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One-step concentration and partial purification of *Aspergillus kawachii* non-acidic polygalacturonases by adsorption to glass fiber microfilters

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Abstract

The non-acidic polygalacturonases produced by *Aspergillus kawachii* in a glucose/tryptone medium were adsorbed to a glass fiber microfilter that was used to clarify the fermentation broth. Maximum adsorption occurred at pH 3 under low ionic strength conditions. The adsorbed activity could be readily released with a buffer solution at pH 5. Based upon these observations, a separation process was developed which enabled the broth to be clarified and, at the same time, the non-acidic polygalacturonases to be concentrated 20-fold and purified 100-fold in a unique filtration step. The practical advantage of recovering polygalacturonases by a filtration process lies in the simplicity and efficiency of the operation involved.

Introduction

Fungal polygalacturonases either alone or in combination with other pectinases have several commercial applications in the food and beverage industries (Pilnik & Voragen 1993). Recent reviews have also highlighted the potential use of these enzymes in the pulp industry, wastewater treatment and for retting and degumming of fiber crops (Kashyap *et al.* 2001). In order to characterize and study the properties of polygalacturonases, the enzymes must be purified. This is usually carried out by a multi-step process involving biomass separation, concentration, primary isolation, purification and polishing as the main unit operations (Asenjo 1990). The conventional procedures for the initial recovery of polygalacturonases from the culture broth include concentration under reduced pressure, ultrafiltration, fractionation with salts or solvents, adsorption on inorganic supports and aqueous two-phase systems (Rombouts & Pilnik 1980, Bailey & Ojamo 1990, Sakai *et al.* 1993, Wu *et al.* 2001, Sathyanarayana *et al.* 2003). These procedures are usually laborious and time consuming although enzyme recovery may be high. Furthermore, recovery or disposal of materials used in the separation process may increase the cost of the separation step or result in environmental pollution. Therefore, development of alternative separation process for the initial isolation of these enzymes from the cultivation media is always needed. In a previous work, we reported the purification of an acidic endo-polygalacturonase (PGI) from the culture filtrate of *Aspergillus kawachii* grown in a glucose-trytone medium (Contreras Esquivel & Voget 2004). The PGI purification scheme includes the filtration of the fermentation broth through a glass fiber microfilter (GFM) in order to obtain a particle-free protein solution prior to enzyme recovery. The GFM was routinely used for that purpose due to

its high capacity for sub-micron particles and resistance to clogging. The non-acidic polygalacturonase activity produced by *A. kawachii* in the glucose/tryptone medium was adsorbed to the GFM during the filtration process. The adsorption phenomenon was unexpected since there was no previous report on the adsorptive properties of this type of microfilter towards proteins. The adsorbed activity could subsequently be released from the filter by elution with a buffer solution resulting in a high recovery of the enzyme activity. Based upon these observations, a simple separation process has been developed which enabled to perform broth clarification together with the concentration and partial purification of two non-acidic polygalacturonases in a unique filtration step. In this way the overall purification process of these enzymes was greatly enhanced, as compared to previously reported methods.

Materials and methods

Microorganism, culture conditions and biomass separation

Aspergillus kawachii IFO 4308 was cultured at 30 °C in a 1-litre flask filled with 100 ml GT medium containing 10 g glucose/l and 5 g tryptone/l (Contreras Esquivel *et al.* 1999). The initial pH was adjusted to 4.0 and the cultivation was carried out for 35–40 h. At that time, glucose was almost consumed and culture pH reached the lowest value (around 2.5). Separation of the fungal mycelium was achieved by filtration of the whole broth through a cheese cloth and the obtained permeate kept frozen at −20 °C until use. The mycelium-free fermentation broth is herein referred as FB.

Filtration procedure

Filtration was carried out by using a vacuum filtration unit equipped with a 47 mm polysulfone filter holder. The effective filtration area was $\sim$10.5 cm^2. Unless otherwise stated, the borosilicate glass fiber microfilter was obtained from Osmonics Inc (47 mm, nominal pore size 0.45 μm, G15WP04700). These filters contained an acrylic resin as a binder (Osmonics, technical information). The filter was used as received, without any treatment. Filtration was carried out at 20–25 °C in the normal flow filtration mode at a flow rate of 20–25 ml/min. To ensure equilibrium conditions, samples were filtered three times through the same filter (by recycling the permeate). The obtained permeate was kept refrigerated until use.

Activity assays

Polygalacturonase (PG) activity was assayed by measuring the release of reducing groups by the dinitrosalicylic acid method. The reaction mixture contained 2 ml 0.5% polygalacturonic acid solution in NaCP buffer and 50 μl of enzyme solution (NaCP buffer contained 50 mM citric acid/25 mM Na_2HPO_4 adjusted to pH 6 with NaOH and then recalibrated to the desired pH with HCl.). β-Glucosidase was measured with 1 mM *p*-nitrophenyl-β-D-glucopyranoside in NaCP buffer, pH 5 (Iwashita *et al.* 1998), acid-protease with denatured hemoglobin at pH 3 (Yagi *et al.* 1986) and amylase with 1% soluble starch in NaCP buffer, pH 5 (Mikami *et al.* 1987). Enzyme activities were measured at 37 °C. Protein was determined by the Lowry method using BSA as standard.

Polygalacturonase purification

The purification of polygalacturonases by column chromatography was performed at room temperature by using an FPLC system. The concentrated PG solution obtained after the GFM sorption process was dialyzed overnight against NaCP buffer pH 5 (1/4 strength) and loaded onto a Sepharose SP FF column (1.6×20 cm, 140 ml gel) pre-equilibrated with the same buffer. After washing the column with two volumes of buffer, the bound proteins were eluted with a linear gradient of NaCl (0 to 0.5 M NaCl in buffer). The column was operated at a flow rate of 0.75 ml/min and fractions of 5.0 ml were collected. Active fractions were pooled, dialyzed overnight against buffer and freeze-dried. The solid preparation was dissolved in a minimum amount of deionised water and poured over a Sephacryl S-100 column (XK 16/70, 100 ml gel) pre-equilibrated with NaCP buffer, pH 5.0. The column was eluted with the same buffer at a flow rate of 3 ml/min and fractions of 2 ml were

collected. Active fractions were pooled, dialyzed overnight against NaCP buffer (1/10 strength), pH 5, freeze-dried and kept refrigerated until use. SDS-PAGE and isoelectric focusing were carried out as described elsewhere (Contreras Esquivel & Voget 2004).

Results and discussion

Polygalacturonase adsorption

As previously reported, *A. kawachii* IFO 4308 cultivated in GT medium produced significant amounts of PG activity but activities of other pectinases were not detected (Contreras Esquivel *et al.* 1999). Figure 1 shows the pH vs activity profile of the PG activity measured before and after filtration of FB at pH 3. PG activity in the permeate decreased, as compared to FB, at pH values higher than 2.5 being negligible at pH 5.0. Further experiments showed that this phenomenon was due to the adsorption of the PG activity to the GFM. Based on these results, it was also inferred that at least two groups of PGs were produced by *A. kawachii* in GT medium. One group corresponded to those PGs active in the pH range 2.0–2.5 but inactive at pH 5, which did not bind to the GFM. According to another study, the main component of this group was PGI, an acidic endo-PG (Contreras Esquivel & Voget 2004). The other group corresponded to those PGs active at pH 5 but not at pH 2.0–2.5 (hereafter referred as PG5 or non-acidic polygalacturonases), which were bound to the GFM. PG5 activity in GT medium varied from 1.2 to 5.6 U/ml.

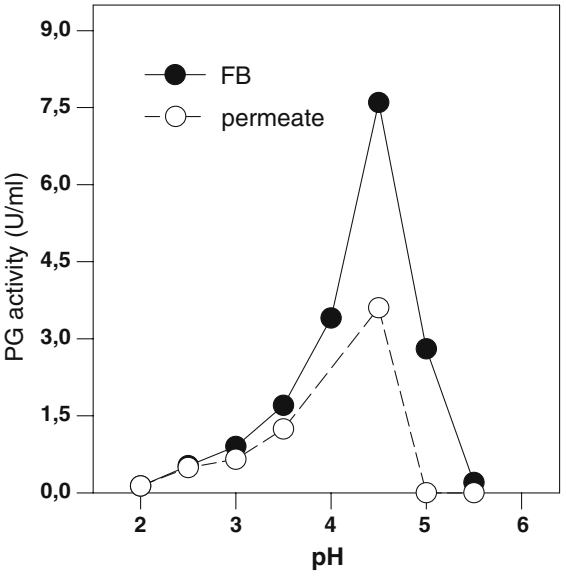

Fig. 1. pH vs activity curve of polygalacturonase before and after filtration of the fermentation broth through a borosilicate glass fiber microfilter (GFM). After the separation of the mycelium, the pH of the fermentation broth (FB) was adjusted to 3 and filtered three times (by recycling the filtrate) through a borosilicate glass fiber microfilter (47 mm, 0.45 μm pore size, OSMONICS) using a 47 mm polysulfone vacuum filtration unit. Filtration was carried out at 25 °C, at a flow rate of 20–25 ml/min. The filtered FB volume was 50 ml. Polygalacturonase activity was determined in FB and the permeate by measuring the release of reducing groups in a reaction mixture containing 2.0 ml of 0.5% PGA solution in NaCP buffer and 50 μl of enzyme solution.

Effect of different variables on PG5 adsorption

Maximum adsorption of PG5 to the GFM occurred at pH 3 and was negligible at pH 5 (Figure 2). On the other hand, the acidic PG activity (measured at pH 2) was not adsorbed in the pH range tested (not shown). The increase of the ionic strength of FB (at pH 3) by adding

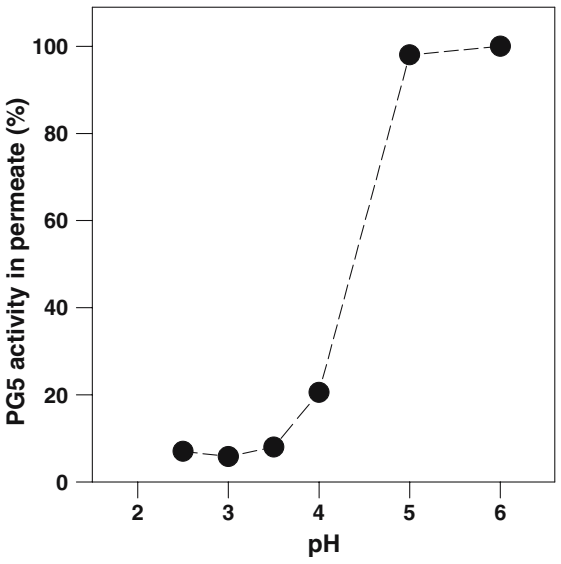

Fig. 2. Influence of fermentation broth pH on the adsorption of PG5 to the GFM. The pH of FB was adjusted to the desired value with HCl or NaOH. One filter was used for each pH experiment. The filtered FB volume was 50 ml and filtration conditions were those described in Figure 1. PG5: polygalacturonase activity measured at pH 5.0.

NaCl resulted in a decrease of PG5 adsorption. At 1 M NaCl, adsorption was completely avoided. Neutral detergents such as octyl-glucoside, Tween 80 and Triton X-100 at 0.1% showed no effect on PG5 adsorption. Similar results were obtained with a GFM without binder (nominal pore size 1.0 μm, G20MP04750), indicating that the binder was not involved in protein adsorption. This result also suggests that the borosilicate glass was the active adsorbent of PG5. Adsorption probably occurred through electrostatic interactions and hydrogen bonding as it has been reported for other inorganic adsorbents (Green & Wase 1986).

Effect of the feeding volume on PG5 adsorption

Figure 3 shows that the amount of PG5 adsorbed to the GFM increased with the increase of the feeding volume and plateaued at a loading level of 750 ml which corresponds to ~500 U/filter. Due to equilibrium conditions, the proportion of adsorbed activity rapidly decreased after a feeding volume of 300 ml.

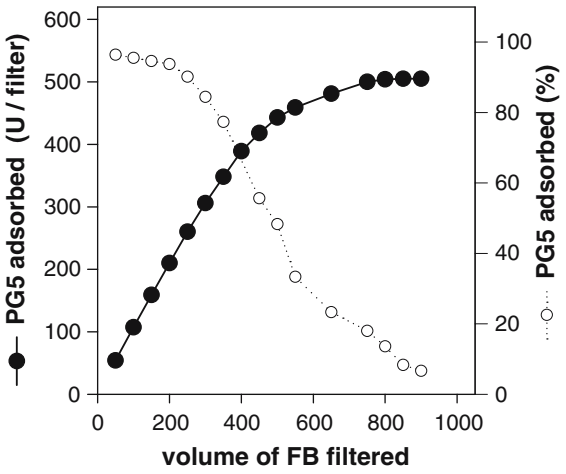

Fig. 3. Effect of the feeding volume on PG5 adsorption. 900 ml of FB at pH 3 was filtered in 50 ml volume fractions. PG5 adsorption after each filtration step was calculated according to: $(A_{CM}–A_P)\times 50$, where A_{FB} and A_P are the FB and permeate PG5 activities respectively. PG5 activity in FB was ~1.2 U/ml. Filtration conditions were those described in Figure 1.

PG5 desorption

PG5 was readily released from the GFM when the original permeate was used as eluant at pH 5 or by using buffer solutions of appropriate pH and ionic strength. Figure 3 shows the desorption process carried out with NaCP buffer, pH 5. Maximum PG5 recovery was 80% of the adsorbed activity. On the other hand, no PG5 activity was released with NaCP buffer (1/4 strength), pH 3.0. This buffer was therefore selected to wash the filter from non-adsorbed components.

PG5 purification

Table 1 summarizes the purification of the non-acidic polygalacturonases. After the sorption process by using the GFM, a 20-fold concentrate PG5 solution was obtained together with a 100-fold increase in specific activity. Some non-pectolytic activities produced by the fungus in GT medium were also present in the concentrated PG5 solution, being the β-glucosidase the most relevant activity (Table 1). Two PG5 fractions, which were designated PGII and PGIII, respectively, were separated by cation-exchange chromatography. The PGII fraction was homogeneous when examined by SDS-PAGE (Figure 5) and by isoelectric focusing (not shown). PGII represented more than 95% of the total PG5 activity and may be considered the main non-acidic polygalacturonase produced by *A. kawachii* in GT medium. PGIII was not detected by SDS-PAGE due to the very low protein concentration present in this sample. Minor impurities were removed from the PGII and PGIII preparations by gel permeation chromatography. The concentration of active fractions by freeze-drying after being eluted from chromatographic columns caused the major losses of polygalacturonase activity. Analysis of the purification process by SDS-PAGE confirmed that in addition to PGII and PGIII, other proteins were separated and concentrated by the GFM sorption process, being a 100 kDa protein the most important contaminant, which may be related to the β-glucosidase activity (Iwashita *et al.* 1998). Neither this protein nor PGII could be observed in the FB sample. On the other hand, some major bands present in FB were absent in both the original permeate and the eluate of the GFM. Further research is needed to

Table 1. Purification of non-acidic polygalacturonases from *A. kawachii.*

purification step	Volume (ml)	Protein (mg)	PG5 Activity (Units)	Specific Activity (Units/mg protein)	Yield (%)	Purification factor
FB	900	105	1570	15	100	1
GFM[a]	30	0.77	1160	1506	74	100
Sepharose SP[b]						
PG-II	40	~0.13	596	4585	38	306
PG-III	30	negligible	16	–	1	–
Sephacryl S-100[c]						
PG-II	40	–	360	–	23	–
PG-III	30	–	4.5	–	0.29	–

[a]Adsorption of PG5 to the GFM was carried out by filtering 300 ml FB at pH 3. After washing the filter with 10 ml of NaCP buffer (1/4 strength), pH 3, the bound activity was eluted with two 5 ml volume fractions of NaCP buffer, pH 5. The eluates from the three batches of filtration were pooled and polygalacturonases purified as described in Materials and methods. Measured activities in the eluate were: PG5: 38.7 U/ml, β-glucosidase 0.65 U/ml (33%), acid protease 2.0 U/ml (1.2%), amylase 0.26 U/ml (4.6%). In brackets: activity recovered in the eluate as % of the FB activity.
[b]Elution of PGII and PGIII occured at 75 mM and 270 mM NaCl respectively.
[c]Active fractions were pooled, dialysed overnight against NaCP buffer (1/10 strength) pH 5, freeze-dried and kept refrigerated until use for the determination of enzyme properties.

determine whether these proteins have been irreversibly bound to the GFM or they could not be released under the chosen conditions.

Enzymatic properties of the purified polygalacturonases

The molecular mass of PGII estimated by SDS-PAGE was 41 kDa while that obtained by gel filtration was 38 kDa. This result indicates that PGII is a monomeric protein. The p*I* of PGII was estimated to be 5.3. The molecular mass of PGIII estimated by gel filtration was 35.5 ± 1.03. The optimum pH for PGII and PGIII towards PGA were 4.7 and 5, respectively (Figure 4).

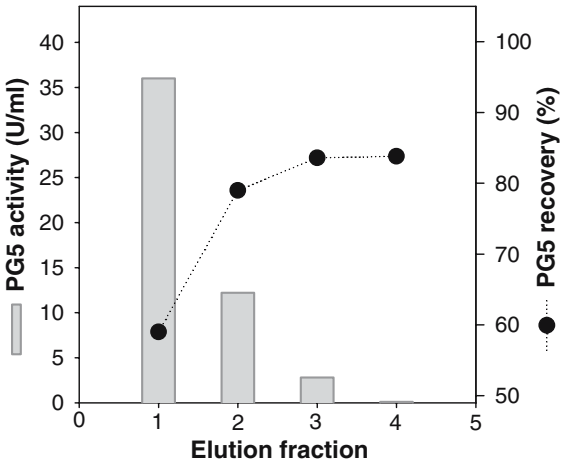

Fig. 4. Elution of PG5 adsorbed to the GFM. 300 ml of FB at pH 3 was filtered through a GFM. After washing the filter with 10 ml of NaCP buffer (1/4 strength), pH 3, the bound PG5 activity was eluted stepwise with 5 ml volume fractions of NaCP buffer, pH 5. PG5 recovery was expressed as % of the adsorbed activity. Filtration conditions were those described in Figure 1.

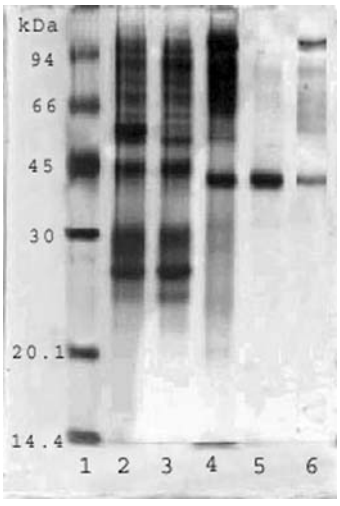

Fig. 5. Progress of polygalacturonases purification visualised on SDS-PAGE gel. Lane 1, molecular mass standard proteins; lane 2: FB (10.5 μg); lane 3: permeate (9.6 μg); lane 4: eluate from the glass fiber microfilter (3.0 μg); lane 5: PGII active fraction from cation-exchange chromatography (1.0 μg protein), lane 6. Idem 4 (1.2 μg). In brackets: amount of protein loaded on the gel. Samples for SDS-PAGE were desalted with a PD-10 column equilibrated with water and concentrated by freeze-drying.

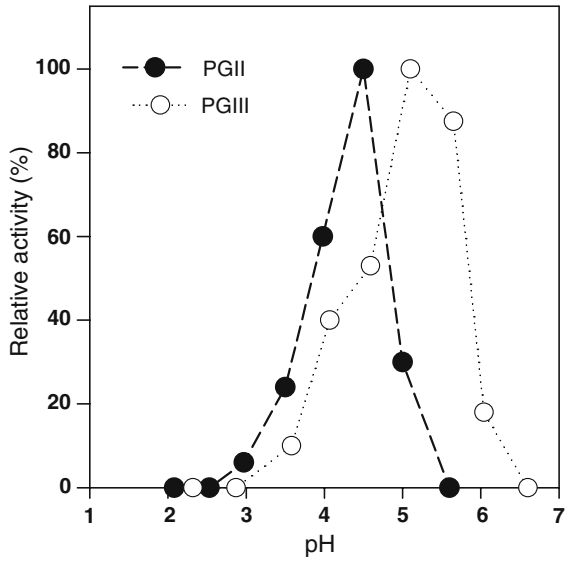

Fig. 6. Effect of pH on the activity of the purified PGII and PGIII polygalacturonases.

Both polygalacturonases reduced the viscosity of a 1% PGA solution by 50% with a less than 10% increase in reducing sugar groups. This indicate an endo-action (Tutobello & Mill 1961). These results suggest that PGII and PGIII are fairly typical fungal endo-PGs (Rombouts & Pilnik 1980, Kester & Visser 1990, Sakai *et al.* 1993, Jayani *et al.* 2005).

Conclusions

Two *A. kawachii* endo-polygalacturonases were recovered from the fermentation broth by adsorption to a glass fiber microfilter. Although adsorption was not completely selective, after the elution process a highly concentrated and purified polygalacturonase solution was obtained. Properties of the purified polygalacturonases involved in the adsorption process suggest that similar enzymes from other fungal strains may also be adsorbed to the glass fiber microfilter. As microfiltration is a common operation unit in the downstream processing of proteins and enzymes, the glass fiber microfilter may be promising to use as an adsorptive medium for polygalacturonase separation and enrichment.

Acknowledgments

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References

Asenjo JA (1990) Selection of operations in separation process. In: Asenjo JA, ed. *Separation Process in Biotechnology*. New York: Marcel Dekker, INC, pp. 3–16.

Bailey MJ, Ojamo H (1990) Selective concentration of polygalacturonase and β-D-glucosidase of *Aspergillus niger* culture filtrate using mineral adsorbents. *Bioseparation.* **1**: 133–139.

Contreras Esquivel JC, Hours RA, Voget CE, Mignone CF (1999) *Aspergillus kawachii* produces an acidic pectin releasing activity. *J. Biosci. Bioeng.* **88**: 48–52.

Contreras Esquivel JC, Voget CE (2004) Purification and partial characterization of an acidic polygalacturonase from *Aspergillus kawachii*. *J. Biotechnol.* **110**: 21–28.

Green S, Wase DA (1986) Practical and theoretical aspects of inorganic adsorbents technology in downstream processing – a review. *Proc. Biochem.* **21**: 200–203.

Ito K, Ogasawara H, Sugimoto T, Ishikawa T (1992) Purification and properties of acid stable xylanases from *Aspergillus kawachii. Biosci. Biotech. Biochem.* **56**: 547–550.

Iwashita K, Todoroki K, Kimura H, Shimoi H, Ito K (1998) Purification and characterization of extracellular and cell wall bound β-glucosidases from *Aspergillus kawachii. Biosci. Biotech. Biochem.* **62**: 1938–1946.

Jayani RS, Saxena S, Gupta R (2005) Microbial pectinolytic enzymes: a review. *Proc. Biochem.* **40**: 2931–2944.

Kashyap DR, Vohra PK, Chopra S, Tewari R (2001) Applications of pectinases in the commercial sector: a review. *Bioresour. Technol.* **77**: 215–227.

Kester HCM, Visser J (1990) Purification and characterization of polygalacturonases produced by the hyphal fungus *Aspergillus niger*. *Biotechnol. Appl. Biochem.* **12**: 150–160.

Mikami S, Iwano K, Shinoki S, Shimada T (1987) Purification and some properties of acid-stable α-amylases from *shochu koji* (*Aspergillus kawachii*). *Agric. Biol. Chem.* **51**: 2495–2501.

Pilnik W, Voragen AGJ (1993) Pectic enzymes in fruit juice and vegetable juice manufacture. In: Reeds G, ed. *Food and Science Technology. Enzymes in Food Processing*. New York: Academic Press, pp. 363–399.

Rombouts FM, Pilnik W (1980) Pectic enzymes. In: Rose AH, ed. *Microbial Enzymes and Bioconversions. Economic Microbiology,* Vol. 5. New York: Academic Press, pp. 227–282.

Sakai T, Sakamoto T, Hallaert J, Vandamme EJ (1993) Pectin, pectinase and protopectinase: production, properties and applications. *Adv. Appl. Microbiol.* **9**: 213–294.

Sathyanarayana N, Gummadi T, Panda (2003) Purification and biochemical properties of microbial pectinases – a review. *Proc. Biochem.* **38**: 987–996.

Tutobello R, Mill PJ (1961) The pectic enzymes of *Aspergillus niger*. II: Endopolygalacturonase. *Biochem. J.* **79**: 57–64.

Yagi F, Fan J, Tadera K, Kobayashi A (1986) Purification and characterization of carboxyl proteinase from *Aspergillus kawachii*. *Agric. Biol. Chem.* **50**: 1029–1033.

Wu YT, Pereira M, Venâncio A, Teixeira J (2001) Separation of endo-polygalacturonases using aqueous two-phase partitioning. *J. Chrom A.* **929**: 23–29.