PREPARATION OF HIGH-MOLECULAR PECTIC ACID BY TOMATO PECTINESTERASE

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Pectin was deesterified by pectinesterase of tomato either by the isolated enzyme or by the crude preparation, using a pH-stat and a continuous titration at pH 7·5 up to the esterification degree DE $< 2\%$. A simple procedure for preparation of high-molecular potassium pectate by enzymatic deesterification in a buffer solution of sufficient capacity was worked out. The final products of esterification degree DE $= 10 \pm 1\%$ were obtained by immobilized pectinesterase of tomato. The molecular weight of the final products was identical with that of the original pectin.

Pectic acid, as well as pectin of a lower esterification degree, obtained by a total or partial deesterification of pectin are important products for food industry, pharmacy and medicine. Pectic acid could be utilized as a substrate when determinig the activity of endo-D-galacturonanases,* the low-esterified pectin as a gel-forming substance enabling to produce foodstufs of low nutritional value and both these products as antidotes in poisoning with some metal cations and also as adjuvants in pharmacy.

The alkaline deesterification of pectin[1,2] leads to the desired deesterification degree or to a totally deesterified pectic acid; this process is associated with a partial cleavage of glycosidic bonds and the mean molecular weight of products drops roughly to the half of its original value.

The enzymatic deesterification of pectin[3,4] catalyzed by pectinesterase (E.C. 3.1.1.11) afforded products having their molecular weight close to the starting pectin, providing that the action of D-galacturonanases was eliminated. This process can be effected *e.g.* with pectinesterases of higher plants; pectinesterases of microbial origin with pH optimum close to that of the accompanying endo-D-galacturonanases have to be prepared in an enzymaticaly homogeneous form[5].

Ambiguity concernig the resulting esterification degree of pectin after action of pectinesterases is met in the literature; this could be caused both by the inhomogeneity of substrates and enzymes[6] and by various methods of determination of methoxyl groups[7,8]. Deesterification catalyzed by pectinesterase does not proceed totally, but stops at a certain degree of esterification: DE $= 3{\cdot}1\%$ was reported for tomato pectinesterase after a 3-day action[7]. The same value was achieved when using alfalfa pectinesterase[8]; DE $= 10$–11% was published[9] for orange pectinesterase. The inhibition effect of D-galacturonan (final product of the reaction) comes into account during the action of pectinesterase[10,11].

* The new expression for polygalacturonases proposed according to the correct name of substrate[6].

This paper deals with the preparation of pectic acid resulting from the treatment of free and immobilized tomato pectinesterases under such a condition of the enzymatic reaction, where the original molecular weight of pectin was maintained.

EXPERIMENTAL

Substrate: the purified citrus pectin (Genu Pectin, Københavns Pektinfabrik, Denmark) of esterification degree DE = 64·3%, of D-galacturonan dry substance 89—90% and of molecular weight close to 60000 (limit viscosity number $[\eta] = 350$ ml/g).

Enzyme: pectinesterase of tomato (*Lycopersicum esculentum* var. *immuna*) prepared according to procedure already described[12]. A crude preparation (M 2) of specific activity 1·5 μmol s^{-1} . mg^{-1} (showing also an endo-D-galacturonanase activity) and isolated specific pectinesterase (M 4) of specific activity 56 μmol s^{-1} mg^{-1} were used.

Immobilization of pectinesterase: product M 4 was immobilized on the CNBr-activated Sepharose 4B (Pharmacia Uppsala, Sweden) according to procedure recommended by the manufacturer; a preparation of activity 1·38 μmol s^{-1} per 1 ml of the gel was obtained[13].

Activity determination: the activity of pectinesterase was determined by the method of continuous titration using an auto-titration apparatus PHM 28/TTT 1 (Radiometer, Copenhagen, Denmark). The activity is expressed in micromoles of esters liberated at pH 7·5 and 30°C within 1 s.

Esterification degree of pectin (DE) and the D-galacturonan content in preparations were determined by the precipitation method of insoluble copper pectinates and pectates[14,15]. The content of methoxyl groups was concurrently estimated by the Zeisel's method[8].

The mean molecular weight of pectin was determined viscometrically in 0·155M-NaCl–0·005M sodium oxalate. The reduction of the limit viscosity number $[\eta]$, which is an exactly defined numerical quantity, on molecular weight has so far not been unambiguously defined[16]. The mean molecular weight is, therefore, in this paper calculated conventionally according to[17] what is the most frequented means. Chemicals were of analytical grade and water redistilled.

RESULTS AND DISCUSSION

Pectin was enzymatically deesterified at a constant pH value close to the pH optimum of the pectinesterase used. Two procedures were employed when using free pectinesterase of tomato:

Deesterification of the 1% pectin solution in a 0·15M-NaCl by means of a pH-stat at a constant pH 7·5 ± 0·1 value by pectinesterase of 6·0 μmol s^{-1} activity (*i.e.* product M 2 (4 mg) or product M 4 (0·107 mg)) per 1 g of pectin. This procedure led within 3 h to a virtually total deesterification of pectin (the final deesterification degree DE = 1·8 ± 0·5% did not change upon further action of the enzyme); cleavage of glycosidic bonds did not take place even with the crude product M 2. The final product had the limit viscosity number $[\eta] = 332 \pm 5$ ml/g (the average value of 4 samples) close to that of the starting pectin $[\eta] = 350$ ml/g corresponding to the decrease of the molecular weight by only 5%. The time dependence of the enzymatic deesterification under these conditions is plotted in Fig. 1 (curve *1*). This curve had a more moderate course when using a 8-fold lesser amount of the enzyme

(Fig. 1, curve 2). The DE = 5% after 3 h and values below 2% vere attained after 5 h of action of the enzyme.

The second method of preparation of pectic acid was based upon an enzymatic deesterification in a phosphate buffer solution of sufficient capacity guaranteeing the constant pH within the whole deesterification of pectin. The presence of a 0·3M phosphate buffer solution contributed, at the same time, to the enhancement of the ionic strength of the medium and therefore, also to the suppression of the inhibition effect of the product of the enzymatic reaction[9]. This simple procedure deesterified pectin to the DE lower than 2% in the same time as described in the first procedure using the same amount of pectinesterase.

Potassium pectate was directly obtained from the 0·3M-KH_2PO_4 buffer solution adjusted with 0·3M-KOH to pH 7·5 in the presence of 0·1M-KCl by precipitation with ethanol. Products obtained by this method did not differ from those prepared by means of pH-stat; also the curves illustrating the deesterification process showed a similar course.

Different results were obtained when using the immobilized pectinesterase: with the enzyme corresponding to activity of 0·6 μmol s^{-1} per 1 g of pectin even after a long lasting (more than 36 h) action the final DE was 10% only when employing either a pH-stat or a buffer solution (Fig. 1, curve 3). The final value of DE = 10 ± 1% did not drop nor after a 4-day treatment of the immobilized pectinesterase (batch method with continuous stirring at room temperature). In contrast to the action of D-galacturonanases, with pectinesterase the magnitude of the substrate molecule does not change and after immobilization of the enzyme, steric and diffusion effects come here into effect in a more pronounced measure. Consequently, the inhibition effect of the product could not be suppressed by the increase of salt concentration or by the increase of pH to 7·5 as is the case with the free enzyme. Lowering of the activity of immobilized pectinesterase was considerably manifested on the course of

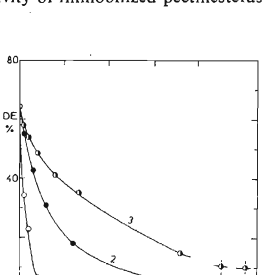

FIG. 1

Course of Deesterification of Pectin by Tomato Pectinesterase

1, *2* the action of free pectinesterase (activity 6·0 or 0·75 μmol. s^{-1} per 1 g of pectin); *3* the action of immobilized pectinesterase (activity 0·6 μmol s^{-1} per 1g of pectin).

the deesterification (Fig. 1, curve 3). The relative activity of pectinesterase decreased after immobilization below 5% in contrast to glycosidases, which maintained more than 30% of the relative activity after immobilization, similarly as with endo-D--galacturonanase of tomato, where up to 40% relative activity was achieved[12].

The presented procedures of enzymatic deesterification of pectin afforded products having the limit viscosity number close to that of the starting pectin with a difference less than 5%, thus evidencing the retention of the original molecular weight of preparations.

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