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INHIBITORY EFFECT OF DI- AND TRIPEPTIDYL ALDEHYDES ON CALPAINS AND CATHEPSINS*

TAKASHI SASAKI, MOTOHARU KISHI, MASAYUKI SAITO,[1] TAKAHARU TANAKA,[1] NAOKI HIGUCHI,[1] EIKI KOMINAMI,[2,†] NOBUHIKO KATUNUMA,[2] and TAKASHI MURACHI[‡]

Department of Clinical Science and Laboratory Medicine, Kyoto University Faculty of Medicine, Kyoto 606,[1]Institute for Fundamental Research, Suntory Research Center, Shimamoto-cho, Osaka 618 and [2]Department of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan

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Eight different di- and tripeptidyl aldehyde derivatives, each having at its C-terminus an aldehyde analog of L-norleucine, L-methionine, or L-phenylalanine with a preceding L-leucine residue, were synthesized and tested for their inhibitory effects on several serine and cysteine endopeptidases. These compounds showed almost no inhibition of trypsin, and only weak inhibition of α-chymotrypsin and cathepsin H, while they exhibited marked inhibition of cathepsin B < calpain II ≃ calpain I < cathepsin L, being stronger in this order. The mode of inhibition of these cysteine proteinases was competitive for the peptide substrate used and inhibitor constants (K_i) were calculated from the Dixon plot. The best inhibitors found were: 4-phenyl-butyryl-Leu-Met-H for calpain I (K_i, 36 nM) and calpain II (K_i, 50 nM); acetyl-Leu-Leu-nLeu-H for cathepsin L (K_i, 0.5 nM); acetyl-Leu-Leu-Met-H for cathepsin B (K_i, 100 nM).

KEY WORDS: Calpain, cathepsin, peptidyl aldehydes, proteinase inhibitor.

INTRODUCTION

Cysteine endopeptidases, which include papain, calpains and cathepsins, are known to be strongly inhibited by leupeptin and antipain.[1,2] These *Actinomycete* products are tripeptide analogs, each having at its C-terminus an argininal residue (Arg-H), a residue in which the carboxylic acid group is reduced to a formyl group. In contrast to alkylating inhibitors such as peptidyl chloromethyl ketones[3,4] and diazomethanes,[5,6] these aldehyde analogs are essentially reversible inhibitors. By virtue of this, aldehyde inhibitors can be bound to the enzyme almost instantaneously and they can be used for reversible modification of the enzyme. Such usefulness could be amplified,

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†Present address: Department of Biochemistry, School of Medicine, Juntendo University, Tokyo 113, Japan

‡To whom correspondence should be addressed.

Abbreviations: Z-, benzyloxycarbonyl-; Leu, L-leucine residue; nLeu, L-norleucine residue; Met, L-methionine residue; Phe, L-phenylalanine residue; PB-, 4-phenylbutyryl-; -OMe, methyl ester; AMC, 7-amino-4-methyulcoumarine; MCA, 4-methylcoumaryl-7-amide; WSCD, *N*-ethyl-*N'*,N'-diethylaminopropyl carbodiimide; MeOH, methanol; EtOH, ethanol; EtOAc, ethyl acetate; $CDCl_3$, chloroform-d_1; TMS, tetramethylsilane; t-BuOH, 2-methyl-2-propanol; DMSO, dimethylsulfoxide; Suc, succinyl.

if different peptidyl aldehyde derivatives could be synthesized each specifically acting on one of the different cysteine proteinases in a discriminative fashion.

We attempted to synthesize several di- and tripeptidyl aldehydes, aiming at obtaining some derivatives which would fulfill the specificity requirement of calpain.[7] Besides leupeptin and antipain, several synthetic peptide inhibitors for calpain have been reported,[4] but they are also effective on other cysteine poteinases. The peptidyl aldehydes we synthesized were found to inhibit strongly both calpain I (a low-Ca^{2+}-requiring form) and calpain II (a high-Ca^{2+}-requiring form), but they failed to be specific for calpains, also showing fairly strong inhibition of cathepsins L and B. Thus, the primary objective of our study was not reached, but the kinetic data obtained provided us with several new lines of information on the specificity of calpains and cathepsins.

MATERIALS AND METHODS

Materials

Calpains I and II were isolated from pig erythrocyte and kidney with specific activities of 185 units/mg and 217 units/mg, respectively, using the method of Kitahara *et al.*[8] Cathepsin B, H, and L were purified from rat tissues by the methods of Towatari *et al.*[9], Kirschke *et al.*[10], and Bando *et al.*[11], respectively. Trypsin (Type I, 1100 units/mg) and α-chymotrypsin (Type II, 59 units/mg) were purchased from Sigma, St. Louis, MO. Boc-Val-Leu-Lys-MCA, Z-Phe-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Arg-MCA were obtained from the Protein Research Foundation, Osaka, Japan. All other chemicals were of reagent grade.

Synthesis of inhibitors

The synthesis of Z-Leu-nLeu-H, Z-Leu-Met-H, PB-Leu-nLeu-H, and PB-Leu-Met-H was described earlier,[12] and the synthesis of Z-Leu-Phe-H and PB-Leu-Phe-H was carried out by the same methods. The following analytical data were obtained. Z-Leu-Phe-H, $C_{23}H_{28}N_2O_4$ Calc.: C,69.68; H,7.12; N, 7.07. Found: C,69.55; H,7.06; N, 7.10; ^{1}H-NMR (δ – ppm from TMS in $CDCl_3$) 0.88 (6H,m), 1.24 ~ 1.72 (3H,m), 3.12 (2H,d,J = 7Hz), 4.16 (1H,m), 5.08 (2H,s), 5.12 (1H,m), 5.64 (1H,m), 7.16 ~ 7.34 (10H,m), 9.56 (1H,s). PB-Leu-Phe-H, $C_{25}H_{32}N_2O_3$, Calc.: C, 73.50; H, 7.89; N, 6.86. Found: C, 73.33; H, 7.92; N, 6.88.^{1}H-NMR (δ – ppm from TMS in $CDCl_3$) 0.92 (6H,d,J = 6Hz), 1.52 ~ 2.26 (7H,m), 2.57 ~ 2.72 (2H,m), 3.12 (2H,d,J = 7Hz), 4.40 ~ 4.76 (2H,m), 5.72 (1H,d,J = 7Hz), 6.68 (1H,d,J = 6Hz), 7.14 ~ 7.26 (10H,m), 9.58 (1H,s).

The derivatives of acetyl tripeptidyl aldehyde that were used in this study were Ac-Leu-Leu-nLeu-H and Ac-Leu-Leu-Met-H. The synthesis of Ac-Leu-Leu-R-H, with R representing nLeu or Met, was started by the deprotection of the Z-group of Z-Leu-R-OMe obtained in the course of the synthesis of Z-Leu-R-H.[12] The Z-group of Z-Leu-nLeu-OMe (4.0 g) was removed by catalytic reduction with a small amount of 5% palladium on carbon in absolute EtOH (100 ml) in an atmosphere of hydrogen to obtain H-Leu-nLeu-OMe quantitatively. H-Leu-nLeu-OMe (2.6 g) and Z-Leu-OH (2.6 g) were coupled by WSCD (2.0 g) in dry dichloromethane (100 ml) to give Z-Leu-Leu-nLeu-OMe in 85% yield (4.3 g) after purification by recrystallization from

10% EtOAc in ether. The R_f on TLC with 10% MeOH in chloroform was 0.80. The Z- group of Z-Leu-Leu-nLeu-OMe was removed by catalytic reduction with a small amount of 5% palladium on carbon in absolute EtOH (100 ml) in an atmosphere of hydrogen to give H-Leu-Leu-nLeu-OMe quantitatively. The H-Leu-Leu-nLeu-OMe (2.9 g) was acetylated by acetic anhydride (10 ml) in dry chloroform (50 ml) and dry benzene (20 ml) to afford Ac-Leu-Leu-nLeu-OMe in 90% yield (2.9 g) after purification by recrystallization from 10% EtOAc in ether. The R_f on TLC with 10% MeOH in chloroform was 0.75. Ac-Leu-Leu-nLeu-OMe (2.2 g) was then reduced to the alcohol using sodium borohydride (0.5g) in t-BuOH (50 ml) with absolute MeOH (8 ml) under reflux at 90°C in an atmosphere of nitrogen. The reaction mixture was quenched with 40 ml of water after 1h to give Ac-Leu-Leu-norleucinol in 42% yield (840 mg) after purification by recrystallization from EtOAc. The R_f on TLC with 10% MeOH in chloroform was 0.50. Ac-Leu-Leu-norleucinol (770 mg) was oxidized to the aldehyde by a sulfur trioxide pyridine complex (1.3 g) with triethylamine (800 mg) in anhydrous DMSO (16 ml). After quenching with water (100 ml), the final compound Ac-Leu-Leu-nLeu-H was obtained in 47% yield after purification by crystallization from EtOAc. The R_f on TLC with 10% MeOH in chloroform was 0.65. The analytical data for Ac-Leu-Leu-nLeu-H are as follows: $C_{20}H_{37}N_3O_4$, Calc.: C, 62.63; H, 9.72; N, 10.96. Found: C, 62.70; H, 9.81; N, 10.94. $[\alpha]_D^{28} = -91.6°$ (c = 1.6, MeOH); mp 182–184°C; MS (EI) (m/z) $[M]^+ = 384$; ^{1}H-NMR (δ – ppm from TMS in $CDCl_3$) 0.93 (15H,m), 1.16 ~ 1.89 (12H,m), 2.02 (3H,s), 4.37 ~ 4.66 (3H,m), 6.25 (1H,d,J = 8Hz), 6.89(1H,d,J = 8Hz), 7.00(1H,d,J = 8Hz), 9.62 (1H,s).

The synthesis of Ac-Leu-Leu-Met-H was carried out in the same manner as for Ac-Leu-Leu-nLeu-H except with the addition of boron trifluoride etherate during the deprotection of the Z-group of Z-Leu-Met-OMe of Z-Leu-Leu-Met-OMe because in the absence of boron trifluoride etherate, the sulfur atom of the Met residue would poison the hydrogenation catalyst. The analytical data for Ac-Leu-Leu-Met-H are as follows: R_f 0.55 (10% MeOH in chloroform); $C_{19}H_{35}N_3O_4S$, Calc.: C, 56.69; H, 8.76; N, 10.44. Found: C, 56.33; H, 8.99; N, 10.81. $[\alpha]_D^{28} = -74.3°$ (c = 0.98, MeOH); mp 189–191°C; MS (EI) (m/z) $[M]^+ = 402$; ^{1}H-NMR (δ – ppm from TMS in $CDCl_3$) 0.95 (12H,m), 1.45 ~ 1.85 (8H,m), 2.02 (3H,s), 2.07 (3H,s), 2.42 ~ 2.62 (2H,m), 4.44 ~ 4.64 (3H,m), 6.30 (1H,d,J = 8Hz), 6.98 (1H,d,J = 8Hz), 7.29 (1H,d,J = 8Hz), 9.66 (1H,s).

Assays for inhibitory activities

Calpains : The reaction mixture (0.5 ml) contained 110 mM imidazole (pH 7.5), 5 mM cysteine, 5 mM $CaCl_2$, 0.01–0.5 mM Boc-Val-Leu-Lys-MCA, 0–0.4 μM synthetic inhibitor, 6% DMSO, and 5.3 μg of calpain (I or II). The reaction was started by adding the enzyme and continued for 5 min at 30°C. It was terminated by adding 50 μl of 100 mM EGTA. AMC released was fluorimetrically determined at λ460 nm for emission and at 380 nm for excitation. Control runs were performed by omitting Ca^{2+}.

Cathepsins : The method of Barrett and Kirschke[13] was employed. The reaction mixture (1 ml) contained 0.3 ng of cathepsin B or 2.5 ng of cathepsin L, 100 mM acetate (pH 5.5), 1 mM EDTA, 8 mM cysteine, 0.01–0.05 mM Z-Arg-Arg-MCA for cathepsin B or 0.01–0.05 mM Z-Phe-Arg-MCA for cathepsin L, and 0–0.4 μM synthetic inhibitor. The reaction was allowed to proceed at 37°C for 6 min, and

terminated by adding a mixture of 1 ml of 100 mM acetate buffer (pH 4.3) containing 100 mM $ClCH_2COONa$ and 0.5 ml of water. AMC released was fluorimetrically determined as previously. For cathepsin H (40 ng per assay), 100 mM sodium phosphate buffer (pH 6.8), containing 1 mM EDTA, 0.01–0.05 mM Arg-MCA, and 0–50 μM synthetic inhibitor was used.

Trypsin and α-chymotrypsin : Trypsin (4.5 μg) or α-chymotrypsin (0.3 μg) was incubated at 30°C for 5 min in 0.5 ml of the assay mixture which contained 100 mM imidazole (pH 7.5), 6 mM $CaCl_2$, 7% DMSO, 1 or 50 μM synthetic inhibitor, 0.3 mM Z-Phe-Arg-MCA (for trypsin) or 0.3 mM Suc-Leu-Leu-Val-Tyr-MCA (for α-chymotrypsin). The reaction was terminated by adding 50 μl of 200 mM HCl. AMC released was fluorimetrically determined. Control runs were performed without enzyme.

RESULTS AND DISCUSSION

The newly synthesized peptidyl aldehydes were all found to be inhibitory towards calpains I and II and cathepsins B and L. They showed only weak inhibition of cathepsin H. Lineweaver-Burk plots indicated that the mode of inhibition of calpains I and II is competitive for the substrate. The inhibitor constant, K_i, was calculated from the Dixon plot, several examples being shown in Figure 1. The K_i values obtained are listed in Table I. Table II shows that all the compounds tested were almost ineffective on trypsin and only weakly inhibitory on α-chymotrypsin. Even with the best α-chymotrypsin inhibitor, PB-Leu-Phe-H, the inhibitor concentration at which the enzyme activity was half of the unihibited value (IC_{50}) was estimated to be

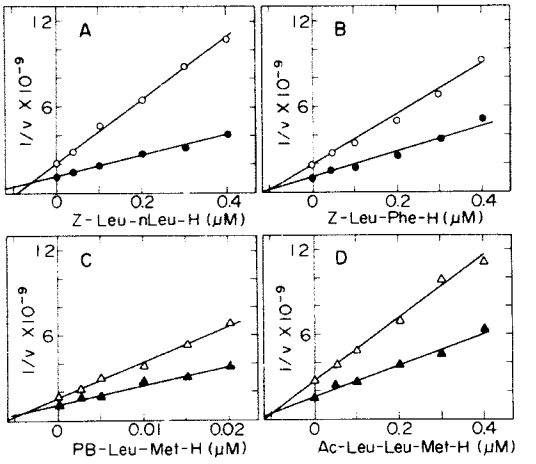

FIGURE 1 Dixon plots for the inhibition of calpains and cathepsins by peptidyl aldehydes. The assay was carried out at 30°C (for calpain) or 37°C (for cathepsin) and at two different concentrations of the substrate with varying amounts of the inhibitor in the assay system as described in "Materials and Methods." The rate of hydrolysis, v (mol/s/l), was calculated from the amount of AMC released in 5 min. A, calpain I; B, calpain II; C, cathepsin L; D, cathepsin B. Concentrations of the substrate used are: ○, 0.3 mM; ●, 0.5 mM; △, 0.04 mM; ▲, 0.08mM.

TABLE I
Inhibition of calpains and cathepsins by di- and tripeptidyl aldehydes

Inhibitor	K_i (μM)				
	Calpain I	Calpain II	Cathepsin L	Cathepsin B	Cathepsin H
Z-Leu-nLeu-H	0.067	0.062	0.0034	0.13	> 10
PB-Leu-nLeu-H	0.065	0.068	0.0033	0.22	> 10
Ac-Leu-Leu-nLeu-H	0.19	0.22	0.00050	0.15	> 10
Z-Leu-Met-H	0.036	0.068	0.013	0.25	> 10
PB-Leu-Met-H	0.036	0.050	0.0045	0.16	> 10
Ac-Leu-Leu-Met-H	0.12	0.23	0.00060	0.10	> 10
Z-Leu-Phe-H	0.060	0.10	0.020	2.40	> 10
PB-Leu-Phe-H	0.038	0.078	0.0014	0.43	> 10

TABLE II
Inhibition of trypsin and α-chymotrypsin by di- and tripeptidy aldehydes

Inhibitor	Concentration (μM)	Activity remaining (%)	
		Trypsin	α-Chymotrypsin
Z-Leu-nLeu-H	1	100	100
	50	98.8	57.4
PB-Leu-nLeu-H	1	100	97.4
	50	84.9	18.2
Ac-Leu-Leu-nLeu-H	1	100	100
	50	98.2	71.7
Z-Leu-Met-H	1	100	88.8
	50	92.0	60.8
PB-Leu-Met-H	1	100	95.3
	50	92.9	23.8
Ac-Leu-Leu-Met-H	1	100	93.9
	50	97.6	53.3
Z-Leu-Phe-H	1	100	75.1
	50	94.7	14.0
PB-Leu-Phe-H	1	100	70.9
	50	92.5	3.3

approximately 4 μM, a value much higher than the IC_{50} of this analog against calpin I or II (0.08 μM or less).

The initial objective of the present study was to synthesize some potent inhibitor peptide analogs which were specific for calpain. The aldehyde derivatives were designed so as to match the substrate specificity of calpains I and II reported earler.[7] A Leu residue was always present at P_2 position, since this residue was known to be the most preferred by either calpain I or II.[7] Several kinds of bulky amino acid residues were placed at the P_1 position, also conforming to the previous knowledge that such residues are favored by calpain both as peptide substrates[4] and as peptidyl chloromethylketone inhibitors.[4] Aldehyde derivatives were chosen in order to study steady state kinetics rather than the reaction rate which had earlier been studied with chloromethylketone derivatives.[5]

All of the 8 compounds were found, however, not to be specific for calpains, but to quite strongly inhibit also the cathepsins. From direct comparison of K_i values, the strength of affinity of the cysteine proteinases tested with these peptidyl aldehydes generally increases in the following order: cathepsin H $<<$ cathepsin B $<$ calpain II $\simeq$ calpain I $<$ cathepsin L. However, when the K_i values are compared with the K_m values for the respective enzymes, some different views can be obtained. For example, the best (or the smallest) K_i values found for calpain I is 0.036 μM with either Z-Leu-Met-H or PB-Leu-Met-H, and that for cathepsin L is 0.0005 μM with Ac-Leu-Leu-nLeu-H (Table I). A K_m value of 5.92 mM for calpain I was reported with the substrate used in this study, Boc-Val-Leu-Lys-MCA,[7] and K_m of cathepsin L for Z-Phe-Arg-MCA was shown[13] to be 7 μM. Thus, the ratio K_i/K_m calculated for calpain I (6.08×10^{-6}) is 1(11.7) 1.7-times smaller than that for cathepsin L (7.14×10^{-5}), implying higher relative specificity of inhibition of calpain I than of cathepsin L. Therefore, the apparent large difference between K_i values for calpain I and for cathepsin L, listed in Table I, may not be interpreted as showing much less capability of the peptide analogs to inhibit calpain I, but it may reflect inherent difference in affinity to the peptide moiety between these two enzymes.

It was earlier reported[7] that the substrate specicificity of calpain I is closely similar to but not identical with that of calpain II. This was shown also to be true when the peptidyl aldehydes are used as inhibitors (Table I). For example, about twice the difference in K_i values between calpains I and II were noted with Z-Leu-Met-H, AC-Leu-Leu-Met-H, Z-Leu-Phe-H, and PB-Leu-Phe-H, whereas almost equal K_i values were obtained with Z-Leu-nLeu-H, PB-Leu-nLeu-H, and Ac-Leu-Leu-nLeu-H. No general rule may be deduced from these variations.

The data shown in Table I also gives some information on the subsite specificity of cathepsins L and B. With a Met or Phe residue at P_1 position, the nature of the P_3 residue showed a profound influence of K_i, while such an influence was not seen when P_1 was nLeu. Besides, elongation from di- to tripeptide analogs by inserting one Leu residue at P_3 position strongly increased the affinity of the peptide analogs to cathepsin L, but not to cathepsin B. All these lines are in accord with the known importance

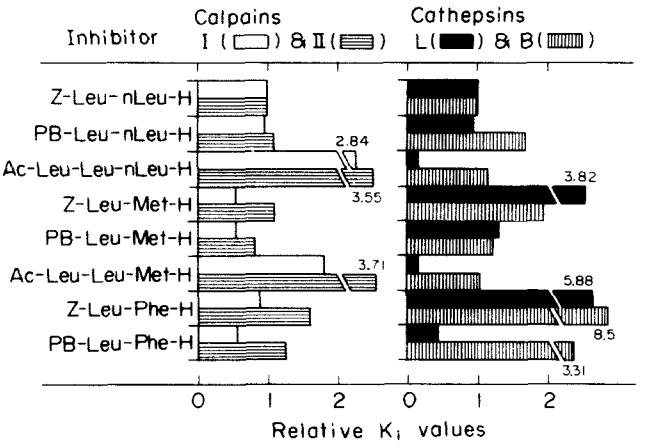

FIGURE 2 Profiles of inhibitory potencies of several peptidyl aldehydes on calpains and cathepsins. K_i values for Z-Leu-nLeu-H were taken as unity.

of the P_3 residue in determining the specificity of these cathepsins.[14] The nature of the P_3 residue also influenced calpains I and II, but not always in the same ways as those for cathepsins. The presence of a Leu at P_3 always resulted in a remarkable decrease in affinity of the inhibitor to calpains.

The fact that the aldehyde derivatives used for the present study all showed stronger inhibition of cathepsin L and calpains I and II compared with cathepsin B (Table II) is in full accord with the recent report by Crawford *et al.*[6] who showed that several peptidyl diazomethanes inactivated cathepsin L and calpains rapidly, but cathepsin B only slowly. However, this may not necessarily be interpreted as representing closer similarity of specificities between calpains (I and II) and cathepsin L than between calpains and cathepsin B. Thus, if the profile of affinities (in terms of K_i values) is depicted in such a way after normalization by placing K_i for Z-Leu-nLeu-H for each enzyme as unity (Figure 2), comparative degrees of dissimilarity can be seen between any one of the three pairs out of calpain (I or II), cathepsin L and cathepsin B. It should be noted, however, that cathepsin B also exhibits a peptidyl-dipeptidase activity[15] whereas the action of calpains is thought to be exclusively endopeptidatic[7] as that of cathepsin L is.[16]

References

1. Ishiura, S., Murofushi, H., Suzuki K. and Imahori K. (1978) *J. Biochem. (Tokyo)*, **84**, 225–230.
2. Murachi, T., Tanaka, K., Hatanaka, M. and Murakami, T. *Adv. Enzyme Regul.*, **19**, 407–424.
3. Kettner, C., and Shaw, E. (1981) *Meth. Enzymol.* **80**, 826–842.
4. Sasaki, T., Kikuchi, T., Fukui, I. and Murachi, T. (1986) *J. Biochem. (Tokyo)*, **99**, 173–179.
5. Green, G.D.J., and Shaw, E. (1981) *J. Biol. Chem.* **256**, 1923–1928.
6. Crawford, C., Mason, R.W., Wikstrom, P. and Shaw, E. (1988) *Biochem. J.*, **254**, 751–758.
7. Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N. and Murachi, T. (1989) *J. Biol. Chem.*, **259**, 12489–12494.
8. Kitahara, A., Sasaki, T., Kikuchi, T., Yumoto, N., Hatanaka, M., Yoshimura, N. and Murachi, T. (1984) *J. Biochem. (Tokyo)*, **95**, 1759–1766.
9. Towatari, T., Kawabata, Y. and Katunuma, N. (1979) *Eur. J. Biochem.*, **102**, 279–289.
10. Kirschke, H. Langner, J., Wiederanders, B., Ansorge, S., Bohley, P. and Hanson, H. (1977) *Acta Biol. Med. Germ.*, **36**, 185–199.
11. Bando, Y., Kominami, E., and Katunuma, N. (1986) *J. Biochem. (Tokyo)*, **100**, 35–42.
12. Tsujinaka, T., Kajiwara, Y., Kambayashi, J., Sakon, M., Higuchi, N., Tanaka, T. and Mori, T. (1988) *Biochem. Biophys. Res. Comun.*, **153**, 1201–1208.
13. Barrett, A.J. and Kirschke, H. (1981) *Meth. Enzymol.*, **80**, 535–561.
14. Towatari, T. and Katunuma, N. (1983) *J. Biochem. (Tokyo)*, **93** 1119–1128.
15. Aronson, N.N. Jr. and Barret, A.J. (1978) *Biochem. J.*, **171**, 759–765.
16. Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S. and Bohley, P. (1977) *Eur. J. Biochem.*, **74**, 293–301.