

## Enzymatic Breakdown of Polymetaphosphate

### V. Purification and Specificity of the Enzyme

H. MALMGREN

*Institute of Biochemistry, University of Uppsala, Uppsala, Sweden*

Phosphatase activity on a great number of phosphorus compounds has been found in extracts from different species of *Aspergilli*, and there exists a rather rich literature in this field of research. Bamann and Salzer<sup>1</sup> have studied the phospho-esterase activity of *A. oryzae* (substrate:  $\alpha$ - and  $\beta$ -glycerophosphate), Fleury and Courtois<sup>2</sup> reported pyrophosphatase activity; Neuberg and Fischer<sup>3</sup> and recently Neuberg, Grauer and Mandl<sup>4</sup> investigated its activity on tripolyphosphate  $\text{Na}_5\text{P}_3\text{O}_{10}$ . Kitasato<sup>5</sup> found in 1928 that "taka-phosphatase" from *A. oryzae* was also capable of liberating orthophosphate from metaphosphate. During his investigation on phosphorus metabolism in mould Mann<sup>6</sup> observed metaphosphatase activity in extracts from *A. niger*. Mann also made the observation that extracts from *A. niger* exhibit activity on a great number of phosphorus compounds, *e.g.* pyrophosphate, metaphosphate,  $\alpha$ - and  $\beta$ -phosphoglycerol, 1-phosphoglucose, 6-phosphoglucose, aneurin diphosphate and adenosine triphosphate (all three atoms of phosphorus). Also Krishnan<sup>7</sup> recently reported apyrase activity in mycelial mats of *A. niger*. The polymetaphosphatase-activity (substrate: potassium metaphosphate of molecular weight more than one million) in extracts from *A. niger* has been investigated by the author and Ingelman<sup>8-11</sup>, who also succeeded in isolating a metaphosphate of high molecular weight from the mycelium of *A. niger*<sup>12-14</sup>. The question arose, however, as to whether *A. niger* possesses a specific polymetaphosphatase or if the activity towards all substrates used by Mann and the polymetaphosphate used by the author was the result of one or a few non-specific enzymes. The purpose of the work presented in this paper was to decide whether there exists a specific polymetaphosphatase or not and — in the former case — to purify the enzyme as much as possible.

## MEASUREMENTS OF THE ENZYME ACTIVITY

As the substrate for measurements of the polymetaphosphatase activity a polymetaphosphate  $(\text{KPO}_3)_n$  — designated K15 — was used the molecular weight of which is more than one million. Colloids of this type have been described in previous papers<sup>15, 16</sup>. The enzyme activity was determined by means of viscosity measurements at 25° C. As a relative measure of the enzyme activity in comparison experiments (the same substrate and substrate concentration) a quantity  $z$ , defined by

$$(z = \eta_{sp})_{t=0} \times \frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt}$$

is used, where  $\eta_{sp}$  = specific viscosity and  $t$  = time. To 5 ml 0.5 % substrate solution in acetate buffer of ionic strength 0.3 and pH = 5.4 was added 1 ml enzyme solution. For a more detailed discussion of this method of calculation see Ingelman and Malmgren<sup>9</sup>.

The enzyme activity on substrates of low molecular weight was measured by determining the amount of orthophosphate liberated in a certain time of incubation (orthophosphorus in percent of total phosphorus). The orthophosphate was determined by the method of Lowry and Lopez<sup>17</sup> (based upon reduction of phospho-molybdate by ascorbic acid at pH = 4). The substrate solutions were always 0.01  $M$  with regard to total phosphorus. The proportion of the substrate solution to the enzyme solution was 5 : 1 and the temperature 25° C. As a blank the corresponding substrate solution plus water was always used instead of the enzyme solution. The enzyme solutions were always made free from orthophosphate by dialysis before being used.

## CULTIVATION OF THE MOULD

The mould used for the enzyme production was, as described in previous works<sup>8, 10</sup>, *A. niger* van Tieghem no. 594 from the National Collection of Type Cultures maintained in Britain by the Medical Research Council. In order to get an optimal production of enzyme a systematic investigation on the relationship between the polymetaphosphatase production and the composition of the nutrient solution was carried out by Lindeberg and the author<sup>18</sup>. According to the results of that work it seemed most suitable to cultivate the mould on the following medium: 5 g  $(\text{NH}_4)_2\text{SO}_4$ , 100 g glucose, 2 g  $\text{K}_2\text{HPO}_4$ , 0.5 g  $\text{MgSO}_4 \cdot 7 \text{ aq.}$  and distilled water to 1 liter. Fe and Cu were added at a concentration of 1 mg/liter, Zn at 0.1 mg/liter and Mn at 3 mg/liter.

The solutions were inoculated with a suspension of conidia and tapped into Petri dishes. After 4—6 days at about 30° C most of the polymetaphosphatase produced by the mould has gone into the nutrient solution. The advantage of using  $(\text{NH}_4)_2\text{SO}_4$  as a source of nitrogen is that most of the enzyme is found in the nutrient solution. Extraction of the mycelia is avoided which facilitates the concentration and purification of the enzyme. From this point of view  $(\text{NH}_4)_2\text{SO}_4$  is the best source of nitrogen even if the same total enzyme production can be achieved by using other nitrogen compounds, e. g. nitrates. The pH-value of the final nutrient solution was generally very low, about 1.5—2, and the pH was adjusted to  $\sim 5$  before working up the solution.

## PURIFICATION

*Adsorption.* Mann <sup>6</sup> tried to purify the enzyme by precipitation with acetone in the cold, adsorption on Al-C- $\gamma$  gel and subsequent elution. In that way Mann achieved a purification from notatin, which — according to Mann — always is present in the mycelium and the medium of *A. niger*. The present author made attempts to follow Mann's preparation scheme. However, the precipitation with acetone causes denaturation of the enzyme proteins to a high degree even when working in the cold. After dissolution of the precipitate only a minor part of the activity is recovered. Also on absorption on Al-C- $\gamma$  or  $\text{Ca}_3(\text{PO}_4)_2$ -gel and elution, which is rather difficult to perform, there is a great loss of enzyme activity. Further, there is no separation effected between the different phosphatase activities. This is shown by the following experiment: 15 ml of the crude enzyme solution (A) + 5 ml  $\text{Ca}_3(\text{PO}_4)_2$  gel (5 mg Ca/ml) were shaken for 10 minutes, and the adsorbent was centrifuged off. The sediment was dissolved in 5 ml of ethylenediamine tetra-acetate \* solution (25 mg/ml), dialyzed and the volume made up to 15 ml (B). The two solutions were tested for the activity on several substrates. The activity measurements were carried out in veronal-acetate buffers of optimal activity for the substrate in question (see page 22); time of incubation, 20 hours. The results have been collected in Table 1.

As seen from Table 1 the calcium phosphate gel is no selective adsorbent for any of the enzymes acting on these phosphorus compounds. Nor is it possible to get a better separation by washing the gel with a suitable eluent. In order to remove the adsorbed polymetaphosphatase from the calcium phosphate gel it is necessary to use rather concentrated salt solutions, 50 % saturated ammo-

Table 1. Data of adsorption experiments.

Substrate	Activity (per cent liberated ortho P.)		Activity of (B) / Activity of (A)
	(A)	(B)	
$\text{Na}_4\text{P}_2\text{O}_7$	4.12	1.44	0.35
$\text{Na}_5\text{P}_3\text{O}_{10}$	3.84	1.45	0.37
$\text{Na}_3(\text{PO}_3)_3$	5.29	2.08	0.39
$\beta$ -Phospho-glycerol	19.8	7.22	0.36
Adenosine-monophosphate	14.9	5.28	0.35
K 15	$z = 5\ 600$	$z = 1\ 860$	0.33

\* Kindly supplied by Leo AB, Helsingborg.

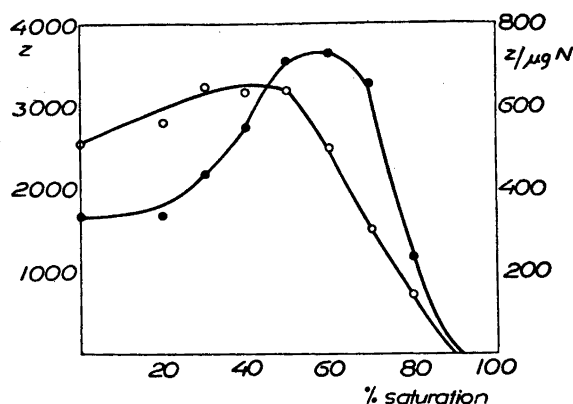


Fig. 1. Remaining activity of supernatants (ordinate to the left; ○) resp. activity /  $\mu\text{g N}$  (ordinate to the right; ●) as a function of various degree of ammonium sulphate saturation.

nium sulphate solution, for instance, and only a small amount of the polymetaphosphatase is recovered — less than what is obtained when using the technic of dissolving the adsorbent. Attempts to adsorb the polymetaphosphatase on the substrate, K 15, and on a gel of the corresponding calcium salt were not successful; these compounds do not act as adsorbents.

*Fractionation with ammonium sulphate.* The wellknown method of fractional precipitation with ammonium sulphate has also been utilised. Equal volumes of dialyzed und buffered crude enzyme solution ( $\mu \sim 0.1$ ) were saturated to various degrees by addition of solid ammonium sulphate. The solutions were left over night in a refringator, the precipitates were centrifuged off, the ammonium sulphate was dialyzed off and the solutions were made up to the same volume before the determination of the polymetaphosphatase activity and analysis of the nitrogen content. The results of such a series at  $\text{pH} = 3.1$  (isoelectric point) is shown in Fig. 1.

As seen from the Figure there is an optimum of the  $z/\text{N}$ -value at about 60 per cent saturation and even at 70 per cent there is still a high  $z/\text{N}$ -value. A higher pH-values the enzyme is more soluble at high salt concentrations than at the isoelectric point. The  $z$ -values increase a little when passing from the blank to about 40 per cent saturation. Probably there is some protein in the crude enzyme solution acting as inhibitor that is precipitated at the addition of ammonium sulphate. If egg albumin is dissolved in the 70 per cent fraction to a concentration of 0.2 % the  $z$ -value is diminished by about 4 % and at a concentration of 0.5 % by about 20 %, which is probably a consequence of the property of proteins to interfere in a system involving metaphosphate.

## PREPARATION

For preparative work it has proved most convenient to add ammonium-sulphate to 70 per cent saturation, discard the precipitate and work up the remaining solution with an electrophoretic purification as the last step. The following preparation scheme proved to be the best. The activity and nitrogen values refer to a preparation of average yield.

1. About 20 liters nutrient solution  $z = 4,000$ ;  $z/\text{mg N} =$   
(after 6 days of cultivation) buffered to pH=5 50,000 (non dialysable nitrogen)
2. Evaporation in vacuo to 3 liters volume; tempera-  
ture not above 25° C  $z = 20,000$
3. Dialysis against calcium-free water \*
4. Evaporation in vacuo to 200 ml; pH adjusted to  
~ 3-4 (acetate buffer)  $z = 190,000$
5.  $(\text{NH}_4)_2\text{SO}_4$  to 70 % saturation; precipitate discarded
6.  $(\text{NH}_4)_2\text{SO}_4$  dialyzed off against calcium-free water \*
7. Evaporation to about 20 ml volume  $z = 770,000$   
(in cellophane sac in streaming air of room  $z/\text{mg N} = 650,000$   
temperature in order to avoid local super-heating)
8. Solution run in electrophoresis at the isoelectric  $z = 600,000$   
point pH = 3.1 for 15 hours; afterwards  $z/\text{mg N} \sim 10^6$   
dialyzed against distilled water

This preparation yields an enzyme solution purified to about twenty times the activity of the crude material. The  $z$ -value 600,000 means that even at a dilution of 1 : 10,000 (corresponding to 0.00006 mg N/ml) there is a measurable activity.

The preparation described above proved to be rather homogenous in the ultracentrifuge. In acetate buffer of pH = 5.4 and ionic strength 0.3 the value  $S_0 = 3.4$  S was obtained; this is in agreement with the value 3.2 obtained from activity measurement after centrifugation in a separation cell<sup>8</sup>. The value of the diffusion constant determined by the usual optical method (Lamms' scale method) is  $5.1 \times 10^{-7}$  c.g.s. which is not in agreement with that

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\* It is of importance to use calcium free water when dialysing off the ammonium sulphate else the polymetaphosphatase is adsorbed on the calcium sulphate precipitate.

obtained by the porous disc method<sup>8</sup>, 8.8. Probably the latter value is to be regarded as the best, because it refers to a certain enzyme activity and not to an impure enzyme.

However, even after the purification previously described the resulting enzyme solutions acts on the same phosphorus compounds as the original solution. It is probably very difficult — if possible — to separate the poly-metaphosphatase from the other phosphatases by means of fractionation with ammonium sulphate or other salts. In fact, all these phosphatases seem to have the same solubility properties. If the supernatants from a series of enzyme solutions which are more and more saturated with ammonium sulphate, are tested for enzyme activity on different phosphorus compounds, it is found that the activity decreases on increasing degree of saturation in almost the same proportion for every substrate. The solutions from the salting out experiments at pH = 3.1 — previously described — were also tested for activity on other phosphorus compounds. The experiments were carried out in veronal-acetate buffers at the pH-optima of the substrates; time of incubation: 20 hours. The activity was put arbitrarily at 10 for the blank (no ammonium sulphate added). The results are collected in Table 2. For comparison the *z*-values have been reduced to the same scale.

As is seen from the table there is too small a difference in solubility between the polymetaphosphatase and the other phosphatases to permit a separation.

Further, the increase of activity in the supernatants up to 50 % saturation might indicate the presence of an inhibitor in the crude enzyme solution from which it is precipitated at rather moderate concentration of ammonium sulphate.

Table 2. Relative solubility of the phosphatases of *A. niger*.

Substrate	percent saturation	Relative enzyme activity of supernatants								
		Blank	20	30	40	50	60	70	80	100
K 15		10	11	12	12	12	10	8	2	0
Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub>		10	11	12	12	12	9	9	3	0
Na <sub>5</sub> P <sub>3</sub> O <sub>10</sub>		10	10	13	13	13	10	6	3	0
Na <sub>3</sub> (PO <sub>3</sub> ) <sub>3</sub>		10	11	13	11	13	8	7	4	0
(NaPO <sub>3</sub> ) <sub>~50</sub>		10	10	12	11	12	10	8	2	0
Glucose-1-phosphate		10	11	11	10	11	10	7	2	0
β-Glycero-phosphate		10	11	12	11	10	10	9	3	0
Adenosine-monophosphate		10	11	11	11	11	10	8	3	0

It is also very difficult to perform an electrophoretic separation. All proteins remaining in the enzyme solution obtained according to the preparation scheme previously described have almost the same electrophoretic properties. Preliminary attempts to get a separation by means of paper electrophoresis were not successful; the moving boundary is not sharp but there is no separation effected — quite in analogy to electrophoresis when using the usual optical methods of observation.

#### SPECIFICITY

Even if it has not been possible to get the polymetaphosphate degrading enzyme in a pure state, there is full evidence for the existence of such a specific enzyme. A comparison between the pH-optima and the conditions of activation and inhibition leads to this conclusion.

The enzymatic breakdown caused by the polymetaphosphates seems to stop when the scission products have reached an average particle weight of  $\sim 480$ , *i. e.* a tetra- or pentaphosphate depending on the dissociation. This result was obtained from equilibrium centrifugations of a polymetaphosphate solution incubated for two days at pH = 5.7 with a strong enzyme solution ( $z = 70,000$ ). After this length of time, less than 0.2 per cent of the total phosphorus had been converted to orthophosphate.

*pH-optima.* As already mentioned Mann <sup>6</sup> showed that the crude extracts of the mycelia (and the remaining nutrient solution) exhibit phosphatase activity towards a great number of substrates; Mann reports an optimal activity in the pH-range 3.7—4.2 without giving the pH-value for every substrate used. In order to get a better picture of the specificity of the phosphatases of *A. niger*, the crude enzyme solution was investigated regarding the pH of optimal activity for the various substrates such as salts of condensed phosphoric acids of various degree of polymerisation and some organic esters of phosphoric acid. The buffers used in these experiments were of the veronal-acetate system. The results are drawn up in Table 3.

Table 3. *pH-optima of different phosphatase activities.*

Substrate	pH-optimum
$\text{Na}_4\text{P}_2\text{O}_7$	2.2
$\text{Na}_5\text{P}_3\text{O}_{10}$	2.3
$\text{Na}_3(\text{PO}_3)_3$	2.0
$(\text{NaPO}_3)_\sim 50$	2.6
K 15	5.7
Glucose-1-phosphate	2.2
$\beta$ -Glycero-phosphate	3.0
Adenosine-monophosphate	4.0

All pH-optima — except that of K 15 — are in a very acid range. Fleury and Courtois<sup>2</sup> report an optimal activity of pyrophosphatase from *A. oryzae* at pH = 7. Neuberg and Fischer<sup>3</sup> found optimal tripolyphosphatase activity in extracts from *A. oryzae* at pH = 2.9; Bamann and Salzer<sup>1</sup> found optimal phosphoesterase activity ( $\alpha$ - and  $\beta$ -glycero phosphate) at pH = 4.2 (*A. oryzae*). The values of *A. niger* given in Table 3 are somewhat lower they are also lower than the values of *A. niger* reported by Mann<sup>6</sup>. However the *A. niger* of the present investigation has been cultivated on a very acid medium which may account for the difference. The most interesting thing of Table 3 is the position of the pH-optimum of the polymetaphosphatase activity, pH = 5.7, which is considerably higher than the other values. It must be pointed out that in measurements of the activity by the analysis of the orthophosphate liberated, the rate determining factor may be the final reaction of a pyrophosphate if the substrate contains a chain of three or more atoms of phosphorus.

*Activation and inhibition.* As reported in a previous paper<sup>10</sup> the polymetaphosphatase is activated by many bivalent metals of which manganese and zinc seem to be most effective; also magnesium and calcium are activators. Under proper conditions the presence of manganese can activate the enzyme about 30 times. However, on no other substrate than polymetaphosphates of high molecular weight can such an effect be recorded. On the contrary, the phosphatases acting on substrates of low molecular weights are rather a little inhibited if the substrate solutions contain  $\text{MnCl}_2$ , see Table 4. However, not only with respect to the activation but also to the inhibition there exists a difference between the polymetaphosphatase and the other phosphatases of *A. niger*. Already Inouye<sup>19</sup> found that the acid phosphatases of *A. oryzae* are inhibited by fluoride ions. This is also the case with all the phosphatases of *A. niger* except that acting on metaphosphates of high molecular weight. Two other substances, viz. phloretin phosphate\* and citric acid have the same effect as sodium fluoride, see Table 4. This property of citric acid is somewhat surprising as — according to Bamann and Salzer<sup>1</sup> — citrate ions do not inhibit the phospho-esterases of *A. oryzae* but cancel out the phosphatase inhibitor of *A. oryzae* (Taka diastase) investigated by — among others — Inouye<sup>20</sup>.

The experiments were carried out in veronal-acetate buffers at the pH of optimal activity of the substrate in question. The substrate solutions were made 0.0025 *M* in NaF,  $\text{MnCl}_2$  and citric acid and 0.1 % in phloretin phosphate respectively. Time of incubation: 20 hours.

\* Phloretin phosphate was supplied by Leo AB, Hålsingborg. It is a condensation product of phosphorylated phloretin. The substance used by the author has a molecular weight of about 15,000. For further details is referred to a coming paper in *Acta Chem. Scand.* by the scientific staff of Leo.



Table 4. Inhibition of the phosphatases of *A. niger*.

Substrate	Inhibitor	percent inhibition			
		NaF	MnCl <sub>2</sub>	Citric acid	Phloretin phosphate
K 15		2	600 activa- tion	10	33
Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub>		98	14	96	83
Na <sub>5</sub> P <sub>3</sub> O <sub>10</sub>		87	4	100	96
Na <sub>3</sub> (PO <sub>3</sub> ) <sub>3</sub>		100	14	100	91
(NaPO <sub>3</sub> ) <sub>3</sub> ~50		100	8	91	97
β-Phosphoglycerol		99	0	100	94
Glucose-1-phosphate		74	9	100	75
Adenosine-monophosphate		98	2	68	75

There exists a difference between the polymetaphosphate degrading enzyme and the other phosphatases not only with regard to the chemical activation and inhibition but also to the heat inactivation; the polymetaphosphatase is more sensitive to heat than the other enzymes. The heat inactivation is, however, not only a question of the heat resistivity of the enzyme as such but also of its purity. A crude enzyme solution is not so sensitive to heat as a purified enzyme probably because the crude solution contains some proteins or lipo-proteins acting as protective colloids.

A series of heat inactivations was carried out by keeping portions of crude enzyme solution at different temperatures (in a water bath) for ten minutes. The solutions were rapidly cooled down and tested for activity on different substrates. The results are seen in the Fig. 2, where the percentage of liberated ortho-phosphate after 4 hours' incubation is plotted against the temperature, the buffers used were veronal-acetate buffers of pH of optimal activities.

As seen from the Fig. 2 there is a sudden drop in the polymetaphosphatase activity at 50° C whereas the other curves show an activity decrease when passing 60° C. After purification the activity decreases already at 35—40° C, but also in this case the polymetaphosphatase is the most heat labile of the phosphatases.

#### OCCURRENCE OF METAPHOSPHATASES IN HIGHER ORGANISMS

Hitherto polymetaphosphatase has only been found in fungi and the bacteria *Proteus vulgaris*<sup>10</sup>. But in tissue extracts from higher animals metaphosphatase activity on substrates of low molecular weight can be demonstrated. The author found metaphosphatase activity in extracts from bovin

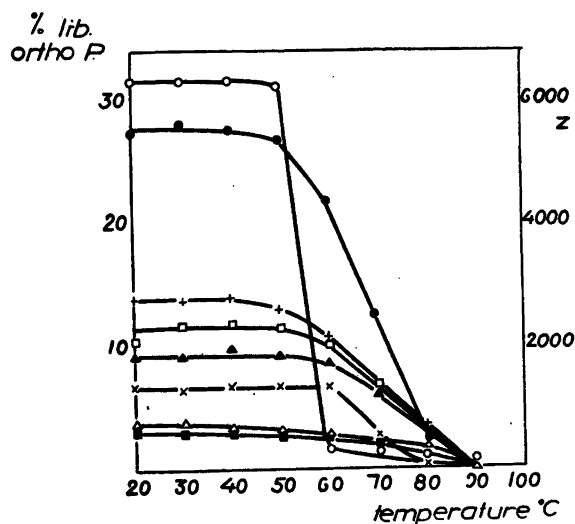


Fig. 2. Activity of heat inactivated enzyme solution as a function of temperature. Ordinata to the left: per cent liberated ortho phosphate; ordinata to the right: z-values.

○ = K15; × =  $\text{Na}_4\text{P}_2\text{O}_7$ ; ■ =  $(\text{NaPO}_3)_3$   
 + =  $\text{Na}_5\text{P}_3\text{O}_{10}$ ; □ =  $(\text{NaPO}_3)_{\sim 50}$ ; ● = glucose-1-phosphate ▲ =  $\beta$ -phos-  
 phoglycerol; △ = adenosine-monophosphate.

liver. (Water extract fractionated with ammoniumsulphate; the fraction 30—60 % saturation was taken, dissolved in distilled water and dialyzed.) This solution was tested for activity on a series of phosphates of various degree of polymerisation, see Table 5. Medium: acetate buffer of pH = 5.2; time of incubation: 4 hours.

The liver enzyme does not split off any orthophosphate from the polymetaphosphate of very high molecular weight, nor does it reduce the viscosity

Table 5. Activity of liver enzyme on various substrates.

Substrate	Per cent liberated ortho-phosphate
$\text{Na}_4\text{P}_2\text{O}_7$	19.7
$\text{Na}_3(\text{PO}_3)_3$	19.3
$(\text{NaPO}_3)_{\sim 50}$	4.8
K 15	0

of the solutions of this colloid (K 15). It is very likely that the metaphosphates of lower molecular weights are broken down by some non specific phosphatase *e.g.* a pyrophosphatase. Metaphosphate has not yet been isolated from tissues of higher animals and there is no reason why specific metaphosphatases should be found in such tissues.

#### SUMMARY

1. It has been proved that *A. niger* possesses an enzyme specifically directed on polymetaphosphate of high molecular weight. The final scission product is a tetra- or pentaphosphate.

2. The turn over number of this polymetaphosphatase is high. There is still good activity in a solution containing only 0.00006 mg *total* nitrogen per ml of which probably the less part belonging to the polymetaphosphatase.

3. The polymetaphosphatase has been purified about twenty times, but it has not been possible to prepare the enzyme in a pure state. The physico-chemical properties of all the phosphatases of *A. niger* are very alike, thus making a separation very difficult; besides it would be necessary to cultivate the mould on a bigger scale, perhaps ten fold or hundred fold that used at present.

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