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Determination of "Strepogenin" with *Lactobacillus bifidus*

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In 1944 Sprince and Woolley [1] suggested the name "strepogenin" for a growth factor required by certain hemolytic streptococci. This factor was originally [2] found to be present in liver but later work [3,4] has shown that also enzymic hydrolysates of certain proteins possess similar activity. In addition to hemolytic streptococci various other lactic acid bacteria have been shown to be dependent on this factor.

The Group A streptococci to which strepogenin is an essential growth factor cannot be used for its assay since the growth obtained is too weak. *Lactobacillus casei*, first employed as the assay organism by Sprince and Woolley [1], gives better results but the growth response has been found to be not specific to strepogenin. Under certain conditions asparagine and glutamine and many synthetic peptides also show "strepogenin"-activity [5-9]. To avoid these defects Wright *et al.*[10] have developed an assay method based on the essential nature of strepogenin for a strain of *Lactobacillus bulgaricus*. A great number of peptides tested as well as asparagine and glutamine had no growth-promoting properties for this organism under the conditions employed. Recently Kodicek and Mistry [11] have again studied the reliability of the *L. casei*-method. Using an improved basal medium of Clegg *et al.*[12] they obtained satisfactory results. Under the conditions of this method, however, glutamine, asparagine and aspartic acid showed a slight strepogenin-like activity.

Since strepogenin has not yet been isolated and identified the selection of a suitable standard for microbiological assays is an important question. Sprince and Woolley [1] in their original studies used an arbitrary liver standard and later workers have selected one or another of their preparations as arbitrary standards. Kodicek and Mistry [11] in their recent work proposed the use of a partial hydrolysate of insulin as the standard, since insulin was known to be a rich source of strepogenin. The insulin standard was prepared by a short-time acid hydrolysis at a low temperature and under carefully controlled conditions.

Studies in our laboratory [13,14] have shown that strepogenin is an essential growth factor for certain strains of *Lactobacillus bifidus*. Its effect on this organism is specific at least in that asparagine and glutamine have no activity. Since the growth response of *L. bifidus* to strepogenin is very pronounced and

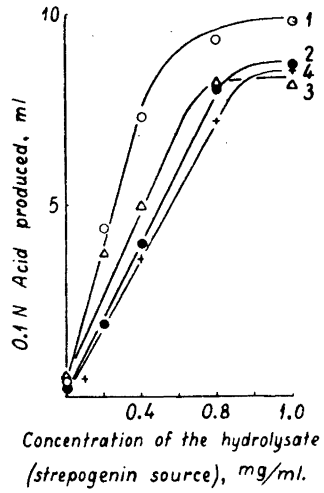

Fig. 1. Growth of strain THH 5 under different cultural conditions. 1. Tubes plugged with paraffin; inoculum 0.1 ml, 2. 0.1 % Sodium thioglycollate; no paraffin plug; inoculum 0.1 ml, 3. Incubated in CO_2 -atmosphere, no paraffin plug; inoculum 0.1 ml, 4. Incubated in ordinary atmosphere, no paraffin plug; inoculum 1 ml.

since this organism can be grown on a very simple medium it seemed to us that a method of determining strepogenin with *L. bifidus* should possess some advantages over the other methods in use. Several experiments were conducted to discover the optimal conditions and test the reliability of such a method.

EXPERIMENTAL

Test organism. Three strains of *L. bifidus* (labelled THH 3, THH 5, and KPH 16) isolated from the faeces of breast-fed infants were used in the experiments. Two of them, THH 3 and THH 5, were from the same child and might thus be considered identical. The isolation was carried out using the medium of Hassinen *et al.*[15], described below, supplemented with 15 g of agar and diluted from 500 to 1 000 ml. Only typical *bifidus* colonies grew on this medium.

A fresh stock culture was prepared every other week in the above medium further supplemented with 0.8 mg/ml of tryptic digest of casein (in terms of the original weight of casein). Incubation was carried out at 37° C for 3 days and the culture was then stored in a refrigerator.

All the strains of *L. bifidus* used by us have shown changes in morphology accompanied with a gradual loss in the ability to develop active growth. This degeneration has usually taken place within 4 to 6 months after isolation. Therefore, when *L. bifidus* is used for the assays fresh strains must be isolated at intervals of 4 to 6 months.

Anaerobiosis. To maintain the anaerobic conditions essential for the growth of *L. bifidus* the test tubes in the first experiments were plugged with paraffin. Although this procedure gave a good growth the method was found to be too laborious because the plugs had to be removed before titration; they also tended to increase the titration errors. Other methods for attaining anaerobic conditions were therefore also tested. They included an addition of 0.1 % sodium thioglycollate to the medium, incubation of the tubes in a CO_2-atmosphere, or using a fairly large inoculum to obtain a rapid fall in the redox potential of the medium. The results of these experiments are given in Fig. 1. It can be seen that strongest growth took place in the paraffin-plugged tubes. Incubation in CO_2-atmosphere and the addition of sodium thioglycollate gave somewhat lower figures. The growth was still weaker in the tubes where the only measure for attaining anaerobic

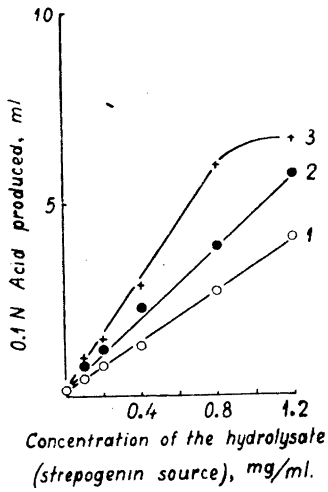

Fig. 2. Effect of the incubation time on the growth of strain THH 5. 1. Incubation time 48 hours, 2. 72 hours, 3. 96 hours. Inoculum in all tubes 0.4 ml.

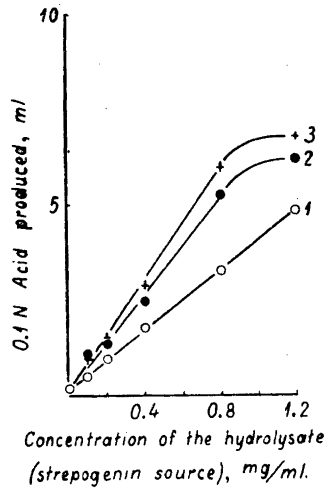

Fig. 3. Effect of the size of inoculum on the growth of strain THH 5. Incubation time 72 hours. 1. Inoculum 0.1 ml, 2. 0.4 ml, 3. 1 ml.

conditions had been the use of large inocula. Since, however, the growth curve even in this case had a good shape and covered a suitable range, this simplest procedure was selected for further experiments.

Fig. 2. shows the influence of the time of incubation and Fig. 3 that of the size of inoculum on the growth curves. It is noteworthy that the blanks are always very low, even in the cases where large inocula have been employed. On the basis of these results an incubation time of 72 hours and inocula of 1 ml were employed in the final procedure.

Procedure. The basal double-strength medium had the following composition (*cf.* Hassinen *et al.*[15]):

Lactose	35	g
Sodium acetate, anhydrous	25	»
Ammonium acetate	2	»
K_2HPO_4	2.5	»
Cysteine	0.1	»
Ca-pantothenate	0.4	mg
Biotin	0.004	»
Salt solution B ($MgSO_4 . 7 H_2O$ 10 g, $FeSO_4 . 7 H_2O$ 0.5 g, NaCl 0.5 g, $MnSO_4 . 4 H_2O$ 0.337 g in 250 ml of water)	10	ml
Distilled water	to 500	»
pH of the medium was 6.8		

Inocula for seeding the tubes were grown in the above medium supplemented with a tryptic digest of casein at a level corresponding to 0.8 mg of casein per ml of the medium. The cells of a 3-day, 10-ml culture were collected by centrifuging and suspended in 50 ml of sterile saline. 1 ml of this suspension per tube was used for inoculation.

The preparations to be investigated were dissolved in distilled water in a concentration of 4 mg/ml (tryptic hydrolysates were obtained by hydrolysis of the corresponding solutions with trypsin under toluene for 24 hours at 37°) and these solutions were then measured in test tubes at 5 different levels. 5 ml of the double-strength basal medium was added and the volume made up to 10 ml with distilled water.

After autoclaving for 20 min. at 105° C the tubes were inoculated and incubated at 37° C for 72 hours. The growth was measured titrimetrically using 0.1 *N* NaOH with bromothymol blue as the indicator.

In the selection of a suitable strepogenin standard we finally decided on peptonized milk (Bacto Difco). Available commercially and requiring no further hydrolysis, it possesses definite advantages compared with the enzymatic or acid hydrolysates of different proteins so far used as standards. The activity of peptonized milk was taken as 100 and relative activity values were calculated for the other materials from the standard curve obtained with peptonized milk.

RESULTS AND DISCUSSION

The strepogenin-activities of the following preparations were determined with the method described above: Peptonized milk, yeast extract, trypsin 1 : 250, pepsin 1 : 10 000, egg albumin, blood albumin, casein, gelatin, and tryptic hydrolysates of pepsin, egg albumin, blood albumin, casein, and gelatin. Suitable insulin preparations could, unfortunately, not be obtained.

The results of the determinations expressed as mean values of 8 or 9 separate experiments together with the standard mean deviations are given in Table 1. It can be seen that the mean deviation is between 5 and 10 % except in the cases where the activity has been very low. The accuracy of the method is thus of the order of magnitude generally occurring in microbiological assays. The results obtained with different strains of the test organism agree in general quite satisfactorily. In some cases, however, particularly where the tryptic hydrolysates are concerned, strain KPH 16 has given values distinctly higher than those obtained with the other strains.

A comparison of our results with those reported by other authors is very difficult because of the different techniques used. It seems that the *L. bifidus*-method gives higher activity values for the hydrolysates of albumins and lower values for the hydrolysates of casein than the *L. bulgaricus*-method (Wright *et al.*[11]). On the other hand, our values seem to be in good agreement with those of Kodicek and Mistry[12] obtained by the *L. casei* method. The differences between the results obtained by various methods is understandable

Table 1. The strepogenin-activities of different preparations determined with three strains of L. bifidus.

Preparation	Strain employed for determination		
	THH 3	THH 5	KPH 16
Peptonized milk (Bacto-Difco)	100	100	100
Yeast extract (Bacto-Difco)	625 ± 29.0	600 ± 33.0	470 ± 34.0
Trypsin, 1 : 250 (Difco)	76 ± 3.3	95 ± 6.3	69 ± 3.7
Pepsin, 1 : 10 000 (Difco)	31 ± 2.6	36 ± 2.7	52 ± 2.8
Pepsin, tryptic hydrolysate	36 ± 3.6	36 ± 1.8	88 ± 7.7
Egg albumin, soluble, (Difco)	14 ± 1.8	13 ± 1.9	14 ± 2.9
Egg albumin, tryptic hydrolysate	72 ± 4.6	64 ± 2.8	90 ± 7.0
Blood albumin, (B.D.H.)	17 ± 1.7	14 ± 2.1	14 ± 3.0
Blood albumin, tryptic hydrolysate	155 ± 11.5	150 ± 8.4	165 ± 14.3
Casein, vitamin-free, (Difco)	23 ± 3.1	19 ± 2.3	25 ± 3.9
Casein, tryptic hydrolysate	44 ± 5.2	49 ± 4.7	76 ± 7.3
Gelatin, (Gurr)	8 ± 0.6	8 ± 0.6	7 ± 0.5
Gelatin, tryptic hydrolysate	16 ± 2.8	19 ± 2.3	28 ± 4.3

considering the fact that there might be a variety of "strepogenins" with somewhat different combinations of amino acids. Little wonder, then, that various strepogenin-requiring species and strains of bacteria seem to respond somewhat differently to these different "strepogenins".

SUMMARY

An assay method for strepogenin based on the essential nature of this substance to *Lactobacillus bifidus* is described. The use of *L. bifidus* as the test organism has the following advantages: the blank values are always very small, the growth response is pronounced, and the basal medium is very simple containing in addition to lactose, sodium acetate, dipotassium phosphate and the salts B only ammonium acetate, cysteine, biotin and pantothenic acid. The anaerobic conditions required by *L. bifidus* are established by using a large inoculum. Figures for the activity of 13 different preparations are given, using peptonized milk as the strepogenin standard.

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