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Articles

Microanalysis of GABA: An application for evaluating GABA production in yeast strains and the effect of spice extracts on glutamate decarboxylase activity

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A high performance liquid chromatography (HPLC) system was optimized for quantitating sub-nmol levels of γ-amino butyric acid (GABA) by using cation exchange column chromatography and *O*-phthalaldehyde (OPA) fluorescence post-column derivatization. This system was used for routine analysis of glutamate decarboxylase (GAD) activity from yeast for the endogenously and exogenously expressed enzymes. Although the levels of GABA and GAD activity highly depend upon yeast strains, significant amounts of GABA as well as GAD activity are found in cultured yeast cell extracts. The system is applied to evaluate (1) the GABA producing ability of yeast cells and (2) the effectiveness of the spice extracts on GABA-producing ability of GAD.

Key words: GABA, GAD, spice, yeast

γ-Amino butyric acid (GABA), synthesized by glutamate decarboxylase (GAD) from L-glutamic acid,[1] is an inhibitory neurotransmitter in central nervous system (CNS).[1] It also involves important biological functions, such as inducing hypotensive and diuretic effects.[2, 3] Because of the GABA's role, there is a great interest in raising the amount of GABA contents in food products. Yeast can be an ideal candidate to be used for producing GABA-containing health food, since it has been used for major food production including bread and alcohol beverages; however, we have limited information on both GABA contents and GABA production in yeast. Our first aim of this study is to evaluate various yeast strains for their ability in producing GABA and GAD.

The physiological function of GAD is to produce GABA and its role in neuronal system is well reported; however, very little is known in non-neuronal system. The presence of GABA in small intestine suggests a possible role of GAD in food digestive system.[4] We have suspected that certain food components, such as spices, may interact directly with enzymes found in the food digestive system like GAD, and may alter the catalytic role of these enzymes. Our second aim in this study is to test this hypothesis. The effects of spices on GAD activity were investigated by our HPLC system.

Materials and methods

Materials - Strain of *Saccharomyces cerevisiae* (pWIG32-2) which carries rat brain GAD65[5] was described previously.[6] Spices were gifts from Somatech Center of House Foods Co., Japan. Other yeast strains, *Candida lipolytica* (No. 1066), *Debaryomyces hansenii* (No. 1104), *Saccharomyces sake* (No. 1460), *Saccharomyces rosei* (No. 1465), *Schizosaccharomyces pombe* (No. 1490) were from the collection of this laboratory.

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Abbreviations : AET, 2-aminoethyl isothiouronium bromide hydrobromide; CNS, central nervous system ; DTT, dithiothreitol ; EDTA ; ethylenediaminetetraacetic acid; GABA, γ-amino butyric acid; GAD, glutamate decarboxylase; HPLC, high performance liquid chromatography; OPA, *O*-phthalaldehyde; PCA, perchloric acid; PLP, pyridoxal 5'-phosphate

O-Phthalaldehyde (OPA) was purchased from Wako Chemical Co. Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim. Other reagents were the highest grade obtained locally.

Yeast cultivation - Yeast cultivation, except for pWIG32-2, was carried out in yeast extract/peptone/dextrose (YPD) liquid medium[7] at 28 °C in the rotary shaker (200 rpm). Pre-cultivation was done in 50 ml medium for overnight. Aliquots of pre-cultivation medium were transferred to 1 l medium to start the inoculation where initial optical density was adjusted to 0.05. Typical cultivation was carried out for 24 h.

For pWIG32-2 strains, after the pre-cultivation, aliquots were transferred to yeast extract/peptone/acetate (YPA) medium (pH 6.0) to induce GAD production which was under the control of isocitrate lyase gene promoter.[6] The growth was continued for 70 h.

Preparation of yeast extract - Cell pellets were collected by centrifugation at room temperature, washed once with distilled water, and stored at -20 °C until further use. Defrosted cell pellets were suspended in lysis buffer containing one tablet of protease inhibitor cocktail, 1 mM AET, 0.5 mM PLP, 0.1 mM DTT, 1 mM EDTA in 60 mM sodium phosphate buffer, pH 7.1. The suspension was subjected for cell disruption either by ultrasonication (Heat Systems) or beads beater (Biospec) and centrifuged at 4 °C. Supernatant was used for endogenous GABA determination, GAD assay and incubation with various spice extracts. When the supernatant was not used immediately, it was kept at -80 °C, under which conditions enzyme activity was maintained.

Protein determination - Protein concentrations in yeast extracts were estimated with Bradford method by using a protein assay kit from BioRad, where serum albumin was used as a reference protein.

Enzyme assay - Crude protein extract was passed through a Pharmacia Desalting column to remove endogenous GABA and other low molecular weight materials, which potentially disturbed GAD assay. Enzyme solution was then incubated with assay mixture, containing 20 mM sodium glutamate, 0.1 mM PLP in 100 mM sodium phosphate buffer, pH 7.1, at 30 °C for 30 or 60 min.[6] At the end of incubation period, 60% perchloric acid (PCA) was added to stop the reaction and the mixture was centrifuged. Supernatant was subjected for GABA analysis on HPLC. Amount of endogenous GABA found in the extract was expressed as μmol/mg protein. A unit of GAD activity was expressed as nmol of GABA produced/min/mg protein.

Spice extracts - Grounded powder of randomly selected five different spices among popularly used kinds in Japan, turmeric, ginger, peppermint, Japanese pepper, and sage, was weighed and mixed individually with either distilled water or 70% ethanol to make up 0.2 g/ml suspension. Extraction was carried out by gently rotating the container for over night at 4 °C. Centrifugation of the mixture gave an extract that was kept at room temperature under dark. Effect of spice extract on GAD activity was evaluated by incubating the water or ethanol extract of spice (3% v/v) with desalted enzyme extract, obtained from the culture of pWIG32-2 strain, and GAD assay mixture for 30 min at 30 °C. After the addition of 60% PCA and following centrifugation, the supernatant was applied for HPLC analysis. In place of spice extract, just water or ethanol was added for taking control GAD activity that was referred as 100%.

HPLC conditions - High performance liquid chromatography (HPLC) system was constructed with two Shimadzu model LC-6A pumps, autoinjector (model SIL-6A), column oven (at 30 °C), Shimadzu system controller (model SCL-6A), Gilson fluorometer (Model 121) and Shimadzu LC600 pump which delivered OPA solution (made up with 1.54 g

OPA, 2 ml 2-mercaptoethanol, and 25 g boric acid in 1 liter of water to adjust pH 10.4). Elution buffer of 0.1 N sodium citrate, pH 3.4, was delivered to cation exchange column (Shiseido Capcell pack SCX UG80, 4 x 250 mm) at 0.7 ml/min. OPA solution was delivered at 0.4 ml/min. Chromatography was recorded on Shimadzu Chromatopac C-R3A where peak area corresponding to authentic GABA was integrated and compared with standard GABA solution (1 nmol).

To protein extract, as well as GAD assay solution, was added 60% PCA to precipitate protein components. After brief centrifugation, supernatant was applied to HPLC for GABA analysis.

Results and Discussion

Analysis of GABA contents - Fig. 1 shows a typical separation profile of the mixture of glutamate and GABA. Under our experimental conditions, glutamate and GABA were eluted at 6.3 and 27.6 min, respectively. There are other conditions for separating these two compounds; however, they are impractical for our purpose: the time span that two peaks appear have to be longer than 10 min apart to avoid the interference of glutamate. Although an assay method employing radioactive substrate has been extensively used for GAD activity measurement,[8] we did not use the method as it does not allow to quantitate the endogenous GABA in the sample.

Yeast endogenous GABA expression and GAD activity - Five strains of yeast were randomly selected from our yeast stock. As shown in Fig. 2, *Schizosaccharomyces pombe* exhibited the highest amounts of GABA in its cell lysate whereas the highest GAD activity was found with *Candida lipolytica*. There seems to be no direct correlation between the GAD activity and endogenous GABA level in the cell lysate. It is of interest to point out that two similar strains, *Saccharomyces sake* and

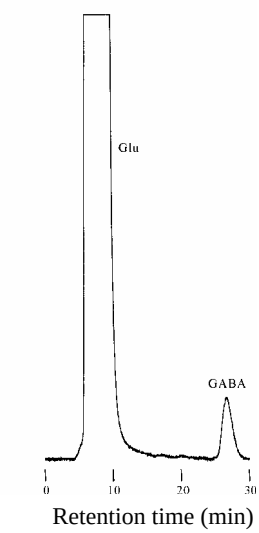

Fig. 1. Elution profile of glutamate and GABA. 10 μl of a mixture of glutamate (20 mM) and GABA (0.1 mM) was injected.

Saccharomyces rosei, showed GAD activities at the similar level but significantly different in GABA contents. Although endogenous GABA contents in yeast cell lysates have not been thoroughly studied yet, there was a report of GABA production into medium by lactic acid bacterium,[9] where 0.01-5.2 g of GABA was

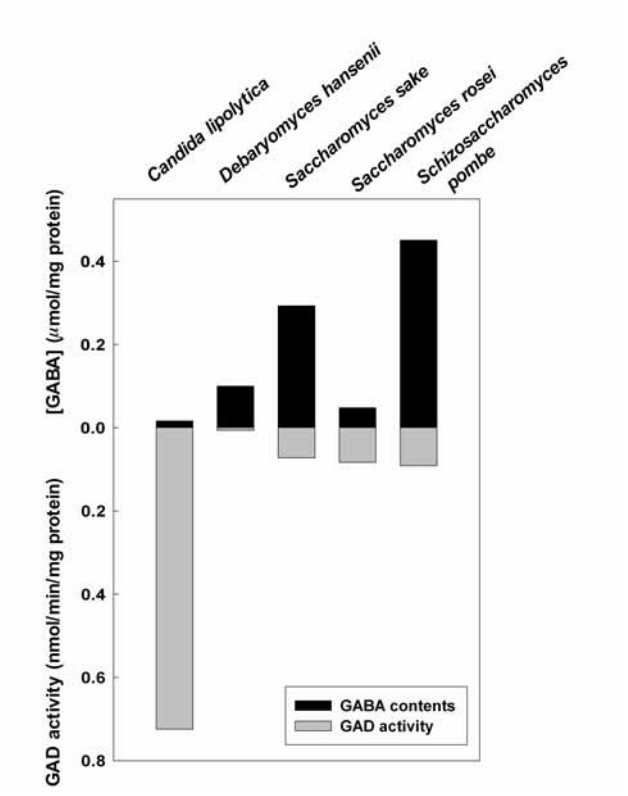

Fig. 2. Endogenous GABA level and GAD activity in the crude extract from yeast. Data shown here are average of three experiments.

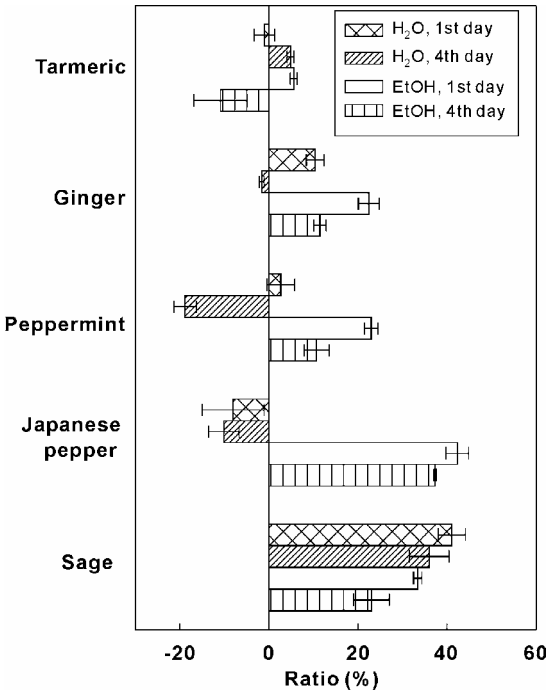

Fig. 3. Effects of spices on GAD activity. Ratio was calculated as per cent inhibition or activation in the presence of spice extract vs. control. Positive ratio indicates inhibition and negative ration indicates activation.

found in one liter of culture medium. There is little information available regarding to yeast originated GAD. Present study suggests that yeast produces GABA and its synthetic enzyme, GAD. It is not clear from our results the metabolic role of GABA in yeast system. Further characterization of GAD is warranted in order to classify the yeast enzyme, whether it is a mammalian type or bacterial type.[5)]

Effect of spice on GAD activity – The five randomly selected spices were evaluated for their potential in affecting GAD activity. A system expressing rat brain GAD65[5)] in *S. cerevisiae* was constructed previously.[6)] For this experiment, partially purified GAD was used. Fig. 3 summarizes our results in which the effects of both water and ethanol extracts on GAD activity were indicated. The inhibitory effects were shown to the right direction and activation effects to the left, where both effects were expressed as % of control. The spices affect GAD activity significantly and the patterns appear quite complex. Alcohol extracts tend to inhibit GAD activity and water extracts showed both inhibition and activation. It is of great interest to ask which components of water or alcohol extracts are responsible for affecting GAD activity. The difference between 1st day and 4th day extracts suggests that some of the effective components may be sensitive to oxygen in air.

In conclusion, our HPLC method is highly useful for searching high GABA-producing yeast strains and evaluating the effect of spices on GAD activity.

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