

Studies of Growth Factors for *Streptococcus faecalis* Occurring in Marine Algae

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It has been reported from this laboratory that water extracts of marine algae contain factors that stimulate the growth of *Streptococcus faecalis* ATCC 8 043¹. The nature of these factors was not further investigated and no attempt was made to release possible bound forms of the active substances. Different methods of extracting the growth factors and of separating and identifying the active substances bioautographically have now been tried.

EXPERIMENTAL

The algae investigated are *Sphacelaria fastigiata*, *Laminaria saccharina* and *Fucus vesiculosus* of the division *Phaeophycophyta* and *Furcellaria fastigiata*, *Rhodomela subfusca* and *Polysiphonia nigrescens* of the division *Rhodophycophyta*¹. The algae were dried at room temperature and ground.

The Difco folic acid assay medium with addition of 1.6 % Bacto-agar was used in an agar cup plate method with *Streptococcus faecalis* ATCC 8 043 as test organism². The same medium and the same organism were employed for the bioautographic studies. For the chromatographic separation of the factors different solvent systems were tried, *i. e.* (I) water-saturated sec. butanol containing 3 % acetic acid and 25 mg KCN/l, (II) *n*-butanol-water-acetic acid 125 : 125 : 30 v/v, (III) 70 % ethanol, and (IV) 5 % water solution of Na₂HPO₄ in *iso*-amyl alcohol (aqueous phase). Whatman No. 1 paper was used and the chromatograms were run at 21° C for 7—18 hours, depending on the solvent system used.

Six different methods of extracting the growth factors for *S. faecalis* were tried, *i. e.* water extraction for 24 hrs at 20° C and at 37° C, boiling the algal material under reflux at 100° C for 30 min., treatment with chicken pancreas homogenate, with hog kidney homogenate and with papayotin. The samples were heated at 100° C for 5 min. before extraction in order to destroy the algal enzymes.

The water extracts were prepared by adding 25 ml of water to 2 g of dried and ground algal material. The chicken pancreas and the hog kidney were removed and frozen immediately after the animals had been killed. The organs were homogenized in a Waring blender to give suspensions containing about 400 mg fresh tissue material per ml. The homogenates were then centrifuged to remove solid particles. To 2 g of alga 1 ml of these enzyme preparations was added and the volume was taken to 25 ml with 0.2 *M* phosphate or acetate buffers. For the chicken pancreas enzyme a pH of 7.5 (phosphate buffer) was

used, for the hog kidney the pH was 4.5 (acetate buffer). The buffer for the latter enzyme was 0.01 M in respect of cysteine hydrochloride. — In the experiment with papayotin 10 ml of a 0.4 % papayotin solution in 0.2 M phosphate buffer having a pH of 6.5 was employed per 2 g of alga, and the solution was taken to 25 ml with the same buffer.

An example of the effects obtained with these different methods of extraction is given in Table 1, which shows the results with one brown (*Laminaria saccharina*) and one red alga (*Furcellaria fastigiata*). It can be seen that much higher values are obtained when enzymatic digestion with chicken pancreas or hog kidney homogenate is employed. The activity of the enzyme homogenates was insignificant. The enzyme complex of the chicken pancreas homogenate appears to be the most effective in releasing growth factors for *S. faecalis*. Treatment with papayotin did not significantly increase the total folic acid activity of the algae.

Table 1. Effect of different methods of extraction on the total activity for *S. faecalis*.

Algae	Activity expressed as μg folic acid/g dry weight					
	20° C 24 h	37° C 24 h	100° C 30 min.	Chick.pancr. 37° C pH 7.5 24 h	Hog kidney 37° C pH 4.5 24 h	Papayotin 37° C pH 6.5 24 h
<i>Laminaria saccharina</i>	0.13	a)	0.20	0.40	0.40	0.16
<i>Furcellaria fastigiata</i>	0.10	0.16	0.08	0.87	0.33	0.11

a) Due to liberation of inhibitory substances, no estimation of the folic acid content could be made.

RESULTS

Table 2 summarizes the results of a quantitative estimation of the activity for *S. faecalis* in the algae investigated. The maximum figures obtained after enzymatic treatment of the samples with chicken pancreas homogenate as described above are given. Folvite, Lederle, was used as a standard. The activity found corresponds to from 0.25 to 1.10 μg folic acid per gram dry weight of alga.

Table 2. Quantitative estimation of *S. faecalis* activity in algae.

Algae	Activity expressed as μg folic acid per g dry weight
<i>Sphacelaria arctica</i>	0.90
<i>Laminaria saccharina</i>	0.40
<i>Fucus vesiculosus</i>	0.25
<i>Furcellaria fastigiata</i>	0.85
<i>Rhodomela subfusca</i>	0.90
<i>Polysiphonia nigrescens</i>	1.10

In order to study the type of factors released by various treatments of the algal material and to investigate the naturally occurring forms of the active substances, the growth factors in four of the six different extracts of *Laminaria*

saccharina and *Furcellaria fastigiata* were separated chromatographically. A standard solution containing pteric acid (7-9 113 Lederle; 2 $\mu\text{g/ml}$), folic acid (pteroylglutamic acid, Folvite, Lederle; 0.1 $\mu\text{g/ml}$), pteroyltriglutamic acid (Teropterin 7-9 111, Lederle; 0.2 $\mu\text{g/ml}$), N_{10} -formylfolic acid (Ro 1-5 681, Hoffman-La Roche; 0.1 $\mu\text{g/ml}$), N_{10} -formylptericoic acid (Rhizopterin 7-9 112, Lederle; 0.1 $\mu\text{g/ml}$), N_5 -formyl, 5,6,7,8-tetrahydroformylfolic acid (Leucovorin, Lederle; 0.2 $\mu\text{g/ml}$) thymine-desoxyriboside (thymidine; 100 $\mu\text{g/ml}$) and thymine (100 $\mu\text{g/ml}$) was employed for comparison. The position of the growth zones of the factors in the algal extracts and in the standard solution is shown in Figure 1. Solvent system I was employed.

All extracts, whether treated with enzymes or not, contained factors with the same R_F -values as N_{10} -formylfolic acid, folinic acid, N_{10} -formylptericoic acid and thymidine respectively. A growth factor for *S. faecalis* having a R_F of 0.36 was also found (Factor V) in all samples except those treated with hog kidney homogenate. Pteroyltriglutamic acid but only traces of folic acid were observed in the aqueous extracts. A factor with R_F 0.07 (Factor I) could be detected both in the aqueous extracts of *Furcellaria fastigiata* and in a sample of *Furcellaria* treated with hog kidney enzyme.

Both the brown alga *Laminaria saccharina* and the red alga *Furcellaria fastigiata* contain a factor having a R_F -value of 0.12 (Factor II) after boiling or treatment with chicken pancreas homogenate. Chicken pancreas also releases one factor with R_F 0.17 (Factor III) and another with R_F 0.24 (Factor IV), not found in the boiled samples or in samples that had been incubated with hog kidney homogenate. The hog kidney enzyme appears to release folic acid, whereas little or no folic acid seems to result from the treatment with chicken pancreas. An intensified growth due to pteroyltriglutamic acid was observed in the algal samples that had been incubated with the hog kidney enzyme. The enzyme complex of the hog kidney homogenate also gave rise to a spot having a R_F of about 0.40 (Factor VI).

Factors with the same R_F -values (solvent system I) as pteroyltriglutamic acid, N_{10} -formylfolic acid, folinic acid, N_{10} -formylptericoic acid and thymidine were found in the aqueous extracts of all the seaweeds investigated. Factor I was observed in the aqueous extracts of *Sphacelaria arctica*, *Fucus vesiculosus* and in *Furcellaria* as mentioned above, Factor II in all seaweeds except *Fucus vesiculosus*. Factor V appeared in the aqueous extracts of *Sphacelaria* and *Fucus* as well as in *Laminaria* and *Furcellaria* as just described.

An increased activity due to the formation of Factor III and Factor IV was observed in all seaweeds after treatment with chicken pancreas homogenate, which also released a factor with the same R_F -value as Factor V.

All algal extracts were also chromatographed in solvent systems II and III. Factors with the same R_F as pteroyltriglutamic acid, N_{10} -formylfolic acid, folinic acid, N_{10} -formylptericoic acid and thymidine were found. This agrees with the results obtained with solvent system I. Thymidine has the same R_F -value as thymine in solvent system I, but separates from thymine in system III. No thymine was found in the algal extracts.

When the algal samples were treated with chicken pancreas enzyme and chromatographed in solvent system III, factors with low R_F -values appeared which is in agreement with the observations made with solvent system I.

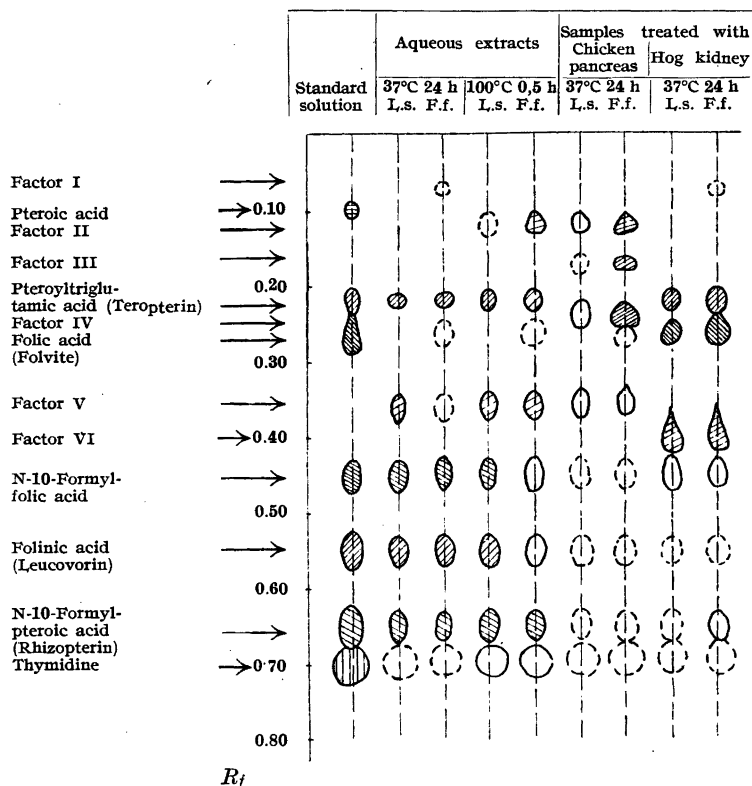


Fig. 1. Chromatographic separation of growth factors for *Streptococcus faecalis* (ATCC 8 043) in different extracts of *Laminaria saccharina* (L.s.) and *Furcellaria fastigiata* (F.f.). Solvent system I was employed. Thymine has the same R_f -value as thymidine in this solvent.

Cutting chromatograms lengthwise through the original spots and placing one half on plates seeded with *S. faecalis* and the other on plates seeded with *Leuconostoc citrovorum* revealed that the same areas on the chromatograms were active both for *S. faecalis* and *L. citrovorum*. This was observed both when solvent I and solvent III were employed. The existence of growth factors — other than folic acid and thymidine — active both towards *S. faecalis* and *L. citrovorum* is thus probable.

A preliminary study of the action of chicken pancreas homogenate on several pure substances has been carried out. For that purpose solutions of crystalline pteric acid, folic acid, pteroyltriglutamic acid, folic acid, N₁₀-formylpterotic acid and N₁₀-formylfolic acid were incubated with chicken pancreas homogenate at 37° C for 24 hours at pH 7.5, and the solutions thus obtained assayed bioautographically with *S. faecalis* and *L. citrovorum*. The treated compounds were converted into several new growth factors for these two microorganisms.

No significant release of folic acid from pteroyltriglutamic acid could be observed. Instead a factor with the same R_F as Factor IV had been formed. Formylation of pteronic acid and folic acid appeared to have taken place as well as a conversion of some factors for only *S. faecalis* to growth factors for both *S. faecalis* and *L. citrovorum*. Treatment of plant extracts with chicken pancreas homogenate can thus not be used when the naturally occurring forms of growth factors for *S. faecalis* and *L. citrovorum* are to be studied.

DISCUSSION

The results of the present investigation establish the occurrence in marine algae of a number of compounds that can stimulate the growth of *S. faecalis* in a medium used for the estimation of folic acid². Pteroyltriglutamic acid, N_{10} -formylpteronic acid, N_{10} -formylfolic acid, folinic acid, thymidine and small amounts of folic acid as well as three other factors — Factors I, II and V — appear to be present in the aqueous extracts. Factor II was observed first when boiling was used in the extraction process.

Treatment of the algae with chicken pancreas or hog kidney homogenates gives rise to three new factors, provisionally called Factors III, IV and VI. Factors III and IV appeared only after incubation with chicken pancreas. Factor VI only after treatment with hog kidney homogenate. This demonstrates a different action of the enzyme complexes of chicken pancreas and of hog kidney homogenates, a phenomenon that was also observed by Doctor and Couch, in their recent study of a conjugated form of the citrovorum factor³. To what extent this depends on the differences in the pH at which the two enzyme mixtures were employed has not been further investigated.

At least twelve different growth factors for *S. faecalis* in addition to thymine have thus been observed. Chromatography of the extracts in different solvent systems and comparison of the activity of the factors for *S. faecalis* and for *L. citrovorum*, indicates that the number of naturally occurring substances that can support the growth of *S. faecalis* may be still higher. This can be expected considering the fact that formylated (position N_5 or N_{10}) and/or reduced (di- or tetrahydro-) forms of pteronic acid, pteroylglutamic acid, and the pteroylpolyglutamic acids can act as growth factors for this microorganism. It seems likely that at least some of the unidentified growth factors in marine algae are such formylated and/or reduced forms of known factors.

Wieland *et al.* have studied the natural occurrence of folic acid and the citrovorum factor⁴. They mention only two substances other than folic acid and thymidine (found in mouse liver homogenate and in the charcoal eluate of a liver preparation) that can stimulate the growth of *S. faecalis*. They also demonstrate the multiple nature of the *L. citrovorum* activity, but do not describe any factor other than folinic acid and thymidine that are active for both microorganisms. The existence of such substances seems likely, considering the results of the present study.

The action of chicken pancreas or hog kidney homogenate (or other enzyme preparations) on different substrates is generally referred to as only a release of folic acid or folinic acid from bound forms⁵⁻¹⁰. Such enzyme mixtures

appear, however, to carry out several different reactions that result in a number of active compounds.

Dabrowska *et al.* have shown that digestion of pteroyltriglutamic acid with the chicken pancreas enzyme leads to pteroyldiglutamic acid¹¹. This compound is thus likely to be one of the unidentified growth factors (Factors I to VI) that are present in the algae investigated.

SUMMARY

Aqueous extracts or marine algae have been shown to contain at least nine factors stimulating the growth of *S. faecalis*, among them pteroyltriglutamic acid, N₁₀-formylptericoic acid, N₁₀-formylfolic acid, folinic acid, thymidine and small amounts of folic acid as well as three other as yet unidentified factors. Three further factors appeared when the algal samples were treated with chicken pancreas or hog kidney homogenates. It was also observed that some crystalline substances stimulating the growth of *S. faecalis* were converted to new growth factors for this microorganism and also to growth factors for *L. citrovorum* on incubation with chicken pancreas homogenate. As the chicken pancreas homogenate causes not only a release of growth factors but also a conversion of some factors to others, it cannot be employed in studying the naturally occurring factors for *S. faecalis* or *L. citrovorum*. Evidence has been gathered for the existence of factors, other than folinic acid and thymidine, stimulating the growth of both *S. faecalis* and *L. citrovorum*.

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