THE MECHANISM OF ACTION OF MALT BETA-GLUCANASES

II. Separation and Characterization of Malt Endo-Beta-Glucanases[1]

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ABSTRACT

Ammonium sulfate fractionation of an extract of barley green malt yielded three fractions containing endo-beta-glucanase activity. The endo-beta-glucanase in fraction A_I was characterized by being half-inactivated by heating for 0.7 hours at 40°C. The optimum pH for enzyme action was 4.75, and the apparent ionization constants of the active form of the enzyme suggested the involvement of aspartic acid and histidine in the active site.

The endo-beta-glucanase activity in fraction A_{II} was not well defined. A part of the activity was lost rapidly when the preparation was diluted with distilled water. Reduced glutathione and other compounds stabilized the enzyme. When stabilized, the pH optimum and heat stability of the enzyme(s) present in A_{II} were quite similar to those for the endo-beta-glucanase present in A_{III}.

The endo-beta-glucanase in fraction A_{III} was characterized by being half-inactivated by heating for 5.2 hours at 40°C. The optimum pH for enzyme action was 4.55. The apparent ionization constant on the acid side of the active form of the enzyme suggested the involvement of aspartic acid in the active site; the apparent ionization on the alkaline side did not fall in the ranges published for common amino acids.

Investigations on the beta-glucan-degrading enzymes in green malt suggest that complete degradation of barley beta-glucan requires a combination of endo-beta-glucanase, exo-beta-glucanase, and disaccharase actions (1,3,8,9). These conclusions are based generally on the action of relatively crude malt extracts. Even though barley beta-glucan is known to contain at least two different linkages (beta-1,3- and beta-1,4-), there have been few attempts to establish whether there is more than one endo-beta-glucanase (or exo-beta-glucanase) present. The first definite separation was made by Luchsinger, Cochrane, and Kneen (7), using ammonium sulfate fractionation. These workers reported that green malt contained at least two endo-beta-glucanases which differed in heat stability. Bass and Meredith (2), using column separation on activated alumina, obtained a fraction which contained mainly endo-beta-glucanase activity and a second fraction containing exo-beta-glucanase and disaccharase activities.

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Endo-beta-glucanase activity is characterized by a rapid decrease in viscosity of the substrate resulting from hydrolysis of internal glucosidic bonds (with a concurrent increase in reducing power). In contrast, exo-enzymes remove terminal groups and typically cause a rapid increase in reducing power accompanied by only a small decrease in viscosity. Except in unusual cases the decrease in viscosity due to the action of exo-enzymes will not interfere with the investigation of the endo-enzymes.

Study of the endo-enzymes has been handicapped by a lack of substrates containing specific linkages. Barley beta-glucan contains both beta-1,3- and beta-1,4-, and perhaps beta-1,6-linkages. Thus a decrease in viscosity could be caused by hydrolysis of any of the bonds. Laminarin, which contains mainly beta-1,3-glucosidic bonds and some beta-1,6-bonds (10), is useful for measuring exo-activity. However, solutions of available preparations do not have viscosities high enough to measure endo-activity by a decrease in viscosity. Basing the assay on an increase in reducing power leads to difficulties in distinguishing endo- from exo- activities. Modified cellulose substrates are available for studying enzymes which hydrolyze beta-1,4-glucosidic bonds. Even so, it is not certain that an enzyme specific for the beta-1,4-glucosidic bonds in barley beta-glucan or in cellulose would be able to effectively attack the beta-1,4-linkages in a substrate such as carboxymethyl cellulose.

In view of these difficulties the work was directed toward separating and purifying the malt beta-glucanases. If the enzymes could be obtained pure, the mechanism of their action could be determined even on the complex substrates. Either reducing power or viscosity data would suffice for kinetic studies, and where needed, analysis of the products of the reactions would show the specificities of the enzymes. It is the purpose of this paper to present the results of further purification and characterization of green malt endo-beta-glucanases.

Materials and Method

Enzymes. Extracts of kilned malts were prepared by mashing finely ground malt for 2 hours in a 0.5% solution of sodium chloride at room temperature. Extracts of green malts were prepared by blending the ice-cold materials for 1 to 1.5 minutes in a Waring Blendor, followed by a 2-hour mashing at room temperature. The insoluble material was removed by filtration or centrifugation (6).

Substrate. A 1.72% solution of Kindred barley gum prepared as described previously was used as the general substrate (6). Other

substrates used in the investigation are described in the text.

Activity Determination. Enzyme activity was determined by measuring the change in viscosity of the substrate at pH 4.7 and 30°C. in an Ostwald No. 200 viscometer. Endo-beta-glucanase activities were expressed as the change in reciprocal specific viscosity per 30 minutes per g. of dry malt (6), or as relative activities based on the change in reciprocal specific viscosity per 30 minutes per ml. of enzyme solution.

Results

Separation and Purification of Barley Malt Endo-Beta-Glucanases. Solid ammonium sulfate (ACS grade) was added to 14 liters of a 0.5% sodium chloride extract of green barley malt. The fraction which was soluble in 30% but insoluble in 40% saturated ammonium sulfate, and the fraction which was soluble in 50% but insoluble in 60% saturated ammonium sulfate were collected by centrifuging. These fractions corresponded respectively to fractions II and IV previously isolated (7) and were so designated. Fraction II which contained 11% of the original endo-beta-glucanase activity was dissolved in 330 ml. of distilled water. Fraction IV, which contained 22% of the original endo-beta-glucanase activity, was dissolved in 365 ml. of distilled water. Aliquots of 200 ml. were dialyzed at 0° to 4°C. against 2-l. aliquots of distilled water. The water was replaced after 2, 2.5, and 2.5 hours. The final dialysis proceeded for 12 hours. Containers were subjected to gentle agitation during dialysis.

About ¾ to ⅞ of the solids initially present precipitated from both samples (estimated visually from the size of the pads after centrifuging). The precipitates were suspended in 0.5% sodium chloride solution. The endo-beta-glucanase and cellulase activities (7) of the supernatants and precipitates were as follows:

Sample	*Total Relative Activities* × 10^{-3}	
	Endo-beta-glucanase	*Cellulase*
Fraction II	575	...
Dialyzed fraction II		
Supernatant	550	0
Precipitate	75	1.4
Fraction IV	1,040	...
Dialyzed fraction IV		
Supernatant	810	18.0
Precipitate	107	9.5

The supernatant from dialyzed fraction II was refractionated twice; the portion soluble at 30% and insoluble at 40% saturated

ammonium sulfate was collected and labeled A_I. Excessive losses occurred as only 15% of the activity was recovered. Dialysis as described above caused no further loss.

The supernatant from dialyzed fraction IV was refractionated twice. Two fractions were collected, that soluble at 50% and insoluble at 60% saturated ammonium sulfate was labeled A_{II}, and that soluble at 60% but insoluble at 90% saturated ammonium sulfate was labeled A_{III}; both were dialyzed as described above. Again there were excessive losses of activity as only about 10% of the activity was recovered in both A_{II} and A_{III}. (For the remainder of this paper, the labels A_I, A_{II}, and A_{III} will refer specifically and only to the endo-beta-glucanase activities present in the respective fractions.)

Heat Stability of the Malt Endo-Beta-Glucanases. The three preparations were heated at 40°C. and aliquots were removed at several time intervals. The log of the activity remaining after heating was plotted against the time of heating (7). A_I was half-inactivated in 0.7 hours (Fig. 1) showing its identity with the endo-beta-glucanase

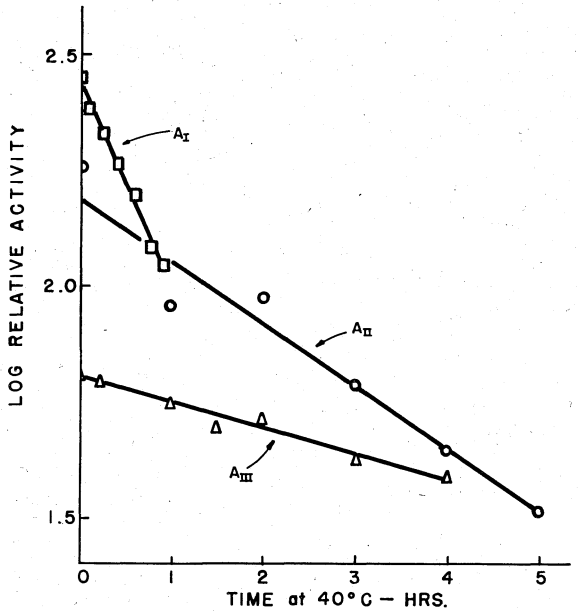

Fig. 1. Heat-inactivation of malt endo-beta-glucanases at 40°C.

A_L reported previously (7). A_{III} was half-inactivated in 5.2 hours showing its identity with the endo-beta-glucanase A_S reported previously (7).

Since A_{II} was half-inactivated by the intermediate heating time, 2.2 hours, it seemed possible that A_{II} was a mixture of A_I and A_{III}. However, repeated experiments consistently gave very erratic results for the activity remaining after the first 2 to 3 hours of heating (note curve in Fig. 1). In addition, the results showed that the activity of A_{II} before heating varied more than could be accounted for by procedural errors. It seemed that the diluted enzyme was being rapidly inactivated while standing in the ice bath before being assayed. To test this possibility 1 ml. of A_{II} was diluted 70-fold with distilled water and placed in an ice bath; and 1-ml. aliquots of the diluted enzyme were added to three viscometers at 2-minute intervals. The relative activities of the three samples respectively were 220, 150, and 110. Indeed, part of the activity was lost very rapidly even when the samples were held in an ice bath. Such a rapid loss of activity, about one-half the activity in 4 minutes, had not been encountered previously. A_I, when diluted 10-fold with distilled water and kept at ice-bath temperature, loses activity at a rate of 1.4% per hour. A_{III} apparently is stable for relatively long periods under similar conditions. The rapid losses might be sustained under these conditions if the enzyme were being inactivated by diluting a stabilizing agent present in the enzyme preparation, or less likely, by a trace compound present in the distilled water.

Effects of Stabilizing Agents on the Malt Endo-Beta-Glucanases. The effects of reduced glutathione (GSH), cysteine, substrate, and sodium chloride on diluted A_{II} are shown in Table I. All the re-

TABLE I

EFFECTS OF GSH, CYSTEINE, SUBSTRATE, AND SODIUM CHLORIDE ON THE STABILITY OF DILUTED A_{II} AT 0° TO 4°C.[a]

DILUTING AGENT	RELATIVE ACTIVITY			
	Time in ice bath			
	0 hours	1 hour	2 hours	3 hours
GSH, 0.01*M*	320	330	330	...
Substrate, 0.35%	330	350	...	340
Distilled water	320	170	...	170
Sodium chloride, 0.01*M*	300	250[b]	..	280
GSH, 0.001*M*	290	...	250	...
Cysteine, 0.001*M*	300	290	280	...

[a] One ml. of enzyme diluted with 40 ml. of reagent.
[b] Solid GSH to a concentration of 0.01*M* was added at 1 hour.

agents, including sodium chloride, stabilized the enzyme against the inactivation caused by dilution with water.[3]

[3] The reader will notice that the activity of a given enzyme, when expressed as relative activity, may vary from experiment to experiment. This usually is caused by variations in the susceptibility of substrate which occasionally occur unless special precautions are taken (6).

Although GSH protected the diluted enzyme from inactivation in the ice bath, it did not restore lost activity (Fig. 2). Solid GSH was

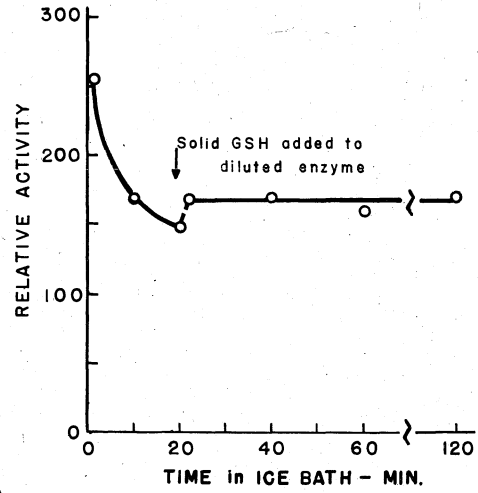

Fig. 2. Action of GSH on partially inactivated A_{II}.

added at 22 minutes to eliminate the need for a volume correction. Separate experiments showed that 0.01*M* GSH solution, which had a pH of 3.1, was as effective in protecting the enzyme as GSH solution adjusted to pH 4.7 or 5.5.

The effects of dilution with 0.01*M* GSH on the heat stability of the three preparations are shown in Fig. 3. A_I and A_{II} were diluted tenfold with 0.01*M* GSH, pH 4.7, and A_{III} threefold with the GSH solution. The GSH had no effect on the stability of A_I and A_{III} at 40°C. On the other hand, the stability of A_{II} in the presence of 0.01*M* GSH approached that of A_{III}.

Substrate also exerted a protective action on A_{II} at 40°C. A_{II} was diluted 20-fold with 0.35% substrate solution before heating. Initially the inactivation appeared to roughly parallel the rate when A_{II} was diluted tenfold with 0.01*M* GSH. The rate of inactivation then increased steadily until, after about 30 minutes (the substrate would be extensively hydrolyzed), the activity dropped rapidly to a lower level which appeared to represent a residue of more stable activity present in fraction A_{II} (Fig. 3).

Effect of pH on Malt Endo-Beta-Glucanases. The endo-beta-glucanase activities of A_I and A_{III} were measured over pH ranges of 2.7 to 6.5 and 7.1, respectively. The pH values found immediately after the reaction are reported. Each point represents the average of

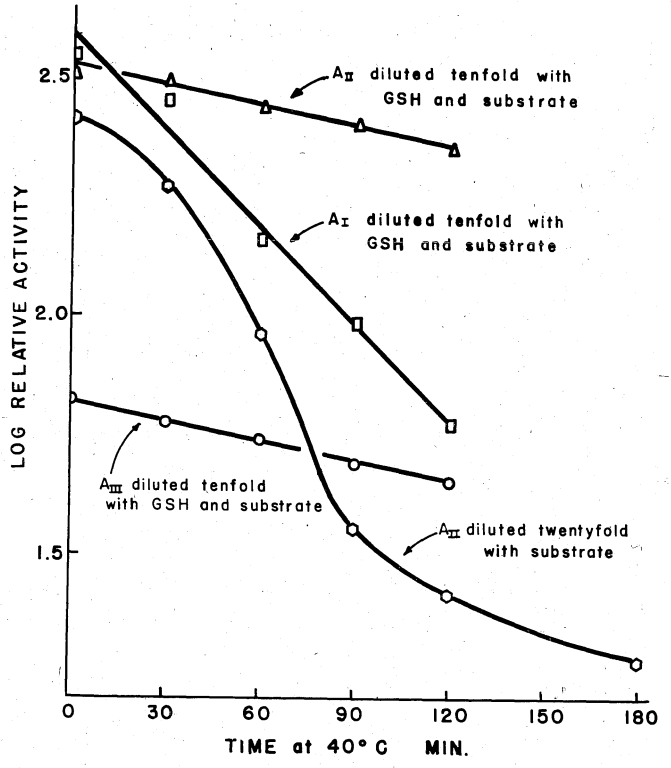

Fig. 3. Effects of GSH and substrate on the stability of A_I, A_{II}, and A_{III} at 40°C.

two or more determinations. The results for A_I are given in Fig. 4. The pH-activity curve for A_{II} stabilized with 0.01*M* GSH over a range of pH 4.3 to 5.7 is included with that for A_{III} in Fig 5. A_I exhibited its optimum at pH 4.75. The optima for A_{III}, and A_{II} stabilized with GSH, both appear to be pH 4.55. Thus the optima for A_I and A_{III} fall within the ranges previously reported for A_L and A_S (7).

The pH-activity curves for A_I and A_{III} cover sufficient ranges to allow calculation of the acid and alkaline ionization constants of the active form of the enzyme (K_{ES1} and K_{ES2} according to the terminology of Dixon and Webb, 5). Proof that the enzymes were saturated at all pH values was not obtained. However, a 1.72% solution of beta-glucan was used for the substrate, which is considerably over the value of 1.15 to 1.2% required for maximum activity of the endo-beta-glucanases of kilned malt (6). Thus, it is assumed that the enzymes were saturated at all pH values.

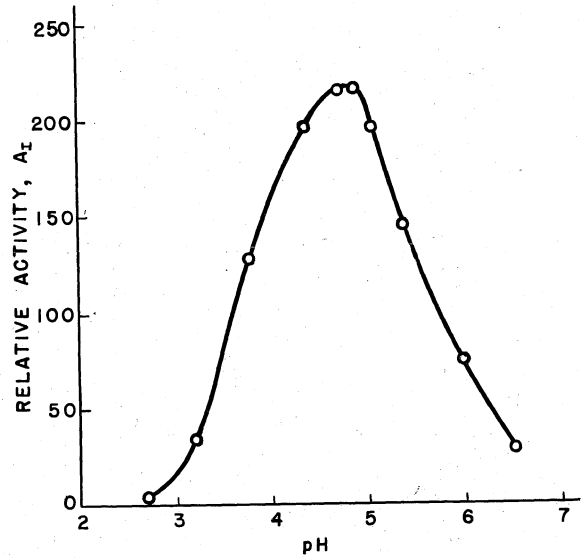

Fig. 4. Effect of pH on the activity of A_I.

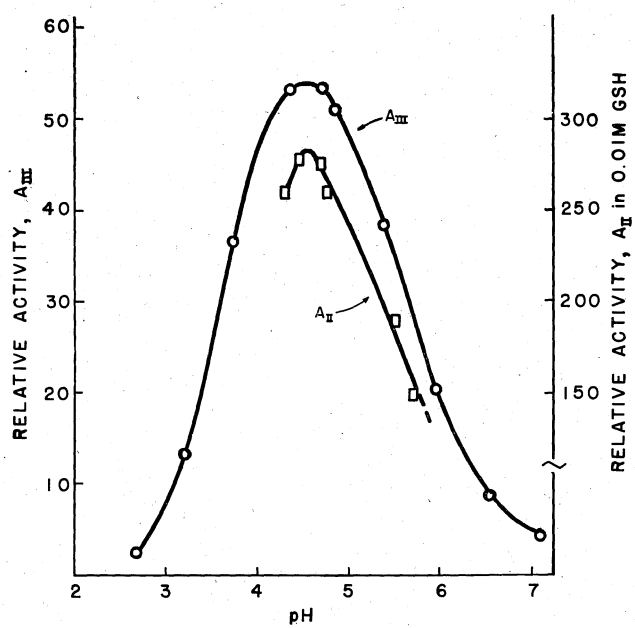

Fig. 5. Effect of pH on the activities of A_{II} and A_{III}.

The pH optimum for A_I is 4.75 and the midpoints on the acid and alkaline sides of the curve are respectively pH 3.64 and pH 5.65 (Fig. 4). The ionization constants for the active form of the enzyme-substrate complex, calculated from these data, are 1.60×10^{-4} (pK = 3.80) and 1.98×10^{-6} (pK = 5.70). The constant on the acid side falls within the range reported for the beta carboxyl of aspartic acid (pK = 3.0 to 4.7); that on the alkaline side in the range for the imidazole nitrogen of histidine (pK = 5.6 to 7.0) (4).

The pH optimum for A_{III} is 4.55 and the midpoints on the acid and alkaline sides of the curve are respectively pH 3.55 and pH 5.76 (Fig. 5). The ionization constants for the active form of the enzyme-substrate complex, calculated from these data, are 1.71×10^{-4} (pK = 3.77) and 4.65×10^{-6} (pK = 5.33) respectively. The constant on the acid side falls within the range reported for the beta carboxyl of aspartic acid (pK = 3.0 to 4.7); that on the alkaline side does not fall into the range published for common amino acid side groups (4).

The pH-activity curve for A_{II} in the presence of GSH is shown in Fig. 5. The optimum appears very close to pH 4.55. The curve does not cover a sufficient range to determine the midpoint on the acid side, but that on the alkaline side can be estimated to fall at pH 5.84. If the pK for the acid side is assumed to be the same as that for A_{III} (note that the values for the acid sides of A_I and A_{III} are almost equal), the ionization constant for the alkaline side of A_{II} is 4.65×10^{-6} (pK = 5.33), which is identical with that found for A_{III}.

Discussion

A combination of ammonium sulfate fractionation and dialysis yielded what appeared to be three endo-beta-glucanases from a sodium chloride extract of green malt. The fractions were labeled A_I, A_{II}, and A_{III} on the basis of their solubilities in ammonium sulfate.

A_I exhibited heat stability and solubility in ammonium sulfate identical to the enzyme designated A_L in an earlier publication (7). The enzyme appeared essentially free of other endo-beta-glucanase enzymes as reflected by the kinetics of its heat inactivation. A_I rapidly decreased the viscosity of barley beta-glucan solutions but had no effect on the viscosity of carboxymethyl cellulose solutions.[4] Although this might lead to the speculation that the enzyme is specific for the beta-1,3-linkages, such a conclusion is not warranted at this time. It is possible that an enzyme able to attack beta-1,4-linkages in barley beta-glucan might not be able to attack beta-1,4-linkages in carboxy-

[4] Luchsinger, W. W.: unpublished results.

methyl cellulose. For example, the presence of the carboxymethyl groups might make the polymer resistant, or the enzyme might require the configuration resulting from an adjacent beta-1,3-linkage.

The apparent ionization constants of the active form of A_I suggest that aspartic acid and histidine are involved in the active site of the enzyme.

A_{II} seemed to contain two endo-beta-glucanase activities, one of which was rapidly inactivated even at ice-bath temperatures when the enzyme was diluted. The second activity appeared to be relatively stable under these conditions (Table I). The lability of the enzyme to dilution could be counteracted by the presence of GSH, cysteine, substrate, and even sodium chloride. Although it prevented inactivation of the enzyme, GSH did not effect any significant reactivation of the enzyme. In the presence of GSH the stability of A_{II} at 40°C. approached that of the quite stable activity A_{III}. In addition, GSH had no effect on the heat-stability of either A_I or A_{III} (Fig. 3). In view of these considerations and the similarity of the pH curve of A_{II} stabilized with GSH to that of A_{III}, it seems possible that A_{II} might be a labile form of A_{III}. Some stabilizing component might have been lost during purification, since, even though neither enzyme can be considered pure, the specific activity of A_{II} is several times greater than that of A_{III}. A_{II} had a relative activity of 125/mg. solids as compared to 33/mg. solids for A_{III}. In addition, probably more than half the activity of A_{II} was lost before its instability was recognized. Based on a preliminary assay of A_{II}, the figure would be approximately 250/mg. solids. The estimates of the solids present were made by freeze-drying portions of the enzyme solutions.

The cause for the inactivation of A_{II} upon dilution is not clear. GSH prevented its inactivation but did not effect any significant reactivation which might have been expected if maintenance of a free –SH group were involved. The possibility that GSH acted by binding heavy metals seems doubtful in view of the protection afforded by substrate and sodium chloride. Furthermore, the distilled water used for dilution in Table I had been passed through ion-exchange resins in the acid and base forms, which would have removed essentially all cations as well as anions.

Another possibility might be stabilization due to the ionic strength. Undiluted A_{II}, even after dialysis, contains considerable solids, and is reasonably stable until it is diluted (one-half its activity lost in about 2 hours at 40°C.). Furthermore, the specific activity of A_{III} is much lower than A_{II}, which means that if, when assayed, it were diluted to a solids content equal to that of A_{II} when assayed,

the activity would be below the sensitivity of the procedure. Thus under truly similar conditions A_{III} might prove unstable.

A similar response to dilution was observed with the original malt extracts. A tenfold dilution with distilled water decreased the over-all stability of the endo-beta-glucanases at 40°C. On the other hand, a tenfold dilution with 1% by weight ammonium sulfate solution effected a slight increase in the over-all stability (6).

A_{III} exhibited heat-stability identical to that of the enzyme designated A_S in an earlier publication (7). It appeared free of other endo-beta-glucanases as reflected by the kinetics of its heat-inactivation at 40°C. The preparation exhibited a low level of cellulase activity which was not affected by heating for 5.25 hours at 40°C. These results agree with earlier evidence that neither A_I nor A_{III} is identical with malt cellulase (7). The apparent ionization constant of the active form of the enzyme on the acid side of the pH curve suggests the involvement of aspartic acid in the active site. The apparent ionization constant of the alkaline side does not correspond to published values for any of the common amino acids. This might result from the presence of an atypical group, the interaction of the side groups of two amino acids, or a second active form of the enzyme. It might also be that the pH curve is an artifact of the isolation and purification procedure. The enzyme may have been disrupted in such a manner as to change its characteristics but not cause complete inactivation.

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