Volume 227, number 2, 209-214 FEB 05508 January 1988

Evidence for a Ca^{2+}-independent association between calpain II and phospholipid vesicles

Christophe Garret, Patrick Cottin, Jean Dufourcq[+] and André Ducastaing

Laboratoire de Biochimie et Technologie des Aliments, Dépt Alimentation et Nutrition, Université de Bordeaux I, Avenue des Facultés and [+]*Centre de Recherche P. Pascal, CNRS, 33405 Talence Cedex, France*

Received 21 October 1987; revised version received 27 November 1987

Possible interactions between calpain II and phospholipids such as phosphatidylinositol, phosphatidylserine and phosphatidylcholine were studied using fluorescence and gel filtration techniques. Changes in fluorescence intensity of purified calpain II show that the enzyme strongly interacts with phosphatidylinositol and phosphatidylserine and to a lesser extent with phosphatidylcholine. These results are corroborated by the gel filtration technique which permits the isolation of the enzyme/phospholipid complex. Association between calpain II and various phospholipid vesicles can occur in the absence of calcium. Such binding occurs without any observable change of the molecular mass of the two subunits on SDS-polyacrylamide gel electrophoresis.

Calpain II; Phosphatidylinositol; Phosphatidylserine; Phosphatidylcholine

1. INTRODUCTION

Calcium activated neutral protease (mM CaANP, calpain II, EC 3.4.22.17) contains two subunits; a large one (M_r 80000) and a smaller one (M_r 28000). Although calpain II appears to play important roles in various cellular events [1–4], the mechanism of its in vivo proteolytic activity remains to be elucidated since the enzyme is inactive at physiological intracellular Ca^{2+} concentrations. However, calpain II can be converted to a different enzymatic form by limited autolysis in the presence of calcium [5–8]. Since autolysis greatly reduces the Ca^{2+} dependence of catalytic activity, it may represent an important initial step in the activation of the enzyme. This activation step may be initiated or regulated by a combination of Ca^{2+} and phospholipids because some phospholipids such as phosphatidylinositol and, to a lesser extent, a metabolic derivative dioleoylglycerol stimulate in vitro the Ca^{2+}-dependent autolysis by reducing the amount of Ca^{2+} necessary [8–10]. The mechanism of enzyme phospholipid interactions is unknown at present. According to recent regulation models [11,12] the presence of Ca^{2+} is necessary for calpain II-membrane binding. In order to investigate this phenomenon two techniques were used, the first (fluorescence) providing information on structural changes induced by the association with phospholipids, the second (gel filtration) permitting the recovery of the complexes. The studies described here show that purified calpain II can bind phosphatidylinositol, phosphatidylserine and phosphatidylcholine in the absence of Ca^{2+}: the dissociation constants (K_d) are respectively 4×10^{-9}, 5×10^{-9} and 10^{-8} M. These calpain II-phospholipid associations were confirmed by gel filtration techniques using a Sepharose 4B column: our results demonstrate that calpain II can only bind small unilamellar phospholipid vesicles. In the presence of phosphatidylinositol, phosphatidylserine and phosphatidylcholine, respectively 75%, 75% and 25% of calpain II was found to be associated.

Correspondence address: P. Cottin, Laboratoire de Biochimie et Technologie des Aliments, Département Alimentation et Nutrition, Université de Bordeaux I, Avenue des Facultés, 33405 Talence Cedex, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2. MATERIALS AND METHODS

Unless indicated, all chemicals and reagents were from Sigma in the highest grade available. Casein was obtained from Merck (art.2244). L-3-Phosphatidyl[2-^{3}H]inositol (17.9 Ci/mmol), 1,2-dioleoyl-L-3-phosphatidyl-L-[1-^{14}C]serine (30 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C]linoleoyl-L-3-phosphatidylcholine (50 mCi/mmol) were purchased from Amersham. Phenyl-Sepharose 4B and MonoQ, (HR 5/5) were obtained from Pharmacia.

Calpain II was prepared from rabbit skeletal muscle using the procedure of Penny et al. [11] partly modified by the use of an HPLC anion-exchange column (MonoQ, HR5/5) for the final purification step. The specific activity of purified calpain II was 9000 U/mg. Protein was determined by the method of Bradford [13] using the Bio-Rad reagent and BSA as standard. Proteolytic activity was measured with alkali-denatured casein as substrate according to Penny et al. [11]. The enzymatic unit was defined as described [14].

The phospholipids were dried from the organic solvent under a stream of nitrogen and then suspended in a 20 mM Tris-HCl buffer, pH 7.50, containing 1 mM EDTA (buffer A) and subjected to a direct probe sonication (60 s). This procedure resulted in the production of mainly single unilamellar small vesicles.

Binding of calpain II to the phospholipid vesicles was estimated from changes in the protein fluorescence intensity on excitation at λ = 280 nm, using as SLM 8.000 spectrofluorometer and 10 mm cells. The decrease of the maximum fluorescence intensity at 333 nm as a function of phospholipid addition was ascribed to calpain II-phospholipid binding. The data are reported as the relative change in emission intensity $(I_o - I)/I_o$; I, represents the fluorescence intensity of the protein-lipid complex and I_o the intensity of the protein alone. The association constants (K_a) and the number of phospholipid molecules bound per enzyme molecule (PL/E) were calculated according to [15] using the Scatchard method. K_a can be related to the molar ratio (R_i) = (PL/E) by the general relationship: $\alpha/[(1-\alpha)P_oR_i] = K_a\ (1/n - \alpha/R_i)$, where α is the enzyme bound fraction; $(1-\alpha)$ the free enzyme fraction; P_o the total enzyme concentration; and n the number of phospholipid molecules needed for defining a lipid binding site for calpain II.

SDS-gel electrophoresis was performed according to the procedure of Laemmli [16], using a stacking gel of 4% (w/v) acrylamide and a gradient polyacrylamide resolving gel (10–20%, w/v). Radioactivity was measured using Ready-solv EP scintillation fluid (Beckman) in a Beckman LS 1800 scintillation counter.

3. RESULTS

3.1. *Evidence for phospholipid-calpain II interactions by fluorescence*

The direct detection of phospholipid-calpain II binding is reported in fig.1 at a calpain II concentration of 70 nM. As can be seen the fluorescence yield of tryptophan residues of the protein decreases, following the addition of the various

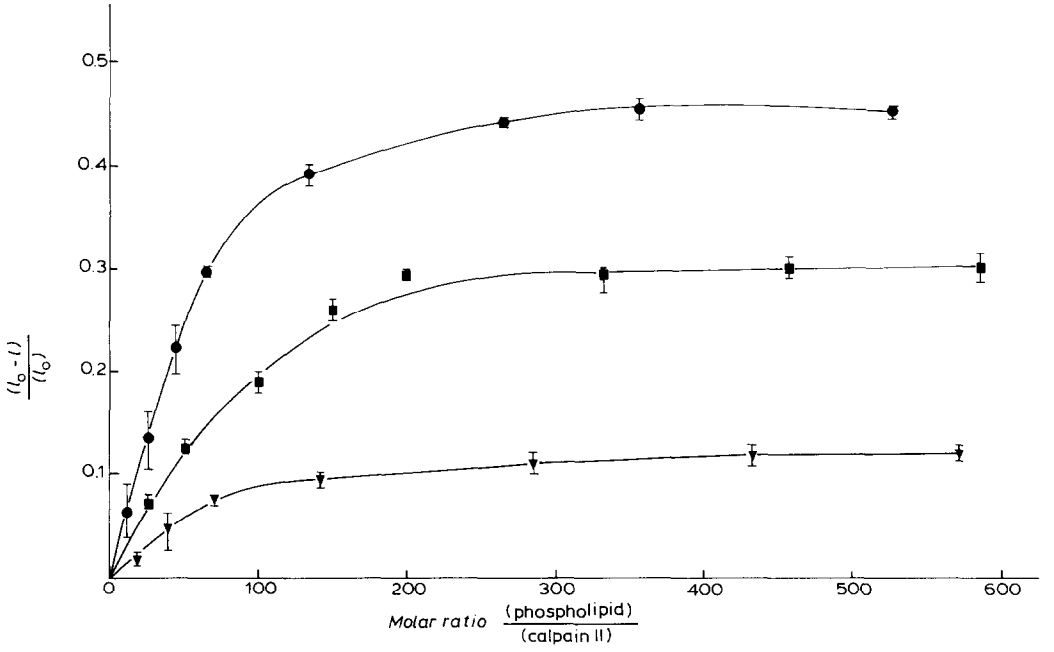


Fig.1. Relative changes in the fluorescence intensity (λ = 333 nm) of calpain II versus the (phospholipid)/(enzyme) molar ratio. Calpain II concentration was 70×10^{-9} M in buffer A. (●—●) Phosphatidylinositol vesicles, (■—■) phosphatidylserine vesicles, (▲—▲) phosphatidylcholine vesicles. All the experiments were carried out at 10°C and each value is an average of 3 experiments.

phospholipids. Structural changes of calpain II show that each phospholipid can interact with the protein. Whatever the phospholipid used, the binding curve reaches a saturation plateau for high molar ratios (PL/E). The final quenching of fluorescence for acidic phospholipids is stronger (30–40%) compared to the very weak, although significant quenching observed with phosphatidylcholine. Plotting these data according to the Scatchard method (cf. section 2), allows the estimation of K_d values which are rather similar for both phosphatidylinositol and phosphatidylserine-calpain II complexes (fig.2) about 5×10^{-9} M). Despite a large uncertainty the affinity for phosphatidylcholine is about an order of magnitude weaker (10^{-8} M) (fig.2). Experiments carried out at different calpain II concentrations (25×10^{-9} M, 90×10^{-9} M) are consistent with these determinations. So far, for phosphatidylinositol and phosphatidylserine, binding appeared to be of a relatively high affinity and the association rate was rapid on the time scale of the experiments. The number of phospholipid molecules needed to define a binding site for calpain II varies from about 100 negatively charged phosphatidylserine or phosphatidylinositol molecules to a lower value of about 40 for phosphatidylcholine. Thus, both from the quantum yield of the bound state and the binding parameters it appears that calpain II binds differently to these two types of phospholipid interfaces.

3.2. *Recovery of enzyme-phospholipid complexes: elution profiles of the Sepharose 4B column and SDS-gel electrophoresis analysis*

Fig.3A–C, shows that each labelled phospholipid is eluted from the Sepharose 4B column in two different peaks. According to Huang [17] and Dufourcq et al. [18], these two peaks correspond, respectively, to multilamellar phospholipid vesicles ($M_r > 10^{-7}$) eluted in the void volume and unilamellar phospholipid vesicles eluted in the inclusion volume (fractions 32–33) with a diameter of about 200 Å. As can be seen in fig.3A, in the presence of phosphatidylinositol vesicles, about 75% of bound calpain II molecules, based on protein concentration, were eluted in the unilamellar phospholipid vesicle elution volume of the column

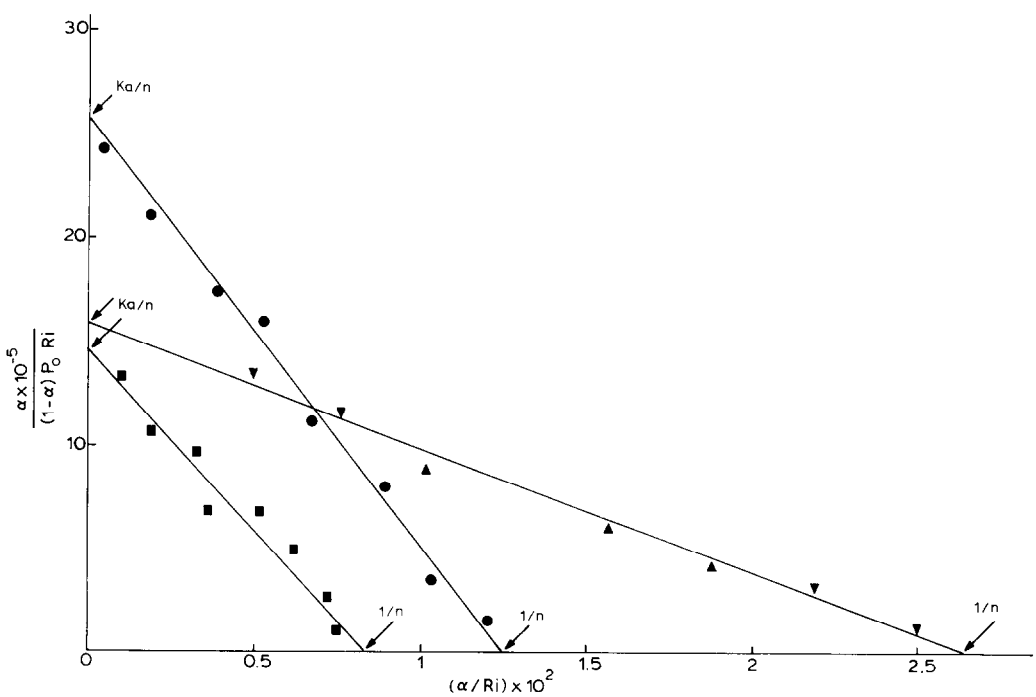
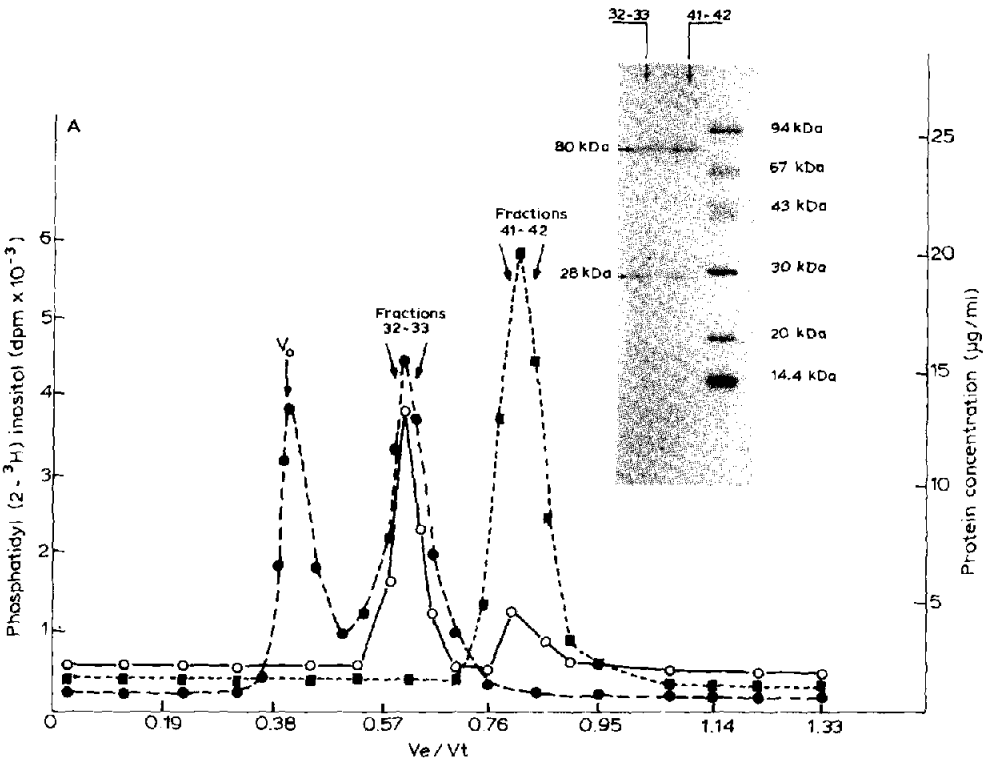
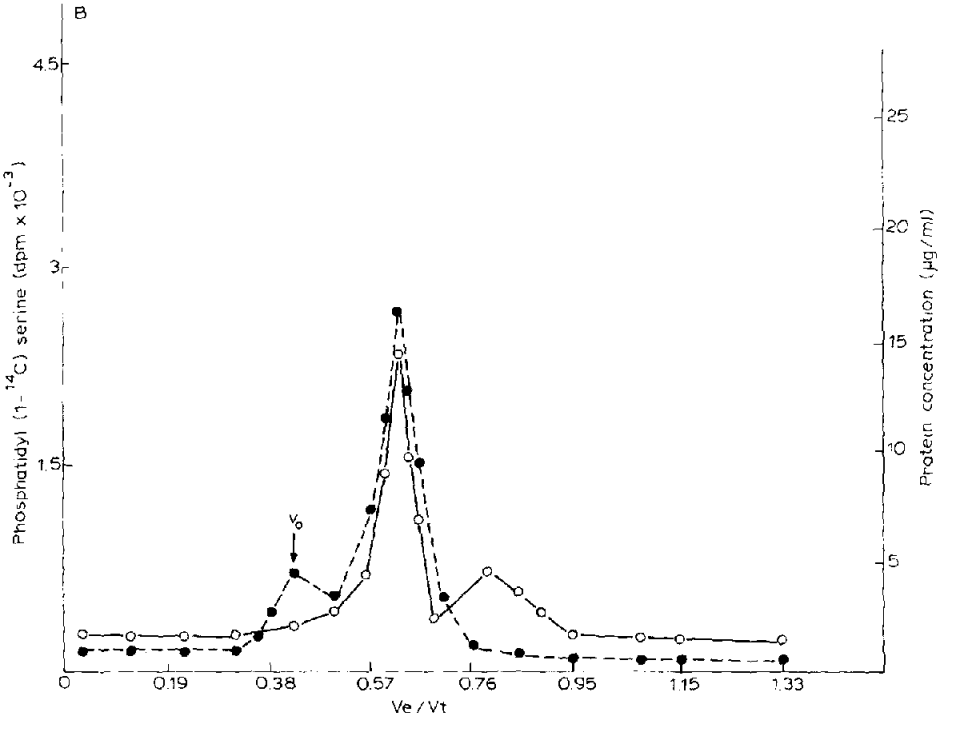


Fig.2. Scatchard plots of the data from fig.1 concerning the calpain II-phospholipid interactions. (●—●) Phosphatidylinositol, (■—■) phosphatidylserine, (▲—▲) phosphatidylcholine.


A
32-33
41-42
80 kDa
28 kDa
94 kDa
67 kDa
43 kDa
30 kDa
20 kDa
14.4 kDa
Fractions 41-42
Fractions 32-33
V_o
Phosphatidyl (2-^{3}H) inositol (dpm x 10^{-3})
Protein concentration (µg/ml)
Ve / Vt
0 0.19 0.38 0.57 0.76 0.95 1.14 1.33


B
V_o
Phosphatidyl (1-^{14}C) serine (dpm x 10^{-3})
Protein concentration (µg/ml)
Ve / Vt
0 0.19 0.38 0.57 0.76 0.95 1.15 1.33

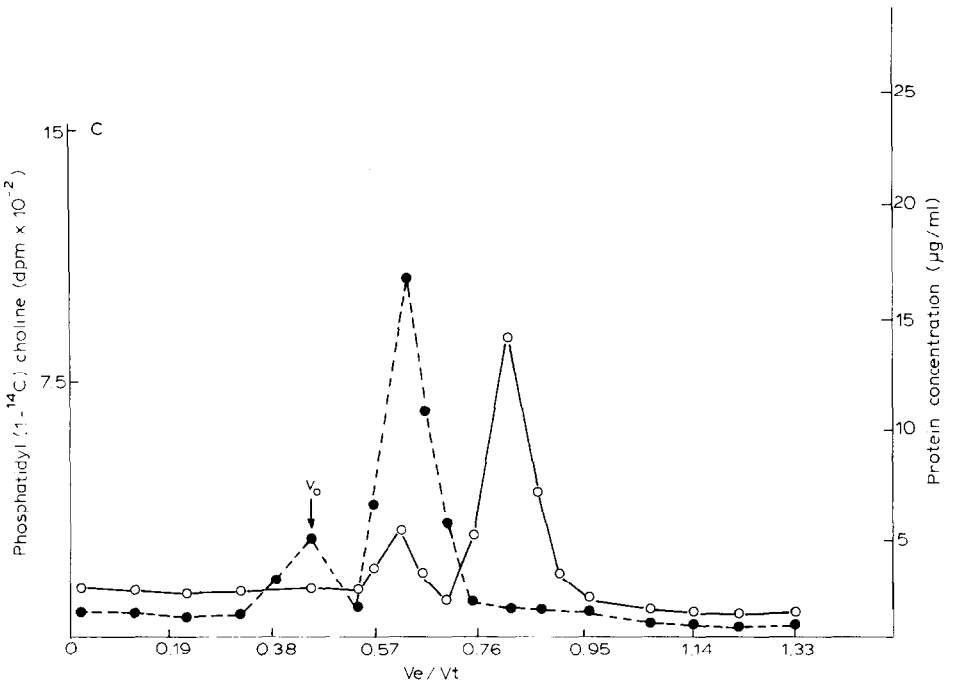


Fig.3. Sepharose 4B elution pattern of calpain II (A) and calpain II-phospholipid complexes (A, B and C). After sonication, 50 µl of the appropriate phospholipids (4 mg/ml) labelled with L-3-phosphatidyl-[2-^{3}H]inositol (800 dpm/nmol) or 1,2-dioleoyl-L-3-phosphatidyl-L-[1-^{14}C]serine (450 dpm/nmol) or 1-palmitoyl-2-[1-^{14}C]linoleoyl-L-3-phosphatidylcholine (150 dpm/nmol) in buffer A were incubated at 10°C with 750 µl of purified calpain II (0.080 mg/ml) and applied to a Sepharose 4B column (40 × 1 cm) previously equilibrated with buffer A. The column fractions (600 µl) were assayed for (■---■) protease concentration, when calpain II is chromatographed alone (A) or for both (○—○) protein concentration, and (●—●) radioactivity measures (A,B,C) when calpain II was chromatographed in the presence of phospholipids. 50 µl aliquots from tubes 32–33 and 150 µl from tubes 41–42 were heated at 100°C for 2 min in the presence of 1% SDS and subjected to electrophoresis (inset). (A) Phosphatidylinositol vesicles; (B) phosphatidylserine vesicles; (C) phosphatidylcholine vesicles.

and 25% of free calpain II molecules in the free calpain II elution volume. From the inset (fig.3A) bound (fractions 32–33) and free calpain II (fractions 41–43) consist of 80 and 28 kDa subunits. From the phosphatidylserine and phosphatidylcholine vesicle elution profiles (fig.3B and C, respectively), it can be observed that 75% and 25% of bound calpain II molecules were in the unilamellar phospholipid vesicle elution volume. So far calpain II molecules were found to be bound only with small unilamellar phospholipid vesicles.

4. DISCUSSION

In vitro the autolytic process of calpain II involving phospholipids and Ca^{2+} is relatively well known whereas in vivo the mechanism of regulation remains hypothetical [10,19,20]; the first activation step involving Ca^{2+} dependence of the enzyme-membrane interactions is still unclear. In order to clarify the calpain II membrane association, we studied the binding of calpain II to charged and neutral phospholipid vesicles. Our fluorescence data demonstrate that Ca^{2+} are not necessary for the binding of the enzyme to phospholipids. Assuming a simple equilibrium between free and bound enzyme the affinity of calpain II for the interface was estimated to be about 5×10^{-9} M for phosphatidylserine and phosphatidylinositol with about 100 phospholipid molecules being required for defining a site. This affinity for the uncharged lecithin interface is weaker. Gel filtration results have shown that about 75% of calpain II was bound to charged phospholipids and 25% was bound to non-charged phospholipids: in the same experimental conditions but with mixed phospholipid vesicles (con-

taining 20% of PI, 20% PS and 60% PC) the interactions were also found to be significant (not shown). Taken together these results suggest that ionic and hydrophobic interactions could be involved in the calpain II-phospholipid complexes. This also confirms results indicating that calpain II possesses in its native form Ca^{2+}-independent weakly hydrophobic accessible region [21]. Thus, even in the absence of Ca^{2+}, it can be suggested that intracellularly a fraction of calpain II molecules could be permanently bound to membrane phospholipids. Local increases of Ca^{2+} concentration, producing some structural modifications of calpain II such as exposure of strongly hydrophobic areas, could modify the membrane-enzyme interactions [8] (with for example possible accessibility to the biological substrates such as kinase C [19]) and promote the autocatalytic activation of the enzyme according either to currently proposed or as yet unknown mechanisms.

Acknowledgements: We thank Bruno Varichon for the iconography and Cynthia Dubourg for her skilful secretarial assistance. This work was supported in part by a grant from the National Institute for Agricultural Research (INRA).

REFERENCES

[1] Murachi, T. (1983) Trends Biochem. Sci. 8, 167–169.
[2] Suzuki, K., Kawashima, S. and Imahori, K. (1984) in: Calcium Regulation in Biological Systems (Ebashi, S. et al. eds) pp.213–229, Academic Press, New York.
[3] Kay, J. (1984) in: Proteases: Potential Role in Health and Disease (Hörl, W.J. and Heidland, A. eds) pp.519–531, Plenum, New York.
[4] Savart, M., Belamri, M., Pallet, V. and Ducastaing, A. (1987) FEBS Lett. 216, 22–26.
[5] De Martino, G.N., Huff, C.A. and Groall, D.E. (1986) J. Biol. Chem. 261, 12047–12052.
[6] Inomata, M., Hayashi, M., Nakamura, K., Imahori, K. and Kawashima, S. (1985) J. Biochem. 98, 407–416.
[7] Hathaway, D.R., Diane, K.W. and Haeberle, J.R. (1981) J. Biol. Chem. 257, 9072–9077.
[8] Pontremoli, S., Melloni, E., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. and Horecker, B.L. (1985) Biochem. Biophys. Res. Commun. 129, 389–395.
[9] Coolican, S.A. and Hathaway, D.R. (1984) J. Biol. Chem. 259, 11627–11630.
[10] Imajoh, S., Kawasaki, M. and Suzuki, K. (1986) J. Biochem. 100, 633–642.
[11] Penny, I.F., Taylor, M.A., Harris, A.G. and Etherington, D.J. (1985) Biochim. Biophys. Acta 829, 244–252.
[12] Pontremoli, S., Sparatore, S., Melloni, E., Michetti, M. and Horecker, B.L. (1984) Biochem. Biophys. Res. Commun. 123, 331–337.
[13] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[14] Azanza, J.L., Raymond, J., Robin, J.M., Cottin, P. and Ducastaing, A. (1979) Biochem. J. 183, 339–347.
[15] Dousseau, F., Faucon, J.F. and Dufourcq, J. (1986) in: Surfactants in Solution (Mittal, K.L. and Bothorel, P. eds) vol.5, pp.907–916, Plenum, New York.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Huang, C. (1969) Biochemistry 8, 344–351.
[18] Dufourcq, J., Bernon, R. and Lussan, C. (1976) Biochim. Biophys. Acta 433, 252–263.
[19] Suzuki, K., Imajoh, S., Emori, Y., Kawasaki, H., Minami, Y. and Ohno, S. (1987) FEBS Lett. 220, 271–277.
[20] Pontremoli, S., Melloni, E., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. and Horecker, B.L. (1985) Biochem. Biophys. Res. Commun. 128, 331–338.
[21] Gopalakrishna, R. and Barsky, S. (1986) J. Biol. Chem. 261, 13396–13942.