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(54) Title: METHOD FOR REDUCING THE SERUM SEPARATION IN A PECTIN CONTAINING AQUEOUS MASS

(57) Abstract

A method for reducing the serum separation of an aqueous mass containing pectin, comprising the steps of: a) providing an aqueous mass substantially free from pectin depolymerizing enzymes, b) adding an effective amount of pectinesterase, preferably substantially free from pectin depolymerizing enzymes, and c) incubating said mass in the presence of divalent cations, is disclosed. The method has been found to be advantageous with products wherein the pectin containing aqueous mass is derived from broccoli, pepper, mustard, apples, tomatoes, oranges, lemons, grapes, lime, pears, carrots, peas, cauliflower, and berries, such as blackcurrant, blue-berries, strawberries, and raspberries to obtain products, such as jam, marmalade, jelly, juice, paste, soup, dressing, sauce, condiment, ketchup, salsa, chutney, pudding, mousse, or other deserts.

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Title: Method for reducing the serum separation in a pectin containing aqueous mass

FIELD OF THE INVENTION

The present invention relates to a method for reducing the serum separation/syneresis in a pectin containing aqueous mass by the use of certain enzymes, and enzyme compositions therefore.

BACKGROUND OF THE INVENTION

Dicotyledonous plants comprise some of the major crops cultured by man, such as beans, peas, beets as well as most other fruits and vegetables.

The primary cell wall and middle lamella of these plants usually have a high content of pectic substances. The pectic matrix consists of smooth regions of polygalacturonic acids and of rhamnogalacturonan.

The polygalacturonic acid areas are usually highly esterified by methoxyl groups. Side groups consisting of araban, galactan and arabinogalactan are attached to the rhamnogalacturonan residues. The presence and distribution of the carboxy-methyl groups in pectin significantly alters its solubility and physico-chemical and gel forming properties.

This is of major importance both in the plant cell wall, where the pectic matrix is a key regulator of porosity and passive diffusion of macromolecules but also in industrial processing of pectin and pectin containing plant material.

Pectinesterase (PE), EC 3.1.1.11 (*Enzyme Nomenclature 1992*, Academic Press, Inc., 1992), hydrolyses the ester linkage between methanol and galacturonic acid in esterified pectin. PE is found both in plants and in microorganisms. That is, PE offers a possibility of completely controlling the properties of pectin by altering the degree of esterification (DE).

Thus, an endogenous highly methoxylated content of pectin (HM pectin) in various fruits and vegetables can enzymatically be modified to a low methoxylated pectin by pectinesterase. In combination with the natural content of calcium ions this is sufficient for an *in situ* gelation or an *in situ* thickening to take place if the PE is substantially free from depolymerizing enzyme

activities (*Calesnik, E.J. et al 1950, Arch. of Biochem., 29, 432-440. Meurens, M., 1978, Rev. Ferment. Ind. Aliment., 33, 95-104*), since the presence of such activities will substantially break down the pectin.

It is a well known problem that several products that are fully or partially vegetable or fruit based, such as ketchup, mustard and jam, "weep" or separate on standing. The processes used result in an indelicate appearance of the product. Furthermore use of the product on *e.g.* bread or rolls results in the bread or roll getting soaked and disintegrate. Similarly other products such as fruit soups and yoghurts separate and develop an unattractive appearance.

It is an object of this invention to provide a method of causing a decrease of the serum separation in an aqueous mass containing methoxylated pectin without the need for addition of stabilising agents, such as externally added hydrocolloids, modified starch etc.

SUMMARY OF THE INVENTION

The present invention relates to a method for reducing the serum separation of an aqueous mass containing pectin, comprising the steps of :

a) providing an aqueous mass substantially free from pectin depolymerising enzymes.
b) adding an effective amount of pectinesterase, and
c) incubating said mass in the presence of divalent cations.

Surprisingly it has been found that no other stabilising agent is needed in relation to the method according to the invention.

Hereby the method according to the invention offers the unique feature that the serum separation can be reduced or even avoided without the addition of any further external agents, whereby the only external agent being added is an effective amount of pectinesterase, and optionally divalent ions, such as alkaline earth ions, especially calcium ions.

Thus, for the first time it has been proven possible to utilise deesterified endogenous pectin in itself for a direct reduction of serum separation.

DETAILED DESCRIPTION OF THE INVENTION

As indicated the present invention relates to a method for reducing the serum separation of an aqueous mass containing pectin, comprising the steps of :

a) providing an aqueous mass substantially free from pectin depolymerising enzymes.
b) adding an effective amount of pectinesterase, and
c) incubating said mass in the presence of divalent cations.

According to one embodiment of the invention the pectin may be endogenous HM.

In an embodiment of the method according to the invention the pectinesterase used is derived from a fungus of the genus *Aspergillus*, preferably *A. japonicus*, (S. Ishii *et al.*, 1979, *Journal of Food Science* 44, p 611-614), *A. aculeatus* , *A. niger* (EP 0 388 593 A1), *A. awamori* (EP 0 388 593 A1), or the genera *Fusarium*, *Sclerotonia*, or *Penicillium*, (Kikkoman: DE 2843351; US 4,200,694). These pectinesterases exhibit a relatively low pH optimum, corresponding to the relatively low pH optimum of many fruits.

The pectinesterase to be used in the invention should preferably be substantially free from pectin depolymerizing enzymes, such as pectinlyases or polygalacturonases. In case these activities are present in substantial amounts the pectin will be degraded.

Such enzymes are obtainable by using a host system for the expression of the enzyme which does not produce any pectin depolymerizing enzymes (WO 94/25575).

The activity of the pectinesterase is indicated in Pectin Esterase Units (PEU) defined as the amount of enzyme which under standardised conditions hydrolyses 1 mmol carboxyl groups per minute. A folder describing the Novo Nordisk assay ABT-SM-0005.02.1 is available upon request.

According to the invention the pectin should be present in the aqueous mass in an amount from 0.1%w/w to 10%w/w preferably from 0.2%w/w to 1.0%w/w.

In further embodiments of the invention it may be advantageous to add sugar, acetic acid and/or salt in amounts from 0-60%w/w, 0-10%w/w, and 0-10%w/w, respectively.

The method of the invention will normally be carried out at a pH value from 2.5 to 7.5, preferably from pH 3 to pH 5, and for a time period for the incubation step from 5 to 60 minutes, preferably from 10 seconds to 30 minutes.

The incubation step will normally be performed at a temperature from 10 to 60°C, preferably from 15 to 50°C.

According to the invention the PE is added in an amount of from 4 to 400 PEU, preferably from 9 to 135 PEU, better from 25 to 110 PEU, still better from 35 to 90 PEU, and more preferably from 45 to 70 PEU/kg VSS (Vegetable Soluble Solids, VSS is measured refractometrically as % Brix).

According to the invention the method is designed for use with a pertinacious mass, especially food, and especially products such as tomato juice, tomato slurry, tomato paste or salsa or ketchup.

The method of the invention has been found to be advantageous with products wherein the pectin containing aqueous mass is derived from broccoli, pepper, mustard, apples, tomatoes oranges, lemons, grapes, lime, pears, carrots, peas, cauliflower, and berries, such as blackcurrant, blue-berries, strawberries, and raspberries.

Products obtained by the process of the invention are such as jam, marmalade, jelly, juice, paste, soup, dressing, sauce, condiment, ketchup, salsa, chutney, pudding, mousse, or other deserts.

MATERIALS AND METHODS

Materials:

Hot break tomato paste
Pectinesterase prepared according to WO 94/25575
Brookfield Viscometer, DVII

DUR Refractometer, Schmidt and Haensch, Germany
Blotter test paper (Bridge & Company)

Methods:

Viscosity Measurement:

The viscosity was measured by Spindel C, specification no. 93. Measurements at shear rate 2.5 rpm and 20 rpm were carried out.

Measurement of vegetable soluble solids (VSS):

A drop of the test solution is applied to the sample well in the DUR refractometer.

The instrument indicates directly the solids content in % Brix.

Blotter Test:

To quantify the serum separation from the solid content the serum separation evaluation procedure of using a blotter test paper was used (Gould W.A., et al 1992, Tomato Production Processing & Technology 3rd edition CTI Publications).

7.0 g of ketchup was weighted out in a metal cylinder (dia 38 mm) placed in the centre of the blotter paper. After 7 min the serum separation was measured at the four rules. The measurement is given as an average of the four figures.

A low value indicates a low degree of syneresis/serum separation.

EXAMPLES

EXAMPLE 1

Hot break tomato paste was diluted from 22.5% VSS to 8.5% VSS and homogenised at 300 bar. Afterwards, eight samples of 455 g were weighed out in 1000 ml containers, the temperature was adjusted to 40°C.

8 x 25 ml enzyme solutions of increasing PE concentrations were added to the above mentioned substrates, the final enzyme protein concentrations were as follows: 0, 17, 34, 59, 68, 85,

102, 136 PEU/kg VSS. The samples were then incubated for 30 min at 40°C.

After the enzyme treatment a ketchup was prepared by adding 300 g of brine to each of the samples. The brine consists of sugar, salt and acetic acid (Skott, W. P. *Die Industrielle Obst- und Gemüseverwertung*, 1970 **55** 229-234). The prepared ketchup were then heat treated, 88°C in 3 min. The samples were then cooled in an ice bath and finally placed in the refrigerator until analysis could take place.

The results from the analysis is shown in Table I

Table I

Pectinesterase Concentration PEU/kg VSS	Blotter Test mm	Viscosity 2.5 rpm cP	Viscosity 20 rpm cP
0	20.7	8305	1455
17	18.2	9027	1543
34	14.4	9527	1660
59	9.7	8745	1595
68	5.8	22900	3330
85	10.3	31965	4250
102	14.2	35505	4780
136	25.0	45415	8340

From the Blotter test results it is clearly seen that the syneresis or serum separation can be controlled by adjusting the concentration of pectinesterase. Not surprisingly, also the viscosity depends on the enzyme concentration. It is noticed that it is possible at the same time to obtain both a reduction of the syneresis and a viscosity increase.

REFERENCES CITED IN THE SPECIFICATION

1. Calesnik, E.J. *et al* 1950, *Arch. of Biochem.*, **29** 432-440.
2. Meurens, M., 1978, *Rev. Ferment. Ind. Aliment.*, **33** 95-104)
3. Gould W.A., *et al* 1992, *Tomato Production Processing & Technology*, 3rd edition CTI Publications
4. S. Ishii *et al.*, 1979, *Journal of Food Science* **44** 611-614
5. EP 0 388 593 A1
6. DE 2843351
7. US 4,200,694
8. WO 94/25575
9. Skott, W. P. *Die Industrielle Obst- und Gemüseverwertung*, 1970 **55** 229-234
10. *Enzyme Nomenclature 1992*, Academic Press, Inc., 1992
11. Novo Nordisk assay ABT-SM-0005.02.1

PATENT CLAIMS

1. A method for reducing the serum separation of an aqueous mass containing pectin, comprising the steps of :

a) providing an aqueous mass substantially free from pectin depolymerising enzymes.

b) adding an effective amount of pectinesterase, and

c) incubating said mass in the presence of divalent cations.

2. The method of claim 1, wherein the pectin is endogenous HM pectin.

3. The method of claims 1 or 2, wherein the pectinesterase is substantially free from pectic depolymerizing enzymes.

4. The method of any of claims 1 to 3, wherein said PE is added in an amount from 5.5 to 550 PEU, preferably from 15 to 135 PEU, better from 25 to 110 PEU, still better from 40 to 95 PEU, and more preferably from 55 to 80 PEU/kg VSS.

5. The method of any of claims 1 to 4, wherein the pectin is present in an amount from 0.1%w/w to 10%w/w, preferable from 0.2%w/w to 1.0%w/w.

6. The method of any of the preceding claims, wherein sugar (0-60%w/w), acetic acid(0-10%w/w) and/or salt (0-10%w/w) is added.

7. The method of any of the preceding claims, wherein said method is performed at a pH value from 2.5 to 7.5, preferably from pH 3 to pH 5.

8. The method of any of the preceding claims, wherein said incubation step is performed for a period from 5 to 60 minutes, preferably from 5 minutes to 30 minutes.

9. The method of any of the preceding claims, wherein said incubation step is performed at a temperature from 10 to 60°C, preferably from 15 to 50°C.

10. The method of any of the preceding claims, wherein the aqueous mass containing pectin is a tomato juice, tomato slurry, tomato paste, salsa, ketchup.

11. The method of any of the preceding claims, wherein the pectin containing aqueous mass is derived from broccoli, pepper, mustard, apples, tomatoes, oranges, lemons, grapes, lime, pears, carrots, peas, cauliflower, and berries, such as blackcurrant, blue-berries, strawberries, and raspberries.

12. A product produced by the process of any of the claims 1 to 11.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00392

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A23L 1/0522, A23L 1/06
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9412055 A1 (GIST-BROCADES N.V. ET AL.), 9 June 1994 (09.06.94), page 4, line 22 - line 38; page 5, line 18 - line 22; page 6, line 3 - line 15, claim 1	1-12
A	US 4200694 A (S. ISHII ET AL.), 29 April 1980 (29.04.80), column 2, line 57 - column 3, line 35, claim 1	1-12
A	WO 9309683 A1 (GIST-BROCADES ET AL.), 27 May 1993 (27.05.93), claims 1-2,6-9	1-12

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Patent Abstracts of Japan, Vol 8,No 110, C-224, abstract of JP,A,59-25673 (KITSUKOO SHIYOKUHIN KOGYO K.K.), 9 February 1984 (09.02.84) --	1-12
A	Patent Abstracts of Japan, Vol 8,No 110, C-224, abstract of JP,A,59-25674 (KITSUKOO SHIYOKUHIN KOGYO K.K.), 9 February 1984 (09.02.84) -- --------	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/10/96

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PCT/DK 96/00392

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9412055	09/06/94	AU-B-	672662	10/10/96
		AU-A-	5650594	22/06/94
		CA-A-	2128249	09/06/94
		EP-A-	0624062	17/11/94
		HU-A-	67664	28/04/95
		HU-D-	9401988	00/00/00
		IL-D-	107791	00/00/00
		NZ-A-	258574	27/08/96
		PL-A-	304693	09/01/95
		ZA-A-	9308949	02/08/94
US-A- 4200694	29/04/80	DE-A,B,C	2843351	12/04/79
		FR-A,B-	2405263	04/05/79
		JP-C-	1147089	12/05/83
		JP-A-	54055784	04/05/79
		JP-B-	57024755	26/05/82
		JP-C-	1132369	27/01/83
		JP-A-	54055792	04/05/79
		JP-B-	57018796	19/04/82
WO-A1- 9309683	27/05/93	AT-T-	140594	15/08/96
		AU-B-	660339	22/06/95
		AU-A-	2924192	15/06/93
		CA-A-	2098117	14/05/93
		DE-D-	69212447	00/00/00
		EP-A,B-	0547648	23/06/93
		HU-A-	65701	28/07/94
		NZ-A-	245135	27/04/94
		PL-B-	169858	30/09/96
		PL-A-	299999	24/01/94
		ZA-A-	9208774	21/06/93