Appl Biochem Biotechnol (2009) 158:398–407
DOI 10.1007/s12010-008-8341-x

Rapid Determination of L-Glutamine using Engineered *Escherichia coli* Overexpressing Glutamine Synthetase

Ni-Ning Hong · Guang Yang · Jian Li · Yao-Ping Zhang · Ji-Lun Li

Received: 28 May 2008 / Accepted: 1 August 2008 /
Published online: 19 August 2008

Abstract A genetically engineered *Escherichia coli* was developed as the source of enzyme for rapidly quantifying glutamine. *E. coli* BL21 (DE3) cells overexpressing a glutamine synthetase from *Bacillus subtilis* were prepared as tube aliquots and used in a small volume of nontoxic mixture. The current method was compared to high performance liquid chromatography analysis, Sigma kit (GLN-1) and Mecke method. The method is applicable to a wide range of glutamine concentrations (0.05–2.5 mM) and correlates well to the detection results obtained from high performance liquid chromatography (Pearson correlation is 0.978 at the 0.01 level). Moreover, the whole assay procedure takes less than 15 min and uses nontoxic reagents, so it can be applied to monitor glutamine production and utilization conveniently.

Keywords *Escherichia coli* · Glutamine synthetase · L-glutamine · Rapid determination

Introduction

L-Glutamine is an essential metabolic precursor and a source of oxidative energy in organisms [1]. It is important for optimal growth of eukaryotic cells in culture [2, 3] and can be a conditionally essential amino acid for the human body [4]. Although many sensitive methods have been developed for L-glutamine determination, such as capillary electro-

N.-N. Hong · G. Yang · J. Li · Y.-P. Zhang · J.-L. Li (✉)
State Key Laboratories for Agrobiotechnology, China Agricultural University, Beijing 100094, People's Republic of China
e-mail: jilunli@gmail.com

N.-N. Hong · G. Yang · J. Li · Y.-P. Zhang · J.-L. Li
Department of Microbiology and Immunology, College of Biological Sciences, China Agricultural University, Beijing 100094, People's Republic of China

Y.-P. Zhang
Department of Bacteriology, University of Wisconsin Madison, Madison, WI 53706, USA

Y.-P. Zhang
Center for the Study of Nitrogen Fixation, University of Wisconsin Madison, Madison, WI 53706, USA

chromatography with a modified photopolymerized sol–gel monolithic column [5], an LC-MS-MS method for the quantitation of underivatized amino acids [6] and an optic biosensor system [7], they need either special devices or complicated procedures. Thus, some routine methods are still widely used in research or industry. High performance liquid chromatography (HPLC) can be applied to many researches [8–10], but it may take time when large amounts of assays are carried out simultaneously. A glutamine detection kit using glutaminase and glutamate dehydrogenase is commercially available (Sigma kit GLN-1), but it is time-consuming and not suitable for samples containing large amounts of glutamate, ammonia, or amide compounds [11]. Mecke [12] developed a method specific to L-glutamine using glutamine synthetase (GS) based on the following "transferase" reaction:

$$\text{Glutamine} + NH_2OH \xrightarrow[Mn^{2+},\ ADP]{GS} \gamma\text{-L-glutamyl hydroxamate} + NH_3$$

The GS can be prepared by *Escherichia coli* fermentation under a nitrogen-limiting condition. Although partially purified GS can effectively catalyze the reaction for normal glutamine determination, insufficient enzyme and low activity resulting from covalent modification have limited the application of this method to large amounts of assays.

In this study, a glutamine synthetase gene of *B. subtilis* was overexpressed in *E. coli* BL21 (DE3). The resulting cells were adopted in a small volume of nontoxic mixture and used to monitor glutamine utilization of eukaryotic cells in culture and glutamine synthesis by microbial enzymatic transformation. Moreover, comparisons of the developed method to several previously reported methods were also done.

Materials and Methods

Reagents and Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Ligase, endonucleases, pMD18-T Simple vector and *E. coli* DH5α were from TaKaRa. Vector pET30a and host strain *E. coli* BL21 (DE3) were from Novagen.

Construction of Engineered *E. coli* for Overexpression of Glutamine Synthetase

glnA gene segment (GenBank accession no. Z99113 REGION: 70518…71852) was PCR-amplified with forward primer 5′-TTACCATATGGCAAAGTACAC-3′ (*Nde*I site underlined) and reverse primer 5′-ATTGTCGACGACATAGACTAAAAGTG-3′ (*Sal*I site underlined) using *B. subtilis* total DNA as template. Firstly, the amplified fragment was cloned into pMD18-T Simple vector to yield pMD18-TG. Subsequently, the plasmid was digested with *Nde*I and *Sal*I, and the released 1.4 kb fragment was then inserted into similarly digested pET30a vector to yield plasmid pETG. 5 ml culture of *E. coli* BL21 (DE3) pETG was inoculated into 495 ml LB medium containing 50 μg/ml kanamycin and incubated at 37°C with shaking of 200 rpm, IPTG was added to a final concentration of 0.4 mM at OD_{600} of 0.4–0.5 and incubation was continued for 4 h to induce GS expression. The induced culture was divided into 2-ml aliquots, centrifuged at 5,000×*g* for 5 min at 4°C. The supernatant was discarded and the tube-aliquot pellet was used immediately or stored at −70°C. Protein was checked by SDS-PAGE electrophoresis [13] and determined by the method of Bradford [14] using bovine serum albumin (BSA) as standard. Glutamine synthetase activity was determined as previously described [15] using crude extracts in the transferase reaction.

Proposed Assay for L-Glutamine Determination

Glutamine assay pre-mixture containing 25 mM imidazole-chloride (pH 7.5), 50 mM NH_2OH, 12.5 mM K_2HPO_4, 0.4 mM $MnC1_2$, and 2 mM ADP was pre-warmed at room temperature. One tube-aliquot cell pellet was suspended with 72 μl pre-mixture and 12 μl suspension was mixed with 38 μl of glutamine standard or sample solution to a total volume of 50 μl. After incubating at 42°C for 9 min, the reaction was stopped with 25 μl stop solution as described [15] (0.37 M $FeCl_3$, 0.67 M HCI, 0.2 M trichloroacetic acid). The mixture was centrifuged at 5,000×*g* for 1 min, and the absorbance at 540 nm of the supernatant was determined with a Beckman DU 640 spectrophotometer. Glutamine standard curve was graphed and the L-glutamine in test sample could be calculated by the standard equation.

Paper Chromatography Assay

An ascending technique was adopted on Whatman 3 MM paper with the developing solvent being *n*-butanol:acetic acid:H_2O at a ratio of 4:1:1 as previously described [16]. The culture mixture was sampled and centrifuged. An aliquot (5 μl) of the supernatant was subjected to paper chromatography together with authentic L-glutamine solution. Spraying with 0.7% ninhydrin and drying at 105°C for 5 min localized the spots of amino acids on the chromatogram.

HPLC Assay

Glutamine was measured as its *o*-phthaldialdehyde/2-mercaptoethanol derivative according to Donzanti and Yamamoto [17]. The samples were pretreated and measured by a Waters 510 system (USA) with a reversed-phase C18 column. Phosphate (20 mM; pH 6.8) with 36% methanol was used as mobile phase at a flow rate of 1.0 ml/min.

Glutamine/Glutamate Determination Kit Assay

A Sigma kit (GLN-1), modification of a procedure described by Lund [11], was used for the spectrophotometric measurement of L-glutamine in liquid preparations as described by the product information.

Monitoring Glutamine Utilization of Eukaryotic Cells in Culture

Baby hamster kidney (BHK) cells were routinely grown at 37°C with a 5% CO_2 atmosphere in Dulbecco's modified eagle's medium (DME) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) and 1.98 g/l L-glutamine. Culture samples were taken at time intervals and glutamine was measured after a 1:10 dilution. Cells were counted using a hemacytometer after being removed from plates by trypsinization.

Microbial Transformation of Monosodium Glutamate to L-Glutamine

The engineered *E. coli* cells per se were also developed to transform monosodium glutamate to L-glutamine using the biosynthetic activity. Transformation reaction was initiated by adding four tube-aliquot cell pellets (ca. equivalent to 3 mg of protein) into 2 ml transformation solution containing 50 mM Tris–HCl (pH 7.5), 15 mM monosodium

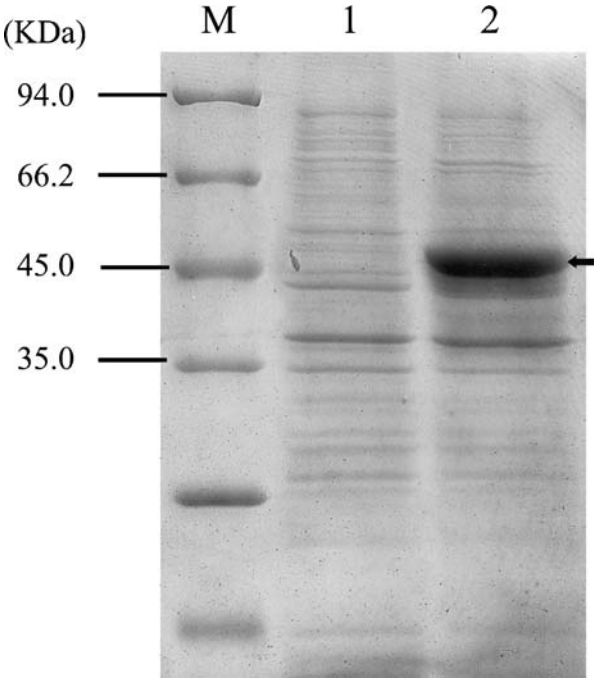

Fig. 1 Analysis of *E. coli* BL21 (DE3) pETG cells by SDS-PAGE. *M*, protein marker; *1*, without IPTG induction; *2*, 4 h of IPTG induction. The 50-kDa band of GS is indicated by an *arrow* on the Coomassie-blue-stained gel

glutamate, 20 mM NH_4Cl, 10 mM $MnCl_2$, 150 mM NaCl, and 7.5 mM ATP. After incubating at 42°C for 9 min, the reaction was stopped by immediately putting the reacting tube into ice. The mixture was centrifuged and the pellet was washed twice with 50 mM Tris–HCl (pH 7.5) buffer, resuspended in fresh reaction solution, and then incubated under the same conditions.

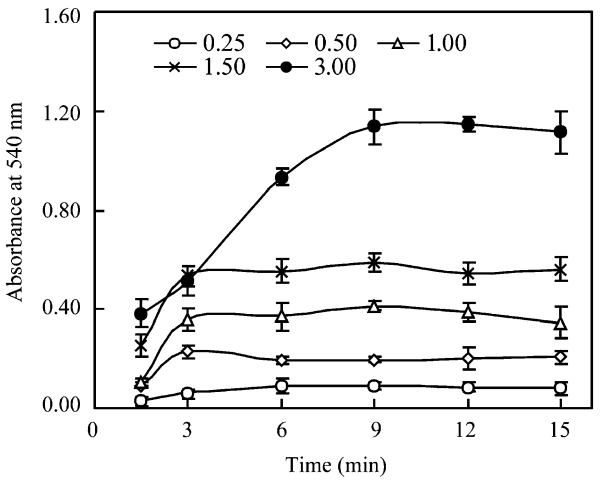

Fig. 2 A full time course assay of L-glutamine samples with concentration from 0.25 to 3.0 mM. The reaction was carried out at 42°C with one-aliquot cells being equivalent to 8.2 U of GS

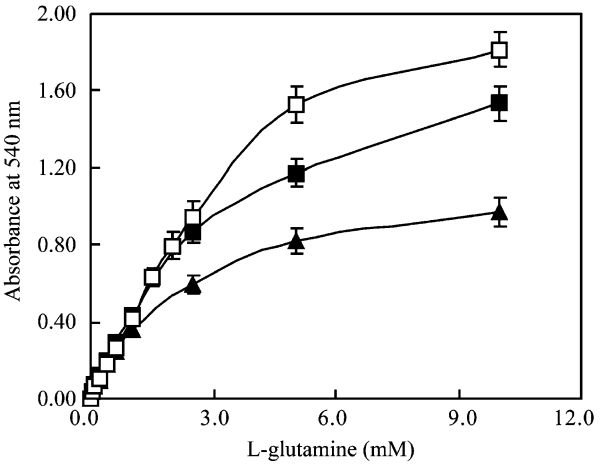

Fig. 3 Effects of cell amount (equivalent to GS activity, *upright filled triangle*, 2.4 U; *filled square*, 4.1 U; *empty square*, 7.4 U) on L-glutamine assay detection range. The assay was carried out at 42°C for 9 min

Results

Preparation of Engineered *E. coli* Cells

E. coli BL21 (DE3) pETG cell pellets were efficiently obtained and one tube-aliquot pellet contained total soluble protein of 0.73±0.03 mg. The GS transferase activity in the soluble parts was 67.8±6.1 U/mg protein. The permeability barrier of the cell pellets was overcome due to overwhelming GS expression (Fig. 1) and cytoplasmic membrane localized GS activity [18].

Optimization of Assay Conditions

The commonly used toxic sodium arsenate was substituted with phosphate and no significant deviation was found when the phosphate concentration rose from 25 to 50 mM.

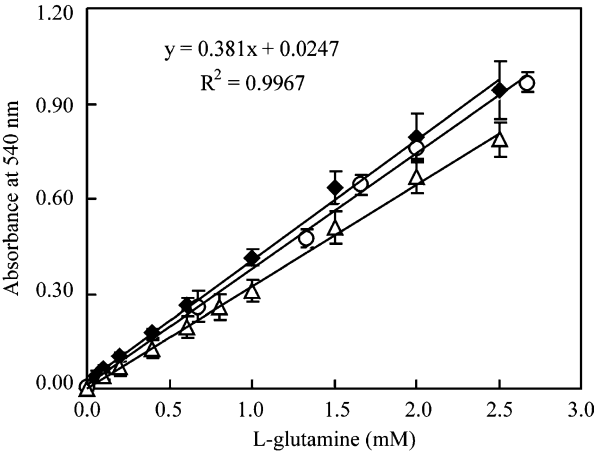

Fig. 4 L-glutamine in pure water (*filled square*), 1/10 diluted transformation (*empty square*) or DME D042 (*empty circle*) solution was assayed to graph the standard curve with relevant regent as blank. The equation of typical standard curve (pure water) is shown. Data are given as the means±SD, n=3–9

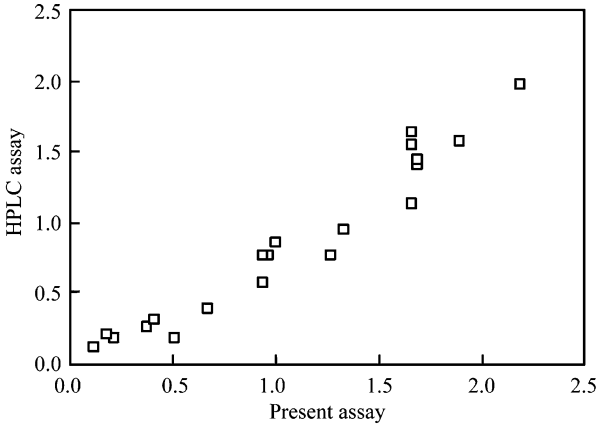

Fig. 5 Correlation of present assay and an HPLC assay. Random selected samples were assayed and the results were analyzed with a SPSS data editor for Pearson correlation

As reported by Mecke, no significant difference was observed within the temperature range from 20 to 40°C, but a higher temperature of 42°C would be optimal under the present conditions. Figure 2 showed a full time course assay of glutamine solutions and it indicated that 9 min was enough to complete reaction for samples with a concentration range from 0.25 to 3.0 mM. Figure 3 showed that when the tube-aliquot cell pellet was divided into smaller aliquots, more than 0.10 mg protein (equivalent to 6.8 U of GS) was sufficient for a broader detection range up to 2.5 mM glutamine. Moreover, when this amount of cells was used, 9 min of reaction time would reduce the discrepancy from hydrolyzation of γ-glutamyl hydroxamate Mecke mentioned.

Curve Calibration

The sensitivity and accuracy of the present assay were evaluated by measuring linearity and the standard curves were shown in Fig. 4. Furthermore, the reliability of the assay was verified by an HPLC assay, and statistical analysis indicated that the results obtained by the present assay were significantly related to those obtained by the HPLC assay (Pearson correlation is 0.978 at the 0.01 level; Fig. 5).

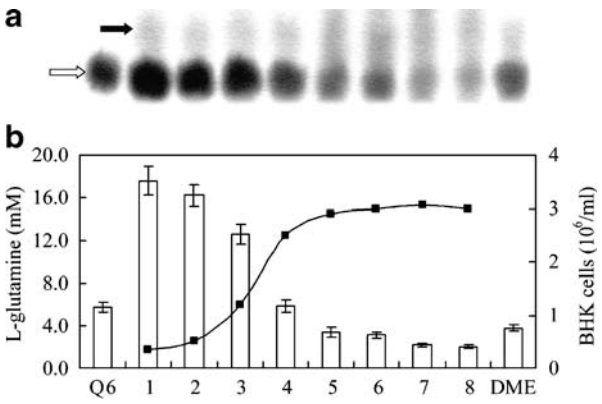

Fig. 6 Monitoring L-glutamine in a process of BHK cells in culture with paper chromatography (**a**) and present method (**b**). Q6, 6 mM glutamine standard in pure water; 1–8, sampling time (h) of 0, 8, 16, 24, 34, 42, 50, and 55, respectively; DME, D0422 plus 4 mM glutamine; *filled square*, BHK cell concentration. *Open arrow* and *solid arrow* indicated L-glutamine and other amino acids, respectively

Table 1 Comparison of several methods for determination of L-glutamine.

	HPLC	Sigma kit (GLN-1)	Mecke	This study
Sample volume (μl)	20	250	1,200	38
Total volume (ml)	0.12	1–2	2	0.05
Range (mM)	0.025–5.00	0.14–2.00	0.4–4.00	0.05–2.50
Reaction time (min)	5–15[a]	100	30	9
Reagents	*o*-Phthaldialdehyde, 2-mercaptoethanol, 20 mM phosphate (pH6.8) with 36% methanol	Glutaminase, glutamic dehydrogenase, NAD^+	*E. coli* GS, sodium arsenate, ADP	*E. coli* cells with *B. subtilis* GS, phosphate, ADP
Equipments	HPLC system, C18 column, etc.	Spectrophotometer	Spectrophotometer	Spectrophotometer

[a] Time including derivatization and elution

Interference Factors

Possible interference from ammonia or amino acids was investigated by assaying L-glutamine in transformation or DME+FBS solutions. Relevant transformation and DME+FBS standard solutions were prepared by reconstituting glutamine standard solution with 1:10 dilution of transformation solution or DME D0422 solution (Sigma product without adding L-glutamine) instead of pure water. Figure 4 showed that the slope of the standard curve for transformation (y=0.3222x+0.0023, R^2=0.9975) or DME+FBS (y=0.3668x+0.0091, R^2=0.9967) was less than the typical one, indicating that the sensitivity was slightly affected. However, with diluted transformation or D0422 solution as a control, the accuracy was not affected by the linear relationship between L-glutamine concentrations and absorbance values at 540 nm.

When applied to monitor glutamine utilization of BHK 21 cells in culture at an inoculation concentration of 3.5×10^5 cells/ml, the concentration of the L-glutamine decreased gradually with an increase in the concentration of BHK cells. After 36 h of growth, there is no obvious change in L-glutamine concentration during culture, for the cell concentration reached 3.0×10^6 cells/ml, and the activity of the cell proliferation reached the maximum (Fig. 6). Furthermore, the reliability of the assay was verified by paper chromatography (Fig. 6a) and the accuracy was also evaluated by assaying the added glutamine in DME+FBS and D0422, i.e., 1 and DME shown on the abscissa (Fig. 6b), and the recovery was determinate as 95.1% and 94.4%, respectively. So, other residual amino acids in the culture mixture showed no significant interference to the present assay.

Moreover, when the present assay was applied to monitor L-glutamine produce, L-glutamine was produced via engineered *E. coli* cells per se by the biosynthetic activity, which showed that repetitive uses for effective L-glutamine produce were possible, although the response gradually decreased due to diminished intracellular metabolic potential. The present assay was suitable to monitor produced L-glutamine and the reliability of the assay was verified by a Sigma kit (GLN-1) (Fig. 7). Furthermore, more than 7.5 mM residual glutamate and ammonia in the transformation mixture showed no significant interference.

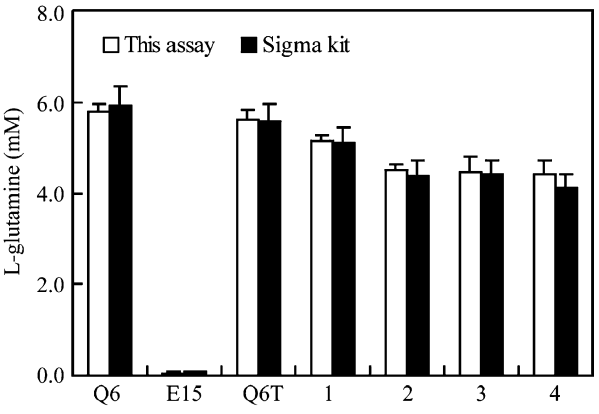

Fig. 7 Monitoring transformation of glutamate to L-glutamine with a Sigma kit and present method. Q6, 6 mM glutamine standard in pure water; E15, 15 mM monosodium glutamate in transformation solution; Q6T, 6 mM glutamine added in 1:10 diluted transformation solution; 1–4, four repetitive transformations using the same *E. coli* cells to test the stability of the cells for continuous application

Discussion

In this study, recombinant *E. coli* cells were used as the source of GS to modify an existing method [12]. It enables less time, fewer samples and more sensitivity, and can be easily applied to assay L-glutamine using a nontoxic reaction mixture (Table 1).

Although many more sensitive methods were available for glutamine determination over the years than listed in the table, these methods need either special devices or complicated procedure [5–7], which limited their wide applications. The present method used recombinant cells with high GS activity to substitute crude or pure enzyme by a relatively simple procedure. It used small-volume samples, routine device and regents. Most importantly, it could be potentially applied to large amounts of assays rapidly.

Adenylylation of GS would occur in *E. coli* by the stimulation of L-glutamine [19], but it was not observed in *B. subtilis* [20] and recombinant *E. coli* overexpressing *B. subtilis* GS. L-glutamine was an essential metabolic precursor and a source of oxidative energy for organism, but there was no significant error in using the *E. coli* cells for quantitative analysis of L-glutamine, which was mainly because the cells have stopped growth due to nutrient deficiency and high GS activity [21]. Therefore, recombinant *E. coli* provided reliable enzyme source for L-glutamine measurement in the present study. Considering the common use of enzymatic analysis in research or industry, the results presented here may be applied to other enzymes, especially those that are commercially not available, for more rapid and reliable assays.

Acknowledgments This work was financially supported by Chinese High Technology Research and Development Program (Grant No. 2006AA02Z233). The authors would like to thank Prof. Ying Li for helpful suggestions and Bin Wang for providing wild-type BHK cells. Thanks also to Fei-Fei Guan, Yong-Xing Wang for HPLC analysis, Xiang Li and Zhong-Huai He for BHK cell culture.

References

1. Tapiero, H., Mathe, G., Couvreur, P., & Tew, K. D. (2002). *Biomedicine and Pharmacotherapy*, *56*, 446–457. doi:10.1016/S0753-3322(02)00285-8.
2. Nishikawa, T., Tomiya, T., Ohtomo, N., Inoue, Y., Ikeda, H., Tejima, K., et al. (2007). *Biochemical and Biophysical Research Communications*, *363*, 978–982. doi:10.1016/j.bbrc.2007.09.082.
3. Tenuzzo, B., Dwikat, M., & Dini, L. (2008). *Tissue & Cell*, *40*, 177–184. doi:10.1016/j.tice.2007.11.005.
4. Lacey, J. M., & Wilmore, D. W. (1990). *Nutrition Reviews*, *48*, 297–309.
5. Kato, M., Jin, H., Sakai-Kato, K., Toyo'oka, T., Dulay, M. T., & Zare, R. N. (2003). *Journal of Chromatography. A*, *1004*, 209–215. doi:10.1016/S0021-9673(03)00451-5.
6. Qu, J., Chen, W., Luo, G., Wang, Y., Xiao, S., Ling, Z., et al. (2002). *Analyst (London)*, *127*, 66–69. doi:10.1039/b108422b.
7. Tolosa, L., Ge, X. D., & Rao, G. (2003). *Analytical Biochemistry*, *314*, 199–205. doi:10.1016/S0003-2697(02)00586-9.
8. Ligthart-Melis, G. C., van de Poll, M. C. G., Boelens, P. G., Dejong, C. H. C., Deutz, N. E. P., & van Leeuwen, P. A. M. (2008). *The American Journal of Clinical Nutrition*, *87*, 1282–1289.
9. Abely, M., Dallet, P., Boisset, M., & Desjeux, J. F. (2000). *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *278*, G789–G796.
10. Wang, L., Maher, T. J., & Wurtman, R. J. (2007). *The FASEB Journal*, *21*, 1227–1232. doi:10.1096/fj.06-7495com.
11. Lund, P. (1986). In H. U. Bergmeyer (Ed.), *Methods of enzymatic analysis, vol. 8* pp. 357–363. Weinheim: Verlagsgesellschaft.
12. Mecke, D. (1981). In H. U. Bergmeyer (Ed.), *Methods of enzymatic analysis, vol. 8* pp. 364–369. NY: Academic.

13. Laemmli, U. K. (1970). *Nature*, *227*, 680–685. doi:10.1038/227680a0.
14. Bradford, M. M. (1976). *Analytical Biochemistry*, *72*, 248–254. doi:10.1016/0003-2697(76)90527-3.
15. Fisher, S. H., & Sonenshein, A. L. (1984). *Journal of Bacteriology*, *157*, 612–621.
16. Satomura, T., Shimura, D., Asai, K., Sadaie, Y., Hirooka, K., & Fujita, Y. (2005). *Journal of Bacteriology*, *187*, 4813–4821. doi:10.1128/JB.187.14.4813-4821.2005.
17. Donzanti, B. A., & Yamamoto, B. K. (1988). *Life Sciences*, *43*, 913–922. doi:10.1016/0024-3205(88)90267-6.
18. Sallal, A. K. (2000). *Journal of Basic Microbiology*, *40*, 127–131. doi:10.1002/(SICI)1521-4028(200005)40:2<127::AID-JOBM127>3.0.CO;2-K.
19. Stadtman, E. R. (2001). *The Journal of Biological Chemistry*, *276*, 44357–44364. doi:10.1074/jbc.R100055200.
20. Deuel, T. F., Ginsburg, A., Yeh, J., Shelton, E., & Stadtman, E. R. (1970). *The Journal of Biological Chemistry*, *245*, 5195–5205.
21. Zhang, Y. P., Pohlman, E. L., Conrad, M. C., & Roberts, G. P. (2006). *Molecular Microbiology*, *61*, 497–510. doi:10.1111/j.1365-2958.2006.05251.x.