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PURIFICATION AND SPECIFICITY OF RNase A_3 FROM *VICIA FABA* ROOT CELLS

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Key Word Index—*Vicia faba*; Leguminosae; broad bean roots; Blue dextran–Sepharose; specificity; ribonuclease.

Abstract—*Vicia faba* root ribonucleases are bound to Cibacron blue F3GA. A Blue dextran–Sepharose column was used to purify RNase A_3, the more abundant enzyme from *V. faba* root. Using dinucleoside monophosphate as substrates, it appears that this enzyme behaves as a cyclizing phosphotransferase. With high enzyme/substrate ratios on prolonged digestion a partial release of a nucleoside 3′ phosphate occurs. The specificity is relatively high since only the purine–purine phosphodiester linkages out of 16 types of possible links are easily cleaved. When a pyrimidine is involved in the phosphodiester bond, a much slower rate of attack (Py in 5′) or no attack (Py in 3′) was detected.

INTRODUCTION

As described in a preceding paper[1], several enzymes showing a nucleolytic activity were extracted from *Vicia faba* roots: three 'anionic' (not retained on CM-cellulose at pH 5.5) enzymes, noted A_1, A_2, A_3, and two 'cationic' (retained at this pH) ones, C_1 and C_2. Specificity was determined by using homopolymers. The four RNases isolated (A_2, A_3, C_1, C_2: A_1 being a nuclease) showed the following specificity[1]: the anionic proteins hydrolyse preferentially the purine homopolymers [poly (A) ≥ poly (I) ≫ poly (U) > > > poly (c)] and the cationic ones attack only pyrimidine homopolymers. In order to get a more precise evaluation of enzyme specificity we used RNase A_3, the most abundant enzyme present and obtained in the purest form according to gel electrophoresis[1]. But the limitation of the ion-exchange chromatography previously used led us to check the purity of this enzyme by using chromatography on Blue dextran–Sepharose columns as the chromophore of Blue dextran, Cibacron blue F3GA, has been shown to bind to several enzymes interacting with nucleotide substrates and coenzymes[2–7].

Digestion of homopolymers only gives an indication of the behavior of enzyme towards four phosphodiester linkages out of 16, and we do not know if the neighbouring sequences or secondary structures in themselves could enhance or decrease the velocity of the enzyme attack. In order to study the differences in the rates of cleavage of nucleotide linkages due mainly to the chemical nature of the bases involved, we chose the following approach: digestion, in well-defined conditions, of the 16 dinucleoside monophosphates and analysis by chromatography of the reaction products on PEI cellulose thin-layer plates or on columns of QAE-Sephadex.

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Abbreviations: X_{OH} = nucleoside; $X_{3'}$ = 3′ nucleoside monophosphate; $X_{5'}$ = 5′ nucleoside monophosphate; Xcy = 2′–3′ cyclic nucleoside monophosphate; XpX = dinucleoside monophosphate, X being any base.

RESULTS

Chromatography on Blue dextran–Sepharose

Preliminary experiments were carried out to evaluate the interaction of root RNases with the dye by comparison of the retention of the anionic fraction with that of commercial RNase A which seems to bind weakly to this matrix[8,9]. Two batches of Blue dextran were used, corresponding to 12 or 32 mg of Blue dextran respectively per g of dry Sepharose. Results obtained showed that RNase A was fixed, but eluted with low concentrations of KCl: 10 or 20 mM respectively (Fig. 1A). Thus binding of RNase A is relatively weak; this was confirmed by the fact that elution was obtained by raising the phosphate buffer concentration from 10 to 50 mM (results not shown). Chromatography on Blue dextran–Sepharose of the anionic fraction, which contains[1] three enzymatic species (A_1, A_2, A_3), led to significantly different results: RNase activities bound to the matrix (over 90% of the total) needed a much higher concentration of KCl for elution. Three peaks were eluted (Fig. 1B) by KCl concentrations ranging from 150 to 375 mM (12 mg Blue dextran g dry Sepharose). Higher molarities of KCl were needed with 32 mg/g dry Sepharose

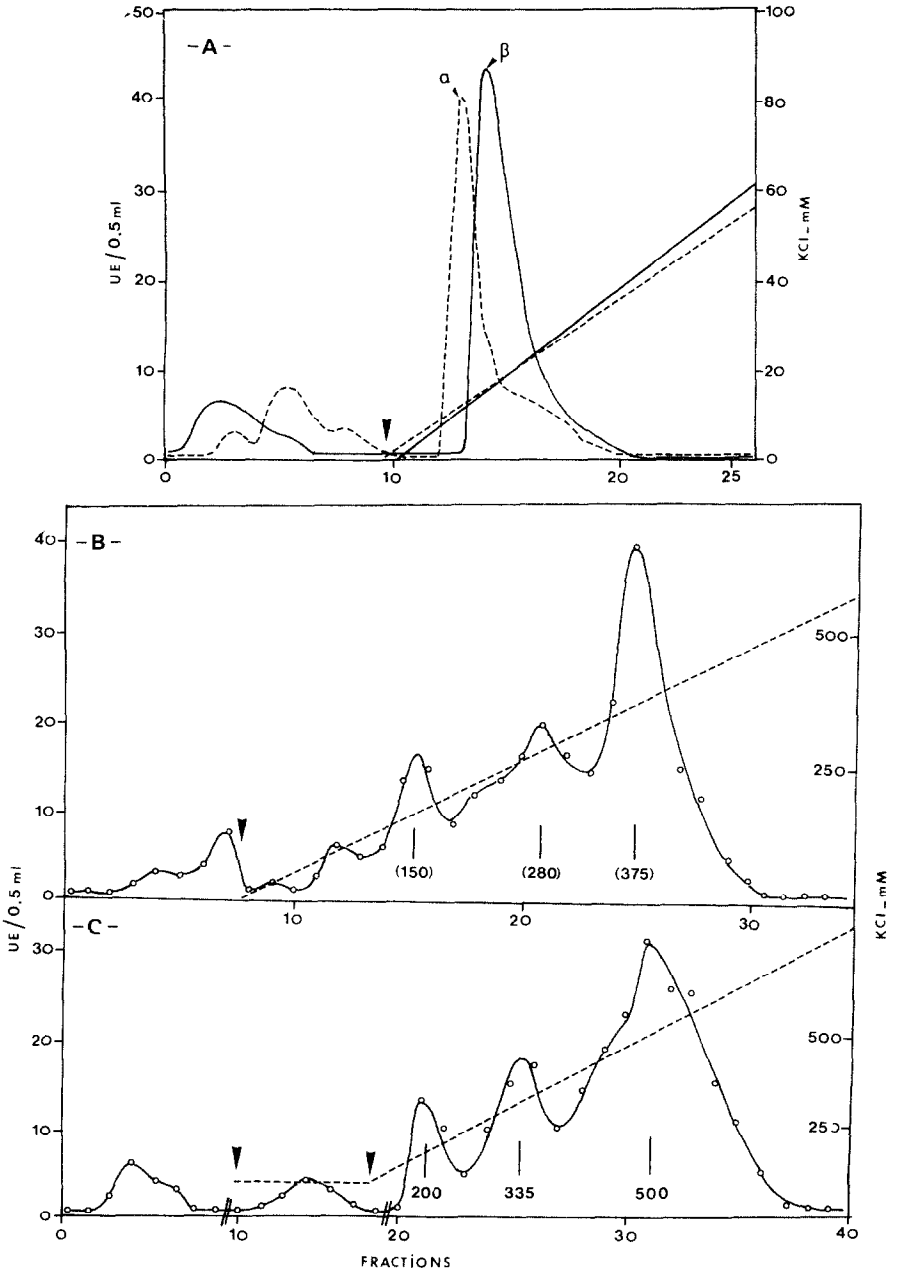

Fig. 1. Fixing of RNase A (A) and *V. faba* anionic RNases (B,C) on Blue dextran–Sepharose and elution by a KCl gradient. 100 UE of RNase A and 300 (B) or 250 (C) of *V. faba* RNases were loaded on to Blue dextran–Sepharose columns containing 12 mg (Aα,B) or 32 mg (Aβ,C) of Blue dextran. Elution buffer gradients were from 0 to 100 mM (A) or to 1 M (B,C) KCl. Enzymatic activity expressed as UE/0.5 ml (volume of each fraction).

(Fig. 1C), indicating a higher fixation of the enzymes onto the dye. Gel electrophoresis analysis (results not shown) showed that most of the proteins of the anionic fraction were unbound and that each peak contained several molecular species. These results led to the conclusion that the nucleolytic activities of the anionic fraction were strongly fixed by the dye but we did not attempt to correlate protein populations present in the three peaks of Fig. 1(B) and (C), and the three previously isolated enzymes.

Chromatography on Blue dextran–Sepharose was then carried out on enzyme A_3 previously purified by several ion-exchange steps[1,10]. More than 90% of the activity was fixed and two peaks were separated (Fig. 2A), eluted at 460 and 600 mM KCl and containing *ca* 1/3 and 2/3 of the total activity, respectively. Fractions of the second peak (i.e. 23–30) were pooled, dialysed and re-chromatographed. All the activity (nearly 95%) was fixed and eluted in a single peak by a buffer containing 550 mM KCl, giving one band on gel electrophoresis (Fig. 2B, insert). As previous results[1,10] have led to the conclusion that A_3 was

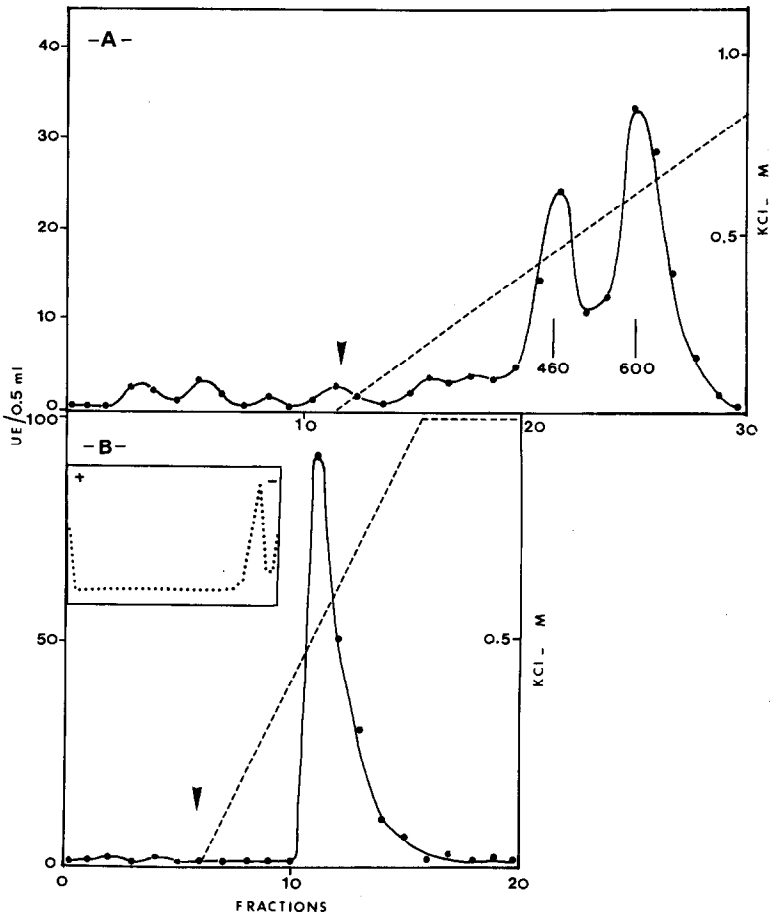

Fig. 2. Elution profile of *V. faba* RHase A_3 from a Blue dextran–Sepharose column by a gradient of KCl. 200 UE of A_3 were loaded in A; fractions 23–30 were pooled, dialysed and loaded (200 UE summing two independent chromatographies) on to a new column (B). Blue dextran: 32 mg/g Sepharose. Enzymatic activity as in Fig. 1. Insert: densitometric tracing of gel from purified A_3.

fairly homogeneous, one may consider that the first peak, eluted at 460 mM, could be a more or less proteolysed form of A_3, eluted at 550–600 mM, since no differences between the properties of these two fractions (pH optimum, attack of homopolymers, gel electrophoresis) could be detected. This last fraction was used for further studies.

Analysis of specificity

Preliminary results obtained by using labelled rRNA from *V. faba* as substrate, allowed us to determine the optimal conditions of the reaction, taking into account the fact that each dinucleoside monophosphate corresponds to 1/16th of the RNA linkages. Thus, 10 μg of each dinucleoside monophosphate were submitted to attack by A_3 in a sealed capillary tube and the reaction products analysed by TLC with several solvents. From the results obtained (some of which are illustrated in Fig. 3) it was concluded that the various linkages show a clear-cut pattern of decreasing susceptibility to the enzyme, the purine–purine dinucleoside monophosphates being the most susceptible base pairs (Fig. 3, lanes for ApA and GpA) whereas pyrimidine–purine or pyrimidine–pyrimidine pairs were not hydrolysed (Fig. 3, lane for UpU). In order to confirm these conclusions and get a quantitative evaluation of hydrolysis of the dinucleoside monophosphates, we used chromatography on QAE-Sephadex A25[11]. Due to the large amount of substrate needed for this technique (200 μg of dinucleoside monophosphate in our conditions) a relatively small degree of hydrolysis can be much more easily detected than by TLC. The results obtained, as illustrated by Fig. 4 and the data of Table 1, confirmed these conclusions. It appeared that ApA and GpG were more susceptible than ApG or GpA (conclusion suggested by Fig. 3) since an enzyme/substrate (E/S) ratio higher than 0.025 was necessary for the ApG and GpA. With purine–pyrimidine linkages, attack of the phosphodiester bond seemed rather low especially when cytidine is present. No attack was detected, in the same conditions, with pyrimidine–purine or pyrimidine–pyrimidine pairs.

TLC on PEI-cellulose allowed analysis of the reaction products which were (Fig. 3, lane for ApA) always a nucleoside and a cyclic nucleotide. By comparing purine–purine linkages, as ApA and GpA,

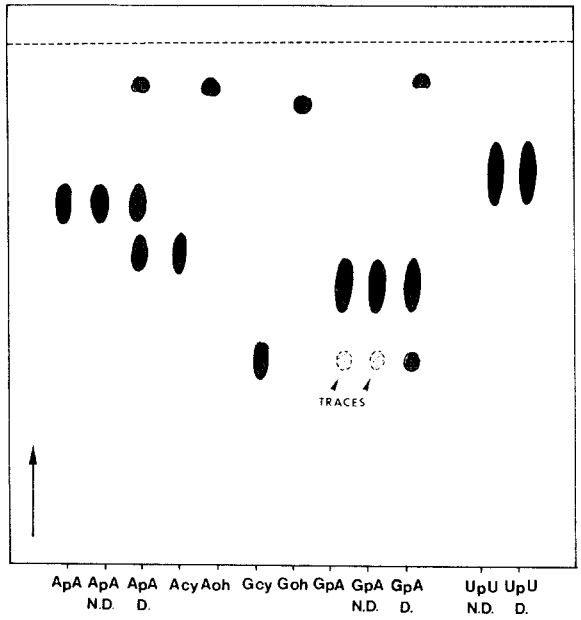

Fig. 3. TLC of some dinucleoside monophosphates submitted to RNase A_3 action. After digestion in sealed capillary tubes and lyophilization, the lyophilizate (3 μl from 10 μl) was deposited on PEI-cellulose plates. Chromatographic conditions: 0.5 M acetic acid (up to 12 cm) and then 0.3 M LiCl (up to 14 cm). The lanes correspond either to standards or to dinucleoside monophosphates incubated without (N.D.) or with A_3 (D.), respectively.

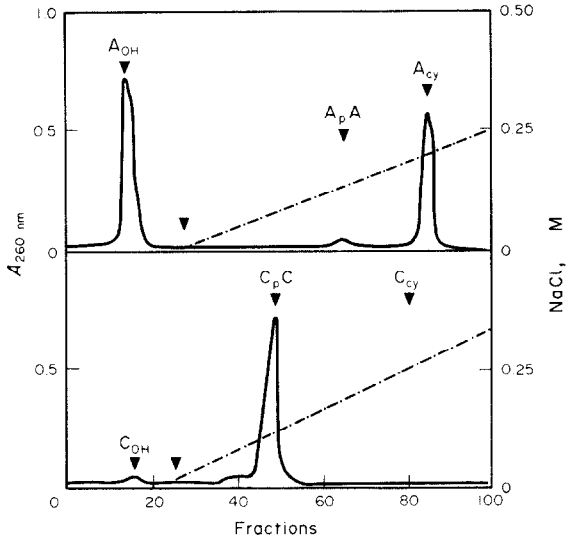

Fig. 4. Elution profiles of ApA and CpC, submitted to RNase A_3 action, from QAE A25 column by NaCl gradient. The incubation mixtures were loaded on to QAE columns; washing off by 4.4 mM Tris–HCl, pH 8, eliminated the nucleoside. Gradient from 0 to 0.5 M NaCl allowed separation of the undigested substrate from the cyclic nucleotide. Condition of incubation: E/S ratio = 1. Standard positions (from independent chromatography) located by arrows.

Table 1. Degree of hydrolysis of several dinucleoside monophosphates after RNase A_3 action and separation of the products by QAE chromatography

Substrate	E/S ratio 0.025	0.1	1
ApA	1–3%	8–15%	50–80%
ApG	—	5–10	25–35
ApU	—	—	5–10
ApC	—	—	—
GpG	1–3	8–15	50–80
GpA	—	5–10	25–35
GpU	—	5–10	20–30
GpC	—	—	1–3

Hydrolysis is expressed as per cent of the starting amount of substrate and the data correspond to extreme values observed in at least two independent determinations.

it was concluded that A_3 splits these doublets by liberating the nucleoside formerly positioned on the 3′ side of the linkage (i.e. A_{OH}) and the cyclic nucleotide corresponding to the nucleoside positioned on the 5′ side (Fig. 1, lane for GpA). In our experimental conditions no decyclization was detected. However at very high E/S ratios and with a digestion time of at least 5 hr the cyclic nucleoside 2′–3′ phosphates can be further partially transformed into the corresponding 3′ phosphate (results not shown).

DISCUSSION

Blue dextran–Sepharose has been used as an affinity chromagraphic tool for studies of enzymes which have a nuclotide or cofactor as substrate [4, 5, 12, 13]. Many of these results imply that the Blue dextran–Sepharose can recognize a polynucleotide-binding site on several proteins which interact with polynucleotides or nucleic acids. As far as *V. faba* root ribonucleases are concerned and although accurate study about the interaction with the dye was not carried out, the high concentration of KCl required for elution of the enzymes and the inadequacy of mononucleotides to elute them (results not shown) suggest an interaction with a domain related to polynucleotide binding site. Our experience indicates that Blue dextran–Sepharose affinity chromatography can be used to improve purification methods for plant RNases. Our results with the *V. faba* root anionic fraction suggest that proteins other than RNases are fixed on the dye and that Blue dextran–Sepharose cannot be used as a one-step purification procedure. However, this technique seems to be very useful as a supplementary step as illustrated for RNase A_3 (Fig. 2).

From the results obtained by using dinucleoside monophosphates as substrates, one can conclude that large differences in the rate of cleavage do exist according to the chemical nature of the nucleic aid bases involved on both sides of the link; RNase A_3 shows a pronounced preference for purine–purine pairs. As a matter of fact, the effect on rates of the base

attached to the oxygen in 3'P of the susceptible internucleotide linkage is particularly striking, no attack being detected in our experimental conditions when this base is a pyrimidine. Moreover, when this is a purine, the occurrence of a pyrimidine in 5'P induces much lower rates of hydrolysis since a low hydrolysis (GpU, GpC, ApU) or no attack (ApC) was detected. From other work devoted to plant RNases and using dinucleoside monophosphates [14–17], it appears that RNase A_3 shows the same general trend: production of a cyclic nucleotide and a nucleoside, indicating that A_3 is a cyclizing phosphotransferase. As far as specificity is considered, comparison with other papers is difficult since only one paper [17] dealt with the 16 dinucleoside monophosphates. *V. faba* RNase A_3 presents, in our experimental conditions, a much higher specificity for purine–purine pairs whereas all other enzymes studied were shown to attack, for example, CpC [15], ApC or UpA [17]. Barley leaf RNase [14] seems to belong to the same class as *V. faba* A_3 since the fastest rate was observed when *G* was present in either side, the presence of cytosine in 3'P led to reduced rates and no cleavage could be detected when two pyrimidines were present.

However, for each ribonuclease species, specificity might be dependent on local environment and such a specificity is *in vivo* probably much narrower than the specificity observed *in vitro*. Secondary structure in itself, the presence of modified or rate bases and neighbouring sequences might induce a modification of the activity.

EXPERIMENTAL

Plant material and preparation of enzyme extracts. Growth of bean roots, preparation of enzyme extract and purification of RNase A_3 were already published [1, 10]. Proteins precipitated between 30 and 80% $(NH_4)_2SO_4$ saturation were fractionated by chromatography on CM-cellulose at pH 5.5 into anionic (not retained) and cationic (retained) fractions. The anionic fraction, freed from phospho-mono and -diesterases by chromatography on Biogel P30, was separated by several chromatographic steps on DEAE-cellulose at pH 5.5 into three fractions called A_1, A_2 and A_3 according to the order of elution.

Blue dextran–Sepharose columns. Blue dextran was covalently bound to CNBr-activated Sepharose as described in ref. [12]. The Blue dextran–Sepharose used had 12 or 32 mg Blue dextran/g dry Sepharose. The gel was packed into polystyrene columns (sterile disposable syringes) to make 1 ml bed vol. and equilibrated with 10 mM Pi buffer pH 6.4. Enzyme solns were loaded and after washing in 5 to 10 ml of equilibration buffer, elution was carried out by applying linear gradients of NaCl and fractions of 0.5 ml were collected. Eluted enxymes were detected by measuring activity and analysed by gel electrophoresis [1].

Digestion of dinucleoside monophosphates. For analysis by TLC, digestion was conducted for 1 hr at 35° in sealed capillary tubes (25 μl, Blaubrand) containing 10 μl of each dinucleoside monophosphate (from Sigma) at 1 mg/ml, 1 μl of 2.2-triethylamine carbonate (TEC) prepared according to ref. [18], 1–10 μl of A_3, H_2O, to a final vol. of 25 μl; final pH: 7.4. Enzyme soln used, contained 130 ng protein and 1 UE/μl. One UE is defined as the amount which will hydrolyse 5 μg/min of *Torula* RNA [1]. For the hydrolysis of dinucleoside monophosphates, the enzyme/substrate (E/S) ratios were considered to be from 0.1 to 1, i.e. 1–10 UE for 10 μg of dinucleoside monophosphate. At the end of incubation, the soln was twice lyophilized in order to eliminate the TEC; the final lyophilisate was dissolved in 10 μl H_2O. For analysis by QAE-Sephadex A25, the incubation mixture contained 200 μl of each dinucleoside monophosphate and 5, 20 or 200 μl of A_3 (E/S ratio = 0.025, 0.1 and 1) was brought to 35° for 1 hr.

Decyclizing activity. The incubation was carried out in a sealed capillary tube as above with incubation time from 3 to 5 hr and by using 2'–3' cyclic nucleoside monophosphates as substrate.

TLC on PEI-cellulose. PEI-cellulose sheets (F1440LS254, Schleicher & Schüll) were previously washed in 0.5 M NaCl, then with H_2O and dried at 55°. The hydrolysate or standard soln (3 μl samples) were separated in two solvent systems. In the first derived form [19], 0.5 M HOAc was run for 12 cm above the starting line, followed by 0.3 M LiCl up to 14 cm without intermediary drying. In the second, 0.3 M TEC was run for 4 cm above the starting line, followed by 0.6 M TEC up to 8 cm.

For measuring the decyclizing activity, chromatograms was developed for 16 hr with *n*-BuOH–Me_2CO–HOAc–NH_4OH (18 M)–H_2O (9 : 3 : 2 : 2 : 4), [20]. Spots were detected by UV light.

QAE A25 chromatography. All the procedure was carried out at 4°. The incubation mixture, brought to 4° for 10 min, was loaded on top of a QAE-Sephadex A25 column (90 × 1 cm) equilibrated with 4.4 mM Tris–HCl pH 8.0 [11]. After washing off the column with the same buffer, elution was conducted by using a NaCl gradient from 0 to 0.5 M with a flow rate of 3 ml/cm² per hr; A_{260} was monitored with an ISCO UA5 analyser, and 4.5 ml fractions were collected. Fractions corresponding to each peak were pooled, concentrated and their A_{260} measured in order to determine the degree of hydrolysis.

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