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Microdialysis clean-up and sampling in enzyme-based methods for the characterisation of starch

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Abstract

Microdialysis was used for sampling enzyme hydrolysis products of starch hydrolysed with β-amylase, pullulanase, and/or isoamylase, to obtain information about the molecular structure of starch. Starches from waxy, normal, and high amylose maize, and from normal and genetically modified potato (amylose deficient) were used, and also commercial potato amyloses. The hydrolysis products were analysed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Simultaneous sampling and sample clean-up were achieved with microdialysis, thus enabling on-line injection into the liquid chromatographic system. The molecular weight cut-off of the membrane allowed for diffusion of small molecules such as oligosaccharides through the membrane, but hindered large molecules, e.g. enzymes and large polysaccharides, from entering the chromatographic system.

With microdialysis sampling, it was possible to investigate the short chain fractions of debranched starch in the presence of amylose without pre-fractionation. The microdialysis–HPAEC-PAD system was also used for determination of the A:B chain ratio and the β-limit value. After β-amylolysis, only liberated maltose diffused through the dialysis membrane, which resulted in on-line sample clean-up from branched β-limit dextrin as well as from the enzyme. The proposed method is fast and easy to handle since clean-up of the hydrolysate is achieved on-line with the chromatographic system.

Keywords: Starch; Microdialysis; β-Limit dextrin; Chain length

1. Introduction

Starches from various plant species have different chemical structures. A well-known significant variation for amylopectin is the chain length distribution pattern (Hanashiro, Abe & Hizukuri, 1996; Koch, Andersson & Åman, 1998). Obviously, the molecular structure strongly influences the properties and thus macroscopic/technological qualities of the starch (Noel, Ring & Whittam, 1993). As a consequence, these parameters need to be investigated by a variety of techniques, commonly including some enzymically catalysed hydrolyses. Starch hydrolysing enzymes with different selectivities are used to obtain information about the molecular structure such as chain length (CL), CL distribution, and A:B chain ratio of the polysaccharides (Manners, 1989).

Sampling of starch hydrolysates should be representative, easy to handle, reproducible, fast, preferably automated, and coupled on-line to the analytical system. An additional criterion, which must be fulfilled prior to injection of starch hydrolysis products into a liquid chromatographic system, is removal of large molecules such as proteins, which will interfere in subsequent analyses. Removal of proteins is usually performed by means of boiling (precipitation), followed by centrifugation or filtration. However, sampling and sample clean-up can be simplified by using on-line microdialysis sampling, which results in significant reduction of the total analysis time.

Microdialysis has been extensively used over the years for in vivo sampling of substances in e.g. brain, tissues, and blood, as recently reviewed in a special issue of Analytical Chimica Acta (1999). The technique has also been used lately for in vitro sampling of bioprocesses (Torto et al., 1998; Torto, Laurell, Gorton & Marko-Varga, 1999) and for determination of starch hydrolysis products (Zook & LaCourse, 1998). There are several advantages of microdialysis sampling of enzymic hydrolysis products; on-line

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sampling can be performed while simultaneously eliminating large polysaccharides, reducing separation time for subsequent chromatographic analysis, and eliminating enzymes.

High-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) has been reported for the determination of CL distribution of debranched amylopectin in several studies (Hanashiro et al., 1996; Koch et al., 1998). Chains of increasing CL are baseline separated between CL of six up to ≈80, where the obtained patterns are assumed to be fingerprints for amylopectin from different plant species (Hanashiro et al., 1996; Koch et al., 1998; Wong & Jane, 1997). Amylose is essentially non-branched/long chain branched and consists of components with different CLs with occasional branching points depending on its botanical origin and the applied fractionation method (Hizukuri, 1991; Takeda, Shirasaka & Hizukuri, 1984). Although it is well-known that amylose contains branches, little is known about its unit chain length distributions.

β-Limit values reflect the extent of non-branched exterior domains in amylose and amylopectin. β-Amylase catalyses the hydrolysis of α-(1 → 4) linkages of starch by the successive liberation of maltose from the non-reducing ends, but cannot bypass the α-(1 → 6) branching points. A strictly non-branched α-(1 → 4) anhydroglucose chain hydrolysed by β-amylase produces only maltose (except for chains with an odd number of glucose units that in addition give one maltotriose molecule), resulting in a β-limit value of 100%. Commonly (Hizukuri, 1991; Hizukuri, Takeda & Yasuda, 1981; Inouchi, Glover & Fuwa, 1987; Takeda et al., 1984) the reducing power of enzyme hydrolysates is determined by chemical reagents such as copper sulphate (Nelson, 1944) or ferricyanide (Hizukuri et al., 1981; Park & Johnson, 1949). However, the reducing power does not distinguish between oligosaccharides of different CL. Maltose can also be determined more selectively with enzyme-based assays (Bertoft, 1989).

The A:B chain ratio is an important parameter, which provides information about the architecture of amylopectin. For determination of the A:B chain ratio, hydrolysis with enzymes with different specificity is used. Reported values have been in the range of 0.8–2.2, depending mainly on the method applied (Bertoft, 1991; Hizukuri, 1986; Hizukuri & Maehara, 1990; Manners, 1989; Zhu & Bertoft, 1996), e.g. examination of the chain profile of debranched amylopectin by size-exclusion chromatography (SEC) (Hizukuri, 1986), successive debranching of β-limit dextrin with isoamylase and pullulanase, followed by separation and quantification of the products by reversed phase liquid chromatography (Bender, Siebert & Stadler-Szöke, 1982; Hizukuri & Maehara, 1990) or SEC (Yuan, Thompson & Boyer, 1993; Yun & Matheson, 1993), or determining the reducing power in the hydrolysates (Marshall & Whelan, 1974).

This investigation shows the possibilities of using microdialysis sampling for monitoring the short chain fraction of debranched starch in the presence of amylose and also in amylose preparations. We suggest a fast and easy method for determination of the β-limit value that employs microdialysis to sample β-amylase hydrolysates, followed by quantification of maltose by HPAEC (with PAD). Furthermore, an assay for determination of the A:B chain ratio is proposed. Additionally, exterior and interior CLs are determined.

2. Experimental

2.1. Chemicals

Potato amylopectin starch (PAP) obtained from genetically modified potato and starch from normal potato were from Svalöf Weibull AB (Svalöv, Sweden) and Lyckeby Stärkelsen (Kristianstad, Sweden). Commercial potato amylose was purchased from Sigma Chemical Co. (St. Louis, MO, USA, type 3, cat. no. A-0512, lot 42H3861 and lot 75F3854, denoted H and F, respectively). Starches from waxy, normal, and high amylose (Hylon® V and Hylon® VII) maize were from National Starch and Chemical Company (Bridgewater, NJ, USA). Isoamylase (EC 3.2.1.68) from *Pseudomonas amylodermosa* was purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan, cat. no. EN 102, lot. no. 412071) and pullulanase (EC 3.2.1.41) from *Klebsiella pneumoniae* was purchased from ICN Biomedicals Inc. (Ohio, USA, cat. no. 32-1721, lot. no. 88637). β-Amylase (EC 3.2.1.2) from barley was purchased from Megazyme International (Bray, County Wicklow, Ireland, cat. no. E-BARBP, lot. no. 50302). The water used in the experiments was purified in a Milli-Q system, Millipore (Bedford, MA, USA).

2.2. Chromatographic systems

A chromatographic system, HPAEC-PAD, with CarboPac PA-100 pre- and analytical columns, GP40 gradient pump, and ED40, all from Dionex Corp. (Sunnyvale, CA, USA), was used for separation and detection of the starch hydrolysis products. The electrochemical detector was used with the following waveform: $E_1 = 0.10$ V ($t_d = 0.20$ s, $t_1 = 0.20$ s), $E_2 = 0.70$ V ($t_2 = 0.19$ s), and $E_3 = -0.75$ V ($t_3 = 0.39$ s) vs a $Ag/AgCl_{(sat)}$ reference electrode (Antec, Amsterdam, The Netherlands) and a gold working electrode. The system was controlled by PeakNet software from Dionex. Elution was performed using a gradient programme with 150 mM NaOH (eluent A) and 500 mM NaOAc prepared in 150 mM NaOH (eluent B).

For determination of the chain length distribution pattern of debranched samples, eluent A decreased linearly and was 70% at 0 min, 60% at 5 min, 55% at 10 min, 48% at 15 min, 33% at 25 min, 10% between 26 and 30 min and finally readjusted to 70% for equilibration of the column (eluent B = 100% − eluent A). For determination of the β-limit value and the A:B chain ratio, the gradient was as follows:

eluent A decreased linearly and was 70% at 0 min, 50% at 2 min, and 0% at 20 min. The flow rate was 1.0 ml/min and the injection volume 20 μl. Identification of the peaks was performed by comparing the retention times of commercial standards (glucose to maltoheptaose) and assuming that the following peaks eluted in increasing order representing one additional glucose unit in CL.

Absolute molecular weights of β-limit dextrins were determined by means of a SEC system with dual detection of scattering intensity (low angle laser light scattering device, LALLS) and mass (differential refractive index detector, DRI) (Huber & Praznik, 1998). The sample solution (200 μl) was separated on a series of SEC-columns (TSK; PWM + G PW6000 + 5000 + 4000 + 3000, each 30 cm with i.d. 0.75 cm, TosoHaas) at a flow rate of 0.8 ml/min and eluted with 0.05 M aqueous NaCl. The individual SEC-separated fractions were detected with respect to their scattering intensity at a low scattering angle (LALLS, TSP/USA, KMX-6; $\lambda = 632$ nm; scattering angle 5°) and with respect to their mass (DRI detector from Wyatt Technology/CA, USA, Optilab 903, $\lambda = 632$ nm). Data acquisition was performed with the software package CODAwin, and data processing and documentation with the software package CPCwin.

2.3. Microdialysis sampling

For the present investigations a previously described microdialysis sampling set-up was used (Richardson, Nilsson, Torto, Laurell & Gorton, 1999; Torto et al., 1998). The microdialysis probe (Laurell & Buttler, 1995), with effective dialysis length of 10 mm, was fitted with a hollow fibre polysulphone membrane (Fresenius A/G, St, Wendel, Germany, membrane type SPS 660, fibre type 6005) with 30 kDa molecular weight cut-off. The perfusion flow rate was 3 μl/min, if not otherwise stated, delivered with a CMA/100 micro-injection pump (CMA Microdialysis, Stockholm, Sweden) with a 2.5 ml micro-syringe (Exmire, ITO Corporation, Fuji, Japan, cat. no. 8309021).

2.4. Debranching of starch and amylose samples

Maize or amylose samples (30 mg) were dissolved in 90% DMSO/water (0.50 ml) at 100°C for 30 min in 5 ml screw-cap reaction vials housed in a heating and stirring module (Reacti-Therm model no. 18791 from Pierce, Rockford, IL, USA). This step was followed by addition of water (4.25 ml) and boiling at 100°C for another 30 min. After cooling, 0.4 M citrate buffer (0.25 ml, pH 6.0) and 20 μl (8 U) of pullulanase were added. Potato starch samples (20 mg) were dissolved in water (4.75 ml) at 100°C for 30 min in the Reacti-Therm. After cooling, 0.4 M citrate buffer (0.275 ml, pH 6.0) and 10 μl (4 U) of pullulanase were added. The samples were debranched for 2–4 h at 40°C and immediately injected into the HPAEC-PAD system to prevent precipitation of amylose. Complete debranching was confirmed by addition of another 4 U of pullulanase and prolonged hydrolysis time in parallel samples. Chromatograms of these samples were compared, as was the reducing power in both samples, determined with the copper sulphate method (Nelson, 1944; Nilsson, Bergquist, Nilsson & Gorton, 1996) by measurement of absorbance at 660 nm (Ultrospec II spectrophotometer from LKB Biochrom, Cambridge, U.K.).

2.5. Preparation of β-limit dextrin

β-Limit dextrins were prepared according to Inouchi et al. (1987) for subsequent determination of A:B chain ratios. Waxy maize or PAP (800 mg dry weight) was gelatinised in 1 M NaOH (120 ml) for 1–2 h at 45°C with continuous stirring. The pH of the solution was adjusted to 6.0 with 1 M citric acid, then diluted to 160 ml with water. The sample solution was incubated with β-amylase (700 U) for 20 h at 37°C with continuous stirring. The enzyme was deactivated by heating the solution in boiling water for 5 min. The β-limit dextrin was precipitated by adding cold methanol (500 ml) under vigorous stirring. The precipitate was allowed to settle (12 h) before rinsing on a G-4 glass filter with hot methanol for removal of maltose and then dried with ethyl ether.

2.6. β-amylolysis limit

For determination of the β-amylolysis limits (β-limit values), smaller sample amounts were used than in the preparations described above. Five milligrams of waxy maize, PAP, or potato amylose (H) were gelatinised in 1 M NaOH (8 ml) for 1 h at 45°C. Citric acid (1 M) was then added to adjust the pH to 6.0. The final sample volume was 10 ml. One fraction (5 ml) of the sample was withdrawn and used as a blank. The remaining 5 ml was incubated with β-amylase (5 U) for 2 h at 37°C with continuous stirring. Complete hydrolysis was confirmed as in Section 2.4. For determination of the maltose liberated, the on-line microdialysis–HPAEC-PAD system was used. Concentrations were determined by the standard addition method, with five successive additions of 5 mM maltose (5 μl) to the sample solution. The copper sulphate method was used as reference, calibrated against maltose.

2.7. A:B chain ratio

β-Limit dextrins for determination of the A:B chain ratio were debranched with pullulanase as follows: β-limit dextrin was dissolved (1 mg/ml) in 20 mM citrate buffer, pH 5.0, for 30 min at 37°C. The sample was divided into three parts: (a) blank (only substrate, no enzyme), (b) sample for determination of maltotriose originating from A-chains, and (c) sample for determination of maltotriose originating from B-chains. Sample (b) (5 ml) was incubated with pullulanase (5 U) for 2 h at 37°C, then sampled with microdialysis and analysed with HPAEC-PAD. For quantification of maltotriose in the hydrolysates, five standard

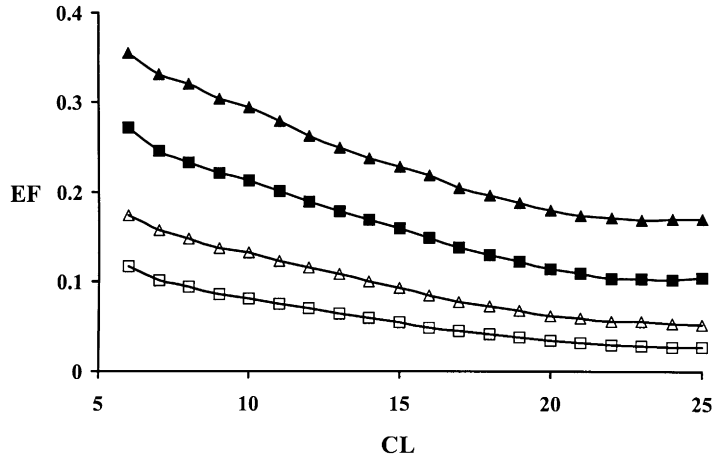

Fig. 1. EF for different unit chain lengths in debranched potato amylopectin starch (PAP) at four different flow rates, using a polysulphone microdialysis membrane with a 30 kDa molecular weight cut-off (□) represents 7 μl/min, (△) 5 μl/min, (■) 3 μl/min, and (▲) 2 μl/min.

additions of 5 mM maltotriose (5 μl) were added to sample (b) and the peak areas were determined. Sample (c) (5 ml) was incubated as described for (b), but additionally mixed with β-amylase (5 U). After 4 h the solution was diluted with water (15 ml) and then injected via the microdialysis probe onto the HPAEC-PAD system. Quantification was performed with standard additions according to (b).

As a reference method, the A:B chain ratio was also determined according to Marshall & Whelan (1974). This method determines the A:B chain ratio by measuring the reducing power with the copper sulphate method in hydrolysates of β-limit dextrin debranched with isoamylase and isoamylase plus pullulanase.

3. Results and discussion

3.1. Extraction fraction of the microdialysis membrane

Microdialysis is based on diffusion of substances due to a concentration gradient across a permeable membrane. The mass transfer aspects of microdialysis sampling can be quantitatively described by Eq. (1) (Bungay, Morrison & Dedrick, 1990; Morrison, Bungay, Hsiao, Mefford, Dykstra & Dedrick, 1991).

$$\mathrm{EF} = \frac{C_{\mathrm{d}}^{\mathrm{out}}}{C_{\mathrm{e}}} = 1 - \exp\left[\frac{-1}{Q_{\mathrm{d}}(R_{\mathrm{d}} + R_{\mathrm{m}} + R_{\mathrm{e}})}\right] \quad (1)$$

The dialysate extraction fraction (EF) is expressed as a function of the perfusion flow rate through the microdialysis probe (Q_{d}) and the sum of mass transfer resistances of dialysate (R_{d}), membrane (R_{m}), and external medium (R_{e}), i.e. the bioreactor (is nil in a well-stirred solution). Parameters R_{d} and R_{m} are functions of cannula and membrane dimensions, porosity, and diffusion coefficients in the membrane and free medium. The EF values were calculated as the ratio of the concentration of analyte in the dialysate ($C_{\mathrm{d}}^{\mathrm{out}}$) to that in the bioreactor (C_{e}) with pure water as the perfusion fluid. Experimentally, the peak areas of the HPAEC-PAD chromatograms obtained from the unit chains of debranched

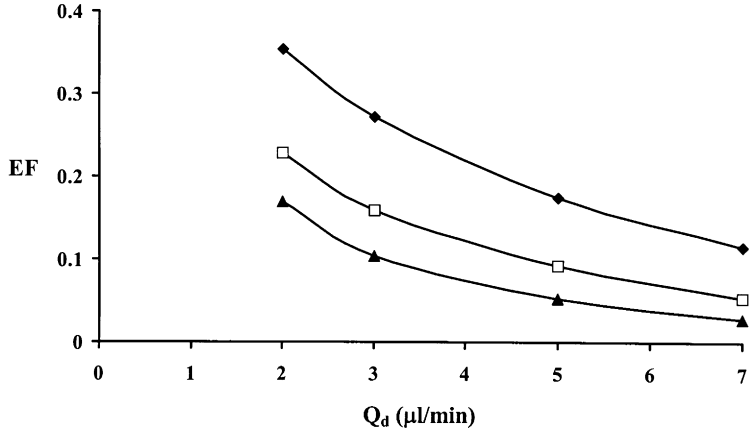

Fig. 2. EF for CL 6(◆), 15(□), and 25(▲) in debranched PAP at different flow rates, using a polysulphone microdialysis membrane with a 30 kDa molecular weight cut-off.

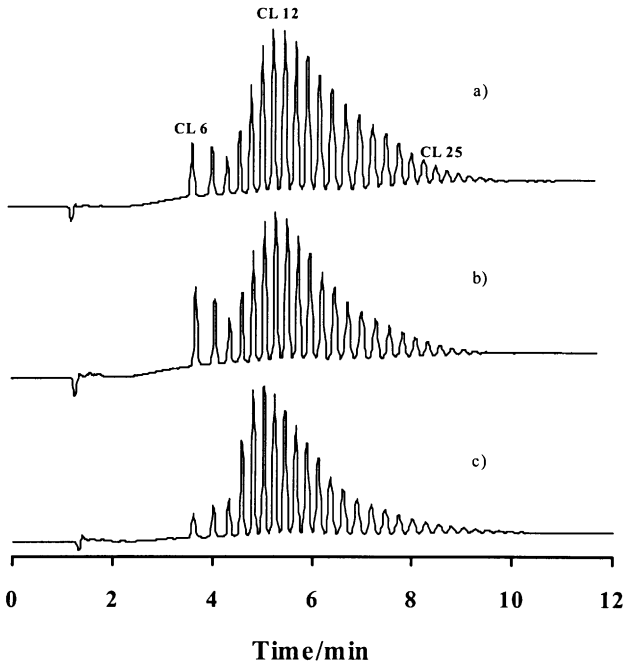

Fig. 3. Chromatograms obtained from HPAEC-PAD with chain length distribution pattern of starch from (a) PAP injected directly onto the HPAEC-PAD system (0.5 ml/mg), (b) PAP injected via the microdialysis probe (2.0 mg/ml), and (c) waxy maize injected via the microdialysis probe (2.0 mg/ml).

PAP from direct injections and injections via the microdialysis probe, were compared

The molecular weight cut-off of the membrane should be small enough to exclude large molecules e.g. enzymes and polymers, but large enough for the analytes, e.g. saccharides, to diffuse through the membrane. There are microdialysis membranes that vary in molecular weight cut-off, porosity, polymeric material, and design (Torto et al., 1998, 1999). The optimal choice of membrane for various applications has previously been investigated and the polysulphone membrane used here, with a molecular weight cut-off of 30 kDa, was chosen after experience from previous work (Richardson et al., 1999; Torto et al., 1998). Microdialysis membranes of various molecular weight cut-offs have previously been investigated with malto-oligosaccharides up to CL 7 (maltoheptaose) (Torto et al., 1998). Here, the EF values at four different Q_d were determined for chains with CL between six and 25 using debranched PAP.

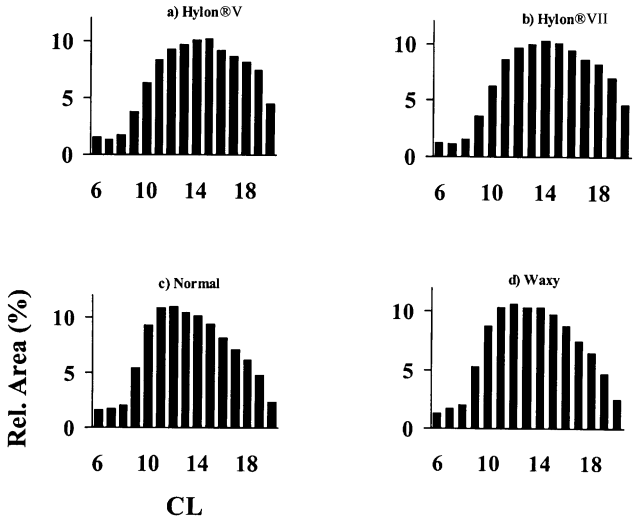

Fig. 4. Chain length distribution pattern of maize starch; Hylon® V (a), Hylon® VII (b), normal (c), and waxy maize (d), as relative response areas obtained from the on-line microdialysis–HPAEC-PAD-system.

EF decreased with increasing CL (Fig. 1), which is in agreement with smaller diffusion coefficients for larger molecules than for smaller ones.

As expected, EF values increased with decreasing Q_d for all CLs (Fig. 2). The higher the Q_d, the more diluted samples will be collected, which means lower EF as demonstrated in Figs. 1 and 2. In microdialysis sampling, the EF is highly dependent on Q_d, as described in Eq. (1); when $Q_d \rightarrow 0$, the EF $\rightarrow 1$. The flux of substances increases with Q_d until it levels off at $Q_d > 5$–10 μl/min, which is probably due to diffusion limitations when the maximal concentration gradient across the membrane is attained (Benveniste & Hansen, 1991).

3.2. Chain length distribution

CL patterns for debranched PAP and waxy maize were obtained with HPAEC-PAD combined with microdialysis sampling (Fig. 3). The high peak for CL 6 and the dip at CL 8, characteristic for potato amylopectin (Hanashiro et al., 1996; Koch et al., 1998), were obtained by samples injected onto the chromatographic system both with, and without, microdialysis sampling (Fig. 3a and b). The waxy maize sampled via the microdialysis probe also showed a typical pattern (Hanashiro et al., 1996; Koch et al., 1998) with low peaks at CL 6–8. Thus, the fingerprints of amylopectin from different botanical origins could be monitored by using on-line microdialysis sampling with HPAEC-PAD without loss of essential information. In this investigation, typical patterns in the CL between six and 25 were studied and the abundance of chains with the same CL were compared, as the PAD response varies with varying CL (Ammeraal, Delgado, Tenbarge & Friedman, 1991; Wong & Jane, 1997; Koch et al., 1998). Koch et al. (1998) reported a linear increase in the pulsed amperometric detector response per mole glucan chains with increasing CL, 3–65.

The unit chain distribution was compared for waxy, normal, and high amylose maize (Hylon® V and Hylon® VII) (Fig. 4a–d). The relative peak areas were plotted after correction with the known EF values. Waxy maize starch is regarded as amylose free, whereas normal maize starch, and Hylon® VII, contain 28, 55, and 70% of amylose, respectively, according to the manufacturers information (National Starch and Chemical Company). The CL distribution patterns of high amylose maize starches (Fig. 4a

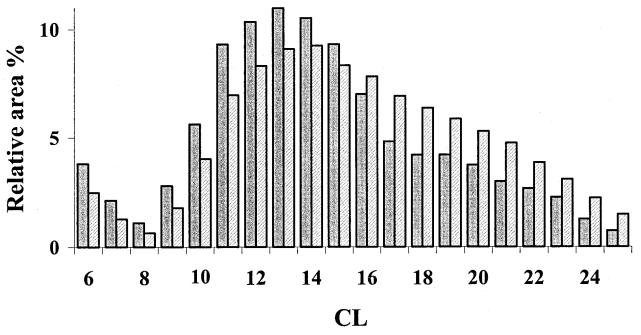

Fig. 5. Comparison of the chain length distribution pattern of potato amylose from two commercial preparations, H (grey bars) and F (striped bars), as relative response areas obtained from on-line microdialysis–HPAEC-PAD.

and b) were similar to that of maize amylopectin, with small peaks for CL 6–8. However, differences were observed in that the high amylose starches contained a higher relative amount of long chains compared with waxy maize starch (Fig. 4).

The quality, i.e. the abundance of short chains, of different amylose preparations was possible to compare by means of microdialysis sampling. Results obtained for the potato amyloses (Fig. 5) showed the high response for CL 6 and the dip at CL 8 (the typical pattern for potato amylopectin); however, differences in the CL distribution were observed for the two preparations. From these results it was not possible to conclude whether the short chain fractions were derived from amylopectin impurities or/and from amylose. Results obtained of amylose samples represent 10–15% of the total sample. It would be impractical to inject amylose chains having CL $>$ 500 (Hizukuri, 1991; Hizukuri et al., 1981; Takeda et al., 1984) onto the HPAEC system since the elution time for CL 80 is approximately 3 h (Hanashiro et al., 1996). Information about the short chains of amylose containing samples was obtained with this system, without time-consuming pre-fractionation of amylopectin and amylose.

The genetically modified potato starch (amylose deficient) PAP was compared with starch from its mother variety. Fig. 6 shows that PAP contained less CL 6 chains than

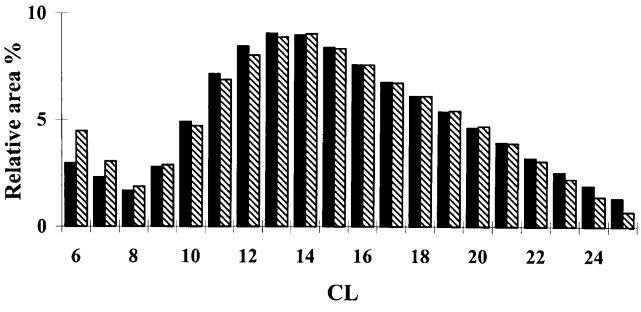

Fig. 6. Comparison of the chain length distribution pattern of starch from PAP (black bars) and normal potato (striped bars) as relative response areas obtained from on-line microdialysis–HPAEC-PAD.

normal potato starch. This difference was significant since the error of the method was less than 5%.

3.3. β-amylase hydrolysates

Results obtained from the investigation of β-amylase hydrolysates showed that the microdialysis membrane pore size selectively excluded the diffusion of the large β-limit dextrin polymer as well as the enzyme. The 244–320 kDa β-limit dextrins of PAP, waxy maize, and potato amylose, as determined by SEC-DRI/LALLS (Table 1), were not expected to diffuse through the microdialysis membrane with a 30 kDa cut-off. Therefore, only maltose was observed after the chromatographic separation and detection of the hydrolysates (Fig. 7).

Additionally, the chromatograms proved the purity of the β-amylase preparation, as only maltose was found as hydrolysis product. This fact may be judged as important progress, as in a previous work with microdialysis sampling, side reactions were observed in commercial preparations of β-amylase and pullulanase (Richardson et al., 1999). Impure β-amylase preparations produced glucose additionally to maltose, which results in erroneously high reducing power with non-specific reagents such as copper sulphate, and consequently too high β-limit values. The β-limit was calculated as the percentage of maltose liberated from the total maltose content in the starch sample. Furthermore, the present investigation demonstrates that combining microdialysis sampling with liquid chromatography provides a rapid and efficient method that can be automated for β-limit determination. Hydrolysis can be carried out unattended to achieve sample clean-up, sampling, and chromatographic separation, as well as detection within a reasonable time (3.5 h).

The result of the β-limit value for potato amylose (H) was 70% and the average CL was 100, as previously determined by ^{1}H-nuclear magnetic resonance spectroscopy (Nilsson et al., 1996). Various values have been reported for β-limit values of potato amylose depending on, for example, the fractionation method. Hizukuri, Takeda and co-workers reported β-amylolysis limits of 68–90% for potato amylose with average CLs of 510–850 (Hizukuri, 1991; Hizukuri et al., 1981; Takeda et al., 1984).

In our approach with microdialysis–HPAEC-PAD, the β-limit value of PAP was determined to be 54% and with the copper sulphate method, 57% (Table 1), both values in agreement with previously published results (Bender et al., 1982; Praznik, Rammesmayer, Spies & Huber, 1992). Praznik et al. (1992) determined β-limit values of amylopectin from two potato varieties to be 57 and 60%, respectively, whereas Bender et al. (1982) determined the β-limit for potato amylopectin to be 56%. Potato amylopectin contains a small proportion of glucose residues with phosphate ester groups (one per 200–300) at C-3 and C-6, located mainly on B-chains (Muhrbeck & Tellier, 1991; Takeda & Hizukuri, 1982). β-Amylase is prevented from

Table 1
Properties of waxy maize, potato amylopectin starch (PAP), and potato amylose. Relative standard deviations (%) are given in brackets ($n = 3$), n.d. = not determined

	Waxy maize	PAP (-94)	Potato amylose (H)
β-limit (microdialysis–HPAEC-PAD)	54(1)	54(3)	67(2)
β-limit ($CuSO_4$)	60(4)	57(5)	70(2)
M_w (kDa)	309	244	320
M_w/M_n	2.75	2.44	2.90
A:B chain ratio (microdialysis–HPAEC-PAD)	1.2(8)	1.2(8)	n.d.
A:B chain ratio ($CuSO_4$)	1.0(11)	1.0(14)	n.d.
CL (Nilsson et al., 1996)	n.d.	24	100
ECL	n.d.	15	69
ICL	n.d.	8	30
Degree of branching (%) (Nilsson et al., 1996)	n.d.	4.2	1.0

bypassing the phosphorylated glucose units (Takeda & Hizukuri, 1981), leading to a small error in the β-amylolysis limit, which is within the range of the experimental error.

β-Limit values for waxy maize were determined to be 54% with microdialysis–HPAEC-PAD and 60% with the copper sulphate method. Again, these values are consistent with previously determined values of 52–57% (Bertoft, 1989; Inouchi et al., 1987; Yun & Matheson, 1993), and are similar to those of PAP.

Application of the copper sulphate method resulted in 105–111% of the values determined with microdialysis–HPAEC-PAD (Table 1). Higher β-limit values determined with copper sulphate than with specific maltose determination have previously been reported (Bertoft, 1989). However, Yun and Matheson determined the maltose concentration of β-amylose hydrolysates by comparing the copper sulphate method with SEC analysis (Yun & Matheson, 1993). Although different β-limits were obtained, neither of the two methods resulted in a consistently higher or lower β-limit. The reason for the higher values obtained by the copper sulphate method herein remains unknown, since the purity of the enzyme had already been confirmed (Richardson et al., 1999).

3.4. A:B chain ratio

β-Limit dextrins are branched molecules consisting of interior chains and short stubs from A- and exterior B-chains. The A-chains remaining after β-amylase hydrolysis consist of maltosyl and maltotriosyl stubs, depending on whether the number of glucose units in the chains is even or odd. Only one (odd chains) or two (even chains) glucose units remain from the exterior B-chains on the β-limit dextrin (Manners, 1989). In the present investigation, the A:B chain ratio was determined after debranching of the β-limit dextrins with pullulanase and/or isoamylase, using two different methods for analysis of the hydrolysis products: microdialysis–HPAEC-PAD, which was compared with the method by Marshall & Whelan (1974), where the reducing power is determined.

Incomplete hydrolysis of the β-limit dextrin from PAP with isoamylase with obvious double peaks is shown in Fig. 8a. The smallest substrate for isoamylase is ^{2}6-α-D-maltotriosylmaltose, whereas that of pullulanase is ^{2}6-α-D-maltosylmaltose. According to Ammeraal et al. (1991), a branched chain will be eluted earlier than a non-branched chain. Therefore, the smaller peaks are assumed to derive from B-chains with remaining A-chain maltosyl stubs (Fig. 8a). When using pullulanase, all branching points were hydrolysed, as indicated by single peaks shown in the chromatogram in Fig. 8b. Maltose and maltotriose liberated during hydrolysis are the debranched maltosyl stubs

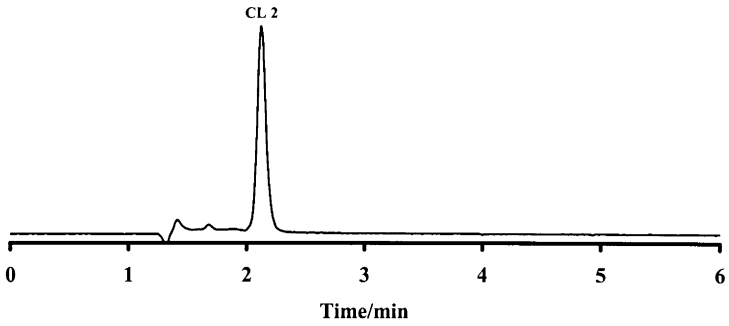

Fig. 7. Chromatogram of the β-amylase hydrolysate of PAP, injected via the microdialysis probe onto the HPAEC-PAD system.

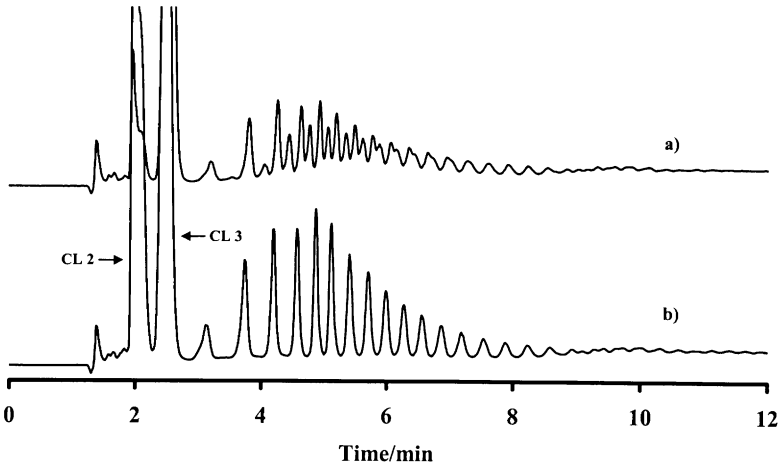

Fig. 8. Chromatograms obtained from the on-line microdialysis–HPAEC-PAD system of debranching products of β-limit dextrin from PAP hydrolysed by (a) isoamylase, and (b) isoamylase plus pullulanase.

from A- and exterior B-chains, and maltotriosyl stubs from A-chains, respectively. The longer chains are interior B-chains, and therefore, the interior chain length pattern was simultaneously monitored, as shown in Fig. 8b.

The A:B chain ratio determined with microdialysis–HPAEC-PAD was calculated according to Eq. (2), which is based on the assumption that the number of chains in amylopectin containing even and odd numbers of glucose units is equal. The maltotriose concentrations after pullulanase hydrolysis [MT_P], and after pullulanase plus β-amylase hydrolysis [$MT_{\beta P}$], were determined using the microdialysis–HPAEC-PAD system with the standard addition method. MT_P originates from the maltotriosyl stubs on A-chains with an odd number of glucose units. $MT_{\beta P}$ is maltotriose from B-chains with an odd number of glucose units.

$$\frac{A}{B} = \frac{2 \times MT_P}{2 \times MT_{\beta P} - MT_P} \quad (2)$$

The results obtained are shown in Table 1. Waxy maize had an A:B chain ratio of 1.2, which is in accordance with values reported previously ranging from 1.1 (Manners, 1989) to 1.3 (Yun & Matheson, 1993). The A:B chain ratio of PAP determined here was 1.2. Bender et al. (1982) obtained an A:B chain ratio of potato amylopectin of 1.1 using successive enzymic hydrolysis followed by HPLC analysis, while Hizukuri (1986) reports a ratio of 0.8 determined by SEC.

The A:B chain ratio was also determined using the copper sulphate method for determination of the reducing power in the hydrolysate (Marshall & Whelan, 1974). The A:B chain ratio was calculated according to Eq. (3) (Marshall & Whelan, 1974), where A_{iso} and $A_{iso+pull}$ are the absorbance at 660 nm after digestion of β-limit dextrin by isoamylase alone, and by the combination of isoamylase and pullulanase, respectively.

$$\frac{A}{B} = \frac{2(A_{iso+pull} - A_{iso})}{2A_{iso} - A_{iso+pull}} \quad (3)$$

The A:B chain values obtained (Eq. (3), Table 1) were lower than those achieved with on-line microdialysis–HPAEC-PAD. One explanation could be the high sensitivity to small experimental errors in the measurement of the reducing power (Atwell, Milliken & Hoseney, 1980). However, the repeatability of both methods was poor (Table 1), and the difference between A:B chain ratio of 1.0 and 1.2 could not be regarded as significant. Optimisation of the experimental conditions is therefore required.

3.5. Exterior and interior CL

From known CL and β-limit values, it is possible to calculate the average length of the exterior chains (ECL), i.e. those chains located outside the branching points (Eq. (4)) (Manners, 1989). If the A:B chain ratio is 1.0, the average length of the exterior stubs is 2.0 (therefore addition of 2 in Eq. (4)). With an A:B chain ratio of 1.2, the change in the average length of the exterior stubs is small and can thus be ignored. However, if the A:B chain ratio is 1.5, the average length of the stubs is 2.1 (Manners, 1989).

The average interior chain length (ICL) can be calculated according to Eq. (5) (Manners, 1989). (Subtraction of 1 in Eq. (5) is due to the glucose unit with both an α-(1 → 4) linkage, and an α-(1 → 6) linkage; this monomer is by definition included in neither exterior nor interior chains.)

$$ECL = CL \times (\%\beta\text{-limit}/100) + 2 \quad (4)$$

$$ICL = CL - ECL - 1 \quad (5)$$

The results of mean CL, ECL, and ICL of potato amylopectin starch and amylose are shown in Table 1. The ECL was calculated using the β-limit values determined with microdialysis–HPAEC-PAD. The CLs were previously determined by ^{1}H-nuclear magnetic resonance spectroscopy (Nilsson et al., 1996). The ECL of PAP was determined to be 15 and ICL to be eight, which are similar to those chain lengths of potato

amylopectin reported by Bender et al. (1982) and Zhu and Bertoft (1996) (14–16 and 6–8, respectively). The errors in the calculations of ECL and ICL are approximated to be ±1.0 glucose residues, estimated from the experimental errors in the determination of CL, β-limit value, and the A:B chain ratio. This is in agreement with previous results (Manners, 1989).

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References

Ammeraal, R. N., Delgado, G. A., Tenbarge, F. L., & Friedman, R. B. (1991). High-performance anion-exchange chromatography with pulsed amperometric detection of linear and branched glucose oligosaccharides. *Carbohydrate Research*, *215*, 179–192.

Analytica Chimica Acta (1999). Analytical applications of microdialysis. *Analytica Chimica Acta*, Vol. 379.

Atwell, W. A., Milliken, G. A., & Hoseney, R. C. (1980). A note on determining amylopectin A-to-B chain ratios. *Starch/Stärke*, *32*, 362–364.

Bender, H., Siebert, R., & Stadler-Szöke, A. (1982). Can cyclodextrin glycosyltransferase be useful for the investigation of the fine structure of amylopectins? Characterization of highly branched clusters isolated from digests with potato and maize starches. *Carbohydrate Research*, *110*, 245–259.

Benveniste, H., & Hansen, A. J. (1991). Practical aspects of using microdialysis for determination of brain intestinal concentrations. In T. E. Robinson & J. B. Justice, *Techniques in the behavioural and neural sciences* (pp. 81–100). Amsterdam: Elsevier.

Bertoft, E. (1989). Partial characterisation of amylopectin alpha-dextrins. *Carbohydrate Research*, *189*, 181–193.

Bertoft, E. (1991). Investigation of the fine structure of alpha-dextrins derived from amylopectin and their relation to the structure of waxy-maize starch. *Carbohydrate Research*, *212*, 229–244.

Bungay, P. M., Morrison, P. F., & Dedrick, R. L. (1990). Steady-state theory for quantitative microdialysis solutes and water in vivo and in vitro. *Life Sciences*, *46*, 46–119.

Hanashiro, I., Abe, J. -I., & Hizukuri, S. (1996). A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. *Carbohydrate Research*, *283*, 151–159.

Hizukuri, S. (1986). Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydrate Research*, *147*, 342–347.

Hizukuri, S. (1991). Properties of hot-water-extractable amylose. *Carbohydrate Research*, *217*, 251–253.

Hizukuri, S., & Maehara, Y. (1990). Fine structure of wheat amylopectin: the mode of A to B chain binding. *Carbohydrate Research*, *206*, 145–159.

Hizukuri, S., Takeda, Y., & Yasuda, M. (1981). Multi-branched nature of amylose and the action of debranching enzymes. *Carbohydrate Research*, *94*, 205–213.

Huber, A., & Praznik, W. (1998). Dimensions and structural features of aqueous dissolved polymers. In W. Praznik & A. Huber, *Carbohydrates as organic raw materials* (pp. 230–246). Vienna: WUV-Universitätsverlag.

Inouchi, N., Glover, D. V., & Fuwa, H. (1987). Chain length distribution of amylopectins of several single mutants and the normal counterpart, and sugary-1 phytoglycogen in maize. *Starch/Stärke*, *39*, 259–266.

Koch, K., Andersson, R., & Åman, P. (1998). Quantitative analysis of amylopectin unit chains by means of high-performance anion-exchange chromatography with pulsed amperometric detection. *Journal of Chromatography A*, *800*, 199–206.

Laurell, T., & Buttler, T. (1995). A microdialysis probe offering arbitrary membrane length and an in situ tunable relative recovery. *Analytical Methods and Instrumentation*, *2*, 197–201.

Manners, D. J. (1989). Recent developments in our understanding of amylopectin structure. *Carbohydrate Polymers*, *11*, 87–112.

Marshall, J. J., & Whelan, W. J. (1974). Multiple branching in glycogen and amylopectin. *Archives of Biochemistry and Biophysics*, *161*, 234–238.

Morrison, P. F., Bungay, P. M., Hsiao, J. K., Mefford, I. N., Dykstra, K. H., & Dedrick, R. L. (1991). Quantitative microdialysis. In T. E. Robinson & J. B. Justice, *Techniques in the behavioural and neural sciences* (pp. 47–80). Amsterdam: Elsevier.

Muhrbeck, P., & Tellier, C. (1991). Determination of the phosphorylation of starch from native potato varieties by ^{31}P-NMR. *Starch/Stärke*, *43*, 25–27.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry*, *153*, 375–380.

Nilsson, G., Bergquist, K. -E., Nilsson, U., & Gorton, L. (1996). Determination of the degree of branching in normal and amylopectin type potato starch with ^{1}H-NMR spectroscopy. *Starch/Stärke*, *48*, 352–357.

Noel, T. R., Ring, S. G., & Whittam, M. A. (1993). Physical properties of starch products: structure and function. *Food Colloids and Polymers*, *113*, 126–137 (Special publication — Royal Society of Chemistry (Stability and Mechanical Properties)).

Park, J. T., & Johnson, M. J. (1949). A submicrodetermination of glucose. *Journal of Biological Chemistry*, *181*, 145–151.

Praznik, W., Rammesmayer, G., Spies, T., & Huber, A. (1992). Characterisation of the (1,4)-alpha-D-glucan branching 6-glycolystransferase by in vitro synthesis of branched starch polysaccharides. *Carbohydrate Research*, *227*, 171–182.

Richardson, S., Nilsson, G. S., Torto, N., Laurell, T., & Gorton, L. (1999). Rapid determination of enzyme purity by a microdialysis-based assay. *Analytical Communications*, *36*, 189–193.

Takeda, Y., & Hizukuri, S. (1981). Re-examination of the action of sweet-potato beta-amylase on phosphorylated (1,4)-D-glucan. *Carbohydrate Research*, *89*, 174–178.

Takeda, Y., & Hizukuri, S. (1982). Location of phosphate groups in potato amylopectin. *Carbohydrate Research*, *102*, 321–327.

Takeda, Y., Shirasaka, K., & Hizukuri, S. (1984). Examination of the purity and structure of amylose by GPC. *Carbohydrate Research*, *132*, 83–92.

Torto, N., Bång, J., Richardson, S., Nilsson, G. S., Gorton, L., Laurell, T., & Marko-Varga, G. (1998). Optimal membrane choice for microdialysis sampling of oligosaccharides. *Journal of Chromatography A*, *806*, 265–278.

Torto, N., Laurell, T., Gorton, L., & Marko-Varga, G. (1999). Recent trends in the application of microdialysis in bioprocesses. *Analytica Chimica Acta*, *379*, 281–305.

Wong, K. S., & Jane, J. (1997). Quantitative analysis of debranched amylopectin by HPAEC-PAD with a postcolumn enzyme reactor. *Journal of Liquid Chromatography*, *20*, 297–310.

Yuan, R. C., Thompson, D. B., & Boyer, C. D. (1993). Fine structure of amylopectin in relation to gelatinization and retrogradation behavior of

maize starches from three wx-containing genotypes in two inbred lines. *Cereal Chemistry*, *70*, 81–89.

Yun, S. H., & Matheson, N. K. (1993). Structure of the amylopectins of waxy, normal, amylose-extender, and wx:ae genotypes and of the phytoglycogen of maize. *Carbohydrate Research*, *243*, 307–321.

Zhu, Q., & Bertoft, E. (1996). Composition and structural analysis of alpha-dextrins from potato amylopectin. *Carbohydrate Research*, *288*, 155–174.

Zook, C. M., & LaCourse, W. R. (1998). Pulsed amperometric detection of microdialysates from the glucose oxidase reaction. *Analytical Chemistry*, *70*, 801–806.