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A Novel Approach to the Determination of Trace α-Amylase by Photographic Assay. The Synthesis and Enzymatic Characterization of an Oligosaccharide Derivative as a Substrate for α- Amylase from Porcine Pancreas

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In line with a new concept for the photographic assay on the basis of chemical amplification by the catalytic Ag° nuclei, an α-amylase substrate containing a new sensitive labeling component and a bioaffinity group was designed and synthesized. The analysis of enzymatic degradation proved that the substrate can release efficiently the labeling component without loss of photographic activity.

Various tools have been developed for the determination of trace organic compounds.[1] One of the most valuable amplification methods uses the combined action of an enzyme and a fluorescent dye as a marker, especially in enzyme immunoassay.[2] On the other hand, a photographic process also includes a sensitive chemical amplification step catalyzed by Ag° nuclei which initiate the formation of silver images.[3] Although the Ag° nuclei are normally produced by exposure to light, we observed[4] that they are formed by treatment of an unexposed negative film with 1-(3-[4-(2-formylhydrazino)phenylaminosulfonyl]-5-mercapto-tetrazole 1 (a nucleating reagent)[5] without the benefit of exposure. Moreover, a linear relationship clearly existed between the optical density of the film developed and the logarithm of concentration of 1 over the range from 4.0×10^{-8} M to 8.0×10^{-10} M. On the basis of the findings, we proposed an imaginary system A as a substrate for the determination of α-amylase by using a highly sensitive photographic assay procedure.[4]

System A

We present herein a successful embodiment of the system A incorporating a more sensitive nucleating reagent 2a as a labeling component. Figure 1 (line A) shows the assay profile of 2a by using a particular negative film having a cationic polymer layer.[6] It is noteworthy that a clear straight line exists between 2.0×10^{-8} M and 8.0×10^{-11} M of 2a in Fig. 1. Although a structural proposal involving a facile ring closure of the analogue 3 to 4 via 6-End-Dig pathway[7] has been advanced[8] to account for the unusual reducing power of compounds of this type, the true nucleating power of 2a may result from both the reducing and adsorptive activities because the lack of the thiocarbonyl moiety in 2a lowers it toward AgBr grains (Fig. 1, line B for 2b).

A plausible α-amylase substrate incorporating d(+)-biotin[9] and 2a was designed as 5. The synthesis was performed in a straightforward manner as described below. The diol 6b, on treatment with 7 and dry p-TsOH under reduced pressure, formed 8a in 70% yield, which was hydrolyzed with $NaOCH_3$ in CH_3OH to yield 8b, quantitatively. Catalytic hydrogenation of 8b on 10% Pd/C in aqueous CH_3OH produced 8c in 92% yield. Transformation of 8c into 5 was accomplished by coupling of 8c to 2c in DMF to afford 5[10] in 45% yield.

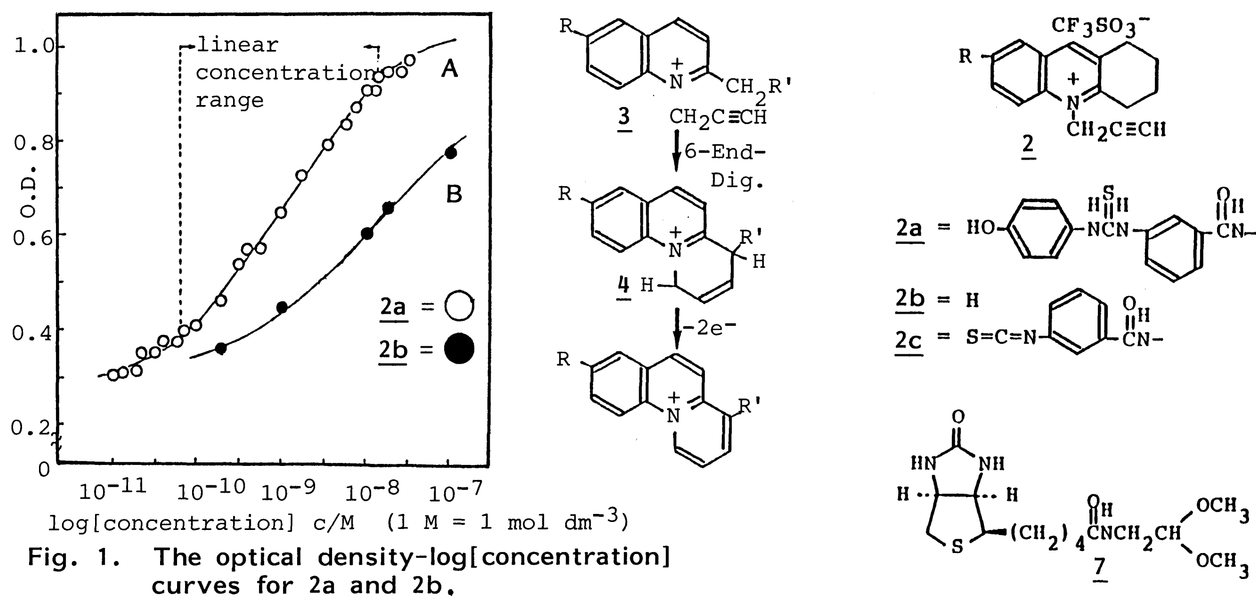
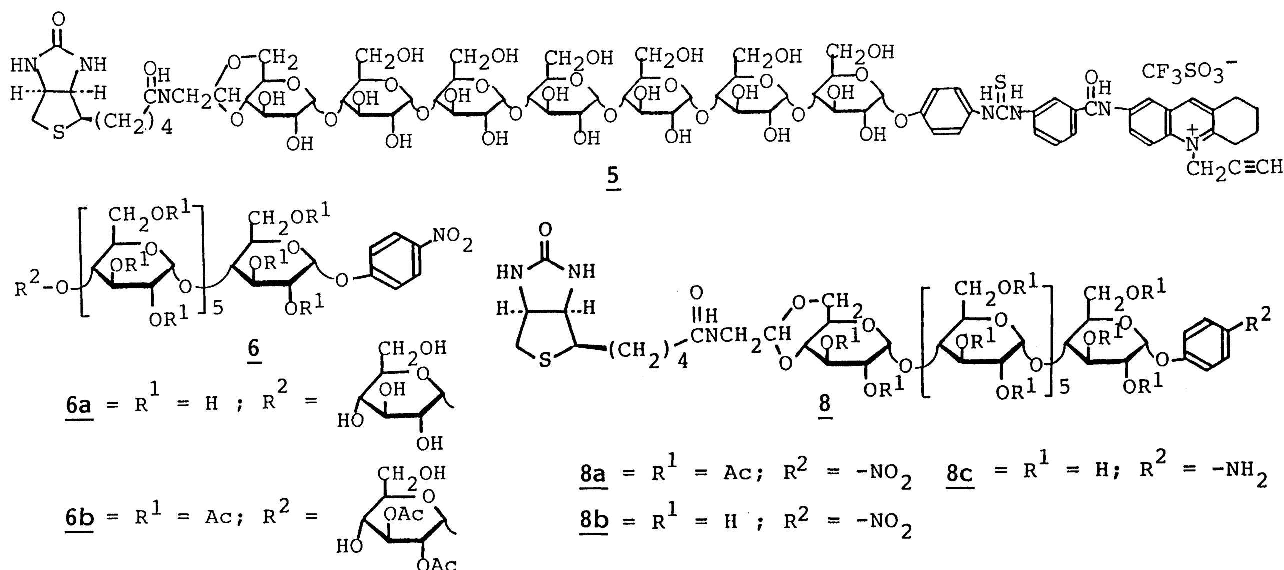


Fig. 1. The optical density-log[concentration] curves for 2a and 2b.



Prior to the continuous enzymatic degradation of 5 with α- amylase, glucoamylase, and α-glucosidase according to the assay procedure,[11] the molecular activities (k_o) of several amylases for 6a were determined in order to select an appropriate endoamylase for the initial cleavage of 5. At the optimum pH, the k_o of α-amylases from porcine pancreas, Aspergillus oryzae, and Bacillus Subtilis[12] were 640 s^{-1}(pH 7), 485 s^{-1}(pH 6), and 200 s^{-1}(pH 6), respectively. These results indicated that α- amylase from porcine pancreas can be employed to advantage. Table 1 shows the cleavage patterns and the k_o of the

amylase for 5, 8b, and 9e. Since the replacement of the reducing end by 2a resulted in the lowering of the k_o in spite of the absence of substituents on the nonreducing end (run 1 and 3), it is considered that α-amylase subsite affinity[13] may be strongly affected by the quaternized structure in 5.

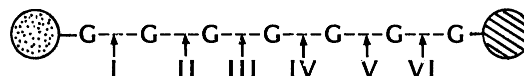
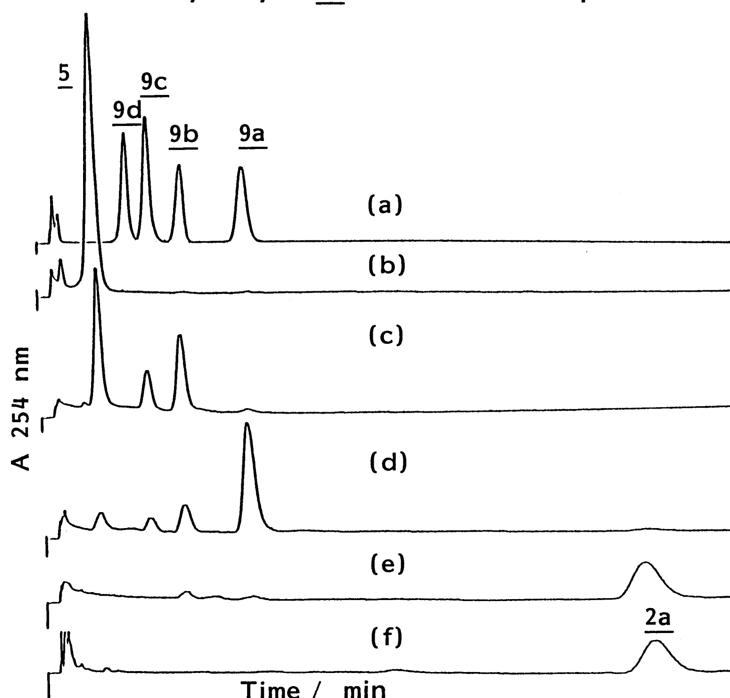


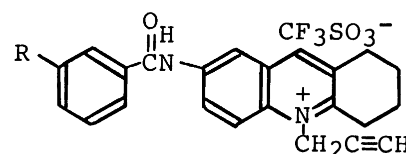
Table 1. Cleavage distribuition of 5, 8b, and 9e and the k_o of α- amylase from p. pancreas[a)]

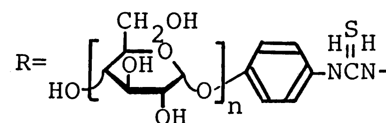
Run	Compound	Distribution/% I	II	III	IV	V	VI	k_o/s^{-1}
1	5				70	27	3	11
2	8b			24	28	45	3	192
3	9e			3	55	36	6	10

a) All reactions were performed in GP buffer (pH 6.5) at 25 °C for 20 min. $[S]=10^{-3}M, [E]=2.0x10^{-9}M$.

As shown in Fig. 2, the HPLC analysis of 5 was performed by direct comparison with authentic fragments (9a-9e). First, 5, consisting seven glucose units, is predominantly cleaved with α-amylase to give 9b and 9c (Fig. 2c), and the subsequent treatment with glucoamylase[12)] exhibiting a simple action pattern splitting off glucose from the nonreducing ends[14)] affords 9a (Fig. 2d). Finally, α-glucosidase[12)] can hydrolyze 9a at the anomeric position to release 2a (Fig. 2e). Thus, the enzy-







9a: n = 1
9b: n = 2
9c: n = 3
9d: n = 4
9e: n = 7

Fig. 2. Continuous enzymatic treatment of the substrate 5.
(a)(b)(f) HPLC analysis of 2a, 5, and 9a-9d. Separation was achieved on a C_{18}-reverse phase column eluted isocratically with $CH_3CN:H_2O$ = 70:30 (v/v%) containing 0.1% $C_7H_{15}SO_3Na$; flow rate 5 cm^3/min. Compounds retention time(r.t.), 9d, 2.7 min; 9c, 3.4 min; 9b, 4.4 min; 9a, 6.4 min; 5, 1.7 min; 2a, 18.9 min. (c) HPLC analysis of the products from 5 degraded with α- amylase. Reaction conditions, 5 ($1.1x10^{-3}$ M, 0.27 cm^3); α-amylase ($3.9x10^{-9}$ M, 0.03 cm^3), 5 min at 30 °C in 50 mM GP buffer (pH 6.5) containing 0.5 M NaCl. Elution conditions, see above; products, r.t., 9c, 3.3 min; 9b, 4.4 min; 9a, 6.4 min. (d) HPLC analysis of the products from Fig. 2c degraded with glucoamylase ($1.9 x10^{-5}$ M, 0.01 cm^3). Reaction time and temperature; 20 min at 30 °C. Elution conditions, see above; products, r.t., 9c, 3.3 min; 9b, 4.4 min; 9a, 6.4 min. (e) HPLC analysis of the products from Fig. 2d degraded with α- glucosidase ($1.9x10^{-5}$ M, 0.01 cm^3). Reaction time and temperature; 30 min at 30 °C. Elution conditions, see above; products, r.t., 9b, 4.4 min; 9a, 6.4 min; 2a, 18.6 min.

matic characterization proved that 5 can serve as as an α- amylase substrate in the photographic assay procedure. The direct assay of graded amounts of the residue after a similar exhaustive degradation of 5 (2.0×10^{-5} M) with enzymes (α- amylase, 10^{-9} M) for 45 min at 30 °C, gave a comparable assay profile to that of 2a without a lowering of photographic activity. Data for the continuous enzymatic treatment of 5 indicated the clear elimination of a serious problem concerning loss of the labeling reagent activity, especially in a highly sensitive assay system. These results are significant because the net concentration of α-amylase can be calculated in principle by maltiplication of the concentration of 2a observed by the apparent k_o for the reaction time, when excess 5 is separated.[15]

In conclusion, This provides the first successful approach for application of a photographic system into a quantitative determination of trace organic molecules. Details of a novel enzyme immunoassay system incorporating this procedure will be appear elsewhere.[16]

References

1) For a review, see: "Bioluminescence and Chemiluminescence Instruments & Applications," ed by K. Van dyke, CRC Press (1985), Vol. 2.

2) For a review, see: "Alternative Immunoassay," ed by W. P. Collins, John Wiley & Sons, New York (1985).

3) For an excellent text book, see: "The Theory of the Photographic Process," Macmillan Publishing Co., Inc., New York (1977).

4) M. Ono, N. Masuda, T. Matsushita, I. Itoh, and M. Kitajima, Chem. Lett., 1990, 215.

5) "A nucleating reagent" is defined as an organic compound which produce the catalytic Ag^o nuclei in AgX grains.

6) The cationic layer mainly consists of a mixture (50:50 w/w%) of gelatin (pI 6.7) and polystyrene quaternized with N-methylpiperidine (styrene:p-chloromethylstyrene: divinylbenzene=47.5:47.5:5.0 (w/w%). The constituents of other layers are described in Ref. 4.

7) J. E. Baldwin, J. Chem. Soc., Chem. Commun., 1976, 734.

8) R. L. Parton, W. S. Gaugh, and K. E. Wiegers, U. S. Patent, 4471044 (1984).

9) The function of the biotin part in 5 is to separate 2a liberated from excess unreacted 5 by using an avidin-immobilized bioaffinity column.

10) 5: mp 252-256 °C; $[\alpha]_D^{24}$ +112 (c 0.5, H_2O); FAB MS, m/z 1910 $(M-CF_3SO_3)^+$. All the new compounds gave satisfactory analytical data.

11) Although several enzymes are employed continuously in the assay, only an α- amylase causes a first degradation of 5 to expose nonreducing ends. Therefore, when other enzymes are employed in excess, a net amount of the released 2a should depend on the α-amylase activity to be determined.

12) α-Amylases were from BM GmbH or SANKYO Co. Ltd.: α- amylase from porcine pancreas (1000 units, suspension in 3.2 M $(NH_4)_2SO_4$); α-amylase from Aspergillus oryzae (120 units, crystalline); α-amylase from Bacillus Subtilis (1350 units, lyophilized powder). Glucoamylase was from SEIKAGAKU KOGYO Co. Ltd. (30 units, lyophilized powder from Rhizopus niveus). α-Glucosidase was from TOYOBO Co. Ltd. (100 units, lyophilized powder from Saccharomyces sp.).

13) K. Hiromi, Y. Nitta, C. Numata, and S. Ono, Biochim. Biophys. Acta, 302, 362 (1973).

14) A. Tanaka, T. Yamashita, M. Ohnishi, and K. Hiromi, J. Biochem., 93, 1037 (1983).

15) An avidin column can remove concentration up to about 10^{-4} M of 5.

16) The C.V. range for this assay was between 2.0 and 3.3% (n=80).

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