Plant α -1,4- Glucan Phosphorylase

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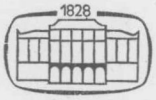

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I. INTRODUCTION

Hardly more than a century has elapsed since urea, a compound present in living organisms, was first prepared in the laboratory by chemical synthesis by the German chemist Wöhler. In so doing he opened the field of study of the vast number of chemical compounds occurring in living organisms. Since Wöhler's work, the role, fate, formation and decomposition of a large number of these compounds have been successfully elucidated. In particular, it has been established that certain biocatalysts, the enzymes, which are capable of performing reactions under extremely mild conditions (such as nearly neutral solution, relatively low temperature) due to their very high transfer number actually play a central role in these processes. The effects of enzymes have been proved to be particularly specific.

Just these specific properties have made possible their widespread use in industry. Research scientists have endeavoured to utilize enzymes under industrial conditions on the basis of their role in living organisms. At present, many problems of industrial production and use have been solved for quite a number of enzymes. Of these, the amylolytic enzymes are quite prominent. Their application offers remarkable advantages in starch degradation processes. As a comparison of the degradation operations carried out with chemical agents such as acids, the hydrolysis of glucosidic bonds is performed by amylolytic enzymes at identical proton concentrations and temperatures 10^9–10^{12} times faster than by hydrochloric acid.

In addition to this extraordinary advantage, however, enzymes also have a number of unfavourable properties which limit their further use in industry. Of these, one of the most inportant is that enzymes are very complicated protein molecules and thus extremely sensitive to external effects such as hydrogen ion concentration, temperature, ionic and non-ionic contaminants, etc. Another problem of industrial importance concerns the repeated use of enzyme catalysts.

Recently, more or less successful attempts have been made to overcome these difficulties by producing insoluble enzyme preparations. Though the problem of the repeated use has been solved by the availability of these preparations, the sensitivity of protein molecules invariably remains high

even in those enzymes which are bound to solid carriers. Further, preparations of this type prove to have very short life times at the optimum temperatures of the catalyzed reactions.

Where is the solution of this problem to be found? In our opinion, the chemical nature of enzymatic catalysis must first be cleared up and then, in the light of this knowledge, the protein-nature of enzymes must be reduced. That this is a realistic conception may be illustrated by two quite extreme examples. One of these is the subject of the present monograph, α-1,4-glucan-phosphorylase of plant origin, with a molecular weight of 200 000 and an extremely complicated ternary and quaternary structure; the other is the α-amylase prepared from *Bacillus thermophylicus*, with a molecular weight of 15 000, with virtually no ternary–quaternary structure.

In the knowledge of the above-discussed and similar facts, attempts to approach this aim have been, and still are being carried out in our Institute. We have set ourselves the task of investigating the overall complexity of the problem rather than one single enzyme, by studying a group of enzymes which are in some way correlated to each other but which, at the same time, differ from each other in their effects. For this reason, we chose amylolytic and starch-synthesizing enzymes for our studies, in particular α- and β-amylases, amyloglucosidase and phosphorylase.

In the selection of the enzyme groups for our investigations, an important aspect was the fact that our Department has a very wide 15 year experience in research into the substrates of the above-mentioned enzymes (in starch research), and also has a research team of adequate training and orientation. The selected enzymes are of importance because they are widely used in industry.

However, the most important reason was the fact that starch, the substrate of the enzymes chosen for study, is a homogeneous polymer (in contrast to proteins and nucleic acids) which lends itself readily to the determination of the actual kinetic constants of enzyme-catalyzed reactions. This is possible since, on employing special labels or on determining the distribution ratios of the products in an extremely precise way, the probability of effective enzyme-substrate collisions can easily be measured for each single enzyme; this known probability is then suitable for the determination of the actual rate constants of the reactions of formation and dissociation of the enzyme-substrate complex studied.

In the present monograph, the findings of earlier publications dealing with α-1,4-glucan-phosphorylase are surveyed.

II. OCCURRENCE OF THE ENZYME

The effect of the enzyme phosphorylase* was first detected in 1925 by Bodnár [9] who observed that during the incubation of pulverized peas with phosphate buffer the inorganic phosphate content of the reaction mixture shows a steady decrease. Later, in 1935, it was proved experimentally by Tankó [94] that the decrease of the inorganic phosphate content is actually due to the formation of organic ester phosphate bonds with reducing sugars.

In 1937, Cori *et al.* first succeeded in isolating phosphorylase (the enzyme which synthesizes polysaccharides) from animal muscle tissues of various origin [13], and then again in 1938 from yeast [14].

Since then, the occurrence of phosphorylase in the leaves, roots and seeds of a great number of higher plants has been proved by many authors. In 1940 Hanes first demonstrated the presence in potato and sugar beet tubers of a plant phosphorylase which is capable of synthesizing amylose from glucose-1-phosphate [32]. Later, the presence of this enzyme in potato tubers was proved. The enzyme has been investigated at various stages of plant development and during storage, and enzyme preparations of various degrees of purity have been produced under in vitro conditions by a number of authors [6, 7, 22, 28, 32, 33, 36, 39, 41, 42, 44, 51, 57, 58, 61, 63, 67, 73, 79, 80, 85, 88, 90, 91, 99]. Phosphorylase has been detected in the tubers of sugar beet [11, 33, 50], in sweet potatoes [1, 12, 43, 66, 84, 86, 92] and in tapioca roots [65]. The presence of the enzyme has been investigated by Krech in the tubers of a number of higher plant types. Phosphorylase activity has been detected in the tubers of *Saxifraga crassifolia*, *Iris sibirica*, *Hyacinthus orientalis*, *Galanthus nivalis* and *Tulipa gesneriana*, but the tubers of various *Allium* varieties did not show phosphorylase activity [47].

The presence of phosphorylase has been observed by a number of authors in the leaves of higher plants, e.g. in the leaves of potatoes [59, 80], tapioca [89, 96], tobacco [26, 52], rice [29, 98], geranium and sugar beet [33, 50, 100] and in the leaves of other higher tuberose plants [47].

* In the following, the term phosphorylase will be used throughout instead of α-glucan phosphorylase (EC 2.4.1.1,α-1,4-glucan orthophosphate glucosyl transferase).

A number of papers deal with the determination of the enzymatic activity, and of the changes in the activity during the development of plants, of phosphorylase present in the plastides of the various plant cells [87], e.g. in rice plant [3, 49, 98], *Vicia faba* [78], cereals [83] and maize [21].

Phosphorylase activity has also been detected in the pollen grains of plants [16, 37, 60, 95].

Many authors have reported the detection and isolation of phosphorylase in fruits and seeds of higher plants such as barley [69, 75], waxy maize [8], sweet corn [72], maize [33, 40, 93], rice [2, 29–31, 101], various cereals [4, 48], peas [28, 32, 102, 103], broad beans [38, 48, 78], Lima beans [28, 68], jack beans [91], soybeans [100], green grain [77], squash [74], pumpkins [43, 66], *Trapa bispinosa* [19] and sea lettuce [45].

Phosphorylase has been detected in various types of algae *(Protophytes)* as well [5, 23–25, 56].

Phosphorylase systems prove to be present in microorganisms such as bacteria [10, 20, 34, 35, 70, 76], protozoa [53, 54], and yeasts [14, 46, 55, 62, 81, 82]. Further, it has been possible to detect phosphorylase in a great number of animal tissues and organs and to isolate it in a very simple way not only in a pure state free from contaminant enzymes but also in a crystalline form, e.g. in brain [14, 15, 17], fat tissue [64], heart [14, 17], liver [14, 17, 71, 97] and muscle tissue [13–15, 18, 27].

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III. PREPARATION

A crystalline enzyme or at least a homogeneous enzyme preparation free of protein contaminants is an indispensable prerequisite for the accurate study of the detailed mechanism of enzymatic synthesis and phosphorolysis, and for the elucidation of the enzyme structure. In the case of plant enzymes (apart from a few exceptional cases), this is a very difficult task which has only become possible during the last few years by the use of high-capacity ion exchangers.

Nearly all the methods of protein chemistry (salting-out based on the differences in the solubilities of proteins; precipitation by solvents; selective separation by heavy metal salts; various adsorption procedures; zone electrophoresis; ion-exchange; and molecular sieves) have been applied to obtain, purify and crystallize plant phosphorylases.

It has been pointed out in Chapter II that a great number of starting materials are available for the preparation of phosphorylase products.

Potato tubers are used by the majority of authors as starting material because enzyme proteins are present in a dissolved state in potato tubers and because relatively mild and simple methods are sufficient to destroy the cells and obtain potato juice rich in enzyme. The drawback of potatoes, however, is that the enzyme content is not stable and the enzymatic activity continually changes during the storage period [22, 47, 48].

The use of other starting materials can lead to problems: on the one hand the removal of plant fats is complex and high losses in activity are faced (e.g. in the case of peas, beans or waxy maize), and on the other hand high losses and poor yields occur during extraction and dissolution of proteins (cereals, rice) due to the presence of enzyme proteins present in the bound state.

On taking into account all these aspects, potato tubers appear to be the most suitable starting material. Hence the various methods of purification are described in detail for this material.

1. Pretreatment and Production of Potato Juice

In the papers dealing with the production of potato phosphorylase a more or less identical technique is suggested for the preparatory and juice-producing operations. Different methods, however, are applied for the inhibition of enzyme contaminants (phenoloxidases and amylases).

In the first step potatoes are peeled and sliced. However, prior to juice production, the effect of phenoloxidases must be inhibited because non-inhibited phenoloxidases induce appreciable changes in enzyme proteins.

No inhibition of phenoloxidases was applied by Hanes [13], Hidy and Day [14], Meyer and Traz [31], Maruo [30], Nakamura [33] or Husemann [19 – 21]. The action of phenoloxidases was inhibited by Green and Stumpf [11] (treatment for 2 hours at room temperature with a 0·005 M solution of potassium cyanide), and by Weibull and Tiselius [45] (treatment at 0°C with a solution of sodium hyposulphite of unknown concentration).

Sodium dithionite has been used by all other authors as inhibitor.
A 0·5% solution of sodium dithionite (20 minutes at room temperature) was applied by Barker *et al.* [2], and by Holló *et al.* [16].

Phenoloxidases were inhibited by Lee [28] and by Staerk and Schlenk [43] during a one-minute homogenization at 3°C by a buffer containing 0·5% sodium dithionite and 0·5% sodium citrate, using 100 ml of buffer for 1 kg of potatoes.

Baum and Gilbert [3] used a 0·7% solution of sodium dithionite for 10 minutes at room temperature.
Potato slices were treated for an hour by Kamogawa *et al.* [25] with a buffer containing 0·7% sodium dithionite and 0·7% sodium citrate.

Inactivation was brought about by Fischer and Hilpert [7] by a 30-minute treatment with 5% sodium dithionite at room temperature.

After the inactivation of phenoloxidases, the potato slices are thoroughly washed and made up to a pulp. Juice is generally produced by pressing [2, 7, 11, 14, 28, 30, 31, 43, 45], filtering through linen sacks [16] and by centrifugation [25]. Pure potato juice was obtained by cooling to a temperature of 0 to 3°C by Hanes [13], Baum and Gilbert [3], Lee [28], Staerk and Schlenk [43], Holló *et al.* [16] and Kamogawa *et al.* [25]; or simply at room temperature, by Green and Stumpf [11], Husemann and Pfannemüller [20], Fischer and Hilpert [7] and Barker *et al.* [2].

In the next step, the potato starch is removed by sedimentation or centrifugation. Subsequently, prior to the fractionation or to other purifying operations, heat treatment has been applied by a number of authors in order to inhibit the effect of amylases.

The removal of amylase contaminants by heat treatment has been investigated in detail by Husemann *et al.* [20] who observed the conditions of heat treatment by control tests. They proved in this way that it is possible to subject potato juice of pH 7·0 to 54–56°C for long periods without any essential deterioration of phosphorylase (Table III/1).

Table III/1

Heat Treatment of Potato Juice [20]

Temperature of 15-minute heat treatment (°C)	Phosphorylase activity* (Units/ml)	Hydrolyzing activity** (sec/60 min)
Untreated	22·5	13·3
37	22·8	12·8
45	22·5	8·4
50	23·2	3·2
54	21·9	2·1
56	18·0	—
60	2·9	—

* Activity measured by the Hidy and Day method [14].
** Hydrolyzing activity (expressed by the decrease in seconds of the time of outflow after inhibition for 60 minutes, referred to hydroxyethylamylose as control standard).

Amylase is thus inactivated as proved by the fact that a sample added to a solution of hydroxyethylamylose did not cause any decrease in viscosity (Fig. III/1).

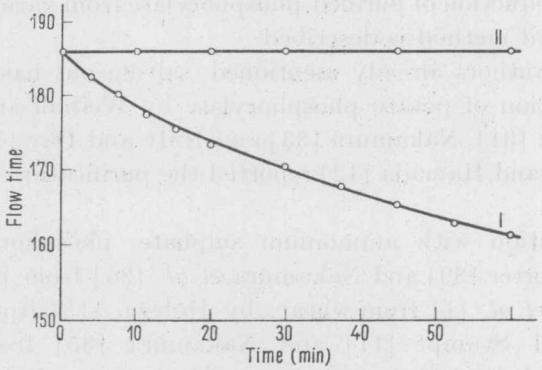

Fig. III/1. Investigation (by viscosimetry, using a solution of hydroxyethylamylose) of the amylase content of heat-treated (II) and untreated (I) potato juice [20]. The heat-treatment was carried out for 10 minutes at 54°C

A five-minute heat treatment at 50°C was applied by Green and Stumpf [11], Weibull and Tiselius [45] and Nakamura [33].

Husemann *et al.* [20] applied at first a treatment of 54–56°C for 10–15 minutes. According to their data, a temperature of 54°C is sufficient for the inactivation of amylases in freshly harvested potatoes. However, in stored potatoes (in late springtime) a temperature of 56°C must be applied. In their later paper [21] a treatment for 40 minutes at 55·5 ± 0·2°C (at pH 7·0) is described. Potato juice was heat-treated by Holló *et al.* [16] for 15 minutes at 54 ± 0·1°C (pH 7·0) while a 10-minute treatment at 55–56°C (pH 7·2) has been applied by Kamogawa *et al.* [25]. According to all the authors, the heat-treated potato juice was quickly cooled in ice-water, the precipitated proteins removed by centrifugation, and the pure supernatant solution was processed in the later operations of enzyme purification.

2. *Salting Out*

Fractionation by ammonium sulphate, the simplest method of enzyme purification known and applied since the earliest time, has been employed by a great number of authors, and it is still used in the most recent and up-to-date techniques as a method of prefractionation.

Green and Stumpf were the first [11] to employ (in 1942) purification with ammonium sulphate for the production of phosphorylase from potatoes and lima-beans. Since then, a large number of papers have been published in which the production of purified phosphorylase from various plants by the above-mentioned method is described.

Besides the authors already mentioned, salting out has been employed for the production of potato phosphorylase by Weibull and Tiselius [45], Meyer and Traz [31], Nakamura [33] and Hidy and Day [14], while Kurasawa *et al.* [26] and Hamada [12] reported the purification of rice phosphorylase.

By fractionation with ammonium sulphate, phosphorylase has been produced by Porter [39] and Nakamura *et al.* [36] from barley; by Shaw [40] and Aimi *et al.* [1] from wheat; by Hobson [15] from broad beans; by Green and Stumpf [11] and Nakamura [35] from lima beans; by Sumner *et al.* [44] from jack beans; by Inoue and Onodera [23, 24], Nakamura [34] and Shoichiro [41] from sweet potato; by Kursanov and Pavlovina [27] from sugar beet; by Inoue and Onodera [23, 24] from squash; by Phillips and Averill [38] from pumpkins; by Srivastava and