[Chem. Pharm. Bull. 33(12)5413—5418(1985)]

Triton-Induced Proteolysis of Rabbit Intestinal Microvillar Proteins: Inhibition by Ethylenediamine *N,N,N′,N′*-Tetraacetic Acid

YASUKO KOZAKI (née NAKASE),*[,a] KUNIO YOKOTA,[a] SACHIKO TAKESUE[b] and YOSHIKI TAKESUE[a]

Research Institute of Environmental Medicine[a] and Biological Institute,[b] Nagoya University, Chikusa-ku, Nagoya 464, Japan

(Received April 10, 1985)

The addition of Triton X-100 to isolated rabbit intestinal microvilli induced proteolysis of some microvillar proteins. The proteolysis, which was conspicuous in actin, was inhibited by ethylenediamine *N,N,N′,N′*-tetraacetic acid (EDTA) effectively at pH 7.0 but partially at pH 8.6. Phosphoramidon and phenylmethylsulfonyl fluoride showed no inhibitory effect. When microvilli solubilized by Triton X-100 were concentrated, trehalase, a microvillar membrane enzyme, became subject to limited proteolysis, which was also almost completely inhibited by EDTA at pH 7.0. These results show that on solubilization of intestinal microvilli with detergent, care must be taken to avoid induced proteolysis. Most of such proteolysis can be prevented by EDTA at pH 7.0.

Keywords——protease; intestinal microvilli; actin; trehalase; EDTA

Plasma membrane-bound proteases have been reported in various cell types, including the intestinal epithelial cell,[2-8] but little has been known about their physiological roles.[9,10] When the membranes are disintegrated or solubilized with detergent, these proteases could induce structural artifacts in coexisting proteins. For example, the heterogeneity of beta-adrenergic receptors observed in lung membranes was reported to be predominantly due to proteolysis.[8] When isolated intestinal microvilli are treated with detergent, some microvillar proteins are digested.[11,12] Gains and Hauser[12] have reported that this detergent-induced proteolysis can be prevented effectively only when ethylenediamine *N,N,N′,N′*-tetraacetic acid (EDTA), diisopropylfluorophosphate (DFP) and iodoacetamide are added together. We have explored simpler and milder conditions for inhibition of the proteolysis, and have found that Triton-induced proteolysis of microvillar proteins, especially of actin and trehalase, is effectively prevented by EDTA alone if solubilization with Triton X-100 is carried out at pH 7.0.

Experimental

Materials——Microvilli were isolated from frozen rabbit small intestines by a Ca^{2+} precipitation method.[13] Rabbit skeletal muscle actin was purified by the method of Spudich and Watt.[14] Azocasein and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., U.S.A., DFP from Fluka AG, Switzerland, and phosphoramidon from Protein Research Foundation, Japan.

Treatment of Microvilli with Triton X-100——Isolated microvilli (1.5 mg protein/ml) were preincubated with 0, 5, or 11 mM EDTA in 50 mM sodium phosphate (pH 7.0) or 50 mM Tris–HCl (pH 8.6) at 37 °C for 15 min, and then 1/10 vol. of 10% Triton X-100 in buffer was added. After further incubation at 37 °C for different times, they were analyzed by SDS–polyacrylamide gel electrophoresis for proteolysis.

Solubilization of Microvilli with Triton X-100 and Concentration of the Solubilized Microvilli——Microvilli (5 mg protein/ml) were incubated with 1% Triton X-100 in 10 mM sodium phosphate (pH 7.0) at 0 °C for 30 min, followed by centrifugation at 75000 × *g* for 60 min to give solubilized microvilli. In the experiments designed to examine limited proteolysis of solubilized trehalase, the Triton-solubilized supernatant was further centrifuged at 250000 × *g* for 5 h;

NII-Electronic Library Service

the upper 9/10 of the resultant supernatant was discarded, and the remaining one-tenth and the pellet were mixed to give concentrated solubilized microvilli. More than 90% of solubilized trehalase was recovered in this fraction.

Sepharose 6B Gel-Filtration——Three ml of the Triton-solubilized microvilli was gel-filtered through a Sepharose 6B column (37 × 1.6 cm) with 0.5% Triton and 0.1 M NaCl in 10 mM phosphate (pH 7.0) as the elution buffer; the eluate was collected in fractions of 1.6 ml. In the case of the concentrated sample obtained as described above, prior to gel-filtration it was incubated at 37 °C for 0 or 2 h in the presence or absence of 5 mM EDTA. The sample (0.2 ml) was then applied to a Sepharose 6B column (85 × 1 cm) and the column was eluted with 0.5% Triton X-100 and 2.5 mM EDTA in 10 mM phosphate (pH 7.0); the eluate was collected in fractions of 0.5 ml.

Phenyl Sepharose Chromatography——The concentrated solubilized microvilli incubated at 37 °C for 2 h in the absence of EDTA showed two peaks of trehalase activity on Sepharose 6B gel-filtration as described later. Fractions of each trehalase peak were pooled and 0.6 ml of the pooled sample was applied to a phenyl Sepharose CL-6B column (2.6 × 0.5 cm) equilibrated with 5 mM EDTA in 10 mM phosphate (pH 7.0). The column was eluted first with 5 ml of the same buffer and then with the buffer containing 2% Triton X-100; the eluate was collected in fractions of 0.5 ml.

Enzyme Assays——Azocasein-hydrolyzing activity was assayed by incubating an enzyme sample with 1.25% azocasein in a total volume of 0.5 ml in 0.1 M Tris–HCl (pH 8.6) at 37 °C for 30 or 60 min, after which the reaction was stopped by the addition of 2 ml of 10% trichloroacetic acid. The mixture was filtered through filter paper (Toyo Roshi No. 2) and the absorbance of the filtrate was read at 340 nm; the activity was expressed in terms of changes in absorbance. The activity of aminopeptidase M was assayed by incubating an enzyme sample with 1.6 mM L-leucyl *p*-nitroanilide as a substrate in a total volume of 1 ml in 50 mM Tris–HCl (pH 7.2) and 50 μM $CoCl_2$ at 37 °C for 15 min, after which 1 ml of 1 M acetic acid was added to stop the reaction and *p*-nitroaniline released was determined by absorbance at 405 nm. With samples from Sepharose 6B gel filtration of the concentrated solubilized microvilli, aminopeptidase M was assayed after the samples had been preincubated with 4 mM $MgCl_2$ at room temperature for 10 min and then with 1 mM $MnCl_2$ at 37 °C for 30 min to overcome inhibition by EDTA. Trehalase[15] and sucrase[16] were assayed as described previously.

Protein Determination——Protein was determined by the method of Lowry *et al.*[17] using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis——SDS-polyacrylamide gel electrophoresis was carried out by the system of Laemmli[18] with 8.4% polyacrylamide gel. Gels were stained with Coomassie brilliant blue. To assess actin hydrolysis the amounts of protein in stained bands were estimated as described previously[19] and changes in the amount of actin were normalized according to the procedure of Gains and Hauser.[12]

Results and Discussion

Azocasein-Hydrolyzing Activity

Isolated microvilli hydrolyzed externally added azocasein. Azocasein digestion was maximal around pH 8.6 and minimal at 6.5—7.0 over the range of pH examined (pH 6.5—9.0); the activity at pH 7.0 was about a half of that at pH 8.6. It was inhibited 70—80% by 5 mM EDTA but negligibly by 0.1 mM DFP or 1 mM iodoacetamide at either pH 7.0 or 8.6. Inhibition by EDTA was slightly enhanced by the addition of DFP and iodoacetamide.

The azocasein-hydrolyzing activity was solubilized more than 80% with 1% Triton X-100, with no increase in the total activity, suggesting that the protease(s) is exposed on the outer surface of the microvillar membrane. On Sepharose 6B gel-filtration, the proteolytic activity was eluted with the other microvillar hydrolases examined (Fig. 1). It was inhibited by EDTA; the extent of inhibition was higher in the earlier than in the later fractions of the peak (Fig. 1). Such a difference in sensitivity to EDTA was also seen with rabbit skeletal muscle actin as a substrate (Fig. 1). Therefore, the microvillar membrane probably has EDTA-sensitive and -insensitive proteases.

Actin Hydrolysis

The addition of detergent to isolated microvilli induces proteolysis of some microvillar proteins, especially of actin, which amounts to about 30% of the total protein of the isolated microvilli.[11,12] This Triton-induced proteolysis of microvilli was examined at pH 7.0 (Fig. 2) or 8.6 in the presence or absence of inhibitors. At the two pHs neither iodoacetamide nor DFP inhibited Triton-induced actin hydrolysis, as reported by Gains and Hauser,[12] but EDTA effectively inhibited it to almost the same extent as a combination of EDTA, DFP and

NII-Electronic Library Service

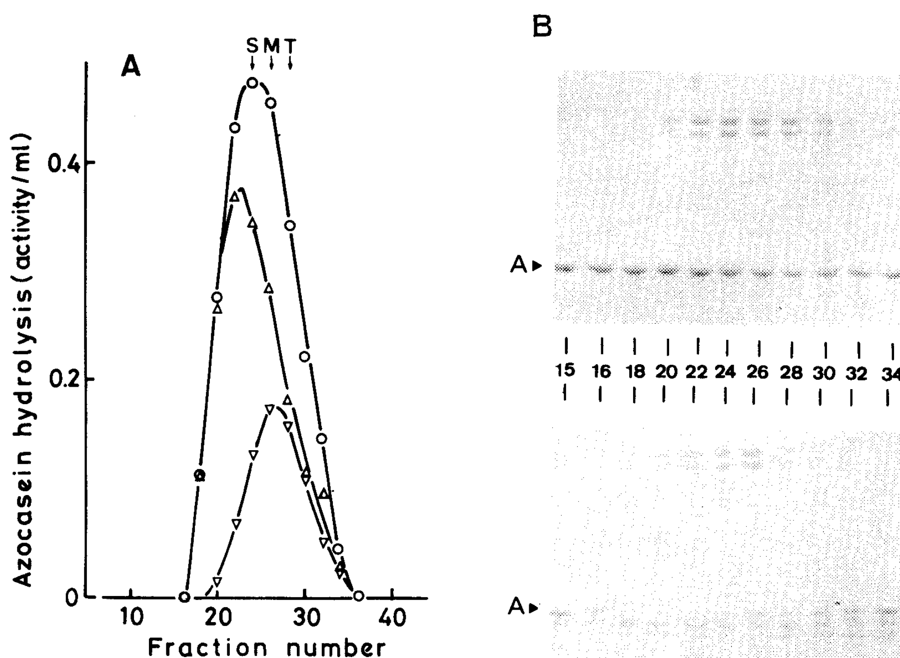

Fig. 1. Sepharose 6B Gel Filtration of the Supernatant from Microvilli Solubilized with 1% Triton X-100

A: azocasein hydrolysis by the eluate at 37 °C for 30 min in the presence (○) or absence (△) of 5 mM EDTA. The difference between the two (▽) is taken as reflecting EDTA-sensitive. The arrows indicate the peak positions of sucrase (S), aminopeptidase M (M) and trehalase (T) activities.

B: rabbit skeletal muscle actin digestion by the eluate in the presence (upper panel) or absence (lower panel) of 5 mM EDTA. The eluate (150 μl) was preincubated with or without 5 mM EDTA in 0.5% Triton X-100, 0.1 M NaCl and 10 mM sodium phosphate (pH 7.0) at 37 °C for 15 min, after which rabbit skeletal muscle actin (15 μg) was added. After further incubation at 37 °C for 90 min, the samples were analyzed by SDS-gel electrophoresis. The numbers indicate the fraction numbers. A, actin.

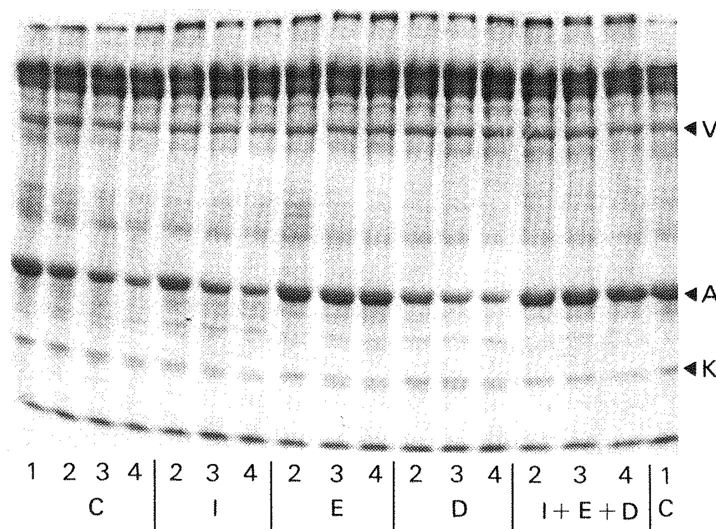

Fig. 2. Effects of EDTA, DFP and Iodoacetamide on Triton-Induced Microvillar Proteolysis

Microvilli (5 mg protein/ml) were preincubated with or without 5 mM inhibitors in 50 mM sodium phosphate (pH 7.0) at 37 °C for 15 min, after which 1/10 vol. of 10% Triton X-100 in buffer was added. After further incubation at 37 °C for various times, the samples were subjected to SDS-gel electrophoresis. The numbers indicate the incubation time (min): 1, 0; 2, 10; 3, 30; 4, 50 min. Inhibitors: E, EDTA; D, DFP; I, iodoacetamide. Bands: A, actin; V, villin; K, 33 kD protein.

NII-Electronic Library Service

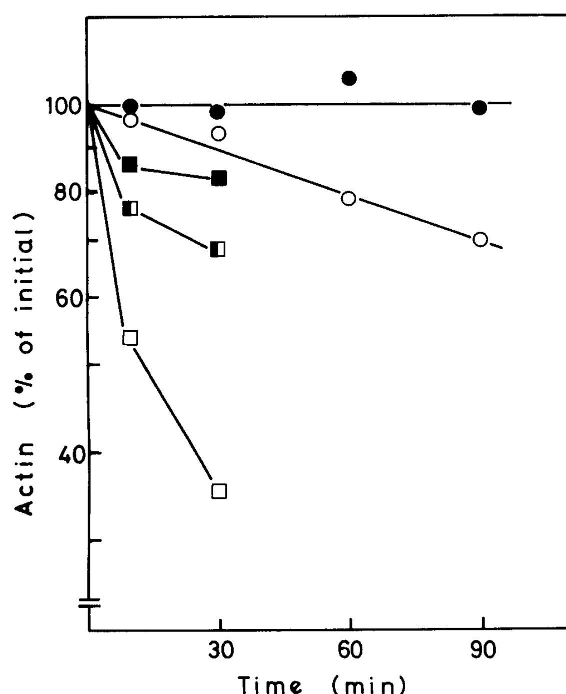

Fig. 3. Effect of pH on the Inhibition by EDTA of Triton-Induced Actin Digestion

Microvilli were preincubated with 0 (○, □), 5 (●, ◧) or 11 (■) mM EDTA in 50 mM sodium phosphate (pH 7.0) (circles) or 50 mM Tris–HCl (pH 8.6) (squares). After addition of Triton X-100 (1%) the samples were further incubated at 37 °C for different times. The amounts of remaining actin in the samples were plotted against the incubation time on a semi-logarithmic scale.

iodoacetamide did. Villin, another microvillar cytoskeletal protein, was also digested on the addition of Triton though to a less extent than actin, and its digestion was also inhibited by EDTA (Fig. 2). The effects of EDTA and pH were estimated by calculating apparent first-order rate constants (Fig. 3). The rate constants in the absence of EDTA were 0.21 and 3.7 h^{-1} at pH 7.0 and 8.6, respectively. EDTA at 5 mM inhibited Triton-induced actin digestion almost completely at pH 7.0, but partially at pH 8.6.

Phosphoramidon at 1 μM has been reported to virtually completely inhibit hydrolysis of (^{125}I)iodo-insulin B chain by pig intestinal and rabbit renal microvilli.[4,20] However, it did not inhibit Triton-induced actin or villin digestion by rabbit intestinal microvilli even at 0.1 mM. Phenylmethylsulfonyl fluoride has often been used to prevent digestion of cytoskeletal proteins during the demembranation of microvilli with detergent.[21–23] This reagent was also found to have no inhibitory effect on Triton-induced actin and villin digestion. Azocazein hydrolysis by microvilli was inhibited about 40% by 5 mM *p*-chloromercuribenzoate. On the other hand, Triton-induced actin digestion was stimulated by preincubating microvilli with 5 mM *p*-chloromercuribenzoate. This stimulatory effect may be due to denaturation of intrinsic actin by the SH blocking agent, because rabbit skeletal muscle actin pretreated with the agent was also digested at a much faster rate than the non-treated one. Dithiothreitol has no stimulatory or inhibitory effect.

Limited Proteolysis of Trehalase

The intestinal microvillar membrane has various enzymes in addition to protease.[24] To examine whether digestion of the membrane-bound enzymes is also induced by Triton X-100, we incubated the concentrated solubilized microvilli in the presence or absence of EDTA and gel-filtered them on Sepharose 6B (Fig. 4). When the sample was not incubated before gel-filtration, each of the enzymes examined, sucrase, trehalase and aminopeptidase M appeared as a single peak. With the sample incubated in the absence of EDTA, however, the trehalase peak split into two, the earlier one corresponding to the original; the total activity remained unchanged. In this case either sucrase or aminopeptidase M was eluted in one peak at the same position as before. When the sample incubated with EDTA was gel-filtered, trehalase, as well as the other two enzymes, was eluted in the same pattern as in the case of the non-

NII-Electronic Library Service

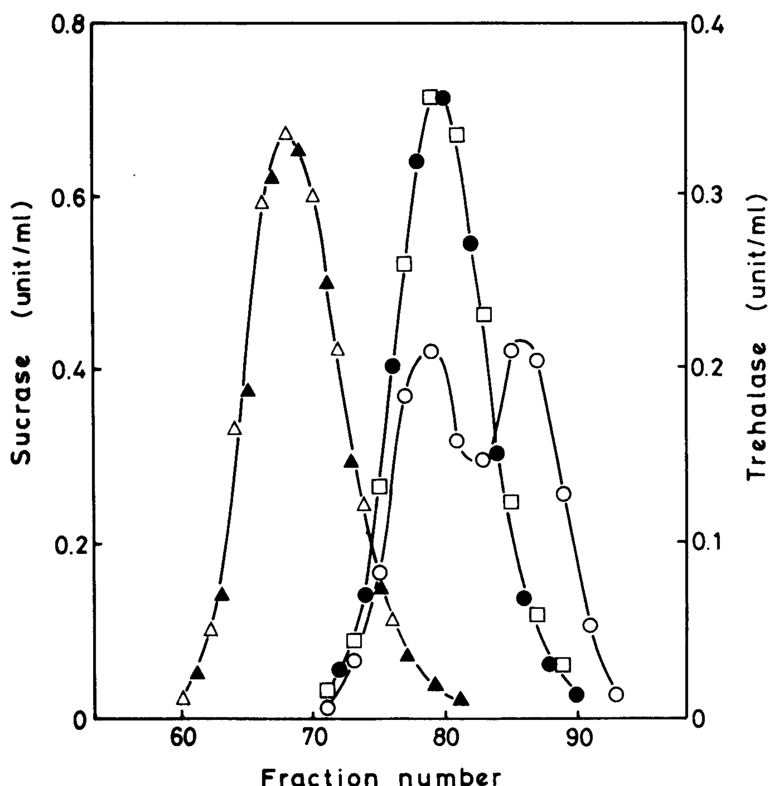

Fig. 4. Limited Proteolysis of Triton-Solubilized Trehalase and Its Inhibition by EDTA

The Triton-solubilized microvillar supernatant was concentrated by centrifugation. Immediately (●, ▲) or after incubation at 37 °C for 2 h in the presence (□) or absence (○, △) of 5 mM EDTA, the sample was gel-filtered on Sepharose 6B. One unit = 1 μmol substrate hydrolyzed/min. ○, ●, □, trehalase; △, ▲, sucrase.

incubated sample.

The two trehalase peaks obtained on Sepharose 6B gel-filtration of the sample incubated without EDTA were separately pooled and each pooled sample was applied to a phenyl Sepharose column, which was then washed with phosphate buffer containing no Triton. The trehalase activity of the first peak was all retained but that of the second one was more than 95% eluted. The retained activity of the first peak could be eluted with buffer containing 2% Triton. These results indicate that the first peak trehalase had a hydrophobic segment but the second peak trehalase did not. It follows that Triton-solubilized amphipathic trehalase was partially converted into a hydrophilic form without loss of enzyme activity by microvillar protease, the action of which could be inhibited by EDTA. It is difficult to detect this change in trehalase by SDS-polyacrylamide gel electrophoresis, because trehalase amounts to less than 0.1% of the total protein of the isolated microvilli.[25] Sucrase and aminopeptidase M were resistant to protease under the present conditions. When the Triton-solubilized supernatant was gel-filtered without concentration by ultracentrifugation, trehalase was eluted in one peak, sometimes followed by another very small peak. Quite similar elution patterns were obtained with non-concentrated samples kept at 4 °C for up to a week (data not shown). These results suggest that limited proteolysis of trehalase was stimulated by concentrating the substrate (trehalase) and the enzyme (protease). Recently, Galand[26] reported the purification of trehalase solubilized from rabbit intestinal microvilli with Triton X-100 without the use of a protease inhibitor. The purified trehalase lacked the hydrophobic segment, most probably due to limited proteolysis occurring during the purification. In fact,

NII-Electronic Library Service

using media containing EDTA at all the purification steps, we have been able to purify Triton-solubilized trehalase in an amphipathic form from rabbit intestinal microvilli.[25]

It should be noted that degradation of some microvillar proteins may occur even in the presence of EDTA, because the results in Fig. 1 show the presence of EDTA-resistant proteolytic activity. The results reported by Gains and Hauser (Fig. 6 of reference 12) also show that proteins of molecular weight in the range of 50000—80000 are digested even in the presence of EDTA, DFP and iodoacetamide together. The 33 kD protein, a microvillar cytoskeletal protein, was also digested on the addition of Triton X-100 but, unlike actin and villin, its hydrolysis was not inhibited by EDTA or by a combination of EDTA, DFP and iodoacetamide even at pH 7.0 (Fig. 2).

The present work shows that EDTA alone can inhibit Triton-induced proteolysis of isolated microvilli effectively, though not completely, if the treatment is carried out at pH 7.0. The present conditions are mild compared with those using EDTA, DFP and iodoacetamide together. The use of EDTA might cause the inhibition of metal enzymes *e.g.* aminopeptidase M. However, the full activity of aminopeptidase M can be recovered by incubating EDTA-treated samples with Mg^{2+} and Mn^{2+} as described under Experimental. It is clear that when dealing with intestinal microvilli, one must take into account the action of intrinsic protease, which is inhibited effectively by EDTA at pH 7.0.

References and Notes

1) This work was supported in part by a research grant from the Ministry of Education, Science and Culture of Japan.
2) M. A. Kerr and A. J. Kenny, *Biochem. J.*, **137**, 489 (1974).
3) S. Pontremoli, F. Salamino, B. Sparatore, E. Melloni, A. Morelli, U. Benatti and A. De Flora, *Biochem. J.*, **181**, 559 (1979).
4) E. M. Danielsen, J. P. Vyas and A. J. Kenny, *Biochem. J.*, **191**, 645 (1980).
5) P. Kocna, P. Frič, J. Slabý and E. Kasafírek, *Hoppe-Seyler's Z. Physiol. Chem.*, **361**, 1401 (1980).
6) J. P. H. Burbach, E. R. De Kloet, P. Schotman and D. De Wied, *J. Biol. Chem.*, **256**, 12463 (1981).
7) L. Guenet, G. Leray, J.-P. Codet, A. Le Treut and J.-Y. Le Gall, *Biochem. Biophys. Res. Commun.*, **108**, 486 (1982).
8) J. L. Benovic, G. L. Stiles, R. J. Lefkowitz and M. G. Caron, *Biochem. Biophys. Res. Commun.*, **110**, 504 (1983).
9) Q. F. Ahkong, G. M. Botham, A. W. Woodward and J. A. Lucy, *Biochem. J.*, **192**, 829 (1980).
10) E. Alhanaty, J. Patinkin, M. Tauber-Finkelstein and S. Shaltiel, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3492 (1981).
11) Y. Takesue, K. Yokota and S. Takesue, *Annu. Reports Res. Inst. Environment. Med. Nagoya Univ.*, **32**, 191 (1981).
12) N. Gains and H. Hauser, *Biochim. Biophys. Acta*, **646**, 211 (1981).
13) K. Yokota, Y. Nishi and Y. Takesue, *Biochem. Pharmacol.*, **32**, 3453 (1983).
14) J. A. Spudich and S. Watt, *J. Biol. Chem.*, **246**, 4866 (1971).
15) M. Nakano, Y. Sumi and M. Miyakawa, *J. Biochem.* (Tokyo), **81**, 1041 (1977).
16) H. Sigrist, P. Ronner and G. Semenza, *Biochim. Biophys. Acta*, **406**, 433 (1975).
17) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
18) U. K. Laemmli, *Nature* (London), **227**, 680 (1970).
19) R. Tamura, Y. Takesue and Y. Nishi, *J. Biochem.* (Tokyo), **92**, 1259 (1982).
20) A. J. Kenny, I. S. Fulcher, K. Ridgwell and J. Ingram, *Acta Biol. Med. Ger.*, **40**, 1465 (1981).
21) M. S. Mooseker, T. D. Pollard and K. Fujiwara, *J. Cell Biol.*, **79**, 444 (1978).
22) A. Bretscher and K. Weber, *Cell*, **20**, 839 (1980).
23) A. Bretscher, *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 6849 (1981).
24) A. J. Kenny and S. Maroux, *Physiol. Rev.*, **62**, 91 (1982).
25) K. Yokota and Y. Takesue, *Annu. Reports Res. Inst. Environment. Med. Nagoya Univ.*, **35**, 222 (1984).
26) G. Galand, *Biochim. Biophys. Acta*, **789**, 10 (1984).

NII-Electronic Library Service