Concerning the Purification of a *p*-Nitrophenylphosphatase from an Oral Strain of *Streptococcus mutans*

MATTI L. E. KNUUTTILA

Institute of Dentistry, University of Turku, SF-20520 Turku 52, Finland

The purification and characterization of a *p*-nitrophenylphosphatase from the cells of *Streptococcus mutans* has recently been described.[1] The purification procedure, which resulted in approximately 3000-fold purification, consisted of ion exchange chromatography and isoelectric focusing as the main steps. A decisive step was also the solubilization of the enzyme.

The aim of this communication is to compare different solubilization methods in the release of the enzyme, and to describe the behaviour of the enzyme in polyacrylamide chromatography. This additional information was considered to be important in successful purification of the phosphatase, as well as in attempts to localize the enzyme.

The culturing of the cells of *Streptococcus mutans* is described elsewhere.[1] The cells harvested at the end of the exponential growth phase from two liters growth media were suspended in 20 ml of cold (+4°C) 0.9 % NaCl, and 4.0 ml of this mixture was transferred to each of five centrifuge tubes. After centrifugation (15 000 *g* for 10 min at +4°C) the resultant pellets were investigated by the five following methods (1–5). In the sixth method the cells were obtained as indicated below.

1. "Cold water wash". The alkaline phosphohydrolase of *E. coli* could be released when EDTA lysozyme spheroplasts were made.[2] The modification of this method, the "cold water wash" was used as described by Neu and Heppel.[3]

2. Deoxycholate method. The treatment of the cell mass with sodium deoxycholate was performed as reported by Hulett-Cowling.[4]

3. Treatment with alkali. Aspen and Wolin [5] have used the treatment with alkali for the solubilization of particulate dehydrogenase from *Vibrio succinogenes*. The following alterations were made to this procedure: The centrifugations were performed as mentioned above, the borate buffer used was 0.05 M (pH 8.8) and the pH of the cell suspension was adjusted to pH 11.0 by the addition of 0.5 M NaOH (other pH values were not studied).

4. The use of magnesium acetate. A solubilization procedure for alkaline phosphatase of *Bacillus licheniformis* using 1 M magnesium acetate as the initial solubilizing agent was performed as described by Hulett-Cowling and Campbell.[6]

5. Ultrasonic treatment. Ultrasonic treatment was carried out as presented by Knuuttila and Mäkinen.[1]

6. The use of chaotropic agents. Hatefi and Hanstein [7] have reported the solubilization of particulate proteins by chaotropic agents. The effect of such compounds in the solubilization of the present phosphatase was studied with cells obtained from one liter culture. The cells were harvested at the end of the exponential growth phase and washed with cold 0.9 % NaCl. The washed cells were suspended in 10 ml of cold (+4°C) 0.01 M tris-HCl buffer, pH 7.0. Solutions consisting of 0.2 ml of the above cell suspension and of 0.5 ml of the chaotropic agents were left to stand overnight at +20°C.

Table 1. Release of enzyme(s), acting on *p*-nitrophenyl phosphate, from the cells of the streptococcus, using different solubilization methods. The zeros indicate that no enzyme activity could be found in the preparation involved.

Solubilization method			Liberated *p*-nitrophenol [M/(min × mg cells, dry weight)] × 10^7
a) "Cold water wash"			0
b) Deoxycholate method			0
c) Treatment with alkali			0.1
d) The use of magnesium acetate			0.4
e) Ultrasonic treatment			0.5
f) The use of chaotropic agents			
Concentration of the agent (M)	Potassium iodide	Sodium chloride	Potassium fluoride
0.0072	0	0	0.2
0.031	0	0	0.3
0.072	0.1	0	0.4
0.31	0.1	0.1	0.3
0.72	0	0.2	0.3
1.44	0	0.2	0.1
2.88	0	0.3	0
3.10	0	0.2	0

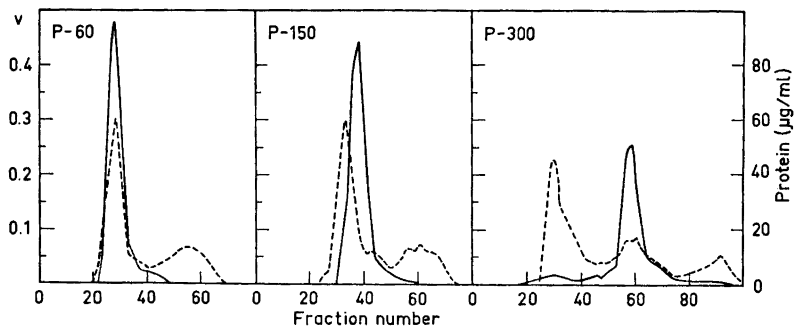

Fig. 1. Molecular exclusion chromatographv of the *p*-nitrophenylphosphatase of *Streptococcus mutans* on Bio-Gel P−60, P−150 and P−300 columns (40×2.0 cm). Elution: 0.01 M tris-HCl, pH 7.0. Flow rate: 0.2 ml/min. Hydrostatic pressure: 30 cm. Temperature: +2°C. Sample: The samples were obtained after ultrasonic treatment, followed by desalting on Sephadex G-25 gel. The stability of the enzyme pools were investigated (*cf.* Fig. 2). – –, protein, ——, enzyme activity (v, in $M \times 10^7 \times min^{-1}$).

After centrifugation the supernatant fluids were dialyzed against water for 15 h at +4°C and the dialysates were used in the determinations of the enzyme activity.

The determination of the enzyme activity and protein, as well as the sources of reagents, have been described earlier.[1] Results of the solubilization studies are presented in Table 1. The most advantageous method seemed to be the ultrasonic treatment, but also the use of magnesium acetate and potassium fluoride at a concentration of 0.072 M yielded almost as good results. No liberation of *p*-nitrophenyl phosphate hydrolyzing enzyme was obtained with the following chaotropic agents: urea and potassium thiocyanate (both at concentrations mentioned in Table 1) ammonium persulphate (excluding the two highest concentrations) and guanidine (excluding the four highest concentrations).

The results from ultrasonic treatment supported the earlier findings:[1] although no intact cells were found in samples after two sonications, a great part of the enzyme remained in a most likely bound form. This would support the idea of a membrane-associated enzyme. "Cold water wash" failed to release the present enzyme, which also supports the above conception. Sodium chloride and potassium fluoride were also suitable in the liberation of the enzyme from the cells, likewise supporting the above mentioned idea. Concerning the last mentioned finding, Hatefi and Hanstein [7] have pointed out that chaotropic ions (those ions which favour the transfer of apolar groups to water), for example chloride and fluoride ions, provide an effective means for the resolution of membranes and multicomponent enzymes and for increasing the water solubility of particulate proteins and nonelectrolytes.

In connection with the purification procedure, it was found that gel permeation chromatography on polyacrylamide-based Bio-Gel, particularly P-300, affected the enzyme protein, leading to a lowering of stability of the phosphatase. The chromatographic experiments were carried out at +4°C according to instructions provided by the supplier (Bio Rad Laboratories, Richmond, Calif. USA).

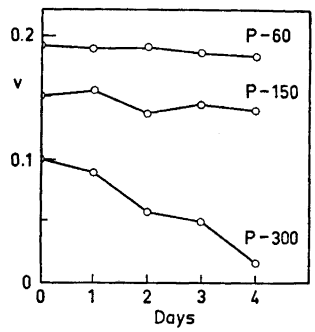

Fig. 2. The stability of the *p*-nitrophenyl phosphate-hydrolyzing enzyme pools, obtained from molecular exclusion chromatography on different Bio-Gel columns (Fig. 1). The enzyme preparations were stored after the chromatography up to 4 days at +2°C in 0.01 M tris-HCl buffer, pH 7.0, before the determination of the stability.

As seen in Fig. 1 the use of Bio-Gel P-300 resulted in a good separation of the enzyme. However, the enzyme activity was gradually lost in the pooled fractions during storage (Fig. 2). Therefore, in the chromatogram of the gel permeation on Bio-Gel P-150 the enzyme peak was only partly separated from the main protein peak which was eluted from the column in the void volume (Fig. 1), but the stability of the enzyme was better than after chromatography on P-300, A corresponding stability as found on P-150 was also found in the case of P-60. The loss of stability of the enzyme was evident only if the main protein peak and the enzyme peak did not coincide (Figs. 1 and 2). This was repeatedly observed in several fractionations. The loss of the activity of the enzyme after P-300 could not be reversed by any of several attempts (for example, the use of thiols, combination with void volume fractions, addition of magnesium or zinc ions to the elution buffer or to the pooled enzyme preparation).

This result may indicate that certain most likely irreversible molecular changes had occurred in the enzyme structure, when using the particular polyacrylamide gel which has a large degree of cross-linkage and a large pore size. Evidently, this also explains why preparative polyacrylamide electrophoresis was found to be unsuitable, in spite of several repeated experiments carried out according to the recommendations provided by Shandon Scientific Company Ltd. (London, England). Also, ion exchange chromatography on hydroxylapatite [8] was found to be unsuitable in the purification of the enzyme. The high phosphate concentrations (0.07 – 0.2 M) at pH 7.5 needed for desorption inhibited the present *p*-nitrophenylphosphatase. The removal of this phosphate would have required additional chromatographic and other steps. Moreover, the degree of the purification was considered to be low when hydroxylapatite was used.

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New Tobacco Constituents — The Structures of Five Isomeric Megastigmatrienones

ARNE J. AASEN, BJARNE KIMLAND, SVEN-OLOF ALMQVIST and CURT R. ENZELL

Research Department, Swedish Tobacco Co., S-10462 Stockholm 17, Sweden

In continuing our studies of the volatile constituents of Greek tobacco (*Nicotiana tabacum* L.) we have encountered five compounds which, as judged by their mass spectra and retention times, appeared to be dehydration products of 3-oxo-α-ionol (*1*), another constituent of Greek tobacco recently discussed by us.[1] The present paper provides evidence for assigning the structures 6ξ-megastigma-4,7E,9-trien-3-one (*2*), megastigma-4,6Z,8Z-trien-3-one (*3*), megastigma-4,6Z,8E-trien-3-one (*4*), megastigma-4,6E,8Z-trien-3-one (*5*), and megastigma-4,6E,8E-trien-3-one