2Cl was the inhibitor with the highest specificity. It is an excellent inhibitor of kallikrein and has a rather low reactivity towards factor XII$_a$ and factor XI$_a$. Therefore, this inhibitor can be used to prevent kallikrein from contributing to chromogenic substrate hydrolysis in factor XII$_a$ and factor XI$_a$ assays. The differences in magnitude and the actual values of the rate constants of inhibition of the three activated contact factors by Dns-Glu-Gly-Arg-CH_2Cl were not large enough to be of practical use as a specific inhibitor in a chromogenic assay. The thrombin inhibitor D-Phe-Pro-Arg-CH_2Cl had a low activity towards contact factors and showed no differences in specificity.

Table 3. *Second-order rate constants of inhibition of factor XI$_a$, β-factor XII$_a$ and plasma kallikrein by chloromethyl ketones*
The second-order rate constants of inhibition by chloromethyl ketones at pH 8.3 at 37°C were calculated from the slopes of the pseudo-first-order plots shown in Fig. 2. Experimental details are given in the legend to Fig. 2

Chloromethyl ketone inhibitor	Factor XI$_a$	β-Factor XII$_a$	Kallikrein
	$M^{-1} s^{-1}$		
Dns-Glu-Gly-Arg-CH_2Cl	63	462	15662
D-Phe-Pro-Arg-CH_2Cl	2271	1160	2299
D-Phe-Phe-Arg-CH_2Cl	110	1389	118129

Simultaneous determination of contact factor activation in a mixture of factor XII, factor XI and plasma prekallikrein

The activation of all three contact factor zymogens at pH 7.3 at 37°C was monitored in a reaction mixture containing 250 nM factor XII, 250 nM prekallikrein and 23 nM factor XI. As assay buffer 100 mM Tris (pH 8.3 at 37°C), 150 mM NaCl, 0.5 mg/ml ovalbumin was chosen. Coagulation factor activation was followed by a combination of chromogenic assays. Kallikrein was determined by its amidolytic activity towards S2302 in the absence and presence of 25μg/ml soybean trypsin inhibitor. The activity sensitive for soybean trypsin inhibitor was used to calculate the amount of kallikrein present in the sample. Thus a possible background due to excess amounts of factor XII$_a$ can be corrected for if necessary. No correction is necessary for factor XI$_a$ since this enzyme hardly contributes to S2302 hydrolysis (cf. Table 1). Factor XII$_a$ was determined by measurements of amidolytic activity towards S2337 in the presence of 25 μg/ml

soybean trypsin inhibitor. This inhibitor effectively blocks the background activity of kallikrein and the major part of S2337 hydrolysis by factor XI_a. Finally the amidolytic activity towards S2366 was determined after preincubation of the sample for 2.5 min with 250 nM D-Phe-Phe-Arg-CH_2Cl. This chloromethyl ketone quantitatively blocked S2366 hydrolysis by kallikrein. The low background activity from large amounts of factor XII_a was, however, not influenced by this treatment. Factor XI_a amidolytic activity was, therefore, calculated after substracting the amidolytic activity of factor XII_a towards S2366. The latter activity was calculated from the amounts of factor XII_a determined with S2377 and the catalytic efficiency of S2366 hydrolysis by factor XII_a (Table 1).

Fig. 3 shows the time course of activation in the reaction mixture as it was determined using these assays. Kallikrein formation was very fast and a plateau was reached within 20 min. Factor XII_a amidolytic activity reached a plateau after 3 h whereas factor XI activation was not complete until after 7 h. Investigation of the earlier time points showed that all curves were simoidal with a lag time of 1 – 2 min for kallikrein, of about 5 min for factor XII_a and about 1 h for factor XI_a. This is consistent with the positive feedback mechanism that occurs between factor XII and prekallikrein. Moreover, the fact that factor XI_a formation only started after substantial amounts of factor XII_a had been formed illustrates the inefficient factor XI_a formation by factor XII_a in the absence of surface. The standard deviation in the values for factor XII_a and kallikrein (determined in the plateau) was 7 nM whereas it was 0.6 nM for factor XI_a.

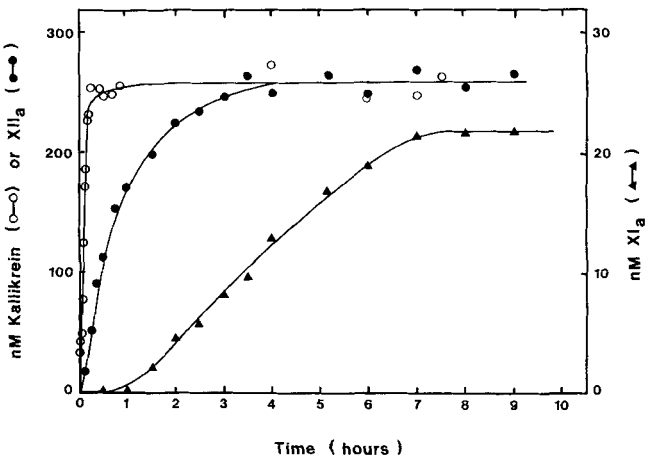

Fig. 3. *Time course of contact factor activation.* 800 µl buffer, containing 225 pmol factor XII, was preincubated at 37 °C in a 1.6-ml Sarstedt snap-cap centrifuge tube. After 5 min reaction was started with the addition of 50 µl storage buffer containing 20.7 pmol factor XI immediately followed by 50 µl storage buffer containing 225 pmol plasma kallikrein. The final reaction conditions were: 100 mM Hepes (pH 7.3 at 37 °C), 150 mM NaCl, 3 mM sodium acetate, 0.5 mM EDTA, 0.5 mg/ml ovalbumin, 250 nM factor XII, 250 nM plasma prekallikrein and 23 nM factor XI. At the time intervals indicated, samples were withdrawn and assayed for contact factor activation by a combination of the following assays: (○—○) Plasma kallikrein was determined after diluting the sample 25-fold in cuvette buffer: 100 mM Tris/HCl (pH 8.3 at 37 °C), 150 mM NaCl, 0.5 mg/ml ovalbumin. 20 µl of this dilution were added to 1-ml semimicro cuvettes with 430 µl cuvette buffer with or without 25 µg/ml soybean trypsin inhibitor present. After an additional 1 min incubation 50 µl S2302 was added to result in a final concentration of 500 µM. The amidolyic activity, sensitive to inhibition by soybean trypsin inhibitor, was used to calculate the amount of plasma kallikrein present in the sample using a calibration curve made under the same conditions with known amounts of plasma kallikrein. (●—●) Factor XII_a was determined by adding 20 µl reaction mixture to a cuvette with 430 µl cuvette buffer containing 25 µg/ml soybean trypsin inhibitor. After an additional 1 min incubation at 37 °C 50 µl S2337 was added to result in a final concentration of 500 µM. The amount of factor XII_a present in the sample was calculated using a calibration curve made under the same conditions with known amounts of β-factor XII_a. (▲—▲) Factor XI_a was determined in a 20-µl sample, which was added to a cuvette with 430 µl cuvette buffer containing 250 nM D-Phe-Phe-Arg-CH_2Cl. After an additional 2.5-min incubation 50 µl S2366 was added to result in a final concentration of 500 µM. The amidolytic activity due to factor XI_a was calculated by subtracting the factor-XII_a-dependent part from the total amidolytic activity measured (the contribution by factor XII_a was calculated from the amount determined as described above and its activity on S2366). The factor XI_a present in the sample was calculated from the residual activity using a calibration curve made under the same conditions with known amounts of factor XI_a

DISCUSSION

The experimental data presented in this paper show that by careful choice of experimental conditions and reagents it is possible to develop methods for the quantification of factor XII_a, factor XI_a and kallikrein in mixtures that contain substantial amounts of each of these enzymes. In order to be of general use we have restricted ourselves to reagents that are commercially available. The chromogenic substrates that we have employed have been shown to be good substrates for activated contact factors. In agreement with the literature S2302 was a good substrate for both kallikrein and factor XII_a, while S2366 was readily hydrolyzed by factor XI_a. S2337 and S2222, chromogenic substrates for factor X_a, can also be used to quantify factor XII_a. However, Table 1 illustrates that the active enzymes show considerable activities towards more than one chromogenic substrate. Therefore, a direct chromogenic assay of a particular contact factor is not possible when substantial amounts of other contact factors are present in the same sample. Kallikrein presents the major source of error in the determination of the other contact factors since it has a relatively high activity towards the chromogenic substrates that have to be used in factor XII_a and factor XI_a assays. Factor XII_a only interferes with kallikrein and factor XI_a assays when present in excess of these contact activation factors, while factor XI_a will hardly interfere with the assays of factor XII_a and kallikrein.

Soybean trypsin inhibitor can be used specifically to inhibit kallikrein in factor XII_a assays. Concentrations of 25 µg/ml completely block kallikrein without affecting factor XII_a. At these soybean trypsin inhibitor concentrations also substantial amounts of factor XI_a are already inhibited. Thus, soybean trypsin inhibitor is an excellent tool in a factor XII_a assay to prevent interference by kallikrein and factor XI_a. It can, however, not be used selectively to block kallikrein in a factor XI_a assay. For this purpose the chloromethyl ketone D-Phe-Phe-Arg-CH_2Cl can be employed. This inhibitor is 1000 times more active towards kallikrein than towards factor XI_a. This means that concentrations of Phe-Phe-Arg-CH_2Cl can be chosen that block the amidolytic activity of kallikrein for more than 99% while the inhibition of factor XI_a will be less than 1%. Thus Phe-Phe-Arg-CH_2Cl can be used to completely block the contribution of kallikrein in a quantitative assay of factor XI_a with S2366.

At present there are no commercially available specific inhibitors of factor XII_a that can be used to inhibit the factor XII_a in samples for a kallikrein or factor XI_a assay. The popcorn trypsin inhibitor [31] may serve this purpose but this inhibitor is not yet commercially available. It is possible, however, to correct for factor XII_a contributions to kallikrein and factor XI_a assays. The amount of factor XII_a present in mixtures of contact activation factors can be calculated by determination of the amidolytic activity towards S2337 or S2302 in the presence of 25 μg/ml soybean trypsin inhibitor. This activity can subsequently be used to calculate and correct for the factor XII_a contributions in kallikrein and factor XI_a assays.

The chromogenic assays were performed at pH 8.3 at 37 °C at a concentration of 150 mM NaCl for the following reasons. At this pH the selectivity for the substrates by factor XII_a and factor XI_a is somewhat favoured since kallikrein amidolytic activity decreases most with increasing pH (see Table 1). More importantly, however, a number of contact activation interactions have been reported to be effectively blocked at pH values higher than pH 8.0 and at high salt concentrations. Thus surface-dependent factor XII activation (both by autoactivation and by kallikrein) is greatly diminished under these conditions [5, 32]. Prekallikrein activation by factor XII_a decreases with increasing salt concentration [33]. Therefore, performing the assays at pH 8.3 and at high salt ensures that these reactions are effectively blocked in the cuvette. A final consideration is that high salt concentrations in the cuvette will prevent large fluctuations in ionic strength when sample size is varied or when the effect of ionic strength in contact activation reactions is studied. It is important to keep this parameter stable since the amidolytic activity of contact factors may be influenced by ionic strenght [23].

We thank AB Kabi Diagnostica for donating part of the chromogenic substrates used in this study. This work was supported in part by the National Institute of Health Grant NHLBI-21544. Guido Tans was supported in part by the Netherlands Organisation for the Advancement of Pure Scientific Research (Z.W.O.).

REFERENCES

1. Griffin, J. H. & Cochrane, C. G. (1982) *Adv. Immunol. 33*, 241–306.
2. Colman, R. W. (1984) *J. Clin. Invest 73*, 1249–1253.
3. Griffin, J. H. (1978) *Proc. Natl Acad. Sci. USA 75*, 1998–2002.
4. Silverberg, M., Dunn, J. T., Garen, L. & Kaplan, A. P. (1980) *J. Biol. Chem. 255*, 7281–7286.
5. Tans, G., Rosing, J. & Griffin, J. H. (1983) *J. Biol. Chem. 258*, 8215–8222.
6. Bouma, B. N. & Griffin, J. H. (1977) *J. Biol. Chem. 252*, 6432–6437.
7. Kurachi, K. & Davie, E. W. (1977) *Biochemistry 16*, 5831–5839.
8. Meier, H. L., Pierce, J. V., Colman, R. W. & Kaplan, A. P. (1977) *J. Clin. Invest. 60*, 18–31.
9. Griffin, J. H. & Cochrane, C. G. (1976) *Proc. Natl Acad. Sci. USA 73*, 2554–2558.
10. Griffin, J. H. & Cochrane, C. G. (1976) *Methods Enzymol. 45*, 56–65.
11. van der Graaf, F., Tans, G., Bouma, B. N. & Griffin, J. H. (1982) *J. Biol. Chem. 257*, 14300–14305.
12. Bouma, B. N., Vlooswijk, R. A. A. & Griffin, J. H. (1983) *Blood 62*, 1123–1131.
13. Bouma, B. N., Miles, L. A., Beretta, G. & Griffin, J. H. (1980) *Biochemistry 19*, 1151–1160.
14. van der Graaf, F., Greengard, J. S., Bouma, B. N., Kerbiriou, D. M. & Griffin, J. H. (1983) *J. Biol. Chem. 258*, 9669–9675.
15. Ward, M. W. N., Owens, C. W. J. & Rennie, M. J. (1980) *Clin. Chem. 26*, 1336–1339
16. Dunn, J. T. & Kaplan, A. P. (1982) *J. Clin. Invest. 70*, 627–631.
17. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680–685.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
19. Mandle, R. Jr & Kaplan, A. P. (1977) *J. Biol. Chem. 252*, 6097–6104.
20. Revak, S. D., Cochrane, C. G., Johnson, A. R. & Hugli, T. E. (1974) *J. Clin. Invest. 54*, 619–627.
21. Fujikawa, K. & McMullen, B. (1983) *J. Biol. Chem. 258*, 10924–10933.
22. Hemker, H. C. (1983) *Handbook of synthetic substrates for the coagulation and fibrinolytic system*, p. 31, Martinus Nijhoff, Boston.
23. Hojima, Y., Tankersley, D. L., Miller-Anderson, M., Pierce, J. V. & Pisano, J. J. (1980) *Thromb. Res. 18*, 417–430.
24. Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715–720.
25. Dixon, M. (1953) *Biochem. J. 55*, 170–171.
26. Wuepper, K. D. & Cochrane, C. G. (1972) *J. Exp. Med. 135*, 1–20.
27. Heck, L. W. & Kaplan, A. P. (1974) *J. Exp. Med. 140*, 1615–1630.
28. Kettner, C. & Shaw, E. (1978) *Biochemistry 17*, 4778–4784.
29. Kettner, C. & Shaw, E. (1981) *Methods Enzymol. 80*, 826–842.
30. Silverberg, M. & Kaplan, A. P. (1982) *Blood 60*, 64–70.
31. Hojima, Y., Pierce, J. V. & Pisano, J. J. (1980) *Thromb. Res. 20*, 149–162.
32. Rosing, J., Tans, G. & Griffin, J. H. (1985) *Eur. J. Biochem. 151*, 531–538.
33. Tankersley, D. L., Fournel, M. A. & Schroeder, D. D. (1980) *Biochemistry 19*, 3121–3127.