Amino Acid-Induced Inhibition and Stimulation of Saccharomyces carlsbergensis

I. Variations in the Response to Pantothenic Acid Induced by Casein Hydrolysat

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The response pattern of Saccharomyces carlsbergensis (ATCC 9080) to pantothenic acid in Atkin’s medium was changed dramatically by adding small amounts of casein hydrolysate (0.032 to 0.32 mg/ml) to the assay medium. Under static, mildly anaerobic conditions, growth at low pantothenic acid levels was reduced by 54 to 69%, whereas at saturating or near saturating pantothenate concentrations marked stimulation of growth (up to 41%) was observed. Under aerobic conditions, inhibition but not stimulation of growth occurred. It is recommended that Atkin’s medium for the assay of pantothenic acid with S. carlsbergensis (ATCC 9080) be modified to include 0.6% acid-hydrolyzed casein (Vitamin Free Casamino Acids, Difco) to prevent erroneous growth responses, which may result if significant amounts of amino acids are present in natural materials being assayed for this vitamin.

During recent studies on pantothenic acid transport, it was deemed necessary to assay cellular extracts of Lactobacillus plantarum for free pantothenate. The assay method of Atkin et al. (1) with Saccharomyces carlsbergensis (ATCC 9080) was employed since this organism responds only to free pantothenic acid (2-4, 6). It was considered very likely that the extracts to be assayed would contain significant quantities of various amino acids since the lactobacilli had been grown in a medium high in amino acid content. Previously, Hertz found that the maximum growth of S. cerevisiae was markedly stimulated by amino acids in Snell’s biotin assay medium (5, 7). Therefore, the effects of various concentrations of amino acids (added as Vitamin Free Casamino Acids, Difco) on the growth response of S. carlsbergensis in Atkin’s pantothenic acid assay medium were studied. The results in this paper demonstrate that small amounts of casein hydrolysate alter the growth response of the organism such that erroneous pantothenic acid values could easily be obtained.

MATERIALS AND METHODS

Organism. S. carlsbergensis (ATCC 9080) was maintained on yeast extract, Casitone, glucose, K2HPO4, agar slants (10, 10, 10, 5, 1.5 g, respectively, per liter of distilled water). The yeast was transferred weekly, incubated overnight at 32 C, and then refrigerated.

Medium. The medium of Atkin et al. (1) was the basic medium used in all of these studies.

Preparation of inoculum. Inoculum cells were grown overnight at 32 C in Atkin’s medium containing 10^-1 \( \mu g \) of pantothenic acid per tube (6 ml). The cells were harvested by centrifugation, washed three times in about 5 ml of sterile distilled water, and resuspended to 0.6 to 0.8 mg (dry weight) per ml.

Assay procedure. Most assays and experiments were carried out in Pyrex tubes (18 by 150 mm). Pantothenic acid levels of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100 \( \times 10^4 \mu g \) per tube were used in each assay except where indicated. Water was added to each tube to bring the volume to 3.0 ml. Then, to each tube was added 3.0 ml of nonsterile, double-strength Atkin’s medium supplemented with various amounts of Casamino Acids (Difco) such that Casamino Acid concentrations of 0, 0.005, 0.05, 0.1, 0.5, 0.6, 0.8, 1.0, and 2.0% resulted after dilution with the pantothenate solutions. The tubes were covered with stainless-steel caps (Belco Glass, Inc., Vineland, N.J.), shaken to mix the contents, heated for 10 min in flowing steam, cooled, inoculated with one drop per tube of the inoculum suspension described above, shaken to mix the inoculum, and incubated in a water bath at 32 C for 20 to 24 hr at which time near maximum growth was obtained. The tubes were then cooled in ice water to stop growth, and the turbidities were determined in a Klett-Summerson photoelectric colorimeter fitted with a red (no. 66) filter and adjusted to zero on a medium blank.

In some experiments, when the cells were grown under highly aerobic conditions, the assay vessels were 25-ml Erlenmeyer flasks. During these experiments,
the flasks containing 10 ml of medium were shaken at 180 rev/min in a controlled environment incubator-shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 32 C. Temperature variation was 0.5 C.

**Chemicals.** Calcium pantothenate was obtained from Sigma Chemical Co., St. Louis, Mo.; casein hydrolysate (Vitamin Free Casamino Acids) was from Difco Laboratories, Detroit, Mich.; and other chemicals required to make the different media were from Mallinckrodt Chemical Works, St. Louis, Mo., Sigma, or Difco.

Nitrogen was determined by nesslerization as described in *Manometric Techniques* (8).

**RESULTS**

**Effect of Casamino Acids on growth of S. carlsbergensis at various pantothenic acid levels.** From Fig. 1A it can be seen that the presence of Casamino Acids mediated two distinct effects on the response of *S. carlsbergensis* (ATCC 9080) to pantothenic acid. At low pantothenate concentrations, growth was dramatically depressed (54% with 0.005% and 60% with 0.05% at 6 × 10⁻³, and 63% with 0.05% at 10⁻³); however, at higher concentrations, those adequate to satisfy most or all of the organism's pantothenic acid requirement, marked stimulation of growth occurred (26 and 41% with 0.1% at 2 × 10⁻¹ and 5 × 10⁻¹, respectively).

The data represented in Fig. 1B show that increasing the content of Casamino Acids from 0.1 to 0.6% modified the growth depression somewhat but did not significantly affect growth stimulation. Further increases up to 1% exhibited little additional effect on growth (Fig. 1C).

Since pantothenic acid is somewhat heat-labile, the possibility existed that casein hydrolysate mediated some pantothenic acid destruction during the heating process and that this effect might be more evident at low concentrations. Accordingly, pantothenic acid concentrations of 10⁻³, 5 × 10⁻³, and 10⁻¹ μg were heated in flowing steam for 20 min in 5 ml of Atkin's medium and in Atkin's medium supplemented with 0.1 and 0.5% Casamino Acids. The media were cooled and assayed, without further heating, for pantothenic acid by using the *L. plantarum* (ATCC 8014) assay method (2). Results of this assay demonstrated that there was no destruction of pantothenic acid during heating in Atkin's medium with or without supplementation with Casamino Acids. Thus, the decreased growth of *S. carlsbergensis* in the presence of casein hydrolysate was due to some effect on the metabolism of the yeast and not on pantothenic acid itself.

**Influence of aeration on the Casamino Acids effect on the growth of S. carlsbergensis.** Since *S. carlsbergensis* grows better under highly aerobic conditions and since most pantothenic assay procedures with this organism call for aerobic conditions, the effect of aeration on the Casamino Acids-induced growth effects were investigated (Table 1).

Marked depression of growth still occurred at low concentrations of Casamino Acids (0.05%), but the stimulatory effect was eliminated under these conditions. Also, at 0.5% Casamino Acids, marked stimulation of growth occurred at pantothenate concentrations (6 × 10⁻³ and 10⁻¹) which had exhibited inhibition under both aerobic...
TABLE 1. Effect of aeration on Casamino Acids-induced inhibition and stimulation of growth

| Amt (μg) of pantothenate per tube (6 ml) | Conc of Casamino Acids in Atkin’s medium | Total amt (mg) of nitrogen per tube (6 ml) \( ^{a} \) at 0.0% | 0.05% | 0.5% |
|------------------------------------------|------------------------------------------|----------------|-------|-------|
|                                          |                                           | 0.0% CA \(^{a}\) | 0.05% CA | 0.5% CA |
| 3 \( \times \) 10\(^{-2} \)              | 15\(^{b}\)                                | 2              |       |       |
| 6 \( \times \) 10\(^{-2} \)              | 86                                        | 37             | 143   |       |
| 1 \( \times \) 10\(^{-1} \)              | 183                                       | 118            | 242   |       |
| 1 \( \times \) 10\(^{0} \)               | 605                                       | 605            | 595   |       |

\(^{a}\) Ten-ml amounts of Casamino Acids-supplemented Atkin’s medium containing the indicated pantothenic acid were shaken in 25-ml Erlenmeyer flasks at 180 rev/min for 2 hr at 32°C. Inoculum was 0.15 mg of cells (dry weight) per flask.

\(^{b}\) Klett readings.

TABLE 2. Comparison of turbidity and nitrogen content of Saccharomyces carlsbergensis grown with various amounts of pantothenic acid and Casamino Acids

| Pantothenate (μg) per tube (6 ml) | Klett readings at | Total amt (mg) of nitrogen per tube (6 ml) \( ^{a} \) at 0.0% | 0.05% | 0.5% |
|-----------------------------------|-------------------|----------------------------------------------------------|-------|------|
|                                   |                   | 0.0% CA \(^{a}\) | 0.05% CA | 0.5% CA |
| 2 \( \times \) 10\(^{-2} \)       | 23\(^{b}\)        | 4              | 10      | 37\(^{b}\) | 5 | 11 |
| 6 \( \times \) 10\(^{-2} \)       | 84               | 14             | 36      | 213    | 11 | 47 |
| 1 \( \times \) 10\(^{-1} \)       | 120             | 56             | 94      | 267    | 135 | 190 |
| 1 \( \times \) 10\(^{0} \)       | 201             | 218            | 253     | 538    | 549 | 706 |

\(^{a}\) Growth was in tubes (18 by 150 mm) under stationary conditions.

\(^{b}\) Cells were harvested, washed, and nesslerized to determine cellular nitrogen.

\(^{c}\) Casamino Acids in Atkin’s medium.

(Table 1; 0 and 0.05% Casamino Acids) and static conditions (Table 2, 0, 0.05, and 0.5% Casamino Acids, respectively).

Comparison of turbidity readings and nitrogen content. While reading turbidities during the course of these experiments, it was noticed that yeast cells growing at low pantothenic acid concentrations, especially in the presence of Casamino Acids, formed large particulate masses consisting of many cells aggregated or otherwise joined together. Therefore, it was reasoned that since the cells were clumped together and not evenly dispersed in the medium, these turbidity readings might be considerably lower than they should be, based on the amount of growth actually present as measured by cellular nitrogen or cell mass. Accordingly, cells were grown under a variety of nutritional conditions both aerobically and in stationary tubes. Growth was measured turbidimetrically and by dry weight and nitrogen content of washed cell suspension.

Comparison of the results obtained from one such experiment (Table 2) shows that the same pattern of inhibition and stimulation was evident from either nitrogen content or turbidity values. Although only nitrogen versus turbidity under static conditions is reported in Table 2, dry weight determinations followed the same pattern, and both dry weight and nitrogen content of aerobically grown cells reflected the turbidity observations reported in Table 1.

Thus, the phenomena observed by turbidimetric determinations were true reflections of the growth of this yeast under the experimental conditions applied.

DISCUSSION

The results presented in this paper suggest the need for modification in the medium used to assay pantothenic acid with S. carlsbergensis (ATCC 9080). As little as 0.3 mg of Vitamin Free Casamino Acids (Difco, 0.005%) per tube (6 ml) markedly affects the growth response of this yeast to pantothenate, and 3 mg (0.05%) per tube results in a dramatic bimodal inhibition and stimulation of growth (Fig. 1A). In fact, when one considers that Casamino Acids is 38% salt, the actual content of amino acid mixture per tube is reduced to 0.186 and 1.86 mg, respectively. Thus, it is quite probable that many natural materials contain quantities of amino acids sufficient to affect the pantothenic acid response of the organism and lead to erroneous results. Incorporation of 0.6% vitamin free acid-hydrolyzed casein eliminates the probability of error from amino acids that may be present in natural material, since further increases in concentration of the amino acid mixture up to 1% have no significant effect on the response of the assay organism at any assayable pantothenic acid level.

Preliminary results indicate that microgram quantities of lysine and methionine are the primary amino acids responsible for growth inhibition, that methionine also stimulates growth at higher pantothenate levels, and that aspartic and glutamic acids primarily cause the marked stimulation of growth at high or low pantothenate levels. The individual amino acid effects, their relationships to pantothenic acid metabolism, and their modes of inhibition and stimulation presently are being investigated as are the morphological changes induced by pantothenic acid deficiency.

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