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Time-co-ordinated control of glycogen synthase, protein phosphatase 2A and protein kinase CK2 during culture growth in *Yarrowia lipolytica* in relation to glycogen metabolism

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Abstract – In the growth course of the lipolytic yeast *Yarrowia lipolytica*, the activities of protein phosphatase 2A (PP2A) and glycogen synthase (GS) rise during the exponential phase and concomitantly glycogen storage occurs in the cells. There is also an increase in the independence ratio (RI) indicating a shift from an inactive phosphorylated GS form to an active dephosphorylated GS form. During the early stationary phase, an increase in protein kinase CK2 (CK2) activity, a reversion of RI variation and a glycogen content decrease are observed. GS activity proved to be a good indicator of early culture growth phase. Experiments carried out with enzymes purified from *Y. lipolytica* show strong RI variations upon the action of CK2 and PP2Ac, and ^{32}P incorporation into GS protein through phosphorylation by CK2. GS activity would be controlled by the sequential action of PP2A and CK2. © 2000 Académie des sciences/Éditions scientifiques et médicales Elsevier SAS

yeast / *Yarrowia lipolytica* / protein phosphatase 2A / protein kinase CK2 / glycogen synthase / glycogen

Résumé – Régulation de la glycogène synthase, de la protéine phosphatase 2A et de la protéine kinase CK2 au cours de la croissance de *Yarrowia lipolytica* en relation avec le métabolisme du glycogène. Au cours de la croissance de la levure *Yarrowia lipolytica*, les activités de la protéine phosphatase 2A (PP2A) et de la glycogène synthase (GS) augmentent pendant la phase exponentielle et d'une manière concomitante le glycogène s'accumule dans les cellules. Se produit également une augmentation du rapport d'indépendance (RI) indiquant le passage d'une forme GS phosphorylée inactive à une forme déphosphorylée active. En début de phase stationnaire, ont lieu une augmentation de l'activité protéine kinase CK2 (CK2), une inversion du rapport RI et une diminution de la concentration en glycogène. L'activité GS est un bon indicateur de la phase exponentielle de croissance. Des expériences réalisées avec les enzymes purifiées montrent des variations importantes de RI sous l'action de la CK2 et de la PP2Ac et une incorporation de ^{32}P dans la GS par phosphorylation avec la CK2.

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L'activité GS pourrait être contrôlée par l'action séquentielle de la PP2A et de la CK2.

levure / *Yarrowia lipolytica* / protéine phosphatase 2A / protéine kinase CK2 / glycogène synthase / glycogène

Version abrégée

Il est indispensable de connaître la régulation du métabolisme du glycogène pour pouvoir contrôler les conditions de croissance des levures d'intérêt biotechnologique. Comme chez les cellules de mammifères, la glycogène synthase (GS) existe sous deux formes chez les levures, une forme phosphorylée (forme D ou GS_D) peu active mais activée par le glucose 6-phosphate (G6P) et une forme déphosphorylée (forme I ou GS_I) active même en absence de G6P. Le rapport d'indépendance RI est défini comme le rapport de l'activité mesurée en absence de G6P sur l'activité mesurée en présence de G6P. Chez *Saccharomyces cerevisiae*, la teneur des cellules en glycogène augmente au début de la phase stationnaire de croissance de même que la quantité de GS totale et l'activité de la forme I. Chez *Yarrowia lipolytica*, nous avons montré une augmentation de l'activité protéine phosphatase de type 2A (PP2A) en fin de phase exponentielle de croissance et de l'activité protéine kinase CK2 (CK2) pendant la phase stationnaire. Le premier but de ce travail est de définir, à côté des activités PP2A et CK2, de nouveaux indicateurs de croissance cellulaire: activité GS ou teneur en glycogène. Une protéine phosphatase de type 2A capable de déphosphoryler la GS a été purifiée chez *S. cerevisiae*. De même des sérine/thréonine protéine kinases sont impliquées dans le métabolisme du glycogène chez *S. cerevisiae* et *Kluyveromyces lactis*. Le deuxième but de ce travail est d'étudier chez *Y. lipolytica* le rôle séquentiel de la PP2A et de la CK2 dans le contrôle du métabolisme du glycogène par déphosphorylation et phosphorylation.

La croissance de *Yarrowia lipolytica* est suivie pendant 30 h sur un milieu appauvri en phosphate. Des prélèvements permettent de mesurer les activités GS, PP2A et CK2 ainsi que la concentration en glycogène sur les extraits acellulaires après broyage. L'activité GS est déterminée à pH 7,8 par incorporation de l'uridine diphospho-[U-^{14}C]glucose dans le glycogène. L'activité protéine phosphatase est mesurée par la déphosphorylation de [^{32}P]caséine à pH 7,0. L'activité CK2 est déterminée après une étape de chromatographie d'affinité sur une colonne HiTrap Heparin par phosphorylation à l'aide de [γ-^{32}P]ATP d'un peptide spécifique des CK2. Le glycogène est extrait par hydrolyse alcaline et après dégradation par l'amyloglucosidase, la concentration en glucose est déterminée par un test enzymatique. Au cours de la croissance de *Y. lipolytica*, les activités GS et PP2A révèlent un pic d'activité à 11 h 30 en fin de phase exponentielle de croissance. Le rapport d'indépendance subit aussi des variations et montre que la forme I est prédominante à 13 h. Parallèlement à ces activités, un pic d'accumulation du glycogène est observé à 13 h. En début de phase stationnaire (16 h–19 h), un maximum d'activité CK2 est observé coïncidant d'une part avec une phosphorylation partielle de la GS illustrée par une diminution du RI, d'autre part avec une diminution de la synthèse de glycogène. L'activité de la GS est donc un bon indicateur de la croissance cellulaire chez *Y. lipolytica* et plus particulièrement de la phase exponentielle de croissance comme la protéine phosphatase 2A. Les résultats sont en accord avec un contrôle séquentiel de l'activité GS : pendant la phase exponentielle de croissance, un pic d'activité PP2A permettrait la déphosphorylation de la GS et son activation, entraînant l'accumulation de glycogène. À l'état stationnaire, l'activité CK2 est maximale de manière transitoire et peut phosphoryler la GS comme le montre la diminution du rapport d'indépendance. La diminution du RI d'une part, de l'activité GS totale d'autre part entraîne une forte diminution de la teneur en glycogène des cellules.

Le contrôle de l'activité de la GS par phosphorylation et déphosphorylation a été réalisé sur les enzymes purifiées à partir de *Y. lipolytica*. Les formes I et D de la GS sont séparées sur colonne échangeuse d'ions (DEAE Toyopearl 650 S) et éluées respectivement par NaCl 0,3 et 0,35 M. L'action de la CK2 sur la GS se traduit par une forte diminution du rapport d'indépendance qui passe de 100 % à 12 % en 1 h et une diminution d'activité de 31 %. À l'inverse, l'action de la sous-unité catalytique de la PP2A (PP2Ac) se traduit par un doublement du RI et une augmentation de l'activité de 30 %. Il a même été vérifié qu'après inhibition par la CK2 l'addition de PP2Ac conduisait à une surstimulation de l'activité GS. Lorsque la glycogène synthase purifiée est incubée en présence de CK2 et de [γ-^{32}P]ATP, le phosphate marqué est rapidement incorporé dans l'enzyme et plus particulièrement dans une unité protéique dont la masse moléculaire apparente a été estimée à 76 kDa par électrophorèse sur gel de polyacrylamide en conditions dénaturantes. Le phosphate incorporé à la GS sous l'action de la CK2 peut être libéré par action de la PP2Ac : 12 % après 15 min, 23 % après 20 min et 32 % après 30 min de réaction. Les expériences réalisées avec les enzymes purifiées confortent l'hypothèse d'une régulation de l'activité GS par phosphorylation/déphosphorylation. En présence de CK2, une forte diminution du RI indique la transformation de la forme

I en forme D par phosphorylation ce qui implique l'existence de sites consensus pour la CK2 comme cela a été confirmé chez *S. cerevisiae*. Inversement, la PP2Ac est capable de déphosphoryler la forme D, d'augmenter le rapport d'indépendance ainsi que l'activité de la glycogène synthase.

1. Introduction

Knowledge of glycogen metabolism regulation is essential to monitor and control culture growth conditions of yeasts used in biotechnology. As in mammalian cells, glycogen synthase (GS) from yeasts exists under two forms interconverted by phosphorylation and dephosphorylation [1]. The GS-phosphorylated form has little activity but is allosterically activated by glucose 6-phosphate (G6P) and is therefore designated as the G6P-dependent form (D-form or GS_D). The GS-dephosphorylated form is active in the absence of G6P (I-form or GS_I). The ratio of independence (RI) is defined as the ratio in percent between activity in the absence of G6P and activity in the presence of G6P. Co-ordinated changes in the activity of protein phosphatases and protein kinases modifying the phosphorylation state of GS could control glycogen biosynthesis in the course of growth.

When *Saccharomyces cerevisiae* grows on phosphate-enriched medium, glycogen content in the cells increases steeply just during the onset of the stationary growth phase concomitantly with the I-form activity and the total GS amount [2]. Work from our laboratory on protein kinases and protein phosphatases from the yeast *Yarrowia lipolytica* has shown that during growth on YPD medium a rapid increase in protein kinase CK2 (CK2) activity occurred at the stationary phase [3] whereas on phosphate-deficient medium strong protein phosphatase 2A (PP2A) activity was observed at the end of the exponential phase [4]. PP2A and CK2 activities could therefore be used as indicators of early and stationary culture growth phases, respectively, in *Y. lipolytica*. The aim of this work was to determine glycogen synthase activity and glycogen content in the course of growth of *Y. lipolytica* in order to identify new markers of cell growth.

It was established [5, 6] that phosphoprotein phosphatases dephosphorylate GS in yeast. Peng et al. [7] purified from *S. cerevisiae* during the early stationary phase a PP2A able to dephosphorylate GS and showing structural and catalytic properties very similar to those of mammalian PP2A. Studies on genomic sequences related to phosphoseryl/threonyl protein phosphatases led to identification of genes encoding protein phosphatases of type 1 [8, 9] and 2A [10], and involved in the control of glycogen accumulation possibly through regulation of the amount of active GS. Serine/threonine protein kinases are closely related to members of the GSK-3 subfamily and are involved in glycogen storage in *S. cerevisiae* and *Kluyveromyces lactis* [11]. We attempt to obtain insights into the role of PP2A and CK2 in the control of glycogen metabolism from *Y. lipolytica*. Our hypothesis is that glycogen metabolism could be regulated sequentially by PP2A and CK2 through dephosphorylation (i.e. activation) and phosphorylation (i.e. inactivation) of glycogen synthase in the course of *Y. lipolytica* growth.

2. Materials and methods

2.1. Chemicals

Chemically dephosphorylated casein (#4765), glucose 6-phosphate, glycogen type II from oyster, uridine 5′-diphosphoglucose were purchased from Sigma. Amyloglucosidase and glucose determination method kit no. 716251 were from Boehringer Mannheim. Uridine diphospho-D-[U-^{14}C]glucose and [γ-^{32}P]ATP were from Amersham. Scintillation liquid (Ecolite TM) was obtained from ICN. TSK diethylaminoethane (DEAE) Toyopearl 650S was from Merck Darmstadt, bactopeptone from DIFCO, Sephadex G-50 Fine gel and HiTrap Heparin fast protein liquid chromatography columns from Pharmacia. All other chemicals were of analytical grade.

2.2. Culture conditions

The yeast *Yarrowia lipolytica* (wild strain W-29, CLIB 89 [CLIB: Collection de levures d'intérêt biotechnologique/ Biotechnological Interest yeast Collection, Centre de biotechnologie agro-industrielle, 78850 Thiverval-Grignon]) was grown on a Pi-deficient medium [12] containing 4 % (w/v) glucose, 1 % (w/v) bactopeptone, 0.2 % (w/v) $MgSO_4$–$7H_2O$ and 0.5 % (w/v) KCl. Cells were incubated at 28 °C in 500 mL Erlenmeyer flasks shaken at 190 rev min^{-1} using a Inford AG shaking agitator (CH4103, Bottringen). Growth was followed by cell number determination and absorbance measurement at 570 nm.

Cells were sampled after different times of culture, spun at 4 400 *g* for 15 min and washed with distilled water to remove culture medium. Aliquots were pipetted into Eppendorf vials for extraction of enzymes and glycogen. Yeast fresh weight was obtained after 30 s centrifugation in a labtop centrifuge (2 000 *g*). Extractions were performed in different buffers as indicated. Proportions for grinding were 1/3/3 (w/w/v) yeast/glass beads (diameter 0.45–0.50 mm, Braun)/buffer. Cells were broken by vortexing for four 30 s pulses [13]. Enzyme determinations were assayed on the supernatant after a 12 000 *g* centrifugation for 10 min at 4 °C. Protein concentration was estimated by the method of Bradford [14] using bovine serum albumin as standard.

2.3. Enzyme assays and glycogen determination

2.3.1. GS activity from Y. lipolytica

GS was assayed using a radioactive-specific assay modified from Thomas et al. [15]. Yeast cells (100 mg) were

ground in 'Tris buffer': 50 mM Tris-HCl (pH 7.5) containing 20 % (v/v) glycerol, 100 mM NaF, 0.1 mM PMSF, 3 mM DTT, 1 mM EDTA, 1 mM benzamidine and 0.025 mM leupeptin. Activity was determined in a 50 mM Tris-HCl buffer (pH 7.8) with 33 mg mL^{-1} glycogen and 1.28 mM uridine diphospho-glucose (UDP-G) containing 23 kBq mL^{-1} uridine diphospho-D-[U-^{14}C]glucose. Incubation was carried out for 30 min at 30 °C in the presence or in the absence of 10 mM G6P, then 85 µL of the mixture were spotted on Whatman ET31 filter paper. In our experiments, it was checked that maximal activity was obtained with 1.28 mM UDP-G. One unit of GS activity is defined as the amount of enzyme that catalyses the incorporation of 1 µmol of glucose from UDP-glucose into glycogen in 1 min at 30 °C.

2.3.2. Protein phosphatase activity

Y. lipolytica cells (100 mg) were disrupted in 'TEA buffer' (20 mM TEA, 50 mM NaCl, 5 % (v/v) glycerol, 1 mM EGTA, 0.1 mM DTT, 0.01 % (v/v) Brij 35, pH 7.0) containing 0.4 mM PMSF. Enzyme activity was assayed using [^{32}P]casein prepared according to McGowan and Cohen [16]. After 20 min incubation at 30 °C with 0.011 mM [^{32}P]casein, proteins precipitated with cold 20 % trichloroacetic acid were discarded by centrifugation and radioactivity was counted in the supernatant through liquid scintillation [4]. This method is very specific, efficient and suitable if interference with proteolysis of the labelled casein by contaminant proteases present in the cell extracts is controlled [17]. We have checked that no interference occurred in our experimental conditions.

2.3.3. CK2 activity

Yeast cells (500 mg) were ground in 25 mM Tris-HCl (pH 8.0) containing 200 mM NaCl, 10 % (v/v) glycerol, 2 mM DTT, 0.5 mM PMSF, 0.5 mM EDTA ('CK2 buffer'). After centrifugation the supernatant was loaded on a HiTrap Heparin column (1 mL) and eluted with a gradient from 0.2 to 1.2 M NaCl (10 mL total volume). After this affinity chromatography step, fraction activity was determined in a 30 µL volume in the presence of 50 mM Mops buffer (pH 6.8) containing 150 mM NaCl and 10 mM $MgCl_2$ with 3 mM of a CK2-specific peptide (HArg.Arg.Glu.Glu.-Glu.Thr.Glu.Glu.GluOH, Mr = 1 206.2, Bachem Biochimie SARL, France) and 0.1 mM [γ-^{32}P]ATP (2159 cpm $pmol^{-1}$) as described by Kuenzel et al. [18]. After 15 min incubation at 25 °C, reaction mix was spotted on phosphocellulose paper (P31 Whatman) precipitated with 100 mM phosphoric acid and counted in a Tricarb 1500 Packard liquid scintillation counter.

2.3.4. Glycogen determination

Glycogen was extracted from *Y. lipolytica* cells according to Becker [19]. Approximately 50 mg of yeast cells were boiled for 1 h 30 min in a water bath in a Pyrex closed vial in the presence of 50 volumes of 0.25 M Na_2CO_3–$10H_2O$. After cooling in ice, 0.2 mL samples were incubated 2 h at 37 °C with 3 M acetic acid (0.2 mL), 0.2 M acetate buffer (pH 4.8, 0.6 mL) and 1.4 U of amyloglucosidase. Reaction was stopped by 5 min boiling in a water bath. After neutralization with 0.2 mL 'KOH buffer' (1 mM TEA–HCl buffer, 10 M KOH and 1 M acetic acid: 3:0.8:0.2), glucose concentration was determined by using an enzymatic method (Boehringer glucose determination kit). In the presence of hexokinase and ATP, glucose was phosphorylated into glucose 6-phosphate. Addition of G6P-dehydrogenase and NADP produced D-gluconate 6-phosphate and NADPH was measured at λ = 340 nm (ε = 6300 M^{-1} cm^{-1}).

2.4. Purification procedures of enzymes from *Yarrowia lipolytica*

2.4.1. Purification of GS_I and GS_D

Yeast cells (10 g), harvested after 13 h culture, were homogenized by using 30 g of glass beads and 30 mL of grinding Tris buffer in a MSK Braun ball homogenizer (Braun Sciencetech, France) cooled under a stream of CO_2, for 4 × 30 s pulses. After 10 min centrifugation at 45 000 *g* the supernatant was batch-processed on a TSK gel Toyopearl (DEAE 650 S) ion exchange chromatography column (2.6 × 30 cm). The gel was previously equilibrated with TEA buffer containing 0.1 mM PMSF. Proteins were eluted with a 50–600 mM NaCl gradient in the same buffer. Fractions were assayed for GS activity in the presence and in the absence of 10 mM G6P (*figure 1*). GS_I and GS_D were eluted at 0.3 and 0.35 M NaCl, respectively. RI of GS_I fractions was about 85–95 %. RI of GS_D fractions was very low (2 %).

The active fractions containing the I- or D-forms were pooled separately and the enzymes were concentrated by precipitation with addition of solid ammonium sulphate to 45 % saturation [20]. After 3 h stirring the precipitate was collected by centrifugation and the pellet dissolved in 4 mL of TEA buffer containing 1 mM 2-mercaptoethanol and spun for 1 h 30 min at 48 000 *g*. Supernatant glycerol concentration was adjusted to 40 % before storage at –20 °C. After concentration with 45 % ammonium sulphate, the purification ratio was about 40-fold and specific activity was near 0.2 U mg^{-1} protein.

2.4.2. PP2Ac purification

Cells of *Y. lipolytica* were harvested after 13 h culture and PP2Ac, the catalytic subunit of protein phosphatase 2A, was purified as described previously [4, 21]. The molecular mass of the catalytic unit was 33 kDa, optimum pH 7–7.3. It was ascertained with effectors, e.g. heparin, that the enzyme was a PP2Ac and not a PP1.

2.4.3. CK2 purification

Cells were harvested after 16–19 h culture and the enzyme purified as described by Chardot and Meunier [22].

2.5. Action of PP2A and CK2 on GS activity measured in the presence or absence of G6P

The dephosphorylating capacity of PP2Ac purified from *Y. lipolytica* on GS was checked on GS purified from

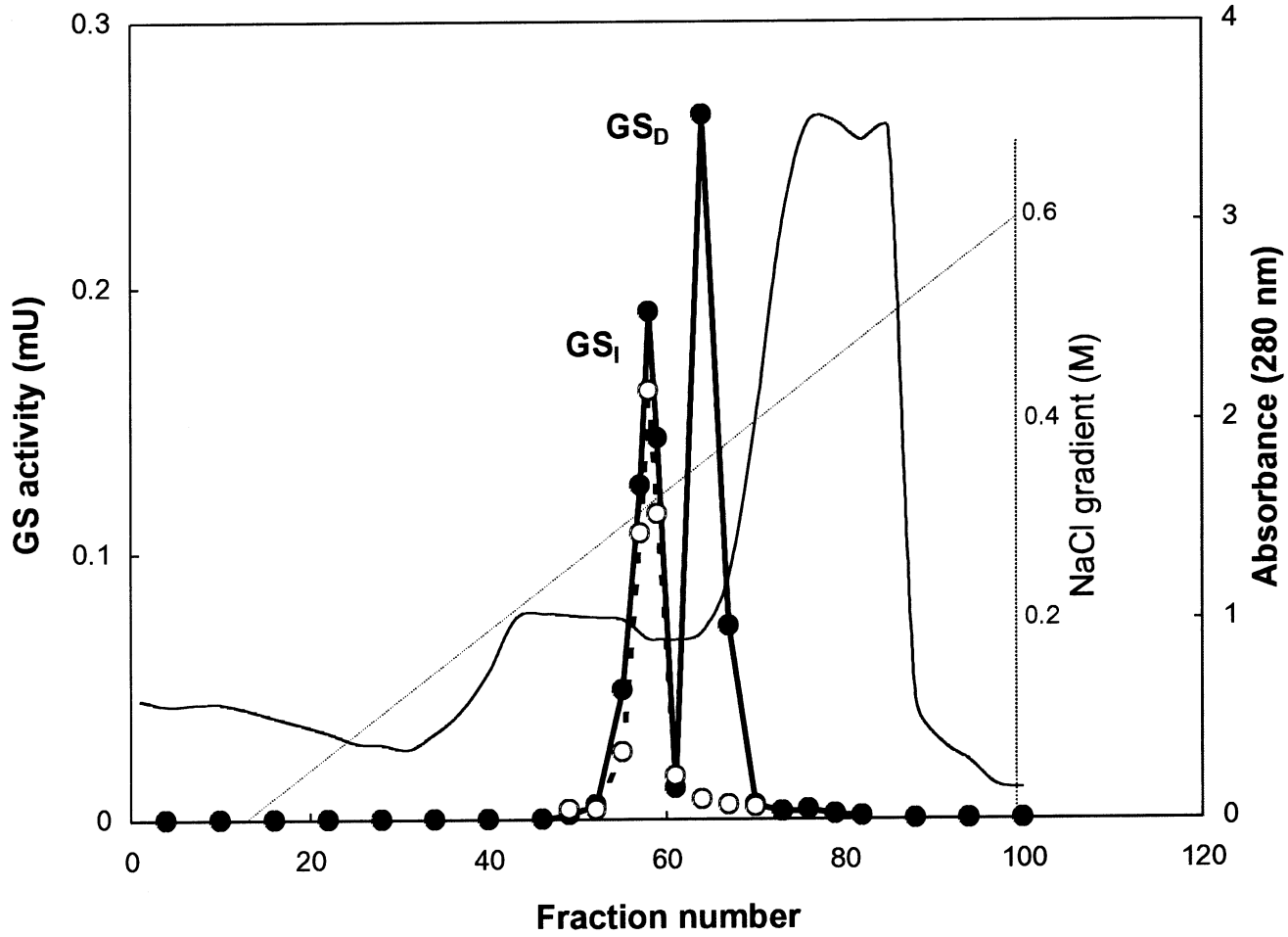

Figure 1. Ion exchange chromatography on DEAE Toyopearl of glycogen synthase extracted from *Yarrowia lipolytica* grown for 13 h on a Pi-deficient medium.
Elution was performed with 50–600 mM NaCl gradient (----). GS activity measurements were carried out as described in experimental section in the presence (●) or in the absence (○) of G6P. Protein elution profile is indicated by the absorbance at 280 nm (—).

Y. lipolytica. It was also assayed on enzyme previously phosphorylated by CK2 purified from *Y. lipolytica*.

GS preparations were incubated with PP2Ac in 50 mM Tris-HCl buffer (pH 7.2). After different incubation times, GS activity was measured in the presence or in the absence of 10 mM G6P. Phosphorylation by CK2 was performed in CK2 buffer containing cold ATP or [γ-^{32}P]ATP (0.1–0.2 mM ATP, 0.8–3.2 10^6 cpm/assay). The study of PP2Ac effect on the phosphorylated GS requires elution of the reaction medium through a Sephadex G50 column in order to remove ATP, a potent inhibitor of phosphatase. PP2Ac is then added to the GS-containing fractions. For the sake of comparison this chromatographic step is also applied to the control reactions (GS alone, GS + CK2). After incubation GS activity is measured with or without G6P in the reaction medium and compared to the controls. Electrophoresis under denaturing conditions was performed by using Novex ready to use 4–20 % gradient gels (Novex, San Diego, CA). Molecular weight determination used Pharmacia standards for SDS-PAGE. Electrophoresis was carried out as described by Laemmli [23]. Gels were stained with Coomassie blue G250. Dried gels were exposed to Phosphor Screen film (Kodak) subsequently scanned with a Storm 620 device (Molecular Dynamics).

3. Results

3.1. Variations in the activity of glycogen synthase, protein phosphatase 2A and protein kinase CK2 in the course of *Yarrowia lipolytica* growth

Growth of *Y. lipolytica* on a phosphate-deficient glucose-sufficientmedium shows an exponential phase up to 14 h then the stationary phase is reached (*figure 2.A*). During the culture pH level was stable around 6.6. Cells were sampled after different times of culture and glycogen synthase, protein phosphatase 2A, protein kinase CK2 activities and glycogen content were determined on aliquots of the same sample through specific assays.

Glycogen synthase activity of both I- and D-forms increased sharply presenting a steep, narrow peak at

11 h 30 min followed by a stable low activity level (*figure 2.B*). The peak appears to be concomitant with active protein synthesis (*figure 2.A*). The ratio of independence RI undergoes strong variations (*figure 2.C*) and reveals that the GS I-form was predominant at 13 h. Glycogen concentration rises steeply from the beginning of growth with a peak at 13 h (*figure 2.D*) in accordance with the increase in GS activity.

Protein phosphatase activity shows a sharp peak between 10 and 13 h culture growth (*figure 2.E*). Former studies established the presence of a major type 2A protein phosphatase concomitant with rapid growth rate [4]. On the whole, results indicate that during the exponential phase (10 h–11 h 30 min) of culture, PP2A activity and GS activity, especially the I-form, rise sharply in synchrony followed at about 13 h by a peak of glycogen accumulation.

The PP2A and GS activity peaks were followed by a maximum in CK2 activity at 16–19 h (*figure 2.F*) leading to partial phosphorylation of GS as deduced from the decrease in RI (*figure 2.C*) observed during the late part of the growth phase. At the same time, glycogen synthesis decreases (*figure 2.D*). As RI is always higher than 50 % except at 10 h, it appears that the decrease in glycogen content might also be ascribed to a decrease in total GS activity.

3.2. Control of GS activity by CK2 and PP2Ac

A kinetic study of CK2 action was performed on total GS extracted at 13 h culture and exhibiting a maximum RI level due to the strong predominance of the I-form (*figure 2.C*). Phosphorylation by CK2 was rapid leading to a minimal RI level (12 %) within 1 h (*figure 3*). The study of PP2A action in the course of time was carried out on total GS with a RI near 65 % indicating a slight I-form predominance. The action of PP2Ac led to an increase in RI and to the transformation of part of GS_D into the dephosphorylated form GS_I (*figure 3*). *Table I* shows GS activity subjected to the action of PP2Ac and/or CK2 and control activity. GS activity was 30 % stimulated by the action of PP2Ac and 31 % decreased by CK2. After CK2 inhibition, addition of PP2Ac led to overstimulation of GS activity.

3.3. Verification of GS phosphorylation by CK2 and dephosphorylation by PP2Ac

Glycogen synthase (I-form) purified from *Y. lipolytica* was incubated with CK2 and [γ-^{32}P]ATP at 25 °C. Aliquots were sampled after 9 min, 30 min, 1 h, 2 h and 4 h and ^{32}P incorporation into the proteins measured after precipitation with 20 % TCA. Results (*figure 4*) show that phosphate was rapidly incorporated into the enzyme and saturation was reached after about 1 h. SDS-PAGE of aliquot fractions prior to TCA precipitation indicated that a 76 kDa form was preferentially phosphorylated (*figure 4*, inset). The labelled protein band at 108 kDa was likely to be a contaminant in the GS preparation obtained in our experimental conditions.

Dephosphorylation by PP2Ac was checked on GS previously subjected to the action of CK2 in the presence of [γ-^{32}P]ATP for 30 min and then separated from ATP through Sephadex G50 chromatography column. PP2Ac was added to phosphorylated GS and removed about 12 % of the bound ^{32}P after 15 min of reaction, 23 % after 20 min and 32 % after 30 min.

4. Discussion

During *Y. lipolytica* growth, we observed strong variations in the activity of several enzymes involved in the metabolism of glycogen, namely glycogen synthase, protein phosphatase 2A, protein kinase CK2 and in endocellular glycogen concentration (*figure 2*). As for PP2A and CK2 activities, GS activity proved to be a good indicator of cell growth. PP2A and GS activities rise in the exponential phase and decrease during the stationary phase when CK2 activity increases. Glycogen storage in the cells was also observed in the exponential phase in contrast to the results of Rothman-Denes and Cabib [2] which indicated glycogen accumulation during the beginning of the stationary phase in *S. cerevisiae* cultures. The data presented here are also consistent with a sequential control of GS activity by PP2A and CK2 during the growth of *Y. lipolytica*. During the exponential phase, a sharp push of PP2A activity would dephosphorylate GS and result in covalent activation of this enzyme into the I-form as indicated by the increase in RI. The peak of glycogen accumulation appears to be in good correlation with the variations observed for PP2A activity. It has been reported in *S. cerevisiae* [2] that the rapid increase in the I-form coincided with an increase in glycogen content, indicating a fundamental role of the I-form in glycogen storage. A similar conclusion may be drawn from our results with *Y. lipolytica*. Transition from rapid growth to progressively slowed down rates coincides with the onset of CK2 activity accompanied by a decrease in glycogen content. After 13 h culture there is also a decrease in total GS concomitant with a decrease in protein synthesis which could also be involved in the decrease in glycogen content.

The two GS forms from *Y. lipolytica* were separated by ion exchange chromatography on DEAE-Toyopearl 650S which appeared to retain GS from *Y. lipolytica* more efficiently than the enzyme from other yeasts. Elution of the I- and D-forms was performed at 0.3 and 0.35 M NaCl, respectively. Peng et al. [20] found that the I-form from *S. cerevisiae* was not bound on DEAE cellulose and that the D-form was eluted at 0.2 M KCl gradient. Huang and Cabib [1] found that I- and D-forms eluted at 0.2 and 0.27 M KCl, respectively. These results could reflect the difference in phosphorylation levels of GS forms.

Results from the experiments carried out with purified enzymes supported our hypothesis of GS regulation through dephosphorylation/phosphorylation. In the presence of CK2, a rapid and strong decrease in RI indicated the conversion of I-form into D-form by phosphorylation. Inversely, PP2Ac is able to dephosphorylate D-form to

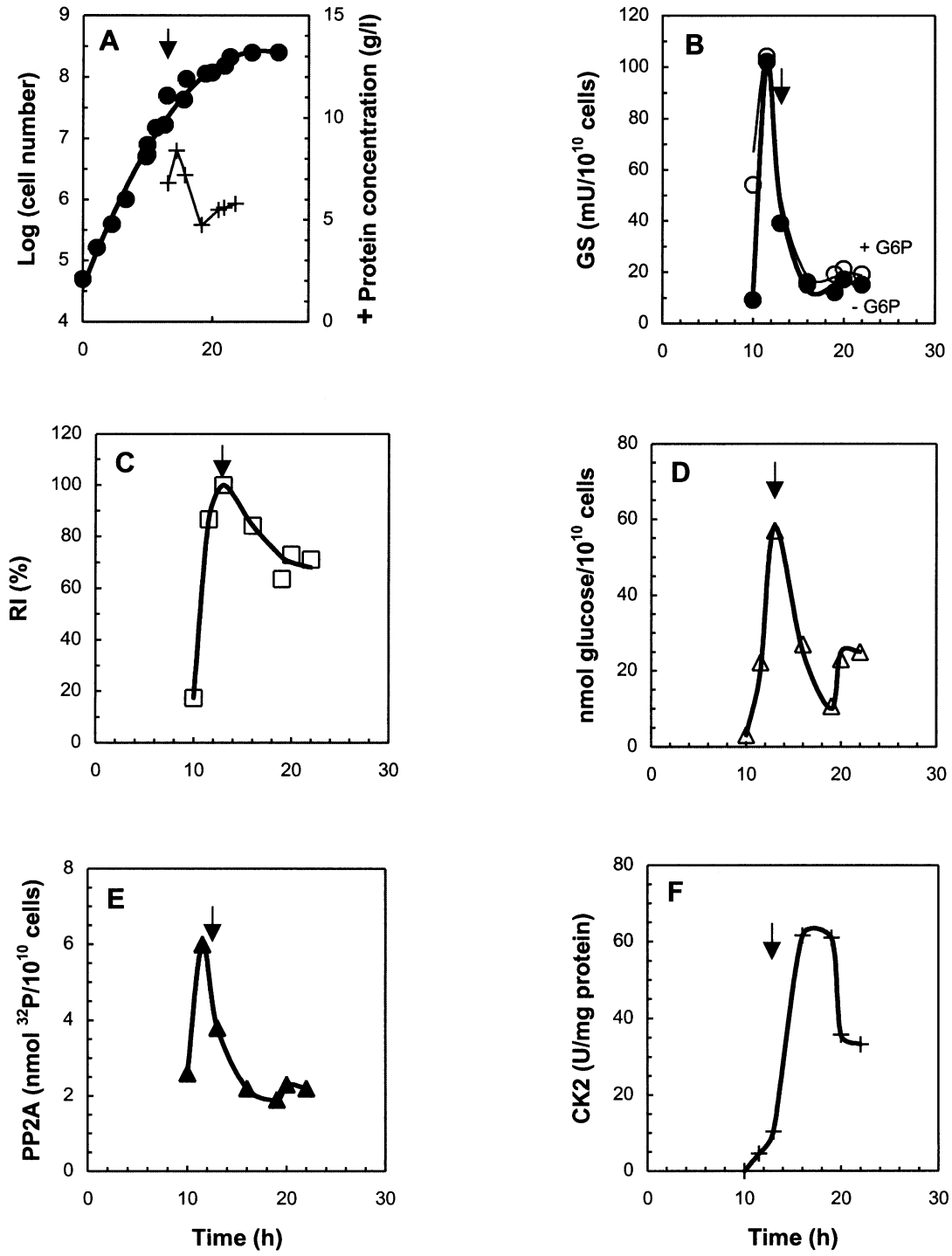

Figure 2. Changes in enzymatic activities and in metabolite contents during cell growth.
Growth expressed as variation in cell number and protein concentration is represented in (A). GS activity measured in the presence (○) or in the absence (●) of G6P (B), PP2A activity (E) and glycogen concentration (D) are expressed in 10^{10} cells. CK2 activity (F) is related to protein concentration. RI (C) is defined as the ratio (in %) of GS activity in the absence of G6P divided by the activity in the presence of G6P. The arrows indicate 13 h. The mean of three experiments is shown and the difference in RI data are significant for all times.

restore independence towards G6P and to increase significantly RI. These results are consistent with those of Peng et al. [20] who reported that the activity ratio of GS_D purified from wild-type or mutant strains of *S. cerevisiae* increased by 15–60 % by incubation with a crude yeast phosphatase preparation. When phosphorylation by CK2 was carried out with [γ-^{32}P]ATP, it was accompanied by ^{32}P incorporation into proteins and mainly into a 76 kDa protein

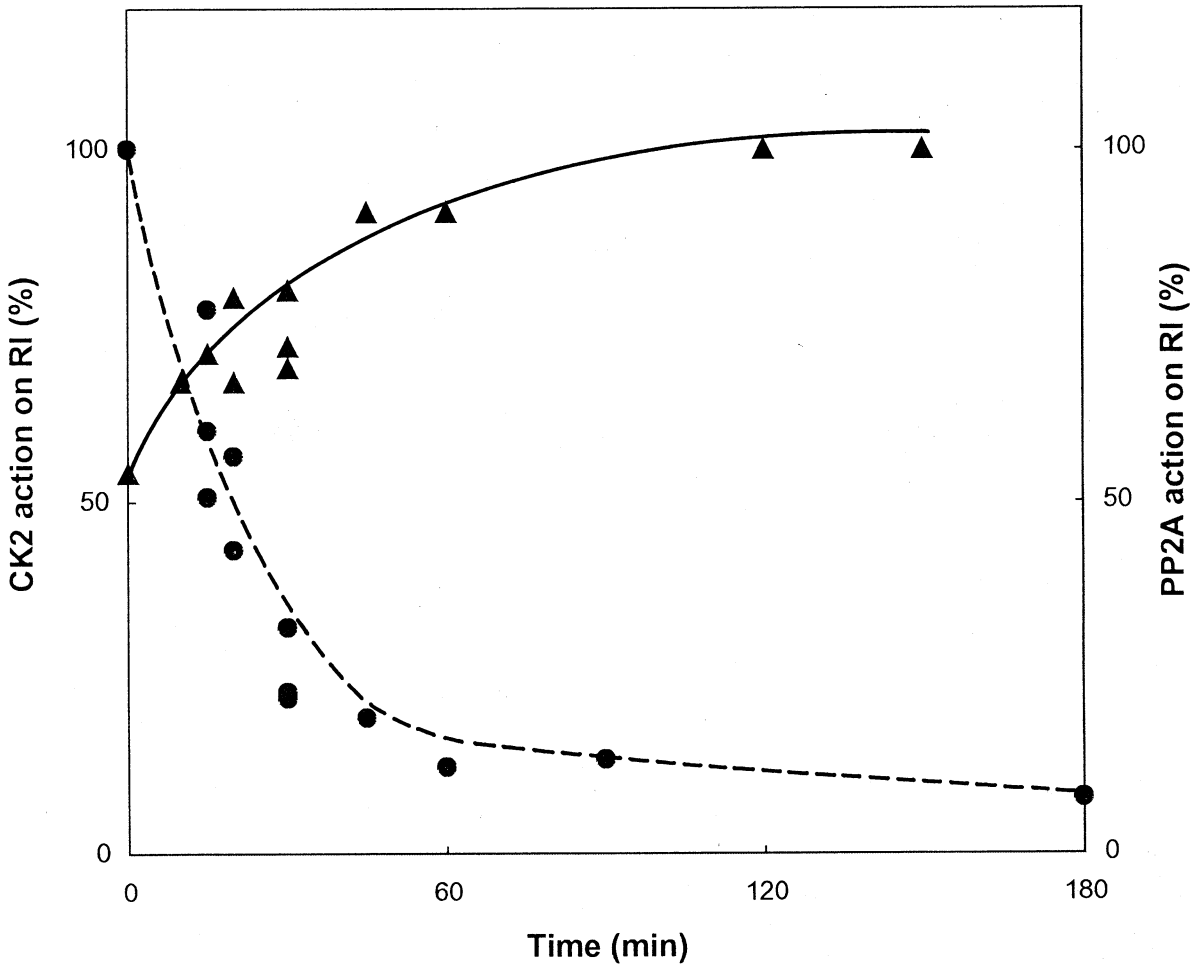

Figure 3. Kinetic studies of the action of CK2 (●) and PP2Ac (▲) purified from *Yarrowia lipolytica* on total GS activity expressed as RI (ratio in % of GS activity in the absence of G6P divided by the activity in the presence of G6P).

band. This 76 kDa protein form is likely to be a GS subunit from *Y. lipolytica.* Other results showed that GS purified from *Y. lipolytica* presented only one protein band at 75 kDa in electrophoresis on polyacrylamide gel under native conditions after Coomassie blue R-250 staining suggesting a monomeric structure (data not shown). Further experiments should be carried out to confirm the monomeric composition of *Y. lipolytica* GS. Glycogen synthase preparations from *S. cerevisiae* contained two polypeptides of molecular mass of 85/83 and 77/76

Table I. Effect of CK2 and PP2A on GS activity.

Activity[a]							
Control GS		GS + PP2Ac[b]		GS + CK2[c]		GS + CK2 + PP2Ac[d]	
10^{-12} kat	%	10^{-12} kat	%	10^{-12} kat	%	10^{-12} kat	%
1.57 (0.43)	100	2.05 (0.38)	130	1.09 (0.38)	69	2.24 (0.16)	143

[a] GS activity was determined for 100 µL enzyme extract in the absence of G6P and expressed as percentage of control GS activity. Data are the mean of seven experiments (± s.d. in brackets). Control was incubated in the same experimental conditions and submitted to the same chromatographic step as the samples (see Materials and methods).

[b]GS (60 µg protein) was incubated with 15 µg purified PP2Ac for 30 min at 30 °C and compared to GS maintained without PP2Ac in the same conditions.

[c]GS was incubated (30 min) with purified CK2 (2 µg) in the presence of 0.2 mM [γ-^{32}P]ATP (final volume 330 µL). After elution on Sephadex G-50 column to discard ATP, GS activity was evaluated with the radioactive assay and compared to control GS incubated without CK2 and eluted from a G-50 column.

[d]Experiment was carried out as above (GS + CK2). After elution on G-50 column, GS was incubated with PP2Ac for 30 min at 30 °C. Control was subjected to the same procedure without CK2 and PP2Ac.

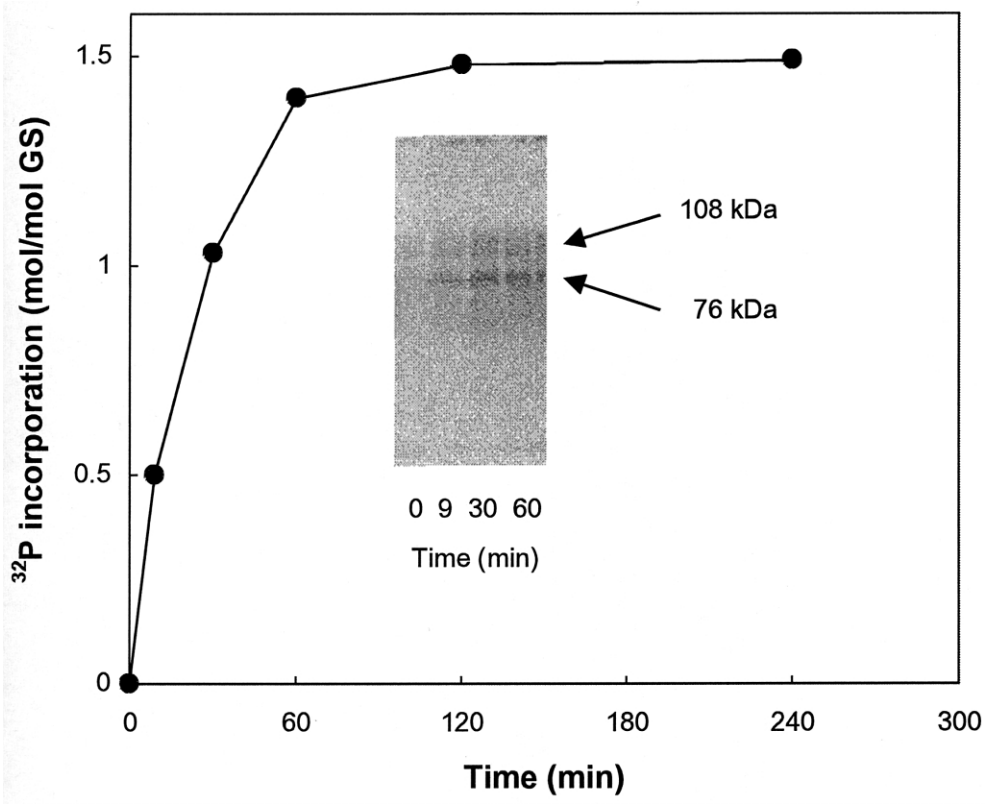

Figure 4. Time course of ^{32}P incorporation into GS by CK2.
120 μg GS_l were incubated with 0.2 mM [γ-^{32}P] ATP and 4 μg CK2 in 660 μL final volume. At different times, aliquot fractions were sampled and proteins precipitated with a 20 % TCA solution containing 100 mM sodium pyrophosphate, then twice with TCA 10 %. The radioactivity in GS was measured in the pellet after centrifugation. Two determinations were carried out for each time. Inset: aliquot fractions were submitted to SDS-PAGE. The gel was dried and exposed to a phosphor screen film.

kDa [20, 24] which would be encoded by two different genes [13]. Phosphorylation of GS from *Y. lipolytica* indicates the existence of CK2 consensus sites in the 76 kDa subunit. Consensus sites have been found in the two glycogen synthase isoforms from *S. cerevisiae* [13, 24].

Glycogen formation and break-down is controlled by glycogen synthase and glycogen phosphorylase, respectively. Fernández-Bañares et al. [25] observed that glycogen synthesis in yeast may occur in the presence of elevated levels of phosphorylase *a* under conditions in which glycogen synthase is highly and fully active. However, it would be interesting to study also the variation of phosphorylase activity during the growth of *Yarrowia lipolytica.*

References

[1] Huang K.P., Cabib E., Separation of the glucose-6-phosphate independent and dependent forms of glycogen synthase from yeast, Biochem. Biophys. Res. Commun. 49 (1972) 1610–1616.

[2] Rothman-Denes L.B., Cabib E., Two forms of yeast glycogen synthase and their role in glycogen accumulation, Proc. Natl. Acad. Sci. USA 66 (1970) 967–974.

[3] Benetti P.H., Kim S.I., Canonge M., Chardot T., Meunier J.C., Cloning of the catalytic subunit of the casein kinase II from the yeast *Yarrowia lipolytica*, Mol. Gen. Genet. 256 (1997) 355–364.

[4] Jolivet P., Queiroz-Claret C., Bergeron E., Meunier J.C., Purification and characterization of a type 2A protein phosphatase from *Yarrowia lipolytica* grown on a phosphate deficient medium, C. R. Acad. Sci. Paris 320 (1997) 441–449.

[5] Mishra C., Evidence for the occurrence of glycogen synthase and phosphatase and kinase in yeasts, FEMS Microbiol. Lett. 18 (1983) 25–29.

[6] Wingender-Drissen R., Becker J.U., Characterization of phosphoproteins phosphatases and phosphorylase phosphatases from yeast, Biochim. Biophys. Acta 743 (1983) 343–350.

[7] Peng Z.Y., Wang W., Wilson S.E., Schlender K.K., Trumbly R.J., Reimann E.M., Identification of a glycogen synthase phosphatase from yeast *Saccharomyces cerevisiae* as protein phosphatase 2A, J. Biol. Chem. 266 (1991) 10925–10932.

[8] Feng Z., Wilson S.E., Peng Z.Y., Schlender K.K., Reimann E.M., Trumbly R.J., The yeast GLC7 gene required for glycogen accumulation encodes a type one protein phosphatase, J. Biol. Chem. 266 (1991) 23796–23801.

[9] Ramaswamy N.T., Li L., Khalil M., Cannon J.F., Regulation of yeast glycogen metabolism and sporulation by Glc7p protein phosphatase, Genetics 149 (1998) 57–72.

[10] Posas F., Clotet J., Muns M.T., Corominas J., Casamayor A., Ario J., The gene PPG encodes a novel yeast protein phosphatase involved in glycogen accumulation, J. Biol. Chem. 268 (1993) 1349–1354.

[11] Rodriguez-Belmonte E., Gonzalez-Siso I., Cerdan E., The *Kluyveromyces lactis* gene KIGSK-3 combines functions which in *Saccharomyces cerevisiae* are performed by MCK1 and MSD1, Curr. Genet. 33 (1998) 262–267.

[12] Galabova D., Tuleva B., Balasheva M., Phosphatase activity during growth of *Yarrowia lipolytica*, FEMS Microbiol. Lett. 109 (1993) 45–48.

[13] Farkas I., Hardy T.A., Goebl M.G, Roach P.J., Two glycogen synthase isoforms in *Saccharomyces cerevisiae* are coded by distinct genes that are differentially controlled, J. Biol. Chem. 266 (1991) 15602–15607.

[14] Bradford M.M., A rapid and sensitive method for quantitation of microgram quantities of proteins utilizing the principle of protein dye binding, Anal. Biochem. 72 (1976) 248–254.

[15] Thomas J.A., Schlender K.K., Larner J., A rapid filter paper assay for UDP glucose-glycogen glycosyl transferase including an improved biosynthesis of UDP^{14}C glucose, Anal. Biochem. 25 (1968) 486–499.

[16] McGowan C.H., Cohen P., Protein phosphatase 2C from rabbit skeletal muscle and liver: an Mg^{2+}-dependent enzyme, Methods Enzymol. 159 (1988) 416–426.

[17] Gopalakrishna R., Gundimeda U., Wilson J.C., Chen Z.H., Multiwell filtration assay for rapid determination of protein phosphatase activity, Anal. Biochem. 212 (1993) 296–299.

[18] Kuenzel E., Mulligan J.A., Sommercorn J., Krebs E.G., Substrate specificity determinant for casein kinase II as deduced from studies with synthetic substrates, J. Biol. Chem. 262 (1987) 9136–9140.

[19] Becker J.U., A method for glycogen determination in the whole cell extract, Anal. Biochem. 86 (1978) 56–64.

[20] Peng Z.Y., Trumbly R.J., Reimann E.M., Purification and characterization of glycogen synthase from a glycogen deficient strain of *Saccharomyces cerevisiae*, J. Biol. Chem. 265 (1990) 13871–13877.

[21] Queiroz-Claret C., Jolivet P., Delérable C., Bergeron E., Meunier J.C., Gel-immobilized protein phosphatase 2A from *Yarrowia lipolytica* dephosphorylates phosvitin and modifies its functional properties, J. Agric. Food Chem. 45 (1997) 2899–2906.

[22] Chardot T., Meunier J.C., Purification and principal properties of the yeast *Yarrowia lipolytica* casein kinase II, Int. J. Biochem. 26 (1994) 1017–1024.

[23] Laemmli U.K., Cleavage of the structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[24] Farkas I., Hardy T.A., DePaoli-Roach A.A., Roach P.J., Isolation of the GSY1 gene encoding yeast glycogen synthase and evidence for the existence of a second gene, J. Biol. Chem. 265 (1990) 20879–20886.

[25] Fernández-Bañares I., Clotet J., Ariño J., Guinovart J.J., Glycogen hyperaccumulation in *Saccharomyces cerevisiae ras2* mutant, FEBS Lett. 290 (1991) 38–42.