Vol. 210, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 5, 1995 Pages 126-132

NOVEL SYSTEM FOR MONITORING AUTOPHAGY IN THE YEAST *Saccharomyces cerevisiae*

Takeshi Noda, Akira Matsuura, Yoh Wada and Yoshinori Ohsumi*

Department of Biology, College of Arts and Sciences, University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153, Japan

Received March 17, 1995

The yeast *S. cerevisiae* imports cytosolic components into the vacuole non-selectively by autophagy and degrades them by vacuolar hydrolases under nutrient starvation conditions. We developed a novel system for monitoring autophagy by constructing cells in which modified vacuolar alkaline phosphatase is expressed as an inactive precursor form in the cytosol. Under starvation conditions, the processing of the precursor to the mature form and phosphatase activity appeared gradually, and the mature form was located in the vacuole. Disruption of *APG1*, an essential gene for autophagy, resulted in no processing or phosphatase activity. These results indicate that the precursor form in the cytosol is transferred to the vacuole by autophagy and converted to the active form by vacuolar proteinases. Thus, autophagy could be determined easily and accurately by measuring the phosphatase activity. © 1995 Academic Press, Inc.

Under starvation conditions, cytosolic proteins are degraded mainly by autophagy in lysosomes of mammalian cells [2, 18]. Autophagy is associated with dynamic movement and interaction of intracellular membranes, indicating that there must be numbers of molecules involved in the process. However, the studies of autophagy in mammalian or plant cells remain mainly at morphological level. Further biochemical and genetic analyses will be necessary for understanding this complex and dynamic membrane interaction and underlying molecular mechanism involved in the autophagy.

In the yeast *S. cerevisiae*, the vacuole is equivalent to the lysosome in mammalian cell [11]. The vacuole is equipped with numbers of hydrolases and its lumen is acidic, so it functions as a lytic compartment. Previously we reported that autophagy in yeast involves dynamic changes of the membranes [1, 22]. Starvation induces formation of autophagosomes, which consist of a double membrane enclosing a portion of the cytosol non-selectively. The outer membrane of the autophagosome fuses with the vacuolar membrane and the resulting inner single membrane structure, the autophagic body, is delivered to the vacuole, where it is rapidly disintegrated depending on vacuolar proteinases, and then its cytosolic contents are degraded by vacuolar hydrolases.

*Correspondence to Yoshinori Ohsumi, Department of Biology, College of Arts and Sciences, University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153, Japan. Telefax:+81-3-5454-4326.

Abbreviations: ADH, alcoholdehydrogenase; ALP, alkaline phosphatase; CPY, carboxypeptidase Y; NPP, nitro-phenyl-phosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl.

0006-291X/95 $5.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Since autophagic import of the cytosolic components into the vacuole is non-selective and no specific marker is available, monitoring of autophagy has been carried out by a combination of indirect ways such as measuring overall protein degradation, decrease of the long-lived cytosolic proteins, and detection of cytosolic proteins in the vacuolar fraction [3, 22, 25]. These methods, however, require a lot of process that prevent quantitative analysis on the autophagy, especially at the early stage of this process. We have developed a novel system for simple and sensitive monitoring of autophagy.

Materials and Methods

Strains, media for yeasts and bacteria . The yeast strains used were as follows: YW5-1B (*MAT***a** *leu2-3,112 trp1 ura3-52*); TN121 (*MAT***a** *leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13::URA3*); TN122 (*MAT***a** *leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13::URA3 prb1::TRP1*); TN123 (*MAT***a** *leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13::URA3 apg1::LEU2*), and TN124 (*MAT***a** *leu2-3,112 trp1 ura3-52 pho8::pho8Δ60 pho13::LEU2*). Bacterial strains were grown in standard media [17]. Yeast cells were grown in YPD, minimal essential medium SD, nitrogen starvation medium S(-N)D, carbon starvation medium S(-C), nitrogen and carbon starvation medium S(-N) or sulfate starvation medium S(-S)D as described previously [22].

Construction of plasmids and yeast strains. Manipulations of *E. coli* cells and DNAs were done essentially based on Sambrook et al. [17]. Plasmid pAL145 [7], which contains 4.4 kbp of *PHO8* region in the *Bam*HI site of pBR322 with *Bam*HI linker, was a gift from Dr. Kaneko of Osaka University. A *Kpn*I site was introduced at 181 bp downstream of the translational initiation codon ATG for *PHO8* by the PCR using a set of primers 5'-TCAGGTACCTCTGCATCACAC-AAGAAG-3', 5'-TTTGTCCTCATTGGAAGCT-T-3', and pAL145 as a template. The PCR product was subcloned into the *Kpn*I/*Hin*dIII sites of pBluescript II SK+ (Stratagene, CA) to generate pTN1. Plasmid pTN2, which contains the *pho8Δ60* lacking the region encoding the N-terminal and transmembrane segments of Pho8p, was constructed by subcloning of 0.8 kbp of *Bam*HI/*Hin*dIII fragment of pAL145 encoding the C-terminal and 3'-untranslated region of *PHO8* into the *Bam*HI/*Hin*dIII sites of pTN1. The *Kpn*I-*Kpn*I fragment (2.5 kbp) of pTN2 was inserted into the *Kpn*I site of pKT10 [21] to connect the *TDH3* promoter. The resulting plasmid, pTN3, was digested with *Bam*HI and *Sal*I, and 2.0 kbp fragment was subcloned into pRS306 [19] to obtain pTN7. Plasmid pTN9 was constructed by ligating the *Bam*HI-*Hin*dIII fill-in fragment (0.8 kbp) of 5' region of the *PHO8* gene in pAL145 into the *Bam*HI site in pTN7 with *Bam*HI linker. Plasmid pST100 contains disrupted *PRB1* gene with the *TRP1* gene [22]. Plasmid pYAP116 contains a disrupted *APG1* gene with the *LEU2* gene (Matsuura et al, manuscript in preparation). Plasmid pTN23, which contains *Eco*RI-*Hin*dIII (1.5 kbp) fragment of *PHO13* gene from pAL55 [9] in pUC119, was partially digested with *Pst*I, and 4.2 kbp of *Pst*I-*Pst*I fragment of the *LEU2* gene from YEp13 was inserted at the *PHO13* region to generate pTN24. Plasmids pAL55 and pPH13 were gifts from Dr. Harashima of Osaka University, and pKT10 was a gift from Dr. Toh-e of University of Tokyo. Yeast strain TN109 was generated by integrating pTN9 into the *PHO8* locus of wild-type strain YW5-1B, and the wild type *PHO8* gene was replaced with the *pho8Δ60* gene by counterselection of Ura$^-$ using 5-fluoro-orotic acid. TN121 and TN124 were generated by integrating pPH13 [9] and pTN24 into the *PHO13* locus of TN109 respectively. TN122 and TN123 were generated by integrating pST100 into the *PRB1* locus and pYAP116 into the *APG1* locus of TN121 respectively.

Preparation of antiserum of Pho8p. A *lacZ-PHO8* fusion gene was constructed by inserting a *Bgl*II-*Sal*I fragment of the *PHO8* gene, which does not contain C-terminal pro-region, into pUR278 [16], and was used to transform *E. coli* strain XL1-blue (Stratagene CA). The fusion protein produced was purified by SDS-PAGE and electro-elution. A rabbit was immunized with the purified fusion protein by Shibayagi (Gunma, Japan). The antiserum was purified on a cyanogen bromide activated Sepharose column coupled with the GST-Pho8p fusion protein. GST-Pho8p fusion protein was obtained by the similar procedure as described above, except for use of the *GST-PHO8* fusion gene in the pGEX-3X [5].

Subcellular fractionation, immunoblotting and alkaline phosphatase assay. Subcellular fractionation was carried out by the method of Herman and Emr [4]. Vacuoles were prepared as described before [22] except for incubation in S(-N) medium as starvation medium. SDS-PAGE was carried out by Laemmli's method [14] and the proteins separated were subjected to conventional immunoblotting analysis. Anti-Vma1p antibody was kindly provided by Dr. Anraku of University of Tokyo. Anti-ADH and anti-CPY sera were obtained as described elsewhere [1, 20] in collaboration with Dr. Nakano of University of Tokyo. Protein was determined with a BCA protein assay kit (Pierce, Ill). A yeast lysate was prepared by disrupting the cells with glass beads in ice-cold reaction buffer (50 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml pepstatin A (Sigma, MI)) and removed the cell debris by centrifugation at 10,000 x g for 5 min. Alkaline phosphatase in the lysate was assayed with p-NPP (Wako, Japan) as a substrate by the method of Torriani [23] in the presence of 1 mM PMSF and 1 μg/ml pepstatin A. One micromole of p-nitro-phenol gives 11.7 units of ΔA_{420}.

Results and Discussion

The structure of Pho8p, vacuolar alkaline phosphatase, is as follows from the N-terminus: a cytosolic tail, a membrane spanning region, a catalytic region, and a pro-region at the C-terminus (Fig. 1) [10]. The analysis on various hybrid proteins of Pho8p and Suc2p showed that the membrane spanning region acts as an uncleavable signal sequence for translocation into the ER membrane and is necessary and sufficient for delivery of Pho8p to the vacuole [12]. Taking advantages of this structural and functional characteristics of Pho8p, for monitoring autophagy, we constructed a gene encoding a truncated form of Pho8p, Pho8Δ60p, which lacks 60 amino acid residues at the N-terminus including the membrane spanning region (Fig. 1). The modified version of Pho8p lacking the membrane spanning region should remain in the cytosol. The *PHO8* gene in wild-type cells (YW5-1B) was replaced with *pho8Δ60* connected under a strong promoter of *TDH3* gene, a gene for glyceraldehyde 3-phosphate dehydrogenase [15]. Disruption of the *PHO8* gene did not affect the accumulation and degradation of autophagic bodies or the growth phenotype (Shirahama and Ohsumi, unpublished observation). We examined the subcellular localization of

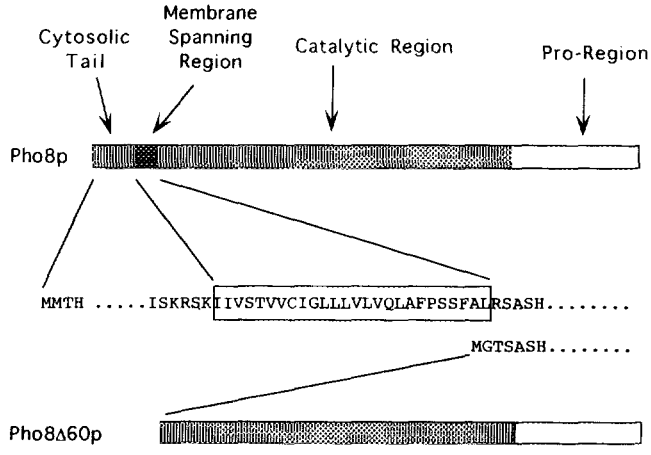


Fig. 1. Structure of Pho8Δ60p. Pho8Δ60p is truncated Pho8p deleted of 60 amino acid residues at the NH_2-terminus, including the membrane spanning region (box).

Pho8Δ60p. Affinity-purified antiserum against Pho8p recognized only Pho8p by immunoblotting (data not shown). A lysate of TN124 cells (*pho8::pho8Δ60*) grown in YPD, nutrient-rich medium, was subjected to centrifugation and proteins of the pellet and the supernatant were subjected to immunoblotting. An authentic Pho8p was localized within the membrane fraction (data not shown). While, Pho8Δ60p was detected in the supernatant fraction with the cytosolic enzyme alcoholdehydrogenase, but not in the pellet fraction with vacuolar H^+-ATPase (Fig. 2A). This result showed that Pho8Δ60p is expressed and remained at the cytosol.

On arrival at the vacuole via the secretory pathway, authentic Pho8p is converted to the mature form with a half time of about 6 min [10] as the result of cleavage of the pro-region at the C-terminus depending on the activity of proteinase A [6, 8, 10]. TN124 cells (*pho8Δ60*) grown in YPD were transferred to nitrogen starvation medium and the lysate was subjected to immunoblotting with anti-Pho8p serum. When grown in YPD, the Pho8Δ60p was detected at approximately 64 kDa, which corresponds to the size deduced from the total length of its open reading frame (Fig. 2B lane 1). When the cells were subjected to nitrogen starvation, a novel band appeared at approximately 59 kDa, and gradually increased in amount during prolonged incubation (Fig. 2B lanes 2 to 5). As the size of the pro-region of Pho8p is about 4 kDa, this processing of Pho8Δ60p coincided with cleavage of the C-terminal pro-region.

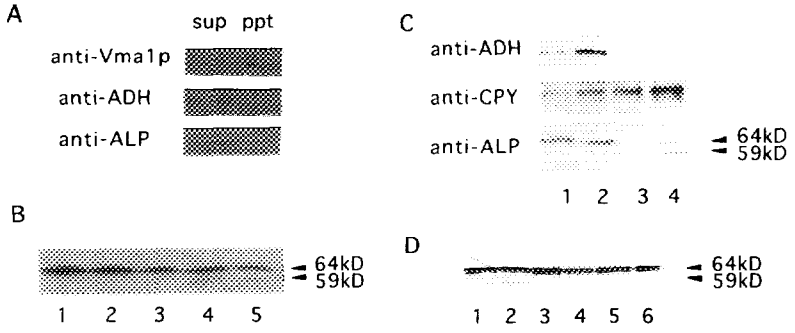
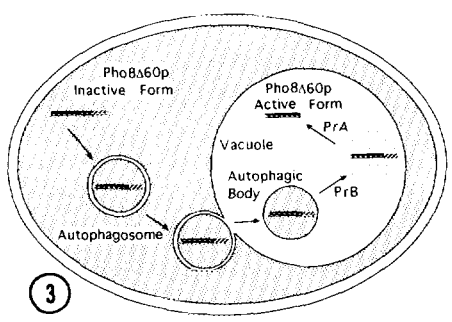


Fig. 2. A. Localization of Pho8Δ60p. TN124 (*pho8Δ60*) cells were grown in YPD medium and disrupted for subcellular fractionation. Sup and ppt indicate the supernatant and pellet fraction, respectively, obtained by centrifugation at 100,000 x g for 30 minutes. Fractions equivalent to the same numbers of the cells were loaded on 10% SDS-PAGE gel and immunoblotted with each antiserum. B. Processing of Pho8Δ60p under starvation conditions. TN124 (*pho8Δ60*) cells were grown in YPD and transferred to S(-N)D. At intervals during incubation, the cells were collected, disrupted and subjected to immunoblotting with anti-Pho8p serum. Samples of 20 μg of protein were loaded in each lane. Lanes 1, 2, 3, 4 and 5 are samples incubated in S(-N)D for 0, 1, 3, 5 and 7 hours, respectively. C. Localization of processed Pho8Δ60p. TN124 (*pho8Δ60*) cells were grown in YPD to a density of 2 x 10^7 cells/ml and transferred to S(-N). Vacuoles were prepared as described in Materials and methods. Samples of 10 μg of protein of each fraction were subjected to immunoblotting with anti-CPY or anti-ALP serum and samples of 0.5 μg of protein with anti-ADH serum. Lanes 1 and 2, spheroplast lysates; lanes 3 and 4, vacuolar fraction; lanes 1 and 3, starvation for 0 hour; lanes 2 and 4, starvation for 4.5 hours. D. TN124 (*pho8Δ60*) , TN122 (*pho8Δ60 Δprb1*) and TN123 (*pho8Δ60 Δapg1*) cells were grown in YPD, transferred to S(-N)D and subjected to immunoblotting with anti-Pho8p serum. Samples of 20 μg of protein were loaded in each lane. Lanes 1 and 2, TN124 (*pho8Δ60*) ; lanes 3 and 4, TN122 (*pho8Δ60 Δprb1*); lanes 5 and 6, TN123 (*pho8Δ60 Δapg1*). Lanes 1, 3 and 5, 0 hour; lanes 2, 4 and 6, 7 hours in S(-N)D.

The intracellular localization of the processed Pho8Δ60p was examined. Pho8Δ60p was not detected in the vacuolar fraction of cells grown in YPD (Fig. 2C). After incubation in starvation medium for 4.5 hours, processed Pho8Δ60p was concentrated in the vacuolar fraction as well as carboxypeptidase Y (Fig. 2C), and most of it was resistant to trypsin treatment (data not shown), indicating that it was located within the vacuole. Proteinase B, encoded by the *PRB1* gene, is localized in the vacuole and necessary for disintegration of the autophagic body [22]. Even after incubation of disruptant of *PRB1* for 7 hours in the nitrogen starvation medium, no processing of Pho8Δ60p occurred (Fig. 2D lane 4). These results indicate that the processing of Pho8Δ60p in starvation conditions is resulted from its delivery to the vacuole, and did not occur outside the vacuole. The *APG1* gene is shown to be essential for autophagy and a disruptant of the *APG1* gene does not accumulate autophagic bodies in the vacuole (24, Matsuura et al, manuscript in preparation). After nitrogen starvation for 7 hours, no processed Pho8Δ60p appeared in *Δapg1* cells (Fig. 2D). This finding indicates that delivery of Pho8Δ60p from the cytosol to the vacuole depends on autophagy (Fig. 3).

Pho8p acquires phosphatase activity when its pro-region is cleaved off in the vacuole. Besides Pho8p, wild-type cells possess Pho13p, a cytosolic alkaline phosphatase that is not essential for vegetative growth [9]. To measure the phosphatase activity derived only from Pho8Δ60p, we disrupted the *PHO13* gene. As shown in Fig. 4A, TN121 cells (*pho8::pho8Δ60 pho13::URA3*) grown in YPD showed negligible phosphatase activity, indicating that non-processed Pho8Δ60p, like authentic Pho8p, does not have phosphatase activity. While, the activity appeared after a lag period of 1 hour and increased during incubation in nitrogen-starvation medium for 7 hours. Furthermore, this induction of phosphatase activity was not seen in *Δapg1* or



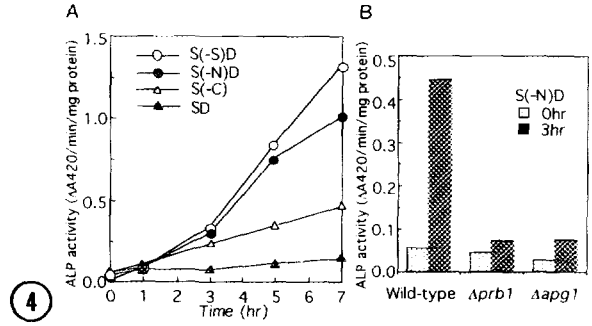


Fig. 3. Schematic drawing of import of Pho8Δ60p into the vacuole by the autophagic pathway. Under nutrient starvation conditions, an autophagosome enclosing Pho8Δ60p in the cytosol fuses with the vacuole. The resulting single membrane structure of the autophagic body is disintegrated depending on vacuolar proteinases. Pho8Δ60p is processed by vacuolar proteinases to acquire phosphatase activity. PrA, proteinase A; PrB, proteinase B.

Fig. 4. Induction of phosphatase activity of Pho8Δ60p under starvation conditions. A. TN121 (*pho8Δ60*) cells were grown in YPD medium and transferred to each medium at 0 hour. After indicated times of incubation, cells were lysed and pNPPase activity was measured as described in Materials and methods. B. Cells of TN121 (*pho8Δ60*), 122 (*pho8Δ60 Δprb1*) and 123 (*pho8Δ60 Δapg1*) were grown in YPD and transferred to S(-N)D. After incubation for 0 or 3 hours, cells were lysed and alkaline phosphatase activity was measured as described in Materials and methods.

Δprb1 cells (Fig. 4B), consistent with the finding that processing of Pho8Δ60p did not occur in these cells. Phosphatase activity emerged also under carbon starvation and sulfate starvation (Fig. 4A). We also observed accumulation of autophagic bodies in the vacuole under these starvation conditions [22]. From these results, we concluded that this phosphatase activity reflects autophagy-dependent processing of Pho8Δ60p.

During nitrogen starvation for up to 7 hours, the amount of the precursor form of Pho8Δ60p did not change significantly in *Δapg1* cells, as determined by immunoblotting (Fig. 2D). In these cells, Pho8Δ60p was not imported into the vacuole, and so its level in the cytosol remains constant during nitrogen starvation. Furthermore, processed Pho8Δ60p was stable in the vacuole, because the phosphatase activity induced under nitrogen starvation remained constant after further incubation in a medium supplemented with ammonium sulfate to stop the autophagy (data not shown). From these results, we concluded that the phosphatase activity shows the extent of autophagy under nitrogen starvation.

There have been several attempts to monitor autophagy so far. One method used was measurement of the radioactivity of amino acids released on degradation of pre-labeled protein [25]. However, by this method, it is hard to distinguish protein degradation by autophagy from the degradation by the other systems. Moreover, released amino acids may be reutilized for synthesis of proteins, which results in under-estimation of the degradation. Another method used was measurement of the decrease of long-lived proteins in the cytosol by immunoblotting [3]. However, this method is not sensitive enough to detect decrease by a few per cent in the total amount of protein in the initial phase of autophagy. There are also reports of quantitation of autophagy in mammalian or yeast cells by subcellular fractionation of autophagolysosomes and measurement of the cytosolic marker [3, 13]. However, this method of analysis involves quite many steps. In contrast, the system presented here allows not only sensitive but simple analysis of autophagy even in the early phase of autophagy.

Acknowledgments

This work was supported in part by a Grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, by Special Coordination Funds for the Promotion of Science and Technology from the Science and Technology Agency of Japan, and a grant from Suntory LTD.

References

1. Baba, M., Takeshige, K., Baba, N. and Ohsumi, Y. (1994) J. Cell Biol., 124, 903-913
2. Dunn Jr., W. A. (1994) Trends in Cell Biol., 4, 139-143
3. Egner, R., Thumm, M., Straub, M., Simeon, A., Schüller, H.-J. and Wolf, D. H. (1993) J. Biol. Chem., 268, 27269-27276
4. Herman, P. K. and Emr, S. D. (1990) Mol. Cell. Biol., 6742-6754
5. Johnson, K. S., Harrison, G. B., Lighttowlers, M. K., O'Hoy, K. L., Cougle, W. G., Dempster, R. P., Lawrence, S. B., Vinton, J. G., Heath, D. D. and Rickard, M. D. Nature, (1989), 338, 585-587
6. Jones, E. W., Zubenko, G. S. and Parker, R. R. (1982) Genetics, 102, 665-672
7. Kaneko, Y., Toh-e, A. and Oshima, Y. (1982) Mol. Cell. Biol., 2, 127-137
8. Kaneko, Y., Tamai, Y., Toh-e, A. and Oshima, Y. (1985) Mol. Cell. Biol., 5, 248-252
9. Kaneko, Y., Toh-e, A., Banno, I. and Oshima, Y. (1989) Mol. Gen. Genet., 220, 133-139

10. Klionsky, D. J. and Emr, S. D. (1989) EMBO J, 8, 2241-2250
11. Klionsky, D. J., Herman, P. K. and Emr, S. D. (1990) Microbiol. Rev., 54, 266-292
12. Klionsky, D. J. and Emr, S. D. (1990) J. Biol. Chem., 265, 5349-5352
13. Kopitz, J., Kise, G., Gordon, P. B., Bohley, P. and Seglen, P. O. (1990) J. Cell Biol., 111,941-953
14. Laemmli, U. K. (1970) Nature (Lond.), 227, 680-685
15. McAlister, L. and Holland, M. J. (1985) J. Biol. Chem., 260, 15013-15018
16. Ruther, U. and Muller-Hill, B. (1983) EMBO J., 2, 1791-1794
17. Sambrook, J., Fritsch, E. F. and Maniatis, T., (1989) Molecular cloning, Cold Spring Harbor Laboratory Press
18. Seglen, P. O. (1987) in Lysosomes (Eds, H. Glamann, J. L. Ericsson and L. Marzella) Academic Press, London, 371-414
19. Sikorski, R. S. and Hieter, P. (1989) Genetics, 122, 19-27
20. Stevens, T., Esmon, B. and Schekman, R. (1982) Cell, 30, 439-448
21. Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matumoto, K. and Toh-e, A. (1990) Mol. Cell. Biol., 10, 4303-4313
22. Takeshige, K., Baba, M., Tsuboi, S., Noda, T. and Ohsumi, Y. (1992) J. Cell Biol., 119, 301-311
23. Torriani, A. (1960) Biochim. Biophys. Acta, 38, 460-479
24. Tsukada, M. and Ohsumi, Y. (1993) FEBS Lett., 333, 169-174
25. Zubenko, G. S. and Jones, E. W. (1981) Genetics, 97, 45-64