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Studies on Aspartase

I. Quantitative Separation of Aspartase from Bacterial Cells, and Its Partial Purification

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In 1926 Quastel and Woolf[1] described a deamination of aspartic acid to fumaric acid in the presence of resting cells of *Escherichia coli*. The enzyme responsible for this reaction, which was reversible, was called aspartase [2] and was found to be present in several facultative anaerobes [3]. Virtanen and Tarnanen [4] obtained a cell-free aspartase preparation by extracting dry bacteria of *Pseudomonas fluorescens* under toluene at 37° C (pH 7). This was the first enzyme to be separated from cells, that caused the synthesis of an amino acid. Attempts to prepare cell-free aspartase solutions from dry bacteria and from an autolyzed bacteria medium of *Propionibacterium* and *Lactobacillus casei* met with no success. From *Pseudomonas fluorescens* only a part of the original aspartase activity found in the dry bacteria could be recovered in solution.

It is a known fact that many enzymes are firmly bound to the cell structure. Different methods have been used in the attempt to break up the association between proteins and insoluble particles in order to obtain larger yields of enzyme, but they have had only a limited application. Recently, however, noticeable improvement seems to have been reached by using *n*-butanol as a reagent in the dissociation of the lipidprotein complex [5].

In the present investigation *n*-butanol has been used in preparing cell-free extracts from dry bacteria of *Pseudomonas fluorescens* and *Propionibacterium peterssonii*, whereby larger enzyme yields were obtained. A partial purification of the enzyme was carried out with this extract.

EXPERIMENTAL

Enzyme material. As enzyme preparation finely ground dry preparations of *Ps. fluorescens* (strain of the Biochemical Laboratory, Univ. of Helsinki) and *Propionibacte-*

rium peterssonii (strain of this laboratory) were used. In culturing *Ps. fluorescens* a nutrient solution of the following composition was employed:

100 l tap water 800 g meat extract-peptone powder (Bacto Nutrient Broth Dehydrated, Difco Laboratories U.S.A.) 300 g K_2HPO_4 100 g $MgSO_4 \cdot 7\ H_2O$	pH 7

The culture of the bacterial mass was performed in an aluminium vat containing 100 litres of nutrient solution at 18° C. After 48 hours the bacteria were harvested by means of a milk separator, and washed with tap water. The bacterial mass was dried on porous plates and the dry mass was ground to a dust-like powder.

The nutrient solution for *Propionibacterium* was prepared as follows:

50 litres of skim milk was coagulated at 35° C with rennet, heated to 96° C to destroy the enzyme, filtered and sterilized at 120° C. After chilling to 45° C the whey was inoculated with *Lactobacillus helveticus* (strain of this laboratory). The lactic acid produced was neutralized with sterilized chalk. The whey culture was incubated at 42° C until all the lactose was fermented to lactic acid, which was checked with the Fehling test. The incubation had to be continued for 5—6 days. The bacteria were then separated from the solution. To 50 litres of fermented whey 1 000 g pressed yeast and 100 g peptone were added. The mixture was heated to 120° C for 30 min. in an autoclave, and centrifuged. The pH was adjusted to 6.5 and the solution was portioned out into 8-litre Erlenmeyer flasks and sterilized at 120° C for 30 min. The culture of the bacterial mass was performed in these Erlenmeyer flasks for the first 20 h at 37° C and later at 25° C. After 45 h the bacteria were separated from the solution with an airdriven Sharples Supercentrifuge. The washed mass was dried on porous plates and ground to a dust-like powder.

Activity determinations. The activity of the preparations was determined by the rate at which aspartic acid was deaminated. The most rapid and convenient method for this purpose is the determination of ammonia.

The experiments were performed in 10 ml measuring cylinders, in which the following test solution was incubated at 37° C for 24 h:

100	mg	dry bacterial mass
26.6	mg	aspartic acid (in 2 ml, pH 7.2)
2	ml	phosphate buffer *M*/15 (pH 7.2)
6	ml	distilled water
10	ml	total volume (0.3 ml toluene was used as antiseptic)

When enzyme extracts were used instead of dry bacteria they were used in amounts corresponding to 100 mg dry bacterial mass.

During the incubation, 2 ml samples were taken for the determination of the liberated ammonia, which was distilled with an apparatus modified from that of Pucher *et al.*[6] The ammonia was trapped in the receiver by 0.01 *N* H_2SO_4, and the excess of acid was titrated iodometrically. The solution to be analyzed was made alkaline in the distillation flask with a sodium carbonate buffer (5 g Na_2CO_3 + 5 g NaCl in 100 ml distilled water) [7].

The rate of reaction is linear at first and then falls off as the substrate concentration decreases and the products are formed. The initial velocities when the reaction rate is

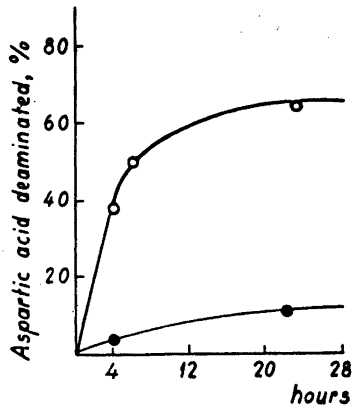

Fig. 1. Deamination of aspartic acid at 37° C by preparations of P s. fluorescens.

○ *dry bacteria*
● *corresponding amount of water extract of dry bacteria*

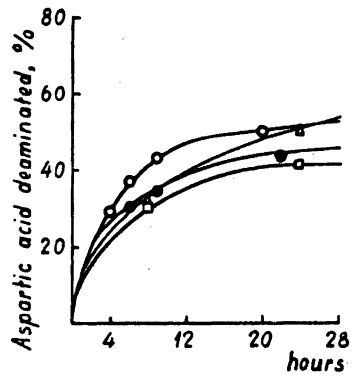

Fig. 2. Deamination of aspartic acid at 37° C by dry bacteria of P s. fluorescens. Bacteria suspension incubated at 37° C.

for 0 days ○
» *3* » ●
» *4* » □
» *5* » △

linear may be expressed as change in aspartic acid concentration per unit time, and as there is a direct correlation between liberated ammonia concentration and aspartic acid concentration, the initial velocity can be expressed in NH_3-N per hour in 10 ml.

Protein-N was determined by the micromodification of the Kjeldahl method. The ammonia was distilled by Klingmüller's method [8] into a receiver containing 10 ml of 0.01 *N* sulphuric acid and titrated iodometrically.

RESULTS

Preliminary experiments were performed according to Virtanen and Tarnanen [4] extracting the dry bacteria with distilled water at 37° C for 24 h. By determining the protein-N in the bacteria and in the extract, it could be shown that 38.3 % of the nitrogen in the dry bacteria was present in the cell extract. Fig. 1 illustrates the poor yield of active aspartase using this extraction procedure. The poor recovery of the active enzyme in the cell extract is not connected with any heat inactivation of the preparation during incubation at 37° C, since no loss of activity could be observed in a suspension of dry bacteria incubated at 37° C for several days, as is shown in Fig. 2.

No improvement in yield was obtained by using larger volumes of water or repeated extractions. Nor did the raising of the pH of the extraction medium have any effect on the extractability of the enzyme. The enzyme is probably

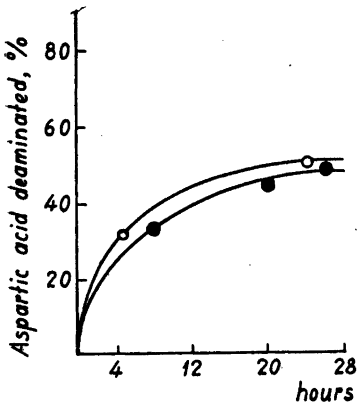

Fig. 3. Deamination of aspartic acid at 37° C by Ps. fluorescens preparations.

○ *dry bacteria*

● *corresponding amount of bacteria extract obtained by butanol treatment*

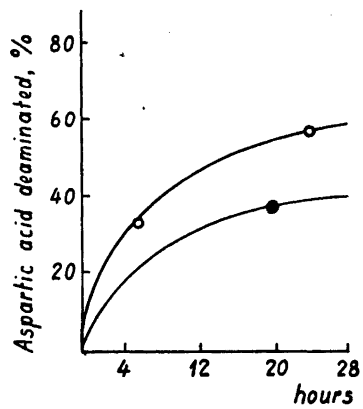

Fig. 4. Deamination of aspartic acid at 37° C by Propionibacterium petersonii.

○ *dry bacteria*

● *corresponding amount of bacteria extract obtained by butanol treatment*

associated with insoluble particles, and till this association is broken the enzyme cannot be extracted with water.

n-Butanol was now used in an attempt to obtain a better yield. The experiments were performed as follows: The dry bacteria were stirred thoroughly with *n*-butanol at a low temperature (0 to −2°) for 30 min. The bacteria were harvested by centrifuging at a low temperature (−2° C), and suspended in water at 0° C. The butanol was removed by dialysing against tap water of low temperature (+ 2° C). After 24 hours' dialysis the bacteria were centrifuged off and aspartase was obtained in a cell-free solution. On determining the protein-N in the dry bacteria and the extract obtained after butanol treatment, it was found that 48.2 % of the nitrogen in the dry bacteria was in the extract.

Fig. 3 shows the practically quantitative yield of active aspartase obtained after *n*-butanol treatment of dry bacteria of *Ps.* fluorescens and Fig. 4 illustrates the yield from *Propionibacterium* after the same treatment.

Precipitation. From *Pseudomonas* extract aspartase was precipitated by lowering the pH to 4.5 according to Virtanen and Erkama [9]. The precipitate was centrifuged off and dissolved in *M*/15 phosphate buffer, pH 7.2. The result of the procedure can be seen in Fig. 5.

With an extract of *Propionibacterium* no precipitation occurred on lowering the pH to 4.5. Thus, the precipitation seems to be specific to *Ps. fluorescens* extract. Fractionation with ammonium sulphate was tried. Solid ammonium

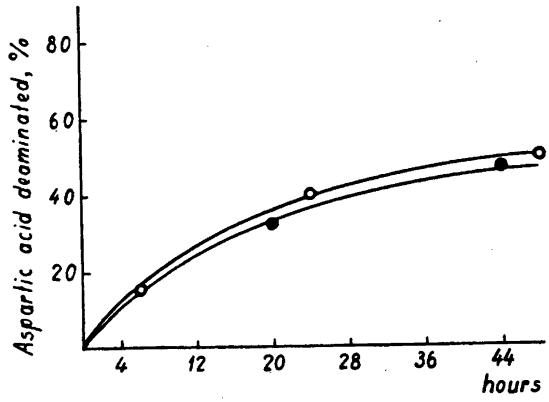

Fig. 5. Deamination of aspartic acid by aspartase preparations.
○ *bacteria extract (butanol treatment)*
● *corresponding amount of aspartase precipitated at pH 4.5*

sulphate was added to the enzyme extract to 6.5 % saturation. After 6 h at 0° the precipitate was centrifuged off. The ammonium sulphate saturation in the mother extract was raised to 30 %, and the extract set aside at 0° for 6 h. The precipitate was again separated, and the ammonium sulphate saturation in the solution was raised to 70 %, *etc.* Thus the extract was fractionated with ammonium sulphate in four fractions of different ammonium sulphate saturation. The different precipitates were dissolved in distilled water, dialyzed overnight against tap water and made up to the same volume. Accordingly, the determinations of the reaction velocities of the different fractions are comparable *inter se.*

Fig. 6 shows the distribution of enzyme activity as a function of ammonium sulphate saturation. Since the recovery of enzyme activity is very poor with ammonium sulphate precipitation (~ 10 %), several other salts were tried ($MgSO_4$, sodium citrate, sodium phosphate) but without any improvement in yield. On the contrary, sodium citrate, for example, gave completely inactivated precipitations though the pH during the precipitation was watched and adjusted to 7.0.

It was found that ethanol precipitation gave poor yields but acetone gave better results. To the enzyme solution was added 0.1 per cent of its volume of a phosphate buffer ($\mu = 0.1$, pH 7.1). The precipitations with acetone were carried out at 0° to −5° C. The precipitate between 28—45 % acetone by volume was taken and dialyzed against tap water for one night. 40 % recovery of the original activity was obtained.

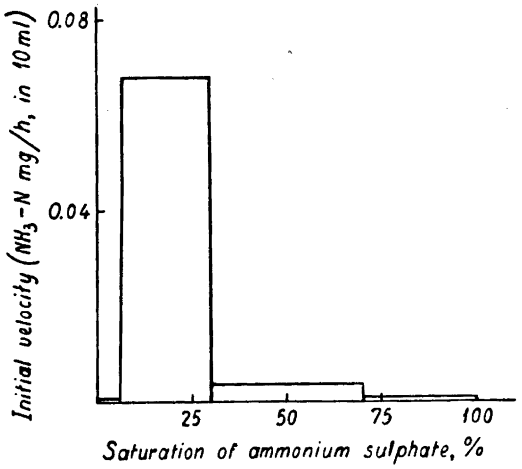

Fig. 6. Distribution of aspartase activity as a function of ammonium sulphate saturation.

DISCUSSION

The results show that aspartase is associated with insoluble lipids in the cell structure. *n*-Butanol, as a somewhat specific reagent in the dissociation of the lipid-protein complex, can successfully be used to separate the water-soluble enzyme from the cells of *Propionibacterium* and *Pseudomonas fluorescens*.

It should be noticed that no inactivation seems to occur during the dialysis of the bacterial suspension after butanol treatment. When, however, preparations precipitated with different salts or with organic solvents were dialyzed, great loss of activity was observed. A quantitative recovery was obtained when the enzyme was precipitated by lowering the pH to 4.5 and dissolving the precipitate formed in neutral buffer without subjecting it to dialysis.

These facts seem to point to the existence of a dissociable active group in the enzyme. In this connection it should be considered that an inactivation of the preparations may be explained as an inhibition of reactive and sensitive groups in the enzyme molecule. Sulfhydryl groups, for instance, are very easily poisoned by traces of heavy metals, which may be added to the solution as impurities with the precipitating salt.

Aspartase from *Ps. fluorescens* was precipitated in the globulin fraction with ammonium sulphate. Here is a contradiction of the results of Gale [10], who reported aspartase I to be precipitated in the albumin fraction (between 50 to 100 % ammonium sulphate saturation).

The precipitation obtained by lowering the pH is largely a precipitation by means of nucleic acids, a method used by Warburg and his school [11]. The difference between *Propionibacterium* and *Ps. fluorescens* in this respect may result from a difference of extractability of nucleic substances from the cells.

SUMMARY

Using *n*-butanol it has been possible to separate aspartase from insoluble particles and obtain the enzyme in solution from the bacteria with a quantitative recovery of the activity. Different precipitation techniques were attempted and acetone precipitation in the cold seems to give the most satisfactory yield.

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