FUNGAL CELLULASES

II. THE COMPLEXITY OF ENZYMES FROM *ASPERGILLUS ORYZAE* THAT SPLIT β-GLUCOSIDIC LINKAGES, AND THEIR PARTIAL SEPARATION

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Summary

Filter paper electrophoresis and paper chromatography have been used to test the homogeneity of the β-glucosidase of *Aspergillus oryzae*. In the crude enzyme preparation there are at least eight components capable of breaking β-glucosidic linkages and showing varying degrees of specificity towards different substrates. These are active not only in splitting simple glucosides but also in depolymerizing sodium carboxymethyl cellulose. The only exception is provided by the enzymes splitting *p*-nitrophenyl-β-glucoside, which are sharply limited to two closely related components. This two-component mixture has been partially purified from accompanying enzymes.

Neither the postulate of a single β-glucosidase nor that of a specific enzyme for each substrate will fit the experimental results. It also appears that the concept of an enzyme (C_x) specific for polymeric β-glucosides and qualitatively different from other β-glucosidases must be abandoned in this instance.

Attempts to separate and purify various enzymes have given results in harmony with the hypothesis of multiple enzyme components.

I. INTRODUCTION

The most recent review of the β-glucosidases is that of Veibel (1950). From 1920 onwards the primary concern of most workers in this field has been the specificity of the enzyme towards different aglycones and the effects of most possible variations in the nature of the aglycone have been investigated. The primary assumption made is that the variations in activity with variation of aglycone are those of a single enzyme, differing according to source, for instance almond emulsin, yeast emulsin, and animal β-glucosidase, but homogeneous from any given source. Veibel states (p. 593), "Originally the concept was that each naturally occurring glycoside required a special enzyme, e.g. amygdalase, salicinase, arbutinase. However, as the artificially prepared glycosides proved to be hydrolysable under the influence of such enzymes as emulsin or invertase, it became clear that the specificity of the enzymes was not as great as at first presumed, but that on the other hand, these preparations were not single enzymes but mixtures of different glycosidases. Further experiments led to the assumption that all β-glucosides are hydrolysed by one enzyme, a β-glucosidase."

In general, proof of the homogeneity of the β-glucosidase used in various studies has been scanty or lacking, and purification and characterization of

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the enzyme itself has been subordinated to the interest in the effect of variation in aglycone on the activity of the enzyme. There seems to be no account of the thorough investigation by all available techniques of the homogeneity of any sample of β-glucosidase. Miwa and Tanaka (1949) have shown that in apricot emulsin the β-glucosidase, β-xylosidase, and β-galactosidase activities, which are often considered by members of the Helferich school to be activities of a single enzyme, can in fact be partially separated by precipitation with ammonium sulphate or methanol, and by absorption on aluminium hydroxide. The nature of the evidence presented—change in the ratio of the activities in fractions prepared by various treatments—is very similar to that presented in this paper, and electrophoresis of such preparations might give similar results. Veibel himself states (p. 588), "Further investigation of the effect of electrophoresis should, however, be of value, as not all the possibilities of this efficient method of separation of (even related) proteins are exhausted by the experiments mentioned."

Pigman (1943) also came to the conclusion that the wide range of specificity of various β-glucosidases could not be explained on the basis of a single enzyme and postulated a class of closely related enzymes all showing specific ability to hydrolyse β-glucosidic linkages.

Any check on the homogeneity of the β-glucosidase present in the *A. oryzae* preparation would also serve as a check on the relationship between the β-glucosidase and the C_x enzyme since these enzyme activities would be expected to segregate independently if they were due to unrelated components of the mixture.

II. Methods

The methods used for determining enzymic activities have been outlined in the preceding paper. Interfering substances present in the crude enzyme material prevented the use of spectrophotometric methods or nitrogen determinations for estimating protein concentrations; a modification of the colorimetric procedure of Stiff (1949) in which very small amounts of protein are estimated through the copper taken up in the biuret reaction was therefore used.

Into each of nine 15 ml. centrifuge tubes were pipetted 8.0 ml. of 5 per cent. trisodium phosphate solution, and 200 mg. of copper phosphate were added to each tube except No. 2. Water and protein solution were added to the tubes according to following schedule:

Tube	1	2	3	4	5	6	7	8	9
Water (ml.)	2·0	0	1·7	1·4	1·1	0·8	0·5	0·2	0
Protein solution (ml.)	0	2·0	0·3	0·6	0·9	1·2	1·5	1·8	2·0

All tubes except No. 2 were now stoppered and shaken for 90 min. in a shaker, then centrifuged and the supernatant filtered off through an open paper (Whatman 541) to remove any floating copper phosphate. From 1.0 to 8.0 ml. of the filtrate (according to the amount of copper expected) was added to 2.0 ml. of 0.5 per cent. sodium diethyldithiocarbamate solution and the whole made up to 20 ml. The colour developed was measured at 440 mμ in a Coleman

spectrophotometer. The amount of copper taken up by the protein in tubes 3-9 could now be calculated and plotted against volume of protein solution. A least-squares line was then drawn to fit the points. The slope of this line (μg. copper dissolved per ml. of protein solution) was reproducible to less than 5 per cent., in spite of erratic variations (±25 per cent.) in single points.

A series of experiments using certain pure proteins in solutions of known strength showed the following ratios for wt. of protein/wt. of copper: haemoglobin 7.1; trypsin 7.2; pepsin 7.3; bovine serum albumin 7.6; giving a mean (7.3) close to the value (7.1) reported by Stiff. The weight of copper dissolved by 1 ml. of protein solution was therefore multiplied by 7.3 to give the weight of protein in 1 ml. of solution. Figure 1 shows some typical results.

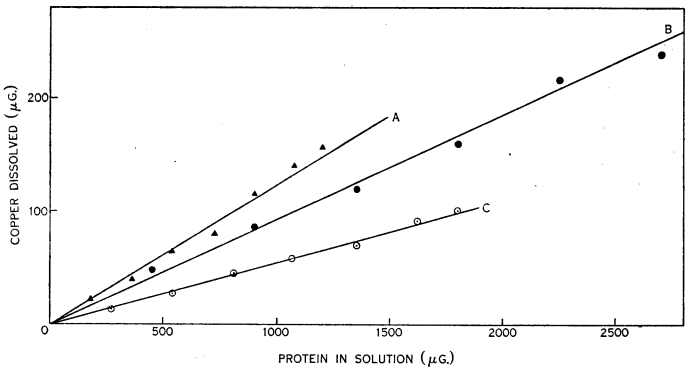

Fig. 1.—Microbiuret estimation of proteins. *A*, haemoglobin; *B*, partially purified *A. oryzae* enzyme (estimated to contain 76 per cent. protein); *C*, crude mould enzyme (estimated to contain 34 per cent. protein). No allowance has been made for the moisture content of the air-dry protein.

The limits of the method are about 1-10 mg. of protein dissolved in a minimum of 11 ml. of H_2O. Its usefulness thus lies in the direct estimation of dilute solutions of protein rather than in the assay of extremely small amounts.

III. Purification of Enzymes

(*a*) *Concentration of* p-*Nitrophenyl-β-glucosidase*

Crude mould enzyme (20 g.) dissolved initially in 750 ml. of pH 7 phosphate buffer was fractionated with ammonium sulphate and the fraction precipitating between 55 and 65 per cent. saturation was retained. This material was refractionated at volumes of 250 and 100 ml. The final material was dissolved in 400 ml. of pH 5 acetate buffer, 500 ml. of 3 per cent. calcium phosphate gel added, and the pellet from centrifugation extracted with three 100 ml. lots of 0.067M disodium phosphate. The extract was adjusted to pH 7 with solid monosodium phosphate, and an equal volume of pH 5 acetate buffer added, followed by 180 ml. of 3 per cent. calcium phosphate gel. The pellet

was extracted with three 30 ml. lots of 0.067M Na_2HPO_4, the extract adjusted to pH 7, dialysed in cellophane overnight against distilled water and the final solution freeze dried. Of the 6.6 g. of original enzyme protein 86 mg. (1.3 per cent.) were recovered as a white powder containing 56 per cent. of protein and 2.8 per cent. of carbohydrate.

TABLE 1

DISTRIBUTION OF VARIOUS ENZYME ACTIVITIES AT DIFFERENT STAGES OF THE CONCENTRATION OF THE *p*-NITROPHENYL-β-GLUCOSIDASE ACTIVITY IN *A. ORYZAE*

Enzyme	After Ammonium Sulphate Fractionation		After Calcium Phosphate Absorption		After Dialysis		
	Activity Recovered (%)	Purification Factor	Activity Recovered (%)	Purification Factor	Activity Recovered (%)	Purification Factor	Loss on Dialysis (%)
Colorimetric glucosidase	23	2·9	19·6	12·7	12·7	9·8	35
Salicinase					14·9	11·5	
C_x enzyme	15	1·9	4·9	3·1	2·2	1·7	55
Cellobiase					2·0	1·5	
Amylase					1·3	1·0	
Sucrase					0·8	0·6	
Esterase					0·13	0·09	
Viscometric protease	1·2	0·15			0·08	0·06	
Recovered protein	7·9		1·54		1·30		16

The inactivation of certain of the enzymes on dialysis forms a serious barrier to their isolation by methods involving solutions of salts. The C_x activity was not absorbed on shaking with cellulose powder at pH values from 5 to 8 for a few minutes and the activity lost on dialysis was not restored by calcium, zinc, manganese, magnesium, or iron ions. The 16 per cent. loss of protein on dialysis suggests that the enzymes may themselves be slowly dialysable through cellophane, although other possibilities, e.g. slow proteolysis by residual protease, cannot be excluded.

Filter paper electrophoresis of the purified enzyme showed that the bulk of the protein material was concentrated in a single spot coinciding in position with that of the *p*-nitrophenyl-β-glucosidase. Determination of other enzyme activities (see next section) showed that the small amounts of protein in other parts of the paper still contained appreciable enzyme activities. The colorimetric β-glucosidase was still inhomogeneous by paper chromatography, showing the same distribution of the two components as the original crude preparation.

Attempts to concentrate or fractionate other enzyme activities by the usual methods (solvent and salt precipitation or adsorption) gave erratic results. The only experiment giving an unequivocal result is described below.

(*b*) *A Demonstration of the Non-Homogeneity of the* C_x *Enzyme*

The C_x activity remained in solution when ethanol was added to a 1 per cent. solution of the crude enzyme to give a final ethanol concentration of 40 per cent. (v/v), and it was found that ammonium nitrate gave satisfactory results as a protein precipitant in this medium. The experiments were carried out at 10°C., the C_x activity being stable for some hours in 40 per cent. ethanol at pH 5.5 at this temperature. The solutions were equilibrated 1 hr. after the ammonium nitrate had been dissolved, the precipitate centrifuged down, and C_x and viscometric protease activities determined in the supernatant. The results are shown in Figure 2 and demonstrate the probable presence of at least two components in the C_x enzyme.

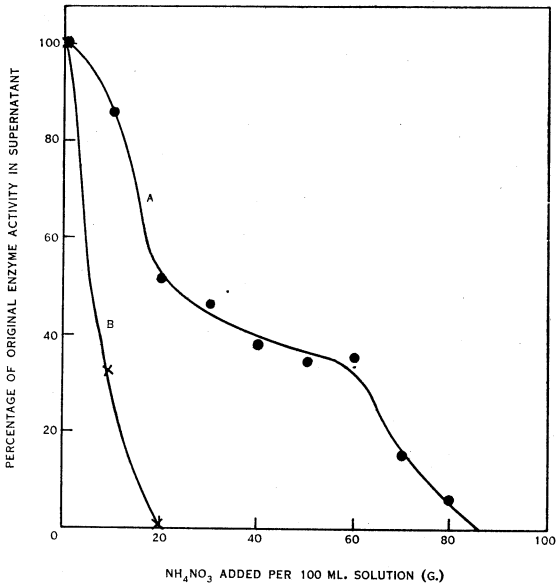

Fig. 2.—Precipitation of enzymes by ammonium nitrate in 40 per cent. ethanol. *A*, C_x enzyme; *B*, viscometric protease.

(*c*) *Activity of* C_x *and β-glucosidase in Various Enzyme Preparations*

It will be shown in the next section that precipitation by 40 per cent. ethanol under certain conditions and passing enzyme solutions through an ion-exchange column lead to differential separation of two components of the *p*-nitrophenyl-β-glucosidase. Material prepared by these methods was tested for activity against a number of substrates and compared with the original crude enzyme and a specimen purified by electrodialysis. The protein concentration of the test solutions was proportional to the amounts of the original protein material retained in each preparation. The relative activities (cellulase = 1) for a given enzyme preparation are the ratios of the number of β-glucosidic linkages split per hour under the standard conditions for any substrate to the number split in SCMC. The results are summarized in Table 2.

IV. Filter-paper Electrophoresis and Chromatography

(*a*) *Filter-paper Electrophoresis*

The method of filter-paper electrophoresis provides a rapid and convenient method of investigating enzyme homogeneity (Cremer and Tiselius 1950). The application of this method to the crude enzyme mixture from *A. oryzae* will form part of a forthcoming paper by Gillespie and Woods. Some indication of the type of results obtained working with the crude *A. oryzae* enzyme has already been given by Gillespie, Jermyn, and Woods (1952). The same standard conditions were employed in all filter-paper electrophoreses (Whatman 3MM paper, veronal buffer of ionic strength 0.025 and pH 8.6, 6 hr. at a potential gradient of 9 V./cm.). Both the crude enzyme and the *p*-nitrophenyl-β-

Table 2

Comparison of the activities of various enzyme preparations against different β-glucosidic substrates

Substrate	Crude Enzyme		Ethanol-Precipitated Enzyme		Enzyme Prepared by Passing Through Ion Exchange Column		Enzyme Prepared by Electrodialysis	
	Activity Recovered (%)	Relative Activity $C_x = 1$	Activity Recovered (%)	Relative Activity $C_x = 1$	Activity Recovered (%)	Relative Activity $C_x = 1$	Activity Recovered (%)	Relative Activity $C_x = 1$
Cellobiose	100	4·1	97	8·1	107	6·9	98	6·3
Salicin	100	2·0	25	1·0	27	0·9	60	1·9
Aesculin	100	6·1	65	8·1	89	8·4	78	7·5
Methyl β-glucoside	100	0·11	38	0·08	70	0·09	70	0·11
SCMC	100	1·0	49	1·0	64	1·0	64	1·0
p-Nitrophenyl-β-glucoside	100	0·2	160	0·6				

glucosidase concentrate were tested for the distribution of the activity against various substrates along the paper. The results are plotted in the form of histograms in Figure 3. The determination of cellobiase was not attempted since it was judged that the activities to be measured were too low to ensure reasonable activity by the available technique.

The degree of variability between different runs is illustrated by the three different results for the "colorimetric β-glucosidase"; the difference between peaks of activity in different runs judged to be due to the same component was not more than 2 cm. Woods (personal communication) finds about the same degree of variability with other enzymes which he has tested; there are too

many small possible sources of variation to expect absolute reproducibility in shape and position of peaks of enzyme activity, and personal judgment enters to some extent into the comparison of the various histograms. No component has been postulated unless a corresponding peak appears on at least two histograms. The existence of only one component actively hydrolysing *p*-nitrophenyl-*β*-glucoside could be checked by observing that a single well-defined yellow spot appeared after the paper had been sprayed with a solution of the glucoside in pH 7.5 phosphate buffer and incubated at room temperature.

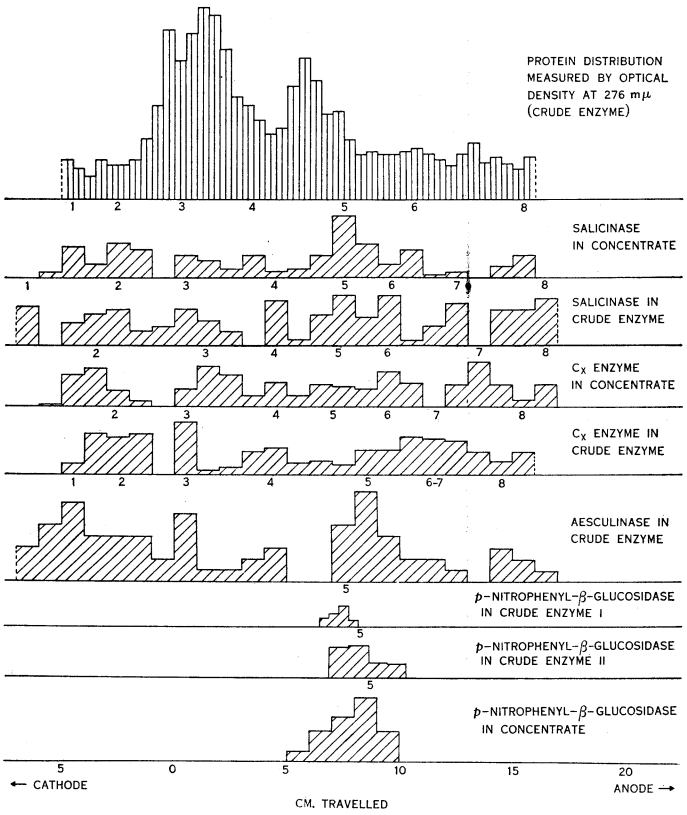

Fig. 3.—Distribution of various enzyme activities of *A. oryzae* enzyme preparations after filter-paper electrophoresis at 9 V./cm. for 6 hr. at pH 8.6 (concentrate = semi-purified *p*-nitrophenyl-*β*-glucosidase). Numbers are assigned to what are believed to be identical components.

(*b*) *Filter-paper Chromatography*

The technique used has already been described (Gillespie, Jermyn, and Woods 1952), and a full investigation of the paper chromatography of the *A. oryzae* enzymes will be made the subject of a later paper. The chromatographic techniques used were based on those of Reid (1950).

The *p*-nitrophenyl-β-glucosidase appears as a single component (No. 5) on filter-paper electrophoresis. Nevertheless, if a solution is spotted on a paper chromatogram, developed using 0.01M pH 5.8 McIlvaine buffer and acetone (60-40 v/v), and the dried paper sprayed with an 0.05 per cent. solution of *p*-nitrophenyl-β-glucoside in 0.2M phosphate buffer at pH 7.5, the presence of two components is revealed by yellow areas against a white background. Component *A* is a well-defined round spot of R_F 0.7 after 20 cm. solvent travel and component *B* a long streak of R_F 0.2-0.55. Both the crude enzyme and the concentrate gave the same pattern. Almond emulsin showed two similar components and a third immobile component. A large number of enzyme preparations were available in this laboratory from various attempts to purify enzyme components. On testing these preparations it was found that one prepared by passing a solution of the crude enzyme through a mixed-bed ion-exchange column was lacking in component *B*, while one prepared from the supernatant after a precipitation with ethanol (final conditions 40 per cent. ethanol, —5°C., and pH 8) was lacking in component *A*. Component 5 is thus further divisible into the two components 5*A* and 5*B*, both of which travel into the same position when investigated by filter-paper electrophoresis.

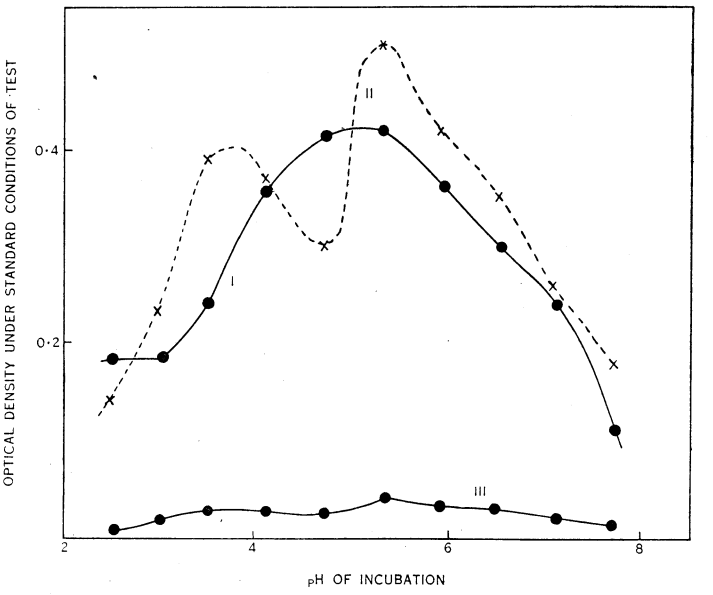

Fig. 4.—pH-activity curves for the *p*-nitrophenyl-β-glucosidase. I, crude enzyme. II, enzyme passed through ion-exchange column. III, curve II reduced in scale for comparison with curve I at equivalent protein concentrations.

Component 5*A* was further investigated by examining its pH-activity curve. The results are shown in Figure 4.

It can be seen that component 5*A* is a very minor fraction of the original component 5. Yet the pH activity curve shows a pronounced double peak and it seems almost certain that further fractionation could again resolve it into

two components $5A_1$ and $5A_2$. The appearance of more than one peak in the pH-activity curves for the *A. oryzae* enzyme acting on various β-glucosidic substrates appears to be quite common (cf. first paper of this series) and is a further argument for the heterogeneity of the enzyme systems involved.

Almond emulsin, which shows a somewhat similar picture to the *A. oryzae* enzyme on chromatography, was subjected to filter paper electrophoresis under the standard conditions. *p*-Nitrophenyl-β-glucosidase appeared as a streak stretching between points 0.5 and 9.0 cm. towards the cathode and showing no signs of resolution into components. Salicinase, which was detected by spraying with salicin solution, incubating, spraying with a solution of *p*-anisidine hydrochloride in ethanol, and heating to develop the colour, appeared as a streak stretching from a point 2 cm. towards the anode to one 6 cm. towards the cathode. Almond emulsin thus does not contain the same β-glucosidase components as the *A. oryzae* enzyme, but may none the less contain a system of components fully as complex.

V. Discussion

Veibel, in the statement quoted in Section I, admits two alternatives — either a battery of β-glucosidases each with its own substrate, or a single β-glucosidase with a wide range of specificity towards various substrates. The present work shows neither of these alternatives to be true for enzymes from *A. oryzae*, where there are a number of β-glucosidases, none of which has absolute specificity for any one substrate. It is evident that these β-glucosidases of *A. oryzae* form a group of proteins of very closely related properties, and that no statement on the specificity of the crude "β-glucosidase" produced by this mould has any real meaning.

It is also evident that the complexity of the material currently available in this laboratory, and the small relative amounts of each component present, make the problem of isolating any one of them and examining its properties almost insurmountable. The mould would have to be grown under conditions leading to maximum β-glucosidase production with as little contaminating protein as possible before any useful attempts could be made in this direction. Even so, the example of *p*-nitrophenyl-β-glucosidase shows the difficulty of proving homogeneity. It appears from the few rough tests made that almond emulsin is also complex, and that statements on its specificity must be viewed with reserve.

Gillespie and Woods (unpublished data) have shown that certain of the enzymes of *A. oryzae*, which appear to be separated into more than one component by filter paper electrophoresis, can actually be fractionated to give preparations showing a single component corresponding to one of the multiple components of the crude enzyme. It must be admitted that the failure to effect such resolution with β-glucosidases makes it possible that the observed components may be artefacts in spite of all evidence to the contrary. Final justification of the conclusions drawn in this paper must therefore await such a resolution in this or a closely related system.

The second conclusion that can be drawn from these experiments also conflicts with many of the conclusions drawn from work carried out with other enzyme systems. It is that in the *A. oryzae* system the enzymes breaking down monomeric β-glucosides do not differ qualitatively from those breaking down polymeric β-glucosides. There is thus no need to assume the existence of a "cellulase" or a "C_x enzyme" to explain the breakdown of long-chain cellulose derivatives in solution. The electrophoretic evidence for the identity of the enzymes capable of breaking down SCMC and β-glucosides is confirmed by the identical values within the experimental error of the observed Michaelis constants and energies of activation of the enzymes when hydrolysing salicin, SCMC, and cellodextrin. Although the evidence is by no means complete at this stage, it is strong enough to suggest that the existence of enzymes of the "C_x" type as a separate species is open to doubt. Variation in specificity towards different substrates among enzymes from different sources is to be expected and cases have been cited by Levinson, Mandels, and Reese (1951) in which near absence of salicinase and cellobiase activity accompanies high C_x activity in fungal culture filtrates. These may represent no more than one extreme in the possible range of specificities, and until the C_x enzyme has been shown in some case to act by a radically different mechanism there seems no justification for removing it from the general class of β-glucosidases. In any case it seems at present most unwise to generalize about the mechanism of "cellulase" action on the results of a set of experiments using enzymes derived from any single organism.

The enzymes splitting *p*-nitrophenyl-β-glucoside appear to be part of a separate system which may not be closely related to the other β-glucosidases. The segregation of the various β-glucosidases together in the purification of *p*-nitrophenyl-β-glucosidase may be no more than a reflection of the fact that carbohydrases form a group of proteins more closely related in properties than the other enzymes. This is in agreement with the work of Niwa (1943) who found that the β-glucosidases from animal viscera splitting *p*-nitrophenyl-β-glucoside were considerably different in their properties from those splitting salicin and phenyl-β-glucoside. The easily applied colorimetric method for β-glucosidase is thus a completely unreliable index of total enzyme activity.

VI. Acknowledgments

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