

A HEAT-LABILE SERINE PROTEINASE FROM *PENICILLIUM CITRINUM*

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Key Word Index—*Penicillium citrinum*; fungus; proteinase; serine protease; specificity.

Abstract—A serine proteinase from *Penicillium citrinum* was purified. The M_r and isoelectric point were determined as about 26 000 and 9.5, respectively. Activity was retained up to above 40° at pH 7 for 30 min, but the enzyme was completely inactivated at 50°. The first amino acids in the *N*-terminal region were ANVVQSNVPSWGLARISSK-RPGTTSYTDSTAGEGVVFGVDTG. The specificity differs from that of other serine proteinases. Kinetic studies on fluorogenic substrates were determined.

INTRODUCTION

It is well known that serine proteinase is widely distributed in various organisms [1]. Although fungal serine proteinase was described more than 40 years ago [2] and was isolated as a pure, electrophoretically homogeneous protein from *Aspergillus oryzae* 27 years ago [3, 4], only a limited number of studies on heat-labile proteinase have been carried out. We have now obtained a purified preparation of a heat-labile serine proteinase from *Penicillium citrinum*, and described its molecular properties and specificities. Homology of *N*-terminal and internal-peptide sequence of the enzyme was compared with other serine proteinases such as *Aspergillus oryzae* serine proteinase [5], *Tritirachium album* proteinase K [6], aqualysin I from *Thermua aquaticus* [7] and subtilisin BPN' [8], and only a low degree of sequence homology with the other serine proteinases was found. A specificity for the oxidized B-chain of insulin was observed which is different from those of other serine proteinase families [1].

RESULTS AND DISCUSSION

Molecular properties

The purified enzyme obtained by successive chromatography migrated as a single band on PAGE at pH 4.3 and SDS-PAGE, respectively. Specific activity of the enzyme was 14 mkat kg⁻¹ protein at pH 7. Yield of the enzyme was ca 15% and the purification ca 105-fold. M_r values of 26 000 and 25 000 were obtained by gel filtration HPLC on Shodex WS-803F and laser small angle scattering method [9, 10], respectively. The value of 29 000 was determined by SDS-PAGE. Electrofocusing showed the isoelectric point to be 9.5. The CD spectrum of the

enzyme is shown in Fig. 1. The contents of α -helix and β -structure of the enzymes were calculated to be ca 11 and 1%, respectively.

The enzyme has the following amino acid composition and a total of 269 residues: Asx₁₉, Glx₇, Thr₂₀, Ser₂₇, Gly₄₅, Ala₄₂, Cys₀, Val₂₈, Met₅, Ile₁₁, Leu₁₄, Tyr₅, Phe₇, Lys₁₄, His₃, Arg₆, Pro₁₂ and Trp₄. *N*-Terminal and internal peptide sequences of the enzyme were determined as shown in Fig. 2. Homology of the enzyme was compared with other serine proteinases, *Aspergillus oryzae* serine proteinase [5], *Tritirachium album* proteinase K [6], aqualysin I [7], and subtilisin BPN' [8]. Homologies of the enzyme with those of *A. oryzae* and *T. album* enzymes were 42% and 44%, and only a low degree of sequence homology with the other serine proteinases was found.

Enzymatic properties

The activity of the enzyme was retained up to ca 40° at pH 7 for 30 min, and the enzyme was completely inactivated at 50°. When the enzyme was incubated at pH 11, the activity was retained up to ca 25° and was completely inactivated at 40°.

The activity was inhibited by 0.1 mM DFP, PMSF [11], chymostatin [12], and elastatinal [13] (each at 0.1 mM). Inhibition studies indicated that the enzyme was a member of the serine proteinase family. The site specific reagents, TLCK, TPCK and ZPCK [14] for active histidine did not inhibit the enzyme. These results indicate that the active site of the enzyme is different from that of subtilisins [15] (see Experimental for abbreviations).

The peptide bond specificity towards the oxidized B-chain of insulin at pH 7 by the enzyme was investigated. Two major peptides, Phe1-Leu15 and Tyr16-Ala30, were

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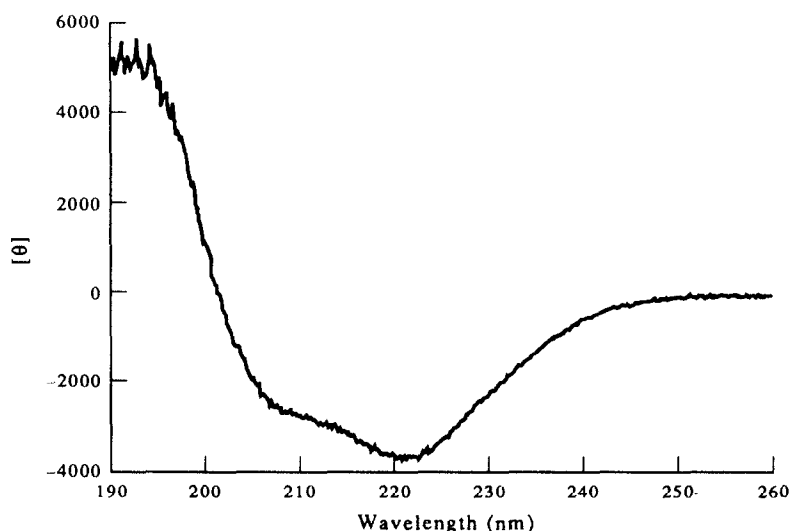


Fig. 1. CD spectrum of the serine proteinase from *Penicillium citrinum* in the region of 190–260 nm.

	1	5	10	15	20	25	30	35	40	*																																
1(1-44)	A	N	V	Q	S	N	V	P	S	W	G	L	A	R	I	S	S	K	R	T	G	T	S	Y	I	Y	D	S	I	A	G	E	G	V	V	I	Y	G	V	D	T	G
2(1-43)	G	L	T	I	Q	K	S	A	P	W	G	L	G	S	I	S	H	K	G	Q	S	T	D	Y	I	Y	D	I	S	A	G	E	G	I	Y	A	Y	V	V	D	S	G
3(1-41)		A	A	Q	I	N	A	P		W	G	L	A	R	I	S	S	T	S	P	G	T	S	I	Y	Y	Y	D	I	S	A	G	Q	G	S	C	V	V	I	D	T	G
4(1-41)		A	I	Q	S	P	A	P		W	G	L	D	R	I	D	R	D	L	P	L	S	N	S	Y	I	Y	I	A	I	G	R	G	V	N	V	V	I	D	T	G	
5(1-34)		A	Q	S		V	P			Y	G	V	S	Q	I	K		-	-	A	P	A	L	H	S	Q	G	Y	I	G	S	N	V	K	V	A	I	D	S	G		

1	S	A	A	H	V	S	I	A	A	S	T	S	I	D	G	S	A	?	P	
2(175-194)	S	A	P	D	A	I	T	V	A	A	I	Q	K	S	N	R	A	S	P	
3(174-193)	S	E	P	S	V	C	T	V	G	A	S	D	R	Y	D	R	S	S	P	
4(169-188)	R	V	A	E	A	L	T	V	G	A	I	T	S	S	D	A	R	A	S	P
5(170-189)	K	Y	P	S	V	I	A	V	G	A	I	D	S	S	N	Q	R	A	S	P

Fig. 2. N-Terminal and internal peptide sequence determination of the serine proteinase from *Penicillium citrinum* and homology of the enzyme with those of other serine proteinases. Asterisk shows the Asp residue corresponding to active-site residue in the subtilisin BPN'. 1, *Penicillium citrinum* serine proteinase. 2, *Aspergillus oryzae* serine proteinase [5]. 3, *Tritirachium album* proteinase K [6]. 4, Aqualysin I from *Thermus aquaticus* [7]. 5, Subtilisin BPN' [8].

obtained for 10- and 120-min digestion. The most susceptible bond of the initial site of cleavage by the enzyme towards the oxidized B-chain of insulin was between Leu15 and Tyr16. After 120-min digestion, four small peptides and four minor peptides were obtained. Additional cleavage of the bonds, Gln4-His5 and Phe24-Phe25 was noted. The minor sites were His5-Leu6 and Phe25-Tyr26. The specificity on the oxidized B-chain of insulin of the enzyme is shown in Fig. 3. It can be concluded that the proteinase from *Penicillium citrinum* has a specificity different from those of other serine alkaline proteinases from *Aspergillus sojae* [16], *Aspergillus oryzae* [17], *Bacillus natto* [18], *Streptomyces rectus* var. *proteolyticus* [19], and subtilisins Carlsberg [20] and Novo (BPN') [20].

The enzyme can hydrolyse the amide bonds between the amino acids and 7-amino-4-methylcoumarin (AMC) in the peptide-MCAs at pH 7. Kinetic parameters are shown in Table 1. Boc-VLK-MCA, which is a synthetic

Table 1. Kinetic parameters of amidase activities towards fluorogenic substrates by the serine proteinase from *Penicillium citrinum* at pH 7

Substrate	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \text{sec}^{-1}$)
Boc-VLK-MCA	0.14	4.6	32
Boc-LSTR-MCA	0.11	1.7	15
Suc-LLVY-MCA	0.13	3.1	24

Boc-, *t*-butyloxycarbonyl-. Suc-, succinyl-. MCA, 4-methylcoumaryl-7-amide. Abbreviations of amino acids follow the alphabetical system.

substrate of plasmin (EC 3.4.21.7), was the best substrate for the proteinase. The enzyme has a different specificity towards peptide-MCAs from those of the enzymes of *Aspergillus sojae* [21], alkalophilic *Bacillus* sp. [22] and

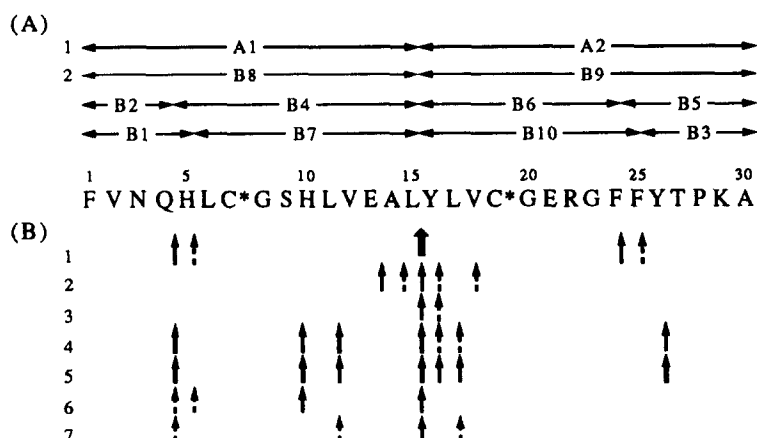


Fig. 3. Summary of the specificities of the serine proteinase from *Penicillium citrinum* and other serine proteinases towards the oxidized B-chain of insulin at pH 7 and 30°. (A) The enzymatic digest of the chains was directly separated with HPLC. Abbreviations of the amino acids follow the alphabetical system. C* indicates cysteine sulphonyl group. The sign (↔) shows the peptides recovered. Perpendicular arrows (↑, ↑, ↑) indicate the bond split, the degree of hydrolysis being: ↑ > ↑ > ↑ (1) 10 min⁻¹; (2) 120 min-digestion by the serine proteinase from *Penicillium citrinum*. (B) 1, Serine proteinase from *Penicillium citrinum*. 2, *Aspergillus sojae* serine proteinase [16]. 3, Aspergillopeptidase B from *Aspergillus oryzae* [17]. 4, Subtilisin Carlsberg [20]. 5, Subtilisin BPN' [20]. 6, Serine proteinase from *Bacillus natto* [32]. 7, Alkaline proteinase from thermophilic *Streptomyces rectus* [19].

subtilisins Carlsberg and Novo (BPN') [21]. The activation energy, E_a , of the enzyme-catalysed action towards Boc-VLK-MCA was calculated from the slope $-E_a/T$ to be 17 kJ mol⁻¹.

EXPERIMENTAL

Materials. Oxidized B-chain of bovine insulin and diisopropylfluorophosphate (DEP) were purchased from Fluka. CM-Biogel was from Bio Rad. CM-Sephadex was obtained from Pharmacia. Bisacrylamide and Folin-Ciocalteu's reagent were from Wako Junyaku Co., Osaka. *N*-Carbobenzoyl-L-phenylalanine chloromethylketone (ZPCK), *p*-chloromercuric benzoate (PCMB), and phenylmethane sulphonyl fluoride (PMSF) were from Sigma. *N*-*p*-Tosyl-L-phenylalanine chloromethylketone (TPCK) and *N*-*p*-tosyl-L-lysine chloromethylketone (TLCK) were from Nacarai Tesque Co., Kyoto. Chymostatin, elastatinal, pepstatin, phosphoramidone, bestatin, Boc-VLK-MCA, *t*-butyloxycarbonyl-L-Leu-L-Ser-L-Thr-L-Arg-MCA (Boc-LSTR-MCA), and succinyl-L-Leu-L-Tyr-MCA (Suc-LLVY-MCA) were from Peptide Institute, Inc., Ina, Minoh-shi, Osaka, Japan. Cytochrome *c* oligomers were from Oriental Kobo Kogyo Co.

Enzyme source. A crude enzyme preparation obtained from a commercial product 'Protease B' from the *Penicillium citrinum* of Amano Pharmaceutical Co., Nishikasugun, Aichi Prefecture, was used in this study.

Assay of serine proteinase activity. The proteolytic activity was measured at pH 7.5 in 1 ml of 2.5% milk casein (Hammarsten, Merck) with 100 μl of enzyme soln at 30° for 10 min. After the addition of 2 ml of the precipitating agent (0.4 M trichloroacetic acid), the sample solns were filtered. The resulting filtrate of 0.5 ml was mixed with 2.5 ml of 0.4 M Na₂CO₃, and 0.5 ml of 6-

fold diluted soln of Folin-Ciocalteu's reagent was then added. One katal of the serine proteinase is defined according to ref. [16].

Fluorometric assay. The substrates, Boc-VLK-MCA, Boc-LSTR-MCA and Suc-LLVY-MCA, were used. For the fluorometric amidase activity of the enzyme the free 7-amino-4-methylcoumarin (AMC) in the enzymatic hydrolysate at pH 7 was measured with a Hitachi fluorescence spectrophotometer model F-3000 as in refs [21, 23]. Excitation and emission wavelengths were 360 and 440 nm, respectively. The instrument was standardized daily so that the fluorescence intensity of 1 μM AMC soln gave a full-scale response on a chart recorder.

Protein concentration. Protein concns were estimated by the method of ref. [24].

Purification of proteinase. The crude enzyme soln was dialysed against 5 mM NaPi buffer, pH 7.5, and was centrifuged at 15000 *g* for 20 min. Ion exchange chromatography was conducted on a CM-Sephadex C 50 column (3.7 × 20 cm) equilibrated with the same buffer. The enzyme was eluted with the same buffer containing 0.5 M NaCl. The eluate was dialysed against 5 mM NaPi buffer, pH 7. CM-Sephadex C 50 (3 × 16 cm) was used for the next step in the purification. The enzyme was eluted with the same buffer containing 10 mM NaCl. In the final stage of the purification for *N*-terminal sequencing, reverse phase column, TSK-gel phenyl-5PW RP (4.6 mm × 5.7 cm), was used for HPLC (TOSOH PX-8010) (10–80% MeCN gradient elution).

Polyacrylamide gel electrophoresis (PAGE). Performed using 10% polyacrylamide gel at pH 4.3 by the method of ref. [25].

Molecular weights. They were determined by SDS-PAGE as described in ref. [26]. Marker proteins of cytochrome *c* from horse and cross-linked cytochrome *c*

oligomers were monomer (12.4 k), dimer (24.8 k), trimer (37.2 k), tetramer (49.6 k) and hexamer (74 k); the values in parentheses are the respective M_r s.

For gel filtration on HPLC with Shodex WS-803F, β -galactosidase (460 k), β -amylase (200 k), aldolase (158 k), bovine serum albumin (67 k), ovalbumin (43 k) and soybean trypsin inhibitor (20 k) from Boehringer were used as markers.

M_r was also determined by low-angle laser light-scattering photometry and precision differential refractometry combined with gel-filtration HPLC (LALLS-HPLC). LALLS-HPLC was performed essentially according to ref. [9]. Samples were applied to a TSK-gel G3000SW_{XL} column (0.78 cm $\times$ 30 cm, TOSOH) equilibrated with 50 mM NaPi buffer (pH 7) containing 0.2 M NaCl according to ref. [10]. The enzyme was sepd by gel filtration HPLC with a TSK-gel G2000SW_{XL} column (0.78 cm $\times$ 30 cm, TOSOH) (in the presence of 1 mM dithiothreitol), and the elution was monitored with a low-angle laser light-scattering photometer (LS-8000, TOSOH) and a precision differential refractometer (RL-8000, TOSOH) connected in series. The molecular mass of the enzyme was determined by the following equation:

$$M = k/(dn/dc) \times (\text{output})_{LS}/(\text{output})_{RI}$$

where k is a constant determined by the instrumental and experimental conditions, dn/dc is a specific refractive index increment, $(\text{output})_{LS}$ and $(\text{output})_{RI}$ are outputs of the light-scattering photometer and refractometer, respectively. The value of $k/(dn/dc)$ was calculated from the values of $(\text{output})_{LS}/(\text{output})_{RI}$ obtained using bovine serum albumin (molecular mass, 67 k) as standard.

Isoelectric focusing. IEF of the enzyme was carried out on one-dimensional PAGE. An IEF gel kit of pH 3.5–9.5 from ACI Japan was used. The following preps were used as standard proteins: phycocyanin (4.65), β -lactoglobulin B (5.1), bovine carbonic anhydrase (6.0), human carbonic anhydrase (6.5), horse myoglobin (7.0), whale myoglobin (8.05), α -chymotrypsin (8.8), and cytochrome *c* (9.6); the values in parentheses are the respective isoelectric points.

Circular dichroism. CD measurements were made on a JASCO automatic recording spectrophotometer, model J-600, at room temp. (22°) and a 1 mm path length quartz cell from 200 to 260 nm. A circular dichroism scale setting of 0.001 m° cm⁻¹ was used in all experiments. Spectra were connected to baseline shifts by running scales of solvent buffer. Results are expressed as mean residue ellipticities (θ) in deg cm² dmol⁻¹. The contents of α -helix and β -structure of the serine proteinase were calculated according to the SSE-338 Program given in ref. [27].

Amino acid analysis. The purified enzyme was hydrolysed by the method of ref. [28]. After dabsylation [29] amino acids were analysed with a Beckman System Gold Programmable Solvent Module 126, analyser. Tryptophan was determined by the NBS titration method of ref. [30]. The number of residues of amino acids was calculated for a M_r of 26 000. Cysteine was determined as reduced carboxymethyl cysteine according to the method

described in ref. [31], except that 7 M guanidine HCl was used to denature the enzyme.

N-Terminal sequence determination. The enzyme purified by reversed phase chromatography was subjected to N-terminal sequence determination on an Applied Biosystems 473A protein sequencer with 610A data analysis system.

Hydrolysis of oxidized insulin B-chain. The oxidized insulin B-chain (0.4 μ mol) was dissolved in 1 ml 5 mM NaPi buffer, pH 7 and the pH was adjusted to 7; 0.25 nmol of the serine proteinase was added to the soln. The mixt. was incubated at 30° for 10 or 120 min. One drop of 11 M HCl was added to inactivate the enzyme. The sample of hydrolysate was dried *in vacuo*. The dried digests of oxidized insulin B-chain were dissolved in milli-Q H₂O.

Separation and identification of peptides. Sepn and identification of the peptides in the dried digests was done by HPLC as described previously [32]. HPLC was performed on a column (4.0 $\times$ 25 mm) of ODS-120T (TOSOH) equipped with a TOSOH model CCPD delivery system. Chromatographic recording was performed at 215 nm with a TOSOH SIC chromatorecorder 12.

Peptide analysis. Peptides isolated by HPLC were dissolved in 7 M HCl containing 10% TFA and 0.1% phenol and were hydrolysed at 158° for 24 min. Hydrolysates of peptides were examined with a Hitachi amino acid analyser, model L-8500 with D2850 integrator using a microcolumn (4.6 $\times$ 60 mm) procedure and MIC buffer L-8500 PF kit (Mitsubishi Kasei Chemical Co., Ltd, Tokyo) system.

Determination of kinetic parameters. The values of K_m and k_{cat} were graphically determined according to the Hanes–Woolf plots [33].

Determination of activation energy (E_a). According to the Arrhenius expression, plots of $\log k_{cat}$ versus $1/T$ gives a straight line of slope $-E_a/T$. The value of E_a was calculated for Boc-VLK-MCA hydrolysis at pH 7.

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REFERENCES

1. Morihara, K. (1974) *Adv. Enzymol.* **41**, 179.
2. Crewther, W. G. and Lennox, F. G. (1950) *Nature* **165**, 680.
3. Subramanian, A. R. and Kalnitsky, G. (1964) *Biochemistry* **3**, 1861.
4. Nordwig, A. and John, W. F. (1968) *Eur. J. Biochem.* **3**, 519.

5. Tatsumi, H., Ogawa, Y., Murakami, S., Ishida, Y., Murakami, K., Masaki, A., Kawabe, H., Amikura, H., Nakao, E. and Motai, H. (1989) *Mol. Gen. Genet.* **219**, 33.
6. Jang, K.-D., Lederer, G. and Mayer, B. (1986) *FEBS Letters* **199**, 139.
7. Kwon, S.-T., Terada, I., Matsuzawa, H. and Ohta, T. (1988) *Eur. J. Biochem.* **173**, 491.
8. Markland, F. S. and Smith, E. L. (1967) *J. Biol. Chem.* **242**, 5198.
9. Maesawa, S. and Takagi, T. (1983) *J. Chromatogr.* **280**, 124.
10. Kamo, T., Shimogawara, K., Fukuzawa, H., Muto, S. and Miyachi, S. (1990) *Eur. J. Biochem.* **192**, 557.
11. Polgar, L. and Bender, M. L. (1966) *J. Am. Chem. Soc.* **88**, 3153.
12. Tatsuka, K., Mikami, N., Fujimoto, K., Umezawa, S., Umezawa, H. and Aoyagi, T. (1975) *J. Antibiot.* **26**, 625.
13. Umezawa, H., Aoyagi, T., Okura, A., Morishima, H., Takeuchi, T. and Okami, Y. (1973) *J. Antibiot.* **26**, 787.
14. Shaw, E. (1972) in *Methods in Enzymology* (Jakoby, W. B. and Griffith, O. W., eds), Vol. 25, p. 655. Academic Press, New York.
15. Ottesen, M. and Svendsen, I. (1970) in *Methods in Enzymology* (Perlmann, G. E. and Lorand, L., eds), Vol. 19, p. 199. Academic Press, New York.
16. Ichishima, E., Hamamatsu, M., Yamamoto, N., Motai, H. and Hayashi, K. (1983) *Food Chem.* **11**, 187.
17. Spadari, S., Subramanian, A. R. and Kalnitsky, G. (1974) *Biochim. Biophys. Acta* **359**, 267.
18. Kamata, H., Yamagata, Y., Nakamura, T., Nakajima, T., Oda, K., Murao, S. and Ichishima, E. (1989) *Agric. Biol. Chem.* **53**, 2695.
19. Ichishima, E. and Matsue, M. (1881) *Curr. Microbiol.* **6**, 17.
20. Johansen, J. T., Ottesen, M., Svendsen, I. and Wybrandt, G. (1968) *Compt. Rend. Trav. Carlsberg* **36**, 365.
21. Iida, H., Takeuchi, M. and Ichishima, E. (1988) *Agric. Biol. Chem.* **52**, 1281.
22. Yamagata, Y. and Ichishima, E. (1989) *Curr. Microbiol.* **19**, 259.
23. Iida, H., Takeuchi, M. and Ichishima, E. (1988) *Agric. Biol. Chem.* **52**, 2313.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
25. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121**, 404.
26. Laemmli, U. K. (1970) *Nature* **227**, 680.
27. Yang, J. T. (1986) in *Methods in Enzymology* (Jakoby, W. B. and Griffith, O. W., eds), Vol. 130, p. 208. Academic Press, New York.
28. Tsugita, A. and Scheffler, J. J. (1982) *Eur. J. Biochem.* **124**, 585.
29. Knecht, R. and Chang, J. Y. (1986) *Analyt. Chem.* **58**, 2375.
30. Spande, T. F. and Witkop, B. (1967) in *Methods in Enzymology* (Hirs, C. H. W., ed.), Vol. 11, p. 498. Academic Press, New York.
31. Crestfield, A. M., Moore, S. and Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622.
32. Ichishima, E., Takada, Y., Taira, K. and Takeuchi, M. (1986) *Biochim. Biophys. Acta* **869**, 178.
33. Price, N. C. and Stevens, L. (1989) in *Fundamentals of Enzymology*, p.144. Oxford University Press, Oxford.