Biochemical Studies on α -Glucosidase from Buckwheat (*Fagopyrum esculentum* Mönch)

Part I. Screening of α -Glucosidase from Plant Sources and Changes of α -Glucosidase Activity during the Germination Stages of Buckwheat*

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Present investigation deals with a search for plant sources which have high α -glucosidase activities. As a result of the screening test on 8 kinds of plant source (spring wheat, maize, buckwheat, malted barley, oat, soybean, azuki bean and kidney bean), buckwheat seed was found to have the highest α -glucosidase (maltase) activity. Changes in α -glucosidase and amylase activities during the germination stages of buckwheat were also examined. It was found that α -glucosidase was rich in seeds and seedlings at initial stages of germination. In addition, the probable existence of glucamylase activity was suggested in the former, and a rapid appearance of α -amylase was recognized in the latter.

It was concluded that ungerminated buckwheat seed was excellent as the material for the preparation of plant α -glucosidase.

α -Glucosidase is widely distributed throughout the plants, animals and microorganisms.¹⁾ Especially, detailed studies have been carried out with purified enzymes from molds and yeasts and it is known that the enzymatic specificity differs from each other according to its source,^{2~4)} including transglucosidase activity and glucamylase activity.⁵⁾ In comparison with molds and yeasts, previous inves-

tigations of plant α -glucosidase have dealt almost exclusively with its existence¹⁾ and some properties^{6~8)} and detailed studies have not yet been carried out. Such a state of affairs appears to be mainly due to its low activity, which leads to difficulty of purification and subsequent investigation.

For the purpose of biochemical investigation of plant α -glucosidase, the present investigation was undertaken to look for a plant with high α -glucosidase activity and also to examine the changes of its activity during germination stages of that plant. A comparative investigation of maltase from plant sources was made in 1913 and it was shown that buckwheat,

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2) J. Larver, "The Enzyme" (P. D. Boyer, H. Laady and K. Myrback, eds.), Academic Press, New York, 1960, Vol. 4, Part A, p. 374.

3) Y. Tsujisaka and J. Fukumoto, *Nippon Nogeikagaku Kaishi*, **37**, 747 (1963).

4) S. Sugawara, Y. Nakamura and T. Shimomura, *Agr. Biol. Chem.*, **23**, 156 (1959).

5) H. Okazaki, "Kôso Kenkyuhô" (S. Akabori, ed.), Asakura Press, Japan, 1957, Vol III, p. 684.

6) H. Pringsheim and J. Leibowitz, *Biochem. Z.*, **161**, 456 (1925).

7) H. Tauber and I. S. Kleiner, *J. Biol. Chem.*, **105**, 679 (1934).

8) D. H. Hutson and D. J. Manners, *Biochem. J.*, **93**, 545 (1964); *ibid.*, **94**, 783 (1965).

maize and millet were maltase-rich grains.⁹⁾ In this investigation, 8 kinds of plant source (spring wheat, maize, malted barley, buckwheat, soy bean, azuki bean, kidney bean and oat) were taken up. The results of a screening test indicated that buckwheat seeds had a higher α -glucosidase (maltase) activity than other cereals tested.

Subsequently, the changes of α -glucosidase activity, amylase activities and starch content were examined with seeds and seedlings of buckwheat. As a result, it was recognized that the activity on maltose was higher in ungerminated seeds and seedlings at an initial stage of germination. Also it was suggested that an enzyme preparation from buckwheat might have glucamylase activity, in addition to maltase activity.

MATERIALS AND METHODS

Seed samples. Spring wheat (Nōrin 75 gō), maize (flint corn), buckwheat (Botan soba), soy bean (Nagahajiro), azuki bean (Maruba 1 gō), kidney bean (Chūnage uzura) and oat were kindly supplied from Hokkaido Experimental Station of Agriculture and malted barley, from Sapporo Brewery, Sapporo Breweries Corporation. Buckwheat seeds used in germination experiments were produced in Hokkaido in 1964. Maltose and soluble starch were obtained from commercial sources.

Preparation of enzyme solutions from various plants. Fifty g of each kind of seed was mashed in a mortar with 100 ml of distilled water, 0.1 M acetate buffer (pH 5.0) or 1.0 M acetate solution (pH 7.0 and pH 8.0), respectively, for five hours at room temperature with frequent stirring. The homogenate was centrifuged, dialyzed against running tap water at 10~11°C for twenty-four hours and made up to 150 ml with distilled water, which was used as the enzyme solution.

Preparation of the enzyme solution from the seedlings of buckwheat. Buckwheat seeds sterilized by a 0.5% solution of bleaching powder were germinated on a filter paper moistened with distilled water in the dark at 26~28°C. After three or four days the seedlings were transplanted in an enamel-

ware, steeped in distilled water up to the roots, and supported by a vinylon net. The water was renewed every other day. At different germination stages 50 pieces of seedling were extracted with 20 ml of 0.1 M acetate buffer (pH 5.0) by mashing in a mortar and the mash was allowed to stand for four hours at room temperature with frequent stirring. The homogenate was centrifuged and the precipitate was washed by centrifugation with 10 ml of distilled water or the same acetate buffer as used in homogenization. The combined supernatants were dialyzed against running tap water at 10~11°C for twenty-four hours, made up to 50 ml, and used as the enzyme solutions. Two other groups of 50 pieces of seedlings which were obtained under the same culture conditions were used to examine the changes of dry weight and sugar contents and the results were already reported.¹⁰⁾

Determination of enzymatic activities on maltose and soluble starch. In screening assays, maltase activity was determined as follows. A mixture of 2 ml of 1% maltose, 2 ml of McIlvaine's buffer (pH 6.0) and 2 ml of the enzyme solution was allowed to react at 37°C. After twenty-four hours the reaction mixture was heated at 100°C to inactivate the enzyme and the degree of hydrolysis was examined by means of thin layer chromatography.

With enzyme solutions from the seedlings of buckwheat, maltase activity and saccharogenic and dextrinogenic activities on soluble starch were determined in the following way. A mixture of 2 ml of 1% substrate solution (maltose or soluble starch), 1 ml of 0.1 M acetate buffer (pH 5.0) and 2 ml of the enzyme solution was allowed to react at 37°C for 1 hr (Fig. 2) or various time intervals between 3 min to 24 hr (Fig. 4).

The increase in reducing power was measured by a modification of Somogyi's copper reagent method by Kobayashi *et al.*¹¹⁾ and expressed as glucose. Enzymatic activity was defined as percentages of hydrolysis of maltose or soluble starch under the condition described above.

For the determination of dextrinogenic activity, 1 ml was pipetted from the reaction mixture and put into 10 ml of 0.5 M acetic acid to inactivate the enzyme. To this was added 10 ml of N/3000 iodine

10) M. Takahashi and T. Shimomura, *Eiyō to Syokuryō (Japan)*, **19**, 361 (1967).

11) T. Kobayashi and T. Tabuchi, *Nippon Nogei-kagaku Kaishi*, **28**, 171 (1954).

9) Z. Wierzbowski, *Biochem. Z.* **57**, 125 (1913).

solution and absorption at 700 m μ was measured.¹²⁾ Iodine value was expressed as the percentage of the optical density of the control containing 2 ml of distilled water in place of the enzyme solution. Dextrinogenic activity was defined as the reciprocal of the time (minutes) which was required for iodine value to reach 40% (violet).

As it is known that the optimum pH of plant α -glucosidases was between 4.5~5.5,¹⁾ these reactions for buckwheat were carried out at pH 5.0. But McIlvaine buffer (pH 6.0) was used tentatively in screening experiments.

Thin layer chromatography of the reaction products. Kieselgel G was used as the adsorbent on a chromatoplate. The samples were developed three times with a solvent system consisting of *n*-butanol-isopropanol-water (10 : 5 : 4 by vol.)¹³⁾ and the sugars

were detected with the anisaldehyde reagent¹⁴⁾ by heating at 115°C for ten minutes.

RESULTS AND DISCUSSION

α -Glucosidase activity of various plants

Maltase activity was compared using enzyme solutions from various plants by means of thin layer chromatography. The result is shown in Fig. 1. Only the enzyme solution from buckwheat could hydrolyze maltose into glucose completely under this condition. Malted barley was next to buckwheat in maltase activity, especially on an alkaline side and about a half of maltose added was hydrolyzed by enzyme extracts from maize and oat. A slight hydrolysis was measured in each of spr-

FIG. 1. Thin-layer Chromatograms of Maltose Hydrolysates by Extracts from Various Plants.

Solutions used for preparing enzyme extracts are as follows: A: distilled water, B: 0.1 M acetate buffer (pH 5.0), C: 0.1 M acetate solution (pH 7.0), D: 0.1 M acetate solution (pH 8.0), As to C and D, pH was adjusted with 1N ammonia. Reaction time was twenty-four hours at 37°C. Other condition, see text.

12) B. Hagihara, "Jikken Kagaku Kōza", Maruzen Press, Japan, 1958, Vol. 24, p. 279,

13) S. Chiba and T. Shimomura, *Agr. Biol. Chem.*, **29**, 486 (1965).

14) E. Stahl, "Dünnschicht-Chromatographie", Springer-Verlag, 1962, p. 514.

ing wheat, soy bean and kidney bean, while no hydrolytic activity was observed with azuki bean. Therefore, in the case of buckwheat it was examined which solution of A, B, C and D in Fig. 1 was the best for extraction of α -glucosidase activity. The reaction time was shortened in order to find out the difference in enzyme activities among those extracts, and B, that is, acetate buffer (pH 5.0) proved to be a far better extracting medium than the others.

Changes of enzymatic activity during the germination of buckwheat

As described above, it was found that buckwheat had a higher α -glucosidase activity than other cereals and the extract solution with 0.1 M acetate buffer (pH 5.0) showed the highest activity. Now, changes of enzymatic activities were examined in the extracts with distilled water or 0.1 M acetate buffer (pH 5.0)

at different germination stages of buckwheat. Changes of enzymatic activities on maltose and soluble starch are shown in Fig. 2. Changes in dry weight and starch content per one individual are also shown. It was shown that the activity on maltose was higher in ungerminated seed and seedling at the initial stage of germination, followed by a gradual decrease afterward. On the other hand, the activity on soluble starch was weak in seed, increased rapidly with the development of the germination and decreased again as in the case of maltose. It was observed that the decrease of starch content in seedlings agreed quite well with the increase of activity on soluble starch. During the initial stage of germination enzymatic activities were higher in extracts prepared with acetate buffer than those with distilled water, but this situation was rather reversed in later stages. These facts seem to indicate that in

FIG. 2. Changes of Enzymatic Activity of Extracts from Buckwheat during the Germination.

Water extracts { —●—: activity on maltose
—▲—: activity on soluble starch
Acetate buffer { ---○---: activity on maltose
(pH 5.0) extracts { ---△---: activity on soluble starch
--×--: dry weight; --□--: starch content.

Values of dry weight and starch content are expressed as mg per one individual.

Reaction time: 1 hr. Other reaction condition, see text.

ungerminated seeds and seedlings at the initial stage of gremination, a part of α -glucosidase may be in an insoluble state in distilled water but soluble in acetate buffer. However, in later stages of germination a neutral or an alkaline medium is presumably more effective for the extraction of enzyme than the acetate buffer (pH 5.0), since α -glucosidase was best extracted at pH 8.5~9.0 from malted barley¹⁵⁾ and at pH 7.5 from seedlings of alfalfa;¹⁶⁾ also in Fig. 1, malted barley was extracted better on alkaline side.

The dextrinogenic activities of these extracts are shown in Fig. 3. A remarkable increase of activity was observed at the same stage as that when saccharogenic activity on soluble starch increased, but it disappeared rapidly at the later stage.

Figure 4 shows the relation between saccharogenic activity and iodine value on soluble starch by 0.1 M acetate buffer (pH 5.0) extracts at the different germination stages. It is generally recognized that the relation between those two activities suggests the type of amylase.^{17,18)} The straight line obtained with ungerminated seeds was very similar in type to that of glucamylase, while other curves with seedlings showed a characteristic type of α -amylase.

Figure 5 shows the hydrolysis products of soluble starch and maltose by enzyme solutions from buckwheat at different germination stages. Glucose was the sole product from soluble starch obtained by the action of the enzyme from ungerminated seeds throughout the reaction time, while by the action of from seedlings, glucose and malto-oligosaccharides were formed. Accordingly, it is possible that the former case was due to glucamylase and

the latter was mainly due to α -amylase.

On the basis of these results, it was concluded that ungerminated buckwheat seeds had a glucamylase activity in addition to a high α -glucosidase activity and in seedlings at an initial stage of germination a rapid appear-

FIG. 3. Changes of Dextrinogenic Activity of Extracts from Buckwheat during the Germination.

—●—: water extract, ---○---: acetate buffer (pH 5.0) extract. Dextrinogenic activity was expressed as the reciprocal of the time (minutes) which was required for iodine value to reach 40%. Other condition, see text.

FIG. 4. Curves showing Changes in the Iodine Value and Saccharogenic Activity on Soluble Starch by Extract from Buckwheat during the Germination.

Number shows the days after planting. A certain amount was pipetted from the hydrolysate of soluble starch at suitable intervals (3 min~24 hr) and measured for saccharogenic activity and for iodine value. Other reaction condition, see text.

15) B. B. Jørgensen and O. B. Jørgensen, *Acta Chem. Scand.*, **17**, 1765 (1963).

16) D. H. Hutson and D. J. Manners, *Biochem. J.*, **94**, 783 (1965).

17) L. L. Phillips and M. L. Caldwell, *J. Am. Chem. Soc.*, **73**, 3563 (1951),

18) J. Fukumoto, *Science and Industry (Japan)*, **39**, 386 (1965).

FIG. 5. Thin-layer Chromatograms of Substrates Hydrolysates by Buckwheat Extracts during the Germination.

S: soluble starch;

M: maltose

ance of α -amylase occurred together with a decrease of α -glucosidase activity. It was also recognized that the appearance of α -amylase was accompanied by a decrease of starch content in seedlings.

The changes of amylase activity during the germination stages of buckwheat were studied by Ōtakara¹⁹⁾ and Haneda and Yamagishi²⁰⁾ and the latter recognized the existence of α -amylase and β -amylase in seed and the increase of α -amylase activity during germination. But no attention has been paid to α -glucosidase in these reports. Concerning rice seeds,²¹⁾ it was reported that maltase activity increased together with the development of germination.

On the other hand, more recently the presence of glucamylase activity in plants has begun to be reported by some workers.^{15,16,22,23)} The evidence that buckwheat α -glucosidase showed both glucamylase activity and maltase activity and glucamylase activity was not caused by a coexistence of β -amylase and maltase will be discussed in the next paper dealing with the purification of α -glucosidase from ungerminated buckwheat seeds, which was found to be an excellent source for the preparation of plant α -glucosidase in this paper.

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20) M. Haneda and E. Yamagishi, *Naganoken Tandai Kiyō (Japan)*, **13**, 20 (1958).

21) G. Yamagishi, *Nippon Nogeikagaku Kaishi*, **18**, 1082 (1942).

22) A. Kasugai, Abstracts of Papers, Annual meeting of the Agr. Chem. Soc. of Japan, Tokyo, April, 1966, p. 83.

23) F. Akimoto and T. Shimomura, *Memoirs of the Fac. of Agr. Hokkaido Univ.*, **6**, No. 3, (in press),