Food Chemistry **17** (1985) 131–142

Isolation and Characterization of 1,4-β-Glucan 4-Glucanohydrolases (EC 3.2.1.4) from a Technical *Trichoderma viride* Cellulase

Ruth Kittsteiner-Eberle, Mechtild Höfelmann & Peter Schreier

Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, D-8700 Würzburg, West Germany

(Received: 28 November, 1984)

ABSTRACT

The following enzyme activities were detected in a Trichoderma viride *cellulase (Röhm 2230 B): 1,4-β-D-glucan cellobiohydrolase (C_1), 1,4-β-D-glucan 4-glucanohydrolase (C_x), β-glucosidase, β-galactosidase, polygalacturonase, proteinase, xylanase, amylase, esterase and 'polyphenoloxidase'. Isolation of cellulolytic enzymes was performed starting with adsorption chromatography on Avicel SF, leading to separation of more than 90% of non-cellulolytic enzymes and 96% of β-glucosidase activity (= fraction A). In fraction A, 30% of the C_x activity was determined whereas, in a separated fraction, B, the remaining C_x and the total C_1 activity was established. Further fractionation of B using ion-exchange and gel chromatography resulted in the separation of three purified enzyme fractions, PI to PIII, with endo C_x activities. Additionally, C_1 activity was found in PIII. PI-PIII were characterized by means of their pH optima, isoelectric points and molecular weights.*

INTRODUCTION

Some years ago, Pilnik *et al.* (1975) extended fruit processing methods by introducing the liquefaction of fruit pulps by means of technical pectinolytic and cellulolytic enzymes. Recently, we described the results

Food Chemistry 0308-8146/85/$03·30

obtained with this technique in tropical fruit processing (Kittsteiner-Eberle, 1984; Schreier & Idstein, 1984). In these studies, the importance of cellulolytic activities for the liquefaction of pulps has been clearly demonstrated. As our knowledge about the enzyme activities used for the liquefaction of fruit pulps is still limited, we investigated a technical cellulase preparation we had successfully employed in our previous study (Kittsteiner-Eberle, 1984; Schreier & Idstein, 1984). In this paper, the results of these investigations are described.

MATERIALS AND METHODS

Enzyme

Cellulase 2230 B was kindly provided by Röhm GmbH, Darmstadt.

Protein determinations

Protein concentrations were measured by the method of Lowry *et al.* (1951) with crystalline serum albumin as standard. Protein concentrations in column effluents were estimated from the absorbance at 280 nm.

Qualitative enzyme assays

Except for the assays of β-glucosidase (Bailey & Nevalainen, 1981), β-galactosidase (Kuby & Lardy, 1953), esterase (Jacks & Kircher, 1967), lipoxygenase (Heimann *et al.*, 1973), peroxidase (Délincée & Radola, 1972) and catalase (Hale & Renwick, 1981) qualitative enzyme assays were carried out using agar diffusion plate tests (Haberman & Hardt, 1972), as already described in detail elsewhere (Höfelmann *et al.*, 1983).

Quantitative determinations of cellulolytic activities

C_1 and C_x activities were determined using the dinitrosalicylic acid (DNS) method (Miller, 1959). DNS reagent: A solution was prepared using 1 g of DNS in 30 ml of distilled water and dropwise addition of 20 ml 2N NaOH.

Thirty grams of K-Na-tartrate were added to this solution, which was finally adjusted to 100 ml with distilled water.

C_x activity

A mixture of 9 ml of substrate solution (0·5 g carboxymethylcellulose dissolved in 25 ml 0·3M acetate buffer (pH 5·0) and 65·0 ml distilled water) with 1 ml of enzyme solution (appropriately diluted) was incubated at 40 °C. After 0, 2, 4, and 6 min, two 1-ml samples were withdrawn and the content of reducing sugars determined by the DNS method at 530 nm with glucose as a reference (Miller, 1959). Determinations of v_0 were performed by graphical extrapolation drawing μMol glucose/ml enzyme solution against reaction time. Definition of activity: U/ml = μMol reducing groups formed per minute by 1 ml enzyme solution.

C_1 activity

For the determination of C_1 activity, a mixture of 18 ml of substrate solution (1 g of Avicel SF dissolved in 25 ml of 0·3M acetate buffer (pH 5·0) and 65 ml of distilled water), 1 ml of enzyme solution (appropriately diluted) and 1 ml of acetate buffer (pH 5·0) was incubated at 40 °C. After 0, 4, 8 and 12 min, three 1-ml samples were withdrawn and heated for 15 min at 100 °C. After cooling to ambient temperature and filtration on Avicel SF, 2-ml samples were each used for the determination of reducing groups by the DNS method as described above. Definition of activity: U/ml = μMol reducing groups formed per minute by 1 ml enzyme solution.

β-Glucosidase

The substrate for the determination of β-glucosidase activity was 4-nitrophenyl-β-D-glucopyranoside and the spectrophotometric method (Bailey & Nevalainen, 1981) was used. Definition of activity: U/ml = μMol 4-nitrophenolate formed per minute (at 405 nm; $\varepsilon = 18{\cdot}5$ 1 $mMol^{-1}$ cm^{-1}) by 1 ml of enzyme solution.

Ultrathin-layer Isoelectric Focusing (UDIEF)

UDIEF was carried out using 50 μm polyacrylamide gels (Radola, 1980). The experimental details for the preparation of ultrathin gels and

ultrathin-layer agar gels (for enzyme print technique) have been previously described elsewhere (Höfelmann *et al.*, 1983).

Adsorption Chromatography on Avicel SF

A modification of the method of Li *et al.* (1965) was used consisting of a twofold increase of the amount of Avicel SF in relation to protein content of raw enzyme employed. The adsorbent was equilibrated in 0·05M acetate buffer (pH 5·0) and a column (2·6 × 18 cm) was packed. As sample, 10 ml cellulase 2230 B solution (1 g + 9 ml 0·05M acetate buffer (pH 5·0)) was applied. The flow rate was 50 ml/h and 5-ml fractions were collected (4 °C) by washing with 0·05M acetate buffer (pH 5·0) and (after fraction 40) by eluting with distilled water. Two fractions were separated by combining eluates 15–54 (A) and 55–100 (B).

Ion-exchange chromatography of fraction B

Further fractionation of B was achieved by ion-exchange chromatography on DEAE-Trisacryl M (Pharmacia). The adsorbent was equilibrated in 0·05M acetate buffer (pH 5·0) and a column (2·6 × 22 cm) was packed. Twenty-two millilitres of ultrafiltered fraction B were applied. The flow rate was 50 ml/h and 10-ml fractions were collected (4 °C) by washing with 200 ml 0·05M acetate buffer (pH 5·0) and then eluting with 500 ml of a linear NaCl gradient (0–0·5M in 0·05M acetate buffer (pH 5·0)).

Gel chromatography

The ultrafiltered (5 ml) combined fractions 32–45 of Avicel SF chromatography (PI), 28–39 (PII) and 7–11 (PIII) of ion-exchange chromatography were applied to a column (1·6 × 70 cm) packed with Ultrogel ACA 54 (LKB), which had been equilibrated with 0·05M acetate buffer (pH 5·0). The flow rate was 10 ml/h and 10-ml fractions were collected (4 °C). Determinations of molecular weights were achieved using a mixture of protein markers (MS II, Serva).

Thin-layer chromatography (TLC) of carbohydrates

Three millilitres of carboxymethylcellulose substrate (*cf.* determination of C_x activity) were incubated for 1 h with each of 100 µl of PI, PII and

PIII. The reaction products were studied by TLC using precoated plates (20 × 20 cm, Merck) with a 0·25 mm layer of Kieselgel 60. The chromatograms were developed with *n*-butanol + acetic acid + diethyl ether + water (9 + 6 + 3 + 1). After developing, the plate was sprayed with anisaldehyde–sulfuric acid reagent and heated at 105 °C for 10 min.

Ultrafiltration

Ultrafiltrations were carried out at 4 °C and 3·5 bar N_2 using a TU-AN 4045. 380 membrane (8000d, Kalle) in an Amicon cell.

RESULTS AND DISCUSSION

Isolation and purification of cellulolytic enzymes

The technical *Trichoderma viride* cellulase under study (2230 B, Röhm) showed a protein content of 25 %. The qualitative investigation of enzyme activities in the raw enzyme revealed that 1,4-β-D-glucan cellobiohydrolase (C_1), 1,4-β-D-glucan 4-glucanohydrolase (C_x), β-glucosidase, β-galactosidase, polygalacturonase, proteinase, xylanase, amylase, esterase and 'polyphenoloxidase' were present in the preparation. Using ultrathin-layer isoelectric focusing (UDIEF), six C_x activities were detected exhibiting isoelectric points (IEP) at pH 3·6, 3·8, 4·0, 4·4, 4·6 and 5·5/5·6, respectively. The quantitatively determined cellulolytic activities are outlined in Table 1.

The isolation of cellulolytic enzymes from the technical cellulase preparation was performed starting with adsorption chromatography on Avicel SF (Li *et al.*, 1965), leading to the separation of more than 90 % of

TABLE 1
Specific Activities of β-Glucosidase, C_1 and C_x Activities in the Technical Cellulase Preparation (2230 B, Röhm)

	Specific activity (U/mg)
β-glucosidase	0·6
C_1 activity	3·4
C_x activity	0·7

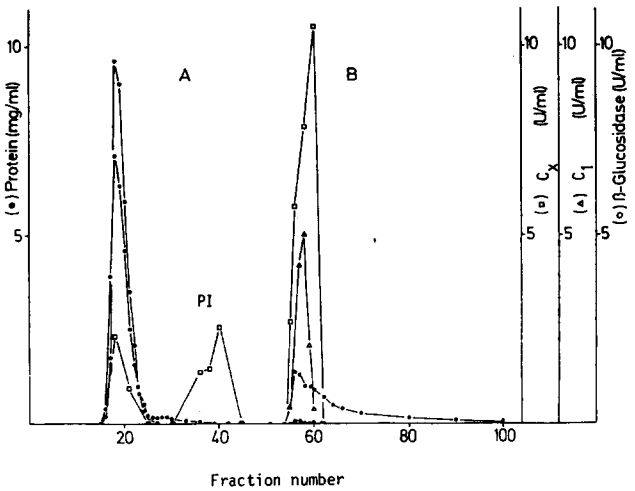

Fig. 1. Separation of cellulolytic enzymes from cellulase 2230 B (Röhm) by adsorption chromatography on Avicel SF. Column, 2·6 × 18 cm. Sample, 10 ml enzyme solution (1·0 g + 9 ml 0·05M acetate buffer (pH 5·0)). 5-ml fractions (50 ml/h). Elution with 0·05M acetate buffer (pH 5·0) and, after fraction 40, with distilled water. A: 'buffer fraction' (fractions 15–54). B: 'water fraction' (fractions 55-100).

the above-mentioned non-cellulolytic enzymes and 96 % of β-glucosidase activity (Fig. 1, fraction A; Table 2).

The fractions in A showing C_x activity (Fig. 1, 32–45 = PI) were combined and concentrated by ultrafiltration before further study by gel chromatography. The separation of enzymes in B was performed by ion-exchange chromatography and subsequent gel chromatography. The fractionation of cellulolytic enzymes by DEAE-Trisacryl M chromatography is shown in Fig. 2. The unbound active protein (Fig. 2, PIII)

TABLE 2

Separation and Purification of Cellulolytic Enzymes in Cellulase 2230 B (Röhm) by Avicel SF Adsorption Chromatography

	Volume (*ml*)	*Total* (*mg*)	*Protein* (%)	C_x U_{total}	*Yield* (%)	C_1 U_{total}	*Yield* (%)	*β-Glucosidase* U_{total}	*Yield* (%)
Cellulase 2230 B	10	250·0	100·0	857·0	100·0	179·0	100·0	172·0	100·0
A (fraction 15–54)[a]	200	158·0	63·2	142·0	16·6	0·0	0·0	200·0	117·0
B (fraction 55-100)[a]	276	74·5	29·8	419·0	48·9	141·0	78·8	7·0	4·0

[a] *cf.* Fig. 1.

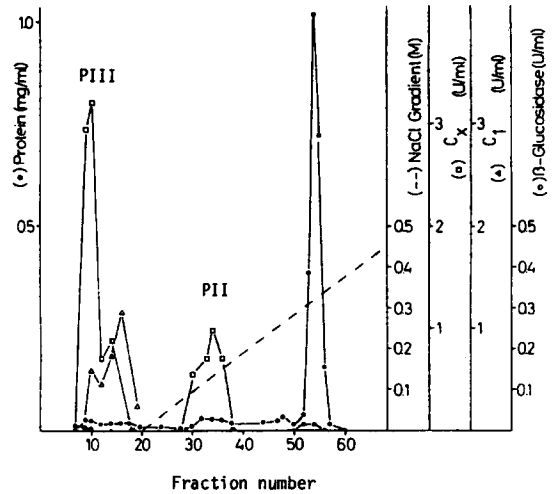

Fig. 2. Separation of cellulolytic enzymes in the 'water fraction' B (fractions 55–100 of Avicel SF chromatography (*cf.* Fig. 1) by DEAE-Trisacryl M ion-exchange chromatography. Column, 2·6 × 22 cm; 0·05M acetate buffer (pH 5·0). Sample, 22 ml. 10-ml fractions (50 ml/h). Washing with 0·05M acetate buffer (pH 5·0) and elution with a linear NaCl gradient (0·0–0·5M in 0·05M acetate buffer (pH 5·0)).

contained C_1 and C_x activities. A bound C_x activity could be eluted by 0·1M NaCl (Fig. 2, PII). The major portion of protein (80%) (Fig. 2, 52–57), eluted by 0·3M NaCl did not show any cellulolytic activity. In these fractions the remaining activities of β-glucosidase, xylanase, proteinase, polygalacturonase and amylase were eluted.

The fractions PII (Fig. 2, 28–39) and PIII (Fig. 2, 7–11) were combined and, after ultrafiltration, further purified by gel chromatography. The purification of fractions PI–PIII achieved by the three above-mentioned chromatographic steps is summarized in Table 3. Table 4 shows the corresponding data for the C_1 activity in PIII. As can be seen from the results outlined in Tables 3 and 4, the C_x activities could be enriched 5-, 29- and 37-fold, whereas, for the C_1 activity, an enrichment factor of 25 was achieved. These enrichment factors are similar to results obtained in studies on cellulolytic enzymes from *Aspergillus aculeatus* (Murao *et al.*, 1979).

Purity and characterization

The purified enzyme fractions PI-PIII were characterized by means of their pH optima, molecular weights and IEP. PI-PIII did not differ in

TABLE 3
Purification Scheme of C_x Activities (PI, PII, PIII) in Cellulase 2230 B (Röhm)

Purification	*Volume (ml)*	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Enrichment*	*Yield (%)*
Raw enzyme	10·0	250·0	857·0	3·4	1·0	100·0
Avicel SF						
B	276·0	74·5	419·0	5·6	1·6	48·9
PI (32–45)	70·0	5·5	90·3	16·4	4·8	10·5
PI (used for purification)	35·0	2·8	45·2	16·4	4·8	5·2
Ultrafiltration						
B-UF	50·0	66·5	333·5	5·0	1·5	38·9
B-UF (used for purification)	22·0	29·3	146·7	5·0	1·5	17·1
DEAE-Trisacryl M						
PII (28–39)	120·0	3·1	85·2	27·3	8·0	9·9
PII (used for purification)	87·5	2·3	62·1	27·3	8·0	7·2
PIII (7–11)	50·0	1·1	84·5	76·8	22·6	9·8
PIII (used for purification)	34·0	0·7	57·5	76·8	22·6	6·7
Ultrafiltration						
PI-UF	6·0	0·8	40·1	50·1	14·7	4·7
PI-UF (used for purification)	5·0	0·7	33·4	50·1	14·7	3·9
PII-UF	5·0	1·6	30·4	19·1	5·6	3·5
PII-UF (used for purification)	4·5	1·4	27·4	19·1	5·6	3·2
PIII-UF	4·5	0·6	54·0	90·0	26·5	6·3
PII-UF (used for purification)	4·0	0·5	48·2	80·3	26·5	5·6
Gel chromatography						
PI	20·0	0·2	31·0	129·1	37·6	3·6
PII	30·0	1·5	27·0	18·0	5·3	3·2
PIII	30·0	0·5	49·8	99·6	29·3	5·8

TABLE 4
Purification Scheme of C_1 Activity (PIII) in Cellulase 2230 B

Purification	*Volume (ml)*	*Total protein (mg)*	*Total activity (U)*	*Specific activity (U/mg)*	*Enrichment*	*Yield (%)*
Raw enzyme	10·0	250·0	179·0	0·7	1·0	100·0
Avicel SF						
B	276·0	74·5	141·0	1·9	2·7	78·8
Ultrafiltration						
B-UF	50·0	66·5	131·1	1·9	2·7	73·2
B-UF (used for purification)	22·0	29·3	57·7	1·9	2·7	32·2
DEAE-Trisacryl M						
PIII (7–11)	50·0	1·1	22·0	20·0	28·6	12·3
PIII (used for purification)	34·0	0·7	15·0	20·0	28·6	8·4
Ultrafiltration						
PIII-UF	4·5	0·6	13·1	21·8	31·2	7·3
PIII-UF (used for purification)	4·0	0·5	11·7	21·8	31·2	6·5
Gel chromatography						
PIII	10·0	0·4	7·1	17·7	25·3	4·0

their pH optima and molecular weights. Each showed maximal activity at pH 5·0 and a molecular weight of about 42 000d (determined by gel chromatography). In the literature, molecular weights between 12 500d and 76 000d can be found for C_x activities from *Trichoderma viride*; mostly, molecular weights between 40 000d and 50 000d have been described (Lee & Fan, 1980). As to C_1 activity of *Trichoderma* sps., molecular weights between 42 000d and 62 000d have been reported (King, 1965; Emert *et al.*, 1974; Gum & Brown, 1976; Ogawa *et al.*, 1982).

The results of UDIEF analyses of PI-PIII are outlined in Figs 3 and 4. From these Figures it is obvious that only PIII was obtained as UDIEF homogeneous enzyme. Thus, except for the major protein band showing cellulolytic activity, in PI non-active protein bands were detected at pH 3·5–3·8 (Fig. 3). Furthermore, low C_x activity was found at pH 4·5. In PII C_x activities were visualized at pH 3·5 and 4·2 besides five non-active protein bands with IEP between 3·5 and 4·2. PIII showed three

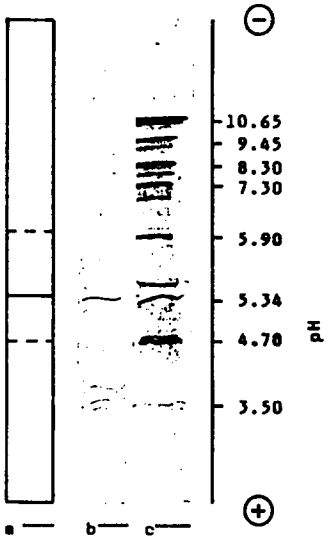

Fig. 3. Ultrathin-layer isoelectric focusing of PI with visualization of C_x activity. a: C_x activity; b: protein staining; c: staining of marker proteins. Experimental data, see 'Material and Methods' section.

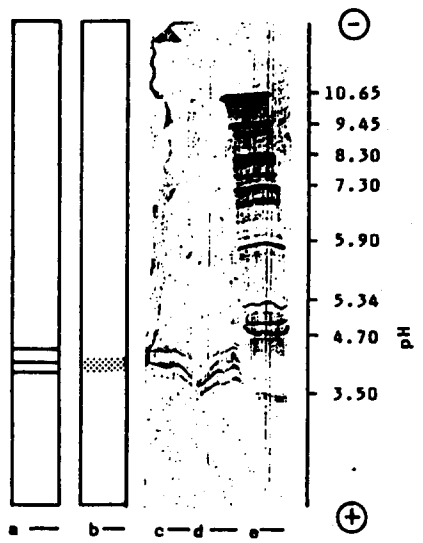

Fig. 4. Ultrathin-layer isoelectric focusing of PII and PIII with visualization of C_x activity. a: C_x activity in PIII; b: C_x activity in PII; c: protein staining of PIII; d: protein staining of PII; e: staining of marker proteins. Experimental data, see 'Material and Methods' section.

enzymatically active protein bands at IEP 3·6, 3·8, and 4·0, respectively. Non-active proteins could not be detected in this enzyme fraction. IEP data of cellulolytic enzymes are rather scarce in the literature. Berghem *et al.* (1976) described an IEP of 3·79 for a cellulolytic enzyme from *Trichoderma viride*, whereas IEP of 4·32 and 5·09 have been reported for the enzymes from *Aspergillus aculeatus* (Murao *et al.*, 1979).

Finally, thin-layer chromatographic studies of the reaction products formed by the catalysis of enzyme fractions PI-PIII revealed that, with carboxymethylcellulose as substrate, these enzymes mainly catalyzed the formation of cellobiose, cellotriose and cellotetrose. Thus, PI-PIII can be included in the series of endo C_x cellulases (Mandels, 1982).

ACKNOWLEDGEMENTS

We thank the Deutsche Forschungsgemeinschaft and the Dr Otto Röhm-Stiftung for supporting this work.

REFERENCES

Bailey, M. J. & Nevalainen, K. M. H. (1981). Induction, isolation and testing stable *T. reesei* mutants with improved production of solubilizing cellulase. *Enzyme Microb. Technol.* **3**, 153–7.

Berghem, L. E. R., Petterson, L. G. & Axiö-Frederiksson, U. B. (1976). The mechanism of enzymatic cellulose degradation. Purification and some properties of two different 1,4-β-glucan–glucanhydrolases from *Trichoderma viride*. *Eur. J. Biochem.* **61**, 621–30.

Délincée, H. & Radola, B. (1972). Detection of peroxidase by print technique in thin-layer isoelectric focusing. *Anal. Biochem.* **48**, 536–46.

Emert, G. H., Gum, Jr., E. K., Lang, L. A., Lin, T. H. & Brown Jr., R. D. (1974). Cellulases. *Adv. Chem. Ser.* **136**, 79–101.

Gum Jr., E. K. & Brown Jr., R. D. (1976). Structural characterization of a glycoprotein cellulase, 1,4-β-D-glucan cellobiohydrolase C from *Trichoderma viride*. *Biochim. Biophys. Acta*, **44**, 371–86.

Habermann, E. & Hardt, K. L. (1972). A sensitive and specific plate test for the quantitation of phospholipases. *Anal. Biochem.* **50**, 163–73.

Hale, A. J. & Renwick, J. H. (1981). A method for the identification of catalase in starch gels. *Biochem. J.*, **80**, 49P–50P.

Heimann, W., Dresen, P. & Schreier, P. (1973). Über das Lipoxygenase-'Lipoperoxidase'—System in Cerealien. Abtrennung von zwei Proteinkomplexen mit Lipoxygenase- und Linolsäurehydroperoxid-Abbau-Aktivität aus Hafer und Sojabohnen. *Z. Lebensm. Unters.-Forsch.* **152**, 147–51.

Höfelmann, M., Kittsteiner-Eberle, R. & Schreier, P. (1983). Ultrathin-layer agar gels—A novel print technique for ultrathin-layer isoelectric focusing of enzymes. *Anal. Biochem.* **128**, 217–22.

Jacks, T. J. & Kircher, H. W. (1967). Fluorometric assay for the hydrolytic activity of lipase using fatty acyl esters of 4-methylumbelliferone. *Anal.* Biochem. **21**, 279–81.

King, K. W. (1965). Enzymatic attack on highly crystalline hydrocellulose. *J. Ferment. Technol.* **43**, 79–92.

Kittsteiner-Eberle, R. (1984). *Untersuchung eines technischen Cellulase-Präparates: Ein Beitrag zur enzymatischen Verflüssigung von Fruchtpulpen.* Dissertation Univ. Würzburg, 1984.

Kuby, S. A. & Lardy, H. A. (1953). Purification and kinetics of β-D-galactosidase from *Escherichia coli*, strain K-12. *J. Amer. Chem. Soc.* **75**, 890–6.

Lee, Y. H. & Fan, L. T. (1980). Properties and mode of action of cellulase. *Adv. Biochem. Eng.* **17**, 101–29.

Li, L. H., Flora, R. M. & King, K. W. (1965). Individual rôles of cellulase components derived from *Trichoderma viride*. *Arch. Biochem. Biophys.* **111**, 439–47.

Lowry, O. H., Roseborough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol chem.* **193**, 265–75.

Mandels, M. (1982). Cellulases. *Ann. Rep. Ferment. Process.* **5**, 35–78.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* **31**, 426–8.

Murao, S., Kanamoto, J., Sakamoto, R. & Arai, M. (1979). Purification of two carboxymethyl cellulose hydrolyzing enzymes from *Aspergillus aculeatus*. *J. Ferment. Technol.* **57**, 157–62.

Ogawa, K., Toayama, H. & Toayama, N. (1982). Nature cellulose hydrolyzing cellulase of *Trichoderma reesei*. *J. Ferment. Technol.* **60**, 349–55.

Pilnik, W., Voragen, A. G. J. & de Vos, L. (1975). Enzymatische Verflüssigung von Obst und Gemüse. *Flüssiges Obst.* **42**, 448–51.

Radola, B. J. (1980). Ultrathin-layer isoelectric focusing in 50–100 μm polyacrylamide gels on silanized glass plates or polyester films. *Electrophoresis*, **1**, 43–56.

Schreier, P. & Idstein, H. (1984). Untersuchungen über die Aromastoffzusammensetzung enzymatisch verflüssigter Guava (*Psidium guajava*, L.)- und Mango (*Mangifera indica*, L., var. Alphonso) Fruchtpulpen. *Dtsch. Lebensm. Rdsch.* **80**, 335–40.