8. Per² combines also with methylcarbylamine, forming a compound very similar to the carbylamine compounds of Hb and Mb.

9. Per²CN undergoes a reversible photodissociation which can easily be followed by direct micro-spectroscopic observation. The light sensitivity of this compound is of special interest considering that hitherto the only compounds of haems known to be light-sensitive were their CO compounds.

10. A method is described for quantitative spectrophotometric study of the kinetics of reversible dissociation of per²CN. The photodissociation was effected by means of Hg-blue light the wavelength of which (435.8 mμ.) lies close to the intense γ-band of per²CN.

11. Examination of MbCN by the same method revealed a less marked but nevertheless distinct light sensitivity.

12. An attempt to calculate, from the spectrophotometric experiments, the quantum efficiency in photodissociation of per²CN gave an approximate value of $\phi = 0.8$ (moles per²CN dissociated per mole light quantum absorbed). In view of the uncertainties in the calculation this result indicates only that the efficiency is of the order of 1.

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Transfructosidase from *Agave vera cruz* Mill

By I. S. BHATIA, M. N. SATYANARAYANA AND M. SRINIVASAN

Division of Quality Control, Central Food Technological Research Institute, Mysore

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The nature and distribution of carbohydrates in *Agave vera cruz* have been reported on by Srinivasan & Bhatia (1953, 1954a). The isolation and properties of an enzyme present in the stem of this plant, capable of building glucofructosans from sucrose, are described in this paper. So far, the only other plant sources shown to contain such an enzyme are Jerusalem artichoke (Edelman & Bacon, 1950, 1951; Dedonder, 1952) and *Polianthes tuberosa* (Bhatia & Srinivasan, 1954), the latter yet another glucofructosan-bearing material (Srinivasan & Bhatia, 1954b).

EXPERIMENTAL

Crude extract. The outer skin was scraped from an agave stem (see Srinivasan & Bhatia, 1953) and the bottom and top ends (3 in. each) were discarded. The remaining stem was then sliced. Some slices representing different sections of the stem were used for examination of microflora. The rest of the slices were treated with CHCl₃-water, grated and pressed through cloth. The pressed juice was centrifuged (2000 rev./min., 20 min.) and filtered. The filtrate, hereafter referred to as the crude extract, was stored over CHCl₃ in the cold room (3°). The slices, gratings and the crude extract were found to be free from microflora, when tested as follows.

Slices (in the form of cubes about 0.5 in. side) and gratings were dipped successively for 1 min. in 90% ethanol, sterile distilled water, 0.01% HgCl_2 , and washed in sterile distilled water. The material was then transferred to potato-dextrose-agar and nutrient-agar Petri dishes under aseptic conditions in an inoculation chamber and incubated at room temp. (26–28°) for 96 hr. At the end of this period, no microbial growth was observed in any of the media. The crude extract (1 ml.) as such or after dilution with sterile distilled water (1:10, 1:100, 1:1000), plated as above and similarly incubated, gave no growth.

Enzyme enrichment. Precipitates obtained from the crude extract with ethanol (30, 50 and 70%, v/v, final ethanol concentration) did not show any significant activity. On the other hand, with the use of ammonium sulphate, an enriched enzyme preparation could be obtained thus.

$(\text{NH}_4)_2\text{SO}_4$ (30 g./100 ml.) was dissolved in 2.5 l. of the crude extract, previously chilled to 3°, and the solution allowed to stand in the cold room (3°) for about 4 hr. The precipitate which formed had little enzyme activity and was centrifuged off (2000 rev./min., 30 min.). A further quantity of $(\text{NH}_4)_2\text{SO}_4$ (15 g./100 ml. original volume) was dissolved in the clear supernatant. The solution was left overnight at 3° to complete precipitation and filtered. The inactive filtrate was discarded and the precipitate was washed with 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ till the washings gave no reaction to Seliwanoff's test. The washed precipitate was dissolved in 250 ml. distilled water and filtered. The filtrate, which was found to be free from microflora (by plating), formed the stock enzyme solution. This, with CHCl_3 added, was stored at 3°. Portions were dialysed as and when required.

Dialysis. Dialysis of the stock enzyme solution was carried out in the presence of CHCl_3 in a cellophan bag, against repeated changes of distilled water (3°), till free from SO_4^{2-} . Dialysis of 100 ml. solution took about 36 hr. for completion and was accompanied by a volume increase of about 50%. A small precipitate appearing during dialysis was filtered off. The resulting filtrate was used as the enzyme. Its activity in comparison with the crude extract or quantity recovered in the final preparation was not determined.

Incubation procedure. AnalaR sucrose (25 g.) was dissolved in and made up to 100 ml. with dialysed enzyme solution. The reaction mixture and the boiled control, in the presence of CHCl_3 , were incubated at room temp. (26–28°). The system at the end of the incubation period (96 hr.) was found to be free from microflora. Samples (10 ml.) drawn at known intervals, into small test tubes were heated in a boiling-water bath for 5 min. to inactivate the enzyme. The inactivated samples were cooled and filtered. The filtrates, with CHCl_3 added to each, were stored in the cold room (3°), until taken for use in the following experiments.

Optical rotation. This was measured in a 2 dm. tube on each of the above samples stored for 16 hr.

Chromatography. (a) *Qualitative.* Reaction mixture at known time intervals was spotted on a filter sheet (Whatman no. 1), the starting line having been previously streaked with 0.01 M- HgCl_2 solution and dried (Bealing & Bacon, 1953). A mixture of fructose, glucose and sucrose as reference and the control (sucrose + boiled enzyme) were also spotted. The paper was irrigated with *n*-butanol-acetic acid-water (4:1:5, by vol.) mixture (Partridge, 1948) by the ascending technique for 48 hr. Finally, the dried paper was sprayed with benzidine-trichloroacetic acid (Bacon &

Edelman, 1951). (b) *Quantitative.* Using a calibrated capillary tube, 15.6 $\mu\text{l.}$ of sample for each time interval were spotted in sextuplicate on a filter sheet (Whatman no. 1), which was then developed by descending flow of the butanol-acetic solvent (triple run, 16 hr. for each run). After locating the separated carbohydrates on a guide with benzidine-trichloroacetic acid spray, the corresponding areas of the unsprayed chromatograms were marked and cut.

Methods for the determination of carbohydrates. (a) *In the reaction mixture.* Free reducing sugars, expressed as glucose, were determined by the method evolved by Hagedorn & Jensen and then Hanes, as modified by Sobotka & Reiner (1930), and glucose by the hypiodite method of Klein & Acree (1930).

(b) *In the cut areas of paper carrying the separated carbohydrates.* The carbohydrates were extracted as described previously by Srinivasan & Bhatia (1954a). Equivalent empty areas were used as blanks. The average paper areas of spot 4, sucrose, glucose and fructose were approximately 22, 19, 17, 11 cm.^2 . As spot 5 (formed after 52 hr.) was faint in intensity, this was combined with spot 4 for analysis. Because of the variation in the carbohydrate content of each spot with time (decrease in sucrose, increase in glucose and oligosaccharides), the volume of water used for extraction was also varied in direct proportion to the carbohydrate content. The amount of total reducing sugars in 1 ml. of the filtered extract were determined by the anthrone method (Yemm & Willis, 1954), using a Beckman spectrophotometer (620 $\text{m}\mu$). The determination was carried out in triplicate, and the variation in optical density between the lowest and highest in any set did not exceed 3%. The blank values for paper, corrected for areas, were deducted from the corresponding experimental results. For 1 cm.^2 of the paper, the average blank value for the colour with anthrone reagent was equivalent to 0.38 $\mu\text{g.}$ sucrose, determined spectrophotometrically. From the net optical densities of the test extract and of the reference standard which was included and determined along with each sugar being estimated, the carbohydrate content in the unknown was calculated. Sucrose was used as the standard for carbohydrates with R_F values lower than that of sucrose. The sugar standard in every case was so chosen that the optical density of the known and the unknown did not differ by more than 5%. The recovery of total carbohydrates (sum of spot contents for each time interval) was 98–104% of the concentration of sucrose employed. Fructose in spot 4 was determined spectrophotometrically (520 $\text{m}\mu$, Roe, Epstein & Goldstein, 1949).

RESULTS

The optimum conditions for action by the dialysed enzyme were found to be pH 5.6–5.8 and 37° (temperature of inactivation, 55°). The optimum sucrose concentration was 30% (w/v). The enzyme preparation did not contain any chromatographically detectable carbohydrate. Addition of boiled, undialysed enzyme did not increase the activity of the dialysed enzyme. Thus there appeared to be no dialysable co-factors involved in the action of the transfructosidase from agave. The dialysed enzyme was free from phosphate as tested with the Fiske & Subbarow (1925) reagent. Addition of phosphate

Table 1. *Transfructosidase activity of Agave vera cruz extract*

25% (w/v) Sucrose in dialysed enzyme incubated at room temp. F.R.S. = free reducing sugars. Quotients refer to changes in sugar concentrations, measured from zero time.

Time (hr.)	$\alpha_{25}^{26^{\circ}}$ ($^{\circ}$)	Sugar content						Glucose Sucrose (0.26†)	Spot 4 Sucrose (0.74†)	Glucose Spot 4 (0.36†)
		Of reaction mixture by macro methods		Of component carbohydrates separated by paper-chromatographic fractionation of 15.6 μ l. of reaction mixture						
		F.R.S. (%)	Glucose (%)	Fructose (μ g./ μ l.)	Glucose (μ g./ μ l.)	Sucrose (μ g./ μ l.)	Spot 4 (μ g./ μ l.)			
0	+32.95	Nil	0.1	Nil	0.9	258.8	Nil	—	—	—
6	+31.70	0.5	0.7	Nil	6.6	236.9	16.2	0.26	0.74	0.35
21	+29.95	1.4	1.6	Nil	14.9	199.6	40.8	0.24	0.69	0.34
52	+25.85	3.0	2.8	Nil	27.2	148.8	80.4*	0.24	0.73	0.33
76	+23.75	3.9	3.9	5.0	33.9	110.4	95.7*	0.22	0.64	0.34
96	+22.35	4.6	4.4	10.0	45.4	83.9	108.6*	0.25	0.52	0.41

* Includes spot 5.

† Theoretical quotients as required by Eqn. 1.

Fig. 1. Carbohydrate make-up of the reaction mixture at different time intervals. Reaction mixture: 25% (w/v) sucrose in dialysed enzyme incubated at room temp. (26–28°); chromatogram developed with butanol-acetic acid-water (4:1:5, by vol.) by ascending technique for 48 hr. *M* = mixture of fructose, glucose and sucrose; *C* = boiled control (sucrose + boiled enzyme). The faint second spot in boiled control is due to glucose present as an impurity in sucrose. Spots 4 (up to 45 min.), 5 and fructose were too faint to be photographed and so their outlines were marked before photographing.

Table 2. *Analysis of spot 4*

Spot 4 was separated on paper from 15.6 μ l. of a 21 hr. sample of the reaction mixture.

Total reducing sugars (μ g./ μ l.)	Fructose (μ g./ μ l.)	Glucose (by difference) (μ g./ μ l.)	Fructose/Glucose
40.8	27.0	13.8	1.96

to the dialysed enzyme did not enhance its transfructosidase activity.

Progress of the reaction. Fig. 1 is representative of the carbohydrate make-up of the reaction mixture at different time intervals up to 96 hr. It was observed that within 15 min. of incubation, two sugars appeared in the reaction mixture, viz. one

corresponding to spot 4 in the chromatogram and the other with the R_f value of glucose. Both these spots progressively increased with time. Spot 5, of which there was a suggestion of formation at 52 hr., was more definite after 67 hr. and onwards. Simultaneously, a spot with an R_f value equivalent to that of fructose became noticeable. Quantitative measurements are given in Table 1.

Substrate specificity. Agave fructosan I (see Srinivasan & Bhatia, 1953) incubated for 24 hr. with the enzyme did not give rise to glucose or oligosaccharides, as tested by chromatography. On repeating the experiment with a mixture of fructosan I and sucrose, the quantity of glucose formed was identical with the quantity formed under similar conditions with sucrose alone. By contrast with sucrose, no higher oligosaccharide was synthesized from raffinose which, however, showed signs of hydrolysis on prolonged incubation with the enzyme. Also, glucose and fructose, alone or in mixtures, did not act as substrates.

Carbohydrate corresponding to spot 4. On hydrolysing the contents of spot 4 and rechromatographing, there appeared two spots with R_f values of fructose and glucose. Analysis of the spot (Table 2) suggested an empirical formula F_2G (see Eqn. 1) for this carbohydrate.

DISCUSSION

Under the conditions of the experiment, up to 52 hr. of incubation, glucose formed is one-quarter of and F_2G three-quarters of the diminution in sucrose (cf. Table 1). Thus, for this period the mechanism of reaction is represented by Eqn. 1 (cf. Dedonder, 1952)

From 52 hr. onwards, a third oligosaccharide and fructose begin to form. From now on the reaction

is of a complex type, and changes in sucrose content hereafter cannot be reasonably correlated with any single factor, though formation of F_2G is still the dominant feature.

Considering only plant transfructosidases, Edelman & Bacon (1951) could not observe the synthesis of glucofructosans from sucrose alone by the action of enzyme from the tubers of Jerusalem artichoke unless inulin was also present. Dedonder (1952), repeating these experiments, found, on the contrary, that sucrose could be acted upon independently and that in the presence of inulin higher oligosaccharides were produced and more rapidly. The dialysed enzyme from the agave stem does not contain chromatographically detectable polyfructosans or lower carbohydrate fragments, but is still able to build glucofructosans from the sucrose alone. Also, this activity is not enhanced by agave fructosan. So far as the agave enzyme is concerned, there seems to be no necessity, therefore, to assume that the enzyme can only act in a coupled system consisting of fructosans or intermediates as fructose donors and sucrose as the acceptor.

We are not in a position to say yet whether the *in vitro* formation of glucofructosans from sucrose by the action of agave enzyme is a step in the *in vivo* mechanism of the biosynthesis of the agave fructosans.

SUMMARY

1. The stem of *Agave vera cruz* contains a transfructosidase which builds up glucofructosans from sucrose with simultaneous release of glucose. With the progress in incubation, small quantities of fructose are released.

2. The enzyme has been partially purified and concentrated by precipitation with ammonium sulphate and dialysis.

3. No dialysable cofactors seem to be involved in the enzyme action.

4. The enzyme acts on sucrose as the single substrate without requiring the interaction of agave fructosans or intermediates. No higher homologues are formed from raffinose, which is, however, slightly hydrolysed during prolonged incubation with the enzyme.

5. Inorganic phosphate does not influence the enzyme action.

6. The conditions for optimum action are: pH 5.6–5.8; temperature 37° and a sucrose concentration of 30 %. The enzyme is inactivated at 55°.

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Gas-Liquid Partition Chromatography: the Separation and Micro-estimation of Volatile Fatty Acids from Formic Acid to Dodecanoic Acid

By J. H. VAN DE KAMER, K. W. GERRITSMA AND E. J. WANSINK
Central Institute for Nutrition Research T.N.O., Utrecht, The Netherlands

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James & Martin (1952) published a method for the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid, making use of gas-liquid chromatograms. In this method the mixture of the fatty acids dissolved in ether is brought on to a kieselguhr column, the liquid phase consisting of DC 550 silicone, containing 10 % of

stearic acid. By heating the column to 100° or 137° the fatty acids are volatilized, separated with the aid of a nitrogen current and titrated successively.

However, the authors do not describe how a mixture of the lower volatile fatty acids (formic up to valeric acid) and the higher volatile fatty acids