Step 2: The 2 supernatants (pH ~ 6) are combined, heated in presence of 5 mM fructose-1,6-P_2 to 48 °C within 2–3 min and after 2 min at this temperature cooled with ice water. The precipitate formed is removed by centrifugation for 60 min at 20,000 × g and 4 °C. The supernatant is dialyzed against 5 l 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride (pH 7.5) at 4 °C for 15 h.

Step 3: The dialyzed supernatant (150 ml, ~ 20,000 U aldolase activity) is chromatographed on a DE 52 column (1.9 × 43 cm). The column is packed under pressure (0.7 kp cm^{-2} = 8.75 psi) and equilibrated with 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride (pH 7.5) at 4 °C. Following application of the sample (57 ml/h), the column is washed with 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/50 mM NaCl (pH 7.6) until the absorbance at 280 nm of the eluate has decreased to zero. This fractionation step removes both unspecific alkaline phosphatase and fructose-1,6-bisphosphatase from the aldolase activity containing fractions which are subsequently eluted with 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/90 mM NaCl (pH 7.6) (figure 1). If not removed, contaminating phospho esterase activity would hydrolyze the immobilized phospho ester ligand used in affinity chromatography.

Step 4: The fractions containing aldolase activity were pooled and subjected to affinity chromatography on a fructose-1,6-P_2 agarose column (0.9 × 6.5 cm) equilibrated with 10 mM Tris-Cl (pH 7.5). The apparent maximum aldolase binding capacity of fructose-1,6-P_2 agarose is 3000 U/g dry weight; after binding of this amount of aldolase activity, the activity concentration of the eluate is the same as that of the solution pumped onto the column (~ 50 U/ml). In order to obtain satisfactory purification, the amount of aldolase applied to the column should not exceed $^1/_3$ of the maximum capacity, i.e., 1000 U per run on the present 4 ml-column. For elution a linear phosphate gradient from 10 mM Tris-Cl/90 mM NaCl (pH 7.6) to 10 mM Tris-Cl/90 mM NaCl/100 mM sodium phosphate (pH 7.6), total volume 2 × 150 ml, was applied (figure 2). Elution with fructose-1,6-P_2 instead of inorganic phosphate did not improve the separation effect. The retardation of aldolase appears to be due to a specific interaction with the immobilized phospho ester ligand. Retardation by ion exchange effects seems to be excluded in view of the isoelectric point of yeast aldolase at pH 5.5[10]. AH-Sepharose 4B without linked fructose-1,6-P_2 did not retard aldolase and brought no purification of the applied enzyme solution. A summary of the purification procedure is given in the table. The yield given for the affinity chromatography is the sum of individual runs with fractional quantities of the enzyme preparation. Amino acid analyses of the purified aldolase closely corresponded with previously reported data[10]. The enzyme proved homogeneous on starch gel electrophoresis stained for protein and enzymatic activity and on polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed several lighter fragments, possibly indicative of a sodium dodecyl sulfate activated protease as observed in the purification of another yeast enzyme[18]. The purified aldolase when stored at –20 °C has, after 1 month, still 90% and, after 3 months, 50% of its initial activity. Addition of 100 mM 2-mercaptoethanol or 0.2 mM phenylmethylsulfonyl fluoride or storage as a suspension in 90% saturated ammonium sulfate does not increase stability.

In conclusion, reduction of the Schiff base formed with aminated agarose is a useful procedure for immobilizing fructose-1,6-P_2 and possibly other glycolytic substrates. Affinity chromatography of a prepurified yeast extract yielded an aldolase preparation comparable to that obtained by other procedures[9]. The non-coincidence of the protein and activity concentration curves in the elution profile (figure 2) appears to indicate, however, a possibly general limitation of the application of sugar phosphates as ligands in affinity chromatography, viz. ion exchange effects and/or unspecific retardation of other enzymes operating on phopho ester substrates.

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Action of iodine on the tomato pectinesterase

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Summary. The organophosphates and carbamates did not inhibit the isolated tomato pectinesterase. Therefore this enzyme cannot be defined as serine-type esterase. The enzyme is inhibited by iodine and the inhibition (irreversible and non-competitive) is dependent on the degree of enzyme purification.

The previous papers[1,2] described a resistance of pectinesterase (pectin pectyl-hydrolase, EC 3.1.1.11) against chemical agents such as iodine, cyanide, formaldehyde and others. In these works, crude preparations of tomato and microbial pectinesterase were used. From the esterase-inhibitors of DIP-type, the use of diisopropylphosphorfluoridate on microbial pectinesterase had a negligible effect[3]. The present paper describes the action of various inhibitors of serine-type esterases, as well as the action of iodine on the tomato pectinesterase.

Material and methods. Pectinesterase was prepared from ripe tomatoes (Lycopersicum esculentum, var. Immuna) as described previously[4]: the crude product was gained after extraction, fractional salting out with ammonium sulfate, dialysis and desalting on Sephadex G 25 column. The crude product was further purified on DEAE Sephadex A 50 and by chromatography on Sephadex G 75 column. The fraction with maximal pectinesterase activity was desalted on Sephadex G 25 column, and this product represented the purified pectinesterase. The isolated

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form of pectinesterase was obtained by chromatography of the purified product on CM Sephadex C 50 column. This product represents 1 of 5 multiple forms of pectinesterase present in tomatoes in dominant amount and having a mol. wt of about 27,000[5,6].

The activity of pectinesterase was determined by continuous titration method on automatic titrator (Radiometer Copenhagen, Denmark) at a constant temperature in a glass vessel under nitrogen atmosphere at pH 7.0 and 30 °C. Purified citrus pectin with a degree of esterification 65% was used as a substrate.

Inhibitors: Diisopropylphosphorofluoridate (Calbiochem, Switzerland). Diethyl-p-nitrophenylphosphate (Bayer, Federal Republic of Germany). Isopropyl-methylphosphonofluoridate (supplied by Dr J. Vachek). 1-naphtyl-N-methylcarbamate and 1-naphtyl-N, N-dimethyl carbamate (prepared by Dr J. Socha, VŠCHT Pardubice, Czechoslovakia). Iodine (Merck, Federal Republic of Germany) used, was resublimated and dissolved in sodium iodide. 5, 5'-Dithiobis-(2-nitrobenzoic acid) (Ellman's reagent, product of Fluka AG, Switzerland). All inhibitors were preincubated with the enzyme at room temperature and 0.1–1.0 ml aliquots of the mixture were added to 15 ml of 0.5% pectin solution in 0.15 M NaCl adjusted to pH 7.0. Inhibitory effect of iodine was stopped by addition of an excess of 0.1 M $Na_2S_2O_3$.

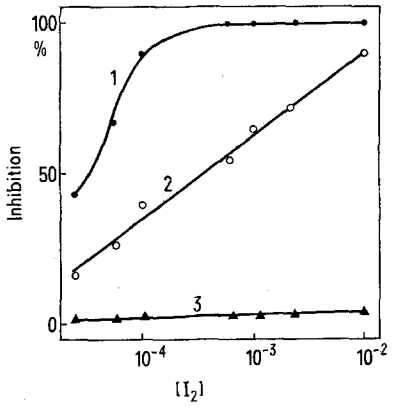

Fig. 1. Inhibitory effect of iodine concentration on the tomato pectinesterase. 1) 1.5×10^{-7} M pectinesterase (isolated form, specific activity 710 µmole ester hydrolyzed $xmin^{-1}xmg\ prot.^{-1}$). 2) 0.8 mg of the purified pectinesterase in 100 ml (specific activity 365 $\mu molxmin^{-1}xmg\ prot.^{-1}$). 3) 7.3 mg of crude pectinesterase in 100 ml (specific activity 47 $\mu molxmin^{-1}xmg\ prot.^{-1}$). Enzymes were preincubated with iodine for 20 min.

Results and discussion. Properties of the active site of tomato pectinesterase were studied in respect to inhibition caused by organophosphates, carbamates and iodine. No inhibition occurred when diisopropylphosphorofluoridate was used in concentrations of $1.5 \times 10^{-5} - 1.5 \times 10^{-2}$ M with the isolated form of pectinesterase in 2.1×10^{-7} M concentration. The activity of the enzyme was not changed when the preincubation with inhibitor was performed at pH 5.2, 5.7, 6.5, 7.0 and 7.8 in 0.1 M acetate or phosphate buffers in the course of 24 h. No inhibitory effect was shown when diethyl-p-nitrophenyl-phosphate, isopropyl-methyl-phosphonofluoridate, 1-naphtyl-N-methylcarbamate and 1-naphtyl-N, N-dimethylcarbamate were used. Based on these results, the tomato pectinesterase cannot be defined as serine-type esterase.

The action of iodine (NaI_3^-) showed an increasing inhibition parallel with purification of tomato pectinesterase. In accordance with previous papers[1,2], relatively high concentration of iodine (10^{-2} M I_2) caused an inhibition of crude pectinesterase less than 5%. Concentration of 10^{-4}–10^{-5} M I_2 caused more than 50% inhibition when purified pectinesterase was used, and the same concentration of iodine caused 90–100% inhibition, its isolated form in concentration 1.5×10^{-7} M in 20 min (figure 1). Action of a constant concentration of iodine on the isolated form and on the purified pectinesterase as a function of time showed initial sharp increasing of inhibition during first 30 min. 2.0×10^{-4} M I_2 caused 5% inhibition of the crude pectinesterase in 100 min, meanwhile 1.1×10^{-5} M I_2 caused 50% inhibition of the purified enzyme in 60 min and 90% inhibition of the isolated form in 60 min (figure 2).

From these results, it can be concluded that inhibition of pectinesterase by iodine is dependent on the degree of enzyme purification. This inhibition was found to be irreversible. All the dialyzed samples of pectinesterase treated with iodine for 60 min did not show any increase in activity against the value of inhibition of the sample before dialysis. As the K_m value of the inhibited and non-inhibited enzyme were found to be identical

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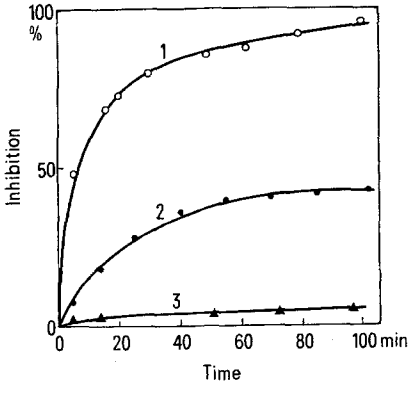

Fig. 2. Time course of inhibition of tomato pectinesterase by iodine. 1) 1.1×10^{-5} M I_2 with 1.5×10^{-7} M pectinesterase (isolated form). 2) 1.1×10^{-5} M I_2 with purified pectinesterase (0.8 mg in 100 ml). 3) 2.0×10^{-4} M I_2 with crude pectinesterase (7.3 mg in 100 ml).

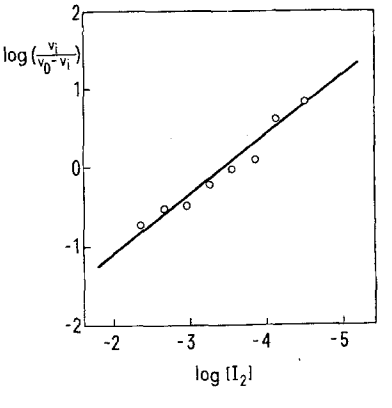

Fig. 3. Inhibition of the tomato pectinesterase by iodine. Data are presented in the form of a Hill plot, with the straight line drawn by the method of least squares. The Hill coefficient 0.71 was computed from the slope of this line.

(1.33×10^{-6} M methyl D-galactopyranuranosyl residues), and the value of the V_{max} of the inhibited one was 1.8×10^{-6} mol min^{-1} and of the non-inhibited one 2.33×10^{-6} mol min^{-1}, the action of iodine on the isolated form of pectinesterase can be characterized as non-competitive.

In order to determine the number of molecules of iodine binding to the enzyme, a Hill plot was used. When log $[v_i/(v_o-v_i)]$ was plotted against the log $[I_2]$, where v_i is the enzyme activity in the presence of inhibitor and the v_o the activity without the inhibitor, a straight line was obtained (figure 3). The slope of this line (0.71) indicates that 1 molecule of iodine is involved in the interaction with the catalytic site of the pectinesterase.

The reaction of iodine with proteins can cause SH oxidation, or iodination of tyrosyl and histidyl residues; tyrosyl residues are iodinated more readily than histidyl residues[7]. To ascertain if the inhibition of pectinesterase with iodine could be due the oxidation of free SH-groups, the action of Ellman's reagent on the isolated form of pectinesterase was examined. Nevertheless Ellman's reagent in concentrations of 10^{-4}–10^{-3} M in phosphate buffer pH 8.0 was not found to be an inhibitor of 1.1×10^{-7} M pectinesterase. Based on the results obtained with the iodination of tomato pectinesterase, the presence of tyrosine in the enzyme's active site can be assumed.

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Different salt concentration requirement of nuclear and cytoplasmic Mn^{++}-stimulated poly(A) synthetase activities from rat liver

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Summary. Mn^{++}-stimulated poly(A) synthetase activity of dialyzed ammonium sulfate fraction (0–50% saturation) of nuclear extracts from rat liver is dependent on salt addition to the incubation mixture. The corresponding soluble cytoplasmic activity is inhibited by the salt concentration required for nuclear activity.

Poly(A) synthetase activities (poly(A) polymerase, terminal riboadenylate transferase) have been found in prokariotes as well as in the nucleus[1-3] and the cytoplasm[3-6] of eukaryotic cells. Enzymes from both subcellular compartments of the latter organisms have been extensively purified[2-3]; the question, however, still remains, whether they are separate entities or the same enzyme undergoing redistribution upon subcellular fractionation. We have found that Mn^{++}-stimulated poly(A) synthetase activities of dialyzed ammonium sulfate fractionated extracts from nucleus and cytoplasm of rat liver cells, behave differently with regard to salt concentration in the test system, thus providing a means for distinguishing the 2 activities.

Methods. Male rats of the Wistar strain, 3 months old and weighing about 300 g were used. 3 animals, fasted for 24 h, were killed for each experiment, their livers were washed and pooled. All the subsequent operations were conducted at 0–2 °C. The organs were minced and homo-

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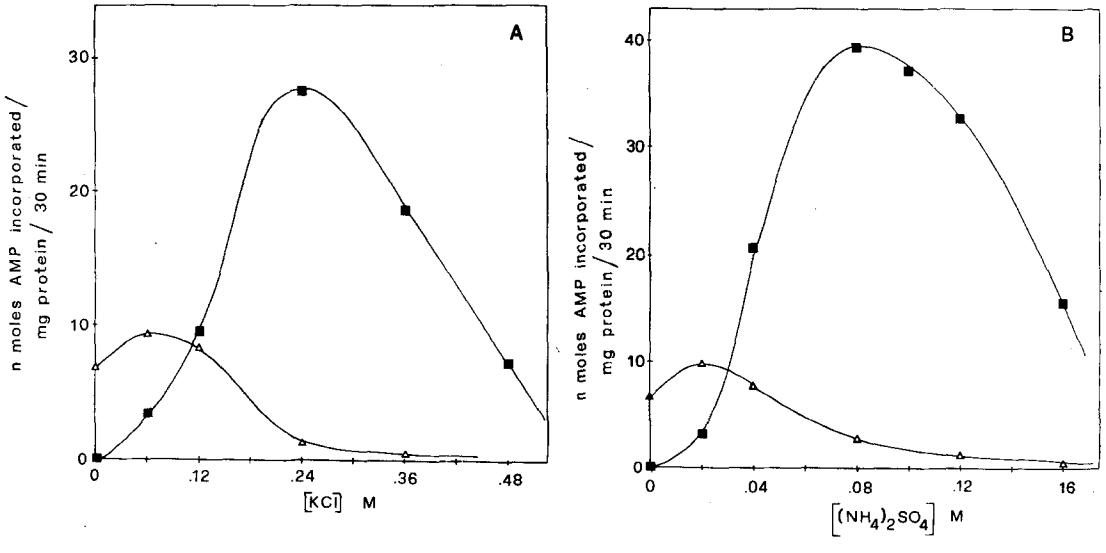

Effect of increasing concentrations of KCl *A* or $(NH_4)SO_4$ *B* on dialyzed Mn^{++}-stimulated poly (A) synthetase activity of ammonium sulfate fractions (0–50% saturation) of nuclear and cytoplasmic extracts from rat liver. The experimental conditions are described in the text. Each point represents the average of 4 determinations on 4 different pools of livers. The SEM was less than 10%. △—△, cytoplasmic activity; ■—■, nuclear activity.