J Sci Food Agric 85:86–90 (2005)
DOI: 10.1002/jsfa.1930

Study of albedo and carpelar membrane degradation for further application in enzymatic peeling of citrus fruits

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Abstract: The enzymatic activity of four commercial enzymatic preparations (Peelzym I, II, III and IV) on citrus pectin, polygalacturonic acid and carboxymethylcellulose was determined (measured as the decrease in relative viscosity). In addition, the effectiveness of these preparations in the enzymatic degradation of the albedo and the segment membrane from Cimboa fruits was assessed. The highest activity on citrus pectin was shown by Peelzym II, although Peelzym I and IV activities were also elevated, 94.5 ± 6.2% and 88.7 ± 8.3% respectively of Peelzym II activity, and no relevant differences were found between them. Peelzym II also showed the highest activity for polygalacturonic acid, which was approximately 25% more than that of Peelzym I and IV, and more than double that of Peelzym III. Peelzym IV showed 40% more EM-cellulase activity than Peelzym I, II and III. Segment membrane solution was degraded mainly by the enzymatic preparations Peelzym I and II. Thus, the most effective activities for the degradation of the carpelar membrane from Cimboa were those activities which act mainly on pectin and especially on polygalacturonic acid. However, the albedo was degraded to the greatest extent by Peelzym II and, in turn, the most important activities for albedo degradation were those which act on polygalacturonic acid. In addition, the concentration of the enzymatic preparation for the degradation of the carpelar membrane was lower than that required for albedo degradation.

Keywords: albedo; carpelar membrane; enzymatic degradation; citrus fruit

INTRODUCTION

Enzymes have been used for centuries to modify the flavour, texture, appearance or storage stability of food. Citrus fruits are ideally suited for peeling by vacuum infusion of pectinases because the edible portion of the fruit is relatively solid and free of voids, whereas the albedo and core of the fruit are extremely porous. Thus, the enzyme solution is preferentially drawn into these porous tissues, maximising its effectiveness. The flavedo needs to be scored or otherwise perforated to permit the entry of the enzyme solution.[1] Bruemmer *et al*[2] were the first to use this enzymatic method in the peeling process of grapefruit by vacuum infusion of commercial pectolytic solutions, showing that the sections obtained maintained their original taste and texture, with higher peeling efficiency and quality than those obtained by conventional chemical peeling methods. In a similar way, Berry *et al*,[3] using the method developed by Bruemmer *et al*,[2] proved that, in both segments and the entire grapefruit enzymatically peeled, the loss of juice was lower than in fruits conventionally peeled. In order to understand better the enzymatic degradation process, Ben-Shalom *et al*[4] put forward evidence of the importance of evaluating the effect of commercial enzymes on the substrates needing to be degraded. This was later confirmed by other authors.[5,6]

Enzymatic peeling potentially allows different citrus presentations to be obtained. Thus, if only the albedo is degraded, peeled entire fruits would be produced while, to obtain peeled segments, the degradation of the carpelar membrane is necessary.[7] The enzymatic degradation of the albedo and the segment membrane requires the combination of many enzymatic activities, depending on structural polysaccharide composition.[8] According to most authors,[2,3,8] polygalacturonase activity is the most important for the enzymatic peeling of citrus. However, owing to the close inter-connection between the cell wall polysaccharides, the enzymatic modification of any of them may cause important effects on complete tissue structure. Therefore, the presence of other pectolytic and cellulolytic activities in the commercial enzymatic solutions could improve

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(*Received 12 March 2003; revised version received 30 April 2004; accepted 1 July 2004*)
Published online 24 September 2004

the effectiveness of the enzymatic peeling process.[4,8] The role of pectinases in the enzymatic peeling of orange is related to the desegregation of pectins that act as cementing agents in the cell walls, while cellulases could be necessary for the pectin liberation.[6]

The commercial pectolytic preparations that are used in the enzymatic peeling of citrus contain a variable proportion of enzymatic activities that act on pectins, polygalacturonic acid and carboxymethylcellulose. The selection and combination of those activities depends on the type of tissue to be degraded. According to Ben-Shalom,[9] pectin and cellulose degradation could be used as a way to measure the peeling effectiveness of an enzyme preparation.

In this work, the enzymatic activity (measured as the decrease in relative viscosity) of four commercial enzymatic preparations (Peelzym I, II, III and IV) on citrus pectin, polygalacturonic acid and carboxymethylcellulose was determined. In addition, the effectiveness of these preparations in the enzymatic degradation of the albedo and the segment membrane of Cimboa fruits was assessed. This allowed a determination of enzymatic activity which is more appropriate for the enzymatic degradation of each fruit tissue. In this procedure the relative activities of the enzymatic preparations on standard substrates (such as pectin, polygalacturonic acid and carboxymethylcellulose) were compared with the activity of the same preparations on a saturated solution of the albedo and the segment membrane from Cimboa. These citrus fruits were selected because they have an elevated quantity of albedo and segment membrane, which could easily be obtained and, therefore, could serve as a model for other citrus fruits.

EXPERIMENTAL

Standard substrates

Citrus pectin, polygalacturonic acid from orange fruits and carboxymethylcellulose were purchased from Sigma Chem Co (St Louis, USA).

Plant substrate preparation

Albedo and segment membrane from five *Citrus maxima* Burm. Merrill variety 'Cimboa' fruits were prepared as follows. Fruits were harvested at the optimal ripening stage (average respiratory rate $9.12 \pm 1.87\,mg\,CO_2\,kg^{-1}\,h^{-1}$, ethylene production rate $0.09 \pm 0.01\,nl\,g^{-1}\,h^{-1}$, titratable acidity $1.69 \pm 0.39\,g\,100\,g^{-1}$ and soluble solids content at 20 °C 11.62 ± 0.34 °Brix). Fruits were manually peeled and samples from albedo and carpelar membrane were carefully obtained to avoid contamination by fruit juices. Then, they were lyophilised, ground and passed through a sieve of 0.125 mm mesh size to obtain an homogeneous sample of each tissue.

Commercial enzyme preparations

The pectolytic enzyme preparations used, provided by the company Novo Nordisk Ferment Ltd, (Dittingen, Switzerland) were: Peelzym I, II and III produced by *Aspergillus niger*, and Peelzym IV produced by *A niger* and *Trichoderma reesi*. All enzymatic preparations contained mainly pectinases, hemicellulases, cellulases and arabinanases.

Determination of the relative enzymatic activity

Relative enzymatic activity was calculated taking into account global viscometric activity, using standard substrates[10] which were prepared as follows. To 10 ml of substrate solution ($5\,mg\,ml^{-1}$) in sodium acetate buffer 0.1 M at pH 4.0, were added 0.2 µl (for citrus pectin) or 2 µl (for polygalacturonic acid and carboxymethylcellulose) of the corresponding Peelzym solution. The fall time of the solution in a capillary viscometer type 100 Cannon-Fenske (ASTM D445, IP-71, BS188), kept at 40 °C by a thermostat, was recorded. One unit of viscometric global activity was considered as the amount of enzyme that produced a 50% viscosity decrease in 1 ml of substrate solution under optimal conditions.[10–12] Results were expressed as relative enzymatic activity (%) of each Peelzym, considering 100% of enzymatic activity as that of the Peelzym showing the highest activity for a particular substrate. Each reaction was repeated four times.

Determination of fluidity using substrates for plant tissue

To 10 ml of membrane or albedo solution in 0.1 M sodium acetate buffer ($20\,mg\,ml^{-1}$), pH 4.0, were added 2 µl of each Peelzym solution. The time the solution mixture took to pass between two marked points on the viscometric tube (fall time) along with reaction time was recorded.[11] Fluidity was calculated according to the method of Levinson and Reese:[13]

$$\text{Fluidity} = 1/\eta_{sp} = 1/[(t_c - t)/t]$$

where η_{sp} is the specific viscosity, t_c the fall time of the reaction mixture and t the fall time of the 0.1 M sodium acetate buffer, pH 4.0. The reaction for each combination of substrate and Peelzym was performed four times, and results are the mean of them.

Statistical analysis

The data were treated for multiple comparison by analysis of variance with least significant difference (LSD) between means determined at the 5% level.

RESULTS AND DISCUSSION

Relative enzymatic activity of four commercial enzymatic preparations

The relative enzymatic activity of four commercial preparations (Peelzym I, II, III and IV), using as substrate citrus pectin, polygalacturonic acid and carboxymethylcellulose, are shown in Fig 1a,b and c, respectively. The highest activity on citrus pectin was shown by Peelzym II, although Peelzym I and IV activities were also elevated, $94.5 \pm 6.2\%$ and

88.7 ± 8.3% respectively, and no relevant differences were found between them (Fig 1a). Peelzym II also showed the highest activity on polygalacturonic acid, which was approximately 25% more elevated than Peelzym I and IV, and more than double that of Peelzym III. These differences in enzymatic activity on pectin and polygalacturonic acid between the four Peelzym preparations could cause variations in their effectiveness in the degradation of albedo and segment membranes since, according to most authors,[2,3,8,14] these activities play a principal role in enzymatic peeling. Thus, Bruemmer *et al*[2] and Berry *et al*,[3] studying enzymatic peeling of grapefruit, reported that polygalacturonase activity was the most important factor in the degradation process. Coll[8] observed that, although the presence of other activities, especially pectolytic, considerably improved the degradative capacity of the membrane segments, the loss of cellular cohesion could be obtained through the isolated action of pure polygalacturonases, the participation of other types of activity not being necessary.

Peelzym IV showed 40% more cellulase activity than Peelzym I, II and III. Significant differences were not found between these three (Fig 1c). Therefore, if cellulase activity were the most important for the degradation of citrus albedo and carpelar membrane, Peelzym IV should produce the highest degradation on the natural substrates. Literature is controversial in relation to the importance of cellulase activity on citrus enzymatic peeling. Ben-Shalom[9] proved that there was a synergistic effect on the combination of pectinases and cellulases for the degradation of the membrane segments of grapefruit. In addition, Ben-Shalom[9] holds that the hydrolysis of pectic substances by pectolytic enzymes makes the access of degradative enzymes to hemicelluloses and celluloses easier. However, Bruemmer[14] found that cellulase did not improve peeling effectiveness in grapefruit and orange. Coll[8] indicated that the degradation of the cell wall in Satsuma mandarin due only to the action of cellulases would be very difficult because cellulose is placed in a matrix that makes it practically inaccessible. Therefore, according to Ben-Shalom,[9] Peelzym IV would be a good enzymatic preparation for the degradation of albedo and segments membrane, because it presented the highest cellulase activity and a high pectinase activity which is necessary for the degradation of pectic substances that form the cell wall matrix in which cellulose is found. However, according to most authors[2,3,8,14] Peelzym II would be the most appropriate for albedo and carpelar membrane degradation since it presents the highest polygalacturonase activity.

Comparing the relative activities of enzymatic preparations on standard substrates, such as pectin, polygalacturonic acid and carboxymethylcellulose, with the activity of those preparations on albedo and segment membrane, it could be inferred which enzymatic activity is the most important for each plant tissue. Thus, this work has attempted to determine the efficacy of those preparations on enzymatic degradation of albedo and segment membranes.

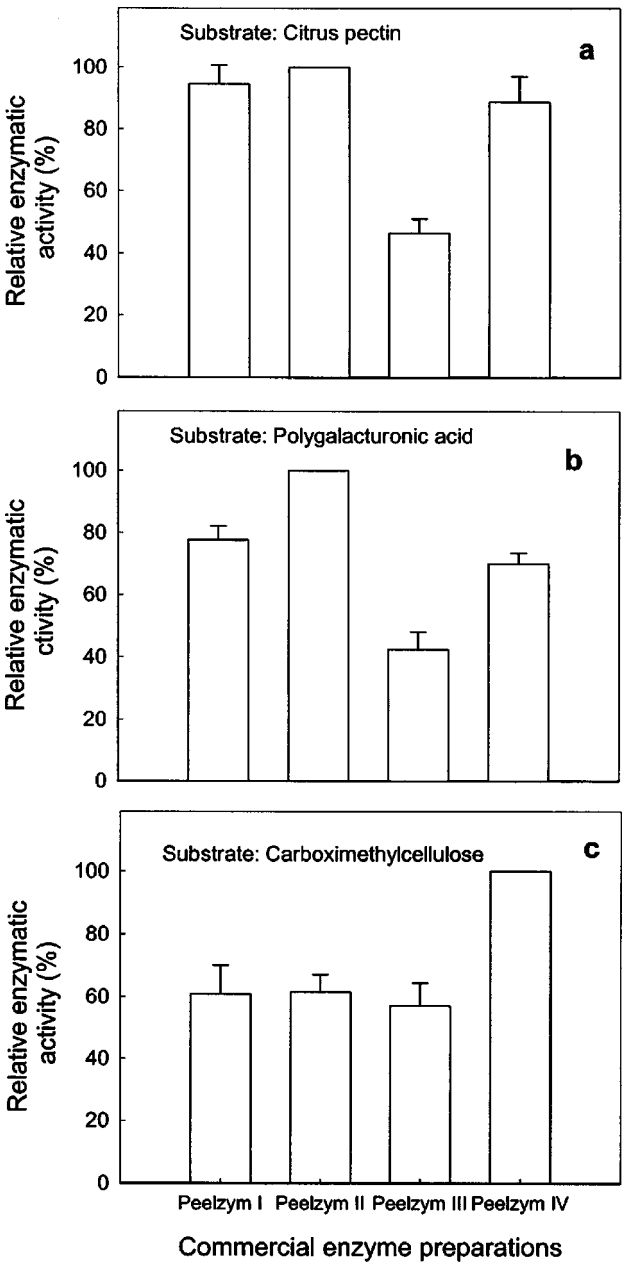

Figure 1. Relative enzymatic activities of enzymatic preparations (Peelzym I, II, III, IV) on different substrates: a, citrus pectin; b, polygalacturonic acid and c, carboxymethylcellulose. Relative activities are expressed in relation to the highest activity for each substrate. Pectinase activity of Peelzym II: 2360 ± 165 U ml^{-1}. Polygalacturonase activity of Peelzym II: 2193 ± 142 U ml^{-1}. Cellulase activity of Peelzym IV: 1634 ± 101 U ml^{-1}.

Enzymatic degradation of carpelar membrane

The degradative capacity of the four Peelzym commercial preparations on a saturated concentration of lyophilised segment membrane obtained from Cimboa is shown in Fig 2. With Peelzym I and II, after 15 min of reaction, 2.35 and 2.56 fluidity units were obtained, respectively, twice that obtained with Peelzym III and IV. Peelzym I had a high activity on pectin (94.5%) relative to Peelzym II,

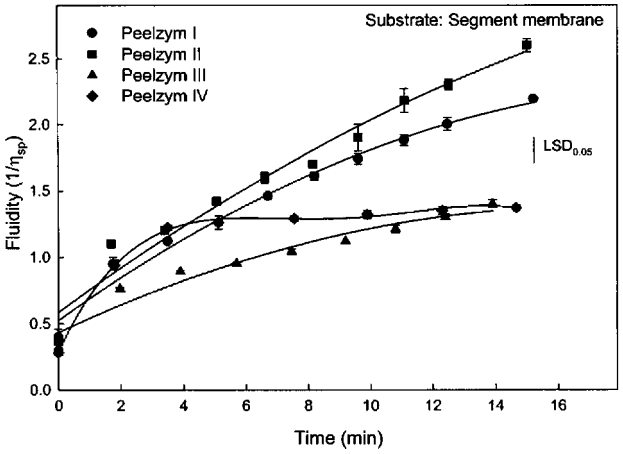

Figure 2. Evolution of fluidity of a solution of segment membranes from Cimboa (20 mg ml^{-1}) against time. Considering as minimum fluidity that shown by the solution before adding the corresponding enzymatic preparation: Peelzym I, II, III or IV. Reaction medium: 10 ml of a solution of carpelar membrane (20 mg ml^{-1}) in sodium acetate buffer 0.1 M, pH 4.0 and 2 µl of a solution of the corresponding enzymatic preparation (Peelzym I, II, III or IV). Data are the mean of four replicates and the $LSD_{0.05}$ value is shown.

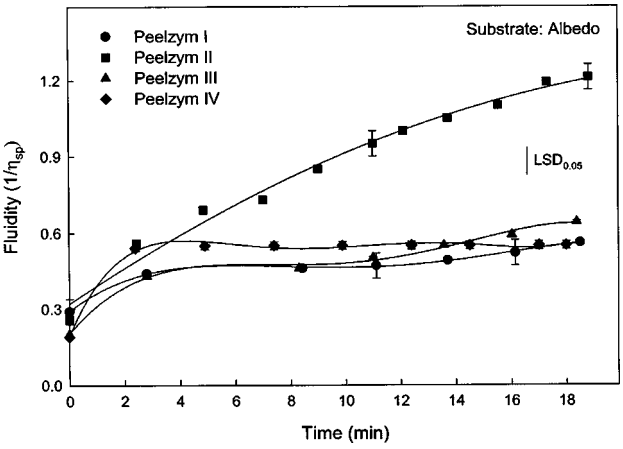

Figure 3. Evolution of fluidity of a solution of albedo from Cimboa (20 mg ml^{-1}) against time. Considering as minimum fluidity that shown by the solution before adding the corresponding enzymatic preparation: Peelzym I, II, III or IV. Reaction medium: 10 ml of a solution of albedo (20 mg ml^{-1}) in sodium acetate buffer 0.1 M, pH 4.0 and 2 µl of a solution of the corresponding enzymatic preparation (Peelzym I, II, III or IV). Data are the mean of four replicates and the $LSD_{0.05}$ value is shown.

which was the enzymatic preparation of the four studied with the maximum pectinase activity (Fig 1a). However, it only reached 77.6% of activity on polygalacturonic acid in relation to Peelzym II, which was the enzymatic mixture with the maximum polygalacturonase activity (Fig 1b). Therefore, if we consider the relative activities of the four Peelzym preparations on pectin, polygalacturonic acid and carboxymethylcellulose (Fig 1a,b and c, respectively), it could be concluded, in agreement with previous reports,[2,3,8,14] that the most important activities for the degradation of the carpelar membrane are those activities which act mainly on pectin and especially on polygalacturonic acid. However, in the industrial process of enzymatic peeling, the combination of hemicellulolytic, cellulolytic and pectolytic enzymes could cause the breakdown of the cellular wall, although the degradation of all its components would not be reached. In the experiment reported here, the presence of high cellulase activity caused a rapid desegregation of tissues, indicated by the rapid increase in fluidity observed during the first 3 min of the reaction time (Fig 2). In turn, the rapid elimination of membrane remains after enzymatic peeling and during rinsing of the segments could be favoured.

Enzymatic degradation of albedo

Enzymatic degradation of albedo, as well as citrus segment membrane, requires the combination of many enzymatic activities, depending on the composition of structural polysaccharides of the tissue to degrade.[15] To determine the real importance of each type of activity on albedo desegregation, the degradative capacity of Peelzym commercial preparations on a solution of a saturate concentration of lyophilised albedo was studied. Figure 3 shows the increase in fluidity produced by the degradation of albedo solution from Cimboa against reaction time. With Peelzym II 1.10 fluidity units were obtained after 15 min, while Peelzym I, III and IV only produced around 0.50 fluidity units after this time. However, with Peelzym IV, the maximum fluidity increase was achieved after 3 min of reaction. Therefore, given that Peelzym II obtained the maximum fluidity, and that it was the Peelzym with the highest polygalacturonase activity, this would be the most important activity for albedo degradation in citrus. This is in agreement with previous reports.[2,3,14] It is probable that the loss of cellular cohesion can be achieved in albedo through the use of pure polygalacturonases.[8] However, the presence of other activities, especially pectolytic, could improve the degradative capacity of the enzymatic preparation and achieve the degradation of the outermost fibres, favouring the separation of the skin in the industrial process of enzymatic peeling.

From the results presented, it can be concluded that the most effective activities for the degradation of segment membrane from Cimboa were those activities which act mainly on pectin and specially on polygalacturonic acid while, for albedo degradation, it was those which act on polygalacturonic acid. In addition, the enzymatic concentration required for carpelar membrane degradation was lower than that required for albedo degradation since, under the same reaction conditions, more than double the fluidity units were obtained from segment membranes other than from albedo. This information will be useful to compare the enzymatic peeling of citrus fruit in future researches, although the differences between segment membrane and albedo intact tissues and the ground-up samples used in this report could be important in considering how an enzymatic preparation would act on a whole fruit.

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