FEBS Letters 351 (1994) 357–359

FEBS 14487

Biochemical evidence that the *Saccharomyces cerevisiae THR4* gene encodes threonine synthetase

Cayo Ramos**, Isabel L. Calderón*

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Ap. 1095, E-41080 Sevilla, Spain

Received 25 July 1994

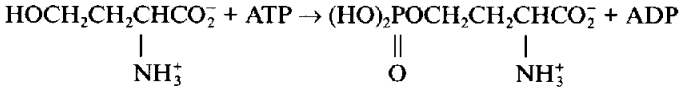
Abstract In yeast, the assignment of the threonine synthetase activity to the *THR4* gene has been inferred from different data, but never really proved enzymatically. In this work, an assay system for threonine synthetase activity in yeast crude extract is reported. The method is based on the quantification by reverse-phase high-performance liquid chromatography, of the threonine formed from *O*-phosphohomoserine. Using this method we have determined that this activity depends on the presence in the cell of an active form of the *THR4* gene, thus demonstrating the univocal relationship between them.

Key words: Threonine synthetase; *O*-Phosphohomoserine purification; *THR4* gene; Yeast

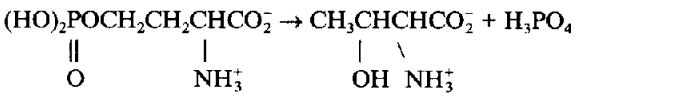
1. Introduction

In yeast, threonine formation from homoserine is catalyzed in two steps. In the first one, homoserine is phosphorylated by homoserine kinase (EC 2.7.1.39) at the expense of ATP, to render *O*-phosphohomoserine (Reaction 1). Threonine synthetase (EC4.2.99.2) catalyzes the second step by an elimination of orthophosphate to yield threonine (Reaction 2) [19].

Reaction 1:



Reaction 2:



In *Saccharomyces cerevisiae*, the *THR1* gene has been assigned to homoserine kinase by enzyme assay [3]. However, assignment of the *THR4* gene to threonine synthetase has only been inferred from the following data: (i) auxotrophy due to mutation in the *THR4* and the *THR1* genes are the only ones satisfied by threonine and not by homoserine [3] and (ii) as deduced from the nucleotide sequence, the *THR4* gene encodes a protein which has extensive homology with *Escherichia coli* and *Bacillus subtilis* threonine synthetases [1,9]. However, levels of threonine synthetase in wild-type or mutant strains of *S. cerevisiae* have not been reported. It is, thus, not possible to establish the relevance of this step in the regulation of the threonine biosynthesis. This lack of information limits the capacity of manipulation of this route in order to obtain yeast strains that overproduce this amino acid. Such strains can be used as a threonine-rich supplement in both animal and human food (Martín-Rendón, E., Farfán M.J. and Calderón, I.L., submitted).

Several methods have been described to measure threonine synthetase activity in crude extract from baker yeast or bacteria. These methods are based on the determination of inorganic phosphate released from *O*-phosphohomoserine [5,17] or of threonine formation from the same substrate [2,4,14,16]. They involve coupled assays or the use of radioactive substrates, which is usually tedious and time consuming. We have developed a fast, simple and reliable procedure for the enzymatic assay of threonine synthetase activity in yeast crude extract using high-performance liquid chromatography (HPLC). Using this method with *THR4* and *thr4* strains we have determined the univocal relationship between this gene and the threonine synthetase activity.

2. Materials and methods

2.1. Strains

Strain X2180-1A [MATa SUC2 mal gal2 CUP1] was provided by the Yeast Genetics Stock Center (Berkeley, USA), strain F4 [MATa thr4] was provided by 'La Cruz del Campo S.A.' (Sevilla, Spain), strain XMR9-9A [MATa his4-417 leu2-3,112 thr1] transformed with the multicopy plasmid pMR1-4 [LEU2 THR1] [10] was provided by Dr. E. Martín-Rendón, Departamento de Genética, Universidad de Sevilla, Spain.

2.2. Media

Usual minimal medium (SD) was used throughout this work; when necessary, minimal medium was supplemented with the appropriate requirements [15].

2.3. Chemicals and enzymes

Dowex AG 1 × 8 was obtained from Serva (Heidelberg, Germany). *E. coli* alkaline phosphatase, *O*-phthaldialdehyde, DL-homoserine, β-mercaptoethanol and all other biochemicals were from Sigma Chemical Co. (St. Louis, MO). Inorganic compounds were from Merck AG (Darmstadt, Germany).

2.4. Amino acid determination

Homoserine, *O*-phosphohomoserine and threonine were determined by the absorbance at 340 nm of their *O*-phthaldialdehyde derivatives measured after separation by reverse-phase HPLC [11]. A Waters Cromatographer, a Novapack C18, 18 × 100 mm column and a manual injector were used. Data integration and processing was done by the Baseline 810 program of an IBM AT computer.

*Corresponding author. Fax: (34) (5) 455 7104.

** *Present address:* Departamento de Bioquímica, Estación Experimental del Zaidin, CSIC, Ap. 419, E-18008 Granada, Spain.

SSDI 0014-5793(94)00874-4

2.5. Preparation of yeast crude extracts

Yeast cells were grown with vigorous stirring, at 30°C in minimal (SD) medium supplemented with the appropriate amino acids. When the culture reached an OD_{660} of 0.7 (late exponential phase), the cells were harvested by centrifugation at room temperature, washed twice and resuspended in TED buffer and disrupted in a Braun homogenizer with 0.5 mm diameter glass beads. Cells debris was removed by 20 min centrifugation at 8000 × *g* and 4°C. TED buffer contains 40 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, pH 7.5; 1 mM EDTA and 1 mM dithiothreitol. Total protein concentration was determined by the method of Lowry et al. [8].

2.6. Preparation of O-phosphohomoserine

O-Phosphohomoserine was prepared enzymatically from homoserine and ATP using yeast homoserine kinase. Homoserine kinase was partially purified as described by Ramos et al. [13] from strain XMR9-9A(pMR1–4). The reaction mixture consisted of 100 mM HEPES pH 7.5, 200 mM KCl, 30 mM $MgSO_4$, 70 mM ATP and 100 mM DL-homoserine. Yeast homoserine kinase was added and the reaction mixture incubated at 30°C. After 3 h, the reaction was stopped by boiling and precipitated proteins were removed by centrifugation.

O-Phosphohomoserine was first separated as barium salt from the deproteinized supernatant. Phosphonucleotides and barium excess were removed by Acid-washed Norit A and precipitation with SO_4Na_2, respectively [18]. The supernatant was applied to a 1 × 24 cm column of Dowex-AG anion exchange resin (200–400 mesh, formic form) and *O*-phosphohomoserine was eluted with 0.5 M formic acid. Ninhydrin-positive fractions [12] were combined and concentrated by rotary evaporation.

The synthesized and purified compound was demonstrated to be *O*-phosphohomoserine by the following criteria: (i) the compound migrated with the retention factor (Rf) described for *O*-phosphohomoserine (R_f = 0.04) in descending thin layer chromatography (0.1 mm cellulose) and 80% phenol as solvent; and (ii) removal of the phosphate group by alkaline phosphatase treatment gave a compound migrating with R_f values identical as those obtained for DL-homoserine in 80% phenol or in 65% ethanol and 1% acetic acid as solvents (R_f = 0.43 and R_f = 0.58, respectively). *O*-phosphohomoserine was quantified as described in section 3.

2.7. Threonine synthetase assay

Threonine synthetase activity was measured in yeast-crude extracts. The standard assay mixture, adapted from Szczesiul and Wampler [17], contains: 50 mM of *N*-tris-(hydroxymethyl)methyl-2-amino ethane-sulfonic acid, pH 8; 70 μM pyridoxal 5-phosphate; 1 mM *O*-phosphohomoserine; yeast crude extract (25 to 30 mg protein/ml), and distilled water to a volume of 4 ml. Reaction mixtures was incubated at 30°C during 60 min. Periodically, 1 ml samples were taken and the reaction stopped by boiling in a water bath during 4 min. The samples were cooled down, centrifuged at 13 000 rpm during 15 min and the sediment was discarded. Threonine formation and *O*-phosphohomoserine content was measured in the supernatant after filtration through 0.45 μm nitrocellulose.

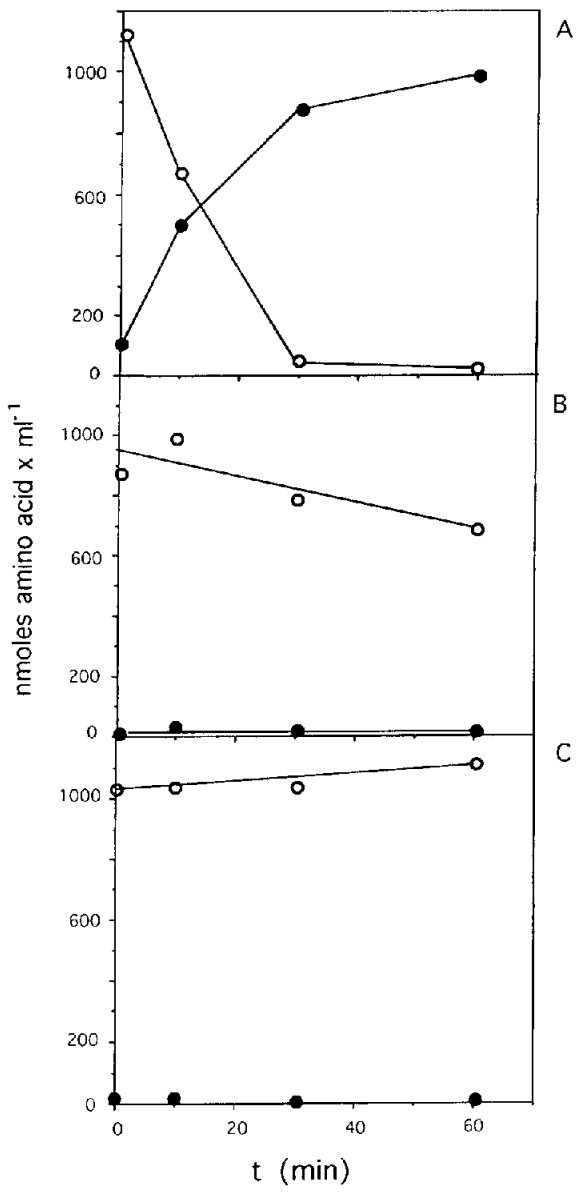


Fig. 1. Threonine formation from *O*-phosphohomoserine by threonine synthetase. Threonine synthetase was assayed in reaction mixtures containing crude extract from: A, strain X2180-1A (*THR4*); B, strain F4 (*thr4*); C, no extract. Closed symbols correspond to threonine; open symbols, to *O*-phosphohomoserine.

3. Results and discussion

In *S. cerevisiae*, threonine biosynthesis from aspartic acid has been the subject of extensive genetic and biochemical studies (for a review, see [7]). However, there have been very few studies devoted to threonine synthetase due to the difficulties found in its enzymatic assay. In the first place, *O*-phosphohomoserine, the substrate for threonine synthetase, is not commercially available; chemical or enzymatic synthesis of this compound is, thus, required. Furthermore, the different methods used for assaying threonine synthetase present many drawbacks. For instance, coupled enzymatic assays can be inappropriated for kinetic studies, since the constants of the coupled system may interfere with the constants under study [4]. Direct measurement of *O*-phosphohomoserine disappearance or of threonine formation is, undoubtedly, more convenient, although according to the assays developed up to now, it requires the preparation of a radioactive substrate [2]. The isocratic elution of amino acid derivatives by HPLC offers a fast and simple alternative to the radioactive detection.

In this work, *O*-phosphohomoserine was synthesized enzymatically from homoserine and ATP using partially purified homoserine kinase from strain XMR9-9A(pMR1–4). This strain, which carries the *THR1* gene encoding homoserine kinase in a multicopy plasmid, contains levels of homoserine kinase 60 times higher than the wild-type strain [10]. The enzymatically synthesized *O*-phosphohomoserine, and pure solutions of L-threonine and DL-homoserine were analyzed by HPLC using the method of Martínez-Force and Benítez [11].

The amino acid derivatives could be completely resolved in less than 10 min. The following retention times (R_t) were obtained: 0.78 min for *O*-phosphohomoserine, 8.31 min for homoserine and 9.04 min for threonine. Threonine and homoserine were quantified by comparing their peak areas with those obtained with solutions of known concentrations of the same amino acids. To calculate the absolute concentration of *O*-phosphohomoserine, aliquots of the synthesized product were treated with alkaline phosphatase [6]. Peaks, with the same R_t of the homoserine (8.31 min) was obtained and quantification was carried out as as stated before.

By using the method described above, we have developed an assay for threonine synthetase in yeast crude extract. Threonine synthetase was assayed in reaction mixtures containing crude extract from strains X2180-1A (wild type) or F4 (Thr4$^-$). The same volume of extract, containing equivalent amounts of total proteins, was used for both strains. A reaction containing no extract was run as control. In the mixture containing extract from the wild-type strain, a consumption of *O*-phosphohomoserine, which was concomitant with the formation of threonine, was observed. After 60 min of incubation, no *O*-phosphohomoserine could be detected in the mixture. Threonine formed was equimolar with the *O*-phosphohomoserine consumed (Fig. 1A). In the reaction containing extract from the *thr4* strain, only a small fraction of the *O*-phosphomoserine disappeared. However, no threonine formation was detected (Fig. 1B). Spontaneous hydrolysis of *O*-phosphohomoserine cannot explain this phenomenon since this compound has been proved to be resistant to hydrolytic breakdown [18]. On other hand, no variation in the amino acid composition of the mixture containing no extract was observed during the 60 min reaction time (Fig. 1C). This disappearance must be, thus, due to the action of an enzyme/s activity/ies present/s in the crude extract. An unspecific phosphatase activity could unlikely be responsible of this consumption, since it was not concomitant with homoserine formation. The direct convertion of *O*-phosphohomoserine to α-ketobutyrate, which is not detectable to our assay, by the action of an *O*-phosphohomoserine deaminase could explain this result. In *B. subtilis* this activity has been reported to be associated with threonine synthetase. Moreover, in this bacterium, single mutational events affecting threonine synthetase also affect the *O*-phosphohomoserine deaminase activity [15,17]. However, this activity has not been described in yeast.

A comparison between Fig. 1A and B leads to the conclusion that the threonine synthetase activity depends on the presence in the cell of an active form of the *THR4* gene. This gene has been cloned by complementation of a Thr4$^-$ mutation [1]. The extensive homology found between the sequences of the *THR4*-gene product (as deduced from its nucleotide sequence) and the threonine synthetase from *E. coli* and *B. subtilis* [1,9], rule out the hypothesis that *THR4* could code for a regulatory protein. Our results demonstrate for the first time the univocal relationship between this gene and its product.

The enzymatic assay described in this paper is sufficiently specific and efficient as to be an useful tool in the quick characterization of the threonine synthetase activity of any yeast strain and, thus, for the determination of the role played by this enzyme in the overall regulation of the route. We also propose that this method could be adapted in order to assay the enzymes which either form or consume homoserine, *O*-phosphomoserine or threonine, in a simple, fast and unambiguous way.

Acknowledgements: This work was supported by the Spanish CICYT (Grant BT90-0010) and the Junta de Andalucía (PAI-3109). We thank Dr. E. Martín-Rendón for providing strain XMR9-9A(pMR1-4), Drs. C. and J.M. Gancedo for the help with *O*-phosphohomoserine synthesis; all of them and Dr. M.A. Delgado are also thanked for the many helpful discussions, and Drs. S. Marqués and I. Martínez, for critical reading of the manuscript.

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