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Improved Viscosity of Tomato Fruit Paste and Serum by Genetic Engineering

Poboljšanje viskoznosti pulpe i seruma plodova rajčice genetičkim inženjeringom

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Summary

Tomato processors aim to produce tomato paste with maximum viscosity. The viscosity of the paste is determined largely by the pectin components of the fruit cell walls. During processing the action of two endogenous hydrolases – pectinmethylesterase (PE) and polygalakturonase (PG) – can extensively degrade the pectin and this results in a singificant reduction in paste viscosity. One approach to reduce this problem is to heat the fruit prior to pulping – the so called »hot break« process. This unactivates endogenous enzymes.

With the advent of recombinant DNA techology it is now possible to down regulate enzyme expression in plant tissue. Tomato plants have been genetically engineered with antisense genes to reduce the endogenous levels of both PE and PG. Paste produced from these transgenic fruit has improved serum viscosity and pulp viscosity, respectively.

Sažetak

Prerađivači rajčice nastoje proizvesti koncentrat maksimalne viskoznosti. Viskoznost koncentrata pretežno ovisi o pektinskim sastojcima staničnih stijenki rajčice. Tijekom prerade dvije endogene hidrolaze, pektin-metilesteraza (PE) i poligalakturonoza (PG), razgrađuju pektin, što znatno snizuje viskoznost koncentrata. Određeni pristup rješenju tog problema je zagrijavanje plodova rajčice prije pasiranja, što se naziva »hot break« (»vrući«) postupak, a kojim se inaktiviraju endogeni enzimi.

Rekombinantnom DNA tehnologijom danas je moguće bitno sniziti aktivnost enzima u biljnom tkivu. U genom rajčice, genetičkim inženjerstvom, ugrađeni su »antisens-geni« kako bi se snizila razina endogenih PE i PG. Koncentrati proizvedeni iz transgenskih plodova pokazuju poboljšanu viskoznost soka i koncentrata.

Introduction

High viscosity is an important quality attribute in pastes produced from fruit. Viscosity is determined by a variety of factors but in the case of tomato pastes a key determinant would appear to be the nature of the pectin components of the paste. Pectin is a complex molecule consisting of long chains of galacturonic acid interspersed with rhamnose. The galacturonic acid residues can exist as either the free acid or as methyl esters and the rhamnose residues can be substituted with neutral sugar sidechains. The structure of these polymers is covered in detail in the review by McNeil *et al. (1)*. The structural parameters of the pectin are likely to have pronounced effects on the rheological properties of the paste. In particular the degree of methyl esterification and polymerisation of the polymers would be expected to affect viscosity.

Pectins form a major part of the tomato fruit cell wall and are subject to degradation during ripening *(2)*. This degradation is likely to be partly responsible for the softening of the fruit during ripening *(3)*. Pectin degradation is brought about by the action of a range of endogenous hydrolases *(4,5)* but in particular by the action of polygalacturonase and pectinesterase. Polygalacturonase (PG) catalyses the hydrolysis of the glycosidic bond between adjacent galacturonic acids in the pectin backbone. This results in the depolymerisation of the pectin. Pectinesterase (PE) catalyses the hydrolysis of the methyl ester on galacturonic acid residues to generate the free acid. The action of this enzyme thus reduces the degree of esterification of the pectin polymers. Since the action of PG reguires the presence of at least two adjacent free acid forms of galacturonic acid it is thought that PE and

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PG can act synergystically to breakdown pectin. The PE, by deesterifying blocks of galacturonic acid residues on the backbone, could generate sites for depolymerisation by PG. It is apparent however, that the action of these two enzymes in the intact fruit is limited in some way. During ripening the pectin does become smaller but the average relative molecular mass shifts only slightly from about 160,000 in mature green to around 85,000 in ripe fruit *(6)*.

In comparison, when tomato fruits are homogenised, as in processing, the enzyme action is far more extensive, resulting in the production of monomeric and small oligomeric polymers *(7)*. Such differential action of the enzymes *in vivo* and *in vitro* would indicate some form of control of activity in the intact fruit. This control is obviously lost when fruits are homogenised resulting in the extensive degradation of the pectin polymers. This degradation can have an adverse effect on the paste viscosity. Heating fruit prior to processing inactivates the endogenous enzymes and hence reduces pectin degradation. Indeed it is common practice for some processors to heat treat fruit at between 85 °C and 100 °C in what is called the »hot break process«. Such heating has several disadvantages. Firstly, it can add to the costs of processing by increasing the energy input. Secondly, heating itself can actually have an adverse effect on the paste viscosity. Finally the heating could have adverse effects on flavour volatiles or other quality attributes of the paste.

The advent of recombinant DNA techniques or »genetic engineering« can provide an alternative to the »hot break« process for the inactivation of endogenous enzymes. Armed with sufficient knowledge of the molecular biology of an enzyme it is possible, using antisense technology, to down regulate the expression of that enzyme.

Mature green fruits have little or no PG activity but this increases dramatically during ripening *(8)*. The activity in a ripe fruit can be separated into at least three isoforms *(9)* but it is apparent that these arise due to post translational modification of a single gene product *(10)*. The PG protein has been purified *(11)* and fully sequenced *(12)*. In contrast PE activity is constitutive throughout fruit development and ripening *(13)*. The PE activity in fruit can also be resolved into at least three isoforms PE A, PE B and PE C *(14)*. The isoform PE A is predominant throughout fruit development and ripening accounting for at least 80 % of the total enzyme activity. This enzyme is also refered to as PE 2 and has been purified *(15)* and fully sequenced *(16)*. Antibodies raised against PE A show only a weak cross reactivity with either PE B or PE C and N-terminal amino acid sequences of these three isoforms are markedly different (*14* and Zhang and Tucker unpublished). This would indicate that, in contrast to PG, there are three separate genes, possibly with little sequence homology, for the three isoforms of PE found in tomato fruit.

Materials and Methods

Plant Material

Ailsa craig tomatoes, normal or homozygous for antisense polygalacturonase or pectinesterase 2 genes, were grown under glasshouse conditions. Fruit were tagged at the first stage of colour change (breaker) and harvested at mature green (ripening stage 0) and at defined days post breaker (dpb) (7, 14 and 21 days, respectively).

Bostwick tests

Tomato fruit were harvested and homogenised directly using a polytron to give approximately 500 mL of »cold-break« paste. For »hot-break« pastes fruit were heated in a microwave oven at 600 W for 10 min prior to homogenisation. Water loss during heating was measured by loss of weight and water added to the resultant paste to compensate. Paste viscosity was measured by placing the paste in the reservior of a standard Bostwick apparatus. This apparatus consists of a long rectangular trough which has a dammed partition at one end. The tomato paste is placed into this partition, the dam removed and the paste allowed to flow freely along the length of the trough. The distance moved in 30 s was then monitored.

Obviously the more viscous the paste the slower it will flow and hence it will give a low Bostwick value.

Serum viscosity tests

Pastes were produced as described for the Bostwick tests. The insoluble colloidal material was separated out by centrifugation. The resultant serum, or supernatant, was then assessed for viscosity using standard Ostwald viscometers. In this instance the time (τ) taken for a fixed volume of serum to flow through a capillary tube was determined and as such high values in this test indicate high viscosity.

Results and Discussion

Tomato cDNAs clones have been isolated for both PG *(17)* and PE 2 *(18)*. These cDNAs have been used to construct antisense genes for PG *(19)* and PE 2 *(20)*. These antisense genes when used to transform tomato plants resulted in the down regulation of either PG or PE activity in the fruit. Thus tomato plants containing an antisense PG gene had less than 1 % normal PG activity, whilst plants with the antisense PE 2 gene exhibited about 10 % of normal PE activitiy in the fruit. The inability of the antisense PE 2 gene to reduce the level of PE activity in the fruit to below 10 % lies in the nature of the PE isoforms in the fruit. Thus expression of PE 2 is almost completely abolished in the transgenic fruit whilst the activities of the other two isoforms remain unaffected or indeed may even increase slightly (Zhang and Tucker unpublished).

The down regulation of the endogenous PG had a marked effect on pectin degradation during ripening. The depolymerisation of the pectin normally associated with ripening was markedly inhibited in the antisense PG fruit *(21)*. Other aspects of ripening such as colour change and ethylene synthesis were unaffected. Similarly the down regulation of PE 2 activity had a pronounced effect on the degree of esterification of the total fruit pectin, the pectin being about 10–20 % more heavily esterified at all stages of development and ripening.

The down regulation of PG appeared to have only marginal effects on the softening of intact fruit. Compression testing detected no difference in the rate of initial softening between normal and transgenic fruit *(22)*.

However, it was apparent that subsequent oversoftening was markedly delayed in the antisense PG fruit *(10)*. It was also clear that the antisense fruit were more resistant to damage during transportation.

Normal and transgenic fruit were grown at the Horticultural Research International Station in Littlehampton on the South coast of Britain. The fruit were harvested at various stages of ripening and transported by rail about 200 miles to Nottingham University. Fig. 1 shows the percentage of cracked fruit upon arrival in Nottingham. It is clear that at all stages of ripening the proportion of antisense PG fruit damaged during transport was much reduced. This property enables fruit to be harvested later than normal and since vine ripened fruit attain a better flavour has important consequences for the quality of fresh fruit. The down regulation of PE 2 had no real detectable effect on the ripening of intact fruit.

The down regulation of PG and PE 2 did however, appear to result in significant benefits for tomato fruit processing. The viscosity of tomato paste is determined commercially by the application of the Bostwick test. The results of such Bostwick tests on normal and antisense PG tomato fruit are shown in Fig. 2. Considering the cold break results it can be seen that mature green fruit (ripening stage 0) gave high viscosity pastes even if the fruit were not heated before paste preparation. However, as the fruit ripened the quality of the paste declined rapidly resulting in a very »runny« paste. In contrast pastes made from antisense PG fruit showed only a marginal decrease in viscosity as the fruit ripened.

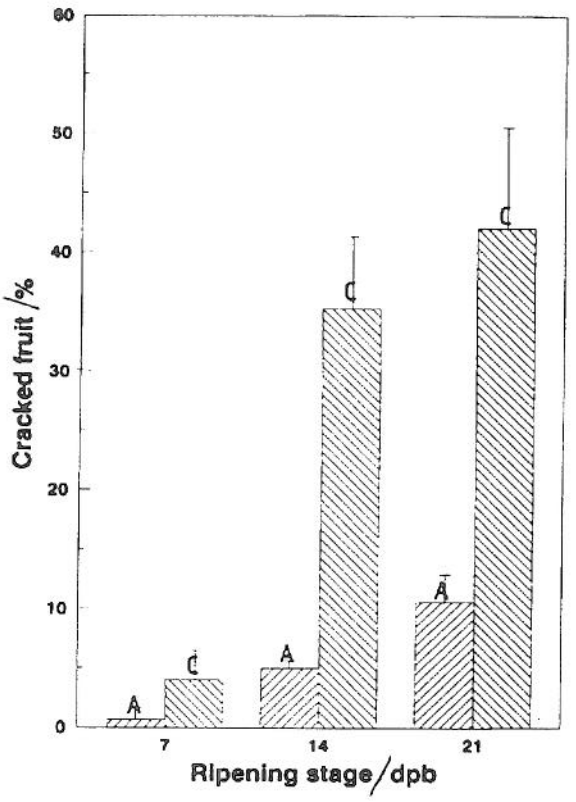

Fig. 1. Percent of cracked fruits normal (C) and antisense PG (A)
Slika 1. Postotak neoštećenih plodova s normalnom (C) i »antisens« PG (A)

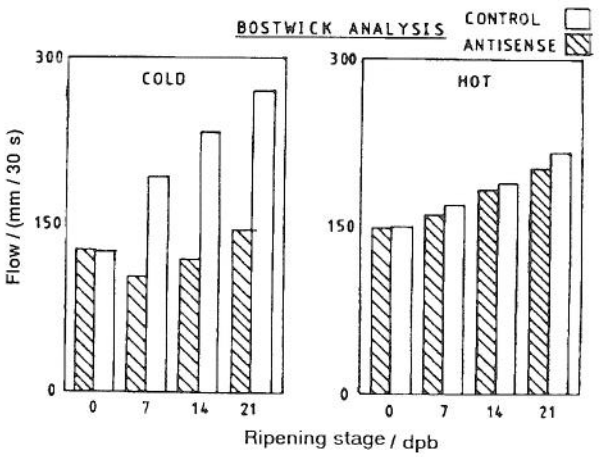

Fig. 2. Bostwick analysis of paste from normal and antisense PG fruit
Slika 2. Bostwickov test koncentrata rajčice normalnih i »antisens« PG plodova

Pastes made from pre-heated fruit displayed a markedly different pattern of viscosity changes. The viscosity of pastes from mature green fruit was slightly lower than from the unheated equivalent. This illustrates the adverse effect of heating on paste viscosity. The paste viscosity again declined as fruit ripened but in this instance there is little difference between normal and antisense PG fruit. These results could be interpreted to indicate the key role of PG and hence presumably the degree of polymerisation of pectin in determining the quality of tomato paste.

The effect of antisense PE 2 on the Bostwick value of the paste was difficult to assess. In some trials there seemed to be little or no effect, the consistency of paste from unheated antisense PE 2 fruit being the same as that from control fruit. However, in the most recent trial the PE 2 antisense fruit produced paste with an apparent increase in consistency (Errington and Tucker unpublished). The significance of this finding is being pursued. Another recent interesting observation concerns the cold processing of fruit which had been stored frozen. Normal and antisense PG fruit in this instance appeared to result in high Bostwick values, these both being around 325 mm/30 s. This would suggest that freezing the tissue masked the beneficial effect of antisense PG found in fresh fruit. However, in this instance the antisense PE 2 fruit when defrosted maintained their integrity better than control or antisense PG fruit and produced a paste with a lower Bostwick value of around 175 mm/30 s.

A second important quality characteristic of tomato paste is the serum viscosity. The serum viscosities of pastes from normal and transgenic fruit are shown in Fig. 3. Again two treatments have been tested, namely cold and hot break. Normal fruit, if unheated prior to paste production, resulted in pastes with very low serum viscosities. The use of antisense PG tomatoes had little effect on the serum viscosity but antisense PE 2 fruit produce a serum viscosity which was much higher than normal. Heating the fruit resulted in a general increase

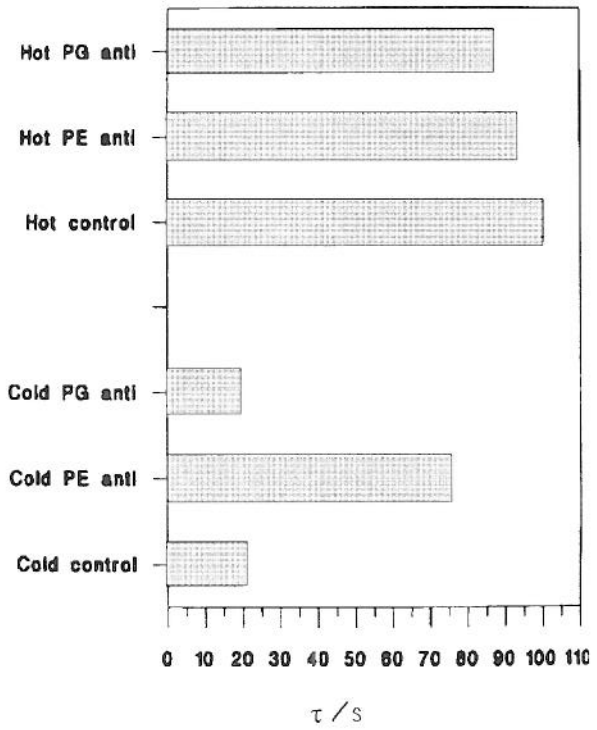

Fig. 3. Time (τ) of fixed volume of serum and pastes from normal and transgenic fruit
Slika 3. Vrijeme (τ) određenog volumena seruma i pulpe normalnih i transgenskih plodova

in serum viscosity which tended to eliminate the differences between the normal and transgenic fruit.

Recently it has been possible to obtain transgenic fruit in which both PG and PE 2 are down regulated *(23)*. Some preliminary processing trials have been carried out with this fruit. There would seem to be no effect on the consistency of paste made from these fruit. Unheated control fruit gave a Bostwick reading of around 225 mm/30 s whilst the corresponding fruit with neither PG or PE 2 gave a reading of around 300 mm/30 s. Similarly the serum viscosities, relative to water, of these two pastes were both around 2. These results are preliminary but would seem to indicate that any benefits derived from either antisense PG or PE 2 are not apparent when both enzymes are down regulated together. At present the detailed structures of the pectin components of all the pastes and sera are being determined. It is hoped that a comparison of pectin structure within the colloidal and soluble fractions of the paste will correlate with the varied rheological parameters already determined. In this case it should be possible to explain the rheological behaviour of the tomato paste in molecular terms and perhaps to be able to predict the effect of any future biochemical manipulations of the pectin components of the paste.

Conclusions

Antisense technology has been successfully employed to down regulate expression of cell wall hydrolases such as polygalacturonase (PG) and pectinesterase (PE) in tomato fruit. This down regulation results in significant alterations to the structure of the pectin in the intact fruit but has little effect on fruit softening. Despite this antisense PG fruit display a marked resistance to cracking during transportation. PG antisense fruit also gave tomato pastes with a significantly higher Bostwick viscosity whilst antisense PE fruit gave pastes with higher serum viscosity. These alterations in paste properties represent a distinct advantage to the processor.

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