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A NEW GROWTH AGENT FOR PROPIONIC BACTERIA

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An agent largely accelerating the growth of propionic acid bacteria (*Propionibacterium shermanii* 1; *Propionibacterium freudenreichii* 3 and *Escherichia coli* 113-3, a mutant showing deficiency of Vitamin B_{12} and methionine) was separated from enzymatic hydrolysate of casein and from autolysate of brewer's yeast cell biomass.

Introduction

In the course of searching for factors enhancing growth of auxotropic *Propionibacterium shermanii* mutants it was observed that a factor indispensable for growth of these strains was present in a water extract of casein, in an enzymatic hydrolysate of casein, and in brewer's yeast extract.

It was assumed that the factor present in the above materials could have a relationship with the antinecrotic liver degeneration factor, separated by Schwarz [11, 12] from casein and yeast and given the name Factor 3, since it was the third in turn factor preventing liver necrosis (after Vit. F and cystine). His subsequent research [13, 14] showed that selenium (Se) is an integral part of Factor 3 responsible for the latter's biological activity. The degree to which this statement is of interest is that, as others have proved it [10, 3], selenium can play a significant role in metabolism of certain bacteria, including *Escherichia coli*.

The aim of the present investigations was to isolate Factor 3 from an enzymatic hydrolysate of casein and from an autolysate of brewer's yeast to be analysed subsequently as to its properties of a growth factor for the propionic acid bacteria and for the auxotrophic mutant *Escherichia coli* 113-3.

MATERIALS AND METHODS

Bacteria. The investigations were performed on bacteria from this institute's own collection of strains. *Propionibacterium shermanii* 1 and *Propionibacterium freudenreichii* 3 were kept in stab cultures on yeast-lactate medium [7]. *Escherichia coli* 113-3, the methionine dependant mutant, was kept on slant agar [1].

The enzymatic hydrolysate of casein and the casein medium had been prepared in a manner described elsewhere [7].

The autolysate of brewer's yeast cells was prepared from a yeast slurry, supplied by the brewery of Poznań, and washed three times. 250 g of freshly rinsed yeast cell biomass was suspended in distilled water at 1:1 ratio (w/v) and then placed in a thermostat at 44°C. After 16 hours the non-autolysed parts of cells were centrifuged, filtered through cellulose pulp on a Büchner's filter and the obtained liquid was poured into flasks. It was then neutralized at 110°C for 10 minutes. The resulting autolysate was stored at 4°C.

Isolation of Factor 3 from the enzymatic hydrolysate of casein and from the autolysate of brewer's yeast was performed after Schwarz [11], with only some minor modifications. 300 ml hydrolysate of casein, or of the yeast autolysate, at pH 8.7 (added with 6N NaOH) was put in a shaker at 25°C and added with 50 g active carbon (Carbopol H2). It was then put on a Büchner's filter. The filtrate was brought up to pH 8.7 and again added with active carbon (25 g), put in a shaker and filtrated again. The carbon on the filter, and then in the flask, was rinsed with double-distilled water. Following the removal of water the carbon was transferred to an Erlenmeyer flask, added with 250 ml ethanol (96%) at 55°C, and shaken for 10 min, the flask being put into a water bath (55°C).

Following the last filtration the carbon on the filter was washed with alcohol. The alcohol eluate was evaporated dry in nitrogen at the boiling point of ethanol. The substance remaining after the evaporation of alcohol was dissolved in 60 ml warm double-distilled water, added with total of 350 ml ethanol put in by portions, and put in a shaker again. Following the filtration on the Büchner's filter through homogenized paper pulp a clear greenish-yellow filtrate was obtained. It was condensed to 40 ml by heating in a water bath in ambient nitrogen, initially at 86°C and after evaporation of alcohol — in a boiling water bath. The resulting concentrate was named a "raw" preparation of Factor 3. Following neutralization (20 ml) at 110°C for 10 min it was stored in a cold storage room at 4°C. The remaining 20 ml "raw" Factor 3 preparation from the casein hydrolysate was additionally purified by putting it through a column filled with resin Dowex 50 Wx8 — Fluka *). The adsorbed substance was eluated with 2N NH_4OH down to 20 ml, and evaporated dry on a water

bath under lowered pressure. The dried substance was dissolved in 20 ml double distilled water. Following filtration on the Büchner's filter the clear liquid was neutralized at 110°C for 10 minutes. This product was then labelled "purified" Factor 3.

The content of selenium in Factor 3 preparations was determined by the fluorometric method [15].

The growth activity of the Factor 3 preparations as regards propionic bacteria was tested on a rich casein medium [7] as well as on a semi-synthetic medium after Delwiche [4]. The inoculum had been prepared from a three-day culture (10 ml) of the bacteria on a casein medium. The bacteria, separated from the liquid base by centrifuging, were washed and again suspended in 10 ml physiological solution of salt. The resulting suspension was put at portions of 0.1 or 0.05 ml on 6 or 10 ml medium in test tubes which contained Factor 3 preparation (0.1 or 0.05 ml portions). After 24, 48, 72 and 96 hours of incubation at 30°C the growth of bacteria was determined either visually or by means of the SPECOL (Zeiss-Jena) spectrocolorimeter at 520 nm wavelength.

The growth activity of the Factor 3 preparations in regard to *Escherichia coli* 113-3 was determined by comparison with the activity of Vit. B_{12}, for which the strain acts as a test organism. The determinations were performed with the plating method [6, 1] by putting 0.03 ml Factor 3 preparation or a solution of Vit. B_{12} on filter paper rings 10 mm in dia, which were first dried up and then put on agar medium inoculated with the *E. coli* 113-3 suspension. Measurements of diameters of the zones with activated growth of bacteria was performed after 18 hours of incubation at 37°C.

RESULTS AND DISCUSSION

Using the technique described by Schwarz [11] Factor 3 preparations separated from the enzymatic hydrolysate of casein and from autolysed cells of brewer's yeast. The preparation from casein hydrolysate was additionally purified in columns filled with Dowex 50-X8 resin.

The 'raw' preparation of Factor 3 (50 ml) obtained from 28 g casein contained 2.06 μg selenium while in 40 ml obtained from 39.5 g dry mass of brewer's yeast there was 22.93 μg selenium. Even the preliminary determinations showed a high growth promoting activity of the Factor 3 from casein in regard to *Propionibacterium shermanii* 1 and *Propionibacterium freudenreichii* 3 (Table 1). Acceleration of growth in the propionic acid bacteria has a great practical significance, particularly when

*) Column 6 cm × 1.2 cm; filtration rate: ca. 1.8 ml per min.

Table 1. Effects of the yeast and casein preparations on the growth rate of *P. shermanii P. freudenreichii*

Bacteria strains	Incubation time hrs	Control without additions	Growth factors					
			'raw' casein factor 3	'pure' casein factor 3	enzymatic hydrolysate of casein	hydrolysate of casein after isolation of factor 3	yeast extract Difco 100 µg/ml	vitamin B_{12} 200 µg/ml
P. shermanii 1	24	± *)	++	+	+	±	+	±
	48	+	+++	++	+	+	++	+
	72	++	++++	+++	++	++	++	++
	96	++	++++	++++	+++	++	+++	++
P. freudenreichii 3	24	±	+	+	±	±	+	±
	48	±	++	+	+	±	+	±
	72	+	+++	++	++	±	++	+
	96	+	++++	+++	++	+	+++	+

*) Growth of bacteria was determined visually on the basis of non-transparency of culture in test tubes:
± Growth very poor residual
+ Growth poor
++ Growth moderate
+++ Growth profuse
++++ Growth very profuse

using these organisms for industrial biosynthesis of Vit. B_{12}. Propionic bacteria are generally known to develop very slowly, particularly long is their lag phase. Addition of Factor 3 to a culture on a semi-synthetic medium Delwiche [4] stimulated the rate growth of the bacteria very clearly. Within the first 24 hours the rate which was only possible on the medium without Factor 3 not until the end of three days of incubation (Table 1).

The 'purified' Factor 3 had only slightly lowered effect on acceleration of the growth rate of the investigated bacteria. It deserves notice that there was a complete lack of stimulation of growth from the enzymatic hydrolysate of casein which was produced after isolation of Factor 3. As regards other additions only the brewer's yeast extract showed a growth activity approximating that of the 'purified' Factor 3 (Table 1) Factor 3 being added to the culture on the casein medium [7], rich and based on casein hydrolysates, did not show any effect on the rate of growth of the investigated propionic bacteria. The growth actitivty of Factor 3 separated from brewer's yeast, despite its significantly higher content of selenium, was the same as of Factor 3 separated from casein as regards *P. freudenreichii* 3 and only slightly higher as regards *P. shermanii* 1 (Table 2).

Table 2. Effects of raw preparations from yeast and casein on the growth rate of *P. shermanii freudenreichii* The activity was expressed in % of light transparency

Bacteria strains	Control samples without addition of factor 3		'Raw' casein factor 3 0,05 ml = 2.064 mg Se		Factor 3 from brewer's yeast 'raw' 0.05 ml 28.665 m g Se	
	after 72 hrs	after 96 hrs	after 72 hrs	after 96 hrs	after 72 hrs	after 96 hrs
P. shermanii 1	61.8	53.2	9.2	7.0	6.2	4.7
P. freudenreichii 3	52.7	52.7	14.7	8.5	13.0	8.1

Factor 3 also turned out to be a highly active growth factor for the mutant Escherichia coli 113-3 commonly used as a test organism for determination of Vit. B_{12} (Table 3). The mutant, as we know [5], shows one of the ways for synthesis of methionine (the one without the participation of Vit. B_{12}) to be blocked. Thus is reacts on the presence of Vit. B_{12} and methionine in the medium. Perhaps one of the components of the preparation of Factor 3 is selenium-methionine, which in turn may be substituting methionine in the mutant [2, 3]. We cannot exclude the presence of selenium in the enzymatic apparatus of the bacteria. particularly in *E. coli* [10]. As regards the activity of Factor 3

Table 3. Effects of raw preaparations from casein and brewer's yeast as well as vit B_{12} on the growth rate of *E.coli* 113-3

Growth substances	Quantity selenium mg/0.03 ml	Growth reaction; diameter of the zone of stimulated growth mm
Vit. B_{12} 1 μg/ml		30*
0.75 μg/ml		29
0.5 μg/ml		28
0.25 μg/ml		26.5
0.125 μg/ml		24
Factor 3 from enzymatic hydrolysate of casein 'raw'	1.24	41 (4d)**
Factor 3 from brewer's yeast 'raw'	17.19	35 (39)

*' All the above figures are averaged from 6 measurements from two different determinations
**' The figures given in brackets concern the zones of diffusive nature present next to clearly marked zones

as a growth factor for the propionic bacteria, there is another possibility of operation of other than selenium compounds present in it [8]. The new growth promoting factor for bacteria separated in the present study and called Factor 3 after Schwarz can be generally characterized as a substance adsorbing on carbon where it can be eluated from with hot ethanol. It is most likely that these are low-molecular organic compounds. It is a thermostable substance containing selenium.

LITERATURE

1. Bogucka J., Iwanowska J., Kąkol H.: Przem. Chem., 1953, **9**, 14.
2. Cohen G. N., Cowie D. B.: Compt. Rend. Acad. Sci., Paris 1957, **244**, 680.
3. Cowie D. B., Cohen G. N.: Biochem. Biophys. Acta 1957, **26**, 252.
4. Delwiche E. A.: J. Bacteriol., 1949, **58**, 395.
5. Friedmann C. H., Cagen L. M.: Ann. Rev. Microbiol., 1970, **24**, 197.
6. Harrison G. F., Lees K. A., Wood F.: Analyst 1951, **76**, 696.
7. Pędziwilk F.: Pr. Komis. Nauk. Rol. Leś. PTPN., 1962, **11**, 141.
8. Pędziwilk F.: Acta Microbiol. Pol., 1971, **3**, 79.
9. Pędziwilk F.: Roczniki Wyższej Szkoły Rolniczej w Poznaniu 1971a, 34.
10. Pinsent J.: Bioch. J., 1954, **57**, 10.
11. Schwarz K.: Proc. Soc. Exp. Biol. Med., 1952, **80**, 319.
12. Schwarz K.: Modern Brewery Age., December 1954 and January 1955.
13. Schwarz K., Foltz C. M.: J. Amer. Chem. Soc., 1957, **79**, 3292.
14. Schwarz K., Fredga A.: J. Biol. Chem., 1966, **244**, 2103.
15. Waliszewski K., Skupin J.: Bromat. Chem., 1974, **58**, 395.

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Streszczenie

Z enzymatycznego hydrolizatu kazeiny oraz z biomasy zautolizowanych komórek drożdży browarniczych wyodrębniono nowy czynnik wzrostowy dla bakterii. Posługując się nieco zmodyfikowaną techniką [11] dla izolacji tak zwanego faktora 3, uzyskano aktywny preparat przyspieszający w znacznym stopniu wzrost bakterii propionowych w hodowlach na podłożu syntetycznym. Bardzo silną reakcję wzrostową na nowy czynnik wykazywał również szczep *Escherichia coli* 113-3, mutant używany do oznaczania witaminy B_{12}, reagujący również na obecność metioniny w podłożu. Wyodrębniony czynnik wzrostowy jest substancją termostabilną adsorbującą się na węglu i zawierającą w śladowych ilościach selen.