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Kinetic Studies on Strawberry Anthocyanin Hydrolysis by a Thermostable Anthocyanin-β-Glycosidase from *Aspergillus niger*

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ABSTRACT

Several kinetic characteristics of a thermostable anthocyanin-β-glycosidase from Aspergillus niger *have been evaluated. With strawberry anthocyanins as substrate, at pH optimum (4·0) and* t = 30°C, K_m *was found to be* 123 ± 4 μM *and* V_{max}, 1·16 ± 0·06 μmol min^{-1} mg^{-1} *protein. Temperature optimum was observed at about 68°C. The apparent energy of activation was calculated to be* 11 ± 1 kcal/mol. *The inhibitory effect of different sugars and sugar derivatives was examined. Glucono-delta-lactone* (K_i = 2·3 ± 0·1 μM), *gluconic acid* (K_i = 82 ± 2 μM) *and glucose* (K_i = 1·3 ± 0·1 mM) *appeared to be competitive inhibitors of this enzyme.*

INTRODUCTION

Anthocyanins are exposed to several breakdown mechanisms when berries are submitted to the process of juice or jam production. Tantschev & Jontschewa (1975) showed that heat damage of anthocyanins proceeds by a first-order reaction. The thawing process has been studied by Skrede (1981) and the influence of pectolytic enzyme preparations on anthocyanin stability by Blom & Juul (1982).

In production experiments with cherry nectar (Blom & Juul, 1982) we observed that anthocyanins were damaged during pectolytic enzyme treatment with a commercial enzyme preparation from *Aspergillus niger*. Recently, it has been shown (Blom, 1983) that this decomposition was

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due to a contaminating anthocyanin-β-glycosidic activity present in the enzyme preparation.

The action of anthocyanin-degrading enzymes has been described by several authors. Anthocyanase from *Aspergillus niger* has been studied by Huang (1955, 1956), Harborne (1965) and Forsyth & Quesnel (1957), and polyphenoloxidase from *Prunus avium* by Pifferi *et al.* (1979). However, more information is needed in order to judge the significance of these enzymes in the course of the production.

The present work was undertaken to establish the possible rôle of anthocyanin-β-glycosidase in anthocyanin breakdown during the processing of berries. Furthermore, the kinetics and the inhibitory effect of various sugars and sugar derivatives on anthocyanin-β-glycosidase have been studied in order to evaluate the possibility of using these as inhibitors during production.

MATERIALS AND METHODS

Enzyme purification

Preparation of anthocyanin-β-glycosidase was carried out as described by Blom (1983).

Preparation of strawberry anthocyanins

Strawberries (*Senga sengana*) (300 g) were mixed and homogenized in 300 ml rectified ethanol (96 %) made 0·1 N with concentrated HCl. The mixture was filtered through a filtering cloth. The filtrate was concentrated 10 × in a rotavapor, followed by extraction with ethylacetate (3 ×) and petroleum ether (3 ×) to remove non-polar constituents. The glucose present was precipitated at −10 °C by adding 96 % rectified ethanol to a final concentration of 85 %. The sample was centrifuged and the ethanol removed by evaporation. This procedure was performed twice. The resulting anthocyanin stock solution was stored in a refrigerator under N_2 atmosphere for not more than a week.

Before enzyme assay was undertaken, the stock solution of anthocyanin was checked for glucose and aglucones by using HPLC according to the procedure described by Blom (1983) and that of the Bio-Rad Laboratories (1979), respectively. If the glucose content exceeded 5 mM

(corresponding to a maximum 0·2 mM glucose in the reaction solution), the anthocyanin stock solution was discarded.

Enzyme assay

Standard enzyme activity measurements were performed in 0·1 M citrate–HCl buffer (pH 4·0) at 30 °C.

The anthocyanin concentrations were in the range 10 to 400 μM, corresponding to 20–800 μl of the anthocyanin stock solution per 20 ml of reaction solution. The assay was carried out in 1-cm cuvettes at 515 nm using 7 μg enzyme per millilitre of reaction volume. The decolorization was read relative to a blank sample containing the ingredients of the test cuvette without the addition of enzyme solution. A Shimadzu Spectrophotometer, model UV-300 (Shimadzu Seisakusho Ltd, Kyoto, Japan) was used. The scale expansion corresponded to 0·01 A/full scale (noise $\pm$ 0·0001 A). The temperature within the cuvettes was maintained using a water bath with circulation through the cuvette house.

Anthocyanin quantitation

The extinction coefficient used in the calculations was extracted from the work of Ribérau-Gayon (1972) (p. 142).

Inhibition of enzyme activity by different sugars and sugar derivatives

Sucrose, glucose, fructose, sorbitol, rhamnose, glucuronic acid, gluconic acid, glucono-delta-lactone and ascorbic acid were examined with regard to potential enzyme inhibitory effect.

The experiments were performed by dissolving the sugars and sugar derivatives at different concentrations in the assay buffer, adding substrate and accomplishing standard enzyme activity measurements.

Protein quantitation

The protein concentration was determined by a modified biuret reaction according to Yonetani (1961), using bovine serum albumin as standard.

THEORETICAL CONSIDERATIONS

The breakdown of anthocyanins by this particular enzyme is believed to proceed as described in Fig. 1. The reaction products using pelargonidin-3-monoglucoside are mainly pelargonidin and glucose. Pelargonidin is

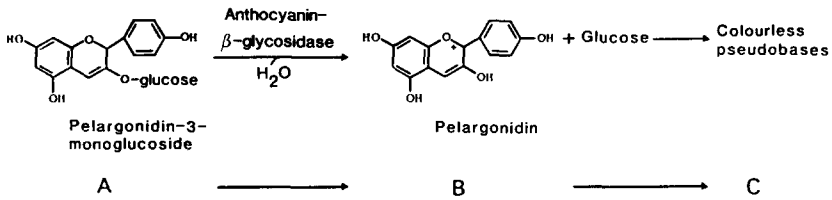

Fig. 1. Enzymatic degradation of pelargonidin-3-monoglucoside to colourless pseudobases (Eskin, 1979).

unstable and is readily converted to colourless compounds, while the glucose is stable and thus may produce some kind of feedback inhibition. Due to instability, it has not been possible to establish whether or not pelargonidin has any such properties.

Figure 2 shows a typical trace at standard enzyme assay conditions. The theoretical considerations in the calculations are based on the following assumptions:

(1) The reverse reactions from aglycone (B) (Fig. 1) to glycone (A) and from colourless compound (C) to aglycone (B) are regarded as insignificant.

$$-\frac{d[A]}{[A]} \ll -\frac{d[B]}{[B]}$$

(2) The total absorbancy of the solution is always due to [A] + [B],

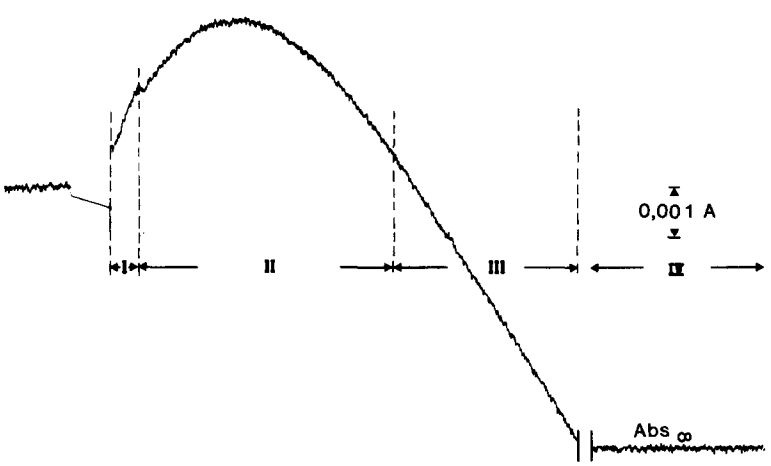

Fig. 2. Typical trace from standard enzyme assay. Four separate phases are indicated.

where the molar extinction coefficient of A is lower than that of B (Ribéreau-Gayon, 1972) (page 154).

(3) When $[A] > 95\%\,[A_0]$, the kinetics of the reaction may be regarded as initial (Segal, 1975).

The overall changes in our reaction solution may then be written as:

$$\frac{dB}{dt} = -\frac{dA}{dt} - \frac{dC}{dt} \tag{1}$$

The reaction sequence, as monitored from absorbancy at 515 nm, could be divided into four distinct phases, as indicated in Fig. 2.

In phase I, a temporary linear increase in absorbancy is observed. [B] is close to zero and, consequently:

$$\frac{dC}{dt} \simeq 0$$

$$-\frac{dA}{dt} = \frac{dB}{dt} \tag{2}$$

[B] is increasing at a rate corresponding to $-dA/dt$.

Since A is converted to B, which has a higher molar extinction coefficient at 515 nm, the initial kinetics of the immediate increase in absorption could be used as a direct measurement of the enzyme activity.

In phase II, $dC/dt \neq 0$. Assuming that $-dA/dt$ is constant and $dC/dt < dA/dt$, eqn. (1) gives a complete description of the reaction.

Consequently [B] is increasing due to generation from $-dA/dt$, while dB/dt is decreasing and the slope of the absorbancy is falling. In this phase, the colorization probably follows second-order kinetics and the total change in absorbancy is thus of a parabolic nature.

In phase III [B] has reached a constant level, i.e. $dB/dt = 0$. From eqn. (1):

$$-\frac{dA}{dt} = \frac{dC}{dt} \tag{3}$$

The contribution of [B] to the total absorbancy of the solution is constant and the linear change in absorbancy may be regarded as a conversion of A to C, depending only on $-dA/dt$, which will be the direct expression of the enzyme activity.

The three phases described usually take place within 10 min from enzyme addition.

Phase IV occurs after a considerable amount of time has elapsed. In this phase we still have a steady-state condition, but lack of substrate, and possible end-product inhibition, slow down the reaction velocity; $-dA/dt$ is decreasing and consequently dB/dt and dC/dt also decrease. The absorbance is changing asymptotically towards an $Abs._{\infty}$ value.

The theoretical considerations outlined above correspond with those reported by Huang (1956), except that, in the latter, calculations were made assuming equal molar extinction coefficients of compounds A

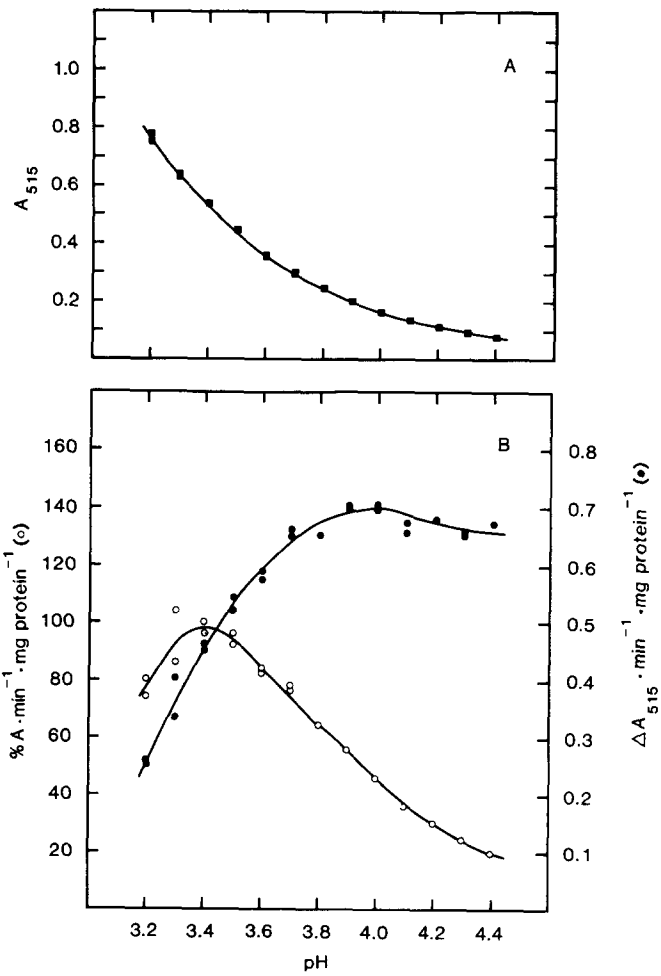

Fig. 3. Absorbance (A_{515}) in anthocyanin substrate solution (A) and enzyme activity (B) as a function of pH. ○, Relative activity; ●, absolute activity.

and B. Huang (1956) also noted that the loss of color from 0–10 min was significantly less than that from 10–20 min, an observation in good agreement with the decolorizing reaction described in Fig. 2—phases I and II.

RESULTS AND DISCUSSION

On the basis of the theoretical considerations outlined in the previous paragraph, the phase III data were chosen to evaluate the kinetic characteristics of the enzyme.

The results presented in Fig. 3B illustrate the effect of pH on the decolorizing activity. When the absolute loss of absorbance is considered, the activity is highest at pH 3·4, while, on the basis of a percentage loss in absorbance (Fig. 3A), the reaction is most rapid at pH 3·9–4·0 (Fig. 3B). These results are in good agreement with the observations made by Huang (1956) on a crude aspergilli-derived enzyme preparation. The standard assay was performed at pH 4·0, although pH values from 3·7 to 4·4 could have been used without significant loss of enzyme activity.

A temperature optimum was observed at 68 °C (Fig. 4) which may relate to the thermophilic nature of this particular *Aspergillus niger* strain (E. Grampp, Röhm GmbH, Darmstadt, West Germany, pers. comm.).

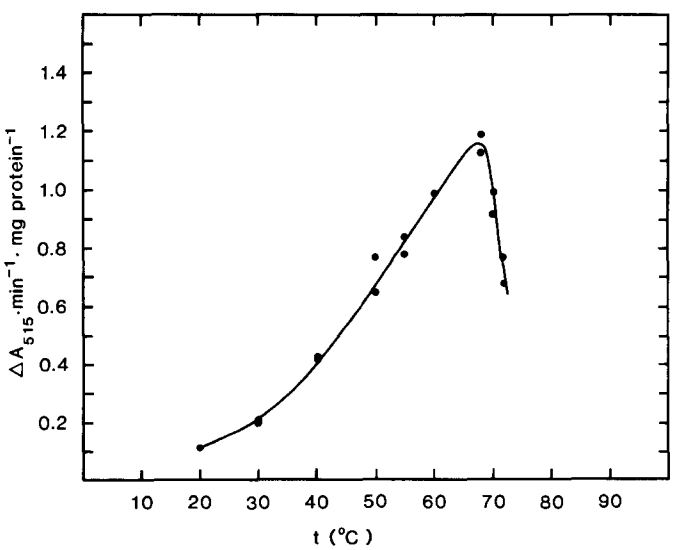

Fig. 4. Enzyme activity as a function of temperature (pH 4·0).

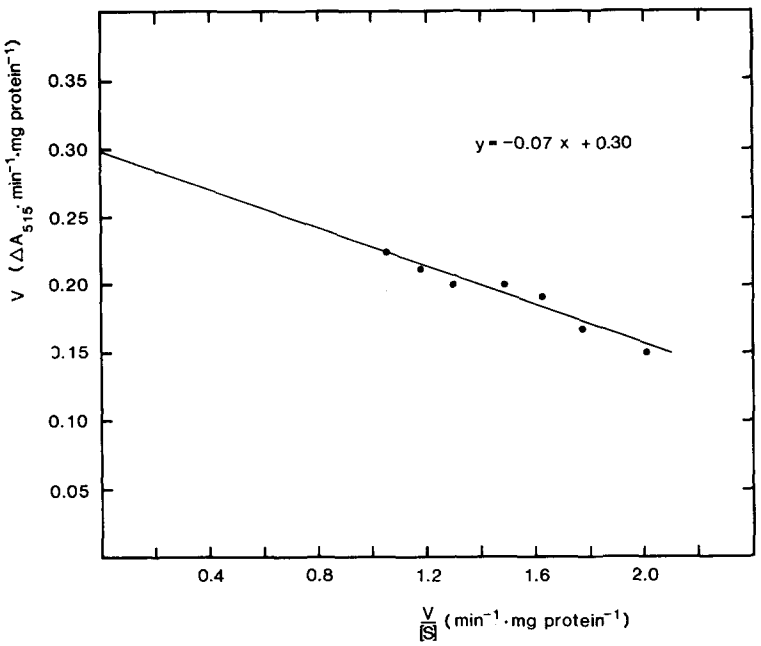

Fig. 5. Woolf-Augustinsson-Hofstee plot of data derived from standard enzyme assay.

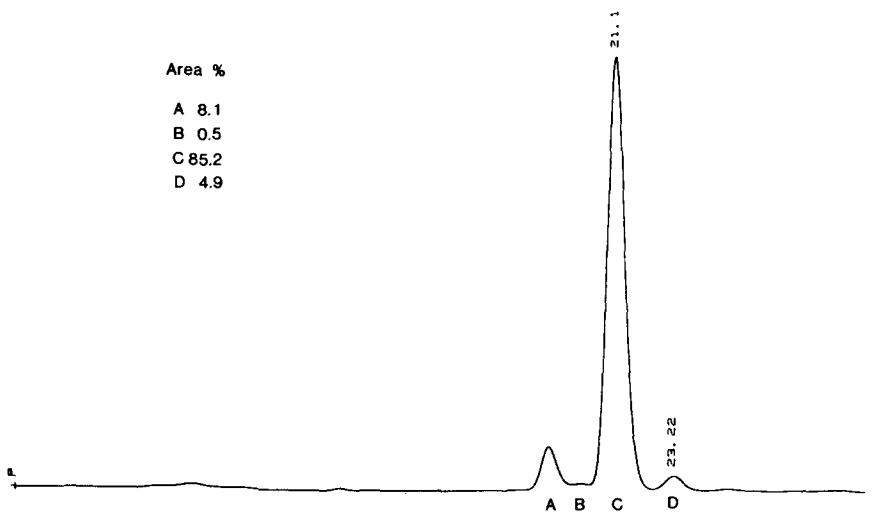

Fig. 6. HPLC chromatogram of substrate anthocyanins. Separation conditions essentially as described by Blom (1983).

At pH 3·2—a pH value frequently encountered in blackcurrant mash (Blom & Skrede, 1984), the temperature optimum was slightly lower (61 °C), but still well above the temperatures actually used during pectolytic enzyme treatment (about 50 °C) before juice production.

At pH 4·0, $t = 30$ °C, a K_m corresponding to 0·0984 ± 0·0016 units of absorbance at 515 nm and a V_{max} corresponding to a ΔA_{515} of 0·31 ± 0·02 units per minute per milligram of protein was obtained (Fig. 5).

Adapting the extinction coefficient $E_{510}^{1\,\mathrm{Mlcm}} = 1000$ of anthocyanins in a citrate-buffered solution at pH 3·9 reported by Ribérau-Gayon (1972) and the effect of pH on the absorbance of the substrate solution shown in Fig. 3A, these values correspond to 123 ± 4 μM and 1·16 ± 0·06 μmol/min per milligram of protein, respectively. The K_m value is in good agreement with that (0·2 mM) reported by Huang (1956) using chrysanthemin chloride (cyanidin-3-monoglucoside) isolated from blackberry as substrate. In strawberries, pelargonidin-3β-monoglucoside is the major anthocyanin (Fuleki, 1969) and, as shown in Fig. 6C, comprises more than 85 % of the anthocyanin content of the substrate used in the present study.

The apparent energy of activation, E_a, was calculated inserting data from 30 °C (T_1) and 50 °C (T_2) into the integrated form of the Arrhenius equation:

$$\log \frac{V_{max} T_2}{V_{max} T_1} = \frac{E_a}{2{\cdot}3R} \frac{T_2 - T_1}{T_2 \times T_1}$$

giving:

$$E_a = 11 \pm 1 \text{ kcal/mol}$$

Experiments were performed to evaluate the potential inhibitory effect of sugars and sugar derivatives. Of the substances tested, glucose, gluconic acid and glucono-delta-lactone appeared to be competitive inhibitors (Fig. 7).

The inhibitor constants, as calculated from Lineweaver–Burke reciprocal plots, where $K_{m\text{apparent}} = K_m(1 + [I]/K_i)$ were:

K_i glucono-delta-lactone	2·3 ± 0·1 μM
K_i gluconic acid	82 ± 2 μM
K_i glucose	1·3 ± 0·1 mM

The results are calculated from five separate experiments.

The K_i for glucose is in good agreement with Huang's (1956) previously observed value (1·25 mM). To the best of our knowledge, the inhibitory

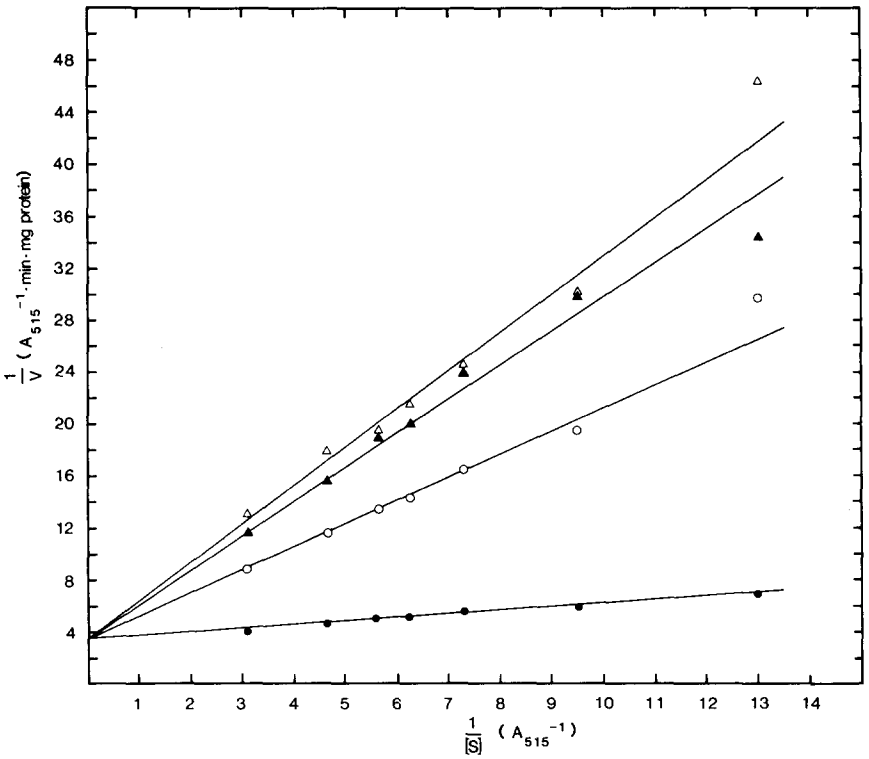

Fig. 7. Lineweaver–Burke reciprocal plot of data derived from standard enzyme assay experiments with different inhibitors. ●, Standard assay without addition of inhibitors. ○, With 5·6 mM glucose. ▲, With 0·5 mM gluconic acid. △, With 20 μM glucono-delta-lactone.

effects of gluconic acid and glucono-delta-lactone observed in the present study have not been reported on previously. However, competitive inhibition of various glycosidases by aldonolactones corresponding in configuration to the substrates has been described (Conchie & Levvy, 1957). The K_i for glucono-delta-lactone obtained in the current study may probably be even lower than indicated, since this molecule is readily converted to gluconic acid (Levvy *et al.*, 1964), which is less inhibitory.

Sucrose, fructose, sorbitol, rhamnose, glucuronic acid and ascorbic acid did not exhibit any inhibitory action other than a decrease of the enzyme activity, probably due to an increase in viscosity, which could be detected when using concentrations of 20–50% of the sugars/sugar derivatives in question.

A consequence of a competitive inhibition is that, at high substrate to

inhibitor concentration ratios, the velocity is almost unaffected by the inhibitor. Glucose is not a strong inhibitor and may, at the substrate concentrations found in anthocyanin-rich berries, be without any significant inhibitory action against this particular enzyme activity.

From our enzyme source—Pectinol D, produced in 1976—we usually gain 7 mg anthocyanin-β-glycosidase per gram of pectolytic enzyme preparation. Using this enzyme preparation on berries with a high anthocyanin content (about 3 mM in blackcurrants), 1 g of pectolytic enzyme preparation will demolish close to 8 μmole of anthocyanin each minute, even though glucose is present, often in per cent quantities.

The strong inhibitory effect of glucono-delta-lactone may prove to be of practical importance. A substrate concentration accomplishing a significant competition to even small amounts of this inhibitor would have to be incredibly high. Theoretically, an addition of glucono-delta-lactone (10–20 g/1000 kg of berries), together with gluconic acid (100–200 g/1000 kg of berries), would accomplish a total inhibition of this *Aspergillus niger* anthocyanin-β-glycosidase, due to the high competitive power of glucono-delta-lactone.

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