The synthesis, kinetic characterization and application of biotinylated aminoacylchloromethanes for the detection of chymotrypsin and trypsin-like serine proteinases

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The synthesis of two biotinylated affinity labels for chymotrypsin and trypsin-like serine proteinases is described, along with their kinetic characterization and application to the detection of these proteinases after PAGE and Western blotting. Thus the chloromethane analogues biotinylphenylalanylchloromethane (Bio-Phe-CH₂Cl; reagent 1) and biotinyl-arginylchloromethane (Bio-Arg-CH₂Cl; reagent 2), have been shown to be potent active-site-directed inactivators of chymotrypsin and trypsin respectively. The apparent overall second-order rate constants ($k_{\text{obs}}/[I]$) for the inactivation of chymotrypsin and trypsin by reagent 1 ($\sim 4.9 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$) and reagent 2 ($\sim 1.0 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$) respectively are comparable with those obtained by other workers with simple urethane-protected analogues and demonstrates that the presence of the bulky biotinyl moiety is compatible with inhibitor effectiveness. Samples of chymotrypsin and trypsin that have been inactivated by reagents 1 and 2 respectively and which have been subjected to SDS/PAGE and Western blotting can be revealed with a streptavidin/alkaline phosphatase label. We can presently detect down to 20 ng of inactivated proteinase by using this system. The utility of the arginine derivative for the detection of the plasma trypsin-like proteinases plasmin and thrombin has also been demonstrated, thus holding out the possibility that this reagent may find general application as an active-site-directed label for this class of proteinase.

INTRODUCTION

Proteinase inhibitors incorporating radiolabels have been prepared for the study of prohormone-processing enzymes (Docherty *et al.*, 1984; Cromlish *et al.*, 1985) and for the identification of active forms of cysteine proteinases (Mason *et al.*, 1989) and serine proteinases (Raubert *et al.*, 1988). Fluorescently labelled inhibitors have been described for labelling tumour-associated proteinases on whole cells (Kozlowski *et al.*, 1984; Steven *et al.*, 1986).

The use of biotinylated affinity labels offers advantages over these techniques as it allows the exploitation of the biotin/streptavidin system for a variety of applications. For example, a biotinylated probe could be used to detect a proteinase blotted from a polyacrylamide gel by detection methods such as streptavidin/peroxidase (Guesdon *et al.*, 1979; Yolken *et al.*, 1983) or streptavidin/alkaline phosphatase (O'Connor & Ashman, 1982). Or, in a similar vein, detection of proteinases on cells or in tissue sections could be achieved using streptavidin/fluorescein (Childs & Unabid, 1982), streptavidin/Texas Red (Berger *et al.*, 1982) for u.v.-microscopy or streptavidin/colloidal gold (Linner *et al.*, 1986) for electron microscopy.

Here we describe the synthesis of two biotinylated peptidyl-chloromethanes, examine their inhibitory activity towards several serine proteinases, and demonstrate their utility for the detection of proteolytic activity in polyacrylamide gels.

EXPERIMENTAL

Materials

The fluorogenic substrates benzyloxycarbonylarginyl-4-methylcoumarin-7-ylamide (Cbz-Arg-NH-Mec), Glutaryl-Phe-NH-Mec (Zimmerman *et al.*, 1977), t-butoxycarbonyl (Boc)-Val-Pro-Arg-NH-Mec (Morison *et al.*, 1977) and Boc-Val-Leu-Lys-

NH-Mec (Kato *et al.*, 1977) were purchased from Bachem Chemicals, Budendorf, Switzerland.

The protected amino acid Boc-Phe-OH was purchased from Biosyn, Belfast, Northern Ireland, U.K. *N*- α -Boc-*N*-G-bis-Cbz-Arg-OH was purchased from Bachem Chemicals. All other chemicals used in the synthesis of the affinity labels were purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Tosylphenylalanylchloromethane ('TPCK')-treated trypsin, thrombin, plasmin and chymotrypsin [thrice-recrystallized] were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Thrombin was stored in phosphate buffer containing 200 mM-NaCl and 0.25% (w/v) poly(ethylene glycol) 6000 as described by Lottenberg *et al.* (1981).

Phosphate-buffered saline (PBS) was obtained in tablet form from Oxoid, Basingstoke, Hants., U.K. Tris-buffered saline (TBS) was 10 mM-Tris/HCl, containing 0.8% (w/v) NaCl. Streptavidin/alkaline phosphatase was purchased from Boehringer Mannheim U.K., Lewes, East Sussex, U.K. Pre-stained molecular-mass markers were from Bethesda Research Laboratories, Paisley, Renfrewshire, Scotland, U.K. Nitro Blue Tetrazolium, 5-bromo-4-chloroindolyl phosphate (Horwitz, *et al.*, 1966), and di-isopropyl fluorophosphate (iPr₂P-F) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Nitro-cellulose (0.2 μm pore size) was obtained from Schleicher and Schüll, Dassel, Germany.

Synthesis of peptidylchloromethanes

N- α -Bio-*N*-G-bis-Cbz-L-Arg. Isobutyl chloroformate (137 μl , 1.10 mmol) was added to a suspension of D-biotin (293 mg, 1.20 mmol) and *N*-methylmorpholine (262 μl , 2.40 mmol) in dimethylformamide (DMF)/tetrahydrofuran (1:1, v/v; 15 ml) at -20°C . After 10 min 3-amino-6-(*N*-bis-Cbz-guanidino)-1-

Abbreviations used: Bio, biotinyl; Cbz, benzyloxycarbonyl; Boc, t-butoxycarbonyl; Mec, 4-methylcoumarin-7-yl; iPr₂P-F, di-isopropyl fluorophosphate; RFU, relative fluorescence units; -CH₂Cl, chloromethane; DMF, dimethylformamide; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

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chlorohexan-2-one hydrochloride [559 mg, 1.1 mmol; prepared by the method of Alpin *et al.* (1983)] in DMF (5 ml) was added. The reaction mixture was stirred for 1 h at -20°C and then for 2 h at room temperature. Ethyl acetate (150 ml) and saturated NaHCO_3 solution (100 ml) were added, the phases were separated, and the organic phase was washed with additional saturated NaHCO_3 (6×100 ml), 10% citric acid (2×100 ml) and 0.1 M-NaCl (2×100 ml). The organic phase was then dried over anhydrous MgSO_4 , filtered and evaporated, yielding an amorphous solid (356 mg, 51%). The product was adjudged pure by t.l.c. (R_F 0.31, chloroform/methanol (19:1, v/v); R_F 0.45, chloroform/methanol (4:1, v/v) and was used directly in the next stage.

Bio-Arg-CH₂Cl. To a solution of liquid HF (15 ml) containing *p*-cresol (200 μl) and anisole (250 μl) was added 3-(*N*-Bio-amido)-6-(bis-Cbz-guanidino)-1-chlorohexan-2-one (698 mg, 0.1 mmol) at 0°C . The mixture was stirred at 0°C for 1.5 h. The HF was then removed by evaporation under reduced pressure and the residue was dissolved in trifluoroacetic acid (5 ml) containing anisole (200 μl). The product was precipitated by addition of dry diethyl ether (15 ml). The mixture was centrifuged and the solvent decanted. The residue was washed thrice with dry diethyl ether, collected by centrifugation, and then dried under high vacuum, yielding a colourless solid (350 mg, 64%), m.p. $110\text{--}113^{\circ}\text{C}$ (decomposition) (Found: C, 41.65; H, 5.28; N, 15.10; Cl, 6.28; S, 5.61; $\text{C}_{19}\text{H}_{30}\text{N}_6\text{O}_5\text{Cl}_{11}\text{F}_3\text{S}_1$ requires C, 41.72; H, 5.48; N, 15.37; Cl, 6.49; S, 5.85).

Bio-Phe-CH₂Cl. Boc-phenylalanyldiazomethane and Phe-CH₂Cl hydrochloride were synthesized essentially by procedures outlined by Green & Shaw (1981). The yield of diazomethane was 92.5% [R_F (chloroform/methanol, 4:1, v/v) 0.88; retention time on h.p.l.c., 12.1 min (C_{18} analytical column; gradient 0–90% acetonitrile in 15 min)].

The Boc group was removed by treatment of the above compound with methanolic HCl to yield Phe-CH₂Cl hydrochloride as a yellow amorphous solid (71.2% yield), m.p. $103\text{--}105^{\circ}\text{C}$, in close agreement with the literature value (Anglikier *et al.*, 1989). R_F (chloroform/methanol, 1:1, v/v) 0.69; R_F (chloroform/methanol, 4:1, v/v) 0.71; retention time on h.p.l.c., 7.3 min (C_{18} analytical column, gradient 0–100% acetonitrile in 10 min). The product was adjudged pure by t.l.c. and h.p.l.c. Isobutyl chloroformate (274 μl , 2.1 mmol) was added to a suspension of D-biotin (500 mg, 2 mmol) and *N*-methylmorpholine (440 μl , 4 mmol) in DMF (35 ml) at -20°C . After 10 min a solution of Phe-CH₂Cl hydrochloride (512 mg, 2.2 mmol) in DMF (5 ml) was added and the reaction mixture left stirring at ambient temperature overnight. The solvents were removed *in vacuo* and the resulting yellowish solid suspended in ethyl acetate and washed with saturated NaHCO_3 (3×50 ml), 10% NaCl (3×50 ml) and water. After drying over anhydrous MgSO_4 , and removal of solvent, a white solid was obtained which was purified by semi-preparative h.p.l.c. (Waters R.C.M. 8 mm $\times$ 10 cm C_{18} cartridge; retention time 11.83 min; gradient 0–100% acetonitrile in 10 min) to yield Bio-Phe-CH₂Cl (overall yield 25%).

Kinetic techniques

Inactivation studies. Inactivation of trypsin and thrombin by Bio-Arg-CH₂Cl. Trypsin (10 μl of a 0.1 μM stock solution) was added to a solution (1 ml) of Cbz-Arg-NH-mec (100 μM) and Bio-Arg-CH₂Cl (0.1–10 μM) in 200 mM-Tris/HCl buffer, pH 7.8, containing 200 mM-NaCl and 1 mM- CaCl_2 maintained at 37°C . The rate of hydrolysis of substrate was monitored continuously by measuring the rate of increase of fluorescence at 455 nm ($\lambda_{\text{excitation}}$ 383 nm) in a Perkin-Elmer MPF-4413 spectrofluorimeter.

The inactivation of thrombin was carried out in an exactly analogous manner, except that the substrate, Boc-Val-Pro-Arg-NH-Mec (5 μM), was used to monitor the activity of the enzyme.

Inactivation of plasmin by Bio-Arg-CH₂Cl. Bio-Arg-CH₂Cl in 1 mM-HCl was added to a solution of plasmin (0.1–1 μM) in 200 mM-Tris/HCl buffer, pH 7.8, containing 20 mM-NaCl and 1 mM- CaCl_2 at 37°C such that the final concentration of inhibitor was 50–250 μM . Samples (~ 50 μl) were removed at timed intervals and assayed for residual activity with Boc-Val-Leu-Lys-NH-Mec. The dilution involved in setting up the assay effectively stopped further reaction with inhibitor.

Inactivation of chymotrypsin by Bio-Phe-CH₂Cl. Chymotrypsin (10 μl of a 0.1 μM stock solution) was assayed in the presence of glutaryl-Phe-NH-Mec (75 μM) and inhibitor (0.5–12 μM) as described for trypsin and thrombin above. In this instance the buffer contained 5% (v/v) DMF to aid the solubility of the inhibitor.

Determination of K_m and V_{max} for the fluorogenic substrates. To determine the K_m and V_{max} for the substrates used in the present studies, substrate concentrations spanning a range 0.2–5 times the K_m were used. For each enzyme/substrate pair studied, it was ensured that the determination of the kinetic constants were carried out under the exact conditions used to monitor the inactivation processes described in the preceding section. The rate of substrate hydrolysis was determined by measuring the rate of increase of fluorescence at 455 nm ($\lambda_{\text{excitation}}$ 383 nm). K_m and V_{max} values were determined by using the least-squares method (Roberts, 1977).

Determination of the operational molarity of the proteinase solutions. Trypsin, plasmin and thrombin were titrated with the spectrofluorometric titrant 4-methylumbelliferyl *p*-guanidinobenzoate, and chymotrypsin by 4-methylumbelliferyl β -trimethylammonium cinnamate according to the procedure of Jameson *et al.*, 1973.

Evaluation of kinetic parameters. The kinetic constants for the irreversible inactivation of chymotrypsin, trypsin and thrombin were determined as previously described (Walker & Elmore, 1984). In this method, data from progress curves for the hydrolysis of a suitable substrate obtained in the presence of an inhibitor are utilized to determine the inhibition constant, K_i , and the first-order rate constant, k_i , for the reaction shown in Scheme 1, where E-I is the inactivated enzyme:

The inhibition of plasmin was evaluated utilizing the Kitz-Wilson (1962) method to determine the apparent second-order rate constant, $k_{\text{obs.}}/[\text{I}]$.

Labelling of proteinases

Trypsin stock solution (1 mg/ml) was made up in 0.1 M-phosphate buffer, pH 7.4. Trypsin stock solution (100 μl) was incubated with Bio-Arg-CH₂Cl (5 μl of a 1.4 mM-solution in 1 mM-HCl) for 15 min at 37°C . The chloromethane was then inactivated by the addition of 4 M-NaOH (5 μl) and the labelled protein denatured by boiling for 5 min in Laemmli (1970) treatment buffer. In the blocking experiment, trypsin was pre-incubated with di-isopropyl fluorophosphate (iPr₂P-F) (5 μl , 1 mM) for 5 min before the addition of the probe. Similarly

plasmin and thrombin were labelled with Bio-Arg-CH₂Cl and chymotrypsin was labelled with Bio-Phe-CH₂Cl.

Electrophoresis and blotting

Electrophoresis in the presence of SDS was performed as described by Laemmli (1970) with 10% slab gels using the Mighty Small Apparatus (Hoefer Scientific). Pre-stained molecular-mass markers (BRL) were included in each gel. Western blotting was carried out by the method of Towbin *et al.* (1979). Transfer of polypeptide from gel to nitrocellulose was performed in 25 mM-glycine/192 mM-Tris/20% methanol using a Hoefer Transphor electrophoresis unit. The running time was 2 h at 200 mA, and a constant temperature of 4 °C was maintained.

Detection of biotinylated polypeptides

After protein transfer, the nitrocellulose sheet was blocked by incubation for 30 min with 3% BSA in PBS. After washing with PBS containing 0.1% Tween, detection of biotinylated proteins was achieved by overlaying with streptavidin/alkaline phosphatase (diluted 1:1000 in TBS) for 1 h at room temperature with shaking. The blot was washed five times with TBS containing 0.1% Tween before alkaline phosphatase activity was detected

Fig. 1. Time course for the release of 7-amino-4-methylcoumarin from the trypsin-catalysed hydrolysis of Cbz-Arg-NH-Mec in the presence of Bio-Arg-CH₂Cl

The curves represent the best fit of the data to the equation for the irreversible inhibition (Walker & Elmore, 1984) of trypsin by Bio-Arg-CH₂Cl in the presence of Cbz-Arg-NH-Mec. The inactivations were carried out with a fixed concentration of substrate (100 μM) and the following concentrations of inhibitor: ■, 0.1 μM; ●, 0.5 μM; ○, 1 μM; □, 10 μM.

with Nitro Blue Tetrazolium (66 μl of a 75 mg/ml solution in 70% DMF) and bromochloroindolyl phosphate (33 μl of a 50 mg/ml solution in DMF) in 10 ml of 0.1 M-Tris/HCl, pH 9.5 containing 0.1 M-NaCl and 5 mM-MgCl₂.

RESULTS AND DISCUSSION

The release of aminomethylcoumarin from Cbz-Arg-NH-Mec by trypsin in the presence of increasing concentrations of Bio-Arg-CH₂Cl is shown in Fig. 1. The observed progress curves are indicative of the action of an active-site-directed irreversible inhibitor (Tian & Tsou, 1982; Walker & Elmore, 1984) and similar profiles were obtained for the inactivation of thrombin by the same inhibitor in the presence of Boc-Val-Pro-Arg-NH-Mec (results not shown). Similarly Bio-Phe-CH₂Cl was found to irreversibly inactivate chymotrypsin. It was thus possible to evaluate the individual kinetic constants (K_i , k_i) for the inactivation of these proteinases with their respective active-site-directed probes.

When plasmin and Bio-Arg-CH₂Cl were incubated together in the presence of substrate (Boc-Val-Leu-Lys-NH-Mec), no discernable inhibition was detected over a period of 30 min, even using concentrations of inhibitor as high as 250 μM. However, incubation with inhibitor in the absence of substrate resulted in the rapid inactivation of the proteinase, the extent of inactivation being dependent on the length of exposure to inhibitor. This suggests that the inhibitor has poor affinity for plasmin (before covalent-complex formation) and that the substrate effectively competes for the active-site, blocking the progress of the inhibitor to the proteinase.

This thesis was supported by analysing the inhibition process by the Kitz-Wilson (1962) method. We found that a plot of k_{obs} against [I] was linear up to inhibitor concentrations of 250 μM, indicating that no non-covalent complex (EI) forms between plasmin and Bio-Arg-CH₂Cl (at least using the concentration range employed in the present study). Consequently it was only possible to evaluate the second-order rate constant ($k_{obs}/[I]$) for the inactivation of plasmin by this affinity label.

The kinetic parameters for the inhibition of trypsin, thrombin and plasmin by Bio-Arg-CH₂Cl and chymotrypsin by Bio-Phe-CH₂Cl are presented in Table 1. The first feature to note is that the arginine derivative is a very effective inactivator of trypsin, plasmin and thrombin, with trypsin being the most readily inactivated of the three. The differences between the overall rates of inactivation of trypsin and thrombin by this reagent can be attributed entirely to the greater affinity of the interaction between the former proteinase and inhibitor (lower K_i) before covalent-complex formation.

It can also be seen that the trypsin-like proteinases are more effectively inactivated by the arginine-derived biotinylated inhibitor than is chymotrypsin by the phenylalanine-derived ana-

Table 1. Kinetic constants for the inactivation of various serine proteinases by Bio-Phe-CH₂Cl and Bio-Arg-CH₂Cl

Values are means ± S.E.M. for four determinations. N.D., not determined.

Enzyme	K_i (μM)	k_i (min ⁻¹)	k_i/K_i (M ⁻¹ ·min ⁻¹)	Kinetic method employed
Chymotrypsin*	98 ± 12	0.40 ± 0.05	4955 ± 568	Walker-Elmore (1984)
Trypsin†	45 ± 5	4.6 ± 0.5	104445 ± 15299	Walker-Elmore (1984)
Thrombin†	520 ± 60	4.1 ± 0.6	74100 ± 8400	Walker-Elmore (1984)
Plasmin†	N.D.	N.D.	52000 ± 6300	Kitz-Wilson (1962)

* Kinetic constants determined for Bio-Phe-CH₂Cl.

† Kinetic constants determined for Bio-Arg-CH₂Cl.

Fig. 2. Western-blot analysis of samples of bovine trypsin inactivated with the active-site-directed affinity label Bio-Arg-CH₂Cl

The samples were inactivated with the biotinylated probe before loading on to a 10%-(w/v)-polyacrylamide gel (run under reducing and denaturing conditions) as outlined in the Experimental section. Lane 1, biotinylated molecular-mass markers; lane 2, trypsin, pretreated with iPr₂P-F (50 μ M, for 5 min at 37 °C) and then incubated with Bio-Arg-CH₂Cl (70 μ M, for 15 min at 37 °C); lane 3, trypsin incubated with Bio-Arg-CH₂Cl (70 μ M, for 15 min at 37 °C). After electrophoretic transfer the biotinylated bands were revealed with streptavidin/alkaline phosphatase as described in the Experimental section.

Fig. 3. Western-blot analysis of human thrombin and plasmin inactivated with the active-site-directed affinity label Bio-Arg-CH₂Cl

The proteinases were inactivated with the biotinylated probe before electrophoresis on a 10%-(w/v)-polyacrylamide gel (run under reducing and denaturing conditions) as described in the Experimental section. Lane 1, thrombin, biotinylated probe omitted; lane 2, thrombin, pretreated with iPr₂P-F (50 μ M, for 5 min at 37 °C) and then exposed to Bio-Arg-CH₂Cl (70 μ M, for 15 min at 37 °C); lane 3, thrombin treated with Bio-Arg-CH₂Cl (70 μ M, for 15 min at 37 °C). Lanes 4, 5 and 6 represent plasmin samples that were treated in the same way as the thrombin samples applied to lanes 1, 2 and 3 respectively. After electrophoretic transfer the biotinylated bands were revealed with streptavidin/alkaline phosphatase as described in the Experimental section.

logue. Indeed, trypsin is inactivated some 30-fold more readily by the arginine analogue than is chymotrypsin by its purpose-designed inhibitor. This increase in inhibitor efficacy is derived from a modest (~ 2 -fold) improvement in affinity (lower K_i) coupled with a 10-fold greater rate of covalent modification of the target proteinase (k_i). Nevertheless, Bio-Phe-CH₂Cl is still an effective inactivator of chymotrypsin, a particularly satisfying finding being that the presence of the long alkyl side chain and central nucleus of the biotin grouping do not appear to adversely affect the interaction of the inhibitor with the active site of the

Fig. 4. Western-blot analysis of active-site-biotinylated chymotrypsin

Samples of Bio-Phe-CH₂Cl-modified bovine chymotrypsin were prepared as outlined in the Experimental section. The samples were then subjected to electrophoresis on a 10%-(w/v)-polyacrylamide gel, run under reducing and denaturing conditions. Amounts of active-site-biotinylated chymotrypsin applied to the gel were as follows. Lane 1, 10 μ g; lane 2, 1 μ g; lane 3, 500 ng; lane 4, 100 ng; lane 5, 50 ng; lane 6, 25 ng; lane 7, 10 ng. After electrophoretic transfer the biotinylated proteins were revealed with streptavidin/alkaline phosphatase as described in the Experimental section.

enzyme. This is borne out by a comparison of the second-order rate constants obtained for the inactivation of chymotrypsin by Cbz-Phe-CH₂Cl and Bio-Phe-CH₂Cl of 4140 $\text{M}^{-1}\cdot\text{min}^{-1}$ (Shaw & Ruscica, 1971) and 4955 $\text{M}^{-1}\cdot\text{min}^{-1}$ (the present study) respectively.

A comparison of our data with the literature values of second-order rate constant quoted for the inhibition of trypsin by tosyl-Lys-CH₂Cl ('TLCK') namely 5.61 $\text{M}^{-1}\cdot\text{s}^{-1}$ at pH 6 (Shaw *et al.*, 1965), suggests that the incorporation of the biotin group into the arginylchloromethane does not appear to adversely affect the efficacy of this inhibitor either. The fact that our biotinylated inhibitors produce higher second-order rate constants than these urethane- and sulphonamido-protected amino acid-derived chloromethanes suggests that perhaps the biotinyl moiety contributes to the enzyme-inhibitor binding. This conclusion should, however, be treated with a certain amount of circumspection, since the inhibition studies were carried out at different pH values and also, in the case of trypsin, comparison was made between a lysine and an arginine analogue.

The irreversible nature of the inhibition obtained with both chloromethanes led to the application of these probes to the localization of several enzyme species on nitrocellulose after blotting from PAGE. It was essential to prove that the probes were labelling the enzymes by active-site-directed inhibition and not by a non-specific reaction at other sites. This was achieved by a blocking experiment in which each proteinase was preincubated with iPr₂P-F, an active-site-directed inhibitor of serine proteinases, followed by incubation with the biotinylated probe. Figs. 2 and 3 show that labelling was completely blocked under these conditions. For example, from Fig. 3 it can be seen that pretreatment of thrombin (lane 2) and plasmin (lane 5) with iPr₂P-F completely abolishes the labelling with Bio-Arg-CH₂Cl. The bands migrated in the gels in accordance with the documented molecular masses for the catalytic chains of these proteinases run under reducing the denaturing conditions. The kinetic data implies competitive irreversible inactivation of the proteinases, as indicated by the progress curve shown for trypsin inhibition (Fig. 1), and the blocking experiment corroborates this (Fig. 2, lane 2).

The limit of detection was determined by incubating differing amounts of enzyme with the same amount of probe under

identical conditions. Fig. 4 shows that 10 ng of chymotrypsin probed with Bio-Phe-CH₂Cl was clearly visible. Two bands were observed in the commercially obtained chymotrypsin irrespective of the source. Similarly, 10 ng of trypsin was detected with Bio-Arg-CH₂Cl (results not shown).

We have demonstrated here how the arginylchloromethane can be used to detect a variety of trypsin-like enzymes. We feel that this offers tremendous potential in those situations where one wishes to establish the presence of multiple trypsin-like activities in a given sample. Should one wish to characterize these enzymes further, there are two possible approaches. One could, for example, design specific biotinylated affinity labels which take into consideration the secondary specificity of a given proteinase. This has been achieved by Williams *et al.* (1989), who designed peptidylchloromethanes to discriminate between enzymes/zymogens involved in the blood-clotting process. However, an equally valid alternative would be the use of the broad-spectrum probe described above in conjunction with unbiotinylated affinity labels of known reactivity. Pretreatment of a sample with each of these reagents in turn would lead to differential incorporation of the biotinylated probe.

Although we have only demonstrated labelling of chymotrypsin by Bio-Phe-CH₂Cl, it is probable that this reagent will also function as a broad-spectrum inactivator of chymotrypsin-like activities, such as mast-cell chymase and cathepsin G.

Finally, the present study has demonstrated the use of biotinylated probes for the detection of serine proteinases. However, Arg-CH₂Cl will probably inactivate trypsin-like cysteine proteinases such as clostripain, which cleaves peptides and proteins after bonds involving arginine residues (Mitchell & Harrington, 1971). In order to discriminate between cysteine enzymes and serine enzymes in a complex mixture, the cysteine proteinases could be inactivated by prior incubation with an irreversible class-specific inhibitor such as iodoacetamide or *trans*-epoxy-succinyl-L-leucylamino-(4-guanidino)butane E-64.

In an accompanying paper (Walker *et al.*, 1992), we describe the synthesis and application of a biotinylated peptidyl diazomethane which should exhibit selectivity for cysteine proteinases.

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