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FAST AND EFFICIENT PURIFICATION OF YEAST PLASMA MEMBRANES USING CATIONIC SILICA MICROBEADS

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A fast and efficient procedure for the purification of plasma membranes from *Saccharomyces cerevisiae* is described. Protoplasts served as starting material. They were coated with cationic silica microbeads. After lysis, the plasma membranes were washed free from debris and cell organelles. This procedure resulted in a high yield (about 85%) of plasma membranes, as judged by measuring vanadate-sensitive ATPase as a plasma membrane marker. The enzyme was enriched 12-fold relative to the homogenate after lysis. Its specific activity was 1.5–2.0 μmol/min per mg protein, the pH optimum was 6.5, and 10 μM vanadate was sufficient to obtain maximum inhibition. Based on the assay of internal markers and electron microscopic studies, we found our preparation essentially free of contamination from other cell organelles.

Introduction

Traditional methods for purification of fungal plasma membranes are based on either harsh cell homogenization techniques or gentle protoplast lysis followed by differential and gradient centrifugation. The results of these different methods have recently been compared in a review by Goffeau and Slayman [1]. With both techniques, relatively pure plasma membranes can be obtained. Yields, however, are often very low and the preparations time-consuming.

To overcome these difficulties, a new approach for plasma membrane isolation has been introduced. This approach is based on the binding of cells via their negative surface charge to positively charged beads [2–8]. Cell binding is followed by a neutralization step, either by the addition of polyacrylate or by a pH shift. This prevents the binding of negatively charged proteins or cell organelles upon lysis, to regions of the bead surface not coated with membrane. After lysis, plasma membrane-coated beads are washed to remove contaminating material.

Recently, a modification of this approach for the isolation of plasma membranes from *Dictyostelium discoideum* was described [9]. Instead of binding cells onto 30 μm beads, microbeads with a diameter ranging between 10 and 50 nm were used to coat the cells. The high density of the microbeads (2.5 g per cm^3) enables the rapid separation of coated plasma membrane sheets from cellular organelles by centrifugation. In the present paper we describe the use of microbeads for the rapid and efficient purification of plasma membranes from yeast protoplasts.

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Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Mes, 4-morpholineethanesulfonic acid.

Materials and Methods

Strain and culture conditions. *Saccharomyces cerevisiae* strain R XII, a kind gift from Dr Kotyk, Prague, was grown in medium I (2% glucose, 1% Difco yeast nitrogen base, 0.5% peptone) at 30°C under agitation.

Preparation of protoplasts. Cells were harvested in the exponential growth phase at a titer of $2 \cdot 10^7$ cells/ml. Protoplasts were prepared using snail gut enzyme [10]. The digestion of the cell wall was monitored using Calcofluor White M2R (disodium salt of 4,4-bis-(4-anilino-bis-diethylamino-*S*-triazin-2-ylaminol)-2,2-stibene-disulfonic acid), a fluorescent dye which stains carbohydrates of the cell wall [11]. For complete digestion of the cell wall the cells had to be incubated for at least 45 min.

Preparation of microbeads. Positively charged silica microbeads were synthesized as described [9]. Immediately before each experiment, the microbeads were diluted with coating buffer (25 mM sodium acetate, pH 5, 0.8 M sorbitol, 0.1 M KCl) to a final concentration of 3% (w/v). The suspension was centrifuged at $1\,500 \times g$ for 10 min to pellet aggregated microbeads. The supernatant was used for coating.

Isolation of plasma membranes. All steps were carried out at 4°C. Freshly prepared protoplasts were washed three times in coating buffer. They were counted and resuspended at a concentration of $1.5 \cdot 10^8$ /ml. Protoplast and microbead suspensions were mixed in the ratio 2 : 1. After 3 min, the coated protoplasts were centrifuged at $500 \times g$ for 4 min. To remove excess microbeads, the pellet was washed once and resuspended in coating buffer at a concentration of $1 \cdot 10^8$ protoplasts/ml. To block free cationic groups on the microbeads, this suspension was diluted 1 : 1 with polyacrylic acid (M_r, 90000; 0.1 mg/ml coating buffer). The protoplasts were centrifuged, washed once with coating buffer and vortexed-mixed under the addition of lysis buffer (5 mM Tris-HCl, pH 8, 1 mM EGTA; 10^8 protoplasts/ml). Lysis was complete after 5 min. The plasma membrane preparation thus obtained was centrifuged at $1000 \times g$ for 5 min and washed 2–3 times in lysis buffer to remove contamination.

Surface labelling of protoplasts. ^{125}I-labelled concanavalin A was prepared according to the procedure of Cuatrecasas [12]. To label protoplasts with ^{125}I-labelled concanavalin A $1.5 \cdot 10^9$ protoplasts suspended in 1 ml coating buffer were mixed with 10 μl of ^{125}I-labelled (0.5 μCi). The protoplasts were washed three times with coating buffer to remove free ^{125}I-labelled concanavalin A. About 14% of the ^{125}I-labelled concanavalin A was bound by the protoplasts.

Enzyme assays. ATPase activity was determined by continuously recording the amount of inorganic phosphate released [13,14]. The standard assay contained 0.5 mM MgCl, 0.5 mM ATP, 80 mM KCl and 10–50 μg protein in a total volume of 5 ml of either 100 mM Mes-Tris, pH 6.5, or 100 mM Tris-HCl, pH 9.0, to assay plasma membrane and mitochondrial ATPase, respectively. Cytochrome *c* oxidase was measured as described by Hodges and Leonard [15]. α-Mannosidase activity was assayed according to the method of Vand der Wilden and Matile [16] and α-glucosidase with the method of Halvorson [17]. Hexokinase, alkaline and acid phosphatase activities were determined as described in Ref. 18. Protein concentrations were measured after trichloracetic acid precipitation using a modification [19] of the procedure of Lowry et al. [20].

Chemicals. The snail gut enzyme was obtained from Reactifs IBF, Villeneuve-la-Garenne, France. Calcofluor White M2R is a product of American Cyanamid Co., Montreal, Canada. All other chemicals were of analytical grade or of the highest purity available. They were purchased from Sigma Chemicals, Serva Heidelberg or Boehringer Mannheim. Positively charged microbeads may be requested from Bruce Jacobson, Department of Biochemistry, University of Massachusetts, Amherst, MA 01003, U.S.A.

Results

Coating of protoplasts. The attachment of microbeads to freshly prepared protoplasts was complete after 3 min. After removing excess microbeads the anionic polymer, polyacrylic acid, was added to neutralize exposed, positively charged regions on the microbead surface. The neutralization step was necessary to avoid adherence of negatively charged cytoplasmic proteins or

organelles which could cause significant contamination of the plasma membrane preparation.

During coating and neutralization procedure, no lysis of the protoplasts occurred. This was confirmed by determining activity of α-glucosidase, a cytoplasmatic enzyme, in the supernatants after each centrifugation. No enzyme activity could be detected.

Lysis of coated protoplasts. Complete lysis of the coated protoplasts was accomplished after 5 min incubation in lysis buffer. The pH of the lysis buffer was adjusted to 8.0 in order to avoid binding of intracellular contaminating material to the plasma membrane fraction. The addition of EGTA was necessary to trap divalent cations. Without the addition of EGTA, severe aggregation occurred and the plasma membranes could not be washed free from entrapped organelles and other contaminants. Fig. 1 shows that after lysis of microbead-coated protoplasts the plasma membranes did not collapse or designate, but retained a shell-like shape.

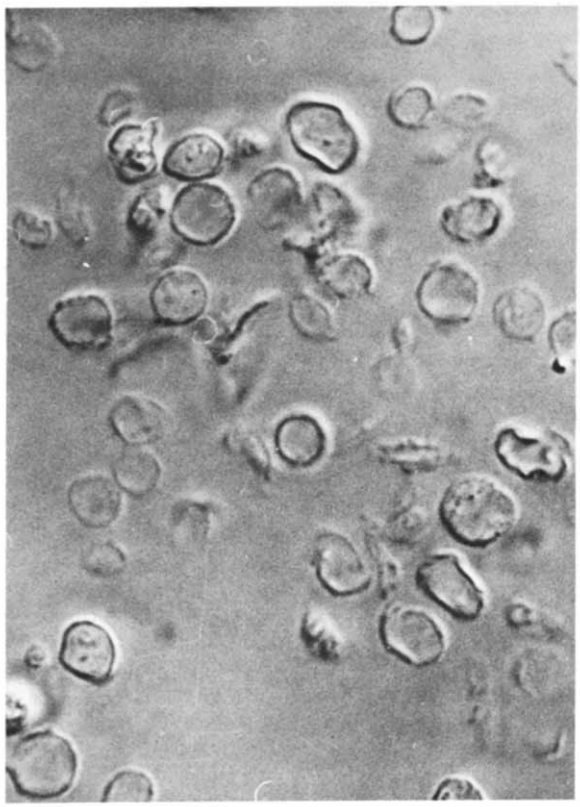

Fig. 1. Typical lysate of microbead-coated protoplasts under Normarski optics (600×).

Labelling of the protoplast surface. To demonstrate the effect of microbeads on the recovery of plasma membranes, protoplasts were labelled with ^{125}I-labelled concanavalin A prior to the coating step. After coating and lysis the lysate was layered on a sucrose step gradient. As a control, the lysate of non-coated ^{125}I-labelled concanavalin A labelled protoplasts was used. After centrifugation at 6000 × *g* for 20 min the sucrose gradients were fractionated and assayed for radioactivity. Fig. 2 demonstrates the difference between microbead-coated and non-coated gradient profiles. Whereas coated plasma membranes migrated through the entire gradient, the uncoated membranes banded between fractions 10 and 15. A centrifugation of 1800 × *g* for 15 min was sufficient to sediment coated plasma membranes through 50% (w/v) sucrose cushions. Uncoated protoplast lysates even did not migrate into the sucrose at that speed.

Purification and studies on marker enzymes. The purification of plasma membranes by successive washing with lysis buffer is shown in Table I.

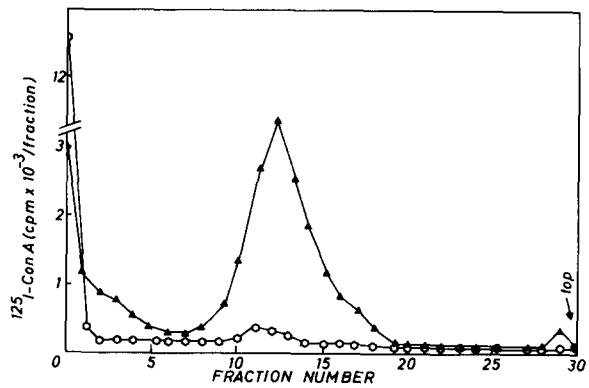

Fig. 2. Distribution of ^{125}I-labelled concanavalin A after sucrose density gradient centrifugation of microbead-coated ( ) and uncoated (▲) protoplast lysates. Protoplasts were labelled and coated as described in Materials and Methods. 7 ml of lysate were layered onto a discontinuous sucrose gradient containing 2 ml of 50, 40, 36 and 30% sucrose. The uncoated protoplast lysate was prepared similarly, except that microbeads were omitted. The gradients were centrifuged at 6000 × *g* in a SW 28.1 (Beckman) rotor for 20 min. After centrifugation, 0.5-ml fractions were collected and assayed for radioactivity.

TABLE I

PURIFICATION OF THE VANADATE-SENSITIVE pH 6.5 PLASMA MEMBRANE ATPase

For the purification of plasma membranes from *S. cerevisiae* freshly prepared protoplasts were treated as described in Materials and Methods. After each washing the resulting pellet was resuspended in the same volume of lysis buffer. Protein and enzyme activities were assayed as described in Materials and Methods. To determine the vanadate-and oligomycin-sensitive part of the total ATPase activity at pH 6.5 or pH 9, sodium vanadate (100 μM) or oligomycin (0.1%) were added to the assay.

	Protein (mg/ml)	Specific activity (μmol/min per mg protein)						% recovery	
		Vanadate-sensitive pH 6.5 ATPase	Oligomycin-sensitive pH 9.0 ATPAse	Cytochrome *c* oxidase	Hexo-kinase	Acid phosphatase	Alkaline phosphatase	Vanadate-sensitive pH 6.5 ATPase	Protein
Protoplast lysate	1.7	0.15	0.14	0.02	0.23	< 0.01	0.01	100	100
Resuspended pellet after first washing	0.24	0.95	0.09	0.01	0.01	–	–	89	14
Resuspended pellet after second washing	0.12	1.87	0.05	0.001	–	–	–	85	7.1

Vanadate-sensitive ATPase activity at pH 6.5 was used as a plasma membrane marker. This enzyme is an accepted marker for fungal plasma membranes [1]. The results show that the ratio of vanadate to oligomycin-sensitive ATPase in the protoplast lysate is close to 1. After washing the microbead-coated plasma membranes twice with lysis buffer this ratio increased by 30-fold. At the same time the specific activity of vanadate sensitive ATPase increased from 0.15 μmol/min per mg protein in the lysate to 1.85 μmol/min per mg protein in the purified plasma membrane fraction, a very good value for purified plasma membranes from *S. cerevisiae* [1].

After washing the pellet twice, the total recovery of pH 6.5 vanadate-sensitive ATPase was 85%. In purified plasma membranes, the vanadate-sensitive ATPase exhibited maximum activity at pH 6.5. A concentration of 10 μM vanadate was sufficient to cause maximal inhibition.

Oligomycin sensitive ATPase at pH 9 and cytochrome *c* oxidase activity were measured to monitor mitochondrial contamination. The results show that these activities were reduced considerably after two washings.

Possible vacuolar contamination was examined by assaying α-mannosidase, which is reported to be a reliable marker for vacuolar membranes in *S. cerevisiae* [16]. No α-mannosidase activity could be detected in the plasma membrane fraction (data not shown).

Other marker enzymes tested included hexokinase and alkaline phosphatase, both cytoplasmic markers, and acid phosphatase, which is localized in the cell wall and lysosomes. None of these activities could be detected in the plasma membrane fraction. The extremely low recoveries of these enzymes indicate that very few contami-

TABLE II

NUCLEOSIDE TRIPHOSPHATE SPECIFICITY

All nucleoside triphosphates were added as the Tris salt at a final concentration of 0.5 in M. Numbers in parenthesis are the percentage of activity as compared to ATP.

Substrate	Nucleoside triphosphatase activity at pH 6.5 (μmol/min per mg protein)	
	Protoplast lysate	Pellet after second washing
ATP	0.163(100)	1.92(100)
GTP	0.078(47.9)	0.03(1.6)
UTP	0.084(51.5)	< 0.01(< 0.5)
ITP	0.118(72.4)	0.02(1.0)

nants are present in plasma membrane preparations isolated by this method.

The specificity of nucleotide triphosphate hydrolysis by the plasma membrane fraction was examined. A high ratio of ATP hydrolysis compared with GTP, ITP and UTP is indicative of highly pure plasma membrane preparations [1]. Table II shows that, relative to ATP, less than 1% of either GTP, ITP or UTP were hydrolyzed by plasma membranes isolated by the microbead technique.

Electron microscopy. Thin sections show that purified plasma membranes (Fig. 3) are free of granular structures. Beside the partially microbead covered plasma membrane ghosts, no contaminations with other membranes or cell organelles could be detected. Fig. 4 shows that the microbeads are not evenly distributed over the plasma membrane surface but are clustered in some spots, leaving large areas of uncovered membrane surface. Fig. 5 demonstrates that the structure of the plasma membrane is preserved during the preparation. The protoplasmic face (PF) shows the regularly arranged regions (R) typical of yeast plasma membranes.

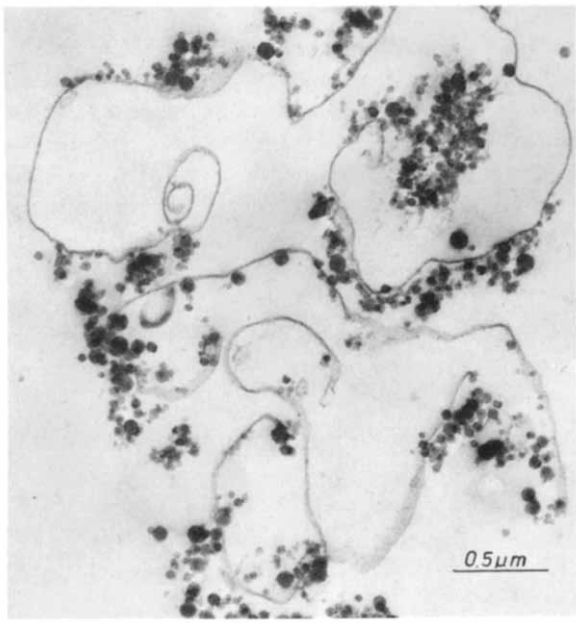

Fig. 3. Thin section of purified plasma membranes. Preparation: cytoplasmic membranes were fixed by a combined aldehyde fixative for 1 h at room temperature; postfixation was with 1.5% osmium tetraoxide + 1.65% potassium bichromate in buffer at room temperature for 1 h; poststaining was with 0.5% uranylacetate; embedding in low viscosity resin and polymerized at 50 and 60°C for over 24 h. Thin sections were prepared with a Reichert OM V3 ultratome. Additional staining of sections by lead citrate for 5 min was performed.

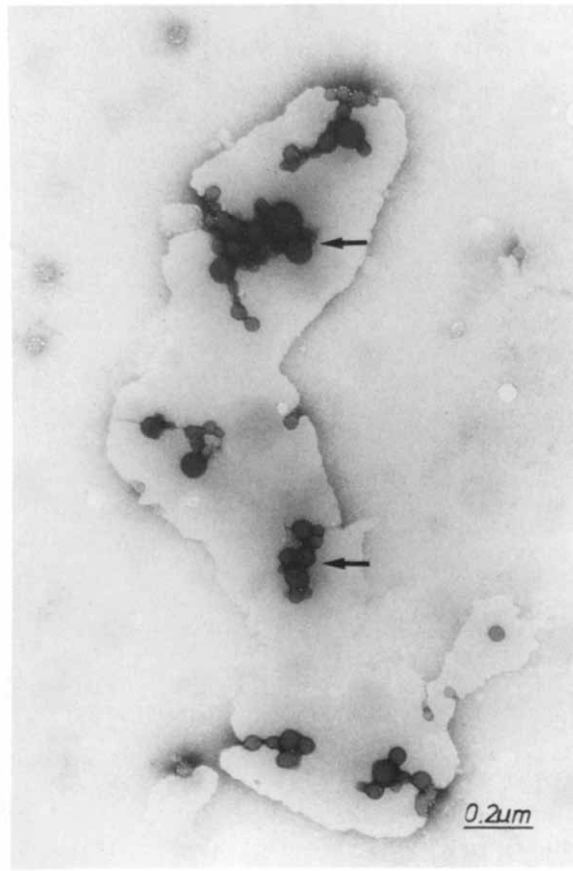

Fig. 4. Negative-stained plasma membranes after purification. Only few sites of the membrane surface are covered with clusters of microbeads (arrows). Preparation: membranes were adsorbed by mica-floated carbon film (Thickness 60 Å). After washing, the material was negative-stained with 1% phosphotungstate acid at pH 7.2.

Discussion

The use of Calcofluor White M2R to monitor the formation of protoplasts proved to be superior to the conventional method, which considers the formation of protoplasts to be accomplished upon complete lysis after transferring cells from diges-

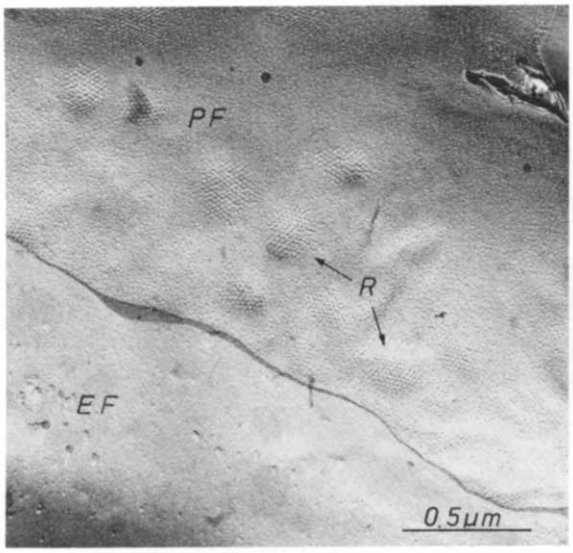

Fig. 5. Freeze-fractured plasma membrane after purification. The typical regularly arranged region (R) of the hydrophobic middle layer (PF) is preserved. EF, external face of the membrane. Preparation: cytoplasmic membranes were frozen in liquid propane without cryoprotectants; replicas were prepared in a Balzers freeze-etching device, BA 500, with turbomolecular pump and electron beam evaporator. Replicas were cleaned in 40% chromic acid. All specimens were examined in a Philips EM 300 or 400.

tion medium into distilled water.

In our preparation after only 15 min incubation 95% of the cells lysed when transferred into distilled water; however, at that time the fluorescence microscope still showed large parts of the cell wall left. Complete digestion of the cell wall requires a 45–60 min incubation. By coating the plasma membranes of protoplasts with cationic silica microbeads of 10–50 nm diameter and a density of 2.5 g/cm^3, the membranes are stabilized and retain a shell-like shape after lysis. Because of their high density they can easily be separated from other cell components by successive washing.

It is very important to maintain a pH value well below 5.5 until lysis. At higher pH values the microbeads as well as microbead-coated protoplasts aggregate, and some of the cells burst.

To ensure that no lysis occurs during the coating procedure, which could cause severe contamination by binding debris or cell organelles to the not yet neutralized microbeads, the activity of α-glucosidase, a cytoplasmatic enzyme, was measured after coating. No activity could be detected. We found that divalent cations cause severe aggregation of the plasma membranes upon lysis. Aggregated plasma membranes could not be purified from other cell components by washing. With the addition of 1 mM EGTA to the lysis buffer we could avoid this negative effect.

Compared to the purification procedure described for plant plasma membranes [9] we had to reduce the concentration of polyacrylic acid drastically, since it had an inhibitory effect on the yeast plasma membrane ATPase.

To ensure that the microbeads do not change the activity of the marker enzymes we tested their activity in the lysate of microbead-coated and uncoated protoplasts. We found the same activity in both lysates.

Table I shows that after the second washing the membranes are already almost free of contamination. In well-purified plasma membranes the ratio of ATPase activity at pH 6.0 to that at pH 9.0, or the ratio of ATP hydrolysis, to ITP or GTP hydrolysis, should be above 20 [1]. Our preparation shows indices of 37 and above 60, respectively.

The specific activity of the plasma membrane ATPase (1.87 µmol/min per mg protein) also indicates a good purification. The values reported for other plasma membrane preparations from *S. cerevisiae* range from 0.4 to 1.8 µmol/min per mg protein [22–24].

Generally, the purification is very reproducible, in some cases the preparations were even free of any oligomycin-sensitive pH 9.0 ATPase activity.

The characteristics of the vanadate-sensitive ATPase are not changed by the coating, blocking or purification procedures. Its properties are comparable to previous reports characterizing the plasma membrane-bound ATPase from *S. cerevisiae* [21–24]. A reduction from 100 µM to 10 µM sodium vanadate proved to be still sufficient to obtain maximum inhibition of the enzyme in purified plasma membranes. They could be stored at −20°C in 50% (v/v) glycerol for 3 months without significant loss of ATPase activity.

The electron microscope studies show that during the purification procedure the plasma membranes are preserved in large sheets partially covered with microbeads (Figs. 3,4). The reason

why the microbeads are clustered in some spots (Fig. 4) and not more or less evenly distributed over the membrane surface is not understood.

Fig. 3, a thin section of the purified plasma membranes, supports our statement about the purity of the preparation, which was based on the assay of marker enzymes. No contamination with other cell membranes could be detected. It is also free of any granular structure which could indicate cytoplasmatic contaminations. The entirety of the plasma membrane structure and its purity after the isolation procedure is demonstrated in Fig. 5. The protoplasmic face (PF) shows the regularly arranged regions (R), a typical marker structure for the yeast plasma membranes.

The advantages of this method are the short time necessary for the preparation of almost pure plasma membranes (2 h) and the yield of 85%, which in some preparations even exceeded 90%.

The purification procedure described could be convenient for the solubilization of the plasma membrane ATPase and for the study of other plasma membrane proteins.

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References

1 Goffeau, A and Slayman, C.W. (1981) Biochim. Biophys. Acta 639, 197–223
2 Jacobson, B.S. and Branton, D. (1974) J. Cell Biol. 63, 153a
3 Jacobson, B.S. (1977) Science 195, 302–304
4 Jacobson, B.S. (1977) Biochim. Biophys. Acta 471, 331–335
5 Cohen, C.M., Kalish, D.I., Jacobson, B.S. and Branton, D. (1977) J. Cell Biol. 75, 19–134
6 Kinoshita, T., Machman, R.L. and Minick, R. (1979) J. Cell Biol. 82, 688–696
7 Jacobson, B.S. (1980) Biochim. Biophys. Acta 600, 769–780
8 Gotlib, L.J. (1982) Biochim. Biophys. Acta 685, 21–26
9 Chaney, L.K. and Jacobson, B.S. (1983) J. Biol. Chem., in the press
10 Eddy, A.A. and Williamson, D.H. (1957) Nature (London) 179, 1252–1253
11 Hughes, J. and McCully, M.E. (1975) Stain Technol. 50, 319–329
12 Cuatrecasas, P. (1973) Biochemistry 12, 1312–1322
13 Ahlers, J. (1974) Biochem. J. 141, 257–263
14 Arnold, A., Wolf, H.U., Ackermann, B.P. and Bader, H. (1976) Anal. Biochem. 71, 209–213
15 Hodges, T.K. and Leonard, R.T. (1974) Methods Enzymol. 32, 392–406
16 Van der Wilden, W. and Matile, P. (1978) Biochem. Physiol. Pflanzen 173, 285–294
17 Halvorson, H. (1966) Methods Enzymol. 8, 559–562
18 Bergmeyer, H.U., Gawehn, K., Graßl, M. (1974) in Methoden der enzymatischen Analyse Vol. 1, (Bergmeyer, H.U., ed.) 3rd Edn., pp. 454–558, Verlag Chemie, Weinheim
19 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
21 Ahlers, J., Ahr, E. and Seyfarth, A. (1978) Mol. Cell Biochem. 22, 39–49
22 Willsky, G.R. (1979) J. Biol. Chem. 254, 3326–3332
23 Borst-Pauwels, G.W.F.H. and Peters, P.H.J. (1981) Biochim. Biophys. Acta 642, 173–181
24 Serrano, R. (1978) Mol. Cell Biochem. 22, 51–63
25 Malpartida, F. and Serrano, R. (1980) FEBS Lett. 111, 69–72