Acta Farm. Bonaerense **19** (4): 257-62 (2000)
Recibido el 26 de mayo de 2000
Aceptado el 19 de junio de 2000

Proteolytic Enzymes from the Latex of *Ficus pumila* L. *(Moraceae)*

Mario PERELLÓ, María Cecilia ARRIBÉRE, Néstor O. CAFFINI & Nora S. PRIOLO *

Laboratorio de Investigación de Proteínas Vegetales (LIPROVE), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CC 711, 1900 La Plata, Argentina.

SUMMARY. The presence of proteolytic activity was detected in fruits of *Ficus pumila* L. *(Ficus repens* Hort.). The crude extract was obtained by clarification of the latex through centrifugation at 16,000 x g for 30 min. Subsequently the supernatant was ultracentrifuged at 100,000 x g for one hour. The crude enzyme preparation showed high proteolytic activity on casein in the presence of 12 mM cysteine, but the activity was inhibited by thiol-specific inhibitors like $HgCl_2$ and E-64, suggesting the proteases belong to the cysteine family. This crude enzyme extract showed maximum caseinolytic activity within an alkaline range of pH (7.0-9.0) and a remarkable thermal stability. The purification was carried out by a simple procedure involving ultracentrifugation, acetone precipitation and cationic exchange chromatography. The main fraction obtained was partially characterized: its optimum pH range is 7.0-9.0, is very stable at high temperatures, showed a Mr = 28.6 kDa (SDS-PAGE) and an isoelectric point higher than 9.3.

RESUMEN. "Enzimas proteolíticas presentes en el látex de *Ficus pumila* L. *(Moraceae).* Se detectó la presencia de actividad proteolítica en frutos de *Ficus pumila* L. *(Ficus repens* Hort.). El extracto crudo fue obtenido por clarificación del látex centrifugado a 16.000 x g durante 30 min y ultracentifugando luego el sobrenadante a 100.000 x g durante una hora. La preparación enzimática cruda mostró elevada actividad proteolítica sobre caseína en presencia de cisteína 12 mM, pero la actividad fue inhibida por inhibidores tiol-específicos tales como $HgCl_2$ y E-64, sugiriendo que las proteasas pertenecen a la familia de las peptidasas cisteínicas. El extracto crudo mostró un rango de pH óptimo en la zona alcalina (pH 7,0-9,0) y una notable estabilidad térmica. La purificación fue llevada a cabo por un simple procedimiento que incluye ultracentrifugación, precipitación acetónica y cromatografía de intercambio iónico. La principal fracción proteolítica ha sido parcialmente caracterizada: su pH óptimo es igual al de la preparación cruda, es termoestable, su Mr (SDS-PAGE) es de 28,6 kDa y tiene un pI mayor que 9,3.

INTRODUCTION

Proteases play a prominent role in plant physiology, being the catalysts of important processes like hydrolysis of storage proteins during seed germination, activation of proenzymes, degradation of defective proteins, etc. [1], but the presence of high concentration of proteolytic enzymes in some tissues is more difficult to explain [2].

Many plants exude a latex containing a high amount of digestive enzymes, mainly cysteine and serine proteinases [2]. It has been known for many years that the milky latex flowing from cuts of the stem, leaves and unripe fruits of several species belonging to the genus *Ficus* contains proteolytic enzymes. The name ficin was coined by Robbins [3] for the purified white powder with antihelminthic activity obtained from any member of the genus. A crystalline preparation from an unnamed species was obtained by Walti [4] and also named ficin. There is more than 1,300 species of *Ficus*, many of which show proteolytic activity [5], sometimes due to the presence of more than one proteinase [6]. The term ficin must therefore be regarded as generic. In 1992 the International Union of Biochemistry

KEY WORDS: *Ficus pumila, Moraceae,* Latex, Enzymes, Plant proteases
PALABRAS CLAVE: Enzimas, *Ficus pumila,* Fitoproteasas, Látex, *Moraceae*

* Author to whom correspondence should be addressed. *E-mail:* priolo@biol.unlp.edu.ar

and Molecular Biology (IUBMB) recommended the name ficain for the major proteolytic component of the latex of the fig, *Ficus glabrata* [7].

The functions of plant latex endopeptidases are not well understood, but they may have a protective role against plant pathogens and herbivorous insects. Though proteases are frequently present in plant laticifers, not all the latex-containing species are good producers of proteolytic enzymes [2].

Proteolytic enzymes of plant origin have received special attention in the field of medicine and industry due to their unique property of being active at a very wide range of temperature and pH. The best known plant proteolytic enzymes of commercial value are papain from *Carica papaya,* ficin from *Ficus* spp. and bromelain from pineapple, which have been called "biological scalpels" because of their specific action on chronic and acute suppurating lesions and in debridement following burns without causing any deleterious action on healthy tissues. Chymotripsin and trypsin have been used as anti-inflammatory agents and a proposal of their mode of action has been described [8].

Proteolytic fractions from fig latex are used for unmasking antigens in serology [9]. The historical interest in ficin originated from its ability to digest gastrointestinal nematodes. Despite more recent evidence that the enzyme(s) may be effective for this purpose [10], ficin has not been adopted widely as a treatment for nematode infections.

The latex of some species of *Ficus (Moraceae)* has been traditionally used as vermifuge in Central and South America. It has been accepted that antihelminthic activity is due to a proteolytic fraction called ficin, but recent studies showing that the latex administered in mice naturally infected produce hemorrhagic enteritis, in addition to a weak antihelminthic efficacy, do not recommend the use of these lattices in traditional medicine [11].

In this paper the isolation and partial characterization of the main protease isolated from the latex of *Ficus pumila* L. *(Moraceae)* is reported.

MATERIALS AND METHODS

Plant Material

Ficus pumila L. fruits were obtained from plants grown in La Plata, Province of Buenos Aires, Argentina. The plant is a vine, with oblong or elliptic leaves, 2-10 cm long; fruits are ovoid, yellow or purple, 5-6 cm long [12]. Voucher specimens were deposited at the LPE 980 herbarium (Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Argentina).

Preparation of the crude extract

The crude extract was obtained from fresh latex collected by superficial incisions of fruits and received on 0.1 M phosphate buffer (pH 7.0) containing 6 mM EDTA and 6 mM cysteine. Gums and other insoluble materials were discarded by centrifugation at 11,000 x g for 30 min at 4 °C.

Enzyme assays: caseinolytic activity

Casein (Hammarsten type, Research Organics Inc.,Cleveland, OH) was the proteolytic substrate used in all the assays. The reaction mixture was prepared by mixing 0.1 mL of enzyme extract with 1.1 mL of 0.1 M Tris-HCl buffer (pH 9.0) containing 1% casein and 12 mM cysteine. The reaction was carried out at 37 °C and 2 min later was stopped by the addition of 1.8 mL of 5 % trichloroacetic acid. Each test tube was centrifuged at 3,000 x g for 20 min and the absorbance of the supernatant measured at 280 nm. An arbitrary enzyme unit ("caseinolytic unity", Ucas) was defined as the amount of enzyme that produces an increase of one absorbance unit per minute in the assay conditions [13].

Protein content

Proteins were determined according to Bradford [14], using bovine albumin (Sigma Chem. Co., St Louis, MO) as standard. In all chromatographic procedures protein concentration was estimated by measuring the absorbance of eluates at 280 nm.

Effect of pH on the enzyme activity

The dependence of enzyme activity of crude preparations and the purified enzyme on pH was measured on casein as substrate within the pH range 6.0 to 11.0, using 0.01 M sodium salts of the following "Good" buffers [15]: MES, MOPS, TAPS, AMPSO and CAPS (Sigma Chem. Co., St Louis, MO).

Effect of inhibitors

The action of some thiol specific proteases inhibitors was evaluated by incubating the crude enzymatic preparation for 10 and 30 min at 37 °C with different chemicals: mercuric chloride (0.1 and 1 mM) and E-64 (2, 8 and 10 μM). The residual caseinolytic activity after each incubation assay was measured as indicated above.

Thermal behavior

Progress curves for different temperatures (37, 45, 55, 65, and 75 °C) were made by measuring the caseinolytic activity along the time (2, 5, 10, 15, 20, and 30 min) for the crude enzyme preparation.

Purification procedure

The crude extract (free of gum and other debris) was ultracentrifuged at 100,000 x g for 60 minutes at 4 °C. Ten mL of this supernatant was loaded onto a CM-Sepharose Fast Flow column (Pharmacia K 15/30) pre-equilibrated with 0.01 M phosphate buffer (pH 7.5). Cation exchange chromatography was developed by adding 60 mL of the starting buffer, followed by 200 mL of a sodium chloride linear gradient (0.0-0.7 M) prepared in the same buffer. Fractions of 1.6 mL were collected. Protein content was monitored by measuring absorbance at 280 nm and the caseinolytic activity of each protein fraction was also determined.

Characterization of the main proteolytic component

Thermal stability

Thermal stability of the purified enzyme was evaluated by measuring the residual caseinolytic activity at 37 °C (pH 9.0) after incubating the samples during 5, 10, 15, 20, 25, and 30 min at 40, 50, 60, 65 and 70 °C. Activity on casein at pH 9.0 after 2 min a 37 °C was taken as 100%.

Isoelectric focusing (IEF)

IEF was developed on immobilized pH gradient gels of polyacrylamide (10%) in the pH range from 3 to 10 (ampholytes Biolyte 3-10, Bio-Rad, Hercules, CA, USA) in a Mini IEF Cell (Model 111, Bio-Rad). The samples were concentrated and deionized by acetone precipitation and further centrifugation at 11,000 x g for 20 min; the precipitates were redissolved with deionized water and the treatment repeated twice. Focusing was carried out under constant voltage conditions in a stepped fashion: 100 V for 15 min, 200 V for the following 15 min and 450 V for the last 60 min. The gels were then fixed and stained with Coomassie Brilliant Blue R-250.

SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Estimation of the molecular mass was performed by SDS-PAGE according to Laemmli [16] in a vertical apparatus (Miniprotean II Cell, Bio-Rad). Samples were precipitated with 3 volumes of acetone, redissolved in the sample buffer containing β-mercaptoethanol and boiled for 3 min, and then loaded on 14% polyacrylamide gels (stacking gel: 5% polyacrylamide). Current was kept constant at 35 mA for 90 min. Gels were stained by Coomassie Brilliant Blue R-250.

Zymogram

Unstained IEF gels were contacted for 45 min at 50 °C with an agarose gel imbibed with a 1% casein solution [17], which was then stained by Coomassie Brilliant Blue R-250, so as to confirm proteolytic activity.

RESULTS AND DISCUSSION

Presence of proteolytic activity was detected in the latex of *Ficus pumila* L. fruits. Crude enzyme preparations (latex devoid of gums and other insoluble materials) showed high proteolytic activity when assayed on casein in the presence of 12 mM cysteine. The enzyme activity was completely inhibited by incubation with low concentrations of $HgCl_2$ (0.1 mM) for 10 min; however, inhibition was reverted by subsequent incubation with 12 mM cysteine during 10 min. On the other hand, the enzyme activity was fully and irreversibly inhibited by E-64 (8 μM) after 30 min incubation. These results suggests that -SH groups could be involved in the mechanism of the enzyme; so, the proteases present in the *Ficus pumila* latex would belong to the cysteine endopeptidases family.

A protease named *ficain p I,* according to current nomenclature trends was purified from the latex of *Ficus pumila* fruits. This procedure involved a simple purification, including ultracentrifugation and ion exchange chromatography. Cation exchange chromatographic profile is shown in Fig. 1. The elution of the column with the starting buffer afforded a fraction with proteolytic activity (fraction I). Subsequent application of a sodium chloride gradient allowed the separation of two additional proteolytically active fractions (fractions II and III). Fraction III *(ficain p I)* exhibited the highest specific activity and was chosen for further analysis. Table 1 shows the purification scheme for *ficain p I.*

Both, the crude extract and *ficain p I* displayed maximum proteolytic activity within a narrow pH range (7.0 to 9.0; Fig. 2).

Thermal behavior of the crude extract is shown in Fig. 3: proteolytic activity increased with temperature, even at 75°C, a temperature that inactivates the majority of enzymes [18]. On

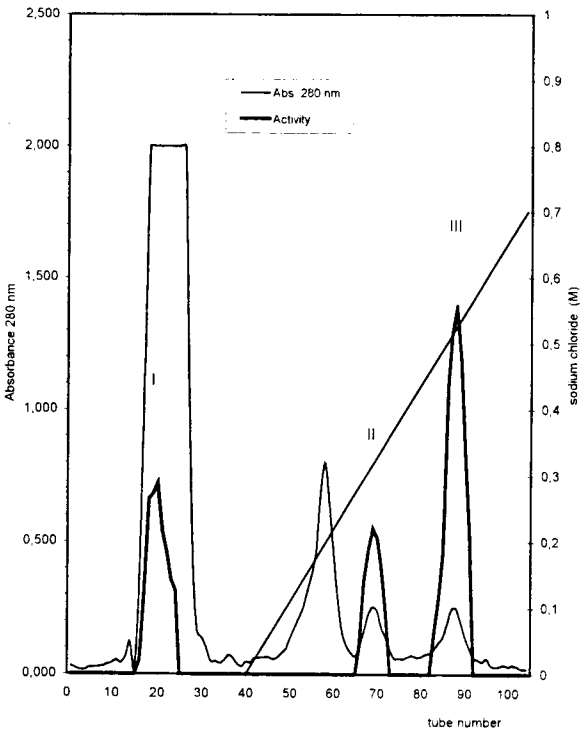

Figure 1. Cation exchange chromatography (CM-Sepharose CL-6B Fast Flow, column Pharmacia K 15/30). Elution buffer: 0.01 M phosphate buffer (pH 7.5). Gradient: sodium chloride 0.0-0.7 M. Fraction volume: 1.6 mL.

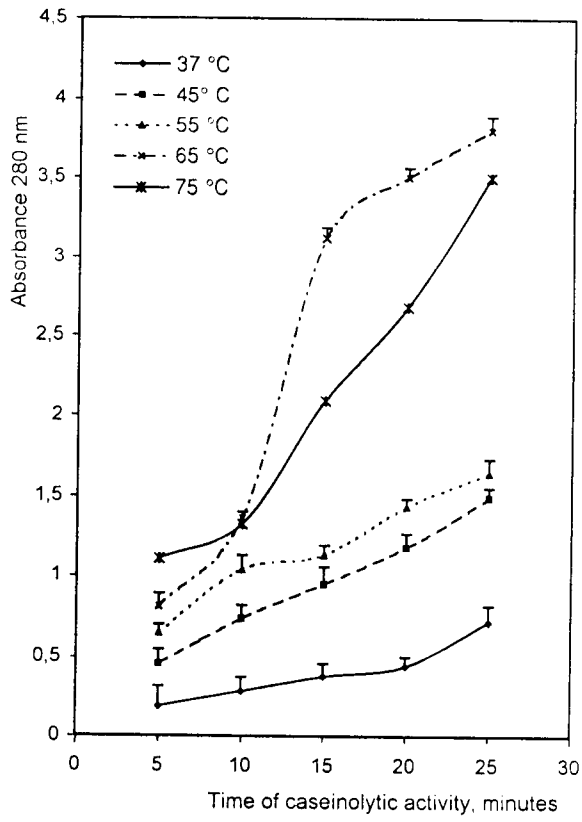

Figure 3. Enzyme activity of crude extract as a function of temperature.

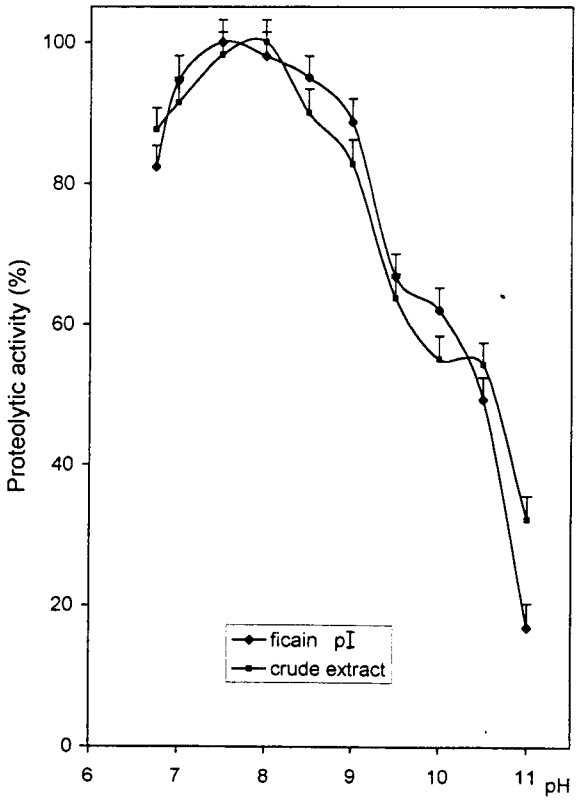

Figure 2. Effect of pH on proteolytic activity of crude extracts and *ficain p I*.

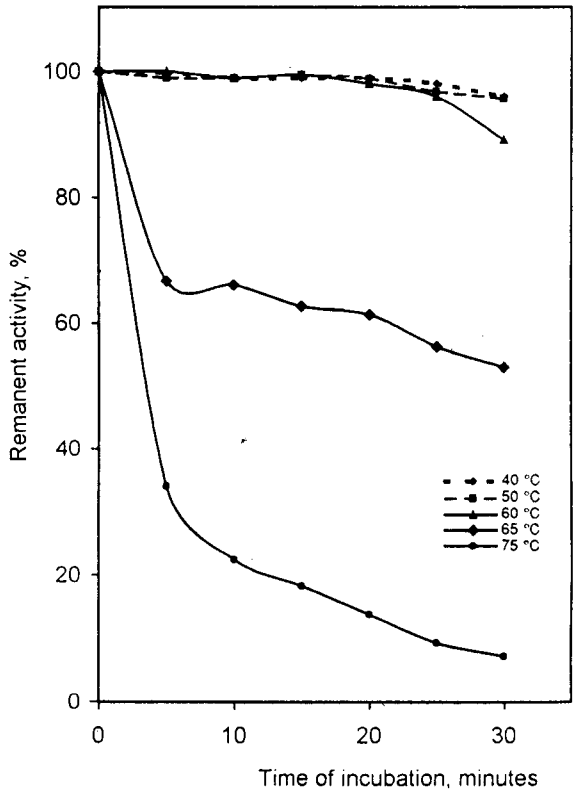

Figure 4. Thermal stability the *ficain p I*. The determinations of activity were made on casein at pH 9.0.

Sample	Protein (µg/mL)	UCAS/mL	Specific Activity (UCAS/mg)	Purification (n-fold)
Crude Extract	86	0.201	2.33	1
Fraction II	37	0.066	1.78	0.76
Ficain p	35	0.159	4.54	2

Table 1. Purification of the proteolytic components present in the latex of *Ficus pumila*.

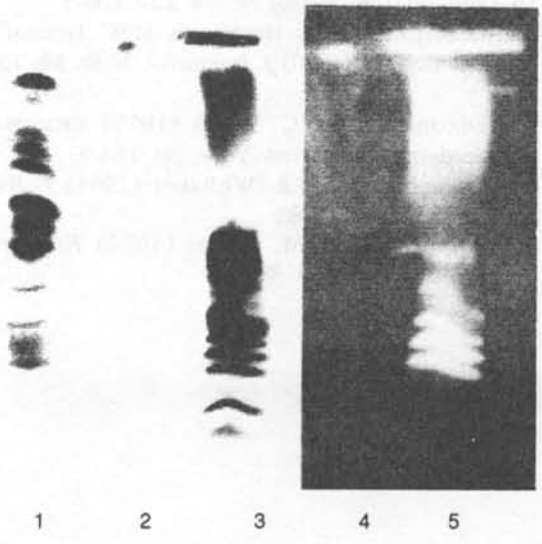

Figure 5. Isoelectric focusing: **Lane 1**: IEF Sigma markers; **Lane 2:** *ficain p I;* **Lane 3:** crude extract. Zymograms: **Lane 4:** *ficain p I,* **Lane 5:** crude extract

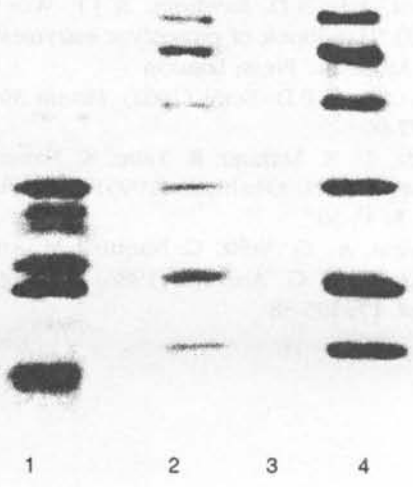

Figure 6. SDS-PAGE. **Lane 1:** crude extract; **lanes 2 and 4:** molecular weight Bio-Rad markers, **lane 3:** *ficain p I.*

the other hand, thermal stability assays with *ficain p I* showed (Fig. 4) that residual caseinolytic activity was notably high even after 30 min at 60 °C (90% of maximum activity), but decayed fast at 65 °C (55% of maximum activity after 30 min incubation) and steeply dropped at 75 °C (about 35% and 7% maximum activity after incubating for 5 and 30 min, respectively). These results suggest, in the case of the crude extract, a protective role played by the substrate and probably by other proteins.

Application of IEF to the crude extract showed that most of the protein fractions exhibited proteolytic activity as evidenced by the corresponding zymogram (Fig. 5); IEF of the chromatographic purified fraction also confirmed its basic nature (pI > 9.3). SDS-PAGE revealed that *ficain p I* appeared as a unique band (Fig. 6), with a relative mass of about 28.6 kDa.

Ficain p I is similar to other enzymes obtained from the genus *Ficus* respecting to optimum pH, thermal stability, behavior against inhibitors, molecular weight and isoelectric point. Kramer and Whitaker [19] showed that *Ficus carica* var. Kadota latex contains ten proteolytic enzymes of basic nature (pI > 9.6) and displays maximum caseinolytic activity within the pH range 6.7-7.5. Ficin A, B, C, D and S were purified from the latex of the *Ficus carica* var Horaishi [20], showing molecular weights ranging from 24.0 to 26.0 kDa, with maximum activity at pH 7.0-8.0, high isoelectric points (8.3-10.2) and similar thermal stability than that of *ficain p I.*

Acknowledgments. N.O. Caffini. and M.C. Arribére are members of the CIC Researcher Career and Support Professional Career programs, respectively. The present work was supported by FONCyT, CIC and University of La Plata.

REFERENCES

1. Rudenskaya, G.N., A.M. Bogacheva, A. Preusser, A.V. Kuznetsova, Ya.E. Dunaevsky, B.N. Golovkin & V.M. Stepanov (1998) *FEBS Lett.* **437**: 237-40
2. Boller, T. (1986) *Roles of proteolytic enzymes in interactions of plants with other organims,* in *Plant Proteolytic Enzymes* (Dalling, M.J. ed.), CRC Press, Boca Ratón, Vol I, pp. 76-86
3. Robbins, B.H. (1930) *J. Biol. Chem.* **87**: 251-7
4. Walti, A. (1938) *J. Am. Chem. Soc.* **60**: 493
5. Williams, D.C., V.C. Sgarbieri & J.R. Whitaker (1968) *Plant Physiol.* **43**: 1083-8
6. Sgarbieri, V.C., S.M., Gupte, D.E. Kramer & J.R. Whitaker (1964) *J. Biol. Chem.* **239**: 2170-7
7. Barrett, A.J., N.D. Rawlings, & J.F. Woessner (1998) "Handbook of proteolytic enzymes" pp. 571, Academic Press, London
8. Atal, C.K. & P.D. Sethi (1962) *Planta Medica* **10**: 77-90
9. Mazda, T., K. Makino, R. Yabe, K. Nakata, K. Fujisawa & H. Ohshima (1995) *Transfusion Med.* **5**: 43-50
10. Hansson, A., G. Veliz, C. Naquira, M. Amren, M. Arroyo & G. Arevalo (1986) *J. Etnopharmacol.* **17**: 105-38
11. de Amorín, A., H.R. Borba, J.P. Carauta, D. Lopes & M.A. Kaplan (1999) *J. Ethnopharmacol.* **64**: 255-8
12. Dimitri, M.J. (1972) Enciclopedia Argentina de Agricultura y Jardinería, Ed. Acme SACI, Buenos Aires, pp. 235
13. Priolo, N.S., L.M.I. López, M.C. Arribére, C.L. Natalucci & N.O. Caffini (1991) *Acta Alimentaria* **20**: 189-96
14. Bradford, M.M. (1976) *Anal. Biochem.* **72**: 248-54
15. Good, N.E. & S. Izawua (1972) *Meth. Enzymol.* **24**: 53-68
16. Laemmli, U.K. (1970) *Nature* **227**: 680-5
17. Westergaar, J.L., C. Hackbarth, M.W. Treuhaf & R.C. Roberts (1980) *J. Immunol. Meth.* **34**: 167-75
18. Dixon, M. & E.C. Webb (1979) *Enzymes.* Academic Press, New York, pp. 164-9
19. Kramer D.E. & J.R. Whitaker (1964) *J. Biol. Chem.* **239**: 2178-83
20. Sugiura, M. & M. Sasaki (1974) *Biochim. Biophys. Acta* **350**: 38-47