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SHORT CONTRIBUTION

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Quantification of intracellular amino acids in batch cultures of *Saccharomyces cerevisiae*

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Abstract The dynamics of intracellular amino acid pools were determined in batch cultures of *Saccharomyces cerevisiae*. Immediate termination of metabolic activity was found to be necessary for accurate quantification of in vivo concentrations of intracellular amino acids, due to significant changes in most intracellular amino acid pools observed during extraction without an instantaneous stop of the metabolism. The method applied to batch-cultures of *S. cerevisiae* on glucose revealed complex dynamics in intracellular amino acid pools. The most drastic changes were observed during the diauxic shift and at the entry into the stationary phase. Even during phases of exponential growth on glucose and ethanol, cells showed significant variations in intracellular amino acid concentrations. The method presented can be used to investigate the physiology of yeast cultures, including industrially relevant batch and fed-batch processes.

Introduction

Intracellular amino acids in yeast are of great interest as precursors of desired products, such as in biomass production for the feed industry (Martinez-Force and Benitez 1995), or are products themselves, as in the production of glutamate, glutamine, lysine, threonine, or tryptophan (e.g. Delgado et al. 1982; Martinez-Force and Benitez 1992a, b). The estimation of intracellular amino acid concentrations is especially important for the elucidation of in vivo kinetics and metabolic regulation. In ^{13}C tracer experiments, amino acid labeling provides valuable information for metabolic flux analysis. Such studies require a fast and reliable method for the analysis of intracellular amino acids. Among the methods available for the analysis of intracellular metabolites, only a few deal with amino acid extraction from yeast (Oshumi et al. 1988; Martinez-Force and Benitez 1995; Gent and Slaughter 1998). With regard to the fact that intracellular free amino acids have turn-over times in the range of seconds (Neidhardt et al. 1990), termination of metabolic activities during sampling seems a rather crucial point, but was not discussed in any of the above papers. The aim of the present work was the development of a suitable protocol for the quantification of intracellular amino acids in *Saccharomyces cerevisiae* and its application to different growth phases in batch cultivations.

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Materials and methods

Organism and growth conditions

The strain *S. cerevisiae* ATCC 32167 was obtained from the American Type Culture Collection (Manassas, Va.) and cultivated in shake-flasks (50 ml) at 30 °C, pH 6.0, and 150 rpm, on a synthetic medium with glucose as carbon source (Theobald et al. 1993).

Analytical methods and chemicals

Biomass was determined via optical density at 660 nm (OD_{660}) or gravimetrically as cell dry weight (CDW) after two-fold washing, centrifugation (15 min, 4,000×*g*, 4 °C), and drying at 80 °C until weight constancy. A linear relationship of OD_{660}=1.77 g CDW/l was observed. The cell volume (V_C) was calculated to V_C=2.38 ml/g CDW (Reich and Selkov 1981). Separation of cells from the medium and extraction of intracellular metabolites was adapted from Gonzalez et al. (1997), with the modifications listed here. A reduced amount of 1.0 mg CDW was sampled. The extraction supernatant was concentrated by lyophilization and the residue was resuspended in deionized water to a final volume of 0.05 ml and stored at –20 °C for 1 week at most. Under these conditions, metabolites were found to be stable. Amino acids were analyzed by HPLC (AQC-fluor reagent, AccQ column (150×3.9 mm); Millipore, Milford, Mass.), as described in the instruction manual, at a flow rate of 1 ml/min, with UV detection at 250 nm. Standard deviations were below 2%. Glutamine/histidine and serine/asparagine cannot be separated by the HPLC method used and, in this paper, they are treated as combined pools. The detection limit was about 1 µM, whereas a reliable quantification required a concentration of at least 5 µM. Bovine serum albumin (BSA; >99%) and leucine-encephaline (>97%) were obtained from Sigma (St.Louis, Mo.). All other chemicals were of analytical grade and purchased from Fluka (Buchs, Switzerland) or Sigma (St.Louis, Mo.), respectively.

Results

Quenching for intracellular amino acid quantification

The necessity of immediate termination of metabolic activities for the analysis of intracellular amino acid pools was studied for exponentially growing yeast cells, by comparing the effects of immediate termination of metabolic activities, either in cold methanol (below –20 °C), or by cooling in cold water (+4 °C) before cell harvest by centrifugation and subsequent extraction with hot ethanol. Substantial differences for most amino acids were observed. In comparison to sampling in cold methanol (–20 °C), sampling at 4 °C led to significantly increased concentrations for phenylalanine (+87%), proline (+79%), tyrosine (+70%), glycine (+56%), leucine (+40%), alanine (+31%), isoleucine (+22%), methionine (+21%), glutamate (+14%), and threonine (+5%), respectively. In contrast, the concentrations of aspartate (- 19%), valine (–14%), lysine (–8%), glutamine/histidine (–7%), asparagine/serine (–6%), and arginine (–1%) were decreased. Further studies were carried out on the stability of amino acids, peptides, and proteins during the incubation in cold methanol. The recovery of proteinogenic amino acids incubated in cold methanol at –20 °C was 98.5% (±1.9%) on average (data not shown). No free amino acids were detected for leucine encephaline (2 mM), or BSA (1.1 mg/ml), respectively, after incubation in cold methanol at –20 °C. These findings indicate that free amino acids, peptides, and proteins are stable under the quenching conditions. Possible losses of intracellular amino acids during quenching were quantified by analyzing the supernatant for amino acids after the quenching of 5 ml of culture (10 mg CDW) exponentially growing on glucose in 45 ml of cold methanol (–20 °C). No amino acids were detected in a 40-fold concentrate of the supernatant. With the detection limit of the HPLC method being 1 µM, the concentrations in the original quenching solution were thus below 25 nM. For example, the complete release of an intracellular amino acid with a concentration of about 50 mM would result in a concentration of about 25 µM in the quenching solution. Accordingly, the loss of intracellular amino acids due to release into the quenching solution was below 0.1%.

Extraction in boiling buffered ethanol

Several studies were performed to investigate whether the protocol with boiling buffered ethanol (Gonzalez et al. 1997) is suitable for the extraction of intracellular amino acids from yeast. All proteinogenic amino acids, the pentapeptide leucine encephaline, and BSA, respectively, were found to be stable during extraction with boiling buffered ethanol. To test for the lack of enzymatic conversion during extraction, amino acids were added to quenched cell suspensions of *S. cerevisiae* after 2.9 h of cultivation (growth on glucose) at amounts in the range of the expected intracellular amounts. The recovery was 90% (Gly), 92% (Ser, Asn), 96% (Arg), 97% (Thr, Lys, Ile, Leu), 98% (Asp, Glu, Gln/His, Ala), 99% (Val), and 103% (Pro, Met), respectively. The higher deviations for cysteine (130%) and tyrosine (66%) are due to the low intracellular concentrations of these amino acids. Phenylalanine and tryptophane were not detected. Similar results were observed for cells after 4.3 h of cultivation (growth on ethanol). Concerning the completeness of extraction, the hot ethanol method resulted in comparable amino acid concentrations throughout different growth phases in batch cultivation of *S. cerevisiae*, as compared to the extraction with boiling water (100 °C, 15 min), commonly used for amino acid extraction in yeast.

Dynamics of intracellular amino acid pools during batch cultivation of *S. cerevisiae*

The dynamics of intracellular amino acid pools were assessed in two parallel cultivations of *S. cerevisiae* ATCC 32167 on mineral medium with 10 g glucose/l. The strain exhibited a diauxic profile of: (1) exponential growth on glucose with ethanol secretion, (2) diauxic shift, (3) exponential growth on ethanol, and (4) stationary phase. After a short delay during the diauxic shift, ethanol was consumed. The intracellular amino acid pools revealed a significant and complex dynamic behavior (Fig. 1A–D). The intracellular amino acid concentrations varied from below the detection limit (equal to about 0.2 mM) to 100 mM, depending both on the amino acid species and on the growth phase. Alanine, aspartate, phenylalanine, and tyrosine varied more then ten-fold throughout the cultivation. In contrast, lysine, proline, threonine, arginine, and glycine exhibited much less variation. The highest concentrations, up to 100 mM, resulted for glutamate, arginine, alanine, aspartate, and glutamine/histidine (Fig. 1A). The majority of amino acids, including serine/asparagine, glycine, threonine, valine, lysine, leucine, and isoleucine were in the range 1–20 mM (Fig. 1A–C). Levels of proline, methionine, and the aromatic amino acids, tyrosine and phenylalanine, were between 0.2 mM and 3 mM (Fig. 1D). The lowest intracellular concentrations, below 0.2 mM, were observed for cysteine and tryptophan. Methionine remained at a low and rather constant level, about 0.5 mM, until the complete consumption of ethanol. With entry into the stationary phase, the concentration sharply dropped. Interestingly, the intracellular concentrations of amino acids did not stay constant during exponential growth on glucose or on ethanol, respectively. For example, the concentrations of alanine, glutamate, and glutamine/histidine decreased significantly during exponential growth on glucose and on ethanol, respectively. A large number of amino acids, including valine, leucine, isoleucine, aspartate, threonine, lysine, phenylalanine, and tyrosine, showed a significant increase at the beginning of the stationary phase. In contrast, glutamate, glutamine/histidine, methionine, and proline decreased. The total intracellular amino acid concentration reached a

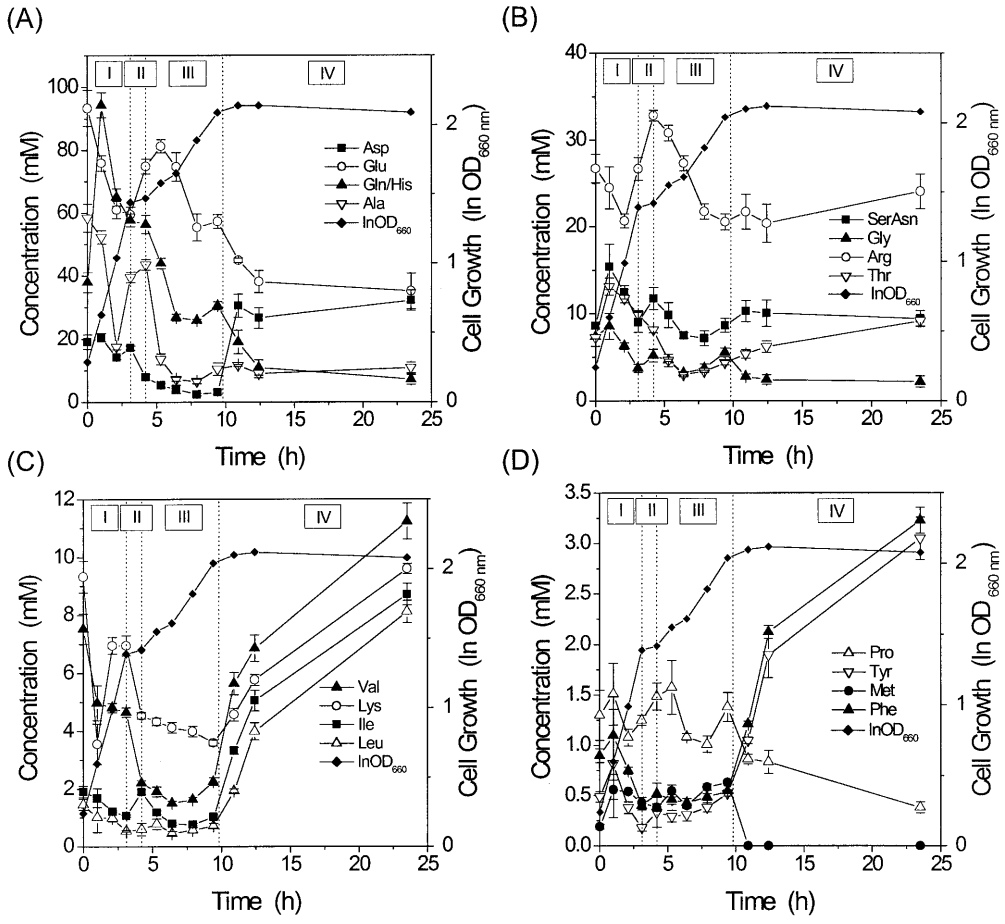

Fig. 1A–D Changes in intracellular amino acid concentrations (mmol/l) during (*I*) growth on glucose, (*II*) diauxic shift, (*III*) growth on ethanol, and (*IV*) stationary phase in batch cultivation of *Saccharomyces cerevisiae* ATCC 32167. Data were obtained from two cultures grown under identical conditions and represent the mean of four separate extractions. Growth of the culture is displayed as log normal optical density at 660 nm. *Ala* Alanine, *Arg* arginine, *Asp* aspartate, *Gln/His* glutamine/histidine, *Glu* glutamate, *Gly* glycine, *Ile* isoleucine, *Leu* leucine, *Lys* lysine, *Met* methionine, *Phe* phenylalanine, *Pro* proline, *Ser/Asn* serine/asparagine, *Thr* threonine, *Tyr* tyrosine, *Val* valine

maximum of 320 mM in the middle of the exponential growth phase on glucose. At this point, 10% of the dry biomass was present as free amino acids. During further cultivation, the total amino acid content decreased gradually to 250 mM at the end of the diauxic shift and 150 mM after ethanol exhaustion. During the stationary phase, a slight increase to 175 mM was observed.

Discussion

The method described displays a suitable tool for the quantification of intracellular amino acid pools in yeast. Our results clearly show that an instantaneous stop of metabolic activities is required for the accurate quantification of in vivo amino acid concentrations. This has not been considered in previously applied protocols (Kitamoto et al. 1988, Martinez-Force and Benitez 1995; Gent and Slaughter 1998). The accuracy of the method is underlined by the good correlation for the intracellular amino acid concentrations, which were obtained from two separate cultivations under identical conditions. Significant and complex dynamics of intracellular pools of amino acids were found during batch-cultivation of *S. cerevisiae*. The most drastic alterations occurred during the diauxic shift and at the entry into the stationary phase, reflecting the accompanying fundamental rearrangements of central metabolism and suggesting a close correlation to the necessary extensive modifications of the protein metabolism (Fuge et al. 1994; Werner-Washburne et al. 1996). The concentrations of glutamate, glutamine, and proline declined when cells entered the stationary phase. Their degradation pathways converge at 2-oxoglutarate, which might be an important entry point for replenishment of the tricarboxylic acid cycle and for energy formation during the stationary phase. Levels of valine, leucine, isoleucine, aspartate, threonine, lysine, phenylalanine, and tyrosine increased markedly, possibly linked to proteolytic activity in the vacuole (Gent and Slaughter 1998). The decrease of methionine, the starting amino acid of translation, at the beginning of the stationary phase might be related to the drastic reduction of protein synthesis (Werner-Washburne et al. 1996). In addition, the phases of exponential growth on glucose and on ethanol, respectively, were also related to changes in various amino acid pools. This indicates that, from the detail of intracellular amino acids pools, growth was not balanced during the exponential growth phases. The presented protocol, including quenching in cold methanol, should be also applicable to other microorganisms. For example, cold methanol was successfully used for the quenching of *Escherichia coli* cells in the analysis of intermediary glycolytic metabolites (Schaefer et al. 1999).

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