A New Factor Stimulating Invertase Production by *Saccharomyces fragilis*

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(Accepted for publication 4 February 1971)

SUMMARY

The isolation of a new factor stimulating invertase production by glucose-repressed *Saccharomyces fragilis* is described. If the incubation time was sufficiently long (7 h.) the amount of invertase produced was proportional to the amount of factor added. With some strains of *S. fragilis* invertase factor could be replaced with arginine and partially with methionine. Its composition, behaviour toward alkali, isatin, ninhydrin and fluorodinitrobenzene, and its electrophoretic properties suggest that invertase factor is the diketopiperazine derivative of arginine and proline.

INTRODUCTION

When *Saccharomyces fragilis* is grown at 25 to 30° for periods up to 48 h. in a yeast extract medium containing 10% glucose the ability of the cells to hydrolyse sucrose is suppressed to a level less than 0.5 μ mole/mg. dry wt yeast/h. If such repressed organisms are washed with water and incubated at 0.1 mg. dry wt/ml. in 0.1 M- K_2HPO_4 + 0.05 M-citric acid buffer (pH 4.5 to 5.5) containing about 0.5 mM glucose, fructose or sucrose, invertase activity increases 10- to 20-fold with no significant change in viable count (Davies, 1953). The increase in invertase activity does not occur in presence of 1 mM- NaN_3 nor in the absence of metabolizable sugar. Under the conditions described invertase production is greatest with young cultures (18 h.) and progressively falls with increasing growth time, cultures grown for more than 40 h. being inactive. The invertase levels attained, though more than sufficient for maximum rate of sucrose fermentation, can be increased a further 10 times by suitable additions to the incubation mixture. The nature of the substance responsible for this stimulated invertase production is the subject of this paper.

METHODS

Organisms, growth media and cell suspensions. *Saccharomyces fragilis* Jorgensen (strain VL) was the strain used previously (Davies, 1953). Various single colony isolates were also used as described in the text. Some experiments were made with *S. fragilis* strain 351. Complex and synthetic growth media, culture techniques and preparation of cell suspensions were as described by Davies (1964). Cultures were grown at 30° for 22 h. unless stated otherwise. For experiments on stimulated invertase production growth media contained 10% (w/v) AnalaR or Kerfoot's bacteriological grade glucose.

Saccharomyces delbrueckii var. *mongolicus* (obtained from Centraal Bureau voor Schimmelcultures, Baarn, Netherlands) for use in manometric estimations of invertase activity was grown at 30° for 24 h. in the basal yeast extract medium of Davies, Faulkner, Wilkinson

& Peel (1951) containing 2% (w/v) glucose. The yeast was harvested, washed twice with water and suspended at about 100 mg. dry wt./ml. in a solution (pH 4.5) containing 0.054 M- K_2HPO_4 , 0.032 M-citric acid, 0.01 M- NaN_3 and 0.3 M-sucrose. The suspension was shaken aerobically at 30° for 2 h. to reduce endogenous CO_2 production to a negligible value.

Estimation of invertase activity. Two methods were used: measurement of increase in reducing sugar at 30° as described by Davies (1953) using Nelson's method (1944); manometric measurement at 30° of the rate of CO_2 evolution when *Saccharomyces fragilis* was incubated aerobically at pH 4.5 with 0.075 M-sucrose in the presence of 2.5 mM- NaN_3 and a large excess (100 mg. dry wt) of the non-sucrose-utilizing *S. delbruekii* var. *mongolicus*. Under these conditions glucose and fructose were fermented almost quantitatively to CO_2 and ethanol. The reaction was carried out in Warburg manometers using the techniques described by Umbreit, Burris & Stauffer (1949). The steady rate of CO_2 production (μ l./h.) was converted to invertase units by dividing by 89.6. Invertase activities are given as μ mole sucrose hydrolysed/mg. dry wt cells/h. at 30°.

Stimulated invertase production. Two procedures were used. (1) Washed *Saccharomyces fragilis* (30 mg. dry wt) grown in 10% (w/v) glucose medium was incubated at 30° in test tubes (15 × 1.5 cm.) in a system containing, in 21 ml., 5.0 ml. buffer pH 4.5 (0.091 M- K_2HPO_4 plus 0.055 M-citric acid), 5.0 ml. 0.5 M-sucrose and other additions as described in the text. After incubation (usually 2 h.) samples were taken, the cells harvested, washed twice with water and resuspended in water; invertase activity of the cells was determined by the reducing sugar method. (2) Unshaken Warburg manometers contained: in the main compartment in a volume of 3.0 ml.: 0.3 mg. dry wt *S. fragilis*, 1.9 ml. buffer pH 4.5 (0.182 M- K_2HPO_4 plus 0.108 M-citric acid), 0.3 mg. glucose, 0.6 mg. sucrose (modified in some experiments), 100 μ g. chloramphenicol and other additions as described in the text. Chloramphenicol was present to prevent bacterial growth during long incubations; it has no effect on invertase production. Bacterial contamination was never observed. After incubation at 30° for the required time, 1.0 ml. of *S. delbruekii* suspension was introduced into the side arm and after tipping into the main compartment the steady rate of CO_2 production was measured. The *S. delbruekii* suspension could not be put into the manometer at the beginning of the experiment because at pH 4.5 sufficient HN_3 to inhibit invertase formation distils from the side arm into the main compartment.

Unit of invertase factor (IF) activity. Stimulation of invertase production by a fixed amount of IF was variable from one *Saccharomyces fragilis* suspension to another. The unit of IF activity is therefore arbitrarily defined as the activity of 1 μ g. of IF preparation C_1 (see text). Specific activities are given as IF units/ μ g. substance.

Estimation of amino acids. Total free amino acids were estimated by the method of Cocking & Yemm (1954) modified by substituting 1.0 ml. sodium acetate buffer (0.2 M-sodium acetate adjusted to pH 4.63 with 0.2 M-acetic acid) for citrate-phosphate; proline by the method of Troll & Lindsley (1955); arginine by the method of Sakaguchi (1950).

Detection of arginine and proline on paper chromatograms and ionograms. Arginine was detected by dipping the papers in 0.02% 8-hydroxyquinoline in acetone, allowing the papers to dry at room temperature and then dipping in sodium hypobromite solution (0.7 ml. Br_2 in 100 ml. N-NaOH). Proline was detected by dipping papers in 0.2% isatin in acetone containing 5% (v/v) acetic acid and heating for a few minutes at 100° (Acher, Fromageot & Jutisz, 1950). Other amino acids were detected by dipping papers in 0.2% ninhydrin in acetone and heating at 100°.

Paper chromatograms and ionograms. These were run on Whatman no. 1 or no. 3 paper. Chromatograms were run with descending solvent flow and ionograms at 25 V/cm.

RESULTS

Enzyme synthesis by suspensions of washed yeast cells is not usually dependent on exogenous nitrogenous substances because of the large intracellular pool of amino acids. In presence of glucose both the rate and extent of invertase formation by washed suspensions of *Saccharomyces fragilis* was increased several-fold by addition of a commercial acid hydrolysate of vitamin-free casein at 1 mg./ml. supplemented with L-tryptophan, L-cysteine and L-methionine each at 100 μ g./ml. Similar results were obtained with fructose or sucrose as energy source, but without sugar the supplemented casein hydrolysate was ineffective (Fig. 1). It was found previously (Davies, 1953) that with glucose or fructose as energy source there was an optimum concentration for maximum invertase production. This was also true

Fig. 1. Effect of casein hydrolysate concentration on invertase production. Incubated for 3 h. Complete incubation system contained 1.4 mg. dry wt glucose-repressed *Saccharomyces fragilis*, K_2HPO_4 + citric acid buffer pH 4.5, 0.12 M-sucrose, casein acid hydrolysate 1.0 mg./ml., tryptophan, cysteine and methionine each at 0.1 mg./ml. ○—○, No sucrose; ●—●, complete system.

for the stimulated invertase production (Fig. 2), although the optimum glucose concentration (0.55 mM) was much lower than that previously observed (13 mM) when the test-tube system was used. This difference was mainly due to the much lower cell concentration (0.1 mg. dry wt/ml.) used in the manometric system compared to the test-tube system (1.4 mg. dry wt/ml.). The maximum attainable invertase activity per mg. dry weight yeast fell rapidly at cell concentrations greater than about 0.02 mg. dry wt/ml. (Fig. 3) and at higher concentrations of cells more sugar was required to achieve a maximum.

The optimum sugar concentration was largely an artefact because at the lower concentrations all the sugar had been metabolized before the end of the incubation. Nevertheless, higher concentrations of sugar were inhibitory. To maintain a supply of glucose at low concentration sucrose was added to a system containing glucose at 0.1 mg./ml. Fig. 3 shows

Fig. 2. Effect of sugar concentration on invertase production. Complete manometric incubation system contained 0.17 mg. dry wt glucose-repressed *Saccharomyces fragilis*/ml., K_2HPO_4 + citric acid buffer pH 4.5, 17 μ g. invertase factor C_1 /ml., sugar as indicated. Incubated 3 h. $\circ$ — $\circ$, Fructose; $\bullet$ — $\bullet$, glucose; $\triangle$ — $\triangle$, sucrose; $\square$ — $\square$, glucose without invertase factor. Fructose, glucose and sucrose $\pm$ invertase factor were different experiments.

Fig. 3. Effect of cell concentration at various sugar concentrations on invertase production. Complete manometric system contained K_2HPO_4 + citric acid buffer pH 4.5, invertase factor C_1 80 μ g./ml., glucose 0.1 mg./ml., glucose-repressed *Saccharomyces fragilis* and sucrose as indicated. $\bullet$ — $\bullet$, No sucrose; $\circ$ — $\circ$, 0.2 mg./ml.; $\triangle$ — $\triangle$, 0.6 mg./ml.; $\square$ — $\square$, 2.0 mg./ml.

that at cell concentrations between 0.05 and 0.15 mg. dry wt/ml. addition of 0.6 mg. sucrose/ml. considerably increased invertase production, whereas 2.0 mg./ml. was inhibitory. All routine assays for invertase factor activity contained 0.1 mg. glucose plus 0.2 mg. sucrose/ml. The poorer invertase production at higher cell concentrations was found at all concentrations of invertase factor (Fig. 4).

Fig. 4. Effect of cell concentration on invertase production at various concentrations of invertase factor. Conditions as for Fig. 3 but with 0.1 mg. glucose plus 0.2 mg. sucrose/ml.; invertase factor C₁ □—□ 90 µg./ml., ○—○ 45 µg./ml., ●—● 15 µg./ml., △—△ 5 µg./ml.

The increase on addition of IF was somewhat variable, from two- to tenfold, mainly because of differences in the amount of invertase produced in the absence of IF. This 'blank' synthesis was dependent on the growth phase at time of harvesting, being high for log phase cells and progressively decreasing towards stationary phase (Davies, 1953). The decrease occurred at about the time at which the glutamic acid and arginine levels in the amino acid pool were also falling (Taylor, 1949). In presence of IF the final level of invertase reached was relatively constant for most of the logarithmic phase but decreased rapidly toward the end (Fig. 5). Attempts to reduce the 'blank' invertase production by aeration in buffer, or buffer plus 10% (w/v) glucose, for several hours, or by growth in a synthetic medium with limiting nitrogen supply, were unsuccessful.

Fig. 5. Effect of growth time on stimulation of invertase production by invertase factor. Complete manometric incubation system contained: glucose-repressed *Saccharomyces fragilis* 0.1 mg. dry wt/ml., K₂HPO₄ + citric acid buffer pH 4.5, glucose 0.1 mg./ml., sucrose 0.2 mg./ml., invertase factor C₁ 33 µg./ml. ●—●, Complete system; □—□, no invertase factor; ○—○, growth.

The strain of *Saccharomyces fragilis* used in early experiments was very pleomorphic and by plating on yeast extract media with glucose, sucrose or lactose many different colonial types were isolated. Most were unstable with respect to colonial form and varied considerably in 'blank' invertase production and in their relative stimulation by arginine and IF. One hundred and seventy single colony isolates were tested and the stimulation of invertase production by IF varied from 1.6 to 18.7 and the relative response to equal amounts of arginine and IF from 0 to 2.19. The isolate used for most of the work reported here was stable with respect to colonial form and had average values for the above ratios of 7.6 and 0.17 respectively. Stock lyophilized cultures were stored at -15° .

Nature of invertase factor

Attempts to replace casein hydrolysate by mixtures of known substances failed. No additions, singly or in combination, of the 18 common amino acids, glutamine, asparagine, hydroxyproline (each at 100 $\mu\text{g.}$ L-isomer/ml.), ornithine, citrulline, creatine, creatinine, glycoamine, glycine anhydride, sarcosine anhydride (at concentrations from 33 to 3300 $\mu\text{g./ml.}$), guanine, adenine, xanthine, hypoxanthine, thymine, uracil (each at 20 $\mu\text{g./ml.}$), pantothenate, pyridoxin, nicotinic acid, thiamin, riboflavin, folic acid (each at 0.5 $\mu\text{g./ml.}$), inositol (2.5 $\mu\text{g./ml.}$), biotin (25 ng./ml.), B₁₂ coenzyme (2 $\mu\text{g./ml.}$), salts of Ca²⁺, Mg²⁺, Fe²⁺, Mn²⁺ nor the ash from casein hydrolysate had any significant effect on invertase production.

Following the observations of Kihara & Snell (1957) that certain amines stimulated growth of *Lactobacillus casei*, the following substances were tested at concentrations from 0.3 to 300 $\mu\text{g./ml.}$: agmatine, cadaverine, putrescine, spermine, spermidine, arcaine, guanidine, methylguanidine, amylamine, ethylenediamine, allylamine and the products from steam distillation at pH 12 of casein hydrolysate. All were without significant effect. Similar results were obtained with *Saccharomyces fragilis* grown in complex or synthetic media.

Table 1. *Effectiveness of different solvents for extraction of invertase factor from casein*

All extractions were for 2 h. Extracts evaporated to dryness *in vacuo* and residue dissolved in water for estimation of invertase factor activity by manometric method.

	Untreated casein	Extractant			
		Water (boiling)	60 % Ethanol 20°	80 % Acetone (boiling)	70 % Ethanol (boiling)
% Casein in extract		12.5	10.7	7.8	9.6
% IF activity extracted		56	67	61	57
Specific activity $\times 10^3$	2.9	63	71	70	17

Material stimulating invertase formation was present in several acid and pancreatic casein hydrolysates. After much of the purification work had been carried out it was discovered that fat-free, vitamin-free casein from British Drug Houses stimulated invertase formation and that 50 to 60 % of the active material, together with about 10 % of the weight of the casein, could be extracted in 2 h. with hot water, 60 % (v/v) aqueous ethanol at 20° or boiling 80 % (v/v) aqueous acetone (Table 1). Genatosan low-vitamin casein used for the initial isolation was devoid of IF activity until subjected to partial acid hydrolysis (12 N-HCl at 37° for 48 h.). Similar partial acid hydrolysis of crystalline lysozyme and protamine sulphate resulted in the appearance of IF activity, not shown by unhydrolysed material. Protamine hydrolysates (specific activity 0.8 after 16 h.) were much more active than lysozyme hydro-

lysates (specific activity 0.016 after 32 h.). Further hydrolysis (6 N-HCl at 105° for 16 h.) completely abolished IF activity in all preparations. Partial acid hydrolysates of crystalline insulin were inactive.

Isolation of invertase factor from partial acid hydrolysates of casein

Genatosan low-vitamin casein was hydrolysed by incubating with five times its weight of 12 N-HCl for 48 h. at 37°. Invertase factor activity was adsorbed on to acid-washed charcoal (Partridge & Brimley, 1952) and eluted therefrom with 5 % (w/v) phenol in 3.5 M-acetic acid. After removal of phenol and acetic acid by ether extraction and repeated evaporation *in vacuo* the preparation was passed through a column of Dowex 2 (OH⁻ form) resin. The active material was not adsorbed. A second passage through Dowex 2 gave preparation C₁ (Table 2) which was subsequently used as a standard reference preparation. When C₁ was subjected to paper chromatography in several solvents the active material appeared to be associated with ninhydrin-negative, Sakaguchi-positive regions of the chromatograms. When subjected to paper electrophoresis in 0.2 M-piperidine (pH about 12.6) C₁ separated into three Sakaguchi-positive bands, one moving slowly toward the anode (ninhydrin-positive), one slowly toward the cathode (ninhydrin-positive) and one rapidly toward the cathode (ninhydrin-negative). All the IF activity was associated with this last band. Complete acid hydrolysates (6 N-HCl at 105° for 16 h.) of active preparations showed arginine and proline to be the principal amino acids with smaller amounts of histidine and lysine.

Table 2. *The relative invertase factor activities of different fractions in the procedure for the isolation of invertase factor from casein hydrolysate*

All extracts freed from volatile extractants by repeated evaporation to dryness *in vacuo*. Residue dissolved in water for estimation of invertase factor activity by manometric method.

Fraction	Weight (g.)	Relative IF activity
A Whole hydrolysate	470	0.7
B Aqueous 5 % phenol + 20 % acetic acid eluate from charcoal adsorbate	119.2	2.3
C Unadsorbed on Dowex 2 (OH ⁻); pH 10.5–11.0 effluent	8.6	31.6
D Unadsorbed on Dowex 2 (OH ⁻); pH 6 effluent	23.8	4.5
E 2N-HCl eluate of Dowex 2 (OH ⁻)	91.5	0.2
C ₁ Fraction C retreated with Dowex 2 (OH ⁻)	2.71	100

Extraction of IF activity from casein by ethanol

Four hundred g. BDH fat-free, vitamin-free casein (9×10^{-3} IF units/ μ g.) was refluxed with 3 l. 60 % (v/v) ethanol for 2 h. and filtered. Trichloroacetic acid to a final concentration of 20 % (w/v) was added to the filtrate. The precipitate was filtered off, extracted with acetone and the extract evaporated *in vacuo*; the residue was extracted with ether until free from trichloroacetic acid. The filtrate was evaporated *in vacuo* to a syrup and extracted with ether until free from trichloroacetic acid. The two ether-extracted residues were extracted several times with boiling 90 % (v/v) aqueous ethanol, the ethanol extracts evaporated to dryness *in vacuo* and the residue extracted with 500 ml. boiling 90 % (v/v) aqueous acetone to give fraction 2526 (2.87 g.; 0.44 IF units/ μ g.).

Paper electrophoresis of fraction 2526 in 0.2 M-piperidine gave a separation into three bands similar to those found for fraction C₁, the fast-moving positively charged component containing 78 % of the IF activity. The other 22 % was associated with the slow-running positively charged material. The bulk of fraction 2526 was subjected to electrophoresis in

0.2 M-piperidine on a column of cotton cellulose (Flodin & Kupke, 1956) and eluted as described by Flodin & Porath (1954) with 0.2 M-piperidine. Eluates containing the fast-moving component were pooled and evaporated to dryness *in vacuo* (fraction F2526; 110 mg.; 3.3 IF units/ μ g.). The residue was dissolved in 10 ml. of a solution containing *n*-propanol + 0.2 M-ammonium acetate pH 5.4 (70 + 30, v/v), applied to a column of cellulose powder (Whatman no. 1) suspended in, and eluted with, the same solution. The active material, lyophilized and redissolved in 0.1 M-(NH₄)₂CO₃ was subjected to electrophoresis in the same solution and the major, rapidly-moving positively charged component eluted with water (fraction G2526; 11.5 mg.; 3.1 IF units/ μ g.). This material gave only one spot (isatin-negative, Sakaguchi-positive) on paper electrophoresis in 0.1 M-Na₂CO₃ or on paper chromatography in the following solvents: *n*-butanol + acetic acid + water (4 + 1 + 5, by vol.) (BAA), *n*-propanol + 0.2 M-ammonium acetate pH 5.4 (70 + 30, v/v), methylethylketone + propionic acid + water (75 + 25 + 30, by vol.) and methylethylketone + *m*-cresol + propionic acid + water (15 + 50 + 20 + 15, by vol.).

Acid hydrolysis (6 N-HCl at 105° for 16 h.) of a sample of G2526 followed by quantitative analysis, paper chromatography and paper electrophoresis showed arginine and proline as the only amino acids in a molar ratio of 0.83. A small amount of carbohydrate (1.5 % by the anthrone method) could have arisen from the unwashed papers used for chromatography and electrophoresis.

Isolation of invertase factor from casein by extraction with buffer. Three hundred and ninety g. BDH fat-free, vitamin-free casein was suspended in 3900 ml. potassium phosphate buffer pH 8.0 (0.064 M-K₂HPO₄, 0.003 M-KH₂PO₄) and incubated at 37° under a layer of toluene for 48 h. The mixture was filtered, the extract evaporated to dryness *in vacuo*, the residue dissolved in 100 ml. water and adjusted to pH 5.0 with 12 N-HCl. The precipitate was filtered off, the filtrate evaporated to dryness *in vacuo* and the residue dissolved in 40 ml. water and adjusted to pH 3.5 with 2N-HCl. After standing at 4° overnight the small precipitate was filtered off, 900 ml. acetone added to the filtrate and the precipitate removed by filtration. The filtrate was evaporated to dryness *in vacuo* (2.4 g.; 0.55 IF units/ μ g.) and dissolved in 20 ml. water. Two ml. portions were subjected to electrophoresis on a column of cotton cellulose as described above. Eluates containing the fast-moving positively charged component were pooled, evaporated *in vacuo*, the residue extracted with 20 ml. 96% (v/v) ethanol and the extract dried *in vacuo*. The preparation was again subjected to electrophoresis in one batch on cotton cellulose and two fractions were collected on the basis of the intensity of the Sakaguchi reaction: E_{ES}10/22 (73 mg.; 0.43 IF units/ μ g.) and E_{ES}23/43 (132 mg.; 0.52 IF units/ μ g.). The relative IF activities of preparations C₁, G2526, E_{ES}10/22 and E_{ES}23/43 are shown in Fig. 6.

Electrophoresis of E_{ES}10/22 and E_{ES}23/43 on paper in 0.1 M-Na₂CO₃ gave similar patterns for both fractions: a fast-moving, positively charged, ninhydrin-negative, Sakaguchi-positive band in a position similar to preparation G2526; and a ninhydrin-positive, Sakaguchi-positive band remaining close to the origin. Both components had IF activity, the fast band about twice that of the slow band. After paper chromatography in BAA the patterns were very different and only E_{ES}23/43 contained a component with chromatographic properties similar to those of G2526 (Table 3). Although IF activity was not confined to one component the major part of the activity was associated with ninhydrin-negative, Sakaguchi-positive material.

Fraction E_{ES}23/43 (61 mg.) was further fractionated by paper chromatography in BAA and the bands corresponding to components 3 and 5 plus 7 (Table 3) were eluted with water to give fractions F2 (*R_F* 0.05 to 0.21; 28 mg.; 0.15 IF units/ μ g.; ninhydrin-positive,

Sakaguchi-positive) and F4 (R_f 0.31 to 0.53; 19 mg.; 0.94 IF units/ μ g.; ninhydrin-negative, Sakaguchi-positive). On rechromatography in the same solvent F2 showed only one main component (R_f 0.15) and F4 one component (R_f 0.46). On electrophoresis in 0.1 M- Na_2CO_3 , F4 migrated rapidly as a single positively charged component whereas most of F2 migrated slowly as a single negatively charged component. There was also in F2 a small amount of positively charged component migrating like F4. Fraction F4 was dried *in vacuo* and the residue extracted with 80 % (v/v) aqueous acetone, the insoluble material (Sakaguchi-negative) being discarded. The extract was dried *in vacuo* to give fraction F4A (17 mg.; 1.04 IF units/ μ g.; ninhydrin-negative, Sakaguchi-positive). Fraction F2 was similarly dried and extracted with 80 % (v/v) aqueous ethanol (it was only slightly soluble in 80 % (v/v) acetone or absolute ethanol) and the extract dried *in vacuo* to give fraction F2E (17 mg.; 0.25 IF units/ μ g.; ninhydrin- and Sakaguchi-positive).

Fig. 6. Stimulation of invertase production by various preparations. Manometric system as for Fig. 5 with invertase factor as indicated. Incubated for 7 h. ○—○, G2526; ●—●, C₁; △—△, E_{BS}23/43; □—□, E_{ES} 10/22; ▲—▲, G2526 after 30 min. at 37° in 0.33 N-NaOH.

After acid hydrolysis (6 N-HCl at 105° for 16 h.) both F4A and F2E contained arginine and proline as the only amino acids detectable after chromatography or electrophoresis. Quantitative analysis gave values of 1.04 (F4A) and 1.00 (F2E) for the molar ratio proline: arginine.

Properties of fractions G2526, F4A and F2E

Fractions G2526 and F4A were strongly basic substances with similar chromatographic and electrophoretic properties, similar reactions with ninhydrin, isatin and Sakaguchi reagents, and which yielded equimolar amounts of arginine and proline on acid hydrolysis. Fraction F2E was much less basic, had different chromatographic and electrophoretic properties,

a much lower specific IF activity and gave a purple colour with ninhydrin and isatin, but still had equimolar amounts of arginine and proline.

The IF activity of G 2526 and F 4A was easily destroyed by alkali, 85 % destruction being caused in 30 min. by 0.33 N-NaOH at 37° (Fig. 6). Following alkali treatment the ninhydrin-negative, Sakaguchi-positive component disappeared and was replaced by ninhydrin- and isatin-positive (purple), Sakaguchi-positive material which remained close to the origin on electrophoresis in 0.1 M-Na₂CO₃ (Fig. 7) and ran with an R_F of 0.14 on paper chromatograms in BAA, properties very similar to those of fraction F2E. Treatment of F2E with alkali had little effect on its properties; its electrophoretic behaviour was unchanged and it ran slightly slower in BAA, but this could have been a salt effect. IF activity and chromatographic and electrophoretic properties of G 2526 and F 4A were unchanged after incubation at 25° with trypsin (pH 8.0; 43 h.) or carboxypeptidase (pH 7.8; 72 h.).

Fig. 7. Electrophoresis of invertase factor preparations in 0.1 M-Na₂CO₃. A. Sakaguchi reaction of preparation E_{ES} 23/43/3 before and after treatment with N-NaOH at 37° for 1 h.; B. Ultraviolet absorption of preparation G 2526 (3), FDNB-treated G 2526 (4), and G 2526 treated first with 1 N-NaOH and then with FDNB (5). C. Sakaguchi reaction of preparations B 3 (C 7), B 4 (C 6) and B 5 (C 8).

Effect of fluorodinitrobenzene (FDNB)

Reaction with FDNB was carried out as described by Sanger & Thompson (1953): 0.4 ml. 5 % (v/v) FDNB in ethanol was added to 1.0 mg. G 2526 dissolved in 0.2 ml. 1 % (v/v) triethylamine in water and the mixture shaken for 2.5 h. at 25°; 1.0 ml. water was added, residual FDNB extracted with ether and the aqueous layer evaporated to dryness *in vacuo* over solid KOH. The residue was dissolved in 0.2 ml. 50 % (v/v) aqueous ethanol and a sample chromatographed on paper in BAA. A second sample was subjected to electrophoresis

in 0.1 M- Na_2CO_3 . The distribution of IF activity and the bulk of the Sakaguchi-positive material appeared to be largely unaffected by FDNB treatment (Table 4), but a small amount of a DNP derivative was produced which migrated like DNP-arginine in 0.1 M- Na_2CO_3 (Fig. 7). Eight mg. of fraction $E_{23/43}$ was treated with FDNB and the product chromatographed in BAA. The unchanged active material was eluted with water (fraction $E_{23/43/3}$; 1.6 mg.; 1.8 IF units/ μg .) and was chromatographically and electrophoretically identical with fraction F4A. Fraction F4A did not react with FDNB.

Table 4. *Effect of fluorodinitrobenzene (FDNB) treatment on invertase factor (IF) activity*

IF preparation G2526 treated with FDNB as described in text and excess FDNB removed by ether extraction. Residue chromatographed on paper in *n*-butanol + acetic acid + water (4 + 1 + 5, by vol.) or subjected to electrophoresis on paper in 0.1 M- Na_2CO_3 . Papers sectioned, eluted with water and IF activity measured.

Section (cm.)	Sakaguchi reaction		Invertase factor units	
	- FDNB	+ FDNB	- FDNB	+ FDNB
Chromatogram				
-1.0 to +1.5	—	—	12	0
1.5 to 5.0	—	—	13	0
5.0 to 8.0	+	+	46	59
8.0 to 11.5	+	+	64	58
11.5 to 15.5	++++	++++	111	103
15.5 to 27.0	—	—	25	48
27.0 to 34.0	—	+	0	1
34.0 to 42.0	—	—	0	1
Ionogram				
-11.0 to -8.0	—	—	22.5	18.3
-8.0 to -5.0	++++	++++	100	123.4
-5.0 to -2.0	—	—	31.3	19.7
-2.0 to +0.5	($\pm$)	++	7.5	9.8
+0.5 to +3.5	($\pm$)	—	10.0	0
+3.5 to +6.5	—	—	0	0

When G2526, $E_{23/43/3}$ or F4A were heated for 30 to 60 min. at 37° in N-NaOH and the products treated with FDNB as described above, all were converted to DNP derivatives which ran slightly faster than DNP-arginine on chromatograms in BAA and were coincident with it on electrophoresis in 0.1 M- Na_2CO_3 (Fig. 7). The u.v. spectrum of this derivative had maximum absorption at 355 nm. and a value of 0.575 for E_{390}/E_{360} . According to Fraenkel-Conrat, Harris & Levy (1955) this ratio has a value of 1.05 for DNP-prolyl peptides and 0.5 to 0.55 for other DNP peptides. Acid hydrolysis (12 N-HCl at 105° for 4 h. or 6 N-HCl at 105° for 16 h.) of the DNP derivative liberated proline and DNP-arginine, but no free arginine or DNP-proline.

Analysis of complete acid hydrolysates by methods relatively specific for arginine and proline gave values close to 1.0 for the molar ratio proline: arginine. This value was confirmed for G2526 by treating a 6 N-HCl hydrolysate with FDNB, separating the DNP-arginine and DNP-proline by ether extraction followed by paper chromatography, eluting the DNP amino acids and measuring the absorption at 360 nm. (DNP-arginine) or 390 nm. (DNP-proline). The value so obtained for the molar ratio DNP-proline:DNP-arginine was 0.95.

The combined weights of arginine and proline accounted for only 52 % (F4A), 69 % (F2E) and 77 % (G2526) of the material analysed and the preparations contained less than 1 % carbohydrate (anthrone method) and no lipid or phosphorus. Preparations F4A and

F2E could have been in the form of acetate salts. F4A was converted into the free base form by passage through Dowex 2 (OH⁻). Analysis of the product gave a value of 0.96 for the ratios proline:arginine, but these two amino acids still made up only 63 % of the material analysed. The nature of the rest is not known.

Stimulation of invertase production by extracts of micro-organisms

Material capable of stimulating invertase production by *Saccharomyces fragilis* under the conditions already described has been extracted from *S. fragilis* (boiling 70 % (v/v) ethanol), *Salmonella typhimurium* (boiled Hughes's press extract: Hughes, 1951) and *Bacillus megaterium* (hot water extract) (Table 5). Glycine incorporation factor at stage 6 (Gale & Folkes, 1958) also had IF activity but this was largely lost after passage through ECTEOLA cellulose (Gale & Folkes, 1962). The activity of the material from *B. megaterium* was increased two to three times by heating in 6 N-HCl at 100° for 30 to 60 min. or in 12 N-HCl at 37° for 24 h. The preparations from *B. megaterium* and glycine incorporation factor were alkali labile, but less so than that from casein (Table 5). The other preparations were not tested in this way.

Table 5. *Invertase factor activity from natural sources*

Ethanol extracts evaporated to dryness *in vacuo* and residue dissolved in water for estimation of invertase factor activity by the manometric method.

Source	Method of extraction	Invertase factor activity	% Initial IF activity after 1 N-NaOH for	
			1 h. at 37°	10 min. at 100°
<i>Saccharomyces fragilis</i>	Hot 70 % ethanol	4.5*		
<i>Bacillus megaterium</i> (lyophilized)	Hot water	50.0*	72	38
<i>Salmonella typhimurium</i>	Hughes's press	10.0*		
Glycine incorporation factor stage 6		16.0†	55	34
Glycine incorporation factor (ECTEOLA cellulose eluate)		1.0†		

* IF units/mg. dry wt cells. † IF units/unit glycine incorporation factor.

Kinetic effects of invertase factor

The effect on invertase production of IF concentration and of incubation time is shown in Fig. 8. For incubation times up to 2 h. the initial rate of synthesis was independent of IF concentration above 25 µg./ml. but if incubation was prolonged until invertase production ceased the final level of invertase was proportional to the amount of IF added.

The optimum pH for stimulated invertase formation was 4.0 to 4.6, similar to the optimum for unstimulated ('blank') synthesis. Induced β -galactosidase production was not changed in presence of IF.

Invertase production was completely inhibited by 17 µg. canavanine/ml. and by 33 µg. cysteine/ml. Both inhibitions were partially reversed (50 %) by IF preparation C₁, and that by canavanine was also lessened by arginine. Growth of this strain of *Saccharomyces fragilis* was not inhibited by 500 µg. cycloheximide/ml., nor was invertase production.

Effect of IF on uptake of some amino acids

The uptake and distribution of radioactivity from [U-¹⁴C]arginine, -proline, -leucine, -glutamic acid, -methionine and -alanine were studied by trichloroacetic acid fractionation, chromatography and autoradiography of complete 6 N-HCl hydrolysates. The pattern was

unchanged in presence of IF over periods between 15 min. and 4 h. and there was no change in the rate or extent of uptake of radioactivity.

Stimulation of invertase production in other yeasts

Invertase production by two strains of *Saccharomyces cerevisiae* and one strain of *Saccharomyces carlsbergensis* was repressed by growth at 25° for 22 h. in media containing 10% (w/v) glucose. Incubation of the repressed cells at pH 4.5 with 0.1 mg. glucose/ml. resulted in production of invertase which was stimulated about twofold by addition of a

Fig. 8. Effect of invertase factor on rate and extent of invertase production. (a) Invertase production at various concentrations of invertase factor C_1 : $\circ$ — $\circ$, 33 $\mu\text{g./ml.}$; $\square$ — $\square$, 17 $\mu\text{g./ml.}$; $\blacksquare$ — $\blacksquare$, 8.3 $\mu\text{g./ml.}$; $\bullet$ — $\bullet$, 3.3 $\mu\text{g./ml.}$; $\triangle$ — $\triangle$, 1 $\mu\text{g./ml.}$ (b) Effect of incubation time; $\bullet$ — $\bullet$, 2 h.; $\blacksquare$ — $\blacksquare$, 3 h.; $\square$ — $\square$, 5 h.; $\circ$ — $\circ$, 7 h.

Fig. 9. Inhibition of invertase production by *Saccharomyces fragilis* strain 351 by cysteine and its reversal by arginine, methionine and invertase factor C_1 . Manometric system with cysteine (33 $\mu\text{g./ml.}$) plus competitor added at zero time. Incubated for 3 h. and invertase activity measured. $\circ$ — $\circ$, C_1 ; $\bullet$ — $\bullet$, arginine; $\triangle$ — $\triangle$, methionine; $\square$ — $\square$, cysteine only.

mixture of the 18 common amino acids. Casein hydrolysate containing IF activity was no more stimulatory than the amino acid mixture. Several methionine- or lysine-requiring and/or adenine-requiring mutants of *S. cerevisiae* strain YEAST FOAM were isolated from X-irradiated (6000 rad. for 30 min.) haploid clones. In several of these invertase production by glucose-repressed cells was increased in presence of the amino acid and/or adenine requirement, but in no case was there a special requirement for IF-like substances.

IF preparations also stimulated invertase production by glucose-repressed *Saccharomyces fragilis* strain 351, but with this yeast arginine alone was 45 to 60 % as effective as IF preparation C₁ and methionine alone about 25 to 45 %. Cysteine was strongly inhibitory and the inhibition was reversed by C₁ and, less effectively, by arginine and methionine (Fig. 9).

DISCUSSION

Invertase synthesis by glucose-repressed *Saccharomyces fragilis*, under suitable conditions (low cell concentration, 0.1 mg. glucose/ml. and pH 4 to 5) was stimulated by an unknown substance (invertase factor) which could be extracted from casein, *S. fragilis*, *Salmonella typhimurium* and *Bacillus megaterium*. There is no proof, however, that the stimulatory substances from all these sources are the same. With some strains of *S. fragilis* stimulation of invertase production was relatively specific for IF but others would respond almost equally well to arginine and less well to methionine. The composition of the invertase factor from casein is not known with certainty but the following properties of purified preparations strongly suggest that it is the cyclic anhydride (diketopiperazine) of arginine and proline:

1. Arginine and proline were the only amino acids present and they were present in a molar ratio of 1.0.
2. It was very basic, being still positively charged in 0.2 M-piperidine (at a pH of about 12.6).
3. It gave no reaction with ninhydrin or isatin but a typical Sakaguchi reaction for arginine.
4. It was fairly resistant to acid hydrolysis but very sensitive to alkali, which converted it to a much less positively charged substance which gave a purple reaction with ninhydrin and isatin and still retained the Sakaguchi reaction.
5. It gave no DNP derivative when treated with fluorodinitrobenzene (FDNB) and retained its invertase factor activity.
6. After mild alkali hydrolysis IF activity was largely lost and the product reacted with FDNB to give a single DNP derivative which, on acid hydrolysis, yielded DNP-arginine and free proline in a molar ratio of 1.0.
7. A mixture of arginine and proline, when subjected to chemical procedures that might have been expected to yield cyclic amino acid anhydrides, acquired invertase factor activity (R. Davies, unpublished).

Invertase factor activity also appeared to be associated with ninydrin-positive (purple), Sakaguchi-positive material that had only a small positive charge in 0.1 M-Na₂CO₃. The probable products of mild alkali hydrolysis of the diketopiperazine from arginine and proline are arginyl-proline and prolyl-arginine. The latter would give a blue colour with isatin; the observed colour was purple, indicating that the hydrolysis product was arginyl-proline.

In other experiments prolyl-arginine methyl ester was inactive as invertase factor. Arginyl-proline has not yet been tested.

The mechanism by which a cyclic dipeptide could stimulate synthesis of a specific protein is not known. Koaze (1958) reported that germination of rice seeds was stimulated by the cyclic dipeptide of valine and leucine, and he has also described the isolation of cyclic dipeptides of proline and valine and of proline and leucine from *Streptomyces* protease hydrolysates of casein, peptone and gelatin and the isolation of the cyclic dipeptide of glycine and proline from similar hydrolysates of glycyl-prolyl-leucine (Koaze, 1960*a*, *b*).

If the stimulated synthesis of invertase described in the present paper was specifically due to a cyclic dipeptide of proline and arginine then the observation that stimulatory material could be extracted from *Saccharomyces fragilis*, *Bacillus megaterium* and *Salmonella typhimurium* by simple procedures could indicate a possible biological function for cyclic amino acid anhydrides. One might be that transport of amino acids into or within the cell involves the formation of cyclic anhydrides, which are much more lipid-soluble than the free amino acids.

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