Phytochemistry, Vol. 23, No. 12, pp. 2949–2950, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

SHORT REPORTS

INTERFERENCE BY EDTA AND CALCIUM IONS OF THE 3,5-DINITROSALICYLATE REDUCING SUGAR ASSAY

DAVID W. M. LEUNG* and TREVOR A. THORPE†

Plant Physiology Research Group, Department of Biology, University of Calgary, Calgary, Alberta, Canada T2N 1N4

(*Revised received* 23 *April* 1984)

Key Word Index—EDTA; calcium ions; 3,5-dinitrosalicylate reducing sugar assay; amylases.

Abstract—The ability of EDTA and calcium ions to interfere with the 3,5-dinitrosalicylate reducing sugar assay is reported. The antagonistic effects of Ca^{2+} and EDTA are evaluated.

INTRODUCTION

Generally, α-amylases are different from β-amylases in that the α-type is sensitive towards EDTA and requires Ca^{2+} ions for stability [1]. Using these two criteria, many studies with plant extracts have demonstrated the presence of α-amylase as a component of the total amylase activity. This is measured via the production of reducing sugars from a starch substrate, using the 3,5-dinitrosalicylate (DNS) reagent (e.g. [2–4]). In this paper, we show that measurements of reducing sugars using the DNS reagent are subject to interference by various concentrations of EDTA, including those normally used to inhibit α-amylases. Additionally, we have confirmed that the reducing sugar assay is also subject to interference by calcium chloride [5], and further we show that this effect of Ca^{2+} is antagonized by EDTA.

RESULTS AND DISCUSSION

The production of reducing groups from amylolysis of starch is most frequently followed by the 3,5-dinitrosalicylate (DNS) method, since it is the simplest assay to use [6]. This method, however, may be subject to interference by substances which are commonly used in amylase assays and not removed prior to measurements of the reducing sugars, using the DNS reagent [7]. In the course of our studies on α-amylase from tobacco callus, we used several inhibitors of well-characterized plant α-amylases. We observed interference by EDTA (9 mM), a particularly well-known inhibitor of α-amylases, with the measurement of various concentrations of an authentic reducing sugar, namely maltose, using DNS, although linearity between concentration and absorbance was maintained (Table 1). EDTA has, however, no apparent effect on the background absorbance of the DNS reagent in the absence of the sugar. Thus the artefactual problem of using EDTA can create a severe limitation for discriminating between α- and β-amylase activities present in plant extracts.

Based on this sensitivity to EDTA, we decided to determine whether concentrations of EDTA, in the range of 1–5 mM, which are commonly used to inactivate α-amylases, can also reduce the colour development between DNS and maltose artificially. In fact, we found that as little as 2 μM EDTA could interfere with the measurement of the reducing sugar and this interference increased with the concentrations of EDTA (Table 2). At the highest concentrations tested (45 mM), complete inhibition of colour development was observed. The inhibitory effect of EDTA was still observed when the DNS reagent was allowed to pre-incubate for 15 min with the reducing sugar solution before addition of EDTA. Prolonged heating, for example, 10 min instead of the normal 5 min for colour development (see Experimental), did not eliminate the interference by EDTA. With other reducing sugars, for instance galactose, similar interference by EDTA with the DNS reaction was observed. By contrast, EDTA at concentrations up to 5 mM had no effect on the reducing sugar assay using Nelson's reagents [8].

The production of maltose by a purified β-amylase

Table 1. Effect of EDTA (9 mM) on the reaction between DNS and various amounts of maltose

	A_{540nm}	
Maltose (μg)	−EDTA	+EDTA
0 (water control)	0.012	0.013
90	0.067	0.039
180	0.133	0.078
360	0.273	0.163
720	0.518	0.283

*Present address: Centro de Genetica, Colegio de Postgraduados, Chapingo, Mexico 56230.
†To whom correspondence should be addressed.

Table 2. Effect of various concentrations of EDTA on the reaction between DNS and maltose (360 μg)*

Sugar	EDTA (mM)	A_{540nm}
None	None	0.010
+	None	0.270
+	0.002	0.240
+	0.020	0.221
+	1.125	0.190
+	5.550	0.176
+	9.000	0.158
+	45.000	0.010

*EDTA was dissolved in sodium acetate buffer (pH 5), but the buffer alone had no effect on the DNS reaction.

Table 3. Effect of calcium chloride (2.78 mM), or EDTA at various concentrations or both on the reaction of DNS with reducing sugars (maltose 360 μg)*

Assay	$CaCl_2$	EDTA (mM)	A_{540nm}
I. 1	–	–	0.265
2	+	–	0.396
II. 3	–	1.11	0.202
4	–	2.22	0.197
5	–	3.33	0.187
III. 6	+	1.11	0.370
7	+	2.22	0.312
8	+	3.33	0.217

*Calcium chloride had no effect on the blank, i.e. reaction mixture minus maltose.

from sweet potato (no detectable β-amylase activity) acting on amylose appeared to be quite sensitive to 5 mM EDTA, though it is well established that such purified α-amylase has no interaction with EDTA [7]. However, this apparently anomalous situation could be explained on the basis of the interference of EDTA with the reducing sugar assay using DNS. Therefore, due caution is necessary in the interpretation of other data concerning the specific inhibition of α-amylase by EDTA in the presence of β-amylase in plant extracts.

Ca^{2+} ions used to stabilize amylases are also known to interfere with the reaction between reducing sugars and DNS [5]. We have also observed the interference resulting in an abnormally higher absorbance with a particular concentration of maltose in the presence of calcium chloride (Table 3, I, assays 1 and 2). Since we have already shown that the interference of EDTA is to decrease the absorbance of a reducing sugar solution with DNS, here we tried to determine if Ca^{2+} and EDTA would antagonize each other in terms of their effects on the reducing sugar assay. The data are shown in Table 3, II and III. By comparing I with any pair of assays, namely 3 and 6, or 4 and 7, or 5 and 8, it is clear that 2.78 mM calcium chloride antagonized the effect of EDTA at various concentrations in the reducing sugar assay. This finding could explain the apparent anomalous observation reported at times in the literature that the inhibition of amylase activity (based on measurements of new reducing sugars produced using DNS) by EDTA could be partially reversed by the addition of calcium chloride (for examples, see [4, 9]).

The basis for the interference caused by EDTA or calcium chloride is not clear. In a preliminary experiment, we found that citrate at 0.05 M and pH 5, another divalent ion-chelating agent like EDTA, did not cause any interference with the DNS reducing sugar assay, although it resembles EDTA in being a potent inhibitor of the α-amylase activity of pea cotyledons [1].

EXPERIMENTAL

Reducing sugar assays. Reducing sugars were measured using the alkaline 3,5-dinitrosalicylate (DNS) reagent [6]. The assay mixture consisted of 0.2 ml DNS reagent and 0.7 ml reducing sugar soln (maltose as standard), with or without other reagents as indicated in the appropriate tables. This mixture was boiled at 95 ± 2° for 5 min, allowed to cool, diluted with 2 ml deionized H_2O, and read at 540 nm.

Enzyme assay. 0.2 ml purified β-amylase (2.5 mg/ml, α-amylase-free, Calbiochem) in NaOAc buffer, pH 5 [10] was incubated with 0.2 ml amylose (2.5 mg/ml) in the NaOAc buffer and 0.3 ml NaOAc buffer with or without EDTA (5 mM) at 37° for 10 min. The reaction was stopped by the addition of 0.2 ml DNS reagent, and the reducing sugar content was then determined. For the enzyme controls, the enzyme and the substrate were incubated separately at 37° for 10 min and then the DNS reagent was added, followed by the enzyme.

Acknowledgement—This work was supported by NSERC of Canada, Grant No. A-6467 to T. A. Thorpe.

REFERENCES

1. Swain, R. R. and Dekker, E. E. (1966) *Biochim. Biophys. Acta* **122**, 75.
2. Tarrago, J. F. and Nicolas, G. (1976) *Plant Physiol.* **58**, 618.
3. Davis, B. D. (1979) *Plant Physiol.* **63**, 1010.
4. Koshiba, T. and Minamikawa, T. (1981) *Plant Cell Physiol.* **22**, 979.
5. Robyt, J. R. and Whelan, W. J. (1965) *Biochem. J.* **95**, 10P.
6. Bernfeld, P. (1955) *Methods Enzymol.* **1**, 149.
7. Thoma, J. A., Spradlin, J. E. and Dygert, S. (1971) in *The Enzymes* (Boyer, P. D., ed.), Vol. 5, pp. 115–189. Academic Press, New York.
8. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
9. Hsiu, J., Fischer, E. H. and Stein, E. A. (1964) *Biochemistry* **3**, 61.
10. Gomori, G. (1955) *Methods Enzymol.* **1**, 138.